Mycorrhizal induction of phenolic compounds and antioxidant properties of fungi and seedlings during the early steps of symbiosis

FILIPA S. REIS,[a,b] ISABEL C.F.R. FERREIRA[a,b,*], LILLIAN BARROS,[a,c] CELESTINO SANTOS-BUELGA[c], ANABELA MARTINS,[b]

[a]*CIMO-ESA, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal*.

[b]*Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal*.

[c]*Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.*

* Author to whom correspondence should be addressed (e-mail: iferreira@ipb.pt telephone +351-273-303219; fax +351-273-325405).

Abstract

Ectomycorrhizal (ECM) symbiosis plays a major role in nutrient cycling and the functioning of forest ecosystems. Trees with well developed ectomycorrhizas are more resistant to environmental stresses such as drought and to biotic stresses such as root pathogens. The establishment of ECM symbiosis is triggered by signals produced by both partners. These signals lead to morphological changes and a complex development of specific structures in both the plant root and the fungus. The development of the ECM fungi, *Paxillus involutus* and *Pisolithus arhizus*, in presence and absence of the symbiont – *Pinus pinaster*- was evaluated, as well as their antioxidant properties and phenolic compounds composition in response to the symbiotic association. ECM fungi grew less in the presence of *Pinus pinaster*, with *Pisolithus arhizus* being less affected in growth and thus being more adapted to this association. Protocatechuic acid was found only in *Paxillus involutus*, while *Pinus pinaster* roots, both in association and isolated, proved to have other phenolic acids, such as *p*-hydroxybenzoic and *p*-coumaric acids. The symbiosis between *Paxillus involutus* and *Pinus pinaster* had no major effects on the symbionts, while the association between *Pisolithus arhizus* and *Pinus pinaster* seems to generally decrease the antioxidant effects of both symbionts, despite the increase in *p*-coumaric and cinnamic acids in the ECM fungi.

Keywords: Antioxidant activity · Ectomycorrhizal fungi · Phenolic compounds · *Pinus pinaster* seedlings · Symbiosis.

Introduction

Ectomycorrhizal (ECM) symbiosis is an important feature in the life and health of trees in boreal and temperate forests where it plays a major role in nutrient cycling and the functioning of the forest ecosystem. Trees with well developed ectomycorrhizal root tips are more resistant to environmental stresses such as drought and to biotic stresses such as root pathogens (Smith and Read 2008; Finlay et al. 2008). The establishment of ECM symbiosis is triggered by signals produced by both partners (Ditengou and Lapeyrie 2000; Martin et al. 2001). These signals lead to a complex development of specific structures in both the plant and the fungus perceptible by morphological changes. ECM associations are characterized by the successive development of three structural components: a mantle or sheath of fungal tissue that encloses the root, the intraradical network of hyphae, called the Hartig net, and an extraradical mycelium, which extends into the soil surrounding the root and is responsible for water and nutrient uptake. The Hartig net is the site of metabolite exchange between fungal and root cells (Smith and Read 2008). The ontogenesis of a functional ECM symbiosis requires finely regulated cross talk in time and space between plant and fungal genetic programs involved in sensing the environment, and in cell-to-cell communication (Heller et al. 2008).

In plant-pathogen compatible interactions, a fungal pathogen either eludes or suppresses recognition, and plant disease ensues. In contrast, in incompatible host interactions as well as in non-host interactions, plants respond to pathogen infection by activating mechanisms of disease resistance, and consequently, no disease symptoms develop (Mysore and Ryu 2004). An oxidative burst is generally defined as a rapid production of high amounts of ROS (reactive oxygen species) in response to external stimuli and has

been considered as one of the earliest responses of plants to pathogenic microorganisms (Lamb and Dixon 1997). The release of ROS was observed in roots of *Castanea sativa* during the early steps of mycorrhizal association with *Pisolithus arhizus*. Superoxide dismutase and catalase activities were concomitantly increased in plant roots of this system (Baptista et al. 2007). During ECM establishment, the mycobiont has the ability to recognize and become associated with host roots. In addition, the fungal symbiont must be able to escape host defense surveillance and establish bi-directional nutrient transfers across the root-fungus interface. Thus, the establishment of this symbiotic association requires a highly regulated and coordinated sequence of events, initiated by an exchange of specific signaling compounds between both partners (Martin et al. 2001; Podila 2002).

In mycorrhizas of *Picea abies*, *Larix decidua* and *Pinus sylvestris*, a lower concentration of soluble and cell wall bound phenolics than in uninoculated roots was observed (Münzenberger et al. 1990; Münzenberger et al. 1995). Later, Münzenberger et al. (1997) observed that laccase and peroxidase activities differed between mycorrhizas and uninoculated roots of *P. abies* and *L. decidua*. In both species, mycorrhizas contained the highest laccase activity and the lowest peroxidase activity. The high laccase activity could induce the polymerisation of soluble phenolics contributing to decreasing concentration of monomers. The low peroxidase activity would inhibit oxidative rigidification of cell wall. These reactions would favour root colonization by ECM fungi.

