Mycorrhization of Quercus Mongolica Seedlings by Tuber Melanosporum Alters Root Carbon Exudation and Rhizosphere Bacterial Communities

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Abstract

Aims To study how ectomycorrhizas (ECMs) mediate plant performance and rhizosphere soil bacterial communities via altered physiological characteristics and root carbon exudation.

Methods Tuber melanosporum-colonized and uncolonized *Quercus mongolica* seedlings were grown on a substrate consisting of 41 % peat, 41 % pumice, 9 % pine bark and 9 % lime. Gas exchange fluorescence system, inductively coupled plasma atomic-emission spectrometer, high-performance liquid chromatography, gas chromatography and mass spectrometry, and 16S rRNA sequencing were used to analyze photosynthetic and nutritional characteristics, and rhizosphere carbon exudates, and bacterial communities.

Results Tuber melanosporum mycorrhization increased leaf photosynthetic rate (by 69 %) and phosphorus concentration (94 %); increased rhizosphere pH (0.4 units), total organic carbon (TOC, 76 %) and acid phosphatase activity (33 %); but decreased leaf potassium concentration (26 %) and rhizosphere organic anions (50 %). Additionally, sugars like galactose were present in rhizosphere extract of colonized, but not uncolonized seedlings. Mycorrhization altered rhizosphere bacterial communities, with only ~10 % operational taxonomic units (OTUs) shared by both colonized and uncolonized seedlings; *T. melanosporum* enriched the phylum actinobacteria and the OTU of amb-16S-1323, IMCC26256 and PLTA13, but reduced SWB02. The abundances of different OTUs were differently affected by *T. melanosporum* colonization, and they were correlated with different physiological and/or rhizosphere factors.

Conclusion Our results demonstrate that *T. melanosporum* ECM colonization can regulate carbon economy and rhizosphere bacterial communities of *Q. mongolica* seedlings grown in a previously sterilized peat-based substrate, to promote plant growth and nutrient cycling.

Introduction

In temperate and boreal forests, ectomycorrhizas (ECMs) are often symbiotically formed between trees and soil fungi. Typically, ECMs are short lateral roots covered in a thick mantle made of fungal hyphae, some of which penetrate the epidermis and grow between cortical cells forming the Hartig net (Peterson et al. 2004), where plants receive water and mineral nutrients from fungi and in turn fungi obtain carbon (C) from plants (Smith and Read 2008). ECMs can produce extra-radical mycelia emanating from the mantle surface into their surrounding soil to absorb nutrients and then transfer them to their host plants (Anderson and Cairney 2007). ECMs can also release C and nitrogen (N) exudates including amino acids, organic acids and enzymes to mobilize plant less-available nutrients, thus promote soil C and other nutrients cycling, as well as plant nutrients uptake (Cairney 2011; Smith and Read 2008; Wang and Lambers 2020).

Plants have evolved various strategies (e.g. changes in root architecture, root exudates and root symbionts) to improve nutrients acquisition, whilst they are likely to exhibit trade-offs in

photosynthetically assimilated C allocation among different adaptive strategies (Wang and Lambers 2020). Take phosphorus (P) uptake for example, root symbiosis with soil mycorrhizal fungi and increased root exudates are two responses to low P availability but both strategies increase P acquisition at significant C cost (Lynch et al. 2005; Raven et al. 2018); hence, plant roots colonized by mycorrhizal fungi may result in decreased root-exuded organic anions (e.g. Ryan et al. 2012; Nazeri et al. 2013). In addition, root exudates such as organic anions and amino acids are a key factor shaping rhizosphere bacterial communities (Haichar et al. 2014; Landi et al. 2006; Zhalnina et al. 2018), and considerable evidence indicates that soil bacterial communities play important roles in plant resistance to biotic and abiotic stress (Bulgarelli et al. 2013). To date, the effects of ECMs on root-released organic anions have been tested in a few studies but showed contrasting results (Casarin et al. 2004; Cairney 2011; Meier et al. 2013; van Scholl et al. 2006), and there is little information on ECM-associated bacterial communities. Moreover, our current precepts on the function of mycorrhizas are mainly based on assumptions from experimental data of studies using limited species under certain conditions, hereby researchers are encouraged to revisit mycorrhizal dogmas, especially for their non-nutritional benefits (Albornoz et al. 2021). Therefore, ECM-associated C exudation and its effects on soil bacterial structure warrant further study.

Tuber melanosporum, the Périgord black truffle, is probably the most economically important truffle in Europe (Donnini et al. 2013), but it is not naturally present in China. Truffle plantations have been established worldwide (Donnini et al. 2013), and recently in China especially in Guizhou, Yunnan and Sichuan Provinces (Wang 2012). Although *T. melanosporum* ECM synthesis and cultivation have made great progress, there are very few studies focusing on the effect of *T. melanosporum* colonization on the rhizosphere physiological processes of its host, which limit our understanding of the eco-physiological significance of its artificial cultivation. We have inoculated *T. melanosporum* onto different Chinese indigenous *Quercus* species and among them *Q. mongolica* showed the highest mycorrhizal receptivity and stability as well as accelerated bud-break and vigorous growth under greenhouse conditions (Wang et al. 2019). However, the underlying eco-physiological mechanisms of *T. melanosporum*-promoted host growth remain poorly explored.

