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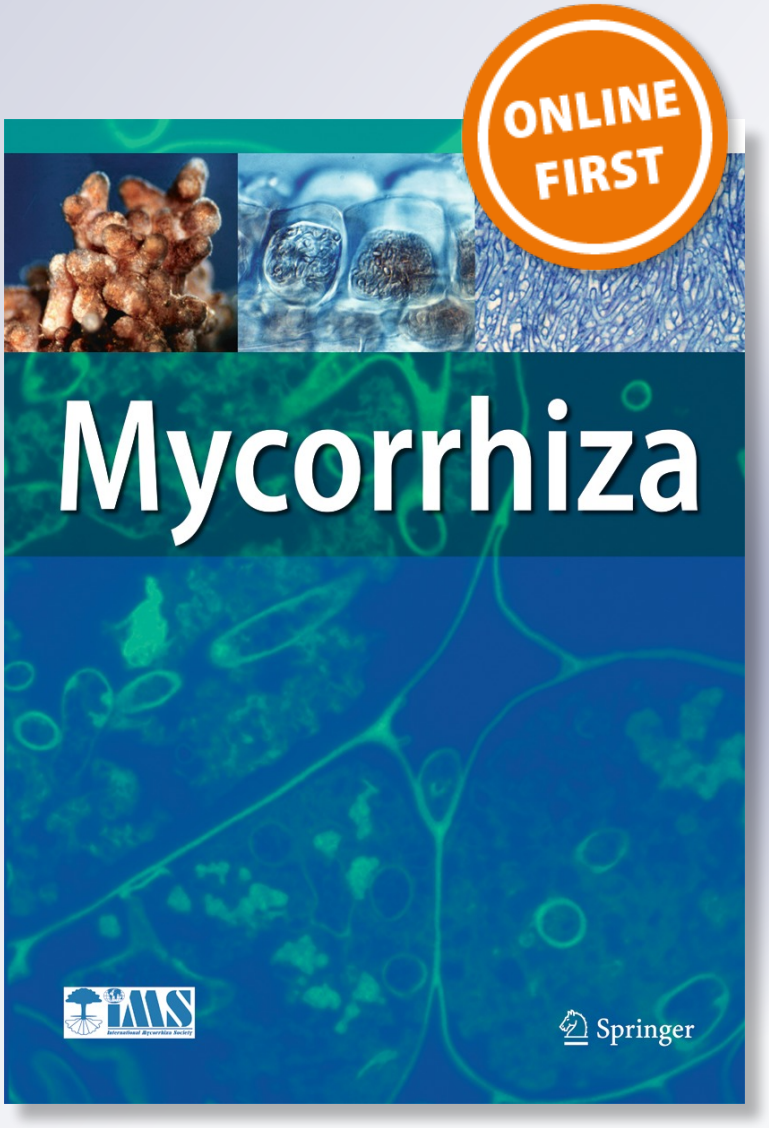

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ORIGINAL PAPER

Pattern of *Tuber melanosporum* extramatrical mycelium expansion over a 20-year chronosequence in *Quercus ilex*-truffle orchards

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Abstract Successful cultivation of black truffle (*Tuber melanosporum*) requires a long-term investment and the maintenance of the symbiosis throughout its preproductive and productive years. Monitoring the symbiosis over time is challenging, as it requires methods that can detect the belowground proliferation of the fungus associated with its host tree. In this study, we used a chronosequence design to study the expansion pattern of this fungus as the host tree grows. We hypothesize that this expansion can be estimated by monitoring *T. melanosporum* DNA from soil beneath host trees of different ages (3, 5, 7, 10, 14, and 20 years old) and at different distances from the trunk of the trees (40, 100, and 200 cm). We also wished to evaluate the presences of *Tuber brumale* and *Tuber indicum*, potentially problematic truffle species, in these plantations. To detect the mycelium of *T. melanosporum* in these soils, we extracted DNA and performed polymerase chain reaction (PCR) with *Tuber* species-specific primers, and to estimate DNA amount, we measured relative band intensities from the amplicons in agarose gels. Both age and distance were related to *T. melanosporum* DNA quantity, which was more abundant in the oldest age classes, reaching a plateau in 5–7 years. At 40 cm from the tree, there were no differences in *T. melanosporum* DNA amounts in orchards of different ages, but at 100 and 200 cm, younger orchards had less *T. melanosporum* DNA. We did not detect DNA from *T. brumale* or *T. indicum* in any of our samples.

