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ORIGINAL ARTICLE

Chinese white truffles shape the ectomycorrhizal microbial communities of *Corylus avellana*

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Abstract

Here, we investigated the influence of Chinese white truffle (*Tuber panzhihuanense*) symbioses on the microbial communities associated with *Corylus avellana* during the early development stage of symbiosis. The microbial communities associated with ectomycorrhizae, and associated with roots without *T. panzhihuanense* colonization, were determined via high-throughput sequencing of bacterial 16S rRNA genes and fungal ITS genes. Microbial community diversity was higher in the communities associated with the ectomycorrhizae than in the control treatment. Further, bacterial and fungal community structures were different in samples containing *T. panzhihuanense* in association with *C. avellana* compared to the control samples. In particular, the bacterial genera *Rhizobium*, *Pedomicrobium*, and *Herbiconiux* were more abundant in the ectomycorrhizae, in addition to the fungal genus *Monographella*. Moreover, there were clear differences in some physicochemical properties among the rhizosphere soils of the two treatments. Statistical analyses indicated that soil properties including exchangeable magnesium and exchangeable calcium prominently influenced microbial community structure. Lastly, inference of bacterial metabolic functions indicated that sugar and protein metabolism functions were significantly more enriched in the communities associated with the ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense* compared to communities from roots of cultivated *C. avellana* without *T. panzhihuanense*. Taken together, these results highlight the interactions among ectomycorrhizal fungi, soil properties, and microbial communities that are associated with host plants and further our understanding of the ecology and cultivation of the economically important *T. panzhihuanense* truffles.

Keywords *Tuber panzhihuanense* · *Corylus avellana* · High-throughput sequencing · Microbial communities · Soil properties

Introduction

Truffles belong to the *Tuber* genus (Ascomycota, Pezizales) and are ectomycorrhizal fungi that are characterized by hypogeous fruiting bodies (Bonito et al. 2010; Kirk et al. 2008). *T. panzhihuanense* is one of ten new white truffle species that were discovered in 2010 and 2011 in southwestern China and is closely related, morphologically and phylogenetically, to another white Chinese truffle species, *T. latisporum* (Li et al. 2017; Liu et al. 2013). *T. panzhihuanense* is abundant in southwestern China, is edible, and exhibits a strong, pleasant aroma, which all contribute to its high economic value and commercial production potential (Liu et al. 2013; Wan et al. 2015a; Wan and Liu 2014). Truffle value has been increasingly recognized by global consumers leading to a rapidly growing market demand, and consequent stimulation of truffle resource plundering. Thus, there has been a gradual decrease of truffle production in the Panzhihua Province of China (Tang 2005; Wan et al. 2015a). Establishing truffle plantations with artificially synthesized mycorrhizal seedlings is an efficient way to compensate for endangered truffle resources (Lefevre and Hall 2001). Like other ectomycorrhizal fungi, truffles require infection of host plants and the development of a symbiotic relationship with them in

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order to complete their life cycle (Kües and Martin 2011; Shu-Chao et al. 2017). Truffles can form symbiotic relationships with host trees of several genera including *Corylus*, *Quercus*, *Abies Pinus*, *Populus*, and *Salix* (Healy et al. 2016; Wan et al. 2015a; Chen 2002). However, little is known of the mycorrhizal symbioses of Chinese white truffles (Liu et al. 2014). *T. panzhihuanense* is frequently collected from natural forests of *Panisea yunnanensis* in between late autumn and winter. In addition, *T. panzhihuanense* forms symbiotic associations with *Castanea mollissima* and *Pinus armandii* under greenhouse conditions (Wan et al. 2015a, 2015b). *C. avellana* is typically the host plant of *T. macrosporum* Vittad and *T. melanosporum* ectomycorrhizae (Benucci et al. 2012; Bradshaw 2005). However, the host association of *C. avellana* with *T. panzhihuanense* is rarely reported.

Ectomycorrhizal fungi play important roles in ecosystems. The mutualistic exchange of nutrients between microsymbionts and their host plants is crucial to the success of ectomycorrhizal interactions (Martin et al. 2010). On the one hand, Ectomycorrhizae can promote plant productivity by enhancing plant growth or resistance to abiotic stressors (Dominguez et al. 2012; Lenoir et al. 2016). On the other hand, plants can promote rhizosphere microorganism growth via their root secretions, which may also induce the formation of mycorrhizae (Berendsen et al. 2012). Moreover, plant genetic backgrounds and soil types are also a particularly important drivers of microbial community structural variation. In particular, truffle ectomycorrhizae and fruiting bodies harbor diverse microbial communities consisting of bacteria, yeasts, and filamentous fungi, and the microbial communities that are associated with truffle grounds vary by season, region, and truffle species (Deveau et al. 2016; Li et al. 2017; Mello et al. 2013; Splivallo et al. 2015).

In addition to the associations described above, the development of mycorrhizal fungi and plant symbiosis can be influenced by rhizosphere bacteria and fungi (Mello et al. 2010). The diversity and functions of microbial communities within ecosystems have recently become an area of intense research interest. Soil microorganisms, particularly rhizosphere microorganisms, play important roles in plant growth and development. Endophytic microorganisms can influence the metabolic processes of the host and produce bioactive compounds that contribute to plant secondary metabolites (Ludwigmüller 2015). In addition, endophytic microorganisms have an impact on host plant yields, but it is unclear how they interact with each other, and how these interactions affect host plants (Esmaeili et al. 2017; Ling and U 2013). Various microorganisms have been isolated from the hyphae of ectomycorrhizal fungi and fruiting bodies (Wan and Liu 2014; Zhou and Wei 2013). However, few studies have investigated the endophytes associated with the ectomycorrhizae of truffle hosts.

In this study, we synthesized ectomycorrhizal relationships by inoculating *C. avellana* with *T. panzhihuanense* under greenhouse conditions to explore how *T. panzhihuanense* influences the endophyte microbial communities associated with the ectomycorrhizae. The endophyte microbial community structures associated with the ectomycorrhizae were determined with next-generation sequencing of community 16S rRNA genes for bacteria and ITS genes for fungi. In addition, the associations of the communities with the physical and chemical properties of rhizosphere soils were analyzed. Concomitantly, we investigated the effects derived from the symbiotic ectomycorrhizal relationships on the diversity of the microbial communities. These data are used to explore the interactions among ectomycorrhizal fungi, soil microorganisms, and host plants.

Materials and methods

Cultivation of *C. avellana* seedlings

C. avellana seedlings were cultivated in greenhouses according to previously described methods (Li et al. 2017). Briefly, *C. avellana* seeds that were purchased from a local seed company were surface sterilized with 30% H_2O_2 for 4 h and washed three times with sterile water. Surface-sterilized seeds were sown in a plastic container (the bottom is a square with a side length of 5.5 cm, and the mouth is a square with a side length of 7.5 cm and a height of 14 cm) filled with substrate materials (vermiculite, perlite, organic soil, and water at a ratio of 1:1:1:0.9, *v*/*v*/*v*/*v*) that were autoclaved for 90 min at 121 °C (Li et al. 2017). The soil to be sterilized is required to ensure sterility when inoculated with truffle spores; otherwise, other bacteria or fungi may affect the inoculation effect. The organic soil refers to the soil used for cultivating seeds and containing the nutrients required for seed growth. The pH of the homogenized substrate was adjusted to 7.5 by addition of calcium hydroxide. Pre-treated *C. avellana* seeds were then cultivated in greenhouses for two months and allowed to germinate. *C. avellana* seedlings without significant differences in plant heights, and of sufficient growth, were selected for experimentation. A total of 30 qualified *C. avellana* seedlings were selected as experimental materials.

