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Soil Physicochemical Properties and the Rhizosphere Soil Fungal Community in a Mulberry (*Morus alba* L.)/ Alfalfa (*Medicago sativa* L.) Intercropping System

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Received: 24 January 2019; Accepted: 13 February 2019; Published: 16 February 2019

Abstract: A better understanding of soil fungal communities is very useful in revealing the effects of an agroforestry system and would also help us to understand the fungi-mediated effects of agricultural practices on the processes of soil nutrient cycling and crop productivity. Compared to conventional monoculture farming, agroforestry systems have obvious advantages in improving land use efficiency and maintaining soil physicochemical properties, reducing losses of water, soil material, organic matter, and nutrients, as well as ensuring the stability of yields. In this study, we attempted to investigate the impact of a mulberry/alfalfa intercropping system on the soil physicochemical properties and the rhizosphere fungal characteristics (such as the diversity and structure of the fungal community), and to analyze possible correlations among the planting pattern, the soil physicochemical factors, and the fungal community structure. In the intercropping and monoculture systems, we determined the soil physicochemical properties using chemical analysis and the fungal community structure with MiSeq sequencing of the fungal ITS1 region. The results showed that intercropping significantly improved the soil physicochemical properties of alfalfa (total nitrogen, alkaline hydrolysable nitrogen, available potassium, and total carbon contents). Sequencing results showed that the dominant taxonomic groups were *Ascomycota*, *Basidiomycota*, and *Mucoromycota*. Intercropping increased the fungal richness of mulberry and alfalfa rhizosphere soils and improved the fungal diversity of mulberry. The diversity and structure of the fungal community were predominantly influenced by both the planting pattern and soil environmental factors (total nitrogen, total phosphate, and total carbon). Variance partitioning analysis showed that the planting pattern explained 25.9% of the variation of the fungal community structure, and soil environmental factors explained 63.1% of the variation. Planting patterns and soil physicochemical properties conjointly resulted in changes of the soil fungal community structure in proportion.

Keywords: Agroforestry; Fungal community; MiSeq sequencing; Mulberry; Alfalfa

1. Introduction

Soil microorganisms are a vital component of soil ecosystems and play an important role in terrestrial ecosystem processes, especially the regulation of carbon and nutrient cycles. Soil microorganisms depend on carbon resources supplied by plant litter and root exudates and they can be influenced by changes in plant-derived organic matter [1,2]. There are differences in plant litter chemistry and root exudates among different vegetation types, and their effects on below-ground

ecosystem processes vary [3,4]. Therefore, changes in land use can affect the structural and functional characteristics of the soil microbial community [5,6].

Agroforestry is defined as "an approach to land use" that incorporates trees into farming systems and allows woody perennials to be managed on the same unit of land as agricultural crops, livestock, or both [7–9]. Compared to conventional monoculture farming, agroforestry systems have obvious advantages in improving land use efficiency [10] and maintaining soil physicochemical properties, reducing losses of water, soil material, organic matter and nutrients, and ensuring stability of yields [11,12]. Moreover, an agroforestry system has relatively high diversity and stability of soil microbial communities, especially the diversity and abundance of beneficial microbial populations, such as arbuscular mycorrhizal (AM) fungi that can enhance the productivity of crop plants by increasing the availability and supply of limiting nutrients [13–16]. Agroforestry practices have been widely applied in Chinese agriculture [17]. Recently, researchers showed a strong interest in these types of cropping systems due to their capacity to produce higher yields and to counteract resource degradation [18]. However, previous studies on the effects of intercropping on soil microorganisms mainly concentrated on bacteria, with only limited studies on fungi [5,6,13].

Fungi are widely distributed species in the soil system; they have a high ability to degrade complex substrates of plant origin, representing up to 90% of net primary productivity in most terrestrial ecosystems. In addition, the usually mutualistic symbioses known as mycorrhizae between many fungi and plant roots have huge impacts in both ecological and economic terms [19,20]. Fungi are spatially ubiquitous and are more susceptible than bacteria to disturbance, such as land use change [21]. Intercropping mulberry (*Morus alba* L.) with alfalfa (*Medicago sativa* L.) is an important planting type for increasing forage yield in the semiarid and salt-alkali soil regions in the Songnen Plain of northern China. The use of a mulberry/N-fixing crop intercropping system has been demonstrated to improve crop yields and to modify salt-alkali soil, as the N-fixing crop provides natural N fertilizer for tree growth and the trees improve the soil total nitrogen and carbon status [22]. Therefore, a better understanding of soil fungal communities is very useful to reveal the effects of an intercropping system and would also help us to understand the fungi-mediated effects of agricultural practices on the processes of soil nutrient cycling and crop productivity. In this study, we attempted to investigate the impact of a mulberry/alfalfa intercropping system on the soil physicochemical properties and rhizosphere fungal characteristics (such as the diversity and structure of the fungal community) and to analyze possible correlations among the planting pattern, the soil physicochemical factors, and the fungal community structure.

2. Results

2.1. Physicochemical Properties of the Rhizosphere Soil of the Mulberry and Alfalfa Intercropping System

Intercropping significantly affected the contents of total N, available K, and total C of the crop rhizosphere soils (Table 1). The total N and available K significantly decreased in the rhizosphere soil of intercropped mulberry ($p < 0.05$). However, the total N, available K, and total carbon significantly increased in the rhizosphere soil of intercropped alfalfa ($p < 0.05$).

Table 1. Physicochemical parameters of different treatments.

Sample	TN g/kg	AHN mg/kg	TP g/kg	AK mg/kg	TC g/kg
MM	0.91 ± 0.00 c	148.88 ± 6.96 ab	0.41 ± 0.06 a	5.02 ± 0.7 c	2.23 ± 0.00 a
IM	0.54 ± 0.00 b	123.07 ± 10.88 a	0.46 ± 0.02 a	3.35 ± 0.00 b	2.22 ± 0.00 a
MA	0.79 ± 0.04 a	111.37 ± 3.37 a	0.42 ± 0.00 a	2.99 ± 0.07 a	2.16 ± 0.00 a
IA	0.93 ± 0.06 c	227.34 ± 87.84 b	0.46 ± 0.01 a	6.26 ± 0.21 d	2.52 ± 0.00 b
Results of two-way ANOVA					
Int	29.15 **	2.73	7.29	144.90 *	11.49 **
Crv	94.29 **	3.23	0.029	710.75 **	9.00 **

Values are means ± standard deviation ($n = 3$). Different lowercase letters indicate significant differences among different samples according to Duncan's multiple range test ($p < 0.05$). Note: MM, IM, MA, IA represent monoculture mulberry, intercropped mulberry, monoculture alfalfa, and intercropped alfalfa, respectively. TN, AHN, TP, AK, and TC represent total nitrogen, alkaline hydrolysable nitrogen, total phosphate, available potassium, and total carbon, respectively. Int and Crv represent the intercropping treatment and crop variety, respectively. * $p < 0.05$, ** $p < 0.01$.

