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Effects of truffle inoculation on a nursery culture substrate environment and seedling of Carya illinoinensis

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Abstract

We inoculated *Tuber aestivum* and *T. sinoaestivum* on *Carya illinoinensis* to explore the effects of inoculation on host plant growth, enzyme activities, the physicochemical properties of rhizosphere soil, the denitrifying bacterial community in the rhizosphere, and the distribution of mating type genes in the rhizosphere. We found that the *Tuber* spp. inoculation increased the height of the host plant and that the stem circumference of the host was greater two months after inoculation. Six months after inoculation, the peroxidase activity of the seedlings inoculated with *T. sinoaestivum* was higher than that of the control. At four and six months after inoculation, the superoxidase dismutase activities of the seedlings inoculated with *T. aestivum* were higher than those of the seedlings inoculated with *T. sinoaestivum*. Six months after inoculation, nitrate nitrogen content was lowest in the control and highest in the *T. sinoaestivum* treatment. Among the *nirS*-type denitrifying bacteria community, the relative abundances of Proteobacteria were high. *T. aestivum* and *T. sinoaestivum* inoculation did not affect the diversity of denitrifying bacteria. The mating type genes MAT1-1-1 and MAT1-2-1 were detected in the rhizosphere of *C. illinoinensis* inoculated with *T. sinoaestivum* and *T. aestivum*, and MAT1-1-1 dominated over MAT1-2-1.

Keywords

Tuber; rhizosphere; host plant growth; mating type gene; denitrifying bacteria diversity

Introduction

Truffles (*Tuber* spp. in the class Ascomycetes) are edible fungi that form symbiotic associations with certain plants (Singer 1961). The genus *Tuber* has been estimated to contain at least 180 species worldwide (Bonito *et al.* 2013). The popularity of truffles stems from their distinct aroma and unique taste (Talou *et al.* 1987; Vahdatzadeh *et al.* 2015). The nutritive and nutraceutical value of truffles is high due to their protein and mineral levels that are higher than those of other edible fungi (Beuchat *et al.* 1993). In addition, truffles are relatively rich in beneficial components, e.g. micronutrients, polysaccharides, sphingolipids, fatty acids, and flavonoids (Akyuz 2013; Pattanayak *et al.* 2017).

The ascospores of truffles need to germinate under appropriate conditions to form mycelium and then infect the root tip of a host plant to form a symbiotic system known as ectomycorrhiza (Kües & Martin 2011). The development of ectomycorrhiza may be induced by plant root exudates that are, together with the host plant genotype and soil type, an important driving force in determining the rhizosphere microbial community structure (Berendsen *et al.* 2012). The growth and development of ectomycorrhiza is influenced by several environmental factors such as soil pH, soil moisture content, ambient temperature, and nutritional status (Hause *et al.* 2002). In return, ectomycorrhiza can improve the soil structure and promote the absorption of nutrients, such as phosphorus and nitrogen, by host plants (Dominguez-Núñez *et al.* 2012). The ectomycorrhiza increases the total surface area for nutrient absorption (Liu *et al.* 2020), and ectomycorrhizal roots may secrete organic acids to break down surrounding primary minerals (Arocena & Glowa 2000).

Sexual development in sexually reproducing fungi is controlled by the mating type (MAT) locus (Ni *et al.* 2011). The mating type loci MAT1-1 and MAT1-2 are called idiomorphs due to their dissimilar sequences and genes (Metzenberg & Glass 1990; Rubini *et al.* 2010). MAT1-1 idiomorph carries mating type gene MAT1-1-1 that encodes a protein with an α-box-domain, and MAT1-2 idiomorph carries MAT1-2-1 that encodes a protein with an high mobility group (HMG) transcription factor (Martin *et al.* 2010; Rubini *et al.* 2010). Heterothallic fungi require two strains for mating; one strain carries either the MAT1-1 or MAT1-2 locus (Rubini *et al.* 2010).

Under extreme environmental conditions, the excessive reactive oxygen produced in plants can damage the cell membrane system, and even cause cell senescence or death (Thompson *et al.* 1987). However, enzymes such as superoxide dismutase (SOD) and peroxidase (POD) in plant cells can scavenge reactive oxygen, and thereby protect the cells (Heath and Packer 1968). The activity and content of POD and SOD in plants correlate with their abilities to adapt to adverse environments and to tolerate stress (Olmos *et al.* 1994; Sreenivasulu *et al.* 2000). Generally, as plants age, the peroxide content in tissues increases and SOD activity decreases (McRae and Thompson 1983; Thompson *et al.* 1983; Dhindsa *et al.* 1981).

The summer truffles *Tuber aestivum*, a truffle species widely distributed over Nordic and Mediterranean regions (Jeandroz *et al.* 2008), and *T. sinoaestivum*, one of the major truffle species

in Panxi region, China (Fu *et al.* 2016; Zhang *et al.* 2012), are heterothallic (Payen *et al.* 2014). The American pecan *Carya illinoinensis* is an important cash tree widely cultivated in the truffle producing areas. Both *T. aestivum* and *T. sinoaestivum* can form mycorrhiza with *C. illinoinensis* (Zhang *et al.* 2012). Currently, truffle cultivation relies on planting artificially mycorrhized seedlings (De Miguel *et al.* 2014). We inoculated *T. aestivum* and *T. sinoaestivum* on *C. illinoinensis* to explore the effects of inoculation on host plant growth, POD and SOD activities, the physicochemical properties of rhizosphere soil and the community structure of nitrogen-cycle related microorganisms in the rhizosphere. In addition, since truffle fruiting body formation requires two parental strains, one with MAT1-1 and the other with MAT1-2 locus (Rubini *et al.* 2014), we analyzed the distribution of the mating genes MAT1-1-1 and MAT1-2-1 in the rhizosphere.