Truffle inoculation experiments

T. panzhihuanense were provided by the Panzhihua Agriculture and Forestry Scientific Research Institute (Fig. 1). Spore powder of *T. panzhihuanense* was obtained by blending ascocarps that were surface sterilized with 75% alcohol and soaked in sterile water (Li et al. 2017). Blending of ascocarps was conducted to incite the release of spores and germination (Wan et al. 2015a). About 1.5–2 g of spore powder was inoculated into the substrate containing the seedlings (approximately 500 g of substrate in each plastic container). An equal number of non-inoculated *C. avellana* seedlings were used as controls. The inoculated and non-inoculated seedlings were cultivated for eight months prior to sampling.

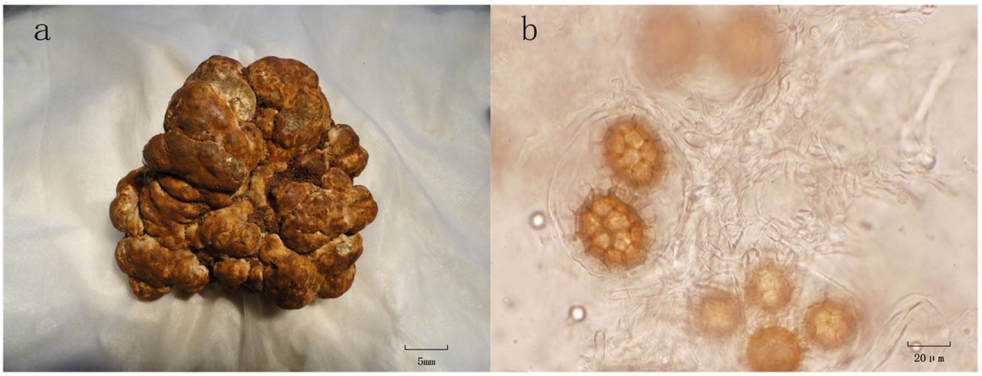

Fig. 1 Fruiting bodies of *T. panzhihuanense* (**a**) and spores of *T. panzhihuanense* (**b**)

Sample sampling and soil analyses

After eight months of cultivation, the *C. avellana* were removed from the plastic container. They were shook gently with hands and slowly tapped to expose the roots from the bulk soil. Then they were soaked in water for 15–20 min, the roots were gently washed, and they were washed with water 2–3 times until the roots are completely separated from bulk soil. The physical and chemical properties of rhizosphere soils were then measured. Each treatment group included three replicates. Soil properties were analyzed according to previously described methods (Li et al. 2016). Briefly, soil pH was determined using a glass electrode (1:5, soil/water volume ratio), organic matter content was measured using the Tyurin method (Ruma et al. 2014), and total nitrogen content was determined using the Kjeldahl method (Page et al. 1982). Other soil parameters were determined using general methods (Ulery and Drees 2008).

C. avellana roots (the main root length is 2–3 cm, the root tips' length is 0.5–3 mm, and the root is roughly 0.2–0.4 mm) were rinsed with sterile water prior to morphological and molecular analyses of ectomycorrhizae. The root tips of *C. avellana* that were mycorrhized with *T. panzhihuanense* were flushed with sterile water and placed in 2-ml centrifuge tubes using sterilized tweezers. *T. panzhihuanense* ectomycorrhizae on *C. avellana* are simple or ramified in a monopodial-pinnate pattern; single ectomycorrhizae tips are straight, fragile, and cylindrical or club shaped with rounded ends. And the mature ectomycorrhizae frequently had a whitish growing tip apex (Fig. 2c, d). Cystidia are the typical of *Tuber* ectomycorrhizae. They are needle-shaped, simple, or connected at the base,

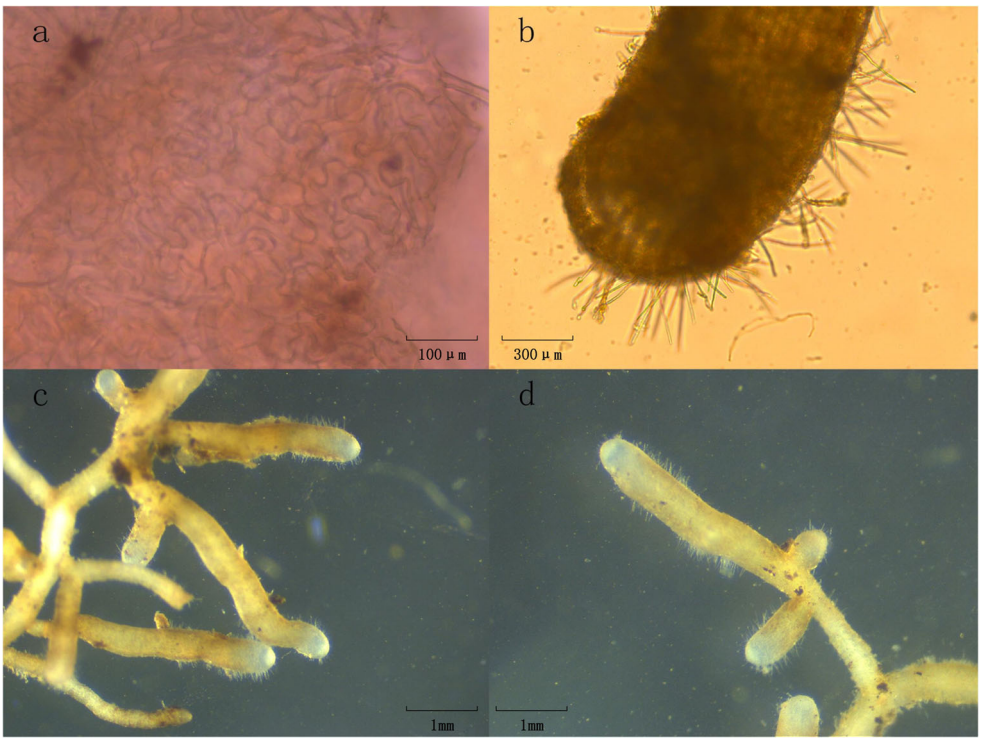

Fig. 2 *T. panzhihuanense* ectomycorrhizal microstructure (**a**) and root tips of *C. avellana* with *T. panzhihuanense* associations (**b**, **c**, and **d**)

smooth, colorless, and generally mono-septate (Fig. 2b). The outer mantle surface is densely short-spiny, composed by epidermoid cells structured in an uneven regular puzzle-like pattern; cells are variable in shape but less in size (Fig. 1a). For the control plants, the non-inoculated roots were removed and placed in 2-ml centrifuge tubes. As many young roots were chosen as possible. Each group comprised five replicates, and each replicate contained greater than 500 mg of ectomycorrhizae (treatment group) or young root tips (control group). Fresh samples were stored at − 80 °C. Root tips of *C. avellana* mycorrhized with *T. panzhihuanense* were designated as "P" and the surrounding soil was designated "P.S." Roots from the control plants lacking *T. panzhihuanense* were designated as "CK" and the surrounding soil as "CK.S."

