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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, -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, 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 by using a microcosm system (Lindahl et al. 1999; Leake et al. 2001; Lindahl 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; Koide and Kabir 2000; Lindahl et al. 2001; 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.

Also, contradictory responses of fungal interactions under natural substrates have been reported. Using a soil microcosm, a clear antagonistic response of ECM (S*uillus 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 occurs 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; Leake et al. 2001; Lindahl 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 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 *Castanea 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 *Castanea sativa* Mill. were harvested in Bragança region orchards. *Hypholoma fasciculare* (Huds.) P. Kumm. was isolated from *Castanea sativa* orchards at Oleiros – Bragança (Northeast Portugal). Fungal isolation was performed on Melin-Norkans (MMN) agar medium at pH 6.6 [NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casamino acids 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L], following Brundrett et al. (1996). The identity of the fungal isolate was molecularly confirmed by the amplification and sequencing of the internal transcribed spacer region (ITS), using the universal primers *ITS1* and *ITS4* (White et al. 1990). *Pisolithus tinctorius* (Pers.) Coker & Couch (isolated 289/Marx) was obtained from the University of Tübingen. This fungus has been used for mycorrhizal formation in seedlings of *C. sativa* (Martins et al. 1997; Martins 2004). Both strains were maintained in MMN agar medium at 25ºC, in the dark, being regularly sub-cultured.

Production of Castanea sativa *seedlings*

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

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 $(NH_4)_2HPO_4$, and no malt extract]. Two-week-old suspension cultures maintained in the dark, at 25ºC, and without agitation, were used for plant inoculations. At the time of transplanting, plants were inoculated (i) with *P. tinctorius*, (ii) with *H. fasciculare*, (iii) with *P. tinctorius* and *H. fasciculare* simultaneously (*P. tinctorius* + *H. fasciculare*), or (iv) with *P. tinctorius* and one month later inoculated with *H. fasciculare* (*P. tinctorius* 30d + *H. fasciculare*). Inoculations were carried out by transferring 100 mL of fungal suspension culture, previously homogenized by hand-shaking for 3 minutes, into the planting hole. For *H. fasciculare* inoculation, performed one month after *P. tinctorius* inoculation, the suspension culture was introduced into a hole made at the root system level. Controls were performed

148 using 100 mL of sterile culture medium. For each treatment and for control 15 pots
149 were prepared, comprising a total of 30 plants per treatment. To reduce the risks of
150 cross contamination, five pots of each treatment were grouped together and kept at a
151 distance of *c.* 60 cm from other treatments. Groups of five from all treatments and
152 controls were arranged at random in the same above-mentioned greenhouse conditions.
153
154 *Sampling and analysis of* Castanea sativa *plants*
155 *Castanea sativa* plants were harvested one year after the first inoculation. Harvesting
156 was performed without damaging the root system, which was carefully washed out of
157 the soil. Fifteen plants per treatment were randomly selected. For each plant, root collar
158 diameter, total shoot height and root length were measured. Increments on shoot height
159 and root collar diameter were evaluated considering the period from inoculation to
160 harvest. During this period, the average growth rate (mm/day) was also determined. The
161 ratio of shoot and root length was calculated at harvesting time.
162 Leaves, stems and roots from the previous 15 plants were separately used to determine
163 fresh weight (fw), oven-dried at 60ºC for four days, and then weighed again to
164 determine dry weight (dw). The ratio of shoot and root dry weight was calculated, as
165 well as the specific root length (cm/g dw), evaluated as the total root length divided by
166 root dw. The effect of fungal inoculation on the leaf water content (LWC) was
167 determined as follows: LWC = [(leaf fw-leaf dw) / leaf dw] x 100 (Wang et al. 2011).
168 The remaining 15 plants were used to determine N, P and K contents. Leaves from five
169 plants were grouped and minced to a fine powder (1 mm mesh size), originating a total
170 of three replicates from each treatment and control. N content determination was carried
171 out by micro-Kjeldahl method using a Kjeltec 1030 distilling unit (AOAC 1990). For
172 the determination of P and K contents, samples were digested using nitric acid and

hydrogen peroxide moisture at 200ºC for 20 min in a microwave (Marspress CEMM). The filtered solution was used for measuring the concentrations of K by atomic-absorption spectrometry (Pye Unicam) and P by spectrophotometry (Genesys 10-UV) following the vanado-molybdate yellow colorimetric method (Jackson 1973).

