Changes in the Density and Composition of Rhizosphere Pathogenic *Fusarium* and Beneficial *Trichoderma* Contributing to Reduced Root Rot of Intercropped Soybean

Xiaoli Chang (✉ xl_chang14042@sicau.edu.cn)

Sichuan Agricultural University

Huiting Xu

Sichuan Agricultural University

Li Yan

Sichuan Agricultural University

Dan Zhu

Sichuan Agricultural University

Wei Wang

Sichuan Agricultural University

Muhammd Naeem

Sichuan Agricultural University

Chun Song

Sichuan Agricultural University

Xiaoling Wu

Sichuan Agricultural University

Guoshu Gong

Sichuan Agricultural University

Huabao Chen

Sichuan Agricultural University

Min Zhang

Sichuan Agricultural University

Taiguo Liu

Sichuan Agricultural University

Wanquan Chen

Sichuan Agricultural University

Wenyu Yang

Sichuan Agricultural University

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Abstract

Background

The dynamic of soil-borne disease is closely related to the rhizosphere microbial communities. Maize-soybean intercropping can suppress soybean root rot as compared to monoculture. However, it is still unknown whether rhizosphere microbial community participates in the regulation of intercropped soybean root rot.

Methods

In this study, the difference of rhizosphere *Fusarium* and *Trichoderma* community was compared between healthy or root-rotted soybean rhizosphere soil from soybean monoculture and maize-soybean intercropping, and the inhibitory effect of potential biocontrol *Trichoderma* against pathogenic *Fusarium* were examined.

Results

The abundance of rhizosphere *Fusarium* was remarkably different between intercropping and monoculture, while *Trichoderma* was largely accumulated in healthy rhizosphere soil of intercropping rather than monoculture. Four rhizosphere *Fusarium* species identified were all pathogenic to soybean but displayed distinct composition and isolation proportion in the corresponding soil types. As the dominant and most aggressive species, *F. oxysporum* was more frequently isolated in diseased soil of monoculture. Furthermore, of three *Trichoderma* species identified, *T. harzianum* dramatically increased in the rhizosphere of intercropping rather than monoculture as compared to *T. virens* and *T. afroharzianum*. For in-vitro antagonism test, *Trichoderma* strains had antagonistic effects on *F. oxysporum* with the percentage of mycelial inhibition ranging of 50.59%-92.94%, and they displayed good mycoparasitic abilities against *F. oxysporum* through coiling around and entering into the hyphae, expanding along cell-cell lumen and even dissolving cell walls of target fungus.

Conclusion

These results indicate maize-soybean intercropping significantly increase the density and composition proportion of beneficial *Trichoderma* to antagonist the pathogenic *Fusarium* species, thus contributing to the suppression of soybean root rot under intercropping.

Introduction

Soybean root rot is one of the detrimental soil-borne fungal diseases in soybean production worldwide and can often be infected by several pathogens mainly *Fusarium* spp. and *Phytophthora sojae* resulting in severe yield losses by 10%-60% (Bandara et al. 2020; Díaz-Arias et al. 2013; Hartman et al. 2015). This disease is remarkably affected by the resistance level of soybean cultivars as well as cropping pattern (Chang et al. 2020; Nelson et al. 1997; Wei et al. 2014; Zhang et al. 2013). In Northeast China, *Fusarium*

root rot is very popular because of long-term continuous soybean monoculture and has been recognized as one important limited factor of soybean production (Wei et al. 2014). With the increasing of planting area in Southwest China, this disease has potentially threatened local soybean production (Chang et al. 2018, 2020). Currently, the control of soybean root rot is mainly dependable of agricultural practices such as crop rotation or intercropping, soil tilling, effective and durable resistant varieties as well as chemical agents (Akamatsu et al. 2019; Gao et al. 2014; Zhang et al. 2013). Although resistance breeding of soybean has largely developed with modern molecular techniques, cost-effective and durable resistant varieties are still lack for application (Nelson et al. 1997; Naeem et al. 2021; Sepiol et al. 2017; Zhang et al. 2013). Chemicals is very helpful to prevent pathogen infection, but it often fails to cure a plant once infection occurs and otherwise sometimes affects beneficial soil microbes following excessive accumulation in soil environment (Naseri 2013, 2014). Recently, biological control has attracted considerable attention to the sustainable management of soybean root rot due to its advantages on balance crop production and agricultural environment protection (Berendsen et al. 2012; John et al. 2010; Wang et al. 2015).

