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Insights into the Fungal Community and Functional Roles of Pepper Rhizosphere Soil under Plastic Shed Cultivation

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Abstract: The rhizosphere fungal community is essential for determining plant health and improving crop productivity. The fungal community structure and functional roles in the plastic shed soils were explored using high throughput sequencing and FUNGuild in this study. The fungal community structures shifted between the rhizosphere and non-rhizosphere soils. The greatest abundance variation was observed for the rare fungal members with relative abundances <0.1%. In the rhizosphere soil of pepper, the abundance of the genera *Purpureocillium*, *Metacorgyceps*, *Arthrobotrys*, *Cephalotheca*, and *Scedosporium* increased significantly, among which, *Purpureocillium*, *Arthrobotrys* and *Metacorgyceps* exhibited biocontrol characteristics. Co-occurrence network analysis revealed different interactions of fungal communities in the rhizosphere and non-rhizosphere soils, both of which were dominated by low abundance members. More positive correlation was identified among the rare members, the fungal pathotroph functions and phthalate acid ester in the rhizosphere soil. This study highlights the important niche of the rare fungal members in soil microbial ecology under plastic shed cultivation.

Keywords: co-occurrence network; fungal community; fungal functional role; plastic shed cultivation; rhizosphere

1. Introduction

Plastic shed cultivation was developed in response to the increasing global population and food demand. The area occupied by global plastic shed cultivation has increased 4.3 times in the past two decades [1]. Furthermore, the total area of plastic shed cultivation in China accounts for more than 80% of that in the world [2]. Pepper is a main plastic shed cultivated vegetable in China with 1.33 million hm^2 of planting area, yielding 28 million tons annually [3,4]. Plastic shed cultivation supplies a great number of off-season vegetables for human consumption. However, the extensive use of plastic films results in phthalate acid ester (PAE) contamination in plastic shed soils [5]. Additionally, the large agricultural input, high soil temperature and airtight environment associated with plastic shed cultivation usually leads to decreased soil quality [6] and microbial diversity [5]. Soil microbiomes

exert a decisive role during carbon and nitrogen cycling; terrestrial ecosystem decomposition; and other multifunctional aspects [7].

Fungal community is an indispensable component of microbiomes and it plays an important role in mediating the terrestrial ecology, such as nitrogen and carbon cycling, parasitism, and pathogenicity [7,8]. The fungal community could impact plant growth by interacting with pathogens and plants [9]. The shift of fungal community structure could be affected by soil properties and cultivation patterns [10]. During plastic shed cultivation, the special planting environment has a significant impact on the fungal community structure and could even inhibit the growth of fungi, thereby disturbing the ecological balance of the microorganisms [11]. It has been reported that when compared to open soil cultivation, the microbial biomass decreases and the fungal community degenerates in plastic shed cultivated soil [11]. Moreover, our previous research, which was based on a twenty-year cultivation study, confirmed that the plastic shed cultivation mode reduces fungal richness and diversity [5]. Meanwhile, it remains unclear how the fungal community's functional roles shift during plastic shed cultivation, especially within the plant rhizosphere.

The plant rhizosphere is the most sensitive hotspot location in which microbiomes respond to their surroundings in soils [12]. The interaction between the soil microbiome and plant roots dominates the rhizosphere process. Plastic shed cultivation commonly causes stunted plant growth and heavy pest infestation, which are closely related to the plant rhizosphere [13,14]. For example, peanut root exudates greatly impact the fungal community, leading to an increase in relative abundance of soil-borne pathogens, particularly *F. oxysporum* [15]. Furthermore, certain root exudates, such as cinnamic, myristic and fumaric acids, have been shown to increase the presence of fungal pathogens in plant rhizosphere soil [16]. However, the influence of the pepper rhizosphere condition on shaping the fungal community in plastic shed soil has been rarely reported [17].

The combination of microbial community structure and functional roles are at the core of understanding the role of microorganisms within an ecosystem [18]. Recently, the python-based FUNGuild tool was used to identify specific ecological categories of fungi through its simple and convenient data processing [19]. Feng et al. reported that some fungi play an important role in restoring the stability of aggregates with FUNGuild [20]. Other studies have used FUNGuild to elucidate the specific roles played by different fungal communities in rhizosphere soils and compost [21,22]. To our knowledge, there is a lack of reports on the fungal functional roles in the pepper rhizosphere and non-rhizosphere soil under plastic shed cultivation. Therefore, clarifying specific shifts in the fungal community structure and functional roles in plastic shed pepper fields will provide new insight for the management and sustainable agricultural production of plastic shed cultivation [23].

