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The influence of forest surroundings on the soil fungal community of black truffle (*Tuber melanosporum*) plantations

Daniel Oliach[a,b,c,*], Carlos Colinas[a,c], Carles Castaño[d], Christine R. Fischer[a], Francesc Bolaño[a], José Antonio Bonet[c,e], Jonàs Oliva[c,e]

[a] Centre de Ciència i Tecnologia Forestal de Catalunya, Crta. Sant Llorenç de Morunys km 2, 25280 Solsona, Spain
[b] Forest Bioengineering Solutions S. A., Crta. Sant Llorenç de Morunys km 2, 25280 Solsona, Spain
[c] Universitat de Lleida, Dept. de Producció Vegetal i Ciència Forestal, Av. Rovira Roure, 191, 25198 Lleida. Spain
[d] Department of Forest Mycology and Plant Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden
[e] Joint Research Unit Agrotecnio-CTFC, Avda. Rovira Roure, 191, 25198 Lleida, Spain

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ABSTRACT

Black truffles are a highly valued non-wood forest product. The success of truffle plantations is arousing the interest to establish orchards within forest settings. One main concern is that the forest may act as a source of ectomycorrhizal fungi that could displace *Tuber melanosporum* in plantations and impair truffle production. We studied the effects of host tree distance to the surrounding forest on *T. melanosporum* development and on the root-associated fungal community. Our research was carried out in a 5-year old holm oak (*Quercus ilex*) plantation established in an abandoned pasture surrounded by a *Q. ilex* forest in the Pyrenees. The spatial distribution of different fungal guilds as well as of *T. melanosporum* mycelium quantity and mating types frequency was correlated with the distance to the forest and the diameter of the trees. We found a higher relative abundance of non-*T. melanosporum* EcM fungi associated with the trees closer to the forest. Larger root collar diameter trees had greater biomass of *T. melanosporum* mycelium and displayed fungal community compositions less affected by the distance to the forest. No associations between the biomass of *T. melanosporum* mycelium in the soil and the distance to the forest or the abundance of non-*T. melanosporum* EcM fungi were observed. Our results indicate that *T. melanosporum* inoculated oaks planted in areas surrounded by forests may be colonised by other ectomycorrhizal species, and develop a distinct microbial community from those usually established in agricultural lands. Further investigations should be carried out to determine whether a different fungal community may affect truffle production in the future, but to date, truffle mycelium does not seem to be impaired.

1. Introduction

Black truffle (*Tuber melanosporum*) plantations are being developed across all the Mediterranean-climate regions around the world i.e. Spain, France, Italy and Australia (Reyna and Garcia-Barreda, 2014). The current global production is about 120,000 kg yr^{-1} which generates an annual turnover of ca. 50 million euros to truffle growers (Oliach et al., 2020). Besides direct revenues, the black truffle production has a positive impact in the areas where they are collected, favouring the development of auxiliary economic activities such as nursery production of mycorrhized seedlings with *T. melanosporum*, technical consulting, trade of truffled products, agro-tourism and research (Büntgen et al., 2017). In France, where annual production of truffles is about 43,000 kg yr^{-1} (2013–2017) (Oliach et al., 2020), for which Escafré and Rousset (2006) estimated a total economic impact of 67 million euros yr^{-1}.

One of the main drivers for establishing plantations has been the decline of wild truffle production in the last 100 years (Callot, 1999). Both lower summer rainfall (Büntgen et al., 2015, 2019; Thomas and Buntgen, 2019), and forest canopy closures seem to be the main causes (Le Tacon et al., 2014). In contrast, plantations are thriving (Sánchez and Sánchez, 2019). Truffle orchards are produced by planting one-year old nursery inoculated seedlings of several oak species (*Quercus* spp.) and hazelnut (*Corylus avellana*) at a density of ca. 250–300 trees per hectare. Truffles begin to fruit after 5–7 years of plantation, depending on site conditions and management intensity. The establishment requirements for truffle orchards in agricultural land and cultural practices in terms of irrigation, weed control and fertilization have been

* Corresponding author.
E-mail address: daniel.oliach@ctfc.cat (D. Oliach).

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studied (Colinas et al., 2007; Jaillard et al., 2014, 2016, Olivera et al., 2011, 2014a, 2014b; Suz et al., 2010). However, the increasing demand for truffles has raised the interest for establishing truffle plantations in other types of non-economically productive lands such as abandoned pastures and agricultural fields within forested settings.

Plantations in the vicinity of forests have been historically discouraged due to inoculum banks of competing ectomycorrhizal fungi (Frochot et al., 1990; Sourzat, 1997). Ectomycorrhizal (EcM) associations are mutualistic associations between higher fungi like *T. melanosporum* and Gymnosperm or Angiosperm plants belonging to certain families, and the majority of EcM hosts are trees or shrubs (Brundret et al., 1996). Agricultural soils are assumed to be free from ectomycorrhizal inoculum and therefore more receptive to *T. melanosporum*. However, propagules of EcM fungi coming from surrounding forests or already present in the soil before planting can colonize the planted seedlings and eventually outcompete *T. melanosporum* as trees grow (Frochot et al., 1990; Reyna et al., 2006), especially during the early years of the plantation (Águeda et al., 2010; De Miguel et al., 2014). Theory predicts that pre-established fungal species in EcM-free areas, may have a competitive advantage over other incoming EcM species due to priority effects (Kennedy and Bruns, 2005). In nature, *T. melanosporum* colonizes young oaks establishing in treeless areas (Reyna, 2012). This observation suggests that *T. melanosporum* benefits from niche pre-emption (Bogar and Peay, 2017), in which early-arriving EcM species who first occupy the root system can have a competitive advantage over later-arriving fungi (Kennedy and Bruns, 2005; Kennedy et al., 2009). This ecological strategy is the one used by growers when establishing nursery-inoculated *T. melanosporum* seedlings on agricultural land. We know that *T. melanosporum* can survive up to 4 years in forest soils where EcM trees have been supressed by fire or grazing (Garcia-Barreda and Reyna, 2013; Martínez de Aragón et al., 2012). Nevertheless, information about the possible competition between local EcM species and the introduced *T. melanosporum* in a plantation environment surrounded by forest is largely missing.

