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Sequential application of inoculation methods improves mycorrhization of *Quercus ilex* seedlings by *Tuber melanosporum*

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Abstract

The use of mycorrhized seedlings has been critical to the success of modern truffle cultivation, which nowadays supplies most European black truffles (*Tuber melanosporum*) to markets. Ascospore inoculation has been traditionally used to produce these seedlings, but little scientific information is publicly available on the inoculation methods applied or on the possibility of combining them. We evaluated the potential of sequential inoculation for the controlled colonization of holm oak fine roots by *T. melanosporum*, with two different nursery assays and a full factorial design. Three inoculation methods were sequentially applied: radicle inoculation, inoculation of the substrate in seedling trays and inoculation of the substrate in the final pot. Despite the differences in the results of the two assays, which suggest that cultivation conditions and/or the timing of nursery operations may influence the relative effectiveness of inoculation methods, the sequential application appeared as an effective and realistic alternative for commercial inoculation of holm oak seedlings with *T. melanosporum*. The increase in the amount of inoculum applied with each inoculation method improved the mycorrhizal colonization of seedlings, whereas separately none of the inoculation methods appeared clearly superior to the other ones. The depth distribution of truffle mycorrhizae pointed that the inoculation in the final pot was more effective than other methods in lower parts of the root system, whereas the early inoculation appeared more effective to reduce the occurrence of the opportunist ectomycorrhizal fungus *Sphaerosporella brunnea*.

Keywords

Truffle cultivation, *Tuber melanosporum*, ectomycorrhiza, mycorrhizal seedling, inoculation methods

1. Introduction

The European black truffle (*Tuber melanosporum* Vittad.) wild production has been declining in recent decades, due to overexploitation and canopy density increasing, among other causes (Garcia-Barreda et al., 2018; Baragatti et al., 2019). When researchers in the 1970s developed the controlled production of mycorrhizal seedlings, black truffle cultivation had a major boost and nowadays, it has become an important economic alternative for rural areas (Olivier et al., 1996; Chevalier, 2001). More than 40,000 hectares of seedlings inoculated with *T. melanosporum* have been planted in southern Europe, with plantations playing a largely dominant role in the global truffle production and with *Quercus ilex* L. being the main host tree in Spain and also widely used in France and Italy (Bencivenga, 2001; Chevalier, 2001; Reyna and Garcia-Barreda, 2014).

Modern truffle cultivation is based on planting mycorrhizal seedlings on lands with low ectomycorrhizal inoculum potential and appropriate edaphoclimatic conditions for the fungus to complete its life cycle (Sourzat, 2008). The choice of a good quality mycorrhizal seedling is critical to the success of a truffle plantation, since (i) the abundance of truffle mycorrhizae in the early years after plantation is related to colonization levels in the nursery (Bourrières et al., 2005; Garcia-Barreda and Reyna, 2013), and (ii) mycorrhizae act as maternal material for sexual reproduction and ascomata production in productive *truffières* (Rubini et al., 2011; Taschen et al., 2016). Most mycorrhizal seedlings for truffle plantations are produced in commercial nurseries, which use ascospores as the inoculum source, either incorporating them into the potting substrate or concentrating them onto fine roots (Chevalier and Grente, 1978; Palazón and Barriuso, 2007; Iotti et al., 2012). Seedling inoculation with mycelium is not applied in commercial nurseries due to the slow growth of *T. melanosporum* in vitro mycelium cultures (Iotti et al., 2012), although it is used with *Terfezia* species and with *Tuber borchii* Vittad. (Arenas et al., 2018; Leonardi et al., 2020). The high price of truffle ascomata

78 used as ascospore source has led nurserymen to develop different inoculation methods and to
79 fine-tune the amount of inoculum applied per seedling, especially in nurseries where
80 thousands of seedlings are produced (Averseng and Rouch, 2001; Palazón et al., 2007; Iotti et
81 al., 2012). However, for *Tuber* species, little scientific information is publicly available on the
82 efficiency of inoculation methods or on the possibility of combining these methods, often
83 because of patents and confidentiality agreements (Pruett et al., 2008; Pereira et al., 2013;
84 Garcia-Barreda et al., 2017).

85 The quality of mycorrhizal seedlings is assessed by the level of colonization of the fine
86 roots by the target truffle species, with most evaluation methods using calculated or estimated
87 percent root colonization as the measurement variable (Andres-Alpuente et al. 2014; Donnini
88 et al. 2014). However, these seedlings may present mycorrhizae from other non-desired
89 species, the so-called "contaminants", which sometimes are accidentally introduced with the
90 inoculum and sometimes come with the potting substrate or from the surrounding
91 environment (Iotti et al. 2012; De Miguel et al. 2014). Among the ones associated with
92 potting substrates, the facultatively mycorrhizal *Sphaerosporella brunnea* (Alb. & Schwein.)
93 Svrček & Kubička, a pioneer and opportunist species, is the most common fungal competitor
94 in nurseries producing seedlings mycorrhized by *Tuber* species (Bencivenga et al., 1995; De
95 Miguel et al., 2014; Sánchez et al., 2014). This species is frequently present in marketed
96 *Sphagnum* peat (Danielson, 1984; Ángeles-Argáiz et al., 2016) and generally spreads in
97 truffle nurseries during late autumn and winter, under conditions of high substrate moisture
98 and reduced ventilation of greenhouses (Palazón et al., 2005; Garcia-Montero et al., 2008;
99 Sánchez et al., 2014). Inoculation methods that boost early formation of truffle mycorrhizae
100 could reduce the level of colonization by undesired ectomycorrhizal fungi and thus improve
101 the quality of mycorrhizal seedlings. Seedlings are usually inoculated 2-3 months after the
102 acorn germinate, when they begin to form lateral fine roots (Granetti, 2005; Garcia-Barreda et

al., 2017), but there is no publicly available information on whether earlier inoculation could accelerate and improve the process of mycorrhizae formation.

