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Effects of Combined Application of Biological Agent and Fertilizer on Fungal Community Structure in Rhizosphere Soil of *Panax notoginseng*

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Abstract: The fungal community structure and soil fertility in rhizosphere soil have an important effect on the health of *Panax notoginseng* (*P. notoginseng*). The attack of pathogenic fungi and the imbalance of soil fertility can easily lead to diseases. The effect of *Bacillus subtilis* on improving the community structure of soil fungi has been confirmed, and the corresponding biological agent products have been commercialized. A pot experiment carried out in a greenhouse explored the effect of a biological agent and fertilizer on the fungal community in the rhizosphere of *P. notoginseng*. In the experiment, fertilization and the addition of biological agents were set up with three gradients, respectively, and the full coupling experiment was adopted, and the blank control group (CK) was set up at the same time. Therefore, there were thirteen treatments in the experiment. NH4 decreased between 36.42% and 11.56%, AP increased between 6.03% and 92.46%, AK increased between 2.99% and 25.40%, TN increased between 0.10% and 9.41%, and TP increased by 18.25% to 47.73% The addition of *Bacillus subtilis* biological agent decreased the Chao1, Shannon, Simpson, and ACE index of fungi in the rhizosphere soil of *P. notoginseng*. The Chao1 index decreased between 0.39% and 78.22%; the ACE index decreased between 0.43% and 78.24%. The main pathogenic fungi *Cylindrocarpon* and *Fusarium* of *P. notoginseng* were different in the experimental results. *Cylindrocarpon* decreased under F1C1, F2C1, and F3C2 treatments, while *Fusarium* increased under F1C1, F2C2, F3C1, and F3C3 treatments and decreased *Fusarium* content in rhizosphere soil of *P. notoginseng* in other treatments. RDA analysis (Redundancy analysis) showed that NH4-N was negatively correlated with the main pathogen *Cylindrocarpon*, *Fusarium*, and *Ilyonectria*, while AP and AK were positively correlated with *Cylindrocarpon*, *Fusarium*, and *Ilyonectria*. The results of the GRA-TOPSIS analysis showed that the score of F3C2 was the highest, while F2C3 and F2C1 ranked second and third, respectively. The calculation results of the theoretical model based on GRA-TOPSIS analysis showed that the GRA-TOPSIS score was highest when the theoretical optimal fertilizer application rate and bacteria application rate were 116.31 kg hm^{-2} and 15.83 kg hm^{-2}, respectively.

Keywords: *P. notoginseng*; biological agent; *Bacillus subtilis*; rhizosphere soil fungal community

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1. Introduction

P. notoginseng is a perennial herb preferring moisture and shade. The main root has high medicinal value. *P. notoginseng* is mainly produced in southwest China (Yunnan and Guangxi). Previous studies have shown that *P. notoginseng* is mostly used to stop bleeding and treat cardiovascular diseases [1,2]. At the same time, *P. notoginseng* also plays a role

in anti-thrombosis, lowering blood pressure and relieving pain and neuroprotection [3,4]. With the increase of cardiovascular diseases in the world and the modern application of traditional Chinese medicine, the demand for *P. notoginseng* is increasing [5,6]. At present, the research on *P. notoginseng* focuses on water and fertilizer management, pesticide residues, and pest control [7–9]. Water and fertilizer management in the field of *P. notoginseng* is very important for the sustainable cultivation of the crop. Water and fertilizer mismanagement will lead to soil consolidation, nutrient loss, agricultural pollution, and low efficiency of water and fertilizer use. Pesticide spraying is not only the main means to prevent insect pests but also an important aspect to ensure yield. Excessive pesticide spraying will, however, lead to pesticide residues. In the actual planting process, yield was seriously restricted by diseases. Such as root rot, black spot, and round spot [10]. Previous studies have shown that root rot is the main cause of yield reduction, with annual losses of up to 30% [11]. Continuous cropping obstacles not only reduce the quality and yield but also affect the health of planting soil and limit the planting area [12]. However, the main reason for the production issues is the imbalance of fungal structure in soil and the increase of pathogenic bacteria [13]. The main pathogens causing root rot are *Cylindrocarpon*, *Fusarium*, and *Ilyonectria* [14], while Alternaria causes black leaf disease [15].

Rhizosphere soil is the main active area of crop roots [16], so the structure and fertility of rhizosphere soil microorganisms play a very important role in crop growth [17,18]. The accumulation of *P. notoginseng* saponins is greatly affected by soil water content and fertilizer application [19]. Previous studies have shown that the structure of the soil microorganism community changes significantly with the increase in fertilizer application [20]. Fertilization not only changes the content of nutrients in the soil but also changes the structure of the microbial community in the soil, which in turn affects the transport of soil nutrients and inhibits or increases plant morbidity. The relationship between microorganisms and crops in rhizosphere soil is generally divided into positive interaction, negative interaction and non-interaction [21]. Positive microorganisms are not only involved in the growth and development of crops, but also one of the key factors to ensure plant health and soil fertility [22,23]. For example, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi enhance plant nutrition, improve plant growth and protect host plants from pathogen infection [24–26]. Although plants benefit from some rhizosphere microorganisms, some microbes also spread diseases [27]. Plant diseases caused by soil fungi can greatly reduce crop yield and even lead to crop death in serious cases [28]. In summary, in the process of planting *P. notoginseng*, the characteristics of the rhizosphere soil fertility and fungal community jointly affected the health and final yield of *P. notoginseng*.

Many microbes have the ability to promote plant growth [29–31]. These microbes promote plant growth by producing compounds that stimulate plant growth or inducing plants to produce antibiotics [32,33]. *Bacillus subtilis* is a kind of thermophilic and aerobic Gram-positive rod-shaped bacteria. Its endospores are heat-resistant, drought-resistant, and UV-resistant [34]. Previous studies have shown that a variety of secondary metabolites produced by *Bacillus subtilis* have the potential to mediate the production of antibiotics. It is estimated that at least 4% to 5% of the genome of any given group of *Bacillus subtilis* is used to produce antimicrobial compounds (AMCs) [35]. These molecules are mainly antimicrobial peptides (AMPs) and contain special parts, such as D-amino acids (AA) or intramolecular thioether bonds, whose overall structure is usually cyclic and has some hydrophobicity [36]. *Bacillus subtilis* can enhance plant resistance by secreting extracellular polysaccharides to iron carriers to inhibit the movement of toxic ions, maintain the movement of water molecules in plant tissues, and inhibit pathogenic microorganisms in soil [37]. Previous experiments have shown that adding *Bacillus subtilis* to farmland can not only reduce nitrogen and ammonia emissions [38] but also improve soil colony structure [39]. *Bacillus subtilis* performs well in the control of tomato blight [40]. Therefore, we hope to explore whether Bacillus subtilis can inhibit pathogenic microorganisms in the production process of *P. notoginseng*. Due to the potential of *Bacillus subtilis* to secrete

antimicrobial compounds, it is possible to improve the rhizosphere soil microenvironment of *P. notoginseng*. On this basis, we speculated that adding *Bacillus subtilis* could improve the fungal community in the rhizosphere soil of *P. notoginseng* and inhibit a series of pathogenic fungi in the rhizosphere soil.

In this study, we applied biological agents together with fertilizer for the first time to reduce the pathogenic bacteria in *Panax notoginseng* soil through the improvement effect of biological agents on the soil fungal community. Therefore, this study introduced *Bacillus subtilis* biological agent as an exogenous additive bacteria, hoping to explore the possibility of *Bacillus subtilis* in improving the rhizosphere soil microenvironment of *P. notoginseng* by way of co-application with fertilizer. The aims of this study are to explore: (1) the effect of fertilization on the nutrient characteristics in the rhizosphere; (2) the structural characteristics of the microenvironment in the rhizosphere under different bacterial and fertilizer combinations; (3) whether the addition of *Bacillus subtilis* can inhibit the pathogenic bacteria in the rhizosphere; and (4) the potential of adding *Bacillus subtilis* to improve the rhizosphere.