This study aimed to clarify the shifts in the soil fungal community structure and functional roles of the pepper rhizosphere in response to the plastic shed cultivation conditions. The rhizosphere and non-rhizosphere soil samples were collected from a long-term plastic shed vegetable cultivation site. The soil fungal community structures were analyzed by sequencing and then the potential fungal functional roles were predicted using FUNGuild. This study will provide helpful information for the management of the soil microbial ecology during plastic shed cultivation.

2. Materials and Methods

2.1. Soil Sample Collection

The plastic shed soil site, with the soil type of fulvo-aquic soil, was located in Weifang, Shandong province, China. After 4 months of pepper cultivation, which is the maturity period of pepper, all the soils were sampled at a depth of 20 cm from a single greenhouse with an area greater than 2000 m^2. The fertilizer was applied in combination with fertilizer and chicken manure, respectively 1500 kg/ha (N/P/K, 15:15:15) and 7500 kg/ha [5]. The 5 mm of soil attached to the pepper roots were taken as rhizosphere soil. Then, the corresponding non-rhizosphere soil that was 1 m away from the pepper roots was also sampled. Five replicates were sampled to ensure the reliability of the field experiment

data [2] and then all samples were carried to the lab in cool boxes. Subsamples for DNA extraction were stored at −80 °C. The remaining subsamples were used for soil property analysis after being air-dried and then sieved to soil particle size less than 2 mm. The soil properties were determined using the reference standard determination method, while PAE extraction and detection in the soil was conducted according to [5].

2.2. DNA Extraction and High-Throughput Sequencing

The E.Z.N.A. ® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) was used to extract microbial DNA from 0.5 g of soil. The DNA extraction procedure was as follows: First, the soil sample was mixed with glass beads (0.5 g), SLX-Mlus Buffer (1 mL) and DS Buffer (0.1 mL) in a 15 mL centrifuge tube. The samples were vortexed to lyse the cells; and then the tubes were incubated at 70 °C for 10 min with a slight shaking. Next, the tubes were centrifuged at 3000 rpm for 3 min and then the supernatant with the volume of 0.8 mL was mixed with 0.27 mL of P2 Buffer in a 2 mL centrifuge tube and incubated for 5 min under cold conditions. After a subsequent centrifugation, the DNA supernatant was transferred into a new 2 mL centrifuge tube. Next, isopropanol and Elution Buffer were used successively, followed by a 2 min incubation with 0.1 mL of HTR Reagent. A HiBind® DNA Mini Column was used for DNA purification and then the DNA sample finally dissolved in 0.1 mL of Elution Buffer. The DNA samples were stored at −20 °C until downstream analyses. The Nano drop system (Thermo Scientific) was used to determine the purity and concentration of DNA.

The fungi-specific primers ITS1F (-CTTGGTCATTTAGAGGAAGTAA-) and ITS2R (-GCTGCGTTCTTCATCGATGC-) were used for polymerase chain reaction (PCR). The PCRs (20 μL) were performed with DNA sample (10 ng), FastPfu Buffer (4 μL), 2.5 mM dNTPs (2 μL), each primer (0.8 μL), FastPfu Polymerase (0.4 μL), BSA (0.2 μL) and double-distilled H_2O (0.2 μL). Then the PCR was amplified according to the program described by [5]. The amplicons were analyzed by 2% agarose gel. Triplicate amplification was used for each sample and then pooled for sequencing. Amplicon sequencing was carried out on Illumina MiSeq platform (Illumina, San Diego, CA, USA). After sequencing, the raw sequences were analyzed with the QIIME 1.9.0-dev pipeline and FLASH. The data filtering was completed by removing bases with a quality score less than 20 and splice contaminated sequences to obtain high-quality target sequences for subsequent analysis. Operational taxonomic units (OTUs) were classified from the sequences at the similarity of 97% with the USEARCH algorithm (http://drive5/uparse/). Accession number of the raw reads submitted to the Sequence Read Archive database of National Center for Biotechnology Information is SRP182013.

2.3. FUNGuild Analysis

Fungal ecological function prediction and categorization was performed via the FUNGuild v 1.0 database. At the time of analysis, the database contained 9476 entries, of which 66% were at the genus level and 34% were at the species level. The guilds' information was accepted only if the identity and coverage were more than 97%. The evaluation results were then ranked as "highly probable", "probable", or "possible" after comparison in the fungal database, while unmatched taxa were named "unassigned" and subsequently excluded. To interpret the data in an ecological context, the selected OTUs were classified according to the main feeding habits of fungi. Finally, 240 OTUs were assigned to 8 trophic modes as follows: saprotroph, 118 OTUs; symbiotroph, 2 OTUs; pathotroph, 28 OTUs; saprotroph-symbiotroph, 19 OTUs; pathotroph-saprotroph, 7 OTUs; pathotroph-symbiotroph, 4 OTUs; pathotroph-saprotroph-symbiotroph, 47 OTUs and pathogen-saprotroph-symbiotroph, 15 OTUs.

