INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Estudo da interacção do fungo saprófita *Hypholoma fasciculare* com microrganismos filamentosos e seu efeito no crescimento de plantas de castanheiro

Eric Carvalho Pereira

Dissertação apresentada à Escola Superior Agrária de Bragança

para obtenção do Grau de Mestre em Biotecnologia

Orientado por

Prof. Doutora Paula Cristina dos Santos Baptista

Prof. Doutora Maria Teresa Lino Neto

Bragança

2010

“Escolhe um trabalho que gostes e não terás de trabalhar
nenhum dia da tua vida” (Confúcio)

Aos meus queridos pais

AGRADECIMENTOS

Ao entregar este trabalho, é com enorme prazer e satisfação que agradeço a todos aqueles que, de uma maneira ou de outra, me ajudaram na sua realização e conclusão.

Em primeiro lugar gostaria de agradecer aos meus orientadores. À Professora Doutora Paula Cristina dos Santos Baptista, da Escola Superior Agrária, por todo o conhecimento transmitido, por toda a ajuda prestada ao longo da realização do trabalho laboratorial e escrito, incentivo, paciência, permanente disponibilidade e acima de tudo por toda a amizade demonstrada.

À Professora Doutora Maria Teresa Lino Neto, do Departamento de Biologia da Escola de Ciência da Universidade do Minho, por todo o auxílio prestado, pela constante presença e disponibilidade, pelos conhecimentos transmitidos e pelas sugestões e críticas que permitiram melhorar este trabalho.

Aos meus amigos Gabriel Figueiredo e Ivo Oliveira por toda a ajuda prestada, por todo o conhecimento transmitido, assim como a permanente disponibilidade na realização deste trabalho e por toda a amizade demonstrada.

Aos meus amigos de sempre Tiago Mucha, Tânia Ribeiro, Madalena Vaz e Francisca Santos que viveram esta tese como se fosse sua, por estarem sempre ao meu lado nos momentos mais difíceis, por toda ajuda prestada, pela eterna amizade, companheirismo e por todos os momentos bem passados.

Aos meus colegas de laboratório Valentim Coelho, Fátima Martins, Hélio Belo e Juliana Garcia pelo apoio, incentivo, auxílio e conhecimentos transmitidos ao longo do trabalho.

A todos os meus amigos e colegas, que de uma maneira ou outra ajudaram na realização deste trabalho ou simplesmente pela sua amizade.

Por fim agradeço à minha família: aos meus irmãos, sobrinhos e afilhadas Erica e Eva, pelo constante apoio, carinho e incentivo na realização deste trabalho; aos meus pais que batalharam e sacrificaram para poder estar onde estou hoje e pelo seu amor incondicional.

Este trabalho foi efectuado no âmbito do Projecto PTDC/AGR-AAM7099556/2008 "Efeito do fungo *Hypholoma fasciculare* na sustentabilidade de soutos de *Castanea sativa*", financiado pela Fundação para a Ciência e a Tecnologia e pelo Programa Operacional COMPETE, com apoio FEDER

ÍNDICE

RESUMO

Hypholoma fasciculare é um fungo saprófita-lenhícola muito comum nos povoamentos de *Castanea sativa* na região do nordeste transmontano. Nos últimos anos, esta espécie tem sido explorada como agente de controlo biológico, por ser muito combativa contra espécies patogénicas. O presente trabalho tem como objectivo geral avaliar o potencial do *H. fasciculare* como agente de controlo biológico; e o efeito da sua aplicação nos fungos ectomicorrízicos bem como nas plantas de castanheiro e no processo de micorrização pelo *Pisolithus tinctorius*.

A avaliação do potencial do *H. fasciculare* como agente de controlo biológico, foi efectuada em condições *in vitro* pelo método da cultura dupla. O fungo *H. fasciculare* inibiu significativamente o crescimento dos patogéneos *Armillaria mellea* e *Phytophthora cambivora*, e dos fungos micorrízicos *Suillus luteus* e *Pisolithus tinctorius*. Os mecanismos adoptados pelo *H. fasciculare* foram "antagonismo e agonismo à distância", respectivamente para as espécies patogénicas e micorrízicas. Neste processo parecem intervir enzimas líticas, como a amilase, celulase, lacase e lipase, produzidas pelo *H. fasciculare*. Observaram-se ainda alterações morfológicas no *H. fasciculare* possivelmente com o intuito de se tornar mais resistente à invasão da espécie interactuante e/ou mais invasivo. Pelo contrário, nas co-culturas com *Amanita gemmata* e *Colletotrichum acutatum* o crescimento de *H. fasciculare* foi inibido; e com *P. cinnamomi* e *S. bovinus* não se observaram diferenças de crescimento para as espécies interactuantes.

O efeito da aplicação de *H. fasciculare* nas plantas de castanheiro e no processo de micorrização pelo *P. tinctorius* foi avaliada em condições de estufa. As plantas inoculadas com *P. tinctorius* apresentavam um maior crescimento e níveis foliares de azoto, fósforo, clorofilas (a, b e total) e carotenóides face às plantas não inoculadas. O efeito positivo da micorrização foi contudo suprimido pelo fungo *H. fasciculare* quando aplicado simultaneamente com *P. tinctorius*. Este resultado poderá estar relacionado com a inibição da micorrização das raízes pelo *H. fasciculare* e/ou pela competição entre o *P. tinctorius* e o *H. fasciculare* por nutrientes, nomeadamente azoto. O efeito deletério de *H. fasciculare* não se verificou quando aplicado nas plantas isoladamente ou 30 dias depois da inoculação com *P. tinctorius*. Os resultados obtidos evidenciam

uma interacção antagónica entre fungos ectomicorrízicos e saprófitas com repercussões ao nível do crescimento e fisiologia da planta.

Palavras-chaves: *Hypholoma fasciculare*, interacção micelial, enzimas líticas, *Castanea sativa*, interacção planta-fungo, *Pisolithus tinctorius*.

ABSTRACT

Hypholoma fasciculare is a saprophyte-legnicolous fungus very common in communities of Castanea sativa in the northeast region of Portugal. In the last few years this species has been explored as a biological control agent due to its ability to combat pathogenic species. The objective of this study is to evaluate the potential of *H. fasciculare* to act as a biological control agent and the effects of its application on ectomycorrhizal funguses, chestnut plants as well as in the process of mycorrhization by *Pisolithus tinctorius*.

The evaluation of the potential of *H. fasciculare* as a biological control agent was made in *in vitro* conditions and by the double culture method. The H. fasciculare fungus inhibited significantly the growth of the pathogens *Armillaria mellea* and *Phytophthora cambivora* and the micorrizic *Suillus luteus* and *Pisolithus tinctorius*. The mechanisms adopted by *H. fasciculare* to act on pathogenic species and micorrizic species were respectively "antagonism and agonism at a distance". In this process it is observed the intervention of litic enzymes such as amylase, cellulase, lacase and lipase, all produced by *H. fasciculare*. It is also observed morphological alterations of H. *fasciculare* possibly with the intent of becoming more resistant to the possible invasion of the interacting and/or more invasive species. On the other hand, in the co-cultures with *Amanita gemmata* and *Colletotrichum acutatum*, the growth of *H. fasciculare* was inhibited. With *P. cinnamomi* and *S. bovinus* no differences of growth was observed by the interacting species.

The effects of the application of *H. fasciculare* in the chestnut plants and in the process of micorrization by *Pisolithus tinctorius* was evaluated in greenhouse conditions. The plants inoculated with P. tinctorius showed a greater growth, at a foliar level, of nitrogen, phosphorous, chlorophylls (a, b and total) and caratanoids relatively to the non-inoculated plants. The positive effect of the micorrization was however suppressed by the *H. fasciculare* fungus when applied simultaneously with *P. tinctorius*. This result may be related to the inhibition of the micorrization of the roots by H. fasciculare and/or by the competition between *P. tinctorius* and *H. fasciculare* for nutrients, namely nitrogen. The deleterian effect of *H. fasciculare* was not observed when applied to plants in isolation neither after 30 days of inoculation by *P. tinctorius*.

The results obtained show an antagonic interaction between ectomicorrizic and saprophyte funguses with repercussions in the growth and the physiology of the plant.

Keywords: *Hypholoma fasciculare*; mycelial interaction; lytic enzymes; *Castanea sativa;* plant-fungus interaction; *Pisolithus tinctorius.*

Capítulo 1

Introdução Geral

1.1. INTRODUÇÃO GERAL

O solo serve de habitat a uma enorme variedade de formas de vida, sendo constituído por grupos altamente diversificados de organismos, desde bactérias a fungos passando por componentes da fauna, como nemátodes e protozoários (Bardgett, 2005).

De entre os microrganismos presentes no solo destacam-se os fungos, pois desempenham um papel importante ao nível do desenvolvimento, nutrição e sanidade das plantas. As principais acções dos fungos podem ser ao nível da decomposição de matéria orgânica morta, do antagonismo em relação a outros microrganismos, da associação parasita ou patogénica e da associação, mutuamente favorável, com as raízes das plantas superiores (Elsas *et al.*, 2006). Os fungos são muito diversificados, tanto ao nível da estrutura como a nível funcional e adoptam diferentes estratégias tróficas, podendo ser saprófitas, simbiontes, patogénicos ou parasitas.

Os fungos saprófitas constituem o maior grupo de organismos decompositores, apresentando um papel essencial na decomposição de polímeros orgânicos, em especial na degradação e reciclagem de matéria vegetal, constituído na sua maioria por materiais celulósicos. Obtêm os seus nutrientes através da matéria orgânica morta, libertando ainda nutrientes bloqueados que possam ser utilizados por outros seres-vivos. Participam também no ciclo de carbono, actuando na degradação das moléculas orgânicas para a reposição do dióxido de carbono na atmosfera (Carlile *et al.*, 2001; Elsas *et al.*, 2006).

Uma das estratégias adoptada por alguns fungos para a obtenção de carbono foi a sua associação com a raiz de plantas autotróficas, formando as micorrizas (Weber & Webster, 2001), ou com as algas, originando os líquenes. Os fungos que intervêm nesta associação, de carácter mutualista, são designados por simbiontes. A associação micorrízica pode ser dividida em seis tipos (Figura 1), com base na estrutura de associação e das características do fungo e da planta hospedeira envolvidos. As **micorrizas arbusculares**, que constituem a associação micorrízica mais amplamente distribuída na natureza, caracterizam-se pela presença de arbúsculos no interior das células corticais da raiz e pela formação de vesículas (Figura 1) (Smith & Read, 2008). Os fungos que intervêm nesta associação pertencem à classe Glomeromycetes (Walker & Schüßler, 2004). As **micorrizas ericóides**, caracterizam-se por apresentarem, no

interior das células corticais da raiz, espirais de micélio (Figura 1). As plantas que intervêm nesta associação são da família *Ericaceae*, *Empetraceae* e *Epacridaceae*; os fungos pertencem sobretudo à classe Ascomycetes, e em menor extensão à classe Basidiomycetes (Smith & Read, 2008). As **ectomicorrizas** caracterizam-se pela presença de três estruturas: um manto de tecido fúngico que envolve a raiz, um labirinto que cresce entre as células epidérmicas e corticais chamada de rede Hartig e o crescimento no exterior de um sistema de hifas que forma uma conexão essencial entre o solo e o fungo (Figura 1) (Smith & Read, 2008). Os fungos envolvidos nesta associação pertencem às classes Basidiomycetes, Ascomycetes e em menor extensão à Zigomycetes; as plantas pertencem sobretudo às famílias Fagaceae, Betulaceae e Pinaceae (Smith & Read, 2008).

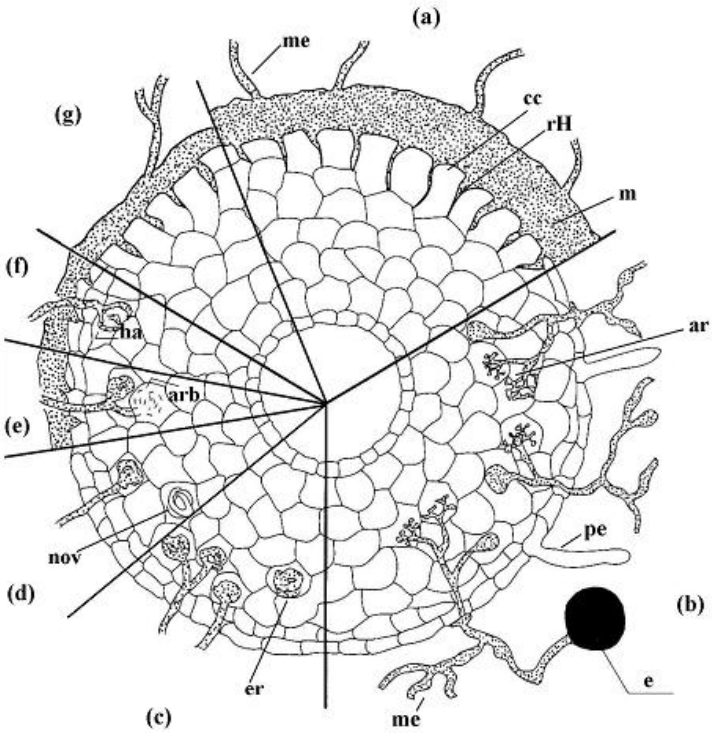


Figura 1 - Representação esquemática dos seis tipos de associação micorrízica. **(a)** ectomicorrizas, **(b)** micorrizas arbusculares, **(c)** micorrizas ericóides, **(d)** micorrizas orquidáceas, **(e)** micorrizas arbutóides, **(f)** micorrizas monotrepóides, **ar** - arbúsculo, **arb** - arbutóides, **cc** - célula cortical, **e** – esporo, **er** – enrolamento, **ha** – haustórios, **m** – manto, **me** - micélio extrarradicular, **nov** – novelos, **rH** – rede de Hartig. **(Adaptado de Azul, 2002).**

As **micorrizas orquidóides** formam-se especificamente entre plantas da família *Orchidaceae* e fungos pertencentes à classe Basidiomycetes. Esta associação é crucial para estas plantas dado que requerem, após germinação, compostos carbonados que não conseguem sintetizar e que são cedidos pelo fungo. Nesta associação formam-se novelos de micélio no interior das células do córtex da raiz (Figura 1) (Smith & Read, 2008). As **micorrizas arbutóides**, ocorre entre fungos da classe Basidiomycetes e as espécies de plantas pertencentes à sub-família Arbutoideae. As **micorrizas monotrepóides**, formam-se em algumas plantas da família *Monotropaceae* e fungos da classe Basidiomycetes (Smith & Read, 2008). Estes dois últimos tipos de micorrízas apresentam características estruturais semelhantes às ectomicorrízas e as micorrizas arbusculares (Figura 1).

O líquen trata-se de uma simbiose entre fungos do filo *Ascomycota* ou *Basidiomycota* com algas, podendo crescer em diversos habitats como em ambientes semi-áridos, sobre rochas ou mesmo em tundras (Elsas *et al*., 2006).

Por fim temos os fungos parasitas e patogénicos, que obtêm o carbono através da estratégia de parasitar plantas, sendo responsáveis deste modo por 70% das doenças em plantas superiores. Obtêm o carbono actuando como patogénico necrotrófico, invadindo e matando o hospedeiro através de toxinas ou enzimas digestivas (Elsas *et al*, 2006).

A interacção microbiana no solo pode ser caracterizada com base nos tipos de organismos que participam na interacção. Segundo Elsas *et al.* (2006) e Tuininga (2005) existem vários tipos de interacção possíveis entre os microrganismos no solo, podendo ser classificadas como interacções positivas, neutras ou negativas. Nas interacções positivas não há prejuízo para as espécies participantes ou então existe vantagem para pelo menos uma delas, sem o prejuízo da outra. Nas interacções negativas ambas ou apenas uma das espécies participantes é prejudicada, podendo existir benefício para uma delas. Nas interacções neutras, nenhuma das duas espécies interactuantes é afectada. Segundo Tuininga (2005) as interacções inter-especificas positivas que podem operar entre microrganismos, incluindo fungos, podem ser de dois tipos: comensalismo e mutualismo. Por sua vez, as interacções negativas são classificadas em antagonismo, co-antagonismo e agonismo; enquanto que as neutras em co-habitação (Tabela 1).

Tabela 1 - Tipos de interacção entre duas espécies fúngicas, suas características e exemplos de mecanismos (adaptado de Tuininga, 2005).

Tipos de interacção	**Resultado**		**Exemplos de mecanismos**
Interacções neutras			
Co-habitação	0 / 0	Nenhuma das duas espécies afecta a outra	Entrelaçamento de hifas
Interacções negativas			
Antagonismo	- / 0	Morte, redução de crescimento de uma das espécies	Lise e vacuolização das hifas, alterações no valor de pH, produção de compostos voláteis e/ou difusíveis (antibióticos)
Co-antagonismo	- / -	Morte, redução de crescimento das duas espécies	Lise e vacuolização das hifas, alterações no valor de pH, produção de compostos voláteis e/ou difusíveis (antibióticos)
Agonismo	- / +	Redução de crescimento de uma das espécies e aumento de crescimento da outra espécie	Produção de compostos voláteis e/ou difusíveis
Interacções positivas			
Comensalismo	0 / +	Aumento de crescimento de uma das espécies sem prejuízo para a outra espécie	Produção de exsudados e entrelaçamento de hifas
Mutualismo	+ / +	Aumento de crescimento de ambas as espécies	Produção de exsudados e entrelaçamento de hifas

0 a espécie não é afectada pela presença da outra
\+ a espécie é beneficiada pela presença da outra
\- a espécie é prejudicada pela presença da outra

A comunidade microbiana presente no solo é influenciada por vários microrganismos antagonistas (Whipps, 2004), assim, a interacção entre os vários tipos de fungos pode resultar na consequente inibição de um deles. Por exemplo, em vários estudos verificou-se que os fungos filamentosos saprófitas influenciavam negativamente a formação de micorrizas (Zadworny, 2007), facto que poderá advir da competição entre

os fungos micorrizicos e saprófitas por recursos nutricionais (Shaw *et al*. 1995; Mrnka *et al*, 2009).

