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Control of Fusarium wilt of lisianthus by reassembling the microbial community in infested soil through reductive soil disinfestation

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ABSTRACT

Continuous monocropping often influences negatively the soil microbial community and leads to the occurrence of soil-borne diseases. In this study, a pre-cultivation soil management strategy, reductive soil disinfestation (RSD), involving amendment by the use of reed straw, bagasse, and rice straw, and creating anaerobic soil conditions, was used to regulate the microbial community in a soil infested by Fusarium wilt of lisianthus and make it suitable for plant cultivation. The results showed that RSD significantly decreased *F. oxysporum* population by 97.1%–99.1% and the incidence of lisianthus wilt disease to 3.0%–14.3% compared with that of the untreated soil. The lowest disease incidence was found in the soil treated with RSD where bagasse was incorporated. The replantation of the host plant differently stimulated the pathogen proliferations across the different soils. MiSeq sequencing and culture-dependent investigation showed that the RSD treatments established distinct microbial communities compared to that of the untreated soil. Furthermore, the relative abundances of representatives of the families Cytophagaceae, Chitinophagaceae, Chaetomiaceae, and an unclassified family within Sordariomycetes, as well as soil microbial activity and the proportions of antagonists were significantly and negatively correlated with the pathogen population increase. Overall, the RSD treatment contributed to the reassembly of the soil microbiome which contained more beneficial agents that successfully controlled the pathogen inoculum level and lisianthus Fusarium wilt disease.

1. Introduction

Lisianthus (*Eustoma grandiflorum*) is a popular ornamental crop, with various varieties and colors. It has been intensively cultivated in southern China and worldwide in recent years to meet the increasing production demand. Due to limitations in the arable land suitable for lisianthus cultivation, farmers usually grow lisianthus continuously in the same soil. However, the release of specific root exudates by the host plant in monocropping usually causes the accumulation of soil-borne pathogens at the expense of plant-beneficial fungi (Li et al., 2014a, 2014b). As a consequence, continuous monocropping of lisianthus often leads to a serious quality and yield decline. Furthermore, this repetitive cultivation practice augments the occurrence of Fusarium wilt, caused by *F. oxysporum* f. sp. *eustomae*, which is considered the most important threat to lisianthus production in China, Italy, and Japan (Hahm, 1998; Gilardi and Gullino, 2006; Tomioka et al., 2011; Bertoldo et al., 2014). Currently, soil disinfestation by chemicals is largely applied in practice for the control of soil-borne diseases. However, chemical fumigation can decrease the total microbial population and diversity, severely interrupting the microbial balance, which is followed by rapid pathogen recolonization (Griffiths, 2000). Besides, increasing concerns about food safety and environmental pollution further limit the use of chemicals for soil-borne disease control. Reportedly, other control methods, such as soil flooding and solarization, have rarely provided consistent and effective control in all production areas (Stover, 1962; Katan, 1981; Ploetz and Ploetz, 1990). Hence, there is an urgent need for the development of effective approaches to control this disease.

In 2000, reductive soil disinfestation (RSD), known also as

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biological soil disinfestation (BSD) or anaerobic soil disinfestation (ASD), has been developed separately in Japan (Shinmura, 2000) and the Netherlands (Blok et al., 2000) as an alternative approach to chemical soil disinfestation, and has proven to be effective in the control of soil-borne pathogens, nematodes, and weeds (Goud et al., 2004). RSD is an environmentally friendly measure that is implemented by flooding soil incorporated with labile organic matters, followed by its covering with a plastic film at an optimum temperature (25 °C – 35 °C) for approximately two weeks (Blok et al., 2000; Momma et al., 2006). Previous studies have demonstrated that the production of antagonistic compounds, such as organic acids, Fe^{2+}, Mn^{2+}, and ammonia, are responsible for the disinfestation effect exerted (Tenuta and Lazarovits, 2002; Momma et al., 2006, 2011). Until now, RSD has been widely applied in the field (Momma et al., 2013) to successfully control soil-borne diseases in many crops, such as spinach Fusarium wilt (Mowlick et al., 2013), cucumber damping-off disease (Huang et al., 2017), and tomato Fusarium wilt (Messiha et al., 2007). In addition, a large number of studies have revealed that the soil microbial community is substantially changed during the RSD process under soil anaerobic conditions (Momma, 2008; Huang et al., 2016). However, only few studies have reported data on the impact of RSD on soil microbial communities during the plant cultivation after the RSD treatment. Using the results of clone library analysis based on 16S rRNA gene sequences, Mowlick et al. (2013) found that most of the bacterial groups, including the phyla Proteobacteria, Bacteroidetes, Acidobacteria, Gemmatimonadetes, TM7. etc., recovered to their initial state in the untreated soil after spinach cultivation in RSD-treated soil, but more detailed information is poorly known. We hypothesized that the promoted disease suppression after the RSD application could have been the result of the combined effects of decreasing pathogen population and alteration of soil microbiota composition (control of pathogen proliferation).

The conventional culture-based approaches provide only limited information regarding the soil microbial community. New molecular tools, such as pyrosequencing and MiSeq sequencing, offer considerable convenience in conducting research on this subject. The combination of new molecular tools and traditional approaches can unravel the interactions not only between potential antagonistic microbes and target pathogens but also among members of the entire soil microbial communities (Cha et al., 2016). In the present study, MiSeq sequencing and culture-based method were used to compare the differences in the microbial compositions of RSD-treated and untreated soils at the end of lisianthus cultivation. Additionally, an assessment was performed of the relationships between the microbial community composition and pathogen or disease suppression.

2. Materials and methods

2.1. Experimental design and soil sampling

The experiment was conducted in Shiping, Yunnan Province, southwestern China (23°40′N, 102°35′E, 1400 m alt.). In the experimental area, lisianthus had been continuously grown for four years and had suffered severely from Fusarium wilt disease before the experiment. Four treatments were examined: untreated soil (CK), without any organic amendment; RSD treatments, in which amendments were performed with 15 t ha^{-1} reed (*Phragmites australis*) straw (RE), sugarcane (*Saccharum officinarum*) bagasse (BA), and rice (*Oryza sativa*) straw (RS). Powdered (< 2 mm) reed straw, sugarcane bagasse, and rice straw contained 427, 442, and 437 g kg^{-1} organic carbon and 6.8, 4.66, and 17.59 g kg^{-1} total nitrogen, respectively. For the RSD treatments, the organic materials were well incorporated into the soil, which was then irrigated with 100 L m^{-2} of water. Finally, the irrigated soils were covered with plastic film (transparent, 80-μm thickness; Baoding Baoshuo Plastic Co., Ltd., Hebei, China). Each treatment included three replicates, each of which covered an area of 90 m^2. After 20 days of RSD treatments with temperatures within the range 25 °C – 40 °C, the plastic films were removed, and the soils were naturally air-dried. Pathogen-free lisianthus seedlings, purchased from Sakata Seed (Suzhou) Co. Ltd. (Jiangsu, China), were routinely cultivated (54 plants m^{-2}) in the experimental site of the greenhouse and under natural conditions (20 °C – 35 °C). The disease incidence was recorded at the flowering stage which occurred five months after planting.