2.2. Fungal Shannon-Wiener Curve

A total of 315,837 ITS sequences were obtained from the eight soil samples by MiSeq analysis of the ITS1 region of fungi. As the read numbers per sample were uneven (ranging from 20,418 to 37,373), all samples were reduced to the same size using MOTHUR software (version 1.39.5, http://github.com/mothur/, Detroit, MI, USA) based on the smallest read number (20,418).

Operational Taxonomic Units (OTUs) were identified at a similarity level of 97%; almost all curves reached the saturated state, indicating that the survey effort was sufficiently large to capture the complete diversity of these communities, as the curves leveled off with increasing sample sizes (Figure S1).

2.3. Fungal Community Richness and Diversity

Among all samples, a total of 5844 OTUs were obtained, ranging from 2848 in the intercropping soils to 2996 in the monoculture soils (Table 2). The coverage of all samples was estimated to reach 99%. The mean community richness (the Chao1 estimator) was higher in the alfalfa rhizosphere soil than in the mulberry rhizosphere soil. The community diversity estimators, including the Shannon index and Simpson index, suggested that the values in the intercropped mulberry soils exceeded those in the corresponding monoculture soils; however, the community diversity indices of monoculture and intercropped alfalfa soils were similar.

Table 2. Fungal richness and diversity of different planting patterns.

Sample	Cluster Distance (0.03)				
	OTUs	Chao 1	Coverage	Shannon	Simpson
MM-1	321	416	1.00	4.08	0.040
MM-2	382	473	1.00	4.10	0.048
MM-3	480	873	0.99	4.20	0.041
IM-1	605	788	0.99	4.34	0.037
IM-2	503	600	1.00	4.34	0.033
IM-3	705	1006	0.99	4.51	0.028
MA-1	486	544	1.00	4.38	0.028
MA-2	522	695	0.99	4.42	0.026
MA-3	657	898	0.99	4.50	0.025
IA-1	321	416	1.00	4.08	0.040
IA-2	382	473	1.00	4.10	0.048
IA-3	480	873	0.99	4.20	0.041
Total	5844	–	–	–	–

Note: The description of abbreviation names is shown in Table 1. MM-1, MM-2, and MM-3 represent three repeats of MM; IM-1, IM-2, and IM-3 represent three repeats of IM; MA-1, MA-2, and MA-3 represent three repeats of MA; and IA-1, IA-2, and IA-3 represent three repeats of IA. The same abbreviations appear below.

Beta diversity analysis showed dissimilarity values among the different treatments based on the biological distances. The results indicated that the degree of fungal community dissimilarity between mulberry and alfalfa was different. Intercropping changed the fungal communities of mulberry and alfalfa. The Bray-Curtis distances were 0.4889 (between MM and IM) and 0.4944 (between MA and IA). In addition, the Bray-Curtis distance between mulberry and alfalfa decreased from 0.5689 (between MM and MA) to 0.4222 (between IM and IA) (Figure S2).

2.4. Taxonomic Structure of Fungal Communities among Different Treatments

All valid sequences from the soil sample libraries were classified from phylum to species based on the Unite Blast database. The results showed differences in fungal community abundance at different phylogenetic levels (Tables S1–S3).

At the phylum level, a total of seven phyla were identified in all the soil samples. The four dominant phyla were *Ascomycota* (59.92%), *Mucoromycota* (18.81%), *Basidiomycota* (12.32%), and unclassified fungi (8.67%); they accounted for 99.82% of the total relative abundance (Figure 1, left). Other sequences belonged to *Blastocladiomycota*, *Chytridiomycota*, and *Zoopagomycota*, which were always found in very low proportions (<2%). The primary fungi (*Ascomycota*) mainly consisted of *Sordariomycetes*, *Pezizomycetes*, *Dothideomycetes*, *Leotiomycetes*, and *Eurotiomycetes* (Figure 1, right).

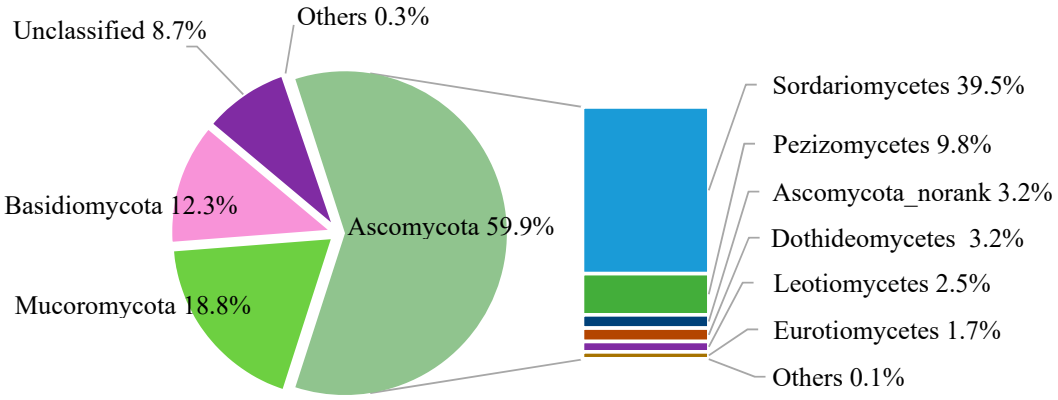

Figure 1. The bar of the pie plot showing the relative abundance of soil fungal phyla (left) and *Ascomycota* classes (right) with different treatments. The four dominant phyla were *Ascomycota* (59.9%), *Mucoromycota* (18.8%), *Basidiomycota* (12.3%), and Unclassified (8.7%), respectively. The primary fungi (*Ascomycota*) mainly consisted of *Sordariomycetes*, *Pezizomycetes*, *Dothideomycetes*, *Leotiomycetes*, and *Eurotiomycetes*.

Comparative analysis of monoculture and intercropped treatments revealed a distinct distribution of the fungal phyla (Figure 2). In the dominant phyla, the relative abundances of *Mucoromycota*, *Basidiomycota*, and *Zoopagomycota* were significantly different among the different planting patterns (Figure S3). For mulberry rhizosphere soil, the relative abundances of *Mucoromycota* and *Zoopagomycota* presented as being significantly higher in the intercropped treatments ($p < 0.05$), whereas *Basidiomycota* showed higher relative abundance in the monoculture treatments than in the intercropped treatments ($p < 0.05$).

