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Effects of Sphaeropsis Blight on Rhizosphere Soil Bacterial Community Structure and Soil Physicochemical Properties of *Pinus sylvestris* var. *mongolica* in Zhanggutai, China

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Received: 16 September 2019; Accepted: 23 October 2019; Published: 26 October 2019

Abstract: *Pinus sylvestris* var. *mongolica* is an important tree species for ecological construction and environmental restoration owing to its rapid growth rate and excellent stress resistance. *Pinus sylvestris* var. *mongolica* sphaeropsis blight is a widespread disease caused by *Sphaeropsis sapinea*. This study was focused on non-infected (CK) and infected (SS) *Pinus sylvestris* var. *mongolica* plants in Zhanggutai area, Liaoning Province, China. Illumina high-throughput sequencing based on the templates of sequencing-by-synthesis working with reversible terminators is a widely used approach. In the present study, systematic differences in relationships among rhizosphere soil physicochemical properties, bacterial community structure, diverse bacterial genera, and alpha diversity indices between the two categories were evaluated. The current findings are as follows: (1) Shannon's index of SS soil was significantly higher than CK, and it was significantly lower in May than July and September ($p < 0.05$). (2) Non-metric multidimensional scaling (NMDS) showed a difference in bacterial community structure during May (spring), July (summer), and September. (3) At the phylum level, no significant difference was found in the bacterial genera between CK and SS soil for three seasons; however, at the genus level, there were about 19 different bacterial genera. The correlation studies between 19 different bacterial genera and environmental factors and α -diversity indicated that bacterial genera of non-infected and infected *Pinus sylvestris* var. *mongolica* were distributed differently. The bacterial genera with CK were positively correlated with soil physicochemical properties, while a negative correlation was found for SS. In conclusion, the differences in nutrient and microbial community structure in the rhizosphere soil of *Pinus sylvestris* var. *mongolica* are the main causes of shoot blight disease.

Keywords: *Pinus sylvestris* var. *mongolica*; rhizosphere soil; bacterial diversity; shoot blight pathology; MiSeq high-throughput sequencing

1. Introduction

Soil bacteria, being one of the most abundant bacteria [1], play a vital role in several biochemical reactions, such as soil nutrient transformation, nutrient metabolism, organic matter decomposition, and humus formation [2–4]. These reactions maintain the soil nutrient balance in order to improve plant nutrient availability and also work as a source and sink of effective nutrients in soil [5,6]. The interaction between soil bacteria and plant root systems leads to the production of chemical compounds, such as auxins, which can stimulate plant root growth and improve root–soil contact.

Therefore, plants can grow profusely and become healthy and stronger [7]. The currently available studies on soil microorganisms have mainly been focused on species, along with structural, functional, and genetic diversity [8]. High-throughput culture, membrane, and micro-encapsulation technology are widely used methods for culturing microorganisms; however, these approaches have their own limitations, as only 0.1–1% of the total microflora can be cultured by such methods. Therefore, other superior and advanced methods were developed, such as DNA fingerprinting, phospholipid fatty acid analysis, and the gene-chip method, for the identification of soil microorganisms. In some earlier reports, although bacterial cultures were obtained successfully, no valid information could be drawn from the soil bacteria [9–13]. Nonetheless, in comparison with high-throughput sequencing, these methods have their own drawbacks, such as low throughput and inadequate information [14]. Based on Solexa, the Illumina MiSeq platform uses a sequence-by-synthesis (SBS) approach and modified dNTPs, which contain a reversible terminator to block further chemical reactions. These methods are high throughput as well as cost effective [15–17].

Pinus sylvestris var. *mongolica* is a geographical variety of *Pinus sylvestris* and is widely planted due to its height and evergreen nature. In addition, owing to its strong ability to withstand poor conditions such as barren soil and a dry, cool climate, it is a crucial ecological species in sandy areas. Due to the similarity with the original habitat, it was introduced into the Zhanggutai area, which features proper physical and chemical soil properties as well as hydrothermal conditions, in 1955 [18–20]. During the seedling stage, *P. sylvestris* var. *mongolica* adapted well and experienced rapid growth; however, its biotic and abiotic resistance potential could not improve. On the other hand, soil fertility declined because of high stand density and the slow process of litter decomposition, which further led to lower stand density and disease breakout. *P. sylvestris* var. *mongolica* shoot blight is a widespread host-mediated disease caused by *Sphaeropsis sapinea*, which belongs to the subphylum *Deuteromycotina*, class *Coelomycetes*, order *Sphaeropsidales*, and family *Sphaeropsidaceae* [21,22]. During winter, the pathogenic bacteria remain [23,24] in the sporulation state, and when the temperature is appropriate, spores become ready for spreading and infection. In northeastern China, there are two peak periods of incidence, i.e., from March to May and from July to August. It can happen for both seedlings (3–5 years) as well as mature trees (older than 20 years). Since the first incidence of an infected tree during sand fixation in 1991 [25], there has been a decline in several *P. sylvestris* var. *mongolica* stands, covering a 1000 hm² sand fixation area and 24,700 hm² within Liaoning Province. Previous studies have greatly advanced our understanding of *P. sylvestris* var. *mongolica*, showing that precipitation is the key factor for its biomass and health [26,27]. In the Zhanggutai area, it rains mainly in June to August, with a maximum evaporation rate in June, which is getting higher. Zeng et al. [28] and Chen et al. [19] showed that soil physicochemical properties varied significantly with seasons, and soil nutrient concentration was highly associated with soil moisture content. Rhizosphere soil pH was lower; however, soil organic matter content and nitrogen concentration from hydrolysis were higher than non-rhizosphere soil. Total and effective nitrogen and the effective potassium of the rhizosphere soil were not limited, but the effective phosphorus was limited, and all indices, including microbial biomass and enzymatic activity, decreased as the infection severity increased [29–31]. However, the studies conducted so far have not yet addressed the soil microbial community structure in Zhanggutai area; therefore, this is the first study of its kind.