Soil samples were collected at the end of the RSD treatments and lisianthus cultivation. Briefly, a composite soil sample for each replicate was collected that contained 10 soil cores (sampling depth 3–10 cm). The soil samples were pooled, and sieved to 2 mm. One portion of the soil sample was kept at 4 °C for microbial isolation and activity analyses, whereas another was stored at −80 °C for DNA extraction and subsequent molecular analyses.

2.2. Determination of total soil microbial activity

The total microbial activity of the soil was determined using the method of the fluorescein diacetate (FDA) hydrolysis described by Adam and Duncan (2001). The results were expressed as μg fluorescein released by gram dry weight of soil per hour.

2.3. Extraction of soil DNA and real-time PCR assay

Soil DNA extraction was performed by the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA). The DNA quality was determined by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Real-time PCR was performed on a CFX-96 thermocycler (Bio-Rad Laboratories Inc. Hercules,

Table 1
Primers and thermal profiles used in this study.

Primers	Sequence(5'-3')	Thermal profile	Reference
ITS1F	CTTGGTCATTTAGAGGAAGTAA	2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 15 s at 58 °C and 20 s at 72 °C	Gardes and Bruns (1993)
AFR308	CGAATTAACGCGAGTCCCAAC		Lievens et al. (2005)
Eub338	CCTACGGGAGGCAGCAG	2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 53 °C and 30 s at 72 °C	Lane (1991)
Eub518	ATTACCGCGGCTGCTGG		Muyzer et al. (1993)
ITS1f	TCCGTAGGTGAACCTGCGG	2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 53 °C and 30 s at 72 °C	Gardes and Bruns (1993)
5.8S	CGCTGCGTTCTTCATCG		Vilgalys and Hester (1990)
I27	AGAGTTTGATCCTGGCTCAG	5 min at 95 °C, followed by 29 cycles of 40 s at 94 °C, 60 s at 52 °C and 90 s at 72 °C, and 10 min at 72 °C	Flanagan et al.(2007)
1492	TACCTTGTTACGACTT		Flanagan et al.(2007)
ITS1	TCCGTAGGTGAACCTGCGG	5 min at 95 °C, followed by 30 cycles of 40 s at 94 °C, 40 s at 56 °C and 60 s at 72 °C, and 10 min at 72 °C	Gardes and Bruns (1993)
ITS4	TCCTCCGCTTATTGATATGC		Gardes and Bruns (1993)
520	AYTGGGYDTAAAGNG	2 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and 5 min at 72 °C	Claesson et al. (2009)
802	TACNVGGGTATCTAATCC		Claesson et al. (2009)
ITS2	GCTGCGTTCTTCATCGATGC		White et al., (1990)

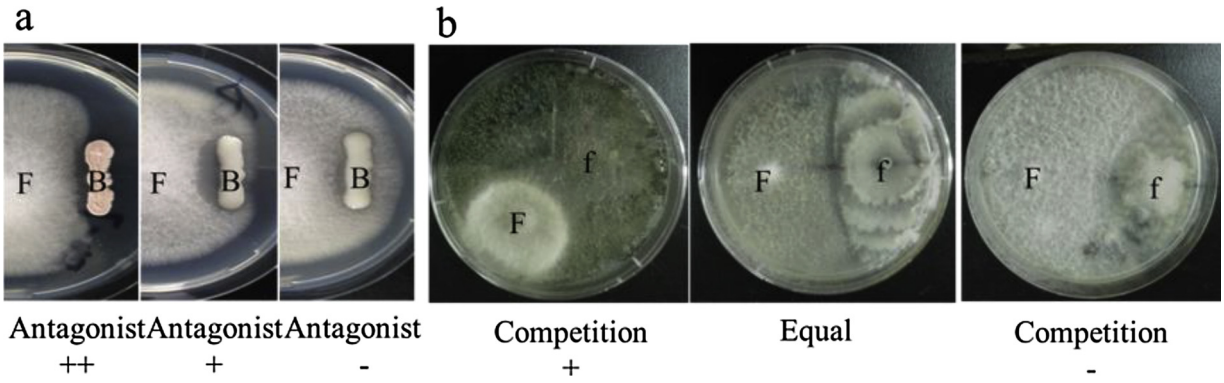

Fig. 1. Inhibition patterns by bacterial (a) and fungal (b) isolates against *Fusarium oxysporum* strain SP in dual-culture tests. F, B and f represent *Fusarium oxysporum* strain SP, bacterial, and fungal isolates, respectively. Antagonist + +, entirely inhibited the growth of *F. oxysporum* strain SP and formed an obvious inhibition zone; Antagonist +, partially inhibited the growth of *F. oxysporum* strain SP and formed a clear inhibition zone; Antagonist-, no influence on the growth of *F. oxysporum* strain SP was exerted. Completion +, Equal, and Competition- mean that the colony area of the fungal isolate was larger, approximately equal, or lower than that of *F. oxysporum* strain SP, respectively.

CA, USA) to determine the copy numbers of bacteria (Eub338/Eub518), fungi (ITS1F/5.8S), and *Fusarium oxysporum* (ITS1F/AFP308) using the specific primer pairs listed in Table 1. The PCR reaction mixture contained 10 μL of SYBR Green *Premix Ex Taq*™ (2×, TaKaRa, Japan), 1 μL of each primer (10 μM), 2 μL of the target DNA (range 28.95–34.55 ng μL^{-1}) and 6 μL of sterile distilled water. The thermal cycling conditions used are presented in Table 1. The amplification efficiencies for bacteria, fungi, and *F. oxysporum* were 107.5%, 95.1%, and 94.8%, respectively.

2.4. MiSeq sequencing and data analysis

MiSeq sequencing was used to characterize the changes in the composition of bacterial and fungal communities occurring in response to the different treatments applied. The DNA extracted from each soil sample served as a template for the amplification of the bacterial and fungal fragments for MiSeq sequencing. The bacterial 16S rDNA V4 region was amplified using the primer set 520 and 802 (Table 1). For fungi, the primers ITS1F and ITS2 (Table 1) were used to amplify the ITS region. The thermal cycling conditions used are presented in Table 1. After successful amplification was achieved, the PCR products were sent to Genesky Biotechnologies, Inc. (Shanghai, China) for Illumina MiSeq sequencing.

Sequence analysis was performed using the Quantitative Insights Into Microbial Ecology (QIIME) software package (version 1.9.1) (Caporaso et al., 2010). In short, the paired-end FASTQ reads were merged and quality controlled using the default arguments in the multiple_joined_paired_ends.py and multiple_split_libraries_fastq.py, respectively, to obtain the quality-filtered sequences. Then, all high-quality sequences were clustered at OTUs at a dissimilarity of < 0.03, and classification information of OTUs was obtained by alignment against the reference sequence database (Greengenes for bacteria, Release 13.8, http://greengenes.secondgenome.com/; UNITE for fungi, Release 5.0, http://unite.ut.ee/index.php). Sequence data were deposited in the NCBI Sequence Read Archive database under the accession number SRP118560.

