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Abstract

Ectomycorrhizae form a multitrophic ecosystem made of the association between tree roots, mycelium of the ectomycorrhizal fungus, and a complex microbiome. Despite the expected importance of these microorganisms for the physiology of the tree host and the functioning of the ectomycorrhizal symbiosis, few studies have carried out in depth analyses of ectomycorrhiza associated bacterial community composition and their temporal dynamics. Our objective was to investigate the composition and the dynamic from summer to winter time of bacterial communities associated with ectomycorrhizae (EcM) from *Tuber melanosporum* in a hazelnut tree *Corylus avellana* plantation. We used 16S rRNA based pyrosequencing to compare the bacterial community structure and the richness in *T. melanosporum's* EcMs with those of bulk soil. The EcMs of *T. melanosporum* harboured distinct bacterial communities from those of the bulk soil, with an enrichment in Alpha- and Gamma-Proteobacteria. In contrast to the bacterial communities colonising truffle ascocarps that deeply varies in composition and richness during the maturation of the fruiting body and to those from the bulk soil, *T. melanosporum* EcM-associated bacterial community composition stayed rather stable from September to January. Our results fit with the recent demonstration performed on the same experimental site at the same period that a continuous supply of carbohydrates and nitrogen occurs from EcMs to the fruiting bodies during the maturation of the ascocarps, that could potentially create a stable niche in the ectomycorrhizosphere while the phenology of the tree changes.

Keywords: *Tuber melanosporum*, bacterial communities, temporal evolution, soil-ectomycorrhizae interface, ectomycorrhizosphere, 16S rRNA based pyrosequencing.

Introduction

Among soil microorganisms, ectomycorrhizal fungi (EcM) that form symbiotic associations with plants are important actors in terrestrial ecosystems. Owing to their ability to forage large volumes of soil where they access nutrients such as phosphorous, nitrogen (N) and inorganic compounds, to degrade organic matter, and to weather minerals, EcM fungi improve the nutrient uptake capacity of their associated trees (Smith and Read 2008; Courty et al. 2010). In exchange, trees provide the symbiotic fungi with carbohydrates that are transferred to the fungus in the ectomycorrhizae, a mixed organ formed by tree roots and fungal mycelium. This particular interface, called the ectomycorrhizosphere, connects tree roots to the surrounding soil and forms an unique environment both in terms of their physicochemistry and microbiology (Rambelli 1973; Linderman 1988). The mycorrhizosphere is comparable to the rhizosphere by its intense bacterial colonisation, but differs from the rhizosphere by the nature of the main chemicals released in soil. Especially, mycorrhizal roots are characterised by elevated concentrations of fungal derivatives such as trehalose, mannitol or N-acetyl glucosamine, which are less represented in the rhizosphere (Martin et al. 1984; Martin et al. 1985). The bacterial communities of the mycorrhizosphere interact physically with mycorrhizal fungi through the symbiotic mantle or the hyphal network, and also chemically by metabolising mycorrhizal root exudates (Frey-Klett et al. 2005; Uroz et al. 2007). Through these physicochemical characteristics, mycorrhizal fungi enrich their vicinity with specific bacterial communities capable of improving fungal and plant growth through the access to inorganic nutrients or the production of hormones and/or vitamins (Uroz et al. 2007; Deveau et al. 2010; Uroz et al. 2013).

Although the enrichment of specific bacterial communities in the mycorrhizosphere has been demonstrated in multiple studies, these analyses were focused only on few fungal species and mostly based on culture-dependent and fingerprinting approaches giving a partial view on the real community composition and structure (Uroz et al. 2007; Izumi et al. 2007; Burke et al. 2008; Izumi and Finlay 2011). The factors that impact bacterial selection, and the parameters that drive their community composition and diversity in mycorrhizosphere are yet to be understood. While there are indications that the ectomycorrhizal fungi themselves might impact the associated bacterial community composition, some studies suggest that fungi alone are not the only drivers in EcM bacterial community structuration (Izumi et al. 2007; Burke et al. 2008; Izumi and Finlay 2011; Uroz et al. 2012). Furthermore, seasonal variations of the associated bacterial communities (diversity, structure, and composition) are expected to occur because the transfer of photosynthates from the tree to the mycorrhizal partner has a direct impact on the fungal metabolism and is known to vary along seasons (Epron et al. 2011; Churchland and Grayston 2014). Although seasonal changes have been evidenced in the rhizosphere of various

plants (Rasche et al. 2011; Collignon et al. 2011; López-Mondéjar et al. 2015), the relative stability or dynamics of the EcM associated bacterial communities are not known.

Determining such temporal evolution of natural mycorrhizosphere bacterial communities remains a technical challenge as it requires to be able to collect samples at different time from the same mycorrhizal species. In general, tree roots are colonised by complex and dynamic EcM fungal communities that evolve along time, and thus reducing the probability to sample the same EcM species through time. To reduce this problem we took advantage of the controled 'mycorrhization' process that reduces the variability owing to the high level of mycorrhization by a single species. We used *Corylus avellana* trees that were highly mycorrhized with *Tuber melanosporum* and planted in the experimental truffle orchard of Rollainville (North of France). This made possible to collect EcMs of the same species at different dates. *Tuber melanosporum* Vittad. is an Ascomycete ectomycorrhizal fungus well known for its production of hypogeous fruiting bodies (ascocarps) called Périgord black truffles. Life cycle of *Tuber* species differs from many other EcM fungi in the duration taken to develop their fruiting bodies (Le Tacon et al. 2015a). Ascocarps take several weeks to few months to grow, compared to merely few days for other well-known epigeous EcM fungi such as *Boletus* sp. or *Cantharellus* sp. The *T. melanosporum* ascocarp is thought to be functionally linked to its host tree through EcMs from its birth in late spring to its maturation during the following winter (Le Tacon et al. 2013; Le Tacon et al. 2015a; Le Tacon et al. 2015b). From the latter studies, we infered that *T. melanosporum* EcMs serve as a nutritional pipeline during the entire duration of the ascocarp development (Le Tacon et al. 2013; Le Tacon et al. 2015b).