Trichoderma is one genus of fungi in the family Hypocreaceae, which is commonly existing in soil, root and foliar ecosystem (Benitez et al. 2004). Since 1932, *T. lignorum* was firstly reported to have antagonistic effect on *Rhizoctonia solani* (Wending 1932), many *Trichoderma* species such as *T. harzianum*, *T. viren*, *T. asperellum*, *T. hamatum*, *T. longibrachiatum* and *T. koningii*, have successively been identified as antagonistic fungi against plant pathogens (Harman et al. 2004; Izquierdo-García et al. 2020), especially soil-borne pathogenic fungi (Anees et al. 2010; Lynch et al. 1991; Lahlali and Hijri 2010; Li et al. 2018). For example, *T. harzianum* and *T. asperellum* are effective biological control agents against *F. oxysporum* causing watermelon wilt (Zhang et al. 2020), and *T. asperellum* isolates significantly suppresses *Fusarium* tomato wilt (Cotxarrera et al. 2002). Some *Trichoderma* strains can confer biocontrol either directly by interacting with pathogens *via* re-parasitism, or by competition for nutrients or root niches, while other strains establish robust and durable colonization of root surfaces and penetrate into the epidermal cells to indirectly induce host resistance mechanism and enhance root growth (Harman et al. 2004; Hermosa et al. 2012). Accordingly, *Trichoderma* has become among the most extensively studied beneficial fungi for agricultural crop improvement (Abdelrahman et al. 2016) and a few species have already been explored as fungal biocontrol agents (BCA), bio-pesticides or bio-fertilizers for the biocontrol of the soilborne fungal diseases (Alfiky and Weisskopf 2021).

Accumulating researches indicate that the cropping pattern can affect soil microbial community, change the composition of beneficial and pathogenic microbes and regulate the occurrence of soil-borne disease (Rolfe et al. 2019). In the *Radix pseudostellariae* rhizosphere, the consecutive monoculture increases the abundance of the pathogenic *F. oxysporum* but decreases *Trichoderma* spp. (Chen et al. 2021). Recently, a maize-soybean relay strip intercropping is widely practised in Southwest China, and this cropping pattern is characterized by two-row maize plants strip intercropped with two-to-four rows of soybean which has positive effects on increasing the land equivalent ratio (Yang et al. 2015), improving the soil nutrients and microbe structure (Chen et al. 2017; Song et al. 2020,2021), reducing weeds and diseases in

the field (Chang et al. 2020; Su et al. 2018) and increasing crop yield (Du et al. 2018; Liu et al. 2017) as compared to soybean monoculture. This intercropping can also reduce the disease severity of *Fusarium* root rot and change the population diversity of the pathogenic *Fusarium* species in diseased soybean roots (Chang et al. 2020). However, the underlying mechanism by this intercropping on regulating soybean root rot is largely unclear.

In the current study, we will focus on rhizosphere microbial communities and their participation in the regulation of soybean root rot in maize-soybean relay strip intercropping as compared to soybean monoculture, and two aspects were performed: (1) the density of *Fusarium* and *Trichoderma* communities were identified and compared from diseased soybean rhizosphere soil and healthy soybean rhizosphere soil of intercropping and monoculture; (2) the antagonistic effects of *Trichoderma* species on the pathogenic *F. oxysporum* of soybean root rot were examined *in vitro*. This study will be meaningful for uncovering the rhizosphere microbial regulation of maize-soybean relay strip intercropping on *Fusarium* root rot and exploring the beneficial *Trichoderma* strains for the biocontrol of soil-borne diseases

Materials And Methods

Maize-soybean relay strip intercropping and soybean monoculture were continuously planted since 2012 at Yucheng District, Yaan City, China (29°59'3.17"N, 102°59'2.57"E) as described by Chang et al. (2020). This field experiment was designed using the randomized complete block for three replicated experimental plots. Maize cultivar "Denghai605" and soybean cultivar "Nandou12" were selected in the intercropping or monoculture, and all plots were not tilled before sowing to remain soil microbial community.

Collection of soil samples

At the R2 growth stage of soybean, soil samples were collected from diseased soybean plants displaying root rot and healthy soybean plants in each experimental plot of intercropping (marked as IDR and IHR) and monoculture (marked as MDR and MHR), respectively. The rhizosphere soil was carefully scraped from soybean root hair using a hairbrush after shaking off the loosely attached soil from the soil block. The soil of 9 plants accounting for about 5% of total plants in each plot were mixed as one sample and kept in an icebox with a sterile polyethylene bag, and then were used for the isolation and identification of *Fusarium* and *Trichoderma* species.