Other factors, such as host growth, may play a role modulating the competition between *T. melanosporum* and local EcM fungi. We know that in plantations, larger trees harbour higher quantities of mycelium than smaller trees, and that truffle burns, i.e. zone around the host tree lacking herbaceous vegetation, appear earlier on fast growing trees of the same age (Lulli et al., 1999; Suz et al., 2008). Gaining insights on the interplay amongst host growth, *T. melanosporum* and other EcM fungi is needed.

In this research, we studied the effects of host tree distance to the forest and *T. melanosporum* development in planted seedlings under conditions of high exposure to inoculum of other EcM fungi. The study was carried out on 5-year old holm oak (*Quercus ilex*) trees that had been inoculated with black truffle spores and outplanted as 1 yr old seedlings in an abandoned pasture, surrounded by a *Q. ilex* forest. Development was measured in terms of mycelium biomass by qPCR and mating type distribution by PCR. These two parameters are typically used to assess the expansion of *T. melanosporum* before the first truffles are produced (Queralt et al., 2017). Soil fungal community was assessed by sequencing amplified fungal markers with PacBio RSII. We hypothesised that (i) trees planted closer to the forest would have higher presence of non-*T. melanosporum* EcM fungi than trees located further from the forest. Furthermore, we hypothesized that (ii) niche pre-emption would be more important than native EcM fungi arriving from the forest, and therefore *T. melanosporum* would dominate irrespective of the distance to the forest. Finally, we expected that (iii) greater tree growth would be positively associated with *T. melanosporum* mycelium abundance.

2. Material and methods

2.1. Experiment site

In May of 2010, we established a 1-ha experimental afforestation plot in a recently abandoned pasture located in the eastern Pre-Pyrenees of Spain (42°02′36.96″N, 1°14′5.62″E). The site plot is located at 996 m above sea level, has a South west aspect with minimal slope and it is surrounded by an extensive *Q. ilex* forest. The plot is located on soil formation from the Jurassic period of the type Lias-malm, formed mainly by calcareous and calcarenite rocks. The site presents a soil with a heterogeneity depth of about 40 cm with loam soil texture (USDA) and pH 8.03 (1:2.5 H_2O). The climate is continental Mediterranean with 700 mm of annual precipitation. Temperatures ranges from an annual average of maximum daily temperatures of 16 °C and minimum of 4.4 °C.

The plantation consisted of 249 one-year-old *Q. ilex* seedlings inoculated with *T. melanosporum* purchased from a commercial nursery (Cultivos Forestales y Micológicos S.L., Torre de las Arcas, Teruel, Spain). Prior to planting, we confirmed *T. melanosporum* seedlings colonisation according to Fischer and Colinas (1996). No other EcM fungi were observed in the seedlings before planting.

The plantation was established following standard procedures: the soil was prepared by ripping to a depth of 60 cm to break up hardpans and promote root penetration (Oliach et al., 2005). Seedlings were planted in a 6 m × 6 m grid. A 2 m × 2 m polypropylene 110 g m^{-2} mulching fabric was placed surrounding each tree in order to prevent herbaceous competition and to maintain soil moisture during the first years after planting following Olivera et al. (2014b). The fabric was double layered: black below to reduce weed germination and white above to reflect solar radiation. The fabric was specifically chosen to allow water and gas exchange in and out of the soil.

2.2. Data collection

Five years after planting, in March 2015, we randomly chose 28 seedlings, for which we measured height, root collar diameter and the minimum distance to the forest. Root collar diameter was calculated as an average of two perpendicular calliper measurements per tree. Tree height was measured from the root collar to the top of the tree. The minimum distance to the forest was obtained from a satellite image of the plantation (Cartographic Institute of Catalonia, www.icgc.cat). From each of the 28 trees, we collected soil samples at 40 cm and 80 cm from the stem (28 × 2 distances = 56 samples) in order to determine the quantity of *T. melanosporum* mycelium, the presence of the two mating types, and to characterize the community of soil fungi (Fig. 1). Each sample consisted of a composite of 3 subsamples (totalling 168 soil cores) collected between 5 and 20 cm deep using a 7 cm diameter soil core (Fig. 1), which was washed with bleach between each sample. Prior to DNA work, the samples were sieved through a 3 mm mesh, homogenized with a pestle and mortar, lyophilized and stored at −20 °C.

2.3. DNA work

DNA extractions were performed from 0.5 g of the homogenized soil with the NucleoSpin® Soil extraction kit (Macherey-Nagel, Duren, Germany), following the manufacturer's instructions. For quantification of *T. melanosporum* DNA we used the qPCR protocol from Parladé et al. (2013) in an iCycler iQ thermal cycler (Bio-Rad). Each plate included three technical replicates per sample, three replicates for each standard and a negative control. Reactions contained 2 × Takara Premix Ex Taq™ (Takara Bio Europe, SAS, France), 0.8 mM of each primer (Forward: Tmelfwd, Reverse: Tmelrev), 0.2 mM of probe, 5 µl of the template DNA and sterile water to achieve a final reaction volume of 20 µl. Cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C