O *Hypholoma fasciculare,* é um fungo saprófita-lenhícola que apresenta uma vasta distribuição geográfica, sendo muito comum em povoamentos de *Castanea sativa* na região do nordeste transmontano (Baptista, 2007). Segundo Kirk *et al.* (2001), inclui-se no reino Fungi, filo Basidiomycota, classe Basidiomycetes, Sub-classe Agaricomycetidae, ordem Agaricales, família Strophariaceae e género: *Hypholoma*.

Trata-se de uma espécie fúngica que se caracteriza por apresentar um crescimento micelial através de cordões (Boddy, 2000), podendo atingir um tamanho superior a 100m de diâmetro (Boddy, 1993). É uma espécie muito combativa contra outras espécies saprófitas (Donnelly & Boddy, 2001), característica que tem incentivado a sua utilização como agente de controlo biológico contra diversos microrganismos incluindo fungos patogénicos/parasitas (Varese *et al.*, 2003; Chapman *et al.*, 2004; Cox & Scherm, 2006) e bactérias (Folman *et al.*, 2008; Boer *et al.*, 2010).

Em Trás-os-Montes, os povoamentos de *Castanea sativa* são habitados por uma grande população de fungos e oomycetas apresentando, algumas destas espécies de microrganismos, efeitos deletérios e benéficos para o castanheiro. De entre os microrganismos com efeitos deletérios destacam-se os oomycetas *Phytophthora cinnamomi* Rands e *P. cambivora* (Petri) Buis, responsáveis pela designada doença da tinta, que constitui a maior causa do decréscimo da área ocupada por esta cultura na região Transmontana. Por sua vez, os fungos micorrízicos apresentam reconhecidos efeitos positivos para o castanheiro. Verificou-se que a micorrização com *Pisolithus tinctorius* promovia um aumento da disponibilidade de nutrientes minerais, do crescimento e da produtividade das plantas de castanheiro (Martins, 2004). Adicionalmente existe um grande número de espécies fúngicas saprófitas, das quais se destaca o *Hypholoma fasciculare,* que não se conhece até à data terem qualquer efeito directo nas plantas de castanheiro.

O presente trabalho tem como objectivo geral avaliar o potencial do *H. fasciculare* como agente de controlo biológico de diversos microrganismos parasitas e patogénicos e o efeito da sua aplicação na população fúngica do solo com acção benéfica para as plantas (e.g. fungos ectomicorrízicos). Pretende-se aainda avaliar o efeito da aplicação

de *H. fasciculare* ,nas plantas de castanheiro e no processo de micorrização pelo fungo ectomicorrízico *Pisolithus tinctorius*.

Assim sendo, os objectivos específicos foram:

- Caracterizar os mecanismos de interacção entre o fungo *H. fasciculare* e diversos microrganismos filamentosos, com acção benéfica ou deletéria para a planta, em condições de *in vitro*. As espécies analisadas foram diversos fungos ectomicorrízicos, tais como a *Amanita gemmata, Pisolithus tinctorius, Suillus luteus* e *Suillus bovinus,* um fungo parasita *Armillaria mellea*, um fungo fitopatogénico *Colletotrichum acutatum* e os oomycetas *Phytophthora cambivora* e *Phytophthora cinnamomi* (Capítulo 2). Espera-se deste modo avaliar o potencial do *H. fasciculare* como agente de controlo biológico de diversas espécies fitopatogénicas e o seu efeito nos fungos ectomicorrízicos com acção benéfica para as plantas.

- Avaliar, em condições de *in vitro*, a produção de enzimas líticas durante o processo de interacção entre *H. fasciculare* e diversos microrganismos patogénicos e simbiontes (Capítulo 2). Espera-se, através dos resultados obtidos, elucidar o papel das enzimas líticas na actividade antagonista exibida pelo *H. fasciculare*.

- Avaliar o efeito da inoculação de plantas de castanheiro com o fungo *H. fasciculare,* aplicado individualmente ou em combinação com o fungo ectomicorrízico *Pisolithus tinctorius*, no crescimento, nutrição mineral, conteúdo de clorofilas e carotenóides e na micorrização, em condições de estufa (Capítulo 3). Os resultados obtidos poderão elucidar, pela primeira vez, o efeito do *H. fasciculare* na planta e no processo de micorrização.

Espera-se, que os resultados obtidos possam contribuir para um melhor esclarecimento do papel do fungo *H. fasciculare* no controlo biológico de determinadas doenças, nomeadamente da doença da tinta, e das repercussões da sua aplicação nos ecossistemas naturais, nomeadamente ao nível das plantas e de fungos ectomicorrízicos.

1.2. REFERENCIAS

Azul, A. & Ramos, V. (2007). Estudo das gramíneas da Serra da Estrela – Diversidade e significado ecofisiológico das comunidades de fungos simbióticos do solo. *Bloco de notas do centro de interpretação da Serra da Estrela*, **19**, 8-9.

Baptista, P. (2007). Macrofungos associados à cultura de castanheiro: aspectos da sua biodiversidade e da interacção de *Pisolithus tinctorius* e *Hypholoma fasciculare* com raízes de *Castanea sativa* Mill. Tese de Doutoramento, Universidade do Minho, Braga, 267 pp.

Bardgett, R.D. (2005). The biology of soil: A community and ecosystem approach. Oxford University Press, USA.

Boddy, L. (1993). Saprotrophic cord-forming fungi: meeting the challenge of heterogeneous environments. *Mycologia,* **91**, 13-32.

Boddy, L. (2000). Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiology Ecology*, **31**, 185-194.

Boer, W., Folman, L.B., Gunnewiek, P.J.A.K., Svensson, T., Bastviken, D., Öberg, G., Rio, J.C. & Boddy, L. (2010). Mechanism of antibacterial activity of the white-rot fungus *Hypholoma fasciculare* colonizing wood. *Canadian Journal of Microbiology*, **56(5)**, 380-388.

Chapman, B., Xiao, G. & Myers, S. (2004). Early results from field trials using *Hypholoma fasciculare* to reduce *Armillaria ostoyae* root disease. *Canadian Journal of Botany*, **82**, 962-969.

Carlile, M.J., Watkinson, S.C. & Gooday, G.W. (2001). The Fungi. Academic press, UK.

Cox, K.D. & Scherm, H. (2006). Interaction dynamics between saprobic lignicolous fungi and *Armillaria* in controlled environments: Exploring the potential for competitive exclusion of *Armillaria* on peach. *Biologicol Control*, **37**, 291-300.

Folman, L.B., Klein Gunnewiek, P.J.A, Boddy, L. & de Boer, W. (2008). Impact of white-rot fungi on numbers and community composition of bacteria colonizing beechwood from forest soil. *FEMS Microbiology Ecology,* **63**, 181–191.

Elsas, J.D.V., Jansson, J.K. & Trevors, J.T. (2006). Modern soil microbiology. *CRC Press Inc. Boca Raton*, NY.

Kirk, P.M., Cannon, P.F., David, J.C. & Stalpers, J.A. (2001). Ainsworth & Bisby's Dictionary of the Fungi. *CABI Bioscience*, UK.

Martins, A. (2004). Micorrização controlada de *Castanea sativa* Mill.: aspectos fisiológicos da micorrização *in vitro* e *ex vitro*. Tese de Doutoramento. Faculdade de Ciências da Universidade de Lisboa. Lisboa

Shaw, T.M., Dighton, J. & Sanders, F.E.T (1995) Interactions between ectomycorrhizal and saprotrophic fungi on agar and in association with seedlings of lodgepole pine (*Pinus contorta*). *Mycological Research*, **99**: 159-165.

Smith S.E. & Read D.J. (2008). *Mycorrhizal symbiosis.* Academic Press, USA.

Tuininga, A.R. (2005). Interspecific interaction terminology: from mycology to general ecology. In The fungal community its organization and role in the ecosystem. Dighton, J., White, J.F. & Oudeman P. edit. *Taylor & Francis*, London.

Varese, G.C., Gonthier, P. & Nicolotti, G. (2003). Long-term effects on other fungi are studied in biological and chemical stump treatments in the fight against *Heterobasidion annosum* coll. *Mycologia,* 95, 379-387.

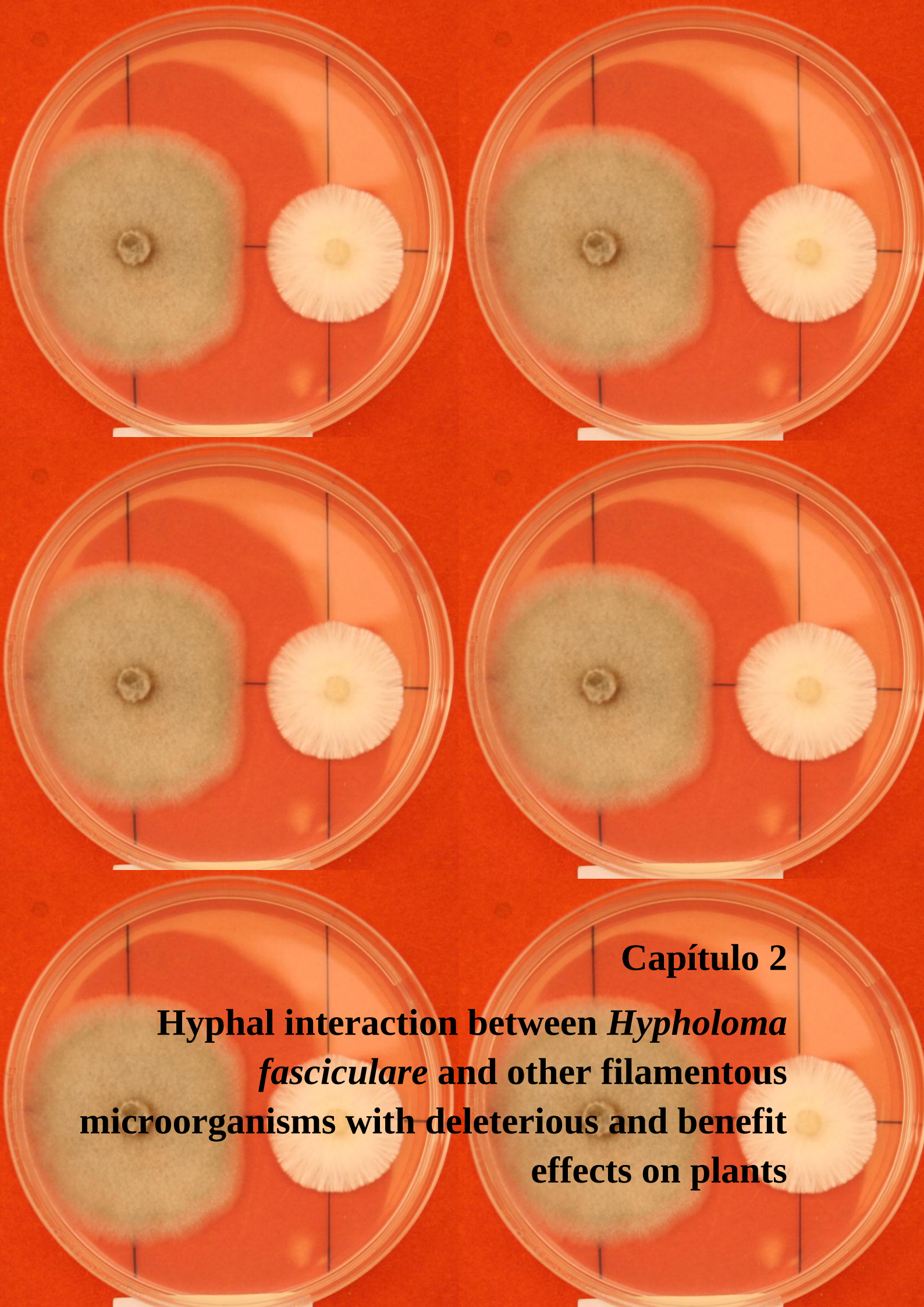
Walker, C., Schüßler (2004), Nomenclatural clarification and new taxa in the Glomeromycota, *Mycological Research News*, **108(9),** 981 – 982.

Weber, R.W.S. & Webster, J. (2001). Teaching techniques for mycology. Mycology, **25(4),** 1-4.

Webster, J. & Weber, R.W.S. (2007). *Introduction to Fungi*. Cambridge university press, UK.

Whipps, J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany***, 52,** 487-511.

Zadworny, M., Tuszynska, S., Samardakiewicz, S. & Werner, A. (2007), Effects of mutual interaction of *Laccaria Laccata* with *Trichoderma harzianum* and *T. virens* on the morphology of microtubules and mitochondria. *Protoplasma*, **232,** 45 – 53.



Capítulo 2

Hyphal interaction between *Hypholoma fasciculare* and other filamentous microorganisms with deleterious and benefit effects on plants

HYPHAL INTERACTION BETWEEN *HYPHOLOMA FASCICULARE* AND OTHER FILAMENTOUS MICROORGANISMS WITH DELETERIOUS AND BENEFIT EFFECTS ON PLANTS

ABSTRACT

Hypholoma fasciculare is a widespread cord-forming basidiomycete specie on chestnut orchards as well as on other agro-forestry ecosystem, in Northeast of Portugal. In an *in vitro* study, interactions between *H. fasciculare* and pathogenic/parasitic microorganisms as well as ectomycorrhizal fungi, were examined in order to evaluate its potential use as biological control agent without causing deleterious effects in beneficial fungi. The involvement of lytic enzymes in the mechanism of interaction was also evaluated. The results obtained showed that interaction development and outcome was species dependent. In dual culture *H. fasciculare* inhibited significantly the growth of the pathogens *Armillaria mellea* (in 49%) and *Phytophthora cambivora* (in 17%) as well as of the ectomycorrhizal fungus *Suillus luteus* (in 43%) and *Pisolithus tinctorius* (in 49%), long before the contact between hyphae of their colonies had occurred. For the first two species the mechanism adopted by *H. fasciculare* was antagonism at distance whereas for the two last species was agonism at distance. The qualitative identification of amylase, cellulase, laccase and lipase produced by *H. fasciculare* in the culture media suggested its involvement in the mechanism of interaction. During interaction *H. fasciculare* formed a dense and compact mycelial, like a barrier (defensive) or cords (invasive), especially in the interacting zone. Different pattern of growth was observed for the species *Amanita gemmata*, *S. bovinus*, *Colletotrichum acutatum* and *P. cinnamomi* when cultured with *H. fasciculare*.

Keywords: *Hypholoma fasciculare*; mycelial interaction; lytic enzymes; phytopathogenic microorganisms; ectomycorrhizal fungi

2.1. INTRODUCTION

Hypholoma fasciculare (Huds.) P. Kumm is a wood-decaying basidiomycete with a worldwide distribution and occurs in various habitats including tropical, temperate and boreal forest (Boddy, 1999). In Trás-os-Montes region (Northeast of Portugal) this specie is commonly present in soils of several habitats including chestnut (Baptista *et al*., 2010) and oak (Branco, 2003) trees as well as in other natural habitats such as wood. Its high mycelial extension rates allow the fungus to colonize extended areas that can reach more than 100 m diameter (Boddy, 1993). This fungus, due to its remarkable growth pattern, plays an important role in mobilization and reallocation of biomass and nutrients, representing a significant nutrient reservoir in woodland ecosystems (Wells & Boddy, 2002). This characteristic also derived from the growth patterns exhibit by *H. fasciculare*. On the forest floor, this fungus produce 'root-like' linear organs - termed cords, which allow translocation of water and mineral nutrients from one location to another, and also provide resistance to antagonistic effects of soil microorganisms (Boddy, 2000). Their mycelial extension rates associated to the formation of linear mycelial organs, suggests that *H. fasciculare* has high combative ability and therefore could be use as biological control agent against phytopathogenic microorganisms. This hypothesis is reinforced by the results obtained by several authors that have been shown antagonistic activity of *H. fasciculare* against *Armillaria* spp., responsible for the Armillaria root rot disease, under axenic conditions (Chapman & Xiao, 2000; Cox & Scherm, 2006) as well as in forest systems (Chapman *et al*., 2004). For example, it was verified in a variety of laboratory conditions that *H. fasciculare* was able to reduced colonization of poplar wood blocks and peach root segments by *Armillaria tabescens* and *Armillaria mellea* (Cox & Scherm, 2006) and to inhibited the growth of *Armillaria ostoyae* both on agar plates and woody substrate (Chapman & Xiao, 2000). Similarly, in the field Chapman *et al.* (2004) have verified a reduction in mortality levels of several forest trees species caused by *A. ostoyae* in the plots treated with *H. fasciculare*. The possible role of *H. fasciculare* to control wood-inhabiting bacteria in a soil microcosm (Folman *et al*., 2008; Boer *et al*., 2010) and *Heterobasidion annosum*, the disease agents of conifers causing root rot and/or butt, on Norway spruce forests (Nicolotti & Varese, 1996; Varese *et al*., 2003), has been also exploited.

