

# Biological Potential of Bioorganic Fertilizer Fortified with Bacterial Antagonist for the Control of Tomato Bacterial Wilt and the Promotion of Crop Yields

Kai Wu<sup>1,2†</sup>, Zhiying Fang<sup>1†</sup>, Lili Wang<sup>3</sup>, Saifei Yuan<sup>1</sup>, Rong Guo<sup>1</sup>, Biao Shen<sup>1\*</sup>, and Qirong Shen<sup>1</sup>

<sup>1</sup>National Engineering Research Center for Organic-based Fertilizers, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, P.R. China

<sup>2</sup>Key Engineering Research Center of Ministry of Education for Sustainable Development and Utilization of Biomass Energy, College of Energy and Environmental Sciences, Yunnan Normal University, Yunnan 650500, P.R. China

<sup>3</sup>Ningbo Academy of Agricultural Science, Zhejiang 315040, P.R. China

Received: April 7, 2016

Revised: June 6, 2016

Accepted: July 1, 2016

First published online  
July 6, 2016

\*Corresponding author

Phone: +86-25-84396104;

Fax: +86-25-84396104;

E-mail: shenbiao@njau.edu.cn

<sup>†</sup>These authors contributed  
equally to this work.

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2016 by  
The Korean Society for Microbiology  
and Biotechnology

The application of *Bacillus* sp. in the biological control of plant soilborne diseases has been shown to be an environmentally friendly alternative to the use of chemical fungicides. In this study, the effects of bioorganic fertilizer (BOF) fortified with *Bacillus amyloliquefaciens* SQY 162 on the suppression of tomato bacterial wilt were investigated in pot experiments. The disease incidence of tomato wilt after the application of BOF was 65.18% and 41.62% lower at 10 and 20 days after transplantation, respectively, than in the control condition. BOF also promoted the plant growth. The SQY 162 populations efficiently colonized the tomato rhizosphere, which directly suppressed the number of *Ralstonia solanacearum* in the tomato rhizosphere soil. In the presence of BOF, the activities of defense-related enzymes in tomato were lower than in the presence of the control treatment, but the expression levels of the defense-related genes of the plants in the salicylic acid and jasmonic acid pathways were enhanced. It was also found that strain SQY 162 could secrete antibiotic surfactin, but not volatile organic compounds, to suppress *Ralstonia*. The strain could also produce plant growth promotion compounds such as siderophores and indole-3-acetic acid. Thus, owing to its innate multiple-functional traits and its broad biocontrol activities, we found that this antagonistic strain isolated from the tobacco rhizosphere could establish itself successfully in the tomato rhizosphere to control soilborne diseases.

**Keywords:** *Bacillus amyloliquefaciens*, *Ralstonia*, biocontrol, tomato bacterial wilt, bioorganic fertilizer

## Introduction

The current system of continuous monocropping causes severe soilborne plant diseases that directly threaten the intensive cropping system used in China. *Ralstonia* is a serious soilborne phytopathogen with a worldwide distribution and a large host range [2, 15]. Crop rotation was found to be an effective strategy to control many soilborne diseases, because most soilborne pathogens can persist for a long time in the soil with infested plant residues [18]. Chemical

pesticides not only cause bacterial resistance but are also harmful to the environment and human health [11]. Therefore, biological control by beneficial microorganisms is considered a potential environmentally friendly pesticide alternative.

Many *Bacillus* spp. have been found to efficiently suppress various soilborne bacterial and fungal plant diseases in greenhouse and field conditions [24, 32]. It was reported that many strains of *Bacillus* could produce different types of antibiotics, such as surfactin, iturin, and polyketides,

that act against the soilborne pathogens [7]. Furthermore, some *Bacillus* strains were also found to induce the production of defense enzymes and systemic resistance in plants, primarily through the mediation of salicylic acid/jasmonic acid/ethylene (SA/JA/ET) pathway signals [27, 34].

However, large-scale use of bio-control agents in fields is still limited because of the variability and inconsistency of the biocontrol activity. In some cases, such variability may be caused by the sensitivity of biocontrol agents to environmental influences such as plant types and soil conditions [10]. Guo *et al.* [14] found that the suppression efficacy of antagonist inoculation against bacterial wilt was inconsistent across field trials and was influenced by environmental conditions. Successful root colonization by *Bacillus* spp. is considered a prerequisite and is directly related to the efficacy of the bacteria in controlling soilborne diseases [4, 33]. Zhang *et al.* [33] also suggested that the application of *Bacillus subtilis* N11 with suitable organic carriers could promote the colonization of strain N11 of the rhizosphere and the roots of banana to effectively control banana wilt. The application of biocontrol agents with organic fertilizer is reportedly more effective in controlling tobacco bacterial wilt than the sole application of biocontrol agents [31]. Hence, understanding the antagonistic mechanisms of the beneficial microorganisms with organic carriers will help us to improve their effectiveness in the future.

In the present study, *Bacillus amyloliquefaciens* SQY 162 was selected to study its antagonistic potential against tomato bacterial wilt under greenhouse conditions. Strain SQY 162 was previously found to effectively suppress tobacco bacterial wilt in the greenhouse and in the field [31]. Therefore, the objectives of this study were (i) to evaluate the potential biocontrol abilities of the strain towards tomato bacterial wilt, and (ii) to investigate its mode of action.

## Materials and Methods

### Bacterial Strains

*Ralstonia solanacearum* ZJ3721 (biovar 3), which can cause tomato bacterial wilt, was kindly provided for the use in this study by Prof. Guo Jianhua (Department of Plant Pathology, Nanjing Agricultural University). The *Ralstonia* strains were grown in CPG medium [16]. If necessary, antibiotics were used in the following concentrations: ampicillin, 100 mg/l; kanamycin, 25 mg/l; tetracycline, 15 mg/l; and gentamicin, 12.5 mg/l. The antibiotics were purchased from Sigma-Aldrich (USA).