ECM symbiosis between the mycelia and the roots of some plants could have important effects in the levels of antioxidants of both parts: fungal mycelium and plant roots. *Paxillus involutus* (Batsch) Fr. and *Pisolithus arhizus* (Scop.) Rauschert are widely

spread fungal species. The present study aimed to reproduce *in vivo* mechanisms of symbiosis between ECM fungi and specific host plants. *In vitro* experiments of mycorrhizal induction between *Paxillus involutus* and *Pisolithus arhizus* ECM fungal mycelium and *Pinus pinaster* seedlings were performed. Phenolic production and antioxidant properties of both partners in the absence and in the presence of each other, and the antioxidant mechanisms involved during the partners recognition process were compared for two different times of co-culture during early steps of *in vitro* mycorrhizal synthesis.

Methods and Material

Biological material

Samples of *Paxillus involutus* (Batsch) Fr. (Fig. 1A) and *Pisolithus arhizus* (Scop.) Rauschert (Fig. 1B) were collected under *Castanea sativa* Mill. in Bragança (Northeast Portugal), in autumn 2008. Taxonomic identification of sporocarps was made according to Benguria (1985) and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança.

Pinus pinaster (Aiton) seeds were obtained from CENASEF (Centro Nacional das Sementes Florestais, Portugal).

In vitro production of mycelia and germination of *Pinus pinaster* seeds

Mycelia of each of the fungi were isolated from sporocarps and cultured on solid modified Melin-Norkrans medium (MMN) pH 6.6 (NaCl 0.025 g/l; $(NH_4)_2HPO_4$ 0.25 g/l; KH_2PO_4 0.50 g/l; $FeCl_3$ 0.0050 g/l; $CaCl_2$ 0.050 g/l; $MgSO_4.7H_2O$ 0.15 g/l; thiamine 0.10 g/l; glucose 10 g/l; agar 20 g/l in tap water) (Marx 1969). The strains

were maintained in Petri dishes (9 cm diameter) containing the same medium at 25ºC in the dark and subcultured every 4 to 6 weeks (Fig. 1C and 1D). The weekly growth of the mycelium was evaluated by measurement of the mycelia radial growth. For the assay, after 45 days of growth, the mycelium was scraped off the medium with a scalpel, and both the mycelium and culture medium, were weighted separately to obtain the fresh biomass (fw).

Pinus pinaster seeds were germinated in agar:water 0.9% in tubes (3 cm diameter) after washes with tap water, superficial disinfection in sodium hypochloride (5%; 10 min), several washes with sterile water, brief contact with hydrogen peroxide (3%) and subsequent washes with sterile water. After inoculating the seeds in the tubes, they were left in the dark at 25ºC for 48 h, and then exposed to light until the mycorrhization induction (Fig. 1E and 1F).

Induction of the mycorrhizal symbiosis

Two *Pinus pinaster* seedlings were introduced in Petri dishes (13 cm diameter) with modified MMN. Inoculums of *Paxillus involutus* or *Pisolithus arhizus* mycelia (3 mm) were laid between the two plants for mycorrhizal induction (Fig. 1G and 1H). The inoculated plants were incubated in a culture chamber with *Daylight* lamps (Phillips, Amsterdam, Netherlands) with a photoperiod of 8 h light (23 ºC) and 16 h dark (18ºC). After 45 days of growth, mycelium and plant roots were recovered from the medium. Mycelium, plant and culture medium were weighted separately to obtain the fresh biomass (fw), and then stored at –40ºC for further analyses. Petri dishes inoculated only with *Paxillus involutus*, *Pisolithus arhizus* and *Pinus pinaster* were used as controls.

Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Lab-Scan (Lisbon, Portugal). Phenolic compounds standards and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Warren, USA).

Preparation of the extracts

Each sample (~0.5 g for mycelia and roots; ~20 g for culture media) was extracted by stirring with 40 ml of methanol (25ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then re-extracted with 20 ml of methanol (25ºC at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness, redissolved in water:methanol (80:20) for analysis of phenolic compounds, and in methanol for antioxidant activity assays (extracts concentrations ranging from 20 to 0.3125 mg/ml for mycelia and roots, and from 100 to 5 mg/ml for culture media).

Analysis of phenolic compounds

The extract solutions were filtered through a 0.22 μm disposable LC filter disk and were analysed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Santa Clara, USA) as previously described (Barros et al. 2009). Separation of phenolic compounds was achieved on a Spherisorb S3 ODS-2 (Waters, Milford, USA) reverse phase C_{18} column (3 μm, 150 × 4.6 mm) thermostated at 25ºC.

