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The Effects of *Suillus luteus* Inoculation on the Diversity of Fungal Communities and Their Structures in the Soil under *Pinus massoniana* Located in a Mining Area

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Abstract: As important decomposers and plant symbionts, soil fungal communities play a major role in remediating heavy-metal-polluted soils. However, the diversity and structures of fungal communities generally remain unclear in mining areas. This study aimed to assess the rhizospheric fungal-community composition of Masson pine (*Pinus massoniana*) in the lead-zinc mining area of Suxian District, Hunan Province, China. This experiment undertook the following three treatments: Masson pine inoculated with or not inoculated with *Suillus luteus*, and bulk soil without plants as a control. The results thereof showed that inoculation of ectomycorrhizal fungi could enlarge plants' capability to absorb heavy metals and secrete soil enzymes. The richness and diversity of fungi in the rhizospheric soil were significantly higher than of those in the bulk soil ($p < 0.05$), but no significant difference was noted between the rhizospheric soils inoculated with and not inoculated with ectomycorrhizal (ECM) fungi as the community structure changed. The rhizospheric fungi belonged to 6 phyla, 25 classes, 65 orders, 115 families, and 150 genera, and the dominant phyla were Chytridiomycota (50.49%), Ascomycota (38.54%), and Basidiomycota (9.02%). Through use of LEfSe and heatmapping, the relative abundances of *Suillus*, *Paraglomus*, *Agaricus*, and *Tulasnella* were found to be the highest in the soil with ECM fungus inoculation. RDA showed that the community structure nearly changed with ECM-fungus inoculation; this was significantly related to soil water content, the carbon–nitrogen ratio, bulk density, available potassium, and soil enzymes. Altogether, inoculation with ECM fungi may change the habitation environments of microorganisms and dominant fungi in soil, providing keystone screenings in heavy-metal-contaminated mining areas.

Keywords: heavy metals; fungal community; bulk soil; rhizosphere; *Suillus luteus*; *Pinus massoniana*

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1. Introduction

Mining activities lead to accumulation of heavy metals such as lead (Pb), zinc (Zn), cadmium (Cd), arsenic (As), mercury (Hg), etc., in soil, creating inhospitable environments and endangering the dynamics of ecosystems so that survival is difficult for most microorganisms [1–3]. Moreover, heavy metals accumulate in living organisms, including human beings; along with the trophic chain, they interfere with the organisms' normal metabolisms, such as soil respiration, microbial growth, and plant photosynthesis [4,5]. Therefore, interactions between soil organisms and heavy-metal-polluted environments have become an issue of international relevance [1].

As a matter of fact, the toxic and barren characteristics of heavy-metal-contaminated soil greatly affect growth of plants and microbial communities present in these polluted

environments [3,6], which poses a great threat to remediation of contaminated soil in mining areas [7]. Fungi are known as an important component of the belowground community, closely related to plant roots and rhizospheric environments [8]. Fungi have been reported to form mycorrhizal symbiosis with more than 90% of vascular plants [9] and support up to 20% net primary productivity through their nutrition metabolisms [10] in different forest ecosystems. Soil fungi are also key decomposers in forest ecosystems, playing an important role in turnover of soil carbon pools and nitrogen sources in soil processes [11]. As Yuan et al. (2019) mentioned, some special soil fungal communities, as important decomposers, could better adapt to their habitation environments and decompose resistant compounds in order to absorb heavy-metal elements more rapidly in their places of origin than outside of those habitation environments [12]. In addition, fungi can significantly affect aboveground vegetation, including growth of plant biomass [13]; resistance against extreme circumstances, such as drought habitats [14]; and heavy-metal biodegradation and transportation [15,16]. Previous studies showed that the majority of fungi, such as ascomycetes and basidiomycetes, were adapt at resisting the toxicity of heavy metals via co-metabolism pathways [17]. However, fungi are easily affected by surrounding soil microenvironments: for example, diversity of plant rhizospheres, soil-composition properties, the carbon–nitrogen ratio (C/N), organic matter (OM), nitrogen (N), the pH, and soil water content (SWC). These lead to variations of fungal-community structures [18]. The interspecific interactions among fungal groups can also influence fungal-community structures [19,20]. Those changes can highly impact fungal-community succession, which is directly linked to the biochemical-cycling functions associated with different fungal guilds [21]. Thus, a major challenge is to disentangle the effects of heavy-metal pollution from environmental covariation (e.g., soil nutrients) on host–rhizospheric–fungus interactions.

Ectomycorrhizal (ECM) symbioses, which form between special groups of fungi and plant roots, are essential for enlarging the absorption areas of host-plant roots [14], binding soil particles together, and promoting absorption of water and nutrient elements [14]. Moreover, in highly polluted mining areas, ECM fungi can help their hosts adapt to heavy-metal-polluted soils through immobilization of heavy metals in plants' roots and limit transference of heavy metals to the shoots of plants [22,23]. Shi et al. (2019) showed that a combined remediation system of *Pinus thunbergii* inoculated with *Pisolithus* sp1 had a higher tolerance index, and the total accumulated chromium (Cr) was 2.83–27.75-fold higher than in non ectomycorrhizal (NM) seedlings in pot experiments [24]. As one of the "early stage" ECM fungi that commonly coexist in pioneer coniferous species [25], *Suillus* species have proven to independently evolve in resistance to excessive concentrations of heavy metals, such as zinc (Zn) and cadmium (Cd) [26–28]. However, how those "early-stage" ECM fungal species help their hosts adapt to heavy-metal-polluted areas and shape rhizospheric environments remains unclear.

In this study, we determined the rhizospheric fungal community of Masson pine with and without ECM fungus (*Suillus luteus*) inoculation in heavy-metal (Pb/Zn)-polluted soils via the high throughput sequencing method on the 18S rRNA region. We aimed to determine (1) whether the root system of trees with *S*. luteus inoculation could significantly reduce the concentrations of heavy metals (Pb/Zn) and (2) whether the fungal diversity and composition in Masson-pine rhizospheric soils with *S*. *luteus* inoculation were significantly higher than those in bulk soil.

