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Development of a mode of application of bioorganic fertilizer for improving the biocontrol efficacy to *Fusarium* wilt

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Abstract More effective ways of applying biocontrol products should be developed based both on the characteristics of the biocontrol agents and the normal practices of the agricultural producer. A new system was developed to improve the biocontrol efficacy of *Fusarium* wilt for watermelon production, and this system was tested in pot and field experiments. Biocontrol was achieved by applying a novel bioorganic fertilizer product (BIO) to *Fusarium*-infested soil. The best biocontrol was obtained by application of a bioorganic fertilizer, BIO, into soil during the nursery phase of watermelon seedling followed by a second application to *Fusarium*-infested soil when watermelon seedlings were transplanted. In comparison with the controls, the incidence of the disease was reduced by 60–100% in the pot experiment and by 59–73% in the field experiment when the BIO was applied during the nursery stage. After application of BIO during the nursery stage, the number of colony-forming units of *Fusarium oxysporum* in rhizospheric soil was significantly ($P < 0.05$) inhibited compared to the controls. An in vitro experiment showed that the antagonist *Paenibacillus polymyxa* in the BIO could effectively colonize the rhizosphere of watermelon and proliferate along the extending plant roots. This inhibited growth of *Fusarium oxysporum* in the rhizosphere of watermelon and protected the watermelon roots from attack by the pathogens. The method used for biocontrol *Fusarium* wilt disease in watermelon should be a useful strategy to improve field efficacy of other biocontrol agents.

Keywords Bioorganic fertilizer (BIO) · Nursery application · *Fusarium* wilt · Watermelon · *Paenibacillus polymyxa*

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Introduction

Watermelon (*Citrullus lanatus* (Trunb.) Matsum and Nakai) is a widely cultivated fruit that is consumed globally. However, the growth of this plant is often threatened by *Fusarium* wilt in soils where watermelon is continuously mono-cropped. *Fusarium* wilt in watermelon is caused by *Fusarium oxysporum* Schleicher: Fr. f. sp. *niveum* (E. F. Smith, W. C. Snyder and H. N.Hans) (Booth 1971). The fungus is found worldwide in soils in temperate, subtropical and tropical regions (Booth 1971). *Fusarium oxysporum* f.sp. *niveum* also causes damping-off, cortical rot and the stunting of watermelon seedlings (Booth 1971). It is considered to be the most important soil borne facultative parasite leading to economically

important losses of watermelon and limits production in many areas of the world (Martyn 1996).

Chemical control of *Fusarium* wilts relies to a large extent on the use of the fumigant methyl bromide in soils (Fuchs et al. 1999). However, methyl bromide is now prohibited in many countries of the world due to environmental and food quality concerns. The development of resistant cultivars is an attractive strategy against *Fusarium* wilts but new virulent races of *Fusaria* can appear within a few years after commercialization of resistant cultivars (Alabouvette et al. 1993; Fuchs et al. 1999). Biological control represents an alternative for protection of plants against *Fusarium* wilts.

The introduction of beneficial microorganisms into soil or the rhizosphere has been proposed for the biological control of soil-borne plant diseases (Cook 1993). There have been many studies on the application of antagonistic microbes for biocontrol of *Fusarium* wilts including the use of *Pseudomonas* spp., *Penicillium oxalicum*, *Bacillus subtilis*, non-pathogenic *F. oxysporum*, *Trichoderma* spp., and *Paenibacillus polymyxa* (Duffy and Weller 1995; Fuchs et al. 1999; Sabuquillo et al. 2006; Hervás et al. 19971998; Dijksterhuis et al. 1999). The incidence of several soil-borne plant pathogens have also been reduced by using composts made of different raw materials (Borrero et al. 2004; Cotxarrera et al. 2002; Hoitink and Boehm 1999; Hoitink and Fahy 1986; Lumsden et al. 1983). Since Hoitink et al. (1975) first suggested compost could be used as a peat substitute to control root pathogens, biocontrol research has increasingly focused on developing the right combination of composts and antagonistic microbes. *Trichoderma asperellum* in combination with composts from agricultural wastes, for example, was used to suppress *Rhizoctonia solani* in cucumber seedlings (Trillas et al. 2006), and *Trichoderma asperellum* and sewage sludge compost were used to suppress *Fusarium* wilt of tomato (Cotxarrera et al. 2002). Mature composts are known to better sustain biological control agents, whereas immature composts do not. Immature composts also can negatively affect the growth of crop plants if they introduce pathogens to the soil or growing medium (Litterick et al. 2004). Currently it is believed 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 (Trillas et al. 2006; Cotxarrera et al. 2002; Sivan and Chet 1992).