*B. amyloliquefaciens* SQY 162 (SQY162, CGMCC Accession No. 7500, China General Microbiology Culture Collection Center),

isolated from the rhizosphere soil of healthy tobacco surviving in a diseased field in Fuquan, Guizhou Province, China, showed strong antagonistic properties against the tobacco bacterial wilt pathogen [31].

The antagonistic activity of strain SQY 162 against *R. solanacearum* was determined on the CPG medium in vitro. The isolates were spotted on the center of the CPG plate with sterile toothpicks. After incubation at 30°C for 18 h, the plates were sprayed with *R. solanacearum* ZJ3721 and subsequently incubated under the same conditions for 24 h.

### Bioorganic Fertilizer Preparation

The organic fertilizer (OF) and bioorganic fertilizer (BOF) were prepared by solid fermentation according to the procedure described by Wu *et al.* [31]. The OF was composed of cattle manure compost and amino acid fertilizer (1:1 (w/w)). Cattle manure compost containing 35% organic matter, 2.51% N, 2.4% P<sub>2</sub>O<sub>5</sub>, 1.13% K<sub>2</sub>O, and 35.5% water was kindly supplied by Jiangyin Lianye Organic Fertilizers Ltd., China. Amino acid fertilizer containing 44.2% organic matter, 12.93% total amino acids, 4.4% N, 3.5% P<sub>2</sub>O<sub>5</sub>, 0.67% K<sub>2</sub>O, and 28.5% water was supplied by Jiangsu Xintiandi Amino Acid Fertilizers Ltd., China.

The BOF was produced as follows: approximately 10% (v/w) SQY 162 culture (10<sup>7</sup> CFU/ml) was inoculated into the OF and fermented at 40–45% moisture for 6 days, and the fertilizer was turned twice per day. After solid fermentation, it was found that the antagonistic strain SQY 162 population could reach to 2.5 × 10<sup>7</sup> CFU/g of fertilizer. The antagonistic agent population was measured with selective medium consisting of polymyxin B (35 µg/ml), lincomycin (5 µg/ml), and cycloheximide (50 µg/ml) [31].

### Pot Experiment Design

To evaluate the suppression efficacy of BOF fortified with the antagonist against tomato bacterial wilt, pot experiments were conducted in a greenhouse. Tomato seeds were surface-sterilized in 70% ethanol for 1 min, washed three times with sterile water, immersed in 2% NaClO for 2 min, and washed three times in sterile water. The seeds were then transferred onto steam-sterilized vermiculite. After 30 days of incubation, the plants were gently transferred into a pot containing 200 g of sterile paddy soil. The pot treatments were designed as follows: (1) Control, the soil was treated with nothing; (2) OF, the soil was treated with 2 g of organic fertilizer; (3) BOF, the soil was treated with 2 g of bioorganic fertilizer fortified with SQY 162. Each treatment was performed with three replicates, and each replicate contained 15 plants. For the tomato pot experiment, a suspension of *R. solanacearum* ZJ3721 was inoculated into the soil (10<sup>7</sup> CFU/g soil) at plant transplantation.

The disease index (di) of tomato bacterial wilt was recorded on the 20<sup>th</sup> day after transplantation according to a method described elsewhere [25], where 0 indicated no wilting, 1 indicated that 1%

to 25% of the leaves were wilted, 2 indicated that 26% to 50% of the leaves were wilted, 3 indicated that 51% to 75% of the leaves were wilted, and 4 indicated that 76% to 100% of the leaves were wilted or dead. The DI was calculated as  $DI = [\sum(\text{number of diseased plants in this index} \times di) / (\text{total number of plants investigated} \times \text{highest di})] \times 100\%$ . After transplantation, the aboveground fresh weight of the plant was also recorded at each treatment for 20 days.

#### Enumeration of Pathogen and Antagonist Populations in the Tomato Rhizosphere

The populations of SQY 162 and *R. solanacearum* in the rhizosphere soil in different pot treatments were counted on the 20<sup>th</sup> day after transplantation. Rhizosphere soils from three plants were collected individually from each replicate as described by Smalla *et al.* [26]. Briefly, the roots were shaken vigorously to separate soil that did not adhere tightly to the roots. Then, the soil still tightly adhering to the roots was harvested as rhizosphere soil. The population of SQY 162 was counted on selective medium as described above.

The population of *R. solanacearum* was counted on semi-selective medium from South Africa (SMSA) [9]. The SMSA (1 L) contained casamino acid, 1 g; peptone, 10 g; glycerol, 5 ml; agar, 20 g; crystal violet, 5 mg; polymyxin B sulfate, 100 mg; bacitracin, 25 mg; chloromycetin, 5 mg; penicillin, 0.5 mg; and cycloheximide, 100 mg. The antibiotics were purchased from Sigma-Aldrich.

#### Defense Enzyme Activity Assay

The plant leaves collected from the pot experiments as described above were used to determine the activities of the defense enzymes. The activities of defense enzymes were determined on the 20<sup>th</sup> day after the inoculation of the pathogen, as described above. Plant leaves were weighed and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized tissue solution was immediately assessed to determine the activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) and the malondialdehyde (MDA) content using the Enzyme Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's procedure.

#### Expression of Defense Genes with Quantitative Real-Time PCR

The plants leaves collected from the pot experiments as described above were used for RNA extraction to determine the expression of the defense genes. The RNA was harvested from the tomato leaves on the 3<sup>rd</sup> and 15<sup>th</sup> days after the inoculation of the pathogen using the RNA Kit according to the manufacturer's instructions (Takara MiniBest Universal RNA Extraction Kit; China). The collected RNA samples were then reverse-transcribed into cDNA in a 20 µl reverse transcription system (TransGen Biotech, China) according to the manufacturer's protocol.