For the alfalfa rhizosphere soil, *Basidiomycota* showed higher relative abundance only in the intercropped treatments than in the monoculture treatments ($p < 0.05$), whereas the differences of other fungal phyla were not significant among the different treatments ($p > 0.05$).

At the class level, the structure of the fungal community also differed between treatments (Figure 3 and Table S2). For the mulberry rhizosphere soil, *Ascomycota_norank* (*Ascomycota*), *Zoopagomycota_norank* (*Zoopagomycota*), Unclassified fungi, *Glomeromycetes* (*Glomeromycota*), *Pezizomycetes* (*Ascomycota*), and *Mucoromycota* (*Mucoromycota*) showed higher relative abundances in the intercropped treatments than in the monoculture treatments ($p < 0.05$), whereas *Geminibasidiomycetes* (*Basidiomycota*), *Tremellomycetes* (*Basidiomycota*), *Leotiomycetes* (*Ascomycota*), and *Sordariomycetes* (*Ascomycota*) showed higher relative abundances in the monoculture treatments than in the intercropped treatments ($p < 0.05$). In the alfalfa rhizosphere soil, *Leotiomycetes* (*Ascomycota*), *Pezizomycetes* (*Ascomycota*), *Eurotiomycetes* (*Ascomycota*), *Cystobasidiomycetes* (*Basidiomycota*), and *Dothideomycetes* (*Ascomycota*) presented marked advantages in the monoculture treatments ($p < 0.05$). In the intercropping system, the differences among the other classes were not significant ($p > 0.05$).

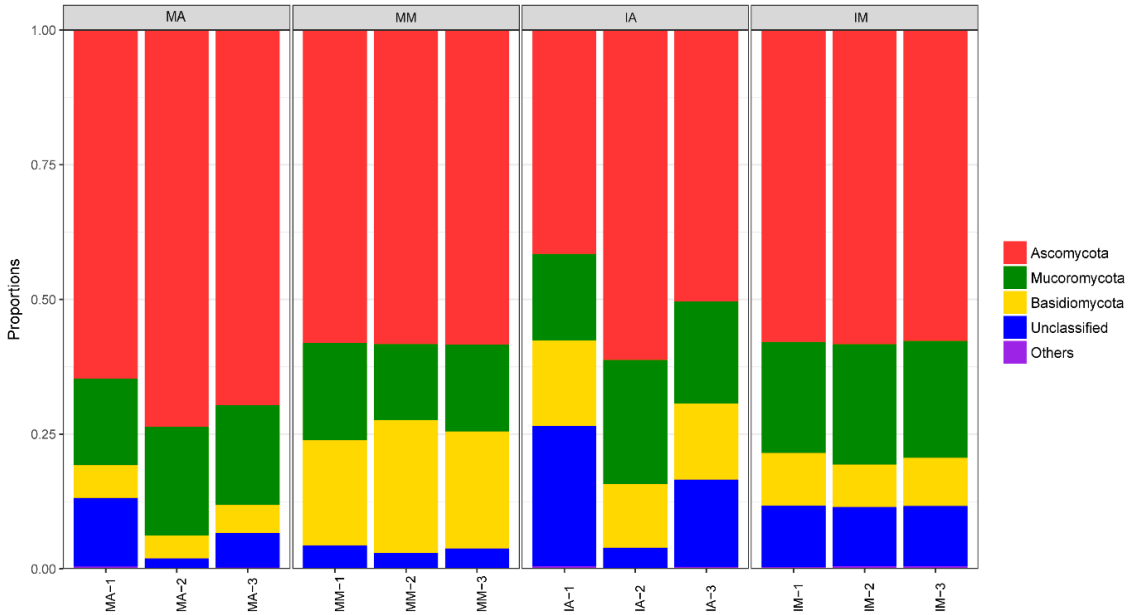

Figure 2. Influence of plant types and planting pattern on the fungal community structure at phyla level in the rhizosphere soil. The dominant phyla were *Ascomycota*, *Mucoromycota*, and *Basidiomycota* in all soil samples. Comparative analysis of monoculture and intercropped treatments revealed a distinct distribution of the fungal phyla. Notes: MM, IM, MA, IA represent monoculture mulberry, intercropped mulberry, monoculture alfalfa, and intercropped alfalfa, respectively. MM-1, MM-2, and MM-3 represent three repeats of MM, IM-1, IM-2, and IM-3 represent three repeats of IM, MA-1, MA-2, and MA-3 represent three repeats of MA, IA-1, IA-2, and IA-3 represent three repeats of IA.

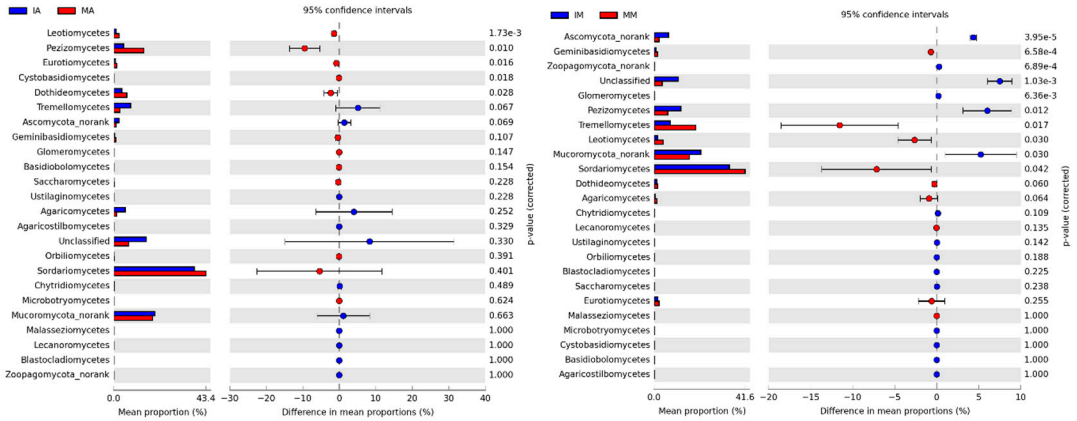

Figure 3. Statistical Analysis of Metagenomic Profiles (STAMP) analysis plots showing the differences in fungal classes in monoculture and intercropping mulberry soil (left) to those in monoculture and intercropping alfalfa soil (right). Welch's *t*-test was applied to test for differences in fungal classes due to planting pattern. The *p*-values are also shown. *Glomeromycetes*, *Pezizomycetes*, and *Mucoromycota* showed higher relative abundances in intercropped mulberry ($p < 0.05$). *Leotiomycetes*, *Pezizomycetes*, *Eurotiomycetes*, *Cystobasidiomycetes*, and *Dothideomycetes* presented higher relative abundances in the monoculture alfalfa ($p < 0.05$).