While biocontrol may be found effective under laboratory or greenhouse conditions, the use of biological control agents often fails to control disease in the field. One reason explaining this failure may be that the biocontrol products are often used in the same way as a chemical product. However, rhizosphere competence of biocontrol agents consists of effective root colonization and the ability to survive and proliferate along extending plant roots over a considerable time period. This is especially important when there are many soil-borne pathogens and strong competition exists between the pathogens and the added biocontrol agents for niches and nutrients (Kamilova et al. 2005; Timmusk et al. 2005). Thus, the suppression of disease by biocontrol agents is not always observed and efficiency varies with the application method, time, and ambient conditions (Alabouvette et al. 2006; Brunner et al. 2005; Georgakopoulos et al. 2002). Therefore, to obtain successful biocontrol, the biocontrol product has to be applied in accordance with sound ecological requirements, and additional work is greatly needed to make the biocontrol agents more efficient and practical under field conditions.

Paenibacillus polymyxa has been found not only to be a plant-growth-promoting rhizobacteria, but also to be an effective biocontrol agent. It can induce plant systemic resistance, and excrete antibiotics and enzymes that can suppress the proliferation of pathogens (Dijksterhuis et al. 1999; Raza et al. 2008). The microbe was also reported to effectively colonize the plant root rhizosphere or even form biofilms on the root surface (Timmusk et al. 2005). We developed a novel bioorganic fertilizer (BIO) by fermenting mature compost with the antagonistic microbe, *P. polymyxa.* This bioorganic fertilizer was previously shown to suppress *Fusarium* wilt disease, and promote growth of watermelon and cucumber plants in greenhouse and field experiments (Zhang et al. 2008; Wu et al. 2008). However, field application of this bioorganic fertilizer is costly for farmers and the way it is applied to the soil–plant system is not in accord with the ecological requirements of the biocontrol agents. The present study was, therefore, carried out to assess biocontrol of *Fusarium* wilt disease by BIO application either into the nursery soil only or into nursery soils and again in the transplanted soils under pot and field experiment conditions. A major goal is to use the information

obtained from these experiments to clarify possible interactions that occur between plant rhizosphere and microbes and to provide guidelines of how to use biocontrol agents more effectively in actual field situations.

Materials and methods

Bioorganic fertilizer preparation

The antagonistic microbe contained in the BIO product is *Paenibacillus polymyxa* (SQR21). The bacterial strain was isolated from the rhizosphere of healthy watermelon plant roots collected from a field where there had been a severe case of watermelon *Fuarium* wilt by the Provincial key lab of organic solid waste utilization, Jiangsu, China. This strain has been found to be highly efficient against *Fusarium oxysporum* causing cucumber and watermelon wilt disease (Zhang et al. 2008; Wu et al. 2008). Screening, purification, morphological and biochemical characterization and phylogenetic analysis of 16SrRNA sequences were also conducted in this laboratory. The antagonist was grown in beef extract and peptone liquid culture (3 g beef extract, 10 g peptone liquid, 5 g NaCl and 1 l sterilized water, pH 7.0–7.2) on a shaker at 170 rpm at 37°C for 2–3 days. This culture was then used directly to prepare the BIO product as described later.

Organic fertilizer, used for the BIO product, was composed of amino acid fertilizer and pig manure compost. Amino acid fertilizer was made from oil rapeseed cakes that were enzymatically hydrolyzed by aerobic microbial fermentation at <50°C for seven days (Zhang et al. 2008). This amino acid fertilizer contained 44.2% organic matter and 12.9% of amino acids, small molecular peptides and oligo peptides. The nutrient content was 4.4% nitrogen (N), 2.3% P_2O_5, and 0.7% K_2O. Pig manure compost was made by Tianniang Ltd. in Suzhou by composting pig manure at a temperature range of 30°C–70°C for 25 days. This compost contained 30.4% organic matter, 2.0% N, 3.7% P_2O_5, and 1.1% K_2O.

The BIO product used in this experiment was obtained by aerobically fermenting a mixture of amino acid fertilizer and pig manure compost (1:1, w/w) with the SQR21 for six days at <45°C. The density of SQR21 was 5×10^9 CFU g^{-1} dry weight (DW) BIO at the end of fermentation. The BIO product was stored at less than 25°C prior to use in experiments.

Experimental designs

To allow for good colonization in the plant root rhizosphere, application of the BIO was made in nursery pots. Watermelon seedlings were grown in the nursery pots until the seedlings had 3–4 true leaves, and then transplanted to pots with infested soil or to infested fields. Transplanting was conducted by transferring all of the nursery soil with the plant seedlings to the new locations (Fig. 1).