In this study, we aim to evaluate the potential of a sequential inoculation method for the controlled colonization of *Q. ilex* fine roots by *T. melanosporum*, as well as the relative effectiveness of each inoculation method. Thus, we combined a radicle inoculation method, an inoculation of the substrate in seedling trays (both performed before the seedling produced lateral fine roots) and an inoculation of the substrate in the final pots (performed when the seedling had already produced fine roots). To obtain a more detailed picture, we evaluated not only the occurrence of *T. melanosporum* mycorrhizae, but also their distribution along the depth of the root system, as well as the occurrence of contaminant ectomycorrhizal fungi. We hypothesized that the cumulative application of different inoculation methods (increasing the total quantity of inoculum applied) would improve the levels of root colonization by *T. melanosporum*, despite the fact that previous studies did not find a positive correlation between inoculum quantity and truffle mycorrhizal rates within the inoculum quantity range commonly used in commercial nurseries (Palazón et al., 2007; Pruett et al., 2008). We also hypothesized that the cumulative application of inoculation methods would decrease the levels of contaminant ectomycorrhizal fungi, with early inoculation methods being more effective. Finally, we hypothesized that early inoculation methods, in which inoculum is applied to a shorter root system than the final pots, would result in mycorrhization levels being more irregular along the depth of the root system in the final seedling.

2. Materials and methods

2.1. Experimental design

A full factorial design was used to evaluate the effect of three different nursery inoculation methods on the root colonization levels by *T. melanosporum*, as well as the possible

128 interactions among these inoculation methods, with nine replicates per treatment (n = 72;
129 Table 1). The three methods used ascospores as inoculum: an inoculation of the seedling
130 radicle (I1), an inoculation of the potting substrate in seedling trays (I2) and an inoculation of
131 the potting substrate in the final pots (I3). Non-inoculated controls were included for each
132 method (Table 1). The experiment was conducted in 2015 and then repeated in 2018 with the
133 same experimental design, in order to confirm the results. There were some differences
134 between the two experiments regarding the timing of inoculation (later in the 2018 compared
135 to 2015, which also implies a change in temperatures during the experiment), the ascospore
136 inoculum dose in I1 (higher in 2015) and the potting substrate used, which was soil based and
137 solarized in 2015 and peat-based and non-disinfested in 2018 (Table 2).

138 The *T. melanosporum* ascomata used as inoculum for each experiment were harvested
139 fresh and mature from several orchards in Huesca province (northeastern Spain) during the
140 fruiting season immediately before setting up each experiment. The ascomata were surface
141 cleaned with a brush under cool water, surface sterilized by immersion in ethanol (70%) and
142 flamed, taxonomically identified by morphological features (Riousset et al., 2001), sliced thin,
143 air dried under room conditions and homogenized with a coffee grinder to obtain a powdery
144 inoculum. Two kilograms of fresh truffles (more than 60 ascomata) were used in each
145 experiment to prepare the inoculum. Only a small part of this inoculum was used in the
146 experiments, but this ensured genetic diversity in the inoculum.

147 *Quercus ilex* was selected as the host plant for the study because it is the most widely
148 used species in Spanish truffle plantations (Reyna and Garcia-Barreda, 2014). For each
149 experiment, we acquired *Q. ilex* acorns of the Spanish provenance region *Sistema Ibérico*
150 from the Centro Nacional de Recursos Genéticos Forestales. They were surface sterilized with
151 a 5% sodium hypochlorite solution for 60 minutes and germinated during winter. The acorns
152 were placed between two layers of wet absorbent paper in laboratory trays covered with

plastic bags to maintain moisture, until germinating acorns were obtained after seven days at an average temperature of 22 ºC. When the acorns had developed a 1-3 cm long radicle, they were removed from the tray and transplanted to the cells of plastic seedling trays (truncated pyramidal cells with square base, 250 ml, 11.5 cm deep, upper section 5 × 5 cm). Only healthy radicles without malformations were included. About two months later, when seedlings in the seedling trays had 6-8 leaves and had formed lateral fine roots, they were carefully removed from the cells without disturbing the root ball (i. e. retaining the integrity of the potting substrate) and transplanted to Full-pot® pots (Acudam, square prisms with 450 ml, 18.5 cm deep, section 5 × 5 cm). The seedlings were cultivated in the CIET greenhouse in Graus (Huesca province) without artificial heating or ventilation and under natural light conditions. They were irrigated until substrate saturation by manually sprinkling water, 2-3 times a week during summer and once each 7-14 days during winter. The pots were placed over greenhouse metal tables, specially designed to fit the plastic grid trays on which the pots are placed, thus leaving 70 cm airspace underneath the pots. The maximum temperatures in the CIET greenhouse were reached in July in both experiments (daily mean 29.5 °C, absolute maximum 44.4 °C in 2015, and daily mean 26.7 °C, absolute maximum 38.9 °C in 2018), while minimum temperatures were also reached in January 2015 and 2019 (daily mean 7.8 °C, absolute minimum - 3.7°C; and daily mean 6.0 °C, absolute minimum - 5.9 °C, respectively).

