Current Microbiology (2020) 77:3085–3094
https://doi.org/10.1007/s00284-020-02071-7

Colonization on Cotton Plants with a GFP Labeled Strain of *Bacillus axarquiensis*

Bo Wang[1] · Chuan-xing Wan[1] · Hong Zeng[1]

Received: 21 January 2020 / Accepted: 30 May 2020 / Published online: 25 July 2020

Abstract

Verticillium dahliae was one of the most important diseases caused Verticillium wilt of cotton. In our previous study, *Bacillus axarquiensis* TUBP1 was screened and found to be an antagonistic strain against *V. dahliae* with 43% biocontrol effect in the cotton field. In order to uncover the functional mechanism of *B. axarquiensis* against *Verticillium* wilt in cotton, the colonization of *B. axarquiensis* labeled with a green fluorescent protein (GFP) was investigated in cotton plants and the rhizosphere soil. Firstly, a plasmid (pHT-315) containing *gfp* gene was successfully transformed into wild *B. axarquiensis* TUBP1 and labeled a green fluorescence by electroporation, which didn't change the bioactivity in vitro. In gnotobiotic conditions, cotton seeds were then inoculated with the *gfp*-labeled strain and grown in green house. Observation with a confocal laser scanning microscope and a scanning electron microscope showed that GFP-labeled *B. axarquiensis* TUBP1 infected cotton roots and widely distributed in epidermis, cortical parenchyma, intercellular spaces, the xylem vessels, and pith cells as well as root hair cells through cracks formed at the lateral root junctions, followed by a slow migration from roots to stems and leaves. Quantitative fluorescence and flow cytometry (FACS) approaches showed a gradual decrease in the number of TUBP1-315*gfp* with increasing inoculation time. However, TUBP1-315*gfp* levels were detectable till 45 days after planting. In contrast, no fluorescence signal was detected in the non-inoculated groups. Therefore, GFP-labeled *B. axarquiensis* TUBP1 exhibited colonization in different parts of cotton plants from the rhizosphere soil.

Introduction

Verticillium wilt is one of the disastrous diseases affecting cotton, which seriously damages the yield and crop quality [1]. In recent years, with the rise in green and organic agriculture, the use of biological agents to control Verticillium wilt in cotton has attracted much attention. Many biocontrol products such as GB03, MBI600, Baikang and Mai Fengning have been developed [2, 3]. These products mainly including *Bacillus subtilis*, which control Verticillum wilt through inhibit plant pathogens directly or can also confer induced systemic resistance (ISR) in plants indirectly. Besides, biocontrol products inhibition of pathogen growth by nutrient competition. *Bacillus axarquiensis* TUBP1 is a Gram-positive biocontrol strain, which was isolated and identified from cotton field soil in Xinjiang, Northwestern China. Studies showed that major active compound was peptide T in *B. axarquiensis* TUBP1 determined by LC–MS/MS, which led to mitochondria-mediated apoptotic cell death in *V. dahliae* [4].The biocontrol effects of *B. axarquiensis* TUBP1 on *V. dahliae* were 43% in the cotton field [5].

Whether biocontrol bacteria colonization in plant or not is one of the main reasons for the biocontrol effects. There are many factors affecting the colonizing ability of biocontrol bacteria in plant roots, including plant type (different root excretions), soil texture, temperature, and soil moisture [6]. Studying the colonization pattern can not only reveal its microecological characteristics, but also help in evaluating the adaptability and stability of bioprotective activity in plant rhizosphere, thereby providing insights into the mechanism of biological control. As we known that *Bacillus* has great advantages, such as forming endospores to resist

Hong Zeng and Chuan-xing Wan contributed equally to this work.

✉ Chuan-xing Wan
wanchuanxing@163.com

✉ Hong Zeng
zenghong0705@163.com

[1] Xinjiang Production and Construction Corps Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin/College of Life Science, Tarim University, Alar 843300, Xinjiang, People's Republic of China

desiccation and UV irradiation, surviving in adverse conditions and producing antimicrobial metabolites to control plant pathogens, potential uses in agriculture. Plant colonization studies revealed that *B. pumilus* SE34, *B. subtilis* MB73/2 [7], *B. subtilis* CB-R05 [8], *B. amyloliquefaciens* FZB42 have different colonization patterns on various plants [9]. Conventional techniques, such as antibiotic labeling and radioisotope labeling, are normally used to investigate the colonization and biocontrol mechanisms [10]. Moreover, green fluorescent protein (GFP) has also been used to study the colonization of Gram-negative and Gram-positive bacteria including plant pathogens, diazotrophs, and different biocontrol strains [8, 11]. In order to uncover the colonization of *B. axarquiensis* TUBP1 in cotton plant and rhizosphere soil against *V. dahliae*, the *gfp* gene was introduced into wild *B. axarquiensis* TUBP1 strain by electrotransformation, and the colonization dynamics of *B. axarquiensis* tagged with *gfp* was investigated in both gnotobiotic and field systems using confocal laser scanning microscopy (CLSM), scanning electron microscope (SEM), fluorescence spectrophotometer (FL), and flow cytometry (FACS).

Materials and Methods

Strains and Growth Conditions

Bacillus axarquiensis TUBP1 wild strain was originally isolated from the soil of 12 consecutive cropping cotton fields in Alar, Xinjiang province. The strain was cultured in Luria–Bertani (LB) medium at 37 °C and 180 rpm min^{-1} for 48 h [12].

Evaluation of Antibiotic Resistance of *B. axarquiensis* TUBP1

To determine the tolerance of *B. axarquiensis* TUBP1 to a variety of antibiotics, the strain was cultured overnight in LB liquid medium (without any antibiotics). Then, 100 µl of *B. axarquiensis* TUBP1 was inoculated on a solid plate containing different concentrations of antibiotics (ampicillin, chloramphenicol, streptomycin, kanamycin, chlortetracycline, erythromycin, and apramycin). The inoculated strains were cultured overnight at 37 °C in inverted positions to observe their growth. The concentration of antibiotics used was similar to the optimum concentration gradient of the antibiotic working solution recorded in Takara Catalog (Bowie Biotech Beijing Co., Ltd.).

Obtaining pHT-315*gfp* Plasmid

Plasmid DNA extraction and verification of pHT-315*gfp*: DH5α carrying pHT-315*gfp* plasmid was grown at Zhongguo Agricultural University. DH5α competent cells were purchased at Beijing Trans Gen Biotechnology Co., Ltd. The strain was grown in LB medium containing 100 µg ml^{-1} of ampicillin.