The solvents used were: (A) 2.5% acetic acid in water, (B) 2.5% acetic acid:acetonitrile (90:10, v/v), and (C) 100% HPLC-grade acetonitrile. The gradient employed was: isocratic 100% A for 10 min, 50% A and 50% B for 10 min, isocratic 100% B for 15 min, 90% B and 10% C for 10 min, 70% B and 30% C for 10 min, 50% B and 50% C for 5 min, 20% B and 80% C for 5 min, 100% A for 5 min, at a flow rate of 0.5 ml/min. Detection was carried out in a diode array detector (DAD), using 280 nm as the preferred wavelength. The phenolic compounds were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The results were expressed as μg per g of extract.

Antioxidant activity

In vitro assays already described by the authors Heleno et al. (2010) were used to evaluate the antioxidant activity of the samples.

DPPH radical-scavenging activity. DPPH scavenging activity is one of the most extensively used antioxidant assay. DPPH is a stable organic nitrogen radical, commercially available, and has a deep purple colour. The assay measures the reducing capacity of antioxidants toward DPPH. Upon reduction, its colour fades and the decreasing in the absorbance can be monitored spectrophotometrically at 517 nm (Amarowicz et al. 2004). This assay was performed in 96-well microtiter plates using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc., Winooski, USA). The reaction mixture in each of the 96-wells of the plate consisted of one of the different concentrations of the extracts (30 μl) and aqueous methanolic solution (80:20, v/v, 270 μl) containing DPPH radicals ($6x10^{-5}$ mol/l). The mixture was left to stand for 60 min in

the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

Reducing power. The transformation of Fe^{3+} into Fe^{2+} in the presence of antioxidants is measured to determine the reducing power ability. Depending on the reducing power of the tested samples, the yellow colour of the solution changes to various shades of green or blue that can be measured spectrophotometrically at 700 nm (Amarowicz et al. 2004). This assay was also performed using microtiter plates and the Microplate Reader described above. Different concentrations of the extracts (0.5 ml) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured into the wells of a 48-well microplate, along with deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

Inhibition of β-carotene bleaching. The β-carotene undergoes a rapid discoloration in the absence of an antioxidant since the free linoleic acid radical attacks the β-carotene molecule, which loses the double bonds and, consequently, loses its characteristic orange color. Inhibition of β-carotene bleaching in the presence of antioxidants can be monitored by spectrophotometry at 470 nm (Gutierrez et al. 2006). Two millilitres of a solution of β-Carotene in chloroform (0.2 mg/ml) were mixed with linoleic acid (40 mg) and Tween 80 emulsifier (400 mg). After chloroform evaporation (40 ºC, under vacuum), distilled water (100 ml) was added and the mixture was vigorously shaking. Aliquots (4.8 ml) of this emulsion were transferred into test tubes containing different concentrations of the extracts (0.2 ml). The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (Analytikjena 200 spectrophotometer, Jena, Germany). β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2 h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

Total phenolics by Folin-Ciocalteu assay

Total phenolics were estimated based on procedures previously described (Heleno et al. 2010). An aliquot of the extract solution (0.5 ml) was mixed with *Folin-Ciocalteu* (FC) reagent (2.5 ml, previously diluted with water 1:10 v/v) and sodium carbonate (75 g/l, 2 ml). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40°C for

colour development. Absorbance was then measured at 765 nm. Gallic acid was used to calculate the standard curve (0.05-0.8 mM), and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Statistical analysis

For each one of the species, three different samples were analysed, three extractions of each sample were performed, and also all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD) or standard errors (SE). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v.16.0 software.

Results and discussion

The formation of mycorrhizas was not observed in all samples for the times here studied. Nevertheless, the effects of the symbionts co-culture were already verified in the radial differential growth of ECM fungi in the presence and absence of *Pinus pinaster* roots and on the antioxidant properties of the three studied partners.

Mycelia growth

The growth of *Paxillus involutus* and *Pisolithus arhizus* mycelia in the absence and in the presence of *Pinus pinaster* seedlings plants is shown in Fig. 2.

Paxillus involutus started to grow only after 8 days of inoculation when growing alone, while in the presence of *Pinus pinaster*, the growth of mycelium began from 0 days. The opposite was observed in the case of *Pisolithus arhizus* growth; isolated mycelium grew immediately, while in the presence of *Pinus pinaster* grew only after 8 days. As it was observed for the isolated mycelia, the radial growth in the presence of *Pinus pinaster* obtained for *Paxillus involutus* was slightly higher than the one obtained for *Pisolithus arhizus*, until 36 days of inoculation. After 43 days, an inversion was observed.

Along the 43 days of growth, *Paxillus involutus* revealed a higher radial growth than *Pisolithus arhizus*, being faster its *in vitro* production. Nevertheless, *Pisolithus arhizus* seems to adapt better to the presence of *Pinus pinaster* showing increasing growth values along the time, despite its slower initial growth. Therefore, *Paxillus involutus* is the most promising to obtain higher levels of mycelium, *eg* for the *in vitro* production of

specific compounds, while *Pisolithus arhizus* seems to be a preferable symbiont for the mycorrhization process with *Pinus pinaster*.