Keywords *Tuber melanosporum* · Ecology · Soil DNA analysis · Chronosequence · Black truffle cultivation

B. Liu · J. A. Bonet · A. Olivera · A. Inchusta · C. Colinas (✉)
Universitat de Lleida/Agrotecnio Center, Av. Alcalde Rovira Roure, 177, 25198 Lleida, Spain
e-mail: carlos.colinas@pvcf.udl.cat

C. Fischer · J. A. Bonet · C. Colinas
Centre Tecnològic Forestal de Catalunya, Ctra. de St. Llorenç de Morunys, km2, 25280 Solsona, Spain

Present Address:
B. Liu
Shenzhen University, Nanhai Ave 3688, 518060 Shenzhen, Guangdong, People's Republic of China

Introduction

The black truffle is the fruit body of *Tuber melanosporum* Vittad., an ascomycete, and ectomycorrhizal hypogeous fungi. Due to the dramatic decline in its wild production over the last century (Callot 1999), orchard cultivation of this economically important fungus has been initiated throughout its natural habitat in France, Spain, and Italy and introduced in new regions around the globe with climatic and soil conditions compatible with the cultivation of this species. Like thousands of other mycorrhizal fungi, *T. melanosporum* has evolved a mutualistic relationship with plants and developed genetic traits that are complementary to those of its hosts (Plett and Martin 2011). Based on observations from natural black truffle sites, the ecological requirements for *T. melanosporum* growth and fructification have been widely documented from France (Delmas and Poitou 1974; Sourzat 1997), Italy (Bencivenga et al. 1990; Raglione et al 2001), and Spain (Reyna 2000; Garcia-Montero et al 2001) during the last 40 years, emphasizing the importance of well-drained, calcareous soils and the adequate timing and quantities of rainfall. The most important abiotic parameters associated with black truffle habitat including soil physical and chemical properties, climate, geography, and geology have been compiled to comprehensively evaluate the suitability of different sites for the cultivation of *T. melanosporum* (Colinas et al. 2007).

With the cloning of the *T. melanosporum* genome (Martin et al. 2010) and the confirmation of this fungus as a heterothallic, outcrossing species (Murat and Martin 2008),

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researchers have access to tremendous amounts of information on the numerous genes involved in the metabolic processes of the different stages in the life cycle (Montanini et al. 2011), as well as the mating genes required for sexual compatibility and reproduction (Rubini et al. 2011a, b), all of which have implications for cultivation. The aromatic compounds produced by *T. melanosporum* have long been recognized as attractions for animals (Claus et al. 1981) and critical for spore dissemination (Maser et al. 2008), but more recently, the importance of these volatile compounds as chemical signals between this fungus and other soil organisms and plant roots has highlighted the ecological role of truffle volatiles (Splivallo et al. 2011). Although many questions remain, we have a much more complex view of the belowground biology of this fungus (Kües and Martin 2011) than when successfully inoculated truffle seedlings first became commercially available for truffle cultivation (Chevalier and Grente 1979).

Successful fruiting of this fungus requires a long-term relationship with the host tree, with ongoing proliferation of the other fungal structures involved in the symbiosis—the ectomycorrhizae and the hyphae forming the extramatrical mycelium, whose physiologic processes, interactions with other organisms, quantification, and distribution are only recently amenable to investigation. With the development of molecular techniques during the last 15 years, polymerase chain reaction (PCR) primers specific for *Tuber* sp. have been designed (Suz et al. 2006; Paolocci et al. 1999; Bonito 2009) providing tools for the detection and distribution of DNA from truffle spores, ectomycorrhizae, and extramatrical hyphae from soils. The development of DNA quantification methods (Suz et al. 2008) and application of real-time PCR techniques (Hortal et al. 2008; Parladé et al. 2013) have further advanced the capacity to examine the belowground expansion and biomass of the extramatrical hyphae.