DNA extraction, PCR amplification, and sequencing

Prior to DNA extraction, the mycorrhizae or control roots of *C. avellana* were washed with distilled sterile water and surface-disinfected with 75% alcohol. Genomic DNA from the tissues and endophytes was extracted using a hexadecyl trimethyl ammonium bromide (CTAB) method (Li et al. 2017). The concentrations of extracted DNA were quantified using an ultraviolet spectrophotometer, and the qualities were assessed by gel electrophoresis on a 0.8% agarose gel.

The 16S rRNA gene amplification primers for bacteria are 515F and 926R (Parada et al. 2016), and the amplification system is 20 μL, including 2 μL of template DNA (1–10 ng); 20 μmol/μL upstream and downstream primers were each 0.4 μL, 10 μL 2 × SYBR Premix Ex Taq (TaKaRa, Japan), and 7.4 μL ddH2O. Reaction procedure: 95 °C for 3 min; 95 °C for 10 s, 55 °C for 20 s, 72 °C for 20 s, 40 cycles. The fungi ITS gene amplification primers are ITS1F and ITS1R (Zhang et al. 2016); the amplification system is 20 μL, including 2 μL of template DNA (1–10 ng), 20 μmol/μL of upstream and downstream primers each 0.4 μL, 10 μL 2 × SYBR Premix Ex Taq (TaKaRa, Japan), and 7.4 μL ddH2O. Reaction procedure: 95 °C for 3 min; 95 °C for 20 s, 53 °C for 20 s, 72 °C for 20 s, 40 cycles. A high-fidelity DNA polymerase (NEB Q5) was used to ensure efficiency and accuracy of PCR amplification (Langenheder and Székely 2011). Successful PCR amplifications were assessed using gel electrophoresis on a 2% agarose gel. PCR amplicons of the right size were excised and purified using an Axygen AxyPrep DNA Gel Extraction kit (AP-GX-500). PCR products were quantified using a Quant-iT PicoGreen dsDNA Assay Kit with a microplate reader (BioTek, FLx800). PCR products were then pooled at equimolar concentrations. An Illumina TruSeq Nano DNA LT Sample Prep Kit (FC-121-4001 or FC-121-4002) was used to construct a library for Illumina sequencing. Final amplicon selection and purification of the library were performed using gel electrophoresis with a 2% agarose gel. Paired-end sequencing (2 × 300 bp) was then conducted on the Illumina MiSeq platform using the V3 MiSeq Reagent Kit over 600 cycles.

Sequence processing and statistical analyses

The QIIME software package v1.8.0 (Caporaso et al. 2010) was used to quality filter the sequencing data. High-quality sequences were obtained after removing chimeric sequences using the USEARCH v5.2.236 algorithm (Edgar 2010). High-quality sequences with ≥ 97% nucleotide similarity were clustered into operational taxonomic units (OTUs) using the UCLUST software, which is a sequence alignment tool implemented in QIIME (Edgar 2010). Representative sequences were chosen for each OTU based on the sequence with the highest abundance in each OTU. OTUs with relative abundances less than 0.001% across all samples were removed (Bokulich and Mills 2013). The taxonomic classifications of bacterial and fungal OTUs were assigned using the RDP 3 classifier and the SILVA and UNITE databases, respectively. Statistical analyses and figures were generated in the R software environment (Team 2009). Shared OTU abundances were visualized using a Venn diagram. Rarefaction curves were calculated in QIIME using four alpha diversity indices including the Chao1, ACE, Shannon, and Simpson indices. The distribution of the 50 most abundant genera among samples was visualized using a heatmap and cluster analysis in R. In addition, the beta diversity (shared OTU composition) of the microbial communities was analyzed using a principal coordinate analysis (PCoA). To investigate the relationships between microbial communities and soil properties, canonical correspondence analysis (CCA) of the communities and soil properties were conducted in R. In addition, a network analysis of soil OTU distributions and soil properties was performed using the Mothur software (Lozupone and Knight 2005; Yatsunenko et al. 2012). Lastly, the PICRUSt software package was used to predict the metabolic functions of the bacterial communities based on the KEGG microbial function database (Langille et al. 2013).

Data are presented as means ± standard deviation (SD) for five biological replicates in each treatment group. Statistical differences were assessed using one-way analysis of variance (ANOVA) tests in the SPSS 19.0 software package. Least significant difference (LSD) tests were performed to determine if the ANOVA results between different treatments were significant at the $P < 0.05$ significance level.

Results

Rhizosphere and ectomycorrhizosphere soil characterization

The physicochemical properties of soils surrounding *C. avellana* root tips differed between treatments with or

without *T. panzhihuanense* (Table 1). The pH of soil samples in the P group were higher than those of the CK group ($P < 0.05$), and varied from 8.10 to 8.48 in P.S. There were no significant differences in the organic matter (OM) and total potassium (TK) content among samples of different treatments. The contents of total nitrogen (TN), available nitrogen (AN), and available phosphorus (AP) were all higher in P.S soils compared to those of CK.S. In contrast, other properties including exchangeable calcium, exchangeable magnesium, and available potassium (AK) were all higher in CK.S compared to P.S.

Bacterial alpha diversity in ectomycorrhizae and roots

A total of 65,202–78,161 bacterial 16S rRNA gene reads were obtained per sample after quality filtering. Thirty-six phyla, 95 classes, and 775 bacterial genera were assigned to OTUs, with 1403–1806 OTUs present in the samples. The numbers of observed species were not significantly different between samples of treatments (Table 2). The other two richness indices (Chao1 and ACE) were higher in ectomycorrhizal communities (P) than in those of the control roots (CK). In addition, bacterial communities were more diverse in ectomycorrhizal communities than in the control root communities, based on the Shannon and Simpson indices.

Fungal alpha diversity in ectomycorrhizae and roots

A total of 38,856–513,507 high-quality fungal ITS reads were obtained per sample, which comprised 10 phyla, 58 classes, and 217 fungal genera. Between 518 and 555 fungal OTUs were observed per sample. Fungal richness indices did not significantly differ between ectomycorrhizae (P) and control root (CK) communities ($P < 0.05$) (Table 2). However, higher fungal diversity was observed in the ectomycorrhizal communities than in those of the control roots (CK), according to the Shannon and Simpson diversity indices.

Taxonomic analyses of bacterial communities

A total of 39 bacterial phyla were observed across all communities, 27 of which were present in all 10 communities (Fig. 3a). Three bacterial phyla, Proteobacteria (46.8–66.4% relative abundance), Actinobacteria (7.1–21.3%), and Firmicutes (0.4–19.5%), were dominant among all samples (Fig. 4a). Proteobacteria were more abundant in the CK group soil communities than in those of the P group ($P < 0.05$). In contrast, the relative abundances of the Actinobacteria were slightly higher in the P group soil communities than in those of the CK group soils ($P < 0.05$). In addition, CK group soil communities contained less Firmicutes than those of the P group ($P < 0.05$).

