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ORIGINAL PAPER

Bio-organic fertilizer application significantly reduces the *Fusarium oxysporum* population and alters the composition of fungi communities of watermelon *Fusarium* wilt rhizosphere soil

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Abstract Watermelon *Fusarium* wilt is one of the most severe soil-borne diseases caused by *Fusarium oxysporum* f. sp. *niveum*. In this study, the population of *F. oxysporum* was quickly monitored by real-time PCR and DNA array in watermelon *Fusarium* wilt infected soils treated with *Paenibacillus polymyxa* SQR21 enhanced bio-organic fertilizer (BIO) at the beginning of nursery growth and/or at the beginning of transplanting. The fungal community composition was investigated by molecular cloning and DGGE techniques. The real-time PCR results showed the *F. oxysporum* population in the rhizosphere soil decreased from 8.56×10^4 colony-forming units (cfu) g^{-1} rhizosphere soil to 9.41×10^3 cfu g^{-1} rhizosphere soil after BIO application and the DNA array detection signals of *F. oxysporum* population weakened. The difference between *F. oxysporum* abundance of BIO amended and not amended bulk soils was lower than 10^4 cfu g^{-1} soil. DGGE profile indicated that BIO application changed the fungal community structure in the rhizosphere soils; the molecular cloning data revealed that consecutive applications of BIO at nursery and transplanting stages not only decreased *Ascomycota* and increased *Basidiomycota* abundance in the rhizosphere soil but also caused the apperance of unique fungal group which were not found in the control. The beneficial fungi *Chaetomium* sp. *Aspergillus penicillioides* were found in the BIO amended treatment, while some harmful fungi such as *F. oxysporum*, *Rhizoctonia solani*, and *Fusarium solani* were only detected in the control. Data from this study indicated that BIO application can control watermelon *Fusarium* wilt by suppressing the population of *F. oxysporum* and changing the fungal community structure in the rhizosphere soils.

Keywords *Fusarium* wilt · Bio-organic fertilizer · DNA array · Fungi diversity · DGGE · Molecular cloning

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Introduction

Watermelon is one of the most important fruits worldwide, but in many areas of China, its yield can be decreased (Ling et al. 2010; Wu et al. 2008) by a widespread diseases caused by the soil-borne pathogen *Fusarium oxysporum* f. sp. *niveum* (Booth 1971; Wu et al. 2008).

Chemical control is one commonly used strategy of disease control, but it is not environmentally friendly and has negative effects on some beneficial microbes (Minuto et al. 2006; Omar et al. 2006). It was reported that consecutive applications of chemical fertilizer lead to a decrease in some beneficial microbes and an increase in developed resistance of target pathogens (Goldman et al. 1994; Tjamos et al. 2004). For this reason, the importance of biological control has been increased over the years and bio-organic fertilizer (BIO) application has become one of the promising biological ways to control various soil-borne disease (Luo et al. 2010; Yang et al. 2011; Zhang et al. 2011). Up to now, many antagonistic microbes have been reported to biocontrol *Fusarium* wilt (Dijksterhuis et al. 1999; Zhang et al. 2011). Although

composts can stimulate proliferation of antagonists in the rhizosphere and reduce the incidence of some soil-borne plant pathogens, it showed inconsistent levels of disease control and less control effectiveness than further manipulation of composts by inoculation or enrichment with specific antagonists (Borrero et al. 2004; Trillas et al. 2006; Zhao et al. 2010). Nowadays, it is widely reported that a combination of antagonistic microbes with mature compost may be more efficient in inhibiting disease than using single antagonistic microbial strains or compost alone (Cao et al. 2011; Huang et al. 2012; Qiu et al. 2012; Shen et al. 2013; Wang et al. 2013; Wei et al. 2011; Yin et al. 2011; Zhang et al. 2011).

Most studies of plant disease have focused on those fungi that affect the above-ground portion of plants (Burdon and Silk 1997). However, many of the most devastating diseases in agricultural systems are caused by below-ground pathogens. The lack of rapid, accurate, and reliable means by which plant pathogens can be detected and identified is one of the main limitations in integrated disease management (Njambere et al. 2011; Zhao et al. 2012). Traditionally, pathogen diagnostic mainly relies on morphological and/or cultural dependent methods, but all these methods are time consuming (Lievens and Thomma 2005; Zhang et al. 2008), because they usually take 1 week or longer to be completed (Zhao et al. 2012). The molecular methods such as real-time polymerase chain reaction (PCR) assay and DNA array have been developed for the rapid detection and quantification of pathogens from environment samples (Gilbert et al. 2008; Lievens and Thomma 2005), including soil samples (Lievens et al. 2003; Zhang et al. 2008). Analyzing fungal communities by denaturing gradient gel electrophoresis (DGGE) with separation of ITS gene or by cloning libraries have become the prevalent method to determine genetic diversity of the rhizosphere fungal communities (Anderson et al. 2003; White et al. 1990). It can reveal a more complete and distinctive picture of soil fungal composition and afford clear information of phylogeny and change in fungal community composition (Martin and Rygiewicz 2005).

The rhizosphere is known to be a research hotspot of plant–microbial interactions and a driving force of soil ecosystems (Fang et al. 2013). The role of fungi in rhizosphere soil is extremely complex and is fundamental to the soil ecosystems because fungi play a very important role in the decomposition of organic matter, enabling and facilitating the transport of nutrients from soil to plant (Bridge and Spooner 2001). Some fungi are known to cause a range of plant diseases, others are known to antagonize plant pathogens and stimulate plant growth, while some fungi can affect the composition of microbial communities by changing host plant physiology directly or indirectly (Marschner et al. 2001).

