Effect of the mycorrhizal symbiosis time in the antioxidant activity of fungi and *Pinus pinaster* roots, stems and leaves

FILIPA S. REIS,[a,b] ISABEL C.F.R. FERREIRA[a,b,*], 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*.

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

ABSTRACT

The ectomycorrhizal (ECM) symbiosis that develops between the roots of host trees and the soil ECM fungi is an important factor towards the survival, health and growth of these trees, as it stimulates their water and nutrient uptake. Ectomycorrhizal colonization can result in the deposition of phenolic compounds in peripheral cortex cells and a similar answer can be recognized as one way of plant defense against pathogenic infections. The aim of the present work was the evaluation of antioxidant potential of the ectomycorrhizal fungi, *Paxillus involutus* and *Pisolithus arhizus*, in presence and absence of the symbiont – *Pinus pinaster* –, in response to the symbiotic association, under different contact periods (45 days and 48 h). Phenolic contents in mycelia, culture media and plant leaves, stems and roots were determined by *Folin-Ciocalteu* assay, and their antioxidant properties were evaluated by three *in vitro* assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power and lipid peroxidation inhibition through β-carotene bleaching inhibition. Comparing the response of the symbionts to the host species, *Pinus pinaster* benefited most with the association, because it generally decreases the phenolics content and the antioxidant activity values in the presence of both ectomycorrhizal fungi along time. *Pisolithus arhizus* also decreased, in general, its phenolics levels and antioxidant properties, alongside with *Pinus pinaster*, unlike *Paxillus involutus* that did not have the same response as it increases its content of phenolics and some of its values of antioxidant activity. These results can be considered as an hypothetical signal of a symbiotic differential compatibility of mycorrhizal fungi for a host.

***Keywords*:** Ectomycorrhizal fungi; *Pinus pinaster*; symbiosis; contact period; antioxidant potential

1. Introduction

Along the time, fungi won a wide range of *habitats*, fulfilling important roles in diverse ecosystems (Dix and Webster, 1995). Their environmental role is that of recycling, having equally important ecological roles as saprophytic, mutualistic symbionts, parasites or hyperparasite (Webster and Weber 2007; Hanson, 2008). Some fungi attack plants, insects and mammals as pathogens, while others are saprophytic and grow on dead matter. Some live in positive symbiotic relationship with a host organism. Therefore, there are mycorrhizal fungi that are associated with plant roots and facilitate the absorption of nutrients by this symbiont (Hanson, 2008) in exchange for their carbon nutrition. In fact, the establishment and growth of most plants requires, or are enhanced by the presence of specialized fungi that form associations in the soil with their roots. Nutrient flow is a reciprocal process in most mycorrhizal associations (Carlile *et al*., 2001). The symbiosis is based on the beneficial exchange of reduced carbon from the plant and mineral nutrients, especially phosphate and nitrogen (Toussaint *et al*., 2004; Jin *et al*., 2005) as well as water from the fungus (Smith and Read, 1997). This intimate association is accompanied with an increased resistance to abiotic stress and to root pathogens (Marx, 1969; Stenström *et al*., 1997; Singh *et al*., 2000; Gianinazzi-Pearson *et al*., 2006; Liu *et al*., 2007; Martins *et al*., 2010). The mycelia of some mycorrhizal fungi can form an exterior sheath covering the roots of plants and are called *ectomycorrhizal* mantle (Stamets, 2000). The most studied ectomycorrhizas (ECM) are those of pine (*Pinus*) and beech (*Fagus*) (Carlile *et al*., 2001).