Isolation and purification of *Fusarium* and *Trichoderma*

After drying and filtered using a 60- μ m sieve, *Fusarium* were isolated from soil samples using sterilized *Fusarium*-selective medium (PPA, 5 g $\cdot$ L⁻¹ Tryptone, 1.0 g $\cdot$ L⁻¹ KH₂PO₄, 0.5 g $\cdot$ L⁻¹ MgSO₄, 1.0 g $\cdot$ L⁻¹ quinozole, 20.0 g $\cdot$ L⁻¹ agar) referred to previous methods (Liu et al. 2008). Rhizosphere soil with different weights (0.020 g, 0.025 g, 0.030 g, 0.035 g, 0.040 g, 0.045 g and 0.050 g) were mixed with PPA medium

poured on 90-mm plates and then incubated at 25°C for 5-7 d. The colony number on PPA plates with the proper soil weight were calculated. Pure isolates of *Fusarium* were obtained through single-spore isolation method and transferred into Potato Dextrose Agar (PDA, 200 g•L⁻¹ potato, 10 g•L⁻¹ *glucosum anhydricum*, and 15 g•L⁻¹ agar) for further analysis.

Isolation of soil *Trichoderma* were conducted using dilution-plate method using *Trichoderma*-selective medium (TSM, glucose 3 g•L⁻¹, monobasic potassium phosphate 0.9 g•L⁻¹, magnesium sulfate 0.2 g•L⁻¹, 1/3000 (V/V) rose-bengal 100 mL•L⁻¹, potassium chloride 0.15 g•L⁻¹, ammonium nitrate 1 g•L⁻¹, agar 20 g•L⁻¹) as described by Elad et al. (1981) with minor revisions. Total 10 g of soil samples were suspended in 90 ml sterilized water containing 0.5% Tween-80 and shaken at 25°C, 150 r•min⁻¹ for 40 min. About 1 mL of soil suspension was then diluted into 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ with sterilized water. And moreover, 1 mL diluted soil suspension were mixed with TSM medium and spread on 9-cm Petri plates. All culture plates were incubated at 25 °C in the darkness for 3-5 days. Colony number of *Trichoderma* in different diluted soil suspensions were calculated. For purification, the single spore of the putative *Trichoderma* colonies was transferred on PDA plates and cultured at 25 °C.

Richness of rhizosphere *Fusarium* and *Trichoderma*

The richness of *Fusarium* and *Trichoderma* in different soil samples were calculated using water content (%) and colony concentration (cfu•g⁻¹) on proper diluted soil suspension by the formula below.

$$\text{soil water content (\%)} = \frac{\text{fresh soil weight} - \text{dried soil weight}}{\text{dried soil weight}} \times 100$$

$$\text{colony units (cfu} \cdot \text{g}^{-1} \text{dried soil)} = \frac{\text{colony number per plate} \times \text{dilution fold}}{1 - \text{soil water content}}$$

DNA extraction and PCR amplification

The mycelia of fungal isolates were collected from 7-day-old isolates on PDA dishes and then grounded in liquid nitrogen with a disposable pellet pestle. Total genomic DNA of all isolates was extracted using a SP Fungal DNA Kit (Aidlab Biotech, Chengdu, China) according to the manufacturer's protocols. The partial sequences of *translation elongation factor 1-alpha (EF-1α)* and *RNA polymerase beta large subunit II (RPB2)* were amplified for the identification of *Fusarium* species using the primer pairs EF1/EF2 (O'Donnell et al. 1998) and RPB2-5f2/RPB2-7cr (O'Donnell et al. 2010,2013), respectively, while *ribosomal DNA internal transcribed spacer (rDNA ITS)*, *EF-1α* and *RPB2* were amplified using primers ITS1/ITS4 (White et al. 1990), EF-728F/EF1LLerev (Jaklitsch et al. 2005) and fRPB2-5F/fRPB2-7cR (Liu et al. 1999) to identify *Trichoderma* species. PCR reaction was conducted in a total volume of 25 μL containing DNA template 1 μL, each primer 1 μL (10 μM), Taq PCR Mastermix (Sangon Biotech, Shanghai, China) 12.5 μL, and DNase free water 9.5 μL. The fragments of *rDNA ITS* were amplified with the conditions of 2 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, initial

extension at 72 °C for 30s, and remaining at 72 °C for 10 min. Amplification conditions for *EF-1α* and *RPB2* were 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, initial extension at 72 °C for 1 min, and kept at 72 °C for 10 min. All primer sequences were listed in Supplementary materials Table S1 and S2. PCR products were checked by 1.5% agarose gel electrophoresis and sequenced using an ABI-PRISM3730 automatic sequencer (Applied Biosystems, Foster, CA, USA) in Sangon Biotech Co., Ltd. (Shanghai, China)

