Sequential application of inoculation methods for the mycorrhization of Quercus ilex seedlings with Tuber melanosporum

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Research Article

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Abstract

The use of mycorrhized seedlings has been critical in the success of modern truffle cultivation, which nowadays supplies most black truffles 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 with black truffle, with two 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. The sequential application appeared as an effective and realistic alternative for commercial inoculation of holm oak seedlings with black truffle. The increase in the amount of inoculum applied with each inoculation method improved the mycorrhizal colonization of seedlings, although separately none of the inoculation methods appeared clearly superior to the other ones. The depth distribution of mycorrhizae levels pointed to the inoculation in the final pot substrate as being 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*. However, the difference of results between both assays suggests that cultivation conditions and/or the timing of the nursery operations may influence the relative effectiveness of these inoculation methods.

1. Introduction

Tuber melanosporum Vittad. wild production is 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 widely used in France and Italy (Bencivenga 2001; Chevalier 2001; Reyna and Garcia-Barreda 2014; Garcia-Barreda et al. 2020).

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 for the success of a truffle plantation, since (i) the abundance of truffle mycorrhizae in the early years after plantation is related to infestation levels in the nursery, and (ii) mycorrhizae act as maternal material for sexual reproduction and fruitbody production in productive *truffières* (Bourrières et al. 2005; Rubini et al. 2011; Garcia-Barreda and Reyna 2013; De la Varga et al. 2017). 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). The high price of truffle fruitbodies has led nurserymen to develop different

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

The quality of mycorrhizal seedlings is assessed with the level of colonization of the fine roots by the target truffle species, with most evaluation methods using percent root colonization as the measurement variable (Andres-Alpuente et al. 2014; Donnini et al. 2014; Murat 2015). However, these seedlings frequently present mycorrhizae from other non-desired species, the so-called “contaminants”, which sometimes are accidentally introduced within the inoculum and sometimes come with the potting substrate (Iotti et al. 2012; De Miguel et al. 2014; Murat 2015). Among the latter the facultatively mycorrhizal *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička, a pioneer and opportunist species, is the most common fungal competitor in nurseries producing seedlings mycorrhized with *Tuber* species (Bencivenga et al. 1995; De Miguel et al. 2014; Sánchez et al. 2014). This species is frequently present in marketed *Sphagnum* peat (Danielson 1984; Ángeles-Argáiz et al. 2016) and generally spreads in truffle nurseries during late autumn and winter, under conditions of high substrate moisture and reduced ventilation of greenhouses (Palazón et al. 2005; Garcia-Montero et al. 2008; Sánchez et al. 2014). Inoculation methods that boost early formation of truffle mycorrhizae could reduce the level of infestation by undesired ectomycorrhizal fungi and thus improve the quality of mycorrhizal seedlings. Seedlings are usually inoculated 2–3 months after the acorn germinates, 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 mycorrhizal 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. Thus, we combined a radicle inoculation method, an inoculation of the substrate in seedling trays (both of them performed before the seedling produces lateral fine roots) and an inoculation of the substrate in the final pots (performed when the seedling has 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 inoculations (increasing the total quantity of inoculum applied) would improve the levels of root infection 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 inoculations 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 smaller root system, would result in mycorrhizal levels being more irregular along the depth of 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 interactions among these inoculations. The three inoculation methods used ascospores as inoculum: an inoculation of the seedling radicle (I1), an inoculation of the potting substrate in seedling trays (I2) and an inoculation of the potting substrate in the final pots (I3). Non inoculated controls were included for each method. The experiment was carried out in 2015 and then repeated in 2018 to confirm the results. A total of 72 seedlings (9 replicates per treatment) were produced in each experiment.

The *T. melanosporum* fruitbodies used as inoculum for each experiment (2 kg, more than 60 sporocarps in each case) were harvested fresh and mature from different orchards in Huesca province (northeastern Spain) during the fruiting season immediately before setting up each experiment. They were surface cleaned with a brush under cool water, surface sterilized by immersion in ethanol (70%) and flamed, taxonomically identified by morphological features (Riousset et al. 2001) sliced thin, air dried under room conditions and homogenized with a coffee grinder.

