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Promoting the Growth of *Pinus sylvestris* var. *mongolica* Seedlings and Improving Rhizosphere Fungal Community Structure through Interaction between *Trichoderma* and Ectomycorrhizal Fungi

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ABSTRACT

In this study, pot experiments were conducted on the seedlings of *Pinus sylvestris* var. *mongolica* to study the influence of *Trichoderma* (*Trichoderma harzianum* E15) and Ectomycorrhizal fungi (*Suillus luteus* N94) on the growth of these seedlings. In particular, the effects of these fungi on the fungal community structure in the rhizosphere soil of the seedlings were investigated. Inoculation with *Trichoderma harzianum* E15 and *Suillus luteus* N94 significantly ($P < 0.05$) promoted the growth of the *Pinus sylvestris* seedlings. The non-metric multidimensional scaling (NMDS) results indicated a significant difference ($P < 0.05$) between the fungal community structures in the rhizosphere soil of the annual and biennial seedlings. In the rhizosphere soil of annual seedlings, the main fungi were Ascomycota, Basidiomycota, Zygomycota. Ascomycota, Basidiomycota, Mortierellomycota, and p-unclassified-k-Fungi were the main fungi in the rhizosphere soil of biennial seedlings. The dominant genus in the rhizosphere soil and a key factor promoting the growth of the annual and the biennial seedlings was *Trichoderma*, *Suillus*, respectively. Both of them were negatively correlated with the relative abundance of microbial flora in the symbiotic environment. *Trichoderma* had a significant promoting effect on the conversion of total phosphorus, total nitrogen, ammonium nitrogen, nitrate nitrogen, and the organic matter in the rhizosphere soil of the seedlings, while *Suillus* significantly promoted the conversion of organic matter and total phosphorus.

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1. Introduction

Pinus sylvestris var. *mongolica* is a geographical variant of *Pinus sylvestris*, which is distributed naturally in the northern of Greater Khingan Mountains (50°10'–53°33'N, 121°11'–127°10'E) and the HulunBuir sandy grassland (47°35'–48°36'N, 118°58'–120°32'E). Seedling blight is a plant disease encountered worldwide and is observed quite commonly in the conifer nurseries in China. In *Pinus sylvestris* var. *mongolica*, seedling blight may be caused by *Fusarium*, *Rhizoctonia*, and *Pythium*, with *Rhizoctonia solani* J.G. Kühn being the main source of infection. *Rhizoctonia solani* J.G. Kühn causes seedling blight mainly in the annual/biennial/triennial seedlings of *Pinus sylvestris* var. *mongolica*. Among the aforementioned seedlings of *Pinus sylvestris* var. *mongolica*, the annual seedlings exhibit the highest incidence rate, with a mortality rate of up to 50% upon disease incidence^[1-2]. Over-reliance on chemical pesticides has caused serious problems such as soil compaction, degradation of soil fertility, increased drug resistance of the pathogens and pests, destruction of soil's micro-ecological environment, increased crop phytotoxicity, and weak seedling growth^[3].

In comparison to the use of chemical pesticides, the application of beneficial microorganisms and microbial metabolites represents a novel environmental-friendly approach for plant management as the latter does not cause pollution, releasing of residues, and killing of natural enemies and also lowers the possibility of drug resistance in pathogens and pests. This approach is conducive to human and animal safety, environment protection, disease prevention and treatment, and production improvement^[4-5]. Beneficial microorganisms promote plant growth through several direct or indirect ways such as by producing hormones, facilitating the absorption of soil nutrients by the plants, and inducing stronger disease resistance in plants. Beneficial microorganisms produce certain concentrations of the plant hormones such as IAA (indole-3-acetic acid), GA3 (gibberellic acid), zeatin, and ABA (abscisic acid) to promote division and differentiation in the plant organs^[6-8]. Improving the absorption of soil nutrients by the plants is another direct way in which the beneficial microorganisms assist in promoting plant growth. Beneficial microorganisms dissolve the inorganic phosphorus by releasing organic acids and degrade the organic phosphorus compounds in the soil by releasing phosphatases, therefore enhance the absorption and utilization of phosphorus by the plants^[9]. Furthermore, the beneficial microorganisms transform the sources of inorganic nitrogen in the soil into sources of organic nitrogen to be absorbed and utilized by the

plants. Most of the beneficial microorganisms exhibit a fast growth rate and strong competitiveness. They can form colonies around the plant roots through competition, where they continue to grow and reproduce, effectively inhibiting the growth and reproduction of harmful microorganisms, improving disease and stress resistance in plants, and promoting healthy growth.

Trichoderma belongs to the subdivision Deuteromycota, class Hyphomycetes, order Hyphomycetes, and family Moniliaceae. Owing to its strong vitality and fast growth/reproduction characteristics, *Trichoderma* is capable of rapidly utilizing limited nutrients and living space. Upon identification of the host plant, the hyphae of *Trichoderma* wind onto the root surface of the host plant to form a cell structure. *Trichoderma* is reported to significantly affect the contents of plant hormones such as auxin, SA (salicylic acid), JA (jasmonic acid), and ET (ethylene) to promote plant growth^[10-11]. *Trichoderma*'s other approach for promoting plant growth is to enhance the absorption of nutrients by the plants. It is reported that after *Trichoderma* colonizes around the plant roots, its external hyphae absorb different forms of nitrogen and transport the assimilated nitrogen to the roots of the host plant to improve the plant's nutrient absorption^[12]. Studies have also demonstrated that *Trichoderma* degrades phosphate in the soil through the secretion of excessive acid to increase the available phosphorus content for plant absorption and utilization^[13]. *Trichoderma* is also reported to enhance the absorption and utilization of trace elements, such as Zn and Fe, by promoting the degradation of organic matter^[14-16]. Studies have suggested that in pine trees, 90% of the nutrients are provided by ectomycorrhizal fungi^[17-19]. Ectomycorrhizal fungi promote plant growth and enhance the disease and stress resistance in the host plant by absorbing and storing nutrients and by effectively separating the pathogens from the plant root^[20].

Introduction of a variety of plant rhizosphere beneficial microorganisms for a synergistic effect produced by their interaction with the plants significantly improves the microbial community structure in the nursery soil, ameliorates the physical and chemical properties of the soil, promotes the plant growth, and enhances the disease and stress resistance in plants^[21-23]. Therefore, it is an effective approach to resolve the multiple problems encountered in nurseries these days. In the present study, seedlings of *Pinus sylvestris* var. *mongolica* were selected as the research object and were co-inoculated with (*S. luteus*) N94 and (*T. harzianum*) E15. The differences in seedling growth, fungal community structure in the seedling rhizosphere soil, and the physical and chemical properties of the seedling rhizosphere soil in seedlings that

received different treatments under *Rhizoctonia solani* J.G. Kühn stress were investigated. The main research questions were: (1) How does the inoculation with Ectomycorrhizal fungus N94 and *T. harzianum* E15 influence the growth of the plants? (2) How does the inoculation with Ectomycorrhizal fungus N94 and *Trichoderma* E15 influence the fungal community structure in the seedling rhizosphere soil? Does the inoculation with *Rhizoctonia solani* J.G. Kühn after the co-inoculation with the Ectomycorrhizal fungus and *Trichoderma* influence the fungal community structure in the seedling rhizosphere soil? What is the relationship between Ectomycorrhizal fungus N94, *Trichoderma* E15, and the fungal community structure? What are the annual change in the fungal community structure and the change in relationships among the bacterial communities under each treatment? (3) How does the co-inoculation with Ectomycorrhizal fungus N94 and *Trichoderma* E15 influence the physical and chemical properties of the soil? What is the relationship between the flora and the physical and chemical properties of the soil?

2. Materials and Methods

2.1 Sources of Plants and Seeds and the Preliminary Preparations

Suillus luteus N94 strain was isolated from the *Pinus sylvestris* var. *mongolica* plantation in Zhanggutai Experimental Forest Farm, Liaoning province. *Trichoderma harzianum* E15 was obtained from the University of Edinburgh, United Kingdom. The *Rhizoctonia solani* JG Kühn strain was isolated in a nursery in Weihe Forestry Bureau, Harbin, Heilongjiang province. *Trichoderma harzianum* E15 and *Rhizoctonia solani* JG Kühn were cultured in PDA medium (potato 200 g/L, glucose 20 g/L, and agar 14 g/L), while the Ectomycorrhizal fungus N94 was cultured in modified PDA (potato 200 g/L, glucose 20 g/L, agar 14 g/L, and peptone 3 g/L). The cultured mycelia colony was cut using a sterile punch with a diameter of 5 mm and subsequently inoculated in 250 mL of PD medium (potato 200 g/L and glucose 20 g/L for *Trichoderma harzianum* E15 and *Rhizoctonia solani* J.G. Kühn, and potato 200 g/L, glucose 20 g/L, and peptone 3 g/L for Ectomycorrhizal fungus N94) in a conical flask. The mixtures were then cultured in the dark under shaking conditions at 25 °C and 150 r/min. The resultant fungal solutions were subjected to homogenizing treatment in a crusher before being used for the inoculation of the seedlings of *Pinus sylvestris* var. *mongolica* (*Suillus luteus* N94 grows slowly and the community may be observed after being cultured for over a month)^[24].

The seeds of *Pinus sylvestris* var. *mongolica* (purchased from Zhanggutai Experimental Forest Farm, Zhangwu county, Liaoning province; stored at -20 °C until use) were disinfected using 0.5% potassium permanganate solution for 30 min, followed by rinsing with sterile water a couple of times. Subsequently, the seeds were wrapped in sterilized wet gauze and placed in a 25 °C artificial climate box to accelerate germination. The seeds were rinsed with sterile water every morning and evening until germination (approximately five days; the germination rate was over 60%). Peat soil, vermiculite, and river sand were mixed in a ratio of 2:1:1 and placed inside a high-temperature high-pressure sterilizer at 121 °C for 2 h. After 7 days, the mixed soil was filled into a nutrition bowl (18 × 18 cm) and soaked with water. Three days later, the seeds were sown in these nutrition bowls (20 seeds per nutrition bowl), following which the bowls were placed in the experimental forest farm shed (day/night thermal regime of 22/30 ± 3 °C, and a 14-h light/10-h dark photoperiod) in the Northeast Forestry University for routine daily management and protection^[25].