Materials and methods

Inoculation of seedlings

The truffles for making the *T. aestivum* inoculum were collected from Station d'Expérimentation sur la Truffe, Cahors, France, and for *T. sinoaestivum* from Huidong, Liangshan Yi Autonomous Prefecture, China. The truffles were identified using microscopic examination and ITS molecular identification (Wedén *et al.* 2005; Zambonelli *et al.* 2012; Zhang *et al.* 2012). The inocula were prepared in sterile conditions as described earlier (Su *et al.* 2012). In brief, mature fruiting bodies were sprayed with ethanol, flame sterilized, ground into fine powder, and suspended into sterile water at the concentration of 12,000 spores ml^{-1}. The control inoculum was incubated at 121 °C for 2h to kill the spores. The inocula were stored in sealed bottles at 4 °C.

The *C. illinoinensis* seeds were from Yangbi Walnut Research Institute of Yunnan Academy of Forestry, China. The seeds were soaked in 100 mg L^{-1} gibberellic acid solution and stirred daily for a week, sterilized with 0.5% potassium permanganate solution and stored in sterilized river sand for 2 months. Seedling substrate containing soil, peat, vermiculite and perlite in a ratio of 4:2:3:1 (v:v:v:v) was blended with distilled water and sterilized. After sand-storage, the seeds were washed with distilled water and sowed in pots filled with the seedling substrate in early spring. The pots were watered with sterilized water thoroughly and kept in a greenhouse for three months at 20-25 °C temperature and 70% to 80% humidity.

The seedlings were inoculated with either *T. aestivum* (AES treatment), *T. sinoaestivum* (SIN treatment) or control inoculum. Growth substrate containing soil, peat, vermiculite and perlite in a 5:2:2:1 ratio (v:v:v:v) was amended with 12% lime and a moderate amount of sterilized water. The three-month-old seedlings were soaked in distilled water to loosen the roots and planted in 1000 ml pots filled with 1/3 of sterilized growth substrate. Ten ml of inoculum (approximately 1.0g ascocarp per seedling) was added dropwise on and around the roots, and the remaining 2/3 of growth substrate was added to the pots and compacted. The pots were watered thoroughly and kept moist by adding sterile water when needed. In total the experiment included 150 seedlings (confirmed to survive after transplantation) per treatment.

Plant Physiological and Biochemical Analyses

To determine infection rate, 15 seedlings per treatment were collected at two, four and six months after inoculation. For the other analyses, triplicate samples were taken at the time of inoculation and at two, four and six months after inoculation.

After the collection of the plant samples, rhizosphere soil was removed from the roots by gentle shaking and rinsing with water, and used filter paper to remove excess water. Taproot length, plant height, stem circumference, and fresh weight of aboveground and underground parts were measured. After deactivating the enzymes in a water bath at 100 °C for 10mins, dry weight was measured by drying to a constant weight at 75 °C, and the root to shoot ratio was calculated.

The infection rate was estimated from five randomly selected lateral roots per plant. The roots were examined under a microscope and the infection rate was calculated as the percentage of ectomycorrhizas (ECMs) on a lateral root using equation R=n/N, in which "n" is the number of mycorrhizas on one lateral root, and "N" is the number of all rootlets on this lateral root (Guo *et al.* 1989).

Root respiration activity was measured from rinsed and water-drained root tips. The tips were cut into small segments of 0-1.0 cm and the activity was measured using the 2, 3, 5-triphenyltetrazolium chloride (TTC) method (Yoshida 1966). Peroxidase (POD) and superoxide dismutase (SOD) activities were measured from fresh leaf samples using a modified guaiacol method (Pochinnok 1981) and the nitroblue tetrazolium (NBT) method (Durak *et al.* 1993), respectively.

Rhizosphere Soil Analyses

Triplicate samples were taken at the time of inoculation and at two, four and six months after inoculation. The seedlings were shaken to remove soil not adhered to the root, after which rhizosphere soil was collected by brushing using sterilized brushes and stored at 4 °C. The pH of the rhizosphere soil was measured using a China Inspection Body and Laboratory Mandatory Approval (CMA) certified pH meter. Organic matter content was measured using the potassium dichromate heating - volumetric method (Simakov 1957; Mebius 1960), total nitrogen content using a modified semi-micro Kjeldahl method (Bremner 1960), available phosphorus content using the 0.5 mol L^{-1} $NaHCO_3$ extraction-molybdenum antimony colorimetric method (Chang and Jackson 1957), available potassium content using a modified flame photometer method (Grimme and Németh 1978), ammonium nitrogen content using a phenol-hypochlorite determination based on Kjeldahl method (Smith 1980), and nitrate nitrogen content using ultraviolet spectroscopy (Cawse 1967).