Due to the large physiological and morphological variability between the ECM fungi, we can expect changes in their behavior during the establishment of symbiosis, causing responses in plants ranging from a very efficient symbiosis to the lack of effects or the

occurrence of deleterious effects on growth (Harley and Smith, 1983). It remains unclear at what point is the recognition between the symbionts and determination of incompatibility or compatibility of the ectomycorrhizal symbiosis, but probably the initial stages of interaction are the main steps for developing an efficient symbiosis (Malajczuk *et al*., 1982). Although there is little evidence of specificity between host plants and mycorrhizal fungi, it appears that there may be more or less compatibility in ectomycorrhizal interactions (Baptista *et al*., 1999). Up and down regulation of several genes belonging to stress or defense responses underlines the complex nature of the ectomycorrhizal interaction (Heller *et al*., 2008). Throughout the natural world there is a chemical language between the fungus and its host which determines the nature of this relationship (Hanson, 2008). ECM symbiosis between the mycelia and the roots of some plants could have important effects in the antioxidants production of both partners. There is increasing evidence that secondary compounds play a significant role in the various interactions occurring between plants and their natural environment (Harborne, 1993). In this respect, antioxidants such as phenolics are known to be of major importance in pathogenic interactions between plants and fungi (Matern and Kneusel, 1988; Dixon *et al*., 1994). Phenolics are known to act as reaction factors; they are reported to be highly reactive on oxidation and may result in the formation of substances highly toxic to pathogens (Mahadevan, 1966; Patil and Dimond, 1967).

Herein, *in vivo* associations between ECM fungi and specific host plants were mimetized by *in vitro* experiments with mycelium of the ECM fungi *Paxillus involutus* and *Pisolithus arhizus*, and germinated *Pinus pinaster* plants, under different contact periods. Therefore, the influence of contact time in the antioxidant potential of both

symbionts (fungi mycelium, and plant leaves, stems and roots) was evaluated and compared.

2. Materials and methods

2.1. In vitro production of mycelia and germination of Pinus pinaster seeds

Mycelia of *Paxillus involutus* (Batsch) Fr. and *Pisolithus arhizus* (Scop.) Rauschert were isolated from sporocarps (collected in Bragança, Portugal) on solid modified Melin-Norkans medium (MMNm) 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. Both mycelium and culture medium were weighted separately to obtain the fresh biomass (fw).

Pinus pinaster (Aiton) seeds (obtained in CENASEF, Centro Nacional das Sementes Florestais, Portugal) were germinated in agar:water 0.9% in tubes (3 cm diameter) after washes with tap water, superficial disinfection in sodium hypochloride (10 min), several washes with sterile water, brief contact with hydrogen peroxide and subsequent washes with sterile water. After inoculation, the seeds were left in the dark at 25ºC for 48 h, and then exposed to light until the mycorrhization assays.

2.2. Induction of the mycorrhizal symbiosis

The induction of the mycorrhizal symbiosis was performed in two different assays.

Assay A: Two *Pinus pinaster* plants obtained after germination were introduced in Petri dishes (13 cm diameter) with MMNm. Mycelia of *Paxillus involutus* or *Pisolithus*

arhizus were inoculated between the two plants (Fig. 1A and 1B). Inoculated plants were incubated at 23ºC/18ºC for day and night photoperiods (16 h/8 h), respectively, in a culture chamber (Gro-Lux, Sylvania) with Daylight lamps (Phillips). After 45 days of growth, mycelium and plants were recovered from the medium. Mycelium, plants and culture medium were weighted separately to obtain the fresh biomass (fw), and stored at –40ºC for further analyses.

Assay B: Two inoculums of mycelia were left to grow for 20 days in Petri dishes (9 cm diameter) with MMNm. After this period, three germinated *Pinus pinaster* plants were added to the grown mycelia (Fig. 1C and 1D) in order to promote symbiosis for a period of 48 h.

Petri dishes inoculated only with *Paxillus involutus*, *Pisolithus arhizus* and *Pinus pinaster* were used as controls in each assay.

2.3. Standards and reagents

All the solvents were of analytical grade purity; methanol was supplied by Lab-Scan (Lisbon, Portugal). The standards used in the antioxidant activity assays: trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic 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 were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.4. Preparation of the extracts

Each sample (~0.5 g for mycelia and plants; 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 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) to dryness, and redissolved in methanol for antioxidant activity assays.

