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SHORT COMMUNICATION

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Mulch removal time did not have significant effects on *Tuber melanosporum* mycelium biomass

İsmail Şen[1,2*], Yasmine Piñuela[2,4], Josu G. Alday[2,3], Daniel Oliach[2,4], Francesc Bolaño[2,4], Juan Martínez de Aragón[4], Carlos Colinas[2,4], José Antonio Bonet[2,3]

[1]Department of Biology, Muğla Sıtkı Koçman University, Muğla, Turkey [2]Department of Crop and Forest Sciences, University of Lleida, Lleida E-251 98, Spain [3]Joint Research Unit CTFC – AGROTECNIO – CERCA Center, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain [4]Forest Science and Technology Centre of Catalonia (CTFC), Ctra. Sant Llorenç de Morunys km 2, 25280 Solsona, Spain

Abstract

Aim of study: We aimed to i) evaluate the effects of mulching on *Tuber melanosporum* mycelium biomass and seedling growth (i.e. root collar diameter and seedling height) and ii) unravel the relationship between growth in root collar diameter and mycelium abundance, in a *T. melanosporum* plantation.

Area of study: The experimental plantation is located in the Pre-Pyrenees mountains in Catalonia, Spain.

Materials and methods: The experimental plantation was established in 2010 using one-year-old *T. melanosporum* inoculated *Quercus ilex* seedlings. Double-layered mulch materials were placed around the seedlings. The mulch materials were removed from randomly selected seedlings in 2015 and 2018. Soil samples were collected in 2018 at 40 and 80 cm distances from seedlings that had mulching during five and eight years, and *T. melanosporum* mycelium biomass was estimated by quantitative Polymerase Chain Reaction (qPCR). Seedling root collar diameter and height were measured simultaneously when mulch materials were removed.

Main results: Mulch removal time did not have significant effects on *T. melanosporum* mycelium biomass or seedling growth. However, mycelium biomass at 40 cm distance tended to be higher on seedlings after eight-year mulching with 0.9 mg/g soil whereas mycelium biomass was 0.4 mg/g soil after five-year mulching. A positive relationship between mycelium biomass and seedling root collar diameter was also found.

Research highlights: Mulching seems to have a positive effect on truffle mycelium biomass, with nearly two times higher quantity of mycelium after eight-years compared with five-years mulching usage. Seedling root collar diameter is a good indicator of mycelium expansion in the plantation.

Keywords: Black truffle; *Quercus ilex*; mulching; tree growth; truffle cultivation.

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Correspondence should be addressed to İsmail Şen: frapesle@gmail.com

Introduction

The Périgord black truffle, *Tuber melanosporum* Vittad., known as the "black diamond of the kitchen", has gained economic and gastronomical importance due to its unique aroma (Bonet *et al.*, 2009). Its demand is continuously increasing worldwide, however, the natural production of this fungus has been decreased in the last few decades (Samils *et al.*, 2008). Therefore, truffle cultivation has become a profitable agricultural alternative in recent years (Bonet *et al.*, 2009). Despite the progress in management practices of truffle plantations (Bonet *et al.*, 2009; Olivera *et al.*, 2011, 2014), there are still uncertainties on how to handle plantations to achieve regular high yields. Several studies have shown that *T. melanosporum* fruiting is strongly related to summer precipitation (Büntgen *et al.*, 2012; García-Barreda *et al.*, 2020). However, climate change scenarios predict that summer precipitation would decline while water evaporation will increase driven by rising temperatures in the close future (Cramer *et al.*, 2018) that will be impacting also truffle cultivation.

Mulching could be a useful tool in truffle plantations to reduce summer drought stress (Le Tacon, 2016) because of its capacity to reduce soil moisture loss (Kader *et al.*, 2017; Bandopadhyay *et al.*, 2018). Moreover, Olivera *et al.* (2014) demonstrated that mulching has positive effects on weed control while improving the survival rates of seedlings in the first three years after truffle plantation establishment. The same authors also found that white double-layered mulching stimulated the development of black truffle mycelium biomass. Nevertheless, long-term use of mulch materials such as plastics, geotextile, or fine-textured organic mulches could cause unnaturally dry soils despite increasing soil water retention at the beginning of its usage (Chalker-Scott, 2007). Similarly, the development of *T. melanosporum* mycelium in response to mid-term mulch presence has not been clearly described yet. Hence, the assessment of mulch removal time has gained importance for sustainable truffle cultivation.

Furthermore, previous studies have concluded that mulching stimulated seedling growth (Olivera *et al.*, 2014) and also that larger trees support higher black truffle mycelium quantities in plantations (Suz *et al.*, 2008; Oliach *et al.*, 2020). Therefore, understanding the interaction between mycelium biomass and seedling growth and how mulching could promote this growth is relevant to improve truffle plantation management practices (Oliach *et al.*, 2020).

