Intraspecific competition results in reduced evenness of *Tuber melanosporum* mating type abundance from the nursery stage

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Abstract

The highly-prized black truffle is a fungus mostly harvested in orchards planted with mycorrhizal seedlings. It is an obligatory outcrossing fungus with a single *MAT* locus containing two alternative mating-type idiomorphs. In the orchards, at the mycorrhizal level, these mating types are frequently spatially segregated. Some studies found that this segregation was pronounced from the nursery stage, whereas others did not find such a marked segregation. Besides, information on the host tree species and nursery conditions used in Spain, one of the main truffle-producing countries, are very scarce. In this study we investigated the temporal dynamics of mating types in nursery seedlings of *Quercus ilex* and *Quercus faginea*, as well as the influence of cultural conditions in the nursery. Our results indicated that at the plant level, there was a trend for one of the mating types to dominate over the other from the first to the second year in the nursery, in both host species and both nursery conditions tested. However, this segregation process was not so sharp as previously reported. Our results support the hypothesis that intraspecific competition results in reduced evenness of mating type abundance from the nursery stage, although almost all seedlings maintained both mating types and, at the seedling batch scale, the occurrence of both mating types was roughly balanced.

Keywords

Truffle cultivation, *Tuber melanosporum*, mycorrhizal seedling, ectomycorrhiza, mating types, MAT genes

1. Introduction

The European black truffle (*Tuber melanosporum*) is an ectomycorrhizal fungus highly appreciated in *haute cuisine* due to its unique organoleptic qualities. Its wild production has decreased in recent decades, so although black truffle grows in oak forests of southern Europe, nowadays most of its production is harvested in orchards planted with seedlings previously inoculated in nurseries under controlled conditions [1, 2]. Part of these plantations have failed to produce truffles, due to factors such as unsuitable soils, dubious climate conditions, poor quality of the mycorrhizal seedlings or inadequate cultural practices [3, 4]. Truffle cultivation is not completely domesticated yet, as the uncertainties around its life cycle remain [5, 6]. Before and after truffle genome was sequenced, many studies have drawn attention to the role of truffle sexuality in the productivity of truffle plantations [7–10]. *Tuber melanosporum* is a heterothallic organism, an obligatory outcrossing species with a single *MAT* locus containing two alternative mating-type idiomorphs (*MAT1-1* and *MAT1-2*) responsible for the mating process [7, 10, 11]. Mycorrhizae and ascospores of *T. melanosporum* are formed by haploid cells, but truffle fruiting (sexual reproduction) requires the concurrent presence of both mating types [11, 12]. During this mating process, the mycelium from ectomycorrhizae behaves as the maternal partner, whereas ephemeral free mycelia present in the soil act as paternal material [8, 12, 13].

In the nursery, where mycorrhizal seedlings are mostly produced using a spore suspension as the inoculum, this ensures the presence of both mating types in the roots, since each fruitbody is likely to produce spores of both mating types at roughly equal ratios [12, 14]. However, once seedlings are planted in the field, it is frequently found a spatial segregation of the mycorrhizal mating types, with host plants in adult *truffières* tending to sustain only one truffle genet or multiple genets of the same mating type [8, 13, 15]. This situation does not

seem to happen during the first year in the nursery, just after seedlings are inoculated. Rubini et al. [12] found that during the first year the abundance of mating types in the seedlings was balanced, whereas in the second year they only found both mating types in half the seedlings due to genet competition and displacement from the roots. However, other studies have not found such a marked trend, with all the analyzed seedlings presenting both mating types [16, 17]. Information about this displacement process and about the possible influence of host species and nursery cultural practices could be useful for improving productivity and sustainability in truffle cultivation.

