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Bio-fertilizer application induces soil suppressiveness against Fusarium wilt disease by reshaping the soil microbiome

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ABSTRACT

Fusarium wilt disease is a growing problem in agriculture systems. Application of bio-fertilizers containing beneficial microbes represents a promising disease control strategy. However, the mechanisms underlying disease suppression remain elusive. Here, in order to assess the importance of direct antagonism and modified soil microbiota on suppression of Fusarium wilt disease, we conducted a pot experiment with chemical, organic and biologically enhanced fertilizers, we tracked the impact of those fertilizer amendments on disease incidence, and measured the pathogen density and changes in soil microbiota. Alterations in bacterial abundance and community structure after bio-fertilizers application were determined to be key factors in constraining the pathogen, *Fusarium oxysporum*. In particular, bio-fertilizer application increased the abundance of indigenous microbial groups with reported antifungal activity, such as *Lysobacter* spp., which could play a keystone role in controlling this pathogen. The microbes introduced in the bio-fertilizer treatments (*e.g. Bacillus* and *Trichoderma* spp.) induced suppressiveness via alteration of the soil microbiome rather than direct pathogen inhibition. These results contrast with the commonly held paradigm of disease suppression using beneficial microbes and open up new perspectives for the promotion of soil health. In addition to seeking antagonistic microbes based on their direct inhibitory activity, disease suppression may also be achieved by introducing keystone species that reshape soil microbiome structure and function.

1. Introduction

Fusarium wilt disease is one of the most serious soil-borne diseases in agriculture systems and is a major limiting factor in many cropping systems (Klein et al., 2016; Wang et al., 2015). This disease can be controlled to some extent with crop rotation (Wang et al., 2015; Xiong et al., 2016) and screening of resistant cultivars (Hwang and Ko, 2004; Pietro et al., 2003; Pinaria et al., 2010) or fungicides (Nel et al., 2007). However, these measures are often impractical due to labor- and monetary-costs or the low efficiency of available chemical control treatments. There is thus a need for alternative control strategies. One promising method is the application of bio-fertilizers, a mix of beneficial microbes combined with organic material. Bio-fertilizers combines the advantages of recycling organic waste, introducing beneficial microbes and providing organic material that will create additional niches for beneficial indigenous microbes (Fu et al., 2017; Qiu et al., 2012).

Bio-fertilizers are often enriched with microbial isolates such as *Bacillus* and *Trichoderma* spp., selected based on their potential ability to suppress Fusarium wilt disease under laboratory conditions (Wang et al., 2013; Zhang et al., 2016). However, direct applications of such potentially beneficial species often results in a poor disease suppression due to their low survival in soil (Saravanan et al., 2003). The secondarily fermented bio-fertilizers, a combination of bio-control agents and mature compost, could enhance resident beneficial microbe density in soil and be more efficient in controlling soil-borne disease than solely antagonistic microbe (Zhang et al., 2011). In addition, compost can stimulate the

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activities of soil microorganisms that are antagonistic to plant pathogens (Akhtar and Malik, 2000; Mehta et al., 2014).

Bio-fertilizers can improve soil health by direct suppression of pathogens or via modification of the indigenous microbial community. While direct suppression is well described, very few is known about the effect of bio-fertilizers on soil microbial communities and their ability to suppress diseases. We hypothesised that changes in disease suppression after bio-fertilizer application is the result of the combined effects of the introduced microbes (direct inhibition of the pathogen) and alterations of soil microbiota.

To investigate this hypothesis, we used Fusarium wilt of vanilla (*Vanilla planifolia* Ames) as a model system. Vanilla is an economically valuable spice crop widely cultivated in tropical regions (Minoo et al., 2008), and Fusarium wilt disease, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *vanillae* (Koyyappurath et al., 2016; Pinaria et al., 2010; Xiong et al., 2015b), is a serious threat resulting in large economic losses in vanilla-cropping regions. In order to assess the relative importance of introduced microbes versus reshaping of the soil microbiome for disease suppression, we amended natural soil with bio-fertilizers containing *Bacillus* (bacterial-enriched biofertilizer) or *Trichoderma* (fungal-enriched biofertilizer), and compared these treatments to the amendment with organic fertilizer without microbial inoculation and nutrient addition via mineral fertilizer. We tracked the impact of the four fertilizer amendments on disease incidence, measured the pathogen density, and examined bacterial and fungal community by Illumina MiSeq sequencing.

2. Materials and methods

2.1. Experimental design

We collected the experimental soil in October 2014 from a 20-year continuously cropped vanilla field showing serious Fusarium wilt disease (before we collected the soil, the field was abandoned for over six months without vanilla cropping). We collected the field soil with a depth of about 20 cm using shovels, mixed it thoroughly and immediately transferred it to a greenhouse with an average temperature of 30 °C and an average humidity of 72% at the Spice and Beverage Research Institute, Wanning City, Hainan Province, China (110°19′E-110°22′E, 18°72′N-18°76′N). A pot experiment was performed using a randomized complete block design with four replicates for each treatment, where each replicate had four pots, and each pot contained 12 kg soil with three vanilla seedlings from the rapid multiplication nursery (please see more details in (Xiong et al., 2015b; Zhao et al., 2015)). Four fertilization treatments were designed as follows: organic fertilizer (OF), which is a mixture of an amino acid fertilizer (Wang et al., 2013) and chicken manure compost in a 1:1 weight ratio (first fermentation), two secondarily fermented bio-fertilizers inoculated with either *Bacillus amyloliquefaciens* W19 (bacterial enriched bio-fertilizer, B_BIO) or *Trichoderma guizhouense* NJAU 4742 (fungal enriched bio-fertilizer, F_BIO) respectively, as previously described (Wang et al., 2013; Yuan et al., 2016). The bio-fertilizers contained approximately 1.0×10^9 and 5.0×10^7 CFU of *Bacillus amyloliquefaciens* and *Trichoderma guizhouense* g^{-1} dry weight of bio-fertilizer, respectively. Each pot was supplemented with 360 g OF or bio-fertilizers. A control treatment was set up with chemical fertilizer (CF) containing the same NPK concentrations (*i.e.*, 13.25 g urea, 13.75 g P_2O_5 and 4.10 g K_2O) as OF. After the fertilization treatments, the vanilla seedlings were planted in pots and covered with some sterile coco fiber. Fusarium wilt disease was based on appearance of typical wilt symptoms, and the cumulative disease incidence was calculated as the percentage of infected plants relative to the total number of plants.

