Interannual dynamics of *Tuber melanosporum* and fungal communities in productive black truffle orchards amended with truffle nests

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Abstract

Truffle growers devote great efforts to improve black truffle productivity, developing agronomic practices such as “truffle nests” (peat amendments that are supplemented with truffle spore inoculum). It has been hypothesized that improved fruiting associated with nests is linked to stimulation of truffle mycelia previously established in soil or to changes generated in soil fungal community. To assess this, we used real-time PCR to quantify black truffle extraradical mycelium during two years after nests installation. We also characterized the fungal community via high-throughput amplicon sequencing of the ITS region of rDNA. We found that neither the abundance of truffle mycelium in nests nor in the soil – nest interphase was higher than in the bulk soil, which indicates that nests do not improve mycelial growth. The fungal community in nests showed lower richness and Shannon index and was compositionally different from that of soil, which suggests that nests may act as an open niche for fungal colonization that facilitates truffle fruiting. The ectomycorrhizal fungal community showed lower richness in nests. However, no negative relationships between amount of truffle mycelium and reads of other ectomycorrhizal fungi were found, thus countering the hypothesis that ectomycorrhizal competition plays a role in the nest effect.

Keywords: *Tuber melanosporum*, truffle nests, extraradical mycelium, ITS amplicon sequencing, fungal community, ectomycorrhizal fungi

1. Introduction

The European black truffle (*Tuber melanosporum* Vittad.) is an ectomycorrhizal fungus that is highly appreciated in *haute cuisine*. It grows wild in southern Europe, although nowadays most of truffle production comes from cultivated orchards established with seedlings previously inoculated in controlled conditions (Olivier, Savignac and Sourzat 2002; Reyna and Garcia-Barreda 2014). Truffle cultivation has spread and advanced greatly in recent decades, although uncertainties regarding mating and the ascocarp development processes still remain (Pacioni *et al.* 2014; Le Tacon *et al.* 2016). Growers devote great efforts to improve the productivity and sustainability of this crop, and empirical cultivation practices are constantly being proposed and tested. Among these is the use of "truffle nests" (also referred to as "truffle traps"), hereafter referred to as "nests" (Reyna and Colinas 2012). Nests are established within the truffle *brûlé* (the truffle-producing soil around the host tree) and typically consist of a peat-based substrate that has been pH adjusted (around pH 7.5) and supplemented with truffle spore inoculum. The spores are thought to play a paternal role in truffle reproduction, serving as male elements in sexual mating (Taschen *et al.* 2016, 2022; De la Varga *et al.* 2017). Further, the physical conditions in the nest, as well as in the soil – nest interphase, are thought to play a role in triggering the ascocarp initiation and development (Garcia-Barreda *et al.* 2020).

Nests frequently account for a large proportion of the truffle ascocarps harvested from orchards where they are used, with growers claiming that they can accelerate and increase truffle production in young plantations (Murat *et al.* 2016; Garcia-Barreda *et al.* 2020; Taschen *et al.* 2022). Their use raises important research questions that may provide insight into the reproductive ecology of this fungus, particularly regarding the biological and environmental mechanisms that trigger truffle fruiting (Murat *et al.* 2016; Garcia-Barreda *et al.* 2020; Taschen *et al.* 2022). Truffle fruiting patterns could be related to the increased

contact between introduced spores and mycorrhizas previously established in the soil, although recent research hypothesized that it may have more to do with soil disturbance and/or with root damage stimulating truffle mycelia previously established in the soil to colonize the introduced substrate (Taschen *et al.* 2022). The peat-based substrate used in nests is characterized by low bulk density, high levels of easily available water and low levels of ectomycorrhizal fungal populations (Argo 1998; Abu-Hambeh and Reeder 2000; Ángeles-Argáiz *et al.* 2016). However, the colonization of nests by truffle mycelium during the time from their installation in the field to truffle fruiting has never been monitored.

The dynamics of truffle mycelial growth in the soil relates not only to changes in the soil microclimate (Queralt *et al.* 2017), but also to soil fungal microbiota activity (Napoli *et al.* 2010; Oliach *et al.* 2022). Communities of ectomycorrhizal fungi compete with cultivated truffles for the colonization of host tree root tips, and impact the vegetative spread of the target fungus into the soil (Zambonelli *et al.* 2012; Garcia-Barreda and Reyna 2013; De Miguel *et al.* 2014). At the same time, both the composition of fungal microbiota and their functional activity are affected by biochemical modifications that black truffles have in productive soils (Napoli *et al.* 2010; Mello *et al.* 2011; Garcia-Barreda and Reyna 2012; Zampieri *et al.* 2016; Oliach *et al.* 2022). Although several microbial species are able to live within the truffle ascocarp, there is little known on the effect of these microbiota on black truffle reproduction (Pacioni *et al.* 2007; Benucci and Bonito 2016; Pacioni and Leonardi 2016). However, for the cultivated truffle *Terfezia claveryi* Chatin, a correlation between the composition of the soil fungal community and plantation yields has been described (Arenas *et al.* 2021). These findings lead to the hypothesis that the relationship between the truffle and the soil fungal community contributes to the anecdotal effects of increased truffle production observed with nests (Murat *et al.* 2016; Taschen *et al.* 2022). Understanding these interactions could help to master *T. melanosporum* cultivation.

In this study, we investigated the spatiotemporal patterns of *T. melanosporum* extraradical mycelium in truffle nests over two years following their installation, in relation to the surrounding bulk soil and the soil – substrate interphase. We also investigated the composition of fungal communities in these nests and explored patterns of co-variation between *T. melanosporum* and the diversity and composition of these fungal communities. We specifically focused on ectomycorrhizal fungi since they directly compete with *T. melanosporum* for root colonization. We hypothesized that vegetative growth of *T. melanosporum* within nests and in the soil– nest interphase would be stimulated to levels higher than those in the bulk soil (Taschen *et al.* 2022). We also hypothesized that nest habitats during the first two years in the field would be characterized by lower ectomycorrhizal and lower overall fungal species diversity, based on previous information on fungal communities in peat and on the low fertility of peat (van Breemen 1995; Ángeles-Argáiz *et al.* 2016). We tested these hypotheses through the use of qPCR for quantifying *T. melanosporum* mycelium and with high-throughput amplicon sequencing to characterize fungal communities across treatments.

2. Material and methods

2.1. Experimental design and sampling

The study was conducted in two *T. melanosporum* truffle orchards in Teruel province (eastern Spain); the first one was in Teruel municipality (1,090 m a.s.l.) and the second was in Mora de Rubielos (1,150 m a.s.l.). The climate is Continental Mediterranean, with respective mean annual rainfalls of 440 and 520 mm and mean annual temperatures of 11.6 and 11.1 °C in Teruel and Mora, respectively (Ninyerola, Pons and Roure 2005). Both soils are calcareous, with Teruel soils being developed on Tertiary conglomerates and clays and presenting sandy clay loam texture, and Mora soils being developed on Tertiary siltstones/sandstones and

presenting sandy loam texture (Table S1) (I.G.M.E. 1972). In the Teruel orchard, the vegetation surrounding the plantation was a mature (6 m height), open *Quercus ilex* L. subsp. *ballota* (Desf.) Samp. forest, whereas in Mora it was a sparse shrubland with scarce ectomycorrhizal plants.