Phylogenetic analysis

Amplified sequences of *EF-1α* and *RPB2* gene from candidate *Fusarium* isolates were blasted on *Fusarium* *MLST* databases, while sequences of *rDNA ITS*, *EF-1α* and *RPB2* gene from candidate *Trichoderma* isolates were blasted on the NCBI database. Species of *Fusarium* and *Trichoderma* were identified based on the sequence similarity as compared to the reference species. For phylogenetic analysis, sequences of *Fusarium* and *Trichoderma* representative isolates, referred isolates and the outgroup isolate were edited and aligned with Clustal X 1.83, and characters were weighed equally. The referred isolates information of *Fusarium* and *Trichoderma* were listed in Supplementary materials Table S2.

For *Fusarium* species, MEGA 5.0 was used to calculate and analyze the differences of their base composition, and phylogenetic trees were constructed using neighbor-joining method based on both *EF-1α* and *RPB2* sequences for *Fusarium* spp., while those were performed for *Trichoderma* spp. based on a combination of either *rDNA ITS* and *EF-1α*, or *rDNA ITS* and *RPB2*, respectively. Clade support was inferred from 1000 bootstrap replicates, and alignment gaps were excluded. Novel sequence data were deposited in GenBank and the alignment in TreeBASE (www.treebase.org).

Pathogenicity test of *Fusarium* species

To test whether these *Fusarium* species identified from soybean rhizosphere are pathogenic, the representative isolates were randomly selected for pathogenicity test on the seedling of soybean cultivar Nandou12 as described by (Chang et al. 2020).

In vitro antagonistic effects of *Trichoderma* strains on the pathogenic *F. oxysporum*

For *in vitro* antagonistic assay, the representative strains of each *Trichoderma* species were randomly selected to test their antagonistic effects on the mycelium growth of *F. oxysporum*, a strong aggressive pathogen of soybean root rot. Both mycelial plugs of *F. oxysporum* and *Trichoderma* strains were inoculated on two opposite sides of 90-mm PDA plates with the distance of 75 mm, while only mycelial plug of *F. oxysporum* isolates inoculated on PDA plates were used as controls. Three plates were prepared for each isolates, and three independent experiments were conducted. All plates were incubated at 25 °C for 5 days in the darkness, and then the distance of *F. oxysporum* and *Trichoderma* on PDA plates was recorded. The percentage of mycelial inhibition (PMI) was calculated by the formula below.

$$PMI\% = \frac{\text{the colony diameter of control} - \text{the colony diameter of treat}}{\text{the colony diameter of control}} \times 100$$

For the visualization of hyphae interaction, during confrontation culture, one sterile cover glass was inserted into PDA medium equidistant from *Trichoderma* and *F. oxysporum* at an angle of 45° and cultured at 28°C in the dark. When both *Trichoderma* and *F. oxysporum* grew on the cover glass, the cover glass was taken out and observed under the compound microscope (Eclipse 80i, Nikon, Japan) and the mycelial interaction between *Trichoderma* and *F. oxysporum* was recorded and taken photos.

Data analysis

All data were recorded and processed using Microsoft office Excel 2010. The data correlation was analyzed using generalized linear model (GLM) with quasipoisson distribution for residuals, and statistical analysis was performed by Duncan's test (P=0.05) with GLM function using SPSS Statistics 20. The composition proportion of *Fusarium* and *Trichoderma* spp. were calculated using the percentage of each species in total isolates from the corresponding soil sample, while isolation proportion of each species was evaluated using the percentage of each species in all isolates from four soil samples. The difference of composition and isolation proportion of *Fusarium* or *Trichoderma* species in each cropping pattern was analyzed by Fisher's exact test.

Table 1 Population richness of *Fusarium* and *Trichoderma* communities in different soil samples from intercropping and soybean monoculture.

Cropping patterns	Soil samples	Sample code	<i>Trichoderma</i> richness (cfu·g ⁻¹)	<i>Fusarium</i> richness (cfu·g ⁻¹)
Intercropping	Diseased rhizosphere soil	IDR	2560.98±2414.51 ab	1046.51±164.44 a
	Healthy rhizosphere soil	IHR	4404.76±168.36 a	329.67±186.49 b
Monoculture	Diseased rhizosphere soil	MDR	523.26±82.22 b	391.62±12.88 b
	Healthy rhizosphere soil	MHR	523.26±82.22 b	458.09±151.62 b

Notes: Different lowercase indicate the significant difference at the level of P=0.05 using SPSS ANOVN.