The primers for quantitative reverse transcription-PCR (qRT-PCR) of tomato defense genes were described previously [21].

qRT-PCR was measured with a SYBR Premix Ex Taq (Perfect Real Time) Kit (Takara). The actin gene was used as the control in the qRT-PCR of tomato defense genes. qRT-PCR was performed with an ABI 7500 system under the following conditions: cDNA was denatured for 10 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 34 sec at 60°C. The qRT-PCR data were analyzed according to the  $2^{-\Delta\Delta CT}$  method described by Livark and Schmittgen [20].

#### Antagonistic Effects of Volatile Organic Compounds (VOCs) on the Survival of *R. solanacearum* in Soil

The antagonistic effects of VOCs on the survival of *R. solanacearum* in soil were determined with a co-culture system. The system consisted of two connected flasks. One flask was used to culture the SQY 162 to produce VOCs. The other flask was used to incubate the *R. solanacearum* in soil. One milliliter of liquid culture with SQY 162 was inoculated into 100 ml of sterile LB broth in the first 250 ml flask. The cultures were incubated at 30°C with 130 rpm shaking to produce the VOCs. The *R. solanacearum* ZJ3721 suspension was inoculated into the sterile paddy soil at a final concentration of  $10^8$  CFU/g soil in the other 250 ml flask. The two flasks were connected with a rubber stopper and rubber tubing. The collected paddy soil had the following characteristics: pH, 7.29; organic carbon, 22.31 g/kg; total N, 2.55 g/kg; total P, 1.02 g/kg; and available K, 272.89 mg/kg. The experiments consisted of three replicates, and each replicate contained five co-culture systems. The population densities of *R. solanacearum* were determined with the selective medium as described above on the 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> days after inoculation.

#### Isolation and Identification of Antibiotics Produced by Strain SQY 162

For the production of antibiotics, cultures inoculated with SQY 162 at a concentration of 1% (v:v) were incubated for 60 h at 30°C with 170 rpm shaking. Then, the cell-free supernatant was obtained through centrifugation (10,000 rpm at 4°C). The supernatant was adjusted to pH 2.0 with HCl. The crude compounds were harvested by centrifugation (10,000 rpm at 4°C for 10 min). The crude compounds were dissolved in methanol and passed through a 0.22 µm filter.

HPLC analysis of the antibiotics was performed at 30°C using an XDB-C18 column (4.6 mm × 250 mm; Agilent Technologies, USA). The mobile phase consisted of 10 mM trifluoroacetic acid:acetonitrile (30:70, for surfactin (v:v)) buffer solution. Each sample solution (10 µl) was eluted for 10 min at a flow rate of 0.8 ml/min and detected at 280 nm. Standard surfactin was purchased from Sigma-Aldrich and chromatographed under the same conditions.

The antagonistic activity against the pathogen at the peak of the HPLC profile at 3.5 min was determined on the CPG medium in vitro. The 10 µl liquid samples collected from the HPLC at 3.5 min were spotted on 8-mm-diameter sterile papers on the center of the CPG plate. Then, the plates were sprayed with *R. solanacearum*

ZJ3721 and subsequently incubated at 30°C for 24 h. A control treatment of 10 µl methanol was also spotted on the papers as described above.

#### Determination of Plant Growth Promotion Compounds Produced by SQY 162

Salkowski reagent was used to examine indolic compounds in this study [13]. SQY 162 was inoculated in 100 ml of LB broth containing tryptophan at a final concentration of 20 µg/ml. After 60 h incubation at 170 rpm at 28°C, cell-free supernatants were collected by centrifuging at 5,000 ×g for 15 min at 4°C. Then, 1.5 ml of the supernatant was added into 1.5 ml of Salkowski reagent (12 g of ferric chloride per liter in 7.9 M sulfuric acid), and the mixture was left in the dark for 30 min at room temperature for color variation. If the mixture became pink or red, this indicated that the strains could produce indolic compounds. For the control treatment, the same volume of LB broth was added into Salkowski reagent.

Production of siderophore by SQY 162 was determined according to the method of Alexander and Zuberer [3]. After incubation at 28°C for 3 days, siderophore production was estimated by a change in the color from blue to orange.

Extraction of IAA was performed according to the method of Karadeniz *et al.* [17] with some modifications. Briefly, the cell-free supernatants were adjusted to pH 2.5 using 6 M HCl and then extracted 3 times with an equal volume of ethyl acetate. The upper phase was collected and the water phase was extracted 3 times with an equal volume of ethyl acetate. The upper solutions were dried with a rotary vacuum evaporator and finally eluted with methanol.

The analysis of IAA was performed by HPLC (Agilent 1200, USA) with a ZORBAX Eclipse XDB-C18 column (4.6 × 250 mm; USA) and a 230 nm UV detector. The mobile phase consisted of 60% solvent A (methanol) and 40% solvent B (water with 0.1% acetic acid) at a flow rate of 0.4 ml/min at 30°C.

#### Data Analysis

The data were calculated with Microsoft Excel 2007 and analyzed using SPSS software (ver. 13.0; SPSS, USA). An analysis of variance was performed and Duncan's multiple tests were used, with a significance level of  $p < 0.05$ .

## Results

#### Control of Tomato Bacterial Wilt by the Bioorganic Fertilizer

The results of the antagonistic assay *in vitro* showed that strain SQY 162 could inhibit the growth of *R. solanacearum* on the plate, suggesting that the strain had strong antagonistic activity against the pathogen (Fig. 1A).

The effects of BOF fortified with the bacterial antagonist SQY 162 on the control of plant soilborne disease were evaluated in pot experiments. The biocontrol efficacy on

plant soilborne disease varied by treatment and by plant growth stage (Fig. 1B). The disease incidences in soil treatments inoculated with BOF were the lowest of each of the three treatments during all growth stages. The disease incidence was 65.18% and 41.62% lower at 10 and 20 days, respectively, after the application of BOF fortified with the antagonistic strain SQY 162 than after the control treatments. Application of OF was also shown to decrease the disease incidence 10 days after transplantation. However, there were no significant differences between the control and OF treatments on the 20<sup>th</sup> day after transplantation.

Owing to the successful suppression of tomato bacterial wilt, the aboveground fresh and dry weights of tomato both increased more in the presence of BOF than in the control condition (Fig. 1D).