2.2 Experimental Design and Seedling Inoculation

The experiments included four treatment groups: the Control, *Pinus sylvestris* var. *mongolica* + sterile PD medium; the PR, *Pinus sylvestris* var. *mongolica* + *Rhizoctonia solani* J.G. Kühn; the PNE, *Pinus sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15; and the PNER, *Pinus sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *Rhizoctonia solani* J.G. Kühn. Each treatment group contained 100 nutrition bowls, each bowl containing 15 seedlings. The fungal solutions were inoculated into the rhizosphere of annual *Pinus sylvestris* var. *mongolica* seedlings through root irrigation^[26]. Each nutrition bowl was inoculated with 100 mL fungal solution (inoculated with Ectomycorrhizal fungi N94 ten days after the emergence of seedlings and subsequently with *Trichoderma* E15 one month later, and both concentrations were 1 × 10⁴ cfu/mL). The nutrition bowls in the control group were inoculated with sterilized blank PD solution.

2.3 Seedling Sampling and Biomass Determination

Ninety days after the emergence of *Pinus sylvestris* var. *mongolica* seedlings, the seedlings with control, PR, PNE, and PNER treatments were sampled randomly. The rhizosphere soil was cleaned using a sterile brush. The seedling samples were placed in a blast drying oven at 85 °C for 5 h, following which the dry weight of each sample seedling was measured.

2.4 Rhizosphere Soil Sample Collection and Processing

The seedling rhizosphere soil was collected in a sterile sample bag using a sterile brush. After being sieved through a disinfected 10-mesh sieve, the collected soil was placed in an incubator along with an ice bag and brought to the laboratory. The soil samples for high-throughput sequencing were stored in sterile centrifuge tubes at -80°C , while the soil samples for determination of the physical and chemical properties were first air-dried in the dark, followed by sieving through 18-mesh and 60-mesh sieves, and were finally stored in a refrigerator at 5°C ^[27].

2.5 Analysis of Physicochemical Properties of Soil

Calibrated HACH HQ30d pH meter was used to measure the pH of soil samples (BANTE, Shanghai, China). Soil Properties Analysis was used to determine soil pH (1:2.5 w/v)^[28]. Organic matter (OM) was measured using the potassium dichromate oxidation heating method^[29]. Total nitrogen (TN) was determined using the Kjeldahl method, potassium chloride (KCl) extraction (BRAN+LUEBBE-AA3, Germany) for NH_4^+ and NO_3^- ^[29]. total phosphorus (TP) was determined using Mo–Sb colorimetry^[29], available phosphorus (AP) was determined using the antimony bismuth anti-colorimetric method with double acid leaching, rapidly available potassium (AK) was measured using an NH_4OAc leaching flame photometer^[29], and total potassium (TK) was determined using aflame photometer^[29].

2.6 DNA Extraction, High-throughput Sequencing, and Biological Information Processing

For the high-throughput sequencing of soil microorganisms, the total genomic DNA was extracted from 0.5 g of soil using a Power Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The DNA sample concentration and quality (A260/A280 ratio) were measured using a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States). The diversity and community structure of soil fungi were determined by the MiSeq sequencing platform (TruSeq™ DNA Sample Prep Kit, Illumina USA). The primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') were used to amplify the ITS1 region of the fungal ITS^[30-31]. Each treatment had three replicates in our experiment. PCR was performed in a 20 μL reaction system: 4 μL of $5\times$ FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, 0.2 μL of BSA, 10 ng of template DNA, and 11.6 μL of double-distilled

water^[32-33]. The PCR conditions were as follows: 95°C for 3 min, 27 cycles of 30 s at 95°C , 30 s at 55°C , and 30 s at 72°C , and with a final extension of 10 min at 72°C (ABI GeneAmp® 9700). In each PCR run, positive and negative control was added to validate the PCR results. The amplicons were pooled, purified, and then quantified using NanoDrop (Thermo Scientific, USA). The resulting PCR products were analyzed with 2% agarose gel and further purified with an AxyPrep DNA Gel Extraction Kit (AxygenBiosciences, Union City, CA, USA), and examined by using QuantiFluor™-ST (Promega, WI, USA). The samples were pooled into proportions for sequencing. Eventually, a fast pfa database was built and IlluminaMiSeq sequencing was carried out^[34]. Then, the purified amplicons were pooled in equimolar concentrations as a single aliquot and employed for library construction, and sequencing was performed on an IlluminaMiSeq sequencer at Majorbio Biotechnology Co., Ltd. (Shanghai, China)^[35]. According to the overlapping relationship between PE reads, the paired reads are spliced into a sequence. Meanwhile, the quality and splicing effect of reads are filtered by quality control, and the effective sequences are distinguished according to the two barcodes and primer sequences. Trimmomatic and FLASH software were employed to quality-filter and merge raw fastq files^[36-37], while UPARSE software (version 7.1, <http://drive5.com/uparse/>) was employed to further analyze the pyrosequencing data. The sequences were then divided into operational taxonomic units (OTUs) with a 97% similarity cutoff, after which chimeras were removed using UCHIME. RDP Classifier (<http://rdp.cme.msu.edu/>) was employed for the taxonomic annotation of each sequence within the confidence threshold of 0.7^[38].

2.7 Data Statistics and Analysis

The significance of differences was determined by one-way analysis of variance (one-way ANOVA) in IBM SPSS Statistical Software Package version 20 (IBM Corporation, New York, USA). Duncan and Games-Howell are used to test the data significance, and the Pearson correlation coefficient is used to analyze the correlation between the indicators. Origin2019b (Origin Lab Corporation, MA, United States) were used for mapping. Shannon index was calculated to analyze the α diversity index of the fungal community^[39]. Using the Bray Curtis distance algorithm of a vegan package in R language to analyze and plot NMDS. Using Bray Curtis distance algorithm of R-language pheatmap package to analyze the horizontal community structure of genus and draw the heat map (version: 3.0.2)^[40]. The data processing and network mapping (Spearman's $\rho > 0.6$ and

significant correlations $P < 0.05$) of annual and biennial rhizosphere soil fungal association analysis network were finished by R language (vegan, igraph package) and Gephi (version 0.9.2) [41]. Data analysis and network mapping of the relationship between annual and biennial rhizosphere soil fungal and soil physical and chemical properties were done by R language ggraph and ggcor package [42-43].

3. Results

3.1 Between-group Differences and Annual Changes in Seedling Biomass

The biomass of the annual *Pinus sylvestris* var. *mongolica* seedlings in PEN and PENR treatment groups was significantly ($P < 0.05$) higher compared to that in the control and PR treatment groups (Figure 1). In particular, the biomass of annual seedlings in the PEN and PENR treatment groups increased by 57.70% and 53.14%, respectively, compared to the control group. The biomass increment was even higher (154.41% and 147.06% for PEN and PENR treatment groups, respectively) when compared to the PR treatment group. Similarly, the PEN and PENR treatments resulted in significantly higher biomass of biennial seedlings ($P < 0.05$), with biomass increments of 81.31% and 26.61%, respectively, in comparison to the control group, and the biomass increments of 262.61% and 153.21%, respectively, in comparison to the PR treatment group. The biomass of *Pinus sylvestris* var. *mongolica* seedlings in each treatment group increased significantly ($P < 0.05$) with the annual change.

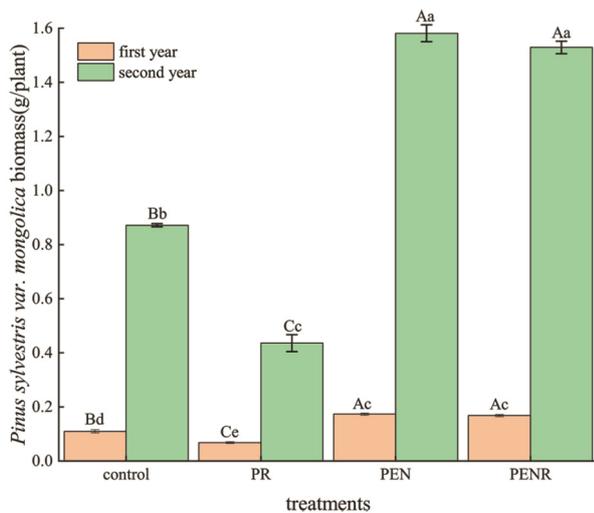


Figure 1. Between-group differences and annual changes in seedling biomass. Control: *P. sylvestris* var. *mongolica* + sterile PD medium; PR: *P. sylvestris* var. *mongolica* + *R. solani*; PNE: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15; PNER: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *R. solani*.

+ *T. harzianum* E15; PNER: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *R. solani*. Data from each treatment were analyzed using a one-way analysis of variance (ANOVA). Bars that do not share a letter are significantly different ($P < 0.05$). The upper case letters in the figure indicate the differences between different treatments in the same year, and the lower case letters indicate the differences between different treatments in the same year ($P < 0.05$).

3.2 Within-group Differences and Annual Changes in the Diversity Index

The Shannon index for the annual seedling rhizosphere soil followed the order control > PR > PENR > PEN, with the Shannon indices for the PEN and PENR treatment groups being significantly ($P < 0.05$) smaller compared to those for the control and PR treatment groups (Figure 2). In regard to the biennial seedling rhizosphere soil, the corresponding Shannon index followed the order control > PENR > PR > PEN. The Shannon index for the control group was significantly greater than those for the PR, PENR, and PEN treatment groups, while the α diversity of the PR, PENR, and PEN treatment groups did not exhibit any significant difference. The Shannon index for the control group increased significantly ($P < 0.05$) with time, while the Shannon index for the PR treatment group exhibited a significant decrease ($P < 0.05$). The α diversity of the PEN and PENR treatment groups did not exhibit any significant difference (i.e., $P > 0.05$).

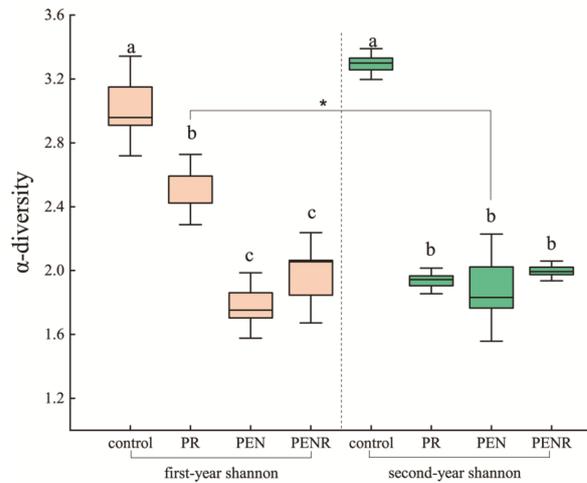


Figure 2. Within-group differences and annual changes in α diversity index. Control: *P. sylvestris* var. *mongolica* + sterile PD medium; PR: *P. sylvestris* var. *mongolica* + *R. solani*; PNE: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15; PNER: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *R. solani*. Pink indicates annual seedling biomass, green indicates biennial seedling biomass. The means with different letters are significantly different at $P < 0.05$.

