

1 **Mycorrhiza (accepted, 28<sup>th</sup> March 2011)**

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

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24 **ABSTRACT**

25 In Northeast of Portugal the macrofungal community associated to chestnut tree  
26 (*Castanea sativa* Mill.) is rich and diversified. Among fungal species, the  
27 ectomycorrhizal *Pisolithus tinctorius* and the saprotroph *Hypholoma fasciculare* are  
28 common in this habitat. The aim of the present work was to assess the effect of the  
29 interaction between both fungi on growth, nutritional status and physiology of *C. sativa*  
30 seedlings. In pot experiments, *C. sativa* seedlings were inoculated with *P. tinctorius* and  
31 *H. fasciculare* individually or in combination. Inoculation with *P. tinctorius* stimulated  
32 the plant growth and resulted in increased foliar-N, -P, and photosynthetic pigment  
33 contents. These effects were suppressed when *H. fasciculare* was simultaneously  
34 applied with *P. tinctorius*. This result could be related to the inhibition of  
35 ectomycorrhizal fungus root colonization as a result of antagonism or to the competition  
36 for nutrient sources. If chestnut seedlings have been previously inoculated with  
37 *P. tinctorius*, the subsequent inoculation of *H. fasciculare* 30 days later did not affect  
38 root colonization and mycorrhization benefits were observed. This work confirms an  
39 antagonistic interaction between ectomycorrhizal and saprotrophic fungi with  
40 consequences on the ectomycorrhizal host physiology. Although *P. tinctorius* is  
41 effective in promoting growth of host trees by establishing mycorrhizae, in the presence  
42 of other fungi it may not always be able to interact with host roots due to an inability to  
43 compete with certain fungi.

44

45 Keywords: *Pisolithus tinctorius*; *Hypholoma fasciculare*; Fungal interaction; *Castanea*  
46 *sativa*; Biomass production

47

## 48 **Introduction**

49 The chestnut (*Castanea sativa* Mill.) agro-ecosystem has been of great social, economic  
50 and landscape importance in Northeast of Portugal. There are multiple resources  
51 associated with this crop, including fruit and wood production and more recently  
52 mushroom harvesting. Two main ecological groups of fungi dominate these habitats, the  
53 saprotrophic and ectomycorrhizal (Baptista et al. 2010), and both are capable of  
54 influencing the plant nutrients acquisition in different ways (Koide and Kabir 2000).  
55 Saprotrophic fungi play an important role in the soil ecosystem as major decomposers  
56 of plant residues, releasing nutrients that sustain and stimulate plant growth (Dighton  
57 2007). Ectomycorrhizal fungi (ECM) increase plant growth, by enhancing the  
58 absorption of mineral nutrients and water, increase plant resistance to pathogens and to  
59 different environmental stresses (Smith and Read 2008). A beneficial effect of ECM on  
60 biological control of larval root herbivores has been also reported (Edda et al. 2010).

61 In spite of their partial spatial separation along the soil vertical axis, ectomycorrhizal  
62 and saprotrophic fungi interact (Leake et al. 2002; Lindahl et al. 2007). Interactions  
63 between ECM and saprotrophic fungi have been observed under axenic conditions  
64 (Shaw et al. 1995; Baar and Stanton 2000; Werner et al. 2002; Mucha et al. 2006;  
65 Sharma et al. 2010), as well as on natural substrates by using a microcosm system  
66 (Lindahl et al. 1999; Leake et al. 2001; Lindahl et al. 2001). A range of responses are  
67 observed depending on the individual species and their combination, nutrients  
68 availability, amount and quality of the carbon substrates from which the fungi grow  
69 (Lindahl et al. 1999; Koide and Kabir 2000; Lindahl et al. 2001; Werner and Zadworny  
70 2003). For example, in pairwise interactions between ECM and saprotrophic fungi, the  
71 suppression of either ECM (Shaw et al. 1995; Zadworny et al. 2004) or saprotrophs  
72 (Baar and Stanton 2000; Werner et al. 2002; Sharma et al. 2010) have been observed.