We demonstrated previously that *T. melanosporum* ascocarps are heavily colonised by complex bacterial communities and the community compositions evolved along the maturation of the ascocarps (Antony-Babu et al. 2014). We also demonstrated that the ectomycorrhizosphere of *T. melanosporum* was highly colonised by complex bacterial communities that differed significantly in terms of structure, diversity and composition from those of the ascocarps (Antony-Babu *et al.* 2014); the study was made at only one sampling date (November 2010) on the Rollainville experimental truffle orchard. EcMs being connected to the ascocarp for the entire period of growth and maturation, we can wonder whether the ascocarpic and the EcM microbial communities are connected and whether they vary similarly along the ascocarp development in autumn and winter. Thus, our objective was to assess whether temporal changes occurred in the composition, richness and structure of the EcM bacterial communities along the development and maturation stages of truffle ascocarps. A subsidiary objective was to confirm that *T. melanosporum* EcMs select in its vicinity specific bacterial communities compared to the surrounding bulk soil and the ascocarps. Our strategy was to collected spatially distant soil samples and ectomycorrhizal roots under the same tree from September 2010 to January 2011, as well as ascocarps

126 (Antony-Babu *et al.* 2014). We analysed bacterial community structure, composition and diversity using
127 16S rRNA gene amplicon pyrosequencing on EcM, ectomycorrhizosphere and bulk soil samples.
128
129 **Materials & methods**
130 **Sampling site**
131 All samples were collected under a single hazel tree (*Corylus avellana)* labelled "A11" in the truffle
132 orchard of Rollainville (48° 21' N, 5° 44' E; Lorraine region, France) during the truffle maturation process
133 from September 2010 to January 2011. Characteristics of the truffle orchard and soil properties are
134 described in Antony-Babu *et al.* (2014).
135
136 **Sampling method and storage**
137 Three types of samples were collected: *T. melanosporum* ectomycorrhizal root tips (EcM), the soil at
138 the interface with the EcM (EcM-I - soil adhering to the ectomycorrhizal root tip) and the surrounding
139 bulk soil (BS). At each sampling date (28th of September 2010, 17th of November 2010, 22nd of
140 December 2010 and 27th of January 2011), samples were collected from three or four independent
141 locations around the tree labelled "A11" and spaced about 2 meters apart from the trunk (Table 1). The
142 samples were stored on ice during transport to the laboratory. At the laboratory, ectomycorrhizal root
143 tips were collected under a microscope in a laminar flow cabinet. Ectomycorrhizae were characterised
144 both by morphotyping and using specific molecular probes as described by Paolocci (Paolocci 1999).
145 One to 5 grams of non adhering bulk soils surrounding ectomycorrhizae (BS) were collected. Soil
146 adhering ectomycorrhizae (ECM-I; 100 to 300 mg) were obtained using needles and forceps. All
147 samples were stored in -80°C directly without addition of any preservative additives. A total of 48
148 samples was obtained (Table 1).
149
150 **DNA extraction**
151 Due to the different type of samples considered in the study (mycorrhizal tips and soil), two different
152 extraction methods were necessary to isolate high quality genomic DNA from the samples compatibles
153 with 16S rRNA gene amplicon pyrosequencing (Antony-Babu et al. 2013). Soil samples were processed
154 using Fast-DNA Spin kit (Q-Biogene, Heidelberg, Germany) and ectomycorrhizal samples were isolated
155 using DNeasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France), according to manufacturer's guidelines.
156
157 **Barcoded pyrosequencing, DNA sequence processing and taxonomic analyses**
158 The extracted DNA samples covering different habitats (BS, ECM and ECM-I) through time were used
159 as templates to generate 16S rRNA gene amplicon libraries. The primers 787r (xxxxx-

ATTAGATACCYTGTAGTCC) (Nadkarni et al. 2002) and 1073F (xxxxx-ACGAGCTGACGACARCCATG) (On et al. 1998) were used to generate PCR fragments of ca 250 bp. where "xxxxx" represents the sample identification barcode sequences. PCR amplifications were carried out with the following conditions: 94°C for 10 min, 30 cycles of 30 seconds at 94°C (denaturation), annealing temperature starting at 48°C for 45 seconds followed by 72°C for 90 seconds (extension). The reactions concluded with final extension for 10 minutes at 72°C. PCR amplicons were purified using Qiagen PCR purification kit or Qiagen gel purification kit for samples showing multiple bands. Quality of the purified PCR amplifications were checked on 1% agarose gels using the Low DNA mass ladder (Invitrogen). PCR product quantifications were performed using both gel electrophoresis and nanodrop spectrometry. The PCR barcoded amplicons were pooled into respective libraries in equimolar concentrations. Amplicon pyrosequencing was performed on the GS-FLX 454 Titanium platform of Beckman Coulter Genomics (Danvers, MA, USA) as half a run.

The sequences obtained were analysed using MOTHUR (Schloss et al. 2009). After demultiplexing and quality control (quality score=30). The sequences were then processed using MOTHUR suite to determine operational taxonomic units (OTUs, 97 % sequence similarity), rarefaction analysis, richness estimates Chao1 and Shannon-Weaver index. Rarefactions curves were generated using the R package (www.r-project.org). After quality trimming and denoising, sets of 16S rRNA sequences were adjusted randomly at 1,245 (corresponding to the smallest sample). Phylogenetic affiliation was performed (80% sequence similarity) with the MG-RAST platform (Metagenome Rapid Annotation using Subsystem Technology, Meyer et al. 2008) and the RDP database (Ribosomal data project, Cole et al. 2009).

Statistical analyses

The different relative values (%) used in this study were transformed by arcsin sqrt for statistical analyses. The habitat and sampling times effects on the structure of the bacterial communities were determined by analysis of variance (ANOVA) at a threshold level of P=0.05 associated with a Fisher test and analyses of similarity (ANOSIM) based on Bray-Curtis distances were performed 16S rRNA pyrosequence data using the anosim function of R Vegan package (Oksanen et al. 2015). Analysis of variance and multivariate analyses were performed with XLStat 2011 (Addinsoft, Paris, France).

Nucleotide sequence accession numbers. The 454 sequences generated have been deposited on the Sequence Read Archive of the NCBI under the the SAMN04103598 to SAMN04103628 and SUB291410 accession numbers.

Results

Pyrosequencing results

A total of 400,965 raw pyrotag reads were obtained across the bulk soil, soil-ectomycorrhiza interface and ectomycorrhiza mantle samples. After quality filtering and chimera removal 180,206 sequences remained to be analysed, showing a number of sequences per sample varying between 1,245 and 8,920. To be able to compare the 48 samples, the same level of surveying effort (n=1,245 sequences per sample) was applied on each sample of the dataset. A total of 33,207 OTUs were identified after bioinformatics analyses, but a detailed analysis revealed that only 422 OTUs were represented by 10 sequences or more across the samples. Regardless of the sampling date, slightly less numbers of OTUs were detected in ECM compared to the two other compartments (926 +/- 33 vs 1100 +/- 19 observed OTUs ; ANOVA $p<0.001$). In contrast, the number of OTUs did not vary significantly between BS and ECM-I notwithstanding the sampling dates considered. Non-parametric analyses also confirmed this trend. A lower richness was predicted in the ectomycorrhizosphere compared to the bulk soil and the soil-ectomycorrhiza interface by the Shannon and InvSimpson indexes ($P<0.001$ ANOVA, Table 2).