2. Materials and Methods

2.1. Experimental Site and Experimental Setup

Yunnan has more suitable climatic conditions for the growth of *P. notoginseng*. In March 2021, we transplanted *P. notoginseng* from the Intelligent Agriculture Demonstration Greenhouse at Kunming University of Science and Technology (24°50′49.95″ N, 102°5′37.54″ E, 1778.9 m above sea level). Eight hundred grams of soil was loaded into a PVC basin with upper and lower diameters of 18 cm and 13 cm and a height of 15 cm. Fifteen days later, the transplant that did not survive was removed. *P. notoginseng* with similar growth was then selected to be taken further in the experiment. The potting soil was collected from the forest soil of Kunming University of Technology. The soil pH was 7.61, and the soil ammonium nitrogen (NH4-N), total nitrogen (TN), available phosphorus (AP), total phosphorus (TP), and available potassium (AK) were 34.67 mg·kg^{-1}, 38.22 mg·kg^{-1}, 80.27 mg·kg^{-1}, 433.75 mg·kg^{-1}, and 35.24 mg·kg^{-1}, respectively. According to the results of previous studies [41,42], there were three gradients fertilizer application and the *Bacillus subtilis* biological agent in which the fertilizer application rate was 80 kg·hm^{-2} with the low fertilizer application, 110 kg·hm^{-2} with medium and 140 kg·hm^{-2} with high fertilizer application, 10 kg·hm^{-2} with low bacterial application, 15 kg·hm^{-2} with medium bacterial application, and 20 kg·hm^{-2} with high bacterial application. The current experiment used the full combination and set up a blank control group, a total of 10 treatment groups, each treatment group repeated three times. The treatments were as follows (Table 1): low fertilizer and low bacteria (F1C1), low fertilizer and medium bacteria (F1C2), low fertilizer and high bacteria (F1C3), medium fertilizer and low bacteria (F2C1), medium fertilizer bacteria (F2C2), medium fertilizer and high bacteria (F2C3), high fertilizer and low bacteria (F3C1), high fertilizer and medium bacteria (F3C2), high fertilizer and high bacteria (F3C3), and the blank control group (CK). Each treatment of processing was repeated three times. Organic carbon water-soluble fertilizer (21%N − 21%P_2O_5 − 21%K_2O + 6% humic acid + trace elements). The contents of chelated iron(Fe), chelated zinc(Zn), chelated copper(Cu), chelated manganese(Mn), boron(B), and molybdenum(Mo) were 0.05%, 0.05%, 0.017%, 0.05%, 0.1%, and 0.007%, produced by Sichuan Shifang Demi Industrial Co., Ltd., Shifang City, China, and *Bacillus subtilis* (enhanced microbial agent, implementation Standard: GB 20287-2006). A bacterial fertilizer produced by Shandong Suyuan Ecological Agriculture Development Co., Ltd., Dezhou City, China was used in the experiment.

2.2. Determination of Soil Physical and Chemical Properties

After the completion of the harvest, three replicates of the soil from each treatment were collected and brought back to the laboratory for related data determination. The indexes using fresh soil were determined as soon as the soil arrived in the laboratory. The indexes that could be determined from air-dried soil were placed in a ventilated and

sheltered place to be tested later. All samples were tested at the same time to ensure that the results were not disturbed by temperature, humidity, or chemical batch variation. pH was determined by PHS-3C using a pH acidity meter, NH4-N by extraction colorimetry, TN by Kjeldahl nitrogen meter, AP and TP by molybdenum antimony sulfate colorimetric method, The content of AK was determined by using an ammonium acetate extraction atomic absorption spectrophotometer.

Table 1. The amount of fertilizer and biological agents.

Treatments	Fertilizer (kg·hm^{-2})	Biological Agents (kg·hm^{-2})
CK	0	0
F1C1	80	10
F1C2	80	10
F1C3	80	10
F2C1	110	15
F2C2	110	15
F2C3	110	15
F3C1	140	20
F3C2	140	20
F3C3	140	20

2.3. DNA Extraction and Amplification

The rhizosphere soil was quickly frozen and preserved in liquid nitrogen at −80 °C. Fungus DNA was isolated using a DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and integrity were measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. Fungal ITS diversity identification corresponding region: front primer: ITS1 FCTTGGTCATTTAGAGGAAGTAA'; back-end primer: ITS2 GCTGCGTTCTTCATCGATGC. The PCR products were detected by electrophoresis, purified by magnetic beads, purified as a second round of PCR template, and then amplified by two rounds of PCR and detected again by electrophoresis. After detection, magnetic beads were used to purify the PCR products. After purification, the PCR products were quantified by Qubit. The same amounts of samples were mixed according to the concentration of PCR products and sequenced on the computer. The Amplicon quality was visualized using gel electrophoresis. The PCR products were purified with Agencourt AMPure XP beads (Beckman Coulter Co., Brea, CA, USA) and quantified using Qubit dsDNA assay kit. The concentrations were then adjusted for sequencing. Sequencing was performed on an IlluminaNovaSeq6000 with two paired-end read cycles of 250 bases each. (Illumina Inc., San Diego, CA, USA; OEBiotech Company; Shanghai, China)

2.4. Bioinformatic Analysis

Raw sequencing data were done in FASTQ format. Paired-end reads were then preprocessed using Cutadapt (Version 2.6) software to detect and cut off the adapter. After trimming, paired-end reads were filtered low-quality sequences, denoised, merged, and detected and cut off the chimera reads using DADA2 with the default parameters of QIIME2 [43] (November 2020). The final product was the representative reads and the ASV abundance table.

The representative sequences of each ASV are selected by using the QIIME 2 software package compared with the Unite database (https://unite.ut.ee/repository.php (accessed on 17 March 2023)). Species comparison annotations were analyzed using default parameters of q2-feature-classifier software (Version 2022.2.0-1).

The soil's physical and chemical properties were processed by Microsoft Excel 2016 and SPSS 22 to use in the statistical analysis via the Duncan method [44]. The vegan package in R (Version 3.5.1) was used to draw heat maps and the composition of fungal communities for principal coordinate analysis (PCoA). The effects of physical and chemical

properties of the rhizosphere soil on fungal microorganisms in rhizosphere soil were analyzed by redundancy analysis (RDA) in Canoco 5.0. Indicator analysis was completed by R (Version 3.5.1); random forest analysis was drawn by R package randomForest (Version 4.7-1.1); GRA-TOPSIS analysis was completed by Python 3.9; theoretical model was established and solved by MATLAB (r2020a); theoretical model function diagram was drawn by Origin 2018.

3. Results

3.1. Physical and Chemical Properties of Rhizosphere Soil of P. notoginseng

The nutrient characteristics of the rhizosphere soil of *P. notoginseng* are shown in Table 2. Generally, the soil pH was between 7.32 and 7.55 and slightly alkaline. The results indicated that the pH value of the soil of the CK treatment decreased after adding biological agents and fertilization. Duncan's analysis showed a significant decrease in the pH of the F3C2 treatment ($p < 0.05$). The content of NH4-N in soil was between 38.44 mg·kg^{-1} and 24.44 mg·kg^{-1} for the CK treatment was 38.44 mg·kg^{-1}. The results showed that NH4-N decreased significantly under all treatments compared to the CK treatment. The NH4-N content in the F2 C3 treatment was 24.44 mg·kg^{-1} and showed the biggest decrease. The total content of AP was between 65.66 and 126.37 mg·kg^{-1}. The content of AP increased in all treatments compared with the control, with the highest increase in treatment F3C2 (126.37 mg·kg^{-1}). The AP content increased significantly in treatments F2C2, F3C1, F3C2, and F3C3 ($p < 0.05$). The AK content ranged between 24.76 mg kg^{-1} and 31.05 mg·kg^{-1}. Compared with the control, the content of AK was increased in some of the treatments, although there were no significant differences. Treatment F3C2 had the highest improvement effect (31.05 mg·kg^{-1}). The soil TN content ranged between 368.90 mg·kg^{-1} and 403.68 mg·kg^{-1}. The same trend as was the case with AK was evident in TN content. The TP content ranged between 335.15 and 495.13 mg·kg^{-1}. All treatments, except F1C2, showed significant improvements ($p < 0.05$), with the highest in treatment F3C1 (495.13 mg·kg^{-1}).

Table 2. Physical and chemical properties of rhizosphere soil of *P. notoginseng*. (a, b, c, d indicate significant differences ($p < 0.05$) among treatments based on Duncan's mean test).

