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Truffle species strongly shape their surrounding soil mycobiota in a *Pinus armandii* forest

Dong Liu[1] · Mariana Herrera[1] · Peng Zhang[1] · Xinhua He[1,2] · Jesús Perez-Moreno[3] · Caspar C. C. Chater[4] · Fuqiang Yu[1]

Abstract

Truffles contribute to crucial soil systems dynamics, being involved in plentiful ecological functions important for ecosystems. Despite this, the interactions between truffles and their surrounding mycobiome remain unknown. Here, we investigate soil mycobiome differences between two truffle species, *Tuber indicum* (Ti) and *Tuber pseudohimalayense* (Tp), and their relative influence on surrounding soil mycobiota. Using traditional chemical analysis and ITS Illumina sequencing, we compared soil nutrients and the mycobiota, respectively, in soil, gleba, and peridium of the two truffle species inhabiting the same *Pinus armandii* forest in southwestern China. Tp soil was more acidic (pH 6.42) and had a higher nutrient content (total C, N content) than Ti soil (pH 6.62). Fungal richness and diversity of fruiting bodies (ascomata) and surrounding soils were significantly higher in Tp than in Ti. Truffle species recruited unique soil mycobiota around their ascomata: in Ti soil, fungal taxa, including *Suillus, Alternaria, Phacidium, Mycosphaerella, Halokirschsteiniothelia,* and *Pseudogymnoascus,* were abundant, while in Tp soil species of *Melanophyllum, Inocybe, Rhizopogon, Rhacidium,* and *Lecanicillium* showed higher abundances. Three dissimilarity tests, including *adonis, anosim,* and MRPP, showed that differences in fungal community structure between the two truffle species and their surrounding soils were stronger in Tp than in Ti, and these differences extended to truffle tissues (peridium and gleba). Redundancy analysis (RDA) further demonstrated that correlations between soil fungal taxa and soil properties changed from negative (Tp) to positive (Ti) and shifted from a moisture-driven (Tp) to a total N-driven (Ti) relationship. Overall, our results shed light on the influence that truffles have on their surrounding soil mycobiome. However, further studies are required on a broader range of truffle species in different soil conditions in order to determine causal relationships between truffles and their soil mycobiome.

Keywords Hypogeous ectomycorrhizal fungi · Truffles · Soil nutrient · Fungal community · Mycobiota

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✉ Dong Liu
liudongc@mail.kib.ac.cn

✉ Fuqiang Yu
fqyu@mail.kib.ac.cn

[1] Key Laboratory for Fungal Diversity and Green Development, The Germplasm Bank of Wild Species, Kunming, Kunming Institute Botany, Chinese Academy of Sciences, Qingsong Road 21#, Kunming 650201, Yunnan, China

[2] Department of Land, Air and Water Resources, University of California at Davis, Davis, CA 95616, USA

[3] Colegio de Postgraduados, Campus Montecillo, Microbiología, Edafología, 56230 Montecillo, Texcoco, Mexico

[4] Royal Botanic Gardens, Kew, Richmond TW9 3AE, Surrey, UK

Abbreviations

ANOVA	Analysis of variance
PCR	Polymerase chain reaction
QIIME	Quantitative insights into microbial ecology
OTU	Operational taxonomic unit
rRNA	Ribosomal RNA

Background

Truffles (*Tuber* spp.) are hypogeous ectomycorrhizal fungi that produce fruiting bodies with high economic value and are one of the most expensive foods worldwide (Le Tacon et al. 2016; Zambonelli et al. 2016). Due to this, truffle research has extensively increased over the last two decades, including work on truffle mycelia, species diversity, host trees, and soil biological characteristics (Bonito et al. 2010;

Liu et al. 2020; Innangi et al. 2020; Piñuela et al. 2021). During their life span, truffles inhabit diverse and complex biotic environments, interacting as free mycelium, ascoma (fruiting body), and/or mycorrhizal symbiont (Wenkart et al. 2001; Pacioni et al. 2014). Truffle development starts with a mycelial pellet that gradually grows into a globular ascoma in which the outward cells differentiate into a protective layer (peridium) with pores as authentic entryways (Pacioni 1990; Zarivi et al. 2015). In most cases, truffle development inhibits herbaceous growth and reduces soil fungal biodiversity (De Miguel et al. 2014; Mello et al. 2015) due to phytotoxic metabolites secreted by some truffle species (*Tuber aestivum, T. melanosporum,* and *T. indicum*) (Pacioni et al. 1990) and allelopathic actions (Streiblová et al. 2012). The herbicidal effect results in the above-ground "brûlé" (burnt) effect indicative of the below-ground truffle zone.

Inter-specific competition among mycelia for soil nutrients and water might also be an important ecological explanation for the phenomenon of biodiversity reduction (Piñuela et al. 2021; Sourzat 2018), and such competitive ability could vary among truffle species. Research on truffle ectomycorrhizal induction and the ecology of truffle-colonized seedlings under controlled conditions has demonstrated variation in soil nutrient acquisition among truffle species. For example, *T. melanosporum* inoculation on *Carya illinoinensis* seedlings had a weak influence on soil N content (Zhang et al. 2019), but *T. borchii* and *Tuber panzhihuanense* strongly modified easily-available soil nutrients (i.e. N and P) and exchangeable cations (Li et al. 2019; Yang et al. 2019; Zhang et al. 2020). These experiments also showed that truffles could shape ectomycorrhizosphere soil microbial community composition (Li et al. 2019; Yang et al. 2019; Zhang et al. 2019, 2020), and that this influence was stronger for bacterial than for fungal communities (Li et al. 2019; Yang et al. 2019; Liu et al. 2020). These studies have deepened our understanding of complex "truffle-plant-soil-microbe" interactions, but as they are limited to greenhouse experimental conditions and focus on commercial truffle cultivation methods, in situ changes of truffle–soil–microbe interactions in natural/field soil conditions have not been well captured.