Several antagonistic mechanisms may play a vital role in disease suppression by *H. fasciculare*. The combative mechanism most frequently adopted is hyphal interference, a form of antagonism that cause cessation of growth and subsequent vacuolation (swelling), granulation and lysis of hyphae of the other interacting species when come in close proximity (Cox & Scherm, 2006). However, cord-forming basidiomycetes could adopt other combative strategies involving antagonism at a distance, gross mycelial contact and mycoparasitism (Boddy, 2000). A variety of compounds have suggested to produced during interaction, which included antibiotics, non-enzymic diffusible metabolite and toxins, extracellular cell wall-degrading enzymes, volatile compounds and proteins (Gloer, 1995; Boddy, 2000; Hynes *et al*., 2007).

The ability of naturally occurring microorganisms to inhibit the growth of deleterious microorganisms has been in fact studied during the past century and continues to inspire research in many fields, especially in crop protection. Interest in biological control, and particularly in the exploitation of fungi to control pests, diseases and weeds, is demonstrated by the number of fungi based products already on the market and also in development. This increased interest is, in part, due to the desire to enhance the sustainability of agriculture and also because biocontrol may provide control of plant diseases that can not, or only partially, be managed by other strategies (Cook *et al*., 1996). However, many concerns were done by the use of biological control organism, since they may have detrimental impacts on other organisms present in the ecosystem to which they are applied (Brimner & Boland, 2003). For example, it was observed on some studies of antagonistic interactions the suppression of saprotrophic fungi on ectomycorrhizal fungal growth and mycorrhization (Shaw *et al.*, 1995; Lindahl *et al*., 2001). This could cause loss of incomes from symbiosis, namely plant fitness and health, and also changed the fungal community.

Keeping in mind the eventual use of *H. fasciculare* as a biological control agent it is needed to understand better its mechanism of action towards both pathogenic microorganism and beneficial fungus. Thus, the purpose of our work was to characterize the interspecific interaction between *H. fasciculare* and various pathogenic/parasitic microorganisms, including *Armillaria mellea, Colletotrichum acutatum, Phytophthora cambivora* and *P. cinnamomi,* as well as ectomycorrhizal fungal species, namely *Amanita gemmata, Pisolithus tinctorius, Suillus bovinus* and *S. luteus,* under *in vitro*

conditions. The fungus *C. acutatum* causes anthracnose and blight in agriculturally important hosts such as almond, avocado, peach, blueberries, citrus, mango, olive and strawberry (Wharton & Diéguez-Uribeondo, 2004). *P. cinnamomi* and *P. cambivora* occurs world-wide and causes Ink disease in European Chestnut trees. *P. cinnamomi* causes also severe root rot and dieback in many ornamental and fruit species, as well as of some 900 other woody perennial plant species (Ferraris *et al*., 2004). Since one of the most important factors implicated in the antagonistic features of fungi are the hydrolytic enzymes (Srinom *et al*., 2006) we also intended to evaluated the production of extacellular hydrolytic enzymes by the microorganisms during hyphal interaction. The enzymes analysed were amylase, cellulase, laccase, lipase, protease and tyrosinase. It is expected to ascertain the potential of *H. fasciculare* as antagonist-mediated biological control without any risk to ectomycorrhizal fungus.

2.2. MATERIALS AND METHODS

2.2.1. Microorganisms

The microorganisms used in the present study are indicated in Table 1. The mycelium of *H. fasciculare*, *A. gemmata*, *A. mellea*, *S. bovinus* and *S. luteus* were isolated from sporocarps. Fungal isolation was performed on Melin-Norkans (MMN) agar medium at pH 6.6 [NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casamino acids 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L], following Brundrett *et al.* (1996). *C. acutatum* isolate was obtained from naturally infected olive fruits collected in Mirandela (Northeast of Portugal). Fungus isolation was performed on potato–dextrose agar (PDA) medium supplemented with 0.01% (w/v) chloramphenicol (Oxoid). Pure cultures of each isolate were obtained by sub-culturing the mycelium in the same medium where fungal isolation was done. The identification of all fungal isolates was molecularly confirmed by amplification of the internal transcribed spacer region (ITS), using the universal primers *ITS1* and *ITS4* (White *et al*., 1990). The product obtained from each isolate was sequenced and compared to those in GenBank; BLAST searches for all isolates had nucleotide identities of 99%, which confirmed the identifications. The obtained isolates were deposited in the culture collection of the School of Agriculture of the Polytechnic Institute of Bragança. *P. tinctorius* was obtained from the University of Tübingen. *P. cambivora* and *P. cinnamomi* are both locally isolated strains and were obtained from the laboratory of phytopathology (School of Agriculture, Polytechnic Institute of Bragança, Portugal). All isolates were maintained by subculturing on MMN medium (pH 6.6) at 25 ± 1ºC in the dark, for routine production of inoculum.

Table 1 - Organisms used in the study.

Organisms	Strain[1]	Origin
Ectomycorrhizal fungi		
Amanita gemmata (Fr.) Bertill.	Ag	Isolated from sporocarps under *Castanea sativa* in northeast of Portugal
Pisolithus tinctorius (Pers.) Coker & Couch	289/Marx	University of Tübingen
Suillus bovinus (Pers.) Roussel	Sb	Isolated from sporocarps under *Pinus pinaster* in northeast of Portugal
Suillus luteus (L.) Roussel	Sl	Isolated from sporocarps under *Pinus pinaster* in northeast of Portugal
Saprotrophic fungi		
*Hypholoma fascicul*are (Huds.) P. Kumm.	Hf	Isolated from sporocarps under *Castanea sativa* in northeast of Portugal
Plant pathogen		
Parasitic fungi		
Armillaria mellea (Vahl) P. Kumm.	Am	Isolated from sporocarps under *Castanea sativa* in northeast of Portugal
Phytopathogenic fungi		
Colletotrichum acutatum J.H. Simmonds	Ca	Isolated from *Olea europaea* fruits collected in northeast of Portugal
Oomycetes		
Phytophthora cambivora (Petri) Buisman	Pcam	Collection of the laboratory of phytopathology (School of Agriculture, Polytechnic Institute of Bragança, Portugal)
Phytophthora cinnamomi Rands	Pc	Collection of the laboratory of phytopathology (School of Agriculture, Polytechnic Institute of Bragança, Portugal)

[1] Isolates obtained were deposited in the culture collection of School of Agriculture, Polytechnic Institute of Bragança (Portugal).

2.2.2. Establishment of dual culture on agar medium

H. fasciculare was challenged by ectomycorrhizal fungal species (*A. gemmata*, *P. tinctorius*, *S. bovinus*, *S. luteus*), parasitic fungus (*A. mellea*), phytopathogenic fungus (*C. acutatum*) and plant pathogen oomycetes (*P. cambivora* and *P. cinnamomi*). All these microorganisms were produced in 9 cm Petri dishes containing MMN medium at pH 6.6, for 15 days at 25 ± 1ºC in the dark, in order to provide mycelium for the establishment of dual cultures. After that, mycelial discs (5 mm diameter) were removed aseptically from the colony margins and inoculated 4 cm apart on the surface of Petri dishes (9 cm diameter) containing 10 ml of MMN agar medium at pH 6.6. Controls consisted of agar plates containing two inocula of the same taxa. The plates were sealed with parafilm and incubated at 25 ± 1ºC in the dark. Five replicate of each combination were performed and the experiment was repeated twice. During interaction, the radial growth towards (internal radius) the interacting fungus was measure and the outcome of interactions was assessed. The interactions was described according to the following categories: (1) contact inhibition, when growth of both species stops at the line of contact (no clear zone is formed); (2) inhibition at distance, when neither species can enter the area inhabited by the other (a clear zone is formed); (3) overgrowth of a mycelium over the other; (4) intermingling of both mycelia without any damage. Macroscopic characteristics of the colonies were also registered and include mycelium texture, colour and border appearance of the colony, aerial growth, medium coloration and exudates production.

2.2.3. Screening for extracellular hydrolytic enzyme activities

Production of extracellular hydrolytic enzymes by the microorganisms during hyphal interaction was assessed only for dual cultures established between *H. fasciculare* and *P. cinnamomi*, *P. cambivora*, *P. tinctorius* or *S. bovinus*. The enzymes analysed were amylase, cellulose, laccase, lipase, protease and tyrosinase. These were detected on MMN agar media, containing the respective enzyme substrate, by placing mycelial discs (5 mm diameter) of 2-week-old mycelia, 4 cm apart. Microorganisms pairings of the same isolate were used as controls. After an incubation period, at 25 ± 1ºC in the dark, the zone of degraded substrate formed around the colony was registered. Five replicate of each combination were performed. The procedure utilized

to assess the production of extracellular enzymes was based on Maria *et al*. (2005). Briefly, amylase activity was assessed on medium with 2% (w/v) soluble starch (Riedel-de-Haën). After incubation the plates were flooded with a solution of 1% (w/v) iodine (Analar) in 2% (w/v) potassium iodine (Prolabo). The formed clear zone surrounding the colony indicated amylase activity. For cellulase activity dual cultures were established on medium supplemented with 0.5% (w/v) Na-carboxymethyl cellulose (CMC) (Alta Aesar). After incubation the plates were flooded with 0.2% (w/v) aqueous Congo Red (Merck) and distained with 1M NaCl (Merck) for 15 minutes. The clear zone surrounding the colony indicated cellulase activity. Laccase activity was assessed on medium amended with 0.005% (w/v) 1-Naphthol (Merck) at pH 6.0. The oxidation of 1-Naphthol by laccase was visible with changing color of the medium, from clear to blue. For lipase activity it was used medium supplemented with 1% (v/v) Tween 20 (Aldrich). A clear zone around the colony indicated lipase-positive fungi. Protease activity was assessed on medium amended with 0.4% (w/v) gelatin (Prolabo) at pH 6.0. After incubation, plates were flooded with saturated aqueous ammonium sulphate (Prolabo) and the undigested gelatin precipitated with ammonium sulphate. The digested area around the colony was clear. For tyrosinase activity, dual cultures were established and after seven days of incubation a mixture of 0.11% (v/v) *p*-cresol (Merck) and 0.05% (w/v) glycine (Merck) was overlaid on the surface of colonies. The appearance of red brown color around the colonies indicates tyrosinase activity.

2.2.4. Data analysis

Data from radial growth (cm) of mycelium are presented as the mean of five independent experiments displaying the respective SE bars. Differences among means were done by analysis of variance (ANOVA), using SPSS v.17 software and averages were compared using Tukey test ($p < 0.05$)

2.3. RESULTS

2.3.1. Effects of hyphal interaction on microorganism growth

Dual cultures between *H. fasciculare* (Hf) and eight microorganisms, *Suillus luteus, Suillus bovinus, Phytophtora cinnamomi, Phytophtora cambivora, Armillaria mellea, Amanita gemmata, Colletotrichum acutatum* and *Pisolithus tinctorius,* were established in solid MMN medium at pH 6.6. Colonies radius towards (internal radius) the interacting fungi were measured along time. The results show that *H. fasciculare* had a marked inhibitory effect on the growth of *A. mellea*, *P. cambivora*, *S. luteus* and *P. tinctorius* long before the contact between hyphae of their colonies had occurred (Figure 1). The internal radius growth of *A. mellea* was significantly inhibited ($p<0.05$), in more than 24% when compared to control (*A. mellea* cultured with *A. mellea*), after 13 days of co-culture. The greatest reduction on internal radius growth of this fungus was reached after 26 days of dual-culture, exceeding 49% ($p<0.001$) from control. After 6-7 days of co-culture, the internal radius growth of *S. luteus, P. cambivora* and *P. tinctorius* was significantly inhibited ($p<0.01$), respectively in 6%, 17% and 26% when compared to respective control. The highest inhibition was reached after 7 days (17%), 12 days (49%) and 15 days (43%) of dual-culture respectively for *P. cambivora, P. tinctorius* and *S. luteus*.

This pattern was not observed in dual cultures established with *A. gemmata, C. acutatum, P. cinnamomi* and *S. bovinus* (Figure 1). In dual culture with *A. gemmata* and *C. acutatum* the internal radius growth of *H. fasciculare* was significantly reduced ($p<0.05$) respectively in 35% after 12 days and in 12% after 9 days when compared to control (*H. fasciculare* - *H. fasciculare*). However, only a significant increased ($p<0.001$) in internal radius growth was observed for the specie *A. acutatum* (79%, after 5 days) when compared to control *A. acutatum* - *A. acutatum*. Although a significantly increase ($p<0.01$) in the internal radius growth of *H. fasciculare* was observed (23%, after 13 days) when challenged with *S. bovinus*, no significant differences were noted on the growth of the last fungal specie (Figure 1). In the co-culture *P. cinnamomi – H. fasciculare* no significant differences were found on internal radius growth of both microorganism when compared to respective controls.

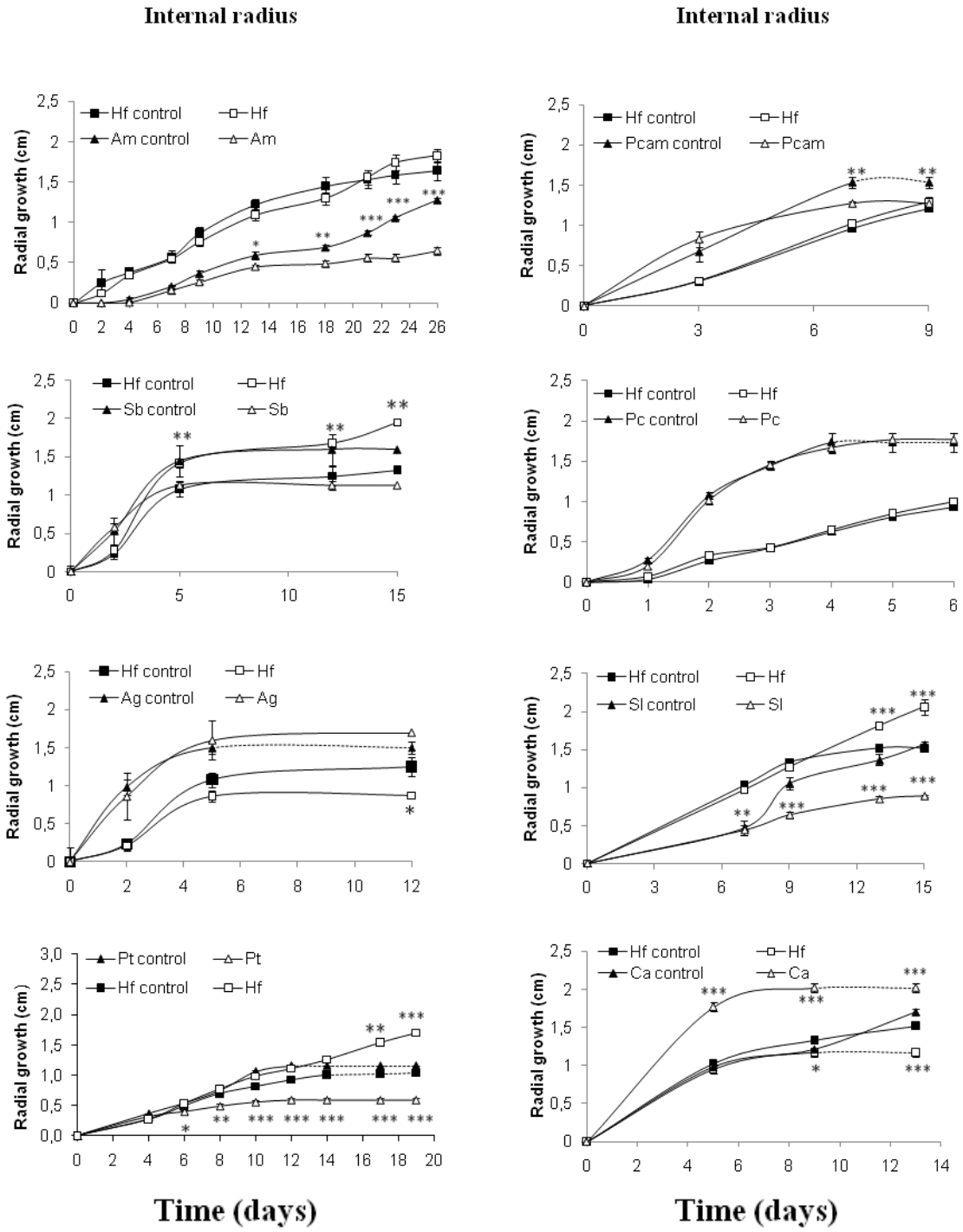


Figure 1 – Internal radial growth (mean ± SE, n=5) of microorganisms in co-culture. *H. fasciculare* (Hf) in co-culture with *A. mellea* (Am), *P. cambivora* (Pcam), *P. cinnamomi* (Pc), *S. bovinus* (Sb), *A. gemmata* (Ag), *S. luteus* (Sl), *P. tinctorius* (Pt) and *C. acutatum* (Ca); and controls. Dashed lines indicate when radius of colonies touches. Asterisk represents statistically different values from control at * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

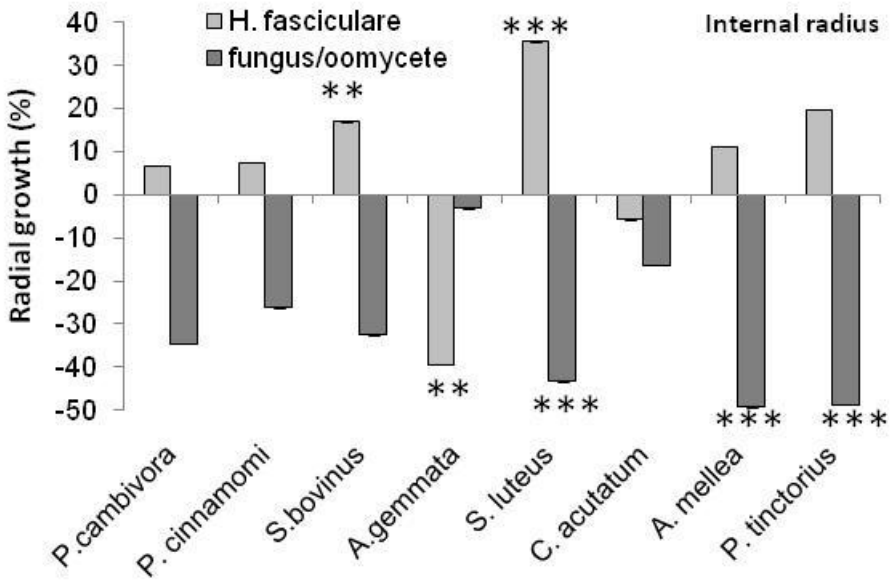


Figure 2 - Differences in the internal radius growth (mean ± SE, n=5) of microorganisms in co-culture in relation to respective controls**.** Asterisk represents statistically different values from control at ** $p<0.01$; *** $p<0.001$.