The Teruel orchard was planted in 1999 and the Mora orchard in 2001. The Teruel orchard is planted with *Q. ilex* subsp. *ballota* as host trees, whereas the Mora orchard is planted with both *Q. ilex* subsp. *ballota* and *Quercus faginea* Lam., although for homogeneity purposes we selected an experimental plot with only *Q. ilex*. In both orchards, trees were planted at a density of 278 trees ha^{-1} (6 × 6 m). The seedlings were produced in commercial nurseries and their mycorrhizal status was controlled by public authorities following INIA-Aragón methods (Andrés-Alpuente *et al.* 2014). In both orchards the soil was tilled once a year during the pre-productive stage and the trees were pruned annually beginning in the fifth year. The Teruel orchard began to produce truffles at age 10 and the Mora orchard at age 6. Since then, the Teruel orchard was no longer tilled, whereas the Mora orchard continued to be tilled once a year in late April. Both orchards were irrigated during the productive stage with a sprinkling system, from April to October.

In April 2016, when the orchards were 19 and 17 years old respectively, we randomly selected 13 productive truffle trees per orchard and established six truffle nests around each tree. The setting up of the nests involved digging holes about 25 cm deep, filling them with 1.5 l of a European *Sphagnum* peat-based substrate (Turbatruf ® from Projar: a black peat – white peat – coconut fiber – perlite mix 11–5–3–1, and pH raised to 7.5 with lime) and re-covering the substrate with soil. Ground ripe truffle ascocarps were previously mixed with the substrate (0.1 g dry ascocarp per liter of substrate) and the mix was thoroughly homogenized before being used. Nests were arranged at regular intervals along a 1.5 m-radius circumference centered on the tree trunk, within the truffle *brûlé*.

The two truffle orchards were sampled twice: in April 2017 (one year after setting up the nests) and in April 2018 (two years after setting up the nests). The same trees were sampled in both years. In each year and for each sampled tree, three samples were collected: one inside the nest (peat-based substrate), one in the soil – substrate interphase and one in the neighboring bulk soil within the *brûlé* (at 20-30 cm from the interphase). Each sample was composed of two sub-samples corresponding to two nests in the same tree, which were mixed. A soil borer 10 cm depth and 3 cm diameter was used for sampling inside the nest and in the bulk soil, resulting in samples of ca. 70 cm^3. To obtain the interphase samples, we removed peat from the wall of the nest and sampled only soil from this wall, carefully with a spatula until ca. 70 cm^3 of sample was collected. All the sampling material was cleaned and disinfected with diluted ethanol between samples.

Not all experimental trees could be sampled, due to external factors related to plot management. In the Teruel orchard we sampled 12 trees in 2017 and 13 in 2018, whereas in the Mora orchard we sampled the same 10 trees both in 2017 and 2018. This resulted in a total of 135 samples (22 trees were sampled in 2017 and 23 in 2018, with three positions sampled in each tree each year). Additionally, in April 2016 two samples of the peat-based substrate (one for each orchard) were taken after adding the ground truffle ascocarps but before setting up the nests, as a reference of the initial conditions and fungal community in the substrate, and to account for the baseline quantity of spores and tissue used as inoculum for the nests.

2.2. Molecular methods

All samples were air-dried at room temperature and then were sieved through a 2 mm mesh to homogenize, washing the sieve with soapy water and ethanol between samples.

Approximately 250 mg of dried sample were used to extract genomic DNA with the

MagAttack PowerSoil DNA Kit (Qiagen) on a KingFisher robot. The same extracted DNAs were used to both quantify *T. melanosporum* mycelium in the sample and for internal transcribed spacer (ITS) amplicon sequencing.

2.2.1. Extraradical mycelium of *Tuber melanosporum*

The quantification of *T. melanosporum* mycelium in the samples was carried out using species-specific DNA primers described in Parladé et al. (2013) and a StepOne™ Real-Time PCR System machine provided with the StepOne software v. 2.3 (Life Technologies, Carlsbad, CA). DNA samples and standards were prepared for real-time PCR using the 2× Takara Premix Ex Taq™ Perfect Real-Time (Takara Bio Europe, SAS, France), the TaqMan probe (200 nM) and primers (800 nM each) described by Parladé et al. (2013), 5 μL of the template DNA and HPLC water to a final reaction volume of 20 μL. Thermocycling profile was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Triplicate real-time PCR were performed on each sample. Standards were prepared using fresh immature *T. melanosporum* ascocarps. The standard curve was generated by plotting serial tenfold dilutions against the corresponding initial amount of ascocarp as described in Parladé et al. (2013). Absolute quantification of mycelium biomass of *T. melanosporum* was expressed in mg of mycelium per g of sample. The two reference measures of the substrate before it was used in the orchards showed a mean of 8×10^{-4} mg of *T. melanosporum* per substrate gram, which accounts for the *T. melanosporum* spores and tissue used as inoculum for the nests.

2.2.2. Amplicon sequencing and bioinformatic analysis

The fungal ITS region of the ribosomal DNA (rDNA) was amplified using ITS1F-ITS4 primers (White *et al.* 1990; Gardes and Bruns 1993). Briefly, a first step PCR was used to enrich the target fragment, a second step PCR was used to add 1-6 bp random frameshift to

improve clusters differentiation during Illumina sequencing, and a third step PCR to ligate a 10bp barcode and Illumina adapters to the final PRC products. PCR products size and concentration were determined on a QIAxcel Advanced machine with a DNA Fast Analysis kit (Qiagen). Sample libraries were then normalized with a SequalPrep normalization plate kit (Thermo Fisher Scientific) and pooled. The generated amplicon library was then concentrated at approximately 20:1 with Amicon Ultra 0.5-mL 50K filters (EMDmillipore, Germany), according to previous studies (Benucci *et al.* 2019; Longley *et al.* 2019; Noel, Chang and Chilvers 2020). Libraries were then sequenced on a MiSeq Illumina platform using the v3 300PE kit. Raw forward ITS reads (ITS1) were used in the downstream analysis. Briefly, we demultiplexed the raw Illumina reads according to the barcode index in QIIME (Caporaso *et al.* 2010). We removed the Illumina adapters and sequencing primers in Cutadapt 4.2 software (Martin 2011). We filtered the demultiplexed reads according to quality (max expected errors = 1.0), trimmed them to 215 bp (Edgar and Flyvbjerg 2015; Edgar 2016), de-replicated them, removed singleton sequences, and clustered them into operational taxonomic units (OTUs) based on 97% similarity using the UPARSE algorithm (Edgar 2013). Taxonomic assignments were performed in CONSTAX2 (Liber, Bonito and Benucci 2021) against the UNITE database (Kõljalg *et al.* 2013) version 7.1 2016-08-22.

2.3. Statistical analysis

The resulting OTU tables, representative sequences, sample data and taxonomic classification files were imported into the R statistical environment (R Core Team 2022) with the phyloseq package (McMurdie and Holmes 2013). Before starting the analyses, we removed PCR and sequencing contaminants with the decontam R package using the negative control samples included in the MiSeq libraries (Davis *et al.* 2018). Non-fungal and undetermined taxa were

filtered out. To avoid biases, OTUs were normalized with a Gaussian model (cumulative sum scaling), with the R package metagenomeSeq (Paulson *et al.* 2013).

