

Effect of competitive interactions between ectomycorrhizal and saprotrophic fungi on *Castanea sativa* performance

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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, foliar-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* · Fungal interaction · *Castanea sativa* · Biomass production

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, including 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 influencing the plant nutrients acquisition in different ways (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 (ECM) increase plant growth, by enhancing the absorption of mineral nutrients and water, and increase plant resistance to pathogens and to different environmental stresses (Smith and Read 2008). A beneficial effect of ECM on biological control of larval root herbivores has been also reported (Edda et al. 2010).

In spite of their partial spatial separation along the soil vertical axis, ectomycorrhizal and saprotrophic fungi interact (Leake et al. 2002; Lindahl et al. 2007). Interactions between ECM 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 on natural substrates using a

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microcosm system (Lindahl et al. 1999, 2001; Leake et al. 2001). A range of responses are observed depending on the individual species and their combination, nutrients availability, amount and quality of the carbon substrates from which the fungi grow (Lindahl et al. 1999, 2001; Koide and Kabir 2000; Werner and Zadworny 2003). For example, in pairwise interactions between ECM and saprotrophic fungi, the suppression of either ECM (Shaw et al. 1995; Zadworny et al. 2004) or saprotrophs (Baar and Stanton 2000; Werner et al. 2002; Sharma et al. 2010) have been observed. Moreover, contradictory responses of fungal interactions under natural substrates have been reported. Using a soil microcosm, a clear antagonistic response of ECM (*Suillus variegates* and *Paxillus involutus*) extending from pine seedling roots was detected against the saprotroph *H. fasciculare* extending from wood blocks (Lindahl et al. 1999, 2001). By contrast, in a similar microcosm experiment, Leake et al. (2001) found that the ECM *Suillus bovinus* mycelium vigour was reduced when in contact with the saprotroph *Phanerochaete velutina*. These contradictory results could be partially explained taking into account the differences on the bi-directional translocation of carbon and minerals that occur between ectomycorrhizal and saprotrophic mycelia. Current evidences indicate that this translocation occurs from areas of high nutrient availability to those of high nutrient demand and are independent of mycelial growth (Lindahl et al. 1999, 2001; Leake et al. 2001). However, regarding their antagonist mechanisms, much variation exists among ECM and saprotrophic fungi and even within species.

Taken together, these experiments revealed that saprotrophic and ECM compete with each other for soil nutrients, as well for territory or space. These interactions may result not only in changes on fungal community (by biomass reduction of one or both competitors) but also on community functioning, namely, in nutrients reallocation (Boddy 2000) with consequences for plant growth and health (reviewed by El-Shatnawi and Makhadmeh 2001). Furthermore, the inhibition of ectomycorrhizae formation by saprotrophic fungi, as already observed in some antagonistic interaction studies (Shaw et al. 1995; Lindahl et al. 2001), may cause additional losses of benefits from symbiosis (plant fitness and health). The contradictory responses obtained from different interaction studies using these groups of organisms suggest that their relations are complex and difficult to study and, therefore, are scarcely known.

In this work, it is aimed to assess the effect of saprotrophic (*Hypholoma fasciculare*) and ectomycorrhizal (*Pisolithus tinctorius*) fungi on *C. sativa* growth. These fungal species are commonly present in *C. sativa* orchards in the Trás-os-Montes region (Northeast of Portugal) and are usually found in the same soil (Baptista et al. 2010).

This study intends to provide knowledge on the influence of co-occurring mycelia of *P. tinctorius* and *H. fasciculare* on chestnut seedlings and elucidate their influence on formation and functioning of the ECM symbiosis.

Materials and methods

Biological material

Seeds of *C. sativa* Mill. were harvested in Bragança region orchards. *H. fasciculare* (Huds.) P. Kumm. was isolated from *C. sativa* orchards at Oleiros–Bragança (Northeast Portugal). Fungal isolation was performed on Melin–Norkans (MMN) agar medium at pH 6.6 [0.025 g/L NaCl, 0.25 g/L (NH₄)₂HPO₄, 0.50 g/L KH₂PO₄, 0.050 g/L FeCl₃, 0.50 g/L CaCl₂, 0.15 g/L MgSO₄·7H₂O, 0.10 g/L thiamine, 1.0 g/L casamino acids, 10 g/L malt extract, 10 g/L glucose and 20 g/L agar], 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). *P. 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 25°C, in the dark, being regularly sub-cultured.