2.5. In vitro antioxidant activity assays

In vitro assays already described by the authors (Reis *et al.*, 2011a) were used to evaluate the antioxidant activity of the samples.

2.5.1. DPPH radical-scavenging activity

This assay was performed in 96-well microtiter plates using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). 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 (6×10^{-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.

2.5.2. Reducing power

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, as also 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.

2.5.3. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40ºC under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 ml) were added to the flask with vigorous 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). β-Carotene

bleaching inhibition was calculated using the following equation: (β-carotene content after 2h 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.

2.5.4. 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.

2.6. Statistical analysis

For each one of the species three different samples were analysed 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.

3. Results and discussion

In order to verify the chemical response time and to evaluate the symbiosis process, *P. pinaster* plants and ECM fungi were inoculated and left in contact for two different periods: 48 h and 45 days. After each period different parts of both symbionts were analyzed: fungi mycelium, plant roots, stems and leaves, and culture medium where the symbiosis was established.

The results of antioxidant activity obtained after 45 days are shown in Table 1. *Pinus pinaster* was the species that revealed the highest phenolics content (leaves: 72.65 mg GAE/g extract), followed by *Paxillus involutus* (59.47 mg GAE/g extract) and finally *Pisolithus arhizus* (9.48 mg GAE/g extract). The same results were obtained in antioxidant activity assays showing *Pinus pinaster* samples, especially the leaves, the lowest EC_{50} values and *Pisolithus arhizus* the highest ones. These results suggest that *Pinus pinaster* seems to have higher antioxidant properties than the studied ectomicorrhizal species. In fact, *Pinus pinaster* has been reported to contain important antioxidants such as procyanidin oligomers and bioflavonoids with important activities against reactive oxygen species, enhancing the antioxidant defences (Pinelo *et al.*, 2004).

Culture media revealed the lowest concentrations of phenolics and the lowest antioxidant activity which leads us to believe that there is not a significant release of antioxidant compounds to the medium.

Along the 45 days of contact, total phenolics increased in *Paxillus involutus* mycelium, did not change in *Pisolithus arhizus* mycelium and decreased in *Pinus pinaster* (especially in roots). The symbiosis did not affect the antioxidant properties of the studied species with regard to free radical scavenging activity, while decreased the

reducing power of the plant (mainly in roots) and *Pisolithus arhizus*, and the ability to inhibit β-carotene bleaching in the latter species. The only exceptions were *Paxillus involutus* and *Pinus pinaster* roots for their ability to inhibit β-carotene bleaching that increased in symbiosis. Plant stems and leaves do not exhibit significant differences as the trials were too short in time to be conclusive on that regard although some stem parameters behave similarly to roots, mainly phenol contents.

After 48 hours of contact, the species *Paxillus involutus* significantly reduced the concentration of total phenolics maintaining, somehow, its antioxidant properties (Table 2). Regarding the effects of this symbiont in the plant, there were no significant differences.

In symbiosis with *Pisolithus arhizus*, the plant seems not to induce significant effects on its antioxidant properties and a reduction in mycelium phenolics content was observed. Analyzing the effects of this fungus in the plant, a considerable increase in the concentration of phenolics in roots of *Pinus pinaster* was noticed, and there was not a great variability in the antioxidant properties. Thus, it can be concluded that a chemical response is illustrated in this interaction mainly by the plant, especially in the first 48 hours, while it has not identified the fungus as non-pathogenic.