#### Rhizosphere Antagonist and Pathogen Populations

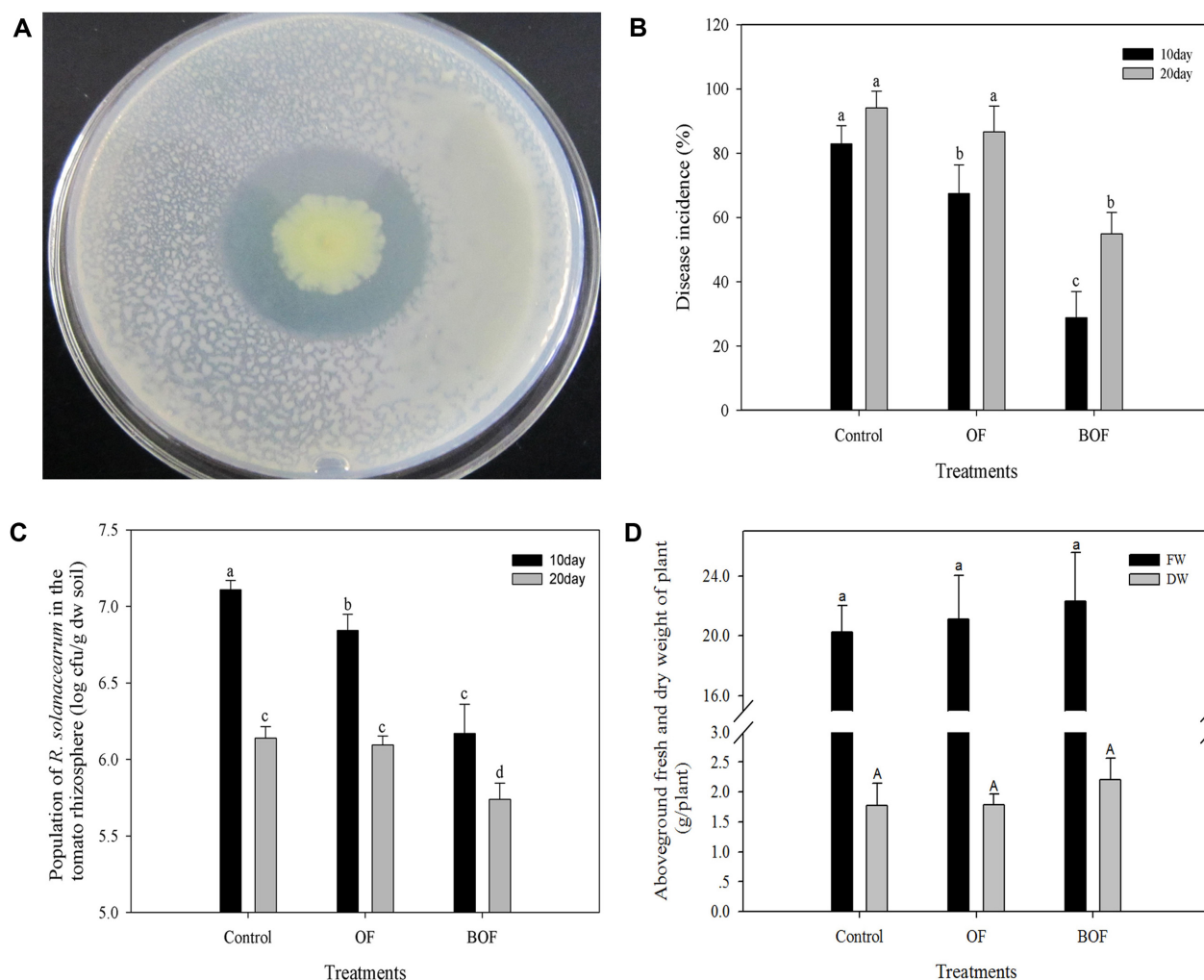
The antagonist population was monitored on the 10<sup>th</sup> and 20<sup>th</sup> days after inoculation, as described above. The SQY 162 population in the tomato rhizosphere decreased as plant growth increased. On the 10<sup>th</sup> day after inoculation, the SQY 162 population in the tomato rhizosphere decreased to 5.76 log CFU/g dry weight soil (log CFU/g dw soil). After being inoculated for 20 days, the bacterial population in the tomato rhizosphere remained at 5.33 log CFU/g dw soil.

The pathogen populations were also counted on a selective medium on the 10<sup>th</sup> and 20<sup>th</sup> days after inoculation, as described above. The *R. solanacearum* population in the tomato rhizosphere differed by treatments (Fig. 1C). The *R. solanacearum* populations in the BOF treatments were the lowest among the three treatments, with 6.16 and 5.74 log CFU/g dw soil on the 10<sup>th</sup> and 20<sup>th</sup> days after inoculation, respectively. At the beginning of plant growth, the *R. solanacearum* population in the tomato rhizosphere was 45.10% lower after the application of OF than after control treatment. However, the results showed few differences in *R. solanacearum* population size between the control and OF treatments 20 days after transplantation.

#### Effect of BOF on the Activities of the Defense Enzymes

Based on the biocontrol efficacy of BOF on tomato bacterial wilt, the influence of BOF on the activities of tomato defense enzymes (SOD, CAT, and POD) and on the tomato MDA content were evaluated. The results showed that the SOD enzyme activity was 9.32% lower after the application of BOF than after the control treatments (Table 1). The CAT activity was the lowest in BOF treatments among the three treatments, being 34.05% and 29.93% lower than





**Fig. 1.** Effects of SQY 162 on the pathogen in vitro (A), disease incidence (B), population densities of *R. solanacearum* (C), and aboveground fresh and dry weight of the tomato plants (D).

Different letters above the bars indicate significant differences ( $p < 0.05$ ).

the CAT activity observed in the control and OF treatments, respectively. The results also showed that the POD activity and MDA content in the BOF treatments were 53.33% and 24.21% lower, respectively, than in the control treatments. However, the activities of the three enzymes in the OF treatments were equivalent to or slightly lower than those in the control treatments.