The pHT-315*gfp* plasmid was extracted according to the following procedure. Single colony of DH5α carrying pHT-315*gfp* plasmid was inoculated in LB medium. Overnight culture was carried out at 37°C and 180 rpm min^{-1}. The cultured DH5α cells were centrifuged for 10 min at 12,000×*g*. The precipitate obtained after centrifugation was re-suspended in 200 µl of solution I and mixed. This was followed by the addition of 400 µl of solution II, along with mild shaking. Then, 300 µl of solution III was added, mixed well by shaking, and centrifuged for 10 min at 12,000×*g* after milky flocculent appearance. The supernatant was absorbed in a fresh 1.5 ml EP tube, to which 5 µl of RNase and 100 µl of chloroform were sequentially added. The supernatant was mixed by oscillation and further centrifuged at 12,000×*g* for 10 min. This was then absorbed into another fresh 1.5 ml EP tube, and an equal volume of isopropanol was added. The supernatant was kept at – 20 °C for 30 min and centrifuged at 12,000×*g* for 10 min. The resulting supernatant was discarded and the pellet was washed twice with 70% ethanol. The pellet was dried and dissolved in 30 ml of sterile water. The size of pHT-315 DNA was estimated by gel electrophoresis, and the presence of the *gfp* gene in the plasmid was verified by double enzyme digestion. The enzyme digestion system had a total volume of 20 µl (including plasmid DNA 10 µl, *Hin*d III 1 µl, *Eco*R I 1 µl, K buffer 2 µl, ddH_2O 6 µl).

Bacillus axarquiensis TUBP1 Electrotransformation

The wild *B. axarquiensis* TUBP1 competent cells were prepared as follows. Single colony of *B. axarquiensis* TUBP1 was cultured in 2 ml LB medium and grown overnight at 37 °C with shaking at 180 rpm min^{-1}. 500 µl of the overnight *B. axarquiensis* TUBP1 was taken into a 250 ml triangular flask containing 50 ml LB medium. This was cultured at 37 °C, with shaking at 200 g for 2–3 h. Subsequently, the bacterial cells were centrifuged at 4 °C and 5000×*g* for 15 min, followed by addition of a small amount of ultra-pure water (sterilized and pre-cooled). The cells were re-suspended and centrifuged. Then, 10% glycerol solution was added into each centrifuge tube and the total volume was made up to 1 ml. After suspension of the precipitate, 40 µl aliquots of the bacterial cell suspension were placed in each 1.5-ml centrifuge tube. The *B. axarquiensis* TUBP1 competent cells were stored at − 80 °C for future use. For electrotransformation, 2 µl of the purified pHT-315*gfp* plasmid was added to the competent cells in a 1-mm electrode cup. These were subjected to an electric shock, with a shock parameter of 1.8 kV for 5–6 ms. 1000 µl SOC liquid medium (room temperature) was added to the electrode cup immediately after

the shock, and the cells were resuscitated for 1 h at 37 °C and shaking at 150 g. The 200 µl of transformed product was coated with 300 µg ml^{-1} erythromycin and cultured overnight in a 37 °C incubator after complete absorption of the bacterial solution.

Screening and Validation of Positive Clones

Single colony was selected and inoculated in LB medium containing 300 µg ml^{-1} of erythromycin, followed by incubation with shaking at 180 rpm min^{-1} for 12 h at 37 °C. Cells were collected through centrifugation (12,000×*g*, 30 s, room temperature) and plasmid DNA was extracted. Transformants were screened and verified with CLSM, PCR (Taq enzyme 0.5 µl, Taq enzyme buffer 2 µl, primer PF+R 2 µl, dNTPS 2 µl, DMSO 22 µl, and ddH_2O 11 µl) and sequenced by Sangon Biotech (Shanghai).

Comparative Analysis of the Properties of Wild *B. axarquiensis* TUBP1 and *B. axarquiensis* TUBP1-315*gfp*

The transformed TUBP1-315*gfp* single colony was inoculated in 10 ml LB medium (containing 300 µg ml^{-1} erythromycin) and cultured at 37 °C, 180 rpm min^{-1} for 48 h. 2 ml of the bacterial suspension was aliquoted into two EP tubes. In one tube, the supernatant obtained by centrifugation was mixed in 1 ml sterile water. Then, the emission of green fluorescence from the tube on excitation at 488 nm wavelength was checked with a confocal microscope. The supernatant obtained by centrifuging the second tube of bacterial suspension was used in a dark box ultraviolet analyzer to observe whether the bacteria could emit green fluorescence.

Genetic stability of plasmids was checked by the following protocol: 10 µl TUBP1-315*gfp* was transferred to 10 ml LB medium (containing 300 µgml^{-1} erythromycin) and cultured at 37 °C and 180 rpm min^{-1} for 48 h. 2 ml of this bacterial solution was taken in an EP tube, centrifuged, and the supernatant was discarded. Then, 1 ml sterile water was added, mixed well, and stored at – 20 °C for reserve. The inoculation with 10 µl of TUBP1-315*gfp* was repeated and transferred to 10 ml LB (containing 300 µg ml^{-1} erythromycin) for more than 30 generations. The prepared samples were tested for the relationship between fluorescence intensity and passage times with an ultraviolet FL spectrophotometer. The inhibitory activity of *B. axarquiensis* TUBP1 on *Verticillium*-induced wilt of cotton before and after transformation was determined by the agar diffusion method.