Based on these premises, the aims of this study were (i) to evaluate the effects of five- and eight-years mulching treatment on *T. melanosporum* mycelium biomass; (ii) to describe the influence of mulching treatment on seedling root collar diameter and height; and (iii) to evaluate the interaction of seedling root collar diameter and its growth with *T. melanosporum* mycelium biomass. We hypothesized that white double-layered mulching will stimulate mycelium development and seedling growth (root collar diameter and height) supporting results obtained by Olivera *et al.* (2014). Furthermore, we expected greater *T. melanosporum* mycelium biomass beneath larger trees in accordance with previous observations made by Oliach *et al.* (2020).

Material and Methods

The experimental plantation was established in May 2010 at the eastern Pre-Pyrenees area in Catalonia, Spain (42°02′36.96″N, 1°14′5.62″E). The altitude of the experimental site is 996 m a.s.l. Soil is calcareous with calcarenite rocks and a pH of 8 (1:2.5 H_2O). The climate is continental Mediterranean with mean daily temperature ranging from 4.4 to 16 ºC and average annual precipitation of 700 mm, thus, the ecological characteristics of the area are suitable for black truffle cultivation (Colinas *et al.*, 2007; Bonet *et al.*, 2009).

The experimental plot was planted with 249 seedlings by using 6 m × 6 m grid in a 1-ha abandoned pastureland (for further description see Oliach *et al.*, 2020). One-year-old holm oaks *(Quercus ilex* L.) inoculated with *T. melanosporum* were planted, which were obtained from a commercial nursery (Cultivos Forestales y Micológicos S.L., Torre de las Arcas, Teruel, Spain). Before planting, the ectomycorrhizal status of the seedlings was evaluated according to the methodology described by Fischer & Colinas (1996), to be sure that the seedlings were properly mycorrhized with *T. melanosporum* and overall adequate for truffle cultivation. Other ectomycorrhizal fungi were not observed in the seedlings roots.

At the time of planting, we placed 2 m × 2 m pieces of double-layered antiweed polyethylene fabric (110 g/m^2 density, Projar, Valencia) around each plant: a black layer underneath to reduce herbaceous competition and a white layer above to reflect solar radiation. This material was chosen because it is permeable letting the rain reach the soil and allowing gas exchange (Oliach *et al.*, 2020). In this experimental plantation, irrigation, weed control, or soil tillage interventions were not performed.

Mulch materials of randomly selected seedlings were removed at two different times to understand suitable mulch removal time and its effects on truffle mycelium biomass. First, 50% of mulch materials (124 seedlings) were removed five years after the plantation establishment (*i.e.* May of 2015). Second, 25% of initial mulches (62 seedlings) were removed eight years after the plantation establishment (*i.e.* May of 2018) and simultaneously the soil sampling was performed. Hereafter, five and eight-year mulching treatments are mentioned as "m5" and "m8" respectively.

In May 2018, 18 seedlings belonging to m5 and 18 belonging to m8 treatments (in total 36 seedlings) were randomly selected for soil sampling. Soil samples were collected at 40 and 80 cm distance from seedlings trunk to determine truffle mycelium biomass in response to different mulch removal time. We were interested in the abundance of truffle mycelium at two different distances from the seedlings to determine how truffle mycelium development responds to mulching because truffle mycelium expansion changes with the age of the seedlings in truffle plantations (Liu *et al.*, 2014). Soil subsamples were taken with a soil core (7 cm Ø) in three different orientations (5 to 20 cm deep). Afterward, the three subsamples collected at each distance were pooled into one sample (Oliach *et al.*, 2020). The lyophilized samples were sieved through a 3 mm mesh, homogenized with porcelain mortars, and stored at –20 ºC in the laboratory until DNA extraction.

DNA extraction was performed of 0.5 g of soil per sample by using NucleoSpin® soil extraction kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. The genomic DNA was stored at –20°C until ready to perform soil mycelium quantification. Following the qPCR protocol of Parladé *et al.* (2013), three technical replicates of each sample, standards, and negative control were included in each reaction. The reaction contained 5 µl of template, 2 x iTaq™ Universal Probes Supermix (Bio-Rad®), 800 nM of each oligo, 200 nM of the hydrolysis probe, and ultrapure water to obtain a final reaction volume of 20 µl. PCR cycling conditions were as described by Parladé *et al.* (2013) and reactions were carried in a Bio-Rad®CFX96™ thermocycler. Bio-Rad CFX™ Manager 3.1 was used for analyzing the data. Standard curves were generated from different known amounts of the targeted truffle added to control soil (control soil was obtained in an area adjacent to the experimental units and it was previously checked by qPCR assay to be free from *T. melanosporum* DNA). The standard curve was built by using ten-fold serial dilutions of gDNA from a mixture of 0.02 g internal glebal tissue of freeze-dried *T. melanosporum* sporocarp and 0.48 g of dried and lyophilized control soil from the study area. The absolute soil mycelium biomass was estimated by interpolating the Ct values obtained from each soil sample on the standard curve.