Although there are many species susceptible to mycorrhization by *T. melanosporum*, the consolidation of the symbiosis in the field and the harvesting of truffles over the years occurs mainly with oak species [1, 18, 19]. Therefore, in Spanish nurseries, *Quercus ilex* and *Quercus faginea* are the most commonly used species, the latter being a faster-growing species that produces more fine roots in the nursery [20]. The carbon that the fungus obtains from the plant through these fine roots is a major trophic resource for which ectomycorrhizal fungi compete [21]. Not only the host species, but also the environmental conditions and the cultural practices in the nursery influence the number of root tips produced by the seedling and the timing of fine-root production [22]. This could also influence the intraspecific competition in truffle, since in the first year in nursery primary infection (from spore-generated mycelium) seems to be the dominant process, while in the second year secondary infection (from associated-to-mycorrhizae mycelium) gains weight, as suggested by the fact that mycorrhizae from a given root branch are frequently genetically identical [23]. Finally, the genetic diversity of the truffle fruitbodies used as inoculum and its dose could also play a role, since they may influence the genetic diversity of the mycorrhizal population established

on the seedling roots, as suggested by genotyping studies in orchards inoculated with mycelium and orchards outside the natural distribution area [16, 24].

In the present study, we investigated the temporal dynamics of *T. melanosporum* mating types in nursery seedlings and the influence of host plant species and cultural practices on these dynamics. We evaluated the relative abundance of mating types during the first and second year in the nursery for the two most frequent host plants in Spanish truffle plantations: *Q. ilex* and *Q. faginea*. We raised the seedlings in two different locations, using different substrates, containers and inoculation doses (all of them within the standard cultural practices in commercial nurseries), in order to obtain contrasting seedling characteristics that would improve the generalizability of the results. We hypothesized that mating types would show a more unbalanced distribution in older seedlings. We also hypothesized that *Q. faginea* and seedlings with faster root growth would show a more balanced representation of mating types, since a major trophic resource for which the mycorrhizal fungi individuals compete (the root tips from which they receive the plant carbon) would be less limiting [25].

2. Materials and methods

2.1. Experimental design

A full factorial design was used to evaluate the effect of three independent variables (plant species, time from inoculation and nursery) and their interactions on the relative abundance of the mating types in the roots of the nursery seedlings. Two plant species (*Q. ilex* and *Q. faginea*) were evaluated at two different moments (6 and 18 months from inoculation). The seedlings were raised at two distant nurseries (El Toro and Graus) with different growth medium, container volume and inoculum application rate. We did so in order to obtain contrasting seedling characteristics that improve the generalizability of the results. A total of

96 seedlings (12 replicates for each of the 8 treatments) were produced, although only 73 of them were finally included in the study due to seedling mortality or to extreme defects in seedlings morphology (e. g. stem die-back or lack of fine roots): 8 for *Q. faginea* in Graus at month 6, 10 for *Q. ilex* in El Toro at month 6, 10 for *Q. faginea* in El Toro at month 18, and 9 for all the remaining combinations of host tree, nursery and time from inoculation (Table S1). The *T. melanosporum* fruitbodies used as inoculum (2 kg, more than 60 fruitbodies) were harvested fresh and mature from plantations in Huesca province (northern Spain). They were surface cleaned with a brush under cool water, surface sterilized by immersion in ethanol (96%) and flamed, taxonomically identified by morphological features, sliced thin, air dried under room conditions and homogenized with a coffee grinder. The oak acorns were acquired from the Spanish provenance region *Sistema Ibérico*, surface sterilized with a 10% sodium hypochlorite solution for 30 min, and germinated in January 2016 in a vermiculite tray. In May 2016, when most seedlings had 6-8 leaves and had formed lateral roots, they were removed from the tray, mechanically root-pruned at the tap root to eliminate defects when they existed, inoculated and transplanted to containers (650 mL Quick-pot® in El Toro nursery and 450 mL Full-pot® in Graus). Seedlings with malformations, poor development or scarce fine roots were excluded. The inoculation was performed by root powdering with a talcum powder (hydrated magnesium silicate) carrier, following Garcia-Barreda et al. [20]. The inoculum quantity was adjusted to obtain a rate of 2.5 g fresh truffle per seedling in El Toro nursery and a rate of 1.0 g in Graus. The potting substrate in the El Toro nursery consisted of 12:6:1 (v/v) calcareous sandy loam soil, base-fertilized *Sphagnum* white peat (Kekkila® White 420W), and limestone coarse sand, while in Graus it consisted of 3:2:1:1 (v/v) calcareous clay loam soil, Prohumin® 5050 Substrate (50% *Sphagnum* black peat, 50% *Sphagnum* white peat), limestone coarse sand and perlite.