3.3 Soil Fungal Community Structure

To evaluate the diversity and annual change in the fungal community composition within each treatment group, the Bray-Curtis distance matrix algorithm was used to perform the NMDS cluster analysis. As showed in Figure 3, the fungal community structure in the rhizosphere soils of the annual (Figure 3A) and biennial (Figure 3B) seedlings exhibited significant ($P < 0.05$) differences among the various treatments that the seedlings were subjected to.

Ascomycota, Basidiomycota, Zygomycota, and unclassified-k-Fungi were the main fungi in the rhizosphere soil

of the annual seedlings, accounting for greater than 98% of the total fungal abundance (Figure 4A). The abundance of Basidiomycota was significantly greater in the PR treatment group compared to the other treatment groups, while the relative abundance of Ascomycota was significantly higher in the PEN treatment group. In the rhizosphere soil of the biennial seedlings, the main fungi were Ascomycota, Basidiomycota, Mortierellomycota, and p-unclassified-k-Fungi, accounting for greater than 99% of the total fungal abundance (Figure 4B). The relative abundance of Mortierellomycota was significantly higher in the control and PR treatment groups, while the relative abundance of Basidiomycota was determined to

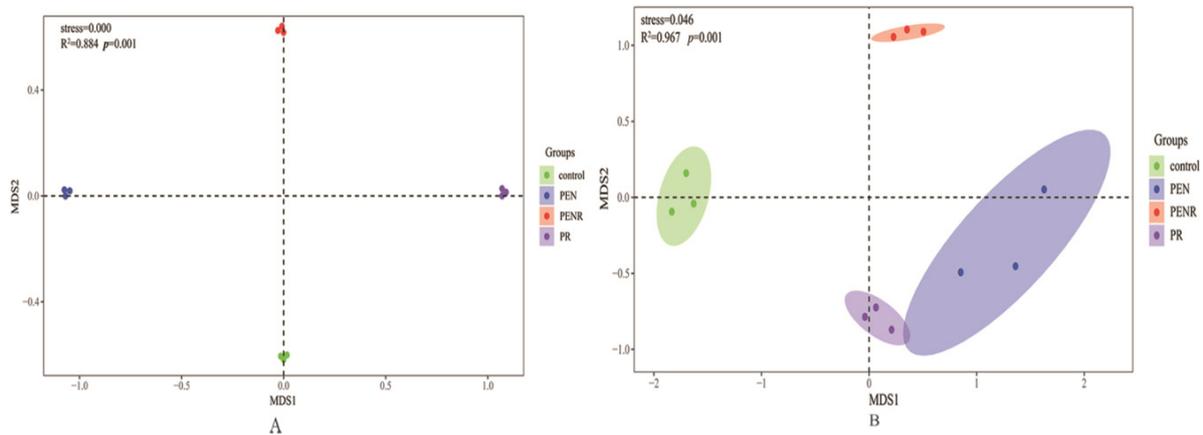


Figure 3. NMDS analysis of fungal community structure in rhizosphere soil of annual (A) and biennial (B) seedlings. Control: *P. sylvestris* var. *mongolica* + sterile PD medium; PR: *P. sylvestris* var. *mongolica* + *R. solani*; PNE: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15; PNER: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *R. solani*.

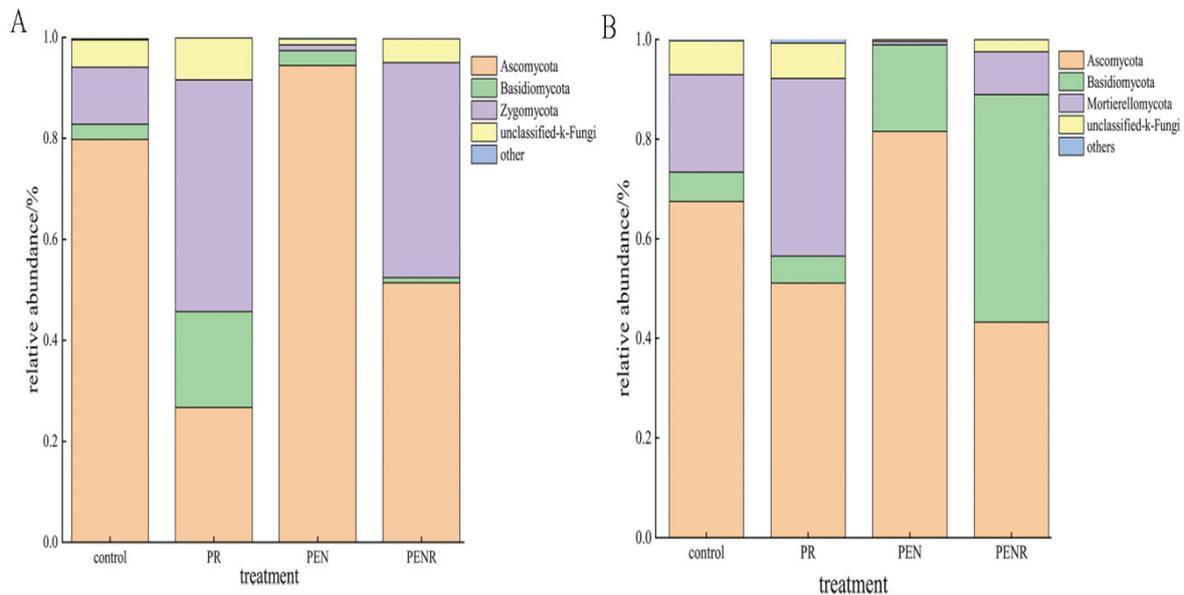


Figure 4. Relative abundance of fungi in rhizosphere soil of annual (A) and biennial (B) seedlings. Control: *P. sylvestris* var. *mongolica* + sterile PD medium; PR: *P. sylvestris* var. *mongolica* + *R. solani*; PNE: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15; PNER: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *R. solani*.

be significantly greater in the PEN and PENR treatment groups. The relative abundance of Ascomycota in control, PEN, and PENR treatment groups was observed to decrease gradually with time, while the relative abundance of Basidiomycota increased gradually with the annual change in each treatment group.

To compare the fungal genera in different treatment groups and observe the annual changes of the fungal genera in each treatment group, a hierarchical clustering method based on the Bray-Curtis distance algorithm was applied to generate a heat map (Figure 5). A significant difference in fungal genera was observed among the different treatment groups, and the annual change in each treatment group was also significant ($P < 0.05$). A heat map was generated with the top 50 fungal genera, *Thanatephorus* (sexual propagules of *Rhizoctonia solani* J.G. Kühn), and *Suillus* (*Suillus luteus* N94) in the rhizosphere soil of the annual seedlings (Figure 5A). *Mortierella* (growth-promoting gene) was the most abundant genus (23.52%), with relative abundance significantly greater in the PR and PENR treatment groups compared to the control and PEN groups. It is noteworthy that the relative abundance of *Gibberella* in the PEN treatment group (32.80%) was significantly higher compared to its relative abundance in the other treatment groups. The relative abundances of plant pathogens such as *Fusarium*, *Alternaria*, *Rhizopus*, *Penicillium*, *Cladosporium*, and *Fusicolla* were significantly higher in the control group. In the PEN and PENR treatment groups, a significantly higher relative abundance of *Trichoderma* was detected in comparison to the control and PR treatment groups. Moreover, the PR treatment group presented a significantly greater relative abundance of the Ectomycorrhizal fungus *Sphaerospora* (0.98%) compared to the other treatment groups. The relative abundances of *Thanatephorus* and *Suillus* in the annual rhizosphere soil were extremely low (0.008% and 0.006%, respectively), with no significant difference among the different treatment groups. *Trichoderma* and *Suillus* in the annual rhizosphere soil were related negatively to the relative abundance of the flora in the symbiotic environment (Figure 6A). Figure 5B depicts the heat map generated with the top 50 fungal genera and *Thanatephorus* in the rhizosphere soil of the biennial seedlings. *Sphaerospora* (Ectomycorrhizal fungi) was the most abundant genus in the rhizosphere soil of the biennial seedlings, with relative abundances in the PEN and PR treatment groups significantly higher than those in the control and PENR treatment groups (the relative abundance of *Sphaerospora* in the PR treatment group was significantly greater for the biennial

seedlings compared to the annual seedlings; the relative abundances of *Sphaerospora* in the annual and biennial rhizosphere soils were 0.315% and 26.65%, respectively). The relative abundances of *Mortierella* (growth-promoting bacteria) in the PEN and PENR treatment groups were significantly lower than those in the control and PR treatment groups (the relative abundance of *Mortierella* in the PR treatment group was significantly lower for the biennial seedlings compared to the annual seedlings, while the relative abundance of *Mortierella* in the control group was significantly higher for the biennial seedlings compared to the annual seedlings). The PEN and PENR treatment groups presented significantly higher relative abundances of *Trichoderma* compared to the control and PR treatment groups. Furthermore, the relative abundance of *Trichoderma* in the rhizosphere soil of biennial seedlings declined significantly compared to that in the rhizosphere soil of the annual seedlings (the relative abundances of *Trichoderma* in the rhizosphere soils of the annual and biennial seedlings were 21.18% and 5.10%, respectively). Similarly, compared to the control and PR treatment groups, the PEN and PENR treatment groups presented significantly greater relative abundances of *Suillus* (the relative abundances of *Suillus* in the PEN and PENR treatment groups were significantly higher for the biennial seedlings compared to the annual seedlings; the relative abundances of *Suillus* in the rhizosphere soils of the annual and biennial seedlings were 0.006% and 4.17%, respectively). The relative abundances of the pathogens such as *Fusarium*, *Pustularia*, *Ilyonectria*, *Gibberella*, *Cladosporium*, and *Chalara* were significantly higher in the control group than in the other treatment groups. The abundance of *Thanatephorus* was extremely low, with no difference observed among the different treatment groups. In the rhizosphere soil of biennial seedlings, *Trichoderma* correlated positively with *Plenodomus*, *Sarocladium*, and *Myrmecridium*, and negatively with *Chalara*, *Clonostachys*, *Pustularia*, *Guehomyces*, *Peziza*, *Mrakia*, *Auricularia*, *Ilyonectria*, *Cladosporium*, *Rhizopogon*, and *Hebeloma*; *Suillus* correlated positively with *Myrmecridium* and negatively with *Rhizophlyctis*, *Rhizophlyctis*, *Olpidium*, *Mortierella*, and *Serendipita* (Figure 6B).