Production of *Castanea sativa* seedlings

C. 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 5–10°C, for 2 months. After germination, the radicle tips were removed, to promote root ramification, and seedlings were separately transferred to plastic pots (each with 300 cm³), filled with sterile vermiculite/topsoil/sand (3:1:1, v/v/v) mixture. Seedlings were automatically sprayed during 10 s, every 40 min, and were kept under greenhouse conditions (day/night thermal regime of 23°/18°±2°C, 10 h light/14 h dark photoperiod, and 70±10% relative humidity) for 4 months. Uniform plants were then selected and transplanted to plastic pots of 2 L (two seedlings per pot) filled with the same growth mixture as before. During this process, seedlings were inoculated with fungi.

Fungal inoculation of *Castanea 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 $(\text{NH}_4)_2\text{HPO}_4$ and no malt extract]. Two-week-old suspension cultures maintained in the dark, at 25°C , and without agitation, were used for plant inoculations. At the time of transplanting, plants were inoculated (1) with *P. tinctorius*, (2) with *H. fasciculare*, (3) with *P. tinctorius* and *H. fasciculare* simultaneously (*P. tinctorius* + *H. fasciculare*), or (4) with *P. tinctorius* and 1 month later inoculated with *H. fasciculare* (*P. tinctorius* 30 days + *H. fasciculare*). Inoculations were carried out by transferring 100 mL of fungal suspension culture, previously homogenized by hand-shaking for 3 min, into the planting hole. For *H. fasciculare* inoculation, performed 1 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 about 60 cm from other treatments. Groups of five from all treatments and controls were arranged at random in the same above mentioned greenhouse conditions.

Sampling and analysis of *Castanea sativa* plants

C. sativa plants were harvested 1 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 (millimeters per 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 60°C for 4 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 (centimeters per gram 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: $\text{LWC} = [(\text{leaf fw} - \text{leaf dw}) / \text{leaf dw}] \times 100$ (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 Kjeltex 1030 distilling unit (AOAC 1990).

For the determination of P and K contents, samples were digested using nitric acid and hydrogen peroxide moisture at 200°C 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 milligrams per gram fw.

Assessing the *Pisolithus tinctorius* colonization

Mycorrhizal colonization was evaluated in 15 root samples randomly selected from each treatment. The presence of ECM roots was based on visual recognition of mycorrhizal roots, which are characterized by swollen root tips, presence of the typical *P. tinctorius* mantle of golden color, and by the absence of root hairs. The percentage of colonized roots was determined by estimating the number of colonized lateral roots in the total number of lateral roots of the root system. Five abundance classes of root colonization were considered (0%, 1–25%, 26–50%, 51–75%, and 76–100%).

Data analysis

Data from plant analysis (growth parameters, water and photosynthetic pigment contents, and nutritional status) are presented as the mean of three to 15 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 ($p \leq 0.05$).

Results

Influence of *Hypholoma fasciculare* on *Pisolithus tinctorius* chestnut root 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 1 year after the *P. tinctorius* or *H. fasciculare* inoculation, and *P. tinctorius* + *H. fasciculare* or *P. tinctorius* 30 days + *H. fasciculare* inoculation (Fig. 1). As expected, the formation of mycorrhizae was not detected in plants that have been inoculated only with *H. fasciculare*.