Quercus ilex was selected as the host plant for the study because it is the most widely used species in Spanish truffle plantations (Reyna and Garcia-Barreda 2014). For each experiment, we acquired selected *Q. ilex* acorns (Spanish provenance region *Sistema Ibérico*) from the Centro Nacional de Recursos Genéticos Forestales, surface sterilized them with a 5% sodium hypochlorite solution for 60 minutes and germinated them during winter. The acorns were placed between two layers of wet absorbent paper in laboratory trays covered with autoclavable plastic to maintain moisture, until the pre-germinated acorns were obtained after seven days at an average temperature of 22 ºC. When the acorns had developed a 1–3 cm long radicle (Table 1), they were removed from the tray and transplanted to the cells of plastic seedling trays (250 ml, 11.5 cm deep). 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 (Table 1), 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 (450 ml, 18.5 cm deep). The seedlings were cultivated in the CIET greenhouse in Graus (Huesca province) without artificial heating or ventilation, under natural light conditions and manual sprinkle irrigation to saturation 2–3 times a week during summer and once each 7–14 days during winter.

Table 1
Dates of inoculations, inoculum rates applied (g fresh truffle per seedling) and potting substrate used in the experiments.

	2015 experiment	2018 experiment
Radicle inoculation		
Date	December 2014	March 2018
Inoculum rate	0.26	0.10
Inoculation in seedling tray		
Date	December 2014	March 2018
Inoculum rate	0.80	0.80
Inoculation in pot		
Date	April 2015	June 2018
Inoculum rate	0.80	0.80
Potting substrate	Calcareous loam soil, Prohumin® substrate [1], limestone coarse sand, perlite, 4:4:2.5:1 (v/v)	Gramoflor ® substrate [2], perlite, 9:1 (v/v)

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

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

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 directly onto the inoculum, whereas for the other two inoculation methods the inoculum was thoroughly mixed with the potting substrate until a homogeneous mixture was obtained. In the radicle inoculation, the rate of inoculum quantity per seedling was inevitably limited by the radicle size, and thus the applied rates were lower than for the substrate inoculations (Table 1). Besides, the control of the inoculum rate was more difficult in the radicle inoculation, due to the different size of each radicle, thus causing different rates between the 2015 and the 2018 experiment (Tables 1, 2). Both experiments also presented some differences in the timing of the inoculations and the potting substrate (Table 1).

Table 2
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.

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

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 (Andres-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-mycorrhizal or mycorrhizal, 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 GenBankR 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 (64) showed *T. melanosporum* mycorrhizae in their roots, whereas none of the non-inoculated seedlings (8) did (Table S1). A 12% of the seedlings presented mycorrhizae of *S. brunnea* (Genbank accession number OP847397), colonizing a 0.9% of the root tips (standard deviation, SD: 2.4). A 5% of the seedlings presented mycorrhizae of *Pulvinula constellatio* (Berk. & Broome) Boud. (Genbank accession number OP847398), colonizing a 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 (I1 + I2, I1 + I3) showed significantly higher *T. melanosporum* levels than seedlings receiving only the radicle inoculation, with the remaining treatments being in an intermediate situation (Fig. 1).

The frequency of occurrence of *S. brunnea* was significantly affected by I1 (z = -2.1, P-value = 0.033), I2 (z = -2.6, P-value = 0.010) and I3 (z = -2.1, P-value = 0.033, Table S3). In all cases, the frequency of occurrence was higher in seedlings that had not received the inoculation that in those that had received it (Table 3). No significant effect of I1, I2 or I3 on the frequency of occurrence of *P. constellatio* was found (Table S4).

Table 3
Frequency of occurrence of *S. brunnea* in the seedlings of the 2015 experiment (mean predicted values and standard error, n = 72) according to the binomial model. In each 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

When the distribution of *T. melanosporum* colonization levels along the depth profile was taken into account, the interaction between I3 and depth significantly affected percent root colonization by *T. melanosporum* (F = 3.8, P = 0.026, Table S5, Fig. S1). The seedlings that received I3 showed significantly

higher *T. melanosporum* levels in the upper and the lower part of the root system, whereas no significant differences were found in the central part (Fig. 2).

