

Response of the rhizosphere soil fungal community to root rot infection of *Plukenetia volubilis* L

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Research Article

Keywords: *Fusarium* spp., *Rhizopus* spp., rhizosphere fungal community, Root rot, *P. volubilis*

Posted Date: October 23rd, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3435002/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at International Microbiology on December 29th, 2023. See the published version at <https://doi.org/10.1007/s10123-023-00470-x>.

Abstract

Background

Root rot disease caused by soilborne pathogens is a threat to agriculture, affecting plant survival and leading to yield losses. The soil in which plants grow is inhabited by beneficial microbiome playing essential roles in various aspects of host functionality and fitness. However, this microbiome composition may change based on plant health status.

Methods

In the current study, we tested the pathogenicity of pathogenic fungi isolated from root rot infected *P. volubilis* plant tissues by inoculating them into healthy *P. volubilis* seedlings. Metagenomic sequencing was used to assess fungal community shifts in the rhizosphere of *P. volubilis* after root rot infection.

Results

Four *Fusarium* isolates and two *Rhizopus* isolates were found to be root rot causative agents in *P. volubilis* as they induced typical root rot symptoms in healthy seedlings. The metagenomic sequencing data showed that root rot infection altered the rhizosphere fungal community. In root rot infected soil, the richness and diversity indices increased or decreased depending on pathogens. The four most abundant phyla across all samples were Ascomycota, Glomeromycota, Basidiomycota, and Mortierellomycota. In infected soil, the relative abundance of each phylum increased or decreased depending on pathogen and functional taxonomic classification.

Conclusions

Based on our results, we concluded that *Fusarium* spp. and *Rhizopus* spp. cause root rot infection of *P. volubilis*. In root rot infected *P. volubilis*, the response of the rhizosphere fungal community was pathogen dependent. These findings may serve as a key point for a future study on biocontrol of root rot in *P. volubilis*.

Introduction

Plukenetia volubilis Linneo, also referred to as sachá inchi, Inca peanut, wild peanut, sachá peanut, or mountain peanut, is an oleaginous plant in the family *Euphorbiaceae* (Chirinos et al. 2013; Gutiérrez et al. 2011; Kodahl et al. 2021; Norhazlindah et al. 2022; Tran et al. 2021; Wang et al. 2018). *P. volubilis* L. is native to the Amazon's tropical rain forest, including regions of Peru and northwestern Brazil, where it is grown at altitudes between 200 and 1500 m above sea level (Chirinos et al. 2013; Gutiérrez et al. 2011; Kodahl et al. 2021; Norhazlindah et al. 2022; Tran et al. 2021; Wang et al. 2018). Due to its excellent

potential as an economic crop, *P. volubilis* has been introduced into Southeast Asia (Norhazlindah et al. 2022; Tran et al. 2021). Altogether, *P. volubilis* is cultivated almost in 20 different countries, predominantly in Latin America, Peru being the largest producer, with nearly 1200 tonnes of seeds for annual production (Tran et al. 2021). In Latin American, in addition to Peru, *P. volubilis* is further cultivated in Colombia, Brazil, Ecuador, Bolivia, Nicaragua, Guatemala, Costa Rica, Mexico, and Cuba (Tran et al. 2021). In Asia, *P. volubilis* is mainly cultivated in China, but also in Cambodia, Thailand, and Laos (Tran et al. 2021). *P. volubilis* was first introduced to China to be cultivated and grown in Xishuangbanna, Yunnan Province, southwest China by the Xishuangbanna Tropical Botanical Garden (XTBG), part of the Chinese Academy of Sciences, in 2006 (Wang et al. 2018). In 2014, nearly 13.5 ha had been used for *P. volubilis* to be cultivated as an under-forest cash crop under *Areca catechu* trees in the Chinese provinces of Lingshui and Hainan (Wang et al. 2018; Yang et al. 2017). *P. volubilis* is being cultivated because of its commercial role and pharmacological value. The commercial role of *P. volubilis* is due to its seeds, which are a good source of 35–60% edible oil (by dry weight), a content comparable to that of sunflower seeds and peanuts, as well as being high in protein (approximately 27% or more by dry weight) and rich in vitamins A and E (Chirinos et al. 2013; Liu et al. 2020). *P. volubilis* pharmacological value is based on extracts from its seeds and leaves which have been used to lower and reduce lipids and cholesterol in people with postprandial lipemia and high blood cholesterol, respectively (Garmendia et al. 2011; Tran et al. 2021). In addition, a study conducted by Nascimento et al. (2013) showed that the extracts from fresh leaves of *P. volubilis* had antioxidant and anti-proliferative activities against HeLa cells, and were also capable of cell proliferation in fibroblast cells-3T3. Furthermore, *P. volubilis* is also used in traditional medicine due to its potential to regulate cholesterol, improve digestion, and have an anti-diabetic effect (Lock et al. 2016). In the past five years, *P. volubilis* cultivation in XTBG is facing challenges due to the invasion of pathogens that cause fungal disease infections. Chai et al. (2017) reported the root and basal stem rot in *P. volubilis*. Infected plants were marked by rotting of the root and basal stem, growth retardation, wilting, leaf yellowing, and plant death. In 2021, a severe root rot infection was observed in some *P. volubilis* cultivated fields. The main symptoms in infected plants included yellow leaves, necrosis, fall of leaves, and rotten roots with brown and black lesions in internal tissues of the roots. A field survey showed that the disease incidence varied between (a) 60 to 70% based on infection symptoms in the foliage, and (b) 100% based on infection symptoms in the underground part of the plants.

The importance of the composition of the rhizosphere microbiome on plant health and productivity has been increasingly recognized. The rhizosphere is inhabited by a diversity of microorganisms greatly influenced by plants (Araujo et al. 2020; Berendsen et al. 2012; Raaijmakers et al. 2009; Wu et al. 2016). The rhizosphere is considered a playground and infection court, where the parasitic relationship between pathogen and plant is established (Raaijmakers et al. 2009). In addition, the rhizosphere is a conflict zone of interactions between microorganisms and pathogens that influence the pathogen infection outcome (Raaijmakers et al. 2009). The diversity and dynamics of rhizosphere microorganisms drive the suppression of plant diseases by acting in direct and indirect ways to suppress the growth of plant pathogens (Chen et al. 2018; Garbeva et al. 2004; Hong et al. 2023). On the other hand, the plant health status affects the composition and diversity of the rhizosphere microbiomes (Xiong et al. 2021).

Pathogen invasion is the most influential factor that affects the rhizosphere's microbiome (Chapelle et al. 2016; Fernandez-Gonzalez et al. 2020). Therefore, this study aims to understand the impact of root rot disease infection of *P. volubilis* on the rhizosphere's fungal community. We hypothesized that fungal pathogens causing root rot of *P. volubilis* may influence the rhizosphere's fungal community in a way associated either with disease infection or with suppression.

Materials and methods

Origin of root rot pathogenic fungi used in this study

The pathogenic fungi used in this study were isolated from *P. volubilis* plants infected by root rot in XTBG (21° 55' N, 101° 16' E). Fungal isolation was performed according to the method by Uwaremwe et al. (2021) in the following steps: samples of plant material including roots, leaves, and fruits were prepared and surface-sterilized twice using sterile water. They were cleaned using 75% ethanol for 30 s and immediately transferred to 3% sodium hypochlorite for 5 min, and finally washed three times with sterile water. Prepared samples were then cut into small, thin blocks 0.5 × 0.5 cm, and placed on Rose Bengal agar culture medium (purchased from Qingdao Hope Bio-Technology Co., Ltd). All petri plates (90 mm × 15 mm) were incubated at 25°C for 5–7 days. Fungal pure cultures were developed by subculturing a single mycelium arisen from explants, on potato dextrose agar (PDA, purchased from Qingdao Hope Bio-Technology Co., Ltd). Molecular identification of fungal isolates was performed by amplification and sequencing of internal transcribed spacer (ITS) region (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'; ITS4: 5'-TCCTCCGCTTATTGATATGC-3' primers) (Schoch et al. 2012 ; Zarrin et al. 2016), 18S rRNA gene (NS1: 5'-GTATCATATGCTTGTCTC-3' ; NS6: 5'-GCATCACAGACCTGTTATT-3' primers) (Banos et al. 2018), RNA polymerase II large subunit (RPB1) gene (F: 5'-GCGACGAAGAAGATGGTTTAGA-3' ; R: 5'-CATCATCCATTCAGGTCTGGCA-3' primers) (Stockinger et al. 2014) and the translation elongation factor (*TEF1-α*) gene region primers (TEF1F: 5'-GTCACCTTGATCTACCAGTGC-3'; TEF1R: 5'-TACCAATGACGGTGACATAG-3' primers) (Hall 1999 ; Uwaremwe et al. 2021).

Pathogenicity test of fungal isolates

To fulfill Koch's postulates, we conducted a pathogenicity test in green house conditions at a temperature of 27 °C and 78% relative humidity, from April to the end of August 2022. Six fungal isolates belonging to *Fusarium* spp. and *Rhizopus* spp. were tested for their root rot disease pathogenicity in *P. volubilis* seedlings. The *Fusarium* spp. included *F. solani* isolate F2P2Rg, *F. graminearum* isolate F4P5Rk, *F. proliferatum* isolate F3P1Ra from root tissues, and *F. solani* isolate F2P1Fd from fruit tissues. The *Rhizopus* spp., included *R. oryzae*, isolate F4P3Rd and *R. oryzae* isolate F4P3Rg from root tissues. The six fungal isolates were inoculated into three-month-old healthy *P. volubilis* seedlings obtained from seed germination. For inoculum preparation, fungi were first grown on PDA medium for ten days. The inoculum was obtained by pouring 30 mL of sterile water into each of the petri dishes and a sterile toothbrush was used to scrape the mycelium on the medium surface. The mixture was then filtered using sterile cheesecloth. The concentration of conidia in the suspension was determined using a

haemocytometer. The final inoculum concentration was adjusted to 5×10^7 conidia/mL. To inoculate the pathogens into seedlings, the main root of each seedling was first wounded using a sterilized blade. Afterward, 50 mL of spore suspension was gently poured on the wounded part of the root and in the rhizosphere. For control, the seedlings' roots were first wounded as well, and treated with sterilized water (50 mL per/seedling). This experiment was conducted according to a randomized complete design composed of three replicates per treatment and three replicates per control (in total 72 seedlings per treatment and control).

