

Division - Soil Processes and Properties | Commission - Soil Biology

Effects of *Meloidogyne incognita* on the fungal community in tobacco rhizosphere

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ABSTRACT: The often widespread and serious Root-Knot Nematode (RKN) disease is an important soil-borne disease affecting tobacco production. This study aimed to understand micro-ecological changes caused by RKN disease and interactions between disease and rhizosphere soil fungal communities. The 18S rRNA gene sequencing was used to study changes in rhizosphere fungal community of tobacco plants having RKN disease. In June 2018, a paired comparison was performed between rhizosphere fungal community structures of healthy tobacco plants and those with RKN disease in Yuxi and Jiuxiang, Yunnan Province, China. Compared with uninfested soil, the OTU abundance, Shannon, ACE and Chao1 indexes of infested soil in the two tobacco areas showed a decreasing trend. Principal Coordinate Analysis showed fungal communities of infested soil and uninfested soil in the two tobacco areas were clustered in different areas, and the community composition was significantly different. Moreover, the dominant fungi community and relative abundance are significantly different at phylum, genus and species levels. More beneficial fungi, such as Penicillium and Aspergillus, were found in soil samples of healthy plants, whereas more pathogenic fungi, such as Phoma and Alternaria, were found in soil samples of diseased plants. In conclusion, changes in fungal community structure and decreases in species diversity and richness were important characteristics of rhizosphere soils from diseased tobacco plants. Disequilibrium in the tobacco rhizosphere micro-ecosystem may allow the development of RKN disease and other more complex diseases.

Keywords: root-knot nematode disease, soil-borne disease, soil microorganism, diversity.

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INTRODUCTION

Meloidogyne spp. is an important group of plant parasitic nematodes, having many species and a wide host range (Back et al., 2002; Jeger et al., 2018). Members including Meloidogyne incognita, Meloidogyne hapla, Meloidogyne javanica and Meloidogyne arenaria are causal agents of tobacco RKN disease, an important soil-borne disease that negatively impacts tobacco production. This desease occurs in 13 major tobacco producing areas in Yunnan, Henan and Shandong provinces of China. For example, in Yunnan, more than 26,000 hectare is affected by the disease, resulting in yield losses of 30 to 50 % (Chen et al., 2015). Furthermore, the Meloidogyne spp. often infect roots and cause mechanical damage, and the tiny wounds greatly assist in infection by other pathogenic microorganisms, such as Ralstonia solanacearum and Phytophthora (Cui et al., 2021). Multiple infections form complex infectious diseases that seriously affect the yield and quality of tobacco leaves. Flue-cured tobacco is an important economic crop in Yunnan, which contains one-third of the total planting area, and produces a corresponding yield in China. Thus, this region plays an important role in China's tobacco industry (Tong et al., 2016). The RKN disease has become an important factor restricting the development of the Yunnan tobacco industry.

Occurrences of soil borne diseases are closely related to imbalances in soil micro-ecosystems (Liu et al., 2016; Kim and Anderson, 2018). Plant rhizosphere microorganisms and soil-borne pathogens grow in the same environment; consequently, their competition for limited resources, such as nutrients, and niches directly or indirectly affects the occurrence and development of plant diseases (Li and Wang, 1989). Some parasitic, symbiotic or antagonistic relationship exists between *Meloidogyne* spp. and soil microorganisms, and some microbial communities have inhibitory effects on *Meloidogyne* spp. (Ruby et al., 2008; Haegeman et al., 2009). At present, *Meloidogyne* parasitoids and antagonistic bacteria have been identified in *Bacillus*, *Pseudomonas*, *Streptomyces* and *Pasteuria* (Siddiqui et al., 2002; Tian et al., 2015). For example, *Pasteuria penetrans* has a highly specific parasitic capability and is an effective biocontrol agent against *M. incognita* and *M. javanica* (Stirling et al., 2011; Ciancio et al., 2016). *Pseudomonas* can inhibit *Meloidogyne* spp. by destroying their eggs, reducing their hatching levels and specifically activating peroxidase and phenylalanine ammonia lyase (Norabadi et al., 2014; Cao et al., 2015).