Observed OTU richness, evenness and Shannon's diversity index were selected as α-diversity metrics and determined using the *plot_richness* function in the phyloseq package. Diversity patterns were then tested for statistical differences across plots (Teruel and Mora orchards), positions with respect to the nest (nest, bulk soil and soil – substrate interphase) and year of sampling (2017 and 2018). For this, we selected the statistical model by comparing: (i) a linear model, (ii) a linear mixed model with "year" as a repeated measures variable, and (iii) a linear mixed model with “year” as a repeated measures variable and including a random effect for the “tree” variable to model tree-specific intercepts (Zuur, Ieno and Smith 2007). The fit of these models to data was assessed with the Akaike information criterion. For richness and Shannon's index, model (i) was selected. For the abundance of *T. melanosporum* extraradical mycelium the same statistical procedure was applied, and model (ii) was selected. The R package nlme was used for these analyses (Pinheiro, Bates and Team 2022). The assumptions of normal distribution, constant variance and linearity were tested in the final models, and thus mycelium abundance was log-transformed to meet these assumptions.

The β-diversity of the fungal microbiome was assessed with a principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity metrics, using the function *ordinate* from the phyloseq package. The differences in the observed community composition across plots, positions and years were assessed with a permutational analysis of variance (PERMANOVA) in the vegan package with function *adonis* (Oksanen *et al.* 2022). Since differences in the PERMANOVA can be related to both differences in the centroid location and in the dispersion of each group samples, the 95% confidence ellipse of the centroids was assessed and data were tested for homogeneity of variances with the function *betadisper* (Anderson

2001). An indicator species analysis was performed to identify OTUs significantly associated with particular positions, with the *multipatt* function in the indicspecies package (De Cáceres and Legendre 2009).

To assess the relation between *T. melanosporum* and the α-diversity of the fungal community, the abundance of *T. melanosporum* mycelium was correlated against richness and Shannon's index of the fungal community using bootstrapped Pearson correlations with a 95% confidence interval. The correlation was calculated separately for each position to check that the three showed the same behavior.

The α- and β-diversity patterns of the communities of ectomycorrhizal fungi were also investigated, to test whether the presence of *T. melanosporum* affected them differentially, since ectomycorrhizal fungi directly compete with truffles for root colonization. For this, functional guilds were assigned to each taxon by using the FUNGuild online tool (Nguyen *et al.* 2016). This was used to obtain an initial list of ectomycorrhizal OTUs that were subsequently confirmed with literature when possible. The α-diversity measures of the ectomycorrhizal community were correlated against truffle mycelium abundance.

Finally, the relation between *T. melanosporum* abundance and that of other ectomycorrhizal fungi was assessed. Truffle mycelium abundance was used as a proxy for the former, whereas the number of reads of the remaining ectomycorrhizal fungi in the amplicon sequencing was used as a proxy for the latter. Linear regression was used to assess this relation, including position as a predictor to test whether the direction of the relation was the same in the three positions.

3. Results

3.1. Extraradical mycelium of *T. melanosporum*

The abundance of *T. melanosporum* extraradical mycelium was significantly affected by the interaction between plot, position, and year (F-value = 3.6, P = 0.031, n = 135, conditional R^2_{GLMM} = 0.509, Table S2, Fig. S1). This resulted in complex patterns of variation. In the Teruel orchard, the mycelium was more abundant in the bulk soil and the interphase than in nests only for year 2017, whereas in Mora only for year 2018 (Fig. 1). In the Teruel orchard, the mycelium increased significantly from 2017 to 2018 in nests, but not in the bulk soil or the interphase, whereas in Mora the mycelium significantly increased from 2017 to 2018 in the soil and the interphase, but not in nests (Fig. 1). As a result, two years after setting up the nests, the mycelium abundance was significantly lower in nests compared to the soil and the interphase in the Mora orchard, but not in Teruel (Fig. 1). In both orchards, the mycelium abundance in nests was significantly higher than 8×10^{-4} mg g^{-1} substrate (mean value for nests before being installed) in year 2018, but not in 2017.

3.2. Fungal community ITS sequencing

After data processing, amplicon sequencing resulted in a total of 3,289 ITS rDNA sequences generated from the 135 samples taken in the field (mean number of OTUs per sample: 266, standard deviation: 136). The total number of reads generated from these 135 samples was 6,152,212 (mean per sample: 45,572, standard deviation: 27,769). In the two peat samples before being installed, 9 and 80 OTUs were found (10,464 and 49,448 reads respectively). Ascomycota was the most abundant phylum, accounting for 54% of the total number of reads, followed by Mortierellomycota with 31% and Basidiomycota with 11%. *Tuber melanosporum* represented 1.6% of the total number of reads.

3.2.1. Fungal community α-diversity

Fungal OTUs richness was significantly affected by position (F-value = 147.0, P < 0.001, n = 135), plot (F-value = 12.5, P < 0.001) and year (F-value = 39.7, P < 0.001), as well as some of their two-way interactions (R^2 = 0.75, Table S3, Fig. S2). Variance partitioning according to the sum of squares indicated that 71% of the variation in richness was explained by the combined effect of position (60.6%), year (8.2%) and their interaction (2.1%). For both 2017 and 2018, OTU richness was significantly higher in the interphase than in the bulk soil and significantly higher in the soil than in nests. In all positions, richness was higher in 2018 than 2017 (Fig. 2a, Table S4).

The Shannon's index of the fungal community was significantly affected by position (F-value = 115.2, P < 0.001, n = 135), year (F-value = 52.5, P < 0.001) and their interaction (F-value = 13.1, P < 0.001), with the linear model presenting an R^2 = 0.72 (Table S5, Fig. S3). For both 2017 and 2018, Shannon's index was significantly lower in nests than in the bulk soil or interphase, with no significant differences between the soil and interphase (Fig. 2a, Table S6). In the nests, the Shannon's index significantly increased from 2017 to 2018, whereas in the soil and the interphase it did not (Fig. 2a, Table S6).

The evenness of the fungal community was significantly affected by the interaction between position and plot (F-value = 22.5, P < 0.001, n = 132, Table S7, Fig. S4), as well as by year (F-value = 7.7, P = 0.006). No significant differences between the soil of the two plots were observed, or between the interphase of the two plots, whereas the nests of Mora showed higher evenness values than those of Teruel plot (Fig. 2a, Table S8).

3.2.2. Fungal community β-diversity

The main patterns of dissimilarity showed by the PCoA in the taxonomic composition of the studied samples corresponded with differences associated to position, according to the first component, and to differences between orchards, according to the second component (Fig. 3).

The PERMANOVA showed significant differences in the fungal community composition associated with position (F-value = 11.9, $P < 0.001$, n = 132), year (F-value = 7.3, $P < 0.001$) and orchard (F-value = 12.1, $P < 0.001$), as well as their two- and three-way interactions (Table S9). However, most of the variability explained by the model (34.8%) was related to position (13.0%), orchard (6.6%) or their interaction (5.0%) (Table S9). The analysis of centroids and dispersion for this interaction indicated that the PERMANOVA results are due to differences in both the location and dispersion of the different groups. Nest samples showed significantly different centroid locations and higher dispersion than the corresponding soil samples, although the dispersion effect was only significant for the Mora orchard (Fig. S5, Tables S10-S11).

3.2.3. Fungal community composition

The fungal communities in the studied nests, interphases and bulk soils were dominated by sequences of saprotrophic fungi including *Mortierella*, *Candida*, *Chrysosporium*, *Humicola*, *Pseudogymnoascus* and *Solicoccozyma* spp. (Fig. 4a). Among the 30 most abundant genera characterized, 14 were significantly associated with either soil and interphase or with nests and interphase (Fig. 5, Table S12).