Assessment of root rot infection in *P. volubilis*

After inoculating the six fungal isolates into *P. volubilis* seedlings, we assessed disease infection degree by looking at disease incidence (DI) and disease severity (DS). The DI was assessed as the percentage of infected seedlings out of the total of all treated seedlings for each fungus (Ao et al. 2022) as shown in the following formula:

$$DI = \frac{\text{total number of infected seedlings}}{\text{total number of inoculated seedlings}} \times 100$$

The DS in the foliage was evaluated using a rating scale from 0 to 5, as reported in Han et al. (2019). Based on different root rot disease observed symptoms, 0 = seedlings with no symptoms, 1 = moderate leaf yellowing, 2 = severe leaf yellowing, 3 = leaf loss, 4 = wilting, and 5 = seedling death. The DS values were obtained from the averages of these scores.

Metagenomic sequencing of fungal community

Thirty-three days after fungal inoculation, rhizosphere soil from the three replicate pots was collected from all treatments (21 samples in total) and sieved (a 2 mm sterilized sieve). Briefly, the roots were lightly shaken to remove loosely attached soil. The soil that was still tightly adhering to the roots was harvested as rhizosphere soil and frozen at -80°C . Total soil DNA was extracted using Tiangen TGuide S96 Magnetic Soil Stool DNA Kit-190430, following all steps provided in the kit handbook. Genomic DNA concentration and purity were measured using a Qubit fluorometer (Thermo Fisher Scientific, United States). The fungal community composition was assessed by sequencing the internal transcribed spacer (ITS) region of the rDNA using a set of primer consisting of ITS1F 5' CTTGGTCATTTAGAGGAAGTAA-3' and ITS4R 5'-TCCTCCGCTTATTGATATGC-3 (Han et al. 2019 ; Wang et al. 2022). PCR conditions were initiated at 95°C for 5 min, followed by 30 cycles of denaturation at 98°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, followed by a final elongation at 72°C for 5 min. The PCR products were pooled and visualized on 1% agarose gel, purified using a MinElute PCR Purification Kit according to the manufacturer's instructions, and quantified using QuantiFluorTM-ST (Promega, United States). High-throughput sequencing was carried out on the Illumina MiniSeq platform NovaSeq 6000 (BioMarker Technologies Co., Ltd., China). The original subreads were first corrected to generate circular consensus sequences (CCS) (SMRT Link, version 8.0), then Lima software (v1.7.0) was used to identify the CCS of different samples through their barcoded sequences, and UCHIME1 (version 8.1) was used to remove the

chimera bodies for high quality CCS sequences. Using USEARCH 4 (version 10.22) with a cutoff of 97% similarity, the Operational Taxonomic Units (OTUs) were clustered and the taxonomic classifications were performed using RDP Classifier (Version 2.2, based on Bergey's taxonomy) with the classification threshold set at 0.5. To annotate the functions of different rhizosphere fungal groups, we used the FUNGuild database. The FUNGuild software annotates taxonomic data in the OTU table with relevant data in its online database, including guild, trophic mode, and growth morphology (Nguyen et al. 2015).

Statistical Analysis

Kruskal–Wallis one-way analysis of variance by ranks was used for comparing DI and DS. The rarefaction curve, corresponding to the observed OTUs at different sequencing depths, was examined using QIIME software to determine whether the depth was reasonable. Chao1 and abundance-based coverage estimator (ACE) indices were used to calculate the evenness of each sample (Bokulich et al. 2013), while the Shannon and Simpson indices were used to measure diversity (Hong et al. 2015). Beta diversity among samples was determined by principal component analysis (PCA) using R software². Significant differences in fungal community composition between paired samples were determined using the Metastats analysis and Mothur program which counted taxa in five classified levels (Lu et al. 2016). Pearson correlation analysis between disease incidence, disease severity and fungal community composition was performed using SPSS software version 23.

Results

Isolation and molecular identification of root rot pathogenic fungi

In total, 75 isolates were identified based on the ITS and 18S rRNA molecular identification. Among all the 75 isolates, *Fusarium* spp. were the most abundant (28%), followed by *Rhizopus* spp. (24%), *Aspergillus* spp. (13%), *Penicillium* spp. (9%), and *Trichoderma* and *Daldinia* spp. at 5% respectively (**Figure S1**). The translation elongation factor (*TEF1-a*) gene and the RNA polymerase II large subunit (RPB1) gene were further used to identify the *Fusarium* and *Rhizopus* isolates at species level.

Pathogenicity test of fungal isolates

The pathogenicity test result proved that all the six isolates (**Table 1**, Fig. 1) were root rot disease causative agents in *P. volubilis* plants, as they induced typical root rot disease symptoms in inoculated seedlings, the same symptoms we observed in the fields (Fig. 2). In the foliage, the first root rot disease symptoms appeared 19 days post-inoculation (DPI). Infected seedlings were marked by moderate and severe leaf yellowing, leaf loss and wilting. None of above-mentioned symptoms was recorded in water treated seedlings (**Figures S2, S3, Table S1**). The disease infection progressively became serious in term of incidence and severity. The six fungi were recovered from infected seedling roots confirming Koch's postulates.

Table 1 Six fungal isolates used in this study and their GenBank accession numbers

Isolate code	Source of the isolate	Name of the isolate	ITS	GenBank accession numbers		
				18S rRNA	<i>TEF1-α</i> gene	RPB1 gene
F2P2Rg	Root	<i>Fusarium solani</i>	OL981323	ON312923	OR134602	**
F4P5Rk	Root	<i>Fusarium graminearum</i>	OM959558	ON026105	OQ605233	**
F3P1Ra	Root	<i>Fusarium proliferatum</i>	OR242482	ON026121	OQ605234	**
F4P3Rd	Root	<i>Rhizopus oryzae</i>	OM959554	ON026101	**	OQ077573
F4P3Rg	Root	<i>Rhizopus oryzae</i>	OM959555	ON026102	**	OR174798
F2P1Fd	Fruit	<i>Fusarium solani</i>	OM033375	ON312930	OQ605232	**

The symbol ** means that the identification type was not concerned. The *TEF1-α* gene was used to identify *Fusarium* isolates at species level whereas the RPB1 gene was used to identify *Rhizopus* isolates at species level.

Disease incidence and severity assessment

To assess the degree of root rot infections in *P. volubilis* plants, we recorded disease incidence (DI) and disease severity (DS) at three time points after pathogen inoculation in terms of infection progress. At 19 days post-inoculation (DPI), the highest DI of 35% was recorded in seedlings treated with *R. oryzae* isolate F4P3Rd, followed by DI of 23% and 21% in seedlings treated with *R. oryzae* isolate F4P3Rg and *F. solani* isolate F2P1Fd, respectively. The lowest DIs (16%, 15%, and 13%) were recorded in seedlings treated with *F. proliferatum* isolate F3P1Ra, *F. solani* isolate F2P2Rg, and *F. graminearum* isolate F4P5Rk, respectively (Fig. 3A). The highest DS was recorded in *R. oryzae* isolate F4P3R treated seedlings, whereas the rest of isolates displayed an equal DS (Fig. 4A). Assessment conducted at 26 DPI showed that there was no increase in DI in seedlings treated with *R. oryzae* isolate F4P3Rd and *F. proliferatum* isolate F3P1Ra. However, an increased DI by 28%, 24%, 25%, and 26% was recorded in seedlings treated with *F. solani* isolate F2P2Rg, *F. graminearum* isolate F4P5Rk, *F. solani* isolate F2P1Fd, and *R. oryzae* isolate F4P3Rg, respectively (Fig. 3B). The DS increased in all treatments, except in seedlings treated with *F. solani* isolate F2P1Fd. The highest DS was recorded in *R. oryzae* isolate F4P3Rd treatment in which seedling death was recorded (**Figure S3**). A significant difference was observed between F4P3Rd and F2P1Fd treatments (Fig. 4B). The last assessment was conducted at 33 DPI, and the results showed that the DI increased in all treatments, with the highest DI recorded in *R. oryzae* isolate F4P3Rd treatment (Fig. 3C). The DS

assessment showed an increased DS in *F. solani* isolate F2P1Fd treated seedlings, whereas no increase was observed in all other treatments (Fig. 4C). When seedlings were uprooted, root observation showed that the roots of infected seedlings had brown-black lesions in the internal tissues and on the outside surface, consistent with the same observation in the fields. In contrast, the roots of water treated seedlings were free of lesions (Figure S4).