Analysis of Denitrifying Microbial Community

The diversity of denitrifying microbial community was analysed from rhizosphere samples taken six months after inoculation. DNA was extracted using a FastDNA Spin Kit for Soil (Bio-Rad Co, USA). The quantity and quality of extracted DNA was determined using a fluorescence spectrophotometer and electrophoresis in 1% agarose gel. *nirS* gene fragments were amplified using primers nirS-4f (5'-TTCRTCAAGACSCAYCCGAA-3') and nirS-6r (5'-CGTTGAACTTRCCGGT-3') (Braker 2000). Amplified fragments were purified using 1.5% agarose gel electrophoresis and a Gel Extraction Kit (Axygen, USA). The fragments were quantified on a Microplate Reader (BioTek, FL×800) using a Quant-iT PicoGreen dsDNA Assay Kit and sequenced on Illumina MiSeq sequencing platform in Shanghai Personalbio Biotechnology Company (China).

Low-quality and short sequences were removed using QIIME software (Caporaso *et al.* 2010) installed with BIOCONDA (Grüning *et al.* 2018). The remaining sequences were merged using UCLUST sequence alignment tool (Edgar 2010) and classified into operational taxonomic units (OTUs) at 97% similarity level. The *nirS* community composition and abundance were analyzed

and visualized in R (R Core Team 2019). Variation in *nirS* communities was visualized using non-metric multidimensional scaling (NMDS) based on unweighted UniFrac distances.

Mating Gene Analysis

Genomic DNA from the youngest fruiting bodies collected from Station d'Expérimentation sur la Truffe, Cahors, France, and from Huidong, Liangshan Yi Autonomous Prefecture, China, to make standard curves. DNA was extracted using an Extraction Kit (Vazyme Biotech Co., Ltd). Mating genes MAT1-1-1 and MAT1-2-1 were amplified using specific primers (Table S1) as described previously (Molinier *et al.* 2016). The PCR products were purified using 1.5% agarose gel electrophoresis and AxyPrep DNA Gel Kit (AXYGEN, USA). The purified PCR products were cloned into pMD18-T Vector (pMD18-T Vector cloning, Kit TaKaRa, Japan); a mixture of 5 μL PCR products, 0.2 μL pMD18-T carrier and 5 μL ligation solution was incubated in a 16 °C water bath for 2h and at 4 °C for 2h. The vector was transformed into JM109 competent cells, prepared using the Competent Cell Preparation Kit GK6031 (Shanghai Generay Biotechnology co., Ltd, China), by thermal stimulation. After unfreezing on ice, 10 μL ligation reaction and competent cells were mixed and placed on ice for 30mins, incubated in a 42 °C water bath for 90 s and on ice for 30 min. The transformed JM109 cells were cultured in a 2×YT fluid medium at 37 °C for 1h, spread on a 2×YT medium containing ampicillin, and cultured at 37 °C for more than 12h. Successful transformation of plasmids containing target gene segments were verified by sequencing. Plasmid DNA was extracted using Plasmid Extraction Kit D6943 (Omega Inc., USA). The concentration of the plasmid in the extract was measured, and a dilution series from 10^{-2} to 10^{-10} was prepared to make a standard curve for quantitative PCR (qPCR).

The abundances of mating genes MAT1-1-1 and MAT1-2-1 in the rhizosphere DNA extracts was measured using qPCR. A 20 μL qPCR reaction included 1 μL DNA extract (5-20 ng μL^{-1}), 10 μL SYBR Premix Ex TaqTM II, 7 μl ddH_2O, and 1 μL of forward and reverse primer (10 μM). The amplification program was 3 min at 95 °C, followed by 39 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 20 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 30 s. The fluorescence was measured twice per second.

For all the statistical analysis in our experiment, the statistical significance of the differences were tested using analysis of variance (ANOVA) and post hoc LSD Duncan tests. Differences were taken as statistically significant at $p \leqslant 0.05$.

Results

The Effects of Inoculation on Seedlings

Two months after inoculation with *T. aestivum* and *T. sinoaestivum*, no ectomycorrhizas (ECMs) were detected on the lateral roots of *C. illinoinensis* seedlings. At both four and six months after inoculation, the infection rate of seedlings inoculated with *T. aestivum* was lower than that of seedlings inoculated with *T. sinoaestivum* ($P < 0.05$) (Table 1). There was no ECM on the roots of the control seedlings, the rootlets were slender, and the root tips were dark brown (Fig. S1). The morphology of the *T. aestivum* and *T. sinoaestivum* ECMs were roughly the same (Fig 1).

At the time of inoculation, the average height of the seedlings was approximately 31 cm (Table 2). Six months after inoculation, the height of the inoculated seedlings was higher than that of the control seedlings ($P < 0.05$ p). At two months after inoculation, the stem circumference of the inoculated seedlings was greater than that of the control seedlings ($P < 0.05$ p) (Table 2).