Comparison of bacterial communities associated to Bs, EcM-I and EcM

The overall analysis revealed that the most abundant phyla that are found across different compartments were Proteobacteria (15 to 17%), Actinobacteria (17 to 20%), Bacteroidetes (8 to 10%), Firmicutes (1 to 5%), Verrucomicrobia (1 to 5 %), Acidobacteria (0.2 to 4%), Planctomycetes (0.4 to 2%), Chloroflexi (0.3 to 1%) and Chlamydiae (0.2 to 1%) as shown by the taxonomic assignment (Figure 1). Among the Proteobacteria, Alpha-proteobacteria were the most represented (4.1 to 9.4 %) followed by Delta- (2.0 to 7.1 %) Beta- (2.3 to 5.6 %), Gamma- (1.0 to 5.9 %), and Epsilon-proteobacteria (0 to 0.1 %).

Nevertheless, the above distribution was not uniform among the compartments as highlighted by multivariate and ANOSIM analyses performed on the relative distribution of these phyla. In all the time points that were considered, the EcM compartment was distinct from those of BS and EcM-I according to the first axis of the PCoA that explained 50 % of the variance in the data at the class level (Figure 2, ANOSIM $p<0.05$). Such similar pattern was observed also at the genus level. Regardless of the taxonomic hierarchy used, the BS and ECM-I compartments were more closely related to each other than to the EcM compartment. When the taxonomic resolution of the analyses were increased it was observed that sequences from Alpha- and Gamma-proteobacteria and especially the sequences assigned to the *Bradyrhizobium* genus, and the Caulobacterales and Rhodospirillales orders were more prevalent in the EcM compartment than the other two (Table 3). In contrast, sequences related to Delta-

proteobacteria, Acidobacteria (*Acidobacterium* and *Candidatus Koribacter*) Flavobacteria (*Flavobacterium*), Firmicutes (*Bacillus*, *Finegoldia*) Verrucomicrobia (*Pedosphaera, Verrucomicrobium*), Planctomycetia (*Pirellula*) were less in the EcM compartment. Actinobacterial genera such as *Mycobacterium, Nocardioides, Rubrobacter* were less frequent in the EcM compartment while representatives of the genus *Thermoleophilum* were enriched in the EcMs. Furthermore, reads that were unassigned to any known taxa were more common in EcM than in the other two compartments, representing 20 to 40 % and 4 to 15% of the reads, respectively.

Temporal evolution of bacterial communities associated to BS, EcM-I and EcM

Richness of the bacterial communities in the three compartments considered did not vary along the sampling season regardless of the compartment considered (Table 2). However, the constituents of the bacterial assemblages differed from September 2010 to January 2011 and varied according to the compartment considered. According to PCoA, the bacterial community dynamics in the BS compartment was more affected by seasonal changes than those associated with the EcM compartment. Indeed, BS and EcM-I samples were separated by sampling time according to the second axis of the PCoA (18 % of the variance), but not the EcM one's (Figure 2). Three phyla showed statistically significant differences to temporal variations in BS and EcM-I compartments: Acidobacteria, Bacteroidetes and beta-Proteobacteria. Each of these three taxa exhibited different abundance patterns along the sampling time. Since BS and EcM-I samples showed similar patterns, only BS samples are given in figure 3a to simplify the reading. Beta-proteobacteria were highly represented in September in comparison to the other sampling dates. In contrast, Acidobacteria (*Candidatus Koribacter*) followed an opposite trend as their levels increased in November, December and January compared to September. Bacteroidetes (*Miscroscilla*) communities increased in November. Actinobacteria did not show significant variation at the phylum level but different significant patterns of variation were observed depending on the genus considered. While reads pertaining to the genus *Rubrobacter* were more abundant in December and January than in November, genus *Terrimonas* and *Nocardioides* were more abundant in November and December, respectively (data not shown).

Although a temporal differenciation in EcM communities was not explicit in the PCoA analyses, some specific genera were consistently more enriched at some periods (ANOVA, $p<0.05$). For example *Actinobacterial* genera *Thermoleophilum* and *Pseudonocardia* were more frequently represented in EcM than in BS and ECM-I in September and November, and in January, respectively. In contrast, Bacteroidetes representatives of the genus *Microscilla* and *Actinobacteria* from the genus *Nocardioides* were not detected in the EcMs collected in November and in December, respectively. Alpha-

proteobacteria showed a negative trend where it decreased from September to January, albeit this trend was not statistically significant (Figure 3b).

As some of the ascocarpic samples of Antony-Babu *et al.* (2014) were collected at the same dates (November, December and January) and using the same molecular and bioinformatic procedure, we performed comparison of the EcM and ascocarp compartments across time. Both multivariate and ANOVA analyses revealed that the EcM bacterial communities were separated from those of the ascocarp compartments (gleba and peridium) (Figure S1). Whatever the sampling date considered, Actinobacteria, Chloroflexi, Planctomycetes, Delta-proteobacteria and Verrucomicrobia appeared significantly enriched in the EcM compartment compared to the gleba ($p<0.05$; Figure 4). Significant variations were also observed between EcM and gleba compartments for other taxa such as Firmicutes, Alpha-, Beta- and Gamma-proteobacteria at specific time points (Table S1).

Discussion

The recent advancements of high throughput sequencing methods has led to the "re"discovery that almost all eukaryotic organisms host a complex microbiome. It is interesting to note that in most cases the composition and the activity of these microbiomes strongly impact the physiology, health and resilience to pertubations of their hosts (Bulgarelli et al. 2012; Goh et al. 2013; Shafquat et al. 2014; Rädecker et al. 2015). For example, it is now evident that the human gut and skin microbiomes influence human immunity, physiology and even behaviours (Schommer and Gallo 2013; Yano et al. 2015). Similar studies on plant models such as *Arabidopsis thaliana* have also highlighted the strong impact of the plant microbiome on host functioning (Lebeis 2014; Panke-Buisse et al. 2015). In comparison to these examples, studies deciphering the composition and potential role of tree microbiomes are scarce. Although EcMs are known for decades to host specific bacterial communities that physically and metabolically interact with the symbiotic fungus and the host plant (Linderman 1988), only three studies have thoroughly analysed the microbiomes of EcMs using high throughput sequencing: the first one performed on EcMs between *Quercus petrea* and *Xerocomus pruinatus* and *Scleroderma citrinum* (Uroz *et al.* 2012), the second on EcMs of the herbaceous plant *Bistorta vivipara* – one of a few herbs that form EcMs (Vik et al. 2013) - and the third on EcMs between *Tuber aestivum* and *Carpinus betulus* (Gryndler et al. 2013). Through the study presented here we provide description of a fourth system, i.e the bacterial communities of EcMs between *T. melanosporum* and *C. avellana.*

Firstly, this study confirms the selection of specific bacterial communities in the ectomycorrhizosphere of *T. melanosporum,* as previously known for other EcM fungi (Mogge et al. 2000; Assigbetse et al.