3.2. Experiment 2018

Seventy-two seedlings were analyzed. All but one of the inoculated seedlings showed *T. melanosporum* mycorrhizae in their roots (63 out of 64), whereas none of the non-inoculated seedlings (8) did (Table S6). A 17% of the seedlings presented mycorrhizae of *S. brunnea*, colonizing a 1.2% of the root tips (SD: 4.2). The percent root colonization by the inoculated *T. melanosporum* was significantly affected by the three-way interaction between I1, I2 and I3 ($F = 4.0$, $P = 0.049$, Table S7, Fig. S2). The seedlings that received the three inoculations showed significantly higher *T. melanosporum* levels than those receiving only one inoculation, with 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), with the frequency of occurrence 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. wo I1-wo I2 < wo I1-w I2, and w I1-wo I2 < w I1-w I2; Fig. 3a). For the lower part of the root system, only the seedlings receiving both I1 and I2 showed significantly higher *T. melanosporum* levels (Fig. 3a). 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 (Fig. 3b).

Table 4
Frequency of occurrence of *S. brunnea* in the seedlings of the 2018 experiment (mean predicted values and standard error, n = 72) according to the binomial model. In each 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)

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 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). 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 mycorrhizal levels when they applied supplemental inoculation. The fact that different inoculum delivery systems and application moments were combined may be playing a role in increasing mycorrhizal rates. However, as clearly shown in both experiments, the combination of several inoculation methods and the resulting increase of 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 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, as 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 (Andres-Alpuente et al. 2014).

Regarding the different mycorrhization percentages along the root ball, the depth distribution of mycorrhizal levels provided insight into the patterns governing *T. melanosporum* colonization, with each inoculation method affecting depth distribution differently. 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. treatment wo I1-wo I2 in 2018; Fig. 4a). The I3 inoculation was the only one that showed a significant effect on depth distribution in both experiments. Interestingly, this method not only increased mycorrhizal levels in the lower part of the root system, where the other methods did not act, but also in the middle part (2015) or in all depth (2018), despite the fact that in I3 most inoculated substrate was added in the lower depth.

The other inoculation methods showed less consistent results: the I1 inoculation did not affect the depth distribution in 2015, but showed a positive effect on the upper part of the pot in 2018 (w I1-wo I2 vs. wo I1-wo I2; Fig. 4a); whereas the I2 inoculation did not affect the depth distribution in 2015, but showed a positive effect on the upper and middle parts of the pot in 2018 (wo I1-w I2 vs. wo I1-wo I2; Fig. 4a), coinciding with the depth of the seedling trays. This differs from the findings of Garcia-Barreda et al. (2017), where no significant depth patterns in *T. melanosporum* colonization were found with the two

inoculation method used, both of them applied in the final pot. All this indicates that, separately, I1 and I2 are not effective in achieving high levels of inoculation in the lower part of the root system, which could lead to mycorrhizal levels being more irregular along the depth of the final seedling, at least during the first year in the nursery.

The tested inoculation methods not only improved *T. melanosporum* colonization levels but also decreased *S. brunnea* spread on the seedling roots, suggesting that *S. brunnea* colonization was related to low inoculation levels by the target fungus. This agrees with the pioneer behavior of this fungus, which usually colonizes the roots during late autumn or winter, thus reducing the availability of root tips for the target fungus during the second year in the nursery (Sánchez et al. 2014; Garcia-Barreda et al. 2017). Besides, *S. brunnea* is able of rapidly fruiting as soon as it establishes its first mycorrhizae, which promotes the rapid spread of the fungus in greenhouses in with 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 infection by *S. brunnea*, although 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. constellatio* in Spanish nurseries (Sánchez et al. 2020). This fungus is relatively frequent in Italy (Marozzi et al. 2018). However, in Spain we have only found it in two cases, both of them using the same commercial substrate (which is no longer marketed in Spain).