In the previous study, we reported that the application of a novel BIO by fermenting mature composts with the antagonistic microbe *Paenibacillus polymyxa* can reduce the incidence of the *Fusarium* wilt disease by 60–100 % in pot experiments (Ling et al. 2010). Nevertheless, it is not known if the soil treated with *P. polymyxa* enhanced BIO can suppress *F. oxysporum* populations and change the compositions of soil fungal communities. We have hypothesized that application of *P. polymyxa* enhanced BIO can effectively control watermelon *Fusarium* wilt by reducing the *F. oxysporum* population and altering the composition of fungal communities in the rhizosphere soil. The results of this study can be important to monitoring soil born pathogens' infection in a quick and accurate way, and an improved knowledge of the composition of fungal communities in the rhizosphere soil after BIO application can lead to a better understanding of fungal roles in these soil ecosystems.

Materials and methods

Organic fertilizer preparation

The organic fertilizer was composed of amino acid fertilizer and pig manure compost (1:1); the amino acid fertilizer was obtained from oil rapeseed cakes after enzyme hydrolysis by microbial fermentation at <50 °C for 7 days (Ling et al. 2010). The obtained organic fertilizer (BIO) was inoculated with the SQR21 (with high antagonistic efficiency against *F. oxysporum* causing cucumber and watermelon wilt disease) and incubated on fermentator for 6 days at 45 °C (Ling et al. 2010).

Soils

The nursery soil (300 g) for growing seedlings was sampled from a site without history of watermelon wilt disease, while the soil for the transplanted pot experiment was collected from the surface of 2 years mono-cultivated watermelon plots with serious watermelon *Fusarium* wilt disease located in Jiangyin country, Jiangsu province, China. The soil (sandy loam) has pH 6.8, organic C 12.0 g kg^{-1}, total N 1.61 g kg^{-1}, total P 0.37 g kg^{-1}, and total K 6.15 g kg^{-1}.

Seedling nursery

Watermelon seeds (Kangbing Jingxin) were surface-sterilized with 2 % NaClO for 3 min and rinsed in sterile water for several times and then put into 9-cm plates covered with sterile wet filter paper at 30 °C for their germination. The two treatments of the nursery soil were N_{CK} (without BIO) and N_{BIO} with BIO treated at 2 % (w:w). The N_{CK} was treated with chemical fertilizer containing equivalent nutrients (192 mg N, 180 mg P_2O_5, and 54 mg K_2O) of the 2 % BIO

amendment (N_{BIO}). The seedlings were grown under greenhouse conditions at temperature ranging from 26 to 35 °C.

Pot experiment and sampling

The seedlings with 3–4 true leaves were transplanted into pots which contained approximately 10 kg of the fresh soil from the watermelon wilt diseased field. In addition to the two nursery treatments, two more treatments (P_{CK} and P_{BIO}) were designed at the pot experiment stage with soils being amended without or with the BIO at a rate of 5 g kg^{-1}. All four treatments are listed in Table 1. Three plant replicates were sampled randomly from four treatments. The rhizosphere soil from each plant was carefully collected by softly shaking by hands. The other soil samples obtained without shaking roots were considered as the bulk soils (Bakker and Schippers 1987).

DNA extraction and PCR amplification

DNA was extracted in triplicate from watermelon planted rhizosphere and bulk soil (0.5 mg) of each treatment by Ultra Clean™ Soil Kit (MOBIO Laboratories, Carlsbad, CA, USA). The extracts were subsequently pooled. The quality and the concentration of extracted DNA were determined by Nanovue (Gelife sciences, USA). The ITS region of the 18S rRNA gene was amplified with primers ITS4/ITS5 (Gardes and Bruns 1993; White et al. 1990) using a Thermocycler (Bio-Rad, icycler, USA). PCR was carried out in 50-μl reaction volume using 5 μl 10×PCR Buffer (Mg^{2+} Free), 3-μl $MgCl_2$ (25 mM, TaKaRa, Japan), 4-μl dNTP Mixture (each 2.5 mM), 1 unit Takara Taq DNA polymerase (Takara, Dalian, China), 2 μl each primer (10 pmol/μl), 4-μl template, and ddH_2O to reach a volume of 50 μl. The used PCR cycling conditions were 95 °C for 5 min, 35 cycles at 95 °C for 50 s, 57 °C for 1 min, and 72 °C for 1 min, followed by final extension at 72 °C for 10 min. PCR products were purified according to the manufacturer's protocol using the Axyprep™ DNA Gel Extraction Kit.