2.5. Isolation and identification of the F. oxysporum strain SP and antagonistic bacterial and fungal isolates

F. oxysporum strain SP was isolated from the infected lisianthus plants and identified according to its morphological characteristics and ITS sequence. Furthermore, we validated the pathogenic ability of this strain according to Koch's rules. Specifically, the strain SP was cultured in potato dextrose agar (PDA) and then inoculated into the soil, where pathogen-free lisianthus seedlings were planted. The results indicated that 92.6% of the plants grown in the SP-inoculated soil were diseased (Fig. S1), whereas the plants grown in the uninoculated control were not infected. Further, we reisolated the strains from the diseased plants and found that they had morphological characteristics and ITS sequence identical to those of *F. oxysporum* SP (Fig. S1). Finally, the strain was stored on PDA at 4 °C.

Bacterial and fungal strains were randomly isolated from 12 soil samples and purified using Luria Bertani agar (LB) medium and potato dextrose agar (PDA) medium, respectively. The bacterial and fungal isolates were stored on LB and PDA media, respectively, at 4 °C. The antagonistic abilities of the isolated bacterial and fungal strains against *Fusarium oxysporum* strain SP were examined by dual-culture tests performed on soil-extract agar medium. The methods reported by Huang et al. (2011, 2012) were employed for the analyses. The soil-extract agar medium (1 L) contained 500 mL of soil extract, prepared by heating 1 kg soil in 1 L of water at 100 °C for 30 min and filtering the supernatant liquid, 500 mL of deionized water, and 20 g L^{-1} agar. A total number of 221 bacterial and 181 fungal isolates were utilized in the dual-culture tests, and the inhibitory degrees of these isolates against the *Fusarium oxysporum* strain SP were determined according to the visual patterns displayed in Fig. 1. Microbial isolates that effectively inhibited the *Fusarium oxysporum* strain SP were selected and stored at 4 °C for further identification analysis.

DNA was extracted from bacterial and fungal isolates using E.Z.N.A.® Bacterial DNA Kits (Omega Bio-tek Inc. China) and E.Z.N.A.® Fungal DNA Kits (Omega Bio-tek Inc. China) according to the respective manufacturers' protocols. PCR was carried out in the thermocycler with a reaction volume of 25 μL containing 1 μL of each of the primers (10 μM, I27 and 1492 targeted at 16S rDNA of bacteria, ITS1 and ITS4 targeted at ITS region of fungi, Table 1), 12.5 μL of Mix (2 ×, Takara, China), 1 μL of DNA (8.96–12.63 ng μL^{-1}), and 9.5 μL of dd H_2O. The PCR cycling conditions are listed in Table 1. The PCR product obtained was checked by agarose gel electrophoresis to confirm that a single DNA fragment was amplified and was then sent to the GenScript Company (Nanjing, China). The sequences were annotated through BLAST in the GeneBank database.

2.6. Statistical analysis

The data of microbial counts were $\log_{10}$ transformed before the statistical analysis. The significant differences between means were determined by the least significant difference (LSD) test ($P < 0.05$) using SPSS version 18.0 (SPSS Inc. Chicago, IL, USA). Principal coordinate analysis (PCoA) based on the Bray-Curtis distance of OTUs was used to evaluate the differences in microbial community structures among the four treatments. Next, heatmaps were created through HemI

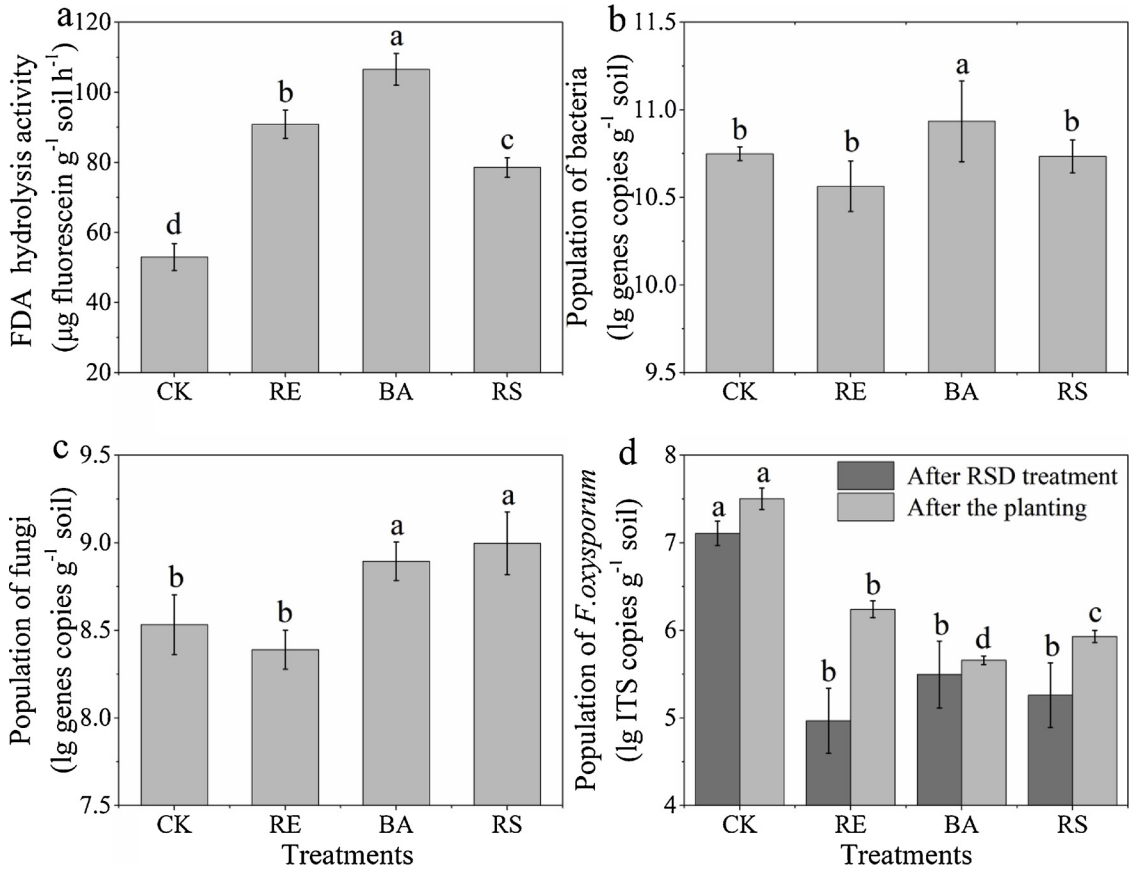

Fig. 2. Fluorescein diacetate (FDA) hydrolysis activities (a), populations of bacteria (b), and fungi (c) in the soils after lisianthus planting, and the numbers of *F. oxysporum* after treatment and after five months of lisianthus planting (d), respectively. Bars with different letters represent significant differences among the four different soils established by the LSD test ($P < 0.05$). Error bars indicate standard deviations. CK, untreated soil; RE, RSD amended with reed straw; BA, RSD amended with bagasse; RS, RSD amended with rice straw.