2.2. Soil sample collection and DNA extraction

After 12 months we removed vanilla plants, and collected 12 random soil cores from pots with a depth of about 5 cm, which were pooled to yield one composite sample per replicate. The soils were passed through a 2 mm sieve, thoroughly homogenized and divided into 2 subsamples. One subsample was air-dried for the analysis of soil characteristics, according to previously established methods (Xiong et al., 2015a), and the other subsample was stored at −80 °C for DNA extraction. For each soil sample (16 in total: 4 treatments * 4 replicates), total DNA was extracted from 0.5 g soil using the MoBioPowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Genomic DNA concentration and purity were measured using a NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE) spectrophotometer.

2.3. Quantification of the Fusarium oxysporum, bacterial and fungal abundances

The total *Fusarium oxysporum*, bacterial and fungal abundances were quantified by quantitative polymerase chain reaction (qPCR), according to previously described protocols (Xiong et al., 2015a, 2016). Briefly, we set up 20 μl reaction mixtures containing 10 μl of the *Premix Ex Taq*™ (2 ×) (Takara-Bio, Japan), 0.4 μl of each primer (10 μM), 0.4 μl of ROX Reference Dye II (50 ×), 2 μl of template DNA and 6.8 μl of ddH_2O. The specific primer sets were as follows: AFP308R (CGAATTAACGCGAGTCCCAAC) and ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Lievens et al., 2005) for *Fusarium oxysporum*; 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACVSGGGTATCTAAT) (Caporaso et al., 2012) for bacteria; and ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS2 (GCTGCGTTCTTCATCGATGC) (White et al., 1990) for fungi. The PCR thermalcycling conditions were set as follows: 30 s at 95 °C for initial denaturation, 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. The standard curves were also generated according to previously described protocols (Xiong et al., 2015a, 2016). The specificity of the amplification products was confirmed by melting curve analysis and agarose gel electrophoresis. Copy numbers were $\log_{10}$-transformed to normalize the values prior to further statistical analysis. Although the used primer set (AFP308R and ITS1F) may also amplify non-pathogenic strains of *F. oxysporum*, we showed in previous studies that the total soil *Fusarium oxysporum* abundance detected with this primer set was highly correlated with vanilla *Fusarium* wilt disease severity (Xiong et al., 2015b, 2016). Therefore, even if we could not differentiate pathogenic and non-pathogenic *F. oxysporum* by the current primers set, our qPCR measurements are a robust indicator for the pathogen (Fu et al., 2017; Qiu et al., 2012).

2.4. PCR amplification and Illumina MiSeq sequencing

We used the general bacterial primers 515F and 806R to amplify the V4 hypervariable region of the bacterial 16S rRNA gene. For fungal communities, the fungal-specific primers ITS1F and ITS2 were used to target the ITS1 region. These primer pairs were modified for sequencing by adding the forward Illumina Nextera adapter, a two basepair “linker” sequence, and a unique 7-bp barcode sequence at the 5′ end of the forward primer, and the appropriate reverse Illumina Nextera adapter and linker sequence at the 5′ end of the reverse primer. PCR amplification was performed following previously established protocols (Xiong et al., 2015a, 2016), 27 and 25 cycles were performed to amplify fungal

and bacterial templates, respectively. Each sample was amplified in triplicate, after purification with a PCR Purification Kit (Axygen Bio, USA), the PCR products were pooled in equimolar concentrations of 10 ng μl^{-1} before sequencing. Paired-end sequencing of bacterial and fungal amplicons were performed on the Illumina MiSeq sequencer at Personal Biotechnology Co., Ltd (Shanghai, China).

2.5. Bioinformatics analysis

After removing the adaptors and primer sequences, the raw sequences were assembled for each sample according to the unique barcode using QIIME (Caporaso et al., 2010). Split sequences for each sample were merged using FLASH V1.2.7 (Magoč and Salzberg, 2011). The sequences retained for each sample were processed following the established UPARSE pipeline (Edgar, 2013). In brief, sequences with a quality score lower than 0.5 or a length shorter than 200 bp were removed. After discarding the singletons, the remaining reads were assigned to operational taxonomic units (OTUs) with 97% similarity threshold. Then, the chimera sequences were removed. Finally, the bacterial representative sequences were matched against the RDP database (Cole et al., 2009; Wang et al., 2007) and the fungal representative OTUs were classified using UNITE database (Kõljalg et al., 2013) using the naïve Bayesian classifier implemented in Mothur with a 80% confidence threshold (Schloss et al., 2009).

We estimated fungal and bacterial diversity using the Chao1 richness and phylogenetic diversity (PD) indices (Faith, 1992). Shannon evenness was calculated to evaluate species evenness. We used weighted (based on abundances of taxa) and unweighted (sensitive to rare taxa) UniFrac metric matrices to explore the variations in bacterial and fungal community structures among all the soil samples (Lozupone et al., 2006). PCoA (Principal Coordinate Analysis) was performed on distance matrices and coordinates were used to draw 2D graphical outputs. Analysis of similarity (ANOSIM) was performed to evaluate the significant differences in bacterial and fungal community structures between the four fertilizer treatments (Clarke, 1993). Heat maps were generated based on the 60 most abundant bacterial and fungal genera in R (version 3.2.2) with the “gplots” package. Venn diagrams were constructed to visualize shared and unique OTUs across the treatments in Mothur.

2.6. Statistical analyses

The fold change of each genus in the OF, B_BIO or F_BIO treatments relative to the CF treatment was calculated using the following formula: (X-CK)/CK, X is the relative abundance of the genus in the OF, B_BIO or F_BIO samples, CK represents the average relative abundance of each genus in the CF treatment. One-way analyses of variance (ANOVA) with the Duncan multiple range test were performed for multiple comparisons, and spearman's rank correlation coefficient between the abundant bacterial and fungal genera with the *F. oxysporum* abundance were calculated using SPSS v20.0 (SPSS Inc., USA). Linear models (LM) to examine the relationships of microbial indicators with *F. oxysporum* abundance and the relative importance for each of the predictors in this model were tested in R (version 3.2.2). For the initial microbial indicators, we chose microbial (bacteria and fungi) abundance, richness, phylogenetic diversity, evenness, weighted and unweighted structure (PCoA1), and relative abundance of the *Bacillus* and *Trichoderma*. We used a stepwise model selection using the Akaike information criteria (AIC) to select the model with best explanatory power (step function in R), and the relative importance for each of the predictors in the model was determined using the “relaimpo” package in R. The linear regression analyses between the *F. oxysporum* abundance with the selected microbial indicators were also tested in R.