The results show that both mycelia had lower radial growths in the presence of the seedlings than in the experiments growing isolated. These results might be explained as due to phenolic compounds accumulated in the root of the plant in response to the invasion. The existence of toxic effects of soluble, low molecular weight phenolics has been reported in other studies for a variety of fungi (Haars et al. 1981; Wacker et al. 1990), including ECM fungi (Lindeberg 1948; Olsen et al. 1971).

Phenolic compounds

Phenolic compounds accumulate as end-products from the shikimate and acetate pathways, and can range from relatively simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group in plants. The intensity of phenolic deposition in response to invasion by EMC-forming fungi and the ability of an ECM fungus to tolerate phenolics could be associated with the symbiotic compatibility of fungus and host associates (Molina and Trappe 1984; Malajczuk et al. 1984). In incompatible pairings, phenolics are accumulated in roots as a result of a hypersensitive reaction. A similar response has been recognized as one mode of plant defense against pathogenic infection (Ebel 1986).

There are several studies reporting phenolic acids as the main phenolic compounds in mushrooms (Barros et al. 2009; Ferreira et al. 2009). In the present work, the main phenolic acids and derivatives previously identified in wild mushrooms (protocatechuic,

p-hydroxybenzoic, *p*-coumaric and cinnamic acids) have been quantified in mycelium of the studied ECM fungi (*ie*, *Paxillus involutus* and *Pisolithus arhizus*) and germinated *Pinus pinaster* plants, in the presence and in the absence of symbiont (Table 1).

Protocatechuic acid was not found in *Paxillus involutus* in the presence of *Pinus pinaster* roots, while no differences were observed in the other three compounds in the absence and in the presence of the roots (similar values for *p*-hydroxybenzoic acid and no detection of *p*-coumaric and cinnamic acids). For *Pisolithus arhizus* in the presence of *Pinus pinaster* roots, *p*-hydroxybenzoic was not detected while *p*-coumaric and cinnamic acids could be quantified (58.98 and 10.85 μg/g extract, respectively). On the other hand, the roots of *Pinus pinaster* in the presence of *Paxillus involutus* or *Pisolithus arhizus* mycelia showed higher levels of cinnamic acid but lower levels of *p*-coumaric acid than the isolated roots. The concentration of *p*-hydroxybenzoic acid in the *Pinus pinaster* roots increased only in the presence of *Paxillus involutus* (51.02 to 80.70 μg/g extract).

Other authors reported a lower concentration of soluble and cell wall bound phenolics in mycorrhizas of *Picea abies*, *Larix decidua* and *Pinus sylvestris* than in uninoculated roots for trials longer than 5 months of mycorrhizal induction (Münzenberger et al. 1990; Münzenberger et al. 1995). Nevertheless, in the present study, after 45 days that effect was only observed for the levels of *p*-coumaric acid.

Total phenolics were also measured by Folin-Ciocalteu assay. The chemistry related to this assay is based on the electron transference from phenolic compounds and other reducing species to molybdenum, in basic medium, giving blue complexes that can be monitored at 750-765 nm (Magalhães et al. 2008). The results can be expressed as

equivalents of different standards (*eg*, catechin, gallic acid, tannic acid, chlorogenic acid, cafeic acid or ferulic acid) that conduce to different order magnitudes and may make results obtained in different studies non-comparable. Regarding phenolic compounds, the obtained absorbance is usually proportional to the number of reactive phenolic hydroxyl groups, depending on the structure of the molecule. There are, however, some limitations of this methodology when used as a measure of the content of phenolic compounds. Thus, the FC reagent is not specific to phenolic compounds and can be reduced by many non-phenolic substances such as ascorbic acid and sugars, which is not reflected in its designation (Karadag et al. 2009). In fact, the assay measures the reducing capacity of a sample, although being performed in aqueous medium it overlooks lipophilic antioxidants. Therefore, the results obtained in this assay should be rather considered as further antioxidant activity data.

Total phenolic (TP) content as determined by the FC reagent increased in *Paxillus involutus* in the presence of *Pinus pinaster* roots, whereas the presence of these latter had no effects on TP contents of *Pisolithus arhizus* mycelium. Regarding *Pinus pinaster*, TP content was higher in isolated roots than in the presence of fungal mycelia (Table 2). This decrease in the presence of symbionts may be related to a decrease in the plant oxidative stress, indicating that the presence of the ECM fungi *Paxillus involutus* and *Pisolithus arhizus* seems to be favourable to *Pinus pinaster* roots. Münzenberger et al. (1997) observed an increase in laccase activity in mycorrhized plants, which could induce polymerisation of soluble phenolics that would also account for their decreasing. Once more, a higher compatibility between *Pisolithus arhizus* and *Pinus pinaster* was verified, since it was not observed a significant increase in the concentration of those secondary metabolites in this fungus.