(version 1.0.0.0, http://hemi.biocuckoo.org/down.php). Finally, the linear regression was calculated using SPSS version 18.0 to determine the relationship between the proliferation of *F. oxysporum* and the microbiological index or the abundance of the bacterial/fungal families.

3. Results

3.1. Soil physicochemical properties, microbial activities, and quantifications

After lisianthus planting, the total carbon in the BA treatment was significantly ($P < 0.05$) higher than in the CK treatment, while no significant differences were found among the CK, RE, and RS treatments. Compared to the CK treatment, the soil pH values in the RE, BA, and RS treatments significantly ($P < 0.05$) increased by 0.19, 0.21, and 0.22, respectively. Soil electrical conductivity in the RE and RS treatments was significantly ($P < 0.05$) lower than that in the CK treatment, while there was no significant difference between the CK and BA treatments (Table S1). Overall, the total microbial activities was significantly ($P < 0.05$) higher in all RSD treatments (RE, BA, and RS) than in the CK treatment (Fig. 2a). The highest microbial activity was observed in the BA treatment, followed by the RE and RS treatments, that is 2.01-, 1.71-, and 1.48-fold increases compared to the CK treatment, correspondingly. The bacterial population increased significantly ($P < 0.05$) in the BA treatment, whereas those in the RE and RS treatments displayed no significant differences to that in the CK treatment (Fig. 2b). In contrast, the populations of fungi considerably ($P < 0.05$) increased in both the BA and RS treatments in comparison to those in the CK treatment, but no obvious difference was detected between the CK and RE treatments (Fig. 2c).

3.2. Microbial diversity estimates and community structure

A total number of 530,998 high-quality bacterial 16S rRNA gene sequences and 589,221 high-quality fungal ITS sequences were generated. These sequences were clustered into 27,975 and 2933 OTUs with 97% sequences similarity for bacteria and fungi, respectively. Bacterial richness indices (the Chao1 richness estimator and abundance-based coverage estimator, Chao and ACE) and the diversity index (Shannon-Wiener index, Shannon) in the RS treatment were significantly ($P < 0.05$) higher than those in CK. In contrast, the Shannon index in the RE treatment was significantly lower than that in CK, and there were no significant differences in the bacterial richness and diversity between the BA and CK treatments. Fungal ACE and Shannon indices in all RSD treatments were significantly lower than that in the CK treatment, and the lowest ACE and Shannon indices were found in the RE treatment (Table 2).

We visualized the differences in microbial community structures among the four treatments using PCoA. PCoA showed that the RSD treatments (RE, BA, and RS) significantly altered the bacterial and fungal community structures as compared to those of the CK treatment (Fig. 3). The soil bacterial community in BA was similar to that in RS, but that in RE was distinct from the others in terms of the first component (Fig. 3a). Furthermore, the fungal communities of RE, BA, and RS were respectively grouped or separated from each other, indicating that the amendments with different organic materials had distinct influence on the regulation of soil fungal community (Fig. 3b). Besides, the clustering analyses based on the top 50 bacterial and fungal genera

Table 2
Diversity estimates of bacterial and fungal communities investigated by Miseq sequencing.

Treatment[§]	Bacteria			Fungi		
	Chao	ACE	Shannon	Chao	ACE	Shannon
CK	2465 ± 36^{ab}	2914 ± 126^{ab}	9.44 ± 0.04^{b}	434 ± 6^{c}	449 ± 14^{c}	4.48 ± 0.06^{c}
RE	2375 ± 65^{a}	2845 ± 58^{a}	9.18 ± 0.10^{a}	296 ± 32^{a}	305 ± 22^{a}	3.16 ± 0.07^{a}
BA	2518 ± 111^{b}	3027 ± 84^{bc}	9.48 ± 0.07^{b}	339 ± 59^{ab}	348 ± 28^{ab}	3.45 ± 0.12^{b}
RS	2654 ± 13^{c}	3108 ± 58^{c}	9.64 ± 0.03^{c}	385 ± 57^{bc}	386 ± 41^{b}	3.52 ± 0.25^{b}

Values (means ± SD, $n = 3$) within the same column followed by different letters indicate significantly different at $P < 0.05$ according to LSD test. The Chao1 richness estimator (Chao) and abundance-based coverage estimator (ACE) represent microbial richness, and Shannon-wiener index (Shannon) represents microbial diversity.

[§] CK, untreated soil; RE, RSD incorporated with reed straw; BA, RSD incorporated with bagasse; RS, RSD incorporated with rice straw.

compositions indicated that the microbial communities in the RSD treatments amended with different organic materials were clustered and separated from that in the untreated soil (Fig. 4).

3.3. Microbial community composition

3.3.1. Bacteria

The bacterial OTUs were assigned into 699 genera of 52 different phyla. Proteobacteria, Actinobacteria, Gemmatimonadetes, Acidobacteria, Chloroflexi, Planctomycetes and Bacteroidetes were the dominant bacterial phyla across all the soil samples. Sequences affiliated within these phyla accounted for more than 85% of the classified sequences within the four treatments (Fig. S2a). At the genus level, the overall compositions of the bacterial communities of the different soils were similar, but the relative abundances of some specific genera were distinct in the four treatments (Fig. 4a). For instance, the relative abundances of an unclassified genus (UC-) belonging to the class Alphaproteobacteria, in the RSD treatments was significantly lower than that in CK and was only accounting for 26.2%–37.2% of that in CK (Fig. S3a). In addition, the relative abundances of UC-Cytophagaceae were significantly ($P < 0.05$) higher in the BA treatment than in the CK, RE, and RS treatments (Fig. 5e). Certain taxa from the Chitinophagaceae family, including *Flavisolibacter* and UC-Chitinophagaceae, had a greater relative abundance in the BA and RS treatments than in the two other treatments (Fig. 5f).

3.3.2. Fungi

Ascomycota was the predominant fungal phylum accounting for more than 95% of the total sequences across all the soils (Fig. S2b). Obviously, in comparison to bacterial community, the soil fungal community had a more pronounced response to the RSD treatments (Fig. 4b). The fungal communities in the RSD-treated soils were different from that in CK in both the fungal compositions and the relative abundances of the dominant fungal species (Fig. S3b). Specifically, the relative abundance of *Fusarium* significantly decreased in the RSD treatments and was only 3.0%–5.6% of that in the CK treatment. UC-Nectriaceae and UC-Microascaceae showed a response to RSD treatments that was similar to that of *Fusarium*. Moreover, ANOVA analysis revealed that the relative abundance of Chaetomiaceae profoundly ($P < 0.05$) increased in all RSD treatments. Within the Chaetomiaceae family, the relative abundance of UC-Chaetomiaceae was higher in the RE and BA treatments than in those in the CK and RS treatments, whereas the relative abundance of *Mycothermus* was significantly ($P < 0.05$) higher in all RSD treatments than that in CK (Fig. 5g). Besides, the relative abundance of UC-Sordaeiomycetes dramatically ($P < 0.05$) increased in the RE and BA treatments (Fig. 5h).