Results

Population richness of rhizosphere *Fusarium* and *Trichoderma* in response to intercropping and monoculture

To uncover the influence of cropping patterns on the density of rhizosphere *Fusarium* and *Trichoderma* communities associated with soybean root rot, the richness of these two fungi were compared between diseased rhizosphere soil and healthy rhizosphere soil, as well as between intercropping and monoculture. As shown in Table 1, *Fusarium* was the most richest with 1046.51 cfu·g⁻¹ in diseased rhizosphere soil of intercropping (IDR) among four soil samples, whereas it had no significant difference in two soil types of soybean monoculture. In contrast, *Trichoderma* richness in two soil types of intercropping (IDR and IHR) was much higher than those of monoculture (MDR and MHR), and meanwhile it was also much richer in healthy rhizosphere soil (IHR) than diseased rhizosphere soil (IDR) of maize-soybean relay strip intercropping but had almost no difference between two soil samples of soybean monoculture. Thus, it is clear that *Fusarium* richness was significantly declined in healthy rhizosphere soil in two cropping patterns which was followed by the increasing of *Trichoderma*, and rhizosphere *Trichoderma* richness is more dramatically affected by cropping pattern while *Fusarium* richness has also remarkable distinct with respect to the occurrence of root rot under the intercropping.

Species identification of rhizosphere *Trichoderma* and *Fusarium* in response to intercropping and monoculture

In this study, a total of 165 *Fusarium* isolates were obtained from four soil samples of intercropping and monoculture. Sequence analysis of *EF-1α* and *RPB2* genes by blasting on the *Fusarium* MLST database showed that these isolates had more than 98% sequence identities with those referred isolates of *F. solani* species complex (FSSC), *F. oxysporum*, *F. incarnatum-equiseti* species complex (FIESC) and *F. commune* (Supplementary material Table S3), respectively. A phylogenetic tree based on *EF-1α* and *RPB2* was constructed with the representative isolates, referred isolates from NCBI and the outgroup isolate *Nectriaceae* sp. (NRRL52754) (Supplementary material Table S2), and results showed that the representative isolates from intercropping were absolutely classified into 3 clades including FSSC, FIESC, and *F. oxysporum* in this tree constructed based on *EF-1α* and *RPB2* (Figure 1A), while those isolates from monoculture were claded into FSSC, *F. oxysporum* and *F. commune*, respectively (Figure 1B), displaying a distinct species composition of *Fusarium* between intercropping and monoculture.

For *Trichoderma* spp., 134 *Trichoderma* isolates were recovered from rhizosphere soil of diseased and healthy soybean plants in two cropping patterns. Blastn analysis of partial *ITS*, *EF-1α* or *RPB2* genes on the NCBI database showed that these isolates had more than 95% sequence identities with *T. harzianum*, *T. virens* and *T. afroharzianum* (Supplementary material Table S4). For phylogenetic analysis in Figure 2, all representative isolates from either intercropping or monoculture were clearly claded into *T. harzianum*, *T. virens*, and *T. afroharzianum*, respectively in the *ITS/RPB2*-based MP trees with ≥99% the bootstrap support value, whereas *T. harzianum* and *T. afroharzianum* were not totally discriminated in the *ITS/EF-1α*-based phylogenetic trees in both cropping patterns (Supplementary material Figure S1). Thus, *Trichoderma* isolates from rhizosphere soil were identified as *T. virens*, *T. afroharzianum* and *T. harzianum*, and there was no difference in species diversity between intercropping and monoculture.

Composition and isolation proportion of *Fusarium* spp. and *Trichoderma* spp. affected by intercropping and monoculture

The composition and isolation proportion of rhizosphere *Fusarium* and *Trichoderma* spp. was further compared between monoculture and intercropping as well as between diseased and healthy soils. As shown in Figure 3A and 3B, FSSC had the highest isolation proportion in all soil types that reached up to 88.89% (40/45) of *Fusarium* composition and 24.24% (40/165) of all isolated *Fusarium* in IDR as compared to 65% (28/43) and 16.97% (28/165) in MDR (Figure 3A and 3B), respectively. *F. oxysporum* accounted for the second largest proportion in different soil samples and had the highest proportion of 32.56% (14/43) in MDR than that of 8.89% (4/45) in IDR (Figure 1A). The FIESC and *F. commune* were specifically isolated species in intercropping and monoculture, respectively, with relatively less proportion in corresponding soil samples (Figure 1A and 1B).