The radicle inoculation and the inoculation in the seedling tray were performed during the transplant to the seedling tray, whereas the inoculation in the pot was performed during the transplant to the pot. For the radicle inoculation, the radicle of each pre-germinated acorn was uniformly impregnated with inoculum (dried, powdered ascomata) by rolling the roots onto the inoculum, whereas for the other two inoculation methods the powdered inoculum was thoroughly mixed with the potting substrate until a homogeneous mixture was obtained

for each pot. In the radicle inoculation, the rate of inoculum quantity per seedling was limited by the radicle size (when dipped into the inoculum, smaller roots were impregnated with less inoculum), and thus the applied rates were lower than for the substrate inoculations (Table 2). The control of the inoculum rate in the radicle inoculation, done by difference in inoculum weight, showed that the radicle inoculation in 2015 presented higher rates than in 2018, due to the higher size of the radicles (Tables 1, 2).

2.2. Data collection and analysis

The seedlings of the 2015 experiment were analyzed in March 2016, whereas those of the 2018 experiment were analyzed in May 2019. The mycorrhizal status was assessed through the INIA-Aragón method, which allows to assess the variability along the depth profile (Andrés-Alpuente et al., 2014). The root system of each seedling was cut into three fragments of roughly the same length (corresponding to 0-6, 6-12 and 12-18.5 cm depth) and root fragments were collected randomly from each sector. For each sector, at least 100 root tips were counted and sorted into non-mycorrhized or mycorrhized, and the latter were classified as *T. melanosporum* or contaminant morphotypes (Rauscher et al., 1995; Agerer, 2002). A sample of each contaminant morphotype was identified by ITS sequencing, using the methodology described in Gómez-Molina et al. (2020). The quality of the obtained sequences was assessed, and low-quality edges were removed with 4Peaks v1.7.2 (2019, https://nucleobytes.com/4peaks). The sequences were registered in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide) (Benson et al., 2005). 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 (Kõljalg et al., 2013).

The effect of the three inoculation methods and their interactions on the percent root colonization by *T. melanosporum* at the seedling level was analyzed with general linear models, whereas the frequency of appearance of the contaminants (proportion of seedlings in which they are present) was analyzed with generalized (binomial) linear models. Significant differences among treatments were identified with a least squares means test, using a P = 0.05 threshold for statistical significance. When the model assumptions were not met, the response variable was transformed. The distribution of *T. melanosporum* colonization levels along the depth profile was analyzed with linear mixed models, considering each depth sector as a different sample and treating depth as a repeated measures variable. All analyses were conducted with R and the emmeans and nlme packages (Makowski et al., 2020; Pinheiro et al., 2022; R Core Team, 2022).

3. Results

3.1. Experiment 2015

Seventy-two seedlings were analyzed. All the inoculated seedlings (63) showed *T. melanosporum* mycorrhizae in their roots, whereas none of the non-inoculated seedlings (9) did (Table S1). Twelve percent of the seedlings presented mycorrhizae of *S. brunnea* (Genbank accession number OP847397), colonizing 0.9% of the root tips (standard deviation, SD: 2.4). Five percent of the seedlings presented mycorrhizae of *Pulvinula convexella* (P.Karst.) Pfister. (=*P. constellatio* (Berk. & Broome) Boud. (Genbank accession number OP847398), colonizing 0.2% of the root tips (SD: 0.9). The percent root colonization by the inoculated *T. melanosporum* was significantly affected by the interaction between I1, I2 and I3 (t-value = -0.86, P < 0.001, Table S2). Seedlings receiving three inoculations and some treatments receiving two inoculations showed significantly higher *T. melanosporum* levels (28.7% for I1 + I2 + I3, 28.4% for I1 + I2, 29.2% for I1 + I3) than seedlings receiving only

227 the radicle inoculation (17.5%), with the remaining treatments being in an intermediate
228 situation (Fig. 1).
229 The frequency of occurrence of *S. brunnea* was significantly affected by I1 (z = -2.1,
230 P-value = 0.033), I2 (z = -2.6, P-value = 0.010) and I3 (z = -2.1, P-value = 0.033, Table S3).
231 In all cases, the frequency of occurrence was higher in seedlings that had not received the
232 inoculation that in those that had received it (Table 3). No significant effect of I1, I2 or I3 on
233 the frequency of occurrence of *P. convexella* was found (Table S4).
234 When the distribution of *T. melanosporum* colonization levels along the depth profile
235 was taken into account, the interaction between I3 and depth significantly affected percent
236 root colonization by *T. melanosporum* (F = 3.8, P = 0.026, Table S5, Fig. S1). The seedlings
237 that received I3 showed significantly higher *T. melanosporum* levels in the upper and the
238 lower part of the root system (14.5% and 9.0% higher than seedlings not receiving I3,
239 respectively), whereas no significant differences were found in the central part (Fig. 2).
240
241 *3.2. Experiment 2018*
242 Seventy-two seedlings were analyzed. All but one of the inoculated seedlings showed *T.*
243 *melanosporum* mycorrhizae in their roots (62 out of 63), whereas none of the non-inoculated
244 seedlings (9) did (Table S6). Seventeen percent of the seedlings presented mycorrhizae of *S.*
245 *brunnea*, colonizing 1.2% of the root tips (SD: 4.2). The percent root colonization by the
246 inoculated *T. melanosporum* was significantly affected by the three-way interaction between
247 I1, I2 and I3 (F = 4.0, P = 0.049, Table S7, Fig. S2). The seedlings that received the three
248 inoculations showed significantly higher *T. melanosporum* mycorrhization levels (45.7%)
249 than those receiving only one inoculation (17.3% for I1, 23.8% for I2 and 17.6% for I3), with
250 seedlings receiving two inoculations being in an intermediate situation (Fig. 3). The frequency

of occurrence of *S. brunnea* was significantly affected by I2 (z = -2.3, P-value = 0.021, Table S8), being higher in seedlings that had not received I2 (Table 4).