2.7. Sequence accession numbers

All raw sequences data are available in NCBI Sequence Read Archive (SRA) database under the accession number SRP090114.

3. Results

3.1. Effect of bio-fertilizers on vanilla Fusarium wilt disease incidence

As compared to the chemical fertilizer (CF) treatment, both the bacterial enriched bio-fertilizer (B_BIO) and the fungal enriched bio-fertilizer (F_BIO) treatments significantly (Duncan test, $P < 0.05$) reduced the cumulative incidence of vanilla Fusarium wilt disease (Fig. 1). The F_BIO treatment showed the lowest cumulative disease incidence (DI) among the four fertilizer treatments with a cumulative DI value of 18.75%. Interestingly, there was no significant difference in cumulative DI between the CF and organic fertilizer (OF) treatments (Duncan test, $P > 0.05$), and no significant difference in cumulative DI between the F_BIO and B_BIO treatments (Duncan test, $P > 0.05$).

3.2. Effect of bio-fertilizers on soil chemical properties

Soil chemical characteristics among the four fertilizer treatments are shown in Table S1. Soil pH did not vary much between the four treatments (ranging from 7.51 to 7.67). OF and the two bio-fertilizer treatments had significantly (Duncan test, $P < 0.05$) higher organic matter and available N contents as compared to the CF treatment. Interestingly, fungal enriched bio-fertilizer (F_BIO) showed significantly (Duncan test, $P < 0.05$) lower organic matter content compared to the OF and bacterial enriched bio-fertilizer (B_BIO). There were no significant differences for available P across the four treatments.

3.3. Effect of bio-fertilizers on microbial abundance, diversity and community structure

Total bacterial and fungal abundances were significantly (Duncan test, $P < 0.05$) higher in the three organic fertilizer treatments (OF, B_BIO and F_BIO) compared to the chemical fertilizer treatment (Table 1). Compared with CF, the three organic fertilizer treatments had significantly (Duncan test, $P < 0.05$) higher Chao1 richness and Faith's phylogenetic diversity (PD) in both bacteria and fungi. The bacterial bio-fertilizer (B_BIO) had the highest Chao1 richness and Faith's PD values of both bacteria and fungi among the four fertilizer treatments. In addition, B_BIO exhibited the highest bacterial evenness, whereas the CF treatment had the highest fungal evenness. Venn diagrams showed 357, 253, 616 and 372 unique bacterial OTUs in the CF, OF, B_BIO and F_BIO treatments respectively, while 210, 185, 242 and 187 unique OTUs were detected for fungi, respectively (Fig. S1). Moreover, the B_BIO and F_BIO treatments harboured the highest number of shared bacterial OTUs (5,727) among the pairwise treatments, whereas B_BIO and OF exhibited the highest number of shared fungal OTUs (1,072) among the pairwise treatments.

Unconstrained PCoA of weighted and unweighted UniFrac distances revealed differences in soil microbial communities across the four fertilizer treatments (Fig. 2). Both weighted and unweighted UniFrac distances revealed that the CF treatment was clearly separated from the other three fertilizer treatments along the first component (PCoA1) for both bacterial and fungal

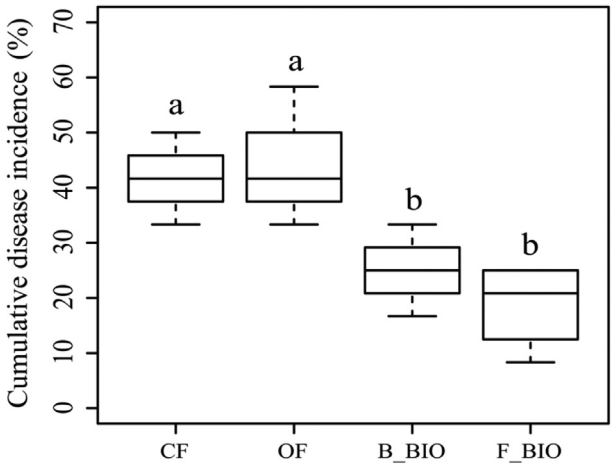

Fig. 1. The cumulative disease incidence of vanilla Fusarium wilt in the four fertilizer treatments.
CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Duncan test.

communities (ANOSIM, CF vs OF, B_BIO and F_BIO, $P < 0.05$). In bacteria, weighted UniFrac distances showed that F_BIO treatment was separated from B_BIO treatment (ANOSIM, F_BIO vs B_BIO, $P < 0.05$) along the second component (PCoA2), whereas F_BIO and B_BIO treatments grouped tightly together based upon the unweighted UniFrac distances (Fig. 2A and B). For fungi, CF, OF, F_BIO and B_BIO treatments showed significant differences based upon the weighted UniFrac distances (ANOSIM, CF vs OF vs F_BIO vs B_BIO, $P < 0.001$), while using unweighted UniFrac distances, OF, F_BIO and B_BIO treatments grouped together (Fig. 2C and D).

3.4. Effect of bio-fertilizers on microbial taxonomic composition

The overall taxonomic complexity of the microbial community at the phylum level is presented in Fig. S2. The *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Firmicutes* were the 6 most abundant phyla across all samples in rank order, together accounting for 71.34% of the total bacterial sequences. Compared with the chemical fertilizer (CF) and organic matter fertilizer (OF), the two BIO-fertilizer treatments had a significantly (Duncan test, $P < 0.05$) higher relative abundance of *Proteobacteria* and significantly (Duncan test, $P < 0.05$) lower levels of *Actinobacteria* abundance. The F_BIO treatment had the highest *Acidobacteria* abundance, whereas CF had the lowest *Acidobacteria* abundance. The *Ascomycota*, *Basidiomycota*, *Zygomycota* were the top 3 most abundant fungal phyla across all samples in rank order. Compared to the CF treatment, the OF and the B_BIO treatments had a significantly (Duncan test, $P < 0.05$) higher *Ascomycota* abundance.