2. Materials and Methods

2.1. Site Description

The study area was located on Mount Manao, Suxian, Hunan, China (113°08′09″, 25°42′05″), which is near the upper reaches of the Xiangjiang River. The mean annual precipitation is 1500–2000 mm; rainfall concentrates in March, June, and August. Within the experimental areas, a former mining area had been previously explored and found to contain Pb (125.33 mg/kg) and Zn (233.40 mg/kg) at concentrations that exceeded safety limits [28]. *Miscanthus* spp., *Equisetum ramosissimum*, and *Esholtzia cypriani* are the dominant

herbaceous plants that grow on Mount Manao and are seriously polluted by heavy metals; worse still, during the rainy season, heavy metals in soil are easily washed away by rain and converge into the Xiangjiang River, resulting in a large area of water pollution.

2.2. Experimental Design and Soil Sampling

Sporocarps of *Suillus luteus* (NCBI: txid5384) provided by the Hunan Academy of Forestry were screened from Masson pine forest soils. The seedlings inoculated with ECM fungi were cultivated according to our previous research [28]. Briefly, *S. luteus* was cultivated on a sterilized potato dextrose agar solid medium for about one week; then, a mycelium cultivated with a modified Kottke method in an aseptic room for another week (25 °C, 120 r/min) was used as an inoculum. Masson pine seedlings were surface-sterilized and cultured in sterilized petri dishes in an incubator (37 °C, 24 h), then transferred to plastic pots in a growth chamber for 4 weeks. The four-week-old seedlings were washed three times with aseptic water to clean the roots, which were immersed in mycelium suspensions as the inoculated plants (WE), and the roots of other seedlings immersed in an autoclaved (121 °C, 120 min) mycelium suspension were the non inoculated plants (NE). The ECM-fungi-inoculated seedlings were cultivated in a greenhouse with a light intensity of 8000 lx, a relative humidity of 55%, and a photoperiod of 13 h, 25 °C in light and 18 °C in the dark for 2 months. Then, the roots with and without ectomycorrhizal fungi were transplanted to the field in November 2016 (Figure 1a,b). We established nine 4 × 5 m planting-space plots with three groups: (i) Masson pine seedlings inoculated with *S. luteus* (WE), (ii) Masson pine seedlings not inoculated with *S. luteus* (NE), and (iii) bulk soil without plants (BS) as a control in the field; each treatment had three replications. For each plant in the WE and NE groups, the planting hole had a size of 30 cm × 30 cm × 40 cm and was covered with soil for 30 cm after the Masson pine seedling was placed in the hole. In October 2017, we surveyed the experiment area, and sporocarps of *S. luteus* were found in plots inoculated with ECM fungi (Figure 1d).

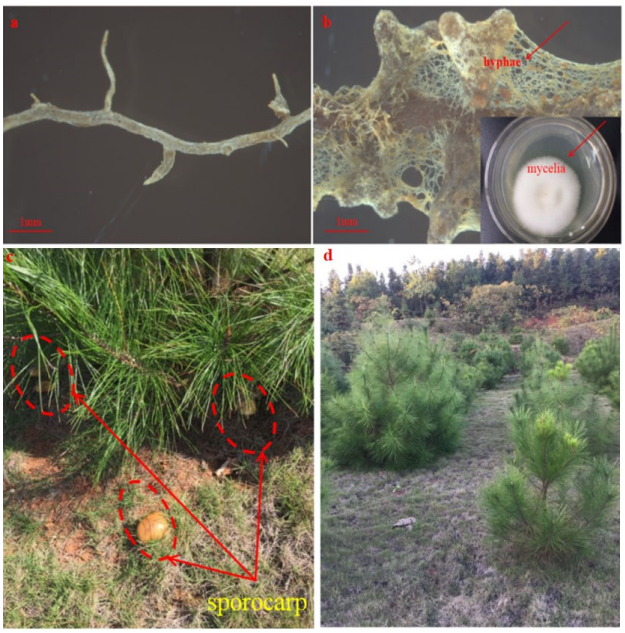

Figure 1. Morphological structures of (**a**) non inoculated fine roots, (**b**) roots inoculated with *S. luteus* (scale bar = 1 mm), plots with (**c**) inoculated roots, and (**d**) non inoculated (control) plots.

We followed the method of Li et al. (2014) to collect rhizospheric soils [29]. Briefly, three root samplings of each plant inoculated with (WE) or without (NE) *S. luteus* were carefully excavated and shaken 5 times; the soil that was still attached to the root surface was rhizospheric soil. Three soil samples were collected in the plots without plants (BS) as a control; their depth was 10 to 20 cm. The soil was divided into two parts; one part was kept

in a liquid-nitrogen canister and later transferred to the laboratory for fungal-community analysis, and the other part was kept in a dry-ice box to determine physicochemical-property analysis of the soil.

2.3. Physicochemical Soil Characterization

The soil samples were air-dried at room temperature for physical and chemical analysis of the soil. For the pHH_2O soil, 5 g of fresh soil collected from the tailing area was suspended in 25 mL of distilled water in a ratio of 1:5 (*w*:*v*) and measured using a pH meter (Sartorius PB-10; Sartorius Scientific Instruments, Beijing, China). The total carbon (TC) and total nitrogen (TN) of the soil were analyzed with an elemental analyzer (VarioEL III; Elementar, Germany) using the combustion method. Available phosphorus (AP) was determined through ammonium molybdate–tartaric emetic–ascorbic acid colorimetry (0.5 mol/L $NaHCO_3$), available potassium (AK) was measured with a flame photometer (1 mol/L NH_4OAc), and available nitrogen (AN) was measured with the alkaline hydrolysis diffusion method [30].