Metagenomic sequencing of rhizosphere fungal community

In total, 274,285 ITS sequences were analyzed throughout 21 rhizosphere soil samples with an average of 13,061 sequences per soil sample (Table S2). Based on a shared nucleotide identity threshold of 97%, these sequences were categorized by OTU, yielding 12,595, 10,515, 10,352, 10,079, 9,944 and 9,762 OTUs at the phylum, class, order, family, genus, and species levels, respectively (Table S3). New taxa were identified based on sequencing depth analysis. A rarefaction curve analysis at 3% dissimilarity showed that the sharp of the curve was smooth and the depth reached a stable level, showing that the sequencing was sufficient (Figure S5). We further assessed the richness (ACE and Chao 1), and diversity (Shannon and Simpson) indices. In comparison to water control, the ACE and Chao 1 indices were found to be higher in F4P3Rg, F3P1Ra, F2P1Fd, and F4P3Rd treatments with a highly significant difference found in F4P3Rg treatment. Although there was no significant difference in the diversity index of all treatments, the highest index was observed in F4P3Rg treatment (Table 2). The principal component analysis of the fungal community across various treatments showed that the PC1 and PC2 explained 17.97% and 17.49% of the total variation, respectively. The rhizosphere fungal communities were relatively close in all isolates (Fig. 5). The average number of microbial groups at the level of phylum, class, order, family, genus, and species was 9, 27, 53, 81, 111 and 132, respectively (Table S4). At phylum and genus levels, all treatments displayed similar composition, but differed in the relative abundances of other taxonomic groups (Fig. 6). Across all treatments, of the 10 most abundant phyla, Ascomycota, Glomeromycota, Basidiomycota, Mortierellomycota, and Rozellomycota were the top five most abundant, and their relative abundances were differentially affected by each treatment in the overall fungal composition of the rhizosphere (Fig. 6A, Table S5). Compared to the control, Ascomycota increased in F2P1Fd, F4P3Rd, and F4P5Rk treatments whereas it decreased in F2P2Rg, F3P1Ra, and F4P3Rg treatments. Glomeromycota decreased in all isolates. A decreased relative abundance in Basidiomycota was found in all isolates except F4P3Rg and F4P3Rd. Mortierellomycota increased in all isolates except F2P2Rg. Rozellomycota increased in all isolates (Fig. 7). At the genus level, the relative abundances were also differently affected by each treatment in the overall rhizosphere fungal composition. Of the 10 most abundant genera, the five most abundant were unidentified followed by *Mortierella*, *unclassified_Ascomycota*, *unclassified_Basidiomycota*, and *Acaulospora* (Fig. 6B, Table S6). In comparison with the control, unidentified and *unclassified_Ascomycota* were found decreased in all treatments whereas *Mortierella*, was increased in all treatments. *Unclassified_Basidiomycota* decreased in all isolates except F4P3Rg. *Acaulospora* increased in F2P2Rg, F3P1Ra, and F4P5Rk, however, it decreased in the rest of isolates (Fig. 8). Moreover, a classification based on their functions showed that, overall, the rhizosphere fungal community was composed of three main functional taxonomic groups including pathotroph, saprotroph, and symbiotroph trophic modes. At the phylum level, in the pathotroph

and saprotroph trophic mode groups, Ascomycota and Basidiomycota were the most represented, whereas Glomeromycota was the most represented in the symbiotroph group (**Figure S6**). At the genus level, *Clonostachys* and *Colletotrichum* were the most represented in the pathotroph group; in saprotroph trophic mode group, *Conocybe* and *Achrocerotosphaeria* were the most represented; and in the symbiotroph group, *Acaulospora* and *Botryotrichum* were the most represented (**Figure S7**). Within treatments, symbiotroph group was the most increased with the highest increase found in the rhizosphere soil of control seedlings. The saprotroph group increased highly in samples from seedlings infected by *R. oryzae* isolate F4P3Rd. However, it decreased moderately in the soil of control seedlings. The pathotroph group decreased the most in the control; in contrast, it increased most in the soil from seedlings infected by *F. solani* isolate F2P1Fd, followed by *R. oryzae* isolate F4P3Rd, and *R. oryzae* isolate F4P3Rg, respectively (Fig. 9).

Table 2
The mean of the richness and Diversity indices with in different treatments

Treatment	Richness index		Diversity index	
	ACE	Chao1	Simpson	Shannon
CK	367.49 ± 11.745 ^b	379.53 ± 11.764 ^b	6.0717 ± 0.0064 ^a	0.9656 ± 0.1789 ^b
F2P1Fd	435.96 ± 25.708 ^{ab}	440.43 ± 23.178 ^{ab}	6.5821 ± 0.0053 ^a	0.9762 ± 0.1948 ^{ab}
F2P2Rg	354.99 ± 10.603 ^b	355.84 ± 4.7477 ^b	6.3308 ± 0.0096 ^a	0.9679 ± 0.2872 ^{ab}
F3P1Ra	491.82 ± 97.409 ^{ab}	487.99 ± 95.558 ^{ab}	6.4934 ± 0.0083 ^a	0.9675 ± 0.2671 ^{ab}
F4P3Rd	410.46 ± 29.733 ^b	428.28 ± 45.919 ^b	6.2877 ± 0.0183 ^a	0.9665 ± 0.4186 ^{ab}
F4P3Rg	598.52 ± 234.81 ^a	599.49 ± 231.86 ^a	6.8998 ± 0.0167 ^a	0.9761 ± 0.8746 ^a
F4P5Rk	358.1 ± 25.229 ^b	362.76 ± 24.297 ^b	6.4579 ± 0.0097 ^a	0.9725 ± 0.2615 ^{ab}

The different treatments include: control CK, *F. solani* F2P1Fd, *F. solani* isolate F2P2Rg, *F. proliferatum* isolate F3P1Ra, *R. oryzae* isolate F4P3Rd, *R. oryzae* isolate F4P3Rg and *F. graminearum* isolate F4P5Rk. Different letters indicate a significant correlation at 0.05 level.

Relative abundance was based on the proportional frequencies of DNA sequences classified at the genus level. Length of a color bar correlates with amount of abundance. Data were averaged from three replicates of each treatment.)

Correlation between root rot disease infection and rhizosphere fungal community

A correlation analysis was performed between the five most abundant fungal phyla and genera, and root rot disease incidence and severity (Table 3). Among the five most abundant phyla, Ascomycota was

negatively correlated with DI, but positively correlated with DS. Glomeromycota was significantly and negatively correlated with DI and DS, while Basidiomycota, Mortierellomycota and Rozellomycota showed positive correlations with DI and DS (Table 3A). At the genus level, *Mortierella* was negatively correlated with DI, but positively correlated with DS. Additionally, *Unclassified_Ascomycota*, *bunclassified_Basidiomycota* and *Acaulospora* showed negative correlations with DI and DS (Table 3B).

Table 3
Pearson correlation between five most abundance fungal phyla (A) and genera (B) and disease incidence and disease severity

	Phylum	CC (DI)	<i>p</i> value	CC (DS)	<i>p</i> value
A	Ascomycota	-0.04	0.932	0.124	0.792
	Glomeromycota	-0.788*	0.035	-0.818*	0.025
	Basidiomycota	0.126	0.787	0.036	0.939
	Mortierellomycota	0.617	0.14	0.72	0.068
	Rozellomycota	0.572	0.18	0.659	0.107
B	unidentified	0.399	0.375	0.222	0.633
	Mortierella	-0.029	0.951	0.008	0.986
	unclassified Ascomycota	-0.142	0.761	-0.227	0.624
	unclassified_Basidiomycota	-0.024	0.959	-0.067	0.886
	Acaulospora	-0.057	0.903	-0.143	0.759

CC (DI) and CC (DS) mean the correlation coefficient in term of disease incidence and disease severity respectively. The * means a significant correlation at 0.05 level.

Discussion

In the current study, pathogenic fungi were isolated from rhizosphere soil and plant material samples from root rot infected *P. volubilis*. Among different fungal isolates, *Fusarium* spp. were the most abundant, followed by *Rhizopus* spp. This result is consistent with previous studies in which *Fusarium* spp. were found to be the most abundant root rot causative fungi in wolfberry and olive plants (Hibar et al. 2017; Uwaremwe et al. 2021). *Fusarium* spp. as phytopathogens are widely distributed and known to cause severe diseases in a wide range of hosts, including economically important crops such as wheat and soybean (Aoki et al. 2014; Arias et al. 2013; Champeil et al. 2004; Parry et al.1995). A pathogenicity test conducted under greenhouse conditions revealed that four *Fusarium* isolates, including two *F. solani* isolates, one *F. graminearum* isolate, and one *F. proliferatum* isolate were root rot pathogens as they induced typical root rot symptoms in *P. volubilis* seedlings. In the foliage, infected seedlings were marked

by moderate leaf yellowing that gradually progressed to severe leaf yellowing and leaf loss. The roots of infected seedlings were rotten with brown and black lesion in internal tissues and external surface. Similar root rot symptoms caused by *F. solani* and *F. graminearum* were found by (Perez et al. 2011) in olive, (Ji et al. 2012) in *Benincasa hispida* (Li et al. 2015), in Okra, (Pastrana et al. 2013), in strawberries, (Melo et al. 2016), in Sunn Hemp, and (Bilgi et al. 2011; Kang et al. 2019) in soybean and dry edible bean, respectively. *F. solani* species are also known as *Fusarium solani* species complex and they have been most extensively studied as plant pathogens (Coleman et al. 2015). *F. proliferatum* is a fungus with a worldwide distribution that has been associated with a variety of diseases in important economic plants such as corn and bananas (Sun et al. 2018). It has been reported as root rot pathogen in soybean in Canada (Chang et al. 2015) and the United States (Diaz Arias et al. 2011); and in Salvia in China (Yang et al. 2021). The two *R. oryzae* isolates tested, also induced root rot disease symptoms in *P. volubilis* including leaf yellowing and fall off, seedling wilting and death, and root decay. Same root rot symptoms due to *R. oryzae* infection were found by Gnanesh et al. (2020) in mulberries, Wang et al. (2017) in sweet potatoes, and Xu et al. (2015) in peanuts. *R. oryzae* is a well-known phytopathogen in different plants and crops worldwide. It infects seedlings, plant parts including flowers, bulbs and tubers, fruits, and vegetables (Al-Dhabaan 2018 ; Khokhar et al. 2019; Kwon et al. 2011).

