Fertilisation of *Quercus* seedlings inoculated with *Tuber melanosporum*: effects on growth and mycorrhization of two host species and two inoculation methods

Sergi Garcia-Barreda (1-2), Sara Molina-Grau (3), Santiago Reyna (4)

Modern truffle cultivation is based on use of inoculated seedlings, which should exhibit highly colonised roots as well as a vegetative quality enhancing field plant performance. However, poor shoot and fine root growth has been a frequent issue in inoculated *Quercus* seedlings production. Fertilisation is a common solution in forest nurseries, but high fertilisation levels have been found to inhibit the formation of ectomycorrhizas of many fungal species. The influence of slow-release fertilisation (52 mg N, 26 mg P and 36 mg K per seedling) on growth and ectomycorrhizal status of *Tuber melanosporum*-inoculated seedlings was evaluated. Most species *Quercus ilex* and *Quercus faginea* and inoculation methods involving root-dipping and root-powdering were tested. Fertilisation increased weight of both host species without significant detrimental effects on ectomycorrhizal colonisation, showing that it can be effectively used in inoculated seedlings production. Both host species showed similar response to fertilisation. The inoculation method affected seedling weight and ectomycorrhizal status, suggesting that some inoculant carriers are able to damage *Quercus* development and *T. melanosporum* colonisation. The study provided an important basis for fine-tuning the use of fertilisers in truffle-inoculated seedling production.

Keywords: Fertilisation, Seedling, Nursery, Ectomycorrhizal, Inoculation

Introduction

The European black truffle (*Tuber melanosporum* Vittad.) is an ectomycorrhizal (EM) fungus extensively cultivated due to its gastronomic value and decline of wild production (Reyna & Garcia-Barreda 2014). Each year, more than 2,000 ha of agricultural lands, with appropriate edaphoclimatic environment for the fungus to complete its life cycle and with low EM inoculum potential (Sourzat 2008).

High quality inoculated seedlings must exhibit a root system abundantly colonised by *T. melanosporum* (Andres-Alpuente et al. 2014, Murat 2015). The commercial production of these seedlings is customarily done with spore inoculum, either concentrating inoculum onto fine roots or incorporating it into substrate (Chevalier & Grente 1978, Palazón & Barriuso 2007). With the high price of sporocarps luring nurserymen into reducing inoculum application rates, selection of carrier materials providing close contact with fine roots and even distribution of inoculum is critical, especially when thousands of seedlings are produced (Averseng & Roux 2001). For *Tuber* species, little scientific information is publicly available on efficiency of the various inoculation methods or on their interaction with other nursery practices, often because of patents and confidentiality agreements (Cartié et al. 2001, Pruett et al. 2008).

The quality of inoculated seedlings is determined not only by abundance of mycorrhizas but also by vegetative quality of seedlings (Fischer & Colinas 1996). Large, nutrient-rich seedlings with high growth potential are likely to perform better in drylands with deep soils (Cortina et al. 2013), such as the agricultural lands where truffle plantations are usually established in Spain (Garcia-Barreda et al. 2007). However, problems of scarce shoot development and stunted lateral root growth have been frequent in the commercial production of *Quercus* seedlings inoculated with *T. melanosporum* (Chevalier & Grente 1978, Averseng & Roux 2001). Some inoculation methods seem to exacerbate this problem (Cartié et al. 1996, Pruett et al. 2008).

In forest nurseries a common solution for low vegetative quality of seedlings is fertilisation, which increases size, nutrient storage and root growth potential (Villar-Sal-
They were surface sterilised with a 20% sodium hypochlorite solution for five minutes, and germinated in January in a tray with perlite and vermiculite. When seedlings had 6-8 leaves and had formed lateral roots (12 weeks after seeding emergence, in late April), they were removed from the tray, mechanically root-pruned at the tap root end to eliminate its defects (Palazón & Barrueto 2007), inoculated, and transplanted to Quick-pot containers (650 ml, 18 cm deep).