In the context of commercial production of truffle-inoculated seedlings, the sequential application of three inoculations 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; Murat 2015). This method 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 single methods and the impact of contingencies in the nursery management by distributing the risk among three inoculations. The early inoculations (I1 and I2) showed positive implications in the management of the opportunist *S. brunnea*, which frequently appears as a serious problem in some nurseries (Sánchez et al. 2014). The third inoculation (I3) showed positive implications in the mycorrhization of roots in the lower depth, which in our experience is frequently the cause of depth irregularity in the mycorrhizal levels of inoculated seedlings, especially during its first year in the nursery. Finally, the transplant of the root ball to the final pot (step from I2 to I3) could also play a role on reducing seedling mortality, which uses to reach 2–10% when nude root transplanting is performed (Palazón and Barriuso 2007).

However, it would be interesting to test whether the mycorrhizal levels of these seedlings are equivalent to those of *Q. ilex* seedlings inoculated with a single method but the same total amount of inoculum, and

whether the sequential inoculation affects the growth and morphology of the plant material. For that matter, it should be considered that in Spain commercial seedlings can be marketed after their first year in the nursery (from about seven months after inoculation) or during their second year (12–19 months after inoculation). For seedlings in their first year, early inoculations could provide some competitive advantage against contaminants colonization. It would also be interesting to investigate how truffle inoculum reaches the fine roots in the radicle inoculation, in order to optimize this method that requires lower amounts of inoculum. Finally, it would also be important to test whether the sequential inoculation could have implications for the relationship between mating types in nursery seedlings. So far, this relationship has only been studied with a single inoculation method applied in the final container, with a tendency for one mating type to dominate over the other from the first to the second year in the nursery (Rubini et al. 2011; Gómez-Molina et al. 2023).

Declarations

Competing interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Authors' 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.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