In this study, total of three *Trichoderma* species were obtained from rhizosphere soils of two cropping patterns, but the species composition and isolation proportion varied over the occurrence of root rot as well as cropping pattern (Figure 3C and 3D). Both *T. harzianum* and *T. virens* were isolated from all soil types, but *T. harzianum* was the most predominant species of *Trichoderma* (Figure 3D). The composition proportion of *T. harzianum* was 95.9 % (71/74) and 76.2% (32/42) in IHR and IDR, whereas it was as low as 66.7% (6/9) and 33.3% (3/9) in MHR and MDR, respectively. As the secondary dominant species, *T. virens* in *Trichoderma* composition was clearly declined in two soil types of intercropping when compared with monoculture, whereas an increase of *T. afroharzianum* appeared in MHR and MDR of monoculture and also in IDR of intercropping. These results indicate that intercropping changes *Trichoderma* composition and remarkably increases *T. harzianum* isolation proportion in comparison to monoculture.

The pathogenicity of rhizosphere *Fusarium* spp. on soybean

Pathogenicity test of rhizosphere *Fusarium* species on soybean seedling showed that all four *Fusarium* species were able to infect soybean roots, and cause stunted, brown, rotted taproots and hair roots but displayed significantly different aggressiveness (Figure 4A). *F. oxysporum* were the most aggressive among all *Fusarium* species with disease severity index (DSI) ranging from 35 to 90 (Figure 4B). The DSI of *F. oxysporum* isolates from monoculture were much higher than those from intercropping, with the highest DSI up to 90 (Figure 4B) followed by serious growth inhibition and rotted taproot (Figure 4A). FSSC as the most isolated fungi had moderate aggressiveness with DIS remained around 50, and there was no significant difference among those isolates from two cropping patterns. In addition, FIESC and *F. commune* separately for intercropping and monoculture were also moderately pathogenic to soybean but different for their aggressiveness.

Inhibition effects of *Trichoderma* spp. on the pathogenic *F. oxysporum* of soybean root rot

As compared to the *F. oxysporum* control, the representative isolates of three *Trichoderma* species grew tropically towards *F. oxysporum* and significantly inhibited mycelial growth of *F. oxysporum* through

spatial competition on PDA confrontation culture plates (Figure 5A), and the inhibition response had almost no difference among the same *Trichoderma* species from intercropping and monoculture. Furthermore, the percentage of mycelial inhibition (PMI) was distinct among three *Trichoderma* species, but 38.10% of isolates had the PMI values in the range of 60%-70% (Figure 5B). *T. harzianum* isolates showed almost same range of PMI between intercropping and monoculture, whereas *T. afroharzianum* and *T. virens* had slightly different central PMI values for tested isolates from two cropping patterns. Under two cropping patterns, there was almost no difference in the mean PMI of *T. harzianum*, which were 58.91% in intercropping and 57.35% in monoculture. The mean PMI for *T. virens* was concentrated at 72.0% in intercropping but it was as high as 60.30% in monoculture (Figure 5C). To be contrast, the mean PMI for *T. afroharzianum* was slightly high up to 73.14% in intercropping as compare to 66.18% in monoculture (Figure 5C). This indicates that *T. virens* and *T. afroharzianum* might be also related to *F. oxysporum* inhibition during rhizosphere fungal interaction with respect to cropping pattern.

To observe the hyphae interaction, *T. harzianum* grew tropically towards (Figure 6A), attached to the hyphae of *F. oxysporum*, and coiled around them (Figure 6B). The strains TRB1-15 and TRB1-7 of *T. virens* coiled around the hyphae, formed brown halos at the contact sites (Figure 6C), and some of them directly entered into the lumen of the target fungus (Figure 6D). As shown in Figure 6E, the hyphae of *T. afroharzianum* has entered into the cell lumen of *F. oxysporum* and expanded along the host hyphae (Figure 6E), and resulted in dissolution of cell walls (Figure 6F). In general, rhizosphere *Trichoderma* strains displays good mycoparasitic abilities against the pathogenic *F. oxysporum* of soybean root rot.