The objective of this study was to determine the response of root C exudation and rhizosphere bacterial communities to ectomycorrhizal colonization, using *T. melanosporum*-colonized *Q. mongolica* seedlings and their uninoculated counterparts. By analyzing leaf photosynthetic parameters, leaf nutrient concentrations, rhizosphere total organic carbon, organic anions and sugars, and rhizosphere bacterial communities, we hypothesized that (1) *T. melanosporum* mycorrhization would result in altered photosynthetic rate, nutrients uptake and rhizosphere carbon exudates, (2) *T. melanosporum* mycorrhization would result in changes in the rhizosphere bacterial communities and (3) variations in bacterial communities would correlate with certain measured plant and soil parameters.

Materials And Methods

Plant material and ectomycorrhizal synthesis

The same seedlings produced by Wang et al (2019) were used in this study, and detailed information for plant cultivation and ECM synthesis can be obtained from the very same reference. Briefly, sterilized and sprouted seeds of *Q. mongolica* (obtained from the Germplasm Bank of Wild Species, Kunming, China) were transplanted into a sterilized mixture of perlite and vermiculite (50:50, v:v) in an environment-controlled indoor chamber. After eight months, seedlings (all free of ECMs) of similar size were selected, water washed and transplanted to sterilized growth media (peat: pumice: pine bark: lime = 9:9:2:2, v: v; pH 8.0, an optimum pH for *T. melanosporum* growth), where the roots of each seedling were inoculated with 10 mL (about 1.5×10^8 spores) of *T. melanosporum* spore slurry (ascocarps sourced from Canterbury, New Zealand). This substrate is, to our experience, favorable to ECM synthesis and acclimation (Wang et al. 2019). The plants were grown in a greenhouse at the Kunming Institute of Botany under natural light; and after three months of inoculation, each pot was fertilized with 2.5 mL of slow-release Osmocote® 5 (ICL SF USA & Canada, Summerville, SC, USA, sold by Lily's gardening in Shanghai) (Crowley et al.1986; Guerin-Laguette et al. 2014), which contains 14 % N (5.5 % NO_3-N + 8.5 % NH_4-N), 13 % P (P_2O_5) and 13% K (K_2O). Ectomycorrhizal development was monitored 6, 9, 12, 24 and 32 months after inoculation, respectively. To identify *T. melanosporum* ECMs, their macro-morphological and anatomical characters were first confirmed using a stereomicroscope (Leica S8AP0) and a compound light microscope (LeicaDM2500); next, the internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using the ITS1F/ITS4 primer pair, and then the PCR products were sequenced and queried against published references of the NCBI public database GenBank. All control and inoculated seedlings were checked by both morphological and molecular methods each time, and the whole root system for each plant was morphological inspected. *Tuber melanosporum* ECMs were successfully produced on all five inoculated seedlings six months after inoculation, and fresh mycorrhizas were constantly observed over at least 32 months. In addition, no contaminant mycorrhizal species were detected, and the DNA extracts of *Q. mongolica* mycorrhizas produced a *T. melanosporum*–specific 440 bp fragment only (Wang et al. 2019). Samplings for the present study were performed 37 months after inoculation in August 2019 when fresh *T. melanosporum* ECMs were still present. Four mycorrhizal and four non-inoculated control seedlings ($n = 4$) were used for this study (as one control seedling had leaves too small to measure photosynthetic parameters and one mycorrhizal seedling was exceptionally small compared to the other four mycorrhizal seedlings, both were excluded from the analysis).

Measurement of leaf photosynthetic parameters and nutrients concentration

A portable gas exchange fluorescence system GFS-3000 (Heinz Walz GmbH, Effeeltrich, Germany) was used to measure the photosynthetic rate (*A*), transpiration rate (*E*), stomatal conductance (GH_2O) and intercellular CO_2 concentration (Ci) in mature leaves (fully expanded, the 2nd top leaf) between 10:00 am-11:30 am, under photosynthetic active light intensity of 1,000 μmol m^{-2} s^{-1} on the upper leaf area. In the measuring head, the external CO_2 concentration, the temperature and relative air humidity were 400 ppm, 25°C and about 60 %, respectively. Photosynthetic parameters were evaluated every 3 minutes until readings were stable, and values of *A*, *E*, GH_2O, and Ci were then recorded.

Two mature leaves from each seedling were sampled. Fresh and dry (65°C for 48 h) weights, and then leaf water contents were determined. The dry leaves were ground into fine powders, which were pulverized to pass through a 0.25 mm sieve. Leaf carbon (C) and nitrogen (N) were determined by a Vario MAX CN instrument (Elementar Analyse system GmbH, Germany). For element determination, the powders were digested with concentrated HNO_3-$HClO_4$, and total phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and iron (Fe) were determined with an inductively coupled plasma atomic-emission spectrometer (IRIS Advantage-ER; Thermo Jarrell Ash Corporation, Franklin, MA, USA).

