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ORIGINAL PAPER

Control of cotton *Verticillium* wilt and fungal diversity of rhizosphere soils by bio-organic fertilizer

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Abstract Cotton *Verticillium* wilt is a destructive soil-borne disease affecting cotton production. In this study, application of bio-organic fertilizer (BIO) at the beginning of nursery growth and/or at the beginning of transplanting was evaluated for its ability to control *Verticillium dahliae* Kleb. The most efficient control of cotton *Verticillium* wilt was achieved when the nursery application of BIO was combined with a second application in transplanted soil, resulting in a wilt disease incidence of only 4.4%, compared with 90.0% in the control. Denaturing gradient gel electrophoresis patterns showed that the consecutive applications of BIO at nursery and transplanting stage resulted in the presence of a unique group of fungi not found in any other treatments. *Humicola* sp., *Metarhizium anisopliae*, and *Chaetomium* sp., which were considered to be beneficial fungi, were found in the BIO treatment, whereas some harmful fungi, such as *Alternaria alternate*, *Coniochaeta velutina*, and *Chaetothyriales* sp. were detected in the control. After the consecutive applications of BIO at nursery and transplanting stage, the *V. dahliae* population in the rhizosphere soil in the budding period, flowering and boll-forming stage, boll-opening stage, and at harvest time were 8.5×10^2, 3.1×10^2, 4.6×10^2, and 1.7×10^2 colony-forming units per gram of soil (cfu g^{-1}), respectively, which were significantly lower than in the control (6.1×10^3, 3.4×10^3, 5.2×10^3, and 7.0×10^3 cfu g^{-1}, respectively). These results indicate that the suggested application mode of BIO could effectively control cotton *Verticillium* wilt by significantly changing the fungal community structure and reducing the *V. dahliae* population in the rhizosphere soil.

Keywords Fungal diversity · Rhizosphere soil · Bio-organic fertilizer · PCR-DGGE · Real-time PCR · SYBR green

Introduction

Verticillium dahliae Kleb. is a ubiquitous destructive vascular wilt soil fungus with a broad host range including trees, ornamental plants, and economically important vegetable and field crops such as cotton and tobacco (Pegg and Brady 2002; Zhu et al. 2007). Management strategies for this disease control are mainly preventative, such as the use of resistant hosts and attempts at biological control practices (Tjamos 1989). Although chemical fungicides seem to be effective, they are not environmentally friendly (Nannipieri et al. 1990). Furthermore, the repeated use of such chemicals generates development of resistance in the target pathogen (Goldman et al. 1994) and has a negative effect on some beneficial organisms. Biological control is one of the most promising ways for suppressing *V. dahliae* since it is safety and environmentally friendly, avoiding the pollution and health hazards resulting from the conventional use of chemical pesticides (Tjamos et al. 2000; Spurrier 1990).

The introduction of beneficial microorganisms into soils or the rhizosphere is sometimes successful for the biological control of soil-borne plant diseases (Cook 1993). A wide variety of microbial strains have been isolated from rhizosphere soils to improve plant growth and health after their inoculation (Bent 2006; Siddiqui 2006; Whipps 2001). The non-symbiotic rhizosphere species that have been used most successfully in the biological control of plant diseases

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are *Bacillus* spp. (Cao et al. 2011; Jacobsen et al. 2004; Luo et al. 2010; Schisler et al. 2004; Zhang et al. 2011), *Pseudomonas* spp. (Weller 2007), and *Trichoderma* spp. (Samuels 2006; Yang et al. 2011).

Composts can stimulate proliferation of antagonists in the rhizosphere, suppressing soil-borne plant pathogens (De Brito Alvarez et al. 1995; Termorshuizen et al. 2006). Termorshuizen et al. (2006) had evaluated the abilities of a wide variety of composts to suppress *Verticillium* wilt and they demonstrated that application of composts of horse manure, unbroken bedding hay+wood shavings and municipal green waste (GR6, originated from Greece) was capable of reducing *Verticillium* wilt in eggplants. However, application of only composts often results in inconsistent levels of disease control (Mazzola 2007; Noble and Coventry 2005). Further manipulation of composts by inoculation or enrichment with specific antagonists shows most promise for soil-borne disease control (Cao et al. 2011; Ling et al. 2010; Luo et al. 2010; Noble and Coventry 2005; Postma et al. 2003; Suárez-Estrella et al. 2007; Wei et al. 2011; Yang et al. 2011; Zhang et al. 2011; Zhao et al. 2011). Composts not only play an important role in providing a suitable substrate but also serve as a growth-promoting medium (Raviv et al. 1998). Recent attempts to produce biological control of *V. dahliae* have indicated that *Bacillus subtilis* strain could effectively reduce the incidence and severity of wilt of cotton plants (Luo et al. 2010) under both greenhouse and field conditions. Therefore developing a new bio-organic fertilizer (BIO) which can help specific and beneficial microbes grow and reproduce in rhizospheric soils or even in bulk soils is a continuous challenge in control of soil-borne disease.

Application of BIOs can affect microbial community in rhizosphere soils and on plant roots. Previous reports correlating bacterial 16S rRNA gene sequences of soil samples to the effect of different treatments have increased our understanding of the dynamics of bacterial communities in the rhizosphere soil (Filion et al. 2004; Ofek et al. 2009). The degree of resolution of the community structure is dependent on the specificity of the primer system and the phylogenetic information contained by the amplified fragments. Fungal 18S rRNA genes vary to a lesser extent than bacterial 16S rRNA genes (Hugenholtz and Pace 1996). Designed PCR primers (Vainio and Hantula 2000) for amplifying fungal 18S rDNA from environmental samples are very powerful tools. Analyzing fungal communities by denaturing gradient gel electrophoresis (DGGE) with separation of 18S rRNA genes fragments can give information of phylogeny and disease.

Although conventional PCR-based techniques are very sensitive, they are neither quantitative nor useful for direct identification of environmental species (Schroeder et al. 2006). Real-time PCR is an accurate, specific, and less time-consuming method for monitoring pathogen infection. Real-time PCR using fluorogenic dyes (such as SYBR Green II dye) (Morrison et al. 1999) measures the intensity of a fluorescent signal that is proportional to the amount of DNA generated during the PCR amplification (Wittwer et al. 1997) and is commonly used to monitor pathogen infection (Filion et al. 2003; Schnerr et al. 2001).

In this study, we used a *B. subtilis*-enhanced BIO to control *Verticillium* wilt (Luo et al. 2010) and tested its effect on the disease incidence in cotton rhizosphere soil. The effects of soil amendments on the fungal diversity of the cotton rhizosphere were analyzed by the PCR-DGGE technique. We also developed a real-time PCR based-assay that provided fast, sensitive, and quantitative detection of *Verticillium* spp. in soils, which is valuable to monitor the pathogen in soils and thus to predict the soil-borne disease appearance.