2.5. Shared Fungal OTUs

The number and differences in OTUs from the soil samples are shown in a Venn diagram (Figure 4). A total of 227 OTUs were common among all soil samples. Moreover, the differences in OTUs demonstrated that each plant rhizosphere had its own fungal population. The number of sequences for each OTU was used to determine the dissimilarity between samples (Figure S4). The IM groups were distinguished from MM, and IA was also distinguished from the MA group, suggesting the clear distinction of the microbial community structure between monoculture and intercropped groups. This conclusion was also supported by the dissimilarity matrix with the Bray-Curtis distance (Figure S2).

Figure 4. Venn diagram showing the shared and unique observed fungal OTUs at 97% similarity among treatments. The unique OTUs demonstrated that a different treatment had its own fungal population. Notes: MM, IM, MA, IA represent monoculture mulberry, intercropped mulberry, monoculture alfalfa, and intercropped alfalfa, respectively.

Hierarchically clustered heatmap analysis, based on the microbial community profiles at the genus level, was used to identify the different structures of these twelve fungal communities (Figure 5). A comparison of the relative abundances at the genus level revealed significant differences in the composition of fungal communities between monoculture and intercropped systems. *Mortierella*, *Schizothecium*, *Fusarium*, *Acremonium*, *Tausonia*, and *Pseudombrophila* were the most abundant genera across all soil samples, representing 20.43%, 11.06%, 8.08%, 7.30%, 5.80%, and 5.16% of all classified sequences in intercropped soils and 17.09%, 4.46%, 9.73%, 12.26%, 8.56%, and 5.86%, respectively, in monoculture soils. The results indicated that these genera might be indigenous. Relative abundances of the dominant genera varied significantly between monoculture and intercropped soils. *Mortierella* (21.77%), *Schizothecium* (11.25%), *Pseudombrophila* (7.45%), *Tetracladium* (6.52%), *Fusarium* (5.94%), *Peziza* (4.66%), and *Humicola* (1.70%) showed higher relative abundances in the mulberry intercropped soil than in the corresponding monoculture soil, whereas *Acremonium* (17.53%), *Tausonia* (16.17%), *Phialophora* (2.18%), *Solicoccozyma* (2.09%), *Neonectria* (1.88%) and *Aspergillus* (1.83%) showed the opposite trend. *Mortierella* (19.59%), *Schizothecium* (10.86%), *Tausonia* (6.52%), *Bolbitius* (2.89), *Tetracladium* (2.56%), *Humicola* (1.32%), and *Nectria* (1.19%) were present in higher proportions in the alfalfa intercropped soil than in the corresponding monoculture soil.

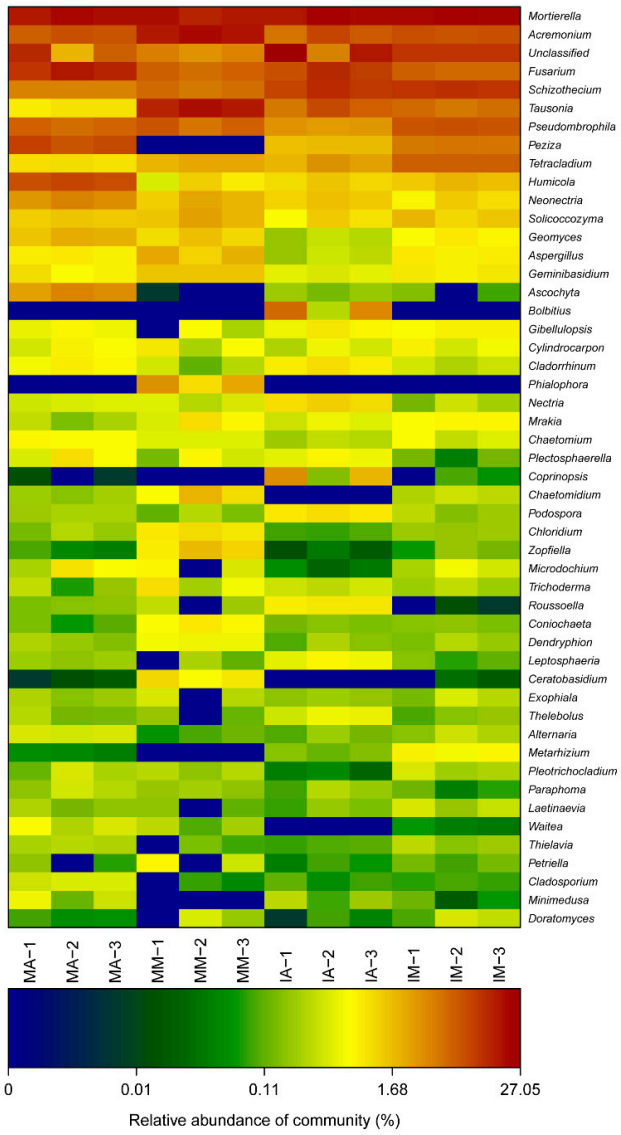

Figure 5. Hierarchical heatmap analysis of 100 predominant fungal communities in different treatments. The OTUs were ordered by genus. Sample communities were clustered based on the complete linkage method. The color intensity of scale indicates the relative abundance of each OTU read. Relative abundance was defined as the number of sequences affiliated with the OTU divided by the total number of sequences per sample. Notes: MM, IM, MA, IA represent monoculture mulberry, intercropped mulberry, monoculture alfalfa, and intercropped alfalfa, respectively. MM-1, MM-2 and MM-3 represent three repeats of MM, IM-1, IM-2, and IM-3 represent three repeats of IM, MA-1, MA-2, and MA-3 represent three repeats of MA, IA-1, IA-2, and IA-3 represent three repeats of IA.

2.6. Relationship of the Soil Physicochemical Properties, Fungal Phyla, and Fungal Communities

Soil TN, TP, and AK were closely correlated with the relative abundances of seven fungal phyla (Table 3). The relative abundances of *Blastocladiomycota* ($r = -0.660$, $p = 0.019$), *Mucoromycota* ($r = -0.656$, $p = 0.021$) and *Zoopagomycota* ($r = -0.925$, $p < 0.001$) were significantly negatively correlated with soil TN. The relative abundance of *Mucoromycota* ($r = 0.599$, $p = 0.040$) had a marked correlation with TP. The relative abundances of *Ascomycota* ($r = -0.726$, $p = 0.008$), *Basidiomycota* ($r = -0.588$, $p = 0.044$) and *Zoopagomycota* ($r = 0.660$, $p = 0.019$) exhibited a highly significant correlation with AK. Additionally, the soil AN and TC had no significant correlation with the abundances of all the fungal phyla. To investigate the relationships between soil fungal community structure and soil physicochemical properties, the OTUs from all soil samples were analyzed using redundancy analysis

(RDA) (Figure 6). Overall, the two RDA axes explained 73.3% of the variation of structure among the different communities.