Table 1 Design of the experiments with or without bio-organic fertilizer (BIO) application; the BIO rates were 10 g kg^{-1} in the nursery soils and at 5 g kg^{-1} in the pot experiments

Treatment	Nursery stage BIO (2 %)	Pot experiments BIO (0.5 %)
$N_{CK}+P_{CK}$	−	−
$N_{CK}+P_{BIO}$	−	+
$N_{BIO}+P_{CK}$	+	−
$N_{BIO}+P_{BIO}$	+	+

Treatment: $N_{CK}+P_{CK}$, both nursery and pot soil were untreated; $N_{CK}+P_{BIO}$, the pot soil was treated with bio-organic fertilizer but not the nursery soil; $N_{BIO}+P_{CK}$, the nursery soil was treated with bio-organic fertilizer but not the pot soil; $N_{BIO}+P_{BIO}$, both nursery and pot soil were treated with bio-organic fertilizer

Real-time PCR amplification

Real-time PCR assays to quantify *F. oxysporum* f. sp. *niveum* DNA were conducted using the primer pair ITS1-F (Gardes and Bruns 1993) and AFP308R (10 mM) (Lievens et al. 2003). Real-time PCR amplification reactions were carried by SYBR® Premx Taq™ (2×TaKaRa Bio technology Dalian Co., Ltd.). Reaction mixture contained 10 μl of SYBR® Premix ExTaq™ (2×), 0.5 μl of each primer, 0.5 μl of ROX Reference Dye II (50×), 2 μl extracted DNA, and double distilled water to reach a 25-μl reaction volume. Samples were preheated to 95 °C for 2 min and then followed by 40 amplification cycles at 94 °C for 15 s, 58 °C for 15 s, and extension at 72 °C for 10 s. After the final amplification cycle, specificity was examined by generating a dissociation curve after amplification.

DNA array probes and array development

Specific oligomer probes used in this study to detect the genus *Fusarium* (Fgn1 and Fgn2) and the species *F. oxysporum* (Fo1, Fo2, Fox1, Fox2), *Verticillium dahliae* (Vda1), *Verticillium albo-atrum* (Val2), *Verticillium* spp. (Vgn1, Vgn2), *Fusarium solani*, and *Rhizoctonia solani* were previously designed and validated by Lievens et al. (2003) and Zhang et al. (2007) (Table 2). All oligomers were dissolved at a concentration of 50 μM in spotting buffer (4 μM sodium carbonate buffer, pH 8.4, 3× SSC [1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.01 % Nlauroyl sarcosine, and 0.004 % bromophenol blue), and each detector oligonucleotide probe was spotted onto Hybond N^+ nylon membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) with four replicates per each probe using a 96-pin tool (V&P Scientific Inc., San Diego, CA, USA). To assess the specificity of detection by the array (Fig. 1), ITS4 (A1), ITS5 (A2), ITS2 (A3), and internal controls from ITS2-1 (A4) to ITS2-5 (A8), which differ from ITS2 at one base of the probes, were spotted to test the cross-reaction against the array (Table 2); negative control were double distilled water (C5) and spotting buffer (C6). The spotted membranes were air dried for 10 min and fixed by UV exposure at 240 mJ/cm^2. After incubation in a 0.5 % SDS at 60 °C for 1 h, membranes were rinsed with 100 mM Tris/HCl (pH 8.0) for 5 min and kept moist at 4 °C until used. The ITS amplicons from soil samples were labeled and hybridized using the Gene Images AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham Biosciences) following the manufacturer's protocol. Chemiluminescence was detected using Kodak

Table 2 Position, name, target, and sequence of probes used in this study

Prob position on the array	Prob name	Probe target	Prob sequence (5′~3′)
A1	ITS4	Fungi	TCCTCCGCTTATTGATATGC
A2	ITS5	Fungi	GGAAGTAAAAGTCGTAACAAGG
A3	ITS2	Fungi	GCTGCGTTCTTCATCGATGC
A4	ITS2−1	Fungi, with a central mismatch	GCTGCGTTGTTCATCGATGC
A5	ITS2−2	Fungi, with a central mismatch	GCTGCGTTCATCATCGATGC
A6	ITS2−3	Fungi, with an end mismatch	CCTGCGTTCTTCATCGATGC
A7	ITS2−4	Fungi, with two central mismatches	GCTGCGTTGATCATCGATGC
A8	ITS2−5	Fungi, with two end mismatches	CGTGCGTTCTTCATCGATGC
B1	Fgn1	*Fusarium* sp.	CACGTCGAGCTTCC ATAGC
B2	Fgn2	*Fusarium* sp.	CCAACTTCTGAATGTTGACC
B3	Fo1	*Fusarium oxysporum*	CGTTCCTCAAATTGATTGGCGGTC
B4	Fo2	*Fusarium oxysporum*	CGTTCCTCAAATTGATTGGCGGTCA
B5	Fox1	*Fusarium oxysporum*	TTGGGACTCGCGTTAATTCG
B6	Fox2	*Fusarium oxysporum*	GTTGGGACTCGCGTTAATTCG
B7	Vgn1	*Verticillium* spp.	GCCGAAGCAACAAT ATGGTT
B8	Vgn2	*Verticillium* spp.	GTTGTTAAAAGTTT TAATAGTTCG
C1	Vda1	*Verticillium dahliae*	AACAGAGAGACTGATGGACCG
C2	Val2	*Verticillium albo-atrum*	CATCAGTCTCTTTATTCATACCAA
C3	Fso1	*Fusarium solani*	ATCAACCCTGTGAACATACCTAA
C4	Rso1	*Rhizoctonia solani*	GCCTGTTTGAGTATCATGAAAT
C5	Spotting buffer	None	–
C6	ddH_2O	None	–

Biomax Light film. Developed films were scanned with a Hewlett-Packard 5300C ScanJet and read by ImageJ 1.33u (National Institutes of Health, MD)(Zhang et al. 2007; Njambere et al. 2011).

