Plant Soil (2011) 344:87–97
DOI 10.1007/s11104-011-0729-7

REGULAR ARTICLE

A new bioorganic fertilizer can effectively control banana wilt by strong colonization with *Bacillus subtilis* N11

Nan Zhang · Kai Wu · Xin He · Shu-qing Li · Zhen-hua Zhang · Biao Shen · Xing-ming Yang · Rui-fu Zhang · Qi-wei Huang · Qi-rong Shen

Received: 13 September 2010 / Accepted: 30 January 2011 / Published online: 20 February 2011

Abstract Fusarium wilt is one of the most serious diseases caused by a soil-borne pathogen affecting banana production. The goal of this study was to evaluate the capability of a novel bio-organic fertilizer (BIO2) that integrated the biocontrol agent *Bacillus subtilis* N11, and mature composts to control Fusarium wilt of banana in pot experiments. The results showed that the application of the BIO2 significantly decreased the incidence rate of Fusarium wilt compared to the control. To determine the antagonistic mechanism of the strain, we also studied the colonization of the natural biocontrol agent on banana roots using a GFP marker. The studies were performed in a hydroponic culture system, a sand system and a natural soil system. The results indicated that the bacteria colonized predominantly by forming biofilms along the elongation and differentiation zones of the roots. The fact that similar observations were obtained in all three systems suggests that colonization by N11 can be studied in a defined system. The population of *B. subtilis* N11 in the rhizosphere and on banana roots was also monitored. We speculate that the colonization pattern of *B.subtilis* N11 can be linked to the mechanism of protection of plants from fungal infection.

Keywords Fusarium wilt · Bio-organic fertilizer (BIO) · Banana · *Bacillus subtilis* · Green fluorescent protein (GFP) · Colonization

Abbreviations

BIO	Bioorganic fertilizer
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
FOC	*Fusarium oxysporum* f. sp. *cubense*
GFP	Green fluorescent protein

Introduction

Fusarium wilt of banana (*Musa* spp.), commonly known as Panama disease, is caused by *Fusarium oxysporum* f. sp. *cubense* (FOC, E.F. Smith) Snyder and Hansen (Snyder and Hanson 1940). It is one of the most serious fungal diseases of banana, and it is reported to be one of the major limiting factors for banana production worldwide (O'Donnell et al. 1998; Lin et al. 2009). Currently, among the options for the control of FOC, including the use of resistant banana varieties, crop rotation, and chemical pesticides, biocontrol is the most promising one for the management of soilborne diseases (Sivamani and Gnanamanickam 1988; Raguchander et al. 1997;

Responsible Editor: Harsh P. Bais.

N. Zhang · K. Wu · X. He · S.-q. Li · Z.-h. Zhang · B. Shen · X.-m. Yang · R.-f. Zhang · Q.-w. Huang (✉) · Q.-r. Shen (✉)
Jiangsu Key Laboratory for Solid Organic Waste Utilization, Nanjing Agricultural University, Nanjing 210095, China
e-mail: qwhuang@njau.edu.cn
e-mail: qirongshen@njau.edu.cn

Getha and Vikineswary 2002; Saravanan et al. 2003; Getha et al. 2005). Because directly deploying the biocontrol agent into the soil may lead to poor activity, integrated approaches that enhance the activity of the biocontrol agents by the addition of organic amendments are more attractive (Saravanan et al. 2003). Saravanan et al. (2003) reported that significant biocontrol of Fusarium wilt of banana could be obtained by the application of a mixture of neem cake and *Pseudomonas fluorescens*. *Trichoderma* spp. and agricultural compost were also used together to suppress rhizoctonia disease in cucumbers (Trillas et al. 2006). Currently, it is believed that a combination of antagonistic microbes with mature compost may be more efficient in inhibiting disease than using single antagonistic microbe (Cotxarrera et al. 2002; Trillas et al. 2006).

There are three main aspects contributing to the biocontrol of soil-borne pathogens by antagonistic bacteria (bio-control agents): (1) the secrection of antibiotics; (2) competition for places and nutrition in the rhizosphere; and (3) the induction of plant resistance against the pathogen (Dunne et al. 1996; Bloemberg et al. 2000; Compant et al. 2005). Root colonization by the added bio-control agents is considered as a prerequisite for successful biological control. For *P. fluorescens* 2–79 and *Bacillus* spp., the soil-borne disease control efficiency was directly related to root colonization by the microbes (Duffy and Weller 1996; Cavaglieri et al. 2005).

Reporter gene technology is very useful in studies of the metabolic status of bacteria introduced into natural environments (Prosser 1994), and different markers have been used to identify cells and assess physiological activities in the rhizosphere; these markers include lacZ (de Weger et al. 1993) and luxAB (Unge and Jansson 2001). Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria,* has been the most revolutionary reporter in biology since its first application as a marker by Chalfie et al. (1994). The major advantage of GFP as a reporter is its noninvasive analysis without the need for exogenous substrates or energy. Therefore, GFP is widely used to identify and quantify specific microbes or activities in a complicated environmental sample such as soil and plant tissues (Chelius and Triplett 2000). The potential for in situ monitoring of GFP-tagged bacteria on plant roots has previously been demonstrated (Timmusk et al. 2005; von der Weid et al. 2005; Liu et al. 2006; Njoloma et al. 2006). However, to our knowledge, the colonization of *B. subtilis* on banana roots has not been reported.

A novel bio-organic fertilizer (BIO) by fermenting mature compost with a mixture of two antagonistic microbes, *Paenibacillus polymyxa* SQR21 and *Trichoderma harzianum* T37 was developed to suppress Fusarium wilt disease of watermelon plants in pot experiments (Ling et al. 2010). Here we isolated one *Bacillus subtilis* strain, N11, from soils in which banana was continuously cropped and the strain could significantly inhibit the growth of the FOC. The present study was, therefore, carried out to assess the capability of the combination of organic fertilizer and antagonistic microorganisms to control Fusarium wilt of banana. In addition, to reveal the antagonist mechanism of the biocontrol agent, the natural isolate of *B. subtilis* N11 was tagged with GFP, and the localization of the bacterium on banana roots in a hydroponic culture system, a sand system and a natural soil system was investigated by confocal laser scanning microscopy (CLSM) to evaluate the root colonization abilities and locations of the strain on plant root. This increases our understanding of the mechanisms of interaction between plant roots and soil microbes.