Determination of rhizosphere pH, phosphatases activity and exudates

The intact seedlings were carefully taken out from pots and roots were gently shaken to remove excess growth media. The growth medium remaining attached to the roots was defined as rhizosphere substrate (Wang et al. 2016). For each plant, about 2 g rhizosphere fresh growth medium were carefully sampled using tweezers and spoons, and were then divided into two parts, one was stocked at -20 ℃ for DNA extraction and another was air-dried at room temperature for phosphatases activity measurements. The activities of rhizosphere acid or alkaline phosphatase (S-ACP or S-ALP) were separately measured using their respective kit (Solarbio®, Beijing, China), following the manufacturer's instructions.

After the subsampling of rhizosphere growth medium, the entire root system with the remaining rhizosphere growth medium was transferred into a flask containing 150 mL 0.2 mM $CaCl_2$ solution to ensure cell integrity. Roots were then gently and carefully dunked for 150 s to get rhizosphere extract (Pang et al. 2015; Wang et al. 2016). Then the roots were removed, the flasks were shaken by hand, and the extract pH (diluted samples, which might underestimate the pH on the root surface) was measured (Wang et al. 2017). Next, 0.01 g L^{-1} Micropur (Katadyn Products, Kemptthal, Switzerland) was added to the extract to inhibit the activity of microorganisms.

A subsample of the rhizosphere extract was centrifuged, and the supernatant was filtered through a 0.45 μm membrane filter and was assessed using a Total Organic Carbon (TOC) analyzer (Shimadzu, Kyoto, Japan). Organic anions were determined through a HPLC (Agilent Technologies, Tokyo, Japan) process. A UV detector (SPD-20A) monitored at 210 nm for the analysis of organic anions, the injection volume was 10 μL and sample components were separated using a ZORBAX SB-Aq (4.6× 250 mm, 5 μm) StableBond analytical column (Agilent, Delaware, USA) at a 35 ℃ column oven temperature and a 10 min running time. The mobile phase was 1 % acetonitrile + 99 % 20 mM $NaHPO_4$, pH 2.2, at a flow rate of 1.0 mL min^{-1}. The organic anions were identified by comparing their retention times with standards and their concentrations were determined according to their standard curves.

GC-MS and HPLC-ELSD analysis of sugars in rhizosphere extracts

Another subsample of 45 mL rhizosphere extract (frozen with liquid nitrogen) was freeze-dried for 2 days; the dried residue was re-suspended in 5 mL of deionized water and freeze-dried again, and then re-

dissolved in 2 mL of cold methanol (Luo et al. 2017). The sample was blown to dryness under a gentle N_2 flow, and then was derivatized by 250 µL salinization solution (Bis(trimethylsilyl)trifluoroacetamid to pyridine ratio, 5:1) under 75°C water bath for 1.0 h (Liu et al. 2015). The solution was filtered through a 0.45-µm membrane filter and then analyzed by GC-MS.

Silylated supernatants were analyzed by GC-MS (Agilent Technologie, Agilent 7890A along with Agilent 5975C, USA) using a DB-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies, USA). The gas chromatographic conditions were as follows: helium was used as a carrier gas at a flow rate of 1.0 mL min^{-1}; the oven initial temperature was 40°C for 2 min, then increased to 200°C at a rate of 5°C min^{-1}, then at a rate of 20°C min^{-1} up to 270°C. Injections of 1µL were made in a splitless mode. The mass spectrometric system was set as follows: the ion source temperature was 230°C, full scan mode with a scan range of m/z 60–640, and mass spectra were generated at 70 eV. Spectrum acquisition was realized 7 min after injection in order to avoid saturation of the detector. Compounds were subjected to NIST11 library search and data were analyzed by using MSD ChemStation software (Agilent, version G1701EA E.02.02.1431).

HPLC-Evaporative Light Scattering Detection (HPLC-ELSD) was further employed (Bernardo et al. 2008; Lindqvist et al. 2018) to identify sugars in rhizosphere extracts, with 1 mg mL^{-1} of glucose, galactose, fructose, sucrose and fucose as standards. A column Hi-Plex Pb (300 mm×7.7 mm, Agilent, Delaware, USA) with HPLC grade water as the eluent at the flow rate of 0.6 mL min^{-1} was used. Sugar compounds were identified by comparison of HPLC retention times with those from standards.

Rhizosphere bacterial DNA extraction and 16S rRNA sequencing

Bacterial DNA was extracted from 0.2 g of fresh rhizosphere growth media using Powersoil™ DNA isolation kits (MoBio, San Diego, CA, USA) according to the manufacturer's instructions. The quality and quantity of the DNA extracts were checked using a spectrophotometer (Nanodrop, PeqLab, Germany). The V3-V4 region of the bacterial 16S rRNA gene was amplified using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Purified amplicons were pooled, and pair-end sequenced on the Illumina MiSeq platform, Miseq-PE250 (Personalbio®, Shanghai, China). The raw reads were analyzed using QIIME software (version 1.7.0, http://qiime.org/) to trim off the low quality reads, adaptors, barcodes, and primers. Sequences were clustered into operational taxonomic units (OTUs) by setting a 97 % similarity (Huang et al. 2017). The Bray-Curtis distance-based dissimilarity distance, Simpson and Chao1 diversity index, principal coordinate analysis (PCoA) and a Venn diagram with shared and unique OTUs were performed on the Genescloud platform of Personalbio® to evaluate the bacterial community differences between control and ectomycorrhizal samples, as well as MetagenomeSeq analyses of enriched core bacterial compositions. Raw sequence data have been deposited in the NCBI Sequence Read Archive database under the bioproject identifier PRJNA662162.