The root activity of seedlings was on the same level in all the treatments (Table 3). Six months after inoculation, the peroxidase (POD) activity of the seedlings inoculated with *T. sinoaestivum* was higher than that of the control ($P < 0.05$) (Table 3). At two months after inoculation, superoxidase dismutase (SOD) activity was highest in the control ($P < 0.05$) (Table 3). At four and six months after inoculation, the SOD activities of the seedlings inoculated with *T. aestivum* were higher than those of the seedlings inoculated with *T. sinoaestivum* ($P < 0.05$).

The Effects of Inoculation on Rhizosphere

The pH and the contents of organic matter, total nitrogen and available phosphorus and potassium were on the same level in all the treatments (Table 4). At the inoculation time, the ammonium nitrogen content was lowest in the *T. sinoaestivum* treatment and at two months after inoculation, in the control treatment ($P < 0.05$) (Table 4). Differences in the nitrate nitrogen content showed no clear trend. At the inoculation time, the nitrate nitrogen content was lowest in the *T. sinoaestivum*

treatment ($P < 0.05$) (Table 4). At two months after inoculation, the nitrate nitrogen content was lowest in the *T. aestivum* treatment and highest in the control ($P < 0.05$); at four months, lowest in the *T. sinoaestivum* treatment and highest in the control ($P < 0.05$); and at six months, lowest in the control and highest in the *T. sinoaestivum* treatment ($P < 0.05$).

Effects of Inoculation on nirS-type Denitrifying Bacteria

The diversity and richness of the *nirS*-type denitrifying bacteria communities were on the same level in all the treatments (Table S2). Out of the 2430 OTUs, 632 were detected in all the treatments, and 300, 535 and 195 were specific for the control, *T. aestivum* treatment and *T. sinoaestivum* treatments, respectively (Fig. S2). The relative abundance of phylum Proteobacteria was over 70% and those of genera *Pseudomonas*, *Pseudogulbenkiania* and *Rhodanobacter* were high in all the treatments (Fig. 3A, B). The differences in relative abundances within treatments were large. In the NMDS ordination, the control samples were slightly separated from the others (Fig. S4).

Mating Type Gene Distribution

In line with the increase in infection rate over time, the copy numbers of MAT1-1-1 and MAT1-2-1 mating genes increased in the rhizosphere of *C. illinoinensis* inoculated with *T. aestivum* (Table 5). In the rhizosphere of *C. illinoinensis* inoculated with *T. sinoaestivum*, the copy numbers of MAT1-1-1 were lowest at two months after inoculation, and those of MAT1-2-1 became slightly lower over time. The ratio of MAT1-1-1 copy number to MAT1 1-1-2-1 copy number increased gradually (Table 5), indicating that the MAT1-1-1 carrying strains became more dominant.

Discussion

Infection rate is one of the most important criteria to measure the success of mycorrhiza formation. The infection rates of *C. illinoinensis* inoculated with *Tuber brumale* and *T. melanosporum* were 37.3% and 34.5%, respectively, in the first year; in the second year, the infection rate of *T. brumale* increased, whereas that of *T. melanosporum* decreased due to other ECM forming fungi, such as *Sphaerosporella brunnea*, *Trichophaea woolhopeia* and *Pulvinula constellation* (Marozzi *et al.* 2017). Similarly, the infection rates of *C. illinoinensis* inoculated with *T. borchii* and *T. aestivum* were 62% and 42%, respectively, after 12 months, yet most of the seedlings were also infected with

S. brunnea with a relative abundance greater than 11% (Benucci *et al.* 2012). In our study, the infection rates of seedlings inoculated with *T. aestivum* and *T. sinoaestivum* were from 45% to 48% six months after inoculation and no ECM contaminants were detected. Possibly the strict humidity control inhibited contamination, as *S. brunnea* is known to thrive in high humidity nursery environment (Bencivenga *et al.* 1995; Donnini and Bencivenga 1995). In addition, the contaminants may have been undetectable due to the relatively short experimental time in our study; 12 months after inoculation, most of the ECM on the roots of *C. illinoinensis* were *Tuber* spp., yet after 24 months the contaminant ECM accounted for the majority of the ECMs (Marozzi *et al.* 2017).

Generally, the growth status of woody plants should be determined by combining plant height and stem circumference. Inoculation with *T. melanosporum* resulted in greater basal diameter and height of *Pinus halepensis* seedlings (Dominguez-Núñez *et al.* 2012). In our study, inoculating with *Tuber* spp. resulted in higher stem circumference two months after inoculation. However, at the end of the experiment the *Tuber* spp. inoculated seedlings were slightly higher yet the stem circumference was at the same level as in the control. Thus, concluding that inoculating with *T. aestivum* and *T. sinoaestivum* would increase the growth of *C. illinoinensis* is only weakly supported.

Peroxidase POD and superoxide dismutase (SOD) activities are related to the stress tolerance ability of plants. Inoculating *Quercus acutissima* with *T. indicum* led to higher root superoxide dismutase (SOD) activity and lower root activity (Zhang *et al.* 2019). Considering the stress tolerance of *C. illinoinensis*, the moderate differences in SOD activities in our study suggested that *T. aestivum* inoculation might be preferred over *T. sinoaestivum* inoculation.