Discussion

Rhizosphere soil microbes play an important role in the regulating soil-borne diseases that are closely correlated with cropping patterns (Hage-Ahmed et al. 2013; Ren et al. 2008; Zhou et al. 2011). Our previous study proved that maize-soybean relay strip intercropping suppressed the occurrence of soybean root rot and changed the diversity of the pathogenic *Fusarium* species (Chang et al. 2020). In this study, we further focused on this intercropping effects on rhizosphere *Fusarium* communities and potential biocontrol *Trichoderma* communities in association with soybean root rot, and results demonstrated that continuous maize-soybean relay strip intercropping caused a significant accumulation of rhizosphere *Trichoderma* but reduced the abundance of the pathogenic *Fusarium* communities, in particular, in healthy soils, whereas consecutive soybean monoculture displayed almost no influence on the density of these two fungi, no matter whether of diseased or healthy rhizosphere soils. This demonstrates that maize-soybean relay strip intercropping is beneficial to induce the accumulation of rhizosphere *Trichoderma* but not *Fusarium* communities.

Fusarium species are the dominant pathogens of soybean root rot in Sichuan Province, the Southwest China (Chang et al. 2020), and these *Fusarium* species often can survive saprophytically and accumulate largely on crop debris, even on or inside cultivated soil, thus serving as primary inocula for soybean infection in rhizosphere microenvironment in the following epidemic year (Beyer et al. 2006). In our study,

four *Fusarium* species including *F. oxysporum*, FSSC, FIESC and *F. commune* were identified from all rhizosphere soil samples and all pathogenic to soybean, but the composition and isolation proportion of these *Fusarium* species in maize-soybean relay strip intercropping were distinct from those in soybean monoculture. Among them, FSSC and *F. oxysporum* were most frequently isolated in both cropping patterns, which is similar to those by Liu et al (Liu et al. 2008). in isolating *Fusarium* spp. from soybean rhizosphere soil of different rotation systems in black soil area of Northeast China. However, *F. oxysporum* rather than FSSC had the highest isolation proportion from soybean rhizosphere soil in black soil area (Liu et al. 2008), indicating an association of the difference in dominant rhizosphere *Fusarium* species with soybean ecological planting areas. Compared to *Fusarium* spp. isolated from diseased soybean root among which FSSC and *F. oxysporum* were the predominant species in both maize-soybean relay strip intercropping and soybean monoculture (Chang et al. 2018, 2020), the diversity of *Fusarium* spp. was remarkably lower from diseased and healthy rhizosphere soils of two cropping patterns, indicating that some of *Fusarium* species causing soybean root rot such as *F. graminearum*, *F. asiaticum*, *F. proliferatum* might come from aboveground inoculums instead of rhizosphere soil-borne pathogens. Moreover, FIESC and *F. commune* were specific to intercropping and monoculture with relatively low isolation proportion, respectively, which is basically consistent with our previous findings that specifically-isolated *F. commune* and a low isolation proportion of FIESC from the diseased soybean roots in monoculture rather than maize-soybean relay strip intercropping (Chang et al. 2020). In addition, compared with maize-soybean relay strip intercropping, soybean monoculture did not change the density of rhizosphere *Fusarium* but dramatically raised the composition proportion and isolation proportion of the most aggressive *F. oxysporum* causing soybean root rot. Therefore, unlike intercropping, more severe root rot in soybean monoculture might depend upon relatively higher proportion of the aggressive *F. oxysporum* but not upon the higher density of *Fusarium* communities. This can be supported by another study that continuous soybean cropping increased the population of *Fusarium* and tended to increase the susceptibility to root rot (Jie et al. 2015).

Beneficial soil microbes have some advantages on suppressing soil-borne pathogenic fungi, promoting plant growth or decomposing plant residues (Setälä and McLean 2004). *Trichoderma* has widely been recognized as biological control of soil-borne diseases in production (Lahlali and Hijri 2010; Li et al. 2018), but *Trichoderma* communities is often affected by cropping patterns (Pérez-Brandán et al. 2014). Previous studies found that compared with soybean-maize rotation, continuous soybean monoculture decreased the populations of *Trichoderma* that may acts as biocontrol alternatives of soil-borne pathogens (Pérez-Brandán et al. 2014)). In contrast, the content of soil *Trichoderma* increased significantly under the intercropping of sorghum and soybean (Lian et al. 2018). In this study, the density of rhizosphere *Trichoderma* communities exhibited an increased trend in the intercropping rather than monoculture, and among three species of *Trichoderma* isolated of two cropping patterns, *T. harzianum* had the highest isolation proportion followed by *T. virens* and *T. afrohazianum* which is basically similar to Wei (2012) that identified three *Trichoderma* species, mainly *T. harzianum* and *T. virens*, and a small amount of *T. viride*, from soybean rhizosphere soil for different rotation years in Heilongjiang Province. In addition, we also observed that these *Trichoderma* species displayed a good mycoparasitic abilities in the