An overview of the 60 most abundant bacterial and fungal genera among the four fertilizer treatments are shown in heat map plot (Fig. S3). Based on the 60 most abundant bacterial genera, F_BIO and B_BIO treatments grouped tightly together and showed different patterns of bacterial community structure relative to the CF and OF treatments. For the fungi, OF and the two BIO-fertilizer treatments had similar community structure patterns. For further analysis of bacterial and fungal genera across the four fertilizer treatments, we used fold change to calculate the variations in the relative abundance of the genera in the OF, B_BIO and F_BIO treatments compared to CF. As shown in Fig. 3A, some taxa from the phylum *Acidobacteria,* such as *Gp6*, *Gp3*, *Gp4*, *Gp17* and *Gp1*, were of greater relative abundance in the OF and the two BIO-fertilizer treatments in comparison with the CF treatment. Moreover, the fold change of *Lysobacter* relative abundance in the OF, B_BIO and F_BIO treatments was significantly (Duncan test, $P < 0.05$) higher 2.7, 5.7 and 6.0 times relative to the CF treatment, respectively (Figs. 3A and 4C). The fold change of *Nitrospira* relative abundance in the two BIO-fertilizer treatments was over 50% compared to the CF treatment (Fig. 3A). For fungi, *Chaetomium* were relatively more abundant in the OF treatment, while *Fusarium* levels were lower in the two BIO-fertilizer treatments (Fig. 3B). Moreover, ANOVA analysis showed that *Bacillus* abundance was significantly higher in the B_BIO treatment compared to the OF and F_BIO treatments (Duncan test, $P < 0.05$), but was not significantly different from the CF treatment (Fig. 4A). The relative abundance of *Trichoderma* did not show significant differences across the 4 fertilizer treatments (Fig. 4B).

3.5. Relationship between microbial indicators and F. oxysporum abundance

Bacterial abundance, bacterial richness, bacterial PD, fungal evenness, fungal PD, bacterial structure (weighted PCoA1), bacterial structure (unweighted PCoA1), fungal structure (unweighted PCoA1), and *Trichoderma* relative abundance were selected from our initial microbial indicators in the linear model, showing the best explanatory power for *F. oxysporum* abundance. *Bacillus* was not retained in this linear model. Importantly, the bacterial abundance ($F = 71.93$, $df = 1$, $P < 0.001$) and weighted bacterial structure ($F = 35.53$, $df = 1$, $P < 0.001$) constrained *F. oxysporum* pathogen density most (Table 2), both having a relative importance of over 10%. In addition, based on the linear regression analyses between the *F. oxysporum* abundance with selected microbial indicators, we found bacterial abundance ($R^2 = 0.48$, $P < 0.01$), weighted

Table 1
Bacterial and fungal abundance and α-diversity indexes for the four fertilizer treatments.

	Treatment	Abundance*	Richness (Chao1)	Faith's PD	Evenness
Bacteria	CF	11.84 ± 0.29 c	6088.24 ± 132.79 c	251.77 ± 7.27 c	0.867 ± 0.002 c
	OF	11.90 ± 0.16 bc	6663.64 ± 117.80 ab	263.66 ± 15.49 bc	0.877 ± 0.002 ab
	B_BIO	12.14 ± 0.07 ab	6830.04 ± 89.61 a	306.61 ± 20.94 a	0.879 ± 0.002 a
	F_BIO	12.23 ± 0.08 a	6632.25 ± 102.99 b	277.04 ± 11.45 b	0.875 ± 0.001 b
Fungi	CF	8.00 ± 0.06 c	1192.47 ± 115.53 c	197.38 ± 22.21 b	0.685 ± 0.004 a
	OF	8.04 ± 0.14 bc	1277.05 ± 76.01 bc	235.30 ± 19.34 a	0.460 ± 0.022 c
	B_BIO	8.20 ± 0.04 a	1424.72 ± 83.82 a	255.30 ± 29.42 a	0.594 ± 0.024 b
	F_BIO	8.16 ± 0.11 ab	1346.84 ± 62.36 ab	234.05 ± 21.04 a	0.606 ± 0.018 b

CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Values are means ± standard deviation (n = 4). * Bacterial or fungal copy numbers were $\log_{10}$-transformed in abundance. Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Duncan test).