144 The seedlings were maintained in the nursery greenhouse and sprinkle irrigated to saturation
145 2-3 times per week during summer and once a week during winter. Both nurseries are located
146 in wild truffle producing regions with Continental Mediterranean climate and a mean annual
147 temperature of 12.0-12.5ºC, although the El Toro nursery is located at 1000 m a.s.l. in
148 Castellón province (eastern Spain) and the Graus nursery is located at 600 m a.s.l. in Huesca
149 province (northern Spain).
150
151 *2.2. Data collection*
152 For each plant species × nursery combination, half of the seedlings were randomly selected
153 and analyzed in October-November 2016 (6 months from inoculation) and the other half in
154 October-November 2017 (18 months). In each sampling, the stem height and the root-collar
155 diameter of seedlings were measured and the number of root tips per seedling, *T.*
156 *melanosporum* mycorrhizae per seedling and proportion of root tips colonized by *T.*
157 *melanosporum* were evaluated.
158 The mycorrhizal status was assessed through random sampling of roots. Fine roots (diameter
159 < 2 mm) were cut under water in portions with length < 1 cm and spread over a grid with 2 ×
160 2 cm square size. A 10% of the grid squares were randomly selected, and the root tips were
161 counted. With the aid of binocular and optical microscopes, tips were classified as non-
162 mycorrhizal or mycorrhizal, and the latter were classified as *T. melanosporum* or contaminant
163 morphotypes [26].
164 Sixteen *T. melanosporum* mycorrhizae were randomly selected in each plant, which is a
165 sample size similar to that of previous nursery studies for this bivalent sexual locus [12, 17].
166 With this purpose, 16 grid squares were randomly selected and one mycorrhiza was taken
167 from each of these squares. These tips were cleaned under the stereomicroscope using fine

forceps, placed in 0.2-mL sterile tubes containing 10 μL of Extraction Solution (Sigma-Aldrich, USA) and stored at -20 ºC for further molecular analysis.

For mating type characterizations, genomic DNA was extracted from each individual ectomycorrhiza: frozen tips were incubated for 10 min at 24ºC and 10 min at 95 °C, following Extract-N-Amp™ (Sigma-Aldrich, USA) recommendations. Then, 10 μL of Dilution Solution (Sigma-Aldrich, USA) were added and tubes were centrifugated at 6.000 rpm for 2 min. A PCR was arranged with primers P19 and P20 to amplify *MAT1-1* and primers P1 and P2 for *MAT1-2* designed for *T. melanosporum* [11]. Each 12,5-μl PCR reaction consisted of 2,5 μL of 5x MyTaq™ Reaction Buffer Red (Meridian Biosciences, USA), 0,5 μL bovine serum albumin (Sigma-Aldrich, USA) at 10 mg mL^{-1} [27], 0.25 μL of each primer at 10 μM, 7 μL of ultra pure water to reach the volume mix of 18 μL and 0,25 μL of MyTaq™ DNA Polymerase (Meridian Biosciences, USA). 1,25 μL of genomic DNA were added for each sample. The PCR reactions were performed in a Applied Biosystems (USA) 2720 thermal cycler with the following thermal profile (adapted from Marozzi et al. [17]): initial denaturation step of 5 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s; and a final extension step at 72 °C for 7 min. A *T. melanosporum* fruitbody was collected and genomic DNA was extracted from its spores [28], to be used as a positive control in PCR to highlight the proper amplification of both mating type genes in every reaction. All PCR experiments included a negative control (no DNA template). Ten μL of PCR products were run on 1.8% (w/v) agarose gel electrophoresis stained with SYBR® Safe DNA Stain (Invitrogen, USA).

ITS regions of competing ectomycorrhizae (not *T. melanosporum*) DNA were sequenced following the conditions detailed in Gómez-Molina et al. [29]. The quality of the obtained sequences was assessed, and low-quality edges were removed with 4Peaks v1.7.2

(https://nucleobytes.com/4peaks). Fungal identification was carried out by searching highly similar sequences in the GenBank and UNITE (https://unite.ut.ee) databases using the megablast procedure and default settings [30].