Because the establishment of black truffle orchards does not always result in truffle production, different approaches for evaluating the development of the symbiosis from the establishment phase to the fruiting phase are of economic interest to truffle growers and to researchers concerned with belowground activity of fungi over time. These approaches include morphologic and molecular studies of *T. melanosporum* ectomycorrhizas in truffle sites (Bonet et al. 2006; Olivera et al. 2011; Belfiori et al. 2012), the detection of extramatrical mycelium in the soil profile beneath truffle-bearing trees (Suz et al. 2006), the quantification of *T. melanosporum* DNA found in soils from truffle-productive and nonproductive trees (Suz et al. 2008; Zampieri et al. 2012), and a comparison of *T. melanosporum* mycelium quantities in natural truffle forests vs. established orchards (Parladé et al. 2013).

T. melanosporum mycelium exerts a phytotoxic activity (Bonfante et al. 1971) by which the majority of herbaceous plants surrounding a tree colonized by this fungus are eliminated. This results in a visual display known as the "burn" or "brûlé" and is recognized as a positive indication that the fungus is active in the soil, although *Tuber aestivum* and *Tuber indicum* can also be responsible for this phytotoxic activity (Streiblová et al. 2012). The burn can be observed to spread radially from the base of the tree, expanding outward for several meters during the first 5–10 years following establishment, and this has been historically the parameter that helped growers gauge the progress of their orchards. Thus, studies that have focused on the ectomycorrhizas and the extramatrical mycelium within the burn help explain some of the belowground activity of *T. melanosporum*. García-Montero et al. (2007a, b) observed that burn size is significantly correlated with truffle production in both *Quercus* and *Cistus* forests although it is not the only factor. Napoli et al. (2010) found a decrease in ectomycorrhiza diversity in the soil fungal community within truffle burns compared to outside the burn. Suz et al. (2008) observed greater quantities of *T. melanosporum* DNA in soils beneath trees with developed burns than those lacking a burn in established truffle orchards. Burn extension is a good indicator of the belowground mycelial expansion, but *T. melanosporum* DNA can also be detected beyond the visual boundaries of the burn (Suz et al. 2006).

Although the differences in presence and amount of truffle mycelium in the soil have illustrated its significance for truffle production, we know that fruit body formation requires the presence of both mating-type genes (Rubini et al. 2011a) and that the distribution of these genes within a truffle orchard appears to be nonrandom, with one type becoming dominant over time (Rubini et al. 2011b; Murat et al. 2013). However, Linde and Selmes (2012) observed that the presence of both mating types in the root system of a single host tree does not ensure fruiting.

The present study applies a chronosequence design to examine the evolution over time and space of the distribution of *T. melanosporum* mycelium in the soil in the region of Teruel, Spain, where truffle cultivation has been increasingly successful during the last 20 years. The objective is to observe the pattern of mycelial growth below *T. melanosporum*-colonized trees from very young orchards (3 years—and lacking visible burns) to trees in fully productive orchards (20 years). Field studies to track *Lactarius deliciosus* mycelium (Hortal et al. 2008, 2009) indicate that quantities can vary significantly with site. By measuring *T. melanosporum* DNA quantities at different distances from the host tree in an array of truffle orchards with a wide spread of age classes in a similar habitat, we have a unique opportunity to observe a broader perspective of patterns of mycelium growth than studies based on fewer sites.

But *T. melanosporum* is not the only *Tuber* species relevant to the truffle industry. *Tuber brumale* Vittad., another winter black truffle native to the calcareous regions of Spain but of lesser economic value, has also been found fruiting in

orchards. The role of *T. brumale* mycelium in orchards is not well understood, and the possibility that it could have a competitive advantage and displace *T. melanosporum* in established orchards is a concern for growers, as also is the risk of introduction and expansion of *T. indicum* Cooke & Massee, the Asian black truffle, in European truffle orchards (Murat et al. 2008).