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

Assessing the Pisolithus tinctorius *colonization*

Mycorrhizal colonization was evaluated in fifteen 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%; 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 fifteen independent experiments. The corresponding standard deviations (SD) values are displayed. The significance of differences among means was tested by analysis of variance (ANOVA), using SPSS v.17 software, in which the averages were compared using Tukey test ($p \leq 0.05$).

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

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 one year after the first inoculations (Table 1). Plants that were only inoculated with *P. tinctorius* displayed the highest increment in shoot height (*c.* 3-fold higher) and the lowest root length (0.84-fold lower) when compared to non-inoculated plants. Similar results were observed in plants first inoculated with *P. tinctorius* and after 30 days inoculated with *H. fasciculare* (*c.* 2-fold higher and 0.90-fold lower than non-inoculated plants, respectively). Accordingly, *P. tinctorius* inoculated plants displayed the highest shoot/root length ratio, and plants inoculated with *P. tinctorius* and 30 days later with *H. fasciculare* the second highest. When *H. fasciculare* was inoculated alone or simultaneously with *P. tinctorius*, plants

223 displayed a non-significant variation in both shoot height and root length compared to
224 non-inoculated plants.
225 Seedlings inoculated with *P. tinctorius* and inoculated with *P. tinctorius* and 30 days
226 later with *H. fasciculare* also displayed the highest shoot/root dw ratios, compared to
227 control plants that presented the lowest value from all fungal treatments. When
228 considering the specific root length, determined as the relation of root length and root
229 dry weigh, significant differences were only detected between plants inoculated with
230 *P. tinctorius* alone and non-inoculated control. Although plants from all treatments
231 exhibited lower specific root lengths when compared to control plants, *P. tinctorius*
232 inoculated plants presented the lowest value (0.48-fold).
233 In plants only inoculated with *P. tinctorius* a significant increase was observed for root
234 collar increment, when compared to non-inoculated control that exhibited the lowest
235 increment. No significant differences were observed between the other treatments.
236 Although all treated seedlings exhibited a higher growth rate when compared to control
237 plants, only plants inoculated with *P. tinctorius* alone showed a significant different
238 growth rate value from non-inoculated plants (3-fold higher). In what concerns leaf
239 water contents no significant differences were found between treatments.
240 The influence of fungal inoculation on photosynthetic pigments content of *C. sativa*
241 plants was evaluated by determining the concentrations of chlorophylls *a* and *b*, and
242 carotenoid content (Table 2). Plants inoculated with *P. tinctorius* alone or with
243 *P. tinctorius* 30 days + *H. fasciculare* exhibited higher contents of all pigments when
244 compared to non-inoculated plants. In contrast, in plants that were simultaneously
245 inoculated with *P. tinctorius* and *H. fasciculare* exhibited the lowest pigments content.
246
247 *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 four-month-old *C. sativa* seedlings inoculated with selected ECM or saprotrophic fungi, or in combination of both. The fungal species, *Pisolithus tinctorius* and *Hypholoma fasciculare*, were chosen as representatives of ECM and saprotrophic basidiomycetes, respectively.

The efficiency of root colonization by *P. tinctorius* is strongly compromised in the presence of *H. fasciculare*. However, if plants had been previously inoculated with *P. tinctorius*, the inoculation of *H. fasciculare* 30 days later did not affect root colonization. This result suggests a competitive interaction between the ECM and saprotrophic fungi, resulting in root colonization inhibition. Accordingly, a reduction in the number of *Pinus contorta* roots colonized by the ECM *Paxillus involutus* in soils containing the saprotrophic fungus *Collybia maculate* was reported (Shaw et al. 1995).