Simultaneously to the mulch removal time in 2015 and 2018, root collar diameter and height were measured for each seedling to evaluate the effects of mulching treatments on tree growth. A caliper was used to measure root collar diameter taking two orthogonal measurements at constant height (1cm above the soil surface), while height was measured with the help of a meter. Root collar diameter per seedling was calculated as the average of both measurements and root collar diameter growth and seedling height increment were obtained by the differences between 2015 and 2018 measurements.

The statistical analyses were carried out with R software environment (version 3.6.2, R Core Team, 2017). Before any analyses, the normality and homogeneity of the data were checked by Shapiro-Wilk (*shapiro.test* function) and Bartlett *(bartlett.test* function) test, and *T. melanosporum* mycelium biomass was square-root transformed to meet the homoscedasticity criteria. Afterward, the effects of mulch removal (factor) on *T. melanosporum* mycelium biomass and plant growth (*i.e.* host tree root collar diameters and heights) were analyzed using ANOVAs (aov function). Likewise, we used the linear regression models (lm function) to relate *T. melanosporum* mycelium biomass with host tree root collar diameters, heights, plant growth, and their interactions (full models). The model that reduced the Akaike Information Criterion (AIC) most, relative to the null model, was selected and the regression determination coefficient was calculated (Alday *et al.*, 2011). Only the best-fit models selected are presented here.

Results and Discussion

At 40 cm distance from the seedlings, the average amount of truffle mycelium biomass was 0.9 ± 0.3 (mean ± standard error, hereafter) mg/g soil (ranging from 0 to 4.4 mg/g soil) and 0.4 ± 0.2 mg/g soil (ranging from 0 to 2.2 mg/g soil) for m8 and m5 treatments, respectively. Even though the average amount of truffle mycelium biomass in m8 treatment is more than twice that of m5 treatment, the high variability among samples did not allow us to detect statistically significant differences between mulch removal times ($F_{1,34} = 0.45$, $p = 0.508$). At 80 cm, we detected *T. melanosporum* mycelium just in 8 experimental units out of 36. At this distance, mulch removal time did not have significant effects on mycelium biomass either ($F_{1,34} = 0.09$, $p = 0.768$), and the average truffle mycelium biomass at this distance varied from 0.07 ± 0.05 mg/g soil (ranging from 0 to 0.9 mg/g soil) to 0.09 ± 0.08 mg/g soil (from 0 to 1.3 mg/g soil) for m8 and m5 treatments, respectively. It seems that black truffle mycelium has not properly colonized the soil at 80 cm from the stem in all plants yet as also observed Liu *et al.* (2014) who noted that the entire colonization process at 80 cm far from seedlings is likely to take more than eight years.

The positive effects of mulching on *T. melanosporum* mycelium biomass have been also observed by other authors that used the same mulching materials. In the first years of truffle plantation establishment, Olivera *et al.* (2014) showed that the use of white double-layered mulch had significant and positive effects on truffle mycelium development at 15 and 30 cm distance from the host tree compared to other mulch materials or bare soil. In our case, the positive effect of mulching at 40 cm follows this

trend although at 80 cm the colonization has not been accomplished in all the seedlings.

Mulch removal time did not have significant effects on seedling growth between 2015 and 2018 ($F_{1,34}$ = 0.03, p = 0.852 for root collar diameter increment; $F_{1,34}$ = 0.07, p = 0.792 for height increment). The mean seedling root collar diameter increments were 7.8 ± 1.2 mm and 8.3 ± 1.3 mm for m5 and m8 treatments, respectively; while the mean height increment was 8.2 ± 2.4 cm for m5 and 6.2 ± 1.6 cm for m8 treatments. In contrast, Olivera *et al.* (2014) observed significant effects of mulch treatment on seedling root collar diameter increment in the first 3 years. Mulching is likely to encourage seedling growth in the first years of truffle plantation establishment, but we observed that more than five years of mulch existence did not have any effects on seedling growth. Likewise, it could be considered that mulching can indirectly encourage mycelium development by providing strong host trees in the first few years of plantation establishment.