Based on Illumina high-throughput sequencing, the difference between rhizospheric soil bacteria community structures and seasonal variations were explored in non-infected (CK) and infected (SS) *P. sylvestris* var. *mongolica*. The correlations among soil chemical and physical properties, bacteria community, and alpha diversity were also examined. This study fosters an understanding of *P. sylvestris* var. *mongolica* shoot blight and suggests measures for disease prevention. We hypothesize that the main reason for the shoot blight disease of *Pinus sylvestris* var. *mongolica* in this area is the difference in nutrient content and microbial community structure in rhizosphere soil. The current investigation involves (1) a comprehensive and comparative study of bacterial community structure in the rhizosphere and its variation across seasons between non-infected and infected *P. sylvestris* var. *mongolica* plants,

and (2) an assessment the difference between the physicochemical properties of two kinds of soil and the investigation of its relationship with bacterial community and alpha diversity.

2. Materials and Methods

2.1. Site Description

The study site is located in Zhanggutai town (N 42°43′–42°51′, E 121°53′–122°22′), southern Korqin sandy land (Figure 1), Liaoning Province, Northeast China. It is an important part of China's 3-North Shelter Forest Program. This region has a semi-arid climate with a relative humidity of about 60.4% and an annual average precipitation and evaporation range from 400 to 550 mm and from 1200 to 1450 mm, respectively. In this area, the annual average temperature is 5.7 °C, and the yearly frost-free time adds up to 154 days. The wind is strong during spring, with a maximum speed of 32 m/s. There are more than 240 windy days with at least 5 m/s speed. The soil type is mainly aeolian sandy soil [32,33], and the height of the sand is from 126 to 128 cm. Drought-tolerant plants are the most prevalent. The other dominant or co-dominant species are *Acer mono* Maxim., *Crataegus pinnatifida* Bunge, *Ulmus pumila* L., *U. macrocarpa* Hance, *Armeniaca sibirica* (L.) Lam, *Lespedeza bicolor* Turcz., *Artemisia halodendron* Pall., *Fraxinus rhynchophylla* Hance, *Cleistogenes chinensis* (Maxim.) Keng., *Salix gordejvii* Y. L. Chang et Skv., *Pennisetum flaccidum* Griseb., *Agropyron cristatum* (L.) Gaertn., *Lespedeza davurica* (Laxm.) Schindl., *Setaria viridis* (L.) Beauv., *Trigonella korshinskyi* Grossh., and *Corispermum* L. The dominant species in woodland are *Populus simonii*, *Pinus sylvestris* var. *Mongolica*, and *P. tabulaeformis*. Arable land is mainly dry land, and the main crops are *Zea may* L., *Arachis hypogaea* Linn., *Glycine max* (L.) Merr., *Oryza sativa* L., and *Sorghum vulgare*, L.

Figure 1. Location of the R&D base of the Sand Fixation and Afforestation Institute in Liaoning Province, China.

2.2. Soil Sampling

Tree shoots and needles are an indicator of the pathogenic condition of a tree. Therefore, about 50 shoots and needles were randomly collected in May 2018 (the first peak of pathogen infection) to observe the presence of pathogenic condition and to determine infected branches and uninfected trees. The sites were divided into non-infected (A) and infected (B) *P. sylvestris* var. *mongolica*. The forest's age is 30 years, and the average diameter at breast height is 14.94 to 17.00 cm. The soil samples were

collected in May, July, and September. At each site, 30 trees were sampled every 30–50 m, and each site was divided into 3 plots, with 10 trees per array. In order to collect the rhizospheric soil, litter and humus layers were first removed, soil was dug up to 15 cm deep, and plants were loosened and uprooted from the ground. Finally, the rhizosphere soil was collected carefully and gently with a sterile brush. The rhizosphere soil samples without roots, leaves, and stones were pooled and filtered with a sterile, 1 mm mesh cell strainer. The soil samples were stored in an incubator when in field and further stored at -80°C until DNA extraction [34–36]. Some samples were air-dried to measure soil physicochemical properties. The numbers 5, 7, and 9 stand for May, July, and September, respectively. Two treatments of CK and SS in May, July, and September, with three replication each ($2 \times 3 \times 3 = 18$ samples), were conducted.