2.4. Determination of Enzyme Activity in Rhizospheric Soil

The urease (URE), saccharase (SAC), and alkaline phosphatase (AKP) activity analyses were measured as referred to by Guan et al. (1986), but modified slightly [31]. Briefly, each 5 g fresh soil sample was homogenized and cultured in an incubator (37 °C) for 24 h, then centrifuged in 4000 r/min for 5 min. The absorbance of the reaction mixture was determined using a UV/visible spectrophotometer (UV-5100B; METASH, China) at relative wavelengths of 578 nm, 508 nm, and 660 nm, respectively, and for catalase (CAT) activity, the soil-reaction mixture was cultured in an incubator (37 °C) for 20 min and recorded at a wavelength of 240 nm, according to Guan et al. (1986) [31].

2.5. DNA Extraction and High-Throughput Sequencing

Microbial DNA was extracted from 0.5 g of soil from each sample plot (n = 3) using a PowerSoil DNA Isolation Kit (MoBio PowerSoil® DNA Isolation Kit; Norcross, GA, USA). Then, the extracted genomic DNA was subsequently analyzed with an agarose gel electrophoresis system (0.8% *w*/*v*) (BIO-RAD, Hercules, CA, USA). Real-time PCR amplification was carried out on a GeneAmp 2720 PCR system (GeneAmp 2720; ABI, Foster City, CA, USA), and the 817F-1196R primer set was used for the 18S rRNA genes, as demonstrated by Rousk et al. (2010) [32]. The reactions of the PCR system were conducted through following the method of Hu et al. (2017) [33]. Each sample was amplified three times, and the final amplified products were mixed and tested with 2% agarose gel electrophoresis. Sequencing was carried out on an Illumina MiSeq platform (San Diego, CA, USA) with Wuhan Frasergen Bio-Pharm Technology (Wuhan, Hubei, China). Low-quality sequences, including overlapped sequences, polyclonal sequences, or sequences shorter than 200 bp, had to be truncated. The SILVA ribosomal-RNA gene-sequence database was used to classify the sequences. After quality control, the obtained sequences were merged and classified into OTUs according to 97% sequence similarity, using QIIME software and the UCLUST sequence-alignment tool [34].

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) was conducted with a confidence level of 95%, using SPSS (version 19.0; SPSS, Chicago, IL, USA). A non metric multidimensional scaling (NMDS) ordination diagram was created to visualize the effects of ECM fungus inoculation on the structure of each soil fungal community. *t*-tests of the intra-group and inter-group distance means of the weighted UniFrac distance matrix were conducted using QIIME software (Quantitative Insights Into Microbial Ecology, v1.8.0, http://qiime.org/, accessed on 22 June 2022; Knight and Caporaso labs, USA), and statistical significance was judged with a 1000 Monte Carlo permutation test. Redundancy analysis (RDA) was used to assess the relationships among the soil fungal community and the environmental

factors; the physicochemical factors of the soil were randomly replaced through Monte-Carlo permutation-test forward selection 999 times to test the similarity of the fungal communities, then visualized with Cano Draw. Effect size measurements (LEfSe) based on linear discriminant analysis (LDA) was used to screen the keystone species among the groups via the Galaxy online analytics platform (http://huttenhower.sph.harvard.edu/galaxy/, accessed on 22 June 2022; Penn State University, USA). The top 50 dominant fungi were clustered and demonstrated in the form of a heatmap using R software (http://www.r-project.org/, accessed on 22 June 2022; Auckland University, NZ).

3. Results

3.1. Heavy Metals and Enzymatic Activities

The concentrations of the heavy metals in the soil with ectomycorrhizal-fungus inoculation were below the Hunan background level, while other treatments were above the Hunan background level (Pb, 29.7 mg/kg; Zn, 94.4 mg/kg) (Table 1). The heavy-metal concentration of Pb ranged from 64.27 to 125.33 mg/kg, and that of Zn ranged from 27.47 to 233.40 mg/kg (Table 1). The heavy-metal concentration of Pb and Zn in the rhizospheric soil inoculated with (WE) or without ectomycorrhizal fungi (NE) was much lower than that of bulk soil (BS) ($p < 0.05$); however, there was no significant variation of Pb between the rhizospheric soils with the WE and NE treatments ($p > 0.05$). All enzymatic activities in the rhizospheric soil with the WE treatment was greater than those of the soil with the BS treatment ($p < 0.05$) (Table 1). The activities of CAT and SAC in the rhizospheric soil with the NE treatment were similar to those in the WE treatment, while the activities of AKP and URE showed a weakly mounting trend compared with those in bulk soil ($p > 0.05$).

Table 1. Heavy metals and enzymatic activities of rhizospheric soil under different treatments.

	Concentration (mg/kg)		Enzymatic Activities (mg/g)			
	Pb	**Zn**	**CAT**	**AKP**	**URE**	**SAC**
WE	64.27 ± 10.85^{b}	27.47 ± 2.55^{c}	2.81 ± 0.14^{a}	0.28 ± 0.02^{a}	0.29 ± 0.02^{a}	23.19 ± 1.61^{a}
NE	66.20 ± 0.81^{b}	193.47 ± 9.51^{b}	1.97 ± 0.25^{b}	0.17 ± 0.03^{b}	0.22 ± 0.02^{ab}	16.37 ± 0.94^{b}
BS	125.33 ± 4.49^{a}	233.40 ± 23.81^{a}	1.08 ± 0.70^{c}	0.12 ± 0.01^{b}	0.13 ± 0.03^{b}	7.28 ± 1.03^{c}

Different letters (a–c) in columns refer to statistically significant differences ($p < 0.05$) among three different sampling areas ($n = 3$); the same goes for tables that follow.

3.2. Rarefraction Measurement

A total of 442,621 quality-filtered gene sequences (ranging from 43,062 to 60,946 sequences) were obtained in nine samples, with an average length of 401 bp. These OTUs belonged to six phyla, 25 classes, 65 orders, 115 families, and 150 genera. These results showed that the rarefaction curve of species diversity tended to smooth gradually with increases in the sequences of samples (Figure 2). Additionally, both the WE and the NE treatment showed increases in species richness compared with that of the treatment without host plants (BS).