73 Also, contradictory responses of fungal interactions under natural substrates have been  
74 reported. Using a soil microcosm, a clear antagonistic response of ECM (*Suillus*  
75 *variegates* and *Paxillus involutus*) extending from pine seedling roots was detected  
76 against the saprotroph *H. fasciculare* extending from wood blocks (Lindahl et al. 1999,  
77 2001). By contrast, in a similar microcosm experiment, Leake et al. (2001) found that  
78 the ECM *Suillus bovinus* mycelium vigour was reduced when in contact with the  
79 saprotroph *Phanerochaete velutina*. These contradictory results could be partially  
80 explained taking into account the differences on the bi-directional translocation of  
81 carbon and minerals that occurs between ectomycorrhizal and saprotrophic mycelia.  
82 Current evidences indicate that this translocation occurs from areas of high nutrient  
83 availability to those of high nutrient demand and are independent of mycelial growth  
84 (Lindahl et al. 1999; Leake et al. 2001; Lindahl et al. 2001). However, regarding their  
85 antagonist mechanisms, much variation exists among ECM and saprotrophic fungi and  
86 even within species.

87 Taken together, these experiments revealed that saprotrophic and ECM compete with  
88 each other for soil nutrients, as well for territory or space. These interactions may result  
89 in changes on fungal community (by biomass reduction of one or both competitors), but  
90 also on community functioning, namely in nutrients reallocation (Boddy 2000) with  
91 consequences for plant growth and health (reviewed by El-Shatnawi and Makhadmeh  
92 2001). Furthermore, the inhibition of ectomycorrhizae formation by saprotrophic fungi,  
93 as already observed in some antagonistic interaction studies (Shaw et al. 1995; Lindahl  
94 et al. 2001), may cause additional losses of benefits from symbiosis (plant fitness and  
95 health). The contradictory responses obtained from different interaction studies using  
96 these groups of organisms suggest that their relations are complex and difficult to study,  
97 and therefore, are scarcely known.

98 In this work, it is aimed to assess the effect of saprotrophic (*Hypholoma fasciculare*)  
99 and ectomycorrhizal (*Pisolithus tinctorius*) fungi on *Castanea sativa* growth. These  
100 fungal species are commonly present in *C. sativa* orchards in the Trás-os-Montes region  
101 (Northeast of Portugal) and are usually found in the same soil (Baptista et al. 2010).  
102 This study intends to provide knowledge on the influence of co-occurring mycelia of  
103 *P. tinctorius* and *H. fasciculare* on chestnut seedlings and elucidate their influence on  
104 formation and functioning of the ECM symbiosis.

105

## 106 **Materials and methods**

### 107 *Biological material*

108 Seeds of *Castanea sativa* Mill. were harvested in Bragança region orchards. *Hypholoma*  
109 *fasciculare* (Huds.) P. Kumm. was isolated from *Castanea sativa* orchards at Oleiros –  
110 Bragança (Northeast Portugal). Fungal isolation was performed on Melin-Norkans  
111 (MMN) agar medium at pH 6.6 [NaCl 0.025 g/L; (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> 0.25 g/L; KH<sub>2</sub>PO<sub>4</sub> 0.50  
112 g/L; FeCl<sub>3</sub> 0.050 g/L; CaCl<sub>2</sub> 0.50 g/L; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.15 g/L; thiamine 0.10 g/L;  
113 casamino acids 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L], following  
114 Brundrett et al. (1996). The identity of the fungal isolate was molecularly confirmed by  
115 the amplification and sequencing of the internal transcribed spacer region (ITS), using  
116 the universal primers *ITS1* and *ITS4* (White et al. 1990). *Pisolithus tinctorius* (Pers.)  
117 Coker & Couch (isolated 289/Marx) was obtained from the University of Tübingen.  
118 This fungus has been used for mycorrhizal formation in seedlings of *C. sativa* (Martins  
119 et al. 1997; Martins 2004). Both strains were maintained in MMN agar medium at 25°C,  
120 in the dark, being regularly sub-cultured.

121

### 122 *Production of Castanea sativa seedlings*

123 *Castanea sativa* seeds were surface sterilized with sodium hypochloride (5%, v/v) for  
124 1 h, followed by washing three times with sterile distilled water. The seeds were then  
125 stratified and germinated in sterile moistened sand, at 5-10°C, for two months. After  
126 germination, the radicle tips were removed, to promote root ramification, and seedlings  
127 were separately transferred to plastic pots (each with 300 cm<sup>3</sup>), filled with sterile  
128 vermiculite:topsoil:sand (3:1:1, v/v/v) mixture. Seedlings were automatically sprayed  
129 during 10 seconds, every 40 minutes; and were kept under greenhouse conditions  
130 (day/night thermal regime of 23°/18° ± 2°C, 10 h light/14 h dark photoperiod and  
131 70 ± 10% relative humidity) for four months. Uniform plants were then selected and  
132 transplanted to plastic pots of two litres (two seedlings per pot) filled with the same  
133 growth mixture as before. During this process, seedlings were inoculated with fungi.

