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Biocontrol potential of antagonist *Bacillus subtilis* Tpb55 against tobacco black shank

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Abstract Tobacco black shank caused by *Phytophthora nicotianae* is a very important oomycete disease of tobacco, and it is widely distributed around the world. In order to develop effective prevention techniques, this study examined the effects of an antagonistic bacterium, *Bacillus subtilis* Tpb55 strain, on the prevention of tobacco black shank in vitro and in vivo. Dual culture test results showed *B. subtilis* Tpb55 strain have a strong antagonism to *P. nicotianae*, inhibit the growth of its hyphae, and produce significant inhibition zones. Scanning electron microscopy showed that the Tpb55 strain can damage the structure of *P. nicotianae* hyphae, cause hyphae deformity, hyphae rupture, and protoplasm leakage. Control effects of Tpb55 strains on tobacco black shank in pot and field experiment can reach up to 70.66 and 59.34 %, respectively. In this study, Tpb55 strain was also labeled with green fluorescent protein (GFP) in order to monitor their rhizosphere colonization of tobacco. The Tpb55 strain's colonization on tobacco roots showed a diffused distribution, largely in the root meristem and elongation zone areas. They can gather focally into microcolonies, forming a biofilm like structure. A small number of these bacteria can colonize at the intercellular space and among vascular bundles. After inoculation, Tpb55-GFP was found to colonize tobacco roots for 30 days or more. The number of bacteria peaked on the 4th day at 1.51×10^7 cfu g^{-1}. By the 12th day, it had dropped to 1.1×10^6 cfu g^{-1}. This study shows that the effect of Tpb55 strains on controlling of tobacco black shank is correlated to their ability to inhibit mycelia growth and ability to successfully colonize tobacco roots.

Keywords Antagonistic bacteria · Colonization · Tobacco black shank · *Bacillus subtilis*

Introduction

Tobacco black shank is caused by *Phytophthora nicotianae*. It is a widespread, destructive disease active in China. The pathogen can survive in soil for many years, and infect tobacco at any stages of plant growth. They have two separate strains with different pathogenicity. Moreover, many of the more popular tobacco varieties are susceptible to black shank disease (Ren et al. 2012). For these reasons, the disease is difficult to control. Currently, the application of chemical fungicides such as metalaxyl and

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propamocarb is the primary method of disease control. Due to long-term, heavy use of fungicides, the fungus has developed resistance, and the chemical residues have caused noticeable environmental contamination. For these reasons, developing other effective, eco-friendly methods of controlling black shank disease is needed.

Biological control using beneficial antagonists to limit plant disease is a promising approach to the protection of plants from soil-borne pathogens and it can reduce the need for chemical fungicides (Cartwright and Spurr 1998; Sukhada et al. 2011; Widmer 2014). *Bacillus subtilis* is a gram-positive, spore-forming bacterium that is widely distributed in the environment. There are a number of reports on the ability of *B. subtilis* to suppress several important soil-borne fungal plant pathogens, including *Fusarium* spp. (Zhao et al. 2013; Cao et al. 2011a), *Rhizoctonia solani* (Kumar et al. 2012), *Sclerotium rolfsii* (de Curtis et al. 2010), *Sporisorium reilianum* (Mercado-Flores et al. 2014), and *Verticillium dahliae* (Li et al. 2013). Today, *B. subtilis* is used for biological control because of its ability to promote plant growth, inhibit the development of pathogens, and induce systemically acquired resistance (Yu et al. 2011; Cao et al. 2011a; Li et al. 2013). The suppression of plant diseases by *B. subtilis* is usually based on competitive root colonization and secretion of antifungal compounds against these soil-borne pathogens (Compant et al. 2010; Cao et al. 2011a, b; Li et al. 2013). It has been postulated that the robust colonization of plant roots directly contributes to biocontrol efficiency (Lugtenberg et al. 2001; Ren et al. 2012). For this reason, not only must the mechanisms underlying antifungal activity be investigated, but a thorough understanding of factors involved in plant colonization by *B. subtilis* is required to improve the efficiency and reliability of inoculant strains.