Materials and methods

Fertilizer preparation and greenhouse experiments

Antagonistic strain

The antagonistic strains, *B. subtilis* HJ5 and *B. subtilis* DF14, were previously isolated from the rhizosphere soil of healthy cotton roots in a field severely affected by *Verticillium* wilt in Dafeng, Jiangsu province, China. The two strains had high antagonistic efficiencies, with 87.4% and 84.7% inhibition rates against the growth of *V. dahliae* (CGMCC no. 3.3757) in previous laboratory experiments (Zhang et al. 2008a) respectively, and the two strains have been registered in the China General Microbiological Culture Collection Center with the assigned accession numbers CGMCC no. 3301 and CGMCC no. 3302, respectively. The strains were stored at −80°C in 20% glycerol and routinely cultured on LB medium at 30°C.

Origin of the organic fertilizer

The organic fertilizer was composed of amino acid and manure composts in a 1:1 weight ratio. Amino acid fertilizer containing 44.2% organic matter, 12.9% total amino acids, and small molecular peptides, 4.4% N, 3.5% P_2O_5, and 0.67% K_2O was kindly supplied by Jiangsu Xintiandi Amino Acid Fertilizers Ltd., China. The amino acid fertilizer was produced from rapeseed meal by solid state fermentation with proteinase-producing bacteria for 7 days (Zhang et al. 2008b). Pig manure compost, which was composted for 27 days and contained 30.4% organic matter, 2.0% total N, 3.7% P_2O_5, and 1.1% K_2O, was kindly provided by Jiangsu Tianniang Ltd., China.

Bio-organic fertilizer

A 1,000-ml suspension containing 10^9 colony-forming units (cfu) of both HJ5 and DF14 per milliliter, and 5 kg of the organic fertilizer were thoroughly mixed in a 500×360×175-mm plastic case for secondary solid fermentation. The mixture was maintained at 40–45% moisture at room temperature (20–31°C) for 6 days and manually turned every day. On the seventh day, the mixture was spread for air-drying in a ventilation room at room temperature for 2 days until the water content was less than 30%. The temperature and bacterial density of the substrates were observed daily during the fermentation. The content of *B. subtilis* HJ5 and *B. subtilis* DF14 in the final product was greater than 1×10^9 cfu g^{-1} dry matter of the formulation and was hereafter referred to as the bio-organic fertilizer (BIO) used for suppressing the growth of *V. dahliae*. The BIO was stored at 4°C prior to use in experiments.

Soils

The nursery soil for growing seedlings was from a paddy field without history of cotton cultivation. The soil for the transplanted pot experiment was collected from a field in Dafeng, Jiangsu province, where the field had been planted with cotton since 1975. The incidence of cotton *Verticillium* wilt reached approximately 60% in the field in 2007 when the soils were collected for this experiment.

Seedling nursery

Cotton seeds (*Gossypium hirsutum* L. Xinluzao no. 8) provided by Xinjiang Shihezi University, China, were surface sterilized with 10% H_2O_2 for 30 min, rinsed three times in sterilized distilled water, and germinated in 9-cm plates covered with sterile wet filter paper at 28°C. Each seedling was grown in a nursery cup (450 ml in volume and 11 cm in height) with 300 g of nursery soil and maintained in a greenhouse for 20 days. Three treatments of the nursery soil were employed: (1) CK, without organic or BIO amendment (the control), (2) OF, amended with the organic fertilizer as described in above section at a rate of 10 g kg^{-1}, and (3) BIO, amended with the BIO as described in above section at a rate of 10 g kg^{-1}. Each treatment had 30 replicates.

Pot experiment and sampling

Approximately 10 kg of the fresh soil from the diseased field was added to pots (12 l in volume and 25 cm in height), and one seedling with its nursery soil was transplanted into the center of each pot. In addition to the three treatments as in the nursery stage, two more treatments were designed at the pot experiment stage where the soils were amended without or with the BIO at a rate of 5 g kg^{-1}. All five treatments are listed in Table 1.

Three blocks were randomly laid out for the replicates of the five treatments, and each treatment with three replicates (pots) was randomly arranged within each block. Thus, each treatment was replicated for nine times. The pot experiment was from 11 April to 12 August 2009.

Rhizosphere soil samples were collected from the five growing seasons: seedling stage, budding period, flowering and boll-forming stage, boll-opening stage, and harvest time. Cotton roots from each pot were carefully separated from the soil and softly shaken by hands. The soils deprived by shaking from the roots were collected as "bulk soils," whereas the soil adhering to the roots was considered "rhizosphere soils" (Bakker and Schippers 1987). All the soil samples were stored at −70°C until DNA extraction.

Disease incidence record

Seedling infection by *V. dahliae* was recorded every day and the cumulative number of infected plants was calculated from the day after transplanting until 100 days. Disease incidence and the percentage disease reduction were calculated by the percentage of diseased plants in each block, in which diseased plants were those when the disease emerged.

DNA extraction, PCR-DGGE, and sequencing

DGGE images for band detection and integrated band area intensities were analyzed with Quantity One computer

Table 1 Design of the experiments using either organic fertilizer (OF) or bio-organic fertilizer (BIO), both applied at 10 g kg^{-1} for OF or BIO in the nursery soils; BIO was applied at 5 g kg^{-1} in the pot experiments

Treatment	Nursery stage		Pot experiments	
	OF	BIO	OF	BIO
$N_{CK}+T_{CK}$	−	−	−	−
$N_{OF}+T_{CK}$	+	−	−	−
$N_{BIO}+T_{CK}$	−	+	−	−
$N_{CK}+T_{BIO}$	−	−	−	+
$N_{BIO}+T_{BIO}$	−	+	−	+

Treatment: $N_{CK}+T_{CK}$, in the profile of both untreated nursery and pot soil; $N_{OF}+T_{CK}$, in the profile of the nursery soil added with organic fertilizer and untreated pot soil; $N_{BIO}+T_{CK}$, in the profile of the nursery soil added with bio-organic fertilizer and untreated pot soil; $N_{CK}+T_{BIO}$, in the profile of the untreated nursery soil and pot soil added with bio-organic fertilizer; $N_{BIO}+T_{BIO}$, in the profile of both nursery and pot soil added with bio-organic fertilizer

software (version 4.6.3, Bio-Rad). Cluster analysis was performed by the unweighted pair group method using arithmetic averages. The relative intensity of a specific band was expressed as the ratio between the intensity of that band and the total intensity of all bands in that lane. The intensities were necessary for determining the Shannon–Wiener diversity index (H) (Luo et al. 2004) and calculated using the formula $H=-\sum p_i \ln p_i=-\sum(n_i/N)\ln(n_i/N)$, where p_i was the ratio between the number in a specific group and the total number, n_i was the intensity of a band and N was the sum of all band intensities in the densitometry profile.

DNA was extracted from 1 g of soil samples, obtained by combining three replicates, with UltraClean™ Soil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The *V. dahliae* isolate was grown in a shaking culture solution at 28°C in 100-ml potato dextrose broth in dark to produce a 3-day-old culture. Genomic DNA from *V. dahliae* solution was isolated as described by the E.Z.N.A.® Fungal DNA Kit's manufacturer's instructions (Omega Biotek Instruments, Inc. USA). DNA yields and purity were determined by UV light spectroscopy.