Overall, an interaction established for a period of 48 hours did not reveal the effects that occurred over time (45 days). Therefore, regarding the mechanisms of oxidative stress in ectomycorrhizal associations with the two studied species (*Paxillus involutus* and *Pisolithus arhizus*), the response of the fungi and the plant to the contact with the host are somehow specific, revealing different forms of mutual recognition. If *Pisolithus arhizus* seems to provoke an initial reaction of stress (48 h) with increasing content of

phenolics in the host roots, after 45 days, this effect disappeared decreasing these compounds (Fig. 2). However, in the fungus, the levels of phenolics are already lower after 48 hours, showing a rapid response to the presence of the host, which is maintained throughout the association. *Paxillus involutus* showed the opposite answer; an initial decrease was observed regarding phenolic content, followed by a further increase (Fig. 2). Equivalent effects were observed in antioxidant properties, which generally decreased in plant and fungus in the case of *Pisolithus arhizus* (Fig. 2) while did not reduce or even increased in *Paxillus involutus* (Fig. 2). We hypothesize that these may correspond to differences in compatibility between these two species of fungi and its host *Pinus pinaster*, once the intensity of phenolic deposition in response to colonization by ectomycorrhizal-forming fungi and the ability of an ectomycorrhizal fungus to tolerate phenolics may be associated with the symbiotic compatibility of fungus and host associates (Molina, 1981; Malajczuk *et al*., 1982; Molina and Trappe, 1982). However, this hypothesis needs confirmation with other contact times and other fungi species.

With our study we can conclude that ectomycorrhizal fungi (especially *Pisolithus arhizus*) could help in keeping their host trees, namely *Pinus pinaster*. It may be quite important for reforestation programs, once *Pinus* is one of the most ecologically and economically significant tree genus in the world (Richardson, 2000), and this work can contribute to improve their *habitats*.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (Portugal) and COMPETE/QREN/EU for financial support through the research project PTDC/AGR-ALI/110062/2009.

References

Baptista, M.J., da Gloria, B.A, Pascholati, S.F, Krugner, T.L. 1999. Produção de compostos fenólicos durante a infecção ectomicorrízica por dois isolados de *Pisolithus tictorius* em *Eucalyptus urophylla in vitro*. Revta Brasil Bot. 22, 309–315.

Carlile, M.J., Watkinson, S.C., Gooday, G.W. 2001. The Fungi. Academic Press, 2nd Ed, Hungary.

Dix, N.J., Webster, J. 1995. Fungal Ecology. Chapman & Hall, London.

Dixon, R.A., Harrison, M.J, Lamb, C.J. 1994. Early events in the activation of plant defenses responses. Annu Rev. Phytopathol. 32, 479–501.

Gianinazzi-Pearson, V., Maldonado-Mendoza, I., Lopez-Meyer, M., Weidmann, S., Harrison, M.J. 2006. Arbuscular mycorrhiza. In: The Medicago truncatula handbook. http://www.noble.org.MedicagoHandbook/.

Hanson, J.R. 2008. The Chemistry of Fungi. Royal Society of Chemistry, United Kingdom.

Harborne, J.B. 1993. Introduction to Ecological Biochemistry. Academic Press, 4th Ed, London.

Harley, J.L., Smith, S.E. 1983. Mycorrhizal symbiosis. Academic Press, United Kingdom.

Heller, G., Adomas, A., Li, G., Osborne, J., Zyl, L., Sederoff, R., Finlay, R.D., Stenlid, J., Asiegbu, F.O. 2008. Transcriptional analysis of *Pinus sylvestris* roots

challenged with the ectomycorrhizal fungus *Laccaria bicolor*. BMC Plant Biol. 8,19.

Jin, H., Pfeffer, P.E., Douds, D.D., Piotrowski, E., Lammers, P.J., Shachar-Hill, Y. 2005. The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytol. 168,687–696.

Liu, J., Maldonado-Mendoza, I., Lopez-Meyer, M., Cheung, F., Town, C.D., Harrison, M.J. 2007. Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. Plant J. 50, 529–544.

Mahadevan, A. 1966. Biochemistry of infection and resistance. Phytopathol. 57, 96–99.

Malajczuk, N., Molina, R., Trappe, J.M. 1982. Ectomycorrhiza formation in *Eucalyptus*. I. Pure culture synthesis, host specificity and mycorrhizal compatibility with *Pinus radiata*. New Phytol. 91, 467–482.