Materials and methods

Strains, plasmids and media

P. polymyxa SQR21 from BIO1, which was isolated and identified in our laboratory and found to be highly efficient against *Fusarium oxysporum* causing cucumber and watermelon wilt disease (Zhang et al. 2008; Ling et al. 2010), was incubated in a beef extract and peptone liquid culture on a shaker at 170 rpm at 30°C.

T. harzianum T37 from BIO1 was also isolated and screened in our laboratory from composts and commonly showed antagonistic effects against *Fusarium oxysporum* in cucumber, watermelon, tomato and banana plants (Chen et al. 2010).

B. subtilis N11 in BIO2 was isolated and identified in our laboratory from the roots of a healthy banana plant surrounded by banana plants that died of

Fusarium wilt disease in a banana field in Ledong county, Hainan province. The strain was found to be specifically and highly effective against *Fusarium oxysporum* f.sp. *cubense* (FOC) race 4, with a 68.2% growth inhibition rate. It was grown in liquid LB medium on a shaker at 170 rpm at 30°C.

The plasmid pHAPII (GenBank accession number HM151400), an *Escherichia coli-Bacillus subtilis* shuttle vector that contains the GFP gene under the control of the strong constitutive promoter *hapII*, was constructed in our laboratory.

Bio-organic fertilizer (BIO) preparation

The BIO1 used in this experiment was described by Zhang et al. (2008) and Ling et al. (2010). The organic substrates in the BIO were composed of amino acid fertilizer and pig manure composts (1:1, w/w). The amino acid fertilizer was made from oil rapeseed cakes that were microbe-enzymatically hydrolyzed for 7 days to obtain a mixed amino acid fertilizer (Zhang et al. 2008). This amino acid fertilizer contained 44.2% organic matter, 12.93% total amino acids, small molecular peptides and oligopeptides, 4.4% nitrogen (N), 3.5% P_2O_5 and 0.67% K_2O. The pig manure compost was made by Tianniang Ltd in Suzhou by composting pig manure at a temperature range of 30–70°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 as follows: 1,000 ml of a suspension of 10^9 ml^{-1} CFU N11, 2.5 kg of pig manure compost and 2.5 kg of the amino acid fertilizer were thoroughly mixed in a 500× 360×175 mm plastic case for secondary 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 7th day, the mixture was spread for air-drying in a ventilation room at room temperature for 2 days until its water contents was less than 30%. During the secondary fermentation stage, the temperature and bacterial density of the substrate were measured daily. The finished bioorganic fertilizer contained N11 more then 1×10^9 CFU g^{-1} DW of N11, and, therefore, was putatively defined as a bio-organic fertilizer (BIO2) especially for suppressing the growth of *F. oxysporum* f. sp. *cubense*. The BIO2 was stored at 4°C prior to use in pot experiments.

Plant material

Banana seedlings (Musa AAA Cavendish cv. Brazil) were used for experiment.

Design of pot experiments

F. oxysporum f. sp. *cubense* was incubated in a PDA liquid culture on a shaker at 170 rpm at 30°C for 3–4 days. Then the culture was filtered with a sterile pledget to obtain a spore suspension.

The soils used for the pot experiments were collected from Yixing, Jiangsu province, China, which had the following properties: pH 5.4, organic matter, 7.3 gkg^{-1}, available N 79.0 mg kg^{-1}, available P 31.0 mg kg^{-1}, and available K 40.0 mg kg^{-1}. The soil was preinoculated with the *F. oxysporum* f. sp. *cubense* spore suspension to obtain a concentration of 10^5 g^{-1} soil.

Banana seedlings were grown in nursery pots with 350 g soil, and one seedling was maintained in each pot. After 20 days, the seedlings with 3–4 true leaves were transplanted into larger pots with 10 kg soil.

Three treatments in the pot experiment were designed as follows:

CK (control): Neither the nursery soil nor the pot soil was supplemented with any bio-organic fertilizer.

BIO1: The nursery soil and the pot soil were supplemented with 2% (w/w) and 0.5% (w/w), respectively, of the bio-organic fertilizer made with SQR21 and *T. harzianum* T37.

BIO2: The nursery soil and the pot soil were supplemented with 2% (w/w) and 0.5% (w/w), respectively, of the bio-organic fertilizer made with *B. subtilis* N11.

Each treatment had three blocks, with ten pots each. The seedlings were grown in the greenhouse under natural conditions and the temperature ranged from 23°C to 30°C.

Disease incidence

Seedling infection by the *F. oxysporum* f. sp. *cubense* was recorded every day, and the cumulative number of infected plants was also recorded begin-

ning the after transplantation for 56 days. The disease incidence was calculated as the percentage of diseased plants over the total number of growing plants in each block and was evaluated when the disease emerged.

Construction of GFP-tagged *B. subtilis* N11

The shuttle plasmid pHAPII was used to introduce plasmid-borne GFP genes into *B. subtilis* by two electroporation methods, the PEB protocol and the high-osmolarity protocol.

The PEB electroporation protocol was carried out as described previously (Brigidi et al. 1991) with some modifications: Cells for electroporation were grown overnight at 37°C in 3 ml of LB broth with shaking. Then 1 ml from the overnight culture was transferred to 50 ml of fresh LB broth and shaken at 30°C until the OD600 reached 0.75–0.85 (about 3–4 h). The cells were harvested by centrifugation at 4°C and 5,000 g for 5 min. After three washes in ice-cold electroporation buffer (PEB buffer: 272 mM sucrose, l mM $MgCl_2$, 7 mM KH_2P0_4, (pH 7.4)), the cells were suspended in 1/50 of the culture volume of the electroporation medium. For electroporation, a 120-μl aliquot of the competent cells was mixed with 1 μl (50 ng/μl) of DNA and then transferred to an ice-cold electroporation cuvette (2-mm electrode gap). After incubation for 1–2 min, the cells were exposed to a single electrical pulse using a Gene-Pulser (Bio–Rad Laboratories, Richmond, CA) set at 25 mF and 2.4 kv, resulting in time constants of 4.5–5.0 ms. Immediately following the electrical discharge, 1 ml of LB medium was added to the cells. After incubation at 30°C for 3 h the cells were plated on LB-agar plates containing 20 μg/ml kanamycin for selection of transformants containing pHAPII.