The microbial diversity of soil samples was determined by PCR-DGGE. For the fungi, the primer pair EF390 (5′-CGA TAA CGA ACG AGA CCT-3′) (Vainio and Hantula 2000) and FR1 (5′-AIC CAT TCA ATC GGT AIT-3′) (Vainio and Hantula 2000) were used to amplify the 5′ end (390_bp) of the 18S rRNA gene. GCFR1 had GC clamps (5′-CCCCCGCCGCGCGGCGGGCGGGGCGGG GGCACGGGCCG-3′) (Vainio and Hantula 2000), which were required for DGGE analysis. PCR was performed using 2.5 μl of 10× Ex Taq buffer (20 mM Mg^{2+}, TaKaRa, Japan), 2 μl of 2.5 mM dNTP mixture, 0.3 μl of 5 units/μl Ex Taq polymerase (TaKaRa), 1 μl of each primer (10 pmol/μl), 1 μl of diluted template, and sterile water to a total volume of 25 μl. Cycle conditions for the fungal PCR were as follows: an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and elongation at 72°C for 2 min and a final elongation step at 72°C for 10 min. The products from the fungal PCR reactions were verified by 1% agarose gel electrophoresis. The amount of DNA in each sample was estimated by image analysis using GeneTools (SynGene) on digital images of the agarose gels obtained with GeneSnap (SynGene) to ensure that equal amounts of DNA from the samples were loaded onto the DGGE gel.

DGGE was performed with the D-GENE™ System (Bio-Rad). Equal amounts of DNA were loaded onto 7.5% (*w*/*v*) polyacrylamide gels (40% acrylamide/bis-solution, 37.5:1, Bio-Rad) with denaturing gradients ranging from 45% to 60% for the fungal DNA. Amplicons of the 18S rRNA gene fragments retrieved from the different samples were loaded in blocks on denaturing gels. Amplicons of the 18S rRNA gene fragments from the genomic DNA of *V. dahliae* were loaded as a control. The amplicons were separated by DGGE in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 18 h at a constant voltage of 50 V and a temperature of 58°C (Vainio and Hantula 2000). The gels were silver stained, dried at 37°C, and scanned as previously reported (Heuer et al. 2001).

Single DGGE bands were excised with a sterile scalpel and the DNA from each band was eluted in 20 μl of sterile water overnight at 4°C. From each DGGE band, 2 μl of the eluted DNA was re-amplified with the primers without the GC clamp using the conditions described above (Hu et al. 2009). The purified PCR products were then ligated to PMD™ 19-T Vector (TaKaRa Biotechnology Dalian Co., Ltd.) according to the manufacturer's instructions. Electro-competent *Escherichia coli* DH5α cells were transformed with the recombinant plasmids. White colonies from each transformation were selected from LB plates containing ampicillin (100 $\mu g ml^{-1}$), IPTG (0.5 mM), and X-GAL (40 $\mu g ml^{-1}$; Sigma-Aldrich Co.). The specific primer pair PMD19RV-M (5′-GAGCGGATAACAATTTCACACAGG-3′) and PMD19M13-47 (5′-CGCCAGGGTTTTCCCAGT CACGAC-3′) was used to verify the identity of the selected colony, which was grown in a shaking water bath at 37°C in 3-ml LB broth overnight and sent for sequencing. The sequences recovered were aligned to bacterial and fungal gene fragments available from databases at the National Center for Biotechnology Information, and searches from GenBank were used to find the closest known relatives to the partial fungal sequences.

Real-time PCR

To target the pathogen, a single real-time PCR primer was chosen and used for amplification in combination with the fungal-specific forward primer ITS1-F, which targeted the 18S rRNA gene. Real-time PCR assays to quantify of *V. dahliae* DNA were conducted using the primer pair ITS1-F (Gardes and Bruns 1993) (5′-CTTGGTCATTTAGAG GAAGTAA-3′) and ST-VE1 (5′- AAAGTTTTAATGG TTCGCTAAGA-3′) (Bart et al. 2006), which produced PCR products of 200 bp.

The quantity of DNA for each sample was determined using SYBR® Premix Ex Taq™ (2×; TaKaRa Biotechnology Dalian Co., Ltd.) with an ABI PRISM 7,500 Sequence Detection System (Applied Biosystems); a total volume of 20 μl containing 10 μl of SYBR® Premix Ex Taq™ (2×), 0.4 μl of each primer, 0.4 μl of ROX Reference Dye II (50×), 2 μl of DNA, and 6.8 μl of sterile water. Real-time PCR was conducted for 15 s at 95°C (one cycle) followed by 5 s at 95°C and 34 s at 60°C (40 cycles). Specificity was examined by generating a dissociation curve after amplifi-

cation. The melting curve was obtained by programming the ABI PRISM 7,500 Sequence Detection System at the end of every run.

Statistical analysis

Changes in diseases incidence and disease reduction percentage and the amounts of *V. dahliae* were statistically determined with Microsoft Excel™ and SPSS Base Ver.11.5 statistical software (SPSS, Chicago, IL, USA). Duncan's multiple range test was applied when the one-way ANOVA showed obvious differences ($p<0.05$). DGGE bands were analyzed with Quantity One computer software (version 4.6.3, Bio-Rad) ($p<0.05$).

Results

The biocontrol effects of BIO on cotton *Verticillium* wilt

Analysis of the disease incidence and disease reduction percentage (Table 2) showed that the application of BIO significantly reduced *Verticillium* wilt disease symptoms in cotton plants relative to the control and OF treatments (Luo et al. 2010; Zhang et al. 2008a). Double application of BIO ($N_{BIO}+T_{BIO}$) in the nursery cups and in the diseased soils of pots was more effective in reducing the disease incidence than single application either in nursery cup soils ($N_{BIO}+T_{CK}$) or in diseased pot soils ($N_{CK}+T_{BIO}$). The disease incidence in the control was 90.0%, whereas it was only 4.4% in $N_{BIO}+T_{BIO}$ treatment; 78.9% of disease incidence was detected in the $N_{OF}+T_{CK}$ treatment, indicating that OF had no significant effect on cotton *Verticillium* wilt. Furthermore, the disease reduction percentage of the $N_{BIO}+T_{BIO}$ treatment was 95.0%, whereas they were 61.3% and 57.9% in the $N_{BIO}+T_{CK}$ and $N_{CK}+T_{BIO}$ treatments, respectively. Application of BIO significantly reduced *Verticillium* wilt disease incidence in diseased soils.