Treatments	PH	NH4-N(mg·kg^{-1})	AP (mg·kg^{-1})	AK (mg·kg^{-1})	TN (mg·kg^{-1})	TP (mg·kg^{-1})	Yield (g)
CK	7.55 ± 0.06 a	38.44 ± 0.94 a	65.66 ± 4.25 d	24.76 ± 1.25 a	368.90 ± 10.27 a	335.15 ± 7.56 c	10.91 ± 0.72 a
F1C1	7.38 ± 0.03 a	34.19 ± 0.50 b	71.34 ± 4.36 cd	26.10 ± 2.28 a	369.27 ± 6.97 a	448.72 ± 32.69 ab	11.09 ± 0.48 a
F1C2	7.41 ± 0.02 a	34.19 ± 1.20 b	73.64 ± 8.82 cd	26.4 ± 2.4 a	373.94 ± 7.32 a	396.32 ± 11.07 bc	11.49 ± 0.29 a
F1C3	7.51 ± 0.01 a	25.22 ± 0.52 c	69.62 ± 10.23 cd	25.50 ± 1.96 a	369.83 ± 8.75 a	449.36 ± 20.03 ab	11.90 ± 0.51 a
F2C1	7.33 ± 0.01 a	25.41 ± 0.45 c	97.98 ± 8.04 abcd	30.48 ± 2.49 a	377.58 ± 11.52 a	445.51 ± 14.69 ab	12.55 ± 1.04 a
F2C2	7.45 ± 0.02 a	25.55 ± 0.29 c	99.42 ± 6.16 abc	30.44 ± 2.09 a	389.94 ± 11.25 a	430.32 ± 14.77 ab	11.99 ± 1.07 a
F2C3	7.36 ± 0.06 a	24.44 ± 1.69 c	91.50 ± 12.82 bcd	30.42 ± 1.18 a	379.77 ± 14.9 a	455.99 ± 20.19 ab	11.66 ± 1.51 a
F3C1	7.40 ± 0.02 a	26.04 ± 0.12 c	120.6 ± 8.99 ab	30.60 ± 0.76 a	399.46 ± 12.07 a	495.13 ± 27.46 a	11.71 ± 0.42 a
F3C2	7.32 ± 0.03 b	27.08 ± 0.46 c	126.37 ± 7.54 a	31.05 ± 1.39 a	403.62 ± 1.96 a	410.43 ± 3.34 b	11.44 ± 0.74 a
F3C3	7.36 ± 0.04 a	25.83 ± 0.48 b	125.46 ± 7.11 a	30.65 ± 3.22 a	400.68 ± 2.48 a	453.64 ± 22.35 ab	13.05 ± 20 a

NH4-N: soil ammonium nitrogen, TN: total nitrogen, AP: available phosphorus, TP: total phosphorus, AK: available potassium. CK: blank control group, F1C1: low fertilizer and low bacteria, F1C2: low fertilizer and medium bacteria, F1C3: low fertilizer and high bacteria, F2C1: medium fertilizer and low bacteria, F2C2: medium fertilizer bacteria, F2C3: medium fertilizer and high bacteria, F3C1: high fertilizer and low bacteria, F3C2: high fertilizer and medium bacteria, F3C3: high fertilizer and high bacteria.

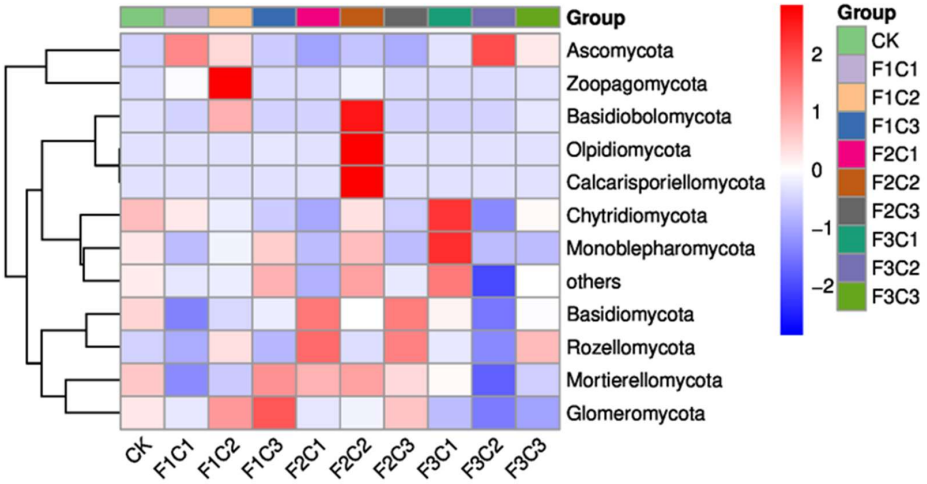
After harvest, the roots of *P. notoginseng* were separated from the stems and leaves, cleaned, and the fresh weight was recorded as the yield. The results showed that the yield increased in all treatments compared with the control (CK), but with no statistical differences.

3.2. Diversity and Structural Characteristics of Fungi in Rhizosphere Soil of P. notoginseng

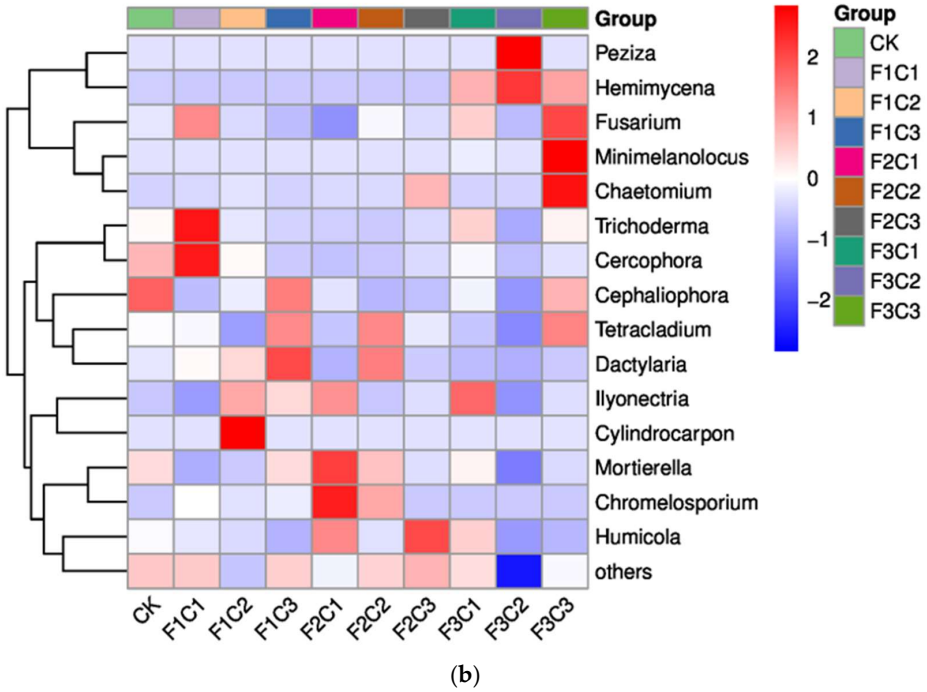
3.2.1. Diversity and Abundance

The average number of fungal ASVs under different treatments ranged from 75 to 342. The average ASV numbers were 342, 224, 310, 306, 232, 341, 233, 320, 75, and 340 for CK, F1C1, F1C2, F1C3, F2C1, F2C2, F2C3, F3C1, F3C2, and F3C3, respectively. The average

number of ASV in the CK treatment was the highest, while that in the F3C2 group was the lowest. From the results of species annotation, the Heatmap map (Figure 1a) of the community structure of each group in the top 15 at the phylum level was selected. The results showed that *Ascomycota*, *Mortierellomycota*, and *Basidiomycota* were the dominant fungal groups in the rhizosphere soil of *P. notoginseng*. Compared with the control, the relative abundance (Table 3) of *Mortierellomycota* increased by 5.23% under F1C3 treatment; the relative abundance of *Mortierellomycota* decreased by 20.43% under F3C2 treatment. The relative abundance of *Basidiomycota* under F1C3 treatment increased by 7.96% compared with CK treatment; the relative abundance of *Basidiomycota* decreased by 15.07% under F3C2 treatment compared with CK treatment.