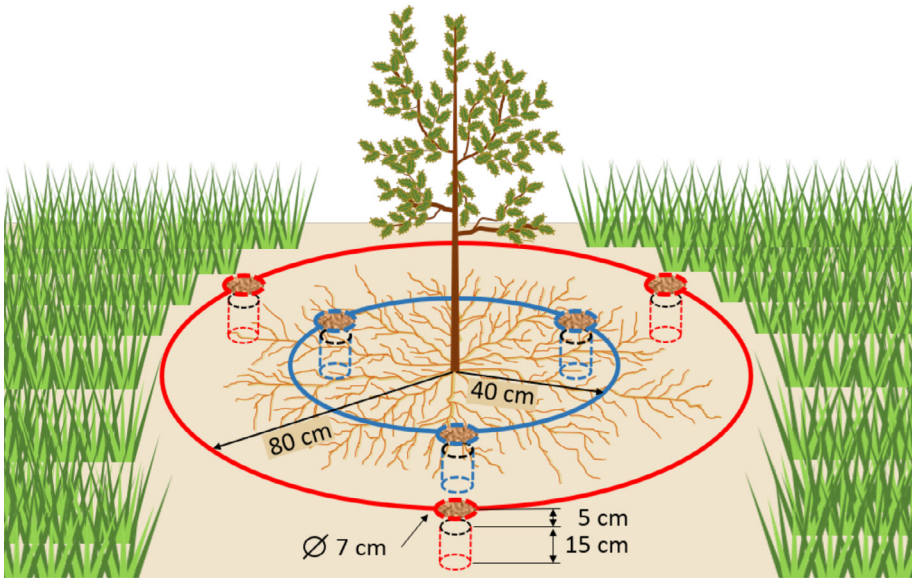

Fig. 1. From each tree sampled (28 trees) we collected soil samples at 40 cm and 80 cm from the stem (28 × 2 distances = 56 samples) in order to determine the quantity of *T. melanosporum* mycelium, the presence of the two mating types, and to characterize the community of soil fungi. Each sample consisted of a composite of 3 subsamples collected using a 7 cm diameter soil core at a depth of 5 to 20 cm.

Table 1
PERMANOVA of the species-level Hellinger transformed community of soil fungi in a truffle plantation, as analysed by sequencing of internal transcribed spacer 2 amplicons. Main factors (DT = distance to the stem (cm); DF = distance to the forest (m); D = root collar diameter of the tree (mm).

Factor	d.f.	R^2	p-value
Distance to the stem (DT)	1	3.50	**0.005**
Distance to the forest (DF)	1	4.44	**0.002**
Root collar diameter of the tree (D)	1	2.75	**0.040**
DT × DF	1	1.09	0.975
DT × D	1	1.23	0.896
DF × D	1	3.15	**0.016**
DT × DF × D	1	0.01	0.989
Total	55		

for 5 s and 60 °C for 34 s. Quantification of *T. melanosporum* mycelium, expressed as mg of mycelium per g of soil, was determined by interpolation of the C_T value on the standard curve. To build the standard curve, we used serial ten-fold dilutions of a DNA extraction from a sporocarp: soil mixture. The sporocarp:soil mixture was made by mixing 0.48 g of dried soil from the study area, previously checked to be free from *T. melanosporum* by Real-Time PCR (> 40 cycles), mixed with 0.02 g of fungal tissue obtained from a dried *T. melanosporum* fruit body.

To amplify the fungal ITS2 region, each sample was PCR-amplified using gITS7 (Ihrmark et al., 2012) and ITS4 primers (White et al., 1990), with 8-bp tags added to both primers to identify each sample. The number of PCR cycles in which a faint band was observed was optimised for each sample in range from 26 to 33 cycles. Cycling conditions were as follows: 5 min at 95 °C, followed by 26–33 cycles of 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C and a final extension step at 72 °C for 7 min. PCR reactions were performed in a volume of 50 µl in a Thermal Cycler (Life Technologies) consisting of 25 ng template, 2.75 mM $MgCl_2$, primers at 0.5 µM (ITS7) and 0.3 µM (ITS4) and 0.025

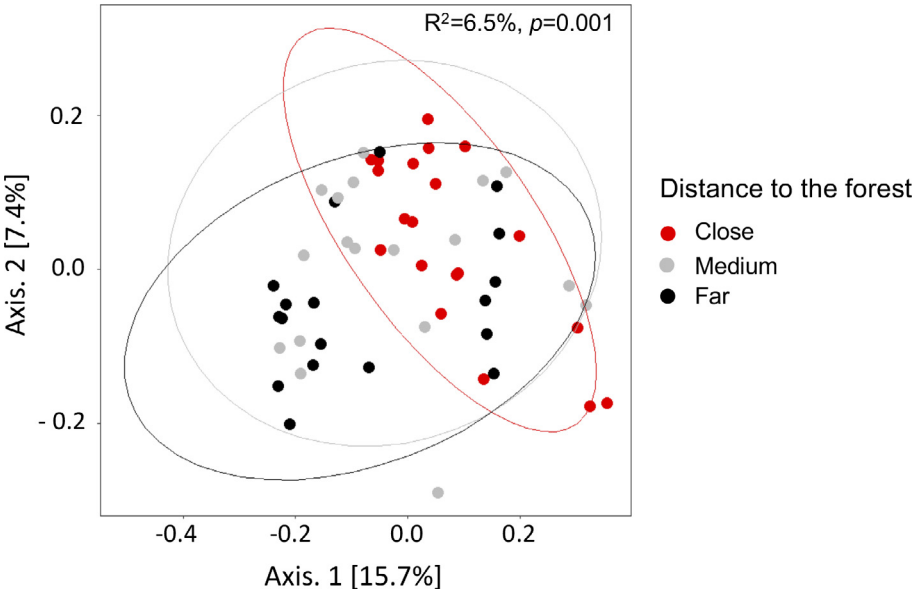

Fig. 2. Fungal community shifts associated with distance to forest. PCoA ordination calculated with a bray-distance matrix. R^2 shown and p-value shown are extracted from a PERMANOVA analysis in which all design variables are included, but distance to the forest is transformed in a category variable, with three levels corresponding to (5.14–12.2 m, 12.2 m–20.8 m and 20.8–32.5 m).