273 *H. fasciculare* has been also referred as a highly competitive saprotrophic fungus that
274 could interfere with the development of new mycorrhizal *Suillus variegatus* mycelia on
275 *Pinus sylvestris* seedlings (Lindahl et al. 2001). In addition, the suppression of ECM has
276 been observed when they are growing in the presence of saprotrophic fungi on agar
277 media (Shaw et al. 1995; Zadworny et al. 2004). However, ECM might occasionally
278 outcompete saprotrophic fungi (Baar and Stanton 2000; Werner et al. 2002). In our
279 study, the fungus *H. fasciculare* seems to have an advantage in the competition
280 compared to the ECM *P. tinctorius*. For this reason, the root colonization was inhibited
281 when both fungi were simultaneously applied. However, if the initial steps of
282 mycorrhizal establishment have already occurred, then the number of ECM roots is not
283 affected, even in the presence of *H. fasciculare* mycelia. Indeed, when *C. sativa* plants
284 were inoculated with *H. fasciculare* 30 days after *P. tinctorius* inoculation, a similar
285 level of mycorrhizal roots was observed compared to plants only inoculated with *P.*
286 *tinctorius*.
287 Although easily macroscopically detected, mycorrhizae formed in *P. tinctorius* 30 days
288 + *H. fasciculare* treatment were not identical to those present in *P. tinctorius* colonized
289 roots. Observation of cross sections from mycorrhizal root tips of chestnut plants
290 inoculated with *P. tinctorius* alone showed the presence of a typical well-developed
291 mantle and elongated epidermal cells (results not shown). Mycorrhizae from *C. sativa*
292 seedlings inoculated with *P. tinctorius* and after 30 days with *H. fasciculare* displayed a
293 layer of hyphae adherent to the epidermal cells, resembling a mantle, but with less
294 elongated epidermal cells (results not shown). This result suggests that the presence of
295 *H. fasciculare* still influences the development of the mycorrhizal association, even
296 when plant-fungus interaction has already started. Albeit not restricting the association,
297 the typical morphological features of *P. tinctorius* mycorrhiza are not fully developed.

298 Thus, the possibility of the saprotrophic fungus to restrict certain interaction processes
299 required for fully developed mycorrhization remains open. Also, the absence of
300 mycorrhizae in simultaneously inoculated plants with both fungi could also be due to an
301 early interaction inhibition promoted by the saprotrophic fungus.
302 In the present study, all fungal inoculations of four-month-old chestnut seedlings
303 induced the plant growth (evaluated as an increase in shoot height increment, shoot/root
304 length ratio, root collar diameter and growth rate), but only the seedlings solely
305 inoculated with *P. tinctorius* exhibited statistically significant increases. Previous
306 studies with the same combination of host and ECM species had already revealed the
307 noteworthy improvement of *C. sativa* growth under *in vitro*, greenhouse and open field
308 conditions (Martins et al. 1997; Martins 2004). Even in other tree species, *P. tinctorius*
309 inoculation has also promoted plant growth (Thomson et al. 1994; Cairney and
310 Chambers 1999; Turjaman et al. 2005). Seedlings growth promotion was suppressed in
311 the presence of *H. fasciculare*, but the severity of this suppression was dependent on the
312 time of fungal application. The adverse effect of *H. fasciculare* on the growth of
313 *P. tinctorius* inoculated plants was mainly noticed when simultaneous inoculation with
314 both fungi was performed. When the *P. tinctorius* mycorrhiza was established prior to
315 *H. fasciculare* inoculation, the adverse effects were greatly reduced.
316 The growth increases observed in plants only inoculated with *P. tinctorius* could be
317 related to the more favourable plant growing conditions promoted by the mycorrhizal
318 establishment (Harris 1992). The changes that occur on root morphology and
319 architecture, associated to the increase of extramatrical ECM mycelium surrounding
320 roots, contribute to a larger volume of soil explored. When *P. tinctorius* was inoculated
321 alone, the lateral roots were shortened by 17% and exhibited 49% higher dry weight as
322 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