2.3. Analysis of Physicochemical Properties of Soil

A calibrated HACH HQ30d pH meter was used to measure the pH of soil samples (BANTE, Shanghai, China). Soil pH was determined at a soil-to- H_2O ratio of 1:2.5 (w/v), the potassium dichromate heating method was used for organic matter (OM), the Kjeldahl method (Kjeldahl distillation unit K9840, Shandong, China) for total nitrogen (TN), potassium chloride (KCl) extraction (BRAN+LUEBBE-AA3, Germany) for NH_4^+ and NO_3^- , the Mo–Sb colorimetric method for total phosphorus (TP), the molybdenum blue method for available phosphorus (AP), the NH_4OAC method for rapidly available potassium (AK), and flame photometry for total potassium (TK)(flame photometer FP6410, Shanghai) [37].

2.4. DNA Extraction

Total soil DNA was extracted following the instructions of E.Z.N.A.[®] soil (Omega BioTek, Norcross, GA, USA) with 1% gel electrophoresis, and NanoDrop2000 was used to examine the DNA concentration and purity. The DNA samples were stored at -80°C [38,39].

2.5. PCR Amplification and Illumina MiSeq Sequencing

Soil bacterial diversity and community structures were determined by Illumina MiSeq sequencing with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') from V3-V4 hypervariable regions of the bacterial 16S rRNA [40,41]. The reactions were performed in triplicate in a 20 μL reaction mixture including 4 μL of 5X FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA [38,39,42,43]. PCR amplification proceeded with the following conditions: initial denaturation at 95°C for 3 min, 27 cycles of 30 s at 95°C , 30 s for annealing at 55°C , 45 s for elongation at 72°C , and a final extension at 72°C for 10 min (PCR: ABI GeneAmp[®] 9700). In each PCR run, a positive and negative control was added in order to validate the PCR results. The resulting PCR products were analyzed with 2% agarose gel and further purified with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and examined by using QuantiFluor[™]-ST (Promega, WI, USA). Based on the need for sequencing, the samples were pooled in proportion. Eventually, a Fastpfu database was built and Illumina MiSeq sequencing was carried out [44].

2.6. Bioinformatic Analysis

FLASH was used to convert the sequence data to single long reads [45], and Trimmomatic [46] was used for quality filtering and merging paired-end reads. Clustering was performed using the UPARSE algorithm with a 97% sequence similarity threshold, generating OTUs (operational taxonomic units). UCHIME was used to remove chimeras [45,47]. Based on the Silva bacteria database [48], taxonomic affiliations were assigned to OTUs using the ribosomal database project (RDP) classifier [49]. Alpha diversity and rarefaction analyses were conducted in Mothur version v.1.30 [50], and Coverage was used for sequencing depth. Chao1 was used for community abundance, while Shannon's

index and Simpson's index were used for bacterial community abundance and community diversity analyses [44,51,52].

2.7. Data Analysis

One-way ANOVA and Duncan's ($\alpha = 0.05$) test were used to compare the microbial diversity and abundance at the phylum level using SPSS 22.0 (IBM Corporation, New York, United States) [53]. A Venn diagram was prepared with R software [54], while non-metric multidimensional scaling (NMDS) based on the Bray–Curtis algorithm and figures were prepared with the Vegan package [55,56]. Variations of species among groups at the genus level were examined with the Kruskal–Wallis test. Pearson's correlation coefficient was used to establish a correlation between different bacterial genera and environmental factors, along with alpha diversity, using the heatmap package [57].

3. Results

3.1. Soil Physicochemical Properties

The soil pH of CK varied significantly with the seasons, while the pH of SS soil in May and July was less than September, and the average pH of SS was significantly lower than CK ($p < 0.05$). The soil pH of CK and SS was highest in the sampling period. The change in soil pH may be related to the seasonal variation of soil nutrients. With the increase of soil environment stress on plants, beneficial microorganisms in the rhizosphere can improve the absorption and utilization of nutrients of plants by secreting organic acids, thus improving the plants' resistance to environmental stress and the soil's pH value. Soil water content varied greatly between seasons, and it was higher in CK than SS ($p < 0.05$). In July, soil water content was highest, followed by September and May. The soil organic matter of CK was higher than SS; initially, it decreased, and then it increased from May to September. In three seasons, the TN, NH_4^+ , NO_3^- , AP, TP, AK, and TK of CK were all found to be higher than SS, and they decreased from May to September (Table 1).