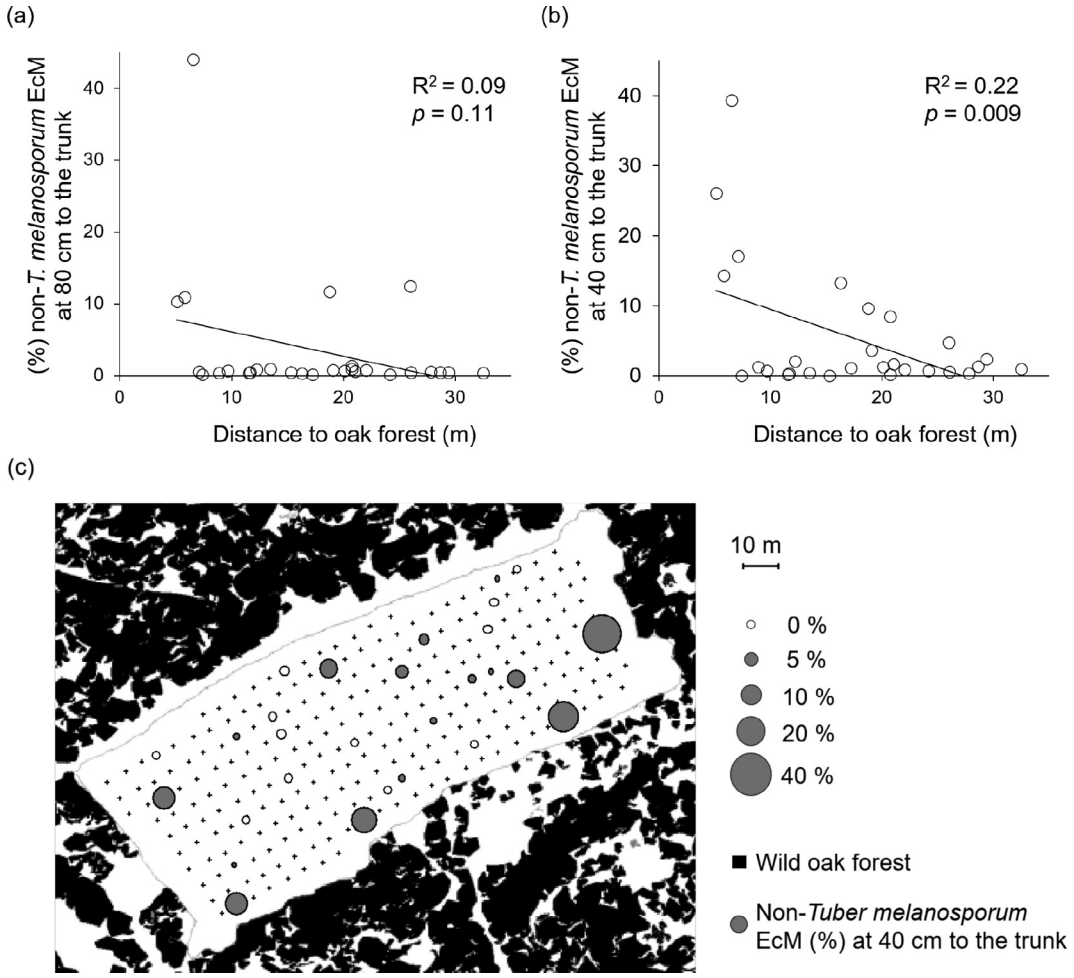

Fig. 3. Relationship between the relative abundance (%) of non-*Tuber melanosporum* ectomycorrhizal (EcM) fungi observed in each tree at 40 cm (a) and (c) and 80 cm (b) to the stem, and the distance of the tree to the forest.

Table 2
Relationship of abundance of guilds with distance to the forest and root collar diameter of the tree at 40 cm and 80 cm. Shown are the positive or negative relation and the significance (p: '*' 0.05, '**' 0.01, '***' 0.001).

Guild	Distance from the stem			
	40 cm		80 cm	
	Distance to the forest	Root collar diameter of the tree	Distance to the forest	Root collar diameter of the tree
EcM (with *Tuber*)	–	–	–	–
EcM (without *Tuber*)	(−)***	–	–	–
Arbuscular	–	–	–	–
Plant pathogens	(+)*	–	–	(+)*
Endophytes	–	(+)*	–	–
Mycoparasites	–	–	–	–
Dung saprotophs	–	–	–	(+)*
Wood saprotophs	–	–	–	–
Soil saprotophs	–	–	–	–
Undefined saprotophs	–	(−)*	–	–
Unknown	–	(−)*	–	–

U μL^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1 × Buffer PCR. Each sample was amplified in triplicate including an extraction negative control and a PCR negative control. Amplified DNA from each sample was pooled and purified using AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled and the mix was purified using EZNA Cycle Pure kit (Omega Bio-Tek). Purified amplicons were subjected to quality control using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a 7500 DNA chip. The samples were sequenced at SciLifeLab NGI, Uppsala, Sweden, in a PacBio RS II system (Pacific Biosciences, Menlo park, CA, USA) using 4 SMRT cells.

For mating-type identification, we used DNA from soil samples collected at 40 cm from the tree. We employed two pairs of primers, one for each mating type, developed by Rubini et al. (2011). PCRs were performed by an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, with a final extension phase at 72 °C for 7 min. PCR amplicons were prepared in 2% agarose GelRed gel electrophoresis and the results were visualized on a UV transilluminator.

2.4. Bioinformatic analysis

Sequences were quality filtered and clustered using the SCATA pipeline (scata.mykopat.slu.se). Sequences < 200 bp were removed and remaining sequences were screened for primers (requiring at least 90% sequence match) and sample tags. After collapsing of homopolymers to 3 bp, sequences were pair-wise compared using 'usearch' clustering algorithm (Edgar, 2010). Pairwise alignments were scored using a mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. Sequences were clustered into operational taxonomic units

Table 3
Indicator species with associated number of OTUs for the community at 40 cm and the community at 80 cm from the tree related to Distance to the forest (m), Root collar diameter (mm) and *Tuber melanosporum* mycelium ($mg \cdot g^{-1}$ soil) (p: '*' 0.05, '**' 0.01, '***' 0.001). (EcM: Ecto; A: Arbuscular; PP: Plant Pathogen; AP: Animal Pathogen; E: Endophyte; S: Saprotroph; DS: Dung Saprotroph; WS: Wood Saprotroph; SS: Soil Saprotroph; UF: Undefined Saprotroph U: Unknown).