2005; Frey-Klett et al. 2005; Uroz et al. 2007; Izumi et al. 2008; Calvaruso et al. 2010). Those communities differed from their respective bulk soil all through the sampling period. Bacterial communities of *T. melanosporum* ectomycorrhizosphere were characterised by the exclusion of taxa such as Acidobacteria, Flavobacteria, Firmicutes and Verrucomicrobia while other taxa among which Alpha-Proteobacteria and Actinobacteria of the genus *Thermoleophilum* were enriched in the EcMs. Vik *et al.* (2013) also observed a significant exclusion of members of Planctomycetes, Firmicutes and Acidobacteria from the ectomycorrhizosphere of *B. vivipara*. However, Uroz *et al.* (2012) observed that Acidobacteria were dominant in EcMs of *X. pruinatus* and *S. citrinum* in temperate and acidic soils. Similar to the study presented here, members of the *Bradyrhizobium* genus were also highly represented in EcMs of *X. pruinatus* and *S. citrinum.* Thereby, these data support the hypothesis, previously proposed based on less exhaustive methods, that EcMs bacterial communities are quite diverse in their composition and that several factors mediate their structuration: the host tree (Calvaruso et al. 2010), the fungal symbiont (Izumi and Finlay, 2011), the soil characteristics and the composition of the soil bacterial communities. However some common trends among bacterial communities from different EcM species and host trees also exist, suggesting a possible core bacterial community (Burke et al. 2008; Uroz et al. 2012). Sampling of a large number of EcMs species is required to test whether EcMs of different species and tree host share a core bacterial community. Such a study would also aid in the identification of factors that drive the composition of the accompanying specific bacterial community.

Our results revealed that the overall composition of *T. melanosporum* EcM bacterial communities was very close to the one obtained in *T. aestivum* EcMs of hornbeam using similar 454 pyrosequencing tools (Gryndler et al. 2013). In both cases, a dominance of Actinobacteria and Proteobacteria among which Alpha-, Beta- and Delta-Proteobacteria, were retrieved. Interestingly, Actinomycetes were isolated from *T. borchii* EcMs (Sbrana et al. 2002). Within the Actinomycetes phylum, members of the Pseudonocardinae sub-order were abundant in both *T. aestivum* and *T. melanosporum* EcMs, and were part of the ten most represented genera in January samples. Members of the Pseudonocardinae order are often found colonising cave walls, thanks to their ability to degrade recalcitrant humic substances and lignocellulose (Stomeo et al. 2008; Porca et al. 2012). They are also found to be associated with leaf cutting ants that culture fungal gardens (Barke et al. 2010; Schoenian et al. 2011). Although their direct fungal helper functions are unknown, they are envisaged to protect the fungi from other competing fungal contaminants (Schoenian et al. 2011). Unfortunately, no cultivable bacterial strains of this order have been isolated from truffles so far, thus making difficult any conjecture on their potential functional interaction with *Tuber* sp. EcMs.

A striking contrast between *T. aestivum* and *T. melanosporum* EcM bacterial communities are their diffference regarding *Bradyrhizobium*. This genus was found to be generally excluded from EcMs of *T. aestivum* while they are particularly enriched in *T. melanosporum* EcMs, as well as in all the truffle species ascocarps analysed so far (Barbieri et al. 2005; Barbieri et al. 2010; Antony-Babu et al. 2014). *Bradyrhizobium* strains were also retrieved at high frequency from other EcM fungi such as *Cenococcum geophilum* (Kataoka et al. 2008). Many of these bacteria, although not all, have the ability to fix N, leading some to hypothesize that these bacteria contribute towards the fungal nutritional requirements for N (Kataoka et al. 2008; Barbieri et al. 2010; Antony-Babu et al. 2014). However, a demonstration of N-fixed transfer from these bacteria to the fungus is missing so far. Functional tests on cultivable bacterial isolates and genomic mining may help to better understand what specifically links this bacterial genus to truffles.

Development of *T. melanosporum* ascocarps occur while the physiology of the tree host changes: it starts at the end of summer/beginning of automn while trees are still photosynthetically active and it ends during the dormant period of trees (November to March). Changes in the tree phenology are coupled to seasonal modifications of edaphic parameters; soil temperature decreases and soil moisture increases at fall (Brockett et al. 2012). These environmental parameters have been shown to strongly impact soil bacterial community composition (Cleveland et al. 2006; Tabuchi et al. 2008; Stres et al. 2008; Rasche et al. 2011; López-Mondéjar et al. 2015). In addition, the fall of leaves in October-November contributes to litter renewal, thereby providing new organic carbon (C) and N sources to the soil microorganisms. Few studies have documented the response of deciduous forest soil bacterial communities to temporal or seasonal variations (Rasche et al. 2011; López-Mondéjar et al. 2015). In line with these two studies, we observed a modification of the soil bacterial communitiy composition from summer to winter. In our study, this change cannot be attributed to the input of fresh nutrients coming from litter decomposition. As part of the experimental set-up, the falling leaves were collected with a net, preventing a leaf litter development. The falling leaves were removed to enable a parallel ^{13}C isotopic labelling of the same hazelnut tree considered in our study so as to assess the heterotrophic behaviour of the ascocarp for C (Le Tacon et al. 2013). Alternatively, an increase of the soil moisture content may have influenced the abundances of Acidobacteria and Beta-proteobacteria in the soil: the Acidobacteria abundance increased from September to January meanwhile Beta-Proteobacteria abundance decreased. Acidobacteria can be particularly favoured by high soil moisture content, in contrast to Beta-Proteobacteria (Rasche et al. 2011). This inverted pattern may also be associated to

changes in the available C concentration in soil since Beta-Proteobacteria are copiotrophic microorganisms while Acidobacteria are oligogotrophic (Fierer et al. 2007).

The composition of EcM-associated bacterial communities was less affected by temporal variations comparatively to the soil and ascocarp compartments, suggesting that a parameter(s) stabilised the ECM communities along time. Such stability may be related to the nutrient exchanges occuring between the tree and its symbiont. We made a $^{13}CO_2$ isotopic labelling of this 'A11' hazelnut tree shoots at the same period. Results indicated that C remobilized from storage compartements of the host tree was continuously transferred from the EcMs to the ascocarps along the growing period of the ascocarp (Le Tacon et al. 2013). This continuous C supply to EcMs during the transition from the photosynthetically active season to the dormant season may create a stable environment in the EcMs. Part of the transferred C may be available to bacterial communities all along ascocarp development and maturation, thus explaining the low variations observed in the bacterial communitiy composition. It also suggests that the fungus could be the main driver of the composition of *T. melanosporum* EcM-associated bacterial communities, in contrast to other few EcM fungi studied so far (Izumi and Finlay 2011; Uroz et al. 2012). Whether this pattern is due to the peculiar long term development of the ascocarps of *T. melanosporum* that would act as continous sink for C from summer to winter or whether it is more widespread among EcMs fungi remains to be determined. Nevertheless, the relative stability of the *T. melanosporum* EcMs bacterial communities composition strongly contrasts with the high dynamic of the bacterial community composition observed in the ascocarps during their maturation.