2.4. Data Analysis and Statistics

The diversity of the fungal communities was expressed by the Chao and Shannon indices with Mothur v.1.30.1. Then the significant difference post hoc comparison was tested at the $p < 0.05$ level by one-way analysis of variance in Statistical Product and Service Solutions V20.0. Venn plots of fungal communities and functional roles were created using the "venerable" package in R 3.4.0.

Significant fungal biomarkers were detected using linear discriminant analysis (LDA) effect size (LEfSe), basing on the assigned fungal taxa at LDA > 3.0. The network of soil fungal community members, functional roles and soil properties was visualized using Gephi 0.9.2. The Spearman correlation index (>0.6) and *p* values (<0.05) were calculated using the "psych" package in R 3.4.0.

3. Results and Discussion

3.1. Shifts in Fungal Community Structure in Soil

A total of 381 fungal OTUs were obtained from sequencing, of which 64 unique OTUs were detected in the rhizosphere and 62 unique OTUs were detected in non-rhizosphere soils, indicating that the plant rhizosphere can drive a unique soil microbial community [24,25]. In addition, both the fungal diversity and richness were higher in the rhizosphere soil than those in the non-rhizosphere soil in the presence of plastic shed condition (Figure 1a).

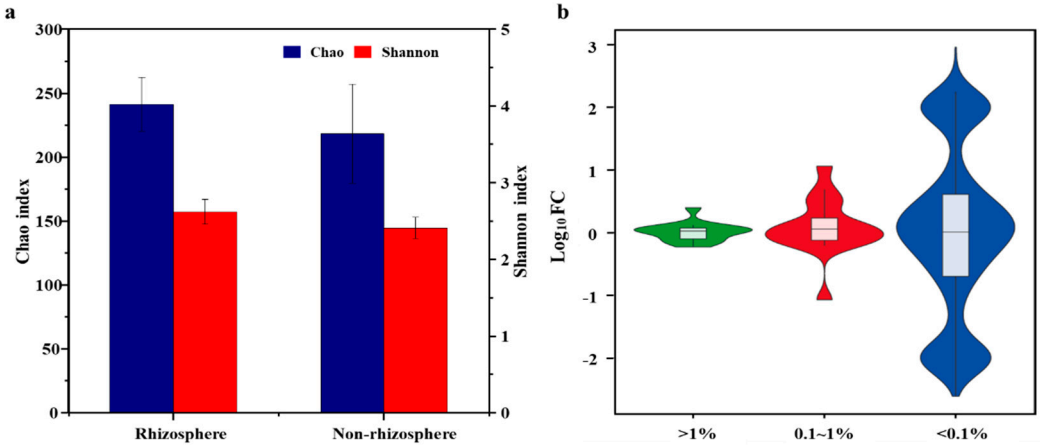


Figure 1. Chao and Shannon indices of the fungal communities in the soils (**a**). The effect of different soil environments (rhizosphere and non-rhizosphere) on the fungal community shift is shown by violin plots (**b**). The relative abundance of the operational taxonomic units (OTUs) are categorized as high-abundance (>1%), medium-abundance (0.1~1%) or low-abundance (<0.1%). FC = the abundance of OTUs in rhizosphere soils/the abundance of OTUs in non-rhizosphere soils.

Ascomycetes and *Zygomycetes* were the two main fungal phyla, accounting for 93.2% and 5.3% of the whole community, respectively (Figure 2a). Through the LEfSe analysis, some fungal community members exhibited a significant shift between the rhizosphere and non-rhizosphere soils. At the genus level, the abundance of *Purpureocillium*, *Metacorgyceps*, *Arthrobotrys*, *Cephalotheca*, *Scedosporium* and *classified-Lasiosphaeriaceae* significantly increased in the rhizosphere environment, while the abundances of *Pseudaleuria* and *classified-Hypocreales* were significantly lower in the pepper rhizosphere than those in the non-rhizosphere soil under the plastic shed condition (Figure 2b). Specifically, *Purpureocillium*, an entomopathogenic fungus within a family of soil-borne micromycetes, increased the most in the pepper rhizosphere soil. This genus was also reported to be beneficial for the defense from nematodes and nutrient uptake by habanero chili pepper *(Capsicum Chinese Jacq.*), thereby facilitating plant growth [26,27]. Meanwhile, other fungal genera, such as *Arthrobotrys* and *Metacordyceps*, reportedly play important roles in defending plants against nematode infestations [25,28], which can damage plant growth and result in lower yields, especially for pepper cultivation [29]. Antagonistic microorganisms that act against nematodes have also been reported in soils with a combination of various planting patterns [30]. Therefore, this result indicates that pepper roots could recruit nematode-resistant fungi in the rhizosphere soil during plastic shed cultivation, thus benefiting its growth. This could be one of the most important ecological functions of fungi within the plant rhizosphere soil, especially for soils subjected to the plastic shed agricultural practice. Additionally, the pepper rhizosphere significantly