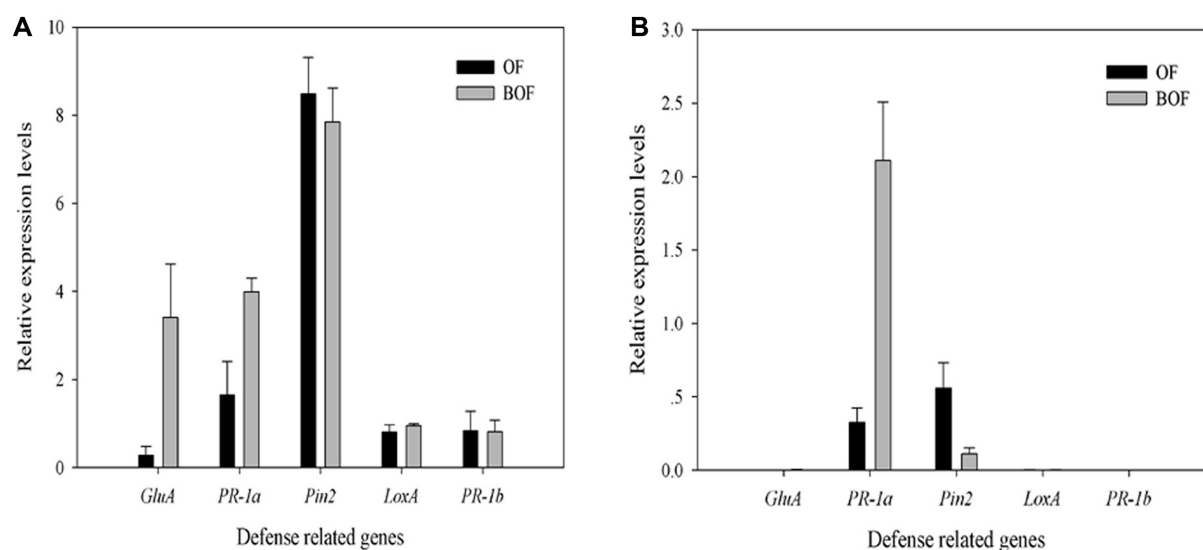
#### Quantification of the Expression of the Defense Genes

To further study the mechanisms governing the biocontrol of the tomato bacterial wilt by BOF, the expression levels of the defense-related genes were determined at different times after transplantation. Three days after application, BOF treatment triggered the expression of several genes in different defense pathways. As shown in Fig. 2A, application

**Table 1.** Effect of BOF on MDA accumulation and defense-related enzyme activities (SOD, CAT, and POD) in tomato plants.

	MDA (nmol/g)	SOD (U/g)	CAT (U/g)	POD (U/g)
Control	66.91 ± 8.83a	233.80 ± 12.74a	42.96 ± 2.35a	10.95 ± 2.06a
OF	57.48 ± 7.73ab	229.15 ± 7.32a	40.43 ± 3.47a	7.20 ± 1.93ab
BOF	50.71 ± 4.79b	212.02 ± 6.69b	28.33 ± 3.61b	5.11 ± 2.38b

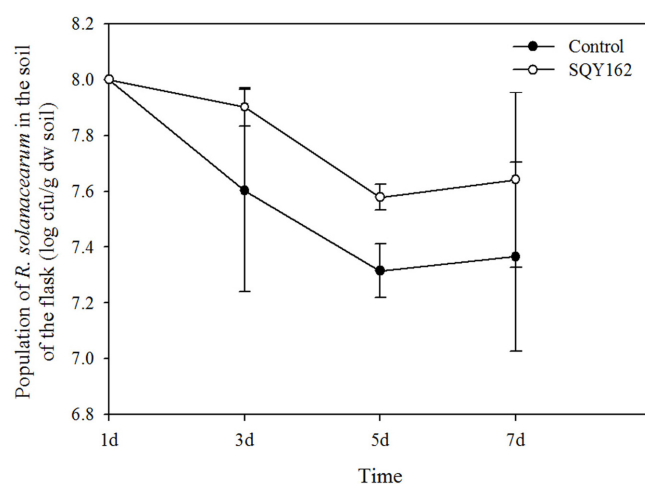
Plant leaves were collected on the 20<sup>th</sup> day after the inoculation of the pathogen. The data are the means of three replicates. The means and standard errors are shown.



**Fig. 2.** Expressions of tomato defense-related genes in tomato leaves following inoculation with SQY 162 on the 3rd (A) and 15th days (B) after inoculation.

GluA and PR-1a are involved in the salicylic acid pathway, Pin2 and LoxA are involved in the jasmonic acid pathway, and PR-1b is involved in the ethylene pathway. The means and standard errors are shown.

of BOF increased the transcription levels of *GluA* and *PR-1a* genes (salicylic acid pathway, SA) 3.41- and 3.99- fold more, respectively, than the control condition. However, no significant differences were found between the OF and control treatment with respect to the transcription levels of *PR-1a*. The transcription levels of *GluA* in the OF treatments were significantly lower than those in the control treatments.



**Fig. 3.** Effect of volatile organic compounds produced by strain SQY 162 on the population density of *R. solanacearum* in the soil.

The means and standard errors are shown.

The results also showed that the application of both OF and BOF could increase the expression of *Pin2*, but not *LoxA*, in the jasmonic acid pathway (JA); the OF and BOF expressions levels were 8.47 and 7.84 times higher, respectively, than in the control treatments. Furthermore, there were few differences in the expression of *LoxA* among the three treatments. The transcription levels of *PR-1b* (ethylene pathway, ET) in the OF and BOF treatments were either equivalent to or slightly lower than those in the control treatments.

The effects of different treatments on the expression levels of defense-related tomato genes at 15 days after transplantation were also determined. Almost all of the expression levels of the genes described above in the BOF treatments decreased significantly, but the expression levels of *PR-1a* remained significantly higher (2.11-fold) than those in the control treatments.