In the high-osmolarity protocol, the overnight culture were transferred to 50 ml LB containing 0.5 M sorbitol and grown to an optical density at 600 nm of 0.85–0.95. The cells were then washed in ice-cold electroporation medium (0.5 M sorbitol, 0.5 M mannitol and 10% glycerol) for three times. The recovery medium was LB containing 0.5 M sorbitol and 0.38 Mmannitol. The remainder of the transformation procedure was preformed using the same method as that described in the PEB protocol (Xue et al. 1999).

Growth curve

Overnight cultures of GFP-tagged N11 and wild-type N11 were adjusted to a suspension of OD600 1.0. Next, 0.3 ml of the suspension was used to inoculate 30 ml of fresh LB broth that was then shaken at 30°C for 72 h. During the first 24 h after inoculation, the OD600s of the cultures were measured every 2 h; after 24 h, the OD600s were measured every 4 h until 72 h after inoculation.

In vitro antagonism against FOC

GFP tagged *B.subtilis* N11 and the wild-type strain were tested for antagonism against FOC on PDA by the dual culture technique as described elsewhere (Anith and Manomohandas 2001). A mycelia plug from actively growing FOC on PDA was taken with a cork borer of 1-cm in diameter and put in the center of the agar medium in a 90-mm Petri plate. Four spots were made on the edges of the plate with an actively growing suspension of the bacterial isolate 24 h after the fungal inoculation. The plates were incubated at 25°C, and the inhibition of fungal growth was noted after 5–7 days.

Inoculation of plants with *B. subtilis* N11

A colony of the corresponding GFP-tagged *B. subtilis* N11 was inoculated into 50 ml of LB broth containing 20 μg/ml kanamycin, and the culture was incubated at 30°C until it reached the stationary phase (about 16 h). The cells were washed twice in M8 buffer (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 100 mM NaCl) and suspended in 500 ml M8 buffer prior to use.

The banana seedlings were soaked in 500 ml bacterial suspension for 30 min at 30°C. Then, the seedlings that had been treated with GFP-tagged N11 were transferred to containers with 200 ml of 1/2 Hoagland culture medium, quartz sand or natural soil. In the sand system and the soil system, the seedlings were irrigated with 1/2 Hoagland medium. The plants were incubated in a growth chamber at 28°C with a 16-h light regimen.

Enumeration of GFP-tagged N11 in the rhizosphere and on banana roots. The numbers of GFP-tagged N11 in the rhizosphere were determined 2, 4, 6, 8 and 15 days after inoculation, and the population of cells on the root surface after treatment was also monitored.

For the collection of rhizosphere soil, the banana seedlings were delicately uprooted from the soil and gently shaken to remove all loosely adhering bulk soil. Then, the roots were soaked in distilled water and slightly shaken to obtain rhizosphere soil samples. The soil suspension from the rhizosphere was serially diluted and plated onto LB medium with 20 μg/ml kanamycin.

For the collection of root-surface samples, 0.2 g of roots were homogenized in 1.8 ml of PBS buffer using a mortar and pestle until a free homogenate was obtained. The homogenates were serially diluted and plated onto LB medium with an appropriate antibiotic. After culture at 30°C for 2 days, the bacterial colonies were examined using fluorescence microscopy (Olympus DP71), and those emitting green fluorescence were counted.

Microscopy

In the hydroponics system, the root samples were monitored at 2 days after inoculation, the roots in the sand system were monitored at 4 days after treatment, and the roots in the soil condition were observed at 2, 4 and 8 days after inoculation.

The collected roots were cut into 1–2 cm in length, put on microscope slides, and visualized using a confocal laser scanning microscopy (CLSM, Leica Model TCS SP2, Heidelberg, Germany) with excitation wavelengths of 488 nm. Emitted light in the range of 500–600 nm was collected for GFP visualization. Images were obtained using Leica confocal software, version 2.61. For SEM, the root samples were cut to 0.5 cm in length and observed under a XL-30 ESEM (Philips, Netherlands).

Statistical analysis

Differences among the treatments were calculated and statistically analyzed using the analysis of variance (ANOVA) and Duncan's multiple range tests ($p < 0.05$). SPSS, version 17.0 was used for statistical analysis (SPSS Inc., Chicago, IL).

Results

Disease incidence of Fusarium wilt

In the pot experiments, at 56 days after transplantation, the incidence of Fusarium wilt in the control plants was 93.3% (Fig. 1). Application of BIO1 significantly decreased the incidence rate to 33.3%. The lowest incidence rate of 16.7% was detected in the BIO2 treatment; thus, the biological control of Fusarium wilt of banana plants by application of BIO2 reached 82%, indicating that this specific bio-organic fertilizer could more effectively control Fusarium wilt of banana plants than the common bio-organic fertilizer (BIO1).

GFP-tagging of the *B. subtilis* isolate

We attempted to transform *B. subtilis* strain N11 cells with GFP-tagged pHAPII by both the PEB method and the high-osmolarity electroporation method. The latter method failed to yield any transformed cells. Although the transformation frequency by the PEB method was also very low (less than 10^2 transformants μg^{-1} DNA), the *B. subtilis* transformants could be easily distinguished by their GFP fluorescence when visualized by fluorescence microscopy. The N11 transformants containing the plasmid pHAPII were chosen for further studies. The growth rate of the N11 transformant on LB medium (Fig. 2a) and its antagonistic ability against the FOC on PDA plate were similar to those of the wild type (Fig. 2b), indicating that the presence of plasmid did not interfere with its normal metabolism.