According to the correlation analysis between the relative abundances of the fungal genera in the rhizosphere soils of the annual and biennial seedlings and the plant biomasses (Table 1), *Trichoderma* was an important factor promoting the growth of the annual seedlings ($R^2 = 1.00^{**}$, $P < 0.05$). *Suillus* also exhibited a promoting effect on the growth of annual seedlings, although the effect was not significant ($R^2 = 0.738$, $P > 0.05$). On the

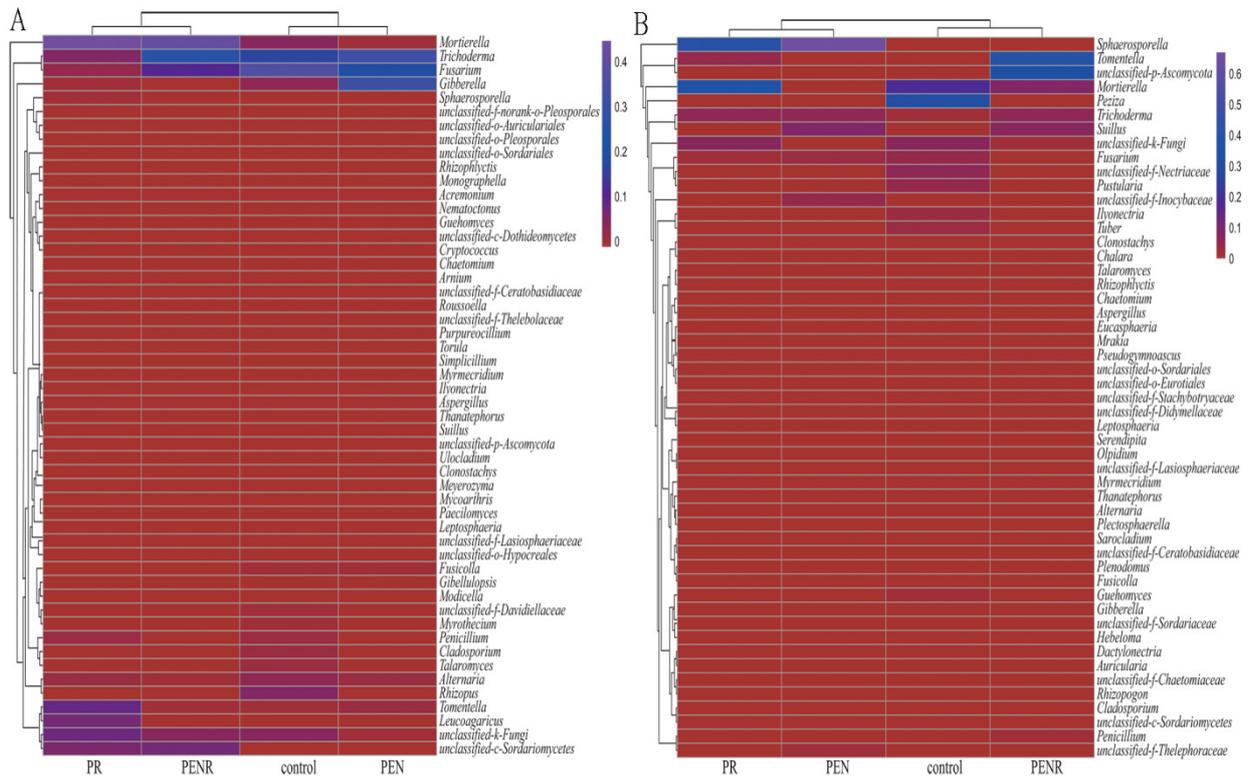


Figure 5. Heat map of the fungal community structure of annual (A) and biennial (B) seedlings. Control: *P. sylvestris* var. *mongolica* + sterile PD medium; PR: *P. sylvestris* var. *mongolica* + *R. solani*; PNE: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15; PNER: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *R. solani*.

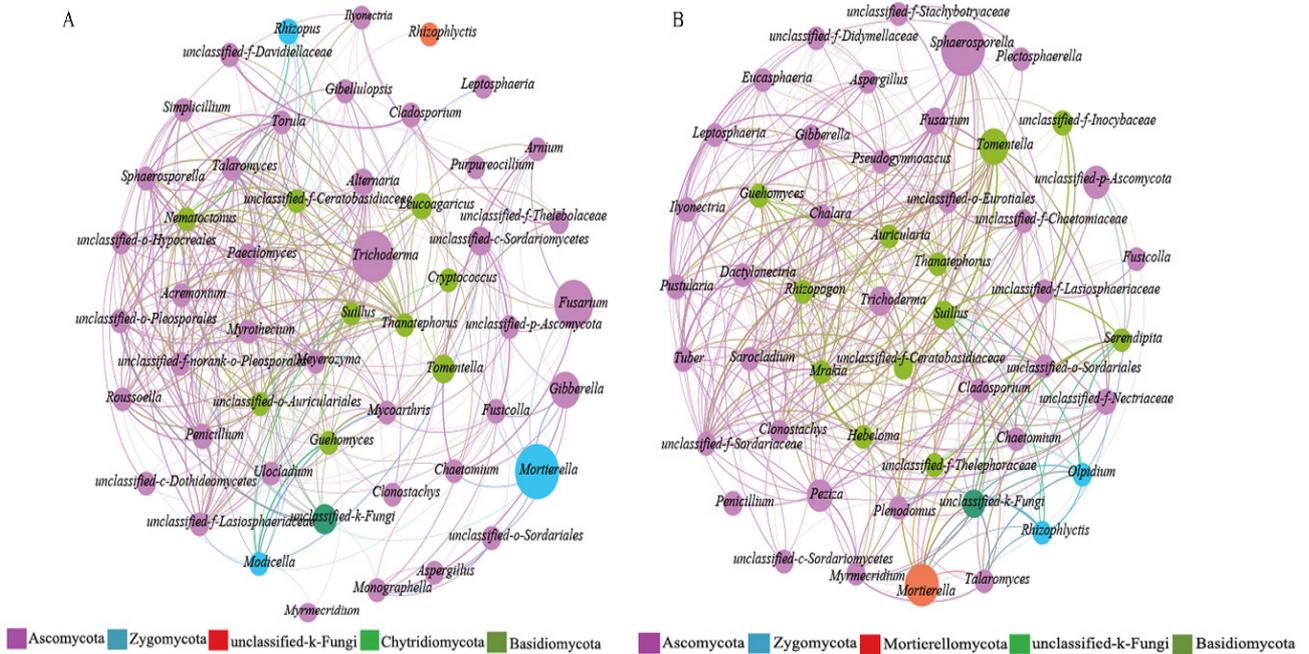


Figure 6. The symbiosis networks of the fungal community structures in the rhizosphere soils of annual (A) and biennial (B) seedlings. The color and size of the circles represent the phylum and the relative abundance of flora, respectively.

Table 1. Spearman correlation coefficients between the relative abundance of fungi taxa and *P. sylvestris* var. *mongolica* biomass

| Genera | | Correlation coefficient | P value | Genera | | Correlation coefficient | P value |
|------------|-----------------------------|-------------------------|---------|----------------------|---------------------|-------------------------|---------|
| First year | <i>Trichoderma</i> | 1.000* | 0.000 | Second year | <i>Mortierella</i> | -1.000** | 0.000 |
| | Unclassified-k-Fungi | -1.000* | 0.000 | | <i>Suillus</i> | 1.000** | 0.000 |
| | Unclassified-o-Pleosporales | -1.000* | 0.000 | | <i>Fusicolla</i> | 1.000** | 0.000 |
| | <i>Suillus</i> | 0.738 | 0.262 | | <i>Rhizophyctis</i> | -1.000** | 0.000 |
| | <i>Thanatephorus</i> | -0.800 | 0.200 | | <i>Trichoderma</i> | 0.600 | 0.400 |
| | | | | <i>Thanatephorus</i> | -0.105 | 0.895 | |

*mean significant different, ** mean very significant different.

other hand, for the growth of biennial seedlings, *Suillus* was an important promoting factor ($R^2 = 1.00^{**}$, $P < 0.05$), while *Trichoderma*, although correlated positively with its growth, did not exhibit any significant effect ($R^2 = 0.600$, $P > 0.05$). *Thanatephorus* correlated negatively with the growth of annual and biennial seedlings ($R^2 = -0.800$ and -0.105 , respectively), although its effect did not reach a significant level ($P > 0.05$).

The contents of available potassium, total potassium, total nitrogen, total phosphorus, ammonium nitrogen, nitrate nitrogen, and the organic matter in the rhizosphere soils of the annual and biennial seedlings of *Pinus sylvestris* var. *mongolica* were significantly greater ($P < 0.05$) in the PEN and PENR treatment groups compared to the control and PR treatment groups (Table 2). Furthermore, the pH values of the soils in the PEN and PENR treatment groups were significantly lower than the pH values in the control group. When the control and PR treatment groups were compared, the contents of total potassium, total nitrogen, total phosphorus, ammonium nitrogen, nitrate nitrogen, and organic matter were significantly greater in the rhizosphere soils of the annual and biennial seedlings of the control group ($P < 0.05$). The physical and chemical properties of soil in each treatment group increased significantly ($P < 0.05$) with the annual change (Table 2). In the rhizosphere soil of the annual seedlings, AK (available potassium) was the most important environmental factor influencing the fungal community structure (Figure 7A). *Trichoderma* significantly promoted the conversion of total phosphorus, total nitrogen, ammonium nitrogen, nitrate nitrogen, and the organic matter in the soil. *Suillus* also played a promoting role in the conversion of soil organic matter and total phosphorus. The microbial community diversity (Shannon) facilitated the conversion of the available phosphorus in the rhizosphere soil of the annual seedlings while being an important factor influencing the pH (Figure 7A). The pH value of the rhizosphere soil of the biennial seedlings was the most important environmental factor influencing the fungal community structure (Figure 7B). *Suillus* demonstrated a significant promoting effect on

the conversion of available potassium, total potassium, available phosphorus, total nitrogen, total phosphorus, ammonium nitrogen, nitrate nitrogen, and organic matter in the rhizosphere soil of the biennial seedlings. *Trichoderma* did not exhibit any significant promoting effect on the conversion of nutrients in the rhizosphere soil of biennial seedlings, which could be related to the significant decrease in *Trichoderma* abundance. The microbial community diversity (Shannon) was an important factor influencing the pH of the rhizosphere soil of the biennial seedlings (Figure 7B).