In general, the fungus *H. fasciculare* inhibited the internal radius growth of all eight isolates (Figure 2). The highest inhibition observed for the species studied were: *A. mellea* (49%), *P. tinctorius* (48%), *S. luteus* (43%), *P. cambivora* (35%), *S. bovinus* (32%), *P. cinnamomi* (26%), *C. acutatum* (16%) and *A. gemmata* (3%). However this inhibition was only statistically significant for the first four species. By contrast, an inhibition of *H. fasciculare* was observed when challenged by *A. gemmata* and *C. acutatum*, which was found only statistically significant for the first species.

2.3.2. Interspecific interactions

Different hyphal interactions were observed depending on the species paired with *H. fasciculare* (Table 2). The most common interaction types were antagonism and agonism, followed by commensalism and co-habitation. The antagonisms was observed in co-cultures established between *H. fasciculare* and *A. gemmata*, *A. mellea* or *C. cambivora* whereas agonism was verified between *H. fasciculare* and *P. tinctorius*, *S. luteus* or *C. acutatum*.

Table 2 - Outcomes of interspecific interactions.

Dual culture between *H. fasciculare* (Hf) and	Type of interaction[1]	Outcomes[2]
A. gemmata (Ag)	Antagonism	0 (Ag) / - (Hf)
P. tinctorius (Pt)	Agonism	- (Pt) / + (Hf)
S. bovinus (Sb)	Commensalism	0 (Sb) / + (Hf)
S. luteus (Sl)	Agonism	- (Sl) / + (Hf)
A. mellea (Am)	Antagonism	0 (Hf) / - (Am)
C. acutatum (Ca)	Agonism	- (Hf) / + (Ca)
P. cambivora (Pcam)	Antagonism	0 (Hf) / - (Pcam)
P. cinnamomi (Pc)	Co-habitation	0 (Hf) / 0 (Pc)

[1] Interspecific interaction terminology was based on Tuininga (2005).
[2] (0 / -) only the growth of one organisms was negatively affected; (- / +) one organism is harmed and the other benefits; (0 / +) one organism is not affected and the other benefits; (0 / 0) neither organisms involved is significantly affected positively or negatively.

2.3.3. Macroscopic characterization of colonies

Macroscopic observation of colonies during interaction was made in order to evaluate morphologic alterations (Figure 3). Different hyphal interactions were observed depending on the isolates species paired with *H. fasciculare*. The most frequent response observed was inhibition at distance, followed by contact inhibition. Inhibition at distance was observed when *H. fasciculare* interacted with *P. cambivora*, *P. cinnamomi*, *S. luteus*, *S. bovinus*, *A. mellea*, *P. tinctorius* or *A. gemmata*. Contact inhibition was observed in the dual culture *H. fasciculare* - *C. acutatum*.

In dual-culture with *P. cambivora* or *P. cinnamomi*, the colonies of *H. fasciculare* were characterized to present aerial hyphae and along the interaction the mycelia thickened especially in the interaction zone, forming a barrier (Figure 3). The mycelia system of *Phytophthora* thickened in the interaction zone. In dual cultures with *S. luteus*, *S. bovinus*, *A. mellea* and *P. tinctorius*, mycelial of *H. fasciculare* appear thickened and in the mycelial margins persistent cords were formed. *P. tinctorius* mycelium was much more dense and compact in the interacting zone with *H. fasciculare* than when grown with *P. tinctorius* (control), on which aerial hyphae predominated (data not shown). Mycelium of *S. luteus*, *S. bovinus* and *A. mellea* in co-culture with *H. fasciculare* had similar appearance to respective controls (data not

shown). The mycelium of *H. fasciculare* cultured with *A. gemmata* had extended radically until 5 days of co-culture; after that hyphae thickened in the interacting zone and produced patches along mycelial cord lengths, especially in the opposite side of the interacting zone. No differences were found on colony appearance of *A. gemmata* in co-culture with *H. fasciculare* and control. In dual cultures of *H. fasciculare* and *C. acutatum*, it was observed following colonies contact lateral *C. acutatum* hyphal grew around *H. fasciculare*, enclosing the latter after 20 days of co-culture (data not show).

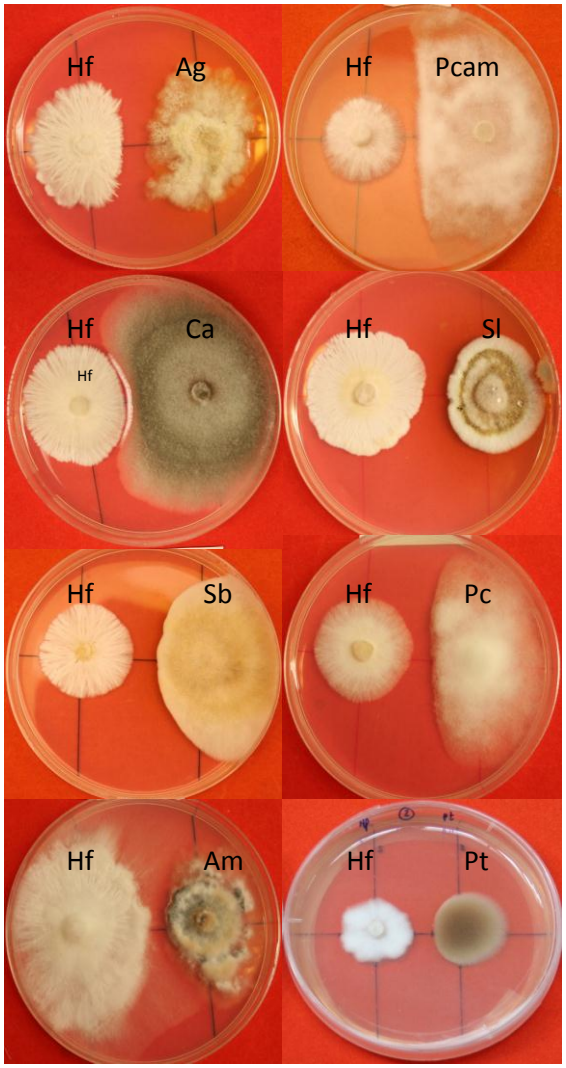


Figure 3 - Macroscopic mycelial interaction between *H. fasciculare* and other microorganisms in co-culture. *H. fasciculare* (Hf), *A. mellea* (Am), *P. cambivora* (Pcam), *P. cinnamomi* (Pc), *S. bovinus* (Sb), *A. gemmata* (Ag), *S. luteus* (Sl), *P. tinctorius* (Pt) and *C. acutatum* (Ca).

2.3.4. Extracellular enzymes

From all the enzymes analyzed only four were produced in co-culture during microorganism interaction (Figure 4, Table 3). Amylase, cellulase, laccase and lipase were produced by *H. fasciculare* when challenged by other species (*P. cambivora*, *P. cinnamomi, P. tinctorius, S. bovinus*) or by the same specie (control). Cellulase production by *P. cambivora* and *P. cinnamomi*, was also observed in co-culture with *H. fasciculare*. None of the isolates studied showed protease and tyrosinase activity.

Table 3 - Enzyme activity of microorganisms during interaction (n = 5).

	P. cambivora		*P.cinnamomi*		*P. tinctorius*		*S. bovinus*		*H. fasciculare*	
	Control	Dual-culture	Control	Dual-culture	Control	Dual-culture	Control	Dual-culture	Control	Dual-culture
Amylase	-	-	-	-	-	-	-	-	++	+
Cellulase	-	+	-	+	-	-	-	-	++	++
Laccase	0	0	0	0	0	0	0	0	++	++
Lipase	-	-	-	-	-	-	-	-	++	++
Protease	-	-	-	-	-	-	-	-	-	-
Tyrosinase	-	-	-	-	-	-	-	-	-	-

No enzyme activity (-), halo < 1 mm. Slight enzyme activity (+), halo 2-3 mm. Moderate enzyme activity (++), halo 4-5 mm.

0 – no growth.

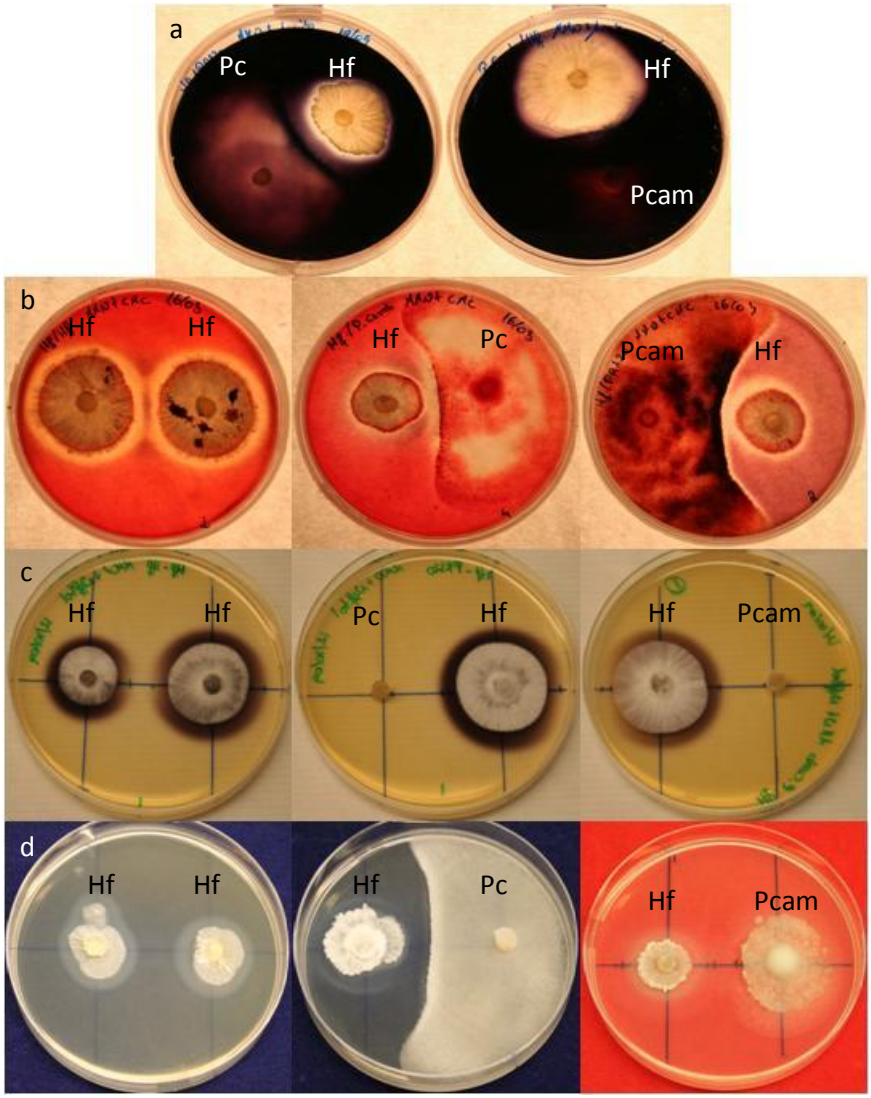


Figure 4 – Screening for extracellular hydrolytic enzyme activities during mycelial interaction between *H. fasciculare* (Hf) and other microorganisms in co-culture: *P. cambivora* (Pcam), *P. cinnamomi* (Pc), *S. bovinus* (Sb) and *P. tinctorius* (Pt). (a) Starch hydrolysis by amylase is indicated by a non-blue halo surrounding the colony; (b) Na-carboxymethyl cellulose hydrolysis by cellulase is indicated by a non-red halo surrounding the colony; (c) 1-naphthol hydrolysis by laccase is indicated by a blue halo surrounding the colony; (d) Tween 20 hydrolysis by lipase is indicated by a white halo surrounding the colony.

2.4. DISCUSSION

Hypholoma fasciculare is a common and widespread cord-forming basidiomycete specie on chestnut orchards as well as on other agro-forestry ecosystem, in Northeast of Portugal (Branco, 2003; Baptista *et al*., 2005). Members of the fungal genus *Hypholoma* have been studied, particularly on their ability to foraging resources (Donnelly & Boddy, 2001; Wells & Boddy, 2002; Heilmann-Clausen & Boddy, 2005) and to act as a biocontrol agent against *Armillaria* spp. (Chapman & Xiao, 2000; Chapman *et al*., 2004; Cox & Scherm, 2006), wood-inhabiting bacteria (Folman *et al*., 2008; Boer *et al*., 2010) and *Heterobasidion annosum* (Nicolotti & Varese, 1996; Varese *et al*., 2003). However, to our knowledge, any risk management study has been conducted yet. If a useful effect is found when *H. fasciculare* limits the growth and spreading of phytopathogenic fungi, a detrimental effect will be expected when the same fungus restricts the growth of beneficial fungi (e.g. ectomycorrhizal fungi). Therefore, to ascertain the consequences of using *H. fasciculare* we have analysed its antifungal activity spectrum against beneficial soil-borne fungi (*A. gemmata*, *P. tinctorius*, *S. bovinus* and *S. luteus*). Due to its recognized ability to act as biological control we also assessed the effect of *H. fasciculare* against several phytopathogenic microorganisms, for the first time for *Colletotrichum acutatum*, *Phytophthora cambivora* and *P. cinnamomi*; and also against *Armillaria mellea*.

2.4.1. Effect of H. fasciculare on growth of other filamentous microorganims

The fungi *H. fasciculare* have shown a marked inhibitory effect on the growth of *A. mellea*, *P. cambivora*, *S. luteus* and *P. tinctorius* long before contact between hyphal of their colonies occurred. The antagonistic activity by saprotrophic fungi against several soil and seed borne plant pathogen in dual-culture has also been reported (Nicolotti & Varese, 1996; Calistru *et al.*, 1997; Perelló *et al.*, 2003; Aggarwal *et al.*, 2004; Cox & Scherm, 2006). The antagonistic saprotrophic fungi studied included some species of the genera *Trichoderma* (Nicolotti & Varese, 1996; Calistru *et al.*, 1997; Perelló *et al.*, 2003) and the species *Chaetomium globosum* (Aggarwal *et al.*, 2004), *Ganoderma lucidum*, *Schizophyllum commune*, *Xylaria hypoxylon* (Cox & Scherm, 2006), *Hypholoma fasciculare* (Nicolotti & Varese, 1996; Cox & Scherm, 2006),

Verticillium bulbillosum, *Phanerochaete velutina*, *Mucor hiemalis* e *Phoma fimeti* (Nicolotti & Varese, 1996). By contrast, there have been a few laboratory studies of interactions between pure cultures of representatives of both ECM and saprotrophs fungi in axenic system. The few studies indicated that the inhibition of ectomycorrhizal fungi by saprotrophs was not always occurs and, usually, is dependent of the species combinations and also of the size of inoculum (Lindahl *et al*., 1999, 2001; Werner & Zadworny, 2003). For example, in dual culture between ectomycorrhizal and saprotrophic fungi have been observed an inhibition of ectomycorrhizal fungi growth (Shaw *et al*. 1995; Murphy & Mitchell, 2001; Zadworny *et al.*, 2004) or, by contrast, of saprotrophic fungi (Baar & Stanton, 2000; Werner *et al*., 2002; Werner and Zadworny, 2003; Sharma *et al*., 2010). The ectomycorrhizal fungi species most studied is *Laccaria laccata*. When grown in co-culture, *L. laccata* inhibits the growth of *Mucor hiemalis* (Werner and Zadworny, 2003), *Trichoderma* spp. (Werner *et al*., 2002; Zadworny *et al*., 2004) and *Tricholomopsis rutilans* (Murphy & Mitchell, 2001).