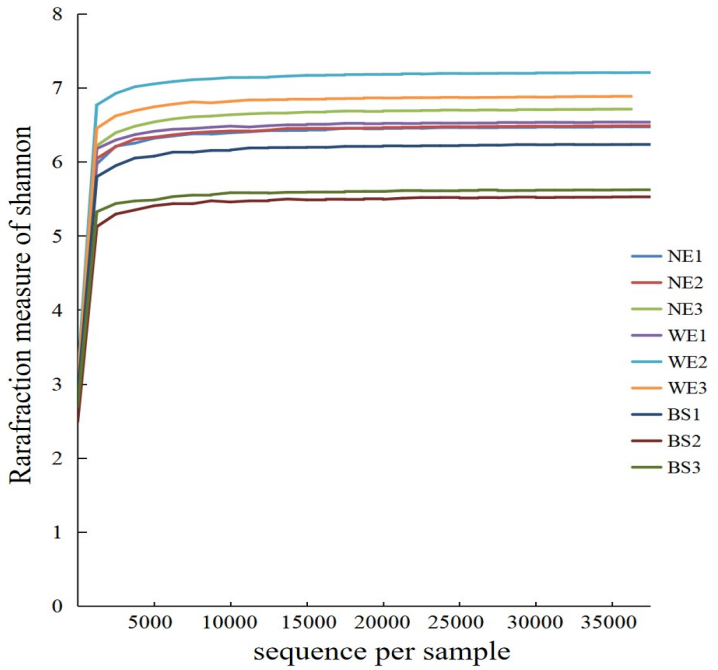

Figure 2. Rarefaction curves of fungal communities based on sequences per sample (label: 0.97). WE refers to soil inoculated with ectomycorrhizal fungi, NE refers to soil inoculated without ectomycorrhizal fungi, and BS refers to bulk soils; numbers 1, 2, and 3 after letters represent three different samples from the same treatment.

3.3. Diversity and Richness

The richness (Chao1 and ACE) and diversity (Shannon) of soil fungal communities were analyzed among nine samples of three different treatments (Table 2). These results showed that the diversity of plants' rhizospheric (the WE and NE treatments) soil fungal communities were significantly higher than that of the BS treatment ($p < 0.05$); however, there was no significant difference between the WE and NE treatments.

Table 2. Statistic of fungal diversity and richness from nine soil samples.

	Simpson	Chao1	ACE	Shannon
Non ECMF	0.95 ± 0.01^{a}	1173.33 ± 75.12^{a}	1174.63 ± 71.85^{a}	6.56 ± 0.08^{ab}
With ECMF	0.96 ± 0.01^{a}	1143.60 ± 49.48^{a}	1049.36 ± 54.31^{a}	6.87 ± 0.19^{a}
Bulk Soil	0.73 ± 0.14^{a}	791.87 ± 154.78^{b}	792.81 ± 154.06^{b}	4.60 ± 0.98^{b}

Different letters (a and b) in columns refer to statistically significant differences ($p < 0.05$) among three different sampling areas ($n = 3$).

3.4. Variation in Fungal-Community Structure

NMDS demonstrated that the triplicate samples of each treatment were clearly clustered in one group (Figure 3a). A multigroup comparison boxplot revealed that the inter-group difference was significantly greater than the intra-group difference ($p < 0.05$) (Figure 3b). Moreover, we conducted a statistical analysis and a Bonferroni-corrected test of the factors that caused the inter-group difference and found that the ECM-fungus inoculation treatment was the main reason for the difference (Figure 3b).

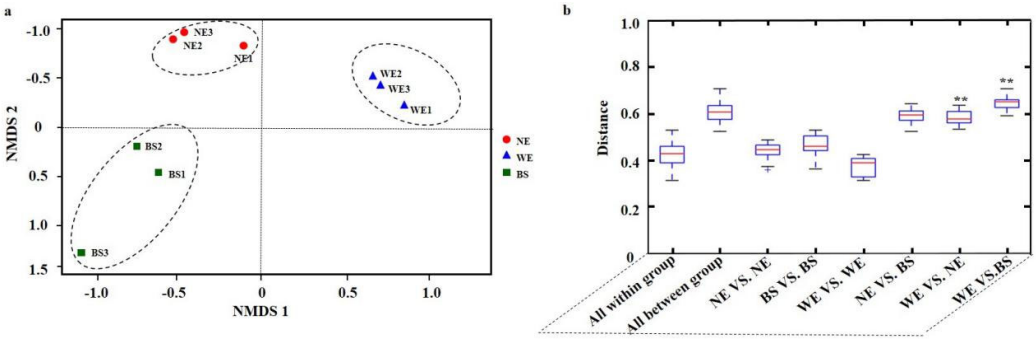

Figure 3. Analysis of fungal–community structures of soil samples. (**a**) Non metric multidimensional scaling (meta NMDS; stress: 0.042) based on BrayCurtis dissimilarity among fungal communities of OTUs that occurred on three sites; NE refers to soil not inoculated with ectomycorrhizal fungi, WE refers to soil inoculated with ectomycorrhizal fungi, and BS refers to bulk soil. (**b**) Boxplot of inter–group and intra–group differences, based on weighted UniFrac distance matrix on soil fungal community under different treatments. ** $p < 0.01$.

3.5. Phylum-Level Taxonomic Affiliation of OTUs

The dominant phyla in the three treatments were Chytridiomycota (50.49%) and Ascomycota (38.54%) (Figure 4). In total, those dominant phyla accounted for 89.03% of the fungal sequences. The relative abundances of Basidiomycota and Cryptomycota were greater than 1% but less than 10%. One-way ANOVA showed that the relative abundances of Basidiomycota and Chytridiomycota were significantly greater in the soil WE sample of than those of the BS, while the relative abundance of Ascomycota was much lower in the soil WE sample. Taken together, these results revealed a great change in the fungal community of the soil inoculated with ECM fungi, which progressively differentiated the dominant phyla in the soil with ECM-fungus inoculation from those of the soil not inoculated with ECM fungi.