Soil microorganisms play an essential role in plant defense (Saleem et al. 2019; Pascale et al. 2020). In particular, rhizosphere microorganisms are very important for nutrient uptake, suppression of pathogen colonization and maintenance of root-associated microecological balance and host immune regulation (Bakker et al. 2018 ; Korenblum et al. 2020 ; Liang et al. 2019 ; Zhang et al. 2017). It is strongly recognized that certain microbiomes of the rhizosphere can act as plant protectors against diseases (Li et al. 2020; Del Barrio-Duque et al. 2019). The composition and diversity of fungi in the rhizosphere are strongly linked to biotic and abiotic factors (Canini et al. 2019; Shanmugam et al. 2018). The main aim of our study was to analyze the relationship between *P. volubilis* root rot disease infection and rhizosphere fungal community composition and functions. We analyzed the richness (ACE and Chao 1), and diversity (Shannon and Simpson) between the rhizosphere soil of root rot infected *P. volubilis* seedlings and healthy seedlings (control). The result revealed that the highest fungal richness was found in the rhizosphere soil of seedlings infected with *R. oryzae* isolate F4P3Rg, *R. oryzae* isolate F4P3Rd, *F. proliferatum* isolate F3P1Ra, and *F. solani* isolate F2P1Fd, with a high significant difference found in *R. oryzae* isolate F4P3Rg treatment. In terms of diversity, we found that fungal diversity was highest in the rhizosphere soil of *P. volubilis* seedlings infected with all fungal isolates, with highly significant differences in Shannon index found in the treatment of *R. oryzae* isolate F4P3Rg. Our results are in agreement with previous studies that have analyzed the relationship between rhizosphere fungal richness, diversity and root rot or other diseases caused by fungi in different plants. A study by Han et al. (2019) revealed that ACE and Chao 1 were significantly higher in the rhizosphere soil of *Fusarium* wilt infected cucumber. Moreover, ACE, chao1, Simpson and Shannon indices were found to be highly significant in the soil samples from the rhizosphere of the root rot susceptible tobacco variety (Ao et al. 2022) and the root rot infected pea (Hossain et al. 2021). On the contrary, we found that the ACE and Chao 1 were higher in rhizosphere soil of healthy seedlings compared to the rhizosphere soil of seedlings

infected with *F. solani* isolate F2P2Rg and *F. graminearum* isolate F4P5Rk. This is in line with the findings of Zhang et al. (2022) where ACE and Chao 1, and Shannon indices of healthy soil samples were significantly higher than diseased samples; and the study of Wang et al. (2022) that found higher fungal richness and diversity in CK treatment than *F. oxysporum* treatment in ginseng.

The relative abundance of the fungal community revealed Ascomycota, Glomeromycota, Basidiomycota, and Mortierellomycota as top four most abundant phyla in the rhizosphere of both healthy and root rot infected *P. volubilis* seedlings. Ascomycota was found to increase in the rhizosphere soil of *P. volubilis* seedlings infected with F2P1Fd, F4P3Rd and F4P5Rk compared to healthy (control) seedlings. However, Ascomycota was higher in healthy soil samples than in infected samples of F2P2Rg, F3P1Ra and F4P3Rg treatments. Furthermore, Ascomycota showed a negative correlation with disease incidence and a positive correlation with disease severity. Similar results were found by Ao et al. (2022) where Ascomycota was found as the most abundant fungal phylum in rhizosphere soil from root rot resistant and susceptible tobacco varieties. In a study by Han et al. (2019), Ascomycota was found the second most abundant phylum in cucumber soil infected with Fusarium wilt caused by *F. oxysporum* f. sp. cucumerinum (FOC), as well as in the healthy cucumber; with higher abundance found in soil of FOC infected cucumber. In addition, Shen et al. (2015) reported that Ascomycota depletion in banana rhizosphere was correlated with Fusarium wilt suppression. Ascomycota is the largest and most diverse true fungal group found in rhizosphere soils (Arafat et al. 2019; Gao et al. 2019). Previous studies have demonstrated that this phylum includes some fungal pathogens (Câmara et al. 2003; Sprague et al. 2007). Moreover, the functional taxonomic group classification revealed that Ascomycota was mainly classified in the pathotroph group in this study. This explains the observed higher abundance of Ascomycota, which may be associated with the increased disease incidence and disease severity in *P. volubilis* seedlings by *F. solani* isolate F2P1Fd, *R. oryzae* isolate F4P3Rd, and *F. graminearum* isolate F4P5Rk. Particularly, the pathography group was found to increase in F2P1Fd and F4P3Rd treatments. In addition, we found that some root rot reported pathogens such as *Alternaria spp.* and *Fusarium spp.* were included in Ascomycota.

Glomeromycota, the second most abundant fungal phylum in our study increased in healthy soil and decreased in all infected soils. Moreover, Glomeromycota showed a significant and negative correlation with disease incidence and disease severity. This is in agreement with a previous study by Huang et al. (2022) in which the abundance of Glomeromycota was found to be higher in healthy soil compared to soil of root rot infected *Atractylodes macrocephala* caused by *F. oxysporum*. Glomeromycota is a group of soil-borne fungi that are among the most important microorganisms on Earth. They form obligate biotrophic associations with a large number of terrestrial plants, with almost 90% of all plants, and form mutualistic associations called endomycorrhizae with the roots of about 70% of the world's plants (Taylor et al. 2015; Vilela. 2021; Volk 2013). Additionally, Glomeromycota has the potential to significantly influence the biodiversity and fitness of plants (Taylor et al. 2015). This emphasizes the highest increase in Glomeromycota abundance that we found in healthy soil. Furthermore, the functional taxonomic group classification revealed that Glomeromycota was mainly classified in the symbiotrophy group. Moreover, the symbiotrophy group increased considerably in healthy soil.

Basidiomycota was the third most abundant fungal phylum in all treatments. Compared to healthy soil samples, the relative abundance of Basidiomycota was higher in rhizosphere soil of F4P3Rg and F4P3Rd infected seedlings, whereas it was lower in seedlings infected with F4P5Rk, F3P1Ra and F2P2Rg. Our findings are similar to those of previous studies that found an increase in Basidiomycota abundance in the soil of root rot infected Ginseng caused by *F. oxysporum* (Wang et al. 2022); and higher abundance in healthy soil compared to the soil from root rot infected *Panax quinquefolius* L. (Jiang et al. 2019). Basidiomycota contains the most important plant pathogens, and their effects on the plant range from reduced yield to death (He et al. 2021; Volk 2001). In the current study, Basidiomycota was found to be positively correlated with both disease incidence and disease severity. Additionally, Basidiomycota was classified in the pathotroph functional taxonomic group. Specifically, the two isolates F4P3Rg and F4P3Rd were found to increase the pathotroph group in the soil, this explains the increased abundance in Basidiomycota found in F4P3Rg and F4P3Rd treatments.

Finally, Mortierellomycota increased in all infected soil samples, except for samples from F2P2Rg treatments. Some Mortierella species have been reported to be hostile to plant diseases such as root rot or potato scab and have exhibited disease suppression characteristics (Li et al. 2022). Mortierellomycota was positively correlated with both disease incidence and disease severity. In addition, Mortierellomycota is a distinct phylum which mostly includes saprotrophs in the soil (James et al. 2006 ; Tedersoo et al. 2018). This explains the increase in Mortierellomycota found in *R. oryzae* isolate F4P3Rd, in which root decay was observed. Especially, the saprotroph group was the most increased in the F4P3Rd treatment

Conclusion

The present study concluded that *Fusarium* spp. and *Rhizopus* spp. cause root rot of *P. volubilis*. The root rot infection of *P. volubilis* altered the rhizosphere fungi community. In the rhizosphere soil of root rot infected *P. volubilis*, the relative abundance of most four top phyla varied depending on pathogens, in a manner related to functional taxonomic groups and lifestyle. Further research is needed to understand the factors that govern the dynamics and distribution of root rot infection of *P. volubilis*, and the development of a biocontrol method for management of root rot in *P. volubilis*.

Declarations

I, Constantine Uwaremwe, declare that the submitted research paper is my original work and no part of it has been published any where else in the past.

Fundings

This research was funded by the National Natural Science Foundation of China (NSFC) (Grant no. 42061144005 and 41877064).

Conflicts of interest

All authors declare that they have no conflicts of interest.

Availability of data and material

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Authors' contributions

Uwaremwe Constantine: Sampling, experiments, writing and submitting. Bao Wenjie, Shen Lingjie and Zhang Xianxian: sampling, inoculation and watering the seedlings. Bachir Goudia Daoura: Primer design and manuscript review. Sandhya Mishra: manuscript review. Xia Shanwen: seeds and soil provision. Yang Xiaodong: project coordinator, supervisor.