2.4.2. Mechanims of interaction adopted by H. fasciculare and involvement of lytic enzymes

The inhibition of the microorganisms studied in the present work by *H. fasciculare* was mostly at a distance and was of two different types, antagonism and agonism. Antagonism, is an interaction that occurs unilaterally by one fungus inhibiting the growth of the other, while continuing to growth uninhibited itself (-/0) (Tuininga, 2005). This type of interaction was verified between *H. fasciculare* and *A. mellea* or *P. cambivora*. Agonism, is an interaction in which one organism is harmed and the other benefits (-/+) (Tuininga, 2005). This type of interaction was verified between *H. fasciculare* and *S. luteus* or *P. tinctorius*. These results provide substantial evidences that the mechanism adopted by *H. fasciculare* against pathogenic and ectomycorrhizal fungi were, respectively "antagonism and agonism at a distance" (Boddy, 2000). The first type of defense mechanism has been earlier reported for *H. fasciculare* against *Heterobasidion annosum* (Nicolotti & Varese, 1996) and for other saprotrohic species (Boddy, 2000). This inhibition could be result from the liberation of volatile compounds (Inbar *et al*., 1996; Boddy, 2000; Hynes *et al*., 2007; Evans *et al*., 2008) and/or diffusible inhibitory substances such as antibiotics (Inbar *et al*., 1996; Calistru *et al*.,

1997; Boddy, 2000) or extracellular enzymes (Heilmann-Clausen & Boddy, 2005) by *H. fasciculare* fungi. Herein, the inhibition of *P. cambivora* and *P. tinctorius* might be caused by production of amylase, cellulase, laccase and lipase by *H. fasciculare*. The production of these hydrolytic enzymes was qualitatively assayed in medium containing starch, carboxymethyl cellulose, 1-naphthol and Tween 20, respectively. *H. fasciculare* produced these enzymes when challenged by other species or by the same specie (control), which suggested that this fungus have a certain level of constitutive production of these enzymes. However, it was noted various levels of enzyme production depending of the interacting specie. This aspect was particularly evident for amylase. An induction of its production was observed in *H. fasciculare* when challenged by a different specie, compared to control *H. fasciculare-H. fasciculare*. These observation, together with the fact that chitin β-1,3-glucan and protein are the main structural components of most fungal cell walls (Peberdy, 1990) suggesting that lytic enzymes produced by *H. fasciculare* may play a role in antagonism of *Phytophthora spp.* and *P. tinctorius* probably by degrading hyphal cell walls. In fact, the complex group of extracellular enzymes have been reported to be a key factor in pathogen cell wall lysis during mycoparasitism (Verma *et al*., 2007). The role of these enzymes has been well documented by several researchers, especially for species of *Trichoderma* parasitizing on other fungal structures (Vazquez-Garciduenas *et al*., 1998; de la Cruz & Llobell, 1999; Zeilinger *et al*., 1999; Pozo *et al*., 2004; Kredics *et al*., 2005). The amylase (Vallier *et al*., 1977), cellulase (Srinon *et al*., 2006), laccase (Velazquez-Cedeno *et al*, 2004; Gregorio *et al*., 2006) and lipase (Diby *et al*., 2005) enzymes have been also described to accumulate in interaction zones where saprotrophic fungi are confronted with other microrganism and/or to have potential to degraded cell wall. Laccase was also described to be a stress responsive enzyme (Gregorio *et al*., 2006). This enzyme is probably involved in passive defence by the formation of melanins or similar compounds, or in the detoxification of xenobiotics (Baldrian, 2008).

2.4.3. Changes on morphology of H. fasciculare mycelium

In our work, using paired cultures of *H. fasciculare* with other microorganisms in MMN medium, we have verified changes on morphology of *H. fasciculare* colonies.

During the interaction with *P. cambivora* or *P. cinnamomi,* and before colonies contact, *H. fasciculare* mycelial become much more dense and compact, especially in the interacting zone, forming a barrier. When in co-culture with *S. luteus, S. bovinus, A. mellea* and *P. tinctorius*, mycelial of *H. fasciculare* appears thickened and formed invasive cords. These mycelial morphology changes during interactions are commonly (Boddy, 2000; Donnelly & Boddy, 2001) and probably could render the *H. fasciculare* colony more resistant to invasion of interacting species.

In conclusion, in dual-culture the fungus *H. fasciculare* triggers antagonistic responses against *A. mellea* and *P. cambivora,* and agonism response against *S. luteus* and *P. tinctorius,* including changes in colony morphology at the interaction zone, growth rate and production of extracellular enzymes. These effects occur prior to colonies contact, suggesting antagonism and agonism at distance. The results obtained gave evidences that *H. fasciculare* could be a useful biocontrol strategy for restricting severe pathogens like *A. mellea* and *P. cambivora*. However, *H. fasciculare* also displays antagonism towards beneficial fungi, like *S. luteus* and *P. tinctorius*, which may cause additional losses of incomes from symbiosis (plant fitness and health). Thus, one strategy that could be explorer is the use of purified antagonist substances or enriched extracts for locally reducing pathogens without the danger of disseminating *H. fasciculare* that has an aggressive behaviour towards some beneficial fungi. However, since our experiments were performed under sterile conditions, interpretation of outcomes and extrapolation to the field must be made cautiously as these approaches do not reflect the natural conditions where individual microoganisms compete and interact with one another. Despite their limited antagonistic activity *in vitro*, therefore, their *in vivo* role warrants further investigation.

2.5. REFERENCES

Aggarwal, R., Tewari, A.K., Srivastava, K.D. & Singh, D.V. (2004). Role of antibiosis in the biological control of spot blotch (Cochliobolus sativus) of wheat by Chaetomium globosum. *Mycopathologia*, **157**, 369-377.

Ali, S., Huang, Z. & Ren, S.X., 2009. Production and extraction of extracellular lipase from the entomopathogenic fungus Isaria fumosoroseus (Cordycipitaceae; Hypocreales). *Biocontrol Science and Technology*, **19**, 81–89.

Annis, S.L. & Goodwin, P.H. (1997). Inhibition of Polygalacturonase Activity Produced by *Leptosphaeria maculans* with Stem Extracts of Canola, and the Relationship of Inhibition to Resistance. *Journal of Phytopathology*, **145**, 217-223.

Baar, J. & Stanton, N.L. (2000). Ectomycorrhizal fungi challenged by saprotrophic basidiomycetes and soil micro fungi under different ammonium regimes *in vitro*. *Mycological Research*, **104**, 691-697.

Baptista, P., Martins, A., Lino-Neto, T. & Tavares R.M. (2005). Effect of soil tillage on diversity and abundance of macrofungi associated with chestnut tree in the Northeast of Portugal. *Acta Horticulturae*, **693**, 685-690.

Boddy, L. (2000). Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiology Ecology*, **31**, 185-194.

Boer, W., Folman, L.B., Gunnewiek, P.J.A.K., Svensson, T., Bastviken, D., Öberg, G., Rio, J.C. & Boddy, L. (2010). Mechanism of antibacterial activity of the white-rot fungus *Hypholoma fasciculare* colonizing wood.

Branco, S. (2003). Macrofungos no Parque natural de Montesinho. Estudo preliminar de inventariação e caracterização. Instituto da Conservação da Natureza.

Brimner, T.A. & Boland, G.J. (2003). A review of the non-target effects of fungi used to biologically control plant diseases. *Agriculture, Ecosystems & Environment*, **100**, 3-16.

Chapman, B. & Xiao, G. (2000). Inoculation of stumps with Hypholoma fasciculare as a possible means to control armillaria root disease. *Canadian Journal of Botany*, **78**, 129-134.

Chapman, B., Xiao, G. & Myers, S. (2004). Early results from field trials using *Hypholoma fasciculare* to reduce *Armillaria ostoyae* root disease. *Canadian Journal of Botany*, **82**. 962-969.

Donnelly, D.P. & Boddy, L. (2001). Mycelial dynamics during interactions between *Stropharia caerulea* and other cord-forming, saprotrophic basidiomycetes. *New Phytologist*, **151**, 691-704.

Calistru, C., McLean, M. & Berjak, P. (1997). In vitro studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. *Mycopathologia*, **139**, 115-121.

Cook, R.J., Bruckart, W.L., Coulson, J.R., Goettel, M.S. Humber, R.A., Lumsden, R.A., Maddox, R.D., McManus, J.V., Moose, M.L., Meyer, L., Quimby, P.C., Stack, J.P. & Vaughn, J.L. (1996). Safety of microorganisms intended for pests and plant disease control: a frame work for scientific evaluation. *BioControl*, **7**, 333-351.

Cox, K.D. & Scherm H. (2006). Interaction dynamics between saprobic lignicolous fungi and *Armillaria* in controlled environments: Exploring the potential for competitive exclusion of *Armillaria* on peach. *Biological Control*, **37**, 291-300.

de la Cruz, J. & Llobell, A. (1999). Purification and properties of a basic endo- 1,6-glucanase (BGN16. 1) from the antagonistic fungus *Trichoderma harzianum*. *European Journal of Biochemistry*. **265**, 145–151.

Diby, P., Saju, K.A., Jisha, P.J., Sarma, Y.R., Kumar, A. & Anandaraj, M. (2005). Annals of microbiology, **55(2)**, 129-133

Evans, J.A., Eyre, C.A., Rogers, H.J., Boddy, L. & Müller, C.T. (2008). Changes in volatile production during interspecific interactions between four wood rotting fungi growing in artificial media. Fungal Ecology, **1(2-3)**, 57-68.

Ferraris, L., Cardinale, F., Valentino, D., Roggero, P. & Tamietti, G. (2004). Immunological discrimination of *Phytophthora cinnamomi* from other *Phytophthorae* Pathogenic on Chestnut. *Journal of Phytopathology*, **152(4)**, 193-199.

Folman, L.B., Klein Gunnewiek, P.J.A., Boddy, L. & de Boer W. (2008). Impact of white-rot fungi on numbers and community composition of bacteria colonizing beechwood from forest soil. *FEMS Microbiology Ecology,* **63**,181–191.

Gloer, J.B. (1995). The chemistry of fungal antagonism and defense. *Canadian Journal of Botany*, **73(1)**, 1265-1274.

Gregorio A.P.F., Da Silva, I.R., Sedarati, M.R. & Hedger, J.N. (2006). Changes in production of lignin degrading enzymes during interactions between mycelia of the tropical decomposer basidiomycetes *Marasmiellus troyanus* and *Marasmius pallescens*. *Mycological Research*, **110**, 161–168.

Heilmann-Clausen J. & Boddy, L. (2005). Inhibition and stimulation effects in communities of wood decay fungi: exudates from colonized wood influence growth by other species. *Microbial Ecology*, **49**, 399-406.

Hynes, J., Müller, C.T., Jones, H.T. & Boddy, L. (2007). Changes in volatile production during the course of fungal mycelia interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. *Journal of Chemical Ecology,* **33**, 43-57.

Inbar, J., Menendez, A. & Chet, I. (1996). Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. *Soil Biology and Biochemistry*, **28**, 757-763.

Kredics, L., Zsuzsanna, A., Szekeres, A., Hatvani, L., Manczinger, L., Vágvölgyl, C. & Erzsébet, N. Extracellular proteases of *Trichoderma* species - a review, *Acta Microbiologica et Immunologica Hungarica*, **52**, 169–184.

Lindahl, B., Stenlid, J. & Finlay, R. (2001). Effects of resource availability on mycelial interactions and 32P transfer between a saprotrophic and an ectomycorrhizal fungus in soil microcosms. *FEMS Microbiology Ecology*, **38**, 43-52.

Lindahl, B., Stenlid, J., Olsson, S. & Finlay, R. (1999). Translocation of 32P between interacting mycelia of a wood-decomposing fungus and ectomycorrhizal fungi in microcosm systems. *New Phytologist*, **144**, 183-193.

Maria, G.L., Sridhar K.R. & Raviraja, N.S. (2005). Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. *Journal of Agricultural Technology*, **1**, 67-80.

Murphy, E.A. & Mitchell, D.T. (2001). Interactions between *Tricholomopsis rutilans* and ectomycorrhizal fungi in paired culture and in association with seedlings of lodgepole pine and Sitka-spruce. *Forest Pathology*, **31(6)**, 331–344.

Nicolotti, G. & Varese G.C. (1996). Screening of antagonistic fungi against air-borne infection by *Heterobasidion annosum* on Norway spruce. *Forest Ecology and Management*, **88**, 249-257.

Peberdy, J. (1990). *Fungal cell wall-a review*, 5- 24. In: Kuhn, P.J., Trinici, A.P.J. Juung, M.J. Goosey, M.M. & Cooping I.G. (Eds.), *Biochemistry of cell walls and membranes in fungi*. Springer-Verlag, Heidelberg, Germany.

Perelló, A., Mónaco, C., Simón, M.R., Sisterna, M. & Dal Bello, G. (2003). Biocontrol efficacy of *Trichoderma* isolates for tan spot of wheat in Argentina. Crop Protection, **22**, 1099-1106.

Pozo, M.J., Baek, J.M., Garcia, J.M. & Kenerley, C.M. (2004). Functional analysis of tvsp1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*, Fungal Genetics and Biology, **41**, 336– 348.

Rodgers, C.J., Blanford, C.F., Giddens, S.R., Skamnioti, P., Armstrong, F.A & Gurr, S.J. (2009). Designer laccases: a vogue for high-potential fungal enzymes?. *Cell Press*, **28(2)**, 63-72.

Sharma, R., Rajak, R.C. & Pandey, A.K. (2010). Evidence of antagonistic interactions between rhizosphere and mycorrhizal fungi associated with *Dendrocalamus strictus* (Bamboo). *Journal of Yeast and Fungal Research*, **1(7)**, 112-117.

Shaw, T.M., Dighton J, Sanders FET (1995) Interactions between ectomycorrhizal and saprotrophic fungi on agar and in association with seedlings of lodgepole pine (*Pinus contorta*). *Mycological Research*, **99**, 159-165.

Srinon, W., Chuncheen, K., Jirattiwarutkul, K., Soytong, K. & Kanokmedhakul, S. (2006). Efficacies of antagonistic fungi against Fusarium wilt disease of

cucumber and tomato and the assay of its enzyme activity. *Journal of Agricultural Technology*, **2(2),** 191-201.

Tuininga, A.R. (2005). Interspecific interaction terminology: from mycology to general ecology. *In The fungal community its organization and role in the ecosystem*. Dighton J.; White J. F.; Oudeman P. edit. Taylor & Francis, London.

Vallier, P., Bata, J. & Colobert, L. (1977). Optimal conditions of alpha-amylase production by *Aspergillus oryzae* in liquid media. *Annals of Microbiology*, **128(3)**, 359-71.

Varese, G.C., Gonthier, P. & Nicolotti, G. (2003). Long-term effects on other fungi are studied in biological and chemical stump treatments in the fight against *Heterobasidion annosum* coll. *Mycologia*, 95, 379-387.

Vazquez-Garciduenas, S., Leal-Morales, C.A. & Herrera-Estrella, A. (1998). Analysis of the Beta-1,3-Glucanolytic system of the biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology, **64(4)**, 1442–1446.

Velazquez-Cedeno, M.A., Farnet, A.M., Ferre, E. & Savoie, J.M. (2004). Variations of lignocellulosic activities in dual cultures of *Pleurotus ostreatus* and *Trichoderma longibrachiatum* on unsterilized wheat straw. *Mycologia* **96**, 712–719.

Verma, M., Brar, K.S., Tyagi, R.D., Surampalli, R.Y. & Valeró. (2007). Antagonistic fungi, Trichoderma spp.: Panoply of biological control. *Biochemical Engineering Journal*, **37**, 1-20.

Wells, J.M. & Boddy, L. (2002). Interspecific carbon exchange and cost of interactions between basidiomycete mycelia in soil and wood. *Functional Ecology*, **16**, 153-161.

Werner, A. & Zadworny, M. (2003). In vitro evidence of mycoparasitism of the ectomycorrhizal fungus *Laccaria laccata* against *Mucor hiemalis* in the rhizosphere of *Pinus sylvestris*. *Mycorrhiza*, **13**, 41-47.

Werner, A., Zadworny, M. & Idzikowska, K. (2002). Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture and in the rhizosphere of *Pinus sylvestris* grown in vitro. *Mycorrhiza*, **12**, 139-145.

Wharton, P.S. & Diéguez-Uribeondo, J. (2004). The biology of *Colletotrichum acutatum*. *Anales del Jardín Botánico de Madrid*, 61**(1)**, 3-22.

Zadworny, M., Werner, A. & Idzikowska, K. (2004). Behaviour of the hyphae of *Laccaria laccata* in the presence of *Trichoderma harzianum* in vitro. *Mycorrhiza*, **14**, 401-405.

Zeilinger, S., Galhaup, C., Payer, K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M. & Kubicek, C.P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host, Fungal Genetics Biology, **26**, 131–140.

Capítulo 3

Effect of the interaction between *Pisolithus tinctorius* and *Hypholoma fasciculare* on *Castanea sativa* Mill. Grown

EFFECT OF THE INTERACTION ECTOMYCORRHIZAL AND SAPROTROPHIC FUNGI ON *CASTANEA SATIVA* PERFORMANCE

Pereira, E. *et al*. (2011) Mycorrhiza (DOI: 10.1007/s00572-011-0379-x)

ABSTRACT

In Northeast of Portugal the macrofungal community associated to chestnut tree (*Castanea sativa* Mill.) is rich and diversified. Among fungal species, the ectomycorrhizal *Pisolithus tinctorius* and the saprotroph *Hypholoma fasciculare* are common in this habitat. The aim of the present work was to assess the effect of the interaction between both fungi on growth, nutritional status and physiology of *C. sativa* seedlings. In pot experiments, *C. sativa* seedlings were inoculated with *P. tinctorius* and *H. fasciculare* individually or in combination. Inoculation with *P. tinctorius* stimulated the plant growth and resulted in increased foliar-N, -P, and photosynthetic pigment contents. These effects were suppressed when *H. fasciculare* was simultaneously applied with *P. tinctorius*. This result could be related to the inhibition of ectomycorrhizal fungus root colonization as a result of antagonism or to the competition for nutrient sources. If chestnut seedlings have been previously inoculated with *P. tinctorius*, the subsequent inoculation of *H. fasciculare* 30 days later did not affect root colonization and mycorrhization benefits were observed. This work confirms an antagonistic interaction between ectomycorrhizal and saprotrophic fungi with consequences on the ectomycorrhizal host physiology. Although *P. tinctorius* is effective in promoting growth of host trees by establishing mycorrhizae, in the presence of other fungi it may not always be able to interact with host roots due to an inability to compete with certain fungi.

