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Cultivation of *Tuber melanosporum* in firebreaks: short-term persistence of the fungus and effect of seedling age and soil treatment

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Abstract

Wildfires are a major threat to Mediterranean forests. Firebreaks are built as a prevention measure, but require a periodic and expensive maintenance. Cultivating the ectomycorrhizal mushroom *Tuber melanosporum* Vitt. in firebreaks could reduce costs and improve their sustainability. But firebreaks are built on forest soil, considered non-optimum for *T. melanosporum* cultivation. A pot experiment was used to study the persistence of *T. melanosporum* in firebreak soils in the short term, as a first step to assess the viability of these plantations. The influence of seedlings, soil heating and liming on *T. melanosporum* was also tested. During the two years after plantation, *T. melanosporum* mycorrhizas increased their number, showing its ability to proliferate. Percent root colonisation by native fungi importantly increased from month 12 to 22; although *T. melanosporum* remained dominant, with a colonisation level similar to those in standard truffle plantations. The age of seedlings at the time of planting influenced *T. melanosporum* poliferation, supporting a key role for nursery-seedling quality in the performance of young plantations. Heating the soil before planting reduced the richness of native fungi, suggesting that this could increase plantation success. The results tend to

support the viability of *T. melanosporum* cultivation in firebreaks, and encourage experimental field plantations.

Keywords: firebreak, truffle plantation, inoculated seedling, soil preparation, ectomycorrhizal fungi

1 Introduction

Wildfires are a major threat to the Mediterranean forests in Europe. A common silvicultural measure to prevent wildfires are firebreaks: in some Mediterranean countries like Spain they usually consist of a central band cleared to mineral earth, surrounded by a reduced fuel zone (with widely spaced, pruned trees). Effective firebreaks require a periodic and expensive maintenance (Plana et al. 2005), and thus grazing and agroforestry have been proposed as secondary uses to reduce costs and increase sustainability. Reyna and Garcia-Barreda (2005) proposed to cultivate the edible ectomycorrhizal (EM) fungus *Tuber melanosporum* Vitt. in the reduced fuel zone of firebreaks, since both uses require open stands. The phytotoxic activity of the fungus inhibiting plant growth around the symbiont tree (Splivallo et al. 2011) could create horizontal discontinuities in ground fuels.

This area devoid of plant cover (called *brûlé*) is formed in most trees producing *T. melanosporum* sporocarps in open stands, whereas it is much rarer in other trees; so the success of the proposal depends on the fungus completing its life cycle. In sandy soils and in the most rainy areas of France and Italy the inhibition of plant growth is lower, thus limiting the potential benefits of these plantations.

Successful *T. melanosporum* cultivation requires planting inoculated seedlings on receptive soils (suitable to complete its life cycle) with low EM infectivity (scant effective inoculum of other EM fungi). Thus Sourzat (1997) recommends establishing *T. melanosporum* plantations on soils

previously cultivated for non-EM plants. In forests dominated by EM trees Frochot et al. (1990) and Reyna et al. (2006) found that the native (soil-borne) populations of EM fungi colonised the roots of inoculated seedling and limited the development of *T. melanosporum* from the first years. This hinders the success of plantations, as they only start producing sporocarps between the 5th–12th year.

The viability of cultivating *T. melanosporum* in firebreaks has not been evaluated. Since the EM fungi obtain carbohydrates from their symbionts, the absence of EM plants negatively affects the EM infectivity of the soil (Dickie and Reich 2005). But in many Mediterranean firebreaks a low density of resprouting EM trees and shrubs usually appears, making the evaluation difficult. De Román and De Miguel (2005) and Martínez de Aragón et al. (2012) found that *T. melanosporum* persisted in recently burned forest soils in the short term despite the presence of resprouters. Reyna and Garcia-Barreda (2005) pointed that it would be interesting to test soil heating and overliming as a means of decreasing the EM infectivity of soil: it is well established that they can damage soil fungi (Erland and Söderström 1990; Neary et al. 1999), and in Spain it was frequent to observe truffle *brûlés* spontaneously forming on recently abandoned charcoal kilns and lime kilns located in forests.

The characteristics of nursery seedlings also influence the success of *T. melanosporum* plantations: the abundance of an EM fungus on the roots can hamper colonisation by other fungi (Kennedy et al. 2009); the seedling attributes at the time of planting influence its early field performance (Del Campo et al. 2009); and the saplings with the higher growth rates in a *T. melanosporum* plantation produce sporocarps and phytotoxic effects earlier (Shaw et al. 1996; Lulli et al. 1999).