Biocontrol agents should be marked to track and monitor their colonization on plants. During the early days, antibiotic-resistant mutant strains were widely used as markers. However, antibiotic-resistant markers cannot reliably identify native microorganisms that have the same ability to resist antibiotics. But then the development of reporter gene technology facilitated the monitoring of microorganisms in various environmental samples (Ramos et al. 2000). Green fluorescent protein (GFP) is an important marker and has been widely used as a reporter for the in situ detection and location of bacterial cells in soil (Hale et al. 2015), in the rhizosphere (Gilbertson et al. 2007; Zhang et al. 2011), and in plants (Ramos et al. 2000; Timmusk et al. 2005; Filho et al. 2013; Dietel et al. 2013).

In previous studies, an antagonist *B. subtilis* Tpb55 was isolated from healthy tobacco phyllosphere and shown to be able to inhibit mycelia growth of *P. nicotianae*. In this study, the ability of the *B. subtilis* Tpb55 to control tobacco black shank is evaluated. In addition, the Tpb55 strain was marked with GFP, and the patterns of colonization in tobacco roots were studied. This is the first report to describe an investigation of the colonization of GFP-tagged *B. subtilis* on tobacco roots.

Materials and methods

Materials

The strain Tpb55 (CGMCC No. 2853) was isolated from tobacco leaves and was described in a previous work (Zhang et al. 2008). *P. nicotianae* JM1 was isolated from tobacco stems in Jimo, Qingdao, Shandong Province in 2011. Tobacco variety Small Gold 1025 is susceptible to black shank disease. The culture medium was lysogenic broth (LB) medium and oats medium (OA).

Preparation of oat medium: oatmeal 30 g with 1000 ml water are heated on a boiling water bath for 1 h, filtered with gauze, and water was added to a total volume of 1000 ml. Then 17 g agar was added and allowed to melt. The sample was then aliquoted and sterilized (121 °C, 20 min).

Antagonism of Tpb55 strain against *P. nocotianae*

The dual culture assay was used (Ren et al. 2012). *P. nicotianae* was cultured until it covered the OA plate. The plate was then punched along the colonies' edge with a 5 mm sterilized puncher to harvest *P. nicotianae*. The fungus cake was placed at the center of a blank OA plate. Two 5 µl drops of Tpb55 culture were placed on both sides of pathogen colony (away from the *P. nicotianae* edge 22.5 mm). The concentration of bacterial culture was 2×10^8 cfu ml^{-1} and it was cultured in a 26 °C incubator to determine antagonistic bacteria mycelial growth at the edge of inhibition zone. Under a scanning electron microscope, the

growth of *P. nicotianae* mycelia that grew far away from Tpb55 was compared with that of *P. nicotianae* mycelia treated with Tpb55.

Preparation of swimming spores of *P. nicotianae*

According to slightly revised version of the method described by Su et al. (2011), the *P. nicotianae* was cultured for 21 days on an OA medium plate. Then 0.1 % KNO_3 solution 10 ml per dish was added. The plate was cultured at 26 °C for 72 h and immediately chilled to 4 °C for 0.5 h. Spore suspension was carefully drawn, adjusted to 10^6 cfu ml^{-1}, and set aside.

Phytophthora nicotianae inoculation was performed using spore suspension for irrigation. Spore suspensions (10^6 cfu ml^{-1}) were prepared for irrigation, 10 ml per plant. Then the plants were incubated at 28 °C for seven days. The disease severity were recorded on the 7th day after pathogen inoculation. Disease severity was scored on a scale of 0–9, where: 0 = no symptoms; 1 = less than one third of the total leaves are wilted; 3 = one third to a half of the total leaves are wilted; 5 = one half to two thirds of the total leaves are wilted; 7 = more than two thirds of total leaves are wilted; 9 = plant was dead. Disease index were calculated by the following formula:

$$\text{Disease index} = \frac{(a \times 0) + (b \times 1) + (c \times 3) + (d \times 5) + (e \times 7) + (f \times 9)}{a + b + c + d + e + f} \times \frac{100}{9},$$

where a, b, c, d, e, and f are the number of plants in each disease category.