134

#### 135 *Fungal inoculation of Castanea sativa seedlings*

136 Suspension cultures of *P. tinctorius* and *H. fasciculare* were obtained by transferring  
137 mycelium inoculum to liquid modified MMN medium [MMN medium containing half  
138 concentration of KH<sub>2</sub>PO<sub>4</sub> and (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, and no malt extract]. Two-week-old  
139 suspension cultures maintained in the dark, at 25°C, and without agitation, were used for  
140 plant inoculations. At the time of transplanting, plants were inoculated (i) with  
141 *P. tinctorius*, (ii) with *H. fasciculare*, (iii) with *P. tinctorius* and *H. fasciculare*  
142 simultaneously (*P. tinctorius* + *H. fasciculare*), or (iv) with *P. tinctorius* and one month  
143 later inoculated with *H. fasciculare* (*P. tinctorius* 30d + *H. fasciculare*). Inoculations  
144 were carried out by transferring 100 mL of fungal suspension culture, previously  
145 homogenized by hand-shaking for 3 minutes, into the planting hole. For *H. fasciculare*  
146 inoculation, performed one month after *P. tinctorius* inoculation, the suspension culture  
147 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 60°C 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:  $LWC = [(leaf\ fw - leaf\ dw) / leaf\ dw] \times 100$  (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 Kjeltac 1030 distilling unit (AOAC 1990). For  
172 the determination of P and K contents, samples were digested using nitric acid and

173 hydrogen peroxide moisture at 200°C for 20 min in a microwave (Marspress CEMM).  
174 The filtered solution was used for measuring the concentrations of K by atomic-  
175 absorption spectrometry (Pye Unicam) and P by spectrophotometry (Genesys 10-UV)  
176 following the vanado-molybdate yellow colorimetric method (Jackson 1973).  
177 Chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoids (car) contents were  
178 determined after methanolic extraction of fresh leaves, following the method of Ozerol  
179 and Titus (1965). Results were expressed in mg/g fw.

180

#### 181 *Assessing the Pisolithus tinctorius colonization*

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

189

#### 190 *Data analysis*

191 Data from plant analysis (growth parameters, water and photosynthetic pigment  
192 contents and nutritional status) are presented as the mean of three to fifteen independent  
193 experiments. The corresponding standard deviations (SD) values are displayed. The  
194 significance of differences among means was tested by analysis of variance (ANOVA),  
195 using SPSS v.17 software, in which the averages were compared using Tukey test  
196 ( $p \leq 0.05$ ).

197



198 **Results**

199 *Influence of Hypholoma fasciculare on Pisolithus tinctorius chestnut root colonization*

200 To determine the influence of *H. fasciculare* on the colonization of *C. sativa* roots by  
201 the ECM *P. tinctorius*, the number of lateral roots displaying mycorrhizae was  
202 determined one year after the *P. tinctorius* or *H. fasciculare* inoculation, and  
203 *P. tinctorius* + *H. fasciculare* or *P. tinctorius* 30d + *H. fasciculare* inoculation (Fig. 1).  
204 As expected, the formation of mycorrhizae was not detected in plants that have been  
205 inoculated only with *H. fasciculare*. Also, the presence of mycorrhizae was not detected  
206 in plants simultaneously inoculated with *P. tinctorius* and *H. fasciculare*. However,  
207 when plants were first inoculated with *P. tinctorius* and after 30 days inoculated with  
208 *H. fasciculare*, chestnut roots displayed a similar level of mycorrhization as plants  
209 inoculated only with *P. tinctorius*. In both treatments, root colonization levels never  
210 achieved more than 75% of the total number of lateral roots.