The stability of the pHAPII plasmid in *B. subtilis* N11 was analyzed by plating a culture of this strain on LB with or without antibiotic. Without antibiotic, approximately 22% of the tagged N11 culture no longer exhibited the GFP phenotype after 72 h incubation in LB medium. In addition, 38% of the plasmid was lost after 15 days when the tagged N11 bacteria were inoculated into soils. Therefore the experiment was carried over 20 days.

The colonization of *Bacillus subtilis* N11 on the roots of banana seedlings

In the natural soil system, 2 days after inoculation with GFP-tagged *B. subtilis* N11, the root systems were examined by CLSM for the presence of the bacterial cells. The GFP-tagged cells could easily be distinguished from the background fluorescence of the root. A biofilm consisting of *B. subtilis* N11 bacterial cells and a semitransparent extracellular matrix had formed on the root surface (Fig. 3).

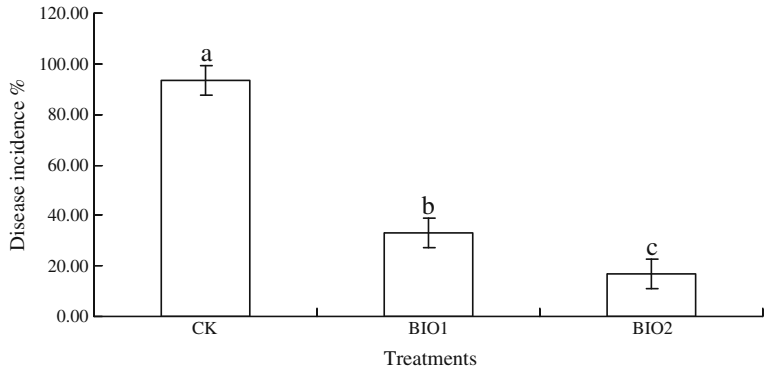

Fig. 1 Effects of different fertilizers on the incidence of Fusarium wilt (56 days after transplantation). *Bars* with different letters indicated statistical differences among the three treatments following Duncan's test ($P<0.05$). Notes: CK (control): Either nursery soil or pot soil were not supplemented with any bio-organic fertilizer application. BIO1: Nursery soil and pot soil were supplemented with 2% (w/w) and 0.5% (w/w), respectively, of the bio-organic fertilizer made with SQR21 and *T. harzianum* T37. BIO2: Nursery soil and pot soil were supplemented with 2% (w/w) and 0.5% (w/w), respectively, of the bio-organic fertilizer made with N11

Colonization occurred preferentially in defined regions of the elongation and differentiation zones of the plant roots, mainly as microcolonies, while only a small number of single cells were found at the root tip (Fig. 3c).

Four and 8 days after inoculation, the colonization pattern of the two GFP-tagged *B. subtilis* N11 strains was similar to that 2 days after inoculation, though the bacterial population seemed slightly decreased (Fig. 4a and c). No bacteria entered the inner parts of the roots (Fig. 4b).

In the hydroponic system and the sand system, the CLSM images showed that the localization of GFP-tagged N11 on the root surface was consistent with the results in the soil system. After inoculation, the bacteria preferred to assemble in defined regions of the elongation and differentiation zones of the banana roots, while only a small

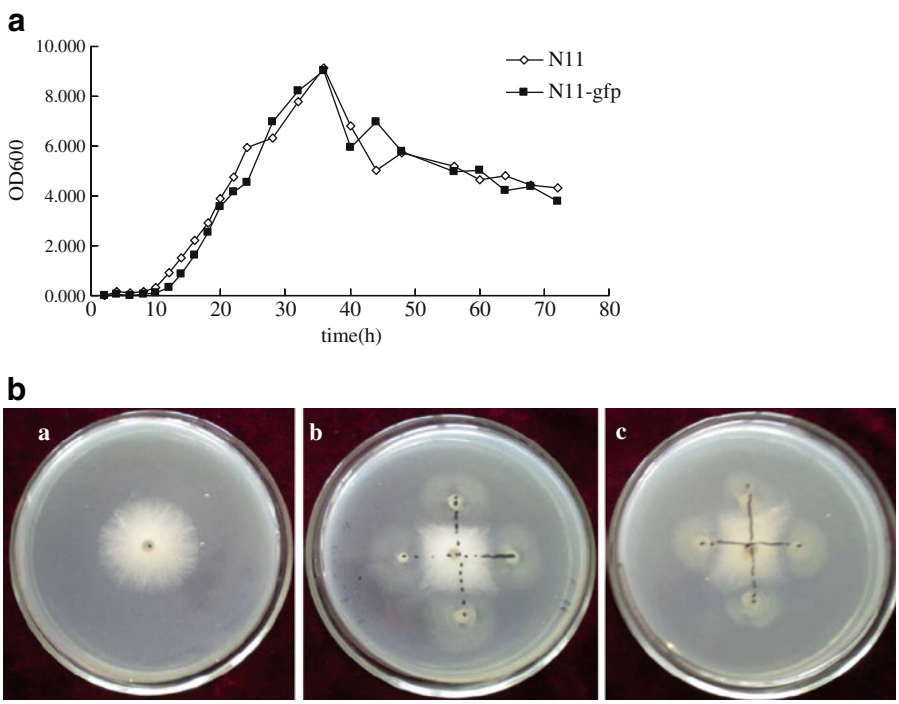

Fig. 2 Comparison of wide-type *B.subtilis* N11 and N11-gfp on growth and antagonism. **a** Growth curve of *B.subtilis* N11 and N11-gfp. **b** In vitro antagonism against FOC by N11 and N11-gfp. (*a*) Control, with no *Bacillus* inoculation. (*b*) Antagonism against FOC by N11. (*c*) Antagonism against FOC by N11-gfp