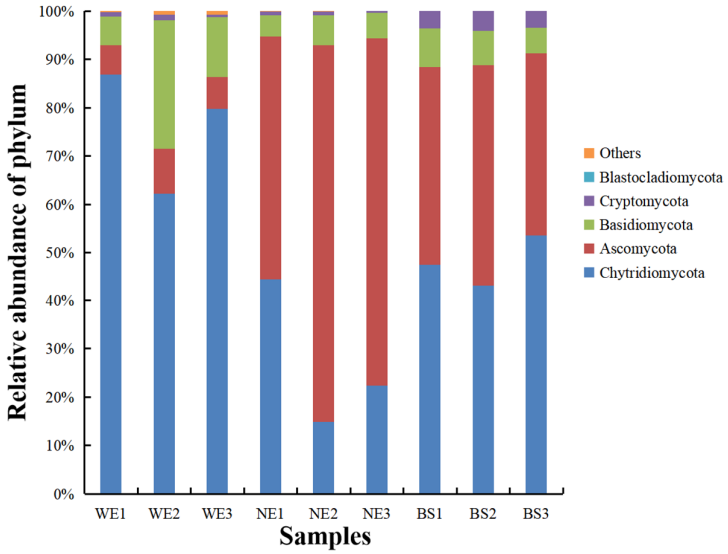

Figure 4. Relative abundance of different fungal phyla in rhizospheric soils under different treatments. WE refers to soil inoculated with ectomycorrhizal fungi, NE refers to soil inoculated without ectomycorrhizal fungi, and BS refers to bulk soil.

3.6. Responses of the Fungal Community in the Soil Inoculated with Ectomycorrhizal Fungi

Another major purpose of our study was to screen out keystone species by comparing fungal communities in different samples. LDA revealed significant variations in soil fungal communities, from the phylum level down to the family (Figure 5). The dominant fungal

species that existed in the soil inoculated with ECM fungi were mainly in the Ascomycota and Chytridiomycota phyla. LEfSe results at the genus level showed that *Agaricus*, *Melanotaenium*, *Suillus*, and *Yamadazyma* were the dominant groups for the treatment with ECM-fungus inoculation; *Brunneosphaerella*, *Saccharomycopsis*, and *Spencermartinsiella* were the dominant groups in NE. Those compositional differences were further confirmed with the Adonis PERMANOVA test (LDA scores of ≥2); there were significant differences in fungal-community composition among the WE soil, NE soil, and BS samples (weighted UniFrac $R^2 = 0.74$, $p < 0.001$; un-weighted UniFrac $R^2 = 0.70$, $p < 0.001$). The top 50 most abundant fungus genera were selected according to their relative abundance information at the genus level in each sample (Figure 6). The clustering relationships among the samples in the columns showed that the experimental groups of Masson pine inoculated with and not inoculated with *S. luteus* were greatly different from the bulk soil without plants. The three samples in the control group (BS1, BS2, and BS3) and the experimental group of Masson pine inoculated with (WE1, WE2, and WE3) and without *S. luteus* (NE1, NE2, and NE3) showed similar evolutionary relationships, respectively. Assignment of fungal communities to the same branch in a row represents that these groups had similar functions (Figure 6). Compared with BS treatment, *Paraglomus*, *Panaeolus*, *Suillus*, *Tulasnella*, *Umbelopsi*, and *Zoophagus* were clustered into one group that was significantly higher in the WE treatment, and *Catenomyces*, *Fimicolochytrium*, *Irineochytrium*, and *Synchytrium* were significantly higher in the NE treatment.

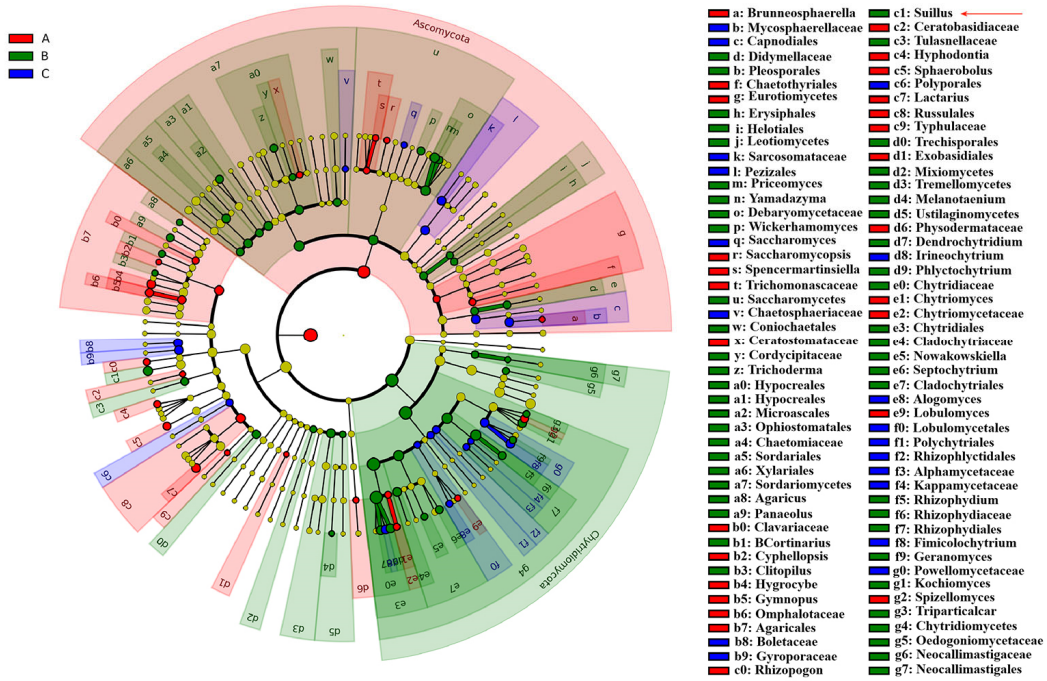

Figure 5. LEfSe identified the most differentially abundant taxon among soil with ectomycorrhizal-fungus inoculation (WE), soil without ectomycorrhizal–fungus inoculation (NE), and bulk soil (BS); the hierarchical relationship of all taxa from phylum to genus (inner to outer circle) in the sample community is shown, node size corresponds to average relative abundance of the taxon, and colored shadows refer to trends of significantly differed taxa. Arrows indicate position of *Suillus*.