Although studies on truffle-associated bacterial diversity are plentiful (Mello et al. 2013; Barbieri et al. 2016; Zampieri et al. 2016; Liu et al. 2021a, b, c), less is known for fungi. In our previous study, over sixty fungal genera were identified within a rare truffle species (*Tuber pseudobrumale*), indicating a high fungal diversity within truffles (Liu et al. 2020). Moreover, endofungal communities closely interact with the truffle peridium by colonizing through peridium pores to the gleba (Pacioni and Leonardi 2016). The quadripartite interactions between host plant, truffle species, soil properties, and fungal communities in truffle soils require further exploration. Under natural conditions,

different truffle species can establish symbiotic relationships with the same host tree species. For instance, both *Tuber indicum* and *T. pseudohimalayense* ascomata can be detected under *Pinus armandii* trees. Despite their shared host tree species, do the distinct truffle species differentially affect surrounding soil properties via recruitment of different soil mycobiota? If so, what are the main relationships between truffle species and soil properties?

To understand how truffle species affect their surrounding soil physicochemical properties, soil mycobiota, and their interactive relationships, two commercially important truffle species (*Tuber indicum* and *T. pseudohimalayense*) were examined by routine soil chemical analyses, Internal Transcribed Spacer (ITS) Illumina sequencing, principal coordinate, and redundancy analysis. These two ectomycorrhizal fungal species share similar morphologies (Manjón et al. 2009) and associate with the same *Pinus armandii* Franch. tree species in southwest China (Juan et al. 2011). This study addressed the following hypotheses: (H1) the surrounding soil properties of the two truffle species could be different; (H2) fungal diversity and community structure could be different in truffle-producing soils and ascomata; and (H3) the determinant factor of the relationship between fungal taxa and soil properties would be different in each truffle's niche.

Results

Soil cation and nutrient changes

Soil moisture was not significantly different among the three soil positions/treatments, regardless of whether soil surrounded the ascomata or not (Table 1). Soil pH values were close to neutral, with the highest and lowest pH values in soils around and below the ascomata of *T. indicum* and the bulk soil, respectively (Table 1). The concentrations of Ca^{2+} and Mg^{2+} were significantly higher ($P < 0.05$) in the truffle soil of Ti, compared to Tp and the bulk soil (Table 1). In contrast, the concentrations of soil organic matter, total carbon, and total nitrogen were similar among the bulk soil, Ti soil, and Tp soil (Table 1). The C:N ratio and the alkaline hydrolyzable nitrogen content were significantly higher ($P < 0.05$) in the truffle soil of Tp and bulk soil, compared to Ti soil (Table 1).

Fungal diversity changes

About 138,854–652,726 raw reads were obtained per sample and read lengths varied from 250 to 438 bp, with an average of 343 bp (Table S1). A range of 130,532–626,071 sequences from individual samples (mean = 317,012) were obtained (Table S1). A total of 6,657,258 high-quality sequences from all 21 samples (soil, gleba, and peridium

of the two truffle species) were represented for 10 phyla, 21 classes, 96 orders, and 179 families of fungi. The number of observed fungal species was around 200 and similar between Ti and Tp ascomata. Fungal community diversity was similar in the peridium and gleba as shown by the Shannon diversity index (considering both richness and evenness) (Fig. 1). Fungal richness and diversity from the soil around fruiting bodies were significantly higher ($P < 0.05$) in Tp than in Ti (Fig. 1). Similarly, the number of unique OTUs in the soil around the truffle was also higher in Tp than in Ti (Fig. 2).

Table 1 Soil physicochemical properties in the soils around the ascomata of *Tuber indicum* and *Tuber pseudohimalayense*

Treatment	Moisture (%)	pH	Ca^{2+} (mg kg^{-1})	Mg^{2+} (mg kg^{-1})	OM (g kg^{-1})	TC (g kg^{-1})	TN (g kg^{-1})	C:N ratio	AN (mg kg^{-1})
S_C	$28 \pm 3a$	$6.29 \pm 0.10b$	$3935 \pm 242b$	$627 \pm 36b$	$80 \pm 4a$	$46.3 \pm 2.2a$	$3.64 \pm 0.06a$	$12.7 \pm 0.4b$	$319 \pm 4a$
S_{Ti}	$29 \pm 1a$	$6.62 \pm 0.05a$	$6035 \pm 221a$	$1116 \pm 75a$	$78 \pm 10a$	$45.1 \pm 6.8a$	$3.68 \pm 0.22a$	$12.2 \pm 0.3b$	$283 \pm 6b$
S_{Tp}	$30 \pm 2a$	$6.42 \pm 0.06ab$	$4421 \pm 303b$	$786 \pm 27b$	$88 \pm 3a$	$51.2 \pm 1.5a$	$3.78 \pm 0.15a$	$13.6 \pm 0.2a$	$316 \pm 5a$

AN alkaline hydrolyzable nitrogen, Ca^{2+} calcium concentration; *C:N ratio* soil total carbon/total nitrogen ratio, Mg^{2+} magnesium concentration, *OM* organic matter, S_C bulk or control soils (10 m away from the *Pinus armandii* trunk to avoid the impact from the truffle's sphere), S_{Ti} soils around and below the ascomata of *T. indicum*, S_{Tp} soils around and below the ascomata of *T. pseudohimalayense*, *TC* total carbon, *TN* total nitrogen

Values (means ± SD, $n = 3$) followed by different letters are significantly different at $P < 0.05$ (ANOVA, *Tukey* HSD)
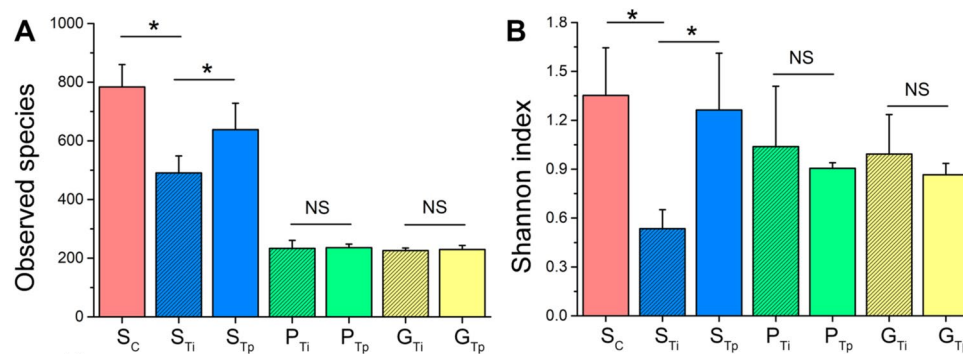
Fig. 1 Fungal species richness (**A**) and Shannon index (**B**) in the control and truffle-surrounding soils, and fungal tissues of *Tuber indicum* (Ti) and *Tuber pseudohimalayense* (Tp) occurring in a *Pinus armandii* forest in Huidong, Sichuan, Southwest China. For individual index boxes, differences between treatments were analyzed by independent samples *T*-tests; *$P < 0.05$. G_{Ti} gleba of *Tuber indicum*, G_{Tp} gleba of *Tuber pseudohimalayense*, P_{Ti} peridium of *Tuber indicum*, P_{Tp} peridium of *Tuber pseudohimalayense*, S_C control soils, S_{Ti} soils around and below the ascomata of *Tuber indicum*, S_{Tp} soils around and below the ascomata of *Tuber pseudohimalayense*