Furhtermore, some fungi can also inhibit *Meloidogyne* spp. through predation or parasitism. More than 30 parasitic fungi of root-knot nematode have been reported (Yang et al., 2004); for example, the hyphae of *Paecilomyces lilacinus* and *Pochonia chlamydosporia* can enter *Meloidogyne* eggs through parasitism and digest and absorb the substances in the eggs, which leads to abnormal egg development (Liu, 2011). Other species such as *Coprinus comatus, Gliocladium roseum, Trichoderma harzianum, Trichoderma viride* can also effectively inhibit *Meloidogyne* spp. (Yang et al., 2004; Cui et al., 2021). Although these previous studies precisely and comprehensively illustrated the involvement of soil microbes in antagonistic interaction with plant-parasitic nematodes, studies identifying the specific group of soil fungus associated with the occurrence of nematodes in the field are still limited (Huang et al., 2020).

Life cycle and development of most plant-parasitic nematodes occur in the rhizosphere of host plants. Rhizosphere is not only the window of efficient utilization of crop nutrients, but also the vector of successful infection of pathogens. It is the key micro domain of plant-soil-microorganism interaction (Yang et al., 2019). In the same plot, with similar natural conditions and soil properties, the existence of both *Meloidogyne incognita* -infected and uninfected plants indicates that there may be specific interactions between plants and rhizosphere soil micro ecological environment, which directly or indirectly affect the occurrence of diseases (Ruby et al., 2008).



We hypothesize that there may be some specific microorganisms regulating *Meloidogyne* spp. activities in the soil. Therefore, Illumina MiSeq sequencing was used to analyze the differences in the fungal community in rhizosphere soil between healthy tobacco plants and those with RKN disease. This study aimed to explore the relationship between the structure and diversity of the fungal community in tobacco rhizosphere soil and RKN disease and provide a scientific basis for the effective prevention and control of RKN disease in tobacco.

MATERIALS AND METHODS

Research area

The experiment was conducted on Gaocang Street, Hongta District, Yuxi City, Yunnan (24° 30′ N, 103° 32′ E) and Jiuxiang Township, Yiliang County, Kunming City, Yunnan (25° 1′ 43″ N, 103° 20′ 31″ E) in June 2018. Average annual rainfall in the Hongta District of Yuxi City is 779.5–989.7 mm. The average annual temperature is 15.6–23.8 °C; the annual frost-free period is 244–365 days; the average annual sunshine is 2,115–2,285 h. The altitude of the site is 1,760 m, the soil type is red loam soil, and the irrigation method is hole irrigation. The previously planted crop was wheat, the land is relatively flat, and the drainage is good.

The average annual rainfall in Jiuxiang Township Yiliang County of Kunming City is 526.9–898.9 mm, the annual average temperature is 16.3–21.7 °C, the annual frost-free period is about 260 d, and the annual average sunshine is approximately 2,032 h. The altitude of the site is 1,560 m, the soil type is red loam soil, the irrigation method is hole irrigation, and the previous crop was corn. Table 1 shows the basic soil properties of the 0.00-0.20 m soil layer in the two test plots before plowing.

After being suspended with water (soil:water ratio = 1:2.5), the soil pH was measured using a pH meter; organic matter with the acidified potassium dichromate ($K_2Cr_2O_7$ - H_2SO_4) heating method; total nitrogen was measured by Dumas Nitrogen Analyzer, available phosphorus was determined with NaHCO₃ 0.5 mol L⁻¹ (pH 8.5); available potassium was determined by CH_3COONH_4 extraction method.

Materials and soil sampling

Tobacco fields with typical and serious RKN disease (*Meloidogyne incognita*) levels were selected in the test areas. In the same plot, with similar conditions and soil properties, plants with typical symptoms of RKN disease (*M. incognita*) and healthy plants were sampled, with three plants per group. The topsoil and whole plants were removed. The loose soil was shaken off, and the soil attached to the root at 0-4 mm was collected as rhizosphere soil. The samples were named as Jiuxiang infested soil (JX_P), Jiuxiang uninfested soil (JX_N), Yuxi infested soil (YX_P) and Yuxi uninfested soil (YX_N). After removing the impurities and residual fine roots, the soil samples were put separately into sterile self-sealed bags, immediately placed in liquid nitrogen, transported back to the laboratory, and stored at -80 °C.