Keywords: *Pisolithus tinctorius*; *Hypholoma fasciculare*; mycelial interaction, *Castanea sativa*, biomass production

3.1. INTRODUCTION

The chestnut (*Castanea sativa* Mill.) agro-ecosystem has been of great social, economic and landscape importance in Northeast of Portugal. There are multiple resources associated with this crop, among them fruit and wood production and more recently mushroom harvesting. Two main ecological groups of fungi dominate these habitats, the saprotrophic and ectomycorrhizal (Baptista *et al*., 2010), and both are capable of independently influencing plant nutrient acquisition (Koide and Kabir, 2000). Saprotrophic fungi play an important role in the soil ecosystem as major decomposers of plant residues, releasing nutrients that sustain and stimulate plant growth (Dighton, 2007). Ectomycorrhizal fungi increase plant growth by enhancing the absorption of mineral nutrients and water (Calvaruso *et al.*, 2010), increase plant resistance to environmental stress (Shi *et al*., 2002; Jourand *et al.*, 2010) and pathogens (Ramachelaab and Therona, 2010) and also have a beneficial effect on biological control of larval root herbivore (Edda *et al.*, 2010).

In spite of their partial spatial separation along the vertical axis (Lindahl *et al*. 2007), interactions between ectomycorrhizal and saprotrophic fungi have been observed under axenic conditions (Shaw *et al*., 1995; Baar and Stanton, 2000; Werner *et al*., 2002; Mucha *et al*., 2006; Sharma et al., 2010), as well as in natural substrates by using microcosm systems (Lindhal *et al*., 1999, 2001; Leake *et al*., 2001). A range of responses are observed depending on individual species and species combinations, on nutrient availability and the size and quality of the carbon substrates from which the fungi grow (Koide and Kabir, 2000; Lindahl *et al*., 1999, 2001; Werner and Zadworny, 2003). For example, in pairwise interactions between ectomycorrhizal and saprotrophic fungi have been observed suppression of ectomycorrhizal fungi (Shaw *et al*. 1995; Zadworny *et al*., 2004) or, by contrast, of saprotrophic fungi (Baar and Stanton, 2000; Werner *et al*., 2002; Sharma *et al*., 2010). Contradictory responses of fungi interaction were also observed under natural substrates. Lindahl *et al*. (1999, 2001) observed in a soil microcosm a clear antagonistic response of the ectomycorrhizal fungi (S*uillus variegates* and *Paxillus involutus*) extending from pine seedling roots against the saprotroph *H. fasciculare* extending from wood blocks. By contrast, in a similar microcosm experiment Leake *et al*. (2001) found that the vigour of the ECM *Suillus*

bovinus mycelium was reduced when it encountered the saprotroph *Phanerochaete velutina*. During ectomycorrhizal and saprotroph fungi interaction, bi-directional translocation of carbon and minerals occurs within mycelia, and current evidence indicates that it occurs from sources to sinks in mycelial systems that are independent of mycelial growth (Lindahl *et al*. 1999, 2001; Leake *et al*., 2001).

All these experiments showed that saprotrophic and ectomycorrhizal fungi compete with each other for soil nutrients, as well for territory or space. These interactions may have effect changes in fungal community (by reduction in biomass of one or both of the competitors), but also in community functioning, namely in reallocation of nutrients (Boddy, 2000) with consequences for plant growth and health (reviewed by El-Shatnawi and Makhadmeh, 2001). On the other hand, the suppression of saprotrophic fungi on ectomycorrhizal formation, observed on some studies of antagonistic interactions (Shaw *et al*., 1995; Lindahl *et al.*, 2001), may cause additional losses of incomes from symbiosis (plant fitness and health). The contradictory responses observed among the several studies of interactions between these groups of organisms suggested that they are complex and difficult to study, and therefore, they are for the most part not exhaustively known.

The aim of the present work was to assess the effect of the individual and combined inoculation of selected saprotrophic and ectomycorrhizal fungi on the growth, mineral nutrition, chlorophyll and carotenoid contents, and on mycorrhizal root colonization of *Castanea sativa* seedlings, under greenhouse conditions. The fungi species chosen were *Pisolithus tinctorius* and *Hypholoma fasciculare*, as representative of ectomycorrhizal and saprotrophic basidiomycetes, respectively. Both species are commonly present in *C. sativa* orchards in the Trás-os-Montes region (Northeast of Portugal) and are found in the same soil (Baptista *et al*., 2010). This study intended to provide knowledge how co-occurring mycelia of *P. tinctorius* and *H. fasciculare* can influence each other and elucidate their influence on formation and functioning of the ECM symbiosis.

3.2. MATERIALS AND METHODS

23.2.1. Biological material

Seeds of *Castanea sativa* Mill. were harvested in Bragança region orchards. *Hypholoma fasciculare* (Huds.) P. Kumm. was isolated from *Castanea sativa* orchards at Oleiros – Bragança (Northeast Portugal). Fungal isolation was performed on Melin-Norkans (MMN) agar medium at pH 6.6 [NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casamino acids 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L], following Brundrett et al. (1996). The identity of the fungal isolate was molecularly confirmed by the amplification and sequencing of the internal transcribed spacer region (ITS), using the universal primers *ITS1* and *ITS4* (White et al. 1990). *Pisolithus tinctorius* (Pers.) Coker & Couch (isolated 289/Marx) was obtained from the University of Tübingen. This fungus has been used for mycorrhizal formation in seedlings of *C. sativa* (Martins et al. 1997; Martins 2004). Both strains were maintained in MMN agar medium at 25ºC, in the dark, being regularly sub-cultured.

3.2.2. Production of C. sativa *seedlings*

Castanea sativa seeds were surface sterilized with sodium hypochloride (5%, v/v) for 1 h, followed by washing three times with sterile distilled water. The seeds were then stratified and germinated in sterile moistened sand, at 5-10ºC, for two months. After germination, the radicle tips were removed, to promote root ramification, and seedlings were separately transferred to plastic pots (each with 300 cm^3), filled with sterile vermiculite:topsoil:sand (3:1:1, v/v/v) mixture. Seedlings were automatically sprayed during 10 seconds, every 40 minutes; and were kept under greenhouse conditions (day/night thermal regime of 23º/18º ± 2ºC, 10 h light/14 h dark photoperiod and 70 ± 10% relative humidity) for four months. Uniform plants were then selected and transplanted to plastic pots of two litres (two seedlings per pot) filled with the same growth mixture as before. During this process, seedlings were inoculated with fungi.

3.2.3. Fungal inoculation of C. sativa *seedlings*

Suspension cultures of *P. tinctorius* and *H. fasciculare* were obtained by transferring mycelium inoculum to liquid modified MMN medium [MMN medium containing half concentration of KH_2PO_4 and $(NH_4)_2HPO_4$, and no malt extract]. Two-week-old suspension cultures maintained in the dark, at 25ºC, and without agitation, were used for plant inoculations. At the time of transplanting, plants were inoculated (i) with *P. tinctorius*, (ii) with *H. fasciculare*, (iii) with *P. tinctorius* and *H. fasciculare* simultaneously (*P. tinctorius* + *H. fasciculare*), or (iv) with *P. tinctorius* and one month later inoculated with *H. fasciculare* (*P. tinctorius* 30d + *H. fasciculare*). Inoculations were carried out by transferring 100 mL of fungal suspension culture, previously homogenized by hand-shaking for 3 minutes, into the planting hole. For *H. fasciculare* inoculation, performed one month after *P. tinctorius* inoculation, the suspension culture was introduced into a hole made at the root system level. Controls were performed using 100 mL of sterile culture medium. For each treatment and for control 15 pots were prepared, comprising a total of 30 plants per treatment. To reduce the risks of cross contamination, five pots of each treatment were grouped together and kept at a distance of *c.* 60 cm from other treatments. Groups of five from all treatments and controls were arranged at random in the same above-mentioned greenhouse conditions.

3.2.4. Sampling and analysis of Castanea sativa plants

Castanea sativa plants were harvested one year after the first inoculation. Harvesting was performed without damaging the root system, which was carefully washed out of the soil. Fifteen plants per treatment were randomly selected. For each plant, root collar diameter, total shoot height and root length were measured. Increments on shoot height and root collar diameter were evaluated considering the period from inoculation to harvest. During this period, the average growth rate (mm/day) was also determined. The ratio of shoot and root length was calculated at harvesting time.

Leaves, stems and roots from the previous 15 plants were separately used to determine fresh weight (fw), oven-dried at 60ºC for four days, and then weighed again to determine dry weight (dw). The ratio of shoot and root dry weight was calculated, as well as the specific root length (cm/g dw), evaluated as the total root length divided by

root dw. The effect of fungal inoculation on the leaf water content (LWC) was determined as follows: LWC = [(leaf fw-leaf dw) / leaf dw] x 100 (Wang et al. 2011).

The remaining 15 plants were used to determine N, P and K contents. Leaves from five plants were grouped and minced to a fine powder (1 mm mesh size), originating a total of three replicates from each treatment and control. N content determination was carried out by micro-Kjeldahl method using a Kjeltec 1030 distilling unit (AOAC 1990). For the determination of P and K contents, samples were digested using nitric acid and hydrogen peroxide moisture at 200ºC for 20 min in a microwave (Marspress CEMM). The filtered solution was used for measuring the concentrations of K by atomic-absorption spectrometry (Pye Unicam) and P by spectrophotometry (Genesys 10-UV) following the vanado-molybdate yellow colorimetric method (Jackson 1973).

Chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoids (car) contents were determined after methanolic extraction of fresh leaves, following the method of Ozerol and Titus (1965). Results were expressed in mg/g fw.

3.2.5. Assessing of **P. tinctorius** *colonization*

Mycorrhizal colonization was evaluated in fifteen root samples selected randomly from plants inoculated with *P. tinctorius* alone and combined with *H. fasciculare*. Since it was not feasible to microscopically verify the presence of the Hartig net in each sample, the presence of ECM roots was based on visual assessment and the percentage of mycorrhized seedlings was determined. We considered mycorrhizal roots when they didn't have root hairs, root tips were swollen and fungal hyphae or mantle is visible that in the case of *P. tinctorius* is characterized to present a yellow gold color. The percentage of roots colonized was visually determined by estimated the number of colonized roots in total root system (number of ectomycorrhizal short roots/total number of short roots × 100). The percentage of roots colonized was converted into abundance classes as follows: 1 = 1–25% colonization; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%.

3.2.6. Data analysis

Data from plant analysis (growth parameters, water and photosynthetic pigment contents and nutritional status) are presented as the mean of three to fifteen independent experiments. The corresponding standard deviations (SD) values are displayed. The significance of differences among means was tested by analysis of variance (ANOVA), using SPSS v.17 software, in which the averages were compared using Tukey test ($p \leq 0.05$).

3.3. RESULTS

3.3.1. Influence of H. fasciculare *on* P. tinctorius *colonization*

To determine the influence of *H. fasciculare* on the colonization of *C. sativa* roots by the ECM *P. tinctorius*, the number of lateral roots displaying mycorrhizae was determined one year after the *P. tinctorius* or *H. fasciculare* inoculation, and *P. tinctorius* + *H. fasciculare* or *P. tinctorius* 30d + *H. fasciculare* inoculation (Fig. 1). As expected, the formation of mycorrhizae was not detected in plants that have been inoculated only with *H. fasciculare*. Also, the presence of mycorrhizae was not detected in plants simultaneously inoculated with *P. tinctorius* and *H. fasciculare*. However, when plants were first inoculated with *P. tinctorius* and after 30 days inoculated with *H. fasciculare*, chestnut roots displayed a similar level of mycorrhization as plants inoculated only with *P. tinctorius*. In both treatments, root colonization levels never achieved more than 75% of the total number of lateral roots.

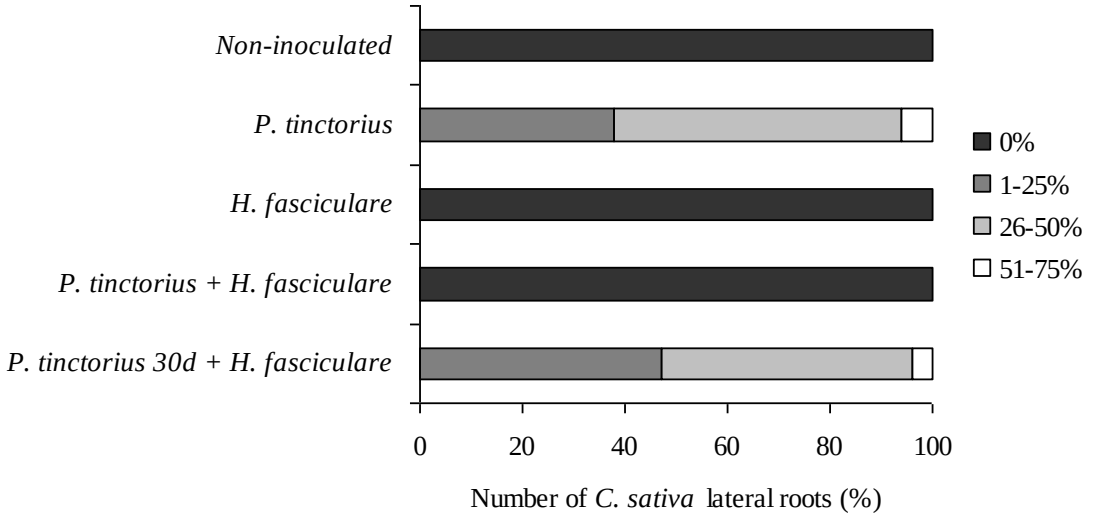


Figure 1 - Effect of the ECM *P. tinctorius* and the saprotrophic *H. fasciculare* on *C. sativa* root mycorrhization. The percentage of *C. sativa* lateral roots displaying *P. tinctorius* mycorrhizae were determined, one year after seedlings had been inoculated with *P. tinctorius*, *H. fasciculare*, simultaneously with *P. tinctorius* and *H. fasciculare* (*P. tinctorius* + *H. fasciculare*), or with *P. tinctorius* followed by *H. fasciculare*, one month later (*P. tinctorius* 30d + *H. fasciculare*). Four abundance classes of root colonization are considered: 0%; 1-25%; 26-50% and 51-75%.

3.3.2. Effect of fungal inoculation on C. sativa growth

The influence of ECM and saprotrophic fungi on *C. sativa* growth was evaluated by the determination of several plant growth parameters one year after the first inoculations (Table 1). Plants that were only inoculated with *P. tinctorius* displayed the highest increment in shoot height (*c.* 3-fold higher) and the lowest root length (0.84-fold lower) when compared to non-inoculated plants. Similar results were observed in plants first inoculated with *P. tinctorius* and after 30 days inoculated with *H. fasciculare* (*c.* 2-fold higher and 0.90-fold lower than non-inoculated plants, respectively). Accordingly, *P. tinctorius* inoculated plants displayed the highest shoot/root length ratio, and plants inoculated with *P. tinctorius* and 30 days later with *H. fasciculare* the second highest. When *H. fasciculare* was inoculated alone or simultaneously with *P. tinctorius*, plants displayed a non-significant variation in both shoot height and root length compared to non-inoculated plants.

Seedlings inoculated with *P. tinctorius* and inoculated with *P. tinctorius* and 30 days later with *H. fasciculare* also displayed the highest shoot/root dw ratios, compared to control plants that presented the lowest value from all fungal treatments. When considering the specific root length, determined as the relation of root length and root dry weigh, significant differences were only detected between plants inoculated with *P. tinctorius* alone and non-inoculated control. Although plants from all treatments exhibited lower specific root lengths when compared to control plants, *P. tinctorius* inoculated plants presented the lowest value (0.48-fold).

In plants only inoculated with *P. tinctorius* a significant increase was observed for root collar increment, when compared to non-inoculated control that exhibited the lowest increment. No significant differences were observed between the other treatments. Although all treated seedlings exhibited a higher growth rate when compared to control plants, only plants inoculated with *P. tinctorius* alone showed a significant different growth rate value from non-inoculated plants (3-fold higher). In what concerns leaf water contents no significant differences were found between treatments.

The influence of fungal inoculation on photosynthetic pigments content of *C. sativa* plants was evaluated by determining the concentrations of chlorophylls *a* and *b*,

and carotenoid content (Table 2). Plants inoculated with *P. tinctorius* alone or with *P. tinctorius* 30 days + *H. fasciculare* exhibited higher contents of all pigments when compared to non-inoculated plants. In contrast, in plants that were simultaneously inoculated with *P. tinctorius* and *H. fasciculare* exhibited the lowest pigments content.

Table 1 - Effect of *P. tinctorius* and *H. fasciculare* on growth parameters of *C. sativa* seedlings one year after inoculation with *P. tinctorius*, *H. fasciculare*, simultaneously with *P. tinctorius* and *H. fasciculare* (*P. tinctorius* + *H. fasciculare*), or with *P. tinctorius* followed by *H. fasciculare*, one month later (*P. tinctorius* 30d + *H. fasciculare*). Means ± SD (n = 15) are shown. In each column different letters mean significant differences ($p \leq 0.05$).