Colonization of Cotton Rhizosphere

The tested cotton variety was upland cotton Xinluzhong 70. The cotton seeds were disinfected with 75% ethanol surface and the disinfected cotton seeds were soaked for 24 h in TUBP1-315*gfp* bacterial solution (10^8 CFU ml^{-1}) cultured for 48 h. *V. dahliae* was cultured in Potato Dextrose Broth (PDB) liquid medium for 10 days, and the spores were collected and diluted with sterile water to make a pathogenic spore suspension (10^8 CFU ml^{-1}). The potted soil was divided into three parts after passing through a 40-mesh sieve. The first part was added with tap water as a control group, and the second part was added with an equal volume of TUBP1 medium (10^8 CFU ml^{-1}) to verify the colonization ability of wild strains on cotton plants in a natural soil environment. In the third, an equal volume of TUBP1-315*gfp* medium (10^8 CFU ml^{-1}) was added to verify the colonization ability of the labeled strain on cotton plants in a natural soil environment. The same weight potting soil was passed through a 40-mesh sieve, and the pathogen spore suspension (10^8 CFU ml^{-1}) was added. After that, the same treatment was performed to verify the colonization ability of wild strains and labeled strains on cotton plants in the environment of pathogenic soil. A total of six treatments were carried out, each treatment was repeated three times. The treated seeds were sown into soils with different treatments with 3 capsules per pot. Seeds were routinely managed after emergence. The cotton plants were regularly irrigated with TUBP1 fermentation broth, TUBP1-315*gfp* supernatant, and tap water, respectively. A 5–10 cm layer of soil around cotton rhizosphere was collected every 7 days, and sampled a total of 6 times. A total of 108 soil samples were obtained. The experiment was maintained in a greenhouse with a 14-h photoperiod and 22/28 °C day/night cycle. Diluted with sterile water, and 100 µl of soil suspension was absorbed to coat the LB erythromycin-resistant plate. The plate was incubated at 37 °C for 24–48 h, depending on the resistant plate. The colony morphology and fluorescence signal observed in the CLSM were used to identify and count the number of TUBP1-315*gfp*. Leaves, roots, and stems of cotton plants were collected and mixed with double volume of sterile water. 100 µl of this suspension was used to coat the LB erythromycin-resistant plate, and the plates were cultured at 37 °C for 48 h. The morphology of colonies grown on the resistant plates and the fluorescence signal observed in the fluorescence microscope were used to identify and count the number of TUBP1-315*gfp*.

Cotton Field Experiment

The tested cotton variety was upland cotton Xinluzhong 70. After the cotton emerged, 720 cotton plants were randomly selected and divided into 4 treatments. Each treatment consisted of 60 cotton plants. Each treatment was repeated 3 times. Different 10^6, 10^8, 10^{10} CFU/ml of *B. axarquiensis* TUBP1-1662gfp medium and LB medium broth (recording as T1, T2, T3, and CK) were applied to the cotton roots at

the cotton seedling stage, budding stage, flowering stage, and bolling stage. The incidence of cotton Verticillium wilt was counted during the bolling stage of cotton and the biocontrol effect of TUBP1-315gfp was calculated.

Statistical Analysis

All data were analyzed in at least three independent assays, and the results were reported as means ± standard deviations. Significant differences between mean values were determined using Tukey test ($P < 0.05$) following a one-way ANOVA. The statistical analysis was performed using the GraphPad Prism 5 software.

Results

Antibiotic Tolerance in Wild Type *B. axarquiensis* TUBP1

The results of wild *B. axarquiensis* TUBP1 sensitivity to antibiotics are shown in Table 1, *B. axarquiensis* TUBP1 was found to be extremely insensitive to ampicillin and streptomycin and was sensitive to chlortetracycline. With the increasing concentration of chloramphenicol, kanamycin, erythromycin, and apramycin in the medium, *B. axarquiensis* TUBP1 gradually showed a sensitive state. Erythromycin was selected as the resistance gene carrying the *gfp* plasmid.

Table 1 *Bacillus axarquiensis* TUBP1 antibiotic resistance test

Antibiotics	Concentration	Results
Ampicillin	100 μg/100 μl	+
	1000 μg/100 μl	+
	5000 μg/100 μl	+
Chloramphenicol	6.8 μg/100 μl	+
	68 μg/100 μl	+
	680 μg/100 μl	−
Streptomycin	20 μg/100 μl	+
	200 μg/100 μl	+
	1000 μg/100 μl	+
Kanamycin	2 μg/100 μl	+
	20 μg/100 μl	+
	200 μg/100 μl	−
Chlortetracycline	6 μg/100 μl	−
	60 μg/100 μl	−
	600 μg/100 μl	−
Erythromycin	6 μg/100 μl	+
	30 μg/100 μl	−
	60 μg/100 μl	−
Apramycin	100 μg/100 μl	+
	1000 μg/100 μl	+
	5000 μg/100 μl	−

+ means that it can grow in the medium containing this concentration of antibiotics, − means that it cannot grow in the medium containing this concentration of antibiotics

Validation of pHT-315*gfp* Gene

The full-length pHT-315*gfp* plasmid was 7705 bp. As shown in Fig. 1a, the size of plasmid strip conformed to the sublength (taking into account the three forms of the plasmid) and theoretical size of the plasmid obtained by double digestion. The value of 6479 bp + 1226 bp was consistent, and confirmed that the pHT-315*gfp* plasmid carried *gfp* gene.

gfp Tagging of Wild *B. axarquiensis* TUBP1

Bacillus axarquiensis TUBP1 transformants expressing the pHT-315*gfp* plasmid were checked for suitability by four methods. First, results of extraction of the TUBP1-315*gfp* plasmid and colony PCR are shown in Fig. 1b. All the bands corresponding to the theoretical band size were obtained. And, compared to the control group, the pHT-315 plasmid including *gfp* was predominantly expressed in wild *B. axarquiensis* TUBP1 transformants. The transformed cells could be easily monitored by confocal microscopy due to the GFP tag (Fig. 2a–f). TUBP1-315*gfp* was observed to emit bright green fluorescence upon excitation at a wavelength of 488 nm, indicating that the pHT-315*gfp* gene was successfully transformed and expressed in wild *B. axarquiensis* TUBP1. The *gfp* expression level was estimated to be 88.57% in *B. axarquiensis* TUBP1 based on FACS analysis (Fig. 2f). Finally, to ensure accuracy, the sequence analysis of PCR product of *B. axarquiensis* TUBP1-315*gfp* was performed. These results were shown in Fig. 2g, the base sequence of *gfp* were same in *B. axarquiensis* TUBP1-315*gfp* and pHT-315*gfp*.