211

212 *Effect of fungal inoculation on Castanea sativa growth*

213 The influence of ECM and saprotrophic fungi on *C. sativa* growth was evaluated by the  
214 determination of several plant growth parameters one year after the first inoculations  
215 (Table 1). Plants that were only inoculated with *P. tinctorius* displayed the highest  
216 increment in shoot height (c. 3-fold higher) and the lowest root length (0.84-fold lower)  
217 when compared to non-inoculated plants. Similar results were observed in plants first  
218 inoculated with *P. tinctorius* and after 30 days inoculated with *H. fasciculare* (c. 2-fold  
219 higher and 0.90-fold lower than non-inoculated plants, respectively). Accordingly,  
220 *P. tinctorius* inoculated plants displayed the highest shoot/root length ratio, and plants  
221 inoculated with *P. tinctorius* and 30 days later with *H. fasciculare* the second highest.  
222 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*

248 No significant differences occurred in the K content of *C. sativa* leaves from all the  
249 plant treatments, in contrast to N and P content that exhibited differences between  
250 treatments (Table 3). Higher contents of N were detected in leaves of *C. sativa* seedlings  
251 inoculated with *P. tinctorius* alone and inoculated with *P. tinctorius* 30 days +  
252 *H. fasciculare* when compared to control plants. In contrast, plants inoculated with  
253 *H. fasciculare* alone or simultaneously inoculated with *P. tinctorius* exhibited the lowest  
254 N content. These results are similar for foliar P, except that no differences in relation to  
255 control plants were detected for those plants treated with both fungi.

256

## 257 **Discussion**

258 The natural benefits of mycorrhization to most agronomical relevant plants, including  
259 European chestnut tree, turns the understanding of interactions between mycorrhizal and  
260 saprotrophic fungi essential. In addition, the influence of saprotrophic fungi on plant  
261 physiology and growth is scarcely studied. In this work, pot experiments were  
262 conducted using four-month-old *C. sativa* seedlings inoculated with selected ECM or  
263 saprotrophic fungi, or in combination of both. The fungal species, *Pisolithus tinctorius*  
264 and *Hypholoma fasciculare*, were chosen as representatives of ECM and saprotrophic  
265 basidiomycetes, respectively.

266 The efficiency of root colonization by *P. tinctorius* is strongly compromised in the  
267 presence of *H. fasciculare*. However, if plants had been previously inoculated with  
268 *P. tinctorius*, the inoculation of *H. fasciculare* 30 days later did not affect root  
269 colonization. This result suggests a competitive interaction between the ECM and  
270 saprotrophic fungi, resulting in root colonization inhibition. Accordingly, a reduction in  
271 the number of *Pinus contorta* roots colonized by the ECM *Paxillus involutus* in soils  
272 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

323 length. Similar results have also been obtained with regard to root length and root dry  
324 weight in *C. sativa* seedlings inoculated with *P. tinctorius* under *in vitro* and open field  
325 conditions (Martins 2004); and specific root length in *Larix gmelinii* (Sun et al. 2010).  
326 The increase of root diameter could be attributed to the cortical cells colonization by  
327 fungal mycelia, as well as to the mantle formation around the root tips. These features,  
328 together with increased lateral roots branching, are general responses to ECM  
329 inoculation (Smith and Read 2008) and ultimately result on a larger available surface  
330 area for the absorption of nutrients and water (Marschner and Dell 1994; Brundrett et al.  
331 1996; Timonen et al. 1996; Jones et al. 1998). In the present study, the inoculation of  
332 chestnut seedlings only with *P. tinctorius* resulted in an increase of N and P foliar  
333 content (21% and 37% higher compared to non-inoculated plants, respectively).  
334 Although the differences are not statistically significant, this result is in accordance with  
335 previous studies using the same (Martins 2004) or other combinations of host and ECM  
336 species (Smith and Read 2008). The increased absorption of N and P due to *P.*  
337 *tinctorius* inoculation could certainly contribute to the enhanced growth response of *C.*  
338 *sativa* seedlings. Better growth responses due to an increase in uptake of P (Jones et al.  
339 1991; Cairney and Chambers 1997) or to enhanced N uptake (Wu et al. 1998; Mari et  
340 al. 2003) were also observed in several mycorrhizal associations. Taking into account  
341 the present results, there seems to be a negative correlation between specific root length  
342 and nutrient uptake in *C. sativa* plants only inoculated with *P. tinctorius*. Similar results  
343 were previously observed in other mycorrhizal associations (Rousseau et al. 1994;  
344 Padilla and Encina 2005).  
345 Plants inoculated with *P. tinctorius* and after 30 days inoculated with *H. fasciculare* also  
346 exhibited enhanced growth when compared to non-inoculated plants. Although not so  
347 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 Zwiasek 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.