Martins, A. 2010. Mycorrhizal inoculation of chestnut seedlings: Effect on survival and growth after transplantation. Acta Hort. (ISHS) 866, 325-334.

Marx, D.H. 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.

Matern, U., Kneusel, R.E. 1988. Phenolic compounds in plant disease resistance. Phytoparasitica 16, 153–170.

Molina, R. 1981. Ectomycorrhizal specificity in the genus *Alnus*. Can. J. Bot. 59, 325-334.

Molina, R., Trappe, J.M., 1982. Patterns of ectomycorrhizal host specificity and potential among Pacific Northwest conifers and fungi. For Sci. 28, 423–458.

Patil, S.S., Dimond, A.E. 1967. Inhibition of Verticillium polygalacturonase by oxidation products of polytotal phenols. Phytopathol. 57, 492–496.

Pinelo, M., Rubilar, M., Sineiro, J., Núñez, M.J. 2004. Extraction of antioxidant phenolics from almond hulls (*Prunus amygdalus*) and pine sawdust (*Pinus pinaster*). Food Chem. 85, 267–173.

Reis, F.S., Ferreira, I.C.F.R., Barros, L., Martins, A. 2011a. A comparative study of tocopherols composition and antioxidant properties of in vivo and in vitro ectomycorrhizal fungi. LWT–Food Sci. Technol. 44, 820-824.

Reis, F.S., Ferreira, I.C.F.R., Barros, L., Santos-Buelga, C., Martins, A. 2011b. Mycorrhizal induction of phenolic compounds and antioxidant properties of fungi and seedlings during the early steps of symbiosis. *Chemoecol*. DOI: 10.1007/s00049-011-0079-1.

Richardson, D.M. 2000. Ecology and Biogeography of *Pinus*. Cambridge University Press. United Kingdom.

Singh, R., Adholeya, A., Mukerji, K.G. 2000. Mycorrhiza in control of soil born pathogens. In: Mycorrhizal Biology. Mukerji KG, Singh J Eds. Kluwer Academic/Plenum Publishers 173-196.

Stenström, E., Damm, E., Unestam, T. 1997. Le rôle des mycorrhizes dans la protection des arbres forestiers contre les agents pathogènes du sol. Rev. For Fr. 121- 128.

Smith, S.E., Read, D.J. 1997. Mycorrhizal Symbiosis. Academic Press, 2nd Ed, San Diego.

Stamets, P. 2000. Growing Gourmet and Medicinal Mushrooms. Ten Speed Press, 3rd Ed, California.

Toussaint, J.P., St-Arnaud, M., Charest, C. 2004. Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus Glomus intraradices Schenck & Smith and Ri T-DNA roots of Daucus carota L. in an *in vitro* compartmented system. Can. J. Microbiol. 50, 251–260.

Webster, J., Weber, R.W.S. 2007. Introduction to Fungi. Cambridge University Press, 3rd Ed, United Kingdom.

Table 1. Phenolic content (mg GAE/g extract) and antioxidant activity EC_{50} values (mg/ml) of the studied samples after 45 days of growth. In each column different letters mean significant differences between results ($p < 0.05$).