Antioxidant properties

Antioxidant properties of phenolic compounds play a vital role in the defence mechanisms of biological systems (Macheix and Fleuriert 1998). In order to understand the effects of the presence of plant roots in the antioxidant properties of ECM fungi mycelia, three assays were performed: DPPH scavenging activity, reducing power and β-carotene bleaching inhibition. The results are given in Table 2.

Analysing the control samples for the assay with *Paxillus involutus* it was possible to conclude that its mycelium and roots of *Pinus pinaster* revealed similar DPPH scavenging effects (EC_{50} values without significant statistical differences; $p < 0.05$), and much higher than the recovered *Paxillus involutus* culture medium. The results for *Pinus pinaster* roots were similar in the presence and in the absence of *Paxillus involutus*. Likewise, the results for *Paxillus involutus* were similar in the presence and in the absence of *Pinus pinaster* (Table 2). Therefore, there were no effects of ECM establishment in fungi (*Paxillus involutus*) or in plant scavenging effects. In the assay with *Pisolithus arhizus*, it was observed that roots of *Pinus pinaster* revealed higher DPPH scavenging effects (lower EC_{50} values) than *Pisolithus arhizus* mycelium and its recovered culture medium. However, the scavenging activity for *Pinus pinaster* roots and *Pisolithus arhizus* when in co-culture were similar to the ones observed in isolated samples (Table 2), also suggesting that ECM synthesis had not relevant effect on this activity.

The results obtained in the analysis of the control samples showed that the mycelium of *Paxillus involutus* possessed the highest reducing power (lowest EC_{50} values), followed

by *Pinus pinaster* roots, and by the recovered *Paxillus involutus* culture medium. The reducing power of *Pinus pinaster* roots was higher in the absence of *Paxillus involutus*; likewise, the reducing power of *Paxillus involutus* was higher in the absence of the plant (Table 2). Therefore, the association seems to decrease the reducing power of plant or fungi (*Paxillus involutus*).

Considering the assay with *Pisolithus arhizus*, it was possible to observe that roots of *Pinus pinaster* revealed higher reducing power (lower EC_{50} value) than *Pisolithus arhizus* and its recovered culture medium. The reducing power of *Pinus pinaster* roots was lower in the presence of symbiont and the reducing power of *Pisolithus arhizus* was also lower in the presence of *Pinus pinaster* (Table 2).

In the assay with *Paxillus involutus* the mycelium revealed the highest β-carotene bleaching inhibition similar to *Pinus pinaster* roots (without significant statistical differences; $p<0.05$), whilst the β-carotene bleaching of *Pinus pinaster* roots and *Paxillus involutus* in co-culture was lower (Table 2). Regarding *Pisolithus arhizus*, both the mycelia and the recovered culture medium showed lower β-carotene bleaching than the roots of *Pinus pinaster*. In this case, the bleaching inhibition of *Pinus pinaster* roots was higher in the presence of *Pisolithus arhizus*, whereas the bleaching inhibition of the fungus was lower in the presence of symbiont (Table 2).

Overall, the interaction between *Paxillus involutus* and *Pinus pinaster* had no major antioxidative benefits for both species since their reducing power decreased, the scavenging activity was similar and the β-carotene bleaching inhibition capacity increased. An increase in total phenolic content was found in *Paxillus involutus* in the presence of *Pinus pinaster*. Furthermore, the levels of *p*-hydroxybenzoic and cinnamic

acids increased in *Pinus pinaster* roots in the presence of *Paxillus involutus*. Otherwise, the association between *Pisolithus arhizus* and *Pinus pinaster* seems to generically decrease the antioxidant effects of both symbionts, despite the increase in *p*-coumaric and cinnamic acids in the ECM fungi, accounting for the hypothesised reduced oxidative stress of the mycorrhizal association induction for both partners.

Acknowledgments

The authors are grateful to the Foundation for Science and Technology (Portugal) and COMPETE/QREN/UE for financial support through the research project PTDC/AGR-ALI/110062/2009 and L. Barros grant (SFRH/BPD/4609/2008). The GIP-USAL is financially supported by the Spanish *Ministerio de Ciencia e Innovación* through the *Consolider-Ingenio 2010* Programme (FUN-C-FOOD, CSD2007-00063), and *Junta de Castilla y León* (Grupo de Investigación de Excelencia, GR133).