#### Effects of Strain SQY 162 VOCs on the Survival of *R. solanacearum* in Soil

The effects of the VOCs produced by SQY 162 on the survival of *R. solanacearum* were determined with a co-culture flask system. The population density of *R. solanacearum* exhibited a decreasing trend with increasing incubation time. Seven days after incubation, the population density of the pathogen agent in the control treatment remained at 7.37 log CFU/g dw soil (Fig. 3). Furthermore, the population

density of *R. solanacearum* in SQY 162 treatment was higher than in the control treatment at all times, indicating that the VOCs of strain SQY 162 could increase the population density of the pathogen in soil to a certain extent.

#### HPLC Analysis of Antibiotic Compounds Produced by Strain SQY 162

The HPLC results for surfactin analysis of the lipopeptide are shown in Fig. 4. By comparing the retention times of the characteristic peaks of the standard surfactin sample, one homolog of surfactin was identified from the crude lipopeptide (Fig. 4A). The results of Figs. 4C and 4D also show that the compound at the retention time of 3.5 min was capable of inhibiting the growth of *R. solanacearum* on the plate.

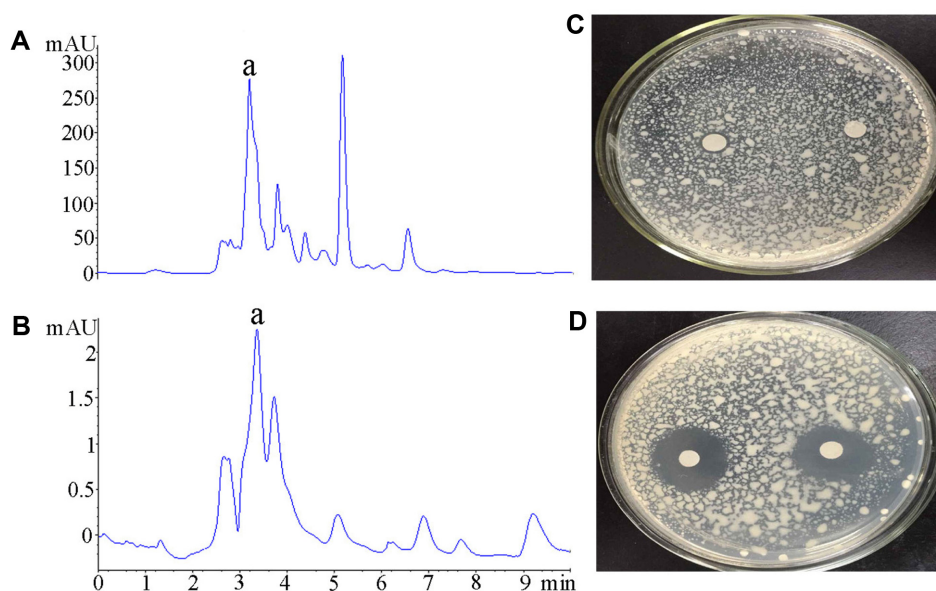
#### Analysis of Plant-Growth-Promoting Compounds Produced by SQY 162

As shown in Figs. 5A and 5B, strain SQY 162 was able to produce siderophores and indolic compounds. For further analysis and quantification of the indolic compounds, strain SQY 162 was incubated in LB broth containing L-tryptophan to produce IAA. By comparing the peak of the standard IAA sample with the retention time of 10.528 min, it was found that strain SQY 162 could produce as much as 11.4 mg/l IAA.

## Discussion

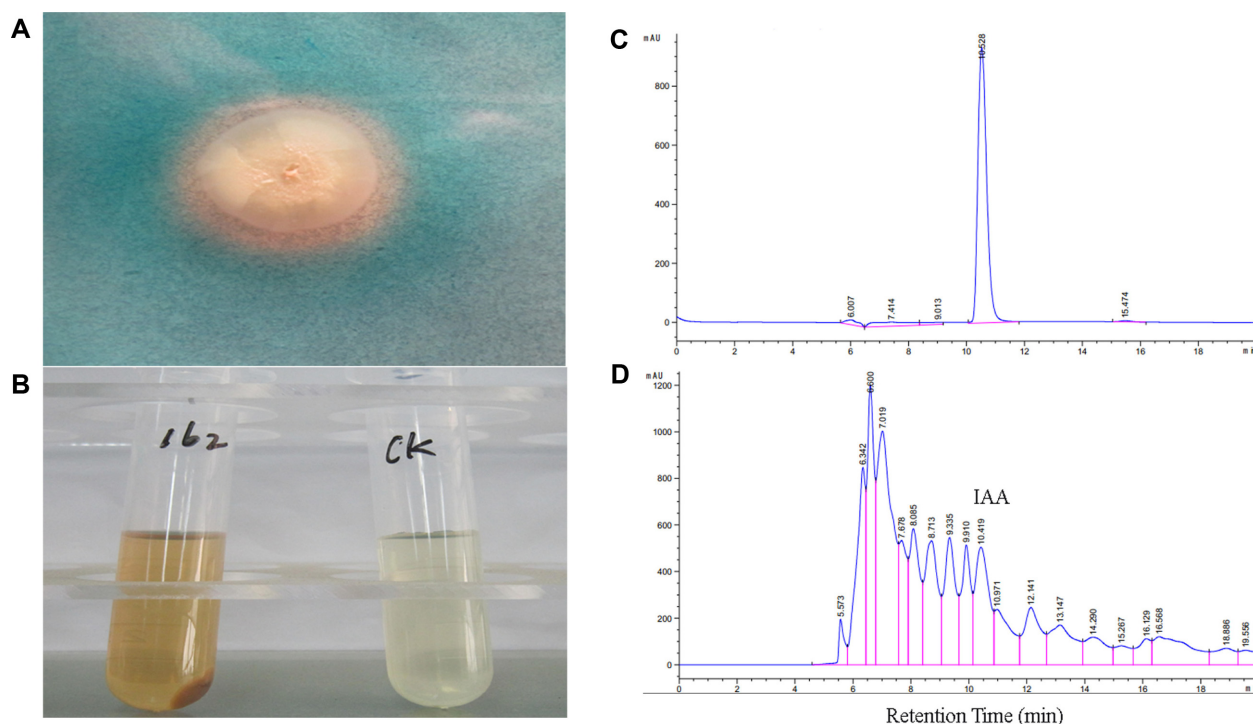
The results showed that SQY 162, isolated as an antagonist against the tobacco bacterial wilt pathogen, could also strongly inhibit the growth of the tomato bacterial wilt pathogen agent in vitro, indicating that SQY 162 has a broader spectrum in the control of plant soilborne diseases. In the pot experiment, the disease incidence of tomato bacterial wilt after the application of BOF fortified with SQY 162 was significantly lower than after the control or OF treatments. The tomato biomass also increased in the presence of BOF. The study further revealed that strain SQY 162 was capable of producing siderophores and indolic compounds such as IAA, which could be one of the reasons for the plant growth promotion in the pot experiments.