3.4. Isolation and identification of the antagonistic isolates

Overall, a total number of 60, 59, 45, and 57 bacterial strains were isolated from the CK, RE, BA, and RS treatments, respectively. These bacterial strains were classified into three groups based on the inhibition degree of *F. oxysporum* strain SP as reveled by the dual-culture assay results. The BA treatment contained the highest proportion of antagonistic bacterial isolates, followed by the RE, RS, and CK treatments (Table 3). Moreover, *Bacillus* spp. were the major antagonistic

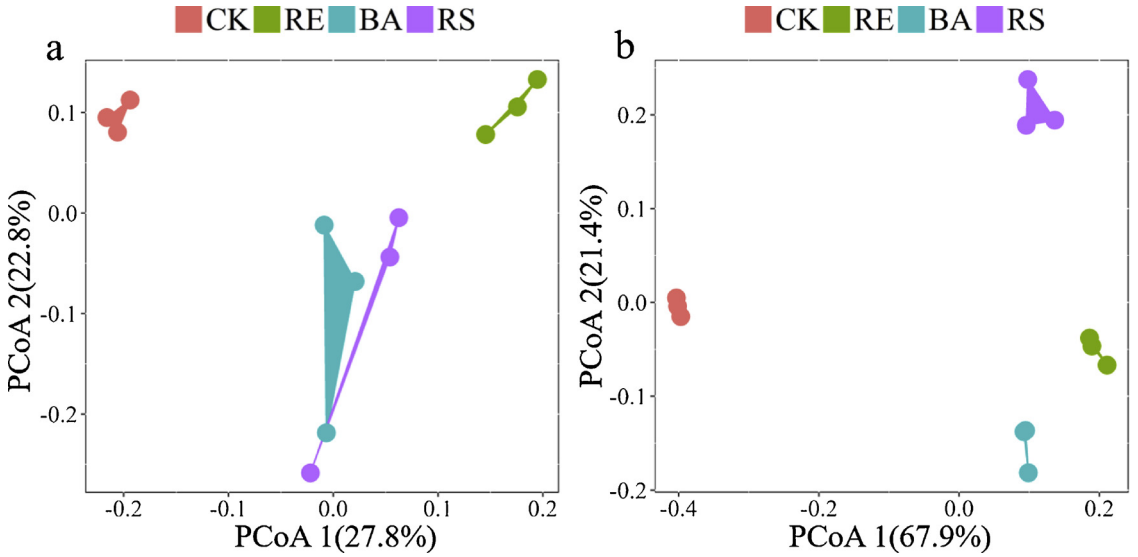

Fig. 3. Visualization for the dissimilarities in soil bacterial (a) and fungal (b) communities among different soils based on Principal coordinate analysis (PCoA). The bacterial and fungal OTUs compositions in different soils after planting were used for PCoA using Bray-Curtis distance. CK, untreated soil; RE, RSD amended with reed straw; BA, RSD amended with bagasse; RS, RSD amended with rice straw.

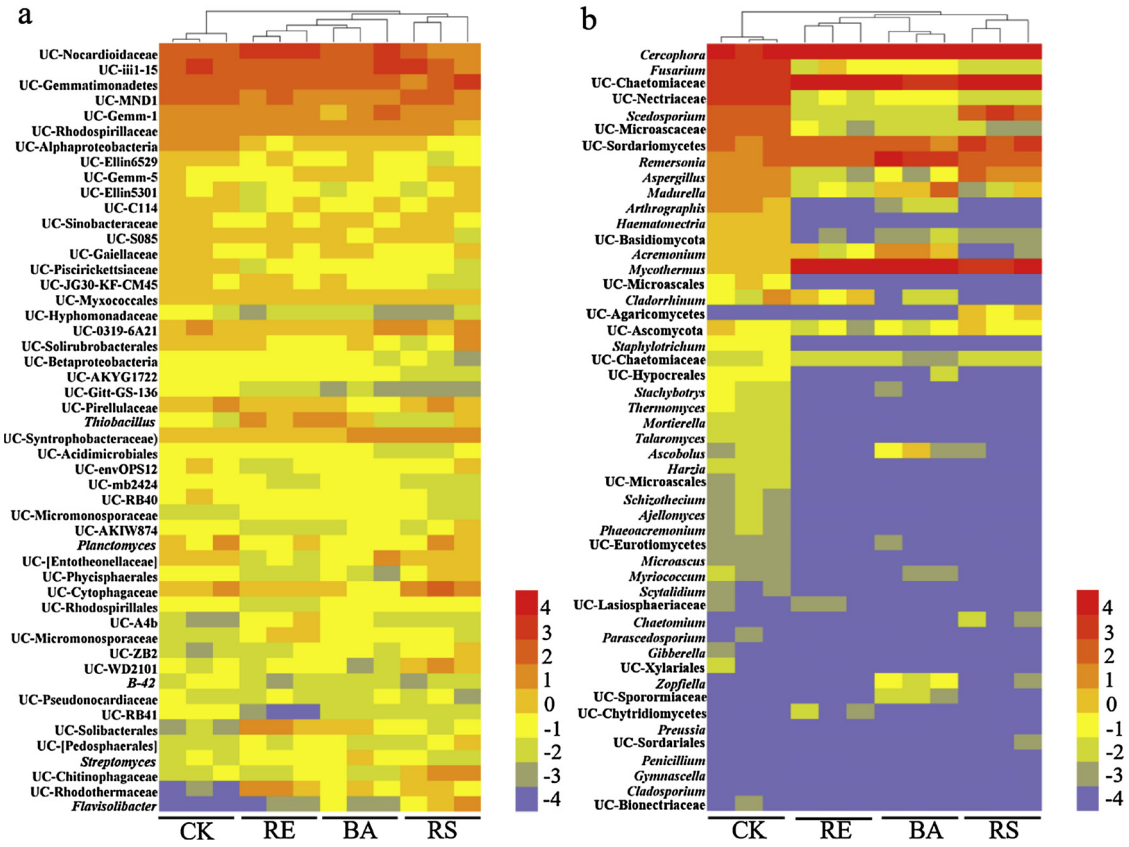

Fig. 4. Heatmap of bacterial (a) and fungal (b) distributions of the top 50 abundant genera present in the microbial communities in the soil samples. Hierarchical cluster analyses were performed using the neighbor-joining method. The relative values for bacterial and fungal genera are indicated by the color intensity. The taxonomic designation following "UC-" represents the most detailed classification of the unclassified genus.

bacterial species isolated from the RSD treatments while *Arthrobacter* spp. were predominant in the CK treatment. Additionally, *Streptomyces zaomyceticus*, defined as Antagonist + +, was isolated only from the RS treatment.