Seedling incubation in nursery pots for pot and field experiments

Watermelon seeds, the cultivar Kangbing Jingxin, were surface-sterilized in 2% NaClO for 3 min, rinsed for three times in sterile water, and then germinated in 9 cm plates covered with sterile wet filter paper at 30°C. The germinated seeds were grown in nursery pots with 300 g healthy nursery-soil (i.e., never planted with watermelon before), and one seedling was maintained in each nursery pot. Two initial treatments were designed for this nursery stage of the experiment. One treatment was growing seedlings in soil without an application of BIO (CKns) and the other treatment involved treating soil

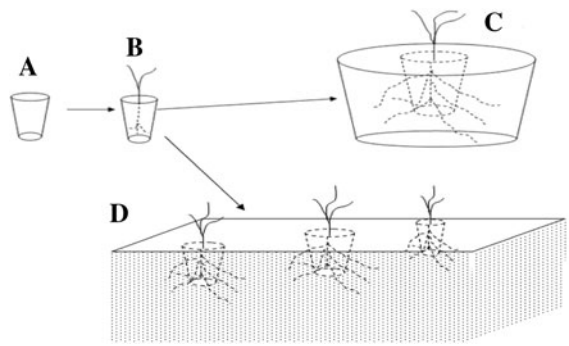

Fig. 1 An illustration of nursery incubation of seedlings. Nursery pots (**A**) with healthy soils to which 2% of BIO was added, **B** where seeds were germinated and seedlings were grown, **C** where seedlings were transplanted into pots containing infested soils and transplanting was conducted by transferring all of the nursery soil with the plant to the new big pot in 2007 and 2008, and **D** where seedlings were transplanted into infested fields and transplanting was conducted by transferring all of the nursery soil with the plant to the infested field in 2008

with 2% (w:w) of BIO (BIOns). The CKns was treated with chemical fertilizer containing equivalent nutrients (192 mg N, 180 mg P_2O_5, 54mgK_2O) of the BIO amendment (2%). The seedlings were grown in the greenhouse with temperature ranging from 26°C to 35°C during the incubation period.

Pot and field design

The seedlings with 3–4 true leaves were transplanted into pots or into fields. The soils in the pots or fields were further assigned to two secondary treatments: (1) the soils of pots or fields into which seedlings were transplanted were not supplemented with BIO (CKts) and (2) the soils of pots or fields into which seedlings were supplemented with BIO (BIOts). The rate of BIO application for the pot experiment was 0.5% (dry weight basis) and the BIO was mixed with the soil prior to transplanting of the watermelon seedlings. For the field experiment, before seedlings were transplanted, 40 g of BIO was added into each hole where one plant seedling was placed. The soils of pots or fields for the control treatment (CKts) were treated with chemical fertilizer containing equivalent nutrients (NPK) of the BIO treatment (0.5%) (pot, 0.90 g N, 0.84 g P_2O_5, 0.25 g K_2O) or 40 g of BIO per plant (field, 1.28 g N, 1.20 g P_2O_5, 0.36 g K_2O), respectively.

Overall, a total of four factorial treatments were created. These treatments included (1) No BIO was applied to nursery pots during seedling stage and to soils of the pots or to the fields at the time the watermelon seedlings were transplanted (CKns + CKts); (2) no BIO was applied to nursery pots during seedling stage but BIO was applied to soil in pots or to the fields at the time the watermelon seedlings were transplanted (CKns + BIOts); (3) BIO was applied to nursery pots during seedling stage but no BIO was applied to pots or to the fields at the time the watermelon seedlings were transplanted (BIOns + CKts); and (4) BIO was applied to nursery pots during seedling stage and also applied to the pots or to the fields at the time the watermelon seedlings were transplanted (BIOns + BIOts).

Pot experiment description

The pot experiment was carried out in a greenhouse located at Nanjing Agricultural University, China during September to November 2007. In 2008, the same pot experiment was repeated at the same stage as 2007 but in a greenhouse located in Yixing, 120 km away from Nanjing. The temperature ranged from 23°C to 30°C and the relative humidity from 60% to 85%. The soil used in pot experiments was collected from the surface of continuous mono-cultivated watermelon plots located in Jiangyin county, Jinagsu province, China. This plot had a two-year history of continuous watermelon cultivation and watermelon plants grown in this soil were infested with *Fusarium oxysporum* f.sp. *niveum*. Each pot (diameter 30 cm, height 35 cm) was filled with 10 kg of the infested soil (44% moisture) and planted with one seedling. The soil in each pot was again inoculated with 10 ml of conidial suspension containing 3×10^6 conidia of *Fusarium oxysporum* f.sp.*nevium* per milliliter before transplanting. This was done to enhance the inoculum density.

Ten replicates were included for each treatment. Disease incidence (DI) was assessed as the percentage of infected plants over the total number of plants. The bioassay of disease incidence was performed 50 days after transplanting when all of the plants in the control had wilted and died. Samples of plant and soil were also collected at this time to determine plant biomass, and pathogenic colonization.

Field experiment description

Field experiments were conducted from March 1 to June 26, 2008 in a plastic greenhouse located in Jiangyin county, Jiangsu province, China. The field had a two-year history of continuous watermelon cultivation, and *Fusarium* wilt disease in watermelon had been prevalent in the previous season. There were four treatments as the design and each treatment had three replicates. The area of each plot was 35 m^2 and 28 watermelon plants were planted in each plot. The treatments were organized in a completely randomized blocks design.