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Figures

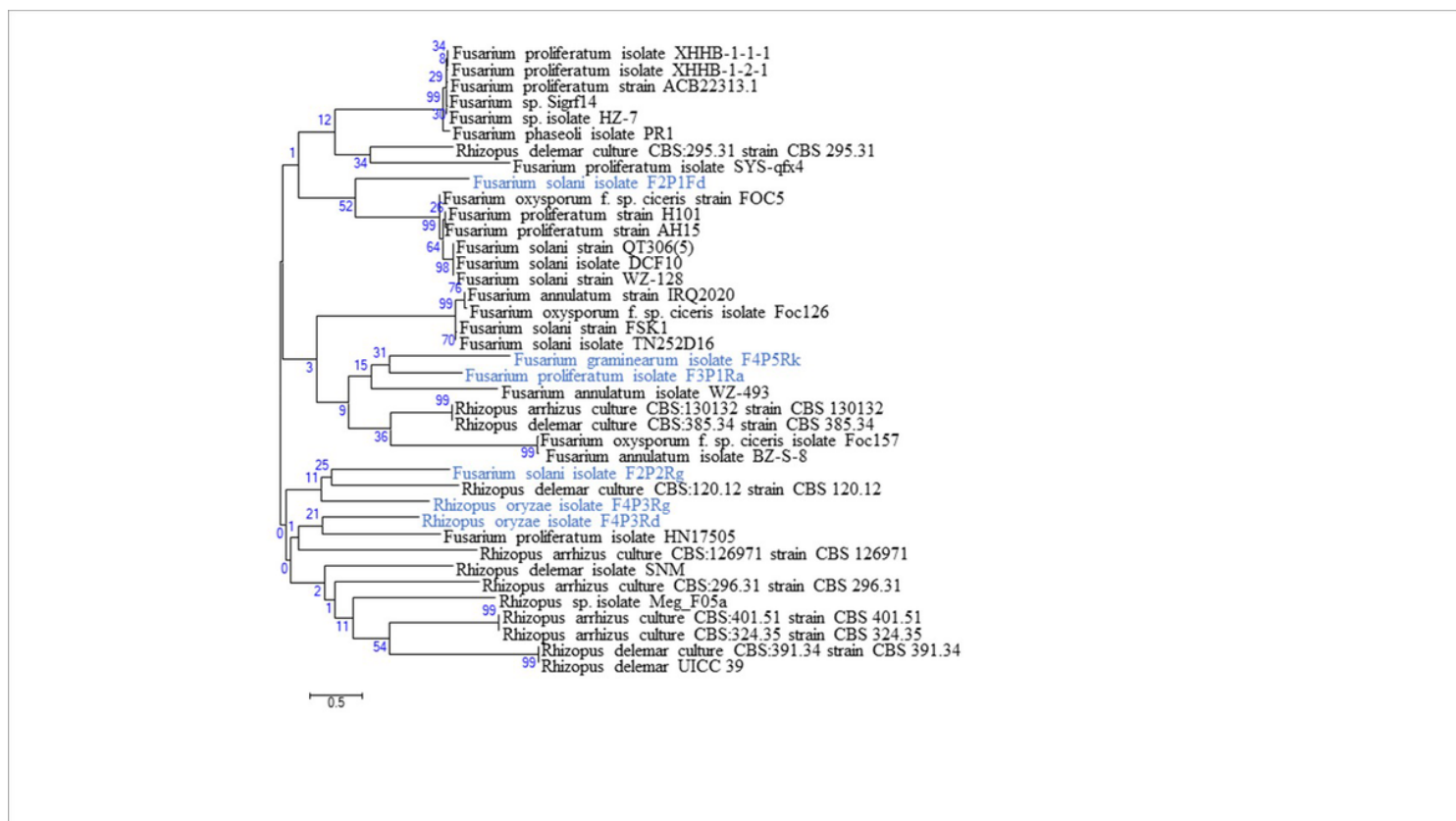


Figure 1

Phylogenetic tree based on ITS showing phylogenetic relationship between the six fungal isolates used in this study and fungi from NCBI. The tree was inferred using the neighbor-joining method and MEGA 5.5 software with 1000 bootstrap replicates (bootstrap values are shown next to nodes).

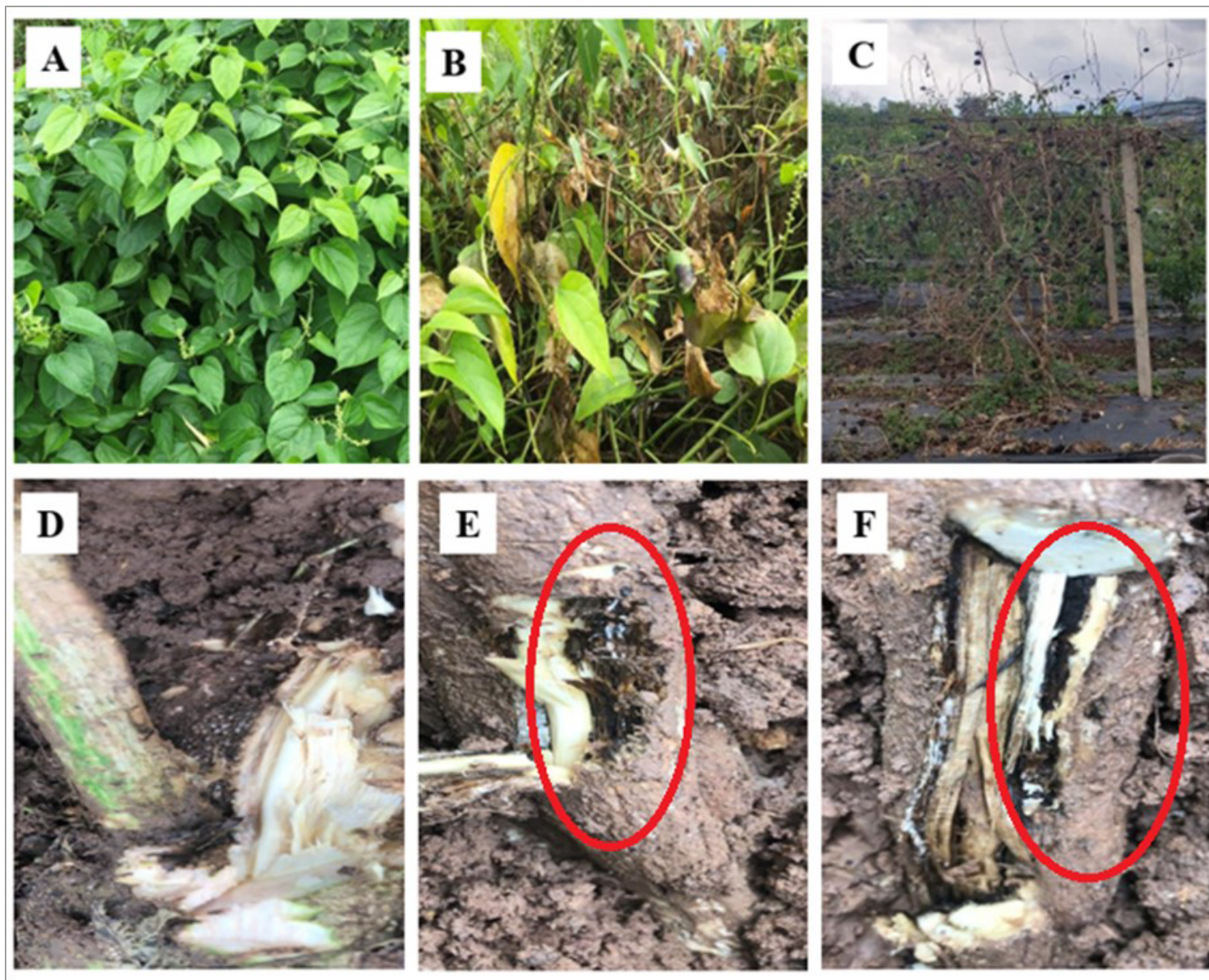


Figure 2

Observed root rot symptoms in the fields. Images of the foliage and root of healthy *P. volubilis* plants (A, D) and the foliage of root rot infected *P. volubilis* plants showing leaf yellowing, necrosis and plant death (B-C), and root of root rot infected *P. volubilis* plants showing black lesions in the internal tissues (E-F).