When the distribution of *T. melanosporum* colonization levels along the depth profile was taken into account, percent root colonization by *T. melanosporum* was significantly affected by the interaction between I1, I2 and depth (F = 8.8, P < 0.001, Table S9, Fig. S3). The seedlings that received I2 showed significantly higher *T. melanosporum* levels in the upper and central part of the root system than the corresponding treatments without I2 (i.e. 35% and 23% higher in without I1-with I2 than in without I1-without I2; and 24% and 13% higher in with I1-with I2 than in with I1-without I2; Fig. 4a). For the lower part of the root system, only the seedlings receiving both I1 and I2 showed significantly higher *T. melanosporum* levels (16% higher; Fig. 4a). Percent root colonization by *T. melanosporum* was also significantly affected by the interaction between I3 and depth (F = 4.2, P = 0.018, Table S9), with I3 increasing percent root colonization throughout all the depth profile, but more markedly in the lower part of the root system (18% higher) than in the rest (10% higher; Fig. 4b).

4. Discussion

Our results show that an increase in the inoculum quantity applied can improve the level of *T. melanosporum* colonization. For the 2018 experiment, this increase happened even in the range from 0.8 (with I2 alone or with I3 alone) to 1.70 g fresh truffle per seedling (I1 + I2 + I3), which is commonly used in commercial seedling production (Granetti, 2005; Hall et al., 2007; Palazón and Barriuso, 2007), as we had hypothesized. However, this increase was barely apparent in the 2015 experiment, agreeing with Palazón et al. (2007) who did not find increases in colonization levels between 1-5 g fresh truffle per seedling with an inoculation method based on a single moment of application, and with Pruett et al. (2008) who could not

increase mycorrhization levels when they applied a supplemental inoculation. The increase in mycorrhizal rates associated to the sequential inoculation may also be related to the fact that different inoculum delivery systems and application moments were combined. However, in spite of the differences between the results of the two experiments, they both clearly showed that the combination of several inoculation methods, and the consequent increase in the amount of inoculum applied, did not have an additive effect on mycorrhizal colonization. On the other hand, none of the three inoculation methods applied separately appeared clearly superior to the other. This is particularly meaningful in the case of the radicle inoculation, which spent three to seven times less inoculum. However, the relative effectiveness of these methods may depend on the cultivation conditions of the seedlings and/or the timing of the nursery operations, as suggested by the differences between the 2015 and the 2018 experiments. Interestingly, the inoculation rates obtained with early inoculation methods (I1, I2, I1 + I2) were similar in both experiments, whereas this did not happen in treatments including an inoculation in the final pot (Figs. 1, 3; Tables S1, S6). Among the latter, treatments with lower inoculum quantity (I3, I1 + I3) seemed to perform better in the 2015 experiment, whereas those with higher inoculum quantity (I2 + I3, I1 + I2 +I3) seemed to perform better in the 2018 experiment. These could be related to the experiment timing, since the 2018 seedlings had the spring of their second year to develop new fine roots. The stronger effect of I3 along the depth profile of 2018 seedlings seems to support this hypothesis. Percent root colonization is the variable generally used to evaluate truffle-inoculated seedlings, but its dynamics relies on the relative rhythm and timing of fine root formation and fine root colonization by truffle (Andrés-Alpuente et al., 2014).

The inoculation methods influenced the depth distribution of *T. melanosporum* mycorrhization levels. In almost all cases the percent root colonization decreased with substrate depth, with the only exception of samples in which there was no I1 or I2 (i.e.,

301 treatment without I1-without I2 in 2018; Fig. 4a). The I3 inoculation was the only one that
302 showed a significant effect on depth distribution in both experiments. Interestingly, this
303 method not only increased the mycorrhization levels in the lower part of the root system,
304 where the other methods did not apply inoculum, but also in the central part (2015) or in all
305 depth (2018), even though in I3 most inoculated substrate is added in the lower depth.
306 The other inoculation methods showed less consistent results: the I1 inoculation did
307 not affect the depth distribution in 2015, but showed a positive effect on the upper part of the
308 pot in 2018 (with I1-without I2 vs. without I1-without I2; Fig. 4a); whereas the I2 inoculation
309 did not affect the depth distribution in 2015, but showed a positive effect on the upper and
310 central parts of the pot in 2018 (without I1-with I2 vs. without I1-without I2; Fig. 4a),
311 coinciding with the depth of the seedling trays. This differs from the findings of Garcia-
312 Barreda et al. (2017), where no significant depth patterns in *T. melanosporum* colonization
313 were found between the two inoculation methods that were tested, both of them applied in the
314 final pot. All this indicates that, separately, I1 and I2 are not effective in achieving high levels
315 of inoculation in the lower part of the root system, which could lead to mycorrhization levels
316 being more irregular along the depth of the final seedling, at least during the first year in the
317 nursery. This is in agreement with our initial hypothesis.
318 The tested inoculation methods not only improved *T. melanosporum* colonization
319 levels but also decreased *S. brunnea* spread on the seedling roots, suggesting that *S. brunnea*
320 colonization was related to low inoculation levels by the target fungus. This agrees with the
321 pioneer behavior of this fungus, which usually colonizes the roots during late autumn or
322 winter, thus reducing the availability of root tips for the target fungus during the second year
323 in the nursery (Sánchez et al., 2014; Garcia-Barreda et al., 2017). Besides, *S. brunnea* is able
324 of rapidly fruiting as soon as it establishes its first mycorrhizae, thus boosting the rapid spread
325 of the fungus in greenhouses in which batches of different ages are kept together (Meotto and