In general, inoculating *C. illinoinensis* with *T. aestivum* and *T. sinoaestivum* did not affect the physicochemical properties of rhizosphere soil. The available nitrogen, i.e. ammonium and nitrate nitrogen, content in the rhizosphere of *Quercus acutissima* was higher with *T. indicum* inoculation than without (Zhang *et al.* 2019). In our study, nitrate nitrogen content was higher in the control at two and four months after inoculation, but after six months the contents were higher in the rhizospheres of *Tuber* spp. inoculated seedlings. Since *C. illinoinensis* grew marginally better and was therefore expected to take up more nitrogen when inoculated with *Tuber* spp., the higher nitrate

nitrogen content at six months could have been to the ECM that is known to increase host plant's access to nutrients (Stuart and Plett 2020).

Another possible explanation for the differences in nitrate nitrogen content at six months is denitrification where nitrate is reduced into nitrous oxide and nitrogen gases that escape into the atmosphere; higher level of denitrification in the control rhizosphere would have resulted in lower nitrate level. To assess the role of denitrification, we analysed the soil denitrifying bacterial community using high throughput sequencing targeting a functional denitrification marker gene, the nitrite reductase-coding gene *nirS* (Braker *et al.* 2000). In the rhizosphere of *Pinus armandii*, *T. indicum* inoculation resulted in lower richness and diversity of bacteria (Li *et al.* 2017). In our study, *T. aestivum* and *T. sinoaestivum* inoculation did not affect the diversity of denitrifying bacteria. Similar to previous studies on ECM and truffle fruiting body associated bacteria (Ye *et al.* 2018; Yang *et al.* 2019), among the denitrifying bacteria the relative abundances of Proteobacteria were high. As the differences in the community composition of denitrifying bacteria were minor, the role of denitrification in the differences in rhizosphere nitrate content remained unclear. In future studies, this could be addressed using qPCR targeting the marker genes of denitrification.

Truffle requires close proximity and interaction of two mating types to complete the fruiting body formation as a part of its life cycle, yet the proximity and interaction of hyphae cannot guarantee the fruiting body formation (Linde and Selmes 2012). The two parents carry different mating type genes, i.e genes MAT1-1-1 and MAT1-2-1. The ECM may act as one of the parents while the other parent has been suggested to originate from free spores in the soil or from relatively short-lived mycelia (Taschen *et al.* 2016). In our study, both mating type genes MAT1-1-1 and MAT1-2-1 were detected in the rhizosphere of *C. illinoinensis* inoculated with *T. sinoaestivum* and *T. aestivum*. Among the *T. melanosporum* ECMs on *Quercus robur*, *Q. ilex*, and *Corylus avellana* roots, MAT1-1-1 incidence was higher than that of MAT1-2-1, suggesting that the MAT1-1-1 strains have a competitive advantage over MAT1-2-1 strains (Linde and Selmes 2012; Osting and Tedersoo 2015). Similarly, in our study MAT1-1-1 dominated over MAT1-2-1 in the rhizosphere of both *C. illinoinensis* inoculated with *T. sinoaestivum* and with *T. aestivum*, possibly due to the competition between ectomycorrhizas with different mating types (Rubini *et al.* 2014). Interestingly, in areas

with high *T. indicum* fruiting body yields the mating type genes were a non-uniformly distributed and MAT1-2-1 dominated (Li *et al.* 2018), whereas MAT1-1-1 dominated in the *T. melanosporum* ECMs of both productive and unproductive trees (Linde and Selmes 2012). Thus, the connection between mating type gene distribution and truffle yield may be truffle and host plant species specific, and as noted by Linde and Selmes (2012), in addition to the mating type, other factors are clearly involved in the formation of fruiting bodies, necessitating further in-depth study to guarantee successful cultivation of truffles.

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Author Contributions

Yue Huang and Jie Zou contributed equally to the work.

Conflict of interest statement

The authors declare no competing financial interests.

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Table 1 Infection rates of *Carya illinoinensis* seedlings inoculated with *Tuber aestivum* and *T. sinoaestivum*

Time after inoculation	Inoculant	Infection rate (%)
2 months	*T. aestivum*	0
	T. sinoaestivum	0
4 months	*T. aestivum*	25.6±1.1[a]
	T. sinoaestivum	30.6±0.8[b]
6 months	*T. aestivum*	45.1±0.4[a]
	T. sinoaestivum	48.3±1.1[b]

*** Different superscript letters at the time points indicate statistically significant difference between inoculants ($p < 0.05$)**

Table 2. Properties of *Carya illinoinensis* seedlings in the inoculation treatments.

Month	Plant height (cm)			Root length (cm)			Root to shoot ratio			Stem circumference (mm)		
	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*
0	31.40±1.21a	31.31±1.22a	31.31±1.13a	8.18±0.48a	8.13±0.18a	7.94±0.29a	0.33±0.04a	0.32±0.04a	0.32±0.04a	2.08±0.22a	2.05±0.14a	2.04±0.07a
2	32.10±0.86a	32.20±1.13a	31.27±1.10a	16.38±1.10a	15.52±1.56a	13.15±2.05a	0.97±0.06a	1.03±0.07a	1.06±0.23a	2.57±0.08b	3.35±0.19a	3.42±0.33a
4	33.73±0.54a	32.33±2.80a	32.84±1.57a	14.98±1.66a	17.35±0.88a	15.19±0.93a	1.31±0.05a	1.35±0.16a	1.18±0.16a	2.27±0.21a	2.40±0.14a	2.25±0.12a
6	36.87±0.56b	38.24±1.00a	38.30±0.97a	20.79±1.43a	21.83±1.14a	18.85±2.24a	1.50±0.09a	1.56±0.13a	1.53±0.04a	2.74±0.33a	2.78±0.24a	2.52±0.19a

Control, inoculated with killed spores. Different letters in the same row indicate statistically significant differences in properties between treatments ($p < 0.05$)

Table 3. Root, peroxidase (POD) and superoxide dismutase (SOD) activities of *Carya illinoinensis* seedlings in the inoculation treatments.