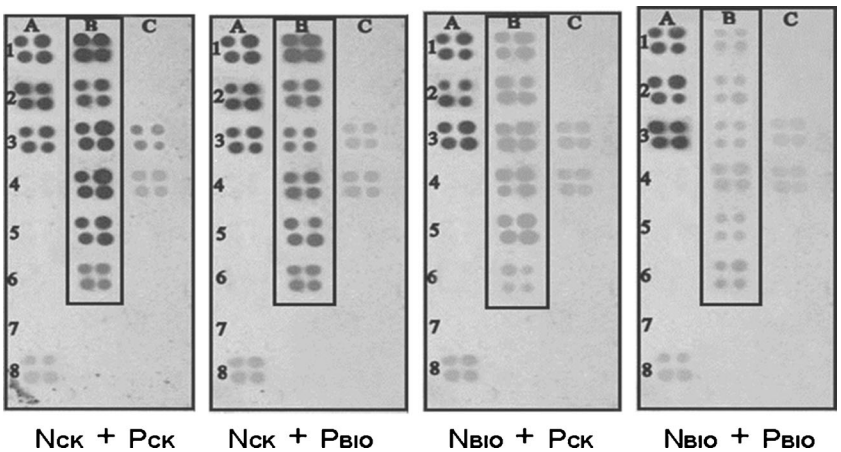

Fig. 1 DNA array design and hybridization results of fungal communities of rhizosphere soil. Positive controls ITS4, ITS5, and ITS2 were spotted in A1, A2, and A3. Internal controls ITS2-1 to ITS2-5 were spotted in A4, A5, A6, A7, and A8. Negative controls (buffer and ddH_2O) were also spotted in C5 and C6. Specific probes for *Fusarium sp.* (B1 and B2), *F. oxysporum* (B3, B4, B5, and B6), *Verticillium* spp. (B7 and B8), *Verticillium dahliae* (C1), *Verticillium albo-atrum* (C2), *Fusarium solani* (C3), and *Rhizoctonia solani* (C4) were spotted four times on the array respectively. $N_{CK}+P_{CK}$, both nursery and pot soil were untreated; $N_{CK}+P_{BIO}$, the pot soil was treated with bio-organic fertilizer but not the nursery soil; $N_{BIO}+P_{CK}$, the nursery soil was treated with bio-organic fertilizer but not the pot soil; $N_{BIO}+P_{BIO}$, both nursery and pot soil were treated with bio-organic fertilizer

Cloning ITS fragment of $N_{CK}+P_{CK}$ and $N_{BIO}+P_{BIO}$ rhizosphere soils

Genomic DNA of rhizosphere soil from $N_{CK}+P_{CK}$ and $N_{BIO}+P_{BIO}$ was amplified with the primer pair ITS1F/ITS4 by the Taq PCR Enzyme Mix (Takara, Dalian, China); PCR products from three separate amplifications per treatments were pooled and used in one cloning reaction, resulting in a total of two cloning reactions. PCR products were purified with Qiaquick gel extraction kit (Qiagen) and cloned into pMD19-T Vector (TaKaRa Biotechnology Dalian Co., Ltd.); the colonies of transformed *E. coli* were isolated from LB media, after incubation for 16 h at 37 °C; the clones were transferred into LB broth and then plasmids were isolated and treated with enzymes (Alu I, Hinf I, and Dra I from New England Bio Lab, Beverly, MA); DNA fragments were then resolved on 2 % agarose. The clones with different band types were sequenced and aligned by Clustal.

DGGE analysis

DGGE analysis was performed using the D-Code DGGE system (Bio-Rad); DGGE profiles were obtained with ITS1-F (Gardes and Bruns 1993) and ITS2 (Anderson et al. 2003; White et al. 1990) primers. A GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) (Muyzer et al. 1993) was added to the 5′ end of the ITS1-F primer to stabilize the melting behavior of DNA fragments. Twenty microliters of PCR products (400 ng) were loaded onto 8 % (w/v) polyacrylamide gels (37.5:1 of 40 % acrylamide/bis-solution, Bio-Rad Laboratories) with denaturing gradients ranging from 25 to 55 % for the fungal DNA. DGGE was performed in 1×Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 60 °C at a constant voltage of 80 V for 16 h; then, DGGE fingerprint were silver stained.

Statistical analyses

Similarly, the microbial communities were expressed using the unweighted pair group method with mathematical averages (UPGMA). Quantity one 4.6.3 (Bio-Rad, USA) was used to construct UPGMA dendrograms and Cluster analyses. The DGGE bands were visualized with UV transillumination and photographed; average intensities of individual bands were analyzed using Quantity One image analysis software, version 4.6.3 (Bio-Rad Laboratories). Shannon index (H') was calculated from DGGE profile (Girvan et al. 2003) using the formula $H'=-\sum P_i \ln pi$, where pi is the percent contribution of the microbial species (intensity of the band i/total intensity of all bands in the lane) and ln p_i is the natural log of p_i. Equitability index (J) was also calculated using the formula $J=H'/(\ln n_i)$, where J is the Shannon Weaver equitability index and ln n_i is the natural log of the total number of species in a fingerprint. Statistical significance was determined by the analysis of replicate samples using analysis of variance (Anova) test. For all experiments a P value of <0.05 was required to establish significant differences between un-amended and amended samples.