Conclusion

This study provides for the first time an exhaustive analysis of the diversity, structure and composition of the bacterial communities associated to EcMs of *T. melanosporum* during the development of its ascocarps. Our data, coupled to the previous ones of Mello *et al.* (2013) and Antony-Babu *et al.* (2014) obtained at other stages of the fungus life cycle, indicate that *T. melanosporum* influences bacterial communities in its vicinity. Based on these studies, we suggest that *T. melanosporum* creates at least four different habitats: the Brulé i.e the soil area almost completely devoid of vegetation around trees colonised by *T. melanosporum* (Mello et al. 2013), the gleba, the peridium (Antony-Babu et al. 2014) and the EcMs (this study). Each of these habitats harbour distinct bacterial communities that are most probably selected from the bulk soil reservoir and that vary along the seasons and depending of the habitat considered. While the bacterial community composition of the ascocarps strongly varies with the stage of development and maturation of the ascocarp, the composition of the bacterial communities colonizing the EcMs remains rather stable along time. We hypothesize that this stability is associated to the continuous supply of nutrients coming from tree host and transferred to the ascocarps through the

EcMs. Thus a rather stable niche would be created in the ectomycorrhizosphere of *T. melanosporum* despite the modification of the tree host physiology and phenology at fall time. The functional properties of the bacteria that colonize these different niches, and the role they play in the life cycle of the fungus remain to be explored. A combination of functional analyses on cultivable strains and metatranscriptomic analyses may help answer these questions.

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564 **Tables**

565 **Table 1**. Types and number of samples analysed in this study. Samples of *Tuber melanosporum*
566 ectomycorrhizal root tips (EcM), of soil at the interface with the EcM (EcM-I - soil adhering to the
567 ectomycorrhizal root tip) and of the surrounding bulk soil (BS) have been collected.
568

	September	November	December	January
BS	3	4	4	4
EcMs - I	3	3	4	4
EcMs	3	4	3	3

569
570 **Table 2**. Alpha-diversity indices expressed as average values of three to 4 samples +/-SE
571

Time	Origin	Phylotypes	Shannon	Inv Simpson
Sept	**BS**	1097 ± 9	6.92 ± 0.01	2828 ± 325
	EcM-I	1110 ± 11	6.95 ± 0.02	3523 ± 326
	EcM	892 ± 26	6.44 ± 0.07	352 ± 70
Nov	**BS**	1104 ± 8	6.94 ± 0.01	3637 ± 244
	EcM-I	1092 ± 6	6.92 ± 0.01	3092 ± 212
	EcM	921 ± 39	6.46 ± 0.19	535 ± 165
Dec	**BS**	1150 ± 15	7.00 ± 0.02	6037 ± 1004
	EcM-I	1111 ± 10	6.95 ± 0.02	3417 ± 376
	EcM	972 ± 47	6.60 ± 0.16	698 ± 330
Jan	**BS**	1121 ± 17	6.97 ± 0.02	4423 ± 473
	EcM-I	1093 ± 15	6.93 ± 0.02	3292 ± 296
	EcM	919 ± 70	6.46 ± 0.19	553 ± 331

572
573
574 **Table 3**. Comparison of compartments at the class, order and genus levels. Class, order and genera
575 appear in a decreasing order of abundance. The 15 more abundant genera representing at least more
576 than 1% in a compartment are figured in the last column. Bold letters are used to highlight statistically
577 significant differences according to a one factor ANOVA followed by Tukey post test ($p<0.01$). Class,
578 order or genus for which a significant higher number of sequences was found in the EcM compartement
579 compared to the other compartment are underlined.
580

Class	Order	Genus
Actinobacteria	Actinomycetales	*Terrimonas*
BS=EcM-I<EcM	BS=EcM-I=EcM	BS=EcM-I=EcM
Bacteroidetes	Sphingobacteriales	*Streptomyces*
BS=EcM-I>EcM	BS=EcM-I=EcM	BS=EcM-I=EcM
Alpha-proteobacteria	Cytophagales	***Mycobacterium***
BS=**EcM-I<EcM**	BS=EcM-I>EcM	BS<**EcM-I>EcM**
Delta-proteobacteria	**Rhizobiales**	***Bradyrhizobium***
BS>**EcM-I>EcM**	BS=**EcM-I<EcM**	BS=**EcM-I<EcM**
Verrucomicrobia	**Verrucomicrobiales**	***Pedosphaera***
BS>**EcM-I>EcM**	BS=**EcM-I>EcM**	BS=**EcM-I>EcM**
Beta-proteobacteria	Clostridiales	***Microscilla***
BS=EcM-I=EcM	BS=EcM-I>EcM	BS=**EcM-I>EcM**
Firmicutes	Burkholderiales BS=EcM-	***Flavobacterium***
BS=**EcM-I>EcM**	I=EcM	BS=**EcM-I>EcM**

Acidobacteria	**Planctomycetales**	***Thermoleophilum***
BS=**EcM-I>EcM**	BS=**EcM-I>EcM**	BS=**EcM-I<EcM**
Planctomycetes	**Flavobacteriales**	*Cytophaga*
BS=**EcM-I>EcM**	BS=**EcM-I>EcM**	BS=EcM-I=EcM
Gamma-proteobacteria BS=EcM-I<EcM	Coriobacteriales	***Candidatus Koribacter***
	BS=EcM-I=EcM	BS=**EcM-I>EcM**
	Bacillales	***Gordonibacter***
	BS=**EcM-I>EcM**	BS<**EcM-I>EcM**
	Thermoleophilales	***Rubrobacter***
	BS=**EcM-I<EcM**	BS=**EcM-I>EcM**
	Rubrobacterales	***Bacillus***
	BS=**EcM-I>EcM**	BS=**EcM-I>EcM**
	Acidobacteriales	***Pirellula***
	BS=**EcM-I>EcM**	BS=**EcM-I>EcM**
		Acidobacterium
		BS=**EcM-I>EcM**

583 **Figure legends.**

584

585 **Figure 1.** Relative abundance of main bacterial classes and Proteobacteria sub classes in bulk soil
586 (black), EcM-soil interface (grey) and EcM samples (white). Data are expressed as the mean values +/-
587 SE. Value with a same letter are not statistically different according to a one-way (sample origin)
588 ANOVA ($p > 0.05$) followed by a Fisher post-hoc test.

589

590 **Figure 2.** Multivariate analysis of the bacterial communities associated to *T. melanosporum* EcM, EcM-I
591 and BS along maturation time. Principal Coordinate axes 1 and 2 explain most of the variance in the
592 data cumulatively (F1 = 49.9 % F2 = 18.4 %). Circles, squares and triangles represent bulk soil, EcM-
593 soil interface and EcM samples, respectively. White, light grey, dark grey and black fillings represent
594 September, November, December 2010 and January 2011 sampling times.