Table 1. Comparative analysis of soil physicochemical properties of CK-5, CK-7, CK-9, SS-5, SS-7, SS-9. CK: Control, soil of non-infected *P. sylvestris* var. *mongolica*; SS: treatment, soil of infected *P. sylvestris* var. *mongolica*; May, July, and September represent spring, summer, and autumn, respectively. Different letters indicate a significant difference at $p < 0.05$ according to Duncan's new multiple range test. MC: moisture content, OM: organic matter, TN: total nitrogen, AP: available phosphorus, TP: total phosphorus, AK: available potassium and TK: total potassium

Sample	pH	MC %	OM g/kg	TN g/kg	NH_4^+ mg/kg	NO_3^- mg/kg	AP mg/kg	TP g/kg	AK mg/kg	TK g/kg
CK-5	5.16 ± 0.18D	6.16 ± 0.06C	21.30 ± 0.45A	0.66 ± 0.01A	8.87 ± 0.02A	5.05 ± 0.01A	177.79 ± 0.39A	0.34 ± 0.00B	101.27 ± 2.67A	3.31 ± 0.00A
	5.39 ± 0.15B	10.53 ± 0.20A	16.74 ± 0.34C	0.48 ± 0.02B	6.77 ± 0.10B	3.39 ± 0.04B	97.37 ± 0.78C	0.36 ± 0.00A	84.70 ± 1.20B	2.90 ± 0.00B
CK-7	5.49 ± 0.03C	8.33 ± 0.03B	19.55 ± 0.57B	0.25 ± 0.00D	5.98 ± 0.05D	2.34 ± 0.01D	60.46 ± 0.39E	0.28 ± 0.00E	75.69 ± 0.85C	2.32 ± 0.00D
	5.52 ± 0.06C	4.19 ± 0.14D	15.34 ± 0.00D	0.38 ± 0.01C	6.24 ± 0.03C	3.32 ± 0.07B	114.08 ± 0.39B	0.33 ± 0.00C	81.62 ± 0.11B	2.72 ± 0.02C
SS-5	5.50 ± 0.12C	8.02 ± 0.05B	13.91 ± 0.00E	0.24 ± 0.00D	5.21 ± 0.02E	2.56 ± 0.03C	70.95 ± 0.39D	0.32 ± 0.00D	70.97 ± 0.28D	2.65 ± 0.05C
	5.63 ± 0.15A	6.20 ± 0.06C	15.27 ± 0.23D	0.19 ± 0.00E	4.12 ± 0.01F	1.66 ± 0.06E	53.86 ± 0.67F	0.27 ± 0.00F	62.78 ± 0.12E	2.13 ± 0.02E
SS-7										
SS-9										

3.2. Bacterial Community Diversity

About 1,083,417 valid sequences were generated from 18 soil samples of CK and SS from three seasons, with an average sequence length of 437.75 bp. The soil samples' sequences were clustered into 3857 OTUs at 97% identity after splitting and removing redundancy. The rarefaction curves of operational taxonomic units (OTUs) were generated from the valid sequences by a random sampling

method (Figure 2). The rarefaction curves of CK and SS across all seasons changed smoothly, i.e., the number of sequences had a slight effect on OTU, which indicates that soil sampling was conducted correctly and the sequence data were valid. This confirms the validity of the investigation of soil bacterial community.

Figure 2. Rarefaction curves of CK-5, CK-7, CK-9, SS-5, SS-7, SS-9 samples (97% sequence similarity). CK: Control, soil of non-infected *P. sylvestris* var. *mongolica*; SS: treatment, soil of infected *P. sylvestris* var. *mongolica*; May, July, and September represent spring, summer, and autumn, respectively.

A Venn diagram showed that the OTU level of CK soil was higher than SS soil in July and September (Figure 3). Particularly, the number of OTUs in CK-7 and CK-9 was higher than SS-7 and SS-9, respectively. The number of OTUs in CK soil was highest in May and lowest in September. Unlike CK, the SS soil had the most OTUs in September and the least in May. The shared number of OTUs in CK and SS soil in the three seasons was 2198, and there were 2584 OTUs for CK-5, CK-7, and CK-9, 2745 OTUs for CK-5 and CK-7, 2782 OTUs for CK-5 and CK-9, and 2955 OTUs for CK-7 and CK-9. About 2584 OTUs were shared by SS-5, SS-7, and SS-9, 2786 OTUs by SS-5 and SS-7, 2868 OTUs by SS-5 and SS-9, and 2899 OTUs by SS-7 and SS-9. In general, the SS soil bacterial community structure was more stable than CK; however, CK and SS soils in July and September were found to be similar.

Figure 3. Venn diagram showing the shared operational taxonomic units (OTUs) of CK-5, CK-7, CK-9, SS-5, SS-7, and SS-9. CK: Control, soil of non-infected *P. sylvestris* var. *mongolica*; SS: treatment, soil of infected *P. sylvestris* var. *mongolica*; May, July, and September represent spring, summer, and autumn, respectively.

ANOVA for α diversity of CK and SS soil in different seasons indicated that the depths for soil samples with coverage ranged from 98–99%, which coincided with the rarefaction curves of OTUs (Table 2). Shannon's indices of CK soil for three seasons were not significantly higher than SS but varied significantly with season. The Shannon's index of SS soil was higher in September than May and July ($p < 0.05$), while the Shannon's index of CK-5 was non-significantly higher than others. These findings show that the soil bacterial community diversity of CK and SS was higher in July than in July and May ($p > 0.05$), and CK-5 diversity was significantly lower than the others. The Ace index and Chao index of SS and CK soil increased slightly with season ($p > 0.05$), which showed that there was no significant difference in bacterial community richness within seasons.