Stability of *B. axarquiensis* TUBP1-315*gfp*

The antifungal activity against *V. dahliae* and growth kinetics of wild *B. axarquiensis* TUBP1 and TUBP1-315*gfp* were measured by the agar diffusion method and ultraviolet (UV) spectrophotometry, respectively. Figure 3a shows that the growth rate of wild ones was higher, compared to the transformed strains. However, there was no significant difference between the two strains in the logarithmic growth phase. Figure 3a shows that the inhibition zone against *V. dahliae* was approximately 0.14 and 0.17 cm for *B. axarquiensis* TUBP1 and *gfp*-tagged TUBP1, respectively. The growth rate and anti-*V. dahliae* activity of *gfp*-tagged TUBP1 cells and wild-type strain were similar, indicating that the presence of pHT-315*gfp* did not interfere with the normal metabolism of *B. axarquiensis* TUBP1. The stability of plasmid pHT-315*gfp* in *B. axarquiensis* TUBP1 was analyzed

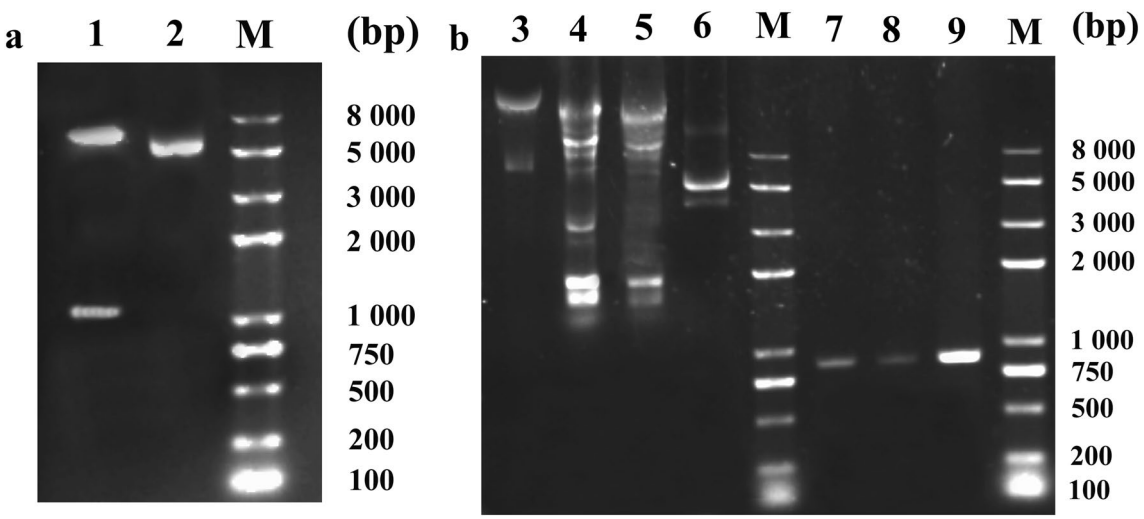

Fig. 1 pHT-315*gfp* plasmid double enzyme digestion verification results and the wild *B. axarquiensis* TUBP1 was successful tagged with *gfp* gene by electrotransformation. Lane 1 is the result of double digestion of pHT-315*gfp* plasmid, lane 2 is pHT-315*gfp* plasmid. The enzyme used is *ECO*R I+*Hin*d III, and the expected band size in lane 8 is 6479 bp + 1226 bp. Lane 3 is the wild strain contrast, lanes 4 and 5 are the *B. axarquiensis* TUBP1-315*gfp* plasmid extraction results, lane 6 is the pHT-315*gfp* plasmid positive contrast, lanes 7 and 8 are the *B. axarquiensis* TUBP1-315*gfp* bacterial solution, and lane 9 is pHT-315*gfp* Plasmid PCR positive contrast. Lane M is Trans 2K Plus II

by plating aliquots of the bacterial culture in LB medium in the presence or absence of antibiotics. The fluorescence intensity or *gfp*-phenotype in *B. axarquiensis* TUBP1-tagged cells decreased gradually with the increasing of incubation time. The degree of loss of pHT-315 *gfp* plasmid following serial subculture in the absence of antibiotics was evaluated for 64 generations in LB, where 40% of the strains were still found to express the pHT-315 *gfp* plasmid.

Population Dynamics of *B. axarquiensis* TUBP1-315 *gfp* in the Rhizosphere Soil of Cotton Plants

The number of *B. axarquiensis* TUBP1 and *B. axarquiensis* TUBP1-315*gfp* in the rhizosphere soil of cotton plants in the presence (inoculated *with* 6×10^8 CFU ml^{-1}) or absence of *V. dahliae* was measured by the dilution plate method and fluorescence spectrophotometry, respectively. Approximately 6×10^8 CFU g^{-1} of *B. axarquiensis* TUBP1-315*gfp* cells were detected immediately after inoculation in rhizosphere soil with and without *V. dahliae* (Fig. 3b). On day 7, the abundance decreased to 10^6 CFU g^{-1} rhizosphere soils of cotton plants. At the end of 2 weeks, the cell counts in the rhizosphere peaked at about 10^5 CFU g^{-1} soil. After 1 month, the *B. axarquiensis* TUBP1-315*gfp* cells counts were about 10^5 CFU g^{-1} in the rhizosphere soil. Although the number of bacteria decreased with the increasing of the days of inoculation, the bacterial counts and fluorescence intensity of *B. axarquiensis* TUBP1-315*gfp* could be detected till 45 days after inoculating (Fig. 3c). In summary, it was also apparent that the wild-type *B. axarquiensis* TUBP1 counts were higher than those of *B. axarquiensis* TUBP1-315*gfp* in the rhizosphere soil of cotton plants with or without *V. dahliae*. *B. axarquiensis* TUBP1-315*gfp* counts were lower in the rhizosphere soil of cotton plants inoculated with *V. dahliae,* compared to those without *V. dahliae*.

Colonization Patterns of *B. axarquiensis* TUBP1-315*gfp* Strains in Cotton Plants

As for the colonization model of *gfp*-tagged *B. axarquiensis* TUBP1, cotton plant root, stem, and leaf samples were quantitatively analyzed every 7 th day after inoculation by the dilution plate method. At the 7th and 28th day after inoculation, cotton plant root, stem, and leaf samples were observed by CLSM and SEM. Figure 3d shows that the number of *gfp*-tagged cells colonized at root, stem, and leaf of cotton plants was about 6.1×10^7 CFU g^{-1} fresh weight after inoculation for 7 days. Compared with the control group, no fluorescent cells were detected in non-inoculated ones. Although fluorescent cells were detected even 45 days after planting, the concentration decreased with the increasing time from 15, 30, to 45 days. CLSM results at the 7th day after inoculation revealed that colonization occurred in the root tip and in defined regions of the elongation and differentiation zones of plant roots.

gfp-expressing *B. axarquiensis* TUBP1 cells were visible in the form of biofilm or at high densities in roots and root hairs of cotton, in contrast to the control group (Fig. 4a). Stem cross-sections of cotton showing colonization by *B. axarquiensis* TUBP1-315*gfp* cells were also detected after