Table 2 Cotton *Verticillium* wilt incidence and percentage disease reduction as affected by treatments

Treatment	Disease incidence (%)[a]	Disease reduction (%)[a]
$N_{CK}+T_{CK}$	90.0±6.7 a	–
$N_{OF}+T_{CK}$	78.9±6.9 b	13.0±12.2 c
$N_{BIO}+T_{CK}$	34.4±5.1 c	61.3±8.6 b
$N_{CK}+T_{BIO}$	37.8±1.9 c	57.9±3.6 b
$N_{BIO}+T_{BIO}$	4.4±1.9 d	95.0±2.1 a

Treatment: $N_{CK}+T_{CK}$, in the profile of both untreated nursery and pot soil; $N_{OF}+T_{CK}$, in the profile of the nursery soil added with organic fertilizer and untreated pot soil; $N_{BIO}+T_{CK}$, in the profile of the nursery soil added with bio-organic fertilizer and untreated pot soil; $N_{CK}+T_{BIO}$, in the profile of the untreated nursery soil and pot soil added with bio-organic fertilizer; $N_{BIO}+T_{BIO}$, in the profile of both nursery and pot soil added with bio-organic fertilizer

[a] Data were expressed as mean±standard error. The data in a column with a different letter differ significantly at Duncan's significance level 0.05

Changes in fungal diversity of soils

Fungal diversity of the rhizosphere soils at the seedling stage

Fungal diversity was investigated in three samples at the seedling stage. The extracted DNA from three replicates was pooled and analyzed with PCR-DGGE to characterize the fungal communities. DGGE profiles were used to compare the fungal communities among the three treatments. The application of BIO significantly changed the observed banding patterns of the fungal communities of the rhizosphere (Fig. 1a). DGGE bands were analyzed with Quantity One computer software (version 4.6.3, Bio-Rad, $p<0.05$) (Fig. 1b), confirming that significant changes occurred in the fungal communities. None of the samples from the three treatments belonged to the same group. Furthermore, *V. dahliae* was not found in any of the three samples at the seedling stage because we used the paddy (healthy) soils without any *V. dahliae* as the nursery soils.

Fungal diversity of the rhizosphere soils at the four stages during the pot experiment

Fungal diversity of soil samples collected from five treatments was investigated during the budding period, flowering and boll-forming stage, boll-opening stage and harvest time, and differences of fungal communities of the samples from the five treatments were observed. Therefore, the application of BIO affected the biomes and composition of the fungal communities. The fungal communities of samples from the five treatments could be divided into three groups (Figs. 2a, b, 3a, b, and 4(A-1, B-1 and A-2, B-2)). The $N_{CK}+T_{CK}$ and $N_{OF}+T_{CK}$ treatments belonged to one group, the $N_{BIO}+T_{CK}$ and $N_{CK}+T_{BIO}$ treatment were the second group, and the $N_{BIO}+T_{BIO}$ treatment was an independent group. Furthermore, *V. dahliae* was found in all the samples from the five treatments at the any sampling date time. DGGE fingerprints revealed that the band corresponding to *V. dahliae* had the highest intensity in the $N_{CK}+T_{CK}$ treatment and the weakest in the $N_{BIO}+T_{BIO}$ treatment.

Analysis of the fungal diversity by the Shannon–Wiener index

DGGE bands were also statistically analyzed by the *H*. A high value of *H* corresponded to great diversity in the

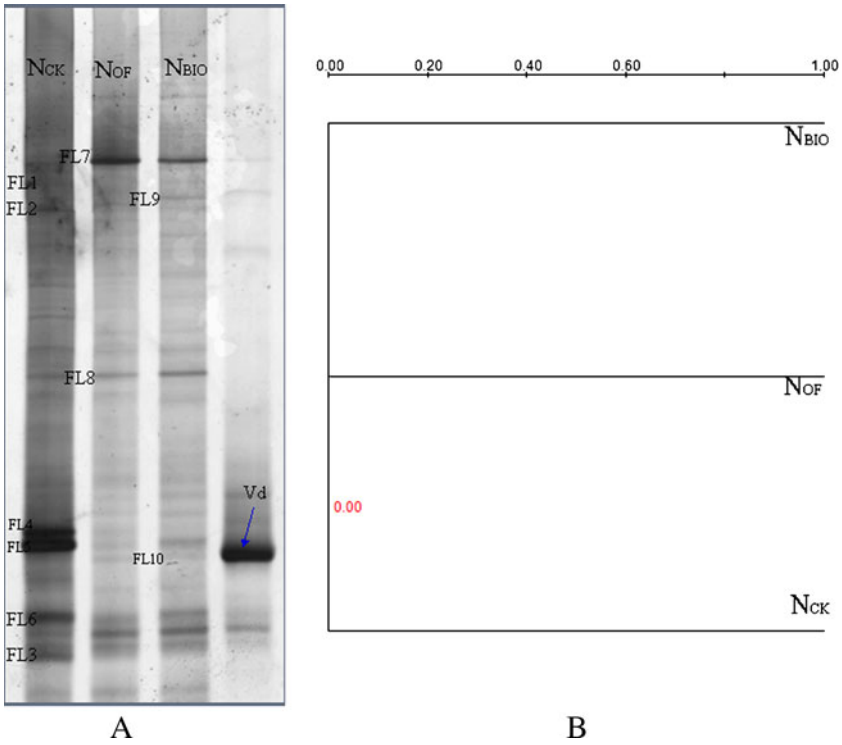

Fig. 1 The DGGE profiles of the fungal community in cotton rhizosphere soil at seedling stage. Bands indicated by numbers FL1 to FL10 were excised, and after re-amplification, subjected to sequencing; Vd is the DGGE profile of *V. dahliae*; N_{CK}, in the profile of the control of nursery stage; N_{OF}, in the profile of nursery soil added with organic fertilizer; N_{BIO}, in the profile of nursery soil added with bio-organic fertilizer

fungal community. At the seedling stage, the *H* of CK was 1.15, which was higher than those of the other two treatments, whereas, the *H* of BIO treatment was only 0.95, with 11 bands detected, compared with 18 of the CK treatment (Fig. 1a). At the budding period, the *H* value of the $N_{BIO}+T_{BIO}$ treatment was 0.83 which was the smallest value, with only nine bands whereas the *H* values of all other treatments ranged from treatment 1.00 to 1.09. At the