Carraturo, 1988; Garcia-Montero et al., 2008). In 2015, the three inoculation methods reduced the occurrence of *S. brunnea*, whereas in 2018 only I2 did. The results in the 2018 experiment suggest that an early inoculation of the substrate was more effective in controlling the non-desired colonization by *S. brunnea*, as we had hypothesized. However, in 2015 –with the inoculation treatments applied 2-3 months earlier than in 2018– the three inoculation treatments were effective. The effectiveness of early substrate inoculation could be related to *T. melanosporum* mycorrhization being spread throughout the roots before the autumn temperature drop and the ensuing period of high and continued moisture.

Regarding to the other contaminating fungus found in the 2015 experiment, our study is the second report of *P. convexella* in Spanish nurseries (Sánchez et al., 2020). Both times the same commercial substrate was used (which is no longer marketed in Spain), thus pointing to an introduction with the potting substrate. Although this species is relatively frequent in Italy (Marozzi et al., 2018), it does not seem to be common in Spain, with the Global Biodiversity Information Facility database only presenting 14 records in 8 locations (www.gbif.org). Nurseries should be cautious about substrate disinfection and pay attention to the appearance of *P. convexella* mycorrhizae and ascocarps, not only due to the damage caused to the commercial quality of seedlings mycorrhized with *Tuber* species, but also due to the risk of this species reaching wild areas and colonizing new ecological niches.

In the context of commercial production of truffle-inoculated seedlings, the sequential application of three inoculation methods appears as an effective and realistic alternative for the inoculation of *Q. ilex* seedlings with *T. melanosporum* (Palazón and Barriuso 2007; Donnini et al. 2014). This strategy is based on (i) putting ascospores in contact with roots before the formation of fine roots (Chevalier, 2001; Granetti, 2005; Palazón and Barriuso, 2007; Garcia-Barreda et al., 2017), and (ii) reducing the deficiencies of a single method and the impact of contingencies in the nursery management by distributing the risk among three

351 inoculations. The early inoculations (I1 and I2) showed positive implications in the
352 management of the opportunist *S. brunnea*, which frequently appears as a serious problem in
353 some nurseries (Sánchez et al., 2014). The third inoculation (I3) showed positive implications
354 in the mycorrhization of roots in the lower depth, which in our experience is frequently the
355 cause of depth irregularity in the mycorrhization levels of inoculated seedlings during their
356 first year in the nursery. Finally, the fact that the transplant from the seedling tray to the final
357 pot is done without disturbing the root ball could also play a role on reducing seedling
358 mortality, which usually reaches 2-10% when nude root transplanting is performed (Palazón
359 and Barriuso, 2007).

360 However, from an economic point of view, sequential inoculation may increase the
361 operation costs of the nursery. It would be interesting to test whether the mycorrhization
362 levels of these seedlings are equivalent to those of *Q. ilex* seedlings inoculated with a single
363 method but the same total amount of inoculum, and whether the sequential inoculation affects
364 the growth and morphology of the plant material. For that matter, it should be considered that
365 in Spain commercial seedlings can be marketed after their first year in the nursery (from about
366 seven months after inoculation) or during their second year (12-19 months after inoculation).
367 For seedlings in their first year, early inoculations could provide some competitive advantage
368 against contaminants colonization. It would also be interesting to investigate how truffle
369 inoculum reaches the fine roots in the radicle inoculation, in order to optimize this method
370 that requires lower amounts of inoculum. Finally, it would also be important to test whether
371 the sequential inoculation could have implications for the relationship between mating types
372 in nursery seedlings. Spore inoculation ensures the presence of the two mating types in the
373 seedlings, but so far, only seedlings with just a single inoculation method applied in the final
374 container have been analyzed, and they show a tendency for one mating type to dominate over
375 the other from the first to the second year in the nursery (Rubini et al., 2011; Gómez-Molina

et al., 2023). This could influence the relative occurrence of mating types in the roots of the truffle orchard.