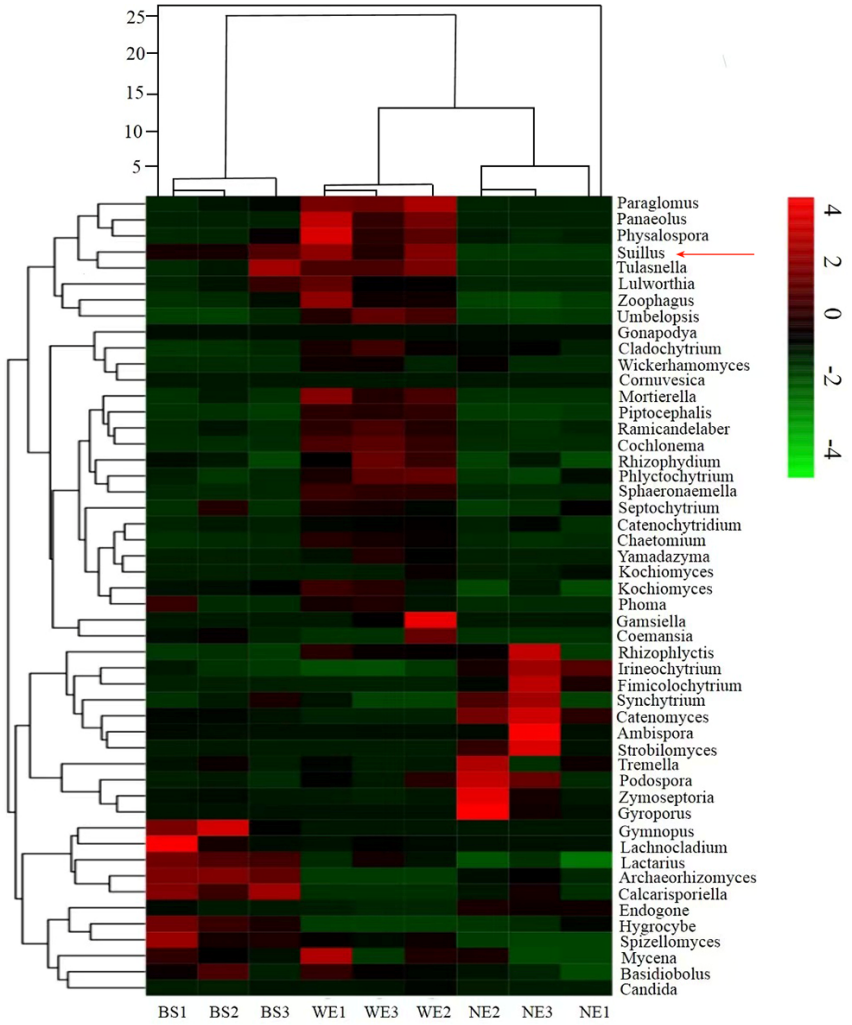

Figure 6. Correlationship of the most fungal communities in soil with ectomycorrhizal–fungus inoculation (WE), soil without ectomycorrhizal–fungus inoculation (NE), and bulk soil (BS)at genus level; arrows indicate position of *Suillus*.

3.7. Relationship between Fungal Communities and Environmental Variables

Seven physicochemical variables (AN, AP, AK, C/N, bulk density, and soil water content) and four kinds of enzyme (urease, saccharase, catalase, and alkaline phosphatase) of soil were selected for biplot RDA (Figure 7). The first axis of the RDA explained 54% of the variation, while the second axis explained 39.8%. In total, these two axes explained 93.8% of the variations between the soil factors and the fungal-community composition. The community structure of the rhizospheric fungi changed positively when the community was inoculated with ECM fungi; this was closely related to saccharase ($F = 8.9$, $p = 0.008$), urease ($F = 6.739$, $p = 0.006$), C/N ($F = 6.036$, $p = 0.016$), catalase ($F = 6.002$, $p = 0.01$), alkaline phosphatase ($F = 5.638$, $p = 0.024$), bulk density ($F = 5.109$, $p = 0.038$), and soil water content ($F = 4.361$, $p = 0.026$).

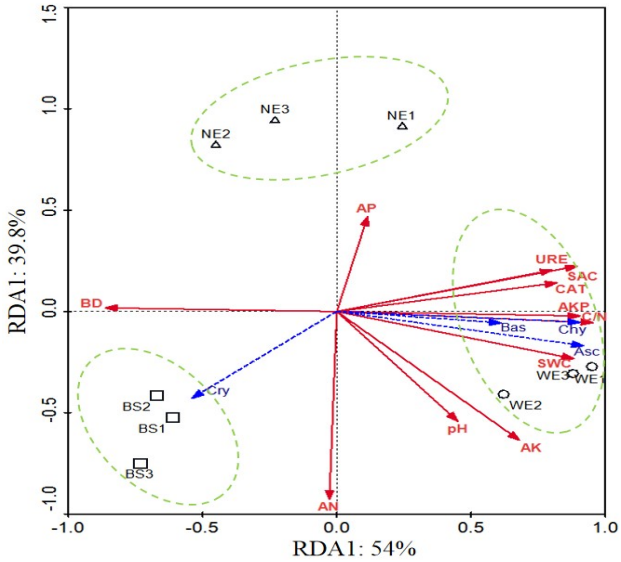

Figure 7. Redundancy analysis of relationships between physicochemical parameters of soil and dominant phyla of fungal communities inoculated with ectomycorrhizal fungi (WE), not inoculated with ectomycorrhizal fungi (NE), and of bulk soil (BS). Red arrows refer to physicochemical properties of soil, available nitrogen (AN), available phosphorus (AP), available potassium (AK), total carbon/total nitrogen (C/N), bulk density (BD), soil water content (SWC), urease (URE), saccharase (SAC), catalase (CAT), and alkaline phosphatase (AKP). Green arrows refer to Chytridiomycota (Chy), Ascomycota (Asc), Basidiomycota (Bas), and Cryptomycota (Cry).