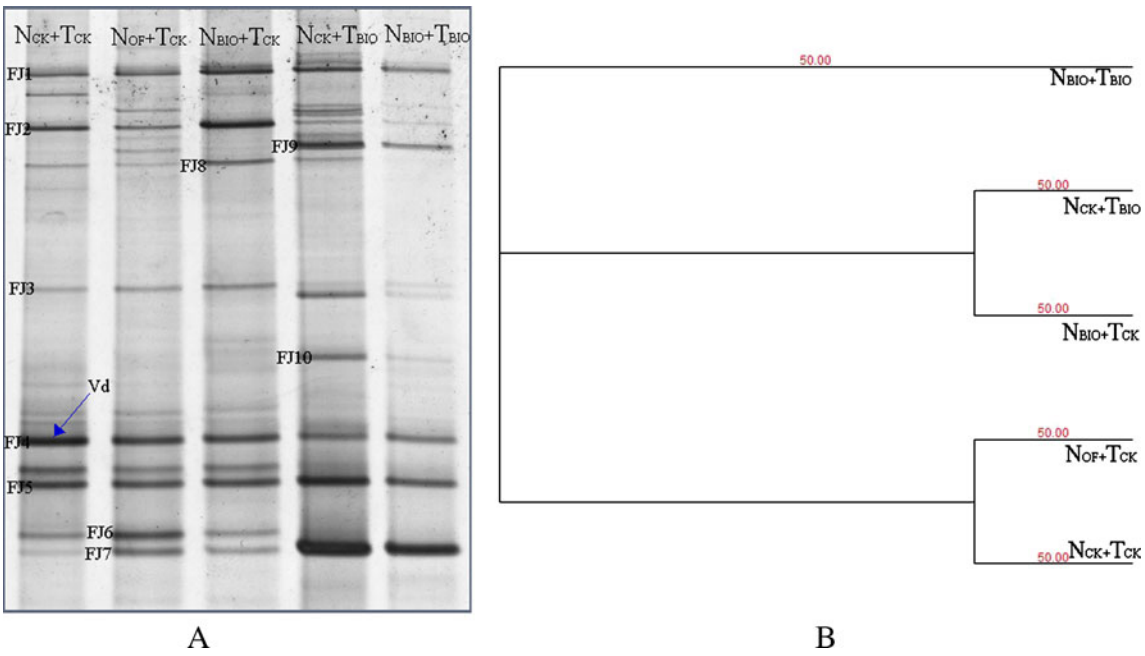

Fig. 2 The DGGE profiles of the fungal community in cotton rhizosphere soil at the budding period. Bands indicated by numbers FJ1 to FJ10 were excised, and after re-amplification, subjected to sequencing; Vd is the DGGE profile of *V. dahliae*; $N_{CK}+T_{CK}$, in the profile of both untreated nursery and pot soil; $N_{OF}+T_{CK}$, in the profile of the nursery soil added with organic fertilizer and untreated pot soil; $N_{BIO}+T_{CK}$, in the profile of the nursery soil added with bio-organic fertilizer and untreated pot soil; $N_{CK}+T_{BIO}$, in the profile of the untreated nursery soil and pot soil added with bio-organic fertilizer; $N_{BIO}+T_{BIO}$, in the profile of both nursery and pot soil added with bio-organic fertilizer

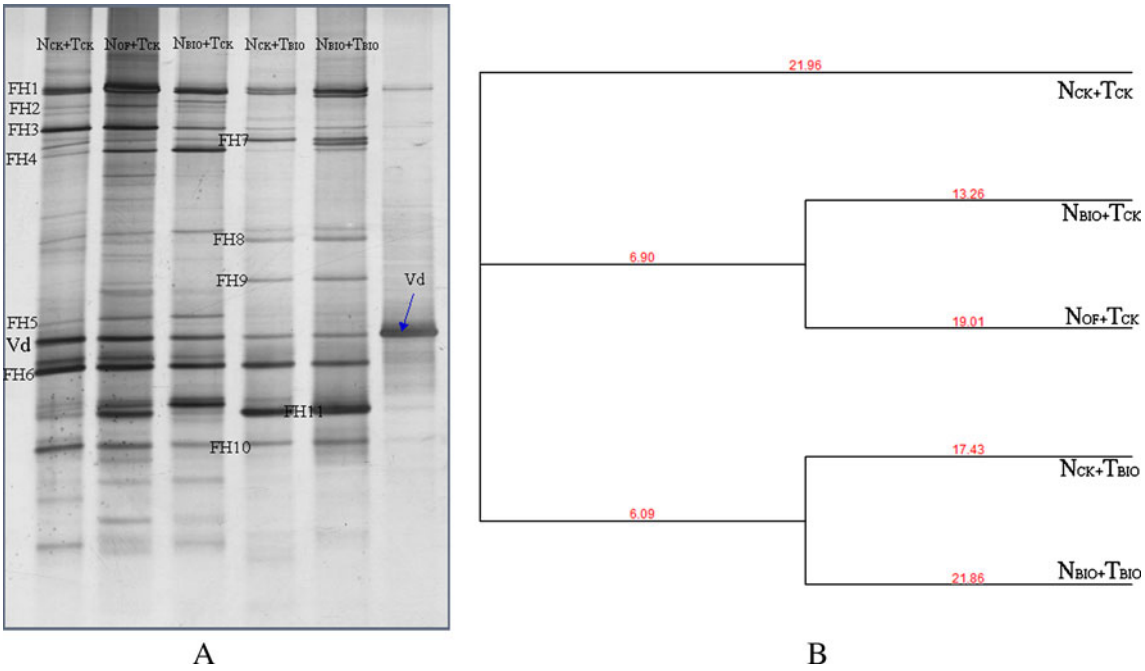

Fig. 3 The DGGE profiles of the fungal community in cotton rhizosphere soil at flowering and boll-forming stage. Bands indicated by numbers FH1 to FH11 were excised, and after re-amplification, subjected to sequencing; Vd is the DGGE profile of *V. dahliae*; $N_{CK}+T_{CK}$, in the profile of both untreated nursery and pot soil; $N_{OF}+T_{CK}$, in the profile of the nursery soil added with organic fertilizer and untreated pot soil; $N_{BIO}+T_{CK}$, in the profile of the nursery soil added with bio-organic fertilizer and untreated pot soil; $N_{CK}+T_{BIO}$, in the profile of the untreated nursery soil and pot soil added with bio-organic fertilizer; $N_{BIO}+T_{BIO}$, in the profile of both nursery and pot soil added with bio-organic fertilizer

flowering and boll-forming stage, the *H* values of the BIO ($N_{BIO}+T_{CK}$, $N_{CK}+T_{BIO}$, and $N_{BIO}+T_{BIO}$) treatments were smaller than that of $N_{CK}+T_{CK}$. At this stage the numbers of bands was 31 in the $N_{OF}+T_{CK}$, 22 in the $N_{CK}+T_{CK}$ and 14 in the $N_{CK}+T_{BIO}$ and $N_{BIO}+T_{BIO}$ treatments (Fig. 3a). It appeared that the BIO treatment inhibited *V. dahliae* and other fungi, with a gradual decrease in abundance and diversity over time. At the boll-opening stage, both the *H* value and the number of bands in $N_{CK}+T_{CK}$ treatment increased, whereas 22, 21, and 18 bands were present in $N_{BIO}+T_{CK}$, $N_{CK}+T_{BIO}$ and $N_{BIO}+T_{BIO}$ treatments, respectively (Fig. 4(A-1)). At harvest time, the *H* value of the $N_{BIO}+T_{BIO}$ treatment was the lowest (1.18) with only 17 bands, compared with 22, 22, 25, and 21 bands, respectively, in $N_{CK}+T_{CK}$, $N_{OF}+T_{CK}$, $N_{BIO}+T_{CK}$, and $N_{CK}+T_{BIO}$ treatments. At this stage, BIO was effective in controlling the growth of *V. dahliae*.