(**a**)



(**b**)

Figure 1. Heatmap diagram of the community structure of each group at the phylum level (Plotting a heatmap requires species relative abundance information. At the phylum level, we selected the top 15 relative abundance species for Heatmap drawing in groups. The Abscissa is sample information, the right ordinate is species annotation information, and the left ordinate is clustering information). (**a**) Heatmap diagram of the community structure of each group at the phylum level; (**b**) heatmap diagram of the community structure of each group at the genus level.

Table 3. The relative abundance of different treatments at the phylum level. Different values in the table represent the percentage of relative abundance, %.

Treatments	Ascomycota	Mortierellomycota	Basidiomycota
CK	50.54	21.48	18.17
F1C1	83.41	5.15	4.22
F1C2	67.17	11.08	11.29
F1C3	49.06	26.71	13.09
F2C1	40.61	23.36	26.13
F2C2	47.53	25.12	14.49
F2C3	42.86	19.77	25.63
F3C1	53.85	16.59	15.75
F3C2	95.32	1.05	3.10
F3C3	63.47	11.53	14.39

Generating the community structure Heatmap (Figure 1b) diagram of the top 15 species abundance at the genus level. The results showed that the relative abundances of *Mortierella*, *Ilyonectria*, *Fusarium*, *Trichoderma*, *Cephaliophora*, and *Cercophora* in the CK group were 8.85%, 1.89%, 2.23%, 1.75%, 3.28%, and 1.77%, respectively, and the relative abundances were all greater than 1% (Table 4).

Table 4. The relative abundance of different treatments at the genus level. Different values in the table represent the percentage of relative abundance, %.

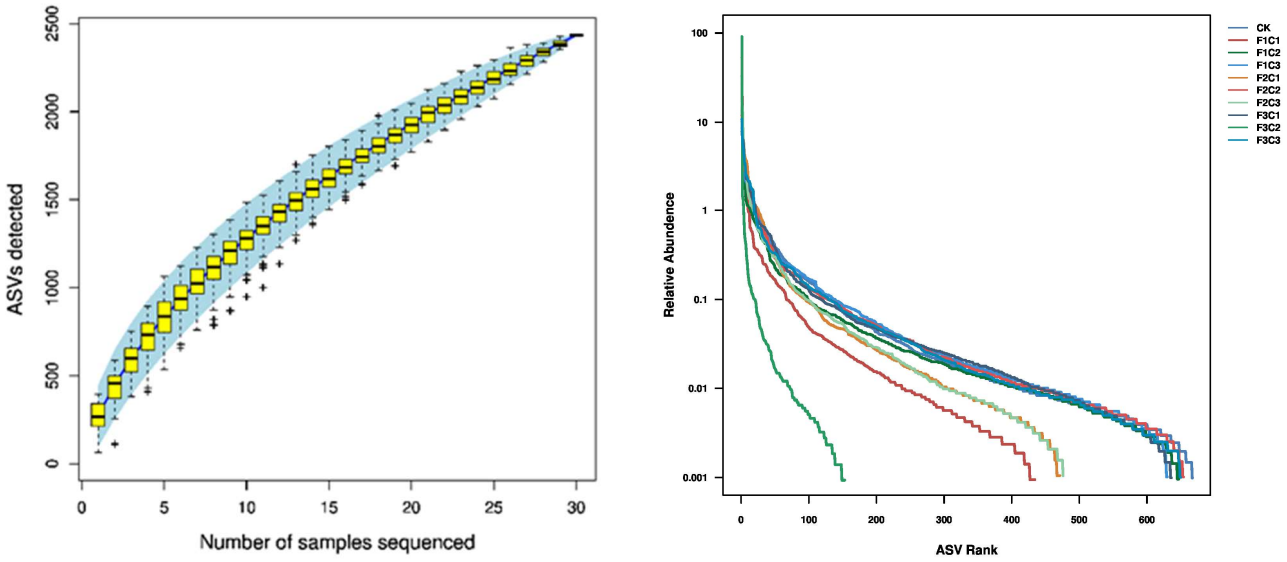
Treatments	Peziza	Mortierella	Ilyonectria	Cylindrocarpon	Fusarium	Trichoderma	Cephaliophora	Minimelanolocus
CK	0.02	8.85	1.89	0.12	2.34	1.75	3.28	0.02
F1C1	0	2.77	0.64	0.07	5.09	5.52	0.59	0.02
F1C2	0.01	4.25	6.04	33.02	2.09	1.25	1.16	0.05
F1C3	0	8.80	4.61	0.30	1.56	0.93	2.95	0.02
F2C1	0.01	17.03	6.63	0.08	0.69	0.86	1.05	0.05
F2C2	0	10.17	1.89	0.19	2.71	0.81	0.50	0.05
F2C3	0.01	5.28	2.55	0.15	2.14	1.06	0.61	0.04
F3C1	0	7.59	7.85	0.26	3.73	2.41	1.23	0.44
F3C2	91.16	0.19	0.44	0.01	1.52	0.26	0.10	0.01
F3C3	0.06	5.05	2.56	0.42	6.38	1.84	2.29	11.72

Analysis of the Alpha and Beta Diversity of Fungi Community

The species accumulation curve (Figure 2a) describes the increase of species with the rise of sampling volume. It is widely used to judge sampling volume adequacy and species richness estimation in biodiversity and community surveys. The results show that as the sample size increases, the curve tends to be stable, which shows that the species in this environment will not increase significantly with the increase in the sample size, and the sampling is sufficient.

The results of the Alpha analysis (Table 5) showed that the ASV, Chao1, and ACE indexes decreased with the addition of fertilization and biological agents. Compared with the control, the ASV decreased by 0.29–78.07%; the Chao1 index decreased between 0.39% and 78.22%; the ACE index decreased between 0.43% and 78.24%. The decreases for ASV, Chao1 and ACE were significant for treatmen F1C1, F2C1, F2C3, and F3C2 ($p < 0.05$). This showed that the number of fungi in the rhizosphere soil of *P. notoginseng* decreased after fertilization and adding biological agents. The Shannon index, which can simultaneously reflect the species richness and evenness of distribution, only increased by 0.33% under the F1C3 treatment while decreasing between 0.33% and 87.15% in the other treatments. The decreases were significant in the F1C1, F1C2, F2C3, and F3C2 treatments. This result indicated that the richness and uniformity of fungal colonies in the rhizosphere soil of *P. notoginseng* decreased after adding fertilizers and biological agents. The Simpson index mainly reflects the diversity of the community. The Simpson index increased slightly by 1.05% in the F2C2 treatment, while the rest of the treatments stayed the same or decreased.

It was only significant in the F1C1 and F3C2 treatments ($p < 0.05$). The Alpha index analysis showed that the number, richness, and uniformity of fungi in the rhizosphere decreased compared with the untreated soil.



(**a**) (**b**)

Figure 2. (**a**) Species accumulation curve. The horizontal axis represents the sample size, and the vertical axis represents the number of ASV detected. (**b**) Rank Abundance curve. Abscissa, which is sorted by ASV according to the number of sequences it contains.

Table 5. Alpha diversity of fungi in rhizosphere soil of *P. notoginseng*. The data in the table are average ± standard error, and those with the same letter after the same column of data indicate that there is no significant difference between processing ($p > 0.05$, Duncan's method).