antagonist against the pathogenic *F. oxysporum* of soybean root rot, mainly through coiling around the hyphae, entering into the the lumen of the target fungus, expanding into the cell-cell area and finally dissolving the cell wall of host fungus, which have been reported as the typical mycoparasitism of *Trichoderma* (Alfiky and Weisskopf 2021; Harman et al. 2004). In other studies, *Trichoderma virens* has been recognized as an aggressive mycoparasite that is capable of competing with pathogens at the site of infection (Djonovic et al. 2007) and also induces JA and SA mediated tomato resistance against *Fusarium* wilt (Jogaiah et al. 2018). From this point of view, *Trichoderma* communities are a important group in response to maize-soybean relay strip intercropping as compared to soybean monoculture. This is also proved in previous study by Chen et al (Chen et al. 2021). That expanded monoculture of *R. pseudostellariae* altered *Trichoderma* communities in the plant rhizosphere leading to relatively low level of antagonistic microorganisms, and among them *T. harzianum* ZC51 could inhibit the pathogenic *F. oxysporum* and induce the expression of defense genes. However, several studies have shown that *Trichoderma* strains with good plate-confrontation effect on pathogens might be not necessarily good, or even no effect, which is predicted to associate with the colonization ability of *Trichoderma* on plants (Sivan and Chet 1989). Therefore, the antagonistic effect of these *Trichoderma* strains against *F. oxysporum* causing soybean root rot should be further verified in the actual field production.

Conclusions

In this study, maize-soybean relay strip intercropping increased the density of beneficial *Trichoderma* but decreased the pathogenic *Fusarium* communities (in rhizosphere diseased soil) as compared to soybean monoculture. Intercropping also affected species composition and isolation proportion of rhizosphere *Fusarium* and *Trichoderma* communities. The change in the pathogenic *Fusarium* might be primarily driven by the beneficial *Trichoderma* strains by re-parasitism interaction. Overall, these findings suggested that maize-soybean relay strip intercropping alters both pathogenic *Fusarium* and beneficial *Trichoderma* communities in soybean rhizosphere soil and lead to antagonistic development of the fungal community structure on plant health, which regulates the suppressive effect of this intercropping on soybean root rot.

Declarations

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Figures

Figure 1

Intercropping were absolutely classified into 3 clades including FSSC, FIESC, and *F. oxysporum* in this tree constructed based on EF-1α and RPB2 (Figure 1A), while those isolates from monoculture were claded into FSSC, *F. oxysporum* and *F. commune*, respectively (Figure 1B), displaying a distinct species composition of *Fusarium* between intercropping and monoculture.

Figure 2

For phylogenetic analysis in Figure 2, all representative isolates from either intercropping or monoculture were clearly claded into *T. harzianum*, *T. virens*, and *T. afroharzianum*, respectively in the ITS/RPB2-based MP trees with $\geq 99\%$ the bootstrap support value, whereas *T. harzianum* and *T. afroharzianum* were not totally discriminated in the ITS/EF-1 α -based phylogenetic trees in both cropping patterns

Figure 3

3A and 3B, FSSC had the highest isolation proportion in all soil types that reached up to 88.89% (40/45) of *Fusarium* composition and 24.24% (40/165) of all isolated *Fusarium* in IDR as compared to 65% (28/43) and 16.97% (28/165) in MDR (Figure 3A and 3B), respectively.

Figure 4

Pathogenicity test of rhizosphere *Fusarium* species on soybean seedling showed that all four *Fusarium* species were able to infect soybean roots, and cause stunted, brown, rotted taproots and hair roots but displayed significantly different aggressiveness

Figure 5

As compared to the *F. oxysporum* control, the representative isolates of three *Trichoderma* species grew tropically towards *F. oxysporum* and significantly inhibited mycelial growth of *F. oxysporum* through spatial competition on PDA confrontation culture plates

Figure 6

To observe the hyphae interaction, *T. harzianum* grew tropically towards (Figure 6A) , attached to the hyphae of *F. oxysporum*, and coiled around them (Figure 6B). The strains TRB1-15 and TRB1-7 of *T. virens* coiled around the hyphae, formed brown halos at the contact sites (Figure 6C), and some of them directly entered into the lumen of the target fungus (Figure 6D).

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