Venn diagrams showed that nest samples contained a limited number of unique fungal OTUs compared to soil and interphase samples (Fig. S6). In the Teruel orchard, the relative abundance of OTUs shared between nests and the other positions was high from one year after setting up the nests (80% in 2017 and 81% in 2018), whereas in Mora it was slightly lower in 2017 (71%) than in 2018 (77%, Fig. S6).

3.2.4. Ectomycorrhizal fungi

The ectomycorrhizal community accounted for 3.4% of the total number of fungal reads, with 86 OTUs. By comparison, arbuscular fungi accounted for 0.03% of the reads and 45 OTUs (Fig. S7), whereas saprotrophs accounted for 28.7% of the reads and 644 OTUs (Fig. S8). Among other FunGuild trophic modes, saprotroph-symbiotroph taxa represented 30.8% of the reads, pathotroph-saprotroph-symbiotroph taxa represented 13.6% of the reads, pathotroph taxa represented 5.4% of the reads and unassigned taxa represented 17.9% of the reads.

The richness of ectomycorrhizal OTUs was significantly affected by position (F-value = 59.1, $P < 0.001$, n = 135), plot (F-value = 72.2, $P < 0.001$) and year (F-value = 32.3, $P < 0.001$), with the linear model presenting an $R^2 = 0.65$ (Table S13, Fig. S9). Variance partitioning indicated that 34% of the variation in richness was explained by the effect of position (with soil and interphase presenting significantly higher richness than nests, according to least-square means) and 20% by plot (with Teruel showing significantly higher richness than the Mora orchard) (Fig. 2b).

The Shannon's index of the ectomycorrhizal community was significantly affected by position (F-value = 18.2, $P < 0.001$, n = 135) and the interaction between plot and year (F-value = 4.7, $P = 0.03$), with the linear model presenting an $R^2 = 0.42$ (Table S14, Fig. S10). According to least-squares means, the bulk soil and interphase presented significantly higher Shannon's index than nests, and the Teruel orchard presented higher Shannon's index than Mora in both years (Fig. 2b).

The evenness of the ectomycorrhizal community was significantly affected by the interaction among position, plot and year (F-value = 5.7, $P = 0.005$, n = 113, Table S15, Fig. S11), with least squares indicating significantly lower values in nests than in soil or interphase, and a significantly more positive time trend in Teruel than in Mora orchard.

The PCoA performed on the ectomycorrhizal OTUs did not show any clear dissimilarity patterns in the taxonomic composition of the samples (Fig. S12). The

PERMANOVA analysis showed significant effects of position (F-value = 1.8, P < 0.001, n = 116), plot (F-value = 4.4, P < 0.001), year (F-value = 1.8, P < 0.001) and several two-way and three-way interactions, but none of these explained more than 3% of the variation among samples at the two plots (Table S16). The most frequent ectomycorrhizal genera in the samples were *Tuber*, *Trichophaea*, *Tomentella*, *Picoa*, *Suillus*, *Genabea*, *Melanogaster* and *Terfezia* (Fig. 4b). Only five genera showed a significant association with a position: *Melanogaster*, *Suillus*, *Pulvinula*, *Rhizopogon* and *Tricholoma*. They all were statistically associated with samples from the soil and interphase treatments (Table S12).

3.2.5. Relations between *T. melanosporum* and fungal communities

The abundance of *T. melanosporum* mycelium showed a significantly positive correlation with the richness of the overall fungal community in the three positions, whereas for the ectomycorrhizal community this positive association was only found in nests (Fig. 6a). In nests, the abundance of *T. melanosporum* mycelium also showed a significantly positive correlation with the Shannon's index of the overall fungal community and the ectomycorrhizal community, which was not observed in the bulk soil or the interphase (Fig. 6b).

The log-transformed abundance of *T. melanosporum* mycelium showed a positive relation with the log-transformed number of reads of other ectomycorrhizal fungi (F-value = 11.2, P = 0.001, n = 129, R^2 = 0.14, Table S17, Fig. S13). The slope of this relation was not significantly different among the three positions (F-value = 0.2, P = 0.78, Fig. 6c).

The log-transformed number of reads of *T. melanosporum* in the amplicon sequencing was significantly correlated with the log-transformed abundance of *T. melanosporum* mycelium (F-value = 311.5, P < 0.001, n = 137, R^2 = 0.90, Table S18, Fig. S14). The slope of this relation was not significantly different in the bulk soil (95% confidence interval: 0.928-

1.130), nests (95% confidence interval: 0.748-0.930) and the interphase (95% confidence interval: 0.747-0.947), thus resulting in very similar regression relations for the three positions (Fig. 6d).

4. Discussion

In this study, we assessed spatiotemporal patterns of *T. melanosporum* extraradical mycelium in truffle nests during the two years following their installation. Two-three years after installation is the time term in which most nests produce truffles (Murat *et al.* 2016; Garcia-Barreda *et al.* 2020; Taschen *et al.* 2022). We hypothesized that during this time *T. melanosporum* mycelium would become more abundant in nest habitats compared to the surrounding bulk soil and the soil – substrate interphase. Yet, contrary to these expectations, our data show this was not the case. We found the quantity of *T. melanosporum* mycelium to be nearly one order of magnitude lower (in milligrams per sample gram) in the nests compared to the bulk soil (Fig. 1). However, it must be considered that the bulk density of the peat-based substrate is almost one order of magnitude lower than that of the studied soils, 0.27 g ml^{-1} for the substrate and 1.1-1.3 g ml^{-1} for the Mora and Teruel orchards, respectively. If we quantified the mycelium abundance in milligrams per liter of soil, the difference between nests and bulk soil would have been higher. Thus, disturbing the soil, damaging roots and adding a new substrate in the truffle habitat does not appear to promote rapid and widespread truffle mycelium colonization as part of the so-called beneficial "nest effect". Mycelium abundance in our nests is similar to that in Taschen et al. (2022), who found a higher abundance of truffle mycelium in nests than in the bulk soil outside the *brûlé*. However, it is important to note that in their study they did not analyze mycelium abundance within the *brûlé*, where nests are installed.

At the soil interphase, where the soil is in direct contact with the nest substrate, we did not observe an increase in truffle mycelium with respect to the bulk soil either. This contrasts with the fact that many ascocarps are observed to be formed at the nest interphase (Garcia-Barreda *et al.* 2020). Thus, our results show that the discontinuity in the soil – substrate interphase does not promote the growth of truffle mycelium. In this regard, Pacioni et al. (2014) suggested that truffle ascocarp initiation may be triggered by a change in soil microenvironmental factors such as soil temperature and water content. The role of the nest interphase could be simply related to providing an abrupt change of soil microenvironment conditions that promotes the entry of black truffle mycelium into the sexual phase (Zampieri *et al.* 2011). It is, indeed, well known that peat substrate differs substantially from mineral soil in several factors, including bulk density, porosity, levels of easily available water, thermal conductivity and nutrients content (van Breemen 1995; Argo 1998; Abu-Hambeh and Reeder 2000). An alternative hypothesis is that, since the main role of spores is fertilization, its effect on mycelium abundance is very localized and limited in time and thus difficult to capture in a sampling. The results of Taschen et al. (2022) suggest that both soil local disturbance and spore addition increased truffle fruiting.