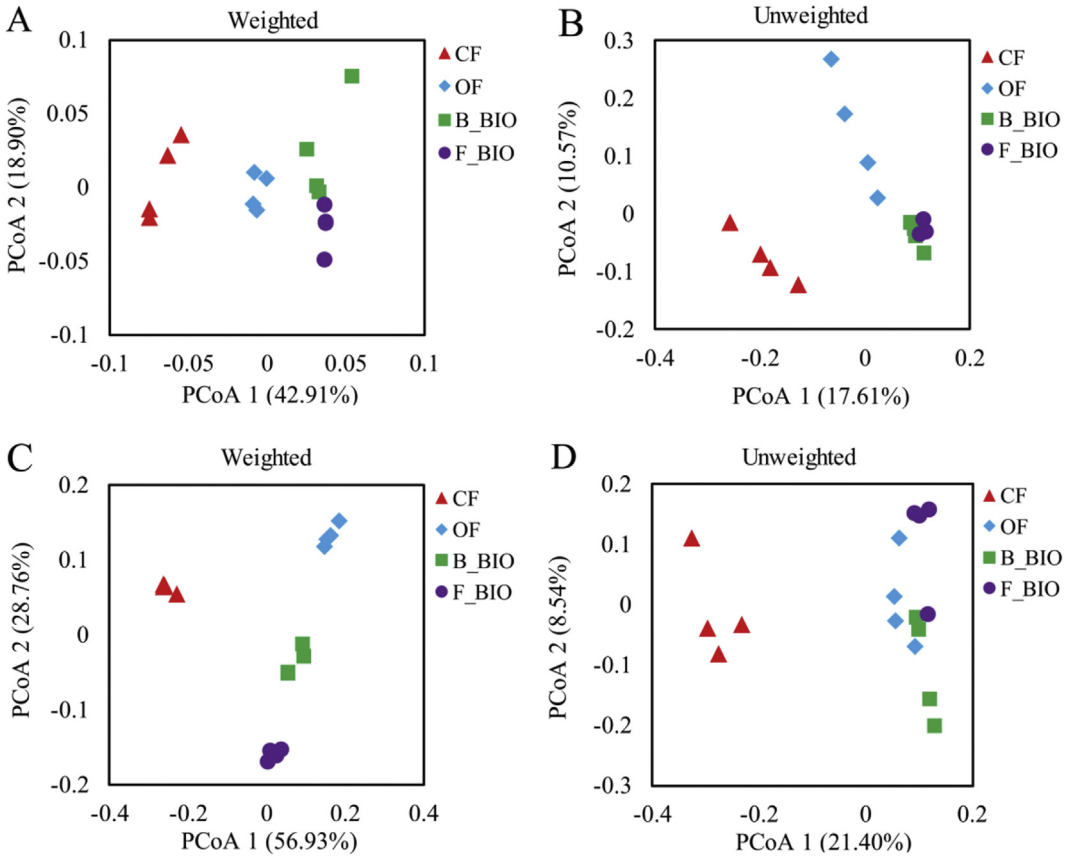

Fig. 2. Microbial community structures in the four fertilizer treatments. UniFrac-weighted principle coordinate analysis of bacterial (A) and fungal (C) community structures, UniFrac-unweighted principle coordinate analysis of bacterial (B) and fungal (D) community structures.
CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer.

($R^2 = 0.38$, $P = 0.01$) and unweighted bacterial structure ($R^2 = 0.41$, $P < 0.01$), and *Trichoderma* relative abundance ($R^2 = 0.31$, $P = 0.02$) have significantly negative relationships with *F. oxysporum* abundance (Fig. 5). Furthermore, we used spearman's rank correlation to evaluate the relationship between the *F. oxysporum* abundance with the 60 most abundant bacterial and fungal genera across the four fertilizer treatments (Table S2). The relative abundance of *Gp6*, *Gp4*, *Lysobacter*, *Nitrospira*,*Trichoderma*, *Penicillium* and *Mortierella* revealed a significantly ($P < 0.05$) negative relationship with *F. oxysporum* abundance, while there was no significantly negative relationship between the *Bacillus* abundance with *F. oxysporum* abundance, even though *Bacillus* levels were highest in the B_BIO treatment.

4. Discussion

In the current study, we sought to examine the mechanisms linking soil amendments (bio-fertilizers) with suppression of *F. oxysporum*. With vanilla used as the wilt-susceptible crop, we observed that the two bio-fertilizer treatments significantly reduced the Fusarium wilt disease incidence as compared to the chemical and organic matter fertilizer treatments. However, no significant difference in disease incidence between the chemical fertilizer and organic fertilizer treatment was observed, suggesting that organic matter amendment alone was not sufficient to induce soil suppressiveness against Fusarium wilt disease. This finding is in line with the findings of Bonanomi et al. (2010) who, using a meta-analysis, showed that organic matter amendment was ineffective or even conducive to other pathogens. Our next question was whether the inoculated *Bacillus* and *Trichoderma* spp. provide a direct suppression against *F. oxysporum* pathogen?

Even though *Bacillus* levels were higher in the B_BIO treatment, there was no significantly negative relationship between *Bacillus* and *F. oxysporum* abundance. In addition, the relative abundance of *Trichoderma* was not different across the four fertilizer treatments, and within the linear models, *Trichoderma* relative abundance did not significantly restrict *F. oxysporum* density. Our results therefore suggest that the introduced microbes (*Bacillus* and *Trichoderma* spp.) had only a limited survival capacity in our study system, and their abundance had only a marginal direct effect on *F. oxysporum* density and thereby the suppression of Fusarium wilt disease.

The soils with the different fertilizer amendments exhibited distinct microbial communities and these differences may be related to the observed patterns of *F. oxysporum* pathogen abundance and Fusarium wilt disease incidence. In our one-year pot experiments, 16S rRNA and ITS gene copy numbers for the OF and two bio-fertilizer treatments revealed higher bacterial and fungal abundance (biomass) compared to the chemical fertilizer treatment, which is consistent with previous observations that organic compost amendment can activate microbial biomass and activity (Jannoura et al., 2013). In addition, the OF and two bio-fertilizer treatments harboured significantly higher bacterial and fungal richness and phylogenetic diversity than CF. Some previous studies support the view that long-term organic compost amendment activates bacterial diversity as compared with conventionally used synthetic chemical fertilizers (Chaudhry et al., 2012). However, some other studies have not revealed any significant effects on microbial richness in organic farms compared to conventional