Before establishing field plantations, and as a first step to design *T. melanosporum* plantations in firebreaks, we examine the ability of *T. melanosporum* mycorrhizas to proliferate on firebreak

soils and to compete against native EM fungi in the first two years after planting. Inoculated holm oak (*Quercus ilex* L) seedlings were grown in pots with firebreak soil and compared to dense forest soil. As a secondary aim, we test if the performance of *T. melanosporum* is influenced by (a) the characteristics of the inoculated seedlings at the time of planting, and (b) two soil preparations aimed at reducing EM infectivity before planting: heating and liming.

2 Materials and methods

2.1 Study site

The study was conducted in the *T. melanosporum*-producing region of El Toro, in the Valencian Community (eastern Spain, 1100 m a.s.l.). The soils are calcixerepts developed on Jurassic hard limestones (loam texture, pH 8.2, organic matter 3.8%). Three firebreaks with over 30 years and distant 2.8, 4.6 and 7.2 km from each other were selected. Subshrubs and herbaceous species (*Santolina chamaecyparissus* L, *Brachypodium retusum* Beauv., *Genista scorpius* AD, *Thymus vulgaris* L) dominated the vegetation, whereas EM trees and shrubs (*Q. ilex*, *Quercus faginea* Lam., *Quercus coccifera* L, and *Pinus nigra* Arnold) were sparse. The forest surrounding the firebreaks was a coppice of *Q. ilex* and *Q. faginea* with 300–900 trees ha^{-1} and a canopy cover 40–90%. According to local harvesters, none of the sampled soils produced *T. melanosporum* in recent years.

2.2 Experimental design and data collection

A total of 48 seedlings were planted in a full factorial design with five independent variables: land use (forest, firebreak), liming (0 and 1 kg m^{-2}), heat treatment (drying oven, microwaves, control), age of the seedlings at the time of planting (one and two years old), and time from plantation to sampling (12 and 22 months).

Two subplots 1×1 m were established in each of the three firebreaks and in the corresponding forest plots. In the firebreaks, the nearest EM tree or shrub was 6–7 m away from the subplots, whereas in the forest it was 2–3 m away. The topsoil (0–20 cm) of the two subplots was mixed. One of the subplots was limed with 1 kg m^{-2} quicklime powder (94% CaO, particle diametre<0.1 mm, Cales Pascual) in October 2006. Immediately after liming, the pH rose from 8.2 to 11.5–12.0, but at the time of planting (five months after liming, with more than 150 mm rainfall) it did not differ from that of the non-limed soil.

In March 2007 the topsoil of the three limed firebreak subplots was collected and pooled. The same was done for the non-limed firebreak, the limed forest, and the non-limed forest subplots. Soils were pooled to reduce the high variability characteristic of EM communities, which often confound the response to the treatments (Marx et al. 1991), although in this way the heterogeneity between sites cannot be tested.

Then the heat treatments were applied: (a) 30 minutes in a drying oven (maximum temperature: 65ºC, time above 60ºC: about ten minutes), (b) 90 seconds in microwaves (nominal power output: 700 W, frequency: 2.45 GHz, maximum temperature: 65ºC, time above 60ºC: about 60 seconds), and (c) control. In all cases the soil was laid out in a 2.5 cm layer to obtain a homogenous temperature. The temperature was measured at 1.2 cm depth. The soil water content at the time of the heat treatment was 10% w/w.

Once the soil cooled down, a mixture of 3.5 l soil and 0.35 l perlite was used to fill the pots in which the seedlings (that came from the nursery in a container Quick–pot® 0.65 l volume and 18 cm depth) were planted. The total volume of the growing medium was 4.5 l, its depth 25 cm, the diameter of top surface 16.1 cm, and the diameter in the open bottom: 13.5 cm.

The seedlings came from a commercial nursery (viveros Alto Palancia) and had been inoculated with a spore slurry containing about 2 g of fresh, mature sporocarps per plant. Two different