In the present experiment, we observed that the root collar diameter and its increment were the best predictors for *T. melanosporum* mycelium with the lowest AIC in our regression models. Thus, *T. melanosporum* mycelium biomass at 40 cm distance from the seedlings was significantly and positively related with root collar diameter (R^2 = 0.37, $F_{1,34}$ = 18.66, p < 0.001, AIC reduction 118.70, Fig. 1a). Similarly, the root collar diameter increment between 2015 and 2018 had a positive relation with mycelium biomass at 40 cm (R^2 = 0.32, $F_{1,34}$ = 14.53, p < 0.001, AIC reduction 11.75, Fig. 1b). But we could not detect any significant relation between mycelium biomass and seedling height ($F_{1,34}$ = 0.43, p = 0.516) or seedling height increment ($F_{1,34}$ = 0.66, p = 0.424). A significant relation between mycelium biomass and seedling root collar diameter and its increment was also observed at 80 cm (R^2 = 0.27, $F_{1,34}$ = 16.97, p < 0.001, AIC reduction 9.17, for root collar diameter; R^2 = 0.24, $F_{1,34}$ = 15.94, p < 0.001, AIC reduction 7.95, for diameter increment). *Quercus ilex* can develop a bushy structure with multiple stems, probably as a strategy to survive heavy browsing and forest fires, even if all seedlings in our plantation have developed without multiple stems. Thus, the height of the highest stem may not reflect the total biomass increment of the sapling as well as the root collar diameter does.

Even though maintaining the mulching longer did not have a significant positive effect on *T. melanosporum* mycelium proliferation, we still believe that it is an advisable management practice since it is clearly not detrimental to the fungus and it reduces the cost of weeding the seedlings and the potential compaction caused by heavy equipment working in the plantation.

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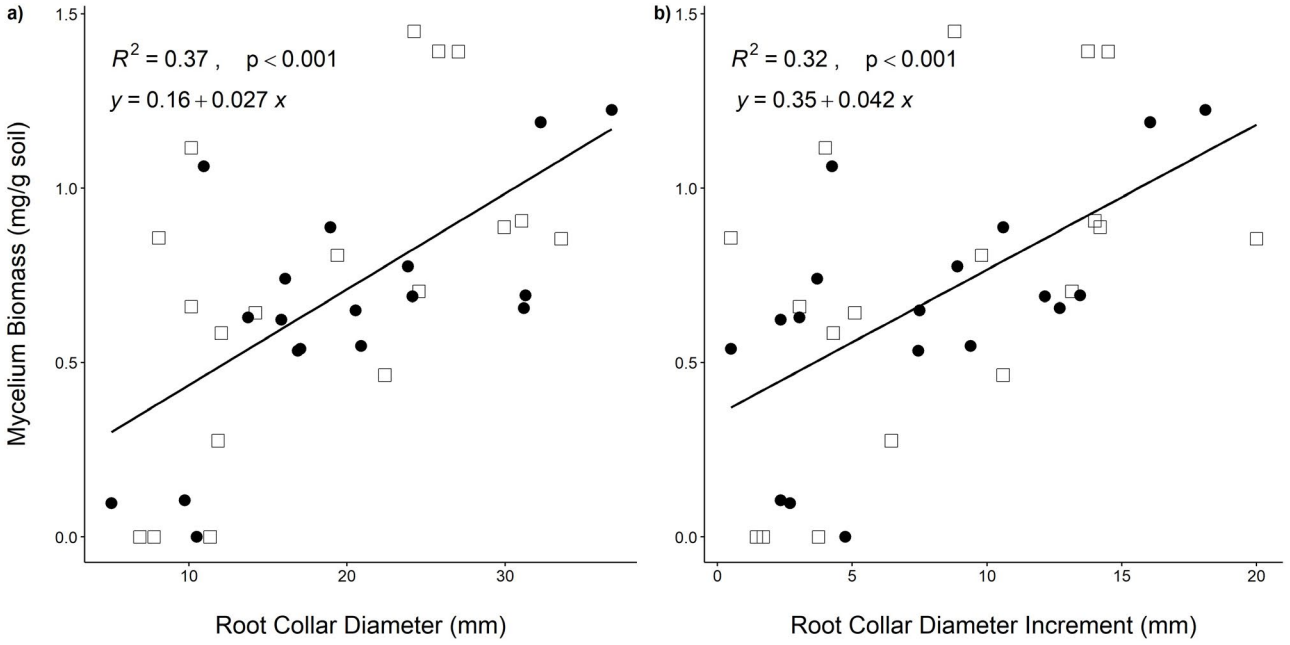

Figure 1. The relationship between black truffle mycelium biomass and (a) host tree diameter in 2018 and; (b) seedling root collar diameter increment from 2015 to 2018 (at 40 cm from the seedlings). The circles indicate m5 treatment, squares indicate m8 treatment. The mycelium data are square-root transformed.

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