Table 1. Basic soil properties of the 0.00-0.20 m soil layer before plowing

Municipality	pH(H₂O)	Organic matter	Total N	Available P	Available K	
	-	g kg ⁻¹		mg kg ⁻¹ ———		
Yuxi	6.62	17.53	0.73	49.11	257.45	
Jiuxiang	5.57	19.69	0.78	41.92	221.37	



DNA extraction and PCR amplification of fungi

Total DNA in the infested and uninfested soil of the two test sites (JX_P, JX_N, YX_P, YX_N) was extracted following the instructions of a FastDNA®SPIN Kit for Soil (USA). The DNA concentration and purity were detected using a NanoDrop2000, and the DNA quality was determined using 1 % agarose gel electrophoresis.

Primers ITS1 F (5'-ACTTGGTCATTTAGGAAGTAA-3') and ITS2 R (5'-BGCTGCGTTCTTCATCGATGC-3') were used to amplify the ITS-1 variable region of the 18S rRNA. The amplification procedure was as follows: pre-denaturation at 95 °C for 3 min, 36 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The amplification system was as follows: 4 μ L 5× FastPfu buffer, 2 μ L 2.5 mmol L⁻¹ dNTPs, 0.8 μ L forward primer (5 μ mol L⁻¹), 0.8 μ L reverse primer (5 μ mol L⁻¹), 0.4 μ L FastPfu polymerase, 0.2 μ L BSA, 10 ng DNA template and water was added until achieve a final volume of 20- μ L.

The PCR products were recovered by 2 % agarose gel electrophoresis and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen, USA). Then, they were sequenced by Shanghai Meiji Biological Medicine Technology Co., Ltd using MiSeq.

Sequence quality control and analysis

The original sequence was spliced using FLASH (Caporaso et al., 2011) software. They were then filtered using Usearch (Edgar, 2013), and the chimeric sequences were removed to obtain the effective sequences. The operational taxonomic units (OTUs) were clustered using UPARSE at a 97 % sequence similarity level. The sequences were annotated by RDP classifier (Wang et al., 2007) and the Silva database (Altschul et al., 1990), and the dilution curve was made using Mothur. The library coverage, Shannon, Simpson, ACE and Chao1 indexes were calculated, and the species diversity and richness indexes were evaluated. A principal coordinate analysis was performed with a Bray-Curtis dissimilarity matrix using Qiime. The rarefaction curves, Veen and community composition histogram and heatmap were generated from the R software.

Data analyses

Differences in the microbial diversity index were compared using Analysis of Variance (ANOVA, IBM SPSS 22.0). Significance was determined at the p<0.05 level and at the 95 % confidence level by Duncan's test.

RESULTS

OTU abundance and the fungal alpha-diversity level

The OTU abundance and alpha-diversity of fungal communities in different samples are shown in table 2. Shannon index dilution curve (Figure 1) showed that the slope of the curve gradually decreased and tended to flatten as the sequencing quantity increased, indicating that the sequencing quantity was sufficient. In this experiment, the coverage rates of the four samples were greater than 99.0 % (Table 2), indicating that the sequencing lengths were sufficient.

Variation trends in the OTU abundances from tobacco rhizosphere soil at different sampling sites were the same, having the following order: *M. incognita* infested soil < uninfested soil (Table 2). Among them, the fungal OTU abundance of JX_P was 6.63 % lower than that of JX_N, and the OTU abundance of YX_P was 17.60 % lower than that of YX_N. The Venn diagram of OTU abundance levels of fungi is shown in figure 2. The number of OTUs shared by JX_N and JX_P was only 587, accounting for 17.96 % of the total OTUs (3,268). The unique number of OTUs in JX N was 1.43 times that of JX P. The common



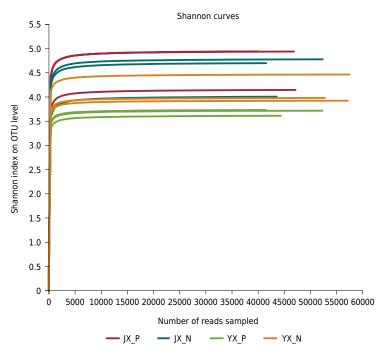


Figure 1. Shannon index-based rarefaction curves on fungus in rhizosphere soils at different samples.