References

Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA (2004) Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem 84:551–562

Baptista P, Martins A, Pais MS, Tavares RM, Lino-Neto T (2007) Involvement of reactive oxygen species during early stages of ectomycorrhiza establishment between *Castanea sativa* and *Pisolithus tinctorius*. Mycorrhiza 17:185–193

Barros L, Dueñas M, Ferreira ICFR, Baptista P, Santos-Buelga C (2009) Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species. Food Chem Toxicol 47:1076–1079

Benguria RL (1985) Mil setas ibéricas. Flash Composition Eds., Bilbao

Ditengou FA, Lapeyrie F (2000) Hypaphorine from the ectomycorrhizal fungus *Pisolithus tinctorius* counteracts activities of indole-3-acetic acid and ethylene but not synthetic auxins in eucalypt seedlings. Mol Plant-Microbe Inter 13:151–158

Ebel J (1986) Phytoalexin synthesis: the biochemical analysis of the induction process. Annu Rev Phytopathol 24:235–264

Ferreira ICFR, Barros L, Abreu RMV (2009) Antioxidants in wild mushrooms. Cur Med Chem 16:1543–1560

Finlay R, Lindahl B, Taylor AFS (2008) Responses of mycorrhizal fungi to stress. In: Avery SV, Stratford M and van West P. (Ed.) Stress in Yeasts and Filamentous Fungi. United Kingdom

Gutierrez RMP, Luna HH, Garrido SH (2006) Antioxidant activity of *Tagates erecta* essential oil. J Chil Chem Soc 51:883–886

Haars A, Chet J, Hüttermann A (1981) Effect of phenolic compounds and tannin on growth and laccase activity of *Fomes annosus*. Eur J Plant Pathol 11:6–76

Heleno SA, Barros L, Sousa MJ, Martins A, Ferreira ICFR (2010) Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity. Food Chem 119:1443–1450

Heller G, Adomas A, Li G, Osborne J, Zyl L, Sederoff R, Finlay RD, Stenlid J, Asiegbu FO (2008) Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus *Laccaria* bicolor. BMC Plant Biol 8:19

Karadag A, Ozcelik B, Saner S (2009) Review of methods to determine antioxidant capacities. Food Anal Methods 2:41–60

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275

Lindeberg G (1948) On the occurrence of polyphenol oxidase in soil inhabiting basidiomycetes. Physiol Plant 1:196–205

Macheix J, Fleuriet A (1998) Flavonoids in health and disease. Marcel Dekker, New York

Magalhães LM, Segundo MA, Reis S, Lima JLFC (2008) Methodological aspects about in vitro evaluation of antioxidant properties. Anal Chimica Acta 613:1–19

Malajczuk N, Molina R, Trappe JM (1984) Ectomycorrhiza formation in Eucalyptus. II. The ultrastructure of compatible and incompatible mycorrhizal fungi and associated roots. New Phytol 96:43–53

Martin F, Duplessis S, Ditengou F, Lagrange H, Voiblet C, Lapeyrie F (2001) Developmental cross talking in the ectomycorrhizal symbiosis: signals and communication genes. New Phytol 151:145–154

Marx DH (1969) The influence of ectotrophic fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathol 59:153–163

Molina R, Trappe J (1984) Mycorrhiza Management in Bareroot Nurseries. In: Duryea ML, Landis TD (Eds.) Forest Nursery Manual: Production of Bareroot Seedlings. M. Nijhoff/Dr W. Junk Publishers. The Hague/Boston/Lancaster, for Forest Research Laboratory, Oregon State University. Corvallis

Münzenberger B, Heilemann J, Strack D, Kottke I, Oberwinkler F (1990) Phenolics of mycorrhizas and non-mycorrhizal roots of Norway spruce. Planta 182:142–148

Münzenberger B, Kottke I, Oberwinkler F (1995) Reduction of phenolics in mycorrhizas of *Larix decidua* Mill. Tree Physiol 15:191–196

Münzenberger B, Otter T, Wüstrich D, Polle A (1997) Peroxidase and laccase activities in mycorrhizal and non-mycorrhizal fine roots of Norway spruce (*Picea abies*) and larch (*Larix decidua*). Can J Bot 78:932–938

Mysore KS, Ryu C-M (2004) Nonhost resistance: how much do we know? Trends Plant Sci 9:97–104

Olsen RA, Odham G, Lindeberg G (1971) Aromatic substances in leaves of *Populus tremula* as inhibitors of mycorrhizal fungi. Physiol Plant 25:122–129

Podila GK (2002) Signalling in mycorrhizal symbioses-elegant mutants lead the way. New Phytol 154:541–551

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

Wacker TL, Safir GR, Stephens CT (1990) Effects of ferulic acid on *Glomus fasciculatum* and associated effects on phosphorus uptake and growth of asparagus (*Asparagus officinalis* L.). J Chem Ecol 16:901–909

Table 1 Phenolic acids and cinnamic acid. In each column different letters mean significant differences between results ($p < 0.05$).