128 seedling stocklots were used: one- and two-year-old *Q. ilex* seedlings. The inoculation technique
129 and the growing conditions were the same, so we consider that the differences reflect the
130 development of the seedling and the fungus during the second year in the nursery. The initial
131 mycorrhizal state of the nursery seedlings was assessed at the time of planting (March 2007)
132 through a volumetric sampling (n=12) to assess both the proportion of root tips colonised and the
133 number of ectomycorrhizas per plant. In each seedling a sample with 8% of the substrate volume
134 (54 ml) was taken; all samples containing more than 104 root tips (mean: 470, standard deviation:
135 275). To cope with heterogeneity across soil depth, every sample consisted of three subsamples:
136 the depth of the container was divided into three equal parts, and in the center of each third (3.5,
137 9, and 15 cm depth) a horizontal core (2 cm diameter) across the container was taken.
138 Once in the pots, the plants were kept outdoors and watered to keep soil water content between
139 15% and 35% w/w (holding capacity: 46% w/w) and simulate the variable soil water content in
140 the field. According to Mamoun and Olivier (1990) and Olivera et al. (2011), moderate irrigation
141 regimes provide optimal conditions for the development of *T. melanosporum* mycorrhizas. High
142 and constant soil water content, and closed greenhouses without ventilation were avoided to
143 reduce the proliferation of nursery-adapted EM fungi. Half of the seedlings were sampled 12
144 months after plantation in the pots (March 2008) and the rest at month 22 (January 2009).
145 The mycorrhizal state at months 12 and 22 was assessed through a volumetric sampling. In each
146 seedling a sample with 5% of the growing medium (219 ml) was taken; all samples containing
147 more than 120 root tips (mean: 935, standard deviation: 498). To cope with heterogeneity across
148 soil depth, each sample consisted of three subsamples: the pot depth was divided into three equal
149 parts, and in the center of each third (6, 13, and 20 cm depth) a horizontal core (2.5 cm diameter)
150 across the container was taken. The deepest core was the only one that did not cross the nursery
151 rootball; thus it only included roots grown after the plantation in the 4.5 l-pots.

152 The samples were kept in water at 4ºC. The length of fine roots (diameter<1 mm) was measured
153 according to Tennant (1975). All root tips (active and senescent) were counted. Active root tips
154 were classified as nonmycorrhizal or mycorrhizal, and the latter were sorted in morphotypes
155 according to the criteria of Agerer (1987-2002) and with the aid of the descriptions in De Román
156 (2003). Their description is given in Table S1. The plants were dried to constant weight at 80ºC.
157
158 *2.3 Statistical analysis*
159 The effect of the independent variables was evaluated through conventional ANOVA, except for
160 the frequencies of appearance (proportion of samples in which a morphotype is present) which
161 were analysed through logistic regression. Significant differences among treatments were
162 identified with a least significant difference test with Bonferroni correction. When the model
163 assumptions were violated, the response variable was transformed. In order to account for within-
164 treatment variability we included root length as a predictor in the ANOVAs of the colonisation
165 level (proportion of active roots colonised) of *T. melanosporum* and the native fungi, and the
166 richness of native fungi.
167 The distribution of *T. melanosporum* along the depth profile was analysed through linear mixed
168 models (LMM). Each core was considered as one different sample and the depth was treated as a
169 repeated-measures variable.
170
171 **3 Results**
172 Before being planted, two-year-old seedlings showed significantly higher dry weight (shoot: $P < 0.001$, root: $P = 0.001$), root tips per plant ($P = 0.002$) and *T. melanosporum* mycorrhizas per
173 0.001, root: P = 0.001), root tips per plant (P = 0.002) and *T. melanosporum* mycorrhizas per
174 plant (P = 0.04) than one-year-old seedlings; whereas the proportion of active root tips colonised
175 by *T. melanosporum* did not significantly differ (P = 0.72, Table 1). Before being planted *T.*

176 *melanosporum* and *Sphaerosporella brunnea* Svrcek and Kubicka were the only mycorrhizas on
177 seedling roots. *S brunnea* was found in 33% of the one-year-old seedlings, colonising 8–34% of
178 the active root tips; and in 50% of the two-year-old seedlings, colonising 0.1–0.7% of the active
179 root tips.

180

181 Table 1 Mean characteristics of the nursery-inoculated seedlings before being planted in the pots.
182 Letters indicate significant differences ($\alpha = 0.05$) between one- and two-year-old seedlings.

	1-year-old seedlings	2-year-old seedlings
Shoot dry weight (g) [a]	0.6 b	1.5 a
Root dry weight (g) [a]	1.8 b	5.3 a
Root tips per plant	2909 b	7528 a
T. melanosporum mycorrhizas per plant [a]	884 b	1477 a
Proportion of active root tips colonised by *T. melanosporum*	0.36	0.32

183 [a] Variables log-transformed
184

185 Once planted in the pots, the shoot and the root dry weight of the seedlings were positively
186 affected by the time from plantation ($P < 0.001$ for both shoot and root), and the age of seedlings
187 at the time of planting ($P < 0.001$ for both shoot and root). The dry weights of shoot and root
188 were significantly higher in the forest soil (shoot: $P = 0.002$, root: $P = 0.01$). Shoot weight was
189 positively influenced by the microwaves treatment ($P = 0.03$) (Table 2).
190 The length of fine roots, the number of root tips, and the number of *T. melanosporum*
191 mycorrhizas per plant were significantly affected by the interaction between seedling age and
192 time from plantation ($P = 0.03$, $P = 0.04$ and $P = 0.04$ respectively): all of them increased with
193 time and were higher in two-year-old seedlings, but one-year-old seedlings showed higher
194 increases of root tips and mycorrhizas from month 12 to month 22, whereas two-year-old
195 seedlings showed higher increases from plantation to month 12 (Tables 1, 3). In the firebreak the
196 number of root tips was higher than in the forest ($P = 0.03$), but the length of fine roots and *T.*
197 *melanosporum* mycorrhizas per plant did not significantly differ.