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Figures

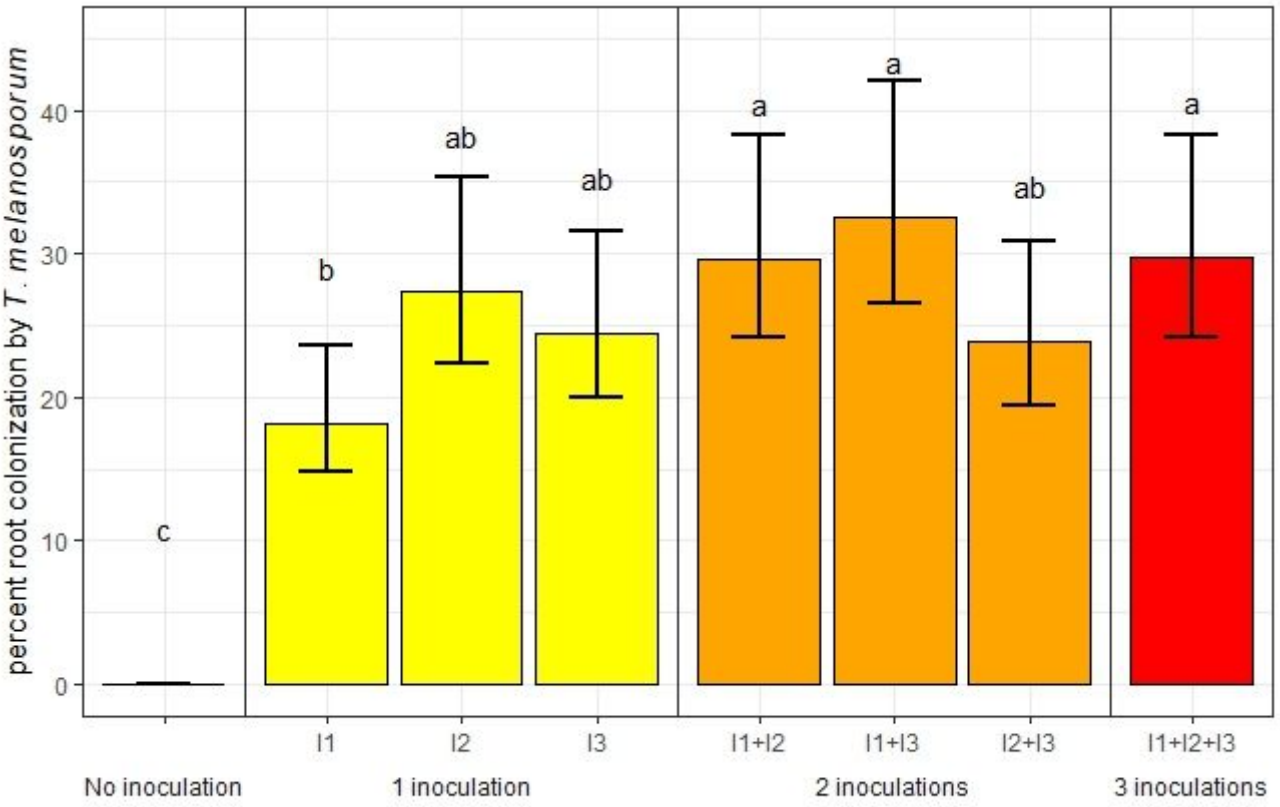

Figure 1

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

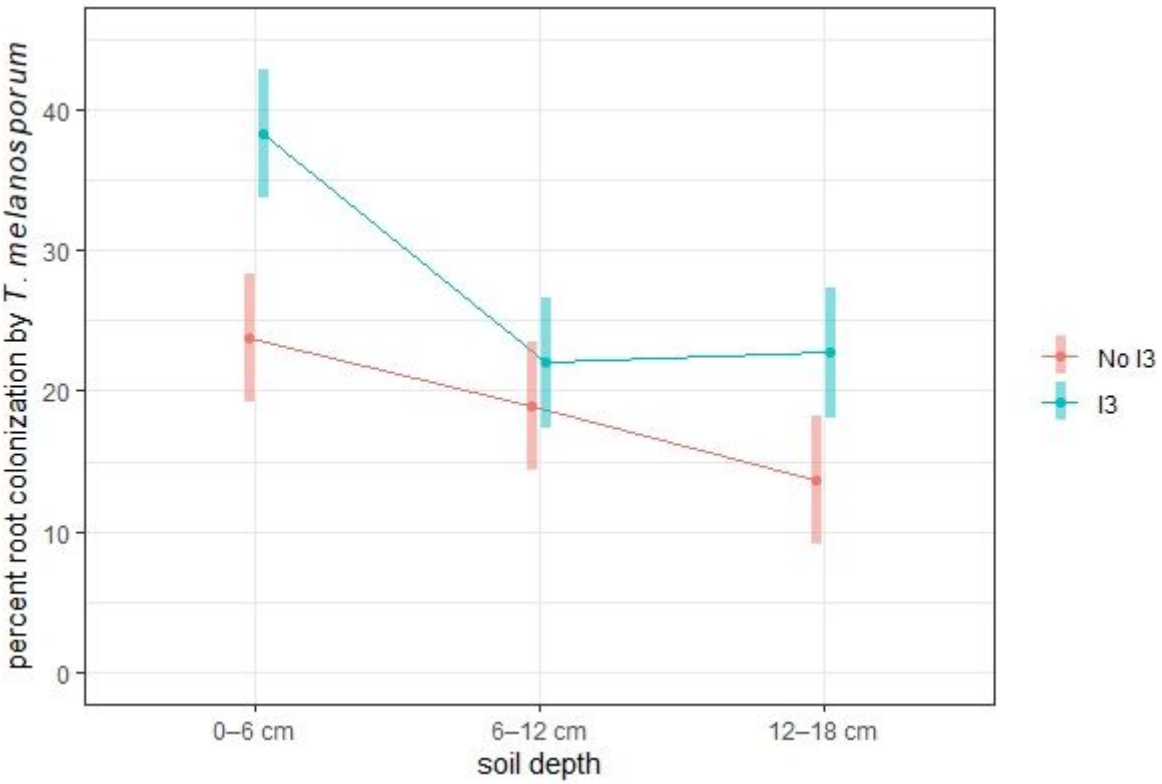

Figure 2

Effect of the interaction between soil depth and inoculation in the pot (I3) on the percent root colonization by *T. melanosporum* 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 (α = 0.05).