Nursery watermelon seedlings prepared as described above were incubated in a plastic greenhouse at a temperature from 18°C to 25°C from January 28 to March 12, 2008. Prior to transplanting, holes were created into which the seedlings were transplanted. These holes had a diameter of about 10 cm and a depth of about 15 cm. The soil that had been removed was then thoroughly mixed with 40 g BIO and then returned to the holes just prior to transplanting of the watermelon seedlings.

After transplanting, the plants were grown at temperatures ranging from 24°C to 38°C and 270 kg N ha^{-1}, 140 kg P_2O_5 ha^{-1} and 300 kg K_2O ha^{-1} were applied with water for 12 times during the whole growth stage as is typical farmering practice. Mature watermelons were harvested and fruit yields were recorded. Disease incidence (DI) was recorded 110 days after transplanting and was expressed as the percentage of infected plants over the total number of plants assigned to a treatment.

Determination of rhizosphere colonization by the pathogen in pot experiment

The number of *Fusarium oxysporum* colony forming units (CFU) in the plant root rhizosphere was determined by making serial dilutions of rhizosphere soil on Komada's medium (Komada 1975) which was specific for isolation of *Fusarium oxysporum* in petriplates. It consisted of 1.0 g K_2HPO, 500 mg KCI, 500 mg $MgSO_2{\cdot}7H_2O$, 10 mg Fe–Na-EDTA, 2.0 g L-asparagine, 20.0 g D-galactose, 15.0 g agar, and 1.0 l water. Then the above mixture was autoclaved and allowed to cool before pouring. The following supplements were added and the medium was mixed thoroughly: 1.0 g pentachloronitrobenzene (PCNB, 75% WP), 500 mg oxgall, 1.0 g $Na_2B_4O_7{\cdot}10H_2O$, and 300 mg streptomycin sulfate. Finally, the pH of the medium was adjusted 3.8 ± 0.2 with a 10% phosphoric acid solution before pouring. The strains belonging to the *F. oxysporum* were identified by their morphological characteristics (Komada 1975) and quantified by counting.

In the pot experiment, three plant replicates were sampled randomly from each treatment. The rhizosphere soil was obtained as described by Hervás et al. (1998). The watermelon plant was carefully uprooted from the pots and shaken gently to remove the soil but some soil remained within the root systems. The roots with some rhizosphere soil were cut into 1-cm segments, and 1 g of these segments was placed into 9 ml of sterile water and sonicated for 15 min to separate bacteria and/or fungi from the roots and rhizosphere soil. Serial 10-fold dilutions of the washings were plated onto Komada's medium (Komada 1975). Colonies were counted after incubation at 28°C for five days and expressed as cfu per g of fresh root tissue.

Colonization capacity of SQR21

Colonization capacity of the SQR21 was tested in another separate in vitro experiment as described below.

Plant material and growth conditions

Surface sterilized seeds of watermelon were placed in a sterilized plate and incubated at 28°C for germination. When the cotyledon emerged, the germinated seeds were again surface sterilized. All the surface sterilization were done in 70% ethanol for 15 s, exposed in 1% mercuric chloride for 5 min and then rinsed six times using sterile distilled water.

The treatment in this experiment was to soak germinated and sterilized seeds in 5 ml of a SQR21 culture (concentration of SQR21 was 10^{11} cfu ml^{-1}) for 24 h at 28°C. The control treatment was to soak seeds in sterile distilled water. All the treated seeds were sown in tissue culture bottles containing an inorganic nutrient medium and subsequently grown for two weeks in a growth chamber at 28°C with a 16 h light regime. The light intensity used was 200 microeinsteins m^{-2} s^{-1}. All the operations were done under sterilized conditions and care was taken to avoid contamination during growth and handling of the plants. The inorganic nutrient medium was prepared as follow: $Ca(NO_3)_2$, 826 mg l^{-1}; K_2SO_4, 607 mg l^{-1}; NH_4NO_3, 53 mg l^{-1}; $MgSO_4$, 370 mg l^{-1}; KH_2PO_4, 181 mg l^{-1}; EDTA-Fe, 5 mmol l^{-1}; Hoagland microelements; Phytagel (Sigma), 3.2 g l^{-1}; pH 6.6–6.8. The medium was sterilized in an autoclave. Before solidified, the medium was treated with iodonitrotetrazolium chloride (INT) solution that had been filter sterilized (0.22 μm). Final concentration of the INT was 10 mg l^{-1}.

Detection of the SQR21 in rhizosphere of watermelon

INT, an electron accepter, becomes red where there is microbial activity. The red reaction observed during plant growth was recorded. After growing for two weeks, the root tip of the watermelon was collected and placed on a glass microscope slide, and then instantly covered by a coverslip. The slide with the root tip was then observed under an optical microscope.

Data analysis

Disease reduction percentage (DRP) was calculated using the following equation:

$$DRP = (1 - D_T/D_C) \times 100,$$

where D_C and D_T were the disease incidence percentages in control and treatments, respectively. Pot experiments were repeated over two years and a very similar trend of response was observed. Therefore only data for the 2007 pot experiment is presented, except for the DRP data which included two years' data (Table 1). Watermelon yields of each treatment in the field experiments were calculated as a cumulative weight of watermelon fruits per ha.