Table 2 Mean dry weight of the plants cultivated on the firebreak and forest soils. Letters indicate significant differences (α = 0.05) among levels.

	Shoot dry weight (g) [a]	Root dry weight (g)
Initial age of seedlings		
1-year-old seedlings	6.4 b	9.8 b
2-year-old seedlings	9.4 a	14.2 a
Time from plantation		
12 months	4.7 j	7.5 j
22 months	12.8 i	16.5 i
Land use		
Forest	8.5 m	13.0 m
Firebreak	7.1 n	11.0 n
Liming		
Control	7.7	11.4
1 kg m^{-2}	7.8	12.6
Heat treatment		
Control	7.0 y	12.2
Drying oven	8.0 xy	11.7
Microwaves	8.3 x	12.1

[a] Variable log-transformed

Table 3 Mean fine root length, root tips per plant and *T. melanosporum* mycorrhizas per plant. Letters indicate significant differences (α = 0.05) among levels.

	Fine root length (m)	Root tips per plant	*T. melanosporum* mycorrhizas per plant [a]
Initial age of seedlings × time			
1-year-old seedling, at month 12	31.5 b	5460 c	1770 b
1-year-old seedling, at month 22	71.8 a	18025 b	4256 a
2-year-old seedling, at month 12	82.6 a	18533 b	5945 a
2-year-old seedling, at month 22	94.0 a	25239 a	6381 a
Land use			
Forest	66.1	15187 j	4411
Firebreak	73.8	18441 i	3832
Liming			
Control	65.5	16379	4129
1 kg m^{-2}	74.4	17250	4094
Heat treatment			
Control	69.6	17565	4312
Drying oven	73.0	15229	3406
Microwaves	67.3	17650	4733

[a] Variable log-transformed

T. melanosporum was present in all the seedlings. The proportion of active root tips colonised by *T. melanosporum* was significantly higher in two- than in one-year-old seedlings (P = 0.005). It was also significantly affected by the interaction between time from plantation and fine root

length (P = 0.003): 12 months after planting the relation between root length and the proportion of active tips colonised by *T. melanosporum* was not significant (P = 0.94); 22 months after planting the relation was significantly negative (P < 0.001) (Fig. 1). None of the soil preparations or land uses showed a significant effect.

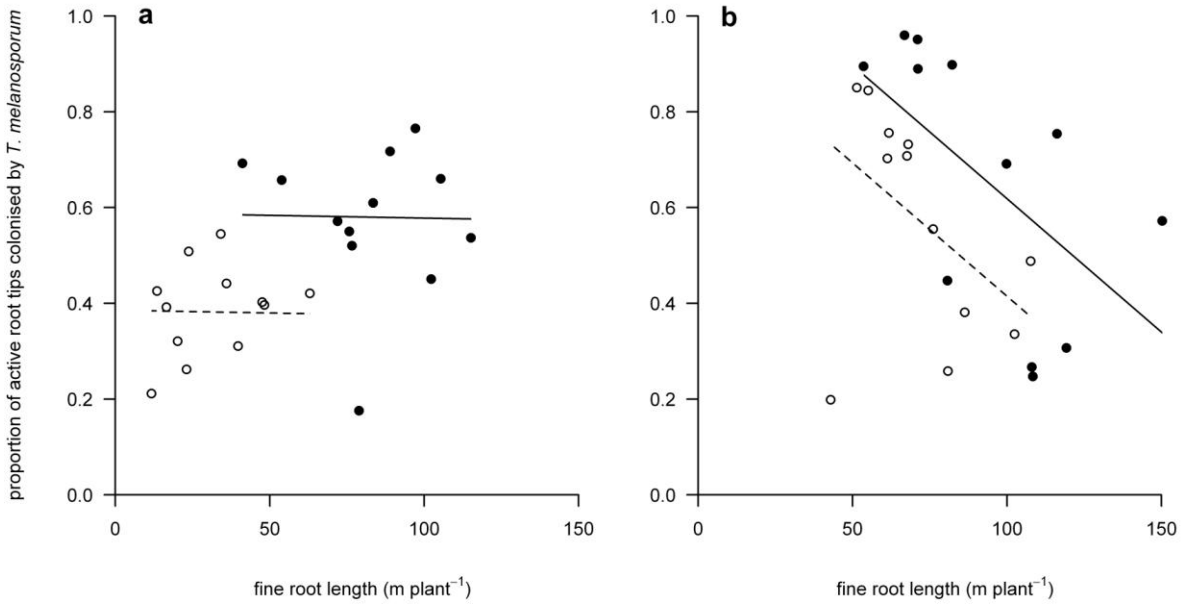

Fig 1 Proportion of active root tips colonised by *T. melanosporum* according to fine root length, at month 12 (a) and at month 22 (b). Open circles and dashed lines correspond to seedlings that were one year old at the time of planting. Full circles and solid lines correspond to seedlings that were two years old at the time of planting.