Preparation of Tpb55 strain liquid culture

Tpb55 was activated on LB slant culture at 28 °C for 24 h. Then, the bacteria cells were flushed with 5 ml sterile water and poured into 100 ml LB broth in a 250 ml flask. After 48 h of growth at 28 °C and 170 r min^{-1}, the fermentation liquid was obtained and the concentration was adjusted with sterile water to 3×10^8 cfu ml^{-1}.

Disease control effect determination with pot experiments

Tobacco plants were planted using a routine method. When they had 2–4 true leaves, they were planted in pots containing sterilized potting soil (10 cm × 10 cm). Tobacco plants that had six true leaves were used for pot experiments. The roots of each plant were irrigated with 10 ml of 3×10^8 cfu ml^{-1} Tpb55 bacterial suspension for three days before exposure to *P. nicotianae*. Control chemical treatment was irrigation of the roots with 58 % metalaxyl manganese zinc wet powder (1:500), 10 ml per plant, 24 h before tobacco was exposed to *P. nicotianae*. Water irrigation served as the blank control treatment. Each group contained 15 plants, and experiments were performed three times in each group.

Determination of disease control efficacy under field conditions

Field experiments were conducted in 2013 and 2014 in a field naturally infested with *P. nicotianae* and under continuous tobacco cultivation for the last 11 years. Fields were located at Jimo tobacco resources and environmental field experiment station of Chinese Academy of Agricultural Sciences (36°27′N, 120°35′E), Qingdao, China. Small gold 1025 tobacco is susceptible to black shank. The roots of the tobacco plants were soaked in 3×10^8 cfu ml^{-1} Tpb55 bacterial suspension for 1 h and then transplanted. On the 7th and 21st day after transplantation, tobacco roots were irrigated twice with 50 ml per plant, 3×10^8 - cfu ml^{-1} Tpb55 bacterial suspension. On the 45th day after transplantation, the disease index was investigated. The control treatment was 1:400 of 58 % metalaxyl manganese zinc to irrigate tobacco 50 ml per plant after transplantation on 25 and 35 days, respectively. The blank control uses water, total three treatments with four replications, plants are randomized planted in blocks, each block is planted three lines, there were 15 tobacco plants per line, individual

row were 40 cm × 100 cm. The disease severity were recorded and disease index were calculated as described above on the 10th day after the second application of Tpb55.

Establishment of green-fluorescent-protein-labeled Tpb55-GFP strain

Bacillus subtilis strain Tbp55 p43 promoter and GFP fusion gene were produced using the method described by Yin et al. (2010). Tpb55 genomic DNA as template, and primers of p43-F: 5′-CCG GCA TGC TGT CGA CGT GCA TG-3′ (underlining indicates SphI restriction sites) and p43-R: 5′-AAG TTC TTC TCC TTT ACT CAT TAA TGG TAG CGC TAT CAC-3′ were used to conduct PCR amplification of p43 promoter Tpb55 sequence. pTKGFP plasmid template and primers of gfp-F: 5′-GTG ATA GCG GTA CCA TTA TAA TGA GTA AAG GAG AAG AAC TT-3′ and gfp-R: 5′-CCG GGT ACC TTA TTT GTA TAG TTC ATC CAT-3′ (underlined indicating KpnI restriction site) were used to construct the PCR-amplified GFP fragment.

The PCR reaction parameters were as follows: 98 °C for 2 min; 98 °C for 10 s, 58 °C for 15 s, 72 °C for 70 s, all reactions are in 35 cycles; 72 °C for 7 min. The PCR product fragment was recovered from electrophoresis agarose gel. The p43 and orfgfp fragments (molar ratio 1:1) were added to the same PCR system to perform SOE-PCR. Cycle parameters were as follows: 98 °C for 2 min; 10 cycles of 98 °C for 10 s and 68 °C for 50 s; 72 °C for 4 min and 4 °C for 10 min (at 4 °C the primers p43-F and gfp-R were added); 98 °C for 2 min; 98 °C for 10 s, 38 cycles of 58 °C for 15 s and 72 °C for 1.5 min; 72 °C for 7 min. The p43-gfp fusion fragment was recovered and digested by SphI, KpnI. The target fragment was recovered and used to produce the p43-gfp expression cassette.