The inoculation was performed by root-dipping, following a traditional method described by Hall et al. (2007) as frequently used in Spain. The bare roots were dipped in a slurry of homogenised sporcars in a sucrose solution (2:1 water: sucrose v/w) aimed to produce a high viscosity suspension. The spore concentration in the slurry was adjusted to obtain an application rate of 2.0 g fresh truffle per seedling (6×10^6 spores per seedling), although some variability in spore density was observed. The method is similar to other inoculation methods, such as root-powdering, with the advantage of reducing the risk of contamination. The spores were applied onto roots with a solid carrier instead of a liquid one (Cartié et al. 2001). A mix of talcum powder (hydrated magnesium silicate) and homogenised sporcars was applied onto seedling bare roots, with spore concentration adjusted to obtain an inoculum rate of 2.0 g fresh truffle per seedling.

The potting substrate consisted of 12:6:1 (v/v) calcareous sandy loam soil, base-fertilised Sphagnum white peat (Kekkila® White 420 W), and limestone coarse sand. It was solarised during summer, and subsequently presented a pH of 7.9, conductivity (15) of 418 mS m⁻¹, 1390 ppm N (Kieldahl), 32 ppm P (Olsen) and 337 ppm K (ammonium acetate extraction), with pH and nutrient levels falling within the common range in Spanish wild truffle soils (García-Barreda et al. 2007). A soil-based potting mix was selected because these are still used with good results in truffle nurseries and research (Cartié et al. 2001, Benucci et al. 2012). Seedlings were cultivated in a greenhouse and sprinkled irrigated to saturation 2-3 times per week during summer and once each 7-14 days during winter.

Following the first shoot flush after inoculation (seven weeks after inoculating, in mid June), slow-release fertiliser Osmocote Exact Mini⁶ (NPK 16-8-11, with a longevity of 3-4 months at 21 °C) was added in the surface of substrates at a dose of 0.5 g L⁻¹, providing 52 mg N, 26 mg P, 36 mg K, 6.5 mg Mg, 1.3 mg chelated Fe, 0.16 mg Mn and Cu, 0.07 mg B and Mo and 0.06 mg Zn per seedling. We selected a slow-release fertiliser because it is the most common in Spanish forest nurseries. The dose was selected following that used by Rincon et al. (2007) for avoiding inhibition of EM formation in containerised Pinus seedlings.

Experimental design

Two experiments were done independently. In 2009 an experiment to compare the effect of fertilisation on two host species (Q. ilex and Q. faginea) was conducted. Four treatments were established in a 2×2 factorial design, with 12 replicates per treatment: unfertilised Q. ilex, fertilised Q. ilex, unfertilised Q. faginea and fertilised Q. faginea. All seedlings were inoculated with root-dipping.

In 2010 an experiment to compare the effect of fertilisation on two inoculation methods was conducted. Four treatments were established in a 2×2 factorial design, with six replicates per treatment: unfertilised root-dipping, fertilised root-dipping, unfertilised root-powdering and fertilised root-powdering. The experiment was conducted with Q. ilex as plant host.

Data collection and analysis

The seedlings of the fertilisation and host species experiment were analysed 12 months after inoculation (in April-May), whereas the fertilisation and inoculation method experiment was analysed 11 months after inoculation (in March). The mycorrhizal status was assessed through a volumetric sampling. In each seedling a sample with 9% of the substrate volume (57 ml) was taken. To cope with heterogeneity across soil depth, each sample consisted of three subsamples: the depth of the container was divided into three equal parts and in the centre of each third (4, 9 and 13.5 cm depth) a horizontal core (2 cm diameter) across the container was taken. Samples were kept in water at 4°C. Length of fine roots (diameter < 2 mm) was measured according to Tennant (1975). Root tips were counted and classified as non-mycorrhizal, T. melanoporum ectomycorrhizas or contaminant ectomycorrhizas. Shoot dry weight, root dry weight, fine root dry weight, stem height and root collar diameter were measured after drying to constant weight at 80 °C.