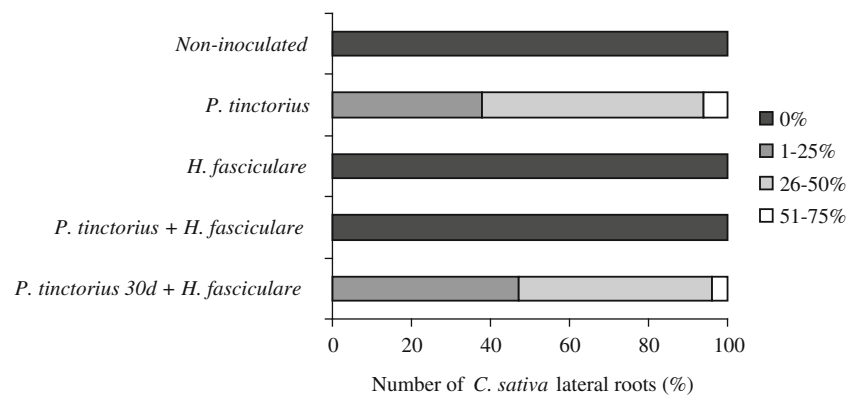


Fig. 1 Effect of the ECM *P. tinctorius* and the saprotrophic *H. fasciculare* on *C. sativa* root mycorrhization. The percentage of *C. sativa* lateral roots displaying *P. tinctorius* mycorrhizae were determined, 1 year after seedlings had been inoculated with *P. tinctorius*, *H. fasciculare*, simultaneously with *P. tinctorius* and *H.*

fasciculare (*P. tinctorius* + *H. fasciculare*), or with *P. tinctorius* followed by *H. fasciculare*, 1 month later (*P. tinctorius* 30 days + *H. fasciculare*). Four abundance classes of root colonization are considered: 0%, 1–25%, 26–50%, and 51–75%

In addition, 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.

Effect of fungal inoculation on *Castanea sativa* growth

The influence of ECM and saprotrophic fungi on *C. sativa* growth was evaluated by the determination of several plant growth parameters 1 year after the first inoculations (Table 1). Plants that were only inoculated with *P. tinctorius* displayed the highest increment in shoot height (about 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* (about 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

Table 1 Effect of *P. tinctorius* and *H. fasciculare* on growth parameters of *C. sativa* seedlings 1 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*, 1 month later (*P. tinctorius* 30 days + *H. fasciculare*)

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±5.5b	45.3±9.2a	0.52±0.22b	0.65±0.13b	8.9±4.6a	3.5±1.6b	0.24±0.15b	209.0±78.8a
<i>P. tinctorius</i>	26.3±14.4a	37.9±5.2b	1.03±0.40a	1.12±0.23a	4.3±1.6b	5.1±2.0a	0.72±0.39a	181.1±44.8a
<i>H. fasciculare</i>	16.7±9.5b	44.8±8.6a	0.75±0.28ab	0.94±0.37ab	6.0±4.2ab	4.4±2.4ab	0.45±0.26b	184.4±54.7a
<i>P. tinctorius</i> + <i>H. fasciculare</i>	13.4±7.7b	46.4±9.2a	0.62±0.06b	0.92±0.20ab	5.5±3.9ab	4.3±1.9ab	0.37±0.21b	194.7±70.6a
<i>P. tinctorius</i> 30 days + <i>H. fasciculare</i>	17.5±8.9ab	40.8±9.1ab	0.89±0.56ab	0.98±0.38a	5.3±2.2ab	4.6±1.9ab	0.48±0.52ab	228.4±96.6a

Means ± SD ($n=15$) are shown. In each column, different letters mean significant differences ($p \leq 0.05$)

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.

Effect of fungal 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.

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 4-month-old *C. sativa* seedlings inoculated with selected ECM or saprotrophic fungi or in combination of both. The fungal species, *P. tinctorius* and *H. 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 *P. 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

Table 2 Effect of *P. tinctorius* and *H. fasciculare* on photosynthetic pigments of *C. sativa* leaves, 1 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*, 1 month later (*P. tinctorius* 30 days + *H. fasciculare*)

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

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*≤0.05)

Table 3 Effect of *P. tinctorius* and *H. fasciculare* on N, P, and K content of leaves of *C. sativa* plants, 1 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*, 1 month later (*P. tinctorius* 30 days + *H. fasciculare*)

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

Means ± SD ($n=3$) are shown. In each column different letters mean significant differences ($p\leq 0.05$)

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. Moreover, 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 4-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 favorable 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 in 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 in 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 *S. variegatus* or *P. 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* 30 days + *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* 30 days + *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.

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