The PRP43-GFP plasmid was constructed and verified as follows: The p43-GFP fusion gene sequences and the shuttle plasmid pRP22 were digested with two different restriction enzymes. After recovery, the purified fragments were ligated overnight and transformed into *Escherichia coli*-JM109-competent cells. The transformed cells were cultured overnight on a LB plate with ampicillin (50 μg ml^{-1}). Clones were picked and positive transformants were identified using primers of gfp-F1: CACATGAGTAAAGGAGAA, gfp-R1: TGTAGAGCTCATCCATGCCATG.

Tpb55-competent *B. subtilis* cells were prepared as described by Cao et al. (2011b). A vial of competent cells 100 μl is added constructed expression vector pRP43-GFP 100 μg, they are incubated on ice for 2 min. In an ice-cold 1 mm electroporation cuvette, cells were given 2.0 kV electric shocks. The cells were immediately plated and cultured on resuscitation medium RM at 30 °C and 200 r min^{-1} for 3 h. Then they were plated overnight on LB plates with 5 μg ml^{-1} chloramphenicol at 30 °C. Transformants were picked. Cell luminescence was checked under a fluorescent microscope. Primers of *gfp*-F and *gfp*-R were used to perform PCR validation for single colonies that fluoresced. The strains that successfully took on green fluorescence are here called Tpb55-GFP.

Labeling the growth curve of Tpb55-GFP strain

The Tpb55-GFP clone was picked and inoculated overnight into 100 ml LB liquid medium with chloramphenicol (5 μg ml^{-1}) at 30 °C and 150 r min^{-1}. The culture was diluted with sterile water to a bacterial content of 2 × 10^8 cfu ml^{-1}. Bacteria were inoculated at 1:100 ratio into 100 ml LB broth with chloramphenicol (5 μg ml^{-1}). They were cultured at 30 °C, 150 r min^{-1}. The original strain Tpb55 served as a control. The cultured Tpb55-GFP strain was measured under the same conditions. Every 2–4 h, OD_{600} value of bacterial culture was measured and the growth curves of the original strain Tpb55 and labeled strain of Tpb55-GFP were drawn.

Determination of stability of Tpb55-GFP strain

Tpb55-GFP strain-single colonies were inoculated in 5 ml of LB medium with chloramphenicol and without chloramphenicol overnight as generation 0. On the next day, 1:100 (v/v) inoculums were transferred into the liquid medium with and without chloramphenicol. Strains were continuously transferred for 30 generations. Every 24 h, bacterial strains were plated on LB plates without chloramphenicol, cultured at 30 °C. Then 100 single colonies were picked and incubated on LB plates with chloramphenicol. Fluorescent colonies were counted under a fluorescent microscope. Standard strains stability was calculated as follows: plasmid stability (stability, Sp) = luminous viable colonies/100.

Dynamic measurement of Tpb55-GFP strain colonization in tobacco rhizosphere

The tobacco seeds were sown in sterilized soil. When tobacco seedlings grow six true leaves, they were transplanted into a matrix (sterile soil:vermiculite = 1:1) in the pots, one plant per pot and 30 rounds per treatment.

Strain Tpb55-GFP colonization in rhizosphere: tobacco plants were irrigated with labeled bacteria strains for 1 h, and then 1 g of the taproot and fibrous root (including small attached soil particles) were collected and cut into pieces of 0.5 cm length. Small pieces of root were soaked in sterile water with shaking for 2 h. The suspension was gradually diluted and plated on LB plates with chloramphenicol (5 μg ml^{-1}). After 24 h of culture, the number of colonies was counted. This was considered day 0 of Tpb55-GFP strain colonization. Then on days 1, 3, 5, 7, 10, 15, and 30, colonies of Tpb55-GFP were sampled and Tpb55-GFP levels were measured to assess bacterial colonization of the tobacco roots. Untreated tobacco plants served as controls.

Observation of colonization status: At different points in time, three tobacco plants were collected. Their roots were washed and dried with clean absorbent paper. It was cut into 1 cm pieces using a clean scalpel. Root pieces were mounted on slides and observed under a confocal laser scanning microscope (Olympus FV1000). The excitation wavelength was 488 nm, and signals were collected within the range of 500-600 nm. Olympus FV1000 software was used to analyze images.