Samples		Protocatechuic acid (μg/g extract)	*p*-Hydroxybenzoic acid (μg/g extract)	*p*-Coumaric acid (μg/g extract)	Cinnamic acid (μg/g extract)
Paxillus involutus	Mycelium	25.98 ± 0.38	33.67 ± 0.43 c	nd	nd
	Culture medium	nd	22.90 ± 3.32 d	nd	nd
Pisolithus arhizus	Mycelium	nd	27.83 ± 1.40 c	nd	nd
	Culture medium	nd	14.07 ± 1.05 e	nd	nd
Pinus pinaster	Roots	nd	51.02 ± 6.01 b	99.73 ± 1.29 a	19.70 ± 0.24 c
Paxillus involutus +	Mycelium	nd	28.16 ± 0.59 c	nd	nd
Pinus pinaster	Roots	nd	80.70 ± 5.84 a	85.29 ± 0.12 b	110.95 ± 1.48 a
Pisolithus arhizus +	Mycelium	nd	nd	58.98 ± 0.32 c	10.85 ± 0.08 d
Pinus pinaster	Roots	nd	45.26 ± 1.49 b	nd	30.50 ± 0.02 b

nd- not detected.

Table 2 Extraction yield, phenolic content and antioxidant activity EC_{50} values. In each column different letters mean significant differences between results ($p < 0.05$).

Samples		Extraction yield [a] (%)	Total phenolics (mg GAE/g extract)	DPPH scavenging activity (mg/ml)	Reducing power (mg/ml)	β-carotene bleaching inhibition (mg/ml)
Paxillus involutus	Mycelium	16.28 ± 0.71	59.47 ± 3.37 b	1.15 ± 0.07 e	0.65 ± 0.03 h	2.21 ± 0.12 d
	Culture medium	2.27 ± 0.08	1.01 ± 0.07 e	99.62 ± 16.78 a	28.56 ± 0.12 a	9.85 ± 0.93 b
Pisolithus arhizus	Mycelium	24.94 ± 2.81	9.48 ± 0.67 d	17.29 ± 0.29 dc	7.29 ± 0.62 d	2.49 ± 0.18 d
	Culture medium	0.95 ± 0.05	2.36 ± 0.07 e	38.56 ± 1.09 b	11.97 ± 0.01 b	4.39 ± 0.05 c
Pinus pinaster	Roots	23.43 ± 1.23	23.24 ± 0.59 c	3.95 ± 0.28 e	1.47 ± 0.06 f	2.26 ± 0.15 d
Paxillus involutus +	Mycelium	12.66 ± 1.09	71.27 ± 6.49a	3.54 ± 0.05 e	0.98 ± 0.02 g	0.56 ± 0.00 e
Pinus pinaster	Roots	26.38 ± 1.78	10.56 ± 0.56 d	9.11 ± 0.66 de	2.96 ± 0.05 e	0.48 ± 0.00 e
Pisolithus arhizus +	Mycelium	65.58 ± 5.65	8.61 ± 0.38 d	19.26 ± 1.03 c	9.79 ± 0.42 c	26.99 ± 1.94 a
Pinus pinaster	Roots	25.74 ± 1.45	11.06 ± 0.84 d	5.27 ± 0.14 e	2.79 ± 0.01 e	0.59 ± 0.00 e

[a]Calculated by the ratio extract weight/dry weight, and then converted to percentage.