Treatments	Shoot height increment (cm)	Root length (cm)	Shoot/root length ratio	Shoot/root dw ratio	Specific root length (cm/ g dw)	Root collar diameter increment (mm)	Growth rate (mm/day)	Leaf water content (%)
Non-inoculated	8.7 ± 5.5^{b}	45.3 ± 9.2^{a}	0.52 ± 0.22^{b}	0.65 ± 0.13^{b}	8.9 ± 4.6^{a}	3.5 ± 1.6^{b}	0.24 ± 0.15^{b}	209.0 ± 78.8^{a}
P. tinctorius	26.3 ± 14.4^{a}	37.9 ± 5.2^{b}	1.03 ± 0.40^{a}	1.12 ± 0.23^{a}	4.3 ± 1.6^{b}	5.1 ± 2.0^{a}	0.72 ± 0.39^{a}	181.1 ± 44.8^{a}
H. fasciculare	16.7 ± 9.5^{b}	44.8 ± 8.6^{a}	0.75 ± 0.28^{ab}	0.94 ± 0.37^{ab}	6.0 ± 4.2^{ab}	4.4 ± 2.4^{ab}	0.45 ± 0.26^{b}	184.4 ± 54.7^{a}
P. tinctorius + *H. fasciculare*	13.4 ± 7.7^{b}	46.4 ± 9.2^{a}	0.62 ± 0.06^{b}	0.92 ± 0.20^{ab}	5.5 ± 3.9^{ab}	4.3 ± 1.9^{ab}	0.37 ± 0.21^{b}	194.7 ± 70.6^{a}
P. tinctorius 30d + *H. fasciculare*	17.5 ± 8.9^{ab}	40.8 ± 9.1^{ab}	0.89 ± 0.56^{ab}	0.98 ± 0.38^{a}	5.3 ± 2.2^{ab}	4.6 ± 1.9^{ab}	0.48 ± 0.52^{ab}	228.4 ± 96.6^{a}

Legend: *P. tinctorius*+ *H. fasciculare* – Inoculation of *C. sativa* simultaneously with *P. tinctorius* and *H. fasciculare*; *P. tinctorius* 30d + *H. fasciculare* - Inoculation of *C. sativa* with *P. tinctorius* and after 30 days with *H. fasciculare*.

Table 2 - Effect of *P. tinctorius* and *H. fasciculare* on photosynthetic pigments of *C. sativa* leaves, one year after inoculation with *P. tinctorius*, *H. fasciculare*, simultaneously with *P. tinctorius* and *H. fasciculare* (*P. tinctorius* + *H. fasciculare*), or with *P. tinctorius* followed by *H. fasciculare*, one month later (*P. tinctorius* 30d + *H. fasciculare*). Contents of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoid (car) are present as means ± SD (n = 7). In each column different letters mean significant differences ($p \leq 0.05$).

Treatments	chl *a* (mg/g)	chl *b* (mg/g)	Carotenoids (mg/g)
Non-inoculated	1.50 ± 0.66^{ab}	0.53 ± 0.31^{ab}	0.30 ± 0.10^{ab}
P. tinctorius	1.85 ± 0.80^{a}	0.73 ± 0.33^{a}	0.38 ± 0.13^{a}
H. fasciculare	1.57 ± 0.46^{ab}	0.59 ± 0.19^{ab}	0.32 ± 0.09^{ab}
P. tinctorius + *H. fasciculare*	1.20 ± 0.55^{b}	0.42 ± 0.21^{b}	0.25 ± 0.10^{b}
P. tinctorius 30d + *H. fasciculare*	1.95 ± 0.67^{a}	0.72 ± 0.28^{a}	0.36 ± 0.13^{a}

Legend: *P. tinctorius*+ *H. fasciculare* – Inoculation of *C. sativa* simultaneously with *P. tinctorius* and *H. fasciculare*; *P. tinctorius* 30d + *H. fasciculare* - Inoculation of *C. sativa* with *P. tinctorius* and after 30 days with *H. fasciculare*.

3.3.3. Effect of fungi inoculation on macronutrient contents of C. sativa leaves

No significant differences occurred in the K content of *C. sativa* leaves from all the plant treatments, in contrast to N and P content that exhibited differences between treatments (Table 3). Higher contents of N were detected in leaves of *C. sativa* seedlings inoculated with *P. tinctorius* alone and inoculated with *P. tinctorius* 30 days + *H. fasciculare* when compared to control plants. In contrast, plants inoculated with *H. fasciculare* alone or simultaneously inoculated with *P. tinctorius* exhibited the lowest N content. These results are similar for foliar P, except that no differences in relation to control plants were detected for those plants treated with both fungi.

Table 3 - Effect of *P. tinctorius* and *H. fasciculare* on N, P, K content of leaves of *C. sativa* plants, after one year of inoculation. Means ± SD (n = 3) are shown. In each column different letters mean significant differences ($p \leq 0.05$).

Treatment	N (mg/g dw)	P (mg/g dw)	K (mg/g dw)
Non-inoculated	8.7 ± 0.6^{abc}	0.60 ± 0.22^{ab}	3.3 ± 0.6^{a}
P. tinctorius	10.5 ± 0.5^{a}	0.82 ± 0.09^{a}	3.3 ± 0.4^{a}
H. fasciculare	8.4 ± 0.6^{bc}	0.51 ± 0.09^{b}	3.5 ± 0.8^{a}
P. tinctorius + *H. fasciculare*	7.4 ± 0.6^{c}	0.64 ± 0.19^{ab}	4.5 ± 0.4^{a}
P. tinctorius 30d + *H. fasciculare*	10.3 ± 0.8^{a}	0.62 ± 0.02^{ab}	4.1 ± 0.7^{a}

Legend: *P. tinctorius*+ *H. fasciculare* – Inoculation of *C. sativa* simultaneously with *P. tinctorius* and *H. fasciculare*; *P. tinctorius* 30d + *H. fasciculare* - Inoculation of *C. sativa* with *P. tinctorius* and after 30 days with *H. fasciculare*.

3.4. DISCUSSION

The natural benefits of mycorrhization to most agronomical relevant plants, including European chestnut tree, turns the understanding of interactions between mycorrhizal and saprotrophic fungi essential. In addition, the influence of saprotrophic fungi on plant physiology and growth is scarcely studied. In this work, pot experiments were conducted using four-month-old *C. sativa* seedlings inoculated with selected ECM or saprotrophic fungi, or in combination of both. The fungal species, *Pisolithus tinctorius* and *Hypholoma fasciculare*, were chosen as representatives of ECM and saprotrophic basidiomycetes, respectively.

The efficiency of root colonization by *P. tinctorius* is strongly compromised in the presence of *H. fasciculare*. However, if plants had been previously inoculated with *P. tinctorius*, the inoculation of *H. fasciculare* 30 days later did not affect root colonization. This result suggests a competitive interaction between the ECM and saprotrophic fungi, resulting in root colonization inhibition. Accordingly, a reduction in

the number of *Pinus contorta* roots colonized by the ECM *Paxillus involutus* in soils containing the saprotrophic fungus *Collybia maculate* was reported (Shaw et al. 1995). *H. fasciculare* has been also referred as a highly competitive saprotrophic fungus that could interfere with the development of new mycorrhizal *Suillus variegatus* mycelia on *Pinus sylvestris* seedlings (Lindahl et al. 2001). In addition, the suppression of ECM has been observed when they are growing in the presence of saprotrophic fungi on agar media (Shaw et al. 1995; Zadworny et al. 2004). However, ECM might occasionally outcompete saprotrophic fungi (Baar and Stanton 2000; Werner et al. 2002). In our study, the fungus *H. fasciculare* seems to have an advantage in the competition compared to the ECM *P. tinctorius*. For this reason, the root colonization was inhibited when both fungi were simultaneously applied. However, if the initial steps of mycorrhizal establishment have already occurred, then the number of ECM roots is not affected, even in the presence of *H. fasciculare* mycelia. Indeed, when *C. sativa* plants were inoculated with *H. fasciculare* 30 days after *P. tinctorius* inoculation, a similar level of mycorrhizal roots was observed compared to plants only inoculated with *P. tinctorius*.

Although easily macroscopically detected, mycorrhizae formed in *P. tinctorius* 30 days + *H. fasciculare* treatment were not identical to those present in *P. tinctorius* colonized roots. Observation of cross sections from mycorrhizal root tips of chestnut plants inoculated with *P. tinctorius* alone showed the presence of a typical well-developed mantle and elongated epidermal cells (results not shown). Mycorrhizae from *C. sativa* seedlings inoculated with *P. tinctorius* and after 30 days with *H. fasciculare* displayed a layer of hyphae adherent to the epidermal cells, resembling a mantle, but with less elongated epidermal cells (results not shown). This result suggests that the presence of *H. fasciculare* still influences the development of the mycorrhizal association, even when plant-fungus interaction has already started. Albeit not restricting the association, the typical morphological features of *P. tinctorius* mycorrhiza are not fully developed. Thus, the possibility of the saprotrophic fungus to restrict certain interaction processes required for fully developed mycorrhization remains open. Also, the absence of mycorrhizae in simultaneously inoculated plants with both fungi could also be due to an early interaction inhibition promoted by the saprotrophic fungus.

In the present study, all fungal inoculations of four-month-old chestnut seedlings induced the plant growth (evaluated as an increase in shoot height increment, shoot/root length ratio, root collar diameter and growth rate), but only the seedlings solely inoculated with *P. tinctorius* exhibited statistically significant increases. Previous studies with the same combination of host and ECM species had already revealed the noteworthy improvement of *C. sativa* growth under *in vitro*, greenhouse and open field conditions (Martins *et al*. 1997; Martins, 2004). Even in other tree species, *P. tinctorius* inoculation has also promoted plant growth (Thomson *et al*. 1994; Cairney and Chambers, 1999; Turjaman *et al*. 2005). Seedlings growth promotion was suppressed in the presence of *H. fasciculare*, but the severity of this suppression was dependent on the time of fungal application. The adverse effect of *H. fasciculare* on the growth of *P. tinctorius* inoculated plants was mainly noticed when simultaneous inoculation with both fungi was performed. When the *P. tinctorius* mycorrhiza was established prior to *H. fasciculare* inoculation, the adverse effects were greatly reduced.

The growth increases observed in plants only inoculated with *P. tinctorius* could be related to the more favourable plant growing conditions promoted by the mycorrhizal establishment (Harris, 1992). The changes that occur on root morphology and architecture, associated to the increase of extramatrical ECM mycelium surrounding roots, contribute to a larger volume of soil explored. When *P. tinctorius* was inoculated alone, the lateral roots were shortened by 17% and exhibited 49% higher dry weight as compared to non-inoculated control, leading to a reduction of 52% in specific root length. Similar results have also been obtained with regard to root length and root dry weight in *C. sativa* seedlings inoculated with *P. tinctorius* under *in vitro* and open field conditions (Martins, 2004); and specific root length in *Larix gmelinii* (Sun *et al*. 2010). The increase of root diameter could be attributed to the cortical cells colonization by fungal mycelia, as well as to the mantle formation around the root tips. These features, together with increased lateral roots branching, are general responses to ECM inoculation (Smith and Read, 2008) and ultimately result on a larger available surface area for the absorption of nutrients and water (Marschner and Dell 1994; Brundrett *et al*. 1996; Timonen *et al*. 1996; Jones *et al*. 1998). In the present study, the inoculation of chestnut seedlings only with *P. tinctorius* resulted in an increase of N and P foliar content (21% and 37% higher compared to non-inoculated plants, respectively).

Although the differences are not statistically significant, this result is in accordance with previous studies using the same (Martins, 2004) or other combinations of host and ECM species (Smith and Read, 2008). The increased absorption of N and P due to *P. tinctorius* inoculation could certainly contribute to the enhanced growth response of *C. sativa* seedlings. Better growth responses due to an increase in uptake of P (Jones *et al*. 1991; Cairney and Chambers, 1997) or to enhanced N uptake (Wu *et al*. 1998; Mari *et al*. 2003) were also observed in several mycorrhizal associations. Taking into account the present results, there seems to be a negative correlation between specific root length and nutrient uptake in *C. sativa* plants only inoculated with *P. tinctorius*. Similar results were previously observed in other mycorrhizal associations (Rousseau *et al*. 1994; Padilla and Encina, 2005).

Plants inoculated with *P. tinctorius* and after 30 days inoculated with *H. fasciculare* also exhibited enhanced growth when compared to non-inoculated plants. Although not so noticeable as observed in *P. tinctorius* treated plants, lateral roots were also shortened (by 10%) and exhibited higher dry weight (47%) as compared to non-inoculated control, leading to a reduction of 40% in specific root length. These results could be related to the existence of mycorrhizal roots in an identical proportion as observed on *P. tinctorius* inoculated plants. Accordingly, plants inoculated with *P. tinctorius* and after 30 days inoculated with *H. fasciculare* display 18% higher N levels compared to non-inoculated plants. However, the regular functioning of these ectomycorrhizae could be compromised by the presence of *H. fasciculare,* as suggested by the presence of only an incipient mantle (microscopic observations, results not shown) and increase of specific root length in relation to *C. sativa* roots infected by *P. tinctorius* (by 23%). Indeed, the presence of *H. fasciculare* reduced the foliar P contents either when applied in combination with *P. tinctorius* (22-24% less when compared to *P. tinctorius*-inoculated plants) or alone (15% less when compared to non-inoculated plants).

The reduction of nutrients in plants only inoculated with *H. fasciculare* (N and P) or simultaneously inoculated with *P. tinctorius* and *H. fasciculare* (N) could be due to the competition of both fungi and roots for nutrient resources. Our results are in accordance with previous results that have reported no increases in shoot N in red pine plants inoculated with *P. tinctorius* in the presence of saprotrophic microbes (Wu et al.

2003). This phenomenon could result from the competitive interaction between *H. fasciculare* and *P. tinctorius* for N, which could lead to a lower nutrient accumulation in *C. sativa* leaves. The competition for nutrient resources is a common phenomenon that occurs between ECM and saprotrophic fungi. It was found that substantial P could be transferred from the ECM *Suillus variegatus* or *Paxillus involutus* to the saprotroph *H. fasciculare*, or vice-versa (Lindahl *et al*. 1999; 2001). These combative interactions could also include N transfers (Koide and Kabir, 2001; Wu *et al*. 2003, 2005).

The effect of fungal inoculation on leaf water status of *C. sativa* seedlings was evaluated through determination of the leaf water content (LWC). Leaf water content is a useful indicator of plant water balance, since it expresses the relative amount of water present on the plant tissues (Wang *et al*. 2011). In the present study, no significant differences in LWC were observed between treatments and control. This result is not surprising since all the plants were grown under well-watered conditions. However, the root system of mycorrhizal plants only inoculated with *P. tinctorius*, despite the smaller root length, supplied a relatively larger shoot with water and mineral nutrients. This is probably related with the increased extension and absorbing surface area of hyphae from mycorrhizal plants (Augé, 2004; Lehto and Zwiazek, 2011), as well as changes on root architecture that may be used to increase the interaction of root and soil (Atkinson 1994; Augé *et al*. 2001). As observed in our study, water contents of non-stressed plants were usually not different in non-mycorrhizal and mycorrhizal plants (Vodnik and Gogala 1994; Bryla and Duniway, 1997), including those with the ECM *P. tinctorius* (Alvarez *et al*. 2009).

The higher growth observed in plants only inoculated with *P. tinctorius* could additionally be attributed to an increase of photosynthetic rate when compare to non-inoculated control (Allen *et al*. 1981; Martins *et al*. 1997; Smith and Read, 2008). This is frequently related with higher chlorophyll and carotenoid contents, which ultimately leads to an improved carbohydrate accumulation (Davies *et al*. 1993; Wright *et al*. 1998). In this work, the inoculation with *P. tinctorius* alone enhanced the contents of chl *a*, chl *b*, and carotenoids in *C. sativa* seedlings (respectively in 23%, 38%, and 27%, when compared to non-inoculated plants). These results are in accordance with those reporting chlorophyll concentration increases in ectomycorrhizal plants when compared

with non-mycorrhizal plants (Huang and Tao, 2004; Alberdi *et al*. 2007). This situation is comparable to plants treated with *P. tinctorius* 30d + *H. fasciculare*, in which increases of 30% (chl *a*), 36% (chl *b*) and 20% (carotenoids) were detected, when compared to non-inoculated plants. The higher chlorophyll contents observed in *C. sativa* leaves inoculated only with *P. tinctorius* or with *P. tinctorius* 30d + *H. fasciculare* could be attributed to the melioration of nutritional status of the host plant, especially in N and P. Indeed, whereas N is an essential element for the formation of chlorophyll (Liu *et al*. 2007), P has an important role as an energy carrier during photosynthesis (Jacobsen, 1991). Similar results were also reported in other studies (Demur, 2004; Zuccarini, 2007; Chen *et al*. 2010). The more reduced growth of *C. sativa* seedlings after being simultaneously inoculated with *P. tinctorius* and *H. fasciculare* could be attributed to some extent to the decreased nutrient acquisition of these plants (particularly N) that will lead to lower photosynthetic pigment contents.

To conclude, the simultaneous inoculation of the saprotrophic fungus *H. fasciculare* negatively affected the interaction between the ECM *P. tinctorius* and *C. sativa* roots. Besides the absence of visible mycorrhizal roots, growth, nutritional and physiological parameter values commonly associated to the mycorrhization benefits were not observed on plants simultaneously inoculated with both fungi. When plants were inoculated with *P. tinctorius* and after 30 days with *H. fasciculare* the same parameter values were very close to those from plants only inoculated with *P. tinctorius*. These results are most probably due to the interaction between *P. tinctorius* and *C. sativa* roots and the ability of mycorrhizal establishment before *H. fasciculare* application. Once formed, the chestnut seedlings are able to take advantage from the mycorrhizal association. Plants exhibit growth improvement, which could be attributed to the enhancement of nutrient acquisition, through an increase in the absorbing surface area. This work confirms the antagonistic interaction between ECM and saprotrophic fungi and demonstrates that fungal interactions affect the physiological processes of the ectomycorrhizal host. Although *P. tinctorius* is an effective colonizer of many tree species, the presence of saprotrophic fungi in the soil could hamper the establishment and functioning of mycorrhizae. The inability of *P. tinctorius* to compete with certain competitive saprotrophic fungi compromises the mycorrhization of host

trees. However, if the initial steps of mycorrhizal symbiosis have already occurred, then the benefits from mycorrhization could be observed, even in the presence of saprotrophic fungi.