Fig. 2 Shared and unique fungal OTUs in the control and truffle-surrounding soils, and fungal tissues of **A** *Tuber indicum* and **B** *Tuber pseudohimalayense* occurring in a *Pinus armandii* forest in Huidong, Sichuan, Southwest China. Abbreviations are shown in Fig. 1
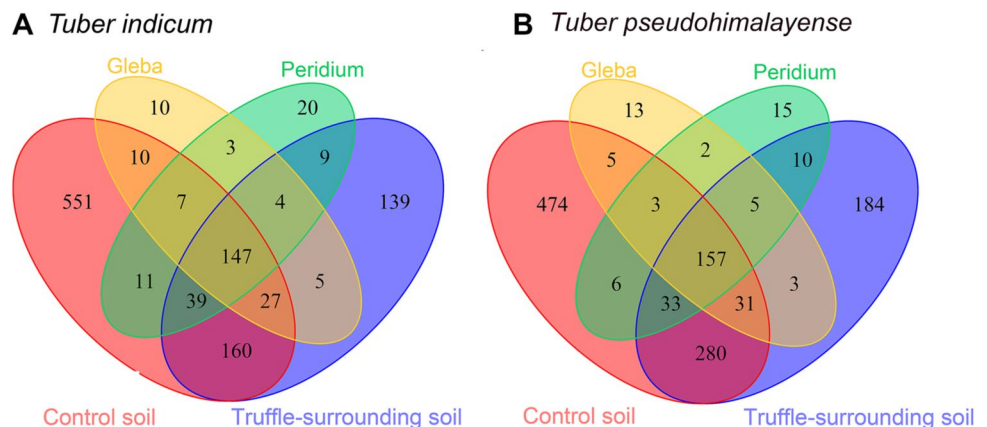
Distribution of fungal taxa and community structure

Two-way cluster analysis of the relative abundance of major fungal genera showed a clear separation of two clusters for truffle-surrounding soils and fruiting bodies (Fig. 3). In Ti soil, six fungal taxa, including *Suillus, Alternaria, Phacidium, Mycosphaerella, Halokirschsteiniothelia,* and *Pseudogymnoascus,* were dominant (Fig. 3, reddish color). Over twenty fungal taxa were identified in the Tp soil, where species of *Melanophyllum, Inocybe, Rhizopogon, Rhacidium,* and *Lecanicillium* showed higher abundances. Of the endofungal fungal genera inhabiting truffle fruiting bodies, *Candida* was most abundant in both truffle species, whereas *Acremonium* and *Mortierella* were more abundant in *T. pseudohimalayense* than in *T. indicum* (Fig. 3).

Various ECM fungi were also identified in the soil samples. For Ti species, there were 15 mycorrhizal species in the bulk soil, and 12 mycorrhizal species in the truffle-surrounding soil (Table 2). Inside the Ti fruiting bodies, there were 6 ectomycorrhizal fungi (*Tuber, Rhizopogon, Inocybaceae, Wilcoxina, Suillus,* and *Tomentella*) and one ericoid mycorrhizal fungus (*Oidiodendron*)

exhibits symbiotroph trophic modes (Rinaldi et al. 2008; Larsson et al. 2009; Newsham 2011). All these soil-derived endofungal fungi may be strongly competitive compared to those in the truffle-surrounding soil: Rhizophagus, Amanita, Chroogomphus, Serendipita, and Geopora (Table 2). For Tp, there were 17 mycorrhizal species in the truffle-surrounding soil (Table 3). 8 ectomycorrhizal fungi (*Tuber, Inocybaceae, Rhizopogon, Suillus, Wilcoxina, Helvella, Tomentella*, and *Cortinarius*) and 1 ericoid mycorrhizal fungus (*Oidiodendron*) co-existed inside the Tp fruiting bodies. Compared to Ti, there were 5 and 2 more mycorrhizal species found in the truffle-surrounding soil and fruiting bodies, respectively (Table 3). Principal coordinates analysis (PCoA) showed that overall fungal community structures (beta-diversity) of control and truffle soils were separated from those of truffle peridium and gleba (Fig. 4). These results were statistically supported by the three dissimilarity tests, including *adonis*, *anosim*, and MRPP ($P < 0.05$). Fungal community structure differences between the truffle and their surrounding soil were stronger in Tp (*anosim*, $r = 0.98$, $P = 0.001$; Fig. 4) than in Ti (*anosim*, $r = 0.48$, $P = 0.01$; Fig. 4). Such differences extended to truffle tissues
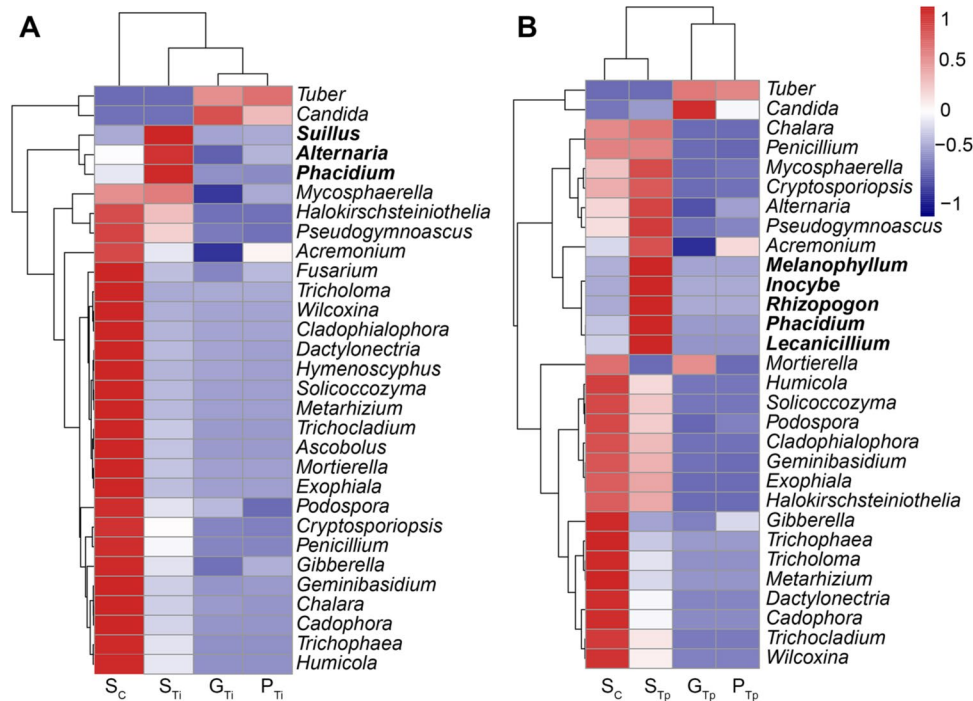
Fig. 3 Cluster analysis-based heatmaps of community composition (top 30 genera) in the control and truffle-surrounding soils, and fungal tissues of **A** *Tuber indicum* (Ti) and **B** *Tuber pseudohimalayense* (Tp) occurring in a *Pinus armandii* forest in Huidong, Sichuan, Southwest China. The horizontal and vertical clusters represent the fungal genera and treatments clustering tree, respectively. In each square, the relative abundance of genera in each row has been stand-