In this study, we are interested in observing the pattern of expansion of the black truffle mycelium during the host tree growth. We hypothesize that this expansion can be observed by monitoring the quantity of *T. melanosporum* DNA from soil beneath host trees of different ages. Our objective is to study the relationship between the quantity of *T. melanosporum* DNA in the soil and the age of the orchards and the distance from the host tree. Further objectives were to detect the possible concomitant colonization of orchards by *T. brumale* and to detect possible introductions of *T. indicum.*

Materials and methods

Study plots

This study was performed in 18 truffle orchards in the province of Teruel, in the east central region of Spain. We have selected a chronosequence of plantations 3, 5, 7, 10, 14, and 20 years old and analyzed three orchards for each age class. All orchards are located in a high open valley between the ranges of Gudar and Javalambre, on fairly homogeneous calcareous soils developed on Tertiary sediments. Before being planted, they had supported cereal crops for decades. The latitudes of the plots range from N 40° 20′ to N 40° 2′ and the longitudes from W 1° 10′ to W 0° 41′ whereas their altitude varies from 843 to 1,124 m a.s.l. The climate is continental Mediterranean with 300–500 mm of annual precipitation concentrated in spring and autumn. Temperatures range from a monthly average of daily minimum temperatures of −2 °C in January to a monthly average of maximum daily temperatures of 30 °C in July. All orchards had Holm oak (*Quercus ilex* L.) as host and had been under similar management.

The orchards were selected randomly through interviews with owners. Any further differences among the orchards other than age were considered part of the error term. This area is well known for both natural and cultivated truffle production with climatic, soil, and geographical parameters conducive to black truffle cultivation (Bonet et al. 2009).

Our experimental unit was the orchard; thus, we selected one tree in each orchard for sampling. In order to avoid unnecessary noise, we excluded from our sampling universe the trees that were clearly different from the most abundant trees in the following two criteria: (a) presence/absence of burn in young age classes and (b) productive/nonproductive in mature age classes. In the 10-, 14-, and 20-year-old classes, most trees were producing, so we randomly selected one productive tree. In the 5- and 7-year-old classes, most trees were not producing but had burns, so we randomly selected one nonproducing tree with a well-developed burn. And, in the 3-year-old class, most trees did not have burns, so we selected one tree without a burn. In each tree, we collected soil samples at three distances (40, 100, and 200 cm) from the trunk.

Soil sampling

For each tree at each of the three distances, we collected three soil subsamples from points located at the vertices of a randomly oriented equilateral triangle in May 2009. Each subsample consisted of a soil core we obtained with a drillable cylinder 25 cm deep and 7 cm in diameter after removing the top 5-cm surface layer of soil that contained leaves and organic debris. We mixed the three subsamples thoroughly to create a single composite soil sample per distance per tree for a total of 54 soil samples. The samples were sieved through a 4-mm mesh to remove stones, roots, and debris, placed on ice, taken to the laboratory, and stored at −20 °C in a sealed plastic bag prior to DNA extraction.

We considered the risk of collecting soil samples with spores that could confound the estimation of mycelium by DNA extraction in the productive orchards. We concluded that since the orchards are fenced, we could not expect spore inputs from big animals, and since mature sporocarps are harvested intensively, we could not expect local small mammals to feed on them and disperse spores. We assumed that if we detected spores, they should have been produced on site, and that would mean that there was mycelium able to produce them, so we would still get the information we were interested in.

DNA extraction and PCR amplification

Fruit bodies of *T. melanosporum*, *T. brumale*, and *T. indicum* were purchased and identified to use as positive controls to detect the presence of mycelium of these *Tuber* species in orchard soils. DNA extractions from sporocarps were carried out using the E.Z.N.A Fungal DNA Miniprep Kit (Omega Bio-Tek, Doraville, GA) following the manufacturer's instructions.

Total soil genomic DNA was extracted from 250 mg of sieved soil samples (fresh weight) using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The extracted DNA was measured spectrophotometrically (NanoDrop 1000, Thermo Fisher Scientific Inc., Waltham, MA, USA) and evaluated for the proportions of proteins and organic compounds based on $OD_{260/230\ nm}$ and $OD_{260/280\ nm}$ ratios.