Differences among treatments were assessed with one-way ANOVA at the end of each assay. Duncan's multiple range test was used when one-way ANOVA revealed significant differences ($P \leq 0.05$). All statistical analyses were performed with SPSS BASE ver.11.5 statistical software (SPSS, Chicago, IL, USA).

Results

Assessment of *Fusarium* wilt disease in experiments

Application of BIO both in the seedling nursery soil and in transplanted soil resulted in the lowest *Fusarium* wilt disease incidence of watermelon in both pot and field experiments (Table 1). As long as BIO was amended into the seedling nursery soil, the disease incidence was far lower than the control for both the pot experiment and the field experiment. The biocontrol efficacies were 60% and 50% in pot experiments in 2007 and 2008, respectively, and 59% in the field experiment. Disease incidence rate was still high in the treatment where BIO was only added to the transplanted soil, reaching 100% and 60% in 2007 and 2008, respectively in pot experiments and 71% in the field experiment ($F = 77.91$; df = 3, 8; $P = 0.0001$). This showed that applying BIO to nursery soil was a significantly effective way to control watermelon wilt disease. However, the best biocontrol efficacy could be obtained if BIO was applied to both the nursery soil and the transplanted soil and the biocontrol efficacies were 100% and 87.5% in pot experiments in 2007 and 2008, respectively, and 73% in the field experiment (see the last column in Table 1). This high biocontrol efficacy was attributed to an intensive colonization of SQR21 on the surface of watermelon roots.

Effect of BIO on watermelon biomass in pot experiment in 2007

BIO application not only suppressed *Fusarium* wilt but also significantly promoted watermelon growth. In the pot experiment in 2007, the lowest plant dry weight (8.73 g plant^{-1}) was found in the control, while the highest was found in plants in the treatment of BIO applied to both the nursery soil and the transplanted soil (16.0 g plant^{-1}, $F = 18.18$; df = 3, 8; $P = 0.0006$). Little difference was observed

Table 1 Disease incidence (DI) rate of watermelon *Fusarium* wilt and disease reduction percentage (DRP) in pot experiments in 2007 and 2008 and field experiments in 2008

Type of experiment	year		Treatments[x]			
		Variable	CKns + CKts (%)	CKns + BIOts (%)	BIOns + CKts (%)	BIOns + BIOts (%)
Pot experiment	2007	DI	100	100	40	0
		DRP	–	0	60	100
	2008	DI	80	60	40	10
		DRP	–	25	50	87.5
Field experiment	2008	DI	85 ± 5a[y]	71 ± 6b	35 ± 9c	23 ± 3d
		DRP	–	16	59	73

[x] CKns, nursery pot with no BIO; CKts, fields with no BIO; BIOts, fields treated with BIO; and BIOns, nursery pot treated with BIO

[y] Values with the different letter within the same line are significantly different at $P < 0.05$ according to Duncan's test. Numbers follow by the "±" are standard errors (SE)

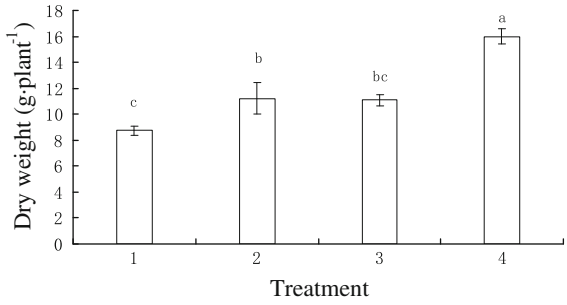

Fig. 2 Effect of BIO on the dry weight of watermelon plants grown for 50 days after transplanting in the pot experiment in 2007. Treatments are 1 (CKns + CKts), 2 (CKns + BIOts), 3 (BIOns + CKts), and 4 (BIOns + BIOts). All values are the means of three replications. Columns with different letters are significantly different at $P < 0.05$ according to post hoc tests (Duncan's test). Bars indicate the standard deviation of the mean

between the CKns + BIOts and BIOns + CKts treatments and the dry weights for these treatments were only 1.28-fold and 1.27-fold greater compared to the control, respectively (Fig. 2).

Effect of BIO on watermelon yield in field experiment in 2008

In the field experiment, the watermelon yield of the treatments with BIO was significantly ($P < 0.05$) higher than in the control (Fig. 3). The highest yield (50.8 Mg ha^{-1}, $F = 71.76$; df = 3, 8; $P = 0.0001$) was obtained in the treatment of BIO applied both in the seedling nursery soil and the transplanted soil in the field. The yield was increases by 4.87 times compared with the control. This yield increment was great enough to than cover the economic cost of the input of the BIO by farmers.

The yields were negatively correlated with *Fusarium* wilt disease incidence of watermelon plants (Table 1 and Fig. 3). These data confirmed that the addition of BIO effectively controlled watermelon wilt disease and increased the watermelon yields in a continuous mono-cultivation system.