422 This work confirms the antagonistic interaction between ECM and saprotrophic fungi  
423 and demonstrates that fungal interactions affect the physiological processes of the  
424 ectomycorrhizal host. Although *P. tinctorius* is an effective colonizer of many tree  
425 species, the presence of saprotrophic fungi in the soil could hamper the establishment  
426 and functioning of mycorrhizae. The inability of *P. tinctorius* to compete with certain  
427 competitive saprotrophic fungi compromises the mycorrhization of host trees. However,  
428 if the initial steps of mycorrhizal symbiosis have already occurred, then the benefits  
429 from mycorrhization could be observed, even in the presence of saprotrophic fungi.

430

431

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- 596
- 597

598 **Figure legends**

599

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

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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  $\pm$  SD (n = 7). In each column different letters mean  
622 significant differences ( $p \leq 0.05$ ).

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

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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  $\pm$  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 $\pm$ 0.6 <sup>abc</sup>	0.60 $\pm$ 0.22 <sup>ab</sup>	3.3 $\pm$ 0.6 <sup>a</sup>
<i>P. tinctorius</i>	10.5 $\pm$ 0.5 <sup>a</sup>	0.82 $\pm$ 0.09 <sup>a</sup>	3.3 $\pm$ 0.4 <sup>a</sup>
<i>H. fasciculare</i>	8.4 $\pm$ 0.6 <sup>bc</sup>	0.51 $\pm$ 0.09 <sup>b</sup>	3.5 $\pm$ 0.8 <sup>a</sup>
<i>P. tinctorius</i> + <i>H. fasciculare</i>	7.4 $\pm$ 0.6 <sup>c</sup>	0.64 $\pm$ 0.19 <sup>ab</sup>	4.5 $\pm$ 0.4 <sup>a</sup>
<i>P. tinctorius</i> 30d + <i>H. fasciculare</i>	10.3 $\pm$ 0.8 <sup>a</sup>	0.62 $\pm$ 0.02 <sup>ab</sup>	4.1 $\pm$ 0.7 <sup>a</sup>

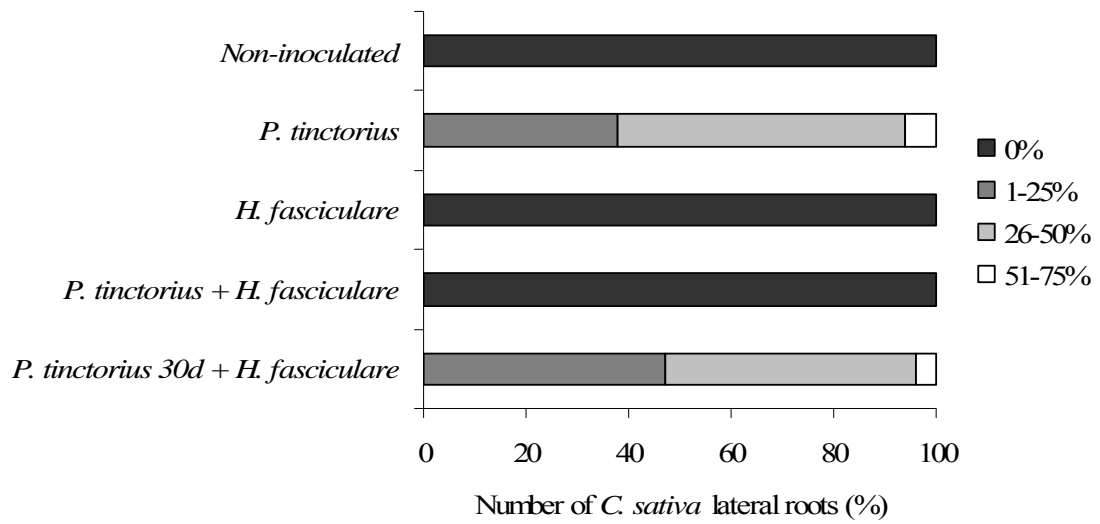
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635 **Figures**

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639 **Fig. 1.**

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