A total of 158 bands were excited for sequencing but only 36 bands gave successful sequences, and the rests failed to be sequenced (Table 3). Several different fungi were identified at the genus or family level. Of the 17 bands at the seedling stage, two bands were identified as *Alternaria alternate* (band FL3) and *Catenomyces* sp. (band FL5), whereas the rest were probably uncultured fungi. Of the 24 bands at the budding period, three bands were identified as *Eimeriidae* (band FJ1), *V. dahliae* (band FJ4) and *Madurella* sp. (band FJ5) whereas the rest were uncultured fungi. Of the 30 bands from at flowering and boll-forming stage, however, only four bands were successfully sequenced; band FH2 was an uncultured fungi, whereas three bands were identified as *Rhizophlyctis rosea* (band FH1), *Spizellomycete* sp. (band FH2), and *Mortierella wolfii* (band FH7), respectively. Of the 32 bands at the boll-opening stage, no identified fungus was found. There were 28 bands at harvesting; six bands were uncultured fungi and two bands were uncultured Eukaryota, whereas nine bands were identified as *Glomus claroideum* (band FF1), *Humicola* sp. (band FF2), *Hemimycena gracilis* (band FF3), *Termitomyces clypeatus* (band FF6), *Chaetomium* sp. (band FF10), *Coniochaeta velutina* (band FF11), *Metarhizium anisopliae* (band FF16), fungal sp. (band FF17), and *Chaetothyriales* sp. (band FF19), respectively. Several bands with identical GenBank matches were detected among the five treatments throughout the entire cotton growing season. For example, uncultured fungi of both FL7 and FL9 bands at the seedling stage had identical GenBank matches as well as uncultured, Chytridiomycota of FL2 band from the seedling stage and FJ2 band from the budding period, uncultured fungi FF5 band from the harvesting time and FJ7 band from the budding period, and uncultured fungi of *soil* FF15 band from the harvesting time and FH2 band from the flowering and boll-forming stage.

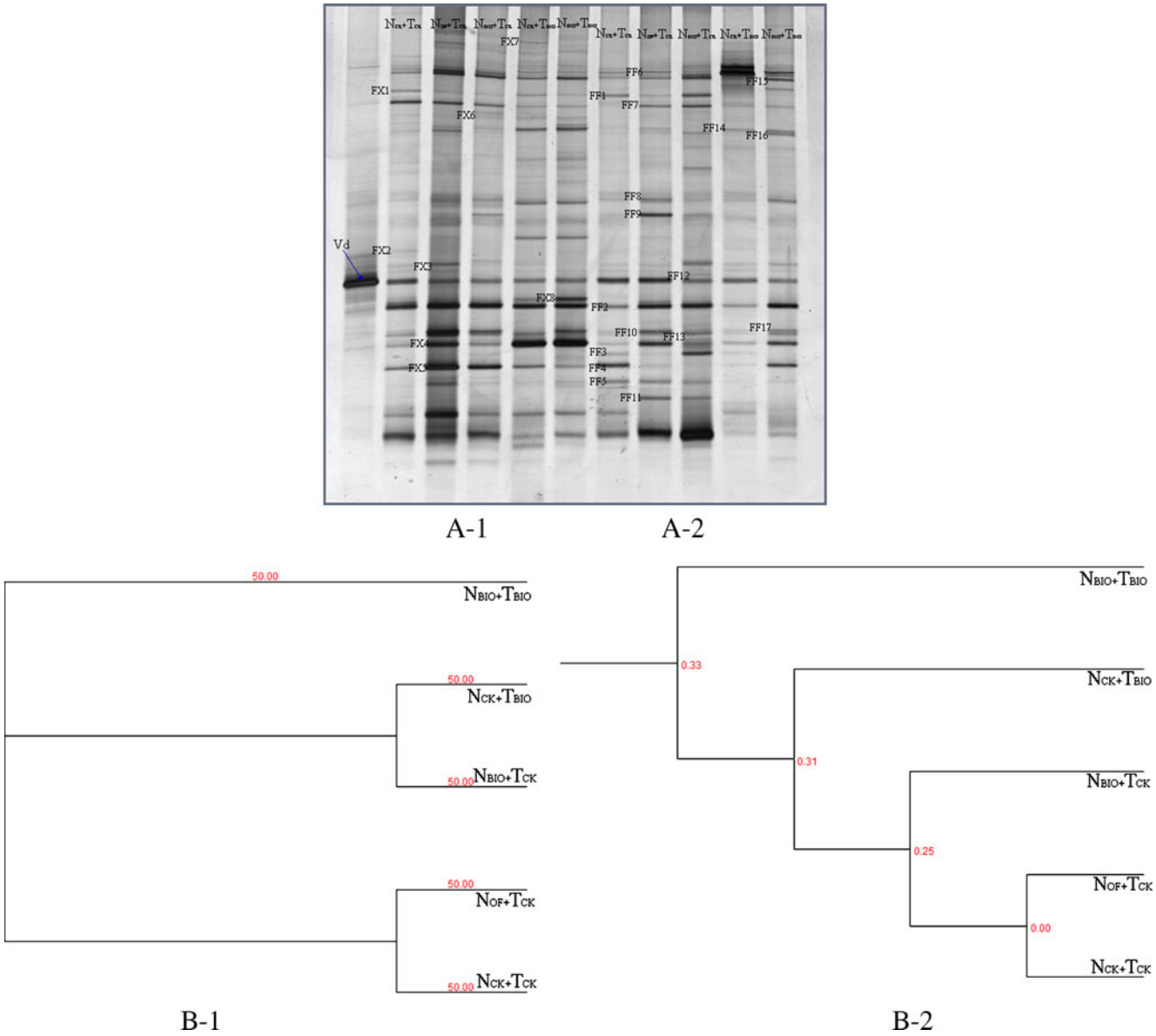

Fig. 4 The DGGE profiles of the fungal community in cotton rhizosphere soil in the boll-opening stage (*A-1*, *B-1*) and harvest time (*A-2*, *B-2*), respectively. Bands indicated by numbers FX1 to FX8 and FF1 to FF19 were excised, and after re-amplification, subjected to sequencing; Vd is the DGGE profile of *V. dahliae*. $N_{CK}+T_{CK}$, in the profile of both untreated nursery and pot soil; $N_{OF}+T_{CK}$, in the profile of the nursery soil added with organic fertilizer and untreated pot soil; $N_{BIO}+T_{CK}$, in the profile of the nursery soil added with bio-organic fertilizer and untreated pot soil; $N_{CK}+T_{BIO}$, in the profile of the untreated nursery soil and pot soil added with bio-organic fertilizer; $N_{BIO}+T_{BIO}$, in the profile of both nursery and pot soil added with bio-organic fertilizer

Furthermore, *V. dahliae* was detected in DGGE fingerprints throughout the growing seasons except the seedling stage.

Adjustment of conditions for real-time PCR assays

A standard curve was established by plotting the logarithmof tenfold serial plasmid dilutions ranging from 3.1×10^8 to 3.1×10^4 copies against Ct values obtained from real-time PCR. Plotting fluorescence intensity against the cycle number resulted in a characteristic sigmoidal kinetic function for various concentrations of target DNA. An average squared regression (R^2) of 0.9992 indicated a good correlation between the amount of template and the Ct values. Dissociation of the PCR reactions consistently produced a single peak, demonstrating the presence of only one product in the reaction.