Table 3. Pearson's correlation (*r*) and significance (*p*) values between the relative abundances of the eight most abundant fungal phyla and the soil physicochemical properties.

Taxonomic Group	TN		AHN		TP		AK		TC	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Ascomycota	−0.295	0.353	−0.150	0.643	−0.391	0.208	**−0.726**	0.008	−0.550	0.064
Basidiomycota	**0.580**	0.048	0.170	0.598	−0.318	0.314	**0.669**	0.017	0.159	0.622
Blastocladiomycota	**−0.660**	0.019	−0.222	0.489	0.434	0.158	−0.336	0.286	−0.194	0.545
Chytridiomycota	0.082	0.800	−0.165	0.607	0.558	0.059	0.205	0.523	0.462	0.131
Mucoromycota	**−0.656**	0.021	0.367	0.240	**0.599**	0.040	−0.224	0.484	0.005	0.988
Unclassified	0.067	0.836	−0.129	0.690	0.507	0.093	0.306	0.333	0.490	0.106
Zoopagomycota	**−0.925**	0.000	−0.307	0.331	0.401	0.197	**−0.588**	0.044	−0.397	0.201

Note: TN, AHN, TP, AK, and TC represent total nitrogen, alkaline hydrolysable nitrogen, total phosphate, available potassium, and total carbon, respectively. Values in bold indicate significant correlations ($p < 0.05$).

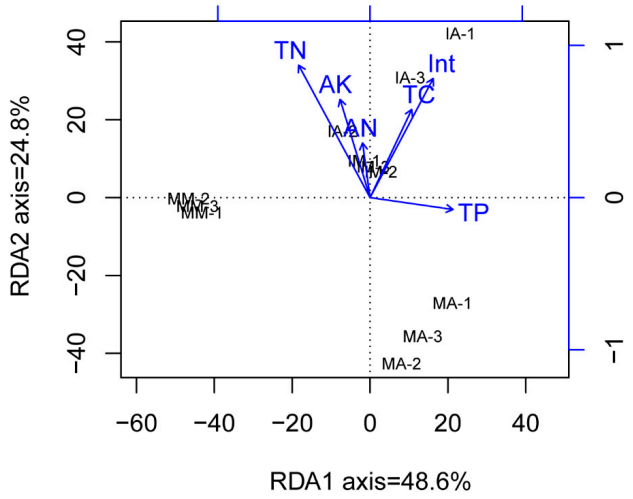

Figure 6. Redundancy analysis (RDA) plot of soil variables, intercropping and soil fungal community in soils from different treatments. The RDA plot was calculated from the relative OTU abundances and soil properties. Arrows of the RDA plot indicate the direction and magnitude of measurable variables associated with fungal community structures. Each word represents a sample. TN had the longest arrow indicating it was the most important in all the shown factors affecting the fungal community. RDA component 1 and 2 explained 48.6% and 24.8% of the total variations of the fungal community structure, respectively. Just based on RDA1, twelve samples were divided into three groups, IM and IA were clustered together, MM and MA were in the different groups. TN, TP, and TC represent total nitrogen, total phosphate and total carbon respectively. Notes: MM, IM, MA, IA represent monoculture mulberry, intercropped mulberry, monoculture alfalfa, and intercropped alfalfa, respectively. MM-1, MM-2, and MM-3 represent three repeats of MM, IM-1, IM-2, and IM-3 represent three repeats of IM, MA-1, MA-2, and MA-3 represent three repeats of MA, IA-1, IA-2, and IA-3 represent three repeats of IA.

A subset of soil environmental parameters of TN, TP, and TC was selected by the BioEnv procedure (Figure 7). The combination of the selected soil physicochemical properties and planting pattern showed a maximum ($r = 0.528$) correlation with the fungal community structure in all the different combinations. These parameters explained 89.0% of the variation of the fungal community structure, leaving 11.0% of variation unexplained. The planting pattern explained 25.9% of the fungal community structure variation. Among the selected soil parameters, TN, TP, and TC explained 29.6%, 17.7%, and 15.8%, respectively, of the fungal community structure variation.

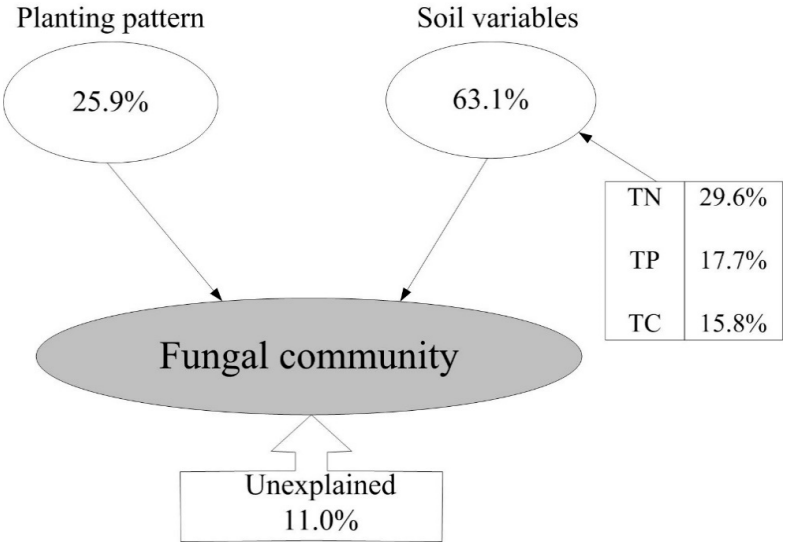

Figure 7. Variance partitioning analysis (VPA) showing the effects of planting pattern and soil variables on the phylogenetic structure of fungal communities based on MiSeq sequencing were calculated from relative OTU abundances and soil chemical properties. The planting pattern explained 25.9% of the variation of the fungal community structure, and soil environmental factors explained 63.1% of the variation. The TN, TP, and TC explained 29.6%, 17.7%, and 15.8% of the variation, respectively.