We first checked for the presence of *T. melanosporum*, *T. brumale*, and *T. indicum* mycelium in all soils prior to quantification. Extracted DNA templates from identified fruit bodies of these three black truffle species and soils of three replicates of six age classes corresponding to three distances were amplified using the species-specific primers ITSB, ITSML, ITSCHCH, and ITS4LNG in multiplex PCR (Paolocci et al. 1999). The amplifications of the rDNA from the ITS region from sporocarp and soil extractions were obtained by PCR containing 1 μl (from a 10 mM stock) of each primer (ITSB, ITSML, ITSCHCH, and ITS4LNG), 1 μl of DNA template containing 10–20 ng of DNA, an Illustra PuReTaq Ready-To-Go PCR bead (GE Healthcare UK Limited), and water to a final volume of 25 μl. PCRs were performed in Biometra T1 Thermocycler (Biometra, Goettingen, Germany) following the cycling conditions performed by Suz et al. (2006). Amplification products were checked by electrophoresis in 1 % agarose gels in 1× TBE buffer (1× TBE=0.09 M Tris–borate, 0.002 M EDTA pH 8) and visualized on a UV-transilluminator after ethidium bromide staining.

Comparison of *T. melanosporum* DNA amounts

For each of the soil extractions, the amplifications were carried out by using *T. melanosporum*-specific primers ITSML and ITS4LNG with the same PCR conditions as described above. To estimate *T. melanosporum* DNA quantity in our soil samples, the relative band intensities obtained on the electrophoresis gel from each soil sample extract were compared with bands that corresponded to standardized quantities of *T. melanosporum* DNA on that same gel (Suz et al. 2006). The standard was prepared using a twofold water dilution series of DNA extractions from fruit bodies of *T. melanosporum* which contained from 600 to 0.058 ng of *T. melanosporum* DNA: 600, 300, 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.937, 0.468, 0.234, 0.117, and 0.058 ng (Fig. 1).

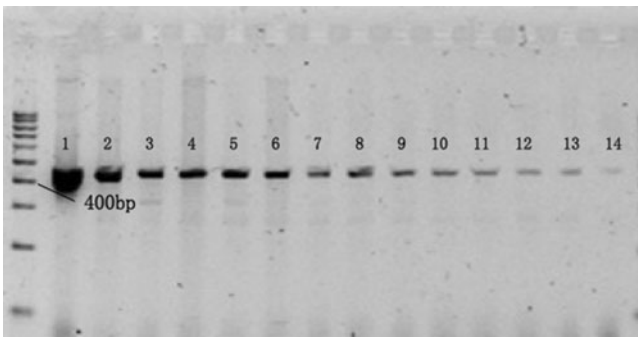

Fig. 1 The standard prepared using a water dilution series of DNA extractions from fruit bodies of *T. melanosporum* which contained from 600 to 0.058 ng of *T. melanosporum* DNA: *1* 600 ng, *2* 300 ng, *3* 120 ng, *4* 60 ng, *5* 30 ng, *6* 15 ng, *7* 7.5 ng, *8* 3.75 ng, *9* 1.875 ng, *10* 0.937 ng, *11* 0.468 ng, *12* 0.234 ng, *13* 0.117 ng, and *14* 0.058 ng

The PCR products were run on 2 % agarose gels together with ten dilutions of the standard that matched the range of our soil samples. We estimated the amount of DNA in the soil samples by comparing their band size and density with those of the standard using the software Gel Doc 2000-QuantityOne (Bio-Rad Laboratories, Hercules, CA, USA). To reduce variability, two subsamples of each soil sample were analyzed (DNA was extracted and quantified) and their results averaged to obtain that sample's datum.

Statistical analysis

We analyzed the data by means of an ANOVA with age as factor for each of the three distances and another one for the mean of the three distances. We used Fisher's protected LSD for multiple comparisons and performed all the calculations with Data Desk (Data Description Inc., Ithaca, NY, US) software. Transformations were used when needed, and the estimates were back transformed to the original scale so they should be considered medians (Ramsey and Schafer 1997).

Results

We did not detect the presence of *T. brumale* or *T. indicum* in any of the soil samples from the research plots. Results from the multiplex PCR yielded expected bands for the three known sporocarps of *T. melanosporum* at 440 bp, *T. brumale* near 700 bp, and *T. indicum* near 140 bp. From the soil samples, only bands corresponding to *T. melanosporum* were observed.