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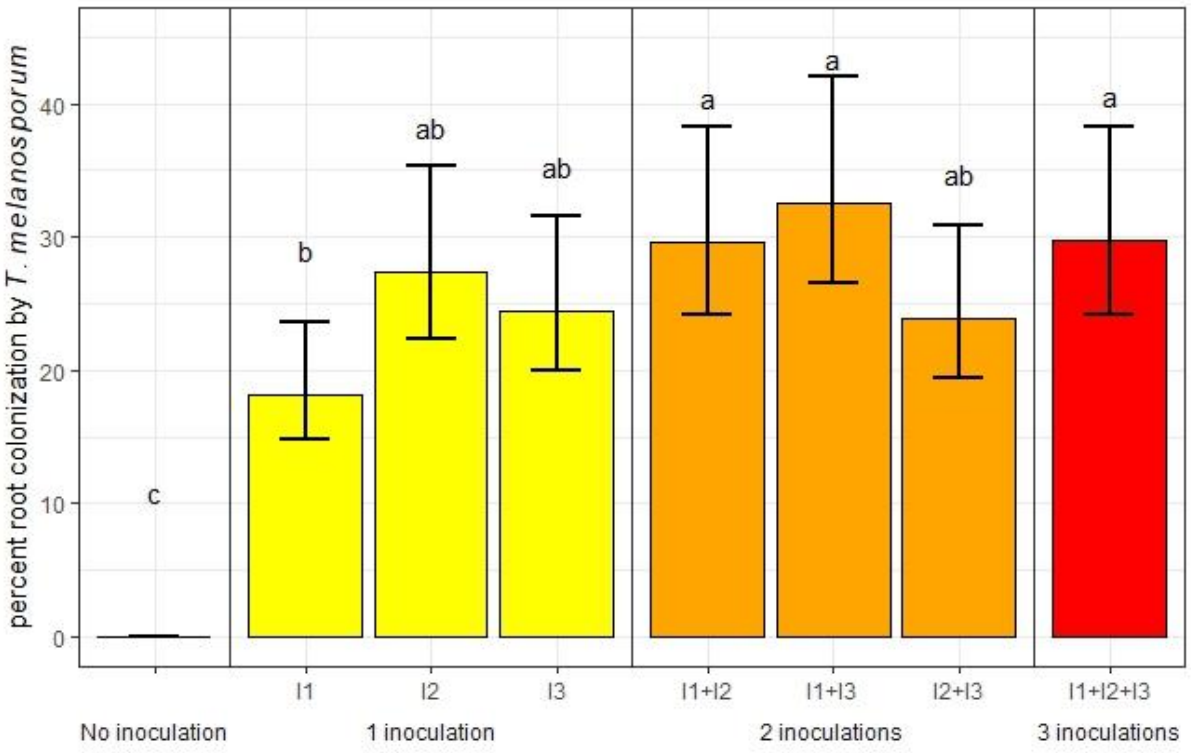


Figure 1. Effect of the sequential inoculation on the percent root colonization by *T. melanosporum* in the 2015 experiment (mean predicted values and 95% confidence intervals, n = 72). Different letters indicate significant differences according to least square means tests ($\alpha = 0.05$). I1: radicle inoculation, I2: inoculation in seedling tray, I3: inoculation in pot.

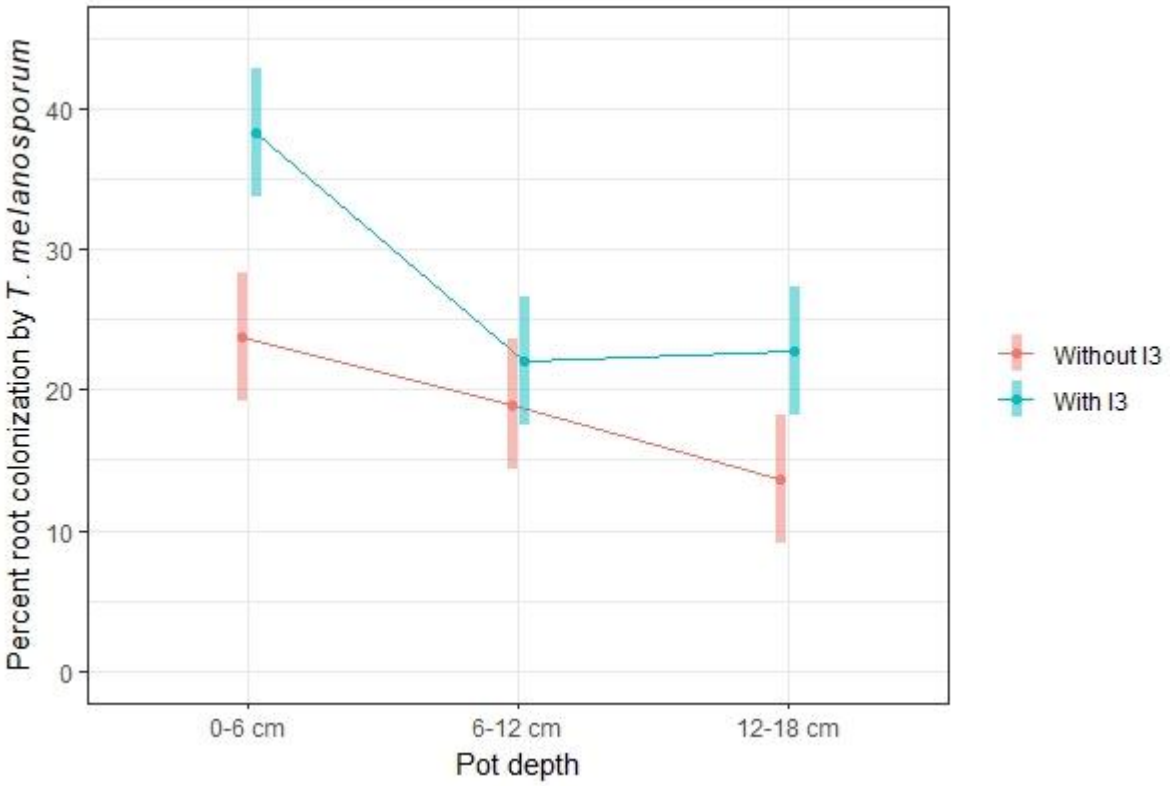
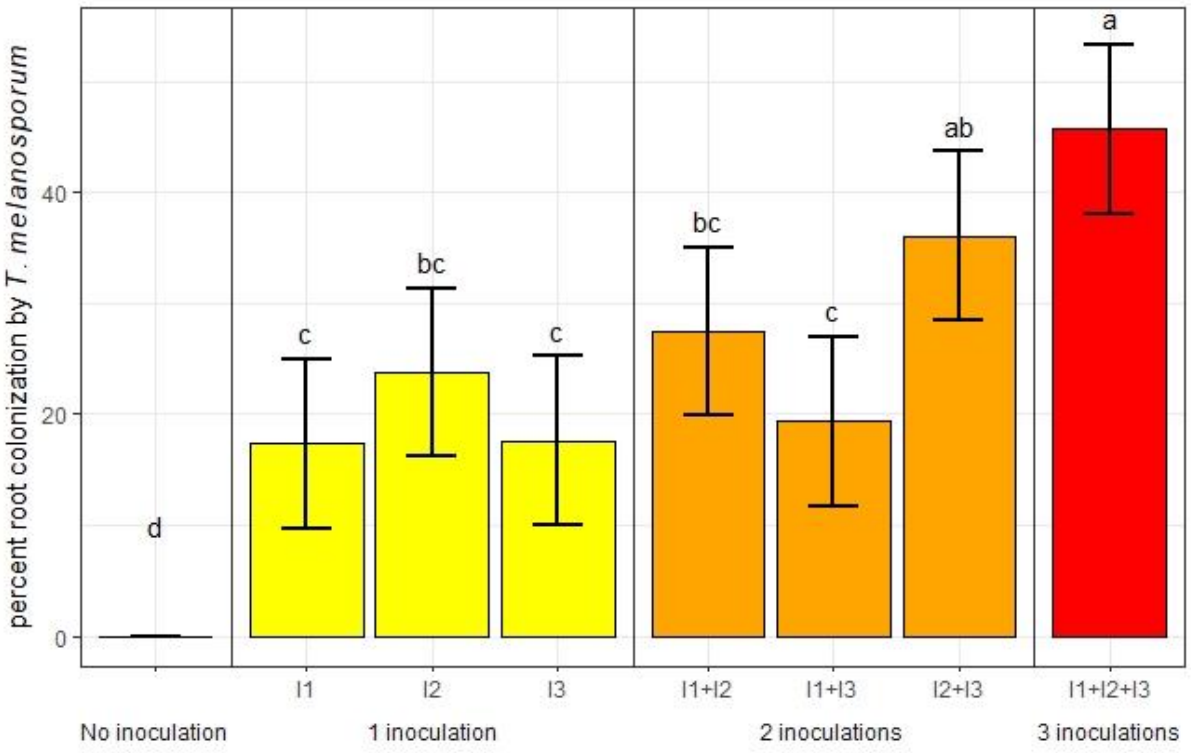