The numbers of fungal isolates in the CK, RE, BA, and RS treatment were 40, 57, 47, and 37, respectively. Fifty-two strains exerted inhibitory effects on the *F. oxysporum* strain SP according to the results of the plate confrontation assay. These isolates were further classified into 15 groups based on the colony and mycelium morphology, and identified according to their ITS sequences. The proportions of antagonistic fungal strains in the RE, BA, and RS treatments were 11.1%, 85.1%, and 7.7%, correspondingly, whereas no fungal antagonist was found in the CK treatment (Table 4).

3.5. Incidence of Fusarium wilt in lisianthus

At the end of lisianthus planting, the incidence of Fusarium wilt disease in the CK treatment was 66.1%, significantly higher than those in all RSD treatments (Fig. 6). The lowest incidence (2.0%) was found in the BA treatment, in which a 33.0-fold reduction was achieved compared to CK. Disease incidences in the RE and RS treatments were 9.5% and 5.5%, respectively.

3.6. Pathogen population and agents inhibiting its proliferation

After the RSD treatments, the *F. oxysporum* populations decreased significantly ($P < 0.05$), and the lowest copy number of *F. oxysporum* was found in the RE treatment (1.13×10^5 ITS copies g^{-1} soil), followed by RS (2.22×10^5 ITS copies g^{-1} soil), and BA (3.89×10^5 ITS copies g^{-1} soil). After the cultivation, the abundance of *F. oxysporum* in all treatments was higher than that before transplanting, but the values of that parameter in the RSD-treated soils were still far below that of the untreated soil. Furthermore, the increases of the pathogen abundance varied across the different RSD-treated soils, and the lowest copy number of *F. oxysporum* was detected in BA (4.6×10^5 ITS copies g^{-1} soil), accounting for only 1.41% of that in the CK treatment (Fig. 2d).

Linear regression analysis indicated that the FDA hydrolysis activity, the percentage of antagonistic bacterial, and fungal isolates were significantly ($P < 0.05$) negatively correlated with the proliferation of *F. oxysporum* (Fig. 7). In addition, the relative abundance of Cytophagaceae, Chitinophagaceae, Chaetomiaceae, and UC-Sordaeiomycetes had a significantly ($P < 0.05$) negative association with the proliferation of *F. oxysporum* (Fig.5).

4. Discussion

Soil microbial community composition is a crucial component of the soil habitat that determines soil health and quality (Garbeva et al., 2006). Unfortunately, continuous monocropping usually causes accumulation of plant pathogens and deteriorates microbial communities and induces the occurrence of soil-borne diseases (Berendsen et al., 2012). Here, we used RSDs combined with the incorporation of different organic substrates to disinfest a soil on which four-year monocropping had been applied. We found that the population of *F. oxysporum* was significantly reduced by RSD, which was consistent with previous observations (Mowlick et al., 2013; Huang et al., 2015; Meng et al., 2018). Consequently, the effective pathogen inactivation should be responsible for the suppression of lisianthus Fusarium wilt in the RSD-treated soils. However, the replantation of the host crop stimulated the pathogen proliferation, which was in accordance with the results of a previous report (Liu et al., 2018). Furthermore, we found that the increase in the pathogen abundance significantly varied across the

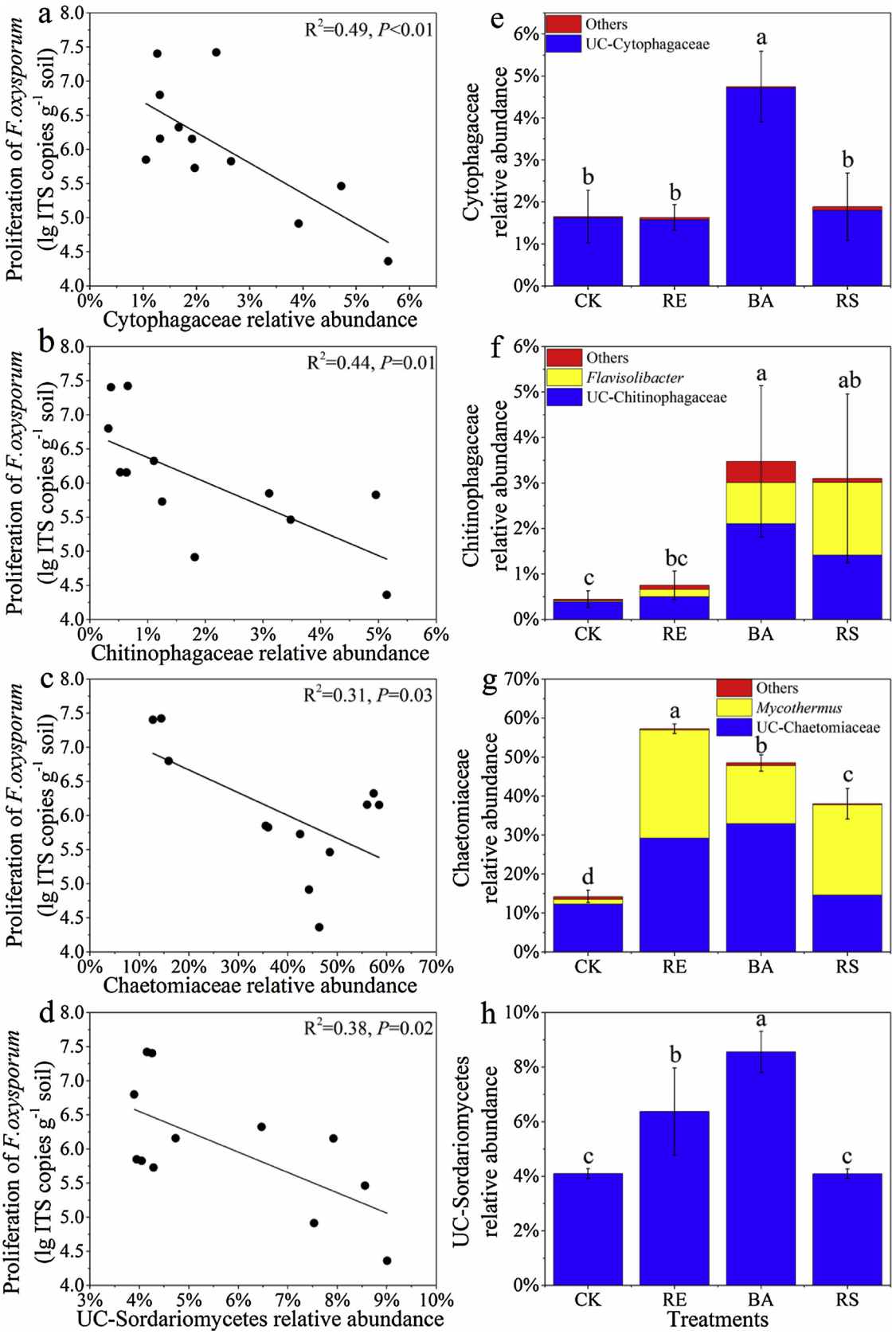

Fig. 5. Linear regression between the relative abundances of dominant microbial groups and *F. oxysporum* proliferation and the distributions of these groups in the four soils studied. The letter following "UC-" represents the most detailed classification of the unclassified family. The bacterial and fungal genera with relative abundance lower than 1% in all the soils was copolymerized in "Other", respectively. Bars with different lower-case letters represent the significant differences among the four different soils assessed by the LSD test ($P < 0.05$). Error bars indicate standard deviations. CK, untreated soil; RE, RSD amended with reed straw; BA, RSD amended with bagasse; RS, RSD amended with rice straw.