348 (by 10%) and exhibited higher dry weight (47%) as compared to non-inoculated control,
349 leading to a reduction of 40% in specific root length. These results could be related to
350 the existence of mycorrhizal roots in an identical proportion as observed on *P. tinctorius*
351 inoculated plants. Accordingly, plants inoculated with *P. tinctorius* and after 30 days
352 inoculated with *H. fasciculare* display 18% higher N levels compared to non-inoculated
353 plants. However, the regular functioning of these ectomycorrhizae could be
354 compromised by the presence of *H. fasciculare*, as suggested by the presence of only an
355 incipient mantle (microscopic observations, results not shown) and increase of specific
356 root length in relation to *C. sativa* roots infected by *P. tinctorius* (by 23%). Indeed, the
357 presence of *H. fasciculare* reduced the foliar P contents either when applied in
358 combination with *P. tinctorius* (22-24% less when compared to *P. tinctorius*-inoculated
359 plants) or alone (15% less when compared to non-inoculated plants).
360 The reduction of nutrients in plants only inoculated with *H. fasciculare* (N and P) or
361 simultaneously inoculated with *P. tinctorius* and *H. fasciculare* (N) could be due to the
362 competition of both fungi and roots for nutrient resources. Our results are in accordance
363 with previous results that have reported no increases in shoot N in red pine plants
364 inoculated with *P. tinctorius* in the presence of saprotrophic microbes (Wu et al. 2003).
365 This phenomenon could result from the competitive interaction between *H. fasciculare*
366 and *P. tinctorius* for N, which could lead to a lower nutrient accumulation in *C. sativa*
367 leaves. The competition for nutrient resources is a common phenomenon that occurs
368 between ECM and saprotrophic fungi. It was found that substantial P could be
369 transferred from the ECM *Suillus variegatus* or *Paxillus involutus* to the saprotroph
370 *H. fasciculare*, or vice-versa (Lindahl et al. 1999; 2001). These combative interactions
371 could also include N transfers (Koide and Kabir 2001; Wu et al. 2003, 2005).

372 The effect of fungal inoculation on leaf water status of *C. sativa* seedlings was
373 evaluated through determination of the leaf water content (LWC). Leaf water content is
374 a useful indicator of plant water balance, since it expresses the relative amount of water
375 present on the plant tissues (Wang et al. 2011). In the present study, no significant
376 differences in LWC were observed between treatments and control. This result is not
377 surprising since all the plants were grown under well-watered conditions. However, the
378 root system of mycorrhizal plants only inoculated with *P. tinctorius*, despite the smaller
379 root length, supplied a relatively larger shoot with water and mineral nutrients. This is
380 probably related with the increased extension and absorbing surface area of hyphae
381 from mycorrhizal plants (Augé 2004; Lehto and Zwiazek 2011), as well as changes on
382 root architecture that may be used to increase the interaction of root and soil (Atkinson
383 1994; Augé et al. 2001). As observed in our study, water contents of non-stressed plants
384 were usually not different in non-mycorrhizal and mycorrhizal plants (Vodnik and
385 Gogala 1994; Bryla and Duniway 1997), including those with the ECM *P. tinctorius*
386 (Alvarez et al. 2009).
387 The higher growth observed in plants only inoculated with *P. tinctorius* could
388 additionally be attributed to an increase of photosynthetic rate when compare to non-
389 inoculated control (Allen et al. 1981; Martins et al. 1997; Smith and Read 2008). This is
390 frequently related with higher chlorophyll and carotenoid contents, which ultimately
391 leads to an improved carbohydrate accumulation (Davies et al. 1993; Wright et al.
392 1998). In this work, the inoculation with *P. tinctorius* alone enhanced the contents of
393 chl *a*, chl *b*, and carotenoids in *C. sativa* seedlings (respectively in 23%, 38%, and 27%,
394 when compared to non-inoculated plants). These results are in accordance with those
395 reporting chlorophyll concentration increases in ectomycorrhizal plants when compared
396 with non-mycorrhizal plants (Huang and Tao 2004; Alberdi et al. 2007). This situation