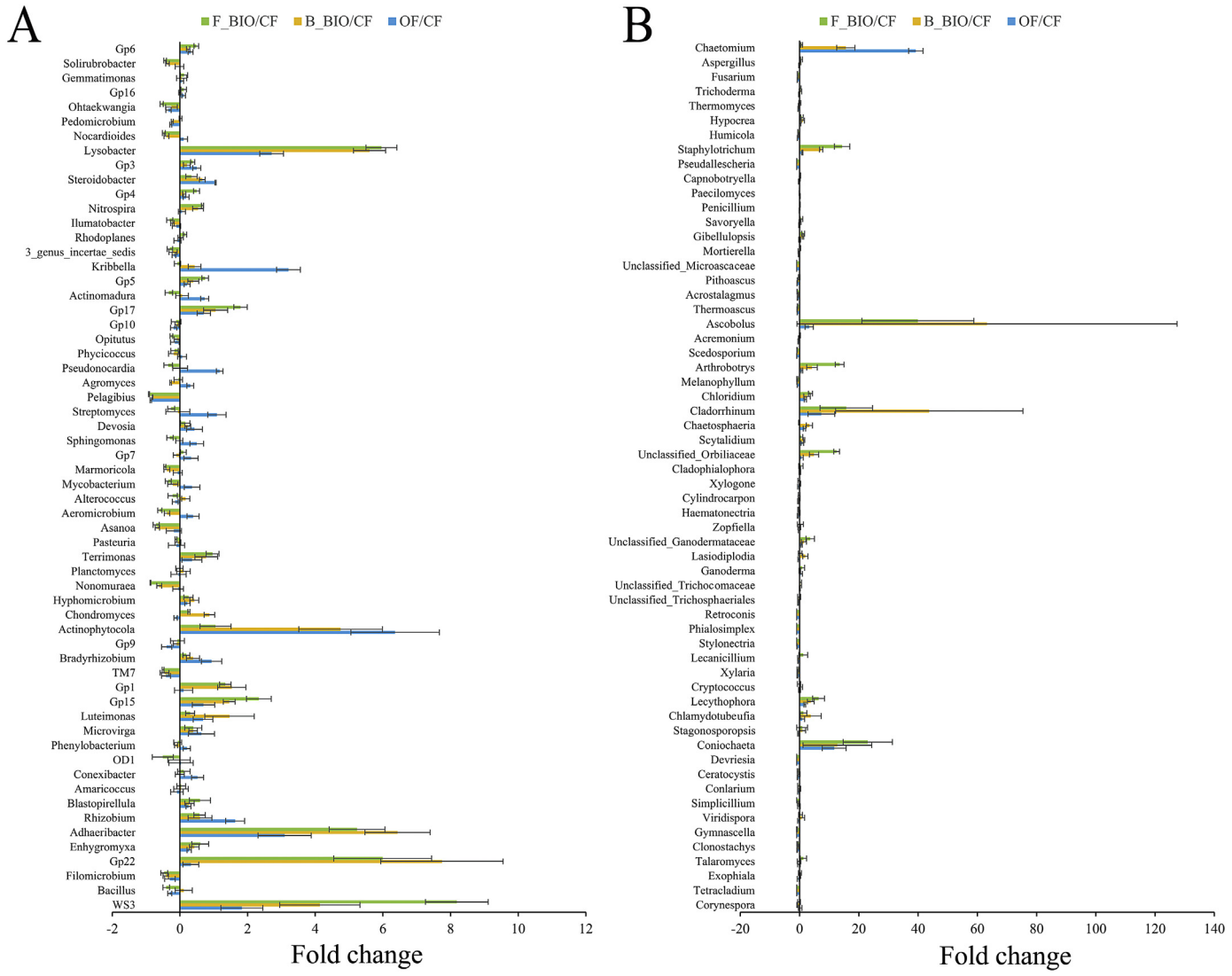

Fig. 3. The fold change of bacterial (A) and fungal (B) genera in F_BIO, B_BIO and OF treatments relative to the control (CF) treatment.
CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Error bars represent the standard deviation of the four replicates.

farms (Sugiyama et al., 2010). In addition, other findings revealed that the alpha diversity of bacteria significantly increased, whereas fungal diversity decreased after two years of bio-fertilizer application (Shen et al., 2015a). Thus, the impacts of chemical versus organic fertilizer on patterns of microbial diversity remain unclear, and differences found between different studies may be related to the use of different experimental systems and management definitions (Hartmann et al., 2015). In our attempt to link microbial diversity or abundance with pathogen density, our linear model analysis showed that bacterial abundance significantly constrained *Fusarium* pathogen density. Higher bacterial abundance or diversity of soil microbial communities can play an important role in the capacity of soils to suppress soil-borne plant diseases (Boer et al., 2003; Elsas et al., 2002).

We observed significant differences in microbial community structure after bio-fertilizers applications, and PCoA results revealed that all four fertilizers treatments harboured structurally distinct microbial communities. The microbial structure within the CF treatment was distinctly different from the OF and the two bio-fertilizer treatments for both bacterial and fungal communities, which is in line with previous observations that organic and inorganic fertilizers often result in distinct soil microbial community structures (Marschner et al., 2003; Zhang et al., 2012). For fungi, the OF, F_BIO and B_BIO treatments showed significant differences based upon weighted UniFrac distances (based on abundances of taxa), although this was not the case when using unweighted UniFrac distances (sensitive to rare taxa). For bacteria, weighted UniFrac distances grouped the F_BIO treatment separately from the B_BIO treatment, and the F_BIO and B_BIO treatments grouped tightly together based upon unweighted UniFrac distances. Taken together, these results suggest that the application of the two bio-fertilizers to soil selects for some of the same soil microbes regardless of the biological agent included. The soil microbial populations stimulated by the bio-fertilizer applications in the two bio-fertilizers may further impact microbial interactions in the soil and may also harbour potential antagonistic capacities to help reduce plant disease (Akhtar and Malik, 2000).

Application of organic fertilizer or bio-fertilizer was a major factor shaping the soil community taxonomic composition, as observed previously in the application of various organic fertilizers