Effect of BIO on the rhizosphere colonization of *Fusarium oxysporum* in pot experiments in 2007

There was a significant difference ($P < 0.05$) in CFU levels of *Fusarium oxysporum* in the rhizosphere between the treatments with (Treatments 3 and 4) or without (Treatments 1 and 2) application of BIO to the nursery soil (Fig. 4). However, if BIO was added to soils at the time the watermelon seedlings were transplanted, there was no significant effect on CFU levels of *Fusarium oxysporum* as can be seen by comparing Treatments 1 and Treatments 3 (Fig. 4). The lowest population of *Fusarium oxysporum* was obtained in the BIOns + BIOts treatment, i.e., 7.0×10^2 cfu g^{-1} fresh root. In contrast, 1.2×10^4 cfu g^{-1} fresh root of *Fusarium oxysporum* were detected in the control, which was much higher than that in the BIOns + BIOts treatment ($F = 18.17$; df = 3, 8; $P = 0.0006$). In the nursery soil treated with BIO, such as the BIOns + CKts or BIOns +

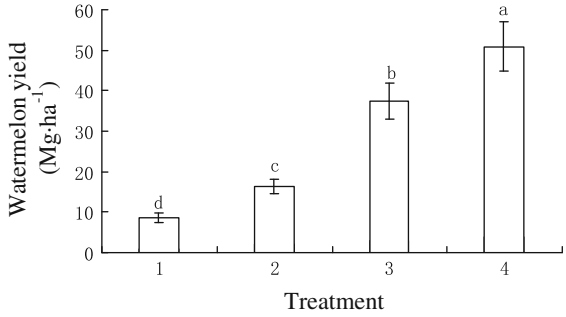

Fig. 3 Effect of BIO on watermelon yield in the field experiment in 2008. Treatments are 1 (CKns + CKts), 2 (CKns + BIOts), 3 (BIOns + CKts), and 4 (BIOns + BIOts). All values are the mean of three replicates. Columns with different letters are significantly different at $P < 0.05$ according to post hoc tests (Duncan's test). Bars indicate the standard deviation of the mean

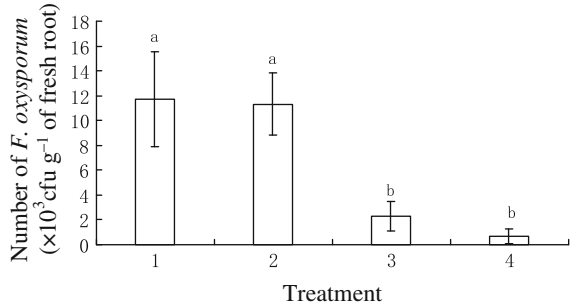

Fig. 4 Rhizosphere colonization by *Fusarium oxysporum* at the CFU levels in the pot experiments in 2007. Treatments are 1 (CKns + CKts), 2 (CKns + BIOts), 3 (BIOns + CKts), and 4 (BIOns + BIOts). All values are the means of three replications. Columns with different letters are significantly different at $P < 0.05$ according to post hoc tests (Duncan's test). Bars indicate the standard deviation of the mean

BIOts treatments, the CFU number of *Fusarium oxysporum* was inhibited and remained within the range of 7.0×10^2–2.3×10^3 cfu g^{-1} in the rhizosphere, which was significantly lower than in control ($F = 18.17$; df = 3, 8; $P = 0.0006$). This indicated that the survival of *Fusarium oxysporum f.sp.nevium* in the rhizoshere was significantly reduced by the presence of the biocontrol agents in the BIO that was applied in the nursery soil.

Assessment of colonization capacity of the strain SQR21

During the growing period of the watermelon plant, the root surface or rhizosphere treated with SQR21 always displayed an acutely red reaction, while that of the control was only slightly red (Fig. 5). The INT could also become red by the activity of roots, themselves, but because the intensity of red reaction of the control was much slighter than the treatment with SQR21, it can be concluded that intense colonization of the root by SQR21 occurred. The optical microscope images also clearly showed intense colonization of SQR21 on root surfaces that had been treated with SQR21 (Fig. 6C and D). In contrast, no microbes were found on root surfaces of the control (Fig. 6A and B).