2.3. Data analysis

The seedling root-collar diameter, stem height, number of root tips, number of *T. melanosporum* mycorrhizae and percent root colonization by *T. melanosporum* were analyzed with general linear models. When model assumptions (homogeneity of variance, normality and linearity) were not met, the response variable was transformed. The proportion of seedling roots colonized by each mating type (*MAT1-1* vs. *MAT1-2*) was analyzed with a generalized (binomial) linear model. We also analyzed the proportion of root tips colonized by the dominant mating type in each seedling (regardless of whether the dominant in that seedling was *MAT1-1* or *MAT1-2*), in order to evaluate the evenness of mating type representation (i. e. to measure how balanced is the abundance of the dominant and the non-dominant mating type in a seedling). This proportion, which ranges from 0.5 (complete evenness) to 1 (no evenness), was also analyzed with a generalized (binomial) linear model. In the generalized linear models, the fit of the binomial error structure was assessed through overdispersion, and when this assumption was not met, a quasibinomial error structure was used. Least-squares means tests were used for post-hoc comparisons, with a $P = 0.05$ threshold for statistical significance.

A post-hoc correlation analysis was conducted to explore seedling characteristics that influence the proportion of mycorrhizae colonized by the dominant mating type. With this purpose, we calculated partial Pearson correlations between the proportion of the dominant mating type and seedling characteristics, controlling for the age of the seedlings.

All analyses were conducted with R and the emmeans and correlation packages [31–33].

3. Results

The root-collar diameter of the seedlings was significantly affected by the interaction between nursery and seedling age ($t = 3.50$, $P < 0.001$, Table S2, Fig. S1), with all seedlings significantly increasing their size over time, although faster in the El Toro nursery (Table 1). The stem height of the seedlings was significantly affected by the nursery ($t = 13.3$, $P < 0.001$), seedling age ($t = 4.4$, $P < 0.001$) and host species ($t = 2.1$, $P = 0.039$, Table S3, Fig. S2), with height being higher in El Toro nursery, older seedlings and *Q. ilex* (Table 2). The number of root tips was significantly affected by the interaction between nursery, age and species ($t = 2.54$, $P = 0.014$, Table S4, Fig. S3), whereas the number of *T. melanosporum* mycorrhizae was significantly affected by the nursery ($t = 5.83$, $P < 0.001$), seedling age ($t = 7.21$, $P < 0.001$) and seedling species ($t = 2.43$, $P = 0.018$, Table S5, Fig. S4). Both root tips and *T. melanosporum* mycorrhizae tended to be more abundant in older seedlings, in *Q. faginea* seedlings and in the El Toro nursery (Tables 2, 3). The percent root colonization by *T. melanosporum* was significantly affected by the interaction between nursery and seedling age ($t = 2.02$, $P = 0.047$, Table S6, Fig. S5). Both nurseries showed high levels of colonization at month 18, but only El Toro nursery presented high levels also at month 6 (Table 1). Besides *T. melanosporum*, two other ectomycorrhizal morphotypes were found in the seedlings: *Sphaerosporella brunnea* in El Toro nursery (in 6.8% of seedlings) and *Telephora ellisii* in Graus nursery (1.4% of seedlings).

More than 1000 ectomycorrhizae were analyzed and the mating type was determined in 862 of them, retrieved from a total of 73 seedlings (466 mycorrhizae with *MAT1-1* and 396 with *MAT1-2*; Table S1). The generalized linear model analyzing the proportion of *MAT1-1* mycorrhizae did not show any significant effect of seedling species, age or nursery on the