Table 1 Physical and chemical properties of *C. avellana* rhizosphere and ectomycorrhizosphere soils

Treatment	H	OM (g/kg)	TN (g/kg)	TP (g/kg)	TK (g/kg)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)	Exchangeable Ca cmol $(1/2Ca2^{+})$/kg	Exchangeable Mg cmol $(1/2\ Mg2^{+})$/kg
P.S	8.46 ± 0.02*	93.97 ± 0.42	3.47 ± 0.01*	1.10 ± 0.01*	25.34 ± 0.13	244.67 ± 2.31*	29.00 ± 0.20*	124.33 ± 0.58*	57.30 ± 1.04*	3.37 ± 0.25*
CK.S	8.14 ± 0.04	94.00 ± 0.36	3.34 ± 0.02	1.17 ± 0.01	24.73 ± 0.45	199.67 ± 1.15	23.83 ± 0.15	153.67 ± 0.58	71.40 ± 0.44	4.63 ± 0.15

OM, organic matter; *TN*, total nitrogen; *TP*, total phosphorus; *TK*, total potassium; *AN*, available nitrogen; *AP*, available phosphorus; *AK*, available potassium; *exchangeable Ca*, exchangeable calcium; *exchangeable Mg*, exchangeable magnesium; *CK.S*, rhizosphere soil; *ECM.S*, ectomycorrhizosphere soil. Each value is the mean of three replicates (± SD). *Significant difference between samples ($P < 0.05$)

Table 2 Richness and diversity of bacterial and fungal communities associated with *C. avellana* roots with or without *T. panzhihuanense* symbionts

	Sample name	Observed species	Shannon	Simpson	Chao1	ACE
Bacteria	P	1806.00 ± 127.28	5.55 ± 0.87*	0.82 ± 0.10*	1382.62 ± 170.36*	1394.32 ± 179.17*
	CK	1403.40 ± 267.29	3.45 ± 1.14	0.54 ± 0.17	999.48 ± 264.20	1012.40 ± 272.26
Fungi	P	518.60 ± 94.43	2.52 ± 0.43*	0.70 ± 0.06*	216.09 ± 76.83	217.05 ± 76.74
	CK	555.20 ± 117.82	2.08 ± 0.67	0.51 ± 0.16	182.90 ± 60.28	185.08 ± 58.17

P, ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense*. *CK*, roots from cultivated *C. avellana* without *T. panzhihuanense*. Each value is the mean of five replicates (± SD). *Significant difference between samples ($P < 0.05$)

Among the 109 bacterial classes that were detected, Alphaproteobacteria (22.0–56.2%), Betaproteobacteria (2.1–22.9%), Actinobacteria (3.8%–18.4%), and Gammaproteobacteria (4.4–10.7%) were dominant (Fig. 3b). P group communities contained less Betaproteobacteria and Gammaproteobacteria than those of the CK group. In contrast, the relative abundances of Alphaproteobacteria, Bacteroidia, and Bacilli were lower in the CK communities than in the P communities ($P < 0.05$) (Fig. 4a).

Among the 856 genera that were identified, 225 were observed in all samples (Fig. 5a). The dominant genera were *Rhizobium* (average relative abundance: 6.7%), *Pedomicrobium* (4.0%), *Streptomyces* (2.6%), and *Woodsholea* (2.0%). *Rhizobium* was more abundant in the P communities than in the CK communities ($P < 0.05$) (Table 3).

Taxonomic analyses of fungal communities

Nine fungal phyla were observed among all root communities (Fig. 3c). The Basidiomycota (average relative abundance, 57.6%) and Ascomycota (40.9%) (Fig. 4b) were dominant in all samples. However, the Basidiomycota were significantly more abundant in the CK communities than in the P communities ($P < 0.05$).

Of the 20 classes detected, Agaricomycetes (average relative abundance, 56.6%), Pezizomycetes (26.0%), and

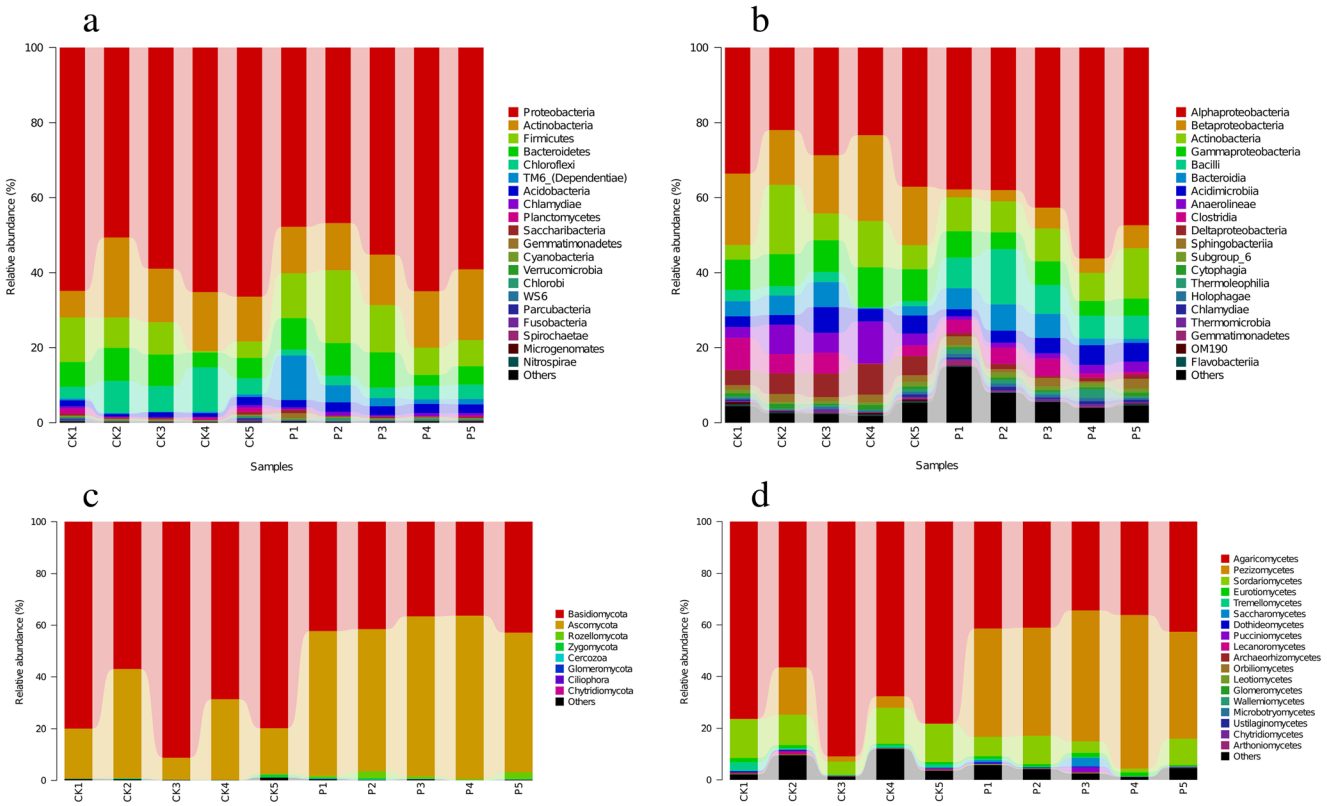

Fig. 3 Taxonomic composition of bacterial and fungal communities associated with *C. avellana* root tips at the phylum and class levels. P, ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense*. CK, roots from cultivated *C. avellana* without *T. panzhihuanense*. **a** Bacterial phyla. **b** Bacterial classes. **c** Fungal phyla. **d** Fungal classes. All experiments were conducted with five replicates