Data analysis

SPSS 16.0 software were used for all data analysis. The data obtained were subjected to one-way ANOVA analysis, and significances between the mean values were determined by Duncan's multiple range statistical tests ($P < 0.05$).

Results

Hyphae inhibition of Tpb55 strains against *P. nicotianae*

The dual culture assay results showed Tpb55 strains to exert stronger inhibition on mycelial growth of *P. nicotianae*. In addition to producing obvious circular zones of inhibition, the growth of *P. nicotianae* was also hindered: mycelium at the edge of colony and the mycelium in the contact area of inhibition zone were dissolved, the entire colony growth was significantly inhibited (Fig. 1a).

Under an electronic scanning microscope, it was observed that Tpb55 ferment substance had harmful effects on morphology of *P. nicotianae* mycelium. Normal *P. nicotianae* mycelia are consistent in shape and size: well-structured single hyphae are clearly visible (Fig. 1b). *P. nicotianae* mycelia treated with Tpb55 was twisted and wrinkled and hyphal tips were deformed and swollen (Fig. 1c) and protoplasm leakage was observerd (Fig. 1d).

Disease severity index under greenhouse and field conditions

P. nicotianae-treated plants in the control pots showed obvious symptoms seven days after exposure. Tobacco disease indexes of Tpb55 treatment and 58 % metalaxyl manganese zinc (1:400) were 21.44 and 19.12 (Fig. 2a), respectively. This showed the Tpb55 strain to have potential in the prevention and control of tobacco black shank. To confirm the preventive effect of tobacco black shank under field conditions, experiments were conducted in a field naturally infested with *P. nicotianae* in 2013 and 2014. Average disease index of Tpb55 strains on tobacco black shank in the field were 21.44 and 14.23, which was lower than that of 58 % metalaxyl MnZn (1:400) (19.12 and 11.71 respectively), but the difference was not significant (Fig. 2b, c).

Establishment of green-fluorescent-protein-labeled Tpb55-GFP strain

The recombinant expression vectors pRP43-GFP is transformed into the Tpb55 competent cells via electronic shock, on the plate with chloramphenicol (5 μg ml^{-1}) at 30 °C overnight culture; positive transformants were picked and transferred overnight to LB broth containing chloramphenicol (10 μg ml^{-1}); 5 μl bacterial culture was drawn and made into slides. Under a fluorescence microscope 40 × objective lens, bacteria emitted bright green fluorescence (Fig. 3a). This *B. subtilis* strain Tpb55 is here called Tpb55-GFP.

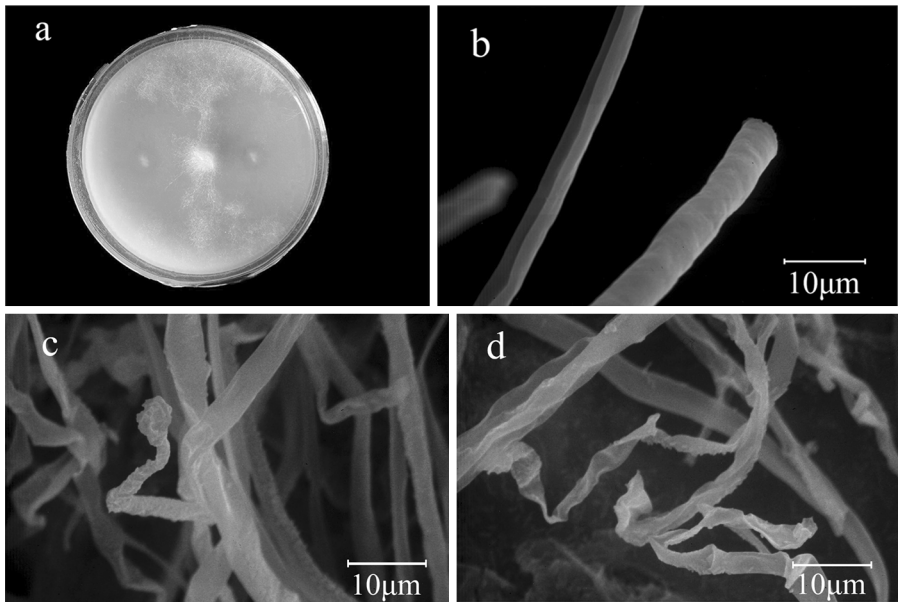

Fig. 1 Inhibitory role of Tpb55 strain on *P. Nicotianae*. **a** Inhibition in a dual culture assay; **b** control of mycelia's growth; **c** Tpb55-treated mycelia showed shrinkage, top deformedly enlargement; **d** Tpb55-treated mycelia appeared dry and ruptured into flakes