240 proportion between mating types (t = 1.78, P = 0.080 for the seedling species; t = 1.39, P =
241 0.169 for the seedling age; and t = -1.96, P = 0.054 for the nursery, Table S7). All seedling
242 batches showed a predicted proportion that was not significantly different from 0.5, which
243 represents a 1:1 ratio, or very close to this value (Fig. 1).
244 The evenness of mating types abundance, computed as the proportion of root tips colonized
245 by the dominant mating type on each seedling, was significantly affected by seedling age (z =
246 2.56, P = 0.010), with no significant effect of seedling species (z = 1.29, P = 0.199) or nursery
247 (z = 0.27, P = 0.788) (Table S8). At month 6, seedlings showed a predicted proportion that
248 was significantly higher than 0.5, but at month 18, this proportion significantly increased by
249 8%, this corresponding to a predicted change from a 2:1 to a 3:1 ratio between the dominant
250 and the non-dominant mating type in each seedling (Fig. 2). Despite these ratios, there were
251 only two seedlings (2.7%) in which just one mating type was found (one at month 6 and the
252 other at month 18).
253 Since the proportion of seedling mycorrhizae colonized by the dominant mating type showed
254 a significant time trend, a post-hoc correlation analysis was conducted to explore its
255 relationship with the seedling morphological characteristics. When controlling for seedling
256 age, the proportion of mycorrhizae colonized by the dominant mating type did not show any
257 significant correlation with the analyzed seedling characteristics (Table 4, Fig. S6).
258

259 **4. Discussion**

260 Our results indicate that the abundance of *MAT1-1* and *MAT1-2* in nursery seedlings was
261 overall balanced from the 6 to the 18 months after inoculation. They also indicate that, at the
262 plant level, there was a trend for one of the mating types to dominate over the other from the
263 first to the second year in the nursery. These results were consistent across two host species

and the contrasting growing conditions in two distant nurseries, agreeing with previous studies on *Q. pubescens* [12] and *Carya illinoinensis* [17], which used lower sample sizes. Although our results broadly agree with those of Rubini et al. [12], they show some differences regarding the extent of the process of displacement of the non-dominant mating type. For seedlings in their second year in the nursery, Rubini et al. [12] only found mycorrhizae of both mating types in 41% of the seedlings (this roughly corresponding to a 7:1 ratio between the dominant and the non-dominant mating type in each plant). Finding only one mating type in the root system of a plant is common in the field, with older plants [12, 15, 16]. However, we found the two mating types in 97% of the seedlings in their second year, whereas Linde and Selmes [16] and Marozzi et al. [17] found both mating types in all their nursery seedlings. Interestingly, Rubini et al. [12] inoculated each seedling with the spores of only one fruitbody, whereas we, Marozzi et al. [17] and probably Linde and Selmes [16] used as inoculum the spores obtained from several fruitbodies, which is a common practice in commercial nurseries. The quicker process of displacement of mating types that Rubini et al. [12] found might be related to the lower genetic diversity of the inoculum and thus of the mycorrhizal population initially established on the plants, which is a frequent outcome with other organisms such as plants [34].

Other factors could also be the cause of this difference with Rubini et al. [12], such as seedling species, inoculum dose, cultural practices in the nursery or size of seedlings (in so far as size may reflect the abundance of root tips and/or the timing of fine root production). However, in our study we did not find differences between *Q. ilex* and *Q. faginea*, despite the fact that the latter produces more root tips and thus more mycorrhizae are formed [20]. Regarding the inoculum dose and cultural practices, previous studies do not detail such factors, thus precluding comparisons. However, in our study we did not find differences

between the two nurseries, despite the fact that in one of them plants formed more root tips and mycorrhizae, especially in the first year. After controlling for the effect of plant age, no correlation was found between the abundance of root tips and the evenness among mating types. Our results suggest that, for the range of root tip abundance in our study, this variable does not condition the competition and displacement process between mating types, in spite of root tips being a major trophic resource for mycorrhizal fungi [25].

The absence of a relationship between the abundance of the trophic resource (root tips) and the intensity of the competition process (with the mating type evenness as a proxy) raises the question of which is the trigger of the displacement process. Rubini et al. [12] results suggest that differences in mating type evenness do not come from differences in the proportions of mating types in the spore inoculum or in the ability of mating types for primary infection, since they inoculated each seedling with spores of only one fruitbody and found a roughly balanced occurrence of mating types in seedlings during their first year in the nursery. This points to some process related with secondary infection (from other mycorrhizae and associated mycelium), which gains importance in the second year in the nursery. Rubini et al. [12] and Selosse et al. [5] hypothesized that the competition for root tip colonization is related to a self/nonself recognition mechanism linked to the *MAT* locus. In vitro dual cultures of genetically different *T. melanosporum* strains showed that hyphae from different strains did not form anastomoses while hyphae from the same strain frequently did, suggesting a vegetative incompatibility based on a mechanism acting before hyphal fusion [35]. In any case, it is likely that this process of self/nonself recognition is not dependent on root tip abundance.