Table 2. Fungus OTU abundance and alpha diversity index in rhizosphere soils at different samples

Camples	Daw number	Effective number	OTII shundanaa	Alpha diversity				Coverance
Samples	Raw number	Effective number	OTO abundance	Shannon	Simpson	ACE	Chao1	Coverage
								%
JX_N	48510	45741	1690	4.80 a	0.0218 b	1307.30 a	1281.67 a	99.39
JX_P	47215	44583	1578	4.36 ab	0.0563 ab	1205.42 a	1190.45 a	99.46
YX_N	58228	55764	1193	4.12 bc	0.0379 ab	1023.62 b	984.02 b	99.58
YX_P	47849	45875	983	3.68 c	0.0669 a	958.86 b	953.19 b	99.51

Different lowercase letters indicate a significant difference between treatments (p<0.05).

OTUs (223) in YX_N and YX_P accounted for 10.25 % of the total OTUs (2,176). The unique number of OTUs in YX_N was 2.11 times that of YX_P, indicating that the occurrence of RKN disease significantly reduced the OTU abundance of the fungi.

Diversity and richness of fungal communities in tobacco rhizosphere soils from the sample sites also showed the same changing trends, which were in the following order: infested soil < uninfested soil. The Shannon index of JX_P was 9.23 % lower than that of JX_N, whereas that of YX_P was 10.59 % lower than that of YX_N. Compared with JX_N and YX_N, the ACE indexes of JX_P and YX_P decreased by 7.79 and 6.33 %, respectively, and the Chao1 indexes decreased by 7.12 and 3.13 %, respectively. In general, RKN disease has a certain impact on the OTU abundance, as well as the diversity and richness, of the fungal community in the rhizosphere soil of flue-cured tobacco, and the same change patterns occurred among the different regions.

Community species and relative abundances of fungi

Fungi relative abundance at the phylum level is shown in figure 3. Fungi in different rhizosphere soils were mainly composed of Ascomycota, Mortierellomycota and Basidiomycota at the phylum level. The relative abundances of JX_P and JX_N in Ascomycota and Mortierellomycota were not significantly different. However, the relative abundance of Ascomycota in YX_P was 12.64 % lower than in YX_N, whereas the relative abundance



of Mortierellomycota in YX_P was 50.03 % higher than in YX_N. The relative abundances of Basidiomycota in all different test sites followed the trend infested soil > uninfested soil. JX_P had 25.39 % more Basidiomycota than JX_N, and YX_P had 49.73 % more Basidiomycota than YX_N.

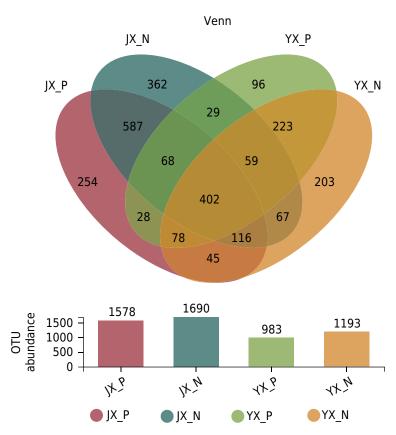


Figure 2. Venn graph of fungus OTUs distribution in rhizosphere soils at different samples.

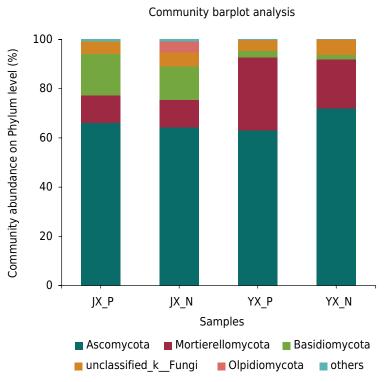


Figure 3. The relative abundance of fungus on Phylum level in rhizosphere soils at different samples.