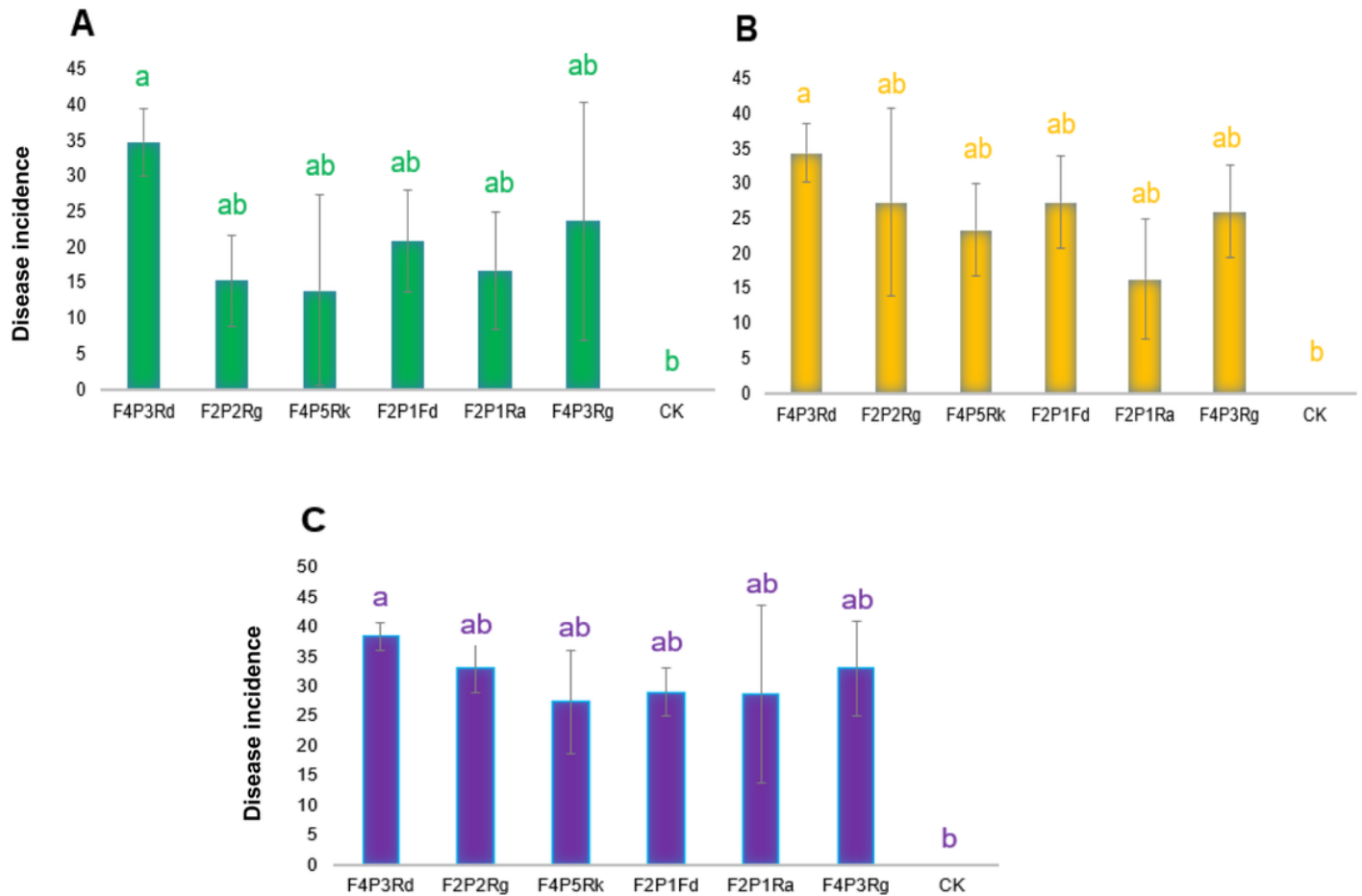


Figure 3

Disease incidence (DI) in seedlings inoculated with different fungal isolates. **(A)**: DI 19 days post fungal inoculation), **(B)**: DI 26 days post fungal inoculation and **(C)**: DI 33 days post fungal inoculation. The different fungi include: *R. oryzae* isolate F4P3Rd, *F. solani* isolate F2P2Rg, *F. graminearum* isolate F4P5Rk, *F. solani* isolate F2P1Fd, *F. proliferatum* isolate F2P1Ra, *Rhizopus oryzae* isolate F4P3Rg. CK represents the control. Letters above each bar indicate significant differences in disease incidence between treatments ($p < 0.05$).

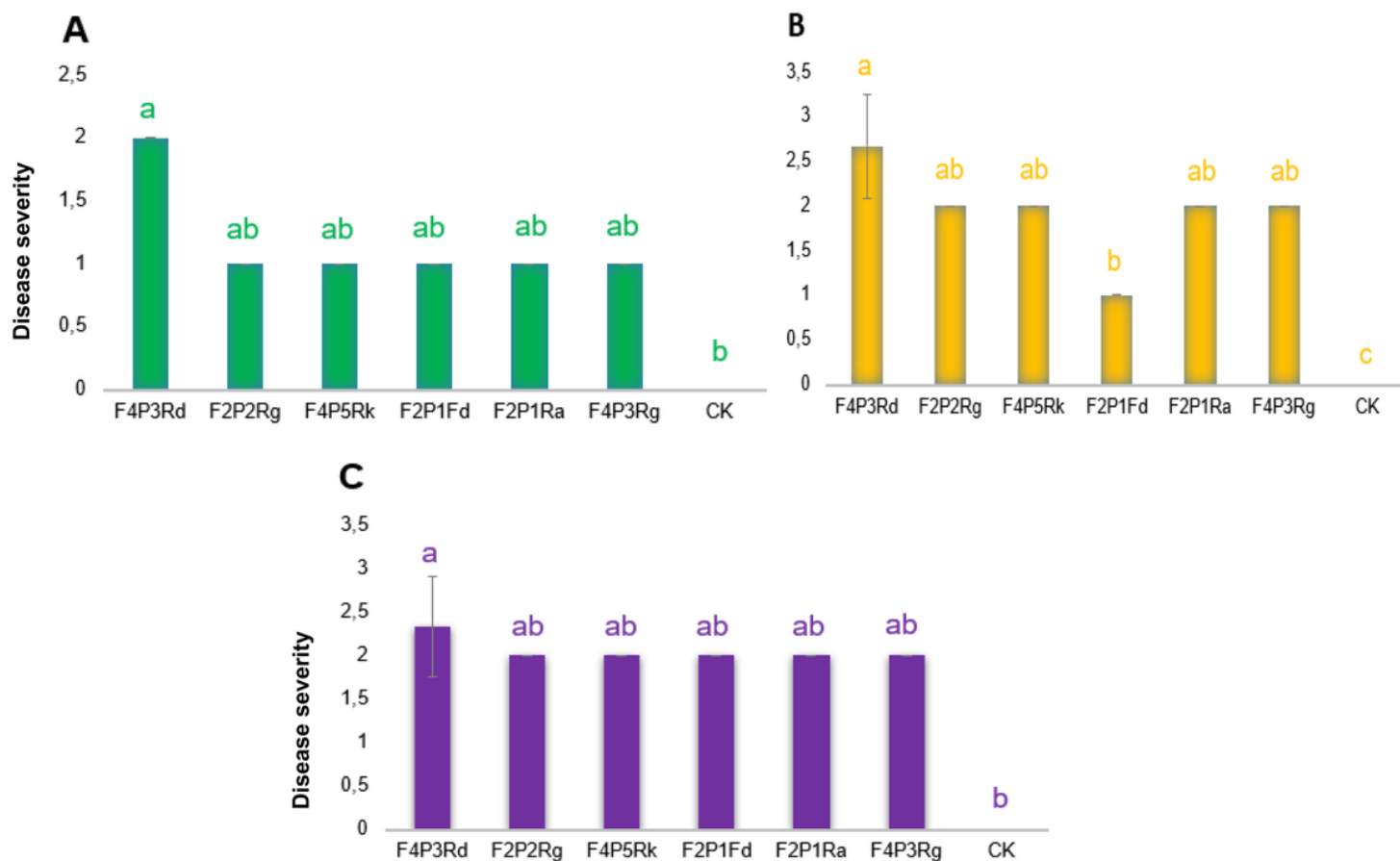


Figure 4

Disease severity (DS) in seedlings inoculated with different fungal isolates. **(A)**: DS 19 days post fungal inoculation, **(B)**: DS 26 days post fungal inoculation and **(C)**: DS 33 days post fungal inoculation. The different fungi include: *R. oryzae* isolate F4P3Rd, *F. solani* isolate F2P2Rg, *F. graminearum* isolate F4P5Rk, *F. solani* isolate F2P1Fd, *F. proliferatum* isolate F3P1Ra, *R. oryzae* isolate F4P3Rg. CK represents the control. Letters above each bar indicate significant differences in disease severity between treatments ($p < 0.05$).