Statistical analysis

Data (means ± SE, $n = 4$) were statistically analyzed by *R* software (version 3.2.3). One-way analysis of variance for independent samples was performed. Pearson correlation analysis (for all analyzed samples, $n = 8$) was used to examine the correlations between the relative abundance of OTUs and measured plant and soil parameters including photosynthetic parameters, leaf nutrient concentrations, leaf water content and rhizosphere pH, phosphatases activity and rhizosphere exudates. Data of leaf C, N, P concentration and leaf water content was transformed to C %, N %, P % and leaf water % (mass ratio) before Pearson correlation analysis. Statistical significance for Pearson correlation was determined by pairwise two-sided comparisons. The PCoA analysis is based on Bray-Curtis distance at the OTU level and the MetagenomeSeq analysis was performed based on –log10 (adj P value) of the relative abundance at the OTU level. The significance α level of 0.05 was used.

Results

Leaf photosynthesis and nutrient concentration responses

Compared to the control, ectomycorrhizal (*T. melanosporum*) colonization enhanced leaf photosynthetic rate (+ 69 %) and P concentration (+ 94 %), decreased K concentration (-26 %), but did not affect leaf stomatal conductance, transpiration rate and intercellular CO_2 concentration, nor leaf C, N, Ca, Mg, Fe concentrations and leaf water content (Fig. 1 and Table S1). The average leaf N: P ratios were 13.03 and 7.87 for the control and ectomycorrhizal seedlings, respectively.

Rhizosphere pH, TOC, organic anions and phosphatase activity

The average rhizosphere pH of mycorrhizal plants was significantly higher by 0.4 units ($P < 0.05$) than that of the control seedlings (Fig. 2a). *Tuber melanosporum* colonization significantly increased TOC in the rhizosphere by 76 % (Fig. 2b). The accumulation of organic anions in the rhizosphere varied significantly and less amounts of organic anions were detected in the rhizosphere of ectomycorrhizal than in that of control seedlings. Tartrate and oxalate were detected with tartrate being the dominant organic anion. Rhizosphere tartrate was significantly decreased under ECM while rhizosphere oxalate was not affected by ECM colonization (Fig. 2c). Rhizosphere alkaline phosphatase activity (ALP) was not affected by ECM symbiosis, but significantly higher (+ 33 %) rhizosphere acid phosphatase activity (ACP) was detected in ECM than in control seedlings (Fig. 2d).

Sugars in the rhizosphere

Since ECM colonization decreased rhizosphere organic anions but increased TOC (Fig. 2b, c), GC-MS was employed to determine the compounds being significantly increased in the extract of ECM rhizosphere. GC-MS detected a major peak corresponding to mannose/glucose/fructose and/or galactose in two of four ECM rhizosphere extracts but not in any of the control samples (Fig. S1). Furthermore, HPLC-ELSD confirmed the presence of galactose and other unidentified sugars in these two ECM samples (Fig. S2).

Rhizosphere bacterial communities

Bacterial community analysis indicated significant differences in β-diversity (dissimilarity distance, Fig. 3a) but not in Chao1 and Simpson index (α diversity, Fig. 3b), between control and ECM samples, and these two groups were clearly defined by the PCoA (Fig. 3c). A total of 29,934 OTUs were displayed and only about 10 % OTUs were shared by both the control and ECM seedlings (Fig. 3d). Compared to the control, significantly greater relative abundance of actinobacteria at the phylum level ($P < 0.05$) was found (Fig. 4a), and significantly more abundant bacterial groups at the OTU level (order or genus equivalent) were shown under ECM by the MetagenomeSeq analysis ($P < 0.05$, Fig. 4b). The Pearson correlation analysis revealed significant correlations between the relative abundance of actinobacteria and rhizosphere amounts of TOC (r = 0.83, $P = 0.01$, $n = 8$) and tartrate (r = -0.72, $P = 0.04$, $n = 8$).

At the OTU level, A4b, Amb-16S-1323, *Bauldia*, Blrii41, *Chryseolinea*, *Falvobacterium*, *Haliangium*, IMCC26256, JG30-KF-CM66, KD4-96, MND1, *Pedomicrobium*, PLTA13, *Pseudomonas*, S085, *Saccharimonadales*, Subgroup_6, Subgroup_17, Subgroup_22 and SWB02 were the top 20 most abundant (Fig. 5a). Among those OTUs, significant different abundances in Amb-16S-1323, IMCC26256, PLTA13 and SWB02 were detected between colonized and un-colonized rhizosphere samples. Correlations between the relative abundance of the top 20 most abundant OTUs and measured plant and soil factors were assessed, and the results were shown in Fig. 5b. A total of 28 significant correlations ($P < 0.05$) were found. SWB02 correlated with eight measured parameters, and *Bauldia*, *Falvobacterium* and S085 correlated significantly with more than three parameters. Of those, rhizosphere TOC, tartrate or oxalate correlated significantly with the relative abundances of OTUs *Bauldia*, Blrii41, *Flavobacterium*, SWB02 or Subgroup_17.