Growth curve of Tpb55-GFP strain

Growth curves of original strain of Tpb55 and labeled strain of Tpb55-GFP on liquid medium are shown (Fig. 3b). The growth trends of the original strain of Tpb55 and labeled strain of Tpb55-GFP were approximately the same: their growth curves were not fundamental different. After 6 h of culture, the bacteria entered into the logarithmic growth phase. After 14 h they entered the stable growth phase. This showed that the expression of green fluorescent protein in original strain Tpb55 did not produce a significant adverse effect on the growth characteristics of Tpb55.

Stability of Tpb55-GFP marker strain

Plasmid stability testing of the Tpb55-GFP strain showed that, in the absence of selective pressure, with increasing passage numbers, the stability of Tpb55-GFP strain tended to decrease. When the culture medium lacked chloramphenicol, the Tpb55-GFP strain was passed for ten generations, and plasmid stability was 83 %. The Tpb55-GFP strain was passed for 20 generations, and plasmid stability was 76 %. Plasmid stability was 69 % after 30 generations. When the culture medium contained chloramphenicol, the plasmid stability of Tpb55-GFP strain was very good. Even after 30 generations, plasmid stability was still 95 %. This shows that the exogenous plasmid in Tpb55-GFP strain can be stably inherited.

Tobacco rhizosphere colonization of labeled strain Tpb55-GFP

After tobacco seedlings were inoculated with Tpb55-GFP, several parts of the root were observed with CLSM. First, 24 h after inoculation, Tpb55-GFP had colonized different parts of the tobacco roots thoroughly, the overall distribution was diffuse. Among them, the largest number of Tpb55-GFP was located at the root meristem and elongation region for colonization. In the local area Tpb55-GFP aggregates into microcolonies, forming a biofilm-like structure (Fig. 4a). After inoculation at the 4th day, a large number of green bacteria were visible with a distribution largely within the intercellular spaces (Fig. 4b). Fluorescent signals were significantly higher in the central pith of the vascular system (Fig. 4c), indicating that Tpb55-GFP bacteria not only successfully colonized the roots of tobacco plants but were also multiplying. As inoculation time continued, fluorescence gradually waned, but even 30 days after inoculation, the Tpb55-GFP strain was still observed in