Plant dry weight, shoot and root dry weight, stem height, root collar diameter, specific root length (SRL, ratio of root length to dry weight of fine roots), number of root tips and number of T. melanoporum tips per seedling were analysed by conventional ANOVA. Propportion of root tips colonised by T. melanoporum and frequency of occurrence of contaminants in seedlings were analysed through generalised (binomial) linear models. When model assumptions were violated, the response variable was transformed. In the model for proportion of roots colonised by T. melanoporum, fine root length was included as a covariate to account for within-treatment variability in fine root development.

Results

Experiment 1: fertilisation/host species

Total dry weight of seedlings was positively affected by fertilisation (P<0.001), with no significant differences between host species (P=0.50 – Tab. 1). Both shoot
and root dry weight followed the same pattern, with a positive effect of fertilisation (P<0.001 in both cases) and no significant differences between host species (P=0.59 and P=0.46, respectively – Tab. 1). Stem height was positively affected by fertilisation (P<0.001), with no significant differences between host species (P=0.59 – Table 2). Root collar diameter was positively affected by fertilisation (P<0.001) and higher in Q. faginea than in Q. ilex (P<0.001 – Table 1). SRL was higher in Q. faginea (P=0.001), with no significant effect of fertilisation (P=0.22 – Table 1). The number of root tips per seedling was higher in Q. faginea (P<0.001) and in fertilised seedlings (P=0.04 – Table 1).

The inoculum of T. melanosporum formed mycorrhizas with all seedlings. The number of T. melanosporum mycorrhizas per seedling and the proportion of root tips colonised by T. melanosporum were higher in Q. faginea (P<0.001 and P=0.01, respectively), with no significant effect of fertilisation (P=0.59 and P=0.24, respectively – Table 1). The only contaminant EM species found in seedlings was Sphaerosporella brunnea Syrcek and Kubicka, showing a higher occurrence frequency on Q. ilex (P=0.02) and no significant effect of fertilisation (P=0.22 – Table 1). The mean proportion of root tips colonised by S. brunnea on Q. ilex was 2.5%.

The distribution of T. melanosporum colonisation levels along the depth profile did not show any significant (α=0.05) interaction with fertilisation.

**Experiment 2: fertilisation/inoculation method**

Total dry weight of seedlings, shoot dry weight and root dry weight were higher in root-powering inoculation than in root-dipping (P=0.004, P=0.003 and P=0.02, respectively). No significant effect of fertilisation was found (P=0.08, P=0.14 and P=0.08, respectively), although the trend with respect to fertilisation was the same as in the fertilisation/host species experiment (Tab. 2). Stem height was not significantly affected by either fertilisation (P=0.28) or inoculation method.

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**Table 1** - Mean growth and mycorrhizal levels in Experiment 1: fertilisation/host species (95% confidence interval in parentheses). Letters indicate significant differences (α=0.05) between treatments according to the respective ANOVA or linear model. (§): Variables log-transformed.

| Variable                        | Host species       | Fertilisation regime |
|---------------------------------|--------------------|----------------------|
|                                 | Q. ilex    | Q. faginea   | Unfertilised | Fertilised |
| Plant dry weight (g)            | 5.34 (4.79, 5.90) | 5.61 (5.05, 6.17) | 4.06 j (3.51, 4.62) | 6.89 k (6.34, 7.45) |
| Shoot dry weight (g)            | 1.51 (1.32, 1.75)  | 1.44 (1.25, 1.66) | 1.02 (0.89, 1.17) | 1.51 A (1.86, 2.46) |
| Root dry weight (g)             | 3.50 (3.13, 3.91)  | 3.71 (3.32, 4.14) | 2.88 j (2.57, 3.21) | 4.50 (4.03, 5.03) |
| Stem height (cm)                | 15.8 (13.9, 17.7)  | 15.3 (13.4, 17.1) | 12.5 j (10.6, 14.4) | 18.6 (16.7, 20.5) |
| Root collar diameter (mm)       | 6.1 b (5.7, 6.6)   | 7.9 b (7.5, 8.4)  | 6.3 b (5.9, 6.8)  | 7.7 b (7.2, 8.1)  |
| Specific root length (m g⁻¹)    | 8.4 b (7.2, 9.8)   | 12.3 b (10.6, 14.3)| 10.9 b (9.3, 12.7) | 9.5 b (8.2, 11.1) |
| Number of root tips (×10³)      | 4.20 b (3.37, 5.24)| 10.04 b (8.05, 12.53)| 5.52 b (4.42, 6.89)| 7.64 b (6.12, 9.53) |
| Number of mycorrhizas           | 1.28 b (0.97, 1.68)| 4.62 b (3.52, 6.06)| 2.31 b (1.76, 3.03)| 2.56 b (1.95, 3.36)|
| Percentage of roots colonised   | 35.0 b (34.1, 35.9)| 45.4 b (44.5, 46.4)| 42.1 b (39.8, 44.4)| 38.3 |
| by T. melanosporum              | § § § § § § § § | § § § § § § § § | § § § § § § § § | § § § § § § § § |
| Frequency of occurrence of S. brunnea | 0.17 b (0.01, 0.33)| 0 b (0) 0 (0)| 0.04 b (0.0, 0.13)| 0.13 b (0, 0.27) |