3. Discussion

3.1. Soil Physicochemical Properties

Soil microorganisms are the principal drivers of soil nutrient cycling processes [23]. Mycorrhizal fungi serve as conduits from the soil to plant roots, transferring water [24,25] and inorganic and organic nutrients to the host plant using a network of extra radical hyphae [26]. In alfalfa rhizosphere soils, the intercropped treatment significantly increased TN, TC, and AK. Tracing the ^{15}N isotope transfer, previous study suggested that arbuscular mycorrhizal fungi (AMF) could improve the efficiency of the biological N fixation of legume plants [27]. However, we found no significant difference in the richness of AMFs (*Mucoromycota*) in the intercropped alfalfa soil. The richness of *Basidiomycota* and *Ascomycota* showed opposite trends in the intercropped treatments. Some reports have shown that EM and ericoid mycorrhizas (*Ascomycota* and *Basidiomycota*) are able to use organic N sources [28]. The decreasing richness of *Ascomycota* could be a reason for the increasing TN content in the intercropped alfalfa soil. Another factor may be the nutrients released by agroforestry trees to meet crop demands [29]. The amounts of TN and AK in intercropped mulberry soil were significantly lower than those in the monoculture mulberry soil. Many plant species form symbiotic relationships with mycorrhizal fungi to augment nutrient uptake from soil [30–32]. In addition, soil physicochemical properties could also be affected by aboveground vegetation types, which cause a change in litter composition (both above- and below-ground) and/or microbial community structure. Therefore, the significant decrease in the contents of TN and AK might be attributed to sufficient utilization of soil nutrients by intercropped mulberry with the help of the increased richness of *Mucoromycota* and *Zoopagomycota*. The changes in yield can partly reflect the nutrient uptake of plants. Based on our results, intercropping significantly increased the yields of mulberry and alfalfa (Table S4). These results indicated that intercropping increased the yields of mulberry and alfalfa through different methods. Intercropping increased mycorrhizal formation, which in turn improved N and K acquisition in mulberry soil; however, the nutrients released by intercropped mulberry appeared to meet the demands of alfalfa growth.

3.2. Fungal Community Diversity and Structure in the Intercropping System

We assessed fungal community diversity and structure between monoculture and intercropped soils. The richness index Chao 1 revealed a richer fungal community in intercropped mulberry soils. In addition, the Shannon index and Simpson index of the soil fungal community in the intercropped mulberry system were higher than in the monoculture mulberry system (Table 2). Beta diversity analysis also showed that the dissimilarity of fungal communities between mulberry and alfalfa rhizosphere soils decreased as a result of intercropping (Figure S2). These observations are supported by the following studies. Agroforestry systems may support a more abundant and diverse AM fungal community compared to conventional monoculture [7,14]. Tree-based intercropping system presented a more heterogeneous vegetation cover and rooting pattern, which increased the diversity of soil microbial communities [15]. Therefore, effects of plant species and intercropping on the richness and diversity of the soil fungal community were observed. Intercropping improved the fungal community richness and diversity of mulberry.

In this study, the dominant taxonomic groups were *Ascomycota*, *Mucoromycota* and *Basidiomycota* (Figure 1, left). They have been depicted as major fungal phyla [33–35]. The *Ascomycota* group mainly included *Sordariomycetes*, *Pezizomycetes*, *Dothideomycetes*, *Leotiomycetes*, and *Eurotiomycetes* (Figure 1, right). The benefit of tree-based intercropping systems is a comprehensive result of the positive and negative interactions between the tree and crop components [36]. The fungal community structures in intercropped treatments differed in mulberry and alfalfa rhizosphere soils (Figure 2).

The relative abundance of *Glomeromycetes* was significantly higher in the intercropped mulberry soil than in the monoculture mulberry soil (Figure 3A and Table S2). AM fungi with the majority of plants, is one of the most ubiquitous terrestrial symbioses [37,38]. AM fungi are able to penetrate the cells of the host plant with their fungal structures (arbuscules, vesicles, hyphae). Our results indicate that the mulberry/alfalfa intercropping system had a positive effect on the relative abundance of AM fungi (*Mucoromycota*) in the intercropped mulberry soil. Some studies have found that tree and legume plant intercropping systems improved the richness of soil microorganisms [14,15,27]. The positive effect seems to result from increased plant diversity; intercropping supports a higher number of possible host–fungal pairings, and there is increased density of plant roots available for colonization [39]. However, the mulberry/alfalfa intercropping system had the opposite influence on AM fungi in the intercropped alfalfa soil. The relative abundance of *Glomeromycetes* was higher in the monoculture alfalfa soil than in the intercropped alfalfa soil (Figure 3B and Table S2). Leal [40] reported a similar result and found that agroforestry sites had considerably lower AM fungal abundance than samples collected from pastures and cropped land.

Ascomycota, *Basidiomycota*, and *Mucoromycota* are known as major phyla in the Kingdom Fungi. The phylum *Ascomycota* is the largest phylum. The saprotrophic capabilities of *Ascomycetes* range from the breakdown of simple sugars to degradation of the lignocellulose complex [41]. The phylum *Basidiomycota* is the second largest phylum, known as white-rot fungi, which have the ability to restrict mainly the decomposition of lignin (a heterologous aromatic polymer) [42]. *Mucoromycota* comprise *Glomeromycotina*, *Mortierellomycotina* and *Mucoromycotina*. *Mucoromycota* is a more derived clade of *Zygomycetes* and mainly consists of mycorrhizal fungi, root endophytes, and decomposers of plant material, which are important for soils [43]. With respect to mulberry, the relative abundances of *Mucoromycota* and *Chytridiomycota* were significantly higher in the intercropped soil than in the monoculture soil, whereas the relative abundance of *Ascomycota* and *Basidiomycota* showed the opposite pattern. The relative abundance of *Basidiomycota* was significantly higher, whereas the relative abundances of *Ascomycota* and *Zoopagomycota* were lower in the intercropped soil of alfalfa than in the monoculture soil of alfalfa (Figure 2 and Table S1). Soil fungi utilize carbon sources provided by plant litter and root exudates; hence, changes in plant-derived organic matter can affect soil fungal communities [1,2]. The results indicate that both the planting pattern and plant species changed the structure of the dominant fungal phyla.