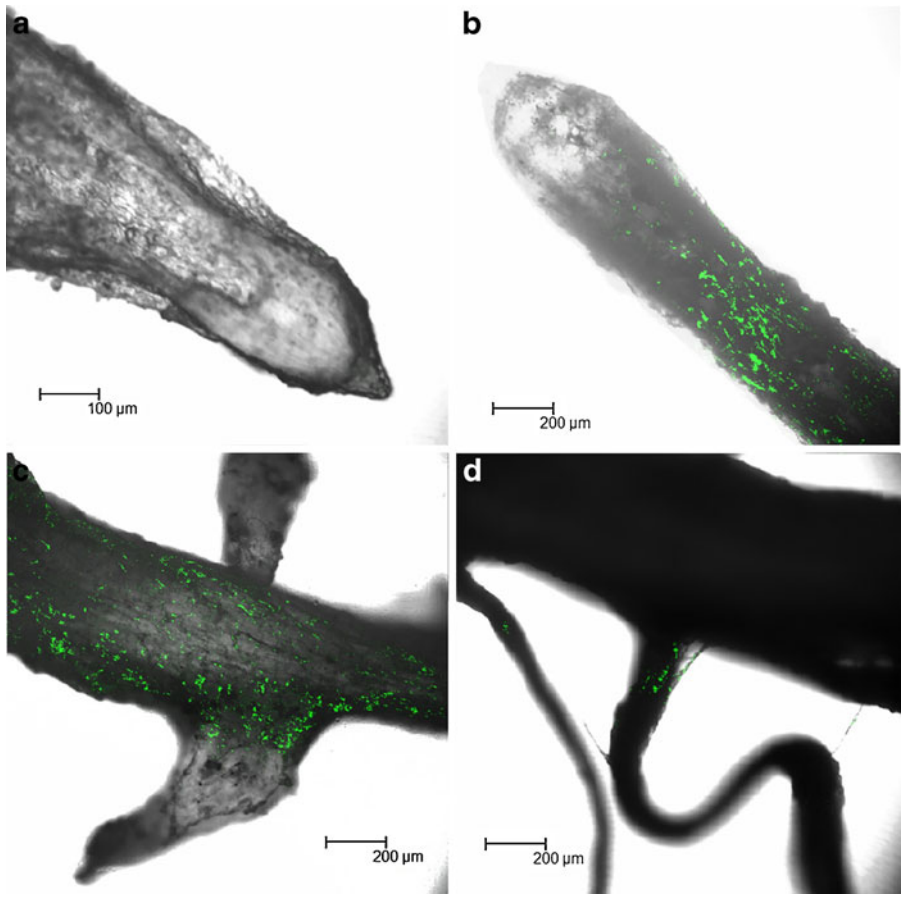

Fig. 3 CLSM micrographs of banana roots colonized by GFP-tagged N11 2 days after inoculation. **a** Control. The N11-gfp strain colonized predominantly along the elongation zones of the roots (**b**), while there were nearly no cells located along the root tip (**b**). The bacteria also colonized along the lateral roots (**c**) and at the junctions between the roots (**d**)

number of single cells was found at the root tip (Data not shown).

Enumeration of the two GFP-tagged *B. subtilis* N11 strains in rhizosphere soil and on banana roots

The enumeration of GFP-tagged *B. subtilis* N11 in the rhizosphere soil and on banana roots was monitored at 2, 4, 6, 8 and 15 days after inoculation, as described in the Materials and methods.

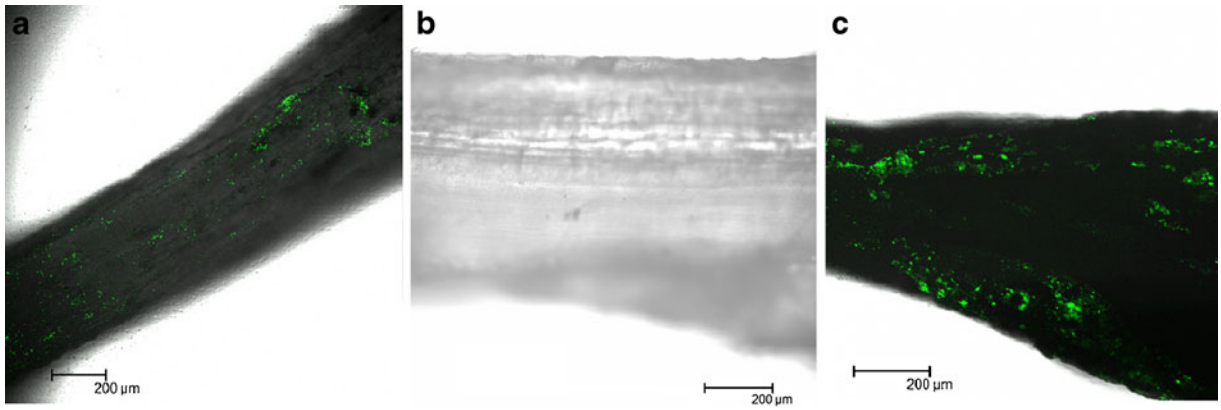

Fig. 4 CLSM micrographs of banana roots colonized by GFP-tagged N11 4 and 8 days after inoculation. The cells showed the same pattern as previous results 4 days after inoculation (**a**), while no cells entered into the root (**b**). By 8 days after inoculation, the number of cells on the roots had slightly decreased (**c**)

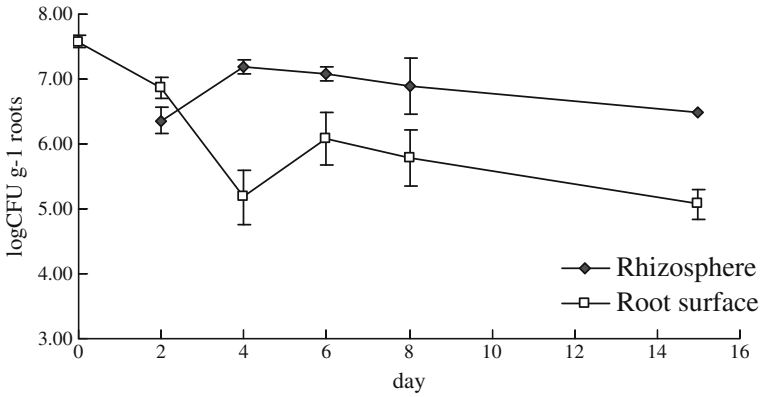

Fig. 5 The population of GFP-tagged N11 in the rhizosphere and on the plant roots. The data are expressed as $\log_{10}$CFU per gram (fresh banana root or rhizosphere). The error *bars* indicate the standard deviations for three samples