Table 2. Soil α -diversity indices of soil bacteria community in CK-5, CK-7, CK-9, SS-5, SS-7, SS-9. CK: Control, soil of non-infected *P. sylvestris* var. *mongolica*; SS: treatment, soil of infected *P. sylvestris* var. *mongolica*; May, July, and September represent spring, summer, and autumn, respectively. Different letters indicate significant difference at $p < 0.05$ according to Duncan's new multiple range test.

Sample	Shannon	Simpson	Ace	Chao	Coverage
CK-5	6.44 $\pm$ 0.03C	0.006 $\pm$ 0.00A	3202.52 $\pm$ 50.50B	3203.13 $\pm$ 38.47B	0.99 $\pm$ 0.00A
CK-7	6.56 $\pm$ 0.06B	0.004 $\pm$ 0.00A	3302.69 $\pm$ 59.63AB	3336.45 $\pm$ 64.93AB	0.98 $\pm$ 0.00A
CK-9	6.78 $\pm$ 0.02A	0.003 $\pm$ 0.00A	3387.62 $\pm$ 54.59A	3388.09 $\pm$ 79.06A	0.98 $\pm$ 0.00A
SS-5	6.63 $\pm$ 0.03B	0.004 $\pm$ 0.00A	3250.22 $\pm$ 50.61AB	3287.08 $\pm$ 39.94AB	0.99 $\pm$ 0.00A
SS-7	6.55 $\pm$ 0.01B	0.004 $\pm$ 0.00A	3281.72 $\pm$ 16.85AB	3286.57 $\pm$ 26.90AB	0.99 $\pm$ 0.00A
SS-9	6.74 $\pm$ 0.00A	0.003 $\pm$ 0.00A	3310.71 $\pm$ 16.13AB	3285.12 $\pm$ 14.68AB	0.98 $\pm$ 0.00A

3.3. Soil Bacterial Community Structure

A cluster analysis was performed with non-metric multidimensional scaling (NMDS) based on the Bray–Curtis algorithm (Figure 4). The results show that CK-5 and SS-7 were relatively close, whereas CK-7, SS-7, CK-9, and SS-9 were clustered together, which shows the similarity between the community structure of CK and SS soils during July and September, and a significant difference in the community structure in July and September.

Figure 4. NMDS (non-metric multidimensional scaling) ordination based on Bray–Curtis similarities of bacteria communities in CK-5, CK-7, CK-9, SS-5, SS-7, SS-9 samples. CK: Control, soil of non-infected *P. sylvestris* var. *mongolica*; SS: treatment, soil of infected *P. sylvestris* var. *mongolica*; May, July, and September represent spring, summer, and autumn, respectively.

The cluster analyses were performed on OTU sequences with a 97% sequence similarity threshold, and about 1099 species belonging to 31 phyla, 85 classes, 165 orders, 312 families, and 539 genera were found. After combining the phyla with a richness level of less than 0.01%, there were only 11 phyla left (Table 3). Proteobacteria, Actinobacteria, and Acidobacteria shared the dominant species for all samples (relative abundance $\geq 10\%$). The relative abundance of Proteobacteria in CK soil was higher than that of SS soil in July and September, and initially, it increased, but thereafter, it decreased across seasons ($p > 0.05$). The relative abundance of Actinobacteria in SS soil was higher than CK soil in May and July, but lower than SS in September ($p > 0.05$). In general, the relative abundance of Actinobacteria in SS soil was higher than CK, and both of them increased across seasons and reached the highest level in September, i.e., 17.38% and 21.13% for CK and SS, respectively. The relative abundance of Firmicutes in CK-5 soil was 12.2%, which was significantly different from others ($p < 0.05$). Likewise, the relative abundance of Nitrospirae in CK soil was 1.57%, which was also significantly different from other samples ($p < 0.05$). The relative abundance of Saccharibacteria in CK soil was higher than SS; it initially increased and then decreased with seasons.

Table 3. Relative abundance (%) of bacterial phyla among CK-5, CK-7, CK-9, SS-5, SS-7, SS-9 samples based on total sequence reads. CK: Control, soil of non-infected *P. sylvestris* var. *mongolica*; SS: treatment, soil of infected *P. sylvestris* var. *mongolica*; May, July, and September represent spring, summer, and autumn, respectively. Different letters indicate a significant difference at $p < 0.05$ according to Duncan's new multiple range test.

