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SHORT COMMUNICATION

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Shifts in soil fungal communities in *Tuber melanosporum* plantations over a 20-year transition from agriculture fields to oak woodlands

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Abstract

Aim of study: To explore the diversity of soil fungi found in black truffle (*Tuber melanosporum*) plantations following the introduction of the mycorrhizal-colonized host tree, (*Quercus ilex*), through the development of the brûlé and production of mature sporocarps.

Area of study: This research was carried out in the province of Teruel, Aragon (central eastern Spain).

Material and Methods: Soil samples from 6 plantations were collected beneath *Q. ilex* trees inoculated with *T. melanosporum*, of 3, 5, 7, 10, 14 and 20 years after out planting in truffle plantations. Soil DNA was extracted, PCR-amplified and sequenced to compare soil fungi present at different ages.

Main results: As tree age increased, we observed an increased frequency of *T. melanosporum* (from 8% to 71% of sequenced colonies) and concomitant decrease in the combined frequency of *Fusarium* spp. and *Phoma* spp. (from 64% to 3%).

Research highlights: There are important shifts in species richness and in functional groups in the soil fungal communities in maturing black truffle-oak woodland plantations. The observed inverse relationship between the frequency of soil endophytic and/or pathogenic fungi and that of the mycorrhizal mutualist *T. melanosporum* provides support to continue a deeper analysis of shifts in fungal communities and functional groups where there is a transition from agriculture fields to woodlands.

Keywords: *Quercus ilex*; ectomycorrhiza diversity; fungal succession; truffle cultivation.

Abbreviations used: Ectomycorrhiza (ECM) fungus; Vesicular arbuscular mycorrhiza (VAM); Operational taxonomic unit (OTU).

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Introduction

The black truffle is the fruit body of the hypogeous ascomycete *Tuber melanosporum* Vitt., an obligate ectomycorrhizal (ECM) fungus, native to Mediterranean calcareous regions. Due to its gastronomic and economic importance, this fungus has attracted long-standing scientific attention in the interest of understanding its life cycle and for cultivation (Kües & Martin, 2011). As an agroforestry endeavour, cultivation of black truffles can be economically profitable in marginal farmlands, promoting increased land-use stability, firebreaks and restoration of open oak woods in Mediterranean landscapes (Reyna & Garcia-Barreda, 2014). Inconsistent harvests of black truffles from plantations established with *T. melanosporum*-inoculated seedlings continue to challenge both scientists and farmers to look for management solutions and insight into belowground processes (Olivera *et al.,* 2011; Zampieri *et al.,* 2012). The belowground landscape for *T. melanosporum* is characterized by dynamic and complex relationships among fungi, plants, microbes and insects, but the multiple players in this community are not well known, including the fungal community composition.

Inquiry into this community began with identification and morpho-typing of approximately 25 frequently observed ectomycorrhizae collected from truffle soils (Donnini & Bencivenga, 1995; Baciarelli-Falini *et al.,* 2006) and has expanded to include 100 species of ECM fungi corresponding to 31 fungal genera (De Miguel *et al.,* 2014). Belfiori *et al.* (2012) observed a negative correlation between the ECM richness and the abundance of *T. melanosporum* mycorrhizae. The hypothesis that *T. melanosporum* is a dominating colonizer is supported by evidence for a decrease in the overall soil fungal diversity with the development of the truffle brûlé (Napoli *et al.,* 2010). The brûlé is an area nearly barren of plant vegetation surrounding a tree colonized by *T. melanosporum* and provides a visible indication that *T. melanosporum* is active in the soil beneath the host tree (Suz *et al*., 2008; Streiblová *et al*., 2012).

This is an exploratory study to provide 1) a preliminary survey of the diversity of soil fungal communities in truffle plantations with the use of Sanger sequencing before applying more costly and in-depth sequencing methods, and to assess 2) changes in the fungal communities with the advancing succession in truffle plantations established in soils previously dedicated to cereal production.

Materials and Methods

The plantations included in this study are part of an on-going long-term project to observe patterns of growth for *Tuber melanosporum* Vitt. in collaboration with truffle farmers who have established successful black truffle plantations in the province of Teruel, (central eastern Spain). As reported in Liu *et al.* (2014): Plantations are located in the valley between the ranges of Gudar and Javalambre, on relatively homogeneous calcareous soils with Tertiary sediments. The latitudes of the plots range from N 40º 20’ to N 40º 2’ and the longitudes from W 1º 10’ to W 0º 41’, with altitudes from 843 to 1124 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 of -2º C in January to a monthly average of maximum daily temperatures of 30º C in July.

All plantations examined had been established on marginal agricultural lands, previously cropped primarily in cereals. Prior to planting the *T. melanosporum*-inoculated *Q. ilex* seedlings, the landowners cultivated the soils thoroughly. After planting they tilled the land periodically to maintain the tree-rooting zone free of competing vegetation to promote seedling growth.