ACKNOWLEDGMENTS

Authors are grateful to Fundação para a Ciência e Tecnologia (FCT) for financial support (Project PTCD/AGR-AAM/102600/2008).

3.5. REFERENCES

Alberdi M, Álvarez M, Valenzuela E, Godoy R, Olivares E, Barrrientos M (2007) Response to water deficit of *Nothofagus dombeyi* plants inoculated with a specific (*Descolea antarctica* Sing) and non-specific (*Pisolithus tinctorius* (Pers.) Coker & Couch) ectomycorrhizal fungi. Rev Chil Hist Nat 80:479-491

Allen MF, Smith WK, Moore TS, Christensen M (1981) Comparative water relations and photosynthesis of mycorrhizal *Bouteloua gracilis* H.B.K. lag ex Steud. New Phytol 88:683-693

Alvarez M, Huygens D, Fernandez C, Gacitúa Y, Olivares E, Saavedra I, Alberdi M, Valenzuela E (2009) Effect of ectomycorrhizal colonization and drought on reactive oxygen species metabolism of *Nothofagus dombeyi* roots. Tree Physiol 29:1047-1057

AOAC (Association of Official Analytical Chemist) Official Methods of Analysis, Association of Official Analytical Chemists. 15th Ed. Gaithersburg, USA: AOAC Press; 1990.

Atkinson D (1994) Impact of mycorrhizal colonization on root architecture, root longevity and the formation of growth regulators. In: Gianinazzi S et al. (eds). Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystem, pp 89-99.

Augé RM (2004) Arbuscular mycorrhizae and soil/plant water relations. Can J Soil Sci 84:373–381

Augé RM, Stodola AJW, Tims JE, Saxton AM (2001) Moisture retention properties of a mycorrhizal soil. Plant Soil 230:87-97

Baar J, Stanton NL (2000) Ectomycorrhizal fungi challenged by saprotrophic basidiomycetes and soil microfungi under different ammonium regimes *in vitro*. Mycol Res 104:691–697

Baptista P, Martins A, Tavares RM, Lino-Neto T (2010) Diversity and fruiting pattern of macrofungi associated with chestnut tree (*Castanea sativa* Mill.) in the Trás-os-Montes region (Northeast Portugal). Fungal Ecol 3:9-19

Boddy L (2000) Interspecific combative interactions between wood-decaying basidiomycetes. FEMS Microbiol Ecol 31:185-194

Brundrett M, Bougher N, Dell B, Grave T, Malajczuk N (1996) Working with Mycorrhizas in Forestry and Agriculture. Australian Centre for International Agricultural Research Monograph 32, Canberra.

Bryla DR, Duniway JM (1997) Growth, phosphorus uptake, and water relations of safflower and wheat infected with an arbuscular mycorrhizal fungus. New Phytol 136:581-590

Cairney JWG, Chambers SM (1999) Ectomycorrhizal fungi-key genera in profile. Springer-Verlag Berlin, Heidelberg

Cairney WG, Chambers SM (1997) Interactions between *Pisolithus tinctorius* and its hosts: a review of current knowledge. Mycorrhiza 7:117–131

Chen L, Zeng J, Xu DP, Zhao ZG, Guo JJ (2010) Macronutrient deficiency in symptoms *Betula alnoides* seedlings. J Trop Forest Sci 22:403–413

Davies FT, Potter JR, Linderman RG (1993) Drought resistance of mycorrhizal pepper plants independent of leaf P-concentration – response in gas exchange and water relations. Physiol Plant 87:45-53

Demur S (2004) Influence of Arbuscular Mycorrhiza on Some Physiological Growth Parameters of Pepper. Turk J Biol 28:85-90

Dighton J (2007) Nutrient cycling by saprotrophic fungi in terrestrial habitats. In: Kubicek CP, Druzhinina IS (eds) The Mycota IV Environmental and microbial relationships, 2nd edn. Springer-Verlag, Berlin Heidelberg, pp 287-300

Edda S, Oddsdottir ES, Eilenberg J, Sen R, Halldorsson G (2010) The effects of insect pathogenic soil fungi and ectomycorrhizal inoculation of birch seedlings on the survival of Otiorhynchus larvae. Agric Forest Entomol 12:319–324

El-Shatnawi MKJ, Makhadmeh IM (2001) Ecophysiology of plant-rhizosphere system. J Agron Crop Sci 187:1-9

Harris RW (1992) Root-shoot ratios. J Arboric 18: 39-42

Huang Y, Tao S (2004) Influences of excessive Cu on photosynthesis and growth in ectomycorrhizal *Pinus sylvestris* seedlings. J Environ Sci 16:414-419

Jackson ML (1973) Soil Chemical Analysis. Prentice Hall, New Delhi.

Jacobsen I (1991) Carbon metabolism in Mycorrhiza. In: Burrock H, Mosser J (eds.) Methods in Microbiology, Academic Press, pp 149-180

Jones MD, Durall DM, Tinker PB (1991) Fluxes of carbon and phosphorus between symbionts in willow ectomycorrhizas and their changes with time. New Phytol 119:99–106

Jones MD, Durall DM, Tinker PB (1998) Comparison of arbuscular and ectomycorrhizal *Eucalyptus coccifera*: growth response, phosphorus uptake efficiency and external hyphal production. New Phytol 140:125-134

Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. New Phytol 148:511-517

Leake JR, Donnelly DP, Saunders EM, Boddy L, Read DJ (2001) Rates and quantities of carbon flux to ectomycorrhizal mycelium following 14C pulse labeling of *Pinus sylvestris* seedlings: effects of litter patches and interaction with a wood-decomposer fungus. Tree Physiol. 21:71–82

Leake JR, Donnelly DP, Boddy L (2002) Interactions between ectomycorrhizal and saprotrophic fungi. In: Van de Heijden MGA, Sanders I (ed) Mycorrhizal Ecology, Ecological Studies, vol 157, Springer-Verlag, Berlin, Heidelberg, pp 346-373

Lehto T, Zwiazek JJ (2011) Ectomycorrhizas and water relations of trees: a review. Mycorrhiza 21:71-90

Lindahl B, Ihrmark K, Boberg J, Trumbore SE, Högberg P, Stenlid J, Finlay RD (2007) Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytol 173:611–620

Lindahl B, Stenlid J, Finlay RD (2001) Effects of resource availability on mycelial interactions and 32P transfer between a saprotrophic and an ectomycorrhizal fungus in soil microcosms. FEMS Microbiol Ecol 38:43–52

Lindahl B, Stenlid J, Olsson S, Finlay R (1999) Translocation of 32P between interacting mycelia of a wood decomposing fungus and ectomycorrhizal fungi in microcosm systems. New Phytol 144:183–193

Mari S, Jonsson A, Finlay R, Ericsson T, Kähr M, Eriksson G (2003) Genetic variation in nitrogen uptake and growth in mycorrhizal and nonmycorrhizal *Picea abies* (L.) Karst. seedlings. Forest Science 49:258-267

Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant and Soil 159:89-102.

Martins A (2004) Micorrização controlada de *Castanea sativa* Mill.: aspectos fisiológicos da micorrização *in vitro* e *ex vitro*. Tese de Doutoramento. Faculdade de Ciências da Universidade de Lisboa, Lisboa

Martins A, Casimiro A, Pais MMS (1997) Influence of mycorrhization on physiological parameters of micropropagated *Castanea sativa* Mill. plants. Mycorrhiza 7:161-165

Mucha J, Dahm H, Strzelczyk E, Werner A (2006) Synthesis of enzymes connected with mycoparasitism by ectomycorrhizal fungi. Arch Microbiol 185 69–77

Ozerol NH, Titus JF (1965) The determination of total chlorophyll in methanol extracts. Trans Ill State Acad Sci 58:15–19

Padilla IMG, Encina CL (2005) Changes in root morphology accompanying mycorrhizal alleviation of phosphorus deficiency in micropropagated *Annona cherimola* Mill. Plants. Sci Hortic 106:360–369

Rousseau JVD, Sylvia DM, Fox AJ (1994) Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine. New Phytol 128:639–644

Sharma R, Rajak RC, Pandey AK (2010) Evidence of antagonistic interactions between rhizosphere and mycorrhizal fungi associated with *Dendrocalamus strictus* (Bamboo). J Yeast Fungal Res 1:112–117

Shaw TM, Dighton J, Sanders FE (1995) Interactions between ectomycorrhizal and saprotrophic fungi on agar and in association with seedlings of lodgepole pine (*Pinus contorta*). Mycol Res 99:159–165

Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Academic Press, San Diego.

Sun Y, Gu J-C, Zhuang H-F, Wang Z-Q (2010) Effects of ectomycorrhizal colonization and nitrogen fertilization on morphology of root tips in a *Larix gmelinii* plantation in northeastern China. Ecol Res 25:295–302

Thomson BD, Grove TS, Malajczuk N, Hardy GE St J (1994) The effectiveness of ectomycorrhizal fungi in increasing the growth of *Eucalyptus globulus* Labill. in relation to root colonization and hyphal development in soil. New Phytol 126:517–524

Timonen S, Finlay RD, Olsson S, Söderström B (1996) Dynamics of phosphorus translocation in intact ectomycorrhizal systems: non-destructive monitoring using a beta-scanner. FEMS Microbiol Ecol 19:171-180

Turjaman M, Tamai Y, Segah H, Limin SH, Cha JY, Osaki M, Tawaraya K (2005) Inoculation with the ectomycorrhizal fungi *Pisolithus arhizus* and *Scleroderma sp*. improves early growth of *Shorea pinanga* nursery seedlings. New Forests 30:167-173

Vodnik D, Gogala N (1994) Seasonal fluctuations of photosynthesis and its pigments in 1-year mycorrhized spruce seedlings. Mycorrhiza 4:277-281

Wang Y, Li G, Zhang L, Fan J (2011) Retrieval of leaf water content of winter wheat from canopy spectral reflectance data using a position index (λmin) derived from the 1200 nm absorption band. Remote Sensing Letters 2:31–40

Werner A, Zadworny M (2003) *In vitro* evidence of mycoparasitism of the ectomycorrhizal fungus *Laccaria laccata* against *Mucor hiemalis* in the rhizosphere of *Pinus sylvestris*. Mycorrhiza 13:41-47

Werner A, Zadworny M, Idzikowska K (2002) Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture and in the rhizosphere of *Pinus sylvestris* grown *in vitro*. Mycorrhiza 12:139-145

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PRC Protocols: A Guide to Methods and Applications, Academic Press, San Diego, pp 315-322

Wright DP, Scholes JD, Read DJ (1998) Effects of VAM colonization on photosynthesis and biomass production of *Trifolium repens* L. Plant Cell Environ 21:209-216

Wu B, Watanabe I, Hayatsu M, Nioh I (1998) Effect of ectomycorrhizae on the growth and uptake and transport of 15N-labeled compounds by *Pinus tabulaeformis* seedlings under water-stressed conditions. Biol Fertil Soils 28:136-138

Wu T, Kabir Z, Koide RT (2005) A possible role for saprotrophic microfungi in the N nutrition of ectomycorrhizal *Pinus resinosa*. Soil Biol Biochem 37:965-975

Wu T, Sharda JN, Koide RT (2003) Exploring interactions between saprotrophic microbes and ectomycorrhizal fungi using a protein–tannin complex as an N source by red pine (*Pinus resinosa*). New Phytol 159:131–139

Zadworny M, Werner A, Idzikowska K (2004) Behaviour of the hyphae of *Laccaria laccata* in the presence of *Trichoderma harzianum in vitro*. Mycorrhiza 14:401-405

Zuccarini P (2007) Mycorrhizal infection ameliorates chlorophyll content and nutrient uptake of lettuce exposed to saline irrigation. Plant Soil Environ 53:283–289

Capítulo 4

Conclusão geral

4.1. CONCLUSÃO GERAL

Hypholoma fasciculare é um fungo saprófita-lenhícola caracterizado por apresentar um crescimento micelial através de cordões e tem sido apontado como uma espécie muito combativa contra outras espécies saprófitas e patogénicas, o que tem incentivado a sua utilização como agente de controlo biológico. Esta espécie apresenta uma vasta distribuição geográfica, sendo muito comum em soutos de *Castanea sativa* do nordeste transmontano. Associado a esta cultura existe uma flora microbiana diversificada de entre os quais se destacam os fungos ectomicorrízicos, com reconhecidos efeitos benéficos para a planta; e os oomycetes *Phytophthora cinnamomi* Rands e *P. cambivora* (Petri) Buis, os principais agentes causadores da doença da tinta do castanheiro.

Recorrendo ao método da cultura dupla em meio de cultura, verificou-se que o fungo *H. fasciculare* inibia significativamente o crescimento dos patogénicos *Armillaria mellea* (em 49%) e *Phytophthora cambivora* (em 17%) assim como das espécies ectomicorrízicas *Suillus luteus* (em 43%) e *Pisolithus tinctorius* (em 49%). Os mecanismos adoptados pelo *H. fasciculare* foram "antagonismo à distância", para as espécies patogénicas; e "agonismo à distância", para as espécies ectomicorrízicas. Contudo, foi observado a inibição no crescimento de *H. fasciculare* quando em co-cultura com *Amanita gemmata* (em 35%) ou com o fungo patogénico *Colletotrichum acutatum* (em 12%). Nestes dois casos os mecanismos de interacção foram, respectivamente "antagonismo e agonismo à distância". Não foram registadas diferenças de crescimento nas co-culturas estabelecidas entre o *H. fasciculare* e as espécies *P. cinnamomi* ou *S. bovinus*, tendo sido os mecanismos de interacção observados a co-habitação e o comensalismo, respectivamente. No antagonismo assim como no agonismo à distância poderão estar envolvidas a produção de enzimas líticas por parte do *H. fasciculare*, como sejam a amilase, celulase, lacase e lipase, com reconhecida acção na lise das paredes celulares fúngicas. A produção de celulase por parte da *Phytophthora spp.* foi igualmente detectada e poderá constituir uma resposta ao antagonismo evidenciado por *H. fasciculare*. Adicionalmente, foram verificadas alterações morfológicas nas colónias interactuantes e, em especial, no *H. fasciculare*. O micélio de *H. fasciculare* ficou mais denso e compacto, em especial na zona de

interacção, possivelmente como forma de tornar a colónia mais resistente à invasão da espécie interactuante. Nalguns casos verificou-se também a formação de cordões, provavelmente com o intuito de tornar a colónia mais invasiva.

Os resultados apresentados constituem uma primeira abordagem ao estudo da interacção entre *H. fasciculare* e fungos ectomicorrízicos, e pela primeira vez com os patogénicos *P. cinnamomi*, *P. cambivora* e *C. acutatum*, sendo necessário a realização de trabalhos futuros que permitam a elucidação da natureza e função dos metabolitos envolvidos nos mecanismos de interacção. Contudo, e apesar de preliminares, os resultados obtidos evidenciam o potencial do *H. fasciculare* como agente de controlo biológico da *P. cambivora*. Estes estudos deverão ser complementados com a realização de ensaios em condições de campo para que se possa compreender melhor o efeito do *H. fasciculare* no controlo deste patogénico e na comunidade fúngica micorrízica, na rizosfera do castanheiro.

A inoculação de plantas de castanheiro com o fungo ectomicorrízico *P. tinctorius*, em condições de estufa, resultou num aumento significativo do crescimento das plantas em altura (em 200%) e diâmetro a nível do colo, comparativamente às plantas não inoculadas. Os níveis foliares em azoto, fósforo, clorofilas (a, b e total) e carotenóides foi igualmente superior (em cerca de 21-38%) em plantas inoculadas face às não inoculadas. Este efeito positivo da micorrização foi, contudo, suprimido pelo fungo *H. fasciculare* quando aplicado simultaneamente com o *P. tinctorius*. A inoculação de plantas de castanheiro simultaneamente com as duas espécies fúngicas resultou num decréscimo de crescimento em altura em 49%, e dos níveis foliares em azoto (em 30%), clorofilas a, b, total e carotenóides (em 30-40%), comparativamente às plantas inoculadas exclusivamente com *P. tinctorius*. Estas plantas, apresentavam ainda as folhas cloróticas e as raízes não se encontravam micorrizadas. Estes resultados sugerem que efeito deletério observado poderá estar relacionado com a inibição da micorrização das raízes pelo *H. fasciculare* e/ou pela competição entre o *P. tinctorius* e o *H. fasciculare* por espaço ou nutrientes, nomeadamente de azoto. Contudo, o efeito deletério de *H. fasciculare* não se verificou quando aplicado nas plantas isoladamente ou 30 dias depois da inoculação com *P. tinctorius*.

Os resultados obtidos evidenciam uma interacção antagónica entre fungos ectomicorrízicos e saprófitas com repercussões ao nível do crescimento e fisiologia da

planta. Apesar do *P. tinctorius* se encontrar descrito como sendo um colonizador primário e eficiente na micorrização de muitas espécies plantas, parece apresentar baixa acção competitiva contra *H. fasciculare*, que pelo contrário é apontada como sendo muito combativa. Assim sendo, o efeito benéfico do *P. tinctorius* só será efectivo quando o estabelecimento da associação simbiótica ocorre previamente à inoculação com *H. fasciculare*.