At month 22 we found native EM fungi on 83% of the seedlings. The proportion of active root tips colonised by native fungi was significantly affected only by the interaction between time from plantation and fine root length (P = 0.01): 12 months after planting the relation between root length and native fungi colonisation levels was not significant (P = 0.87); 22 months after planting the relation was significantly positive (P = 0.003) (Fig. 2). At month 22 the ratio native-to-*T. melanosporum* mycorrhizas was 0.14 for seedlings with mean root length.

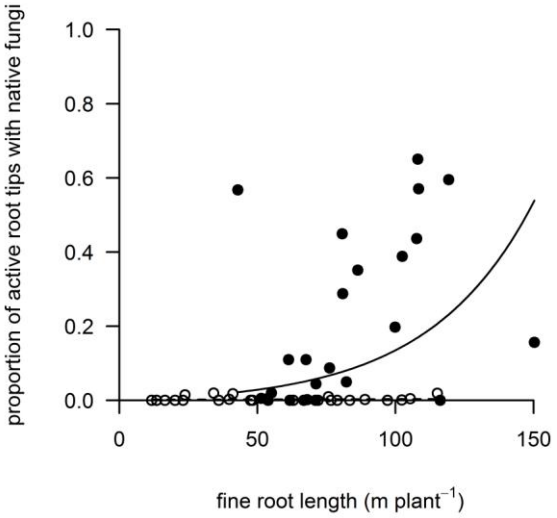

Fig 2 Proportion of active root tips colonised by native EM fungi according to fine root length at month 12 (open circles, dashed line) and month 22 (full circles, solid line).

The richness of native EM morphotypes showed a significant and positive relation with fine root length (P = 0.001) and time from plantation (0.7 types per plant at month 12 and 1.3 at month 22, P = 0.03), although not with their interaction (P = 0.72). The richness was significantly higher in the control heat treatment (1.6 types per plant) than in the drying oven (0.8) and the microwaves (0.7) treatments (P = 0.003); and marginally higher in one- than in two-year-old seedlings (1.3 and 0.7 types per plant respectively, P = 0.05).

Eleven native morphotypes were found in the assay (Table S1), although six of them appeared only on one or two seedlings. The frequency of appearance of four of the five most common morphotypes significantly associated with one land use. In three of them it was significantly lower in the microwaves treatment than in the control (Table 4). Five of the six rarer morphotypes appeared only in the forest (*Tomentella galzinii* Bourdot, type *Hebeloma-Cortinarius*, type *Russula*, type *Pisolithus*, type Thelephoroid), and none of the six appeared in microwaves-treated soil.

Table 4 Frequency of appearance for the most common native EM morphotypes. For each morphotype, letters indicate significant differences (α=0.05) among treatment levels.

	Unidentified 6	Unidentified 1	*Cenococcum geophilum*	Complex *Tuber albidum*	Unidentified 7
Initial age of seedlings					
1-year-old seedlings	0.42	0.13	0.08	0.04	0.08
2-year-old seedlings	0.38	0.13	0.17	0.17	0.08
Time from plantation					
12 months	0.21 b	0.04 b	0.08	0.04	0 b
22 months	0.58 a	0.21 a	0.17	0.17	0.17 a
Land use					
Forest	0.17 j	0.21 i	0.21 i	0.17	0 j
Firebreak	0.63 i	0.04 j	0.04 j	0.04	0.17 i
Liming					
Control	0.38	0.13	0.13	0.13	0.08
1 kg m^{-2}	0.42	0.13	0.13	0.08	0.08
Heat treatment					
Control	0.38	0.25 x	0.31 x	0.06	0.25 x
Drying oven	0.31	0.13 xy	0.06 y	0.13	0 y
Microwaves	0.50	0 y	0 y	0.13	0 y

The frequency of appearance of *S. brunnea* was significantly higher at month 12 (0.42) than at month 22 (0.13, P = 0.02). None of the other predictors showed a significant effect. The proportion of active roots colonised by this fungus ranged from 0 to 20%.

3.1 T. melanosporum *mycorrhizas along the depth profile*

The effect of time and seedling age on the density and proportion of root tips colonised by *T. melanosporum* significantly varied along the depth profile.