In truffle orchards, the process of mating displacement from the plant roots seems to intensify. In young plantations, Linde and Selmes [16] observed that only half of the plants maintained

mycorrhizae of the two mating types, whereas in adult orchards and wild *truffières* Murat et al. [15] and Rubini et al. [12] found that most host trees had only one mating type in their roots. It would be interesting to study whether the number of mycorrhizae formed in the nursery, their genetic diversity, or the mating type evenness in the nursery can influence the process of spatial segregation of mating types observed once the seedlings are planted in the field. In any case, this spatial segregation at the mycorrhizal level does not seem to be so marked at the level of free mycelia in the soil (some of which act as parental material in mating), as suggested by recent research assessing both types of mycelia together [9, 36].

To conclude, at the seedling batch level we found a roughly balanced representation of the two mating types in the mycorrhizae of seedlings inoculated with *T. melanosporum* until month 18 after inoculation. Despite this, at the plant level we found a trend for one of the mating types to dominate over the other during the second year in the nursery. Together, these findings support the hypothesis that intraspecific competition results in reduced evenness of mating type abundance from the nursery stage, corroborating that this process begins in the second year, when the importance of secondary infection increases with respect to primary infection. We did not find differences in this process related to host species (*Q. ilex* versus *Q. faginea*), to cultivation conditions in the nursery or to seedling size. Our study corroborates, with a larger sample size, the results of previous studies, extending them to the more frequent host plants for *T. melanosporum* in Spain and connecting these results with cultivation conditions and seedling characteristics, thus providing helpful information for commercial nurseries producing mycorrhizal seedlings.

Data Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Statements and Declarations

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Author Contributions

E.G.-M., S.S. and S.G.-B. conceived and designed the study. Funding was secured by E.G.-M. and S.S. Laboratory protocols were conducted by E.G.-M., M.P.-P. and S.S. The data were analyzed by S.G.-B. The manuscript was initially written by E.G.-M., along with significant contributions from S.S. and S.G.-B.

Competing Interests

The authors declare no competing interests.

Table 1. Effect of the interaction between nursery and seedling age on root-collar diameter of seedlings and percent root colonization by *T. melanosporum* (mean predicted values and 95% confidence intervals). Letters within each column indicate significant differences (least-squares means, $\alpha = 0.05$, n = 73).

	Root-collar diameter (mm)	Percent root colonization [1]
El Toro		
6 months	4.0 (3.6, 4.3)c	35.4 (28.0, 43.7)a
18 months	6.5 (6.1, 6.8)a	37.3 (29.7, 45.8)a
Graus		
6 months	3.4 (3.0, 3.7)c	11.8 (7.5, 17.1)b
18 months	4.7 (4.4, 5.0)b	29.1 (22.2, 36.9)a

[1] Variable square-root transformed

Table 2. Effect of the host plant, the nursery and seedling age (main effects) on the height of the plant stem and the number of *T. melanosporum* ectomycorrhizae (ECM) per seedling (mean predicted values and 95% confidence intervals). For each predictor variable (host species, nursery, seedling age), letters indicate significant differences (least-squares means, $\alpha = 0.05$, n = 73).

	Stem height (cm) [1]	No of truffle ECM ($\times 10^3$) [2]
Host species		
Q. ilex	17.6 (16.0, 19.4)a	1.4 (1.1, 1.6)b
Q. faginea	15.3 (13.9, 16.8)b	2.2 (1.9, 2.5)a
Nursery		
El Toro	25.8 (23.5, 28.3)i	2.7 (2.3, 3.0)i
Graus	10.4 (9.5, 11.5)j	1.0 (0.8, 1.2)j
Seedling age		
6 months	14.1 (12.8, 15.6)q	0.6 (0.5, 0.8)q
18 months	19.0 (17.3, 20.9)p	3.4 (3.0, 3.8)p

[1] Variable log-transformed

[2] Variable square-root transformed

Table 3. Effect of the interaction between nursery, host plant and seedling age on the number of root tips per seedling (mean predicted values and 95% confidence interval). Different letters indicate significant differences (least-squares means, $\alpha = 0.05$, n = 73).