Discussion

This study found that *T. melanosporum* ECM colonization enhanced C photosynthetic assimilation and TOC exudation, increased rhizosphere pH and acid phosphatase activity, and shifted rhizosphere bacterial communities with enriched actinobacteria of *Q. mongolica* seedlings grown in a peat-based substrate. In addition, the relative abundances of rhizosphere actinobacteria and OTUs *Bauldia*, Blrii41, *Flavobacterium*, SWB02 or Subgroup_17 correlated with rhizosphere amounts of TOC and organic anions. The implications of these findings and other points of interest are discussed below.

Effects of the substrate and plant cultivation system

The present study used a substrate instead of real forest soil to grow plants in a greenhouse. Although the obtained results could not reflect the situation under natural conditions, this plant cultivation system has several advantages, and the results could contribute to underpin the mechanisms being displayed in a real peat-based or even non-peat soil. First, this substrate is reliable for a practical *T. melanosporum* ECM synthesis with oak seedlings, an essential prerequisite for further studies (see Wang et al. 2019), although *Tuber* mycorrhizas might regress or disappear after several to 21 years of transplanting to the

field (Guerin-Laguette et al. 2013). Second, the components of peat moss (Jiffy, The Netherlands, 70 % organic C, 0.8 % organic N, pH 5.0–6.0) and pine bark in the substrate were similar to those found in peatlands or surface layers of forest soils. Finally, the effects of other mycorrhizas that often exist as complex fungal communities in natural conditions (Zhang et al. 2019) could be excluded in our experiment since no other ECM species was detected on any roots (Wang et al. 2019).

T. melanosporum colonization enhances photosynthesis and nutrient acquisition

Studies have reported that ECM colonization can enhance the photosynthesis variables of *Pinus taeda*, *P. densiflora* and *Eucalyptus camaldulensis* seedlings (Choi et al. 2005; Dixon and Hiol-Hiol 1992; Reid et al. 1983), as well as of *Quercus ilex* seedlings colonized by *T. melanosporum* (Nardinia et al. 2000). Similarly, we found that the presence of *T. melanosporum* ECM significantly increased the photosynthetic rate of host seedlings (Fig. 1a) and tended to increase their stomatal conductance and transpiration rate, although not significantly so (Fig. 1b, c). Interestingly, significantly improved C assimilation rates did not result in significantly increased leaf C concentrations in ECM seedlings in our study (Fig. 1e). Since promoted plant growth (Wang et al. 2019) and increased rhizosphere TOC (Fig. 2b) were observed, the assimilated C might have been allocated to biomass production, root exudates, and/or mycorrhizal tissues (Liu et al. 2019).

It is known that ECM colonization improves host plant nutrient acquisition, especially for P (Nehls and Plassard 2018). Indeed, considering the enhanced number of leaves and mean leaf dimension observed in Wang et al. (2019) and the measured nutrient concentration in the present study (Fig. 1f, g and Table S1), we found that *T. melanosporum* ECM improved nutrients (e.g. N, P, Mg and Fe) uptake of *Q. mongolica* seedlings. Similarly to our study, four months old *P. contorta* seedlings inoculated with either *Pisolithus tinctorius* or *Suillus granulatus* showed significantly greater foliar P, but not N concentrations, than non-mycorrhizal seedlings (Reid et al. 1983). Here, with a leaf N: P ratio of 13.03 and a leaf P concentration of 0.88 mg g^{-1}, the non-inoculated seedlings might also have slightly suffered from P deficiency (Güsewell 2004). In previous studies, effect of *T. melanosporum* colonization on leaf K concentration depended on the host plant species (Domínguez Núñez et al. 2006 & 2008). We found decreased leaf K in *T. melanosporum*-colonized *Q. mongolica* seedlings (Fig. 1h).

T. melanosporum mycorrhization alters rhizosphere characteristics

Studies on other mycorrhizal species suggested that mycorrhizas could release protons and C-containing substances like organic anions and phosphatases to mobilize soil nutrients (Cairney 2011; Rigou et al. 1995). Furthermore, the release of oxalate in the rhizosphere of *Rhizopogon roseolus*-colonized *P. pinaster* resulted in a decrease in rhizosphere pH (Casarin et al. 2004). However, our results showed a slight increase of rhizosphere pH by *T. melanosporum* (Fig. 2a), probably due to decreased organic anions that tend to acidify the rhizosphere (Casarin et al. 2004; Hinsinger 2001). We found that increased rhizosphere TOC and acid phosphatase activity were induced by *T. melanosporum* (Fig. 2b, d), which was

consistent with results found in other ECMs (Cairney 2012; Taniguchi et al. 2008). However, less organic anions in the ECM rhizosphere were detected in this study. We see two possible reasons: 1) our uninoculated seedlings might have slightly suffered from P deficiency, hence induced root-released organic anions (Wang and Lambers 2020); 2) mycorrhized plants may exhibit trade-offs in photosynthetically assimilated C allocation (Chen et al. 2016; Wang and Lambers 2000). Moreover, colonization by mycorrhizal fungi could decrease root-released organic anions: this has been experimentally confirmed in both arbuscular (Del-Saz et al. 2017; Nazeri et al. 2013; Ryan et al. 2012) and ectomycorrhizal (Meier et al. 2013; van Scholl et al. 2006) plants. Van Scholl et al (2006) also found that oxalate exudation in *P. sylvestris* roots decreased when these were colonized by *Hebeloma longicaudum* but increased when colonized by *Paxillus involutus* and *Piloderma croceum*, suggesting that the effect of ECM on exudation of organic anions might also be ECM species-dependent. In our study, the increased TOC might be due to exudation of sugars such as galactose (Fig. S2), and mycorrhizal hyphae-exuded sugar like fructose could attract P solubilizing bacteria to promote P mobilization and uptake by plant (Zhang et al. 2018). Meanwhile, sugars derived from the decomposition of mycelia could not be excluded.