Results

Detection of *F. oxysporum* in soils of four treatments by real-time PCR

The real-time quantitative results indicated there was a significant difference ($P>0.05$) in CFU levels of *F. oxysporum* in the rhizosphere soil of BIO treated ($N_{BIO}+P_{CK}$ and $N_{BIO}+P_{BIO}$) or untreated ($N_{CK}+P_{CK}$ and $N_{CK}+P_{BIO}$) nursery cups. The abundance of *F. oxysporum* in nursery cups treated without BIO was 8.56×10^4 cfu g^{-1} rhizosphere soil in the $N_{CK}+P_{CK}$ treatment and 8.08×10^4 cfu g^{-1} rhizosphere soil in the $N_{CK}+P_{BIO}$ treatment, whereas, the abundance of *F. oxysporum* in nursery cups treated with BIO was 16.19×10^3 and 9.41×10^3 cfu g^{-1} rhizosphere soil in the treatments $N_{BIO}+P_{CK}$ and $N_{BIO}+P_{BIO}$ respectively. Then the $N_{BIO}+P_{BIO}$ treatment showed the lowest *F. oxysporum* abundance 9.41×10^3 cfu g^{-1} soil in the rhizosphere soil. In the bulk soils of the four treatments, the number of *F. oxysporum* ranged from 5.72×10^4 cfu g^{-1} soil to 3.93×10^4 cfu g^{-1} soil (Table 3), without significant differences.

Validation of the DNA array

The membrane-based DNA array was validated with DNA extracted from rhizosphere soils of the four treatments. Universal oligonucleotide ITS4, ITS5, and ITS2 (Fig. 1, A1, A2, A3) were used as control being supposed to detect the presence of any fungal species with consistent positive signals in the four treatments; four internal controls ITS2_1 to ITS2_5 (Fig. 1, A4 to A8) that differed from primer ITS2 at one or two bases were not supposed to detect any fungal species with negative signals in the four treatments except A8; which had a

Table 3 Quantification of *F. oxysporum* in soil samples using real-time PCR

Treatment	Rhizospheric soil (10^3 cfu g^{-1})	Bulk soil (10^3 cfu g^{-1})
$N_{CK}+P_{CK}$	85.63±2.9a	57.20±2.7a
$N_{CK}+P_{BIO}$	80.80±5.2a	40.71±1.2c
$N_{BIO}+P_{CK}$	16.19±0.2b	50.97±2.5b
$N_{BIO}+P_{BIO}$	9.41±0.4c	39.33±0.7c

Note: Means±SE. The letters of a, b, and c mean significant difference at $p>0.05$ by Duncan test

very weak cross-hybridization signal. However, there were differences in signal intensities of pathogens between treatments without (Fig. 1, $N_{CK}+P_{CK}$ and $N_{CK}+P_{BIO}$) or with (Fig. 1, $N_{BIO}+P_{CK}$ and $N_{BIO}+P_{BIO}$) BIO application to the nursery cups. The signal intensities of B1, B2 (*Fusarium* sp.) and B3, B4, B5, B6 (*F. oxysporum*) in BIO treatments to the nursery cups (Fig. 1, $N_{BIO}+P_{CK}$ and $N_{BIO}+P_{BIO}$) were weaker than those of treatments without BIO application to the nursery cups (Fig. 1, $N_{CK}+P_{CK}$ and $N_{CK}+P_{BIO}$). Intensities of B1, B2 (*Fusarium* sp.) and B3, B4, B5, B6 (*F. oxysporum*) in the $N_{BIO}+P_{BIO}$ treatment displayed the lowest signals among the four treatments (Fig. 1). The signal intensities of C3 (*Fusarium solani*) and C4 (*Rhizoctonia solani*) of four treatments were positive and showed the same trend of B1, B2 (*Fusarium* sp.) and B3, B4, B5, B6 (*F. oxysporum*) (Fig. 1). No positive signals were detected in the B7, B8 (*Verticillium* spp.), C1 (*Verticillium dahliae*) and C2 (*Verticillium albo-atrum*) position, whereas C5 and C6 displayed negative signals in the four treatments (Fig. 1).

ITS clone libraries of $N_{CK}+P_{CK}$ and $N_{BIO}+P_{BIO}$ rhizosphere soils

A total of 129 fungal sequences were obtained from the $N_{CK}+P_{CK}$ (66 sequences) and $N_{BIO}+P_{BIO}$ (63 sequences) clone libraries, which were classified as 21 types of OTUs after treatment with three enzymes. The Ascomycota was the dominant taxon in the $N_{CK}+P_{CK}$ (35 of 66) and $N_{BIO}+P_{BIO}$ (38 of 63) clone libraries, and the Zygomycota (23 of 66) was the second dominant taxon in the $N_{CK}+P_{CK}$ while the Basidiomycota (13 of 63) became the second dominant taxon in the $N_{BIO}+P_{BIO}$ rhizosphere soil (Table 4). Nine out of 66 sequences had 99 % similarity with sequences of *F. oxysporum* in the $N_{CK}+P_{CK}$ treatment, while there was only one clone showing 99 % similarity with sequences of *F. oxysporum* in the $N_{BIO}+P_{BIO}$ treatment.