ardized to obtain a "*Z*" value. The *Z* value of a genus is the value obtained by dividing the difference between the relative abundance of a sample and the average relative abundance of all samples by the standard deviation of all samples. The redder the square color, the higher the abundance of the genus among samples. Abbreviations are shown in Fig. 1

Table 2 The number of the mycorrhizal taxons and their abundance in the control (Sc) and truffle surrounding soils (STi), and the peridium (PTi) and gleba (GTi) of *Tuber indicum*

Taxon	Trophic mode	Guild	Sc	STi	GTi	PTi
Tuber	Symbiotroph	Ectomycorrhizal	0.01203	0.010905	0.223354	0.250126
Rhizopogon	Symbiotroph	Ectomycorrhizal	0.00011	8.57E–05	0.000036	2.05E–05
Inocybaceae	Symbiotroph	Ectomycorrhizal	0.000113	0.000149	3.58E–05	5.47E–05
Wilcoxina	Symbiotroph	Ectomycorrhizal	0.00127	3.66E–05	4.28E–06	5.87E–06
Suillus	Symbiotroph	Ectomycorrhizal	0.000046	0.002767	0.000004	8.49E–06
Tomentella	Symbiotroph	Ectomycorrhizal	2.07E–06	7.41E–06	0	1.11E–06
Oidiodendron	Pathotroph-Symbiotroph	Ericoid Mycorrhizal	5.37E–05	1.38E–05	0	2.61E–06
Diversispora	Symbiotroph	Arbuscular Mycorrhizal	3.02E–05	0	0	0
Claroideoglomus	Symbiotroph	Arbuscular Mycorrhizal	3.69E–05	0	0	0
Glomeraceae	Symbiotroph	Arbuscular Mycorrhizal	3.43E–05	0	0	0
Paraglomeraceae	Symbiotroph	Arbuscular Mycorrhizal	1.48E–05	0	0	0
Rhizophagus	Symbiotroph	Arbuscular Mycorrhizal	2.07E–06	2.67E–06	0	0
Amanita	Symbiotroph	Ectomycorrhizal	2.86E–05	6.26E–06	0	0
Chroogomphus	Pathotroph-Symbiotroph	Ectomycorrhizal-Fungal Parasite	4.15E–06	6.53E–05	0	0
Serendipita	Symbiotroph	Orchid Mycorrhizal	3.68E–06	2.92E–06	0	0
Geopora	Symbiotroph	Ectomycorrhizal	0	2.67E–06	0	0

Abbreviations are shown in Table 1

Table 3 The number of the mycorrhizal taxons and their abundance in the control (Sc) and truffle surrounding soils (STp), and the peridium (PTp) and gleba (GTp) of *Tuber pseudohimalayense*

Taxon	Trophic mode	Guild	Sc	STp	GTp	PTp
Tuber	Symbiotroph	Ectomycorrhizal	0.012409	0.011821	0.823185	0.78961
Inocybaceae	Symbiotroph	Ectomycorrhizal	0.000116	0.013342	5.14E–05	6.55E–05
Rhizopogon	Symbiotroph	Ectomycorrhizal	0.00011	0.011696	2.62E–05	0.000029
Suillus	Symbiotroph	Ectomycorrhizal	4.59E–05	0.000356	1.12E–05	1.05E–05
Wilcoxina	Symbiotroph	Ectomycorrhizal	0.001269	0.0005	6.12E–06	7.36E–06
Oidiodendron	Pathotroph-Symbiotroph	Ericoid Mycorrhizal	0.000052	0.0002	3.08E–06	5.32E–07
Helvella	Symbiotroph	Ectomycorrhizal	0	0.000306	2.48E–06	2.85E–06
Tomentella	Symbiotroph	Ectomycorrhizal	2.07E–06	8.75E–05	1.23E–06	7.35E–07
Cortinarius	Symbiotroph	Ectomycorrhizal	7.18E–06	0.000107	0	5.32E–07
Helotiaceae	Saprotroph-Symbiotroph	Ectomycorrhizal-Fungal Parasite	0	2.94E–06	0	0
Tricholomataceae	Pathotroph-Symbiotroph	Ectomycorrhizal-Fungal Parasite	0	1.39E–05	0	0
Chroogomphus	Pathotroph-Symbiotroph	Ectomycorrhizal-Fungal Parasite	4.15E–06	9.87E–06	0	0
Meliniomyces	Saprotroph-Symbiotroph	Ectomycorrhizal-Endophyte	0	2.34E–05	0	0
Amanita	Symbiotroph	Ectomycorrhizal	2.86E–05	3.99E–06	0	0
Diversispora	Symbiotroph	Arbuscular Mycorrhizal	3.01E–05	1.35E–06	0	0
Claroideoglomus	Symbiotroph	Arbuscular Mycorrhizal	3.69E–05	1.47E–06	0	0
Glomeraceae	Symbiotroph	Arbuscular Mycorrhizal	4.21E–05	1.29E–06	0	0
Paraglomeraceae	Symbiotroph	Arbuscular Mycorrhizal	1.48E–05	0	0	0

Abbreviations are shown in Table 1

(peridium and gleba). Truffle fungal community structure was also separated in Tp tissues (*anosim*, $R = 0.90$, $P = 0.001$) and in Ti tissues (*anosim*, $R = 0.68$, $P = 0.045$).