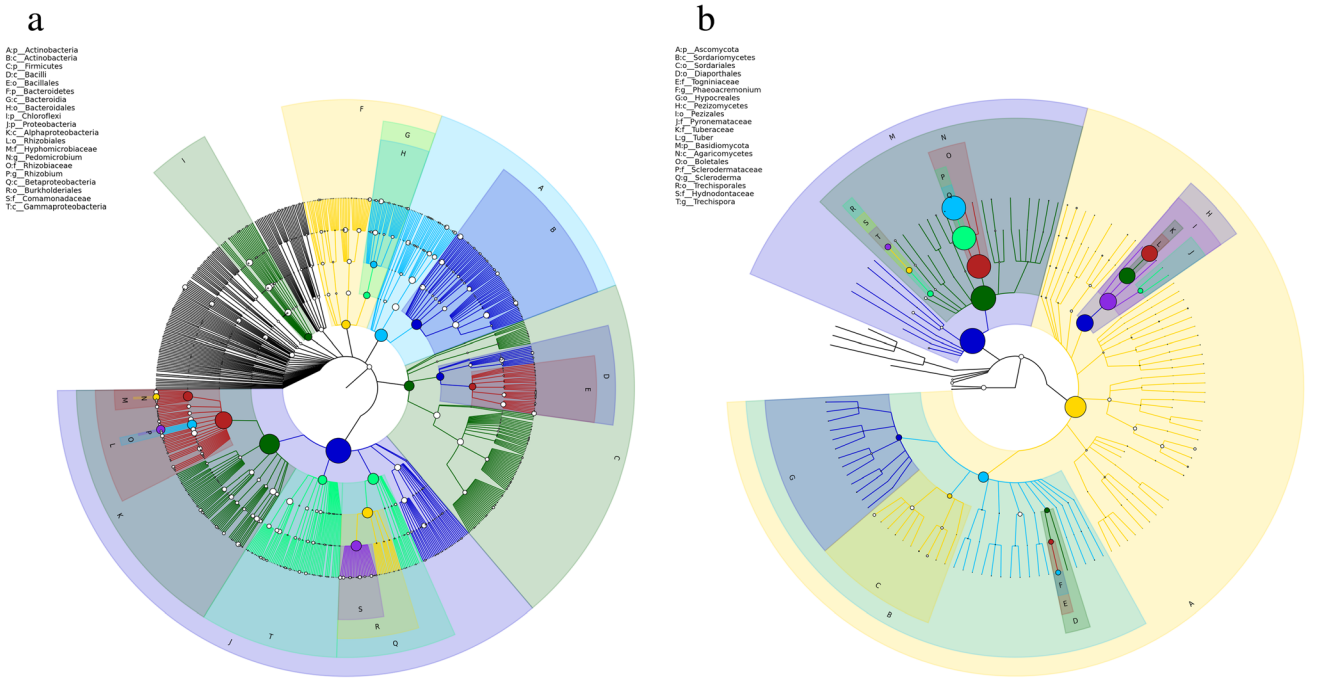

Fig. 4 Overall classification tree map of bacterial (**a**) and fungal (**b**) communities associated with *C. avellana* roots with or without *T. panzhihuanense* symbionts based on GraPhlAn analysis. The node sizes correspond to average relative abundances of taxa, arranged in order from phyla to genus. Shadow colors of the letters are consistent with corresponding node colors

Sordariomycetes (9.5%), dominated the communities (Fig. 3d). The P communities contained more Pezizomycetes and fewer Agaricomycetes than those of the CK group ($P < 0.05$) (Fig. 4b).

A total of 217 fungal genera were observed, 42 of which were detected in all communities (Fig. 5b). The most abundant genera were the *Scleroderma* (average 51.8%), *Tuber* (average relative abundance of 47.0% in P), *Trechispora* (3.2%), and *Phaeoacremonium* (2.4%). *Tuber* were predominant in the roots inoculated with *T. panzhihuanense*. In addition, *Scleroderma* and

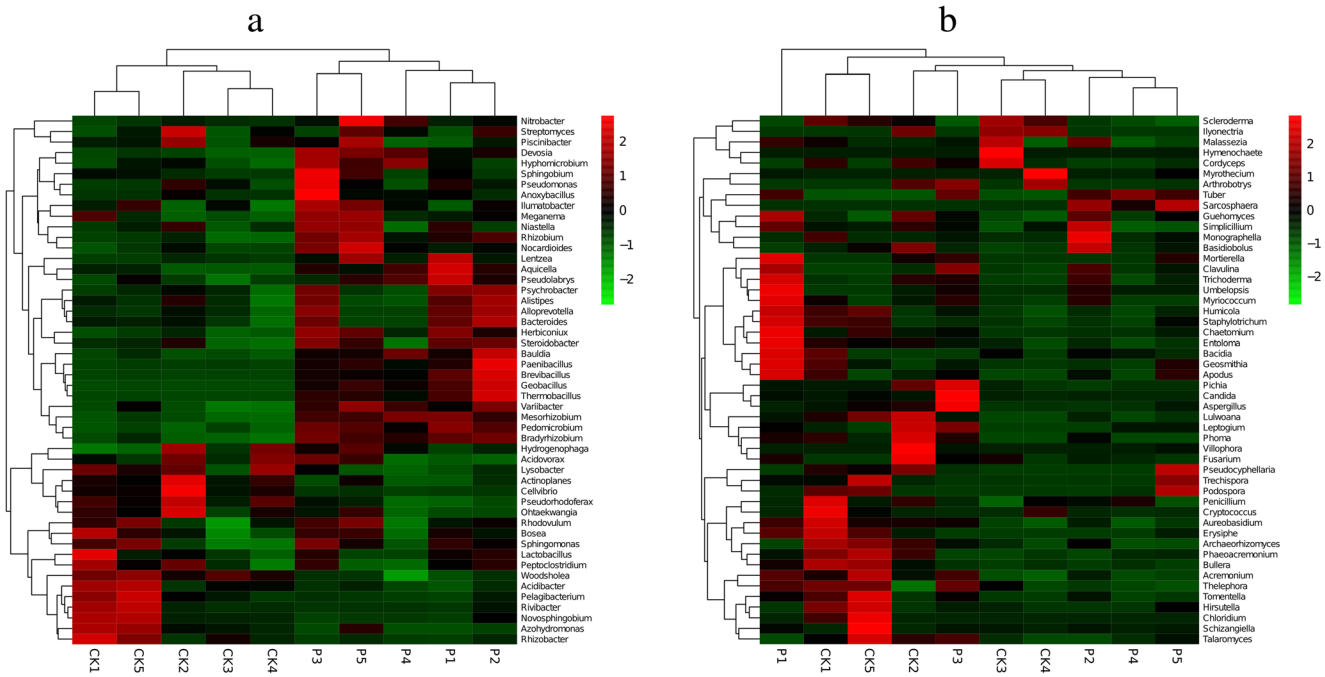

Fig. 5 Heatmap analysis of the 50 most abundant bacterial and fungal genera in *C. avellana* root communities. P, ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense*. CK, roots from cultivated *C. avellana* without *T. panzhihuanense*. **a** Bacterial genera. **b** Fungal genera. The relative abundances of OTUs at the genus level are colored according to the color scales in the upper right of each heatmap