Sample	Proteobacteria	Actinobacteria	Acidobacteria	Chloroflexi	Firmicutes	Verrucomicrobia
CK-5	26.41 ± 1.78A	22.60 ± 1.69AB	15.87 ± 1.75A	6.23 ± 0.62A	12.20 ± 0.62B	4.96 ± 1.09A
CK-7	32.48 ± 4.11A	20.49 ± 1.93B	17.36 ± 2.95A	7.37 ± 1.44A	3.51 ± 0.33A	3.57 ± 1.66A
CK-9	28.72 ± 0.65A	24.06 ± 0.30AB	17.38 ± 0.82A	6.30 ± 0.05A	4.53 ± 0.25A	4.40 ± 0.28A
SS-5	28.51 ± 1.58A	25.59 ± 0.99A	15.18 ± 1.18A	7.18 ± 0.53A	6.14 ± 0.85A	5.00 ± 0.91A
SS-7	29.80 ± 1.87A	22.13 ± 0.80AB	18.53 ± 2.42A	6.68 ± 0.14A	5.01 ± 0.46A	4.33 ± 1.05A
SS-9	25.45 ± 1.21A	20.51 ± 0.26B	21.13 ± 1.45A	7.19 ± 0.62A	4.38 ± 1.59A	6.55 ± 0.96A
Sample	Gemmatimonadetes	Nitrospirae	Saccharibacteria	Bacteroidetes	Planctomycetes	other
CK-5	2.65 ± 0.24B	1.57 ± 0.07B	1.68 ± 0.13C	1.35 ± 0.17AB	1.97 ± 0.44A	2.51 ± 0.23BC
CK-7	4.01 ± 0.46A	2.84 ± 0.47A	3.86 ± 0.28A	1.44 ± 0.25AB	0.84 ± 0.25B	2.22 ± 0.22C
CK-9	3.84 ± 0.20A	2.90 ± 0.21A	2.38 ± 0.11C	1.80 ± 0.09A	0.86 ± 0.03B	2.83 ± 0.13B
SS-5	3.14 ± 0.29AB	2.90 ± 0.15A	1.13 ± 0.12D	1.21 ± 0.10B	1.49 ± 0.27AB	2.52 ± 0.07BC
SS-7	3.80 ± 0.38A	2.97 ± 0.26A	2.15 ± 0.09BC	1.25 ± 0.04B	0.81 ± 0.09B	2.53 ± 0.17BC
SS-9	3.26 ± 0.07AB	3.40 ± 0.24A	2.11 ± 0.08BC	1.48 ± 0.07AB	1.17 ± 0.13B	3.37 ± 0.04A

CK and SS had 35 genera for three seasons, and there were 462, 469, 501, 464, 462, and 503 genera for CK-5, CK-7, CK-9, SS-5, SS-7, and SS-9, respectively. The species with a relative abundance of less than 1% were pooled into one group. The variance analysis of species was conducted by Kruskal–Wallis test (Figure 5). *Acidothermus*, *Acidobacter*, *Bacillus*, *Bradyrhizobium*, *Candidatus-Solibacter*, *norank-f-DA111*, *norank-f-Nitrosomonadaceae*, *norank-p-Saccharibacteria*, *Rhizomicrobium*, and *Sphingomonas* of CK soil for the three seasons had a relative abundance significantly higher than SS, while that of *Burkholderia-Paraburkholderia*, *norank-c-Actinobacteria*, *norank-o-Acidimicrobiales*, *norank-o-Subgroup-7*, *RB41*, and *Streptomyces* in SS soil were higher than CK and varied with the season. *Ruminococcaceae-UCC-002* and *Subdoligranulum* were found in CK and SS soils in autumn ($p > 0.05$). ANOSIM analysis showed that the difference among groups was greater than within groups of CK and SS soil bacteria in different seasons ($R = 0.758$, $p = 0.001$).

Figure 5. Comparative analysis showing the relative abundance of genera levels of CK-5, CK-7, CK-9, SS-5, SS-7, and SS-9.

3.4. Relationship between Soil Bacterial Community Diversity and Soil Properties

Pearson's correlation analysis showed that Simpson's index was significantly positively correlated with NO_3^- , AP, and TK (Table 4). The TN, NH_4^+ , and AK were also positively correlated with

Simpson's index, and TK was significantly negatively correlated with Shannon's index, pH was negatively correlated with Simpson's index, NO_3^- was negatively correlated with Shannon's index, and AP was negatively correlated with Ace index ($p < 0.05$).

Table 4. Correlation analysis of diversity indices and soil properties. The correlation coefficient and significance were obtained using Pearson's correlation analysis. Significant values are shown as: * $p < 0.05$; ** $p < 0.01$.

Diversity Index	pH	MC	OM	TN	NH_4^+	NO_3^-	AP	TP	AK	TK
Shannon	0.784	−0.028	−0.198	−0.801	−0.717	−0.837 *	−0.803	−0.832 *	−0.741	−0.930 **
Simpson	−0.907 *	−0.183	0.500	0.901 *	0.872 *	0.950 **	0.953 **	0.688	0.886 *	0.944 **
Ace	0.576	0.490	−0.084	−0.699	−0.571	−0.754	−0.833 *	−0.654	−0.625	−0.767
Chao	0.507	0.539	−0.128	−0.554	−0.434	−0.609	−0.721	−0.364	−0.471	−0.593