Relationships between soil properties and fungal communities

Redundancy analysis (RDA) showed relationships of the most influential soil properties and fungal genera in both
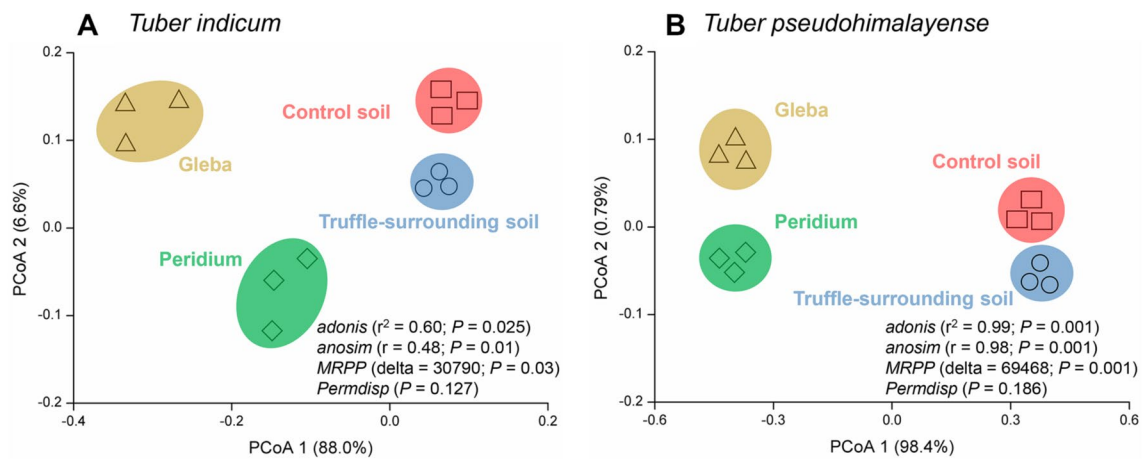
Fig. 4 Fungal community compositions as indicated by principal coordinate analysis (PCoA) of pairwise Bray–Curtis distance in the truffle-surrounding soils and fungal tissues of **A** *Tuber indicum* and **B** *Tuber pseudohimalayense* occurring in a *Pinus armandii* forest in Huidong, Sichuan, Southwest China. Abbreviations are shown in Fig. 1. Bray–Curtis distance-based results of three permutation dis-

similarity tests are presented including analysis of similarity (*anosim*), non-parametric multivariate analysis of variance (*adonis*) using distance matrices, and a Multiple Response Permutation Procedure (MRPP). Permutation test for homogeneity of multivariate dispersion (*Permdisp*) was used to test heteroscedasticity between groups

Ti and Tp soil. For Ti, the first and second axes of the RDA explained 92.7% and 7.3% of the variations in soil fungal taxa (Fig. 5A). The variation in soil fungal taxa was strongly driven by total N, with a contribution (explained fitted variation) of > 90%, and slightly driven by pH (7.5%). Among the 30 top fungal taxa, almost all of them (except for *Chalara*) exhibited positive correlations with soil pH and total N. Species of *Tuber*, *Rhizopogon*, *Inocybe*, and *Acremonium*

showed pronounced correlations as shown by their closer coordinate positions (Fig. 5A) and this was confirmed by significant positive correlations (*P* < 0.001; Fig. S3). In Tp soil, the first and second axes of the RDA explained among 79.0% and 20.1% of the variations for fungal taxa (Fig. 5B), mainly driven by soil moisture (78% variation explained) and partly by total N (21% explained; Fig. 5). In contrast to the relationships of soil properties and fungal genera in Ti,
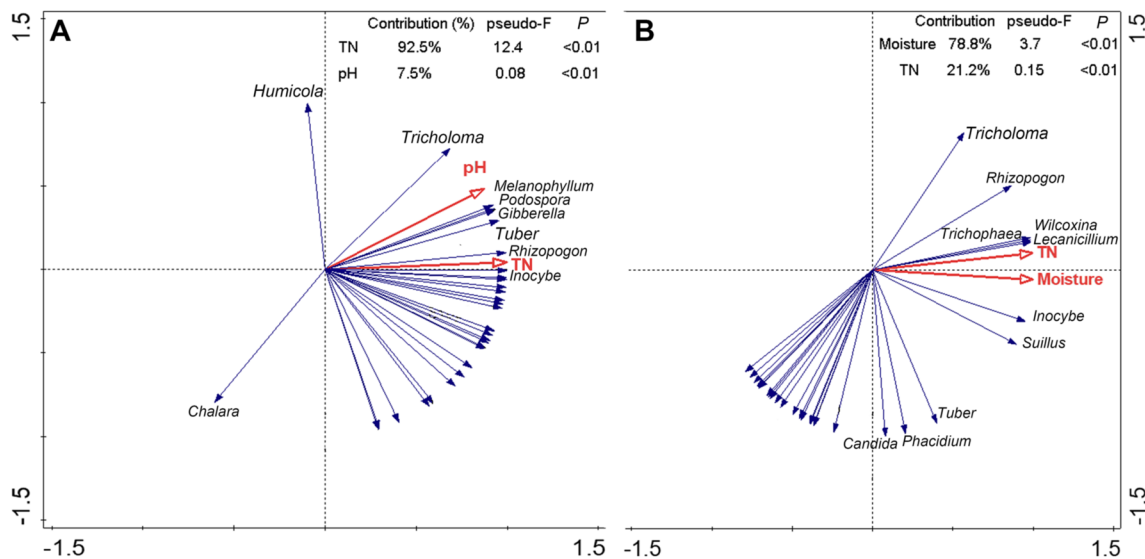
Fig. 5 Redundancy analysis (RDA) showing the relationships of the ecological variables and fungal genera (Top 30) in the below and surrounding soils of *Tuber indicum* (**A**) and *Tuber pseudohimalayense* (**B**) occurring in a *Pinus armandii* forest in Huidong, Sichuan, Southwest China. The measured **nine soil properties** (see Table 1) were used as constraints to select the most influential variables. The

length of arrows represents the strength of the respective soil properties with the fungal genera. The angle between vectors indicates the degree of their associations (smaller angle means higher correlation). To avoid the names of **fungal genera** overlapping, genera with only loose associations with soil major influencing factors are not shown alongside the blue arrow, and can be found in Fig. 3

most fungal taxa in Tp surrounding soil exhibited negative correlations with the soil total N and moisture. There were only nine fungal taxa, including *Tuber,* which showed positive correlations; among them *Lecanicillium, Wilcoxina*, and *Trichophaea* were significant ($P < 0.001$; Fig. S2).