The upper case letters in the figure indicate the differences between different treatments in the same year, and the lower case letters indicate the differences between different treatments in the same year ($P < 0.05$). Control: *P. sylvestris* var. *mongolica* + sterile PD medium; PR: *P. sylvestris* var. *mongolica* + *R. solani*; PNE: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15; PNER: *P. sylvestris* var. *mongolica* + *S. luteus* N94 + *T. harzianum* E15 + *R. solani*. Data are mean values of three replicates \pm standard error (SE).

4. Discussion

Inoculation with *Trichoderma harzianum* E15 and *Suillus luteus* N94 significantly enhanced the growth of *Pinus sylvestris* var. *mongolica* seedlings. The biomass of the seedlings increased significantly with the annual change ($P < 0.05$). *Trichoderma* is present widely at the plant roots in nature and is an important plant growth-promoting fungus (PGPF). *Trichoderma* promotes plant growth through direct and indirect mechanisms, such as competition, hyperparasitism, antibiotic resistance, induction of plant resistance, and plant growth promotion^[44-45]. *Trichoderma* shortens the cell division cycle and facilitates cytokinesis through the synthesis of plant hormones such as auxin, cytokinins (CKs), and gibberellic acids (GAs), promoting plant rooting and the formation and elongation of lateral roots, thereby improving the absorption and utilization of nutrients and water by the plants^[46-50]. Ectomycorrhizal fungi form structures, such as external

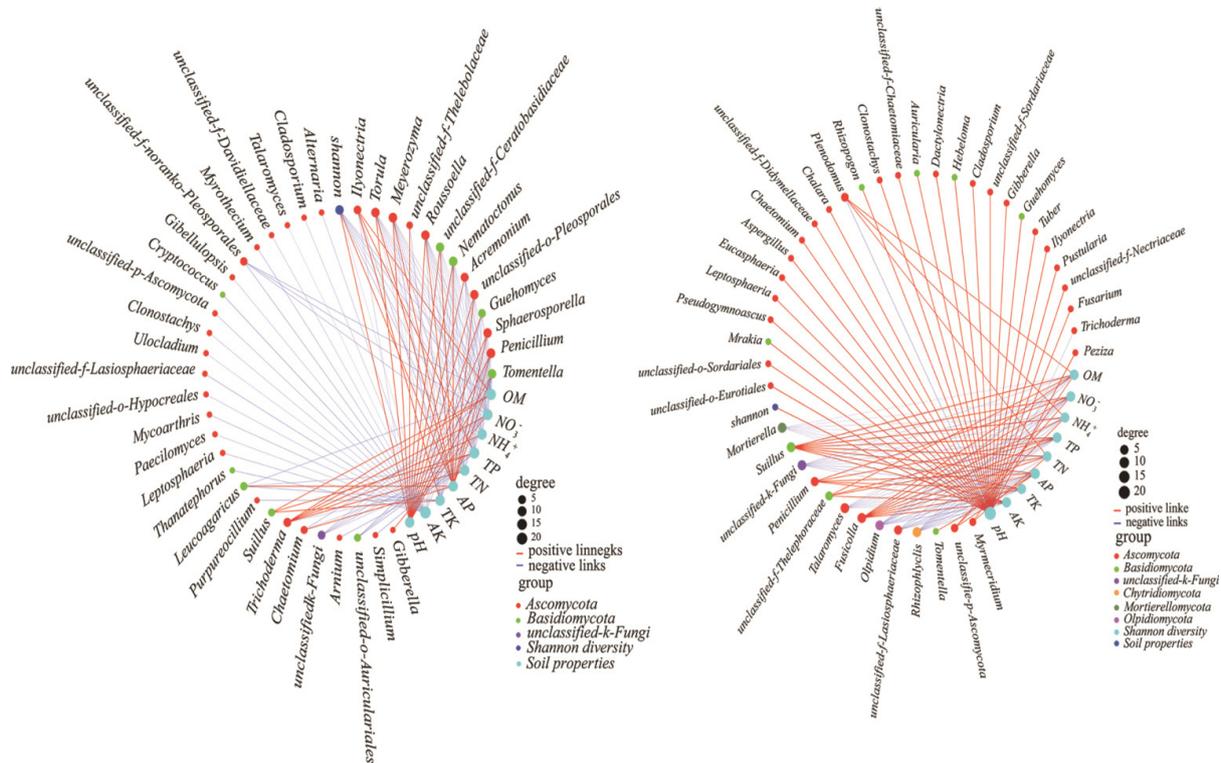


Figure 7. Correlation network of soil environmental factors and species in the rhizosphere of annual (the left) and biennial (the right) seedlings. Circle color represents phylum, soil physical and chemical properties, Shannon index. The circle size indicates the link between species and environmental factors. The red line indicates a positive correlation and the blue line indicates a negative correlation. The connections stand for a strong (Spearman’s $P > 0.6$) and significant ($P < 0.05$) correlations.

Table 2. Soil properties of rhizosphere soils of annual and biennial seedlings

| Treatment | | pH | Available K mg/kg | Total K g/kg | Available P mg/kg | Total N mg/kg | Total P g/kg | Exchangeable NH ⁴⁺ -N mg/kg | NO ₃ -N mg/kg | Organic matter g/kg |
|-------------|---------|---------------|-------------------|---------------|--------------------|----------------|----------------|--|--------------------------|---------------------|
| First year | Control | 6.070±0.006Bc | 114.969±0.335Cc | 3.501±0.033Be | 444.293±5.558Bd | 2.482±0.025Be | 1.247±0.023Be | 0.007±0.000Bd | 0.020±0.0002Cc | 81.415±0.561Be |
| | PR | 6.150±0.021Ac | 129.684±0.578Bd | 3.157±0.080Cf | 461.627±6.557Ad | 2.196±0.0075Cd | 1.166±0.013Cf | 0.005±0.000Cc | 0.0193±0.0003Df | 75.860±0.426Cd |
| | PEN | 5.880±0.006Cd | 138.400±0.580Ac | 3.685±0.027Be | 419.470±2.397Cd | 2.781±0.049Ab | 1.398±0.020Ad | 0.010±0.000Ab | 0.0231±0.0003Ac | 94.345±1.191Aab |
| | PENR | 5.570±0.006De | 141.113±0.335Ac | 4.169±0.103Ad | 415.136±2.461Cd | 2.682±0.057Ab | 1.427±0.004Ad | 0.010±0.000Ab | 0.0222±0.0002Bd | 95.952±0.968Aab |
| Second year | Control | 6.983±0.009Aa | 186.487±1.112Cb | 7.129±0.087Bb | 697.240±1.0424ABCb | 2.549±0.028Bc | 1.9518±0.001Cb | 0.007±0.000Cc | 0.0245±0.001ACBb | 84.025±0.399Cc |
| | PR | 6.440±0.015Bb | 148.364±1.381Dc | 6.629±0.011Cc | 676.358±2.364Cc | 2.227±0.063Cd | 1.875±0.004Dc | 0.0069±0.000Dd | 0.0219±0.000Cd | 79.474±0.478Dd |
| | PEN | 6.483±0.003Bb | 250.646±4.157Aa | 7.713±0.039Aa | 723.244±1.0424Ba | 3.270±0.013Aa | 2.008±0.003Aa | 0.0147±0.000Ba | 0.0270±0.000Ba | 102.156±0.226Aa |
| | PENR | 6.453±0.007Bb | 244.686±1.231Ba | 7.658±0.046Aa | 733.488±2.758Aa | 3.219±0.03Aa | 1.990±0.0019Ba | 0.0150±0.000Aa | 0.0275±0.000Aa | 100.766±0.024Ba |

hyphae, the fungal mantle, Hartig net, and rhizomorph, along with the root system of the host plant, to expand the contact area of the root system for better absorption of water and nutrients. Among the various structures stated earlier, the mantle layer and the Hartig net exert the effect of locking moisture and nutrients, which is further conducive to promoting plant growth^[51-53]. In the rhizosphere soil, Ectomycorrhizal fungi facilitate the colonization of the plant roots by *Trichoderma*^[54] and stimulate the production of plant hormones (such as SA and JA), thereby promoting plant growth^[55]. In the present study, the biomass of the *Pinus sylvestris* var. *mongolica* seedlings co-inoculated with *Trichoderma* and Ectomycorrhizal fungi was significantly higher than that of the uninoculated seedlings. According to the study

conducted by Giuseppe et al.^[56] on lettuce, tomato, and zucchini seedlings, co-inoculation with *Trichoderma atroviride* MUCL 45632 and *Glomus intraradices* BEG72 strains significantly improved the height, shoot dry weight, root dry weight, chlorophyll index (SPAD), and the trace element content of the seedlings compared to seedlings inoculated with *Trichoderma atroviride* MUCL 45632 or *Glomus intraradices* BEG72 alone. With clove (*Syzygium aromaticum* L.) seedlings as the research object, Sutarman^[57] observed that the height, ground diameter, leaf number, and root structure exhibited a significant improvement in the case of the seedlings co-inoculated with *Trichoderma harzianum* Tc-Jjr-02 and Ectomycorrhizal fungi. In addition, a significant difference was observed when comparing the co-inoculated seedlings

with the seedlings that were inoculated with *Trichoderma* or Ectomycorrhizal fungi alone. The study conducted by Badda et al.^[58] indicated that in comparison to the control group, cotton plant inoculated with Ectomycorrhizal fungi (*Acaulosporalaevis*), *Trichoderma viride*, and *Pseudomonas fluorescens* exhibited significantly increased seedling height and biomass, chlorophyll content, phosphorus content in the seedling shoot, phosphorus content in the seedling root, root length, and leaf area.