The interaction among time, seedling age and depth significantly affected the density of *T. melanosporum* mycorrhizas (P = 0.001). The density increased with time in all depth-levels except for the central core of two-year-old seedlings. Twelve months after planting the maximum density was attained in the central core; 22 months after planting it was attained in the lower core, although in the two-year-old seedlings the density was rather uniform (Fig. 3a).

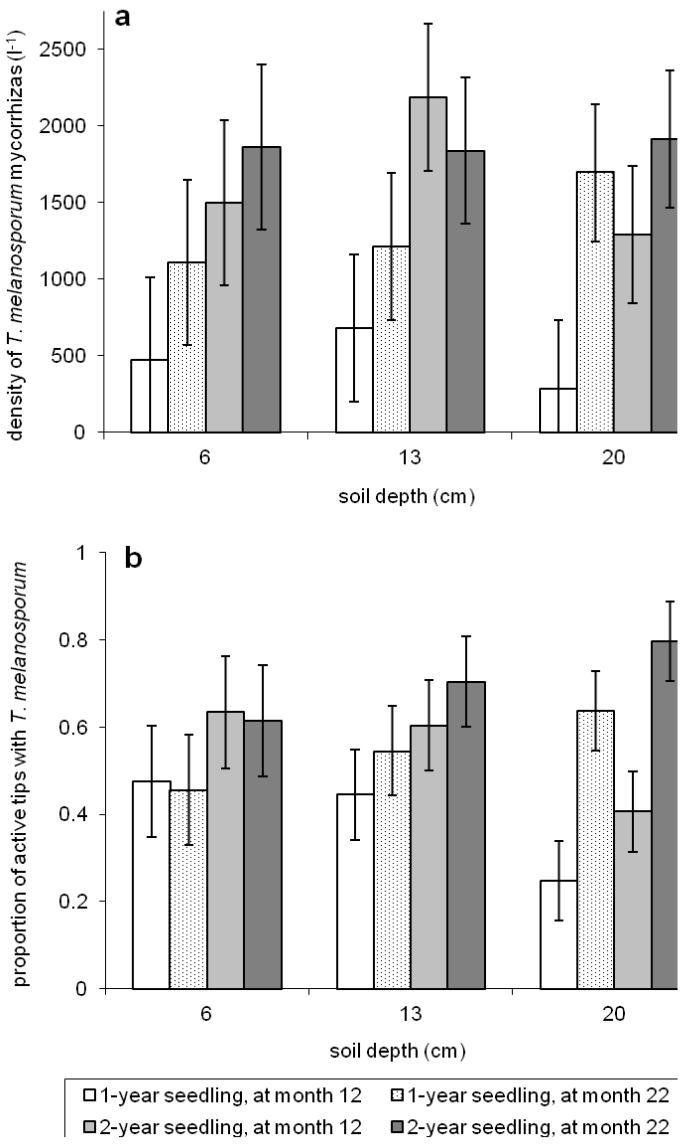

Fig 3 Mean density of *T. melanosporum* mycorrhizas (a) and proportion of active root tips colonised by *T. melanosporum* (b) at the three sampling depths, according to time from plantation and age of seedlings at the time of planting. Error bars correspond to 95% confidence intervals (n=12 for each bar).

The interaction between time and depth significantly affected the proportion of active root tips colonised by *T. melanosporum* ($P < 0.001$), which was also influenced by seedling age ($P = 0.002$). From month 12 to month 22 the colonisation level remained stable in the upper core,

moderately increased in the central core and sharply increased in the lower core, which showed the maximum levels at month 22 (Fig. 3b).

4. Discussion

The proliferation and competitiveness of *T. melanosporum* during the first years after planting can be an early indicator of the viability of *T. melanosporum* cultivation (Martínez de Aragón et al. 2012).

During the study, *T. melanosporum* proliferated in all treatments and almost all depth-levels. The time trends in the abundance of its mycorrhizas were similar to those in fine root length and total root tips (Table 3). At month 22 the maximum density of *T. melanosporum* mycorrhizas occurred in the lower core, where all the roots were produced after plantation in the pot. This supports the receptiveness of these firebreak and forest soils for the *Q. ilex* × *T. melanosporum* symbiosis.

The decrease in the colonisation level of *T. melanosporum* closely associated with the increase of native fungi. After the first year the colonisation level of native fungi was not significant for any value of fine root length, while that of *T. melanosporum* remained similar or higher than initially and was not influenced by fine root length. After the second year fine root length associated negatively with *T. melanosporum* and positively with native fungi. This supports the role of native fungi competition in reducing *T. melanosporum* colonisation levels, and points that they could challenge the success of the plantation.