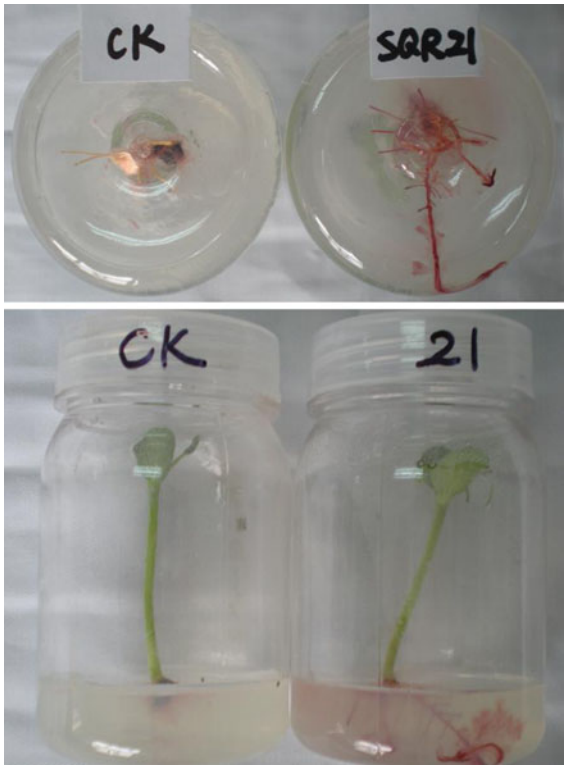

Fig. 5 Observation of the red reaction in plant rhizosphere. Top graph the view from the bottom of the tissue culture bottles. Bottom graph the view from the side of the tissue culture bottles. In each graph the left bottle is the control and the right bottle is the treatment with SQR21

From Fig. 5 we found the root treated with SQR21 developed much better than the control, which also confirmed SQR21 could effectively promote watermelon root growth. The dark red was always found in the rhizosphere of the roots treated with SQR21 during the growing period, inferring that the antagonist, SQR21, could proliferate along extending plant roots.

Discussion

Utilization of composts to minimize organic waste pollution and to reduce the addition of chemical fertilizers and fungicides in crop production is a promising strategy for both the present and the future. Furthermore, many soil-borne pathogens can be reduced by application of composts made of different raw materials (Borrero et al. 2004; Cotxarrera et al. 2002; Hoitink and Boehm 1999; Hoitink and Fahy 1986; Lumsden et al. 1983) and matured composts can sustain biological control agents (Litterick et al. 2004). Based on the above reports, we selected a mixture of amino acid fertilizer and matured pig manure composts as a medium to which the strain, *Paenibacillus polymixa* SQR21 (Zhang et al. 2008; Wu et al. 2008) antagonistic against *Fusarium* wilt, could be added to the fermentation medium. A six-day fermentation process was sufficient to assure good proliferation in the final BIO product. Final populations of SQR21 in the BIO product were 5×10^9 CFU g^{-1} dry weight BIO. The organic substrates or medium we selected could thus provide the SQR21 with good nutrients both during the fermenting process and after application to soils. This made the antagonists in the BIO more competitive in the rhizosphere and on the plant roots than native soil microorganisms. This might protect the plant roots from attack by the pathogens and thus reduce the incidence of *Fusarium* wilt disease.

Usually, a 50% of biocontrol efficacy is acceptable and can be extended in field (Leeman et al. 1996;

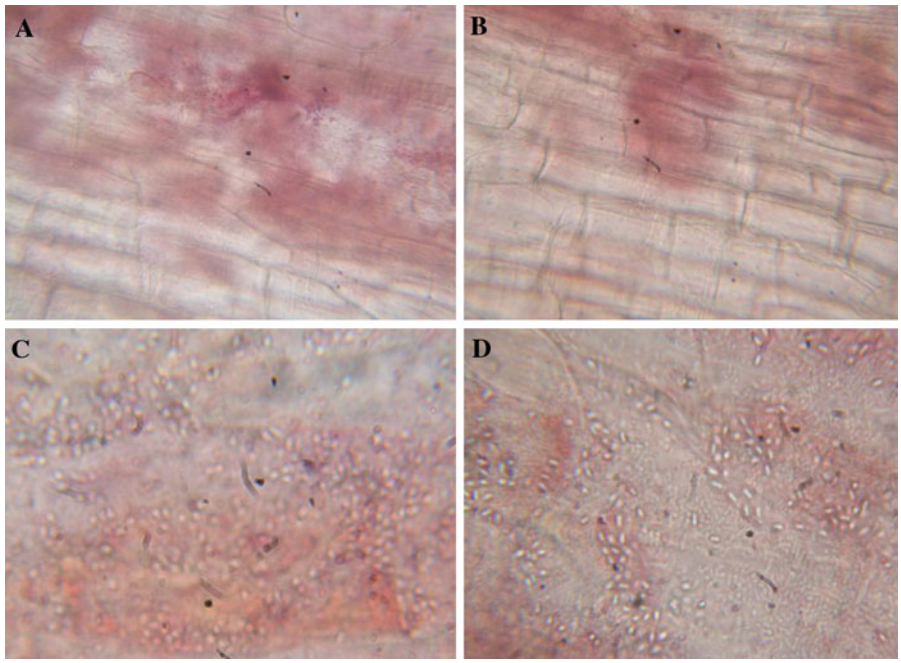

Fig. 6 Optical microscope micrographs of surfaces from control (**A** and **B**) and SQR21treated roots (**C** and **D**). Magnification is 1000×

Minuto et al. 2006; Nemec et al. 1996). The BIO used in this experiment showed a biocontrol efficacy of 73% in the field experiment, and 100% and 87.5% biocontrol in 2007 and 2008, respectively, in pot experiments compared with the control (Table 1). This indicated that our BIO product containing the antagonistic microorganism SQR21 was very promising for the control of *Fusarium* wilt. We have developed a series of BIO products and all the products consistently showed significant biocontrol of *Fusarium* wilt in banana, cucumber and tobacco (Shen et al. to be published).