Species	Sample	Total phenolics	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition
Paxillus involutus	Mycelium[a]	59.47 ± 3.37 c	1.15 ± 0.07 f	0.65 ± 0.03 kj	2.21 ± 0.12 e
	Culture medium[a]	1.01 ± 0.07 i	99.62 ± 16.79 b	28.56 ± 0.12 a	9.85 ± 0.93 c
Pisolithus arhizus	Mycelium[a]	9.48 ± 0.67 h	17.29 ± 0.29 ed	7.29 ± 0.62 d	2.49 ± 0.18 e
	Culture medium[a]	2.36 ± 0.07 i	38.56 ± 1.09 c	11.97 ± 0.01 b	4.39 ± 0.05 d
Pinus pinaster	Roots[a]	23.24 ± 0.59 f	3.95 ± 0.28 f	1.47 ± 0.06 h	2.26 ± 0.15 e
	Stems	31.21 ± 0.34 e	3.58 ± 0.41 f	1.36 ± 0.04 ih	0.59 ± 0.00 fg
	Leaves	72.65 ± 0.76 a	0.77 ± 0.04 f	0.50 ± 0.02 k	0.99 ± 0.21 fg
	Culture medium	3.91 ± 0.21 i	> 200 a	5.20 ± 0.72 e	9.79 ± 0.48 c
Paxillus involutus + *Pinus pinaster*	Mycelium[a]	71.27 ± 6.49 a	3.54 ± 0.05 f	0.98 ± 0.02 ij	0.56 ± 0.00 fg
	Roots[a]	10.56 ± 0.56 h	9.11 ± 0.66 ef	2.96 ± 0.05 g	0.48 ± 0.00 g
	Stems	32.80 ± 0.34 e	1.42 ± 0.12 f	1.26 ± 0.03 ih	0.58 ± 0.02 fg
	Leaves	65.52 ± 3.31 b	1.46 ± 0.04 f	0.68 ± 0.03 kj	0.52 ± 0.00 fg
	Culture medium	2.89 ± 0.09 i	104.57 ± 14.15 b	9.50 ± 1.05 c	28.57 ± 1.11 a
Pisolithus arhizus + *Pinus pinaster*	Mycelium[a]	8.61 ± 0.38 h	19.26 ± 1.03 d	9.79 ± 0.42 c	26.99 ± 1.94 b
	Roots[a]	11.06 ± 0.84 h	5.27 ± 0.14 f	2.79 ± 0.01 g	0.59 ± 0.00 fg
	Stems	49.56 ± 3.08 d	1.20 ± 0.04 f	0.78 ± 0.05 kj	1.53 ± 0.83 fe
	Leaves	73.67 ± 4.13 a	0.70 ± 0.02 f	0.47 ± 0.00 k	0.55 ± 0.00 fg
	Culture medium	19.36 ± 1.09 g	8.94 ± 0.11 ef	4.26 ± 0.30 f	4.65 ± 0.27 d

[a] Reis *et al*., 2011b

Table 2. Phenolic content (mg GAE/g extract) and antioxidant activity EC_{50} values (mg/ml) of the studied samples after 22 days of growth (for ECM fungi) and 48h of interaction fungi-plant. In each column different letters mean significant differences between results ($p < 0.05$).

Species	Sample	Total phenolics	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition
Paxillus involutus	Mycelium	128.40 ± 37.02 a	1.46 ± 0.09 c	0.69 ± 0.03 d	0.89 ± 0.06 hg
	Culture medium	4.87 ± 0.25 ih	38.23 ± 1.00 b	16.78 ± 0.75 b	18.62 ± 1.20 c
Pisolithus arhizus	Mycelium	69.77 ± 3.72 c	3.33 ± 0.20 c	1.35 ± 0.20 d	0.35 ± 0.05 h
	Culture medium	4.19 ± 0.33 ih	41.44 ± 2.72 b	18.87 ± 0.65 b	15.82 ± 1.14 d
Pinus pinaster	Roots	7.93 ± 0.61 ihg	12.11 ± 0.16 c	5.48 ± 0.07 cd	2.26 ± 0.07 g
	Stems	28.83 ± 1.55 fe	4.42 ± 0.15 c	2.14 ± 0.04 d	0.62 ± 0.00 hg
	Leaves	109.36 ± 7.45 b	0.85 ± 0.01 c	0.34 ± 0.01 d	0.28 ± 0.01 h
	Culture medium	0.71 ± 0.06 i	> 75.00 a	> 75.00 a	53.02 ± 4.07 a
Paxillus involutus + *Pinus pinaster*	Mycelium	17.69 ± 1.74 fhg	5.64 ± 0.44 c	2.94 ± 0.09 d	9.53 ± 0.39 e
	Roots	15.81 ± 1.24 ihg	4.40 ± 0.08 c	2.41 ± 0.11 d	4.20 ± 1.48 f
	Stems	37.09 ± 3.24 e	2.29 ± 0.03 c	1.51 ± 0.07 d	0.63 ± 0.05 hg
	Leaves	57.09 ± 3.25 d	2.31 ± 0.06 c	1.06 ± 0.07 d	1.93 ± 0.44 hg
	Culture medium	5.26 ± 0.32 ih	40.69 ± 0.90 b	14.99 ± 0.17 cb	22.14 ± 0.73 b
Pisolithus arhizus + *Pinus pinaster*	Mycelium	23.24 ± 3.26 feg	9.22 ± 0.35 c	4.32 ± 0.10 d	0.56 ± 0.02 hg
	Roots	58.61 ± 4.29 dc	0.78 ± 0.01 c	0.58 ± 0.02 d	0.45 ± 0.00 hg
	Stems	53.76 ± 1.51 dc	1.16 ± 0.02 c	0.87 ± 0.03 d	0.91 ± 0.18 hg
	Leaves	96.85 ± 6.26 b	0.89 ± 0.01 c	0.50 ± 0.01 d	0.26 ± 0.00 h
	Culture medium	12.53 ± 0.69 ihg	14.02 ± 0.27 c	6.58 ± 0.04 cd	1.19 ± 0.02 hg