4. Discussion

4.1. Fungal Diversity of the Rhizosphere

ECM fungi create a highly mutual symbiotic combination of mycorrhizal fungi and host-plant roots [35]. This unique symbiotic relationship can help plants survive in adverse conditions, especially in soil contaminated with heavy metals [17,28]. Our previous study also found that ECM fungi enhanced survival and growth of Masson pine in Pb/Zn tailing areas [36]. In the current study, we used the Illumina MiSeq technique to investigate the diversity and the structures of rhizospheric fungal communities in different inoculation treatments of Masson pine in a mining area contaminated with Pb and Zn. The results thereof indicated that the fungal diversity and richness in the rhizospheric soil of Masson pine were significantly higher than those of bulk soil. However, there was no significant difference between the soil with and without ECM-fungus inoculation (Table 2), which illustrates that the difference in plant rhizospheric fungal diversity was mainly determined by the "rhizosphere effect" of each plant [17,37]. Plants frequently exchange nutrients and water with the surrounding environment of the root system during the growth process, which causes the microenvironment in the rhizosphere to be different from that in non rhizospheric soil. Those differences could have resulted in the great discrepancies of the richness and diversity in the rhizospheric soil of the fungal groups.

Previous studies showed that the fungal ergosterol content was much higher in potato rhizospheric soil than in soil without plants in the growing-season stage [38]. In addition, Thion et al. (2012) found that the diversity of fungi in rhizospheric soil planted with alfalfa was significantly higher than that of non rhizospheric soil in an in situ experiment [17]. Obviously, many factors determine the relative abundance and diversity of soil fungal communities; among them, the plants' roots seem to have changed the physicochemical properties of the soil microenvironment, such as soil organic matter, water content, and enzymes, which greatly impact the growth and number of fungi species indirectly [39]. Those results strongly support that the importance of plants and their "rhizosphere effects" is the main reason for the discrepancies in the diversity and structures of the soil microorganisms. Other studies also showed that ECM-fungus inoculation would decrease the diversity of

fungal communities. According to Li et al. (2017), bacterial and fungal diversity reduced when *Pinus armandii* was inoculated with *Tuberindicum* during the early symbiotic stage, as compared with non ectomycorrhizal-fungus soil [40]. Compared with bulk soil, the great reduction in microbial diversity in the rhizosphere was mainly attributed to large discrepancies between plant host species [37] or related to the surrounding sampling-soil properties of the rhizosphere [39].

Altogether, our results revealed that even in a highly polluted mining area (Pb: 125.33 mg/kg; Zn: 233.40 mg/kg), plants' rhizospheres had a protective effect on the growth of the microbial abundance and the diversity hosted in the plants when compared to the soil without plants, which may be related to a reduction in the toxicity of heavy metals on the rhizospheres.

4.2. Indicators of the Representative Fungal Genus

The differences in the fungal-community structures reflect how mycorrhiza and their metabolites, such as enzymes, extracellular polysaccharides, amino acids, and growth hormones, have great effects on their surrounding environments. The differences in the dominant populations may indicate that some mycorrhizal fungi can adapt to specific environmental stress, especially in rhizospheric soil polluted by heavy metals [22,23]. Although there was no significant difference of rhizospheric fungal-community diversity between the WE and NE treatments, the community composition and dominant species of the rhizospheric fungi were obviously different in the soil inoculated with *Suillus* fungi (Figures 3 and 4). Previous studies in a forest ecosystem found that the phyla of Ascomycota and Basidiomycota were the dominant fungal communities both in rhizospheric soil and in root samples of *Pinus armandii* with *Tuber indicum* inoculation [40]. Through 454 pyrosequencing, Liu et al. (2015) also found that the rhizosphere of Xinjiang jujube was mainly colonized by Ascomycota and, Basidiomycota, whereas Chytridiomycota and Glomeromycota were probably restrained [41]. However, in our research, besides Basidiomycota, more abundant Chytridiomycota existed in the ECM-fungus-inoculated soil compared with the bulk soil, while the relative abundance of Ascomycota was low, which suggests that Basidiomycota and Chytridiomycota might be closely related to the presence of ECM fungus (*Suillus luteus*) synthesis (Figures 5 and 6). It also should be noted that the fungal structures and composition were highly related to the soil environment, and each plant–fungus symbiont was undoubtedly shifted with the soil pH, moisture, and nutrients, which indirectly determine fungal-community composition [40].