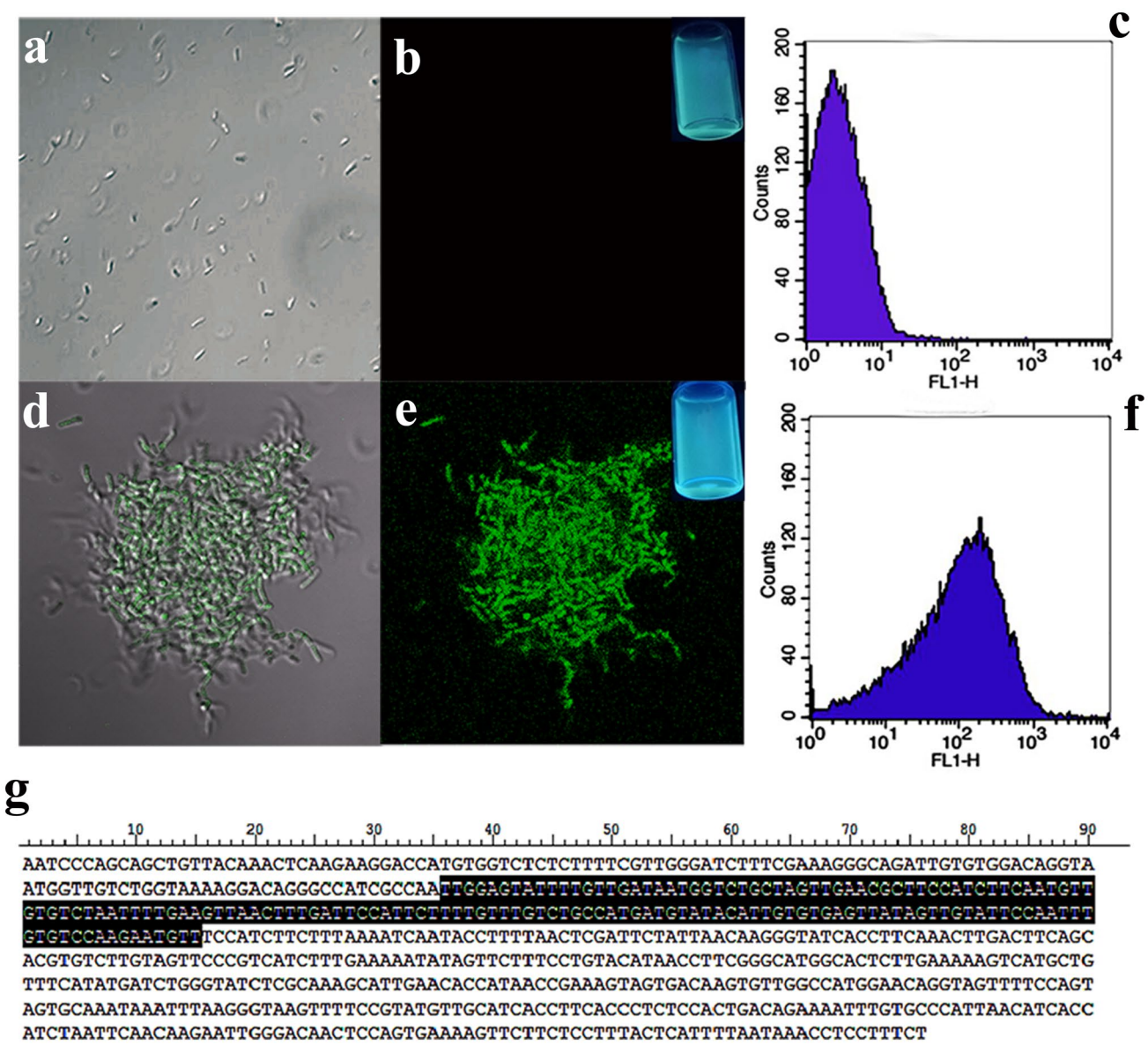

Fig. 2 The results of monitoring the expression of pHT-315*gfp* in TUBP1, **a**, **b** confocal microscopy and UV irradiation of wild strains, confocal microscopy, and UV irradiation of (**d**) and **e** labeled strains. **c** FACS analysis results of wild strains, **f** FACS analysis results of labeled strains. **g** *B. axarquiensis* TUBP1-315*gfp* bacterial solution PCR product sequencing results

7 days of inoculation (Fig. 4b). Few *B. axarquiensis* TUBP1-315*gfp* were observed in leaf tissues after 7 days of inoculation. Although colonization increased 48 h after inoculation with several cells being detected in the leaves, it decreased drastically 72 h after inoculation (Fig. 4c).

SEM results demonstrated that plants were supervised every 7 days after inoculation and *B. axarquiensis* TUBP1-315*gfp* cells were easily monitored and could be distinguished from the background of the root, stem, and leaves. One week after inoculation, *B. axarquiensis* TUBP1-315*gfp* cells were mainly found to colonize at the surface and groove of cotton root tip in the form of biofilm or microcolonies, in contrast with the control group (Fig. 4d). One month after inoculation, *B. axarquiensis* TUBP1-315*gfp* cells were still observed in the form of biofilm at the root base or in the lateral root junctions at high concentration (Fig. 4d). In the stem section, no *B. axarquiensis* TUBP1-315*gfp* cells could be detected in the control group, either 7 or 28 days after inoculation (Fig. 4e). In the treatment group, the number of TUBP1-315*gfp* cells in the xylem of the longitudinal section of cotton stem was higher at the 7th day after inoculation than that at 28th day after inoculation (Fig. 4e). At the leaf surface, compared to the control group, the number of bacteria around stomata of cotton