	No of root tips ($\times 10^3$) [1]
Q. ilex El Toro	
6 months	2.42 (1.78, 3.16)d
18 months	9.59 (8.20, 11.10)b
Q. ilex Graus	
6 months	1.40 (0.90, 2.00)d
18 months	5.46 (4.42, 6.61)c
Q. faginea El Toro	
6 months	5.47 (4.43, 6.62)c
18 months	15.5 (13.8, 17.3)a
Q. faginea Graus	
6 months	2.01 (1.36, 2.77)d
18 months	12.0 (10.4, 13.7)b

[1] Variable square-root transformed

Table 4. Partial Pearson correlations between the characteristics of nursery seedlings and the evenness of mating types abundance (computed as the proportion of seedling mycorrhizae colonized by the dominant mating type in the seedling, square-root transformed), after controlling for seedling age.

	No of seedlings	Partial correlation	P-value
Root-collar diameter	73	-0.03	0.80
Stem height [1]	73	-0.14	0.24
Number of root tips [2]	73	0.09	0.47
Number of *T. melanosporum* mycorrhizae‡	73	0.10	0.40
Percent roots colonized by *T. melanosporum*‡	73	-0.003	0.98

[1] Variable log-transformed

[2] Variables square-root transformed

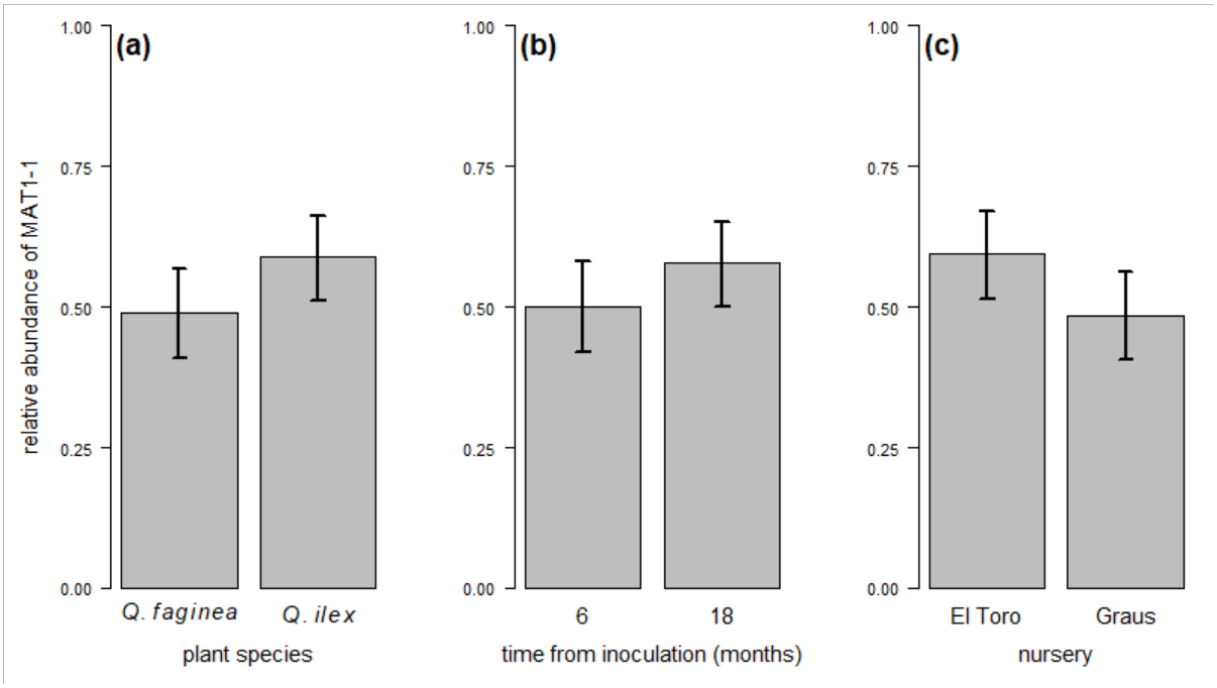