This study was performed in 6 truffle plantations that are owned and managed by collaborating private truffle growers who purchased their Holm oak (*Quercus ilex* L.) seedlings from several commercial nurseries in this and neighboring provinces. These nurseries work with local sources for acorns and truffles, but the precise genetic origin of the plant material and the truffle inoculum is not available. Seedlings were inoculated using spore suspension techniques and, prior to outplanting, evaluated for successful mycorrhizal colonization with *T. melanosporum*. We selected a single tree from plantations of 3, 5, 7, 10, 14 and 20 years old to observe a chronosequence snapshot of soil fungi in developing black truffle plantations.

In each plantation, we selected one tree for sampling: A producing tree in 10-, 14- and 20-year old plantations where most trees were producing truffles; a non producing tree with brûlé in 5-and 7-year old plantations where most trees were not producing truffles but had visible brûlés; and a tree without brûlé in the 3 year-old plantation where most trees did not have brûlés. The 6 trees selected are a subset of the 18 trees sampled to quantify *T. melanosporum* mycelium over 17 years by Liu *et al.* (2014) and soil samples were collected as reported: From each tree we cored three soil subsamples (30 cm deep and 7 cm diameter) 40 cm from the trunk, at the vertices of a randomly-oriented equilateral triangle, and mixed them thoroughly into one pooled sample, which was passed through a 4 mm mesh to remove debris, stones and roots, and then placed on ice and taken to the laboratory where they were stored at -20ºC in a sealed plastic bag.

Soil DNA was extracted and PCR-amplified using the fungal specific primers ITS1F and ITS4. Amplicons were purified, cloned and Sanger sequenced. Data were assembled and edited to remove chimeric data. Sequences were clustered obtaining 228 operational taxanomic units (OTUs) (97% sequence similarity over ≥90 % of the alignment) and queries were made with multiple databases (shown in Table S1 [online supplement]).

Results

Six ITS clone libraries were generated, each corresponding to one tree sampled from each of the six plantations (see Table S2 [online supplement]). The 228 sequences comprised 89 unique OTUs, with 62 singlets occurring only once in the data set. The most abundant species recovered were *T. melanosporum* and *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, which represented 34% and 13% of all sequences. Shannon’s index was calculated based on the number of sequences found for each tree age. With the exception of year 10, Shannon’s diversity index de-

creased with age (see Table S3 [online supplement]). As tree age increased, we observed an increased frequency for OTUs belonging to the genus *Tuber* (from 8% to 71% of sequenced colonies) and concomitant decrease in the combined frequency of the genera *Fusarium and Phoma* (from 64% to 3%) (Figure 1). No other *Tuber* species were recovered other than *T. melanosporum.*

These soils are dominated by Ascomycota (59 of 89 OTUs), with 13 OTUs from the Basidiomycota. The ectomycorhizal basidiomycetes recovered include *Hymenogaster citrinus* Vitt. (age 5), *Hygrophorus latitabundus* Britzelm. (age 7) and *Hymenogaster populetorum* Tul. & C. Tul. (age 14). *H. citrinus* Vitt., a hypogeous member of the Agaricales has been observed repeatedly in truffle grounds (Donnini & Bencivenga, 1995; Belfiori *et al.,* 2012), and fungi from the *Hymenogasteraceae* family have been collected from both productive and non-productive truffle sites (De Miguel *et al.,* 2014; Napoli *et al.,* 2010).

Discussion

This study of the community of soil fungi in black truffle plantations established on abandoned cereal fields allows us to observe an important shift over 20 years as truffle-inoculated oak trees mature and produce truffles. Although we expected to recover vesicular arbuscular mycorrhizal (VAM) fungi from the phylum *Glomeromycota* in the young plantations, their absence here may be due to the intensive tilling practices in these fields, the limitations of the sequencing method and the limitations of a single pooled sample from each site. With the appearance of the truffle brûlé at 5 to 7 years in these plantations, the management techniques shifted to reduce soil disturbance, allowing for occasional grasses and aromatic Mediterranean plants such as *Thymus* sp., which are supported by VAM fungi. The appearance of *Glomus irregulare* in the 10-year-old site and *Scutellospora coralloidea* at the 20-year-old site may represent the slow return of a more mixed VA- and ECM-plant community, typical of more mature truffle beds and Mediterranean open oak woodlands (Belfiori *et al*., 2012; Maremmani *et al.,* 2003).

There were no recoveries of *Cenoccocum geophilum* Fr., one the most frequently observed mycorrhiza from black truffle beds nor did we detect *Trichophaea woolhopeia* (AD-type) also frequently observed in truffle grounds (Rubini *et al.,* 2011). This may be a consequence of the limited sample size. Our sites demonstrate a narrow ECM fungal diversity in comparison to natural truffle sites (Belfiori *et al.,* 2012). Given that this study has a single repetition for each plantation age we cannot make broad conclusions for the species richness of soil fungi in truffle plantations established on abandoned cereal lands, but we believe that these findings will provide guidelines for more in-depth sampling in the future.