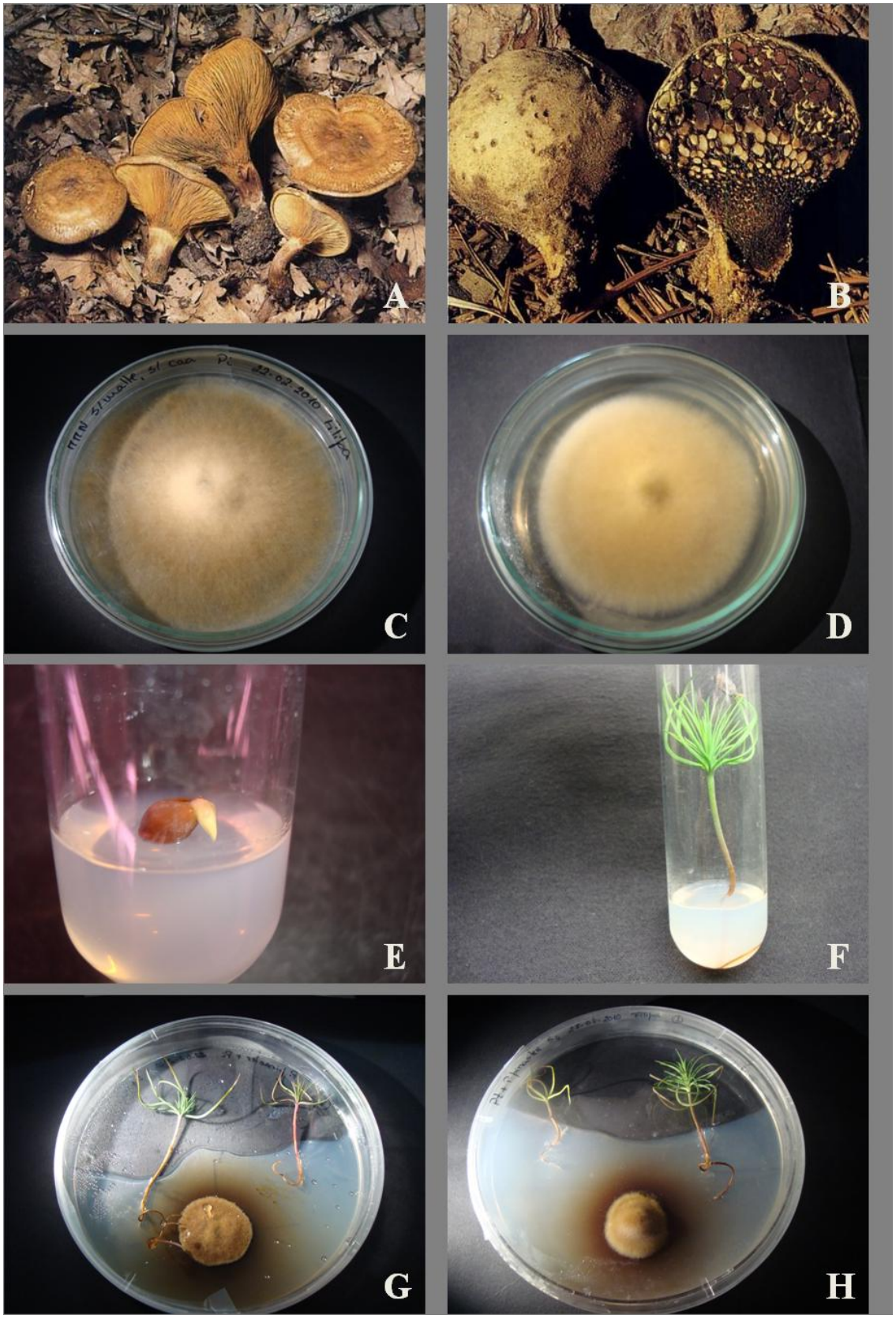
A
B
C
D
E
F
G
H

Fig. 1 A. *Paxillus involutus* fruiting body; B. *Pisolithus arhizus* fruiting body; C. *Paxillus involutus* mycelium; D. *Pisolithus arhizus* mycelium; E. *Pinus pinaster* seeds under germination; F. *Pinus pinaster* seedlings; G - *Paxillus involutus* mycelium - *Pinus pinaster* seedlings mycorrhizal induction *in vitro*; H. *Pisolithus arhizus* mycelium- *Pinus pinaster* seedlings mycorrhizal induction *in vitro*.

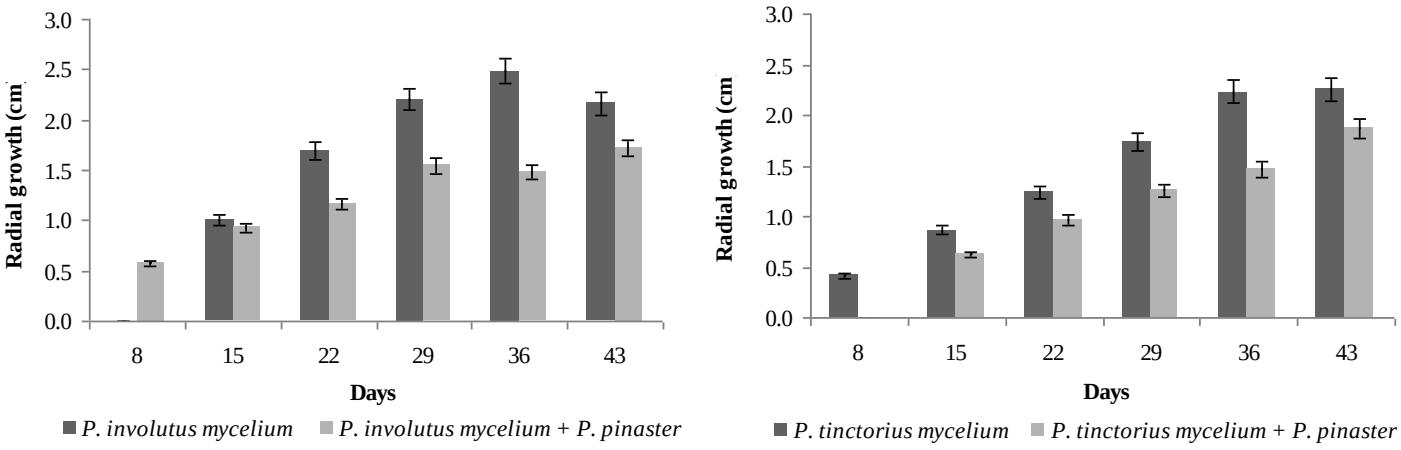

Fig. 2 Mean weekly radial growth of *Pisolithus arhizus* and *Paxillus involutus* in the presence and in the absence of *Pinus pinaster*.