Table 3 The ten most abundant bacterial and fungal genera in *C. avellana* roots with or without *T. panzhihuanense* symbionts

	Bacteria		Fungi	
Sample	P	CK	P	CK
Genera	***Rhizobium* (9.20)**	Uncultured Comamonadaceae (6.74)	*Tuber* (46.96)	***Scleroderma* (68.04)**
	Pedomicrobium (6.22)	Uncultured Anaerolineaceae (6.08)	*Scleroderma* (35.52)	Unidentified Pyronemataceae (4.58)
	Uncultured TM6 (4.10)	*Rhizobium* (4.18)	***Trechispora* (2.86)**	**Unidentified Ascomycota (4.50)**
	Uncultured Methylobacteriaceae (3.84)	Uncultured Sandaracinaceae (4.02)	***Monographella* (2.30)**	***Phaeoacremonium* (3.94)**
	Geobacillus (3.76)	***Streptomyces* (3.24)**	Unidentified Rozellomycota (1.46)	*Trechispora* (3.56)
	Devosia (2.78)	*Woodsholea* (3.22)	Unidentified Ascomycota (1.16)	*Ilyonectria* (2.50)
	Uncultured Bacteroidales_S24-7_group (2.20)	*Actinoplanes* (2.10)	Unidentified Lasiosphaeriaceae (0.86)	*Myrothecium* (1.72)
	Streptomyces (1.92)	Uncultured Bacteroidales_S24-7_group (2.10)	*Phaeoacremonium* (0.78)	Unidentified Auriculariales (1.02)
	Herbiconiux (1.78)	Rivibacter (1.98)	*Podospora* (0.66)	***Podospora* (0.84)**
	Mesorhizobium (1.76)	Uncultured Alphaproteobacteria_Incertae_Sedis (1.96)	*Humicola* (0.50)	*Monographella* (0.82)

Average relative abundances are given in brackets. Each value is the mean of five biological replicates. Genera that were significantly more abundant in a group compared to the other ($P < 0.05$) are highlighted in bold. *P*, ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense*. *CK*, roots from cultivated *C. avellana* without *T. panzhihuanense*

Phaeoacremonium were more abundant in CK communities compared to P communities ($P < 0.05$) (Table 3). Lastly, *Ilyonectria* and *Hymenochaete* were not observed in the P group communities.

Prediction of bacterial metabolic functions

The metabolic functions of bacterial populations are of particular importance in the study of microbial ecology. Thus, we predicted the metabolic functional potential of the bacterial communities using the KEGG Pathway Database, as implemented in the PICRUSt software. The metabolic potentials of the bacterial communities in the P and CK communities were mainly involved in energy metabolism, carbohydrate metabolism, and amino acid metabolism. In contrast, carbohydrate metabolism and amino acid metabolism pathways were more abundant in the P communities than in those of the CK group. Other pathways in low abundance in both the P and CK communities were involved in the metabolisms of cofactors and vitamins, xenobiotics biodegradation and metabolism, lipid metabolism, biosynthesis of other secondary metabolites, glycan biosynthesis and metabolism, and metabolism of other amino acids (Fig. 6).

Principle coordinate analysis of community differences

Differences of the bacterial and fungal communities among samples (based on weighted UniFrac distances) are shown in a PCoA ordination (Fig. 7). Bacterial and fungal communities in the ectomycorrhizae (*C. avellana* mycorrhized with *T. panzhihuanense*, P) were differentiated from the non-inoculated root communities of *C. avellana* (CK).

Microbial community associations with soil properties

CCA was used to determine the relationships between the microbial communities of the root tips and soil properties (e.g., TP, AK, AP, exchangeable Mg, exchangeable Ca, AN, TN, pH) (Fig. 8). Exchangeable Mg, TP, and AK were significantly associated with differences in bacterial community compositions within the root tips (Fig. 8a). Exchangeable Mg, TP, and AK concentrations were positively associated with CK bacterial communities, but negatively associated with P community compositions. In addition, exchangeable Ca, AN, TN, AP, and pH were significantly correlated with fungal microbial community compositions within the root tips. AN, TN, AP, and pH were all negatively correlated with exchangeable Ca (Fig. 8b). Lastly, the CK fungal community compositions were positively associated with exchangeable Ca.