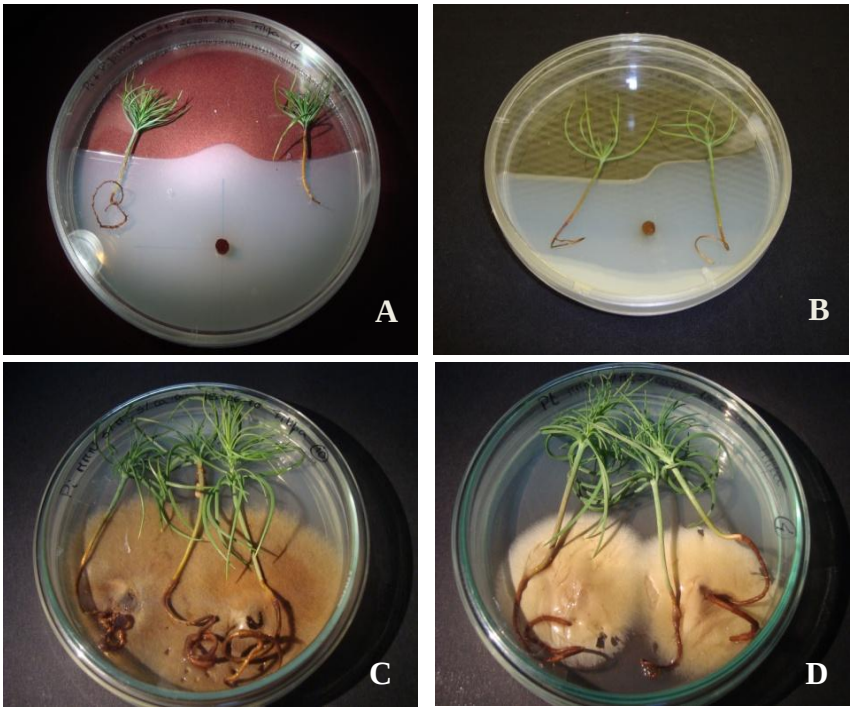

Fig. 1. Assay A: A) *Paxillus involutus* + *Pinus pinaster*. B) *Pisolithus arhizus* + *Pinus pinaster*. Assay B: C) *Paxillus involutus* + *Pinus pinaster*. D) *Pisolithus arhizus* + *Pinus pinaster*.