The soil microbial community diversity is considered important for maintaining the ecosystem function and long-term sustainability of soil^[59]. In the present study, co-inoculation with *Trichoderma harzianum* E15 and *Suillus luteus* N94 significantly reduced the diversity of the soil fungal community ($P < 0.05$). Competition refers to a phenomenon that occurs among biological individuals due to a lack of available nutrients and insufficient living space. Application of plant growth-promoting fungi (such as *Trichoderma* and Ectomycorrhizal fungi), which would compete for the limited nutrients and the living space, is one of the important approaches to cultivate healthy seedlings^[60-62]. A significant difference ($P < 0.05$) was observed in the fungal community structures in the rhizosphere soils of the annual and biennial seedlings, at both phylum and genus levels, among the control, PR, PENR, and PEN treatment groups. Ascomycota, Basidiomycota, Zygomycota, and unclassified-k-Fungi were determined to be the main fungi in the rhizosphere soil of the annual seedlings. In contrast, the main fungi in the rhizosphere soil of the biennial seedlings were Ascomycota, Basidiomycota, Mortierellomycota, and p-unclassified-k-Fungi. Among these, Ascomycota exhibited the highest relative abundance (in the rhizosphere soils of both annual and biennial seedlings), which was consistent with the findings of several previously reported studies^[63]. *Trichoderma* was the dominant genus in the rhizosphere soil of the annual seedlings. The relative abundance of *Trichoderma* in the group that received co-inoculation with *Trichoderma* and Ectomycorrhizal fungi was significantly greater than its relative abundance in the uninoculated groups. The control group exhibited the lowest relative abundance of *Trichoderma* and a relatively higher abundance of the pathogens associated with plant diseases. The relative abundance of *Suillus* and *Thanatephorus* was low in each treatment group, and there was no significant difference among the different groups. The symbiosis network diagram of microorganisms in the annual rhizosphere soil revealed that *Trichoderma* and Ectomycorrhizal fungi inhibited the growth and reproduction of microorganisms in the symbiotic environment. *Trichoderma* is classified

as a saprophytic fungus, with the characteristics of strong vitality and rapid growth and reproduction, enabling it to rapidly utilize limited nutrients and living space and thereby inhibiting the growth and reproduction of the microbial community in the symbiotic environment. Furthermore, upon host identification, *Trichoderma* causes cell wall degradation in the host through the secretion of hydrolytic enzymes and absorbs nutrients resulting in the inhibition of the growth and reproduction of the remaining microbial communities in the symbiotic environment^[64-66]. *Trichoderma* is reported to exert a short-term or long-term inhibitory effect on the soil microbial community structure in the symbiotic environment^[67]. According to the studies conducted by Brimmer and Boland, inoculation with *Trichoderma* inhibits spore germination of Ectomycorrhizal fungi^[68]. Palaniyandi et al.^[69] discovered that inoculation with *Trichoderma* in the rhizosphere soil of pepper (*Piper nigrum* L.) seedlings significantly reduced the relative abundance of symbiotic microorganisms, which is consistent with the results of the present study. The relative abundance of *Trichoderma* in the rhizosphere soil of biennial seedlings in the present study was significantly lower than that in the rhizosphere soil of the annual seedlings. The opposite trend was observed for the relative abundance in the case of *Suillus*. The relative abundance of the plant disease-associated fungi in the group co-inoculated with *Trichoderma* and Ectomycorrhizal fungi was significantly reduced, while the growth and reproduction of other microbial communities in the symbiotic environment were inhibited by *Trichoderma* and Ectomycorrhizal fungi. Enhancing the interaction among the microbial flora in the rhizosphere soil during plant growth leads to increased interaction among the microbial flora in the symbiotic environment^[70]. Studies have demonstrated that microbial flora promotes spore germination of Ectomycorrhizal fungi during plant growth, improving the relative abundance of Ectomycorrhizal fungi in the symbiotic environment and enhancing the ability of the fungi to colonize the rhizosphere and share or compete for the nutrients and the living space in the symbiotic environment^[71-73]. Moreover, Ectomycorrhizal fungi are reported to inhibit the growth and reproduction of saprophytes such as *Trichoderma* and plant pathogens^[74], which is consistent with the results of the present study. The correlation results of the present study suggested that the biomass of annual seedlings is significantly correlated highly positively with the relative abundance of *Trichoderma*, while the biomass of the biennial seedlings is significantly correlated highly positively with the relative abundance of *Suillus*. This might be related to the variation in flora abundance in the

rhizosphere soil.

The nutrient contents in the rhizosphere soils of the annual and biennial seedlings were significantly higher in the PEN and PENR treatment groups (*Trichoderma harzianum* E15- and *Suillus luteus* N94 -inoculated groups), compared to the control and PR treatment groups. In addition, the soil nutrient content increased significantly with the annual change. The results of the network analysis of species and environmental factors suggested AK as the most important environmental factor influencing the fungal community structure in the annual rhizosphere soil, while *Trichoderma* and *Suillus* were determined to play a significant role in promoting the conversion of total phosphorus and organic matter (*Trichoderma* also exerted a significant promoting effect on the conversion of nitrogen). In the rhizosphere soil of the biennial seedlings of *Pinus sylvestris* var. *mongolica*, *Suillus* exerted a significant promoting effect on the conversion of potassium, phosphorus, nitrogen, and organic matter. Rhizosphere beneficial microorganisms improved the absorption and utilization of the plant nutrients by facilitating the degradation of organic matter in the rhizosphere. Greater than 40% of the carbon fixed through photosynthesis is secreted into the rhizosphere, causing the rhizosphere priming effect, i.e., the new organic matter input promotes the degradation of the existing organic matter in the soil, thereby providing macro- and micro-nutrients to the plants [75-77]. The structure of Ectomycorrhizal fungi, such as external hyphae and the fungal mantle, promote the degradation of complex polymer molecules (e.g., chitin and lignin) in the soil through the secretion of enzymes (such as esterase, chitinase, trehalase, phosphatase, etc.), providing organic nitrogen and soluble phosphorus to the host plant [78-80]. In addition, the mycelia absorb nitrogen sources (e.g., NH_4^+ , NO_3^- , and free amino acids) that diffuse and flow into the soil, providing inorganic nitrogen to the host plant [81-82]. *Trichoderma* hyphae absorb nitrogen (present in different states) from the soil and convert it into inorganic nitrogen to be absorbed and utilized by the host plant. In addition, *Trichoderma* degrades the phosphates through the secretion of excessive acid, decomposing the phosphorus that is difficult to absorb and utilize into soluble phosphorus that can be used by the plants. Moreover, the secreted acid may cause pH changes in the soil environment [83-84]. Ahangar et al. [85] demonstrated that inoculation with either *Trichoderma harzianum* or *Laccaria laccata* alone could significantly enhance the contents of nitrogen, phosphorus, and potassium in the rhizosphere soil of *Pinus wallichiana*, while co-inoculation with *Trichoderma harzianum* and *Laccaria*

laccata led to further significant improvement. According to the study conducted by Colla [86], wheat (*Triticum durum* Desf. cv. Avispa) co-inoculated with Ectomycorrhizal fungi and *Trichoderma* exhibited significantly higher plant photosynthetic rate, accompanied with higher K, P, Fe, and Zn contents in the soil.

5. Conclusions

Inoculation with *T. harzianum* E15 and *S. luteus* N94 significantly promoted the growth of *Pinus sylvestris* var. *mongolica* seedlings.

Co-inoculation of *Pinus sylvestris* var. *mongolica* seedlings with *T. harzianum* E15 and *S. luteus* N94 exerted a significant influence on the fungal community structure in the rhizosphere soil of the seedling. Moreover, the fungal community structure changed significantly with the annual change. *Trichoderma* was the dominant genus in the rhizosphere soil of annual seedlings and inhibited the growth and reproduction of microbial flora in the symbiotic environment. In comparison to the relative abundances of microorganisms in the rhizosphere soil of the annual seedlings, the relative abundances of *Trichoderma* and pathogens were significantly reduced, while those of Ectomycorrhizal fungi and the probiotics were significantly increased, in the rhizosphere soil of the biennial seedlings. In addition, Ectomycorrhizal fungi inhibited the growth and reproduction of microbial flora in the symbiotic environment.

Trichoderma and *Suillus* were important for promoting the growth of the annual and biennial *Pinus sylvestris* var. *mongolica* seedlings, respectively. The change in the microbial diversity in the rhizosphere soil of annual *Pinus sylvestris* var. *mongolica* seedlings was the key factor influencing the conversion of available phosphorus and the soil pH. In addition, *Trichoderma* exerted a significant promoting effect on the conversion of total phosphorus, total nitrogen, ammonium nitrogen, nitrate nitrogen, and the organic matter in the rhizosphere soil of the seedlings, while *Suillus* played an important role in facilitating the conversion of organic matter and total phosphorus. The microbial diversity in the rhizosphere soil of the biennial *Pinus sylvestris* var. *mongolica* seedlings was the key factor influencing the soil pH. In addition, *Suillus* exerted a significant promoting effect on the conversion of available potassium, total potassium, available phosphorus, total nitrogen, total phosphorus, ammonium nitrogen, nitrate nitrogen, and organic matter.

Author Contributions

S.H., X.D., and R.Q.S. conceived and designed the study; S.H. and X.D. performed the experiments; S.H., X.D., J.Z., J.X., and X.S.S. contributed to the sample measurement and data analysis; S.H. and X.D. wrote the

paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

References

- [1] Qi JY, Yin DC, Song RQ. Effects of inoculating *Suillusleutus* and *Trichoderma virens* on damping-off resistance of *Mongolian Pine* seedlings. *Journal of Jilin Agricultural University*, 2018, 40, 43-49.
- [2] Lamichhane JR, Dürr Carolyne, Schwanck André, A et al. Integrated management of damping-off diseases. A review. *Agronomy for Sustainable Development*. 2017, 37, 10.
DOI: 10.1007/s13593-017-0417-y.
- [3] John CJ, Jishma P, Karthika NR, et al. *Pseudomonas fluorescens* R68 assisted enhancement in growth and fertilizer utilization of *Amaranthus tricolor* (L.). *Biotech*. 2017, 7, 256.
DOI: 10.1007/s13205-017-0887-2.
- [4] Zhang FG, He YQ, Cobb AB, et al. *Trichoderma* biofertilizer links to altered soil chemistry, altered microbial communities, and improved grassland biomass. *Frontiers in Microbiology*. 2018, 9, 848.
DOI: 10.3389/fmicb.2018.00848.
- [5] Li Y, Chen Z, He JZ, et al. Ectomycorrhizal fungi inoculation alleviates simulated acid rain effects on soil ammonia oxidizers and denitrifiers in Masson pine forest. *Environmental Microbiology*. 2018, 29.
DOI: 10.1111/1462-2920.14457.
- [6] Perrig D, Boiero ML, Masciarelli OA, et al. Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillumbrasilense*, and implications for inoculant formulation. *Applied Microbiology & Biotechnology*. 2007, 75, 1142-1150.
DOI: 10.1007/s00253-007-0909-9.
- [7] Irum N, Asghari B, Hassan TU. Isolation of phytohormones producing plant growth promoting rhizobacteria from weeds growing in Khewra salt range, Pakistan and their implication in providing salt tolerance to *Glycine max* L. *African Journal of Biotechnology*. 2009, 8.
DOI: 10.5897/AJB09.1176.
- [8] Yasar E, Sezai E, Ayhan H, et al. Effects of plant growth promoting rhizobacteria (PGPR) on rooting and root growth of kiwifruit (*Actinidia deliciosa*) stem cuttings. *Biological Research*. 2010, 43.
DOI: 10.4067/S0716-97602010000100011.
- [9] Smith SE, Jakobsen I, Gronlund M, et al. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiology*. 2011, 156, 1050-1057.
DOI: 10.1104/pp.111.174581.
- [10] Contreras-Cornejo HA, Macías-Rodríguez L, Alfaro-Cuevas R, et al. *Trichoderma spp.* improve growth of *Arabidopsis* seedlings under salt stress through enhanced root development, osmolite production, and Na⁺ elimination through root exudates. *Molecular Plant-Microbe Interactions*. 2014, 27, 503-514.
DOI: 10.1094/MPMI-09-13-0265-R. DOI: 10.1073/pnas.1322671111.
- [11] Eugenia Morán-Diez, Belén R, Sara D, et al. Transcriptomic response of *Arabidopsis thaliana* after 24 h incubation with the biocontrol fungus *Trichoderma harzianum*. *Journal of plant physiology*. 2012, 169, 600-620.
DOI: 10.1016/j.jplph.2011.12.016.
- [12] Plett JM, Daguere Y, Wittulsky S, et al. Effector MiSSP7 of the mutualistic fungus *Laccaria bicolor* stabilizes the *Populus* JAZ6 protein and represses jasmonic acid (JA) responsive genes. *Proceedings of the National Academy of Sciences*, 2014, 111, 8299.
- [13] Behie SW, Bidochka MJ. Nutrient transfer in plant-fungal symbioses. *Trends in Plant Science*. 2014, 19, 734-740.
DOI: 10.1016/j.tplants.2014.06.007.
- [14] Sana K, Izzah S, Baig DN, et al. Contribution of zinc solubilizing bacteria in growth promotion and zinc content of wheat. *Frontiers in Microbiology*. 2017, 8, 2593.
DOI: 10.3389/fmicb.2017.02593.
- [15] De SA, Quintero JM, Avilés M, et al. Effect of *Trichoderma asperellum* strain T34 on iron nutrition in white lupin. *Soil Biology and Biochemistry*, 2009, 41, 2453-2459.
- [16] Contreras-Cornejo HA, Macías-Rodríguez Lourdes,

- Vergara AG, et al. *Trichoderma* modulates stomatal aperture and leaf transpiration through an abscisic acid-dependent mechanism in *Arabidopsis*. *Journal of Plant Growth Regulation*. 2015, 34, 425-432. DOI: 10.1007/s00344-014-9471-8.
- [17] Frank B. Über die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. *Berichte Der Deutschen Botanischen Gesellschaft*, 1885, 3, 128-145.
- [18] Smith SE, Read DJ. *Mycorrhizal symbiosis* (Third Edition). London: Academic press, 2008.
- [19] Rousseau JVD, Reid CPP, English RJ. Relationship between biomass of the mycorrhizal fungus *Pisolithus tinctorius* and phosphorus uptake in loblolly pine seedlings. *Soil Biology and Biochemistry*. 1992, 24, 0-184. DOI: 10.1016/0038-0717(92)90276-4.
- [20] Čatská V, Smith SE, Read DJ. *Mycorrhizal symbiosis*. *Biologia Plantarum*, 1997, 40. DOI: 10.1023/A:1000902213906.
- [21] Harrier LA, Watson CA. The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Management Science*. 2004, 60, 149-157. DOI: 10.1002/ps.820.
- [22] Yin DC, Deng X, Song RQ. Physiological responses of *Pinus sylvestris* var. *mongolica* seedlings to the interaction between *Suillus grevillei* N40 and *Trichoderma virens* T43. *Chinese Journal of Ecology*, 2014, 33, 2142-2147.
- [23] Alfred OMO, Patrick OA. Soil fertility and crop productivity in African sustainable agriculture. *Sustainable Agriculture Reviews*. Springer International Publishing. 2015. DOI: 10.1007/978-3-319-09132-7_6.
- [24] Yin DC, Deng X, Chet I, et al. Inhibiting effect and mechanism of *Trichoderma virens* T43 on four major species of forest pathogen. *Chinese Journal of Ecology*, 2014, 7, 1911-1919.
- [25] Yin DC, Song RQ, Qi JY, et al. Ectomycorrhizal fungus enhances drought tolerance of *Pinus sylvestris* var. *mongolica* seedlings and improves soil condition. *Journal of Forestry Research*. 2018, 29, 331-344. DOI: CNKI:SUN:LYYJ.0.2018-06-031.
- [26] Deng X, Song XS, Song RQ. Effect of inoculating *Phialocephala fortinii* D575 and *Suillus luteus* N94 on the growth of *Pinus sylvestris* var. *mongolica* and its resistant to damping-off. *Forest Pest and Disease*. 2017, 36, 21-25.
- [27] Collignon C, Uroz S, Turpault MP, et al. Seasons differently impact the structure of mineral weathering bacterial communities in beech and spruce stands. *Soil Biology & Biochemistry*. 2011, 43, 2012-2022. DOI: 10.1016/j.soilbio.2011.05.008.
- [28] Hussain S, Zhang M, Zhu XX, Khan, et al. Significance of Fe(II) and environmental factors on carbon-fixing bacterial community in two paddy soils. *Ecotoxicology and Environmental Safety*. 2019, 182: 109456. DOI: 10.1016/j.ecoenv.2019.109456.
- [29] Halifu S, Deng X, Song XS, et al. Effects of *Sphaeropsis* blight on rhizosphere soil bacterial community structure and soil physicochemical properties of *Pinus sylvestris* var. *mongolica* in Zhanggutai, China. *Forests*. 2019, 10:954. DOI: 10.3390/f10110954.
- [30] Yang CX, Liu WZ, He ZW, et al. Freezing/thawing pretreatment coupled with biological process of thermophilic *Geobacillus* sp. G1: Acceleration on waste activated sludge hydrolysis and acidification. *Bioresource Technology*. 2015, 175, 509-516. DOI: 10.1016/j.biortech.2014.10.154.
- [31] Buée M, Reich M, Murat C, et al. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist*. 2009, 184, 449-456. DOI: 10.1111/j.1469-8137.2009.03003.x.
- [32] Yong C, Jiang YM, Huang HY, et al. Long-term and high-concentration heavy-metal contamination strongly influences the microbiome and functional genes in Yellow River sediments. *Science of the Total Environment*. 2018, 637-638, 1400-1412. DOI: 10.1016/j.scitotenv.2018.05.109.
- [33] Zhou J, Deng Y, Shen L, et al. Temperature mediates continental-scale diversity of microbes in forest soils. *Nature Communications*. 2016, 7, 12083. DOI: 10.1038/ncomms12083.
- [34] Fu YJ, Zhang JL, Hou XQ. Comparative analysis of fungi diversity in rizospheric and non-rizospheric soil from *Cypripedium macranthum* estimated via high-throughput sequencing. *Acta Agriculturae Boreali-occidentalis Sinica*. 2019, 28, 1-7.
- [35] Anne S, Jacquioid S, Vestergaard G, et al. Analysis of soil microbial communities based on amplicon sequencing of marker genes. *Biology & Fertility of Soils*. 2017, 1-5. DOI: 10.1007/s00374-017-1205-1.
- [36] Magoč Tanja, Salzberg Steven L, Notes Author. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. 2011, 27(21):2957-2963. DOI: 10.1093/bioinformatics/btr507.
- [37] Bolger AM, Marc L, Bjoern U. Trimmomatic:

- a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014, 30, 2114-2120.
DOI: 10.1093/bioinformatics/btu170.
- [38] Kljalg U, Nilsson HR, Abarenkov K, et al. Towards a unified paradigm for sequence-based identification of Fungi. *Molecular Ecology*. 2013, 22, 5271-5277.
DOI: 10.1111/mec.12481.
- [39] Washington HG. Diversity, biotic and similarity indices: A review with special relevance to aquatic ecosystems. *Water Res*. 1984, 18:653-694.
DOI: 10.1016/0043-1354(84)90164-7.
- [40] Dixon P. VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science*. 2003, 14, 927-930.
DOI: 10.1111/j.1654-1103.2003.tb02228.x.
- [41] Xue L, Ren H, Brodribb TJ, et al. Long term effects of management practice intensification on soil microbial community structure and co-occurrence network in a non-timber plantation. *Forest Ecology and Management*. 2020, 459: 117805.
DOI: 10.1016/j.foreco.2019.117805.
- [42] Quan Y, Abdel A, Belkacem OB. Structural analysis for fault detection and isolation in fuel cell stack system[M]// Sustainability in Energy and Buildings. Springer Berlin Heidelberg. 2009.
DOI: 10.1007/978-3-642-03454-1_25.
- [43] Gentleman R, Huber W, Carey VJ. R Language[M]// International Encyclopedia of Statistical Science. Springer Berlin Heidelberg. 2011.
- [44] Ben AM, Lopez D, Triki MA. et al. Beneficial effect of *Trichoderma harzianum* strain Ths97 in biocontrolling *Fusarium solani* causal agent of root rot disease in olive trees. *Biological Control*. 2017, 110,70-78.
DOI: 10.1016/j.biocontrol.2017.04.008.
- [45] López AC, Alvarenga AE, Zapata PD, et al. *Trichoderma spp.* from Misiones, Argentina: effective fungi to promote plant growth of the regional crop *Ilex paraguariensis* St. Hil. *Mycology*. 2019, 1-12.
DOI: 10.1080/21501203.2019.1606860.
- [46] Sharma R, Magotra A, Manhas RS, et al. Antagonistic potential of a psychrotrophic fungus: *Trichoderma velutinum* ACR-P1. *Biological Control*. 2017, 115, 12-17.
DOI: 10.1016/j.biocontrol.2017.08.024.
- [47] Péret B, De Rybel B, Casimiro I, et al. *Arabidopsis* lateral root development: an emerging story. *Trends in Plant Science*. 2009, 14, 399-408.
DOI: 10.1016/j.tplants.2009.05.002.
- [48] Tsukaya H. Leaf morphogenesis: genetic regulations for length, width and size of leaves. *Tanpakushitsu kakusan koso Protein Nucleic Acid Enzyme*. 2002, 47(12 Suppl), 1576-1580.
- [49] Albrecht T, Argueso CT. Should I fight or should I grow now? The role of cytokinins in plant growth and immunity and in the growth-defence trade-off. *Annals of Botany*. 2016, 119, 725-735.
DOI: 10.1093/aob/mcw211.
- [50] Giron D, Frago E, Gaëlle G, et al. Cytokinins as key regulators in plant-microbe-insect interactions: connecting plant growth and defence. *Functional Ecology*, 2013, 27, 599-609.
- [51] Atkinson D, Black KE, Forbes PJ, et al. The influence of arbuscular mycorrhizal colonization and environment on root development in soil. *European Journal of Soil Science*. 2003, 54, 751-757.
DOI: 10.1046/j.1351-0754.2003.0565.x.
- [52] Lilleskov EA, Bruns TD, Dawson TE, et al. Water sources and controls on water - loss rates of epigeous ectomycorrhizal fungal sporocarps during summer drought. *New Phytologist*. 2009, 182, 483-494.
DOI: 10.1111/j.1469-8137.2009.02775.x.
- [53] Ekblad A, Wallander H, Godbold DL, et al. The production and turnover of extramatrical mycelium of ectomycorrhizal fungi in forest soils: role in carbon cycling. *Plant and Soil*. 2013, 366, 1-27.
DOI: 10.1007/s11104-013-1630-3.
- [54] Palaniyandi U, Muthuswamy A, Sailas B. Endophytic interactions of *Trichoderma harzianum* in a tropical perennial rhizo-ecosystem. *Research Journal of Biotechnology*, 2017, 12(3):22-30.
- [55] Ainhoa Martínez-Medina, Antonio R, Albacete A, et al. The interaction with arbuscular mycorrhizal fungi or *Trichoderma harzianum* alters the shoot hormonal profile in melon plants. *Phytochemistry*. 2011, 72, 223-229.
DOI: 10.1016/j.phytochem.2010.11.008.
- [56] Giuseppe C, Youssef R, Elena DM. Co - inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops. *Journal of the Science of Food and Agriculture*. 2015, 95, 1706-1715.
DOI: 10.1002/jsfa.6875.
- [57] Sutarman. Effect of ectomycorrhizal fungi and *Trichoderma harzianum* on the clove (*Syzygium aromaticum* L.) seedlings performances. *Journal of Physics Conference Series*. 2019, 1232, 012022.
DOI: 10.1088/1742-6596/1232/1/012022.
- [58] Badda N, Yadav K, Kadian N, et al. Impact of arbuscular mycorrhizal fungi with *Trichoderma viride* and *Pseudomonas fluorescens* on growth enhancement of genetically modified Bt cotton (*Bacillus thuringiensis*). *Journal of Natural Fibers*.

- 2013, 10, 309-325.
DOI: 10.1080/15440478.2013.791913.
- [59] Kennedy A, Smith K. Soil microbial diversity and the sustainability of agricultural soils. *Plant Soil*. 1995, 170:75-86.
DOI: 10.1007/BF02183056.
- [60] Zhang FG, Huo YQ, Xu XX, et al. *Trichoderma* improves the growth of *Leymus chinensis*. *Biology & Fertility of Soils*. 2018, 54, 685-696.
DOI: 10.1007/s00374-018-1292-7.
- [61] Gary EH, Charles RH, Viterbo A, et al. *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*. 2004, 2, 43-56.
DOI: 10.1038/nrmicro797.
- [62] Hossain MM, Sultana F, Islam S. Plant growth-promoting fungi (PGPF): Phytostimulation and induced systemic resistance. *Plant-Microbe Interactions in Agro-Ecological Perspectives*. 2017, 135-191.
DOI: 10.1007/978-981-10-6593-4_6.
- [63] Summerbell R. The inhibitory effect of *Trichoderma* species and other soil microfungi on formation of mycorrhiza by *Laccaria bicolor* in vitro. *New Phytologist*. 2010, 105, 437-448.
DOI: 10.1111/j.1469-8137.1987.tb00881.x.
- [64] Verena S, Song LF, Erika LQ, et al. Transcriptomic response of the mycoparasitic fungus *Trichoderma atroviride* to the presence of a fungal prey. *BMC Genomics*. 2009, 10, 567.
DOI: 10.1186/1471-2164-10-567.
- [65] Jong JCD, McCormack Barbara J, Smirnoff Nicholas, et al. Glycerol generates turgor in rice blast. *Nature*. 1997, 389, 244.
DOI: 10.1038/38418.
- [66] Szczepaniak Zuzanna, Cyplik Pawe, Juzwa Wojciech, et al. Antibacterial effect of the *Trichoderma viride* fungi on soil microbiome during PAH's biodegradation. *International Biodeterioration & Biodegradation*. 2015, 104, 170-177.
DOI: 10.1016/j.ibiod.2015.06.002.
- [67] Jangir M, Sharma S, Sharma S. Non-target effects of *Trichoderma* on plants and soil microbial communities. *Plant Microbe Interface*. 2019, 239-251.
DOI: 10.1007/978-3-030-19831-2_10.
- [68] Theresa AB, Greg JB. A review of the non-target effects of fungi used to biologically control plant diseases. *Agriculture Ecosystems & Environment*. 2003, 100:3-16.
DOI: 10.1016/s0167-8809(03)00200-7.
- [69] Palaniyandi Umadevi, Muthuswamy Anandaraj, Vivek Srivastav, et al. *Trichoderma harzianum* MTCC 5179 impacts the population and functional dynamics of microbial community in the rhizosphere of black pepper (*Piper nigrum* L.). *Brazilian Journal of Microbiology*. 2017, 49(3).
DOI: 10.1016/j.bjm.2017.05.011.
- [70] Kiers ET, Duhamel M, Beesetty Y, et al. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science*. 2011, 333(6044): 880-882.
DOI: 10.1126/science.1208473.
- [71] Oh SY, Park MS, Lim YW. The influence of microfungi on the mycelial growth of ectomycorrhizal fungus *Tricholoma matsutake*. *Microorganisms*. 2019, 7(6), 169.
DOI: 10.3390/microorganisms7060169.
- [72] Wyatt GAK, Kiers ET, Gardner A, et al. A biological market analysis of the plant-mycorrhizal symbiosis. *Evolution*. 2014, 68 (9), 2603-2618.
DOI: 10.1111/evo.12466.
- [73] Summerbell RC. From Lamareckian fertilizers to fungal castles: recapturing the pre-1985 literature on endophytic and saprotrophic fungi associated with ectomycorrhizal root systems. *Studies in Mycology*. 2005, 53:191-256.
DOI: 10.3114/sim.53.1.191.
- [74] Whipps JM. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. *Canadian Journal of Botany*. 2011, 82, 1198-1227.
DOI: 10.1139/b04-082.
- [75] Singh SP, Gaur R. Evaluation of antagonistic and plant growth promoting activities of chitinolytic endophytic actinomycetes associated with medicinal plants against *Sclerotium rolfsii* in chickpea. *Journal of Applied Microbiology*. 2016, 121: 506-518.
DOI: 10.1111/jam.13176.
- [76] Frank DA, Groffman PM. Plant rhizospheric N processes: what we don't know and why we should care. *Ecology*. 2009, 90, 1512-1519.
DOI: 10.2307/25592653.
- [77] Kamran S, Shahid I, Baig DN, et al. Contribution of zinc solubilizing bacteria in growth promotion and zinc content of wheat. *Frontiers in Microbiology*. 2017, 8, 2593.
DOI: 10.3389/fmicb.2017.02593.
- [78] Hodge A, Storer K. Arbuscular mycorrhiza and nitrogen: implications for individual plants through to ecosystems. *Plant and Soil*. 2015, 386: 1-19.
DOI: 10.1007/s11104-014-2162-1.
- [79] Pritsch K, Garbaye J. Enzyme secretion by ECM fungi and exploitation of mineral nutrients from soil organic matter. *Annals of Forest Science*. 2011, 68, 25-32.

- DOI: 10.1007/s13595-010-0004-8.
- [80] Louche J, Ali MA, Cloutier-Hurteau B, et al. Efficiency of acid phosphatases secreted from the ectomycorrhizal fungus *Hebeloma cylindrosporum* to hydrolyse organic phosphorus in podzols. *FEMS microbiology ecology*. 2010, 73, 323-335. DOI: 10.1111/j.1574-6941.2010.00899.x.
- [81] Wurzburger N, Hendrick RL. *Rhododendron* thickets alter N cycling and soil extracellular enzyme activities in southern Appalachian hardwood forests. *Pedobiologia*. 2007, 50, 563-576. DOI: 10.1016/j.pedobi.2006.10.001.
- [82] Lucas RW, Casper BB. Ectomycorrhizal community and extracellular enzyme activity following simulated atmospheric N deposition. *Soil Biology and Biochemistry*. 2008, 40, 1662-1669. DOI: 10.1016/j.soilbio.2008.01.025.
- [83] De Santiago A, Quintero JM, Avilés M, et al. Effect of *Trichoderma asperellum* strain T34 on iron nutrition in white lupin. *Soil Biology and Biochemistry*. 2009, 41: 2453-2459. DOI: 10.1007/s11104-010-0670-1.
- [84] Altomare C, Norvell WA, Björkman T, et al. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied Environmental Microbiology*. 1999, 65, 2926-2933. DOI: 10.1007/s11837-003-0030-1.
- [85] Ahangar MA, Dar GH, Bhat ZA. Growth response and nutrient uptake of blue pine (*Pinus wallichiana*) seedlings inoculated with rhizosphere microorganisms under temperate nursery conditions. *Annals of Forest Research*. 2012, 55, 217-227. DOI: 10.4996/fireecology.0803104.
- [86] Colla G, Roupheal Y, Bonini P, et al. Coating seeds with endophytic fungi enhances growth, nutrient uptake, yield and grain quality of winter wheat. *International Journal of Plant Production*. 2015, 9, 171-189.