	Distance to the forest (m)			Root collar diameter (mm)			*T. melanosporum* mycelium ($mg \cdot g^{-1}$ soil)	
Group	40 cm	80 cm	Group	40 cm	80 cm	Group	40 cm	80 cm
Closest to the forest (5.14–12.2 m) (n = 18)	Glomeraceae_215 ** (A) Dothideomycetes_69 ** (U) Ascomycota_114 * (U) Onygenales_8 * (U) Eurotiomycetes_73 * (U) Fungi.8 * (U) Zoopagales_261 * (U) Beauveria_sp_200 * (AP) Fungi_133 * (U) X..9 * (U)	Chaetothyriales_379 * (U) Dothideomycetes_139 * (U) Fungi.12 ** (U) Rhizopogon_roseolus_105 * (EcM) Pezizomycetes_26 * (U) Beauveria_sp_200 * (AP)	**Small diameter (5.55 - 10.4) (n = 28)**	X._33 * (U) Ascomycota_282 * (U) Zygomycota_288 * (U) X.2 * (U) Aureobasidium_spp_308 * (US) X_Xylariales_320 * (U) Chytridiomycota_380 * (U)	X._33 * (U) Zoopagales_261 * (U) Ascomycota_179 * (U) Chaetomiaceae_412 * (U)	**Low quantity of mycelium (0-0.0032 $mg \cdot g^{-1}$ soil) (n = 28)**	Mortierella_sp_102 * (US) Zoopagales_261 ** (U) Ascomycota_110 ** (U) Penicillium_sp_174 ** (DS, WS, US) Lyophyllaceae_362 * (U) Mortierella_sp_218 * (US) X.11 * (U) Scolecobasidium_sp_301 * (US) Pleosporales_251 * (U) Mucorales_321 * (U) Tomentella_sp_27 * (EcM) Fungi_103 * (U) Hypocreales_326 * (U)	Penicillium_sp_24 * (A)
Medium distance to the forest (12.2 - 20.8 m) (n = 18)	Clonostachys_spp_285 ** (PP) Ascomycota_41 ** (U) X..11 * (U) Kickxellaceae_148 * (U)	Glomeraceae_sp_81 * (A) Glomeraceae_sp_276 * (A)	**Big diameter (10.4-24.2) (n = 28)**	**Tuber_melanosporum_6 ** (EcM)** Fusarium_sp_9 * (PP, WS, SS) Volutella_ciliata_96 * (PP) Fungi.10 * (U)	Wallemiomycetes_sp_22 * (U) Ascomycota_74 * (U) Cryptococcus_aerius_2 * (AP) Spizellomyces_sp_381 * (PP) Pleosporaceae_297* (U) Diversisporaceae_339 * (U) Fungi_317 *(U) Basidiomycota_175 * (U)	Large quantity of mycelium (0.0032-2.41 $mg \cdot g^{-1}$ soil) (n = 28)	**Tuber_melanosporum_6 ** (EcM)** Fusarium_sp_15 * (PP, WS, SS) Cryptococcus_aerius_2 ** (AP) Fusarium_sp_9 * (PP, WS, SS)	Ascomycota_56 ** (U) Aspergillus_sp_149 **(US) Ascomycota_41 * (U) Glomeromycota_182 * (U) Eurotiomycetes_73 * (U) Glomeraceae_sp_314 ** (A) Pleosporaceae_138 * (U) X..14 * (U) Neonectria_spp_210 * (PP) Glomeraceae_181 * (A) X..4 * (U) Fungi_206 * (U) Agaricomycetes_378 * (U) Lasiosphaeriaceae_336 * (U) Mortierella_sp_218 * (US) Ascomycota_152 * (U) Chytridiomycota_346 * (U)

(*continued on next page*)

Table 3 (*continued*)

	Distance to the forest (m)			Root collar diameter (mm)			*T. melanosporum* mycelium (mg·g^{-1} soil)			
Group	40 cm	80 cm	Group	40 cm		80 cm	Group	40 cm		80 cm
Farthest from the forest (20.8–32.5 m) (n = 20)	Diversisporaceae_339 *** (U) Rhizopogon_mohelnensis_216 ** (EcM) **Tuber_melanosporum_6 *** **(EcM)** Agaricales_269 ** (U) Pleosporales_67 * (U) Fungi_88 * (U) Fusarium_spp._15 ** (PP, WS, SS) X.0.22 * (U) Fusarium_sp._9 * (PP, WS, SS) Tetracladium_sp._20 * (US) Cryptococcus_aerius_2 * (AP) X.0.26 * (U) Zygomycota_145 * (U) Ilyonectria_spp._307 * (PP)	Ascomycota_74 ** (U) Agaricomycetes_129 *** (U) Glomus_sp._361 ** (A) Fungi.3 ** (U) Fungi_206 ** (U) Ascomycota_211 * (U) X_Chytridiomycota_377 * (U) Pyrenochaeta_sp._432 * (WS, US) Glomeromycota_188 * (U) Fungi * (U) Ascomycota_189 * (U) Entoloma_graphitipes_220 * (EcM) Agaricales_269 * (U) Claroideoglomus_sp._443 * (A) Glomeromycota_212 * (U) Ascomycota_392 * (U)								

(OTUs) based on species hypotheses concept (Kõljalg et al., 2013) using single linkage clustering with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Sequence data is archived at NCBI's Sequence Read Archive under accession number PRJNA309233 (www.ncbi.nlm.nih.gov/sra).

2.5. Taxonomic and functional identification

We assigned putative names to the 450 most abundant species hypotheses (SHs), which represented around 91% of the global number of reads. OTUs were annotated manually by selecting the most abundant sequence from each SH for taxonomical identification, using massBLASTer in PlutoF from UNITE (Abarenkov et al., 2010) and International Nucleotide Sequence Database consortium (INSDc) databases. Taxonomic identities were assigned to species level based on a $>$ 98.5% similarity, to a genus level based on a $>$ 97% similarity, to a family level based on a $>$ 95% similarity, to an order level based on a $>$ 92% similarity, to a class order based on a $>$ 90% similarity and to a phylum level based on a $>$ 80% similarity. Plant OTUs represented 3.3% of the first 450 OTUs, and were discarded from the dataset. Functional guilds of SHs were assigned using as FUNGuild (Nguyen et al., 2016), and grouped in ten categories: ectomycorrhizal (EcM), arbuscular mycorrhizal, plant pathogens, endophytes, mycoparasites, soil saprotrophs, wood saprotrophs, dung saprotrophs, undefined saprotrophs and unknown species.