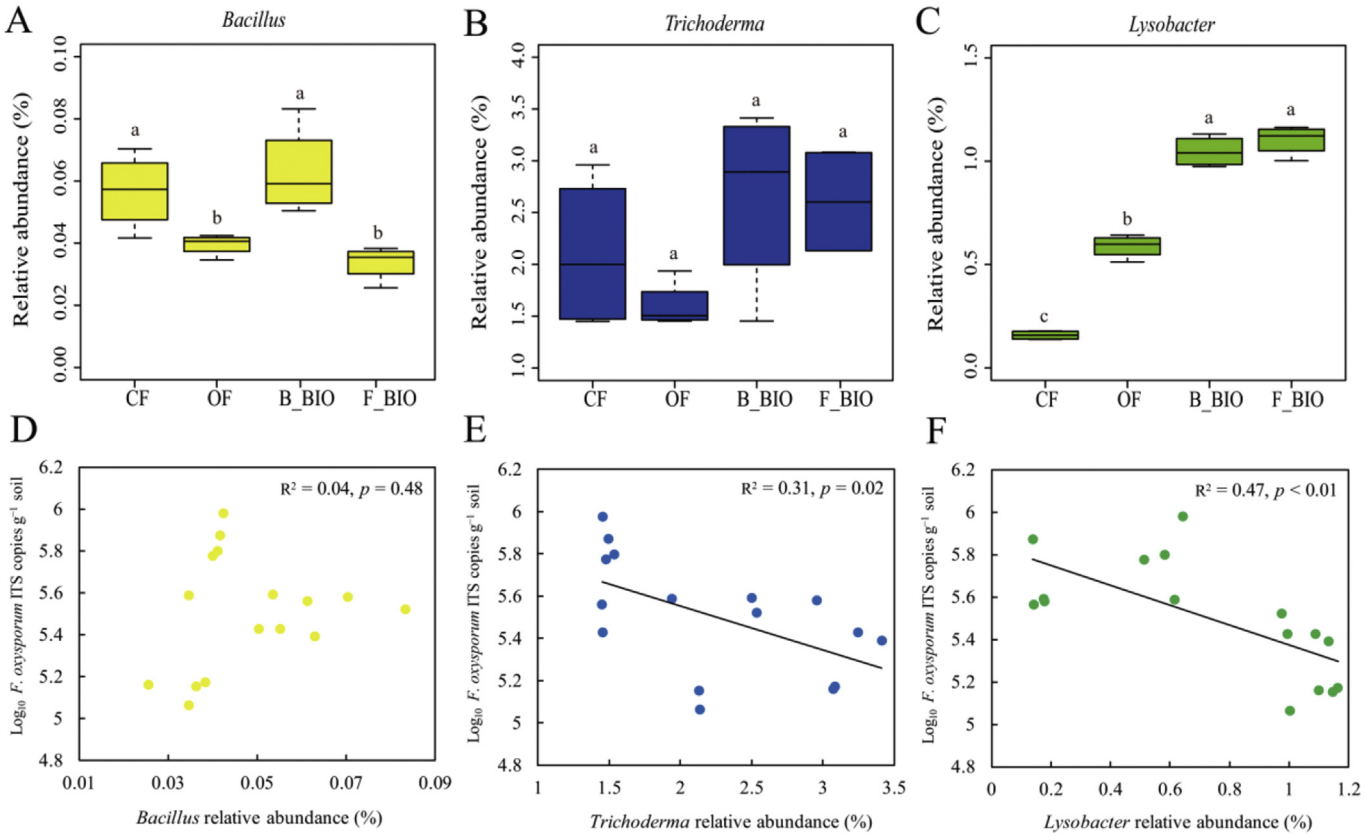

Fig. 4. The relative abundance of *Bacillus* (A), *Trichoderma* (B) and *Lysobacter* (C) genera in the four fertilizer treatments and the linear regression relationship between the *Bacillus* (D), *Trichoderma* (E) and *Lysobacter* (F) genera and the *F. oxysporum* abundance.
CF = chemical fertilizer, OF = Organic matter fertilizer, B_BIO = bacterial enriched bio-fertilizer and F_BIO = fungal enriched bio-fertilizer. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Duncan test.

Table 2
Linear models (LM) for the relationships of microbial indicators with *F. oxysporum* pathogen abundance and the relative importance for each of the predictors in the model.

	Df	F	*P*-value	Relative Importance
Bacterial abundance	1	71.93	<0.001*** ↓	15.36%
Bacterial richness	1	7.52	0.03* ↓	9.55%
Bacterial PD	1	5.04	0.07	4.25%
Fungal evenness	1	7.21	0.04* ↓	14.98%
Fungal PD	1	5.11	0.06	3.29%
Bacterial structure (weighted PCoA1)	1	35.53	<0.001*** ↓	14.27%
Bacterial structure (unweighted PCoA1)	1	4.68	0.07	15.38%
Fungal structure (unweighted PCoA1)	1	2.83	0.14	10.30%
Trichoderma relative abundance	1	2.58	0.16	8.58%
Residuals	6			
Model summary: $R^2 = 0.96$, AIC = −26.56				
Total proportion variance explained by model: 95.96%				

P-values are the results from ANOVAs. *$P < 0.05$, **$P < 0.01$ and *** $P < 0.001$. PD means phylogenetic diversity. "↑" and "↓" behind the *P*-value represent a positive and negative relationship between each predictor and *F. oxysporum* pathogen abundance, respectively.

(Marschner et al., 2003; Shen et al., 2015b; Zhang et al., 2012). Given the fact that the limited read lengths of the Illumina MiSeq sequencing do not allow for robust taxonomic characterization to the species level, we focussed our examination of microbial composition changes at the genus level, as we sought to relate the differences between the four fertilizers treatments to disease suppression. *Lysobacter*, *Gp6* and *Gp4* of the *Acidobacteria* and *Nitrospira* were significantly overrepresented in the F_BIO and B_BIO treatments, and these genera were negatively correlated with *F. oxysporum* abundance. The genus *Lysobacter*, which is known to produce a wide range of extracellular enzymes and other metabolites with antagonistic activities against many soil-borne diseases (Gómez Expósito et al., 2015; Jochum et al., 2006), was six times higher in the two bio-fertilizer treatments compared as compared to the CF treatment. This genus was clearly stimulated in soils treated with the bio-organic fertilizers, and it may play a keystone role in the observed suppression of vanilla Fusarium wilt disease by these bio-organic fertilizers. Some taxa of the *Acidobacteria* that we found to be overrepresented in the bio-fertilizer treatments, such as *Gp4* and *Gp6*, have previously been found to be more abundant in potato common scab disease-suppressive soil as compared to conducive soil (Rosenzweig et al., 2012). Thus, these