397 is comparable to plants treated with *P. tinctorius* 30d + *H. fasciculare*, in which
398 increases of 30% (chl *a*), 36% (chl *b*) and 20% (carotenoids) were detected, when
399 compared to non-inoculated plants. The higher chlorophyll contents observed in
400 *C. sativa* leaves inoculated only with *P. tinctorius* or with *P. tinctorius* 30d +
401 *H. fasciculare* could be attributed to the melioration of nutritional status of the host
402 plant, especially in N and P. Indeed, whereas N is an essential element for the formation
403 of chlorophyll (Liu et al. 2007), P has an important role as an energy carrier during
404 photosynthesis (Jacobsen 1991). Similar results were also reported in other studies
405 (Demur 2004; Zuccarini 2007; Chen et al. 2010). The more reduced growth of *C. sativa*
406 seedlings after being simultaneously inoculated with *P. tinctorius* and *H. fasciculare*
407 could be attributed to some extent to the decreased nutrient acquisition of these plants
408 (particularly N) that will lead to lower photosynthetic pigment contents.
409
410 To conclude, the simultaneous inoculation of the saprotrophic fungus *H. fasciculare*
411 negatively affected the interaction between the ECM *P. tinctorius* and *C. sativa* roots.
412 Besides the absence of visible mycorrhizal roots, growth, nutritional and physiological
413 parameter values commonly associated to the mycorrhization benefits were not
414 observed on plants simultaneously inoculated with both fungi. When plants were
415 inoculated with *P. tinctorius* and after 30 days with *H. fasciculare* the same parameter
416 values were very close to those from plants only inoculated with *P. tinctorius.* These
417 results are most probably due to the interaction between *P. tinctorius* and *C. sativa* roots
418 and the ability of mycorrhizal establishment before *H. fasciculare* application. Once
419 formed, the chestnut seedlings are able to take advantage from the mycorrhizal
420 association. Plants exhibit growth improvement, which could be attributed to the
421 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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598 **Figure legends**

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

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610 **Tables**

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

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

615

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

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

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627 **Table 3 -** Effect of *P. tinctorius* and *H. fasciculare* on N, P, K content of leaves of
628 *C. sativa* plants, one year after inoculation with *P. tinctorius*, *H. fasciculare*,
629 simultaneously with *P. tinctorius* and *H. fasciculare* (*P. tinctorius* + *H. fasciculare*), or
630 with *P. tinctorius* followed by *H. fasciculare*, one month later (*P. tinctorius* 30d + *H.*
631 *fasciculare*). Means ± SD (n = 3) are shown. In each column different letters mean
632 significant differences ($p \leq 0.05$).

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

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634

635 **Figures**

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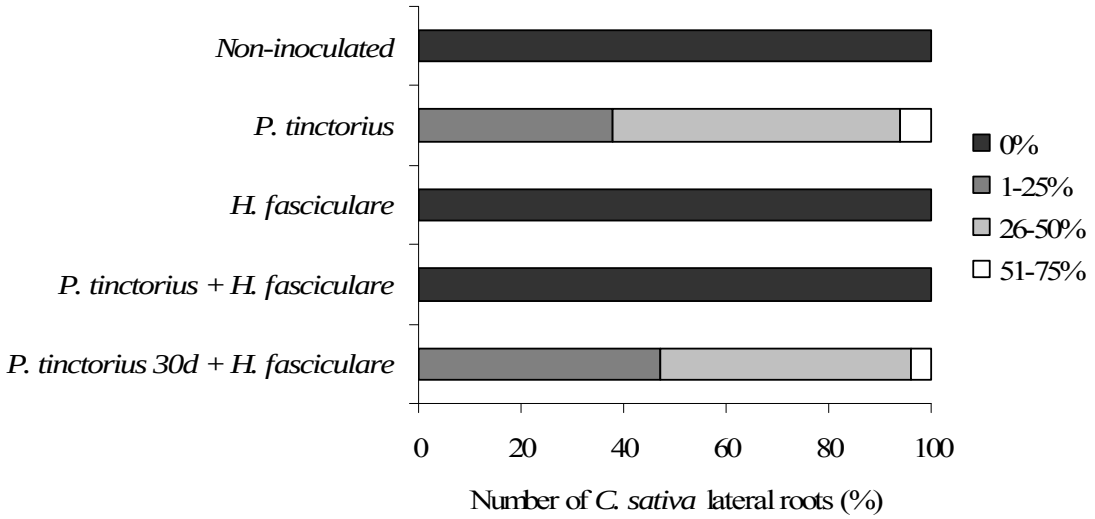

638

639 **Fig. 1.**

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