Treatments	ASV	Chao1	Shannon	Simpson	ACE
CK	342 ± 15.00 a	342.31 ± 14.62 a	5.99 ± 0.13 a	0.95 ± 0.011 a	342.63 ± 14.58 a
F1C1	224 ± 7.00 c	224.14 ± 7.41 c	3.49 ± 0.21 c	0.69 ± 0.052 b	224.07 ± 7.93 c
F1C2	311 ± 36.00 ab	310.65 ± 36.21 ab	4.96 ± 0.44 b	0.86 ± 0.045 a	310.40 ± 36.05 ab
F1C3	307 ± 46.00 ab	306.68 ± 46.25 ab	6.01 ± 0.04 a	0.95 ± 0.007 a	306.83 ± 46.29
F2C1	232 ± 9.00 bc	232.01 ± 9.01 bc	5.43 ± 0.10 ab	0.95 ± 0.004 a	231.82 ± 9.18 bc
F2C2	341 ± 6.00 a	340.98 ± 6.59 a	5.97 ± 0.09 a	0.96 ± 0.005 a	341.16 ± 6.68 a
F2C3	233 ± 7.00 bc	233.33 ± 7.22 bc	4.77 ± 0.36 b	0.87 ± 0.044 a	233.26 ± 7.50 bc
F3C1	320 ± 9.00 a	320.09 ± 9.20 a	5.91 ± 0.20 a	0.95 ± 0.009 a	319.95 ± 9.42 a
F3C2	75 ± 3.00 d	74.54 ± 3.90 d	0.77 ± 0.25 d	0.16 ± 0.065 c	74.56 ± 4.49 d
F3C3	340 ± 15.00 a	340.34 ± 15.50 a	5.87 ± 0.08 a	0.95 ± 0.012 a	340.33 ± 15.48 a

ASV is the Amplicon Sequence Variant (amplified subsequence variation). Each de-duplicated feature sequence is produced by quality control operations such as denoising and splicing by the DADA2 method. The abundance table of these sequences in the sample is called feature table (feature-table); Chao index: The actual number of species in the community was estimated by calculating the ASV of only one or two reads in the community; Shannon index: the higher the species diversity is, the more uniform the species distribution is, and the larger the Shannon index value is (which can reflect both the species richness and the evenness of distribution); Simpson index: the probability that two randomly selected individuals belong to different species. The higher the Simpson index, the higher the community diversity; Ace index: an index used to estimate the number of ASV in a community.

Rank abundance (Figure 2b) is used to simultaneously account for two aspects of sample diversity: the richness and uniformity of the species contained in the sample. The richness of species is reflected by the length of the curve on the horizontal axis; the more significant the span of the horizontal axis of the curve, the richer the composition of species; the uniformity of species composition is reflected by the shape of the curve, and the smaller the span of the vertical axis of the curve is, the higher the uniformity of species composition

is. The results showed that the species richness of each group decreased compared with CK, and the species richness of the F3C2 group was the lowest.

Beta diversity analysis is mainly used to evaluate the differences in species composition between different samples. The more similar the sample composition, the closer the distance reflected in the PCA diagram (Figure 3a). The results of the PCA analysis showed that the explanatory degree of PC1 was 6.34%, the explanatory degree of PC2 was 5.79%, and the total explanatory degree was 12.13%. There was a good aggregation among the samples, and the sample points of different groups were close, which indicated that the fungal community in the rhizosphere soil of *P. notoginseng* did not change significantly after fertilization and biological agent addition.

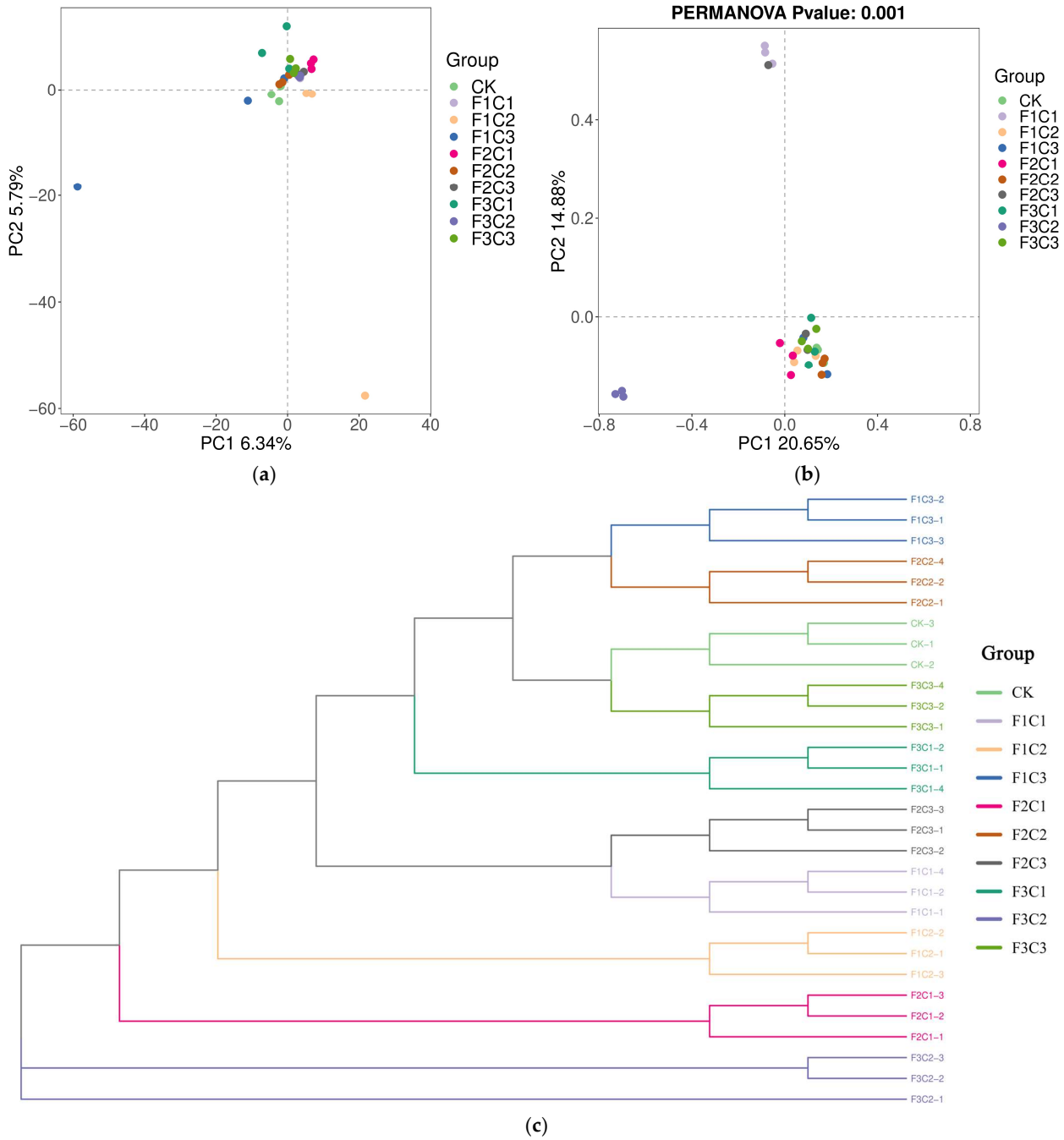


Figure 3. (**a**) PCA analysis; (**b**) PCoA analysis based on the Bray–Curtis distance calculation method (**c**) Clustering tree based on binary-Jaccard distance.

PCoA analysis (Figure 3b) was performed based on the Bray-Curtis distance calculation method. The results showed that the explanation rates of PC1 and PC2 for the differences between groups were 20.65% and 14.88%, respectively, and the repeated distance parameters among the samples were close. The distance between the F1C1 and F3C2 treatments was farther than that of the CK treatment, which indicated that the change of the rhizosphere soil fungal community was more significant than that of other treatments.

The clustering results based on the binary-Jaccard distance algorithm (Figure 3c) show that the groups can be better clustered together, which shows that the grouping effect was excellent. The branching distance reflects the degree of similarity between samples, and the sample most similar to CK is F3C3 processing.

3.3. Relationship between Rhizosphere Soil Microorganisms and Environmental Physicochemical Parameters

The relative abundance of dominant phyla of soil fungi in the rhizosphere of *P. notoginseng* and the RDA analysis (Figure 4a) of soil physical and chemical properties showed that RDA1 and RDA2 explained 27.2% and 2% of the changes in the composition of soil fungi. The relationship between the environmental indicators and RDA1 and RDA2 showed that pH, AK, and RDA1 were negatively correlated; NH4-N, TN, AP, TP, and RDA1 were positively correlated; pH, NH4-N, and RDA2 were positively correlated; TN, AP, TP, AK, and RDA2 were negatively correlated. *Ascomycota* was positively correlated with RDA1; *Rozellomycota*, *Basidiomycota*, *Mortierellomycota*, and RDA1 were negatively correlated; *Mortierellomycota*, *Ascomycota*, and RDA2 were positively correlated; *Rozellomycota*, *Basidiomycota*, and RDA2 were negatively correlated.