2.6. Statistical analyses

Prior to analysis, relative abundance of each OTU was standardized with a Hellinger transformation. We also performed the analyses over non-transformed and binary community data, and the same results were obtained as with the Hellinger transformation. Variables affecting community composition were screened by a PERMANOVA analysis including distance to the tree (two levels: 40 and 80 cm), distance to the forest and root collar diameter with the adonis2 function in the Vegan package (v. 2.5–5) for R (v. 3.6) software. Interactions were investigated by running separate analysis for each of the levels of the factors. In order to visualize the data, and to run indicator species analysis, trees were arbitrarily sorted using the "cut_number" command into three or two different classes with equal number of samples per category. For distance to the forest, the classes were (Closest to the forest: 5.14–12.2 m; Medium distance to the forest: 12.2 m–20.8 m and Farthest from the forest: 20.8–32.5 m), and for diameter two classes (Small root collar diameter: 5.55–10.4 mm; Large root collar diameter: 10.4–24.2 mm) and for mycelium two classes (Low quantity of mycelium: 0.0–0.0032 mg·g^{-1} of soil; Large quantity of mycelium: 0.0032–2.41 mg·g^{-1} of soil). We also used 'vegdist' function to calculate Bray-Curtis dissimilarities of the community matrices and tested for homogeneity of multivariate dispersion 'betadisper' (i.e. multivariate dispersion or beta diversity) using 'permutest' function, in order to test whether data dispersion was different between the tree classes of "distance to the forest". In addition, we tested whether the centroids of the three distance classes were different from each other by passively fitting the "distance to the forest" in the ordination space (using envfit function), using the first two axis scores. Visualization of community composition was done with a principal coordinate analysis (PCoA) based on Bray distances amongst samples. Indicator species analysis was performed using "indicespecies" package (De Caceres and Legendre, 2009), and were run separately for the community at 40 cm and the community at 80 cm from the tree. A separate analysis was performed for analysing the effects of *T. melanosporum* abundance measured by qPCR on the fungal community. Other correlative analyses were done by linear regression in JMP Pro version 14.1.0 for Windows 10.

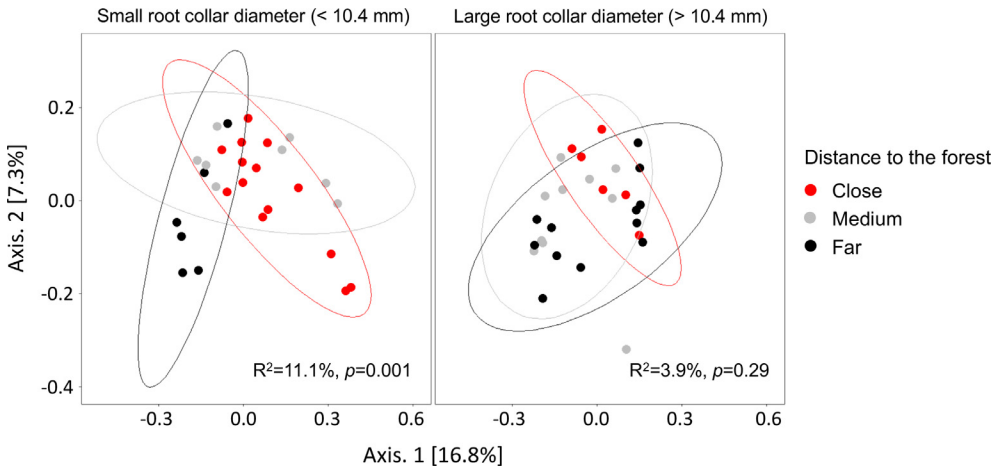

Fig. 4. Soil fungal community shifts associated with the tree root collar diameter and distance to the forest. PCoA ordination calculated with a bray-distance matrix with three levels for the distance to the forest (Closest to the forest: 5.14–12.2 m; Medium distance to the forest:: 12.2 m–20.8 m; Farthest from the forest:: 20.8–32.5 m) and two levels for the root collar diameter (Small root collar diameter: 5.55–10.4 mm; Large root collar diameter:10.4–24.2 mm). R^2 shown and p-value shown are extracted from a PERMANOVA analysis in which distance to the forest and the root collar diameter are analyzed as a continuous variable.

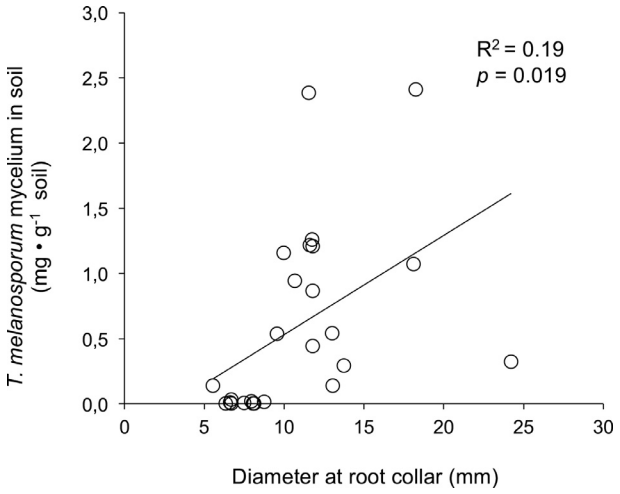

Fig. 5. *Tuber melanosporum* DNA quantity in soil at 40 cm from the stem in relation with the diameter of trees at the root collar.

3. Results

Five years after establishment, average tree height was 74.6 cm (ranging from 28 to 120) with an average root collar diameter of 10.7 mm (ranging from 5.55 to 24.20). No association between root collar diameter or plant height with the distance to the forest was found ($p = 0.21$ and $p = 0.98$, respectively). As expected for the early age of the plantation, *T. melanosporum* mycelium was mainly concentrated in the vicinity of the stem and was seldom detected at 80 cm (on average 0.601 mg·g^{-1} at 40 cm vs. 0.016 mg·g^{-1} soil at 80 cm, $p < 0.0001$). Fungal community DNA was successfully amplified from all 56 samples (28 trees × 2 distances). For the analysis, 37,840 reads after quality control were used. For each tree-distance combination, we obtained an average of 675 reads per sample after restricting our dataset to the 450 most abundant OTUs and taking out plant OTUs.