Month	Root activity, $\mu g \cdot g^{-1} \cdot h^{-1}$			Plant POD activity, U/g FW			Plant SOD activity, u/g FW		
	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*
0	33.21±0.98a	33.30±1.95a	32.89±0.62a	143.78±5.61a	149.33±8.10a	147.68±5.01a	1.68±0.11a	1.62±0.05a	1.66±0.06a
2	35.69±0.50a	35.33±1.16a	33.52±3.03a	155.08±5.15a	156.30±3.58a	155.24±5.18a	3.83±0.90a	1.53±0.46b	1.66±0.28b
4	35.95±0.54a	36.24±2.68a	35.91±1.78a	159.05±0.36a	162.25±9.59a	163.37±7.95a	2.08±0.05ab	2.21±0.23a	1.77±0.17b
6	35.92±0.62a	37.62±1.42a	35.92±0.71a	176.69±11.16b	194.92±8.54ab	210.08±10.18a	1.50±0.52ab	1.99±0.19a	1.03±0.27b

Control, inoculated with killed spores. Different letters in the same row indicate statistically significant differences in activities between treatments ($p < 0.05$).

Table 4. Physico-chemical properties of *Carya illinoinensis* rhizosphere soil in the inoculation treatments.

Month	pH value			Organic matter content, g/kg			Total nitrogen content, g/kg			Available phosphorus, mg/kg		
	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*
0	8.23±0.10a	8.37±0.06a	8.25±0.08a	33.27±3.08a	32.87±2.04a	32.45±1.08a	1.05±0.02a	0.97±0.16a	1.03±0.05a	5.91±0.48a	5.89±0.31a	5.65±0.60a
2	8.25±0.02a	8.39±0.03a	8.27±0.03a	35.75±1.93a	33.14±4.23a	35.86±3.69a	1.05±0.01a	0.96±0.07a	1.08±0.01a	5.70±0.98a	5.81±0.54a	5.64±0.04a
4	8.26±0.03a	8.39±0.03a	8.28±0.07a	36.91±6.66a	35.63±0.98a	35.72±4.17a	1.06±0.08a	1.06±0.11a	1.05±0.03a	5.62±0.39a	5.80±0.51a	5.89±0.50a
6	8.26±0.13a	8.40±0.08a	8.33±0.20a	35.58±4.21a	39.04±1.63a	38.00±2.99a	1.04±0.02a	0.99±0.06a	1.02±0.07a	5.63±0.21a	6.01±0.17a	5.77±0.40a

Month	Available potassium content, mg/kg			Ammonium nitrogen content, mg/kg			Nitrate nitrogen content, mg/kg		
	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*	Control	*T. aestivum*	*T. sinoaestivum*
0	98.92±2.50a	100.37±3.51a	100.85±0.85a	9.25±0.36a	9.59±0.25a	8.67±0.16b	17.11±0.21a	18.69±1.28a	14.78±1.69b
2	102.43±1.10a	103.33±2.63a	101.52±4.41a	8.32±0.73b	9.75±0.35a	10.52±0.54a	19.33±3.17a	9.46±0.11c	15.84±0.43b
4	100.34±5.28a	101.37±4.13a	101.41±4.43a	8.03±0.11a	7.65±0.48ab	7.67±0.42ab	31.59±0.36a	28.83±0.23b	12.87±0.01c
6	95.45±3.83a	101.37±2.94a	99.91±6.07a	6.43±1.88a	6.91±0.17a	5.79±1.44a	4.77±0.30c	15.32±0.35b	18.06±0.19a

Control, inoculated with killed spores. Different letters in the same row indicate statistically significant differences in properties between treatments ($p < 0.05$)

Table 5. Copy numbers of the mating type genes in the rhizosphere of *Carya illinoinensis* inoculated with *Tuber aestivum* and *T. sinoaestivum*

Month	Inoculant	MAT1-1-1 CT value	MAT1-2-1 CT value	MAT1-1-1 copy number	MAT1-2-1 copy number	MAT1-1-1/ MAT1-2-1
2	*T. aestivum*	25.80±0.18	27.48±0.55	2000.00±285.83	1078.67±348.23	1.85
	T. sinoaestivum	25.84±0.57	27.65±0.57	2063.33±984.34	976.00±393.18	2.11
4	*T. aestivum*	25.49±0.46	27.20±0.25	2660.00±966.28	1263.33±209.84	2.11
	T. sinoaestivum	25.34±0.09	27.73±0.42	2876.67±204.04	903.67±266.24	3.18
6	*T. aestivum*	24.96±0.55	26.99±0.13	4156.67±1922.04	1710.67±1200.68	2.43
	T. sinoaestivum	25.40±0.52	27.78±0.37	2866.67±1056.28	870.00±205.18	3.30