Figure 2. Effect of the inoculation in the pot (I3) on the percent root colonization by *T. melanosporum* at different pot depths, in the 2015 experiment (mean predicted values and 95% confidence intervals, n = 216). Overlapping of the confidence intervals indicates lack of significant differences according to the least-squares means procedure ($\alpha = 0.05$).



556

557 **Figure 3**. Effect of the sequential inoculation on the percent root colonization by *T.*

558 *melanosporum* in the 2018 experiment (mean predicted values and 95% confidence intervals,

559 n = 72). Different letters indicate significant differences according to least square means tests

560 (α = 0.05). I1: radicle inoculation, I2: inoculation in seedling tray, I3: inoculation in pot.

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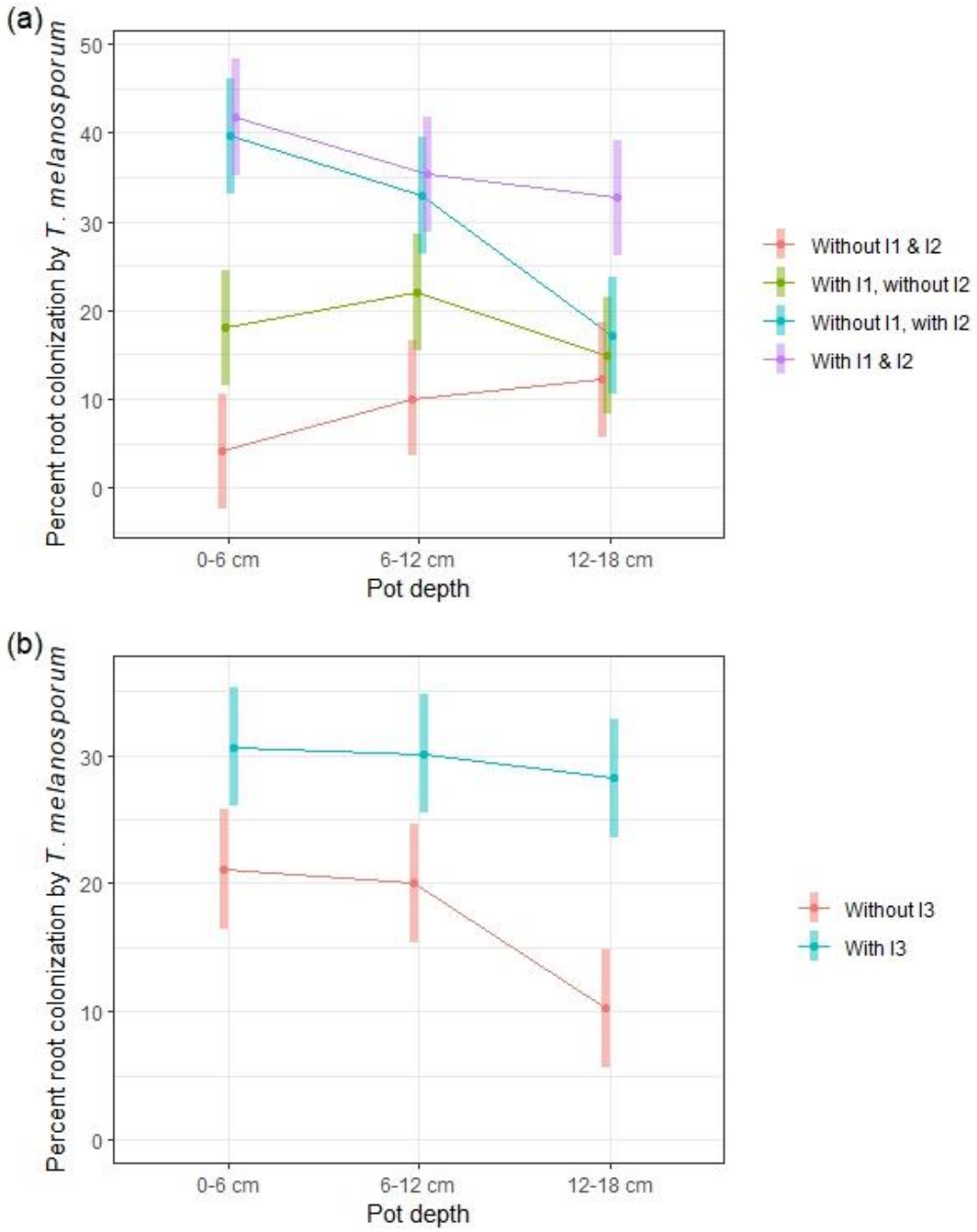