Soil fungal community was different depending on the distance to the forest (Table 1). Trees planted closer to the forest had different communities from those located further away close to the middle of the plantation (Fig. 2). Distance to the forest affected fungal guilds differently. At 40 cm from the stem, where the fungal communities were those belonging to the host tree, the non-*T. melanosporum* EcM fungi were found to increase with the proximity to the surrounding forests (Fig. 3a; Table 2), but no such relationship was found when analysing the communities at 80 cm from the stem, where the fungal communities were those established in the pasture and not the planted trees (Fig. 3b; Table 2). Other guilds showed either the contrary association or no association with distance to the forest e.g. plant pathogens increased with the distance from the forest ($R^2 = 0.22$, $p = 0.011$) (Table 2). The same pattern observed at guild level for pathogens was supported by indicator species analysis, as the OTUs Fusarium_sp_15, Fusarium_sp_9 and Ilyonectria_sp_307 classified by FUNGUILD as pathogens, were associated to trees located far from the forest (Table 3). *T. melanosporum* mycelium measured by qPCR appeared to be unrelated to the distance to the forest ($p = 0.44$), but indicator species analysis showed that *T. melanosporum* was negatively associated with those trees closer to the forest (at 40 cm from the stem) (Table 3).

No association with the abundance of any guild and the distance to the forest was found at 80 cm from the tree. Nevertheless, indicator species analysis showed that at 80 cm from the stem (i.e. pasture community), five out of sixteen OTUs associated with the furthest distance to the forest and two out of two OTUs with the medium distance to the forest corresponded to endomycorrhizal OTUs (considering both Glomeromycota_188 and Glomeromycota_212 also as endomycorrhiza) (Table 3).

Soil fungal community was also different depending on the root collar diameter (Table 1). The effect of the root collar diameter on the fungal composition varied with the distance to the forest (Significant interaction collar diameter × distance to the forest, Table 1). The interaction between root collar diameter and distance revealed that while distance had an influence on the community surrounding small trees, there were no differences for the larger root collar diameter trees (Fig. 4). Root collar diameter was associated with the abundance of some guilds (Table 2). At 40 cm, endophytes were more abundant under larger root collar diameter trees ($R^2 = 0.15$, $p = 0.03$). At 80 cm, plant pathogens ($R^2 = 0.15$, $p = 0.03$) and dung saprotrophs ($R^2 = 0.18$, $p = 0.02$) were more abundant under larger root collar diameter trees (Table 2). At 40 cm, indicator species showed that *T. melanosporum* tended to be more abundant in the soil community from trees with larger root collar diameters ($p = 0.004$). In line with this, the quantity of *T. melanosporum* mycelium at 40 cm increased with the root collar diameter of the tree ($R^2 = 0.19$, $p = 0.01$) (Fig. 5). We did not find differences in beta diversity (i.e. dispersion) between the three distances to the forest (i.e. data dispersion was the same between trees located at short, medium and far distance to forest, F = 1.80, P = 0.166). However, there were marginal differences in beta diversity across between the three distance classes when considering small trees (F = 3.9, P = 0.042), but not for big diameter trees (F = 1.04, P = 0.411). We also confirmed differences in the community centroids between the three distance classes in small trees (P = 0.007) but not in big trees (P = 0.384). Thus, observed differences in fungal communities of small diameter trees were mainly due to differences in centroid dispersion across the three distance classes, but slightly also due to dispersion of the datapoints within treatments.

Besides the distance to the forest, there were compositional differences in soil fungal communities between the samples taken at 40 cm and at 80 cm from the trees (Table 1). At 40 cm, we found that the amount of *T. melanosporum* mycelium affected community composition ($R^2 = 0.05$, $p = 0.046$). Indicator species showed that *T. melanosporum* was the species with the strongest prevalence at 40 cm. No association between composition and the amount of *T. melanosporum* mycelium was found at 80 cm ($p = 0.22$). At 40 cm, soil samples with a higher content of *T. melanosporum* mycelium were those with a smaller relative abundance of arbuscular mycorrhizal fungi ($p = 0.03$) and with a higher abundance of plant pathogens ($p = 0.02$). In fact, two plant pathogens, Fusarium_sp_15 and Fusarium_sp_9 together with *T. melanosporum* appeared as indicator species where the amount of *T. melanosporum* mycelium exceed 0.0032 $mg{\cdot}g^{-1}$ soil (Table 3). No association was found between *T. melanosporum* mycelium and the amount of non-*T. melanosporum* EcM fungi at 40 cm ($p = 0.90$) or at 80 cm from stem ($p = 0.64$).

Mating type frequency showed a random distribution across the plantation. Mating type detection was only successful on samples with > 0.034 $mg{\cdot}g^{-1}$ of *T. melanosporum* in the soil (18 out 28 trees). There was a tendency for MAT 1–1–1 to dominate (83% vs 39%), but the difference was not significant. In 72% of the trees, only one of the mating types was present. The two mating types were only detected under 5 trees. The presence of both mating types correlated with a higher root collar diameter of the tree ($R^2 = 0.41$, $p = 0.02$), but no association was found for presence of MAT 1–1–1 or MAT 1–2-1 individually. Amongst trees with data on mating types, we did not find any association between *T. melanosporum* mycelium abundance or distance to the forest and the presence of either or both mating types.