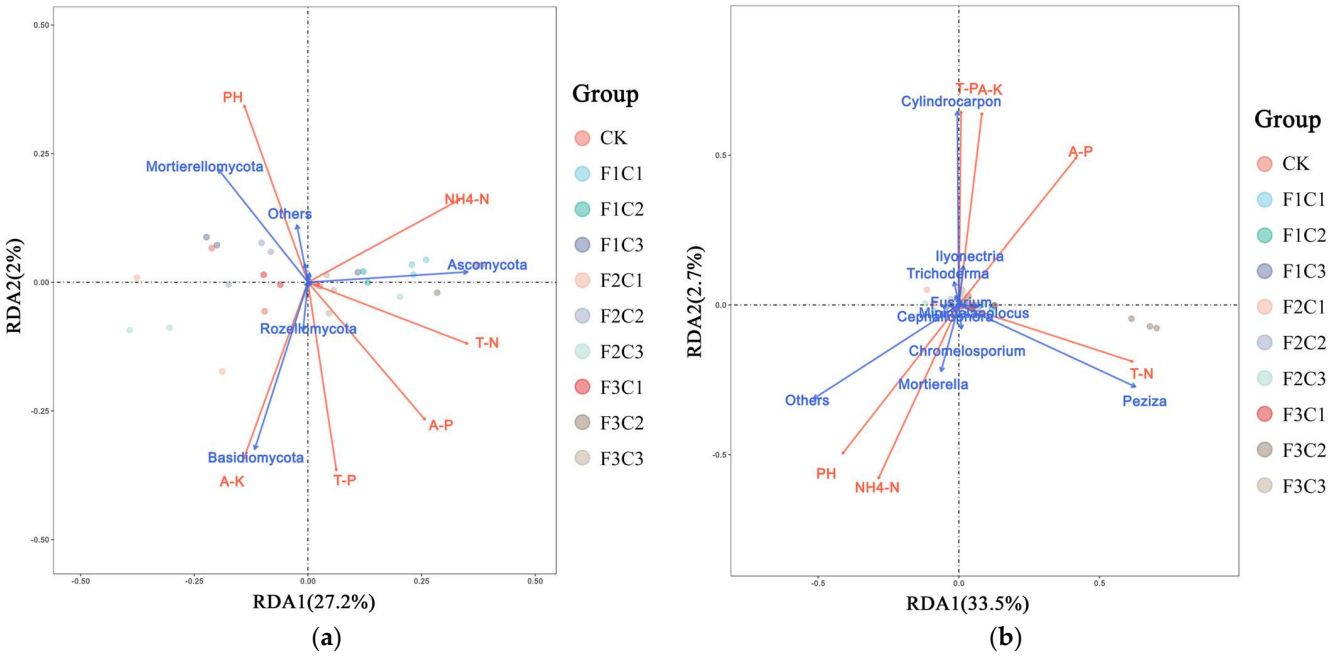


Figure 4. Relative abundance of dominant phylum (**a**), relative abundance of dominant genus (**b**), and redundancy analysis of soil physical and chemical properties in the rhizosphere of *P. notoginseng*. NH4-N: Nitrate nitrogen; T-N: Total nitrogen; A-P: Available phosphorus; T-P: Total phosphorus A-K: Available potassium; PH: Soil pH value.

At the genus level, the relative abundance of rhizosphere fungi Top10 species and soil physical and chemical properties of redundancy analysis (Figure 4b) showed that RDA1 and RDA2 explained 33.5% and 2.7% of the changes in soil fungal colony composition, respectively. AP, AK, TP, TN, and RDA1 were positively correlated; NH4-N, pH, and RDA1 were negatively correlated; pH, NH4-N, and RDA2 were positively correlated; TN, AP, TP, AK, and RDA2 were negatively correlated. *Peziza*, *Ilyonectria*, *Chromelosporium*,

Minimelanolocus, and RDA1 were positively correlated; *Mortierella*, *Cylindrocarpon*, *Fusarium*, *Trichoderma*, and *Cephaliophora* were negatively correlated with RDA1; *Ilyonectria*, *Cylindrocarpon*, *Fusarium*, and *Trichoderma* were positively correlated with RDA2; *Peziza*, *Mortierella*, *Chromelosporium*, *Cephaliophora*, and *Minimelanolocus* were negatively correlated with RDA2.

3.4. Indicator Analysis

Using the R software package(indicspecies_1.7.14), by calculating the indicator value of ASV in each group and then conducting a statistical analysis of the indicator value between groups, the index species of each group were revealed (Figure 5). The index species mainly refer to the biological species, genera, or communities that can have a great impact on the growth environment in a certain area. At the gate level, the indicative species of each group were mainly *Ascomycota*, *Basidiomycota*, and *Rozellomycota*, in which the relative abundance of Ascomycota was the highest; at the genus level, it was mainly *Peziza*, *Cylindrocarpon*, and *Chromelosporium*, in which the relative abundance of *Peziza* was the highest. There were significant differences in the distribution of indicative species among treatments. The main indicative species (Indicator.values > 0.75) were summarized in a table (Table 6).

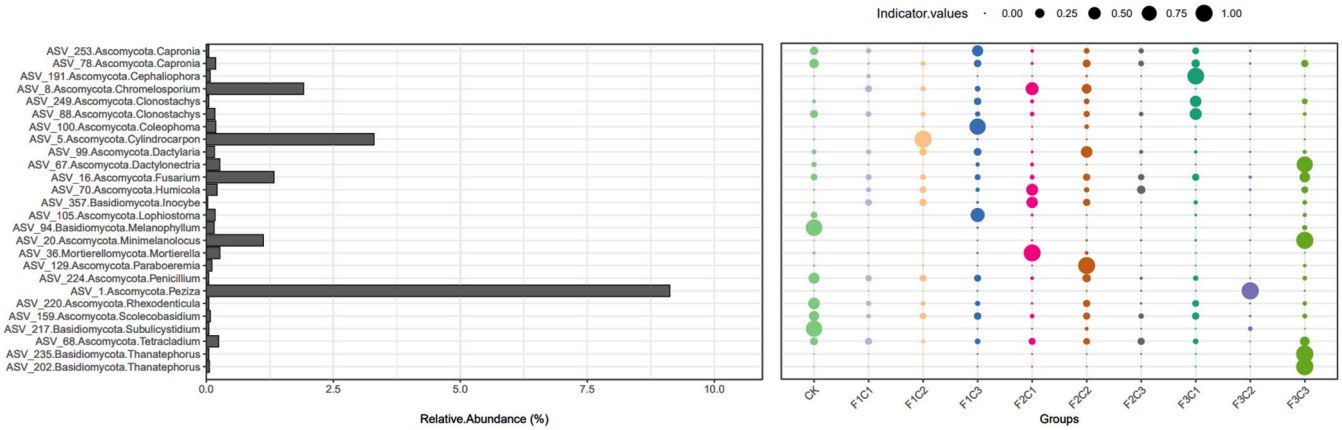


Figure 5. Indicator indicative species map. The first is the species gate level annotation information; the second is the species–genus level annotation information; the third column is the corresponding ASV; the bar chart is the relative abundance of each ASV, the Abscissa of the bubble chart is the sample grouping, and the bubble size represents the indicator value of each species in the sample group, that is, the indicative size of the species in the group.

Table 6. The main indicative species, The "\" in the indicative species column, indicates that there is no indicative species indicator.values > 0.75.

Treatments	Indicative Species	Indicator.Values
CK	ASV_94.Basidiomycota.Melanophyllum	0.93
	ASV_217.Basidiomycota.Subulicystidium	0.90
F1C1	\	\
F1C2	ASV_5.Ascomycota.Cylindrocarpon	0.99
F1C3	ASV_100.Ascomycota.Coleophoma	0.88
F2C1	ASV_36.Mortierellomycota.Mortierella	0.96
F2C2	ASV_129.Ascomycota.Paraboeremia	0.96
F2C3	\	\
F3C1	ASV_191.Ascomycota.Cephaliophora	0.95
F3C2	ASV_1.Ascomycota.Peziza	1.00
F3C3	ASV_235.Basidiomycota.Thanatephorus	1.00
	ASV_202.Basidiomycota.Thanatephorus	1.00
	ASV_20.Ascomycota.Minimelanolocus	0.99
	ASV_67.Ascomycota.Dactylonectria	0.87

3.5. Random Forest Analysis

In order to classify microbial community samples effectively and accurately and to find out the key species that can differ among groups, the genera with the top 30 relative abundance are selected, and R-packet randomForest was used to draw the species importance point map (Figure 6).