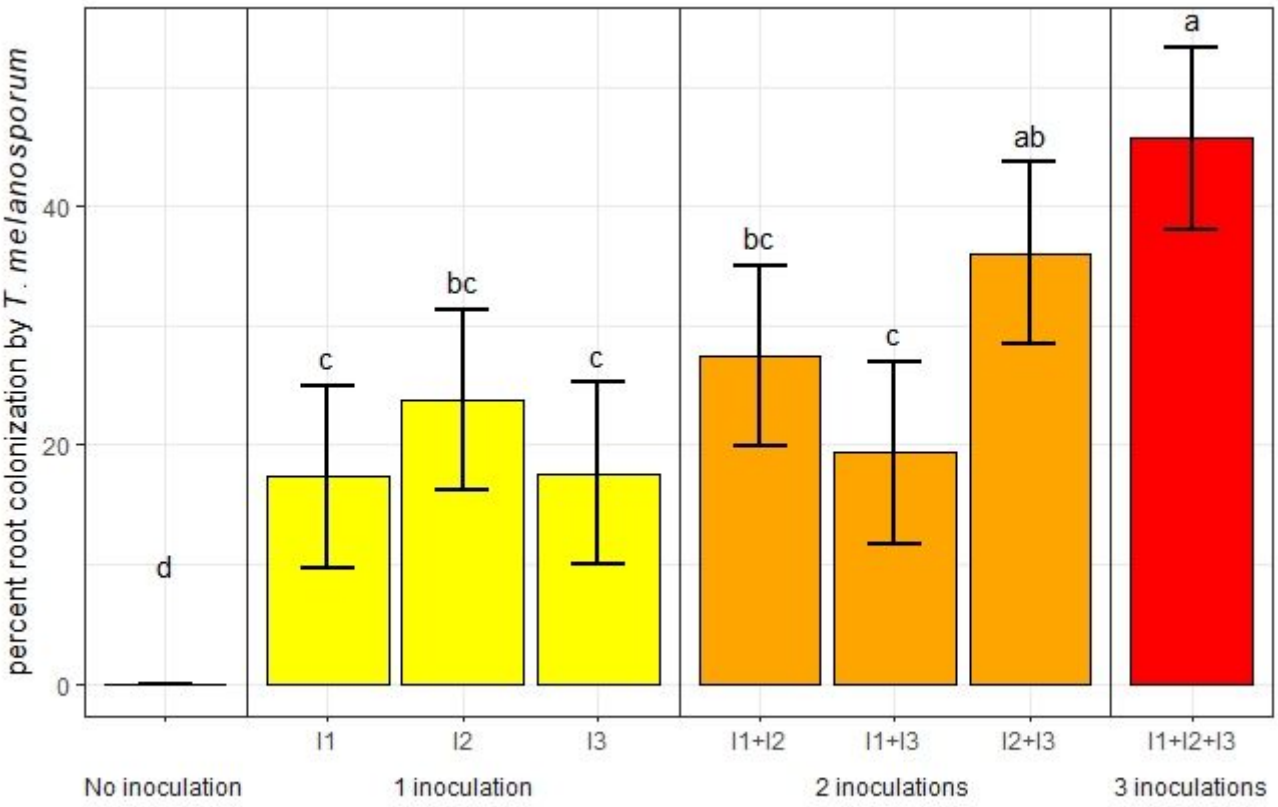

Figure 3

Effect of the sequential inoculation on the percent root colonization by *T. melanosporum*in the 2018 experiment (mean predicted values and 95% confidence intervals, n = 72). Different letters indicate significant differences according to least square means tests (α = 0.05). I1: radicle inoculation, I2: inoculation in seedling tray, I3: inoculation in pot.