Table 3
Inhibition patterns, proportion and classification of the bacterial isolates.

Treatment	Total isolates	Inhibition pattern	Percentage of isolates	Classification	Accession number
CK		Antagonist + +	0	—	—
		Antagonist +	8.3%	*Arthrobacter* sp. (40%)[a]	KY082713, KY082741
	60			*Arthrobacter pascens* (60%)	KY082720, KY082712, KY082721
		Antagonist −	91.7%	—	—
RE		Antagonist + +	0	—	—
		Antagonist +	18.7%	*Arthrobacter globiformis* (18.2%)	KY082722, KY082714
				Bacillus megaterium (18.2%)	KY082715, KY082723
				Arthrobacter sp. (9.1%)	KY082724
	59			*Bacillus firmus* (9.1%)	KY082725
				Bacillus cereus (18.2%)	KY082726, KY082737
				Bacillus sp. (9.1%)	KY082727
				Bacillus nanhaiensis (18.2%)	KY082728, KY082742
		Antagonist −	81.3%	—	—
BA		Antagonist + +	0	—	—
		Antagonist +	22.2%	*Bacillus* sp. (10%)	KY082716
				Bacillus idriensis (20%)	KY082717, KY082739
				Bacillus subtilis (10%)	KY082729
	45			*Arthrobacter* sp. (10%)	KY082730
				Promicromonospora sp. (20%)	KY082731,KY082738
				Bacillus licheniformis (20%)	KY082732, KY082733
				Bacillus firmus (10%)	KY082734
		Antagonist −	77.8%	—	—
RS		Antagonist + +	1.8%	*Streptomyces zaomyceticus* (100%)	KY082735
		Antagonist +	7.0%	*Bacillus subtilis* (25%)	KY082718
	57			*Bacillus idriensis* (25%)	KY082719
				Arthrobacter globiformis (25%)	KY082736
				Promicromonospora sp. (25%)	KY082740
		Antagonist −	91.2%	—	—

[a] The number following the taxonomic name indicates the proportion of each species in each inhibition pattern.

different soils. Since the control of the pathogen increase was critical for the successful management of soil-borne disease (Bonanomi et al., 2010), a further investigation clarifying the reason for the varied increase in the pathogen population is necessary to obtain in-depth insights into the interactions between soil microbes and plant diseases. Takehara et al. (2003) reported that the pathogen rapidly proliferated in the chloropicrin- or hot water-disinfected soils and wilt developed faster than in the untreated soil, showing the importance of soil microbial activity and composition on the pathogen suppression. In the present study, we found that RSDs significantly increased soil microbial activity (FDA hydrolysis), which was consistent with previous observations that crop residues amendments can activate microbial activity (Yulianti et al., 2006). Moreover, Chen et al. (1991) established that the microbial activity in organic soils is often associated with the suppressiveness against soil-borne pathogens, which was further confirmed by the negative relationship between *F. oxysporum* multiplication and FDA hydrolysis activity.

Additionally, RSDs established distinct microbial community

Table 4
Inhibition patterns, proportion and classification of fungal isolates.

Treatment	Total isolates	Inhibition pattern	Percentage of isolates	Classification	Accession number
CK		Competition +	0	—	—
	40	Equal	5%	—	—
		Competition-	95%	—	—
RE		Competition +	11.1%	*Ceriporia lacerate* (16.7%)[a]	KY082745
				Aspergillus niger (33.3%)	KY082744
	57			*Aspergillus tubingensis* (50%)	KY082743
		Equal	0	—	—
		Competition-	88.9%	—	—
BA		Competition +	85.1%	*Ceriporia lacerate* (17.5%)	KY082754, KY082753
				Hypocrea virens (5%)	KY082750
	47			*Myceliophthora verrucosa* (47.5%)	KY082749, KY082747, KY082752
				Trichoderma harzianum (17.5%)	KY082751
				Schizophyllum commune (12.5%)	KY082748
		Equal	4.3%	—	—
		Competition-	10.6%	—	—
RS		Competition +	15.4%	*Fusarium solani* (16.7%)	KY082757
				Purpureocillium lilacinum (16.7%)	KY082755
	37			*Irpex lacteus* (33.3%)	KY082756
				Stachybotrys chartarum (33.3%)	KY082758
		Equal	5.1%	—	—
		Competition-	79.5%	—	—

[a] The number following the taxonomic name indicates the proportion of each species in each inhibition pattern.

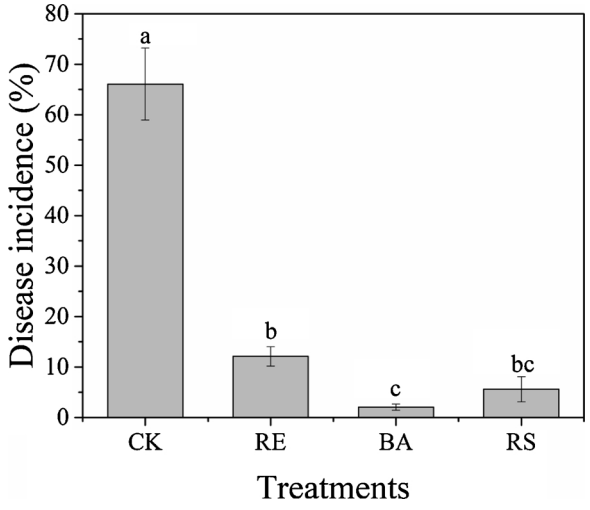

Fig. 6. Fusarium wilt disease incidence of lisianthus after five months of planting in the four treatments. Bars with different letters represent significant differences among the four different soils following LSD test ($P < 0.05$). Error bars indicate standard deviations.