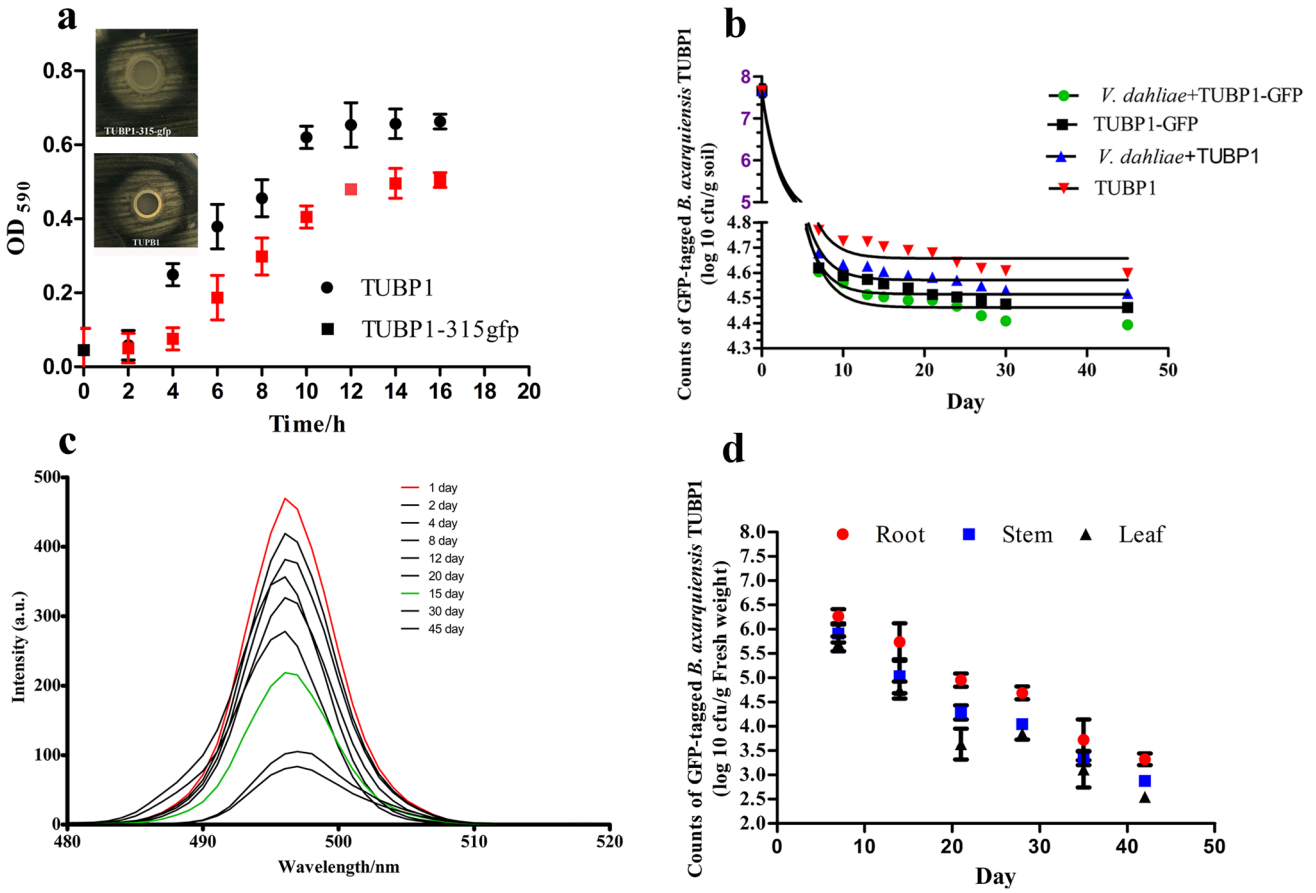

Fig. 3 Activity comparison and colonization dynamics of *B. axarquiensis* TUBP1-315*gfp* and *B. axarquiensis* TUBP1. **a** Growth curve of wild strain and labeled strain. Determination of *V. dahliae* activity by *B. axarquiensis* TUBP1. Determination of *V. dahliae* activity by *B. axarquiensis* TUBP1-315*gfp*. **b** Determination of colony number in cotton rhizosphere soil by plate dilution method and indicating the colonization of *B. axarquiensis* TUBP1 and *B. axarquiensis* TUBP1-315*gfp* in cotton rhizosphere soil. **c** Determination of the genetic stability of pHT-315*gfp* in *B. axarquiensis* TUBP1 by the green fluorescence intensity emitted by *B. axarquiensis* TUBP1-315*gfp*. **d** Colonization of *B. axarquiensis* TUBP1-315*gfp* in various tissues of cotton plants, The number of colonies isolated from the tissues of cotton by dilution plate method was used to describe the colonization of *B. axarquiensis* TUBP1-315*gfp* on cotton plants

leaves on the 28th day after inoculation was significantly less than that on the 7th day after inoculation (Fig. 4f).

Biocontrol Effect of *B. axarquiensis* TUBP1-315gfp in Cotton Field

Figure 5 shows that compared with the control group, the incidence of cotton Verticillium wilt significantly decreased and the biocontrol effect increased significantly with the increase of TUBP1-315*gfp* concentration applied, and the incidence of T3 treatment group was reduced by 57.63% compared with the control.

Discussion

The colonization played an important role in the biocontrol efficiency of various antagonistic bacteria against vascular diseases caused by soil-borne pathogens [13]. It was reported that the colonization of *B. subtilis* SQR9 led to control in *Fusarium* wilt in cucumber roots [14]. A lipopeptide-producing bacterium *B. cereus* F-6 colonized at rhizosphere soil could control stem and root rot disease in vanilla plants [15]. *B. subtilis* B96-II-GFP were detected in high concentration at root and stem and at lower