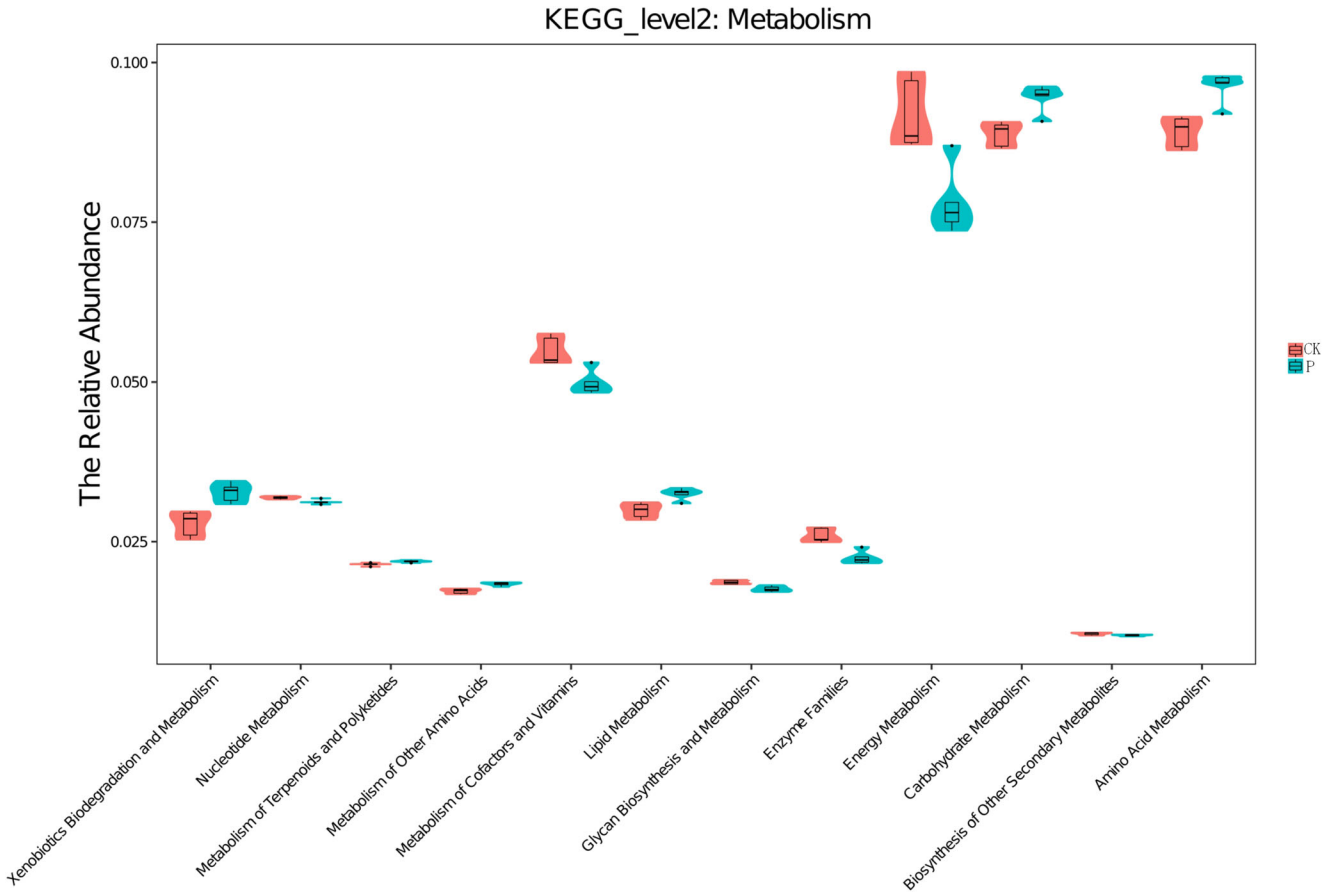

Fig. 6 Relative abundances of inferred metabolic functions of bacterial communities associated with *C. avellana* roots with or without *T. panzhihuanense* symbionts based on KEGG Analysis of PICRUSt metabolic predictions. A, ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense*. CK, roots from cultivated *C. avellana* without *T. panzhihuanense*

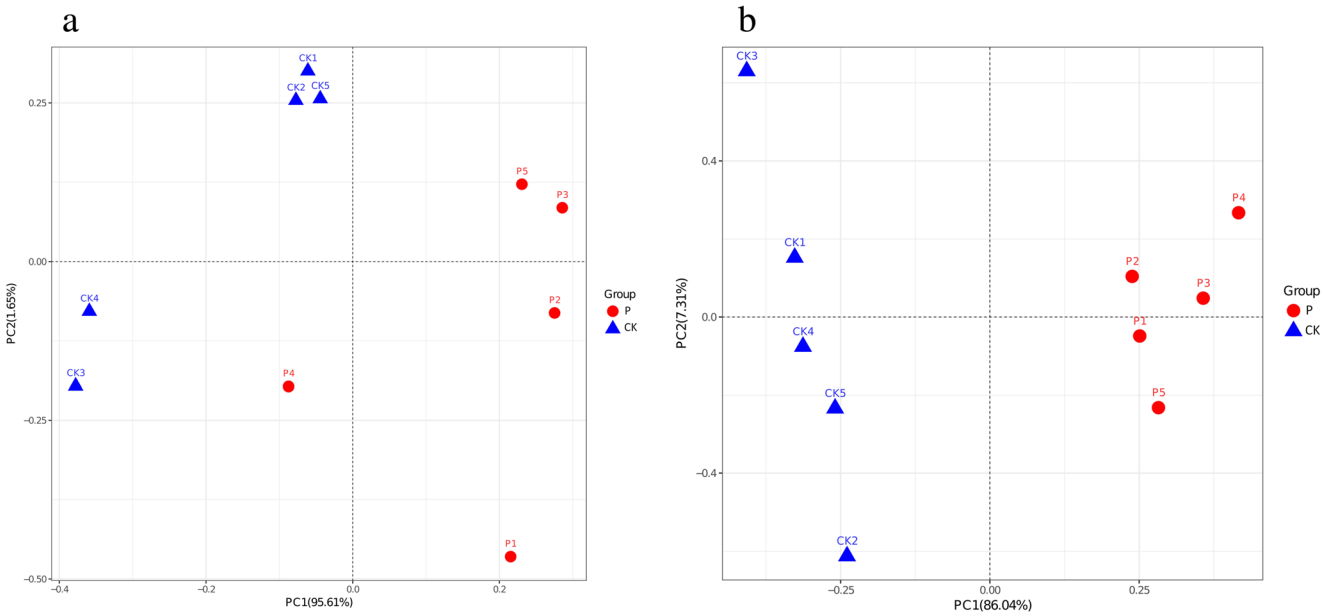

Fig. 7 Principal component analysis (PCoA) of differences among bacterial (**a**) and fungal (**b**) communities associated with *C. avellana* roots with or without *T. panzhihuanense* symbionts based on weighted UniFrac distances. P, ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense*. CK, roots from cultivated *C. avellana* without *T. panzhihuanense*

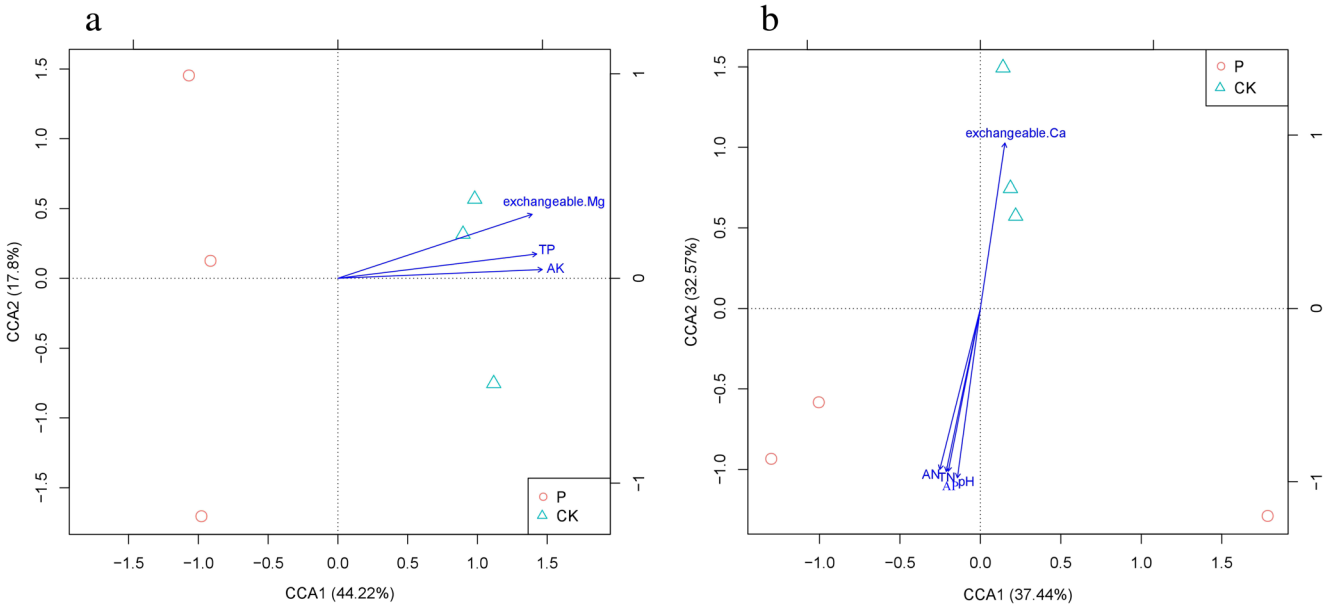

Fig. 8 Canonical correspondence analysis (CCA) of differences in bacterial (**a**) and fungal (**b**) communities associated with *C. avellana* roots with or without *T. panzhihuanense* partner. P, ectomycorrhizae from *C. avellana* mycorrhized with *T. panzhihuanense*. CK, roots from cultivated *C. avellana* without *T. panzhihuanense*. TN, total nitrogen; TP, total phosphorus; AN, available nitrogen; AP, available phosphorus; AK, available potassium; exchangeable Ca, exchangeable calcium; exchangeable Mg, exchangeable magnesium