About 19 genera were significantly different between CK and SS soils, and here the correlations between their abundance and pH, MC, OM, TN, NH_4^+ , NO_3^- , AP, TP, AK, and TK were explored by using Pearson's correlation analysis (Figure 6). *Bacillus* and *Acidothermus* were highly positively correlated with Simpson's indices, TK, AK, NH_4^+ , TN, NO_3^- , and AP ($p \leq 0.001$). *Candidatus-Solibacter* was also highly positively correlated with AK, NH_4^+ , TN, and NO_3^- in a significant manner. Likewise, *norank-f-DA111* was highly positively correlated with AK, NH_4^+ , NO_3^- , and AP. *Bacillus*, *Acidothermus*, *Candidatus-Solibacter*, and *norank-f-DA111* were all significantly positively correlated with OM ($0.001 < p \leq 0.01$). *Rhizomicrobium* was highly positively correlated with OM and NH_4^+ and significantly positively correlated with AK and TN. *Acidobacter* was highly positively correlated with TK, TP, TN, NO_3^- , AK, NH_4^+ , and AP. *Bradyrhizobium* was highly positively correlated with TP, TN, and TK, and significantly positively correlated with AK, NH_4^+ , NO_3^- , and AP. *Sphingomonas*, *norank-p-Saccharibacteria*, and *norank-f-Nitrosomonadaceae* were highly positively correlated with soil water content, and the same was found for *norank-o-Subgroup-7* and pH, while correlations between *norank-o-Subgroup-7* and AK, NH_4^+ , and NO_3^- were highly negative; however, those with TK, TN, AP, and OM were significantly negative. *Norank-c-Actinobacteria* was highly positively correlated with Shannon's index and pH but highly negatively correlated with Simpson's index, TK, AK, NH_4^+ , TN, NO_3^- , and AP, and only significantly negatively correlated with OM. *RB41* was positively correlated with Shannon's index and highly positively correlated with pH, while being highly negatively correlated with TK, AK, NH_4^+ , TN, NO_3^- , AP, Simpson's index, and TP. *Ruminococcaceae-UCG-002* and *Subdoligranulum* were significantly positively correlated with Shannon's index and significantly negatively correlated with TK, NO_3^- , and TP. *Norank-o-Acidimicrobiales* was highly positively correlated with Shannon's index and the Ace index, while being highly negatively correlated with Simpson's index and TP.

Figure 6. Correlation heatmap of soil microbial community composition and environmental factors (note: * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$, red star means the bacteria genus is predominant in CK samples).

4. Discussion

Soil microorganisms, one of the most active components in the forest-soil ecosystem, play a critical role in plant functioning, improving plant health, the cycling of nutrient elements (C, N, P, and S), the maintenance of ecological balance, soil purification, and bioremediation [58,59]. Beneficial rhizospheric microorganisms, which secrete phytohormones such as auxin, gibberellin, and cytokinin, enhance plant growth, root length, and leaf surface area, xylem function, photosynthesis, and stress resistance. In addition, beneficial rhizospheric microorganisms occupy space and trophic niches by competing with other counterparts and improve nutrient uptake, plant health, and growth by establishing interactions with the plant rhizosphere [60–67]. In the present study, NMDS showed that there is no difference between CK and SS soil bacterial community structure, but other sampling showed differences during May and other months. At the phylum level, Proteobacteria, Actinobacteria, and Acidobacteria were common dominant species in CK and SS soil in three seasons (the relative abundance > 10%). The abundance of Proteobacteria in coniferous forests was recorded to be about 29.5% [68], while it was only 25% in the previous report [69]. The relative abundance of Actinobacteria and Acidobacteria in *P. thunbergii* Parl soil has been reported to range from 24% to 32% and from 14% to 51%, respectively [70], which is similar to our findings. At the phylum level, no significant difference was reported in this study. However, of the 19 significantly different bacterial genera, six were classified as beneficial rhizospheric microorganisms, which could be attributed to the stability of soil bacterial community structure and the property of *P. sylvestris* var. *mongolica* sphaeropsis blight, which is host-dependent. A number of studies (national as well as international) have explored certain important measures for the prevention and control of this disease, such as the selection of trees suitable

for the environment, young growth tending, tending felling, pruning and disposal of blighted needles, twigs, and cones, cleaning stands, planting mixed stands, and so on [71,72]. Moreover, the growth and health of *P. sylvestris* var. *mongolica* can be enhanced by filtering the beneficial rhizospheric microorganisms and using them as biological fertilizers. At the same time, these also help to reduce the use of chemical pesticides and the problems associated with these, including widespread disease, polluted soil, weakening of plants, etc. [73–76]. *Bacillus*, with excellent tolerance to leanness, heat, and cold and the ability to produce phytohormone (auxin) could improve plant stress resistance and plant growth. An earlier study proved that *Burkholderia* and *Rhodococcus* enhance the growth and health of *P. sylvestris* var. *mongolica* [77]. *Bacillus cereus* HB12 and HB59 were found to be beneficial for pine growth and disease resistance [78]. *Acidothermus*, with its acid resistance, helps in the degradation of cellulose, hemicellulose, and lignin [79,80]. In addition, *A. cellulolyticus* degraded soluble soils and improved plant growth and biomass by secreting endo- and exoglucanase [81,82]. By virtue of interactions with plant rhizosphere, *Rhizomicrobium* produced auxin and enhanced plant growth, simultaneously promoting plant production of β -glucanase and protease, thereby improved anticoagulant resistance and plant health [83]. Soil nutrient and enzymatic activity were enhanced after *Rhizobium* sp. was inoculated into *Cicer arietinum* Linn, which subsequently improved plant health and biomass [84]. In a separate study, *Bradyrhizobium japonicum* also improved plant and root growth after being inoculated into *Glycine max* [85]. Several studies have reported the successful isolation of mycorrhizal fungi, *Trichoderma*, dark septate endophytes, and bacteria in the Zhanggutai area [86–91]. In the present study, we successfully isolated mycorrhizal fungi, bacteria, and *Trichoderma*, which had antagonistic effect on Sphaeropsis blight and wilt disease in the in vitro condition; afterward, the inoculation experiment was tested with annual and perennial seedlings of *P. sylvestris* var. *mongolica*.