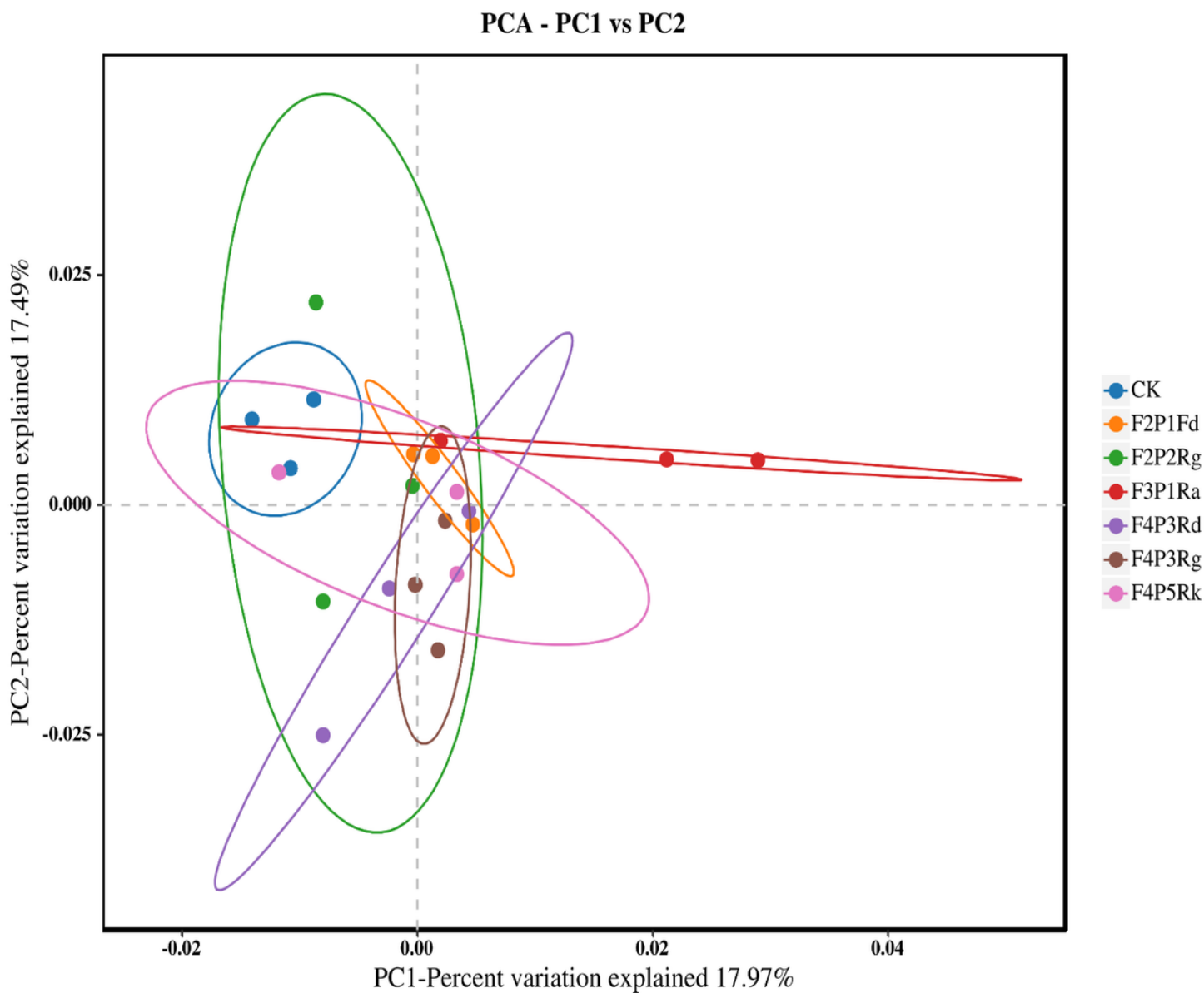


Figure 5

Principal component analysis (**B**) based on the distance matrix calculated using the Bray-Curtis algorithm for soil samples collected from different treatments: (1) CK water control, (2) *F. solani* isolate F2P1Fd, (3) *F. solani* isolate F2P2Rg, (4) *F. proliferatum* isolate F3P1Ra, (5) *R. oryzae* isolate F4P3Rd, (6) *R. oryzae* isolate F4P3Rg and (7) *F. graminearum* isolate F4P5Rk.

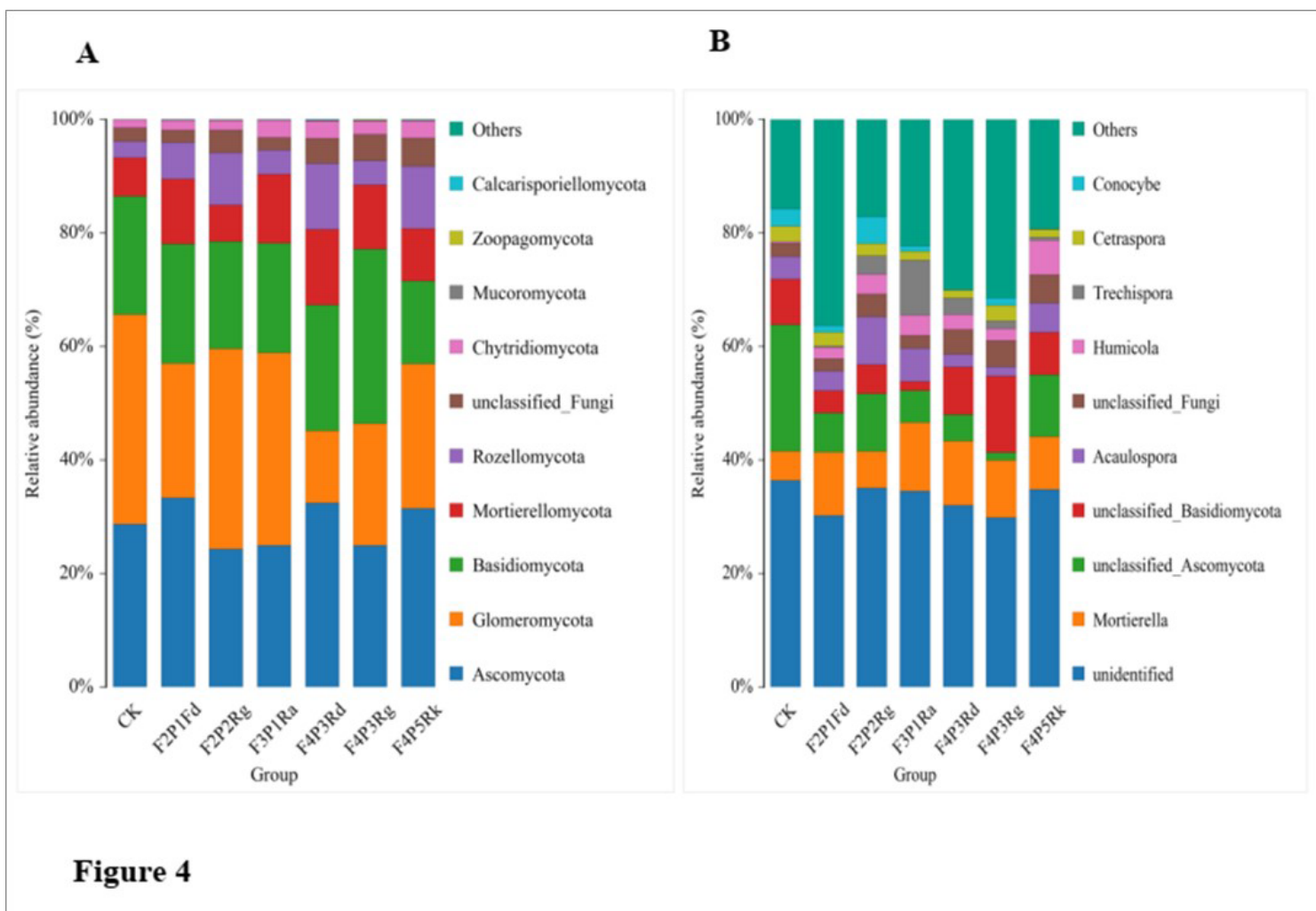


Figure 4

Figure 6

Stacked bar charts showing the relative abundances (in different colors) of the top 10 classified fungal phyla (A) and 10 fungal genera (B) in rhizosphere soil samples treated with different pathogenic fungi and control. The different fungi include: (1) *F. solani* isolate F2P1Fd, (2) *F. solani* isolate F2P2Rg, (3) *F. proliferatum* isolate F2P1Ra, (4) *R. oryzae* isolate F4P3Rd, (5) *R. oryzae* isolate F4P3Rg and (6) *F. graminearum* isolate F4P5Rk. (7) CK represents the control.

Relative abundance was based on the proportional frequencies of DNA sequences classified at the genus level. Length of a color bar correlates with amount of abundance. Data were averaged from three replicates of each treatment.)

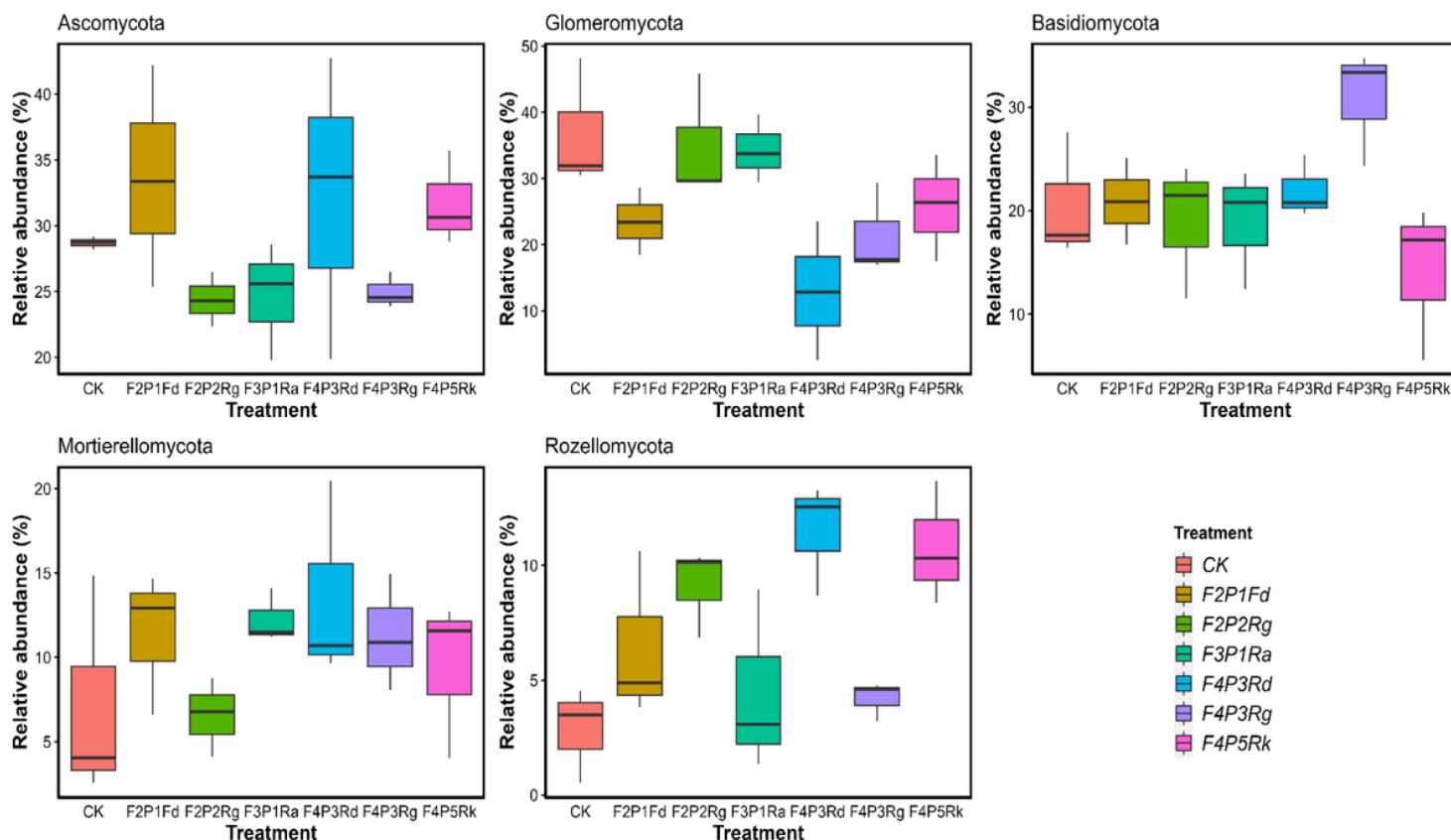


Figure 7

Boxplots showing the relative abundances of the 5 most abundant fungal phyla in rhizosphere soil samples treated with different pathogenic fungi and control. The different fungi include: (1) *F. solani* isolate F2P1Fd, (2) *F. solani* isolate F2P2Rg, (3) *F. proliferatum* isolate F2P1Ra, (4) *R. oryzae* isolate F4P3Rd, (5) *R. oryzae* isolate F4P3Rg and (6) *F. graminearum* isolate F4P5Rk. (7) CK represent the control.