Investigation at the genus level showed that uncultured *Mortierella* accounted for 29 % of the total fungal communities in the $N_{CK}+P_{CK}$ rhizosphere soil (Table 4), suggesting that this was an important soil fungal group in the watermelon *Fusarium* wilt rhizosphere soil. On the contrary, the presence of *Rhodotorula cresolica* increased whereas the presence of *F. oxysporum* and other pathogens decreased in the $N_{BIO}+P_{BIO}$ rhizosphere soil. The results of cloning libraries also indicated that consecutive applications of BIO at nursery and transplanting stages resulted in the presence of fungal group species which were not found in the $N_{CK}+P_{CK}$ ITS clone library; these were uncultured *Hypocreales*, *Fusarium merismoides*, *Bionectria ochroleuca*, *Neonectria* sp., uncultured *Plectosphaerella*, *Chaetomium* sp., and *Aspergillus penicillioides*; both *Chaetomium* sp. and *Aspergillus penicillioides* were considered beneficial fungi. The presence of harmful fungi *F. oxysporum*, *Rhizoctonia solani*, *Fusarium solani*, and *Fusarium* sp. decreased or was suppressed in the $N_{BIO}+P_{BIO}$ treatment. There were nine fungal species that were found in both clone libraries, at varing proportion (Table 4, in bold).

DGGE fingerprints of four treatments rhizosphere soil ITS fragment

A distinct fungal DGGE pattern was observed in the BIO rhizosphere soil of watermelon plants, and there were clear differences in the intensity and number of bands in the DGGE fingerprints of the four treatments (Fig. 2a). The DGGE bands were clustered with Quantity One computer software (version 4.6.3, Bio-Rad, $p<0.05$); three distinct clusters were observed (Fig. 2b). The fungal communities of treatments without BIO application in either nursery or transplanted pot ($N_{CK}+P_{CK}$) and with only BIO application in the pot ($N_{CK}+P_{BIO}$) were in the same cluster, while the fungal communities of only BIO treatment in the nursery ($N_{BIO}+P_{CK}$) and the BIO treatment in both the nursery and pot ($N_{BIO}+P_{BIO}$) formed two different clusters (Fig. 2b). These results confirmed that changes occurred in the composition of fungal communities of different treatments and that the BIO application to both nursery and pot soil had greater effects on composition of soil fungal communities than other treatments.

In fungal community, the Shannon–Wienner index, the equitability index, and number of bands indicating fungal communities were calculated (Table 5). The DGGE profile showed high Shannon–Wienner diversity indices (Table 5) due to high numbers of bands with different intensities. The number of detected bands ranged from 13 to 22 bands but only four bands were present in all samples. The difference between band intensity was assumed to be due to differences in the abundance of the target species. In the BIO treatments ($N_{BIO}+P_{CK}$ and $N_{BIO}+P_{BIO}$), Shannon Weaver (H') diversity values were lower than those of the BIO nursery unamended soil samples ($N_{CK}+P_{CK}$ and $N_{CK}+P_{BIO}$); Shannon Weaver diversity indices of $N_{CK}+P_{CK}$ and $N_{CK}+P_{BIO}$ treatments were 2.86 and 2.82 respectively, while those of the $N_{BIO}+P_{CK}$ and $N_{BIO}+P_{BIO}$ rhizosphere soils were 1.94 and 1.57. The $N_{BIO}+P_{BIO}$ treatment showed the lowest Shannon Weaver (H') diversity value (1.57) among four treatments. Equitability indexes (J') of the four treatments showed the same trend with Shannon Weaver (H') indexes of the four treatments (Table 5), again indicating that BIO application could change fungal diversity and evenness.

Discussion

Plant defense responses to soil-borne pathogens and their roots interactions with soil microbial communities depend on plant species (Lang et al. 2012; Ling et al. 2012; Luo

Table 4 Phylogenetic affiliations of culturable fungi identified on the basis of ITS of $N_{CK}+P_{CK}$ and $N_{BIO}+P_{BIO}$ ITS clone libraries

Closest identified relative of Clones	Phylogenic group	Identity (%)	GeneBank accession	Numbers of clone	
				$N_{CK}+P_{CK}$	$N_{BIO}+P_{BIO}$
Conocybe lactea	Ascomycota	99	FJ481031.1	1	
Fusarium oxysporum	Ascomycota	100	GU445363	9	1
Mortierella sp.	Zygomycota	100	EU877758.1	4	
Fusarium sp.	Ascomycota	100	GQ505717	6	
Rhizoctonia solani	Ascomycota	99	JF817349.1	2	
Fusarium solani	Ascomycota	99	JQ323553.1	5	
Uncultured Hypocreales	Ascomycota	99	FJ554224.1		1
Ascomycota sp.	Ascomycota	98	HM589358.1		2
Fusarium merismoides	Ascomycota	100	AB586998.1		2
Bionectria ochroleuca	Ascomycota	99	HQ607798.1		3
Fungal sp.	/	99	EF031116.1		3
Neonectria sp.	Ascomycota	99	FJ560440.1		4
Uncultured Plectosphaerella	Ascomycota	100	HE977552.1		2
Chaetomium sp.	Ascomycota	99	EU750691.1		6
Aspergillus penicillioides	Ascomycota	99	GU017496.3		8
Uncultured fungus	/	99	AB520304.1	7	3
Cosmospora sp.	Ascomycota	98	JN995629.1	1	6
Rhodotorula cresolica	Basidiomycota	94	AF444570.1	1	13
Uncultured Mortierella	Zygomycota	99	JF831499.1	19	5
Uncultured ascomycete	Ascomycota	100	EU490152.1	4	2
Gibberella moniliformis	Ascomycota	100	JQ277275.1	7	2
Total clone number				66	63

et al. 2010; Wu et al. 2008) because root exudation can be highly specific (Alabouvette et al. 2006; Prieto et al. 2011). BIO (a combination of organic fertilizers with antagonistic microbes) has been reported to have positive effects on controlling soil-borne diseases in many crops such as rice (Yin et al. 2011), cucumber (Chen et al. 2012; Cao et al. 2011; Huang et al. 2012; Qiu et al. 2012), tomato (Wei et al. 2011), banana (Shen et al. 2013; Wang et al. 2013; Zhang et al. 2011), cotton (Lang et al. 2012), potato (Ding et al. 2013), tobacco (Liu et al. 2013), and watermelon (Ling et al. 2012). However, the relationship linking pathogen, plant disease, and composition of soil fungi communities after BIO application to soil are poorly understood.