595

596 **Figure 3.** Relative abundance of main bacterial classes and Proteobacteria sub classes in bulk soil (A)
597 and EcM samples (B). Data for EcM-I samples are not represented to simplify the figure as they show
598 identical pattern to the one's of the BS. September, November, December and January samples are
599 represented by black, dark grey, light grey, white colors, respectively. Data are expressed as the mean
600 values +/- SE. Value with a same letter are not statistically different according to a one-way (sampling
601 time) ANOVA ($p > 0.05$) followed by a Fisher post-hoc test.

602

603 **Figure 4.** Relative abundance of main bacterial classes and Proteobacteria sub classes in bulk soil,
604 EcM, peridium and gleba in November, December and January.

605

606 **Supplemental Figure 1.** Multivariate analyses of the bacterial communities associated to *T.*
607 *melanosporum* EcM, BS and ascocarps (peridium and gleba) in November (A), December (B) and
608 January (C). MBS: bulk soil, MYCO: EcM, AIN: gleba, AOUT: peridium.

609

Figure

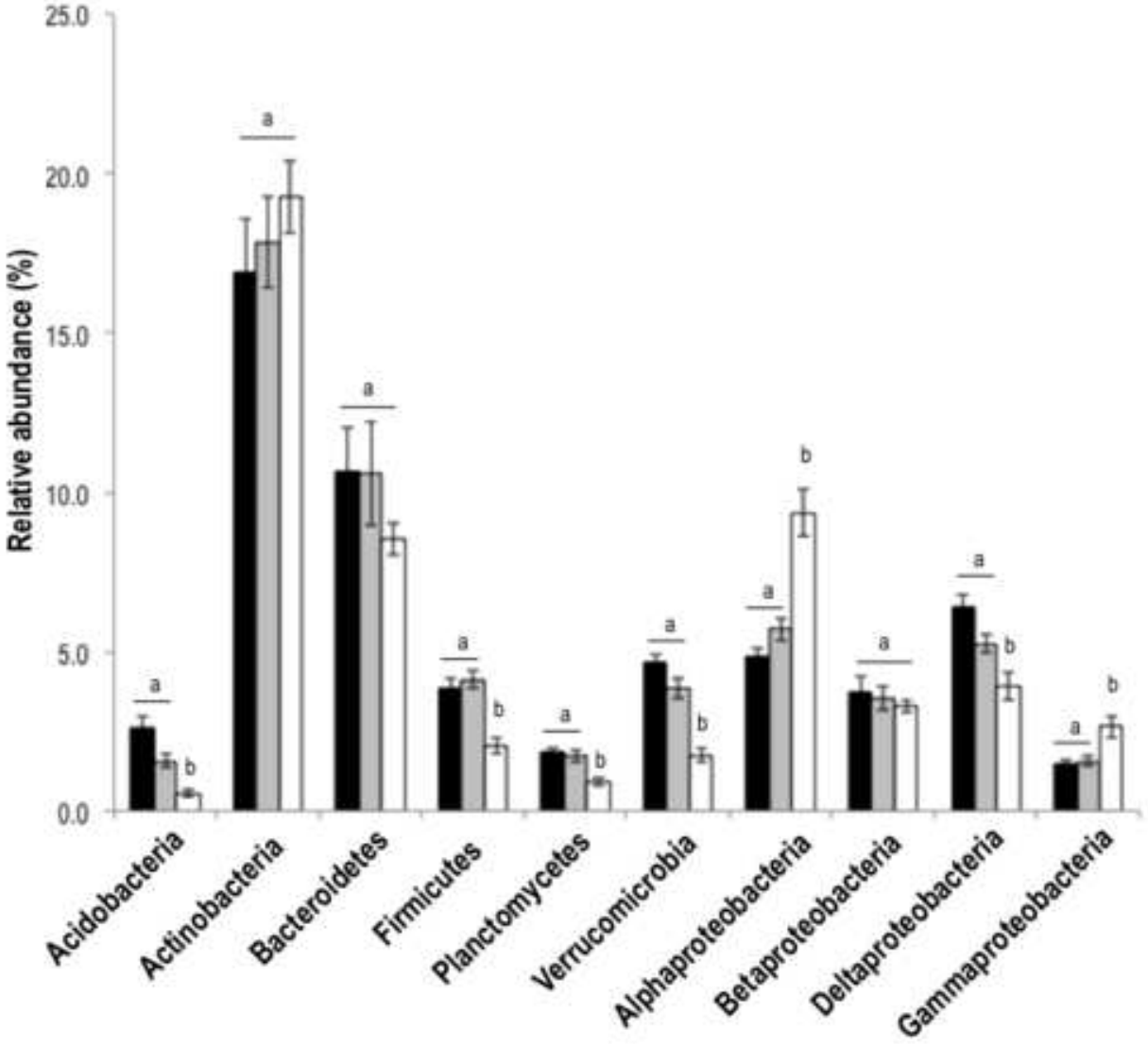


Fig 1 Relative abundance of main bacterial classes and Proteobacteria sub classes in bulk soil (black), EcM-soil interface (grey) and EcM samples (white). Data are expressed as the mean values +/- SE. Value with a same letter are not statistically different according to a one-way (sample origin) ANOVA ($p > 0.01$) followed by a Fisher post-hoc test