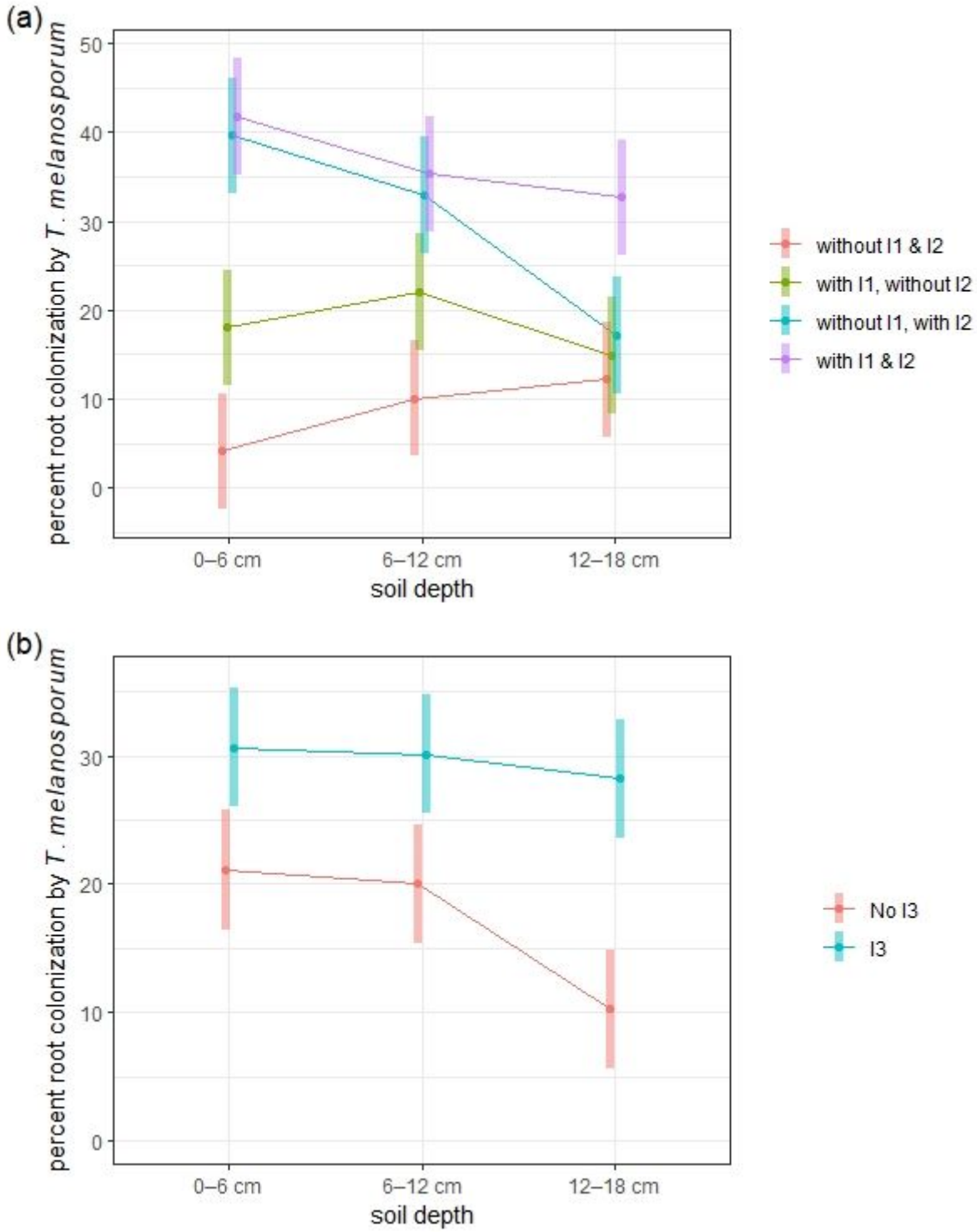

Figure 4

Percent root colonization by *T. melanosporum* along the depth profile in the 2018 experiment (mean predicted values and 95% confidence intervals, n = 216). (a) Effect of the interaction between radicle inoculation (I1), inoculation in the seedling tray (I2), and depth. (b) Effect of the interaction between inoculation in the pot (I3) and depth. Different letters indicate significant differences according to least square means tests (α = 0.05).

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