3.3. Correlation between Soil Physicochemical Properties and the Dominant Fungal Phyla

Not only has soil fungi an essential impact on biogeochemical cycling [44,45], but changes in soil nutrient status affect the fungal community structure closely [46]. To investigate the relationships between the soil fungal community structure of different samples and the measured soil physicochemical properties, the fungal phyla and OTU data were analyzed using Pearson's correlation and RDA, respectively (Table 3 and Figure 6). TN had a significant negative correlation with the relative abundance of *Mucoromycota* ($r = -0.656$, $p = 0.000$), which is in accordance with previous studies reporting that AMF could improve the efficiency of utilization of nitrogen and total plant yield [27]. AK had a negative correlation with the relative abundance of *Ascomycota* and a positive correlation with the relative abundance of *Basidiomycota*, in accordance with previous studies that found that *Tuber melanosporum* (*Ascomycota*, *Pezizomycetes*) showed a marked reduction in K^+ concentration [47]. However, *Rhizopogon* sp. and *S. granulatus* (*Basidiomycota*, *Agaricomycetes*) could be considered important accumulators between soil and trees for K^+ sequestration due to the strong mineral degradation capacities of these two ectomycorrhizal fungi [48,49]. Moreover, voltage-dependent K^+-selective channels were found exclusively in *Basidiomycota* fungi, whereas they were absent in sequenced *Ascomycota* fungi [50]. *Zoopagomycota* fungi had a negative correlation with AK-like *Ascomycota* fungi and a negative correlation with TN. TN, AK, and TP had some positive and/or negative correlations with the fungal phyla, suggesting that these environmental variables affected the dominant fungal phyla.

3.4. Effects of Soil Properties and Planting Pattern on the Fungal Community Structure

RDA results indicate the distinctions in the fungal community structure between the mulberry and alfalfa groups. Intercropped samples (IM and IA) were clustered together and were well separated from monoculture samples (MM and MA), whereas there was a distinction between monoculture and intercropped samples, and monoculture mulberry samples were separated from monoculture alfalfa samples. The results suggested that plant species affected the fungal communities in the soil used to support those plants, while the planting pattern had a greater effect on the fungal community structure. The influences of soil physicochemical properties in the RDA bioplot are indicated by arrows, of which the lengths are proportional to their importance [51]. TN and TP had the longest arrows, indicating that they were the most important in affecting the fungal community, followed by the planting pattern, and then TC, AK, and AN.

Variance partitioning analysis (VPA) quantifies the effects of two or more groups of environmental variables representing some distinct, ecologically interpretable phenomena [52]. VPA clearly demonstrated that the planting pattern and soil physicochemical properties impacted the soil fungal community structure (Figure 7). The basic factors of soil characteristics (63.1%) were more significant than the operational factor of the planting pattern in determining the distribution of the soil fungal communities, in addition to the planting pattern (25.9%) which was also crucial.

4. Materials and Methods

4.1. Site Description and Soil Sampling

In 2011, an experimental field was established by the Institute of Crops, Heilongjiang Academy of Land Reclamation and Agricultural Sciences in Jiamusi City, Heilongjiang Province (46°46′ N, 130°27′ E), P.R. China. The treatments were (1) monoculture mulberry (MM), (2) intercropped mulberry (IM), (3) monoculture alfalfa (MA), and (4) intercropped alfalfa (IA). The soil is a meadow soil with the following characteristics: organic nitrogen 0.58 $g \cdot kg^{-1}$, available phosphorus 128.2 $mg \cdot kg^{-1}$, available potassium 106 $mg \cdot kg^{-1}$, and pH 6.8. The field site had been previously used for monoculture alfalfa. The samples for the study described here were collected in 2013, i.e., the third year after establishment. The same soil preparations, row spacing, fertilization, and harvesting procedures were used for three years. Three plots (replications) of 5 m × 7.26 m were set up for each treatment and were

randomly distributed in the field. All treatments received farmyard manure at an application dose of 30,000 kg·ha^{-1} and ammonium phosphate of 150 kg·ha^{-1}. The manure was applied by a common fertilization machine (Plot seed TC from WINTERSTEIGER, AG, Ried im Innkreis, Austria). The field management was carried out according to routine management practices [53].

The cultivars used were *Morus alba* 'Qinglong' and *Medicago sativa* 'Zhaodong'. Mulberry saplings with a height of 30 cm were cultivated in mid-April (0.67 m inter-plant distance and 11 plants per row), and alfalfa seeds were sown in early April 2012 (the seeding amount was 22.5 kg·ha^{-1}). In the intercropping system, two rows of mulberry trees and two rows of alfalfa (0.66 m inter-row distance) were intercropped, with a total of 12 rows in each plot. The inter-row spacing for monoculture was the same as that for intercropping.

The mulberry and alfalfa rhizosphere soils were randomly sampled using a five-point sampling method in each plot of the monoculture systems, and from the central rows in each plot of the intercropping systems, by uplifting intact roots on 16 August 2013 (by which time alfalfa had generally reached the early flowering stage). After shaking off the loosely adhered soil, the soil tightly adhering to the root surface was brushed off and collected as the rhizosphere soil samples [54]. The rhizosphere soils obtained from 5 plants in each plot were mixed and transported immediately to the laboratory. Each of the soil samples was sieved (<2 mm) and then divided into two aliquots in sealed bags: one aliquot was stored at −80 °C (for DNA extraction) and the other aliquot was stored at 4 °C (for determination of soil physicochemical properties).

Total nitrogen (TN) was determined using a Kjeldahl apparatus (BUCHI Ltd., Flawil, Switzerland) after digestion with concentrated H_2SO_4. Total carbon was examined using a Total Organic Carbon analyzer (TOC, Vario TOC cube, Elementar, Langenselbold, Germany). Total phosphorus (TP), alkaline hydrolysable nitrogen (AHN), and available potassium (AK) were measured as reported previously [55,56].

4.2. Soil DNA Extraction, PCR Amplification and MiSeq Sequencing

Sample DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories, West Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted DNA sample was quantified with 1% agarose gel electrophoresis. The internal transcribed spacer (ITS) region is known as a universal DNA barcode marker for fungi, with the most clearly defined barcode gap between inter- and intraspecific variation [57]. We selected the ITS1 region for PCR amplification and sequence using the sense primer ITSF1: CTTGGTCATTTAGAGGAAGTAA and the antisense primer ITS2-2043R: GCTGCGTTCTTCATCGATGC, with a unique barcode sequence at the 5′-end of each primer [58]. Polymerase chain reaction (29 cycles) was performed in a volume of 20 μL with 1 μL of soil DNA as the template. The PCR products from the same samples were pooled together and quantified by electrophoresis in 2% agarose gels and then recycled and purified with an AxyPrep DNA Purification Kit (AXYGEN Corporation, Union City, CA, USA), after which they were quantified by electrophoresis in 2% agarose gels and quantified with PicoGreen-Invitrogen by QuantiFluor^{TM}-ST (Promega Corporation, Madison, WI, USA). A MiSeq library was constructed, followed by high-throughput sequencing.

4.3. Data Analysis

4.3.1. Sequence Optimization and Data Statistics/Processing the Sequencing Data

MiSeq sequencing was used to obtain the paired-end (PE) reads. According to the overlap relationship of PE reads, pairs of reads were merged to form a complete sequence. Then, the quality of reads and the effects of merging were quality controlled and filtered, and finally, valid sequences were obtained based on the barcodes of both ends of the sequences and different samples owned different primer sequences, the direction of which was revised [59,60].