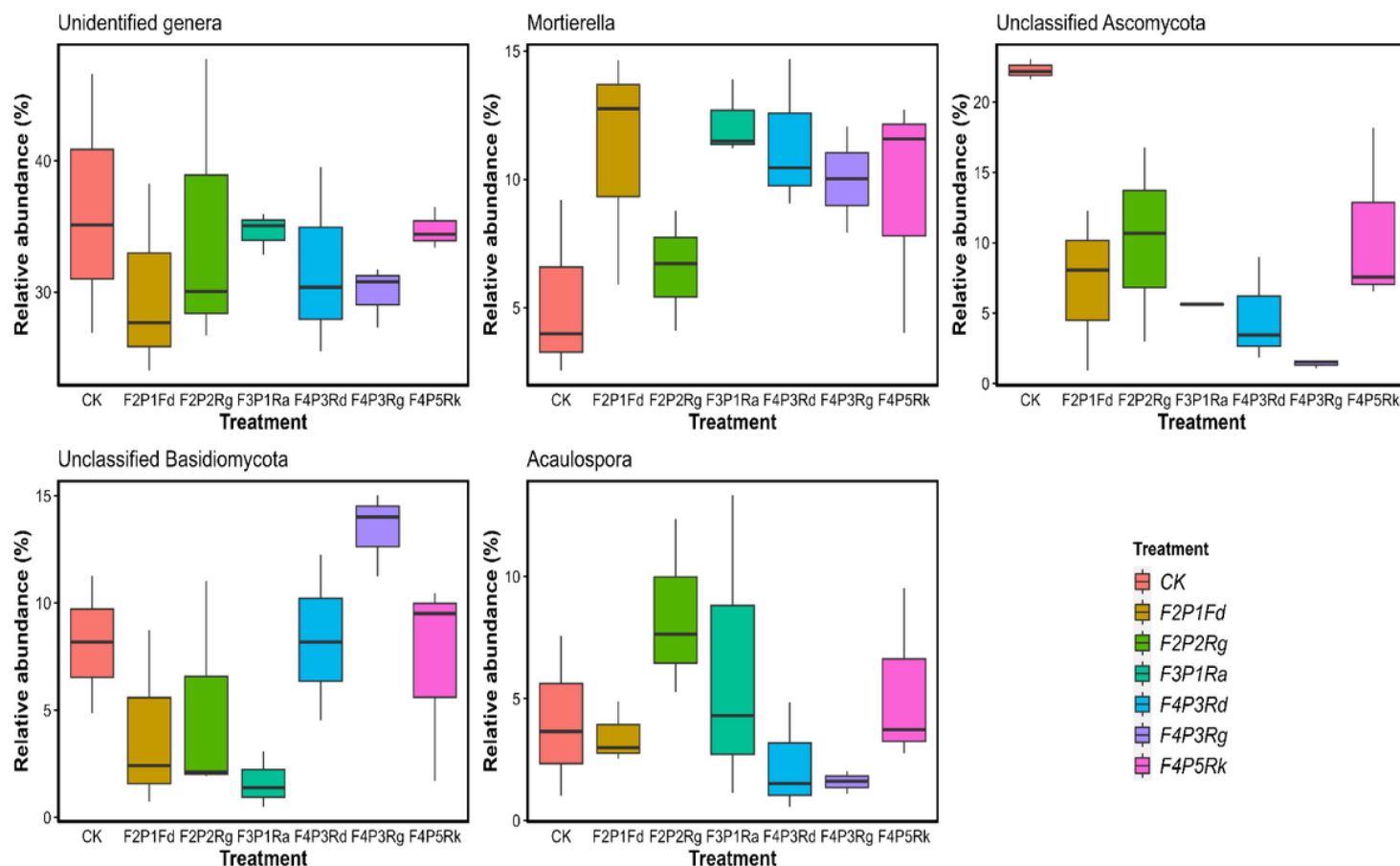


Figure 8

Boxplots showing the relative abundances of the 5 most abundant fungal genera in rhizosphere soil samples treated with different pathogenic fungi and control. The different fungi include: (1) *F. solani* isolate F2P1Fd, (2) *F. solani* isolate F2P2Rg, (3) *F. proliferatum* isolate F2P1Ra, (4) *R. oryzae* isolate F4P3Rd, (5) *R. oryzae* isolate F4P3Rg and (6) *F. graminearum* isolate F4P5Rk. (7) CK represent the control.

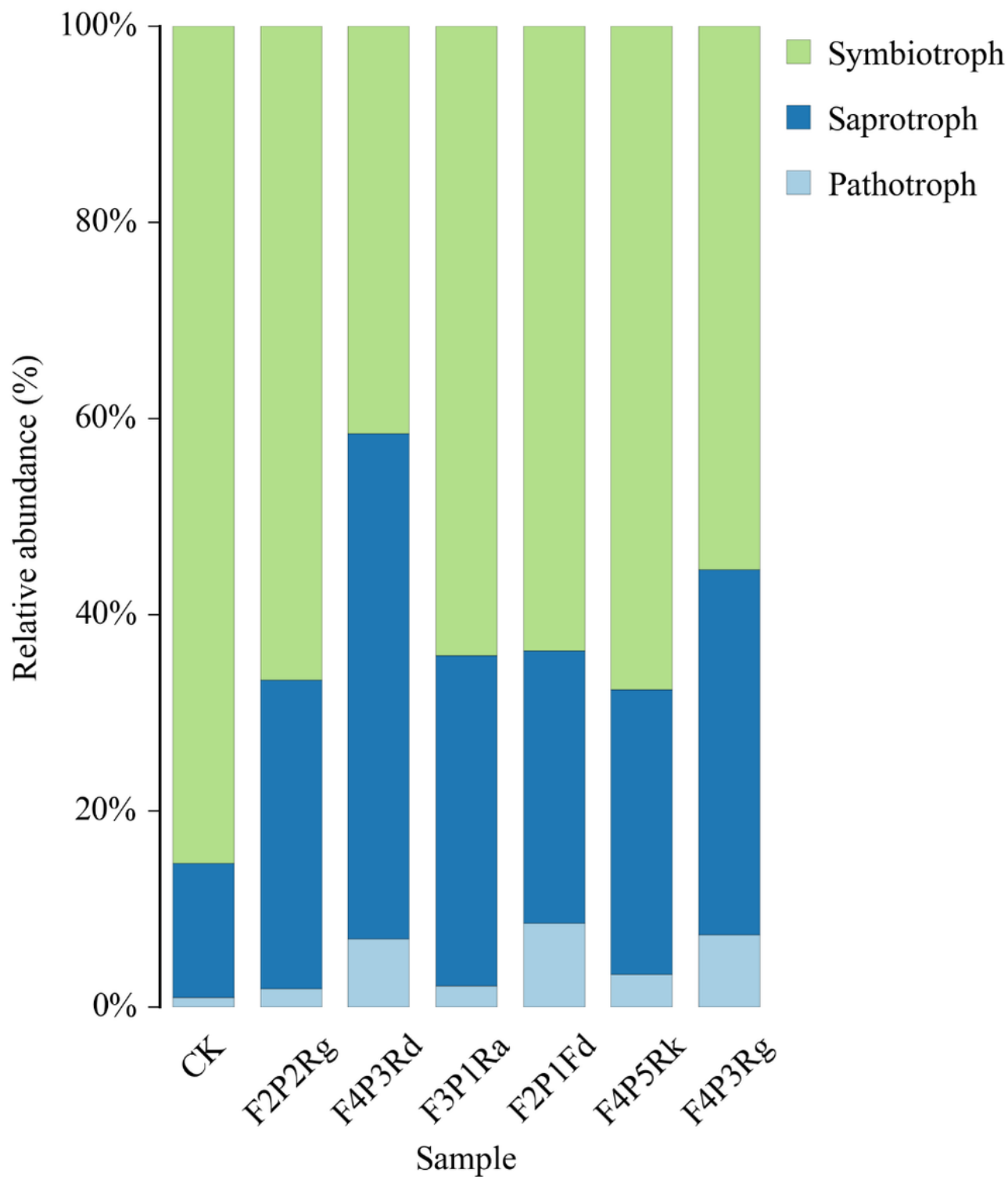


Figure 9

Stacked bar charts showing the relative abundances (in different colors) of functional taxonomic composition of rhizosphere soil fungal community within treatments. The different fungi include: (1) *F. solani* isolate F2P1Fd, (2) *F. solani* isolate F2P2Rg, (3) *F. proliferatum* isolate F2P1Ra, (4) *R. oryzae* isolate F4P3Rd, (5) *R. oryzae* isolate F4P3Rg and (6) *F. graminearum* isolate F4P5Rk. (7) CK represent the control

Supplementary Files

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