Detection of *V. dahliae* in the rhizosphere of cotton plants by real-time PCR

Real-time quantitative assays were used to estimate (and corroborate) the actual of *V. dahliae* population in the five

Table 3 Phylogenetic relationships of cloned sequences

DGGE band	Closest relatives microorganisms (phylogenic affiliations)	Accession number	Similarity (%)
FL1	Uncultured soil fungus	GU568170.1	99
FL2	Uncultured Chytridiomycota	GQ995358.1	99
FL3	*Alternaria alternata*	HM165489.1	99
FL4	Uncultured fungi	DQ865561.1	99
FL5	*Catenomyces* sp.	AY635830.1	98
FL7	Uncultured fungi	DQ865553.1	99
FL8	Uncultured fungi	DQ865522.1	99
FL9	Uncultured fungi	DQ865553.1	99
FJ1	Eimeriidae	EF023211.1	98
FJ2	Uncultured Chytridiomycota	GQ995358.1	98
FJ4	*Verticillium dahliae*	AF104926.1	100
FJ5	*Madurella* sp.	EU815932.1	99
FJ6	Uncultured fungus	GU054062.1	98
FJ7	Uncultured soil fungus	DQ420789.1	99
FJ9	Uncultured fungus	HM104557.1	98
FH1	*Rhizophlyctis rosea*	AF164250.1	100
FH2	Uncultured fungus	HM104558.1	99
FH3	*Spizellomycete* sp.	DQ536490.1	98
FH7	*Mortierella wolfii*	AF113425.1	98
FF1	*Glomus claroideum*	Y17636.2	99
FF2	*Humicola* sp.	EU710839.1	100
FF3	*Hemimycena gracilis*	DQ440644.1	98
FF5	Uncultured soil fungus	DQ420789.1	99
FF6	*Termitomyces clypeatus*	HM036344.1	99
FF7	Uncultured fungus	HM104366.1	100
FF8	Uncultured soil fungus	EU826907.1	99
FF9	Uncultured eukaryote	FJ991634.1	100
FF10	*Chaetomium* sp.	EU710830.1	99
FF11	*Coniochaeta velutina*	GQ154626.1	98
FF12	Uncultured fungus	FJ626924.1	99
FF13	Uncultured eukaryote	FJ990740.1	99
FF14	Uncultured *Chaetomium*	GU055594.1	99
FF15	Uncultured fungus	HM104558.1	99
FF16	*Metarhizium anisopliae*	EU307924.1	100
FF17	Fungal sp.	EU940071.1	100
FF19	*Chaetothyriales* sp.	FJ358320.1	98

treatments of the rhizosphere soil of cotton plants at different growing stages and the results indicated a high degree of uniformity among the different treatments (Table 4). There was a difference between the $N_{CK}+T_{BIO}$ and $N_{BIO}+T_{BIO}$ treatments at the budding period, and the abundance of *V. dahliae* populations were less than 10^3 cfu g^{-1} soil. The abundance of *V. dahliae* was the greatest in the $N_{CK}+T_{CK}$ and $N_{OF}+T_{CK}$ treatments, with intermediate value of 1.60×10^3 cfu g^{-1} in the $N_{BIO}+T_{CK}$ treatment. At the flowering and boll-forming stage, there was no difference of *V. dahliae* populations between the $N_{BIO}+T_{CK}$ and $N_{CK}+T_{BIO}$ treatments, numbers of these treatments were lower than in the $N_{CK}+T_{CK}$ and $N_{OF}+T_{CK}$ treatments but higher than in the $N_{BIO}+T_{BIO}$ treatment. At the boll-opening stage, there were no differences in the abundance of *V. dahliae* among the $N_{BIO}+T_{CK}$, and $N_{CK}+T_{BIO}$, $N_{BIO}+T_{BIO}$ treatments, which had smaller *V. dahliae* numbers than the $N_{CK}+T_{CK}$ and $N_{OF}+T_{CK}$ treatments. At the harvest time, the numbers of *V. dahliae* were highest in the $N_{CK}+T_{CK}$ and $N_{OF}+T_{CK}$ treatments and lowest in the $N_{BIO}+T_{BIO}$ treatment.

Discussion

Inhibition on cotton *Verticillium* wilt by *B. subtilis*-enhanced BIO

BIO containing *B. subtilis* HJ5 and *B. subtilis* DF14 effectively reduced the disease incidence of cotton *Verticillium* wilt (Table 2), in agreement with previous reports (Luo et al. 2010; Zhang et al. 2008a). Application at both nursery and transplanting stages was necessary to achieve the best effect although significant reduction occurred when only nursery soil application was done.

Formulation of the *Bacillus*-based biological control agent with organic matter is important for high biocontrol efficiency. The ability of *B. subtilis* to inhibit plant pathogens and to promote plant growth has been confirmed and reviewed comprehensively (Earl et al. 2008; Nagórska et al. 2007; Stein 2005), but experimental and commercial formulations of *Bacillus*-based biological controls combined with organic fertilizer have only recently been practiced, especially in China (Cao et al. 2011; Ling et al. 2010; Luo et al. 2010; Schisler et al. 2004; Wei et al. 2011; Yang et al. 2011; Zhang et al. 2011; Zhao et al. 2011). There have been some reports of soil-borne disease suppression by the application of organic fertilizer or compost without antagonist inoculation (Bailey and Lazarovits 2003), but the results were inconsistent (Noble and Coventry 2005). In the present study, amending nursery soil only with the organic fertilizer without HJ5 and DF14 inoculation had no significant reduction of disease incidence on the control of *Verticillium* wilt. This suggests that it is needed to supply BIO with specifically functional microorganisms to control soil-borne disease.

Bacillus species can ferment and proliferate on a wide range of organic wastes, such as raw sewage sludge and bark (Chae Gun and Shoda 1990), soybean curd residue (Ohno et al. 1996), seaweed waste (Tang et al. 2007), wheat middlings (Pryor et al. 2007), and matured composts. To make more effective the HJ5 and DF14 strains in the BIO, a

Table 4 Estimation of *Verticillium dahliae* in cotton rhizosphere soil at different growth stages using real-time PCR (10^3 cfu g^{-1})

Treatment	Growth stage			
	Budding	Flowering and boll formation	Boll opening	Harvest
$N_{CK}+T_{CK}$	6.12±0.02 b[a]	3.43±0.52 b	5.17±0.03 b	7.03±0.93 a
$N_{OF}+T_{CK}$	6.71±0.27 a	4.27±0.40 a	10.97±2.59 a	6.76±0.16 a
$N_{BIO}+T_{CK}$	1.60±0.08 c	1.83±0 c	0.81±0.24 c	1.66±0.28 c
$N_{CK}+T_{BIO}$	0.66±0.03 d	1.71±0.13 c	2.90±0.65 b, c	2.88±0.10 b
$N_{BIO}+T_{BIO}$	0.85±0.07 d	0.31±0.19 d	0.46±0.46 c	0.17±0 d

Treatment: $N_{CK}+T_{CK}$, in the profile of both untreated nursery and pot soil; $N_{OF}+T_{CK}$, in the profile of the nursery soil added with organic fertilizer and untreated pot soil; $N_{BIO}+T_{CK}$, in the profile of the nursery soil added with bio-organic fertilizer and untreated pot soil; $N_{CK}+T_{BIO}$, in the profile of the untreated nursery soil and pot soil added with bio-organic fertilizer; $N_{BIO}+T_{BIO}$, in the profile of both nursery and pot soil added with bio-organic fertilizer

Values within the same column followed by the same letter do not differ at $p<0.05$

[a] Values differ at $p<0.05$ at different growing seasons. Mean±SD

good mixture of amino acid fertilizer and pig manure compost was suggested to not only control cotton *Verticillium* wilt but also to promote plant growth.