Discussion

As we hypothesized (H1), soil properties were affected by the truffle species, with different Ca^{2+} and Mg^{2+} concentrations and C/N ratio in the truffles' surrounding soils (Table 1). Ti seemed to mobilize more Ca^{2+} and Mg^{2+} in the surrounding soils, as indicated by significantly higher ion concentrations in Ti soil compared to those of Tp soil (Table 1). Truffles can occur across a wide soil pH range, from slightly acidic, neutral (Li et al. 2017, 2018; Innangi et al. 2020) to alkaline (Mello et al. 2006). In the present study, Ti and Tp surrounding soils were all acidic with minor differences (pH 6.4 and 6.6). Noticeably, although total C and N contents were not significantly affected by truffle species, the C/N ratios were. The fungal species present in Tp soils (many members from Chytridiomycota) may be responsible for the reduced C/N ratio, via mineralizing more organic N than organic C, with consequent variation in organic matter quality (Innangi et al. 2020). In addition, the significantly higher hydrolyzable N found in Tp than in Ti surrounding soil (Table 1) supports this argument.

We hypothesized that a truffle species would form its unique soil fungal community around its ascomata and a difference in fungal taxa might also exist within these truffle fruiting bodies (H2). As expected, Tp surrounding soil exhibited significantly higher fungal richness and diversity compared to Ti (Fig. 1), and this difference was in agreement with the soil C/N ratio and TC contents (Tp > Ti). Fungal diversity differences of the two truffle surrounding soils may relate to (i) a closer association between soil fungi and nutrient content including TC, C/N ratio etc. (Liu et al. 2019; Yang et al. 2020) and/or (ii) the production of various fungal metabolites of Ti and Tp mycelia that may differentially affect their soil fungal communities. Alternatively, the competitive ability of Ti mycelium could be stronger than that of Tp, which might occupy a wider ecological niche and, in turn, lead to a significant decrease in its soil mycobiome diversity. Although relative mycelial abundance of the two truffle species was not recorded in the present study, differences in the number of dominant/abundant fungal taxa (21 in Tp vs. 6 in Ti), as well as the unique fungal OTUs (Figs. 2 and 3), indirectly reflect the fungal competitive environments from the respective surrounding soils.

There were 292 and 283 fungal OTUs identified inside the Ti and Tp ascomata (Fig. 2). Among them, 55% (Ti) and 59% (Tp) of OTUs were shared between the truffle gleba and peridium. Recent studies have demonstrated a disparity in the number of fungal OTUs inside truffle fruiting bodies. For instance, Perlińska-Lenart et al. (2020) reported only six different fungal species inside the ascomata of the European black truffle *T. aestivum*, while Liu et al. (2020) found ~400 fungal OTUs in *T. pseudobrumale*. In the present study, there were ~300 non-truffle fungal OTUs identified in truffles (Fig. 2). Probable reasons for varying fungal OTUs within truffles include the following:

i. *Difference in investigated truffle tissues* Our results showed that the truffle peridium is an important micro-niche harboring similar numbers of fungal OTUs (~230) as the gleba (~220);

ii. *Prevalence of truffle OTUs vs. endofungal OTUs* For example, *T. aestivum* host fungal OTUs represented over 99% of assigned fungal OTUs (Perlińska-Lenart et al. 2020), whereas *T. pseudobrumale* host fungal OTUs accounted for only 70% of OTUs (Liu et al. 2021a, b, c);

iii. *Difference in primers and regions used for amplification of rDNA fragments.* For the same truffle species, observed bacterial OTUs and Shannon's diversity indices were markedly different compared to the results of V1 and V4 bacterial 16S ribosomal DNA amplicon sequencing (Benucci and Bonito 2016).

Trophic modes and intra-fungal competition also contribute to diversity and shifts in fungal OTUs. Among our fungal OTUs, 295 fungal OTUs (69%) were assigned to specific trophic modes. Out of these functionally assigned OTUs, 62 OTUs with the "possible" confidence ranking were removed, and the remaining 43 and 189 OTUs that belonged to the "acceptable" confidence ranking assigned to the "Highly probable" and "probable" type were used for downstream functional analysis. As expected, most of the relative abundance in the gleba of *T. indicum* and *T. pseudohimalayense* corresponded to *Tuber* (99.96% and 99.99%, respectively). However, an interesting finding was the detection of four ectomycorrhizal taxa within healthy ascomata of both evaluated truffles. In the case of the gleba of *T. indicum*, *Rhizopogon*, Inocybaceae, *Wilcoxina,* and *Suillus* were detected, with relative abundances of 0.0200%, 0.0160%, 0.0019%, and 0.0018%, respectively. Meanwhile, in the gleba of *T. pseudohimalayense* the same taxa were detected, but with much lower relative abundances accounting in Inocybaceae, *Rhizopogon*, *Suillus,* and *Wilcoxinia* for 0.010%, 0.003%, 0.001%, and 0.001%, respectively. In order to provide a possible explanation of this finding is important to consider that these four ectomycorrhizal taxa were relatively abundant in bulk soil, soil adhere to peridium, and the peridium itself. As the peridium is not a water-proof structure, during rainy season there is a concomitant complex bidirectional water

movement from outside the gleba and the surrounding soil. Due to the small spore size (ranging from 6 to 16 μm in length and 3 to 8 μm in width) of the four detected ectomycorrhizal fungi, it is possible that a very small amount of some ectomycorrhizal propagules may be moved from the surrounding soil to the gleba and remain there in a quiescent stage, without affecting the functioning or biochemistry of the ascomata. And once the truffles are dispersed by endozoochory or they are rotten, these fungal propagules might start its reproduction. Recently, some ectomycorrhizal taxa belonging to Cantharellales and Pezizales have been also found inside polypores in the subiculum of fruiting bodies growing in *Picea abies* forests (Maurice et al. 2021). A current approach which has been recently demonstrated is that the fungal fruiting bodies, in both Basidiomycota and Ascomycota, contain not only genetic material of a single species, as previously believed, but actually they are "microuniverses" or real holobionts, i.e., reservoirs of a myriad of hundreds or thousands of bacterial and fungal species (e.g., Maurice et al. 2021; Liu et al. 2021a, b, c). However, it is important to point out that these new insights need to be further investigated, in order to have a more holistic comprehension of the interwoven relationships of fungal forest components and its relevance in terms of ecosystem structure and functioning.