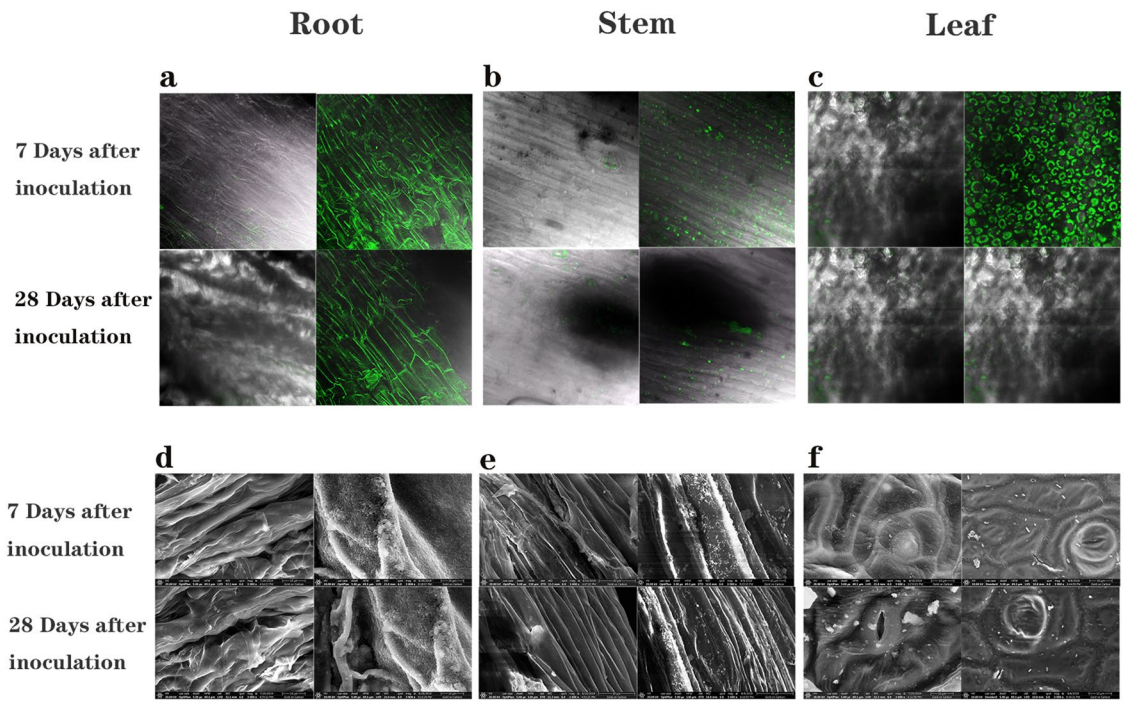

Fig. 4 CLSM and SEM of *B. axarquiensis* TUBP1-315*gfp* colonization on cotton tissue surface. **a**–**c** The results of temporal changes in the colonization of *B. axarquiensis* TUBP1-315*gfp* on the surface of cotton tissues under CLSM observation. **d**–**f** The results of time-varying changes in the colonization of *B. axarquiensis* TUBP1-315*gfp* on the surface of cotton tissues under SEM observation. **a**, **d** The colonization results of cotton root tissue. **b**, **e**, the colonization results of cotton neck tissue. **c**, **f** The colonization results of cotton leaf tissue. The left half of **a**–**f** is the control group, and the right half is the treatment group

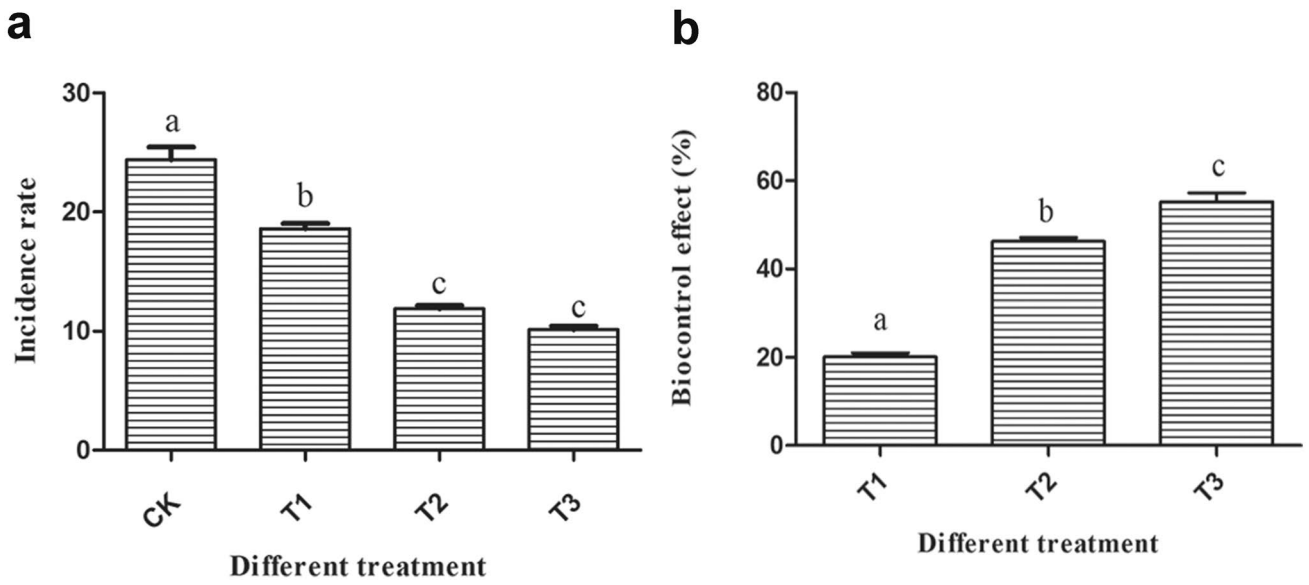

Fig. 5 Biocontrol effect of *B. axarquiensis* TUBP1-315gfp on *V. dahliae*. **a** Changes in the incidence of cotton after applying *B. axarquiensis* TUBP1-315gfp, **b** Biocontrol effects of *B. axarquiensis* TUBP1-315gfp on *V. dahliae* in cotton fields. The concentration of *B. axarquiensis* TUBP1-315gfp administered by T1, T2, and T3 is into 10^6 CFU ml^{-1}, 10^8 CFU ml^{-1}, and 10^{10} CFU ml^{-1}, respectively.

concentration in the leaves of asparagus plants [16]. In our previous study, *B. axarquiensis* TUBP1 was shown to be a potential candidate agent against *V. dahliae* via peptide T-inducing mitochondrial damage and mitochondria-mediated apoptotic cell death [4]. In the cotton field, *B. axarquiensis* TUBP1 showed 43% biocontrol effects on the Verticillium wilt disease [5] while it remained unknown whether the strain could infect and colonize in cotton plant or not. In the present work, the *gfp*-tagged *B. axarquiensis* TUBP1 was obtained by electrotransformation of the pHT-315 plasmid carrying the *gfp* gene, which had a higher copy number in *Bacillus*, ensuring a higher luminescence intensity of the transformed strain [17]. The plasmid pHT-315 carrying *gfp* gene could be stably sustained in the wild *B. axarquiensis* TUBP1 strain for at least 64 generations of TUBP1 strain incubated in LB liquid medium without antibiotics. The pHT-315*gfp* plasmid was a valuable tool to label *Bacillus* besides many other plasmids including plasmids pHAPII-*gfp* in *B. subtilis* HJ5, pHY300-F1*gfp* in *B. brevis* DX01, and pGFP4412-*gfp* in *B. megaterium* C4 [18].