De Román and De Miguel (2005) and Martínez de Aragón et al. (2012) planted mycorrhizal seedlings on soils of burned forests. The former found that the ratio of native-to-*T. melanosporum* ectomycorrhizas was 0.28 after three years, suggesting that the competitive pressure of native fungi could be similar or slightly higher than in our firebreaks. Martínez de

294 Aragón et al. (2012) found a much higher ratio of 1.3 after 4.5 years; despite this, 26% of
295 seedlings displayed a *brûlé* at year ten.
296 The studies comparing the competition by native EM fungi in forest soils and soils cultivated for
297 non-EM fungi (considered optimal for *T. melanosporum* plantation) are scarce. Frochot et al.
298 (1990) planted inoculated seedlings in a recently-cleared forest soil and found native EM fungi in
299 62% of the seedlings after four years, whereas in a soil cultivated with non-EM plants they found
300 native EM fungi in only 24% of the seedlings. Reyna et al. (2006) planted inoculated seedlings in
301 pots with soil from dense forests and cereal crops, and after 21-28 months they found native
302 fungi in 82–92% and 3–27% of the seedlings respectively. Our results are similar to those of
303 forests (83%), suggesting that the EM inoculum of these firebreaks is more effective in early
304 colonisation than that from soils cultivated with non-EM plants.
305 On the other hand, Sánchez-Durán (2012) sampled eight young *T. melanosporum* plantations in
306 Teruel (Spain) on soils previously cultivated with non-EM plants, and found ratios native-to-*T.*
307 *melanosporum* ectomycorrhizas similar to that in our firebreaks: 0.11 in trees three to seven years
308 old, and 0.17 in trees seven to eleven years old, all of them already producing sporocarps.
309 The proliferation of *T. melanosporum* and the low ratio native-to-*T. melanosporum*
310 ectomycorrhizas tends to support the viability of its cultivation in the studied firebreaks and
311 forests. The potential of both land uses appears to be similar, but the experiment does not take
312 into account the hyphae attached to living trees as inoculum source (Jones et al. 2003). These are
313 likely more abundant in the forest than in the firebreak soils (with a much lower density of EM
314 plants), although we did not measure EM fine root densities in the field. Thus, our study is
315 probably underestimating the competitive pressure of the native EM fungi, especially in the forest.
316 The composition of the effective native EM community (fungi able to colonise the seedlings) was
317 quantitatively different in the firebreak and the forest. Dickie et al. (2009) found distinct EM

communities in North American oak savannas and forests. Since the EM species can differ in their competitive ability once they are established on seedling roots (Hortal et al. 2008), the different EM composition in firebreak and forest soils could cause differences in *T. melanosporum* persistence in the long term.

The differences between one- and two-year-old seedlings at the time of planting affected the performance of the seedlings and the introduced fungus after plantation. In the first year two-year-old seedlings produced more root tips, and *T. melanosporum* proliferated more than in one-year-old seedlings. From the first year *T. melanosporum* attained a higher colonisation level in two-year-old seedlings—even though there were not significant differences before planting—and two-year-old seedlings showed a lower richness of native fungi. Bourrières et al. (2005) found that the colonisation level of *T. melanosporum* after four years in the field was positively related with its level in the nursery and with growth rates in the field. This supports a key role for nursery-seedling characteristics in the performance of young *T. melanosporum* plantations, although the relative contribution of early root growth and initial number of mycorrhizas remains unclear.

The second year after plantation appeared to be a critical period in the competition for root colonisation, as already pointed by Frochot et al. (1990) and Reyna et al. (2006) for seedlings inoculated with *T. melanosporum*, and by Pruett et al. (2008) for *Tuber aestivum* Vitt. The colonisation level and richness of native fungi related to fine root length and therefore to the ability of the seedling to explore the soil, which is intrinsic to plant growth. Reducing soil infectivity before planting could be an interesting strategy to enhance the proliferation of *T. melanosporum* while maintaining a high colonisation level.

Our results suggest that heating the soil before planting could be useful to reduce EM infectivity. Heating at 65ºC reduced the richness of native fungi on seedling roots—although not their