4. Discussion

Truffle plantations are a profitable investment, which may be used by forest owners to diversify and increase the revenues of their land (Bonet et al., 2009). However, establishing plantations in forested areas has been traditionally discouraged (Sourzat et al., 2010). The main argument has been that EcM fungi from nearby forest patches may outcompete *T. melanosporum* in occupying the root systems, risking truffle production. Garcia-Barreda et al (2015) suggested the use of soil treatments (Microwaves, quicklime and acetic acid) before planting to reduce the early colonisation by other native EcM fungal competitors in the truffle plantations. However, Domínguez et al (2006) showed that the presence of nursery-associated fungi (e.g. *Laccaria laccata* or *Sphaerosporella brunnea*) or indigenous fungi (e.g. *Tuber brumale*) did not affect colonisation of *T. melanosporum* during the first 2 years after afforestation. Theory predicts that since *T. melanosporum* is introduced first, it would have advantage over putative competitors coming from the forest (Reyna, 2012), notwithstanding the fact that other EcM fungi may colonize the seedlings. By studying inoculated trees planted at different distances from the forest, we observed that the soil fungal community under trees planted closer to the forest edge was different from the community under trees located in the centre of the plantation. We saw that the putative influence of the distance to the forest edge on soil fungal community was smallest in the case of larger root collar diameter trees. Those were also the trees bearing the highest amount of *T. melanosporum* mycelium. Small trees seemed to be less able to sustain *T. melanosporum* and were also more likely to take on the native community in the soil. Despite the sharp difference between the fungal communities around the forest edge and in the centre of the plantation, *T. melanosporum* was able to maintain its establishment throughout the plantation (no correlation between abundance of *T. melanosporum* mycelium and distance to the forest was found).

In this study, we obtained 37,840 high-quality read numbers, which is in the range of what is expected from PacBio RSII platform. Our results indicate an effect of the forest on the fungal community associated with the roots of the planted oaks. However, because our results are based on soil DNA, it could be that the decline of EcM at furthest distances of the forest is only a result of an inoculum arrival gradient. The fact that (i) EcM fungi other-than-*T. melanosporum* was the only guild showing a negative correlation with distance to the forest, and that (ii) this correlation appeared at 40 cm from the stem, where roots were likely to be more abundant, and not at 80 cm, seems to support that surrounding forests have affected the root associated fungal community of the planted trees. Other limitations must be acknowledged if we aim to generalise our results to all truffle producing areas. First of all, the study is established under good conditions for truffle production in terms of climate and soil without irrigation, thus extrapolation to areas were irrigation is used to relieve summer drought must be used with caution. Studies have shown that high doses of irrigation do not affect the proliferation of other EcM fungi in young plantations (Bonet et al., 2006; Olivera et al., 2011, 2014a), but these results may differ with a similar irrigation regime for a black truffle plantation surrounded by native forest. Another limitation of our study, is that it focussed on the first years of the plantation and may be difficult to extrapolate the effects of EcM competitors on future truffle production. Belfiori et al. (2012) have shown that in productive mature plantations there is a negative correlation between EcM species and the abundance of *T. melanosporum*. However, whether other EcM are the cause of the lower *T. melanosporum* colonization remains unclear. The fact that we see small effects of the distance to the forest on the *T. melanosporum* mycelium abundance, but we already see a large mycelium variation across trees of the plantation suggesting that individual tree-level effects may be more important in the long-run than other factors such as distance to the forest.

We found an unexpected association between *T. melanosporum* and the presence of plant pathogens. This same association was found in another preliminary study when comparing young and old plantations (Liu et al., 2016). One explanation for this association may be linked to the formation of the burn. We know that *T. melanosporum* inhibits the development of herbaceous plants in the immediate surroundings of the trees (Splivallo et al., 2007, 2011), so it could be that pathogens proliferate in those weakened plants. The fact that the trees with higher amounts of *T. melanosporum* mycelium and higher presence of plant pathogens are also the largest at the root collar diameter, seems to discard any putative negative effects for the development of the plantation.

Tree growth seems to be a key player in the establishment of *T. melanosporum*. Our results confirm previous findings (Suz et al., 2008), showing that a higher mycelium abundance is found under the largest root collar diameter trees (Fig. 5). The underlying mechanisms by which *T. melanosporum* develops better under big trees are speculative. One possibility, is that trees that cope better with the planting stress and grow more are able to maintain most of the initial root systems colonized by *T. melanosporum*. Growth may also result in a higher host carbon supply to *T. melanosporum* (Le Tacon et al., 2013). A second possibility is that *T. melanosporum* colonization is advantageous to the oaks for the acquisition of water and to reduce competition from herbaceous plants. Further research should investigate the mechanisms behind the interplay between growth and *T. melanosporum* colonization.

Establishing truffle plantations near a forest seems to have little effect on mating type frequency. The relative abundance and distribution of both mating types has been suggested to play a role on the frequency of formation of fruiting bodies (Rubini et al., 2014). In our study area, a single mating type was detected under the majority of trees (72%). Mating type distribution was unrelated to the distance to the forest. The presence of a single mating type is common in young plantations, as found by Linde and Selmes (2012) who showed that 57.1% of the unproductive trees and 50% of the productive trees had one mating type present as mycorrhiza.

5. Conclusions

The results of our study have implications for forest management. Converting abandoned pastures or old agricultural fields into truffle plantations may be a strategy to recover the landscape mosaics characteristic of Mediterranean forests, and for reducing the risk of forest fires (Aquilué, 2019). When considering a truffle plantation in a forest setting, our results indicate that even though the forest vicinity will influence the soil fungal community, this may have a limited effect on the growth of truffle mycelium of a 5-year old holm oak (*Q. ilex*). Our results also highlight the role of tree growth on the appearance of other EcM fungi in the root system of the inoculated trees. Higher *T. melanosporum* mycelium abundance under the largest root collar diameter trees suggests that we improve the best manage practices to promote plant growth. However, further research should investigate the mechanisms behind the interplay between growth and *T. melanosporum* colonization. Concluding, our results indicate that *T. melanosporum* inoculated holm oaks planted in areas surrounded by forests may be colonised by other EcM species, but this has a limited effect on the growth of truffle mycelium. Further investigations should be carried out to determine whether a different fungal community may affect *T. melanosporum* development at 10 years after establishment and the production of truffle sporocarps in the future, but to date, truffle mycelium does not seem to be impaired.

CRediT authorship contribution statement

Daniel Oliach: Writing - original draft. **Carlos Colinas:** Conceptualization, Funding acquisition, Writing - review & editing. **Carles Castaño:** Conceptualization, Methodology, Software. **Christine R. Fischer:** Resources. **Francesc Bolaño:** Resources. **José Antonio Bonet:** Conceptualization, Funding acquisition. **Jonàs Oliva:** Supervision, Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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