In this soil microenvironment with abruptly changing abiotic conditions, biotic factors could also be playing a role in the "nest effect". It is well known that fungal growth and fruiting can be influenced by biotic factors such as competition, as well as beneficial interactions (Kertesz and Thai 2018; Künzler 2018). In truffle plantations, it has been shown that native soil fungal communities change in parallel to plantation age or black truffle dominance (Napoli *et al.* 2010; Liu *et al.* 2016; Oliach *et al.* 2022). In our experimental plots, the fungal community within the nests was less rich, less diverse and clearly dissimilar in composition compared to the bulk soil. These results were consistent across both orchards and during both years of monitoring, suggesting that nests may act as a disturbance providing an

open niche for colonization, and an opportunity for maintaining species diversity (Bruns 1995).

Despite the differences in α- and β-diversity, in the three positions the amount of black truffle mycelium was positively correlated with the fungal community richness, although not always with the Shannon's index. By contrast, in the case of the richness of the ectomycorrhizal fungal community, this positive relation was not found in the soil and the interphase, this suggesting that the positive relation could be related to the activity of particular fungal guilds (e.g. modifying the availability of nutrients) or could simply be an indicator that certain microenvironments are hotspots, favorable for diverse fungal growth. Our results contrast with those of Oliach et al. (2022), who found a negative correlation between truffle mycelium abundance, increasing as the plantation aged, and the diversity of fungal guilds such as arbuscular mycorrhizal fungi, molds, yeast and putative plant pathogens.

Both β-diversity and indicator species analyses indicate important differences in the fungal community composition between the bulk soil and nests. The most common genera in the bulk soil were similar to those previously described in orchard soils of *T. melanosporum* (Orgiazzi *et al.* 2013; Liu *et al.* 2016; Oliach *et al.* 2020; Herrero de Aza *et al.* 2022) and *Tuber indicum* Cooke & Massee (Fu *et al.* 2016; Li *et al.* 2018; Liu *et al.* 2021), with the presence of a diversity of saprophytes and endophytes belonging to *Mortierella, Fusarium, Humicola, Acremonium, Solicoccozyma* and Didymellaceae, molds of *Chaetomium* and *Aspergillus* and soil-borne plant pathogens of *Alternaria* and *Ilyonectria*. On the other hand, the nests were characterized by a higher occurrence of both ascomycetous and basidiomycetous yeasts including *Candida, Apiotrichum, Saitozyma, Tausonia* and *Leucosporidium*, together with species of *Trichoderma* and *Chrysosporium*. Many of these genera are dominant in the *Sphagnum* peat moss microbiome, together with some *Mortierella* and *Solicoccozyma* species, with a high evenness at the initial sampling indicating that this

community was introduced into the orchards with nests (Menkis *et al.* 2016; Taparia *et al.* 2021). The introduction of these taxa to the soil ecosystem may provide a contingent of microbial diversity beneficial for the evolution of certain functional properties. In this sense, yeasts may play a role in nutrient cycling and soil structuring, whereas some *Mortierella* species could promote plant growth (Botha 2011; Ozimek and Hanaka 2021; Vandepol *et al.* 2022). However, studies analyzing the influence those particular taxa have on the productivity of *Tuber* and *Terfezia* in plantations are scarce, with contradictory reports in the case of *Mortierella*, *Fusarium* and *Alternaria* (Mello *et al.* 2010; Oliach *et al.* 2020; Arenas *et al.* 2021; Wang *et al.* 2022).

Regarding the ectomycorrhizal fungal community, spatiotemporal patterns were similar to those of the broader fungal community. Ectomycorrhizal α-diversity was lower within nests, although no clear patterns associated with position were found in β-diversity, indicating a similar ectomycorrhizal community composition in the three positions. Both results could be related to the fact that only one ectomycorrhizal species (apart from *T. melanosporum*) was found in the substrate before being installed in the field. In the bulk soil and the interphase, no correlation between ectomycorrhizal richness and truffle mycelium abundance was found, in agreement with results of Oliach et al. (2022) and suggesting a more negative relationship of *T. melanosporum* with ectomycorrhizal fungi than with other fungal guilds. This could be related to the fact that they directly compete for root colonization. However, in opposition to this hypothesis, the relationship between the abundance of *T. melanosporum* and that of other ectomycorrhizal fungi was positive in the three positions (Fig. 6c). In any case, the low levels of potential ectomycorrhizal competitors in nests may allow pioneer mycorrhizal species to more fully occupy this niche.

Many interactions appeared in our study involving the variables plot and year, showing that spatiotemporal patterns of *T. melanosporum* mycelium and fungal communities

in truffle-producing soils are complex, as previously indicated by Queralt et al. (2017) for truffle mycelium and Castaño et al. (2018) for pine-associated Mediterranean fungal communities. Our results found significant differences between the two orchards regarding the dynamics of *T. melanosporum* mycelium, as well as α- and β-diversity of both the overall fungal community and the ectomycorrhizal community detected in the soil and truffle nests. Some of these between-orchard differences were observed in the nests, even though the same substrate was used in both orchards. These differences could be related to factors such as rainfall in the previous month (Queralt *et al.* 2017) or truffle ascocarp productivity of plantations, which was higher in Mora, although the patterns are in all cases complex. Regarding temporal variability, from 2017 to 2018 we observed an increase in fungal richness not only in soil positions affected by nest installation, but also in the "control" bulk soil, making it difficult to discriminate whether this increase is due to a directional change or to microclimate or other interannual variability. All this complexity clearly indicates that studies not accounting for spatial and temporal replicates (i. e. multiple plots and years of study) should be interpreted with caution.

Finally, at a methodological level, it is important to point out the strong relation between the qPCR-measured mycelium abundance and the number of reads in metabarcoding. While amplicon sequence data is known to have PCR biases, thus considered to be semi-quantitative, our results suggest a power-function relation between these variables, consistent across the three positions. However, such relationships are not reliable for mycelium abundance lower than 0.01 mg g^{-1} sample, close to the detection limit of the qPCR (Ct above 30).

In conclusion, nests are a common strategy used to promote truffle productivity in managed orchards. While we did not find that *T. melanosporum* extraradical mycelium reached higher abundance in nests or in the soil – substrate interphase than in the bulk soil of

brûlés, *T. melanosporum* did appear to dominate the ectomycorrhizal community of the nest habitats. We also found that nests harbored a less rich and less diverse fungal community and ectomycorrhizal fungal community compared to the bulk soil of *brûlés*, and the composition of these two habitats was clearly different, which may improve the chances for pioneer fungal species such as *T. melanosporum* to occupy the niche. Finally, although the ability of nests to stimulate truffle fruiting was not explicitly tested here, our results indicate that this phenomenon is not due to increased mycelium in the nests. Future research may address whether this phenomenon is the result of relaxed competition, differences in soil microenvironmental factors, or the result of adding spores that act as spermatia to initiate truffle fruiting. Investigating the use of nests without spores and the addition of spores in the bulk soil could help disentangle the role of the various underlying mechanisms.

Conflict of 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.

CRediT authorship contribution statement

Conceptualization and methodology, S.S., S.G.-B., P.M., and G.M.N.B.; investigation, P.M., G.M.N.B., J.P., S.S. and G.B.; funding acquisition, S.S., G.B., P.M. and J.P.; formal analysis, G.M.N.B. and S.G.-B.; writing – original draft, S.G.-B., G.M.N.B., J.P., V.G. and I.L.; writing – review and editing, all authors.

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Data Availability

Amplicon sequencing data is available at the SRA: PRJNA938598.