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**Table 2** - Mean growth and mycorrhizal levels in Experiment 2: fertilisation/inoculation method (95% confidence interval in parentheses). Letters indicate significant differences (α=0.05) between treatments according to the respective ANOVA or linear model. (§): Variables log-transformed.

| Variable                        | Inoculation method          | Fertilisation regime |
|---------------------------------|------------------------------|----------------------|
|                                 | Root-dipping | Root-powering | Unfertilised | Fertilised |
| Plant dry weight (g)            | 3.54 b (3.09, 4.05) | 4.73 b (4.14, 5.41)| 3.92 (3.29, 4.30)| 3.88 (3.89, 5.09)|
| Shoot dry weight (g)            | 1.19 b (1.01, 1.40)  | 1.72 b (1.46, 2.02)| 1.31 b (1.11, 1.54)| 1.56 |
| Root dry weight (g)             | 2.33 b (2.03, 2.68)  | 2.99 b (2.60, 3.44)| 2.42 b (2.10, 2.78)| 2.88 |
| Stem height (cm)                | 14.8 (12.0, 17.5)    | 16.9 (14.2, 19.6)| 14.8 (12.1, 17.5)| 16.9 |
| Root collar diameter (mm)       | 5.7 (5.3, 6.1)       | 6.1 (5.7, 6.5) | 5.6 b (5.1, 6.0) | 6.3 b (5.9, 6.7) |
| Specific root length (m g⁻¹)    | 23.6 (20.9, 26.2) | 22.6 (19.9, 23.2)| 23.6 (21.0, 26.3)| 22.5 |
| Number of root tips (×10³)      | 4.84 (3.49, 6.69)  | 6.25 (5.00, 9.57)| 6.25 (4.60, 8.81)| 6.25 (3.80, 7.27) |
| Number of mycorrhizas T. melanosporum (×10³) | 1.62 b (1.24, 2.12)  | 2.74 b (2.10, 3.58)| 2.26 b (1.73, 2.95)| 1.97 |
| Percentage of roots colonised   | 43.7 b (32.6 b) | 37.6 (29.7, 35.5) | 31.6 (27.8, 35.5) | 38.6 |
| by T. melanosporum              | § § § § § § § § | § § § § § § § § | § § § § § § § § | § § § § § § § § |
| Frequency of occurrence of S. brunnea | 0.42 b (0.12, 0.71)| 0 b (0) 0 (0)| 0.25 b (0.0, 0.51)| 0.17 b (0, 0.39) |
The inoculation method (P=0.26 – Tab. 2). Root collar diameter was positively affected by fertilisation (P=0.02), with no significant differences between inoculation methods (P=0.16 – Tab. 2). Neither SRL nor number of root tips per seedling were significantly affected by fertilisation (P=0.54 and P=0.39, respectively) or inoculation method (P=0.59 and P=0.12, respectively – Tab. 2).