In addition, our results showed that the relative abundance of *Suillus*, *Paraglomus*, *Agaricus*, *Tulasnella*, and *Melanotaenium* were functionalities of the abundant fungal genera in the WE-treated soil (Figure 5). Some of these fungal genera are important mycobionts with plants and enforce plant adaptability for growth in stressful conditions, such as metal-contaminated soils. Previous studies showed that *Pinus massoniana* seedlings were significantly more resistant to heavy metals when inoculated with *S. luteus* than were non mycorrhizal plants [28]. Besides *Suillus luteus*, some mycorrhizal-fungus (*Suillus bovinus*)-inoculated plants (e.g., Pinaceae and Fagaceae) have been reported to develop tolerance to heavy metals such as Zn and Cd [15] and improve survival of plants on heavy-metal-contaminated soils [26]. Others, such as *Paraglomus*, a common arbuscular mycorrhizal (AM) fungus that is geographically widespread though locally sporadic in tilled agricultural soils [42], can increase the growth performance of peach seedlings, elevate absorption of essential elements (K, Mg, Fe, and Zn) for plants' growth and photosynthesis, and fix heavy metals, such as Cu^{2+} and Mn^{2+}, to the root system when the plants are potted [43]; *Tulasnella* species can increase carbohydrates and other nutrients for plant uptake in the soil, thus accelerating the adaptation process of plants in stressful environments [44]. These fungal mycobionts that adhere to the roots of plants can form a huge network system underground, inducing variation of the soil microenvironment through formation of a physical barrier or a Hartig network [45] or release of related antioxidant enzymes, such as chitin, melanin, and cellulose, to acquire nutrients from organic compounds [46]. The

unique species that have adapted to these special environments would help plants survive against environmental stress.

4.3. Relationships between Fungal-Community Structures and Soil Properties

Soil processes may be primarily affected by interaction among surrounding environmental factors, the structures of soil microbiota communities, and the reciprocal relationship thereof [47]. Alternatively, this mutual interrelationship has a significant impact on the processes of the ecosystem through feedback mechanism. Fungal communities act as a "bridge" between plants, the soil environmental condition, and the relationship thereof [48], which play vital roles in driving the nutrient- and carbon-cycling processes [11]. Numerous studies have indicated that physicochemical soil properties, such as pH, soil C/N, and soil water content, could shape fungal communities' compositions and structures in plants' rhizospheres [49], especially those in heavy-metal soils [50]. Our results showed that the environmental variables of soil water content, C/N, and bulk density were important factors in determining the composition and diversity of soil fungi (Figure 7).

Previous studies have also indicated that fungal symbionts form an important interface between soil and a plant's rhizosphere, greatly affecting physicochemical soil properties and the microbial community structure in rhizospheric soil [20], which is consistent with our results. As such, the existence of ECM fungi that interact with the rhizospheric environment of a host plant could thus affect fungal abundance, and ECM fungi could influence the fungal community via alteration of physicochemical properties in heavy-metal-polluted mining tailings. According to Luo et al., the ECM fungi that form the first biological barrier of the sheath structure against toxic metal ions, the so-called Hartig net, directly change root morphology to absorb heavy metals and mineral nutrients [51].

In addition, ECM fungi can also enhance host-plant tolerance to heavy metals through the indirect effects of secretion of secondary metabolites. For example, ECM fungi can secrete a large amount of organic acids as a potential ligant to combine with heavy metals, change the soil pH, reduce the effectiveness of heavy-metal elements, and play an important role in the heavy-metal tolerance and the detoxification process of a symbiont [52]. A large number of experiments have proved that ECM fungi can produce a certain amount of oxalic acid, whether under the conditions of pure culture or symbiosis, with plants, and oxalic acid is considered by most scholars to be one of the main products of ECM fungi against heavy-metal stress, with a strong ability to chelate heavy-metal ions [53]. Furthermore, the hormones or hormone conjugates secreted by ECM fungi can induce a systemic resistance-related hormone molecular signaling pathway; regulate the metabolic functions of plant cells; and affect the absorption, transport, and intracellular distribution of heavy metals in plants [54], which enhances the adaptability of symbiotic plants to heavy metal in soil [15].

Extracellular enzymes in soil are another kind of bioactive substance with catalytic abilities, decomposed and released by microorganisms and plants. Soil fungi play an important part in enzyme secretion that is related to nutrient absorption and mobilization, such as of nitrogen and/or phosphorus [11,55]. Among these fungal communities, groups of ECM fungi usually form symbiotic relationships with the roots of plants, especially under the condition of nutrient limitation. ECM fungi could receive carbohydrates from their plant hosts to power nutrient transformation [56] and soil-enzyme expression [11]. In our research, by combining 18S rDNA sequencing with soil-enzyme analyses, we demonstrated relationships between fungal-community structures and soil-enzyme activities with ECM fungi inoculated into Masson pine (Figure 7). Obviously, ECM fungi had a major influence on soil-enzyme activities during restoration processes (Table 1). According to Burke et al. (2011), ECM fungi were positively associated with soil phosphate content and some soil enzymes of phosphatase (organic P degradation) and N-acetyl-β-glucosaminidase (NAG; organic N and chitin degradation) in a field experiment on a northern hardwood forest [57]. Other research also showed that certain ECM fungi, such as the genera Cortinaria and Russula, are significantly associated with lignin-degrading Mn-peroxidase enzyme activity [48] and plant-cell-wall degradation in mineralization of nitrogen and phosphorus for

the mutualistic symbiotic system in deeper layers of humus [58], which is consistent with our research.

It is noteworthy that the discrepancy of diversity in plants' rhizosphere fungal communities might be determined by the "rhizospheric effect" of each plant, while the dominated keystone fungal species chosen by the Suillus–Pinus symbionts showed a more effective way to establish the soil microenvironment system and promote growth of plants in heavy-metal-polluted mining tailings.

5. Conclusions

As a mutual symbiosis system, the Suillus–Pinus symbiont is of great significance to promotion of pioneer tree species for phytoremediation in heavy-metal-contaminated mining areas. This study found that inoculation of ectomycorrhizal fungi further enlarged plants' capability to absorb heavy metals and secrete soil enzymes. In addition, different treatments also significantly affected the composition and structures of rhizospheric fungal communities, among which the fungal-community diversity was highest in Masson-pine rhizospheric soil. The relative abundances of *Suillus*, *Paraglomus*, *Agaricus*, and *Tulasnella* were the highest with ECM-fungus inoculation. The physicochemical properties of the soil under Pinus massoniana, especially SWC, C/N, and bulk density, were also some of the dominant factors that affected fungal-community diversity and structure. In the future, it will be particularly important to further explore mechanisms of ECM fungi that promote host-plant adaptation to heavy-metal-contaminated soil from more perspectives.

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