Various soil microbes are involved in the life cycle of *T. melanosporum* and they may play key roles in the formation of *T. melanosporum* ECMs and ascocarps (Chen et al. 2019; Splivallo et al. 2015). In our study, the substrate was autoclaved before use, the plant cultivation was open to non-sterile environment for more than 3 years and the spore slurry for inoculation was not sterilized, thus the bacterial communities sequenced here were '*de novo*' assembled and enriched from both the environment and ascocarps. A recent study reported that *T. melanosporum* mycorrhization harbored distinct bacterial communities with an enrichment in Alpha- and Gamma-proteobacteria, compared to the bulk soil (Deveau et al. 2016). We found that proteobacteria were the dominant phylum in the rhizosphere of both control and *T. melanosporum* ECM seedlings. Furthermore, the major difference between ECM and control rhizospheres was actinobacteria, which was enriched by *T. melanosporum* mycorrhization (Fig. 4a). Some soil actinobacteria have a strong ability to hydrolyze a wide range of polysaccharides such as cellulose, chitin and xylan (Barka et al. 2016), thus they might contribute to the increased TOC in the ECM rhizosphere, which could partly explain the positive correlation between rhizosphere TOC and the relative abundance of actinobacteria observed in our study. Actinobacteria can fix N and solubilize minerals in soil (Glick 1995), hereby increasing uptake of N and other nutrients in ECM seedlings compared with control plants (Fig. 1 and Table S1). Our results also showed that rhizosphere TOC, tartrate or oxalate correlated with the relative abundances of *Bauldia*, Blrii41, *Flavobacterium*, SWB02 or Subgroup_17 at the OTU level (Fig. 5b). Different components of the microbial community have different membrane transport systems to take up different C-compounds (Jones et al. 1996), therefore changes in rhizosphere TOC and organic anions may lead to changes in overall microbial diversity as shown by the significant difference in β-diversity of bacterial communities between control and ECM samples (Fig. 3a). The genus *Bauldia*, which may have different potential to utilize C source (Yee et al. 2010), was overrepresented in the rhizosphere of ratooning sugarcane (Gao et al. 2019). *Flavobacterium* was quite often detected in plant rhizosphere, and it may increase the carbohydrase activity (Mawdsley and Burns 1994). Blrii41 was abundant in

organic soils and was enriched in maize root under chilling conditions (Beirinckx et al. 2020). SWB02 played important roles in denitrification (Iannacone et al. 2020), and Subgroup_17 showed strong positive correlation with soil available N (Yi et al. 2019). Thus, the high content of organic matter in our substrate, high amounts of rhizosphere TOC and improved N uptake by ECM seedlings could partly explain the increased abundance of Blrii41, *Bauldia*, *Flavobacterium* and Subgroup_17 and decreased abundance of SWB02 detected in the ECM rhizosphere, respectively. Collectively, all these bacteria are important players for soil C and N transformations, and rhizosphere C exudates may affect bacterial communities and N transformations as reported previously (Landi et al. 2006; Meier et al. 2017; Zhalnina et al. 2018).

Conclusions

Tuber melanosporum mycorrhization strongly enhanced leaf C assimilation and root C exudation, promoted nutrient acquisition and altered rhizosphere bacterial communities of *Q. mongolica* seedlings grown in a previously sterilized peat-based substrate. These originally results could provide theoretical and practical bases for a better understanding of C allocation, ECM-facilitated plant growth, and nutrient cycling in both forest ecosystems and truffle orchards.

Declarations

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Code availability: Not applicable

Authors' contributions: YW carried out the data collection and analysis, and wrote the manuscript. YW and FY designed and instructed the study. RW and AGL contributed to plant materials. BL helped with HPLC, HPLC-ELSD and GC-MS analysis. AGL and XH discussed the results and revised the manuscript.