When the average *T. melanosporum* DNA amount for the three distances within each age class was considered, a significant relationship between the age of the orchards and the amount of *T. melanosporum* DNA estimated in the soil samples was evident (Fig. 2). *T. melanosporum* DNA was more

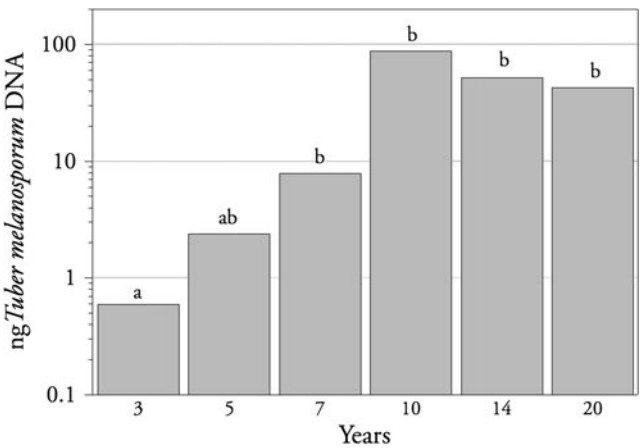

Fig. 2 Estimates of the quantity of *T. melanosporum* DNA (nanograms of DNA per microliter of soil extract, logarithmic scale) observed with increasing age in black truffle orchards. *Bars* with the same *letter* are not significantly different at α=0.05)

abundant in the oldest age classes reaching a plateau in 5–7 years.

However, when we repeated the same analysis for each distance, the results varied. At 40 cm from the tree, there were no significant differences among ages. At 100 and 200 cm, some differences were significant. At 100 cm, 3-year-old orchards had less *T. melanosporum* DNA than those 7-years old or older, and at 200 cm, both 3 and 5-year old orchards had less *T. melanosporum* DNA than the orchards 10 years old and older (Fig. 3).

Discussion

Quantification of mycelia of ectomycorrhizal fungi has been a subject of study because it represents an important fraction of carbon biomass in forests (Högberg and Högberg 2002) and reflects the investment by, or cost to, the photosynthesizing partner (the host tree) of the nonphotosynthesizing partner (the mycorrhizal fungus). This is a dynamic process, related to seasonal changes (Wallander et al. 1997) and nutrient availability (Nilsson et al. 2005). Field estimations of total ectomycorrhizal mycelium in forest soils using in-growth mesh bags and measuring fungal biomarkers (Wallander et al. 2001) demonstrate that ectomycorrhizal mycelial biomass is correlated with periods of maximum fine-root growth.

Laboratory methods to quantify this structure prior to molecular tools included measurements of mycelium by determining levels of chitin or ergosterol (Ekblad and Näsholm 1996; Martin et al. 1990). Colpaert et al. (1992), by oxidizing the mycelium harvested from semi-axenic growth chambers, measured fungal biomass after 6 months and observed different growth patterns among six different strains of ectomycorrhizal fungi. Studies of the structural features of hundreds of individual ectomycorrhizae and their external

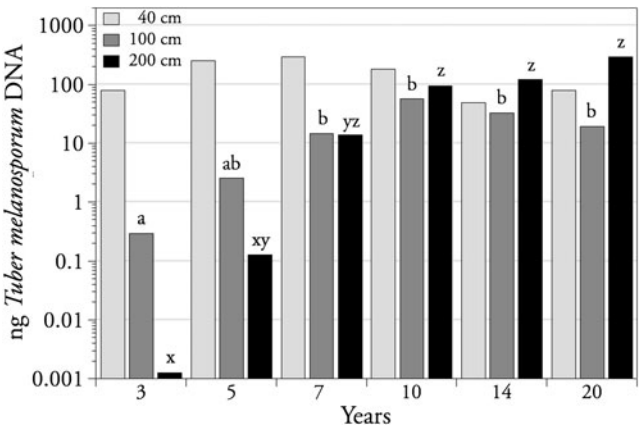

Fig. 3 Estimates of the quantity of *T. melanosporum* DNA (nanograms of DNA per microliter of soil extract, logarithmic scale) observed with increasing age in black truffle orchards at 40, 100, and 200 cm from the tree. *Bars* with the same *letter* within each distance are not significantly different at α=0.05)