Trillas et al. (2006) also reported that significant biocontrol of rhizoctonia disease of cucumber could be obtained by an application of a mixture of agricultural compost and *Trichoderma* spp. The protection of the watermelon plant from *Fusarium oxysporum* f.sp. *niveum* by the BIO was reflected in an increase in plant biomass (Fig. 2) and watermelon yield (Fig. 3). Since it was very difficult to obtain watermelon yields in pot experiments, we used plant biomass as an index to compare growth among the different treatments. Watermelon yield from the treatment with BIO application both in seedling nursery soil and transplanted soil in the field experiments was increased by 4.87 times relative to the control (Fig. 3).

Wilt disease incidence was still high in the treatments only supplemented with BIO in the transplanted soil, i.e., 100% disease in the pot experiment and 71% in the field experiment (Table 1). This might be due to a high population of the pathogen in the originally infected soil plus inoculation with the pathogen, and no propagation of the antagonists in the rhizosphere or on the root surface during the nursery stage. Hervás et al. (1998) demonstrated that seed + soil treatment was more efficient for establishing species of *Bacillus* for biological control than either seed or soil treatment alone. Ryu et al. (2006) reported that the application of *Paenibacillus polymixa* E618 via seed pelleting offered potential to overcome some of the disease problems associated with successive years of sesame cultivation. In our study, nursery application of BIO might also allow good root colonization of the antagonists on watermelon seedlings before they are transplanted. We anticipated nursery application of BIO would help the *Paenibacillus polymixa* to more efficiently establish the antagonistic bacterial population in the rhizosphere during seedling stage. In

watermelon cultivation, the early establishment of the introduced biocontrol agent can play a critical role in the growth of healthy watermelon stands because it allows them to escape disease under subsequent field condition. The wilt disease incidence was obviously lower in the treatments of seedling nursery soil amended with BIO than in the control, with a decrease of 50–100% in the pot experiment and 59–73% in the field experiment (Table 1).

For any biocontrol technology, farmers will need to be convinced that the investment in the biocontrol treatment will result in increased crop yield and economic return. Nursery application of BIO could significantly reduce farmers' input of other disease control measures by 70–80% and as well as increase yield. Thus, the use of BIO could be recommended even in fields that are only slightly infested and where the previous loss from *Fusarium* wilt was less than 20%. Also important is that the biocontrol is consistent from year to year and from one field to another. While additional studies will be needed to confirm this consistency for the BIO product, results obtained to date strongly suggest that if used as described in this paper, biocontrol will be consistently achieved.

Because *P. polymyxa* was able to form biofilms around the root tip and behave as a root-invading bacterium (Haggag and Timmusk 2008; Timmusk et al. 2005), it was reasonable to speculate that the SQR21 in the BIO could form a biobarrier on the root surface or in the rhizosphere at the seedling incubation stage. The biobarrier extended along with the growth of watermelon root in the transplanted soil and protected the roots from infection by pathogens. This could be confirmed by separate in vitro experiment. It is known that microbial activities may be limited in inorganic nutrient medium, but we observed the SQR21 grew very well in the rhizosphere of the watermelon (Fig. 5). We thus conclude that SQR21 could utilize the carbon and nitrogen source from the root exudates of watermelon to support its activities. The result is the formation of a biobarrier on the root surface or in the rhizosphere that protects the watermelon roots from invasion of soil-borne pathogens. This was also indirectly confirmed by the rhizosphere colonization of *F. oxysporum* in the pot experiment. The *F. oxysporum* numbers in the rhizosphere of the treatments that had not been amended with BIO in the nursery soil were all above 10^4 at the CFU level, which were much higher than those of the treatments amended with BIO in the nursery soil (Fig. 4). Additionally, the biobarrier that forms may also exclude *Fusarium oxysporum* f. sp. *nevium* from colonizing the roots due to antibiosis produced by *P. polymyxa* (Dijksterhuis et al. 1999; Timmusk et al. 2005). Studies to obtain further direct evidence of a biobarrier or a biofilm formation on the surface of the watermelon roots by the SQR21 is now being carried out in our laboratory by molecular labeling and other methods and will be reported in the future.

To summarize, the combination of high quality organic substrates with antagonistic microorganisms is necessary to make an effective biocontrol, organic fertilizer product. The highest biocontrol efficacy for controlling *Fusarium* wilt of watermelon was obtained by amending soil with BIO in both seedling nursery soil and transplanted soil. Nursery application of BIO resulted in the effective colonization by SQR21 in both the rhizosphere and on the root surface, thus protecting plant roots from invasion of *Fusarium* pathogens.

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