In the youngest plantation of 3 years, where no brûlé was visible, *T. melanosporum* was recovered in 4 of the 53 sequenced colonies (8%). Its presence increased in number of colonies and proportional frequency considerably by 5 years with 25 of 44 (57% of all colonies)

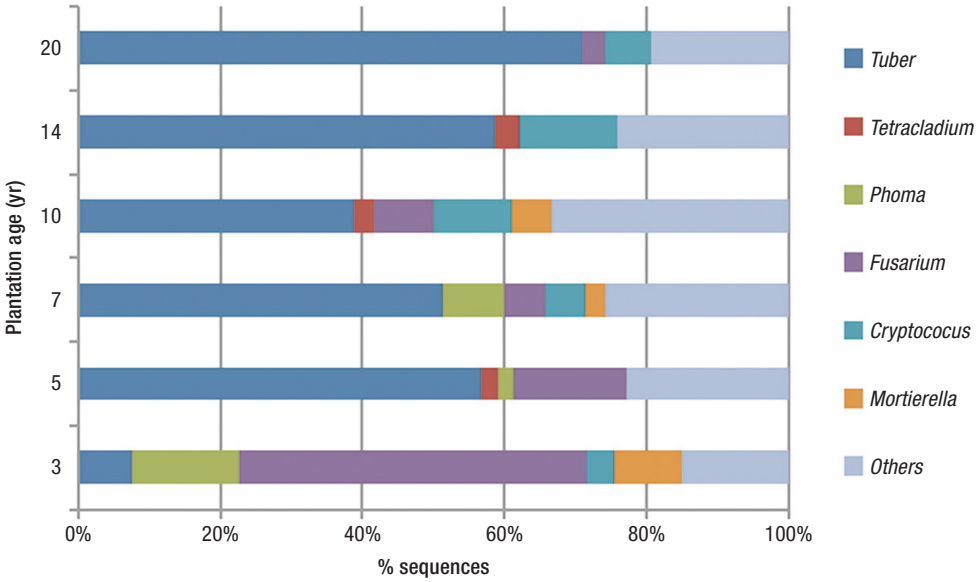

Figure 1. The proportional distribution for the sequences of the most frequent fungal genera detected in *Tuber melanosporum* plantations of six ages. These results correspond to one tree per plantation.

belonging to *T. melanosporum* and, with a fluctuation in the 10-year-old plantation, continued to increase through the 20-year plantation with 22 of 31 (71% of colonies) belonging to *T. melanosporum*, confirming its dominating capacity. This domination was also reflected in the relative quantities of *T. melanosporum* mycelium detected in soils from these plantations in a companion work by Liu *et al.* (2014).

In the 3 yr.-old plantation, the dominant fungi recovered belong to the genus *Fusarium*, representing 26 of the 53 sequences (49%) from this age. The second most frequent at 3 years after plantation establishment belong to the genus *Phoma* (8 colonies), followed by *Mortierella* (5 colonies). All three of these fungal taxa represent complex groups of ubiquitous soil fungi with saprotrophic and/or pathogenic properties. As the plantation age increases we observed a marked increase in the frequency of *Tuber* and the subsequent decrease in *Fusarium* and *Phoma*, with an absence of *Phoma* beginning at the 10 yr.-old plantation, and *Fusarium* declining to 1 of 31 (3% of colonies) in the 20 year-old plantation.

The role of ectomycorrhizae in providing protection against common soil pathogens from the genera *Fusarium* and *Rhizoctonia* has been demonstrated in greenhouse conditions (Chakravarty & Unestam, 1987) and in vitro (Martın-Pinto *et al.,* 2006). *Fusarium oxysporum* and other *Fusarium* species are found in agricultural soils worldwide, and while some are non-pathogenic others are responsible for significant crop damage (Gordon & Martyn, 1997), and serious root rot in forest nurseries (Kim *et al.,* 2012). It is much less frequently reported from forest soils than cultivated soils (Park, 1963; Latiffah & Azaman, 2011), and may be related to the quality of root exudates produced in the mycorrhizosphere of trees (Grayston *et al.,* 1996). Mature truffle orchards are quite different from forests, yet we have captured the reduction in the frequency of OTU's from the genera *Fusarium* and *Phoma* as the *Q. ilex* trees mature and as *T. melanosporum* becomes more dominant.

The mechanisms for ECM fungal-mediated plant protection are not well understood and vary among ECM species (Zengpu *et al.,* 1994). They involve multiple strategies including physical barrier and competition for colonization sites (Smith & Read, 2008), inhibition of *F. oxysporum* spore germination (Chakravarty & Hwang, 1991) and, in the case of *T. melanosporum,* may include the production of specific volatile organic compounds (Splivallo *et al.,* 2011).

Our exploratory study indicates important shifts in species richness and in functional groups in the soil fungal communities in maturing black truffle-oak woodland plantations. The use of Sanger sequencing has given us a glimpse into the changing pattern of decreasing soil fungal diversity as *T. melanosporum* dominates these soils and as the age of the host tree increases. We have identified some of the more important species and functional groups present in these soils. In the future we would recommend examination of the potential biases resulting from the ITS1F/ITS4 primer combination that may influence the diversity of species recovered from these soils (Ihrmark *et al*., 2012). Use of next-generation sequencing tools would permit a deeper query of the diversity and composition of soil fungal communities in maturing truffle orchards with a larger sample size over a greater geographic range.

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