hyphae have shown that they display a wide variability in density, organization, and biomass (Agerer and Raidl 2004). Species-specific molecular primers offer the possibility of quantifying the mycelial biomass of an individual ectomycorrhizal fungal species in laboratory cultures/chambers and in soil samples from nature. Parladé et al. (2007) used real-time PCR to quantify *L. deliciosus* mycelia from potted soils demonstrating that the sampling time and depth were influential factors. Suz et al. (2008), using *T. melanosporum*-specific primers, compared conventional PCR with real-time PCR and found that both methods provided similar results in estimating mycelial abundance in truffle productivity field studies.

In the present study, we observed a pattern of mycelial expansion characterized by maximum colonization of the first 40 cm around the host tree even before the burn was visually apparent. In relative quantity, *T. melanosporum* DNA measured near the stem of young trees was not different from that beneath fully mature and productive trees. The quantity of black truffle DNA seems to stabilize, even as mycelia expand in area, as the trees mature suggesting that there is some limitation to mycelia growth. Surprisingly, this occurred quite early in our orchards. At 40 cm from the tree, the maximum quantity of DNA was measured by the third year (Fig. 3). At 100 cm, this maximum is reached by the 5th or 7th year, and by the 7th or 10th year up to 20 years, the soil at 200 cm from the tree was fully colonized. Suz et al. (2008) observed a similar process with equilibrium of mycelia quantities, burn extension, and tree size in soils beneath productive and non-productive trees in established orchards.

The genotyping and mapping of *T. melanosporum* mycelium (Murat et al. 2013) indicate that the vegetative mycelia grow in small patches with an annual turnover of individual genets. Our detection of the maximum quantity of mycelium at only 3 years at 40 cm from the tree—without significant increase in the older orchards, even as the visual burn expanded—may reflect the turnover of vegetative mycelia and seasonal regrowth rate of *T. melanosporum* genets in these conditions. Guidot et al. (2004) also reported annual recolonization or disappearance of the ectomycorrhizal fungus *Hebeloma cylindrosporum* in a *Pinus pinaster* stand concluding that nutrient quality and competition from other fungi may be important factors. Measuring relative DNA quantities throughout the season to include the various phases in the annual life cycle of a productive truffle burn may show seasonal fluctuations in genet turnover.

The dominant role that *T. melanosporum* exerts in mature natural truffle grounds was demonstrated by Napoli et al. (2010) by comparing fungal species richness inside and outside truffle burns. They found *T. melanosporum* to be dominant within the burn, accompanied by decreases in ectomycorrhiza richness. In the present work, we can highlight the quantitatively similar expansion of the mycelium of

this fungus from close to the tree (40 cm) at 3 years to an extension of 2 m within 10 years.

Our objective was to evaluate the relative quantities of *T. melanosporum* extramatrical mycelium over time and at three distances to be able to look at expansion patterns. We are interested in the relative quantities of extramatrical mycelium among these 18 orchards because it allows us to detect a general pattern of behavior in this type of site—productive truffle orchards established with *Q. ilex*-inoculated nursery seedlings on previously cropped cereal lands. The fairly uniform and consistent pattern that we observed may also reflect a lack of competition from other ectomycorrhizal species since these soils have previously been cropped with VAM-supported plants.

Absolute quantities of DNA from ectomycorrhizal fungi will vary significantly depending on site differences (Hortal et al. 2009), quantification methods (De la Varga et al. 2012) sampling season and host tree (Zampieri et al. 2012), and mycelial strain (Colpaert et al. 1992). We take caution in comparing quantitative results with other studies that measure DNA of ectomycorrhizal fungi from soil for several reasons. One problem is that results are reported in different units: milligrams or micrograms of mycelium per gram soil (Parladé et al. 2007, 2013; Hortal et al. 2009; De la Varga et al. 2012), nanograms of DNA per gram soil (Suz et. al 2008), and nanograms DNA per microliter extract (Zampieri et al. 2012 and this present study). These studies do not always include that he amount of soil in the original sample or the volume of solutions used in the elution process, which can influence the final quantity reported. Another problem is the uncertainty related to reporting DNA vs. mycelium because spores may also be present in the soil samples, and spore concentration can vary with the season of soil collection.