Fungi in different samples also showed significant differences at the genus level (Table 3). *Mortierella* and *Fusarium* accounted for the largest proportions in all the samples, and their relative abundances in JX_P decreased by 1.19 and 17.42 %, respectively, compared within JX_N. Relative abundance of *Fusarium* in YX_P was 11.44 % lower than in YX_N, but that of *Mortierella* increased by 54.35 % in YX_P compared with YX_N. In addition, the relative abundances of *Penicillium* and *Aspergillus* have followed the order of uninfested soil > infested soil. In JX_P, their relative abundances were 29.11 and 4.21 % lower, respectively, than in JX_N; whereas in YX_P, they were 36.48 and 56.98 % lower, respectively, than in YX_N. On the contrary, the relative abundances of *Phoma* and *Alternaria* in uninfested soil were lower than in infested soil. The relative abundances in JX_P were 53.57 and 1156.52 % higher, respectively, than in JX_N, whereas in YX_P, they were 145.12 and 323.08 % higher, respectively, than in YX_N.

Relative abundances of the top 50 species, according to the annotation and relative abundance of each sample, were selected to generate a heatmap (Figure 4). Among them, the fungi in *Phoma* were mainly *Phoma omnivirens*. Its relative abundance in different samples was as follows: $JX_P > JX_N > YX_P > YX_N$. *Purpureocillium lavendulum* relative abundance in different samples was as follows: $YX_P > YX_N > JX_P > JX_N$. Our results showed that the occurrence of RKN disease had a great influence on the fungal distribution and community composition in tobacco rhizosphere soil.

Principal coordinate analysis of the fungal communities

Principal component 1 (PC1) and principal component 2 (PC2) accounted for 50.53 and 11.23 % of the sample differences, respectively, from 61.76 % of the soil samples (Figure 5). The fungal communities of the four samples were separately distributed in the four quadrants, which revealed that the fungal communities in the different samples were quite varied. First, JX_P and JX_N were mainly distributed on the left side of the PC2 axis, whereas YX_P and YX_N were mainly distributed on the right side of the PC2 axis. Additionally, the distance between the clusters was great, indicating that each fungal community's composition was significantly different in the experimental areas.

However, the fungal compositions in RKN infested and uninfested soil showed the same change trends in different experimental areas. Both JX_P and YX_P were concentrated on the upper side of the PC1 axis, whereas JX_N and YX_N were clustered in the lower side of the PC1 axis, indicating that the fungal community compositions in RKN diseased and healthy soils were significantly different, but different regions had the same change trends.

DISCUSSION

Soil microbial communities are influenced by multiple factors such as plant type, climate, soil properties and agricultural practice (Bowen et al., 2017). The results of this study also confirm this point, the fungal communities in the rhizosphere soils of the Jiuxiang and Yuxi experimental sites were distributed on both sides of the PC2 axis, and the distance between them was relatively great, indicating that there was a large difference in the fungal communities at the two experimental sites due to the differences in physical and chemical soil properties, climatic and cultivation management measures (Zhang et al., 2016; Tong et al., 2021). But it is worth noting that the fungal community compositions in rhizosphere soils from diseased and healthy plants in the two experimental plots showed the same trends. The fungal communities in the rhizosphere soils of diseased plants from the two experimental plots clustered on the upper side of the PC1 axis, whereas those of healthy plants clustered on the lower side of the PC1 axis, which supported that RKN disease probably led to the changes in soil microbial community. However, rhizosphere microbial community structure variations may also make the root more susceptible to pathogen infection and RKN disease.