Mello et al. (2011) demonstrated a shift from Basidiomycota- to Ascomycota-dominated fungal communities between control soils to truffle soil zones (*T. melanosporum*) (Mello et al. 2011). We found a relatively higher abundance of Ascomycota (65%) in Tp soil compared to Ti soil (25%), which might reflect a stronger competition between Tp and other Basidiomycota (Napoli et al. 2010). Moreover, when comparing the competition between different mycorrhizal fungi in the soil, we found there were seven more mycorrhizal fungi found in the Tp than in the Ti surrounding soil. Among these, *Cortinarius* and Helotiaceae are typical EMFs with strong infectivity (Rinaldi et al. 2008), as evidenced by their presence in Tp fruiting bodies (Table 3); however, *Helvella* and *Meliniomyces* exhibited more complex trophic modes, as either plant pathogen, fungal parasite, or wood/litter saprotroph (Henrici 2008; Tedersoo et al. 2014). In addition, in the Tp surrounding soils, there were three arbuscular mycorrhizal fungi (*Diversispora, Claroideoglomus,* and Glomeraceae) (Redecker et al. 2013; Tedersoo et al. 2014) which might perform similar functions, including promoting phosphorus absorption and host growth, as they do for host trees. Compared to truffle bacteria, the truffle-surrounding fungi exhibited more complex patterns.

Herein, we propose a conceptual model to explain the effect of truffle species on their surrounding soil mycobiota and soil properties, in the context of shared host tree species and soils (Fig. 6). In support of H2, changes in soil fungal community influenced truffle-inhabiting fungi composition
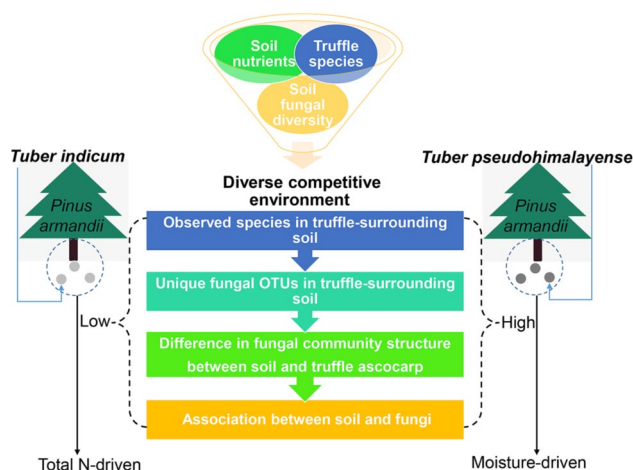
Fig. 6 Changes in the truffle-surrounding soils and fungal tissues of *Tuber indicum* and *Tuber pseudohimalayense* occurring in a *Pinus armandii* forest

and structure (Figs. 3 and 5). On a large scale, using specific PCR amplicon sequencing in tree roots, Gryndler et al. (2017) studied the potential occurrence of mature truffles (*Tuber* spp.). Among the predictor of truffle distribution, soil and climatic conditions were stronger compared to host preferences of the individual *Tuber* spp. So, soil heterogeneity/characteristics can shape the microbial communities, including the *Tuber* species in a specific niche. Changes in truffle-surrounding soil properties and soil fungal diversity might induce further variations in the relationship between fungal taxa and soil properties (Fig. 6). As we hypothesized (H3), the relationship between fungal taxa and soil properties was different between the two truffle species. In Ti and Tp soil, the relationship was mainly related with soil total N content and moisture, respectively (Figs. 5 and 6), with contrasting negative (Ti) and positive (Tp) relationships (Fig. S1). Although soil moisture content did not obviously differ (29 vs 30%) between truffle species soils, RDA identified moisture as a differential driver of the microbiota associated with Tp compared to Ti. The quadripartite interactions between host plant, truffle species, soil nutrients, and fungal diversity therefore shape a complex relationship between soil and fungi. The conceptual model that we propose (Fig. 6) is based on the comparison between the two species within a shared habitat, which highlights the central role of the truffle itself. A systemic investigation of the interactions between truffles, soils, and microbiota is required in further research, with consideration of multivariable properties, such as soil texture, soil enzymes, and climatic factors. Additionally, culture-based and controlled environment methods could be used in combination with Illumina sequencing to confirm the presence of certain fungal taxa and their functional associations with the ascomata.

Conclusions

Our results showed clear differences in soil properties and fungal diversity from soils around the two truffle species (*T. indicum* and *T. pseudohimalayense*) in the same *P. armandii* forest. The presence of *T. pseudohimalayense* appeared to have a stronger modifying influence on the soil environment, leading to changes in soil chemistry (lower pH and higher nutrient contents). Soil nutrients, truffle species, and other soil fungal taxa are interactive. Such influences may form a complex and competitive environment in the truffles' unique ecological niches, and the effect might further extend inside truffle tissues. From an ecological perspective, whether differences in their mycocenosis can affect the entire mycelial biomass of the two truffle species, or indeed their host plant endophytes, requires further exploration to understand the interactions of hypogeous ectomycorrhizal fungi with soil systems.

Methods

Study site and sampling strategy

The sampling site is located in one of the Chinese truffle hotspots in Huidong county (26°22′48″ N, 102°24′36″ E, 2745 m.a.s.l.) (Fu et al. 2016), Sichuan province, Southwest China. The site is a *P. armandii* pine-dominant forest with the following ecological traits: annual variation of air temperature ranges between 11–24 °C; with a mean annual precipitation of 1099 mm (Fu et al. 2016); and sandy loam soil (Haplic Luvisol, FAO Soil Classification System) (Fu et al. 2016). Sampling of soils and truffles was carried out during the truffle production period in December 2018. In order to capture the variation present in the forest, we chose three plots (100 m away from each other; size 200 × 200 m) as field ecological replicates.