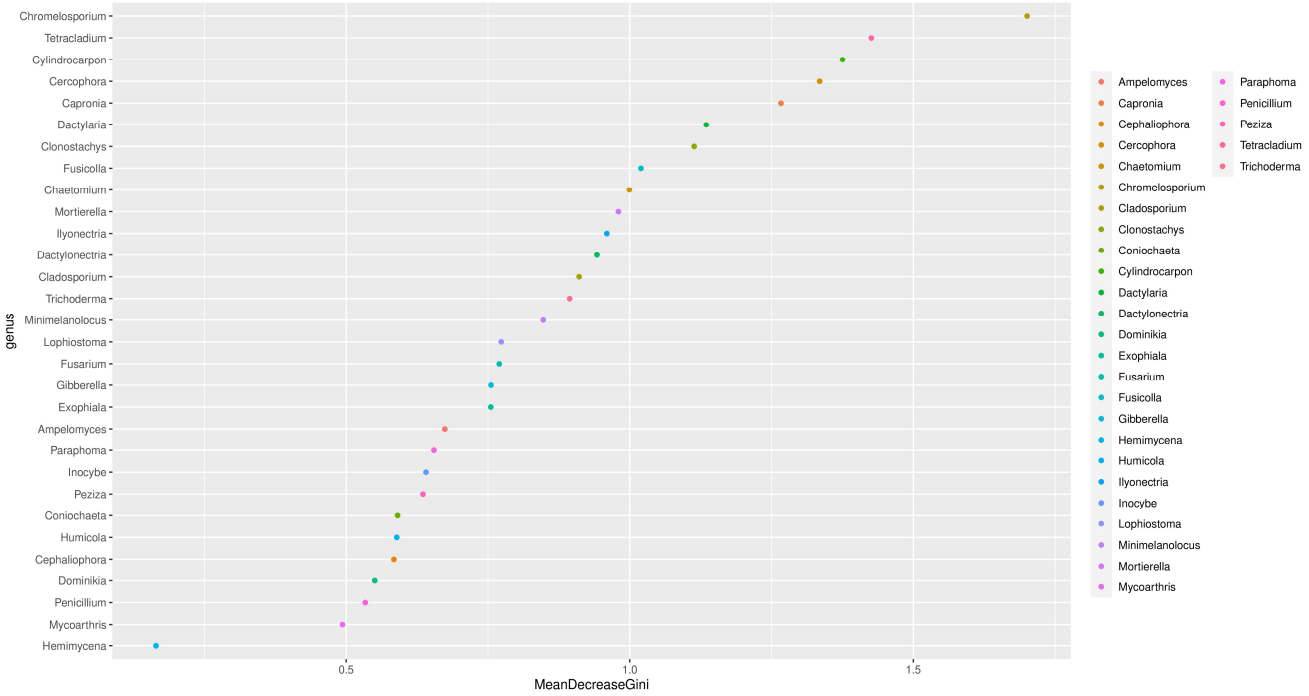


Figure 6. The Abscissa is the measure of importance, and the ordinate is the species name sorted by importance. Standardized importance values are used by default.

3.6. GRA-TOPSIS Analysis

To explore the best combination of bacterial fertilizer, we integrated ten indexes of pH, NH4-N, AP, AK, TN, TP, *Cylindrocarpon*, *Ilyonectria*, *Fusarium*, and yield, and sorted each treatment by GRA-TOPSIS analysis (Table 7). The sort results were displayed in the table.

Table 7. GRA-TOPSIS analysis results.

Treatments	Score	Sort
CK	0.51	6
F1C1	0.48	7
F1C1	0.45	8
F1C2	0.53	5
F2C3	0.57	3
F2C2	0.57	4
F2C3	0.60	2
F3C1	0.42	10
F3C2	0.63	1
F3C3	0.45	9

3.7. Establish the Best Model in Theory

To further determine the response characteristics of biological agents and fertilizer to the rhizosphere and the yield of *P. notoginseng*, we established a theoretical model using MATLAB according to the results of the GRA-TOPSIS analysis. The regression model is as follows:

$$Z = -1.9032 + 0.035192X + 0.059526Y - 0.00014534X^2 - 0.0015591Y^2 - 8.7333 \times 10^{-5}XY$$

In the formula, X represents the fertilizer application amount, kg·hm^{-2}; Y represents ap plication amount of biological agent, kg·hm^{-2}; and Z represents GRA-TOPSIS Score.

The calculation results of the theoretical model were as follows: the maximum value of the GRA-TOPSIS score was 0.61469, and the coordinate of the maximum point of the model was X = 116.31 and Y = 15.83. Generally, the best coupling scheme of biological agent and fertilizer, in theory, was as follows: the amount of fertilizer was 116.31 kg·hm^{-2}; when the number of bacteria was 15.83 kg·hm^{-2}, the score of GRA-TOPSIS was the highest, reaching the comprehensive best.

According to the theoretical model, the corresponding function diagram (Figure 7) was drawn, and the best application interval of biological agents and fertilizer was calculated. The part of the graph with the maximum GRA-TOPSIS score of 0.5% as the optimal interval (GRA-TOPSIS score $\in$ (0.61~0.61)). It was calculated that the optimal interval of fertilization was 111.74~120.86 kg·hm^{-2}, and the optimal interval of adding biological agents was 14.43~17.23 kg·hm^{-2}.

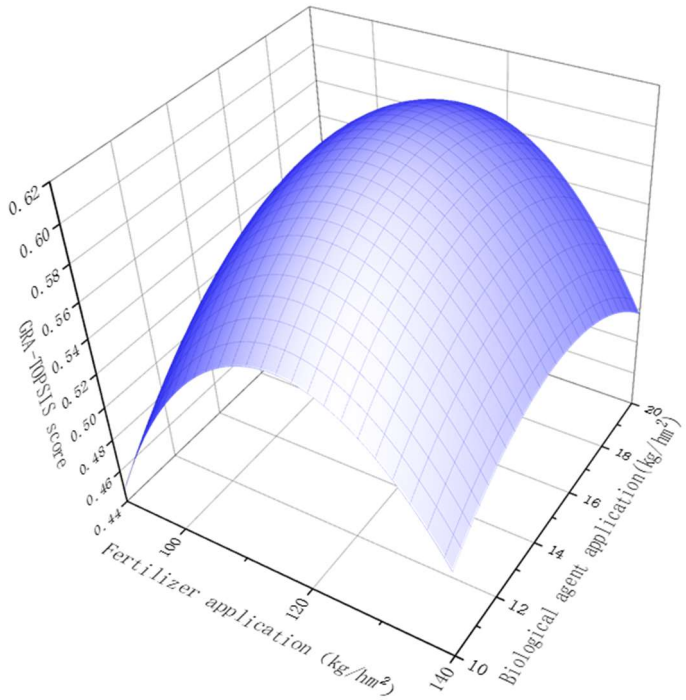


Figure 7. A theoretical model based on GRA-TOPSIS scores. The X-axis is the amount of fertilizer, the kg·hm^{-2}; Y-axis is the amount of biological agents, and the kg·hm^{-2}; Z-axis is the GRA-TOPSIS score.