The ability of antagonistic bacteria to efficiently colonize at the rhizosphere is a key factor in their successful improvement of plant health and the suppression of plant pathogens [8, 33]. Our previous study also showed that the enhanced colonization activity of *B. amyloliquefaciens* SQR9 could increase biocontrol efficacy against cucumber fusarium wilt [29]. These organic fertilizers, consisting of cattle manure compost and amino acid fertilizer, improved the colonization ability and biocontrol efficacy of antagonists more than compost alone [32, 33]. Plate counts showed that SQY 162 was able to survive and persist at a higher level in



**Fig. 4.** Determination of antibiotics produced by strain SQY 162.

HPLC chromatogram of the standard surfactin sample (A) and the extraction sample (B). The inhibitory effects of methanol (C) and the peak collected at 3.5 min (D) on the growth of *R. solanacearum* in vitro. The peak at letter "a" is one of the homologs of surfactin.



**Fig. 5.** Plant-growth-promoting associated compounds produced by strain SQY 162.

Siderophore (A) and indolic compounds (B). The chromatograms of standard samples of IAA (C) and the extraction sample (D). The peak with the retention time of 10.528 min is IAA.

the tomato rhizosphere, directly suppressing the growth of *R. solanacearum* in the tomato rhizosphere during all plant growth stages.

Beneficial microorganisms control plant disease by inducing plant systemic resistance [1]. Kloepper *et al.* [19] indicated that *Bacillus* spp. could induce the systemic resistance of plants to suppress various diseases in a diversity of hosts. Salicylic acid and jasmonic acid were found to be critical components involved in the response of the plant to pathogen invasion [28]. In the present study, application of BOF enhanced gene expression levels in the SA and JA pathways with different patterns, but lowered the gene expression of *LoxA* in tomato JA pathways. This result was in accordance with that of a previous study that demonstrated that *B. cereus* AR156 triggered systemic resistance by simultaneously activating the SA and JA signaling pathways against *Pseudomonas syringae* [23]. When plants experience stress conditions such as injury, pathogen infection, or extreme temperatures, the activities of the plants' defense enzymes generally increase [12]. The results of this study showed that the activities of enzymes such as SOD, CAT, and POD decreased in the presence of BOF significantly more than in the control condition because of

the lower disease incidence. This result agrees with that of a previous study [5, 27], which suggested that the activities of defense-related enzymes increased over time as the disease developed. The plant MDA concentration reveals the degree of damage to plant cell membranes under stress conditions [22]. Wu *et al.* [30] indicated that the MDA content of leaves treated with BOF was lower than that of control leaves. In this study, application of BOF significantly induced the systemic resistance of tomato plants to inhibit the infection of pathogens, resulting in the lower activities of the defense enzymes and the lower MDA content in plants treated with BOF than in plants that received the control treatment.

Multiple factors could be involved in the biological control of plant diseases by the application of beneficial microorganisms [24, 32]. In addition to systemic resistance induction and competition for nutrients in the rhizosphere, the plant-growth-promoting bacterium *B. amyloliquefaciens* also acts as an antagonist of recognized root pathogens, including bacteria, fungi, and nematodes, and has the ability to synthesize lipopeptide and polyketide antibiotics [6, 7]. Here, the nonvolatile antibiotic surfactin produced by SQY 162 was shown to strongly inhibit the growth of



*R. solanacearum* on the plate. However, it was also found that SQY 162 could not produce volatile organic antibiotic compounds to suppress the growth of the pathogen. Moreover, the pathogen was able to use the VOCs produced by SQY 162 as carbon sources to increase its population density to a certain degree when *R. solanacearum* was incubated alone in soil.

In summary, the antagonistic bacterium strain SQY 162 isolated from the tobacco rhizosphere was also able to effectively suppress tomato bacterial wilt in pot experiments, demonstrating strong colonization ability in the tomato rhizosphere and the ability to induce systemic resistance in tomato plants. The results also indicated that the SA and JA signaling pathways, but not the ET pathway, were induced with different patterns after the inoculation of BOF. It was also found that SQY 162 could not produce VOCs to suppress the pathogen but could produce surfactin to inhibit the growth of *R. solanacearum*. Further studies on the dominant mechanism involved in the biological control of *R. solanacearum* are needed.

## Acknowledgments

This research was financially supported by the National Natural Science Foundation of China (41571242), the projects of the Jiangsu Science and Technology Program (BE2014398), the projects of the 111 Project (B12009), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the 58<sup>th</sup> China Postdoctoral Science Foundation (2778), and the Foundation of Education Department of Yunnan Province (2015Y107).

