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

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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, *P. 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. *P. arhizus* also decreased, in general, its phenolics levels and antioxidant properties, alongside with *P. pinaster*, unlike *P. 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.

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, 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

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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 P. pinaster seeds

Mycelia of *P. involutus* (Batsch) Fr. and *P. 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; $MgSO_4 \cdot 7H_2O$ 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 25 °C in the dark and subcultured every 4–6 weeks. Both mycelium and culture medium were weighted separately to obtain the fresh biomass (fw).

P. 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 25 °C 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 *P. pinaster* plants obtained after germination were introduced in Petri dishes (13 cm diameter) with MMNm. Mycelia of *P. involutus* or *P. arhizus* were inoculated between the two plants (Fig. 1A and B). Inoculated plants were incubated at 23 °C/18 °C 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 −40 °C 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 *P. pinaster* plants were added to the grown mycelia (Fig. 1C and D) in order to promote symbiosis for a period of 48 h.

Petri dishes inoculated only with *P. involutus*, *P. arhizus* and *P. 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 (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 20 ml of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (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 50 °C 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