4. Discussion

There is an interactive relationship between soil microorganisms, plants, and the soil environment. In the cultivation of oil peony, as an example, not only the soil's physical and chemical properties of the plants with black root rot were changed, but also the fungal network structure in the soil of diseased plants was more complex than that of healthy plants [45]. Long-term tillage or long-term fertilization will change the soil's physical and chemical properties and soil microbial structure [46,47]. After long-term continuous cropping, the fungal colony of peanuts changed, the pathogenic fungi increased, and the beneficial fungi decreased. At the same time, the bacterial abundance decreased with the increase of continuous cropping years, and the soil microbial structure was destroyed, which led to a decrease in peanut yield [48,49]. There was a similar trend in the planting process of buckwheat and apple [50–53]. Long-term fertilization will also change the soil microbial structure [54]. Long-term application of nitrogen fertilizer will reduce the diversity of soil bacteria [55]. The experiment was carried out in alkaline soil (pH > 7.5), and the soil pH decreased after planting *P. notoginseng*. The experimental results showed

that the pH value decreased significantly only in the F3C2 treatment and CK treatments ($p < 0.05$), and similar results also appeared in previous studies [56,57], mainly due to the selective absorption of ions by crops after fertilization. At the same time, NH4-N was acidic and easily neutralized with alkaline soil [58]. The experimental results showed that with a decrease in soil pH, the soil NH4-N decreased. The soil microbial community will degrade the residual plants deposited in the soil to provide energy for itself, but when the plant residual nitrogen cannot satisfy the microbial community, the microbial community will also absorb nitrogen in soil and fertilizer [59]. This is called nitrogen immobilization. AP and AK were significantly increased with the increase in fertilizer application, which was due to the accumulation of AP and AK in the soil because they were not fully absorbed and utilized by *P. notoginseng* after fertilization. TN and TP increased with the increase in fertilizer application rate, but the increase was not significant ($p > 0.05$). This may be due to the fact that exogenous N and P were not all absorbed and utilized by the roots of *P. notoginseng* after fertilization, and the remaining N and P were fixed and transformed into a steady state by soil. The experimental results are in good agreement with the previous experimental results [60].

Many microorganisms have the ability to promote plant growth and have beneficial effects on root systems and overall plant growth by colonizing the plant rhizosphere [29]. These bacteria have been designated as plant growth-promoting rhizosphere bacteria (PGPR). PGPR promotes plant growth mainly by improving plant abiotic stress tolerance; nutrient immobilization for plant absorption; plant growth regulators; production of iron carriers, and production of volatile organic compounds [61]. It has been confirmed that *Bacillus subtilis* is an excellent biocontrol bacterium in previous reports [62–65]. When applied, it will reduce the diversity of the fungal community in plant rhizosphere soil [66,67]. The diversity index of the fungal community in the rhizosphere soil of *P. notoginseng* decreased after the addition of biological agents in this experiment, which may be due to the antagonism between *Bacillus subtilis* and fungi in the soil after planting inhibiting the increase of fungi in the soil. *Ascomycota*, *Mortierellomycota*, and *Basidiomycota* were the dominant fungal species in the rhizosphere soil of *P. notoginseng*, which was similar to the results of the dominant species during the sitting period of wild ginseng [68]. Furthermore, the results of the genus-level analysis showed that *Cylindrocarpon* and *Fusarium*, which are considered to be the main pathogens causing *P. notoginseng* [14], showed different trends. *Cylindrocarpon* decreased in the F1C1, F2C1, and F3C2 treatments but increased slightly in other treatments. *Fusarium* was increased in the treatment of F1C2, F1C3, F2C1, F2C3, and F3C2. Some studies have shown that many species of *Ilyonectria* can cause plant root rot [69]. The same conclusion was drawn from the comparative experiment on the composition of rhizosphere fungi in healthy and diseased *P. notoginseng* soil [23]. However, the experimental results showed that the co-application of biological agents and fertilizers could not inhibit *Ilyonectria*. Therefore, the effects of biological agents on specific pathogenic fungi in complex soil environments need to be studied in more detail. Based on the diversity of soil fungal community and its inhibitory effect on some pathogenic fungi, the effect of *Bacillus subtilis* on the rhizosphere soil microenvironment of *P. notoginseng* was comprehensively evaluated. *Bacillus subtilis* has application potential.

The increase of soil nitrogen and phosphorus will increase the relative abundance of soil fungal pathogens [70]. Our experimental results showed that the nutrients in the rhizosphere soil of *P. notoginseng* showed a significant upward trend with the increase in fertilizer application rate. RDA analysis showed that NH4-N was negatively correlated with *Rozellomycota*, *Basidiomycota*, and *Mortierellomycota*, while AP was negatively correlated with *Mortierellomycota*. RDA analysis at the genus level showed that NH4-N was negatively correlated with the main pathogenic bacteria *Cylindrocarpon*, *Fusarium*, and *Ilyonectria* of *P. notoginseng*; AP was positively correlated with *Cylindrocarpon*, *Fusarium*, and *Ilyonectria*, the main pathogenic bacteria of *P. notoginseng*. AK in the rhizosphere soil of *P. notoginseng* also increased with the increase in fertilizer application. The results of RDA analysis were the same as AP, and the main pathogenic fungi were proportional to AK. Therefore,

we speculated that excessive fertilization increased the increase of AP and AK in the rhizosphere soil of *P. notoginseng*, which was an indirect cause of *P. notoginseng* disease, which was confirmed in previous reports [71]. Soil pH has significant effects on microbial growth, community structure, and biomass [72–74]. High-pH soil was beneficial to bacterial growth, which led to the inhibition of fungal growth. RDA analysis at the gate level showed that soil pH was negatively correlated with *Ascomycota*, *Rozellomycota*, and *Basidiomycota* but positively correlated with *Mortierellomycota*. RDA analysis at the genus level showed that pH was positively correlated with *Cephaliophora*, *Mortierella*, and *Chromelosporium* and negatively correlated with others, including *Cylindrocarpon*, *Fusarium*, and *Ilyonectria*, the main pathogens of *P. notoginseng* root rot. This shows that the pathogenic fungi in the slightly alkaline soil will be inhibited to a certain extent with the increase in soil pH.

There is competition between pathogenic bacteria and probiotics in soil, which is one of the reasons for the change of colony structure in soil [75]. Previous studies have confirmed that this competition is mainly the competition for space and resources [76]. Strains that can reproduce rapidly tend to occupy more space and resources. When artificially increasing the number of probiotics in the soil can enhance the reproduction speed of probiotics, make them occupy space and resources faster, compress the living space and resources of pathogens, and finally achieve the role of inhibiting pathogens [77]. After long-term planting of a single crop, plant growth will be inhibited, which is also known as soil fatigue [78]. Previous studies have shown that the inhibitory effect of soil on plant growth is weakened after heating and disinfection at 100 °C, which indicates that soil fatigue is related to biological factors in biological soil, especially microbial communities [79]. The space and resources in the soil are often gradually occupied by pathogens with the increase of planting time, and the planting life of *P. notoginseng* is often longer, so *P. notoginseng* diseases caused by the imbalance of soil colony structure are particularly frequent [79]. The microbial structure in the soil is affected by many factors, such as plant root exudates, plant residue decay, external interference, and other factors. Our experimental results showed that the indicative species were different under different treatments, which indicated that the application of biological agents and fertilizers had complex effects on the colony structure in the soil. Therefore, in order to explore the changes of soil colony structure under specific exogenous interference, more detailed and in-depth research should be carried out.

5. Conclusions

The results showed that fertilization increased the nutrient content in the rhizosphere soil of *P. notoginseng*. Excessive fertilization was one of the causes of *P. notoginseng* disease. Co-application of bacterial fertilizer shows that *Bacillus subtilis* biological agent has potential application value in improving the rhizosphere soil fungal community of *P. notoginseng*.

According to the results of the GRA-TOPSIS theoretical model analysis, we established the theoretical model and obtained the theoretical optimal coupling scheme of bacterial fertilizer as follows: the amount of fertilizer application was 116.31 kg·hm^{-2}, and the amount of bacteria application was 15.83 kg·hm^{-2}. Taking part with the maximum score of 0.5% of GRA-TOPSIS as the optimal interval, it was calculated that the optimal range of fertilization was 111.74–120.86 kg·hm^{-2}, and the optimal interval of adding biological agents was 14.43–17.23 kg·hm^{-2}. Bacillus subtilis needs further study on other potential pathogens. Although our experiments have verified that the combined application of *Bacillus subtilis* biological agent and fertilizer has an inhibitory effect on pathogenic fungi in the soil, in the field where the actual situation is complex, the change of colony structure in soil needs further systematic and perfect demonstration.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data are contained within the article.

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