## References

- Adam M, Heuer H, Hallmann J. 2014. Bacterial antagonists of fungal pathogens also control root-knot nematodes by induced systemic resistance of tomato plants. *PLoS One* **9**: e90402.
- Ahn P, Chung HS, Lee YH. 1998. Vegetative compatibility groups and pathogenicity among isolates of *Fusarium oxysporum* f. sp. *cucumerinum*. *Plant Dis.* **82**: 244-246.
- Alexander D, Zuberer D. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biol. Fert. Soils* **12**: 39-45.
- Bais HP, Fall R, Vivanco JM. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol.* **134**: 307-319.
- Chen C, Belanger RR, Benhamou N, Paulitz TC. 2000. Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. *Physiol. Mol. Plant Pathol.* **56**: 13-23.
- Chen X, Koumoutsis A, Scholz R, Schneider K, Vater J, Süßmuth R, et al. 2009. Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. *J. Biotechnol.* **140**: 27-37.
- Chen XH, Koumoutsis A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, et al. 2007. Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat. Biotechnol.* **25**: 1007-1014.
- Chowdhury SP, Dietel K, Rändler M, Schmid M, Junge H, Borriss R, et al. 2013. Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. *PLoS One* **8**: e68818.
- Elphinstone J, Hennessy J, Wilson J, Stead D. 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *EPPO Bull.* **26**: 663-678.
- Fravel D. 2005. Commercialization and implementation of biocontrol 1. *Annu. Rev. Phytopathol.* **43**: 337-359.
- Gamliel A, Austerweil M, Kritzman G. 2000. Non-chemical approach to soilborne pest management – organic amendments. *Crop Prot.* **19**: 847-853.
- García-Limones C, Hervás A, Navas-Cortés JA, Jiménez-Díaz RM, Tena M. 2002. Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f. sp. *ciceris*. *Physiol. Mol. Plant Pathol.* **61**: 325-337.
- Glickmann E, Dessaux Y. 1995. A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl. Environ. Microbiol.* **61**: 793-796.
- Guo JH, Qi HY, Guo YH, Ge HL, Gong LY, Zhang LX, Sun PH. 2004. Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biol. Controls* **29**: 66-72.
- Hayward A. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* **29**: 65-87.
- Hendrick CA, Sequeira L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. *Appl. Environ. Microbiol.* **48**: 94-101.
- Karadeniz A, Topcuoğlu Ş, Inan S. 2006. Auxin, gibberellin, cytokinin and abscisic acid production in some bacteria. *World J. Microbiol. Biotechnol.* **22**: 1061-1064.
- King SR, Davis AR, Liu W, Levi A. 2008. Grafting for disease resistance. *HortScience* **43**: 1673-1676.
- Kloepper JW, Ryu CM, Zhang S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**: 1259-1266.
- Livark K, Schmittgen T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2

- (-delta delta C (T)) method. *Methods* **25**: 402-408.
21. Milling A, Babujee L, Allen C. 2011. *Ralstonia solanacearum* extracellular polysaccharide is a specific elicitor of defense responses in wilt-resistant tomato plants. *PLoS One* **6**: e15853.
  22. Morsy MR, Jouve L, Hausman JF, Hoffmann L, Stewart JM. 2007. Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. *J. Plant Physiol.* **164**: 157-167.
  23. Niu DD, Liu HX, Jiang CH, Wang YP, Wang QY, Jin HL, Guo JH. 2011. The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate and jasmonate/ethylene-dependent signaling pathways. *Mol. Plant Microbe Interact.* **24**: 533-542.
  24. Pérez-García A, Romero D, De Vicente A. 2011. Plant protection and growth stimulation by microorganisms: biotechnological applications of *Bacilli* in agriculture. *Curr. Opin. Biotechnol.* **22**: 187-193.
  25. Scherf JM, Milling A, Allen C. 2010. Moderate temperature fluctuations rapidly reduce the viability of *Ralstonia solanacearum* race 3, biovar 2, in infected geranium, tomato, and potato plants. *Appl. Environ. Microbiol.* **76**: 7061-7067.
  26. Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, et al. 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* **67**: 4742-4751.
  27. Tan S, Dong Y, Liao H, Huang J, Song S, Xu Y, Shen Q. 2013. Antagonistic bacterium *Bacillus amyloliquefaciens* induces resistance and controls the bacterial wilt of tomato. *Pest Manag. Sci.* **69**: 1245-1252.
  28. Thaler JS, Owen B, Higgins VJ. 2004. The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. *Plant Physiol.* **135**: 530-538.
  29. Weng J, Wang Y, Li J, Shen Q, Zhang R. 2013. Enhanced root colonization and biocontrol activity of *Bacillus amyloliquefaciens* SQR9 by *abrB* gene disruption. *Appl. Microbiol. Biotechnol.* **97**: 8823-8830.
  30. Wu HS, Yang XN, Fan JQ, Miao WG, Ling N, Xu YC, et al. 2009. Suppression of *Fusarium* wilt of watermelon by a bio-organic fertilizer containing combinations of antagonistic microorganisms. *BioControl* **54**: 287-300.
  31. Wu K, Yuan S, Wang L, Shi J, Zhao J, Shen B, Shen Q. 2014. Effects of bio-organic fertilizer plus soil amendment on the control of tobacco bacterial wilt and composition of soil bacterial communities. *Biol. Fert. Soils* **50**: 961-971.
  32. Yuan S, Wang L, Wu K, Shi J, Wang M, Yang X, et al. 2014. Evaluation of *Bacillus*-fortified organic fertilizer for controlling tobacco bacterial wilt in greenhouse and field experiments. *Appl. Soil Ecol.* **75**: 86-94.
  33. Zhang N, Wu K, He X, Li SQ, Zhang ZH, Shen B, et al. 2011. A new bioorganic fertilizer can effectively control banana wilt by strong colonization with *Bacillus subtilis* N11. *Plant Soil* **344**: 87-97.
  34. Zhao Q, Dong C, Yang X, Mei X, Ran W, Shen Q, Xu Y. 2011. Biocontrol of *Fusarium* wilt disease for *Cucumis melo* melon using bio-organic fertilizer. *Appl. Soil Ecol.* **47**: 67-75.