Discussion

Analysis of soil physical and chemical properties indicated that mycorrhizal synthesis influenced soil properties, which is consistent with previous studies (Garcíamontero et al. 2006). Some soil properties including TN, AN, and AP content, in addition to pH, were higher in ectomycorrhizosphere soils during symbiosis than in the CK.S soils. The differences in soil properties were significant and they may contribute to the initiation of truffle ectomycorrhizae (Alonso et al. 2014; Garcíamontero et al. 2009; Salerni et al. 2014). Further, some soil properties were significantly correlated with bacterial and fungal diversity patterns in root tips. In particular, exchangeable Mg and exchangeable Ca were the most significant factors related to microbial community compositional differences. Moreover, soil properties including TP, AK, and AN, among others, also had important roles in the growth and reproduction of plants based on CCA. These results illustrate the interactive network that exists among ectomycorrhizal fungi, soil properties, microbial communities, and host plants.

We investigated the effects of *T. panzhihuanense* ectomycorrhizal associations on the microbial communities of *C. avellana* during the early symbiosis development stage. The diversity of ectomycorrhizal-associated bacteria was higher compared to roots without mycorrhization, indicating that the truffle mycelia exert specific effects on the surrounding microbial communities. In addition, PCoA ordination indicated that *T. panzhihuanense* directly, or indirectly, affected the composition of the microbial communities associated with them. The colonizing populations must have originated from the surrounding environment, such as the air or water, and were ultimately significantly differentiated from those of the control soils. Alphaproteobacteria were more abundant in the ectomycorrhizae compared to the CK group roots, indicating a close relationship with the presence of truffle mycorrhizae. Further, these associations indicate that the Alphaproteobacteria may have played a role in ectomycorrhizal synthesis. A previous study (Barbieri et al. 2007) observed that Alphaproteobacteria affiliated with *Sinorhizobium*, *Rhizobium*, and *Bradyrhizobium* spp., in addition to Gammaproteobacteria that mostly comprised fluorescent pseudomonads, were the predominant populations of truffle bacterial communities. We observed higher abundances of *Rhizobium* and *Pedomicrobium* populations in ectomycorrhizal communities compared with those of the CK group, indicating that these genera are closely associated with the presence of tuber mycelia and may also play an important role in the growth and mycorrhizal synthesis of truffles.

Community diversity indices indicated that microbial communities were more diverse in the P treatment soils than in those of the CK treatment. Some bacterial genera including *Rhizobium* and *Pedomicrobium* were enriched in ectomycorrhizae compared to the CK group roots, in addition to the fungal genus *Monographella*. Previous studies have demonstrated that the combination of *Pseudomonas fluorescens* and *T. melanosporum* improve the establishment and functioning of ectomycorrhizal symbiosis (Dominguez et al. 2012). Further, the dominance of truffle mycelia reduces the composition of pathogenic endophytic fungi, such as *Trechispora*, *Phaeoacremonium*, *Hymenochaete*, and *Podospora*, and other mycorrhizal fungi, including *Cryptococcus* and *Ilyonectria*.

These observations support the hypothesis that ectomycorrhizal fungi have a protective effect on the growth of plant hosts (Kennedy 2010; Wang et al. 2015) by reducing the infection of plants by pathogens. In addition, from the richness indexes Chao1 and ACE and the diversity indexes Shannon and Simpson. The abundance and diversity of bacteria are greater than fungi both in P treatment and in CK treatment. This result is consistent with the previous study (Li et al. 2017). The previous study indicated that *T. melanosporum* ascocarps selected specific bacterial communities from the surrounding soil which may contribute to the development, maturation, and even aroma of the truffle (Vahdatzadeh et al. 2015; Deveau et al. 2016). Similarly, the results described here suggest that *T. panzhihuanense* have similar behavior with *T. melanosporum*. *T. panzhihuanense* will select specific bacterial communities from the surrounding soil during the colonization of *C. avellana*, even at the symbiotic stage. This may be the reason for the proportion of bacteria in the ectomycorrhizal microbial community structure is higher than fungi and among fungi yeasts are less represented.

The mycelia of *T. panzhihuanense* were dominant (41.3–59.5%) among ectomycorrhizal fungal communities, although these abundances were lower than those of previous studies. In another investigation, *T. magnatum* ectomycorrhizae were apparently absent, or very rare, when its ascomata formed, and other mycorrhizal fungi were co-localized in the same root tips (Bonito et al. 2011; Lefevre 2012). This discrepancy may be due to differing survival strategies of tubers at different growth periods. Therefore, we can speculate that in the early stages of truffle growth, truffles may compete with other mycorrhizal fungi for nutrition from the host tree and consequently become dominant in a variety of ways. In contrast, when truffles are forming ascocarps, its niche may be occupied by other mycorrhizal fungi because of its inability to produce mycelium. This mechanism may help the ascocarpous produce truffles, since it cannot be excluded that truffles form other types of symbiosis. For example, orchid-like mycorrhizae (Bonito et al. 2011) have been observed for other truffle species or other mycorrhizal fungi that play a certain role in truffle ascocarpous production.

Analysis of the mycorrhizal associations on bacterial metabolic functions indicated that bacterial communities in the P and CK treatments were enriched in functions related to energy, sugar, and protein metabolism, while bacterial communities in the P treatment were significantly enriched in functions related to sugar and protein metabolism compared to those of the CK treatments. Both of these functions are closely related to the growth and development of plants. Consequently, we speculate that the symbiosis between truffles and plants alters the bacterial community structure of plant roots. These community shifts then promote the growth and activity of bacteria with metabolisms associated with sugar and protein metabolism, thereby promoting plant growth and development.

Ectomycorrhizal synthesis is the first step in artificial cultivation of truffles which is extremely important towards subsequent production (Belfiori et al. 2016; Marozzi et al. 2017; Wan et al. 2015a). Other studies have identified factors closely associated with mycelium synthesis including the age of the plantation, the host species, plant productivity, the surrounding environment, and crop management (Bonito et al. 2011). Consequently, it is difficult to compare the differences in microbial communities associated with truffles and observe changes in community structure at different time periods (Streiblová et al. 2012). The development and adoption of high-throughput sequencing methodologies have allowed the in-depth analysis of microbial community structures associated with ectomycorrhizal fungi and interpretation of their role in mycorrhizal growth. Further studies are needed to understand the interactions between truffles and other organisms in the rhizosphere, including mycorrhization helper bacteria (MHB) that are promising targets for enabling the establishment of truffle plantations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This research does not involve human participants and/or animals.

Informed consent Informed consent was obtained from all individual participants included in the study.

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