enriched *Scedosporium*. This may be because its presence is associated with the phosphorus content of the soil, particularly in soils with a moderate and high phosphorus content [31]. In this study, the phosphorus content in the rhizosphere environment soil was indeed greater than that in the non-rhizosphere soil [32]. Meanwhile, the abundance of *Pseudaleuria* was significantly decreased in the rhizosphere soil (Figure 2b). It was reported that manure application increases the abundance of *Pseudaleuria* [33]. Thus, the low abundance of *Pseudaleuria* in the rhizosphere soil of this study may be explained because the turnover of organic fertilizers in the pepper rhizosphere was faster than that in the bulk soil, thereby changing the ideal conditions for *Pseudaleuria*.

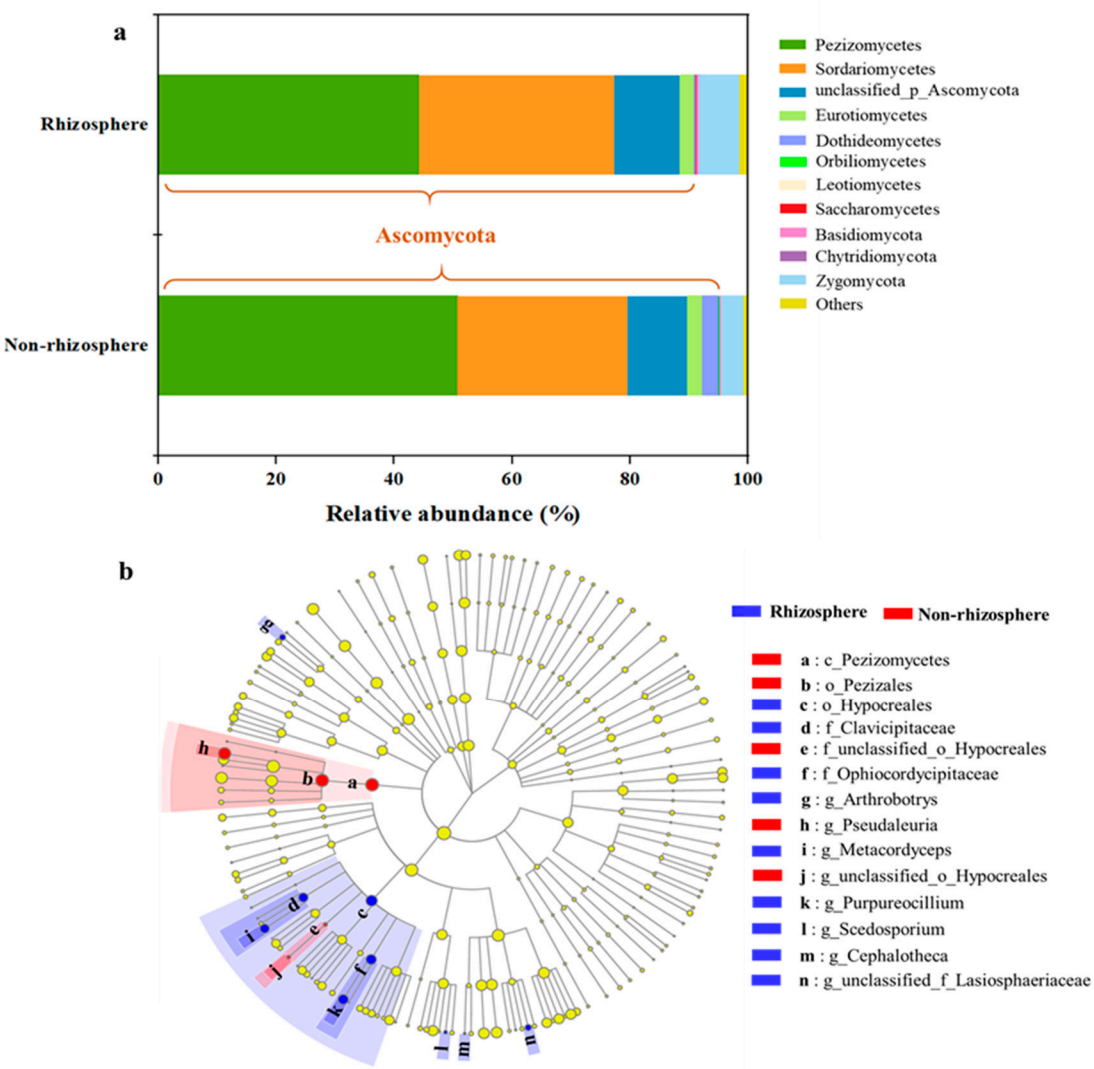


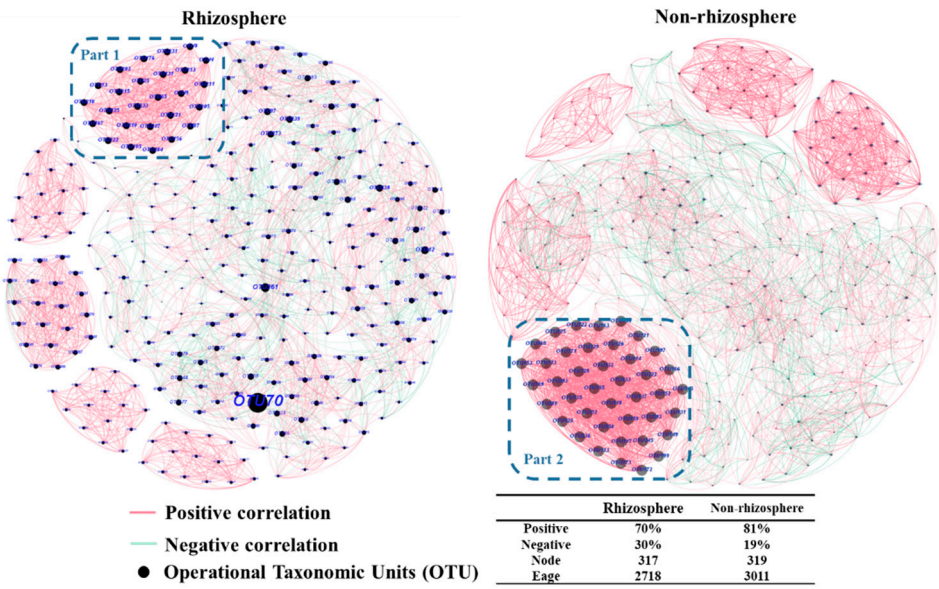
Figure 2. The abundance and composition of the fungal community at the phylum/class levels in soils (**a**). The response of the significantly fungal members from phyla to genera in the rhizosphere (blue) and non- rhizosphere soils (red) (**b**).

3.2. Interaction of Fungal Communities

Determining the abundance distribution of fungi is of great help for understanding the ecological processes of microorganisms [34]. Thus, the effect of the rhizosphere and non-rhizosphere environments on the variation of fungal abundance was determined (Figure 1b). The Log_{10} (FC) > 0 indicates that the abundance of fungi increased in the rhizosphere environment, and vice versa (detailed calculation is shown in Figure 1). High abundance fungi (RA > 1%) were slightly higher in the non-rhizosphere environment, while medium abundance fungi increased in the rhizosphere environment. The extent of variation for low abundance fungi was substantially higher than that of high and medium abundance

fungi. Therefore, fungi with different abundances have different responses to the pepper rhizosphere, among which the low abundance fungi were influenced the most. This is consistent with a previous report indicating that the change of soil environment has a greater impact on low abundance fungi than those of high abundance [35]. Studies have shown that microbiomes with different abundances exhibit different distribution patterns and functions [36]. In the warm and humid environment, the low abundance fungi increased [34]. This also verifies that the low abundance fungi were the main members responsible for the difference between the rhizosphere and non-rhizosphere soils in the special planting environment associated with plastic shed cultivation.