4.3.2. OTU Cluster

Sequencing results of samples were defined as operational taxonomic units by bioinformation statistical analysis with a phylotype threshold of ≥97% sequence similarity. Bioinformation statistical analyses were performed by UPARSE software package using the UPARSE-OTU and UPARSE-OTU algorithms. The used software located in the Usearch platform (UPARSE, version 7.1, Edgar, R.C., http://drive5.com/uparse/, San Francisco, CA, USA) [60].

In an attempt to obtain species taxonomic information of each OTU, we picked a representative sequence for each OTU and used the RDP classifier to annotate taxonomic information for each representative sequence. RDP Classifier algorithm (version 2.2, http://sourceforge.net/projects/rdp-classifier/) [61].

4.3.3. Shannon-Wiener Curves, Alpha Diversity, and Beta Diversity

The trimmed sequences were randomly sampled, and Shannon-Wiener curves were established based on the extracted sequences and the relevant Shannon index (microbial diversity index).

Based on OTU (≥97% similarity) data, alpha diversity was used to reflect richness, and the diversity of the microbial community was assessed by calculating the richness estimator (The Chao1 estimator indicates community richness), the diversity indices (the Shannon index and Simpson index indicate community diversity, including richness and evenness) and the coverage, which shows the sequencing depth.

Beta diversity is based on the biological distance of different samples according to species. The types of biological distances include the Bray-Curtis distance, Euclidean distance, Abund-Jaccard distance etc. We used the Bray-Curtis distance.

4.3.4. Statistical and Bioinformatics Analysis

Significant differences between treatments were assessed by ANOVA using Duncan's multiple range test. Data were subjected to two-way ANOVA using intercropping and crop type as sources of variable. All analyses were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA), SPSS 19.0 (IBM Corp., Armonk, NY, USA) and Vegan Package in R 3.2.3 (Jari Oksanen. vegan: Community Ecology Package. https://CRAN.R-project.org/package=vegan).

5. Conclusions

In conclusion, intercropping improved the richness and diversity of the fungal communities in mulberry and modified the structure of the fungal community by changing the relative abundances of the dominant phyla and habitat. The increased relative abundances of beneficial fungi assisted in improving the sufficient utilization of soil nutrients to satisfy the growth demands of mulberry and alfalfa. Planting patterns and soil physicochemical properties conjointly resulted in the changes in the soil fungal community structure in proportion of 25.9% and 63.1%, respectively.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/2/167/s1, Figure S1: Shannon-Wiener curves of the different treatments at a similarity level of 97%. All curves almost reached the saturated state, indicating the survey effort was large enough to capture the complete diversity of these communities as the curves leveled off with the increasing sample sizes. The description of abbreviation names is shown in Table 1. MM-1, MM-2 and MM-3 represent three repeats of MM, IM-1, IM-2 and IM-3 represent three repeats of IM, MA-1, MA-2 and MA-3 represent three repeats of MA, IA-1, IA-2 and IA-3 represent three repeats of IA. The same abbreviations appear below, Figure S2: The Dis-heatmap plot of fungal community structure in soils from different treatments. The plots based on the Bray-Curtis distance were calculated from relative OTU abundances. MM, IM, MA and IA represent monocultured mulberry, intercropped mulberry, monocultured alfalfa, intercropped alfalfa, respectively. MM-1, MM-2 and MM-3 represent three repeats of MM, IM-1, IM-2 and IM-3 represent three repeats of IM, MA-1, MA-2 and MA-3 represent three repeats of MA, and IA-1, IA-2 and IA-3 represent three repeats of IA. The same abbreviations appear below, Figure S3: STAMP analysis plots showing the differences fungal phyla in monoculture and intercropping mulberry soil (up) and that in monoculture and intercropping alfalfa soil (down). Welch's t-test was applied to test for differences in fungal classed due to planting pattern. The *p*-values are also shown. *Zoopagomycota* and *Mucoromycota* presented marked

advantages in intercropped mulberry ($p < 0.05$). *Basidiomycota* showed significantly higher relative abundances in intercropping alfalfa ($p < 0.05$), Figure S4: Cluster tree plot of fungal community structure in soils from different treatments. The plot showed dissimilarity based on Bray-Curtis distance among different soil samples. Notes: MM, IM, MA, IA represent monoculture mulberry, intercropped mulberry, monoculture alfalfa, and intercropped alfalfa, respectively. MM-1, MM-2 and MM-3 represent three repeats of MM, IM-1, IM-2 and IM-3 represent three repeats of IM, MA-1, MA-2 and MA-3 represent three repeats of MA, IA-1, IA-2 and IA-3 represent three repeats of IA, Table S1: Relative abundances of fungal phylum in the studied soils. Note: Values represent percentages of all sequences assigned to the fungi for soils. The description of sample name is shown in Table 1, Table S2: Relative abundances of fungal class in the studied soils. Note: Values represent percentages of all sequences assigned to the fungi for soils. The description of sample name is shown in Table 1, Table S3: Relative abundances of fungal genus in the studied soils. Note: Values represent percentages of all sequences assigned to the fungi for soils. The description of sample name is shown in Table 1, Table S4: The yields of mulberry and alfalfa in different treatments.

Author Contributions: Methodology, G.S.; Validation, M.Z., N.W. and Y.H.; Investigation, M.Z., J.Z. and D.C.; Resources, J.Z., D.C. and J.G.; Data Curation, M.Z.; Writing—Original Draft Preparation, M.Z.; Writing—Review and Editing—Z.M., Y.H. and G.S.; Supervision, G.S. and D.W.; Project Administration, G.S.

Funding: This research was funded by the National Natural Science Foundation of China, grant number 31500508, and the Natural Science Foundation of Heilongjiang Province, grant number ZD201105 and the Applicant and Developmental Project for Agriculture of Heilongjiang Province, grant number GZ13B004.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 31500508), the Natural Science Foundation of Heilongjiang Province (ZD201105) and the Applicant and Developmental Project for Agriculture of Heilongjiang Province (GZ13B004).

Conflicts of Interest: The authors have no conflicts of interests to declare.

Abbreviations

AMF: Arbuscular Mycorrhizal Fungi; Dis-heatmap, Distance heatmap; ITS1, Internal Transcribed Spacer 1; OTU, Operational Taxonomic Units; RDA, Redundancy Analysis; STAMP, Statistical Analysis of Metagenomic Profiles; VPA, Variance Partitioning Analysis.

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