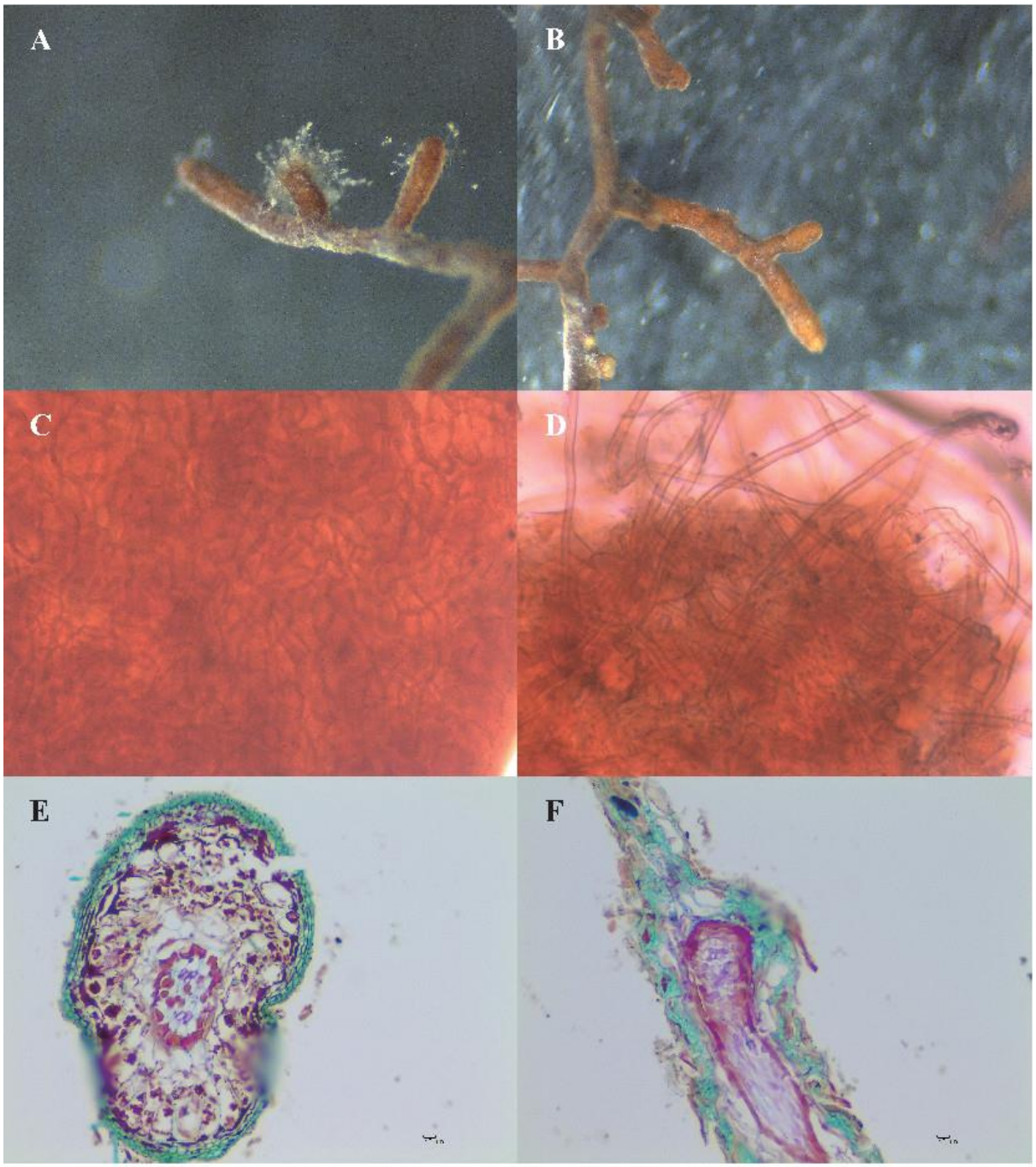

Figure 1 A) Ectomycorrhizas (ECMs) of *T. aestivum* on pecan trees with abundant visible emanating hyphae. **B)** Ramified ECMs of *T. aestivum-C. illinoinensis*. **C)** The outer mantle surface of a *T. aestivum* ECM composed by angular cells (Mantle Type L). **D)** The outer mantle surface of a *T. aestivum* ECM with long, wavy and septated cystidia. **E)** The cross section of an ECM of *T. aestivum-C. illinoinensis*, with Hartig net formation in the intercellular space of the root. **F)** The vertical section of an ECM of *T. aestivum-C. illinoinensis*.