The OTU network relationship of the microbes also showed significant differences (Figure 3), with a positive correlation accounting for 70% within the rhizosphere and 81% within the non-rhizosphere environment. Accordingly, the main nodes of the network diagram were explored. The size of the node reflects the degree to which the fungi were involved in the interaction [37]. Generally, the nodes in the rhizosphere fungal network were greater than those in the bulk soil, leading to a balanced community network in the rhizosphere. *Lasiosphaeriaceae* (OTU70) and part 1 are the dominant members for the communities of the rhizosphere, while part 2 members dominated the fungal community network of the non-rhizosphere. Among them, part 1 members of the rhizosphere included *Pseudeurotium*, *Scedosporium*, *Eurotiales*, *Phialocephala*, *Zopfiella*, *Pseudogymnoascus* and others fungi, while the part 2 members of the non-rhizosphere included *Waitea*, *Acremonium*, *Hypocreales*, *Fusarium*, *Chaetomiaceae*, *Cercophora*. Therefore, it is interesting that the fungi involved in part 1 and part 2 were completely different but all classified as low abundance fungi, leading to the overall changes of the fungal community structure. The study explained that *Lasiosphaeriaceae* is associated with the available carbon sources, and it is also regarded as a potential disease suppressor in plants [38]. Although the abundance of *Hypocreales* was significantly enhanced under the rhizosphere environment, its node appeared in part 2 of the non-rhizosphere soil. This could be because the order *Hypocreales*, which includes the genera *Purpureocillium*, *Metacorgyceps* and *Arthrobotrys* regard as the largest source of biocontrol-related fungi and when the abundance of *Hypocreales* is high in the rhizosphere, its interaction with other fungi are weakened [39]. Thus, the rhizosphere environment can recruit certain fungal members, specifically those of benefit, to alter the fungal community [24].



	Rhizosphere	Non-rhizosphere
Positive	70%	81%
Negative	30%	19%
Node	317	319
Eage	2718	3011

Figure 3. Co-occurrence network of the differential operational taxonomic units in soils.

3.3. Shifts in the Fungal Functional Role in Soil

Each soil environment had some specific functional roles with 41 and 31 unique function roles identified in the non-rhizosphere and rhizosphere environments, respectively. Therefore, the pepper

rhizosphere not only changes the soil fungal community structure but also the functional roles of the fungal community.

Based on the trophic mode of fungal OTU, the fungal community functional roles could be divided into nine categories, including saprotroph, pathotroph, symbiotroph, saprotroph-symbiotroph, pathotroph-saprotroph-symbiotroph and other (Figure 4a). Saprotrophs dominated both the rhizosphere soil and non-rhizosphere soil, accounting for 54.66% and 44.12%, respectively. Within the fungal community, the high fertilizer application and environmental temperature during plastic shed cultivation would be beneficial for the development of saprotroph functions [40]. The next most dominant tropic modes were the saprotroph-symbiotroph and pathotroph-saprotroph-symbiotroph transitional types. However, 36.75% of fungal community functional roles were not classified. Nguyen et al. showed that FUNGuild can provide the main information related to fungal functions, but improving the overall fungal functional information is still in progress [19].