Approximately 10^7 CFU of N11 per g of root were detected immediately after inoculation (Fig. 5). On the 2nd day, the N11 population decreased to 10^6 CFU per g of root, and the rhizosphere soil also contained 10^6 CFU of N11 per g of soil. On the 4th day, the number of the cells on the root surfaces further decreased to 10^5 CFU g^{-1} root, while the population in the rhizosphere reached a peak value of 10^7 CFU g^{-1} soil. After that, on the 6th and 8th days, the bacterial population remained between ca. 10^5 and 10^6 CFU g^{-1} on the roots and approximately 10^6 CFU g^{-1} soil in the rhizosphere, indicting that the bacteria had reached a balance between the root surface and the rhizosphere. However, on the 15th day, the bacterial population on the root surface had decreased to 10^5 CFU g^{-1} root, whereas it had not significantly decreased in the rhizosphere soil.

Discussion

Fusarium wilt of banana plants (Musa spp.) caused by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder and Hansen is a serious and destructive disease worldwide (Ploetz 1990). Increased concern about the impact of chemicals on the environment has resulted in an increased interest in biocontrol strategies (Getha et al. 2005). Here we tested the effects of novel bio-fertilizers, which combined an amino acid fertilizer and mature pig manure compost with the antagonists *Paenibacillus polymyxa* SQR21, *Trichoderma. harzianum* T37 and *Bacillus subtilis* N11 (isolated from the healthy banana roots in a severely Fusarium wilt-diseased field) on the suppression of Fusarium wilt of banana in pot experiments. The bio-organic fertilizers significantly suppressed the incidence of wilt disease, decreasing it by 64–82% by application of BIO1 and BIO2, respectively, compared to the control (Fig. 1). The more effective control of Fusarium wilt than observed in previous studies (Sivamani and Gnanamanickam 1988; Getha et al. 2005) may be due to the application of the antagonists in combination with suitable organic amendments (Saravanan et al. 2003; Trillas et al. 2006). Here we also found that the best biocontrol effect was obtained in the treatment with the BIO2 that contains N11. This strain was isolated and screened from the roots of a healthy plant that was growing in the midst of banana plants that had all died of Fusarium wilt in a field in Ledong county, Hainan province. This finding suggested that the antagonistic microbes specific for wilt diseases affecting different plant species in situ and using suitable organic substrates are very important to achieve good biological control. These results together with other reports (Zhang et al. 2008; Ling et al. 2010; Luo et al. 2010; Zhao et al. 2010) provide a breakthrough in research into the biological control of diseases caused by soil-borne pathogens.

To further study the mechanism of biocontrol of Fusarium wilt of banana plants by *B.subtilis* N11, we tagged the strain with a GFP-marked plasmid and investigated its colonization pattern on the plant roots. Because of the low frequency of transformation of wild *B. subtilis*, and its complexity compared the transformation of gram-negative species (Trevors et al. 1992), we chose two different protocols to transform the plasmid into the bacteria. We were only able to obtain transformants with one method, PEB, suggesting that different transformation methods might have different efficiencies in different strains, though the mechanisms underlying this observation should be studied further.

The GFP-tagged N11 strains could be easily distinguished from the background by their fluorescence under CLSM. The plasmid did not disturb the growth or antagonism of the N11 strains. However, the instability of plasmid pHAPII in the transformant limited its usefulness in long-term experiments. Some researchers have reported that the plasmid was stably maintained for several months in the transformants without selection (Njoloma et al. 2006; Timmusk et al. 2005), while others demonstrated that the plasmid was lost even within several days without antibiotic selection (von der Weid et al. 2005). Thus, different strains and plasmids may show different plasmid maintenance. In further research, we will try to tag cells with a marker that can stably integrate into the bacterial chromosome.

In present study, the biocontrol agent *B. subtilis* N11, tagged with the marker GFP, was monitored in situ during its colonization of banana roots. It has previously been shown that bacteria could colonize plant surfaces, such as roots and leaves, often by forming aggregates or microcolonies (Assmus et al. 1995; Dandurand et al. 1997; von der Weid et al. 2005). Different biocontrol strains are known to colonize different regions of plants (Tombolini et al. 1999; Ramos et al. 2000; Walker et al. 2002). We observed that GFP-tagged *B. subtilis* N11 introduced into soil could be found as aggregates attached to the surface of plant roots after inoculation. The colonization occurred preferentially in defined regions of the elongation and differentiation zones of the plant roots, as well as in the lateral roots and the junctions between the roots. These results are consistent with those of a previous study (Liu et al. 2006), and the colonization pattern observed is predicted to stop the pathogen from invading the plant roots (Compant et al. 2005; Timmusk et al. 2005). Further, we plan to study the interactions of the GFP-tagged strains with FOC in the rhizosphere and on the rhizoplane. The population of the inoculated bacteria attached to the root tip was very low, but this finding is not consistent with some reports that bacterial colonization occurred mostly around the root tip (Ramos et al. 2000; Timmusk et al. 2005).