structures and compositions from the untreated soil. A previous study indicated that specific microbial groups might exert important functions in the pathogen or disease suppression (Cha et al., 2016).Thus, linear regression analyses were used to further search the suppressive agents of pathogen proliferation. The results showed that the presence of Cytophagaceae, Chitinophagaceae, Chaetomiaceae, and UC-Sordariomycetes effectively prevented *F. oxysporum* multiplication. Many of these microbial groups were largely found in previous examinations on RSD-treated soils (Liu et al., 2016; Huang et al., 2016), and the major property shared by these microbes is their remarkable potential to decompose less-degradable organic carbon, such as cellulose and hemicellulose (Savory, 2010; Glavina et al., 2010; Eichorst et al., 2013; Mcbride et al., 2014; Natvig et al., 2015). Furthermore, some of these microbial groups (*e.g.,* Chaetomiaceae) have frequently been reported to be responsible for the suppression of soil-borne pathogen or disease development (Tvett, 1955; Cullen and Andrews, 1984). Our results showed that the RSD treatment profoundly increased the abundances of representatives of Cytophagaceae, Chitinophagaceae, Chaetomiaceae, and UC-Sordariomycetes through the plant residuals amendment, which might have a substantial suppressive influence on the proliferation of *F. oxysporum* in the RSD-treated soil. However, the specific inhibitory mechanisms of action of these microbes are largely unknown and are subjects for future research.

In addition, we employed a culture-based method to further detect the differences in the microbial compositions and functions of these soils. To simulate the natural soil nutrient conditions, we used soil-extract agar medium for the dual-culture tests. Subsequently, the majority of the bacterial antagonists in the RSD-treated soils were identified as *Bacillus* spp. *Streptomyces zaomyceticus*, *Arthrobacter* spp., and *Promicromonospora* spp., whereas the untreated soil contained only *Arthobacter* spp. Obviously, the *Bacillus* species, the prevalent isolated bacterial antagonists in our experiment, have been identities as successful biocontrol agents against bacterial and fungal pathogens and diseases (Schisler et al., 2004; Ongena and Jacques, 2008). Idris et al. (2007) reported that numerous species of *Bacillus* were capable of effectively inhibiting the mycelia growth of *F. oxysporum in vitro*, which is in accordance with our results. Besides, *Streptomyces zaomyceticus* obtained in RS, showed the highest antagonistic activity against *F. oxysporum* strain SP. Cha et al. (2016) isolated a *Streptomyces* strain from a disease-suppressive soil, which synthesized a novel heat-stable antifungal thiopeptide that played an important role in the pathogen and disease suppression. Similarly, *Arthrobacter* spp. and *Promicromonospora* spp. have also been reported as potential antagonists against fungal pathogens (Barrowsbroaddus et al., 1983; Kang et al., 2014). *Ceriporia lacerata*, *Aspergillus* spp. *Hypocrea virens*, *Myceliophthora verrucosa*, *Trichoderma harzianum*, *Schizophyllum commune*, *Fusarium solani*, *Purpureocillium lilacinum*, *Irpex lacteus*, and *Stachybotrys chartarum* were identified in the fungal isolates from the RSD-treated soils in our study, whereas no potential fungal antagonists were found in the untreated soil. Previous reports have indicated that many of the antagonistic species isolated in the present investigation effectively inhibited pathogen and control soil-borne diseases, including *Aspergillus* spp. (Patibanda and Sen, 2005; Aukkasarakul et al., 2014), *Trichoderma harzianum* (Wu et al., 2009; Huang et al., 2017), *Hypocrea virens* (Chaverri et al., 2001), and *Paecilomyces lilacinus* (Kiewnick and Sikora, 2006). Overall, the higher proportions and diversity of microbial antagonists found in the RSD-treated soils might be responsible for the effective control against pathogen proliferation. Moreover, the BA soil contained the highest proportions of bacterial and fungal antagonists, which might have been the reason for the highest effectiveness of the control on the population of the pathogen and disease compared with the effects in the RE- and RS-treated soils.

It is noteworthy that microbial properties, such as microbial population, activity, and composition, in the different organic substrates used as amendments in RSD treatments were different. Since soil properties, such as pH and total organic carbon, were not dramatically different in the RSD treatments, the variations in the microbial properties might have been caused by the heterogeneity of the composition of the organic substrates. A previous investigation (Zhao et al., 2018) using the same soil as this study confirmed that the RSDs incorporated with different organic materials harbored distinct microbial activity, fungal taxa and population, which was in line with many earlier observations that RSD-treated soils amended with different organic materials often have distinct soil microbial community structures and compositions (Mowlick et al., 2012; Huang et al., 2016; Ueki et al., 2018). Thus, to achieve orientated management of disease-suppressive

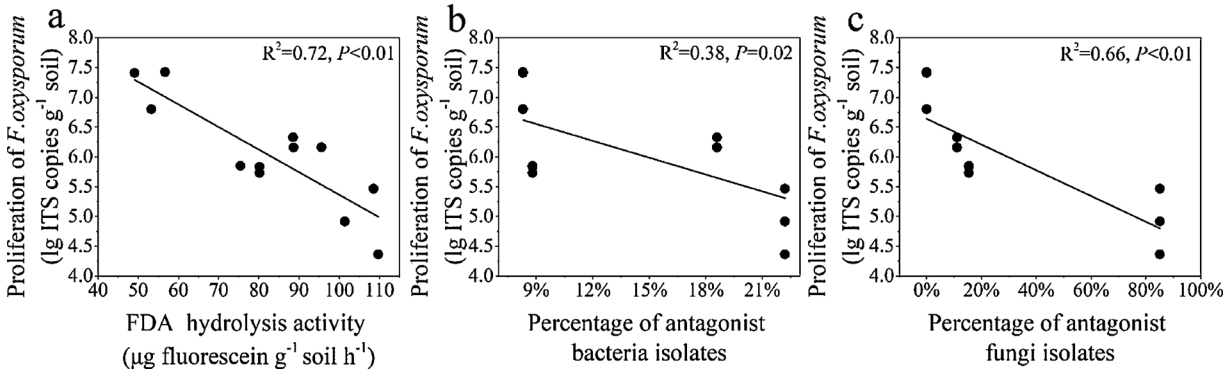

Fig. 7. Linear regression between microbiological indices and the proliferation of *F. oxysporum*.

microbial characteristics, such as microbial activity and percentage of antagonistic microbes, more information regarding the association between these properties and the specific organic sources is essential to be obtained.

Our findings revealed that the RSD-treated soils harbored microbial communities that were distinctive from those in the untreated soil. Moreover, the abundances of Cytophagaceae, Chitinophagaceae, Chaetomiaceae, and UC-Sordariomycetes and the other microbiological indices examined (*e.g.*, FDA hydrolysis activity and percentage of antagonistic bacterial/fungi isolates) were negatively correlated with the pathogen population multiplication. Therefore, it could be speculated that in the RSD treatment, the pathogen was controlled by regulating the soil environment and the formation of a soil microbiome containing more beneficial agents. However, methods for quantification of the changes in the soil environment induced by RSD treatments using appropriate indicators and for determination of the exact specific association between them and the changes in microbial compositions still needs to be further investigated.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors do not have potential conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.12.001.

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