Table 3. The relative abundances of fungus on genus level in rhizosphere soils at different samples

Tayonomic catogory (gonus)	Fungi abundance						
Taxonomic category (genus)	JX_P	JX_P JX_N YX_P Y					
			% ———				
Mortierella	10.76	10.89	29.25	18.95			
Fusarium	4.22	5.11	11.15	12.59			
unclassified_f_Chaetomiaceae	2.86	3.53	10.72	9.85			
unclassified_k_Fungi	5.03	5.81	4.46	6.24			
unclassified_c_Sordariomycetes	2.00	2.47	5.77	3.74			
Penicillium	2.46	3.47	2.42	3.81			
unclassified_p_Ascomycota	1.82	4.95	1.94	2.78			
Phoma	3.87	2.52	2.01	0.82			
Talaromyces	2.78	6.22	0.02	0.06			
unclassified_f_Stephanosporaceae	0.09	8.95	0.03	0.05			
unclassified_f_Nectriaceae	1.19	2.96	1.46	2.51			
unclassified_f_Microascaceae	0.79	1.30	3.32	2.09			
Rhodotorula	7.51	0.07	0.03	0.05			
Zopfiella	3.13	3.63	0.35	0.14			
Cercophora	0.04	0.15	1.81	4.01			
Aspergillus	1.82	1.9	0.37	0.86			
unclassified_f_Didymellaceae	4.36	0.23	0.22	0.13			
Plectosphaerella	0.44	1.08	1.04	1.79			
Trichoderma	2.24	1.92	0.23	0.27			
Olpidium	0.05	4.4	0.02	0.03			
Chaetomium	0.82	0.22	1.06	1.59			
unclassified_o_Pleosporales	1.68	0.91	0.44	0.73			
Thielaviopsis	0.03	0.02	0.02	3.13			
Cladosporium	2.92	0.37	0.38	0.19			
Cladorrhinum	2.37	1.52	0.05	0.12			
Alternaria	2.89	0.23	0.55	0.13			
Geminibasidium	2.50	1.24	0.02	0.11			
unclassified_o_Microascales	0.65	0.46	1.58	0.83			
Purpureocillium	0.24	0.03	2.28	0.81			
Metarhizium	0.69	0.54	0.59	1.29			
unclassified_o_Sordariales	0.58	0.40	1.77	0.38			
Knufia	0.19	0.03	1.20	1.41			
Guehomyces	2.47	0.54	0.03	0.13			
Arthrographis	0.54	1.29	0.05	0.77			
Ochrocladosporium	0.04	0.02	0.02	0.12			
Thielavia	0.03	0.02	1.08	0.84			
Phialemoniopsis	0.03	2.12	0.01	0.16			
Lophiostoma	0.02	1.55	0.01	0.29			
Epicoccum	1.52	0.23	0.02	0.14			
unclassified_f_Clavicipitaceae	0.02	0.01	1.41	0.23			
Cladophialophora	1.45	0.34	0.03	0.26			
Oidiodendron	1.04	0.67	0.02	0.31			
unclassified_o_Pezizales	0.04	0.02	1.51	0.15			
unclassified_o_Agaricales	0.02	0.01	1.32	0.08			
others	19.77	15.66	7.93	15.01			



Community heatmap analysis on Species level

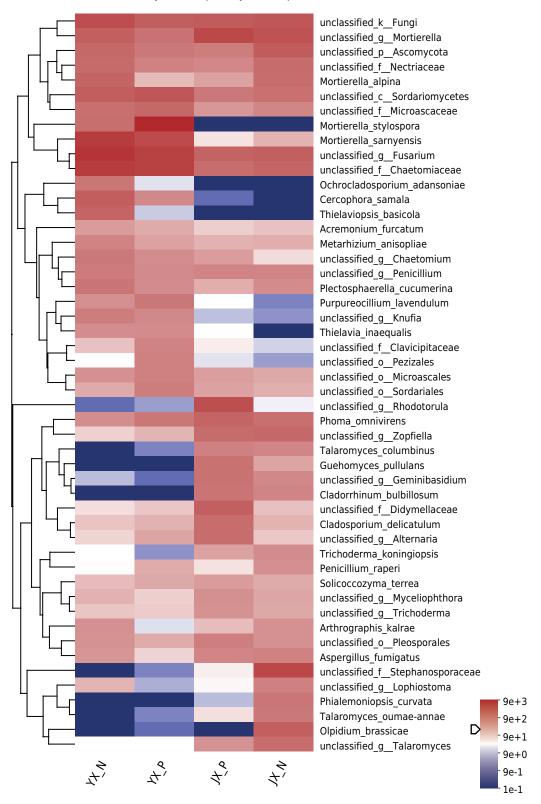


Figure 4. The mcrobial community heatmap on species level in rhizosphere soils at different samples.