One of the most important factors that affected bacterial colonization on the plant roots was root exudation (Walker et al. 2003; Ramey et al. 2004; Compant et al. 2010). Carbon fixed by plant photosynthesis is known to be partly translocated into the root zones and released as root exudates (Bais et al. 2006). The secretion differed along the longitudinal axis from the base to the root tip (Gilroy and Jones 2000). The root cap is a source of hydrated polysaccharides sloughed from the root tip. Apoplastic sucrose could also diffuse from the root tips because of the concentration gradient. Sucrose does not leak from mature root sections because a suberized layer of endodermal cells blocks its diffusion (Jaeger et al. 1999). Microorganisms that prefer this type of nutrition might be attracted to these rich sources of nutrients (Timmusk et al. 2005). However, other studies have indicated that root exudation is greatest at the root tips, where the microbial density is low (Schonwitz and Ziegler 1989). With increasing distance from the root tip, exudation generally decreased and the microbial density increased. Thus, the region of greatest release of root exudates was spatially separated from the region of highest microbial population density (Marschner and Rengel 2007). These different results might be due to the different chemotaxis behaviors of different strains responding to various exudates (Ramey et al. 2004). The detailed mechanisms need to be studied further.

Previous studies had proposed that some *Bacillus.* spp. (Mcinroy and Kloepper 1994; Liu et al. 2006) could be isolated as endophytes. In this study, GFP-tagged N11 cells introduced into the soil could not be found inside the banana root tissue, indicating that this *Bacillus* strain was not an endophyte of banana roots.

Our other aim was to study whether the colonization of roots varied in different culture systems, such as hydroponic, sand and soil systems. The inoculated bacteria showed a similar colonization patterns in these systems, indicating that some models could represent real soil conditions to a certain extent.

We also monitored the bacterial populations on the root surface and in the rhizosphere. We found that the bacteria had colonized banana roots at a level of approximately 10^5 CFU g^{-1} root to 10^6 CFU g^{-1} root and 10^6 CFU g^{-1} soil to 10^7 CFU g^{-1} soil 6 days after inoculation, which was in accordance with earlier reports (Getha et al. 2005; Timmusk et al. 2005). Our study showed that the population of the biocontrol agent had decreased by 15 days after inoculation, which was consistent with a previous study (Hiddink et al. 2005). Perhaps this reduction in the apparent population of the biocontrol agent resulted from competition by the indigenous microorganisms in the soil or the loss of the plasmids.

In summary, our results demonstrated that well-organized combinations of organic fertilizer with the antagonistic strain *Bacillus subtilis* N11 significantly suppressed Fusarium wilt of banana. We also reported here that *B. subtilis* N11 formed biofilms around the elongation and differentiation zones of banana roots. This biofilm formation will be pursued as a possible mechanism underlying the biocontrol of *Fusarium* wilt disease by *B. subtilis* N11.

Acknowledgement We gratefully acknowledge Agricultural Ministry of China (201103004), Nature Science Foundation of China (31070462) and Ministry of Science and Technology of China (2010AA10Z401).

References

Anith KN, Manomohandas TP (2001) Combined application of *Trichoderma harzianum*, *Alcaligenes* sp. strain AMB 8 for controlling nursery rot disease of black pepper. Indian Phytopathol 54:335–339

Assmus B, Hutzler P, Kirchhof G, Amann R, Lawrence JK, Hartmann A (1995) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled rRNA-target oligonucleotide probes, scanning confocal laser microscopy. Appl Environ Microbiol 61:1013–1019

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants, other organisms. Annu Rev Plant Biol 57:233–266

Bloemberg GV, Wijifjes AHM, Lamers GEM, Stuurman N, Lugtenberg BJJ (2000) Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent Proteins in the Rhizosphere, New Perspectives for Studying Microbial Communities. Mol Plant Microb Interact 13:1170–1176

Brigidi P, De Rossi E, Riccard G, Matteuzzi D (1991) A highly efficient electroporation system for transformation of *Bacillus licheniforms*. Biotechnol Tech 5:5–8

Cavaglieri L, Orlando J, Rodriguez MI (2005) Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro, at the maize root level. Res Microbiol 156:748–754

Chalfie M, Euskirchen YTG, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 264:802–805

Chelius MK, Triplett EW (2000) Immunolocalization of dinitrogenase reductase produced by *Klebsiela pneumoniae* in association with *Zea mays* L. Appl Environ Microbiol 66:783–787

Chen LH, Yang XM, Raza W, Li JH, Zhang FG, Tang Z, Liu YX, Shen QR (2010) Trichoderma harzianum SQR-T037 rapidly degrades allelochemicals in rhizospheres of continuously cropped cucumbers. Appl Microbiol Technol (on line)

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases principles, mechanisms of action, future prospects. Appl Environ Microbiol 71:2951–4959

Compant S, Clement C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo-, endosphere of plants: their role, colonization, mechanisms involved, prospects for utilization. Soil Biol Biochem 42:669–678

Cotxarrera L, Trillas-Gay MI, Steinberg C, Alabouvette C (2002) Use of sewage sludge compost, *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. Soil Biol Biochem 34:467–476

Dandurand LM, Schotzko DJ, Knudsen GR (1997) Spatial patterns of rhizoplane populations of *Pseudomonas fluorescens*. Appl Environ Microbiol 63:3211–3217

de Weger LA, Dekkers LC, van der Bij A, Lugtenberg BJJ (1993) Use of phosphate-reporter bacteria to study phosphate limitation in the rhizosphere, in bulk soil. Mol Plant Microb Interact 7:32–38

Duffy BK, Weller DM (1996) Biological control of take-all of wheat in the Pacific North-west of the USA using hypovirulent *Gaeumannomyces graminis* var. *tritici*, fluorescent Pseudomonads. J Phytopathol 144:11–12

Dunne C, Delany I, Fenton A, O'Gara F (1996) Mechanisms involved in biocontrol by microbial inoculants. Agronomie 16:721–729

Getha K, Vikineswary S (2002) Antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f. sp. *cubense* race 4: indirect evidence for the role of antibiosis in the antagonistic process. J Ind Microbiol Biotechnol 28:303–310

Getha K, Vikineswary S, Wong WH, Seki T, Ward A, Goodfellow M (2005) Evaluation of *Streptomyces* sp. strain g10 for suppression of Fusarium wilt, rhizosphere colonization in pot-grown banana plantlet. J Ind Microbiol Biotechnol 32:24–32