We have reported that a *P. polymyxa* strain can enhance the suppressiveness of BIO against *Fusarium* wilt disease by 60 to 100 % and when BIO was applied to nursery and transplanted soils it significantly promoted the growth by 7.27 g plant^{-1} (Ling et al. 2010). Without BIO application ($N_{CK}+P_{CK}$), the culturable abundance of *F. oxysporum* population in the watermelon rhizosphere soil was as high as 8.56×10^4 cfu g^{-1} soil with a high disease incidence (100 %). The most effective treatment in reducing *F. oxysporum* abundance in the rhizosphere soil was the BIO application in both nursery and transplanted soil ($N_{BIO}+P_{BIO}$), confirming previous results (Ling et al. 2010) and indicating that the abundance of the *F. oxysporum* population in the rhizosphere soil is a key factor in the incidence of watermelon disease. The abundance of *F. oxysporum* in the bulk soil of watermelon was stable among four treatments accounting for about 10^3 cfu g^{-1} soil probably because the rhizosphere of watermelon may represent an excellent environment for the fast-growing *Fusarium* species (Timmusk et al. 2005). It has been suggested that the antagonists *P. polymyxa* SQR21 of the BIO can utilize root exudates of watermelon to support its activities (Ling et al. 2010), and thus, it is a more competitive and effective root colonizer than most native microbial species (Haggag and Timmusk 2008; Trillas et al. 2006). Additionally, antibiosis produced by *P. polymyxa* may prevent *F. oxysporum* f. sp. *nevium* colonization of the roots (Dijksterhuis et al. 1999; Timmusk et al. 2005) and thus reducing the incidence of *Fusarium* wilt (Ling et al. 2010; Raza et al. 2008).

Disease management can be improved with rapid and accurate pathogen detection (Njambere et al. 2011; Zhao et al. 2012). The quantification by real-time PCR showed that *F. oxysporum* survives poorly when BIO was applied into nursery and pot soil. The probes for detecting non-target pathogens such as *Verticillium dahliae*, *Verticillium albo-atrum*, and *Verticillium* spp. (Lievens et al. 2003; Zhang