Figure

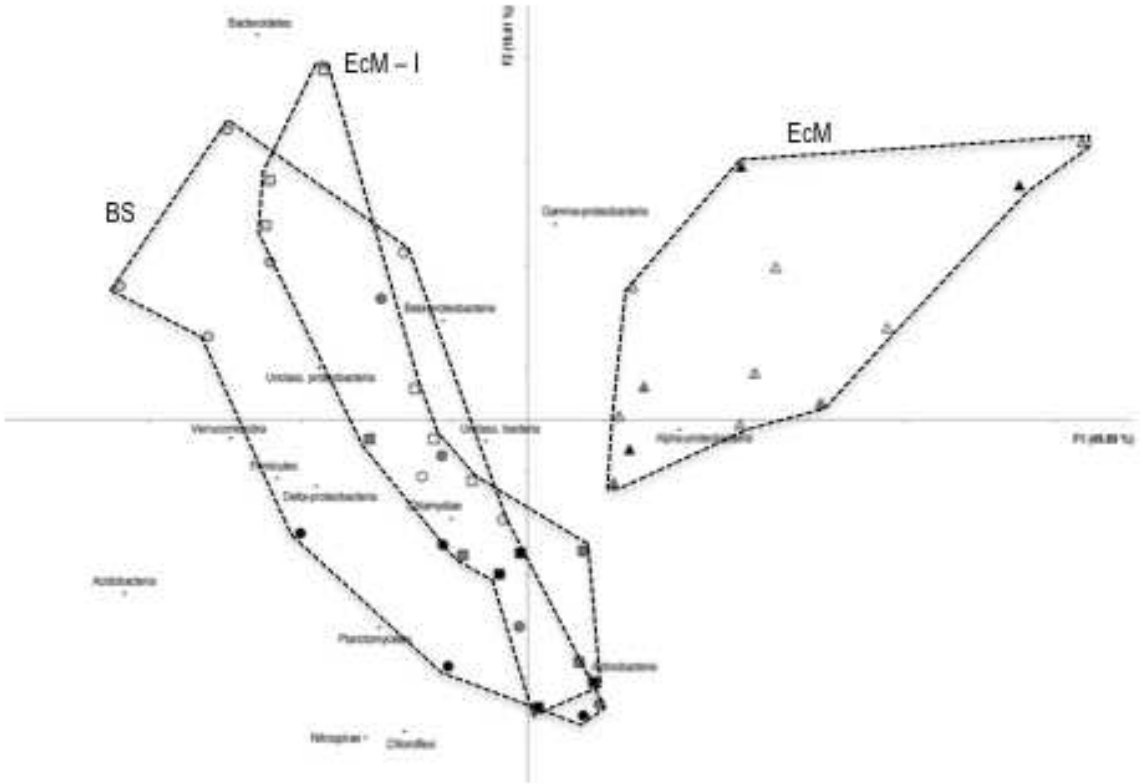


Fig 2 Multivariate analysis of the bacterial communities associated to T. melanosporum EcM, EcM-I and BS along maturation time. Principal Coordinate axes 1 and 2 explain most of the variance in the data cumulatively (F1 = 49.9 % F2 = 18.4 %). Circles, squares and triangles represent bulk soil, EcM-soil interface and EcM samples, respectively. White, light grey, dark grey and black fillings represent September, November, December 2010 and January 2011 sampling times

Figure

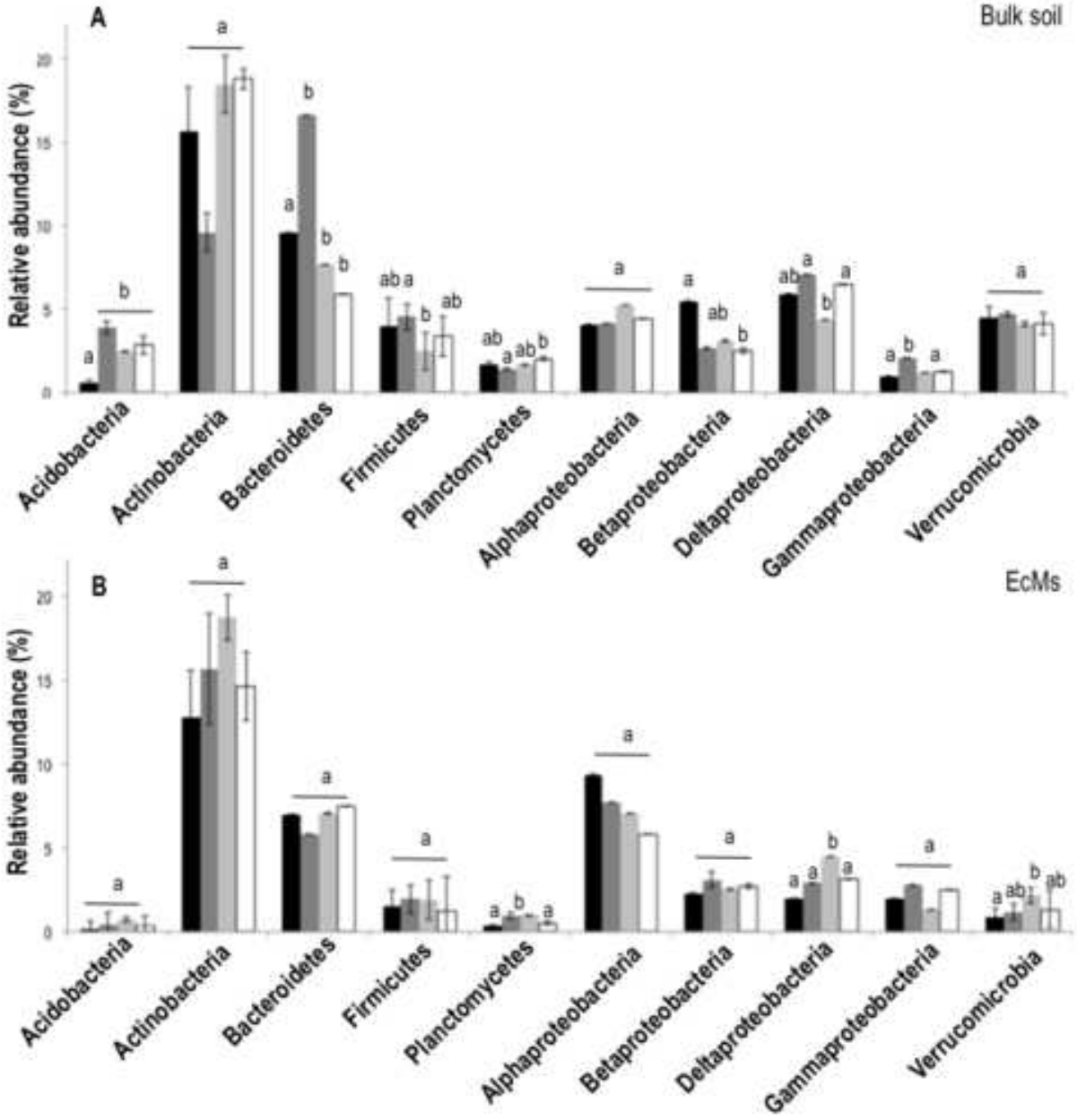


Fig 3 Relative abundance of main bacterial classes and Proteobacteria sub classes in bulk soil (A) and EcM samples (B). Data for EcM-I samples are not represented to simplify the figure as they show identical pattern to the one's of the BS. September, November, December and January samples are represented by black, dark grey, light grey, white colors, respectively. Data are expressed as the mean values +/- SE. Value with a same letter are not statistically different according to a one-way (sampling time) ANOVA ($p > 0.05$) followed by a Fisher post-hoc test