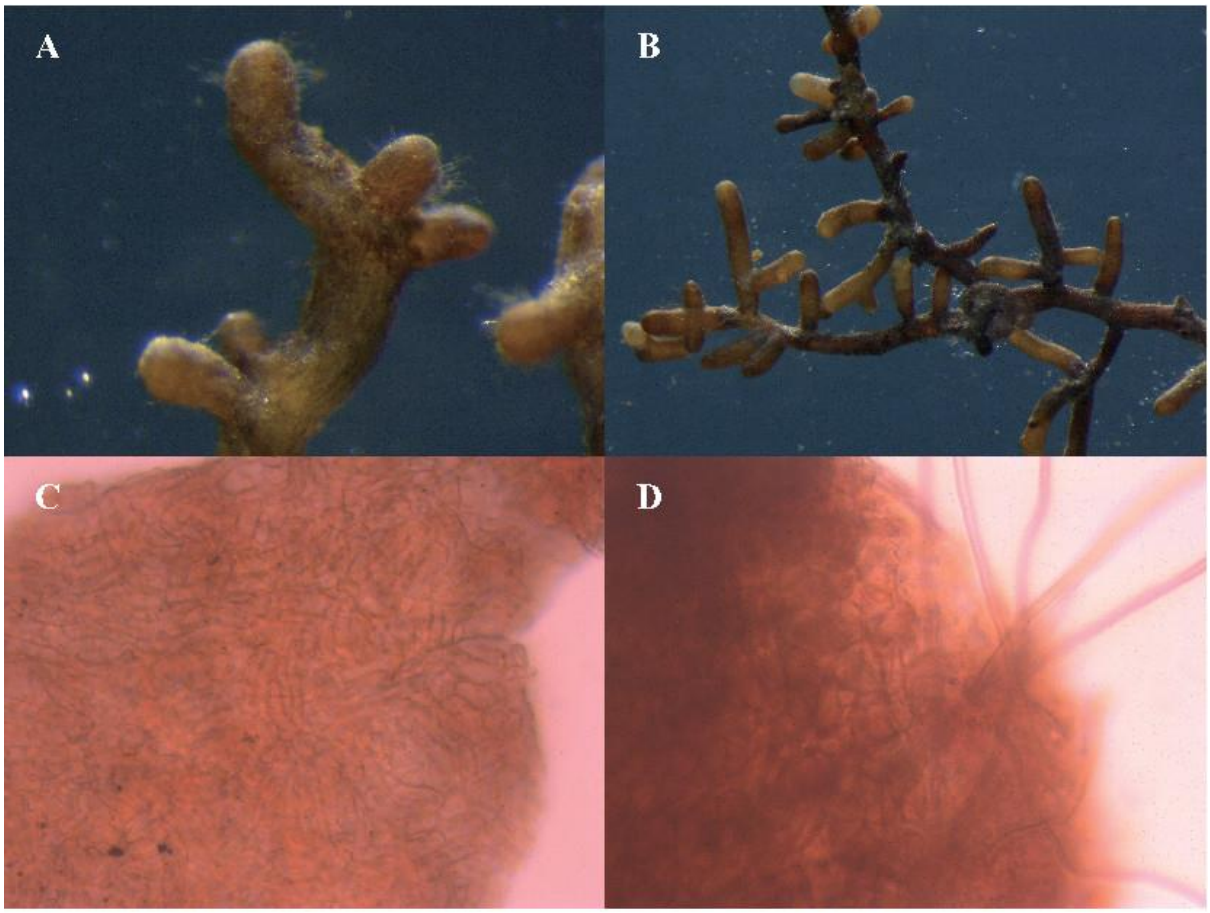

Figure 2 A) Ramified ECMs of *T. sinoaestivum-C. illinoinensis* with abundant visible emanating hyphae. **B)** Complex coral-like ECMs of *T. sinoaestivum-C. illinoinensis*. **C)** The outer mantle surface of a *T. sinoaestivum* ECM composed by angular cells (Mantle Type L). **D)** The outer mantle surface of a *T. sinoaestivum* ECM with relatively sparse, long, wavy and septated cystidia.

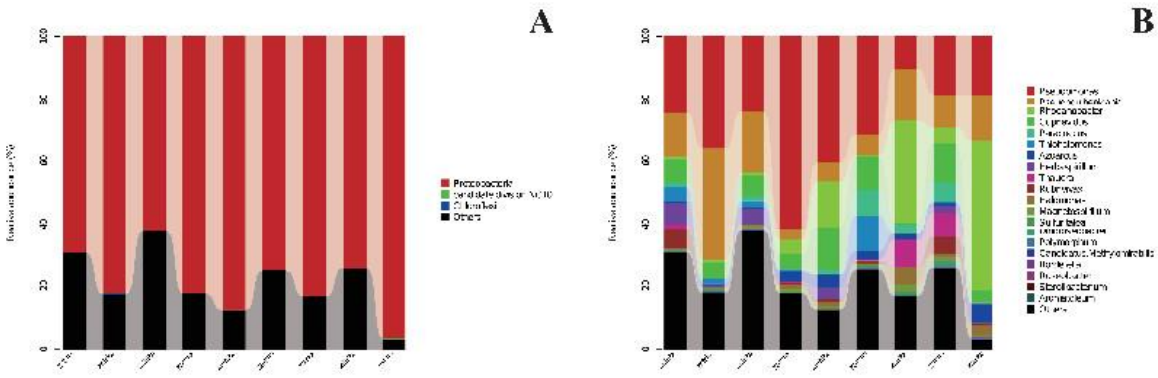

Fig. 3. Distribution of the nirS-type denitrifying bacterial under the different treatments.

A) Distribution at the phylum level. B) Distribution at the genus level.