342 colonisation level—without affecting seedling growth or *T. melanosporum* proliferation. The
343 response to heating was species-specific, agreeing with the findings of Izzo et al. (2006). It would
344 be interesting to test higher temperatures: although Izzo et al. (2006) found that heating the soil to
345 75ºC did not limit root colonisation of bait seedlings, the presence of a nursery-inoculated EM
346 fungus in the roots can hamper colonisation by native fungi (Jones et al. 2003; Kennedy et al.
347 2009).
348 On the other hand, we have not found any significant effect of liming on seedlings or on the EM
349 community colonising the seedlings, in spite of the temporary pH rise. Rineau et al. (2010)
350 pointed that in acidic soils the changes in an EM community after liming were mainly due to a
351 reduction in acidophylic fungi, which are much rarer in calcareous soils like those in our study
352 and in most *T. melanosporum* soils.
353 Some caution is required in extrapolating these results to the field. Although we tried to simulate
354 soil moisture conditions in the field, the Mediterranean region is subject to a more irregular soil
355 water regime with broader ranges. Zambonelli et al. (2000) found that some EM species
356 competed with the nursery-inoculated *Tuber albidum* Pico in greenhouse conditions but not in the
357 field. In our study the occurrence of the pioneer, nursery-adapted *S. brunnea* (Garcia-Montero et
358 al. 2008) decreased with time, suggesting that the experimental conditions were not optimum for
359 such species.
360 Another limitation is the difference in root growth between *Q. ilex* seedlings grown in pots and in
361 the field: Tsakaldimi et al. (2009) found that the fine root length was ten times higher in seedlings
362 germinated in pots than in the field. Similarly, the fine root length in our plants was two orders of
363 magnitude higher than in four- and five-year-old field plantations (Olivera et al. 2011; Martínez
364 de Aragón et al. 2012) and the number of root tips was one order of magnitude higher. The
365 differences in the density and distribution of root tips are likely to affect the proliferation and

competitiveness of *T. melanosporum*. It would be interesting to evaluate the relation between *T. melanosporum* colonisation level and root length in seedlings with lower root lengths, similar to those found in field seedlings.

Despite these limitations, the pot experiment proved useful as a first approach to evaluating the potential of a soil for *T. melanosporum* cultivation. *T. melanosporum* has shown able to proliferate in the firebreak and forest soils of a *T. melanosporum*-producing region, and to maintain high colonisation levels despite the infection by native EM fungi. The next step to design *T. melanosporum* plantations for firebreaks is to assess the viability in field plantations, where the soil environment can be more limiting and the living roots of other trees can colonise the planting holes. The second year after plantation appeared as a critical period in the competition for root colonisation between *T. melanosporum* and native fungi, since the colonisation level of the latter sharply increased. Nursery-seedling quality and heating the soil before plantation are promising for increasing the probability of success of these plantations, whereas liming did not cause any significant effect at the dose applied.

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460 **Table S1** Description and overall frequency of appearance of the EM morphotypes (ordered according to their
461 frequency)

Morphotype	Freq.	Colour	Mantle[a]	Emanating elements[b]
Tuber melanosporum	1	Orange to brown	PS-type M	C: yellowish-reddish, right angle-ramified, non-clamped
Unidentified 6	0.40	Pale yellow or whitish rose	Type B in young mycorrhizas, type P in the older	H: scarce, hyaline, wide, covered by crystals, Y-shaped ramification, enlarged in or between the septa, sometimes with ring-like shapes, non-clamped
Sphaerosporella brunnea	0.28	Yellow to reddish black	PS-type P	H: hyaline to reddish, wide, ramified, constricted in the septa, non-clamped
Unidentified 1	0.13	Pale yellow to brown	PS-type P	C: hyaline, short, non-ramified, capitate, non-clamped H: hyaline-yellow, sometimes with ring-like shapes, ramified, non-clamped
Cenococcum geophilum	0.13	Black	PL-type G	H: dark brown, thick-walled, non-ramified, non-clamped
Complex *Tuber albidum*	0.10	Yellow to brown	PS-type M	C: bristle-like, hyaline to pale yellow, thin, sometimes geniculate base
Unidentified 7	0.08	Reddish black	PS-type O	H: reddish brown, ramified, anastomising, sometimes with warts, with clamped and non-clamped septa R: type C, reddish brown, forming nodia at branching
Tomentella galzinii	0.04	Yellowish to greenish brown	PS-type L	C: bristle-like, enlarged base, yellow below the first septa, clamped H: yellow, ramified, clamped R: type A, yellowish-greenish
Type *Hebeloma-Cortinarius*	0.02	Whitish rose to brown	PL-type B	H: hyaline, ramified, enlarged in the septa, clamped, anastomising R: type A, hyaline, with fan-like connection to the mantle
Type *Russula*	0.02	Witish to yellowish brown	PL-type B	C: hyaline, flask-shaped
Type *Pisolithus*	0.02	Golden-orange	PL-type B	H: yellowish brown, ramified, sometimes ribbon-like, clamped R: type B, brown, ramified, with inflated cells
Type Thelephoroid	0.02	Whitish grey to brown	PL-type D	C: hyaline, awl-shaped, non-ramified, with clamped (when only one) and non-clamped septa H: hyaline, Y-shaped ramification, anastomizing, with clamped and non-clamped septa
Tuber brumale	0.02	Orange to brown	PS-type M	C: yellow, bristle-like, enlarged base, usually without septa

462 [a] PL: plectenchymatous, PS: pseudoparenchymatous (Agerer, 1987-2002)

463 [b] C: cystidia, H: hyphae, R: rhizomorph