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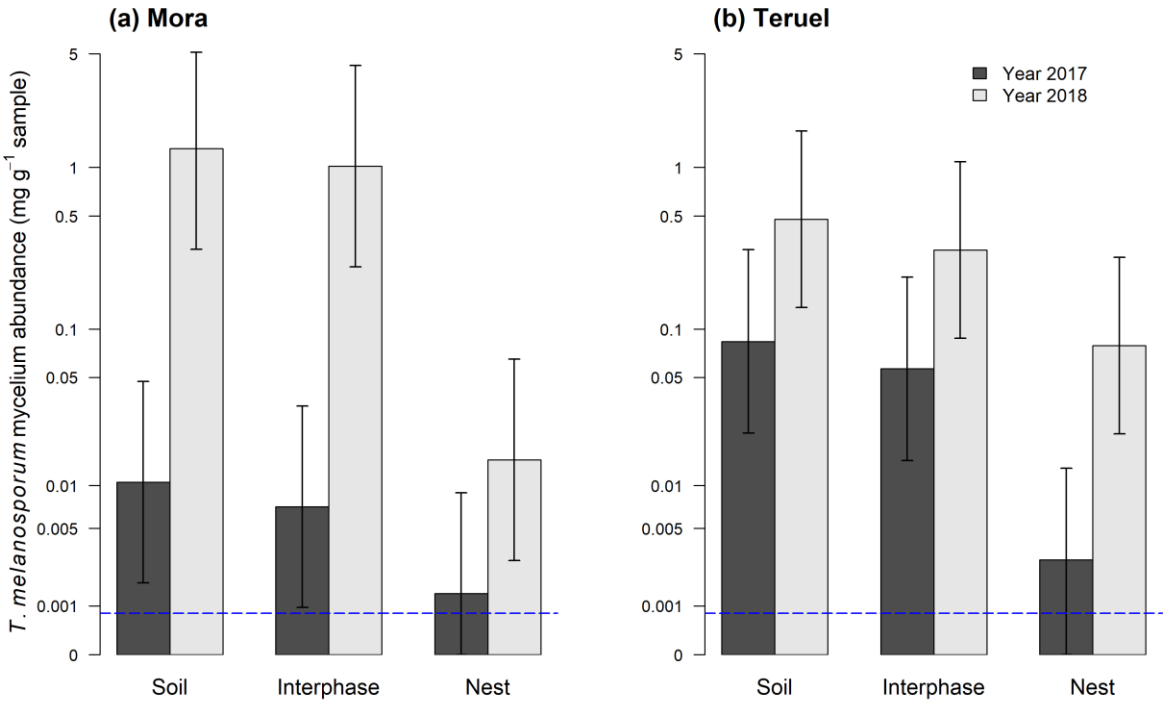

Figure 1. Abundance of *T. melanosporum* extraradical mycelium (log-scaled y-axis, mean predicted values and 95% confidence intervals, $\alpha = 0.05$, n = 135) in the bulk soil, interphase and truffle nests of the Mora orchard (a) and the Teruel orchard (b) one year (year 2017) and two years after setting up the nests (year 2018). The horizontal line marks *T. melanosporum* abundance in the substrate before use (8×10^{-4} mg g^{-1} substrate).

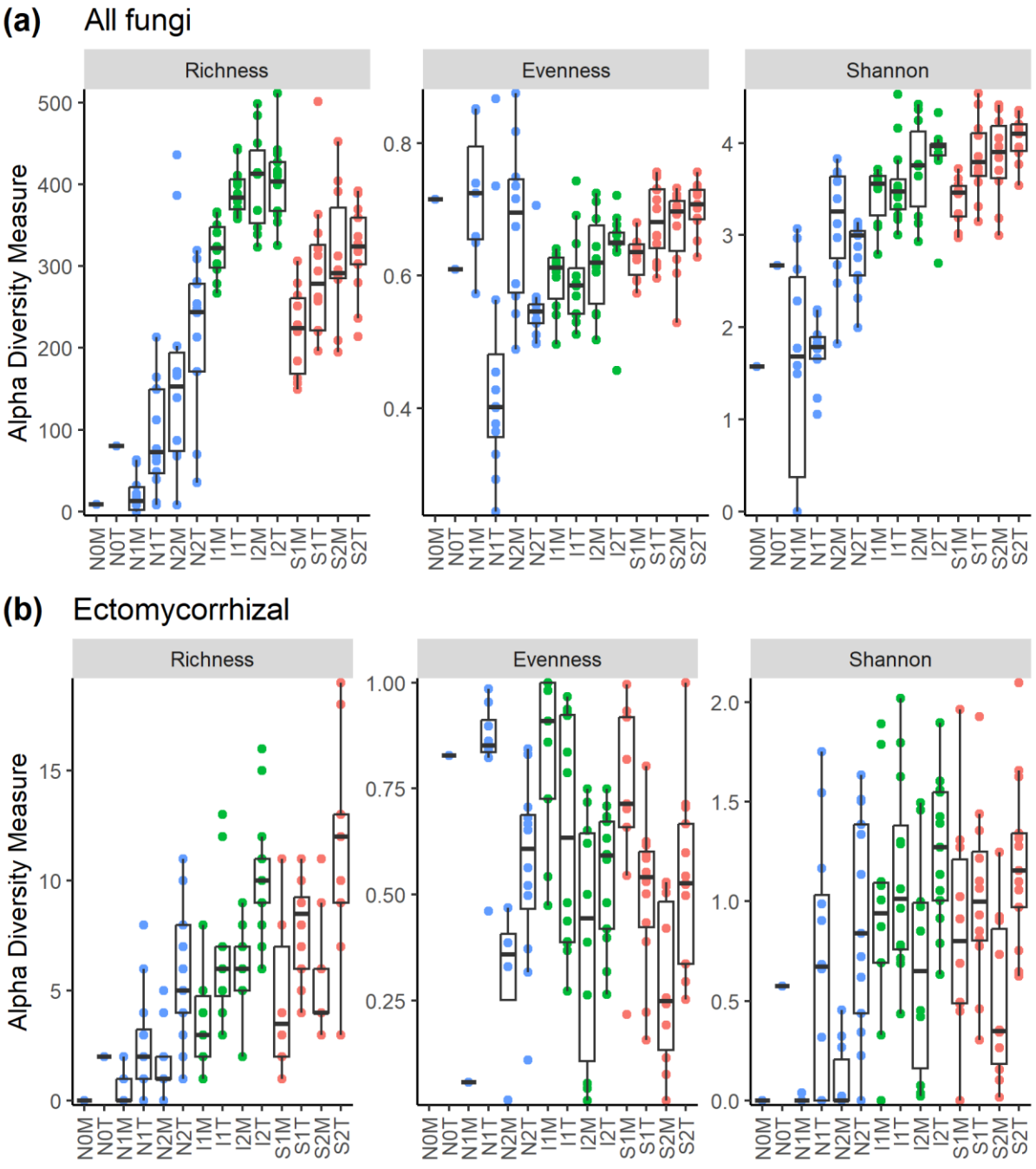

Figure 2. Boxplot of α-diversity measures (observed richness, evenness and Shannon index) for the fungal community (a) and the ectomycorrhizal community (b) in the analyzed samples (n = 137). N: nest, I: interphase, S: soil, M: Mora orchard, T: Teruel orchard. The number next to the initials indicates years from the setting up of the nests (0, 1 and 2 corresponding to years 2016, 2017 and 2018, respectively). In (b), evenness is not calculated for sample N0M because no ectomycorrhizal fungi were found.