If we compare our present results with those of Zampieri et al. (2012) who also quantified DNA of *T. melanosporum* from truffle soils and where results are given in the same units, we see that our range of 0.001–300 overlaps the range that they reported 0.004–4.7 ng DNA/μl extract. They are quite similar at the lower detection level, but our upper level was nearly 100 times greater than what they reported. The differences observed for the maximums reported in the two studies may be due to sampling different soil conditions, different host trees, or the use of different *T. melanosporum*-specific PCR primers: Paolocci et al. (1999) vs. Bonito (2009). Both studies provided results from soil sampling in the spring (May and April) when winter fruit-body formation is over and root expansion is activated by spring weather conditions. Zampieri et al. (2012) reported significant seasonal differences for DNA quantities when comparing truffle-bearing vs. nonbearing trees, but no seasonal differences when comparing DNA quantities in April or October from beneath truffle-bearing *Quercus pubescens* or *Corylus avellana* hosts. This may be related to their small sample size of two trees. In the present study, we had only a spring sample period, and it is likely that quantities of *T. melanosporum* mycelium could vary significantly during other seasons of the year, given that the growth of mycelium coincides with fine-root growth. In the Mediterranean conditions of our sites, where soil moisture is the limiting factor for plant growth, spring is the period of greater rainfall, mild temperatures, and active leaf area increase for *Q. ilex*.

Mycelial detection and quantification allow us to follow extramatrical mycelium activity in soils. Unfortunately, it is difficult to interpret the significance of absolute quantities because they may (Hortal et al. 2008) or may not correlate with sporocarp production or with the presence or abundance of root tips colonized by a specific fungus (Parladé et al. 2007; Suz et al. 2008; De la Varga et al. 2012). Recognizing that different mycobionts have different patterns of mycelial growth, Weigt et al. (2012) have proposed exploration type-specific standard values to quantify mycelia space occupation and biomass. This type of proposal takes into consideration the ecologic significance of the mycelial features and could help to navigate our future studies of mycelial extension patterns and biomass within a biologically meaningful context.

There was no detection of *T. brumale* or *T. indicum* in our study. This is an asset for the growers, although the lack of detection is, by no means, proof of absence. Both *T. brumale*, a European species, and *T. indicum* from Asia are closely related black truffles, which mature in the winter. Because they have similar morphologic characteristics, the sporocarps from these truffles could accidentally be used in a commercial seedling inoculation process, resulting in the cultivation of truffles of lesser gastronomic and economic value. Also, *T. brumale* could be accidentally introduced by wild animals. Further concerns are the unknown consequences of the introduction of a potentially invasive ectomycorrhizal fungal species to a new habitat through the dissemination of host plants (Vellinga et al. 2009). Ectomycorrhizae of *T. indicum* have been reported from *T. melanosporum* orchards in Italy (Murat et al. 2008) and the USA (Bonito et al. 2011). *T. brumale* has been detected in black truffle orchards in Europe including France (Brunel 2000) and Spain (Fischer et al. 2004), as well as outside its native range, in the USA (Lefevre and Hall 2001) and Australia (Linde and Selmes 2012). *T. brumale* can fruit in similar conditions as *T. melanosporum* (Sourzat 2009).

It is interesting to note the coincidence in time between commercial fruiting onset and land occupation by *T. melanosporum.* These orchards are usually planted on a 5×5–6×6-m grid, so shortly after the 10th year, the mycelia from one tree is likely to meet the mycelia from the neighboring trees. This would increase the chance of contacts between thalli of different mating types that would bring about fructification (Rubini et al. 2011b). And usually, it is when the orchards reach 10–12 years of age that the anecdotal early

fruiting turns into commercial-level production of truffles. According to this, denser plantations would start producing earlier, but ultimately, the decision on the density of a plantation should be based on economics.

The results of this work help us better understand the patterns that *T. melanosporum* follows to colonize new soil. This new knowledge contributes to the debate on what should be the appropriate tree density in black truffle orchards in order to optimize the cost-benefit ratio of this crop (Reyna and Colinas 2012).

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