Figure 1. Relative abundance of *MAT1-1* in the ectomycorrhizae of the nursery seedlings (mean predicted values and 95% confidence intervals, n = 73), according to seedling species (a), time from inoculation (b) and seedling batch (c). Overlapping of the confidence intervals indicates lack of significant differences according to the least-squares means procedure.

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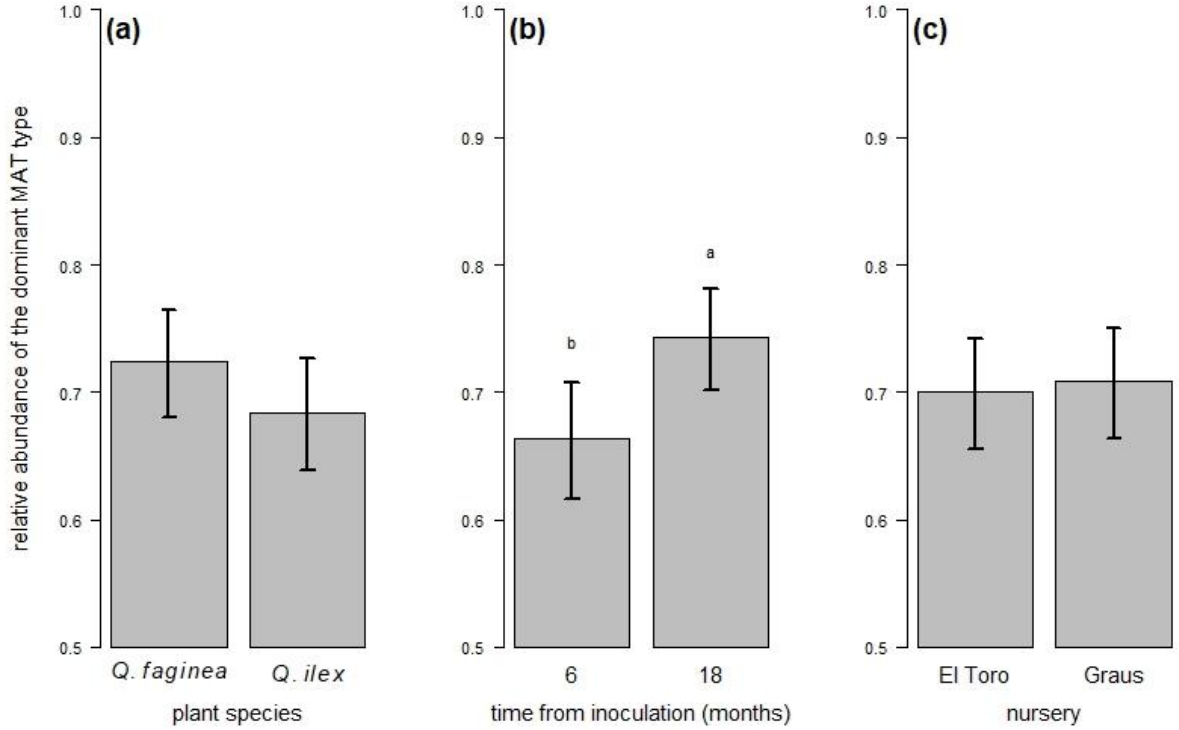

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502 **Figure 2**. Proportion of seedling mycorrhizae colonized by the dominant mating type in the
503 seedling (mean predicted values and 95% confidence intervals, n = 73). A value of 1 means
504 no evenness and a value of 0.5 means complete evenness between the two mating types. (a)
505 Differences between seedling species. (b) Relationship with time from inoculation. (c)
506 Differences between the two nursery batches produced. Letters indicate significant differences
507 according to the least-squares means procedure ($\alpha = 0.05$).