The inoculum of T. melanosporum formed mycorrhizas with all seedlings. The number of T. melanosporum mycorrhizas per seedling and the proportion of root tips colonised by T. melanosporum were higher in root-powdering inoculation (P=0.009 and P=0.049, respectively), with no significant effect of fertilisation (P=0.46 and P=0.68, respectively – Tab. 2). The mean proportion of root tips colonised by S. brunnea in root-dipping inoculation was 5.2%.

The distribution of T. melanosporum colonisation levels along the depth profile did not show any significant (α=0.05) interaction with fertilisation.

Discussion

In modern truffle cultivation the use of inoculated seedlings is fundamental (Hall et al. 2007). The abundance of T. melanosporum mycorrhizas in the early years after plantation establishment has been found positively related to mycorrhizal abundance in the nursery and to plant performance after planting (Bourrières et al. 2005, Garcia-Barreda & Reyna 2013). Nursery practices must be fine-tuned to encourage colonisation by T. melanosporum, but also to improve vegetative quality of seedlings. However, in nursery fertilisation experiments a conflict between optimal seedling growth and EM colonisation has been reported for many EM fungi (Castellano & Molina 1989, Walker et al. 2003, Diaz et al. 2010). Two mechanisms have been suggested to explain this conflict: (i) the host reducing carbon supply to the fungus due to a greater carbon demand by growing shoots, or (ii) the fungus requiring most carbon received from the plant to assimilate the greater N uptake (Wallander 1995).

The fertilisation dose used in the present study increased growth as well as vegetative quality according to Spanish standards (Villar-Salvador et al. 2012a, 2012b), while maintaining T. melanosporum colonisation levels. In the fertilisation/host species experiment, fertilisation increased seedling biomass by 70%, stem height by 49% and root collar diameter by 22%, without significant detrimental effects on fine root traits or EM colonisation levels. In the fertilisa tion/inoculation method experiment, fertilisation increased Q. ilex root collar diameter by 13%. The lower magnitude of the fertilisation effect in the latter experiment could be due to differences in acorn size (Navarro et al. 2006), spore germinability (Falcón & Barrusco 2007) and greenhouse climatic conditions from year to year, although it could also be related to the lower number of replicates decreasing the accuracy of estimation.

Our results disagree with previous experiments with containerised Quercus seedlings. Beckjord et al. (1985) inoculated Quercus alba L. and Quercus rubra L. with Pisolithus tinctorum (Pers.) Coker & Couch EM colonisation levels. In the fertilisation experiment, fertilisation increased seedling maintaining 1995 T. melanosporum survival, root development and Tuber aestivum Vittad. colonisation. Cartié et al. (1996) found that using an alginic solution for root-dipping inoculation provoked an important Q. ilex mortality and a low T. melanosporum colonisation. These results suggest that inoculant carriers forming a sticky coating around the complete root system are able to damage Quercus development and Tuber colonisation.

However, Cartié et al. (2001) found no detrimental effects of alginate solution when the inoculant was applied in a bi-layer, firstly dipping roots in alginate solution and then powdering them with a mixture of inoculum and talcum. Pruett et al. (2008) found no detrimental effect of root-dipping inoculation when it was performed without hydrogel. All this suggests that the damage was due to the combination of sticky carrier and spores in close contact with roots.

Our results show that seedling characteristics and inoculation effectiveness can be affected not only by host species but also by inoculation method. Further research would help to know if fertilisation or inoculation method interact with other nursery practices.

The only EM fungi found in our study other than T. melanosporum was the pioneer, nursery adapted S. brunnea (Sanchez et al. 2014). As expected, its occurrence was higher in treatments with lower T. melanosporum colonisation levels, thus suggesting that it was related to gaps left by the latter.

The inoculation and fertilisation procedures used in the present study proved effective for obtaining EM seedlings with quality levels comparable to commercial standards. All seedlings bore T. melanosporum mycorrhizas, with all treatments showing mean colonisation levels analogous to those in commercial nurseries (Andres-Alpuente et al. 2014). All seedlings met the
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