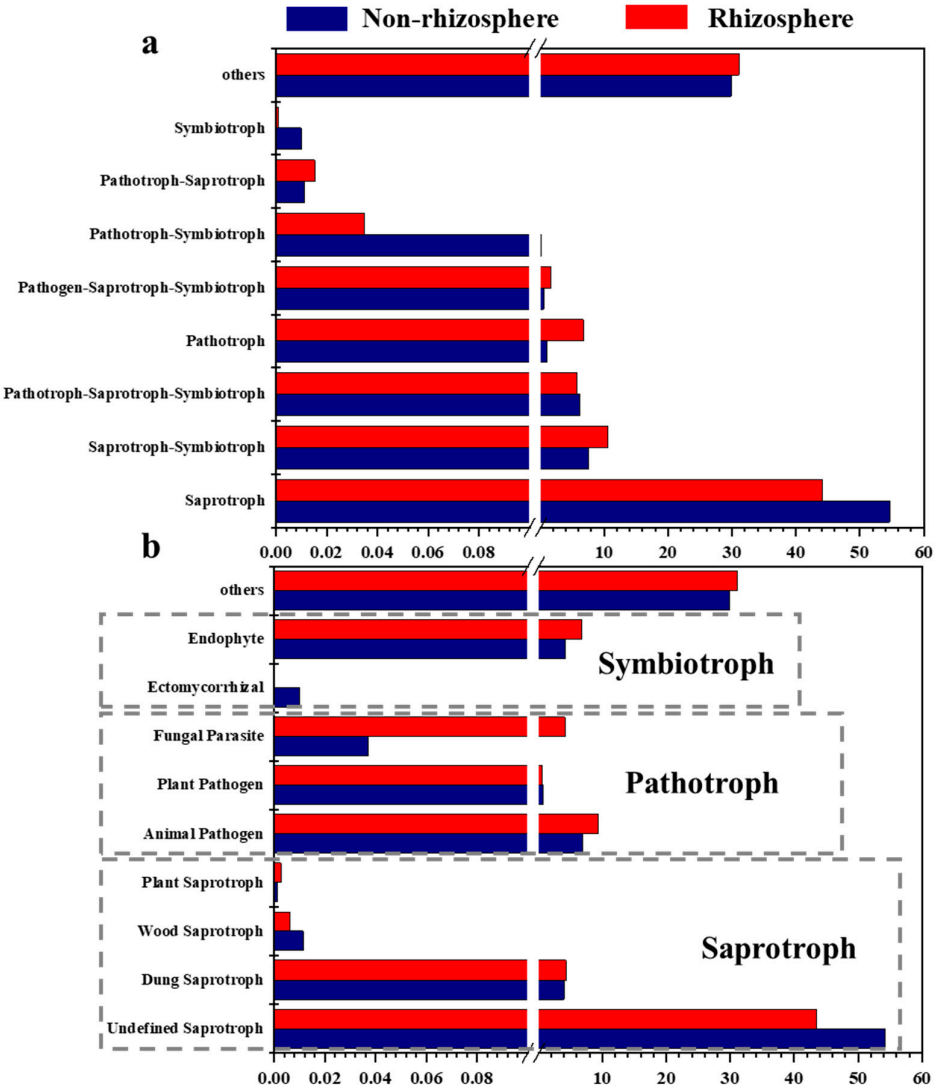


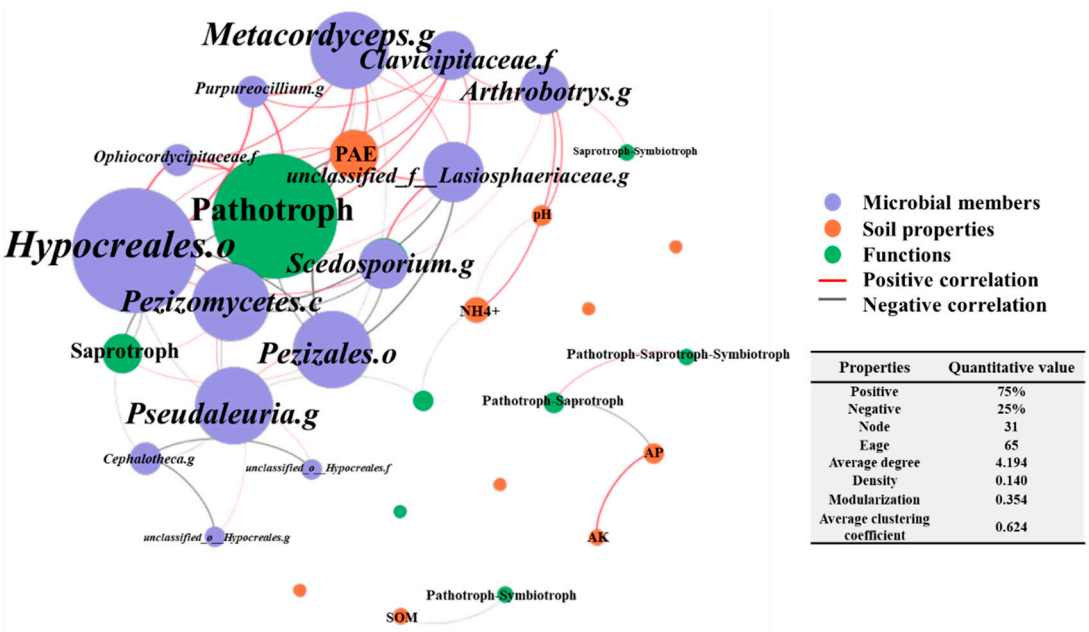
Figure 4. The abundance of different functional roles (**a**) and main guilds (**b**) assigned to the fungal community using FUNGuild.

The abundance of the pathtroph-symbiotroph, saprotroph, symbiotroph and pathotroph-saprotroph-symbiotroph trophic modes were higher in the non-rhizosphere soil than those in the rhizosphere soil, while the pathotroph, pathotroph-saprotroph, saprotroph-symbiotroph and other modes were lower in the non-rhizosphere environment than those in the rhizosphere environment