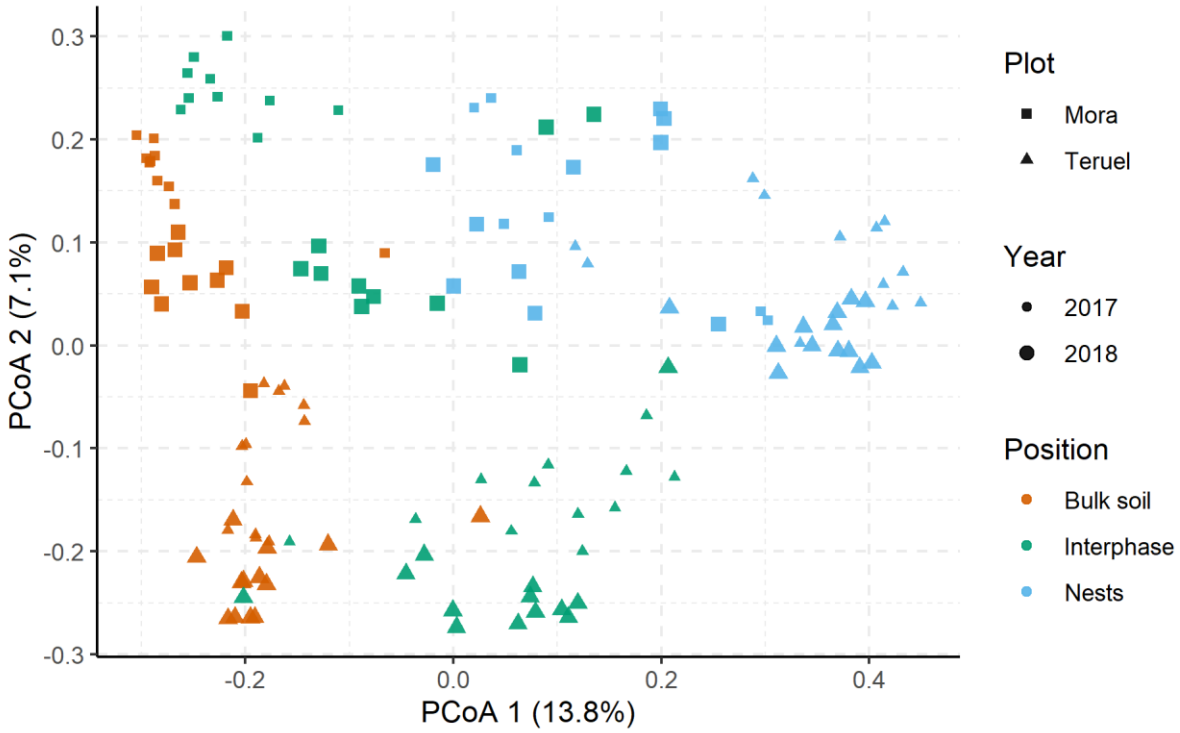

Figure 3. Principal coordinates analysis plot, using Bray–Curtis dissimilarity matrices, of fungal communities in truffle nests, interphase and bulk soil of two truffle orchards one and two years after setting up the nests (2017 and 2018 respectively, n = 132).

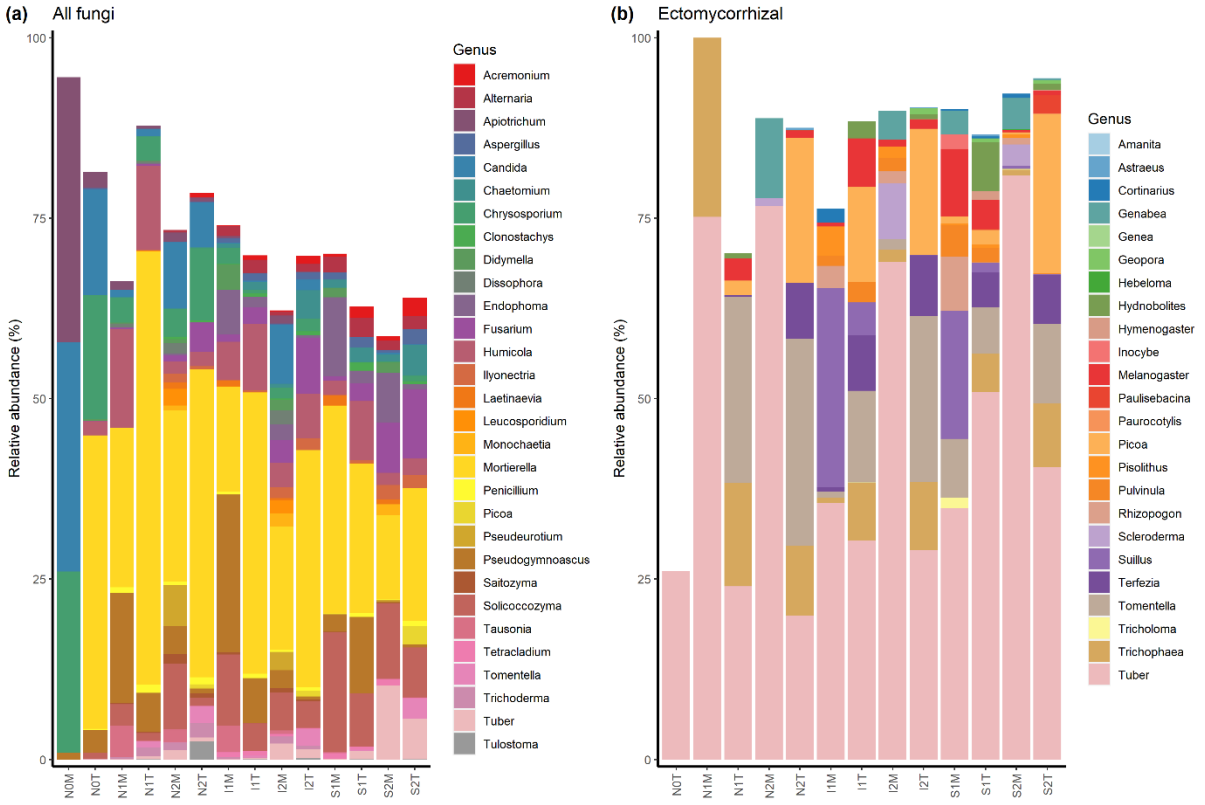

Figure 4. Stacked bar plot showing the relative abundance (%) of the most common genera of the overall fungal community (a), and the most common genera of ectomycorrhizal fungi (b) for each combination of plot, position and year. N: nest, I: interphase, S: soil, M: Mora orchard, T: Teruel orchard. The number indicates years from the setting up of the nests (0, 1 and 2 corresponding to years 2016, 2017 and 2018, respectively). The sample N0M is not shown in (b) because no ectomycorrhizal species was found.