Gilroy S, Jones DL (2000) Through form to function, root hair development, nutrient uptake. Trends Plant Sci 5:56–60

Hiddink GA, van Bruggen AHC, Raaijmakers JM, Semrnoy AV (2005) Effect of organic management of soils on suppressiveness to *Gaeumannomyces graminis* var. *tritici*, its antagonist, *Pseudomonas fluorescens*. Eur J Plant Pathol 113:417–435

Jaeger CH, Lindow SE, Miller W, Clark E, Firestone MK (1999) Mapping of sugar, amino acid availability in soil around roots with bacterial sensors of sucrose, tryptophan. Appl Environ Microbiol 65:2685–2690

Lin YH, Chang JY, Liu ET, Chao CP, Huang JW, Fang P, Chang LD (2009) Development of a molecular marker for specific detection of *Fusarium oxysporum* f. sp. *cubense* race 4. Eur J Plant Pathol 123:353–365

Ling N, Xue C, Huang QW, Yang XM, Xu YC, Shen QR (2010) Development of a mode of application of bio-organic fertilizer for improving the biocontrol efficacy to Fusarium wilt. Biocontrol 55:673–683

Liu XM, Zhou HX, Chen SF (2006) Colonization of Maize, Rice plants by strain *Bacillus megaterium* C4. Curr Microbiol 52:186–190

Luo J, Ran W, Hu J, Yang XM, Xu YC, Shen QR (2010) Application of bioorganic fertilizer significantly affected fungal diversity of soils. Soil Sci Soc Amer J 74:2039–2048

Marschner P, Rengel Z (2007) Contributions of rhizosphere interactions to soil biological fertility. Soil Biological Fertility 81–98

Mcinroy JA, Kloepper JW (1994) Studies on the indigenous endophytic bacteria of sweet corn, cotton. In: O'Gara F, Dowling DN, Boesten B (eds) Molecular ecology of rhizosphere micro-organisms. VCH Press, pp 19–28

Njoloma J, Tanaka K, Shimizu T, Nishiguchi T, Zakria M, Akashi R, Oota M, Akao S (2006) Infection, colonization of aseptically micropropagated sugarcane seedlings by nitrogen-fixing endophytic bacterium, *Herbaspirillum* sp. B501gfp1. Biol Fertil Soils 43:137–143

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear, mitochondrial gene genealogies. PNAS 95:2044–2049

Ploetz RC (1990) Population biology of *Fusarium oxysporum* f. sp.*cubense*. In: Ploetz RC (ed) Fusarium wilt of banana. APS, St. Paul

Prosser JI (1994) Molecular marker systems for detection of genetically engineered micro-organisms in the environment. Microbiol 140:5–17

Raguchander T, Jayashree K, Samiyappan R (1997) Management of Fusarium wilt of banana using antagonistic microorganisms. J Biol Control 11:101–105

Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C (2004) Biofilm formation in plant-microbe associations. Curr Opin Microbiol 7:602–609

Ramos C, Molbak L, Molin S (2000) Bacterial activity in the rhizosphere analyzed at the single-cell level by monitoring ribosome contents, synthesis rates. Appl Environ Microbiol 66:801–809

Saravanan T, Muthusamy M, Marimuthu T (2003) Development of integrated approach to manage the fusarial wilt of banana. Crop Prot 22:1117–1123

Schonwitz R, Ziegler H (1989) Interaction of maize roots, rhizosphere microorganisms. J Plant Nutr Soil Sci 152:217–222

Sivamani E, Gnanamanickam SS (1988) Biological control of *Fusarium oxysporum* f.sp. *cubense* in banana by inoculation with *Pseudomonas fluorescens*. Plant Soil 107:3–9

Snyder W, Hanson H (1940) The species concept in *Fusarium*. Am J Bot 27:64–67

Timmusk S, Grantcharova N, Wagner EGH (2005) *Paenibacillus polymyxa* invades plant roots and forms biofilm. Appl Environ Microbiol 71:7292–7300

Tombolini R, van der Gaag DJ, Gerhardson B, Jansson JK (1999) Colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* MA342 on barley seeds visualized by using green fluorescent protein. Appl Environ Microbiol 65:3674–3680

Trevors JT, Chassy BM, Dower WJ, Blaschek HP (1992) Electrotransformation of bacteria by plasmid DNA. In: Chang DC, Chassy BM, Saunders JA, Sowers AE (eds) Guide to electroporation, electrofusion. Academic, San Diego, pp 265–290

Trillas MI, Casanova E, Corxarrera L, Ordovas J, Borrero C, Aviles M (2006) Composts from agricultural waste, the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. Biol Control 39:32–38

Unge A, Jansson JK (2001) Monitoring population size, activity, distribution of gfp-luxAB -tagged *Pseudomonas fluorescens* SBW25 during colonization of wheat. Microb Ecol 41:290–300

von der Weid I, Artursson V, Seldin L, Jansson JK (2005) Antifungal, Root Surface Colonization Properties of GFP-Tagged *Paenibacillus brasilensis* PB177. World J Microbiol Biotechnol 12:1591–1597

Walker R, Rossall S, Ashe MJC (2002) Colonization of the developing rhizosphere of sugar beet seedlings by potential biocontrol agents applied as seed treatments. J Appl Microbiol 92:228–237

Walker TS, Bais HP, Grotewold E, Vivanco JM (2003) Root exudation, rhizosphere biology. Plant Physiol 132:44–51

Xue GP, Johnson JS, Dalrympl BP (1999) High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis*, *Bacillus licheniforms*. J Microbiol Meth 34:183–191

Zhang SS, Raza W, Yang XM, Hu J, Huang QW, Xu YC, Liu XH, Ran W, Shen QR (2008) Control of Fusarium wilt disease of cucumber plants with the application of a bioorganic fertilizer. Biol Fertil Soils 44:1073–1080

Zhao QY, Dong CX, Yang XM, Mei XL, Ran W, Shen QR (2010) Biocontrol of Fusarium wilt disease for *Cucumis melo* melon using bio-organic fertilizer. Appl Soil Ecol 47:67–75