Control soils (Sc) were collected from the top 0–15 cm soil depth with a stainless-steel (5 cm inner diam.) cylinder, and 10 m away from the nearest *P. armandii* trunk to avoid the truffle's potential sphere of influence. Control sites were also searched by a truffle dog to ensure no ascomata were detected. Six soil cores were obtained from each plot and pooled together to form a representative plot sample, with a total of three pooled soils as ecological replicates per site. After sampling, soils were immediately stored in a cool box and transported to the laboratory where they were sieved (2 mm) to remove stones, roots, and microfauna under aseptic conditions. Half of each pooled soil sample was stored at −20 °C for microbial analysis and the other half was air-dried for chemical analyses.

With the assistance of a trained dog, 20–30 mature truffles were collected from each of the three subplots. In each subplot, twelve (six Ti and six Tp) high-quality, intact, and healthy (with no evidence of damage from fungivory) truffles were selected for truffle-associated compartment separation. Soil samples surrounding the truffles, *T. indicum* (designated S_{Ti}) and *T. pseudohimalayense* (designated S_{Tp}), were those tightly adhering to the truffle surface (< 0.5 cm). In the laboratory, S_{Ti} and S_{Tp} were collected on a sterile petri dish with a sterilized soft-metal brush and then transferred into a 15 ml tube.

All truffles were rinsed and cleaned with sterile milli-Q water. After drying the truffle surface with sterilized absorbent paper, the ascomata were broken by force under axenic conditions and the truffle peridium (P) and inner gleba tissues (G) were collected by sterilized forceps, as described by Liu et al. (2021a, b, c). Three pooled fungal tissue samples (each composed of 18 cut slices from six fruiting bodies of *T. indicum* or *T. pseudohimalayense*) were also, respectively, collected from the gleba or peridium of *T. indicum* (G_{Ti} or P_{Ti}) and *T. pseudohimalayense* (G_{Tp} or P_{Tp}), and then stored in sterilized self-sealing bags (60 mm × 85 mm) at −20 °C for subsequent DNA extraction.

Soil property analysis

Soil pH was determined in a soil:distilled water (1:2.5, W/V) mixture using a Delta 320 pH meter (Mettler-Toledo Instruments, Shanghai, China). Soil moisture was gravimetrically measured by oven drying at 105 °C for 24 h. Soil organic matter was determined with the potassium dichromate external heating method (Guo 2009). Soil total carbon (TC) and total nitrogen (TN) were measured with an elemental analyzer (Vario MAX C/N, Hanau, Germany) (Parkinson and Allen 1975). Determination of alkaline hydrolyzable N, calcium (Ca^{2+}), and magnesium (Mg^{2+}) was based on the Chinese national standard method (Nu 1999).

DNA extraction and PCR amplification

DNA from soil and truffle samples was extracted using the MoBioPower Soil DNA kit (12,888) and the DNeasy Plant Mini Kit (Qiagen SA, Germany), respectively. The ascomata of Ti and Tp were identified by both morphological and molecular techniques in the Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China. Polymerase chain reactions (PCRs) were carried out following the previously described method (Xiong et al. 2016). Internal transcribed spacer 1 (ITS 1) was amplified for fungal community analyses using universal primers ITS5-1737F and ITS2-2043R (Schultz 2005; Jeandroz et al. 2008). For PCR, all the samples were uniformly diluted to 20 ng/μL and PCR reactions were performed in triplicate in a 25 μL mixture

(5 µL of 5×reaction buffer, 5 µL of 5×GC buffer, 2 µL of dNTPs (2.5 mM), 1 µL of each primer, 2 µL of template DNA, 8.75 µL of DNase free water, and 0.25 µL Q5 DNA polymerase (Thermo Fisher, Molecular BioProducts, San Diego, CA, USA). PCR thermal cycling conditions were 94 °C for 5 min (initial denaturation), 30 cycles of 30 s at 94 °C, 52 °C 30 s, and 72° C 30 s, with a final extension for 10 min at 72 °C. Amplicons were extracted from 1% agarose gels and purified with the EZNA Gel Extraction Kit (Omega, Bio-Tech, New York, USA) according to the manufacturer's guidelines and quantified with PicoGreen using a FLUOstar Optima microplate reader (BMG Labtech, Jena, Germany).

Illumina MiSeq sequencing and bioinformatics

Purified amplicons were pair-end sequenced (2×300 bp runs) on the Illumina MiSeq platform (MAGIGE, Guangdong, China) using the MiSeq Reagent Kit v2 (600-cycles-PE, MS-102-3003). Sequences were processed and quality-filtered using the QIIME (V1.9.1) pipeline. Paired-end reads were truncated to 100 bp to remove low-quality sequence tails (average-quality values < 20 over a 10-bp sliding window. The ≥ 10 bp that passed through quality screening overlapping sequences were assembled using the FLASH software (v1.2.11) (Magoč and Salzberg 2011). The remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity cutoff. For species identification, we compared our sequences with those deposited in the UNITE database (for ITS, http://unite.ut.ee/index.php) using a confidence threshold ≥ 0.5. The OTUs assigned to the same phylum, class, genus, and species level were grouped together based on their taxonomic affiliations.

Data processing and statistical analysis

Shannon index and the number of observed species were used to evaluate fungal diversity and richness in soils and ascomata, respectively. One-way analysis of variance (ANOVA) followed by Tukey HSD (at $P < 0.05$) was used to compare significant differences in soil properties of the control, *T. indicum*, and *T. pseudohimalayense* surrounding soils. Independent samples *T*-tests were applied to compare diversity indices between *T. indicum* and *T. pseudohimalayense*. Microbial community beta-diversity between paired samples was determined using the UniFrac metric (Lozupone et al. 2011) in the MOTHUR program (http://www.mothur. org). Principal Coordinate Analysis (PCoA) was performed using the vegan package of R software based on the weighted Unifrac distance matrix, and the obtained coordinate points were plotted using the ggplot 2 package in R. Analysis of similarity (Anosim), non-parametric multivariate analysis of variance (Adonis) using distance matrices, and a Multiple

Response Permutation Procedure (MRPP) were used to examine fungal community differences (Clarke 1993; Van Sickle 1997; Zapala and Schork 2006). Redundancy analysis (RDA) was used to analyze the relationship between fungal communities and soil properties. RDA is advantageous for assessing the explanatory power of each defined variable by parsing out other terms as constraints to calculate its proportion of total variance (O'Connor 1988).

Declarations

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