The rhizospheric nutrient content of non-infected *P. sylvestris* var. *mongolica* was significantly higher than the infected ones. Correlation studies of 19 different bacterial genera and environmental factors indicated that rhizospheric bacterial genera with a high abundance in non-infected and infected *P. sylvestris* var. *mongolica* were differently distributed, and soil nutrient content was significantly positively correlated with the rhizosphere bacterial genera of non-infected *P. sylvestris* var. *mongolica* (Figure 6). In contrast, the rhizosphere bacterial genera of infected *P. sylvestris* var. *mongolica* were significantly negatively correlated with soil nutrient content and significantly positively correlated with pH and diversity index. This clearly indicates that the main cause of Sphaeropsis blight is associated with soil nutrient and ecological functions of rhizospheric bacteria. *Nitrobacter*, *Candidatus-Solibacter*, and *Rhizomicrobium* were negatively correlated with soil nutrient content [92], which disagrees with our study. This could possibly be attributed to the different sampling locations and time. Soil pH, soil organic matter, effective nitrogen, total nitrogen, and soil water content are the main factors affecting the abundance of bacterial community in coniferous forests, and pH influences soil bacterial community structure [93–95], which is in agreement with this study. *P. sylvestris* var. *mongolica*, with tolerance to heat and leanness, is a good alternative for conserving water and soil resources, blocking wind, and fixing sands. Its needles and undergrowth vegetation are the main source of soil nutrients in the Zhanggutai area. Pine needles contain lignin, tannin, resin, wax, etc., which are indissoluble, and soil functional bacteria and plant-growth-promoting rhizobacteria (PGPR) play an important role in nutrient and energy conversion [96]. The PGPRs are capable of fixing N_2 absorbed by plants and forming symbionts with plants, which eventually improves soil nitrogen availability [97,98]. *Bacillus altitudinis*, *Rhizobium daejeonense*, *Azotobacter*, *Azospirillum*, *Bacillus*, and *Klebsiella* also have nitrogen fixation abilities for certain plants [60,99]. Water converts 90% of soil phosphorus, in combination with Fe, Al, and Ca, into insoluble phosphorus, which cannot be reabsorbed by plants. Soil rhizobacteria reduce soil pH by producing organic acids such as formic acid, propionic acid, glycolic acid, fumaric acid, etc., and transform the insoluble phosphorus into soluble form, and therefore, effective phosphorus content is improved in the rhizosphere [100–102]. *Actinomycetes* growing with root secretion release organic phosphorus and increase the soil nutrient content [103,104]. Moreover, earlier studies also

showed that *Bacillus* CECT5105 and *Phyllobacterium* sp. could remarkably enhance nutrient content in the rhizosphere and the overall health of plants [105–107].

5. Conclusions

1. There are significant differences between infected and non-infected *P. sylvestris* var. *mongolica* rhizosphere soil microorganisms, and the beneficial microorganisms are dominant in non-infected *P. sylvestris* var. *mongolica* rhizosphere soil.

2. The physicochemical properties of the rhizosphere of non-infected *P. sylvestris* var. *mongolica* plants were significantly better than those of infected ones. The different bacterial genera of the non-infected *P. sylvestris* var. *mongolica* rhizosphere were positively correlated with the physicochemical properties of soil, whereas the different bacterial genera of the infected *P. sylvestris* var. *mongolica* rhizosphere were negatively correlated with the physicochemical properties of soil.

In future research, we will separate the beneficial rhizosphere microbiology from healthy *P. sylvestris* var. *mongolica* and research the mechanism of beneficial microbiology growth promotion and stress resistance in *P. sylvestris* var. *mongolica* so as to lay a foundation for the microecological control of the shoot blight disease of *P. sylvestris* var. *Mongolica*.

Author Contributions: S.H., X.D. and R.S. conceived and designed the study. S.H., X.D. and Y.A. performed the experiments. S.H., X.D. and X.S. contributed to the sample measurement and data analysis. S.H. and X.D. wrote the paper.

Funding: This research was funded by [National Key Research and Development Program] grant number (2017YFD0600101), [National Natural Science Foundation of China] grant number (31670649, 31700564, 31170597, 31200484).

Conflicts of Interest: The authors declare no conflict of interest.

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