(Figure 4a). Saprotroph is related to the role of fungi in decomposition. The abundance of saprotroph in rhizosphere soil was lower than that in non-rhizosphere soil in this study. Kong et al. also found that the non-rhizosphere condition enriched the relative abundance of saprotroph [37]. The increase of pathotrophic fungi in rhizosphere soil could be ascribed to the plant roots, although some pathogens attack the root system of crops, while others, such as some fungal parasites, could promote the healthy growth of crops [40]. Another study found that pathotrophic fungi could compete with other microbiomes in plant rhizosphere [9]. The relative abundance of fungal parasites and endophytes showed an obvious increase in the pepper rhizosphere soil under the plastic shed condition (Figure 4b). Within saprotroph, undefined saprotroph and wood saprotroph are more common in non-rhizosphere environments (Figure 4b), which is demonstrated by the higher nutrient utilization in this environment [41]. Meanwhile, the rhizosphere and non-rhizosphere soils contain many dung saprotroph, which is closely related to the application of organic fertilizer during plastic shed cultivation. Some studies have suggested that the changes of fungal functional roles were related to many factors, and plant species were also considered as important influencing factors [42,43]. Therefore, the difference between rhizosphere and non-rhizosphere fungal functional roles can be attributed to plant species and environmental factors.

3.4. Co-Occurrence Network among the Fungal Community, Functional Roles and Soil Properties

Soil properties drive the shifts in the soil fungal community structure and functional roles [44]. Furthermore, extensive use of plastic films could result in PAE contamination in the soils [5]. Therefore, to clarify their relationships, co-occurrence network analysis was performed between the soil properties, PAE, fungal functional roles and the significantly different fungal members (Figure 5). The co-occurrence network is composed of 32 nodes and 59 edges (Figure 5). The largest nodes in the network for fungal members, functional roles and soil properties represented *Hypocreales*, pathotroph functional roles and PAE, respectively. In the pepper rhizosphere, the members *Hypocreales*, *Metacordyceps*, *unclassified_f_Lasiosphaeriaceae* showed higher abundances than those in the non-rhizosphere and they positively correlated with PAE and pathotroph function. Conversely, the abundant fungal members *Pezizomycetes*, *Pseudaleuria* and *Pezizales* in the non-rhizosphere soil mainly showed negative correlations with PAE and pathotroph function.



Properties	Quantitative value
Positive	75%
Negative	25%
Node	31
Eage	65
Average degree	4.194
Density	0.140
Modularization	0.354
Average clustering coefficient	0.624

Figure 5. Co-occurrence network of the significantly different fungal members, predicted fungal functional roles and the soil physicochemical properties.

The order *Hypocreales*, in the phylum *Ascomycetes* is considered to be the most abundant source of biocontrol-related fungi, which can inhibit insects, nematodes and other fungal species considered as plant pest populations [39]. Interestingly, *Hypocreales* showed positive correlations with both pathotroph function and PAE. Pathotroph function contains not only plant and animal pathogens, but also fungal parasites whose abundances were significantly greater in the rhizosphere than that in the bulk soils. Schmidt et al. reported that some fungal parasites could confer resistance to plant diseases and insect pests [40]. Therefore, the positive correlation between *Hypocreales* and pathotroph function may primarily be derived from the cooperation between *Hypocreales* and fungal parasites. The rhizosphere microbiomes are greatly affected by plant root exudates. Because of the large amount of organic and chemical fertilizers have been applied to soil under plastic shed cultivation, the differences in the soil properties between rhizosphere and non-rhizosphere were masked [32]. Therefore, common soil properties did not form central nodes in the network analysis (Figure 5). However, PAE, such as diethyl phthalate, di-isobutyl phthalate, di-n-butyl phthalate and di-n-octyl phthalate, have been reported as allelopathic substances of pepper root exudates [45]. It was found that the contents of phthalate, di-isobutyl phthalate, din-butyl phthalate and di-n-octyl phthalate in the pepper rhizosphere soil were greater than that those in bulk soil in our previous study [32]. Therefore, it could be inferred that the positive correlations between biocontrol fungi (*Hypocreales*) and PAE resulted in the positive release of PAE from the pepper root, which recruits certain fungal members. In a word, this case study indicate that the pepper roots recruit special biocontrol functional fungi and low abundance fungal taxa to construct a different and more balanced fungal community network in the rhizosphere, thereby benefitting growth under plastic shed cultivation [46,47].

4. Conclusions

During the intensive planting mode of plastic shed cultivation, low abundance fungi showed a sensitive response to the pepper rhizosphere, leading to a higher community diversity in the rhizosphere relative to the non-rhizosphere soil. *Purpureocillium*, *Metacorgyceps* and *Arthrobotrys*, as well as other unique fungi genera beneficial to plant growth, were recruited by the pepper root, thereby forming a differential network of the fungal community structure and functional roles. The rare members with low abundances of fungi deserved to be concerned for insight into the soil microbial ecology under plastic shed cultivation.

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