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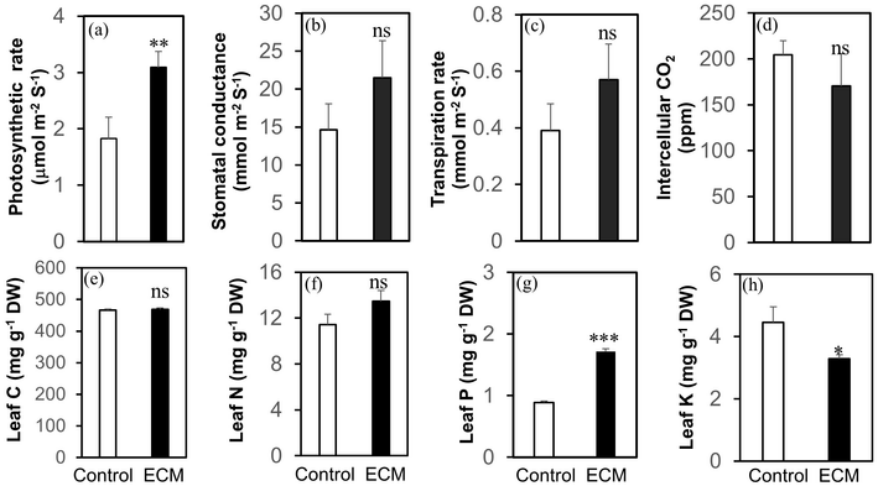
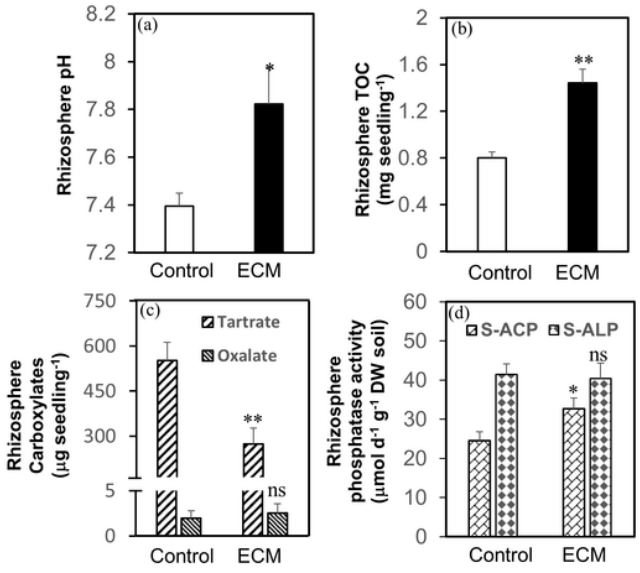
Figures



Wang et al., Fig.1

Figure 1

Mean leaf photosynthetic rate (a), stomatal conductance (b), transpiration rate (c), intercellular CO2 concentration (d), leaf C (e), leaf N (f), leaf P (g) and leaf K (h) of Quercus mongolica seedlings (either non-mycorrhizal (control) or ectomycorrhizal (ECM) with Tuber melanosporum). Error bars indicate SE (n = 4). * P < 0.05; ** P < 0.01; *** P < 0.001; ns, no significant difference.



Wang et al., Fig.2

Figure 2

Rhizosphere characteristics: pH (a), total organic carbon (b), carboxylates (c) and activities of soil acid and alkaline phosphatase (d, S-ACP and S-ALP) of Quercus mongolica seedlings (either non-mycorrhizal (control) or ectomycorrhizal (ECM) with Tuber melanosporum). Error bars indicate SE (n = 4). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, no significant difference.

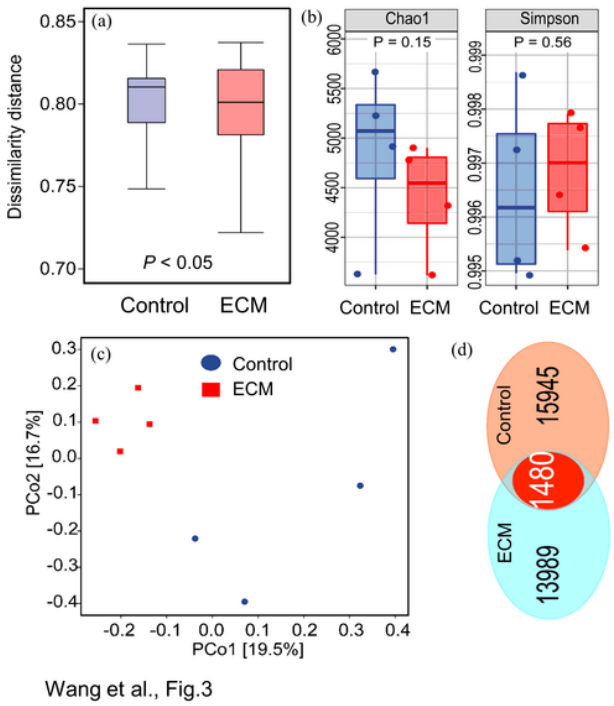
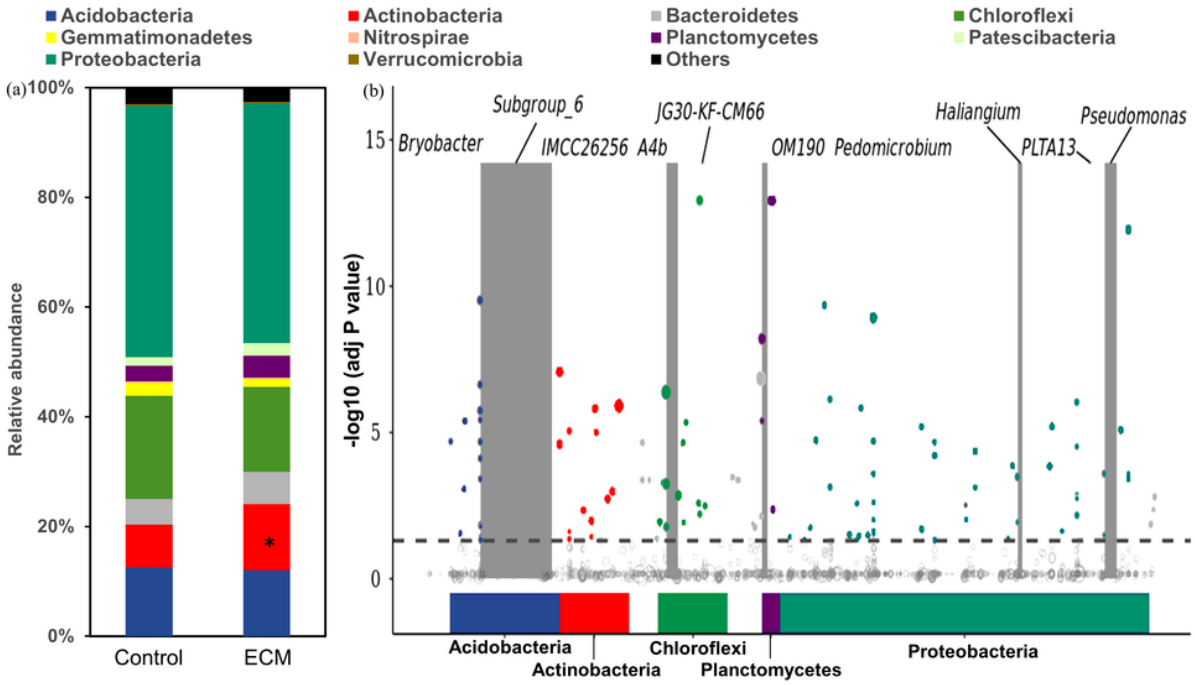