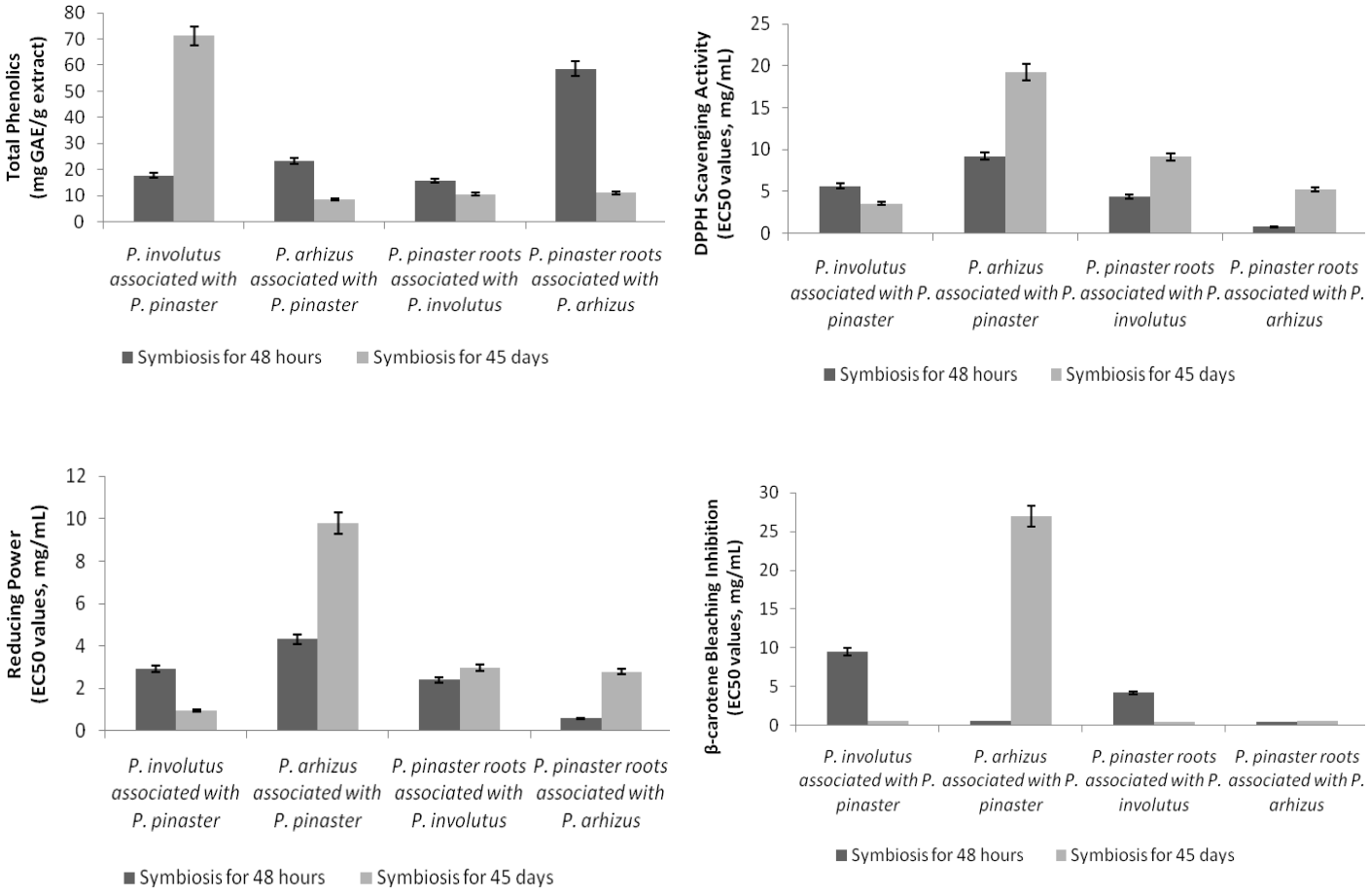

Fig. 2. Phenolic content and antioxidant activity EC_{50} values obtained after 48h and 45 days of contact between fungi and plant roots.