562

563 **Figure 4**. Percent root colonization by *T. melanosporum* along the depth profile in the 2018
564 experiment (mean predicted values and 95% confidence intervals, n = 216). (a) Effect of the
565 interaction between radicle inoculation (I1), inoculation in the seedling tray (I2), and depth.
566 (b) Effect of the interaction between inoculation in the pot (I3) and depth. Overlapping of the
567 confidence intervals indicates lack of significant differences according to least square means
568 procedure ($\alpha = 0.05$).

Table 1. Total inoculum rate (g fresh truffle) received per seedling according to the inoculation treatments applied (n = 9 for each combination of inoculation treatments). I1: radicle inoculation. I2: inoculation of the seedling tray substrate. I3: inoculation of the pot substrate (0: inoculation method not applied, 1: applied inoculation method).

Inoculation			Total inoculum rate per seedling	
I1	I2	I3	2015 experiment	2018 experiment
0	0	0	0	0
0	0	1	0.80	0.80
0	1	0	0.80	0.80
0	1	1	1.60	1.60
1	0	0	0.26	0.10
1	0	1	1.06	0.90
1	1	0	1.06	0.90
1	1	1	1.86	1.70

577

578 **Table 2**. Dates of inoculation, inoculum rates applied (g fresh truffle per seedling) and potting

579 substrates used in the 2015 and 2018 experiments.

Assay		Radicle inoculation (I1)	Inoculation in seedling tray (I2)	Inoculation in pot (I3)
2015	Date	December 2014	December 2014	April 2015
	Inoculum rate	0.26	0.80	0.80
	Potting substrate		Calcareous loam soil, Prohumin ® substrate[1], limestone coarse sand, perlite, 4:4:2.5:1 (v/v). pH adjusted to 7.5 with calcium carbonate powder.	Calcareous loam soil, Prohumin ® substrate[1], limestone coarse sand, perlite, 4:4:2.5:1 (v/v). pH adjusted to 7.5 with calcium carbonate powder.
2018	Date	March 2018	March 2018	June 2018
	Inoculum rate	0.10	0.80	0.80
	Potting substrate		Profi-Substrat ® substrate[2], perlite, 9:1 (v/v). pH adjusted to 7.5 by manufacturer.	Profi-Substrat ® substrate[2], perlite, 9:1 (v/v). pH adjusted to 7.5 by manufacturer.

580 [1] Composed of *Sphagnum* white peat and *Sphagnum* black peat 1:1 v/v (Projar).

581 [2] Composed of *Sphagnum* white peat and *Sphagnum* black peat 3:2 v/v (Gramoflor).

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584

585 **Table 3**. Frequency of occurrence of *S. brunnea* in the seedlings of the 2015 experiment
586 (mean predicted values and standard error, n = 72) according to the binomial model. In each
587 row, different letters indicate significant differences according to the model (α = 0.05).

	Not receiving the inoculation	Receiving the inoculation
Radicle inoculation (I1)	0.251 (0.083) a	0.067 (0.041) b
Inoculation in seedling tray (I2)	0.295 (0.085) a	0.055 (0.036) b
Inoculation in pot (I3)	0.251 (0.083) a	0.067 (0.041) b

588

589 **Table 4**. Frequency of occurrence of *S. brunnea* in the seedlings of the 2018 experiment
590 (mean predicted values and standard error, n = 72) according to the binomial model. In each
591 row, different letters indicate significant differences according to the model (α = 0.05).

	Not receiving the inoculation	Receiving the inoculation
Radicle inoculation (I1)	0.106 (0.053)	0.156 (0.066)
Inoculation in seedling tray (I2)	0.276 (0.075) a	0.054 (0.038) b
Inoculation in pot (I3)	0.129 (0.060)	0.129 (0.060)

592