Figure 3

Dissimilarity distance (a), Chao1 and Simpson (b) indexes showing the differences in microbial community structure between the rhizospheres of Quercus mongolica seedlings (either non-mycorrhizal (control) or ectomycorrhizal (ECM) with Tuber melanosporum). Principal coordinate analysis (PCoA) of the bacterial communities in the rhizosphere (c). Venn figure showing shared and unique operational taxonomic units (OTUs) between control and ECM samples (d).



Wang et al., Fig.4

Figure 4

Bacterial community composition of the rhizosphere at the phylum level (a) and MetagenomeSeq analysis of enriched (color dots indicating significant OTUs) core bacterial composition in the rhizosphere of T. melanosporum colonized seedlings, compared to that of control seedlings (b).

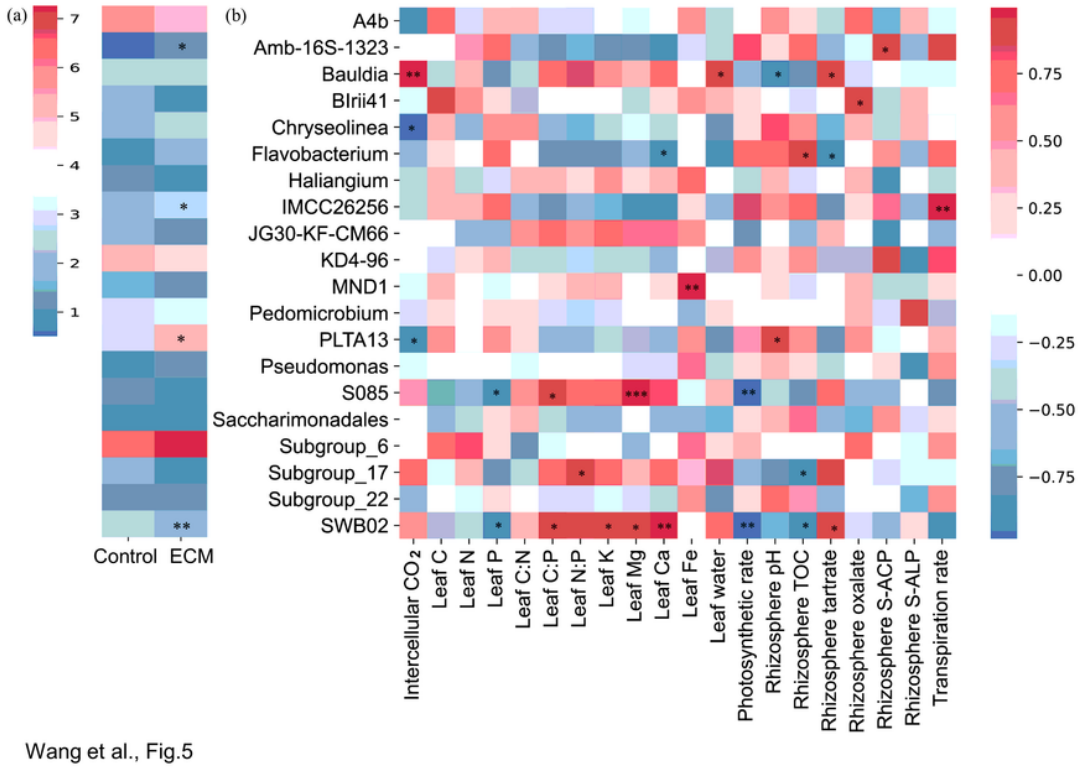


Figure 5

Heatmap of the relative abundance of the TOP 20 most abundant OTUs (agglomerated to genus or order, a) and Pearson correlation analysis between the dominant OTUs and plant or soil parameters (b). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Supplementary Files

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