The colonization and over 45-day survival of the *gfp*-tagged TUBP1 strain in rhizosphere soil and different tissues of plants were found to overlap with the entire period of occurrence of *Verticillium* wilt in cotton. It was showed by CLSM and SEM that *B. axarquiensis* TUBP1-315*gfp* colonized the surfaces of the roots of plants in the form of biofilms and microcolonies in the differentiation zones of plant primary roots, cotton root hair zone, and cotton lateral root junctions, which was similar to other results reporting that the root section and root base of plants were easily accessible locations where colonizing bacteria tended to accumulate [16, 19]. Consistent with these studies, the highest concentration of *B. axarquiensis* TUBP1-315*gfp* cells was found at the root base of cotton plants during the whole growth phase, which reached up to 6.1×10^7 CFU g^{-1} fresh weight after inoculation for 7 days. The *gfp*-tagged *B. axarquiensis* TUBP1 cells could also be observed in the epidermis, the cortical parenchyma, intercellular spaces, xylem vessels, and pith cells, which maybe pass through cracks formed at the lateral root junctions, followed by a slow migration from roots to stems and leaves [15, 18]. The number of *B. axarquiensis* TUBP1-315*gfp* cells gradually decreased from root to stem and leaves along with an increase in the inoculation time length, indicating that *B. axarquiensis* TUBP1-315*gfp* strain migrated into the root system from the rhizosphere soil, and then transferred from root to stem and leaves via an unknown pathway [20]. The labeled strain did not lose its activity against *Verticillium dahliae*. The marked strain showed 55.21% biocontrol effect after being applied to cotton fields, which significantly reduced the incidence of cotton Verticillium wilt. In the soil–plant–microbe complicated environment, different biocontrol strains displayed different colonization patterns depending on their diverse response to components of root secretion [13, 21, 22]. The antagonistic strain of *B. axarquiensis* TUBP1-315*gfp* could form biofilms on the surface of cotton root and survive under the suppression of *V. dahliae*. Future research should be focused on the mechanism of *B. axarquiensis* TUBP1-315*gfp* colonization at cotton plant roots and their interaction with *V. dahliae* in order to uncover the detailed mechanism of bioprotection.

Acknowledgements Our research was supported by the National Science Foundation (31260013), Microbial Resources Utilization Innovation Team in Key Field of XinJiang Production and Construction Corps (2017CB014), and Graduate Innovation Project (TDGRI201810). Hong Zeng and Chuan-xing Wan contributed equally to this work.

Compliance with Ethical Standards

Conflict of interest All authors declare that there have no conflicts of interest.

Research Involving Human and Animal Rights This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Zhang LS, Zhang N, Luo Z, Biao S (2013) Antagonistic *Bacillus subtilis* HJ5 controls verticillium wilt of cotton by root colonization and biofilm formation. Biol Fertil Soils 49:295–303
2. Zhen Y (2003) Genetically marking of natural biocontrol bacterium *Bacillus subtilis* strains with green fluorescent protein gene. Chin J Biotechnol 19:551
3. Shi YW, Li C, Yang HM, Zhang T (2017) Colonization study of *gfp*-tagged *Achromobacter marplatensis* strain in sugar beet. J Microbiol 55:267–272
4. Zeng H, Li T, Tian J, Zhang LL (2018) TUBP1 protein lead to mitochondria-mediated apoptotic cell death in *Verticillium dahliae*. Int J Biochem Cell Biol 103:35–44
5. Zeng H, Chen R, Luo XX, Tian J (2016) Isolation and anti-*Verticillium dahliae* activity from *Bacillus axarquiensis* TUBP1 protein. Process Biochem 51:1691–1698
6. Han QQ, Lü XP, Bai JP, Qiao Y, Paré PW, Wang SM, Zhang JL, Wu YN, Pang XP, Xu WB (2014) Beneficial soil bacterium *Bacillus subtilis* (GB03) augments salt tolerance of white clover. Front Plant Sci 5:525
7. Krzyzanowska D, Obuchowski M, Bikowski M, Rychlowski M, Jafra S (2012) Colonization of potato rhizosphere by GFP-tagged *Bacillus subtilis* MB73/2, *Pseudomonas* sp. P482 and *Ochrobactrum* sp. A44 shown on large sections of roots using enrichment sample preparation and confocal laser scanning microscopy. Sensors 12:17608–17619
8. Ji SH, Gururani MA, Chun SC (2014) Expression analysis of rice pathogenesis-related proteins involved in stress response and endophytic colonization properties of *gfp*-tagged *Bacillus subtilis* CB-R05. Appl Biochem Biotechnol 174:231–241
9. Fan B, Borriss R, Bleiss W, Wu X (2012) Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 colonizes three types of plants in different patterns. J Microbiol 50:38–44
10. Kumar KVK, Yellareddygari SK, Reddy MS, Kloepper JW, Lawrence KS, Zhou XG, Sudini H, Groth DE, Raju SK, Miller ME

(2012) Efficacy of *Bacillus subtilis* MBI 600 against sheath blight caused by *Rhizoctonia solani* and on growth and yield of rice. Rice Sci 19:55–63
11. Huang X, Zhang N, Yong X, Yang X, Shen Q (2012) Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiol Res (Pavia) 167:135–143
12. Zeng H, Yang SQ (2014) Isolation and identification of antagonistic bacteria TUBP1 against cotton *Verticillium dahliae* and its disease prevention effect. Cotton J 26(5):445–451 **(in Chinese)**
13. Barret M, Morrissey JP, O'Gara F (2011) Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. Biol Fertil Soils 47:729–743
14. Cao Y, Zhang Z, Ling N, Yuan Y, Zheng X, Shen B, Shen Q (2011) *Bacillus subtilis* SQR 9 can control Fusarium wilt in cucumber by colonizing plant roots. Biol Fertil Soils 47:495–506
15. Zhao Q, Wang H, Zhu Z, Yu H (2015) Effect of *Bacillus cereus f-6* on promoting vanilla (*vanilla planifolia* andrews.) plant growth and controlling stem and root rot disease. JER 6:1068–1078
16. Hao BQ, Ma LP, Qiao XW (2015) Quantitative analysis of the migration and accumulation of *Bacillus subtilis* in *Asparagus officinalis*. Curr Microbiol 71:357–362
17. Arantes O, Lereclus D (1992) Construction of cloning vectors for *Bacillus thuringiensis*. Gene 108:115–119
18. Liu X, Zhao H, Chen S (2006) Colonization of maize and rice plants by strain *Bacillus megaterium* C4. Curr Microbiol 52:186–190
19. Nybroe O (1999) Green fluorescent protein-marked *Pseudomonas fluorescens*: localization, viability, and activity in the natural barley rhizosphere. Appl Environ Microbiol 65:4646–4651
20. Rengpipat S, Wongtangprasert N, Palaga T (2010) The use of green fluorescent protein as a marker for monitoring a probiotic *Bacillus S11* in the black tiger shrimp *Penaeus monodon*. Aquac Nutr 15:297–305
21. Compant S, Clément C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42:669–678
22. Dennis PG, Miller AJ, Hirsch PR (2010) Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS Microbiol Ecol 72:313–327