Fungal diversity related to the application of the BIO

A distinct fungal DGGE pattern was observed in the rhizosphere soil of cotton plants added with *B. subtilis* HJ5- and *B. subtilis* DF14-enhanced BIO, and the application of BIO affected the composition of fungal communities, which was in agreement with previous reports (Luo et al. 2010). The band corresponding to *V. dahliae* in the $N_{BIO}+T_{BIO}$ treatment was weaker than that in the $N_{CK}+T_{CK}$ treatment (Figs. 1a, 2a, 3a, and 4(A-1, A-2)), indicating that the fungal population in the $N_{BIO}+T_{BIO}$ treatment was smaller than in the $N_{CK}+T_{CK}$ treatment. The Shannon–Wiener index of each treatment exhibited the same trend being increased up to boll-opening then being decreased at harvest (Table 5). This trend may be related to the metabolic activity of the cotton plants and to the ground temperature at different growing stages. A similar phenomenon has been reported previously (Li et al. 1998).

The severity of *Verticillium* wilt on cotton is related to the population of *V. dahliae* in the rhizosphere soil, and the application of BIO was shown to reduce *V. dahliae* and thus result in a consequent reduction of disease symptom. The mechanism by which BIOs reduce the *V. dahliae* population in the rhizosphere could be attributed to the fact that *B. subtilis* can produce a variety of anti-fungal compounds (Earl et al. 2008; Harwood et al. 2001; Nagórska et al. 2007; Stein 2005), and several biocontrol strains of *B. subtilis* can destroy the fungal cell wall (Chaurasia et al. 2005; Manjula and Podile 2005; Romero et al. 2007). We proposed that the HJ5- and DF14-enhanced BIO had a fungistatic effect on the rhizosphere soil, changing its apparent diversity.

Table 5 Shannon–Wiener indexes of the DGGE gel bands at different treatments and different cotton growth stages

Growth stage	Treatment							
	N_{CK}	N_{OF}	N_{BIO}	$N_{CK}+T_{CK}$	$N_{OF}+T_{CK}$	$N_{BIO}+T_{CK}$	$N_{CK}+T_{BIO}$	$N_{BIO}+T_{BIO}$
Seedling[a]	1.15	0.99	0.95	–	–	–	–	–
Budding[a]	–	–	–	1.07	1.09	1.00	1.05	0.83
Flowering and boll forming[a]	–	–	–	1.24	1.39	1.21	1.07	1.10
Boll opening[a]	–	–	–	1.33	1.36	1.29	1.29	1.19
Harvest[a]	–	–	–	1.23	1.20	1.31	1.22	1.18

Treatment: N_{CK}, in the profile of the control of nursery stage; N_{OF}, in the profile of nursery soil added with organic fertilizer; N_{BIO}, in the profile of nursery soil added with bio-organic fertilizer; $N_{CK}+T_{CK}$, in the profile of both untreated nursery and pot soil; $N_{OF}+T_{CK}$, in the profile of the nursery soil added with organic fertilizer and untreated pot soil; $N_{BIO}+T_{CK}$, in the profile of the nursery soil added with bio-organic fertilizer and untreated pot soil; $N_{CK}+T_{BIO}$, in the profile of the untreated nursery soil and pot soil added with bio-organic fertilizer; $N_{BIO}+T_{BIO}$, in the profile of both control at the nursery and pot soil added with bio-organic fertilizer

[a] different cotton growth stages

A total of 36 sequences (Table 3) were obtained from the DGGE gels (Figs. 1a, 2a, 3a, and 4(A-1, A-2)). The band with 100% similarity to *V. dahliae* based on alignments with the GenBank database was not present at the seedling stage indicating that the nursery soil was free of the fungus; however, it was detected at the budding period, the flowering and boll-forming stage, the boll-opening stage and at harvest time after transplanting of the plants to the diseased soils. *M. anisopliae* (Bruck 2009), which has been extensively studied for the biological control of a wide range of insect pests, was shown in the $N_{BIO}+T_{CK}$ and $N_{BIO}+T_{BIO}$ treatments. *Chaetomium* sp. (Suyanto et al. 2003), a thermophilic fungus that can decompose palm-oil mill fibers, was identified in all treatments except the $N_{CK}+T_{CK}$ treatment. These unique results might be related to the metabolic activities of microorganisms during the different cotton growing stages. In addition to soil condition, root exudates have a critical effect on the activity and composition of microbial communities in the rhizosphere (Bais et al. 2006; Nelson and Mele 2007). Indeed, minute changes in root exudates can markedly affect activity and composition of microbial communities in the rhizosphere, although the underlying mechanisms are unclear.

The effects of BIO on the of *V. dahliae* population

Large *V. dahliae* populations were observed in the rhizosphere soils of treatments with high disease incidences. When the *V. dahliae* population in the rhizosphere soil reached to 10^3 cfu g^{-1}, the cotton plants would wilt prior to the appearance of disease ($N_{CK}+T_{CK}$, $N_{OF}+T_{CK}$, $N_{BIO}+T_{CK}$, and $N_{CK}+T_{BIO}$ treatments). Therefore, the size of the *V. dahliae* population in the rhizosphere soil was a crucial factor in the incidence of cotton disease.

The growth of *V. dahliae* in soil was inhibited when BIO was applied both in the nursery and in the transplanted soil. The results suggested that double application of BIO could effectively prevent the occurrence of cotton *Verticillium* wilt disease, reduce the *V. dahliae* population in the rhizosphere soil, and significantly change the fungal community structure of the rhizosphere soils. Both Ling et al. (2009) and Zhao et al. (2010) reported that the experimental application of BIO could reduce both the disease incidence of watermelon Fusarium wilt and the counts of *Fusarium oxysporum* f. sp. *Niveum* in rhizosphere soil, probably due to the application of antagonistic microorganisms with the organic fertilizers. In the presence of available organic and inorganic nutrients, antagonistic bacteria can grow well during nursery stage after added to the soil and can form the so-called "bio-wall" on root surface or even in rhizosphere control the *V. dahliae* in the rhizosphere. The specific mechanism by which the "bio-wall" reduced the *V. dahliae* population need to be further studied.

Conclusions

In conclusion, the application of BIO not only changed the abundance and composition of fungal communities, but also significantly reduced *Verticillium* wilt disease symptoms and the *V. dahliae* population in the rhizosphere soil. Under field conditions it may be difficult to amend soil with a so large amount of BIO to obtain the level of *Verticillium* wilt control. However, nursery application plus a field application with appropriate rates is feasible in practice. The underlying mechanisms of BIO application to control soil-borne disease need to be further studied.

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