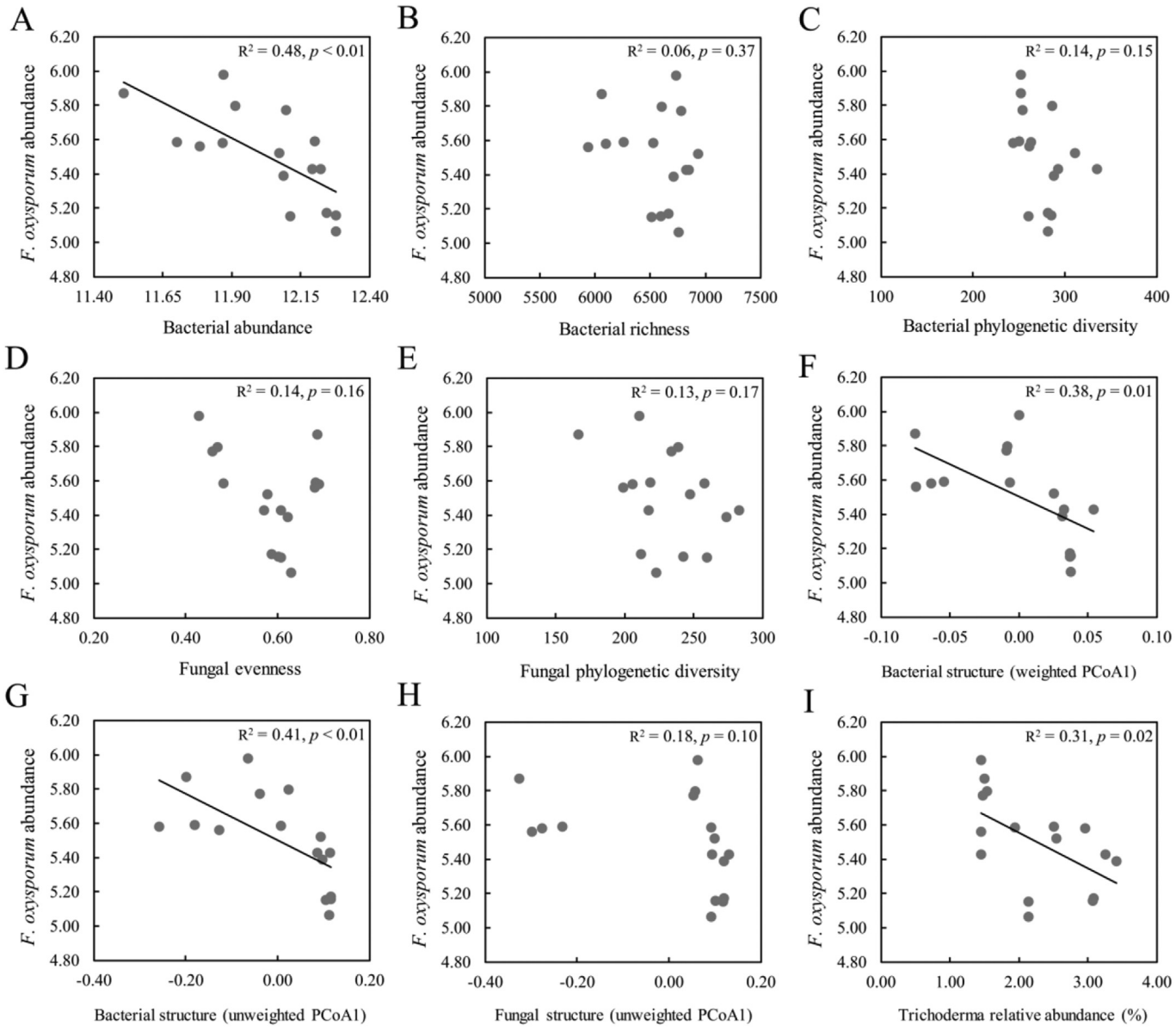

Fig. 5. The linear regression relationship between microbial indicators (seleted from the linear models) and the *F. oxysporum* abundance.
Bacterial abundance (A), bacterial richness (B), bacterial phylogenetic diversity (C), fungal evneness (D), fungal phylogenetic diversity (E), bacterial structure (weighted PCoA1) (F), bacterial structure (unweighted PCoA1) (G), fungal structure (unweighted PCoA1) (H), *Trichoderma* genera relative abundance.

taxa may be indicative of disease suppression, but additional research would be necessary to demonstrate such a role. Genera within the *Nitrospira* (*Nitrospirae*) are known for their nitrite- and ferrous iron-oxidizing activities, which impact the N and Fe cycles in soil (Attard et al., 2010; Xu et al., 2015). However, it is not yet known if such activities help support vanilla plant growth or indirectly enhance the suppression against to Fusarium wilt disease.

Our results also suggest that the microbes introduced into the bio-fertilizers (*Bacillus* and *Trichoderma* spp.) have little direct antagonistic effects on the pathogen. Rather, these introduced species may function as keystone community members that stimulate other potentially antagonistic species already present in the soil. This more complicated picture of bio-control action is in line with the notion that disease suppression is typically a complex phenomenon, most likely determined by complex microbial consortia (Mendes et al., 2011). In addition, the secondarily fermentation may have also changed organic matter structure (Guo et al., 2012), and more studies are needed to focus on the organic matter structure associated with disease suppression after bio-fertilizer applications.

5. Conclusions

The agricultural management of soil ecosystems towards a state of increased disease suppressiveness represents one of the methods by which sustainable disease control may be achievable (Dignam et al., 2016). From our results, the application of the secondarily fermented bio-fertilizers significantly reduced the incidence of vanilla Fusarium wilt disease. Fig. 6 represents a conceptual cartoon summarizing our findings of the relative influence of the inoculated microbes and induced microbial community on *F. oxysporum* pathogen inhibition. According to the results presented above, the abundance of the inoculated *Bacillus* and

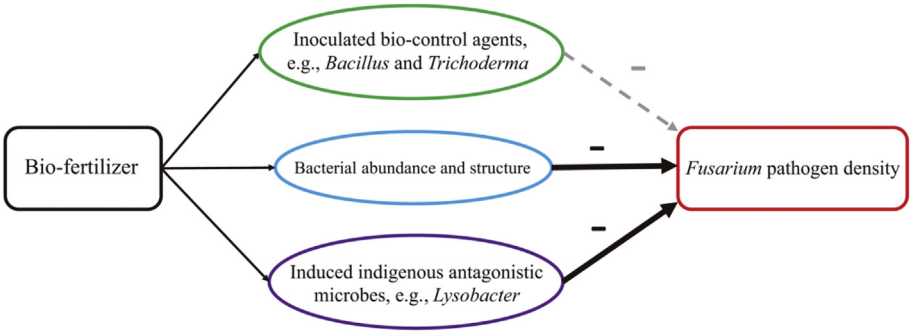

Fig. 6. A conceptual cartoon summarizing the influence of bio-fertilizer treatments on soil microbial community and their influence on *F. oxysporum* pathogen inhibition.

Trichoderma spp. in the bio-fertilizer had only a marginal effect on *F. oxysporum* constriction. In contrast, changes in bacterial abundance and community structure after amendment with bio-fertilizers appeared influence *F. oxysporum* population density. Most strikingly, the density of the indigenous *Lysobacter* spp. populations was negatively correlated with pathogen density (Figs. 6 and 4F). Thus, the effectiveness of bio-fertilizers seems to be a combination of the actual antagonistic activities of the inoculated bio-control agents as well as the promotion of beneficial microbial groups already resident in the soil. We propose that these additional beneficial effects should also be kept in mind in the design of bio-organic fertilizers and their use in sustainable strategies for plant disease protection.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.07.016.

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