In previous studies, the occurrence of RKN disease was closely related to the interaction between soil microbial communities (Echeverrigaray et al., 2010). Studies reported significant differences in the bacterial community richness and diversity between RKN diseased and healthy soil and the presence of different microbial species of the bacteria, but no difference was observed in all aspects of the fungi (Huang et al., 2020). We found



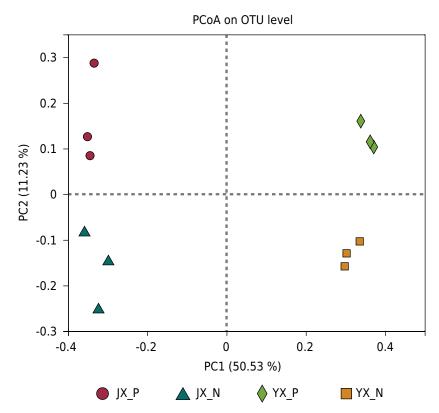


Figure 5. PCoA cluster analysis of fungus community in rhizosphere soils at different samples.

that the soil not infected by *Meloidogyne incognita* had higher fungal diversity than the infected soil, which indicates that the lower microbial diversity may result in high RKN infection. Microbial diversity is an excellent indicator of soil health. Loss of soil microbial diversity contributes to an increase in soil-borne plant diseases; on the contrary, the high microbial, as well as functional diversity and activity, are involved in plant growth promotion, plant defense, and soil-borne disease suppression (Mendes et al., 2015; Raaijmakers et al., 2016). The scientific community has a great interest in developing strategies that reshape the rhizosphere microbial community to attain stable and more diverse conditions to prevent or mitigate pathogen/pest occurrence (Jaiswal et al., 2017). Thus, knowing what kind of microbes and their role is very important.

We found that in rhizosphere soil samples, the abundances of OTUs in soils from healthy plants were higher than in soils from diseased plants. More beneficial fungi, such as Penicillium and Aspergillus, were found in soil samples of healthy plants, whereas more pathogenic fungi, such as *Phoma* and *Alternaria*, were found in soil samples of diseased plants. Some studies have shown that a small class of key groups are present in the rhizosphere microbial community, and they play a leading role in promoting disease occurrence (Berendsen et al., 2012; Lawson et al., 2019). Penicillium participates in the decomposition of organic matter, promotes the cycling of C, N, P and other elements, and degrades a variety of environmentally harmful substances (Luo et al., 2016). Purpureocillium lavendulum is an important nematode biocontrol fungi (Tong et al., 2021). Increases in two kinds of beneficial microorganisms have positive effects on optimizing the rhizosphere fungal community and on improving the micro-ecological environment. Phoma spp. are important plant pathogens that often cause stem rot, branch blight, and leaf and fruit necrosis. Among them, P. omnivirens causes phoma disease by infecting tobacco stems and leaves (Jiang et al., 2018). Alternaria is also a common plant pathogen that can infect tobacco, producing brown spots. The relative abundances of these two genera in the rhizosphere soil of diseased tobacco increased, which may be related to the increase in the incidence rates of complex infectious diseases after infection by Meloidogyne spp. (Li et al., 2013).



CONCLUSIONS

Occurrence of RKN disease in tobacco was closely related to the fungal community and diversity level in the rhizosphere soil. Soil rhizosphere of healthy tobacco plants showed greater fungal diversity and abundance, and the fungal community was more balanced than that of infected plants. The relative abundance of beneficial fungi, such as *Penicillium* and *Aspergillus* in the rhizosphere soil of diseased plants decreased, whereas those of pathogenic fungi, such as *Phoma* and *Alternaria*, increased. The imbalance of rhizosphere micro-ecology may further aggravate the disease state of tobacco. Therefore, reshaping stable and diversified rhizosphere microbial communities to enhance the rhizosphere micro-ecological resilience may help to increase tobacco resistance against *M. incognita*, although it is still a challenge to manipulate the soil traits to reach the ideal community structure.

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