Figure

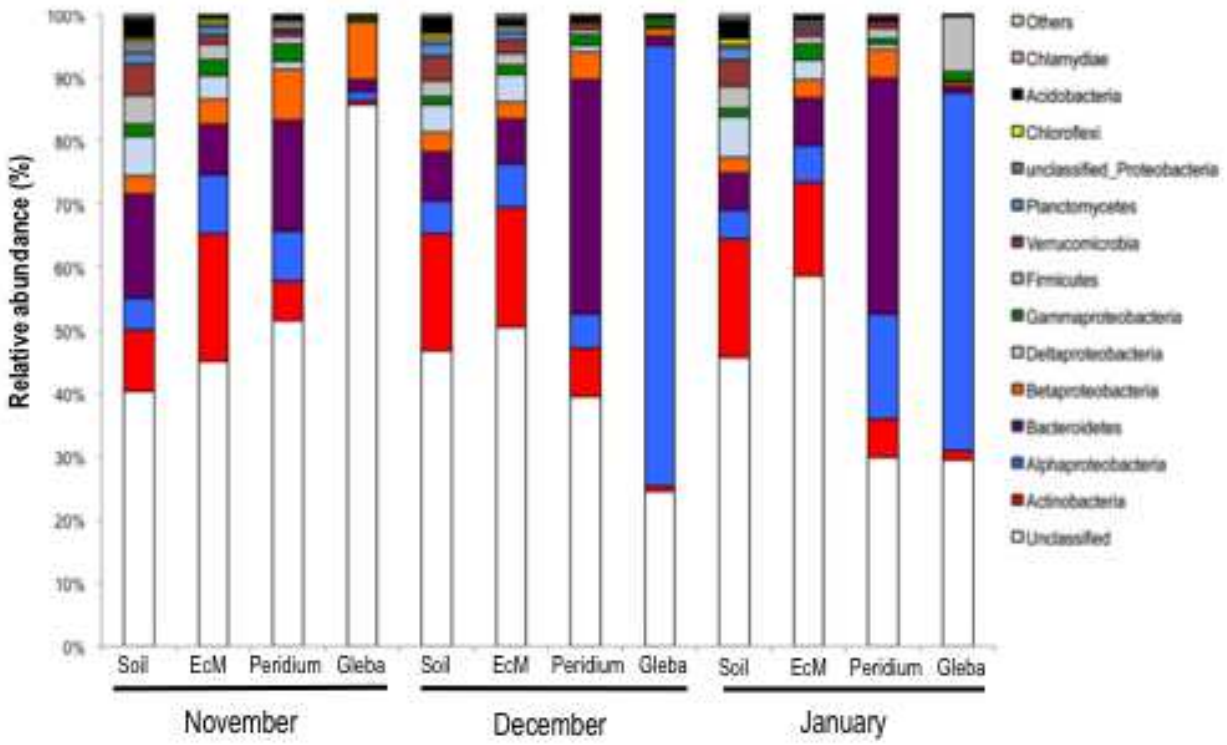
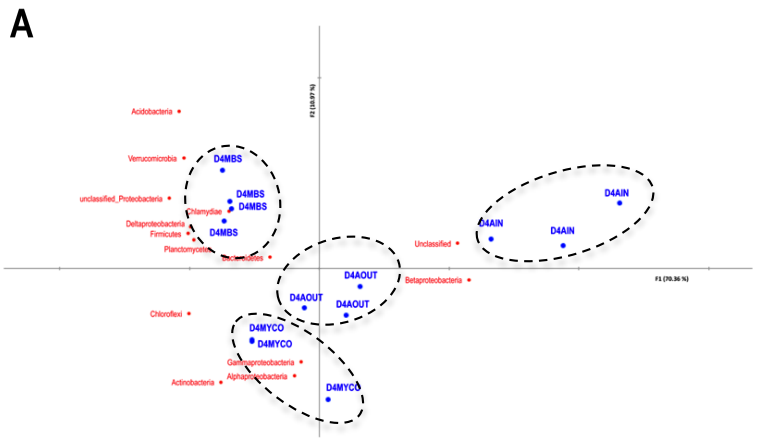
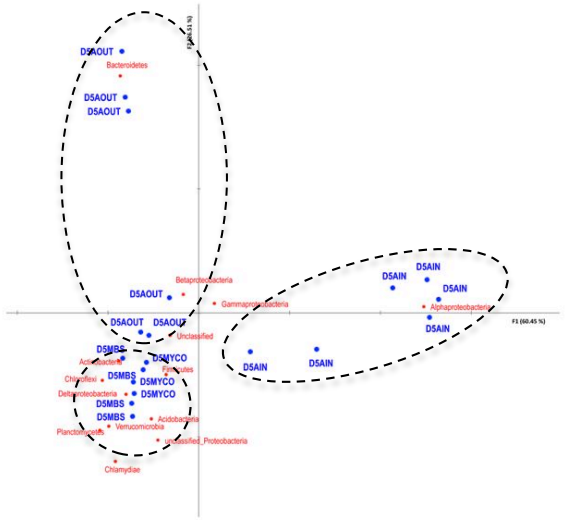
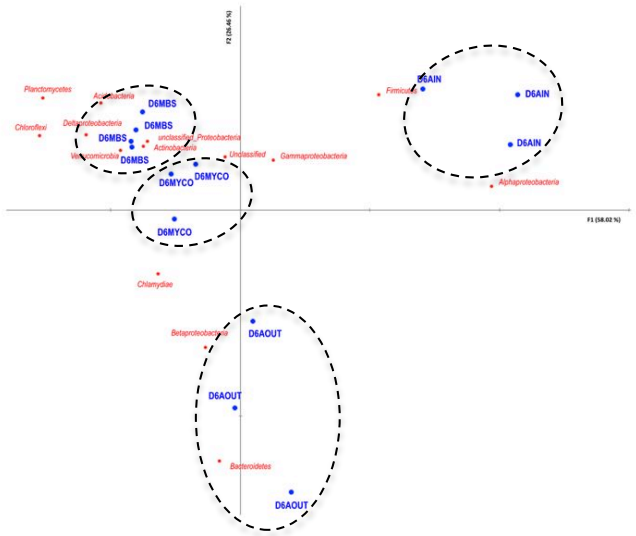


Fig 4 Relative abundance of main bacterial classes and Proteobacteria sub classes in bulk soil, EcM, peridium and gleba in November, December and January







Supplemental Fig 1 Multivariate analyses of the bacterial communities associated to *T. melanosporum* EcM, BS and ascocarps (peridium and gleba) in November (A), December (B) and January (C). MBS: bulk soil, MYCO: EcM, AIN: gleba, AOUT: peridium

Table S1: Comparison of EcM and ascocarps compartments (gleba and peridium). Relative abundance of the main phyla detected in both EcM and ascocarp compartments were used to performed ANOVA analyses. Taxa presenting a significant enrichment whatever the date are presented in column 'all dates' and those presenting significant variations at one or several dates are presented in the column 'specific date(s)'. The symbol '>' means significant enrichment. D4, D5 and D6 mean respectively November, December and January. For ascocarp, data coming from Antony-Babu et al. (2014) have been used.

Taxa	All dates	Specific date(s)
Actinobacteria	EcM > Gleba and peridium	
Chloroflexi	EcM > Gleba	
Planctomycetes	EcM > Gleba	
Delta-Proteobacteria	EcM > Gleba and peridium	
Verrucomicrobia	EcM > Gleba	
Firmicutes		D6 Gleba > D6 EcM ;
		D4 and D5 EcM > D4 and D5 Gleba
Alpha-Proteobacteria		D5 and D6 Gleba > D5 and D6 EcM
Beta-Proteobacteria		D4 Gleba > D4 EcM
		D6 EcM > D6 Gleba
Gamma-Proteobacteria		D4 EcM > D4 Gleba