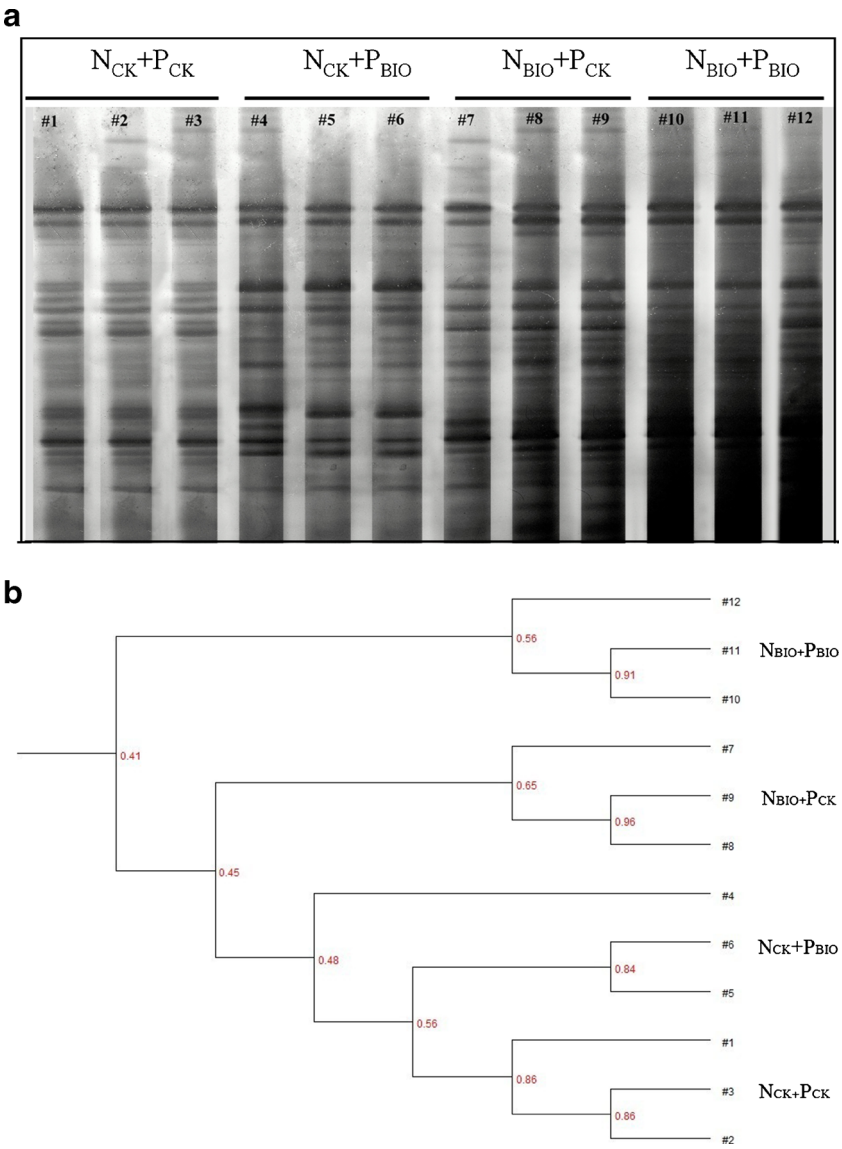

Fig. 2 The DGGE profiles (**a**) and the UPGMA cladograms based on Dice similarity of the composition of fungal communities in watermelon rhizosphere soil (**b**). #1, 2, and 3 were three replicates of $N_{CK}+P_{CK}$ rhizosphere soil; #4, 5, and 6 were three replicates of $N_{CK}+P_{BIO}$ rhizosphere soil; #7, 8, and 9 were three replicates of $N_{BIO}+P_{CK}$ rhizosphere soil; and #10, 11, and 12 were three replicates of $N_{BIO}+P_{BIO}$ rhizosphere soil. $N_{CK}+P_{CK}$, both nursery and pot soil were untreated; $N_{CK}+P_{BIO}$, the pot soil was treated with bio-organic fertilizer but not the nursery soil; $N_{BIO}+P_{CK}$, the nursery soil was treated with bio-organic fertilizer but not the pot soil; $N_{BIO}+P_{BIO}$, both nursery and pot soil were treated with bio-organic fertilizer

et al. 2007) were also included in the DNA assay aiming to assess the specificity and sensitivity of this assay. Even though the DNA assay system used in this study displayed a very weak cross-hybridization of ITS2-5 (Table 4, A8), it detected and monitored the target pathogen *F. oxysporum* DNA. The weak cross-hybridization might be due to highly similar sequences where the mismatched base was located near the end or in a string of the identical bases. This might be prevented by designing dimeric oligonucleotide probes (Njambere et al. 2011; Zhang et al. 2008). The oligonucleotide-based DNA array detection system used in this study is reliable and effective for *F. oxysporum* identification even when multiple pathogens are present in the same soil sample.

Table 5 Shannon diversity and equitability of fungi communities of rhizosphere soils of four treatments. Shannon–Weaver diversity (H'), equitability (J) indices, and number of bands (n) were determined from DGGE profiles

Treatment	H'	J	n
$N_{CK}+P_{CK}$	2.86±0.02a	0.93±0.00a	22
$N_{CK}+P_{BIO}$	2.82±0.01a	0.95±0.02a	19
$N_{BIO}+P_{CK}$	1.94±0.02b	0.68±0.01b	17
$N_{BIO}+P_{BIO}$	1.57±0.03c	0.61±0.01c	13

Values are the means of three times' calculation of each lane with associated standard errors. The letters of a, b, and c mean significant difference at $p>0.05$ by Duncan test

Disease suppression can function as an indicator for a stable and healthy soil ecosystem (Van Bruggen and Semenov 1999), and microbial structural and functional diversity in soil may be important for soil health (Visser and Parkinson 1992). The ability of *P. polymyxa* SQR21 to inhibit plant pathogens and to promote plant growth has been

documented (Ryu et al. 2006; Timmusk et al. 2005), but its effects on composition of soil fungi communities are poorly known. The DGGE and clone library results showed that application of BIO changed the composition of fungal community of the rhizosphere zone. The detectable band number in the $N_{BIO}+P_{BIO}$ treatment was only 13, indicating that the fungal species richness (n) was lower than the other treatments as well as the Shannon Weaver (H') diversity values and equitability (J).

However, the BIO application in both nursery and pot increased the proportion of beneficial fungi such as *Chaetomium* sp. and *Aspergillus penicillioides* and decreased the proportion of some harmful fungi compared with unamended soil (Table 3). *Chaetomium* sp. (Suyanto et al. 2003) is a thermophilic fungus, which can decompose palm-oil mill fibers, while *Aspergillus penicillioides* is a halophile salt tolerant fungal species which inhibit most fungal species (Pitt and Hocking 2009; Tamura et al. 1999). Among the detected pathogenic fungi, *F. oxysporum*, *Rhizoctonia solani*, and *Fusarium solani* were found to co-exist in the rhizosphere soil and both abundances decreased with BIO application. As a result, the presence of pathogenic fungi and potential pathogenic fungi to watermelon was negatively affected by the BIO treatment (Ling et al. 2010). Further, the possible protection mechanism involved in this study may attributed to the increased activities of antioxidases and pathogenesis-related proteins in plants by BIO (Dijksterhuis et al. 1999; Ling et al. 2010), leading to enhanced plant systemic acquired resistance to the pathogen. In addition, the formulation of *P. polymyxa* SQR21 with organic matters changed the microbial composition of the watermelon rhizosphere soil (Dijksterhuis et al. 1999; Raza et al. 2008), and this may have affected soil nutrient availability to watermelons by changing soil enzyme activities (Yang et al. 2007).

In conclusion, our work showed that the application of *P. polymyxa* SQR21 inoculated BIO not only changed the composition of fungal communities but also significantly reduced *Fusarium* wilt disease symptoms by reducing the *F. oxysporum* population in the rhizosphere soil. It is important to have detected changes in the composition of fungal communities of the rhizosphere soil after BIO application because stability and composition of soil microbial communities are important indicators for soil health and sustainability. The underlying mechanisms of *P. polymyxa* SQR21 enhanced BIO application to control *Fusarium wilt* need to be further studied.

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