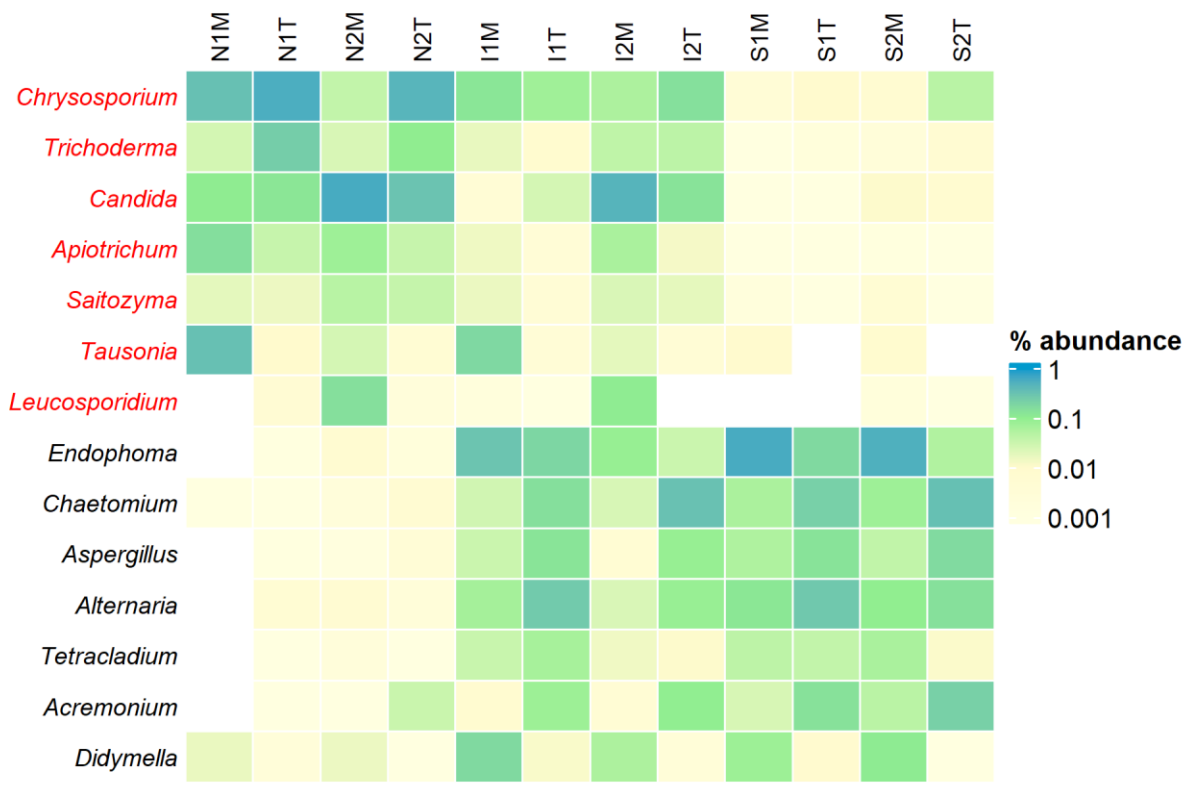

Figure 5. Heatmap showing the most frequent fungal genera with a significant association to nests and interphase (genera in red) or to soil and interphase (genera in black), according to the *multipatt* analysis, and their relative abundance (%) for each combination of plot, position and year. N: nest, I: interphase, S: soil, M: Mora orchard, T: Teruel orchard. The number next to the initials indicates years from the setting up of the nests (1 and 2 corresponding to years 2017 and 2018, respectively).

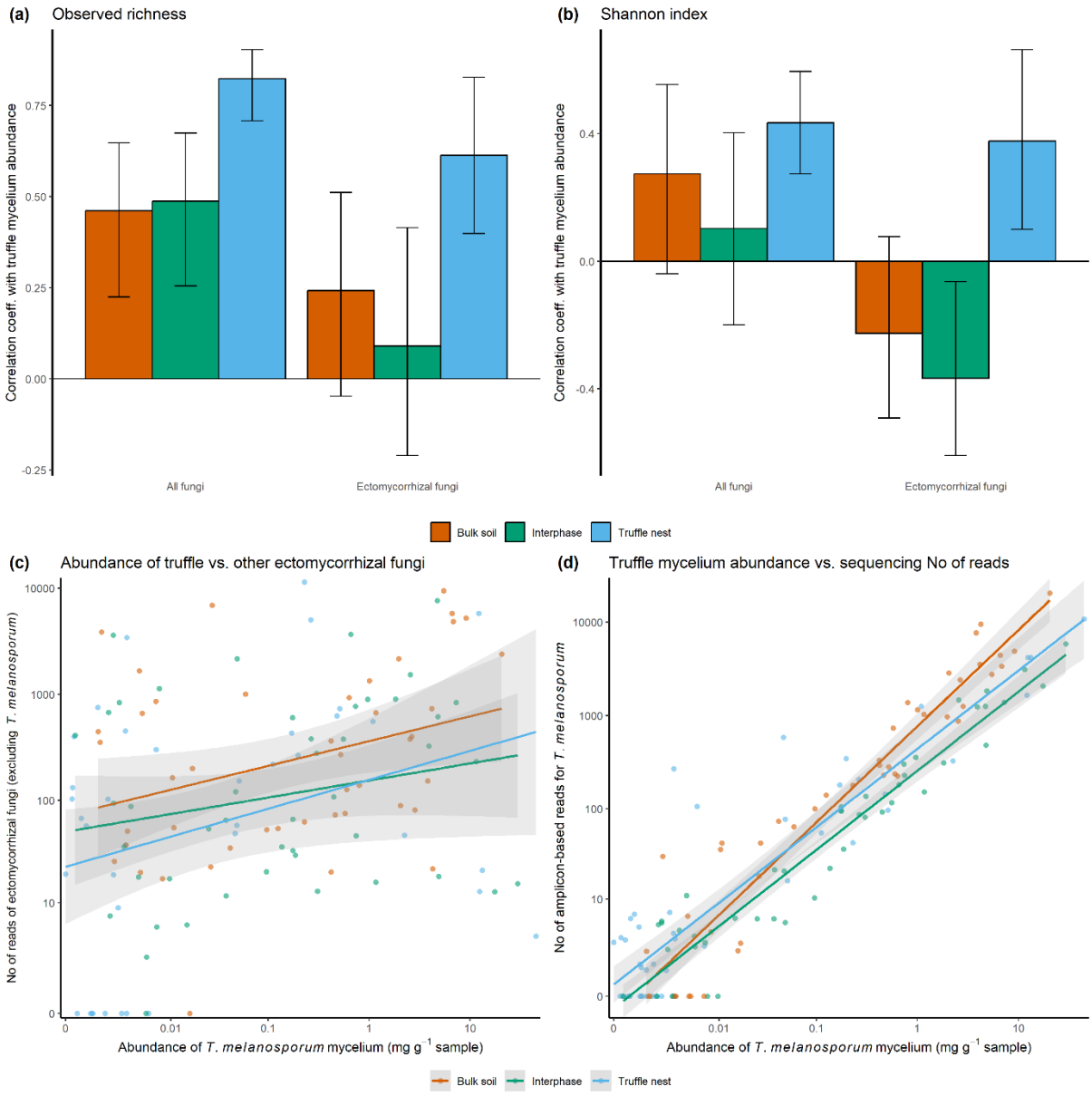

Figure 6. Pearson correlation coefficients of the abundance of *T. melanosporum* mycelium with the observed richness (a) and the Shannon's index (b) of the fungal community and the ectomycorrhizal community in the bulk soil, interphase and nests (mean predicted values and 95% confidence intervals, $\alpha = 0.05$, n = 135). (c) Relation between the abundance of *T. melanosporum* mycelium and the No of read of other ectomycorrhizal fungi in the amplicon sequencing (log-scaled axes, predicted relation and 95% confidence band, $\alpha = 0.05$, n = 129, after excluding samples with no ectomycorrhizal reads). (d) Relation between the abundance of *T. melanosporum* mycelium and the number of reads for *T. melanosporum* according to

amplicon sequencing (both axes log-scaled, predicted relation and 95% confidence band, α = 0.05, n = 137).