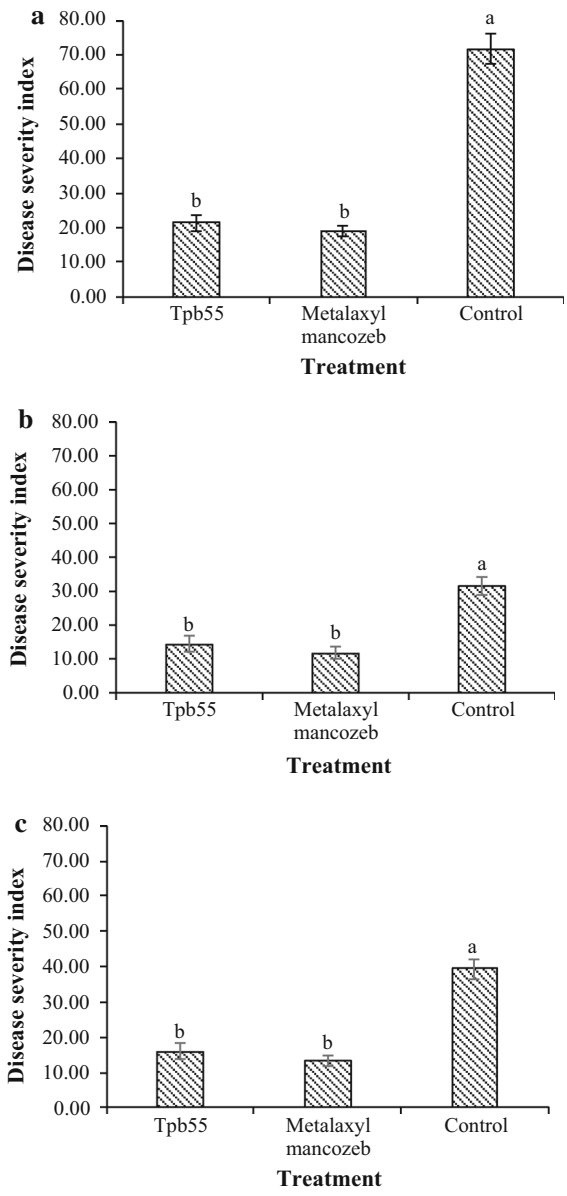

Fig. 2 Disease severity index on tobacco black shank. **a** Pot experiment results ($F = 296.97$, $df = 2, 6$, $P < 0.05$). **b** Field experiment results of 2013 ($F = 62.92$, $df = 2, 6$, $P < 0.05$). **c** Field experiment results of 2014 ($F = 179.79$, $df = 2, 6$, $P < 0.05$). Different *letters* indicate statistically significant differences between treatments ($P < 0.01$). The *error bars* (SD) from the mean for three replicates are shown

tobacco rhizosphere (Fig. 4d), though the intensity of fluorescence had decreased greatly.

Overall, Tpb55-GFP strain colonization around the tobacco rhizosphere initially increased and then decreased (Table 1). On the 2nd day after inoculation,

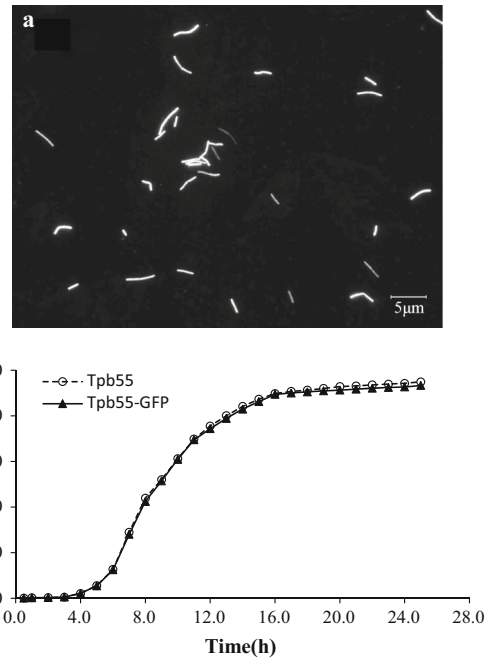

Fig. 3 Establishment of green-fluorescent-protein-labeled Tpb55-GFP strain. **a** Tpb55-GFP showed luminescence under the fluorescent microscope. **b** Growth curve of the wild-type strain of Tpb55 and labeled strain of Tpb55-GFP

the population of Tpb55-GFP in the rhizosphere was 1.08×10^6 cfu g^{-1}. It peaked on the 4th day, at 1.51×10^7 cfu g^{-1}. Then it showed a downward trend lasting until the 12th day, reaching 1.1×10^6 - cfu g^{-1}. It was still detectable on the 30th day, at 6.72×10^5 cfu g^{-1}.

Discussion

The biocontrol mechanisms of *B. subtilis* include production of antifungal substances (Sansinenea and Ortiz 2011), induction of plant resistance, and promotion of plant growth (Chowdappa et al. 2013; Yu et al. 2011; Park et al. 2013). Pathogen antagonism is one of the most important of these methods (Handelsman and Stabb 1996; van Loon et al. 1998). It is often used to screen biocontrol agents. The ability of *B. subtilis* Tpb55 to inhibit fungal growth was tested, and results showed that Tpb55 strains can inhibit the growth of *P. nicotianae* mycelia and form a clear zone of inhibition on the plate. Under scanning electron microscopy, the Tpb55 strain showed visible damaging effects on *P. nicotianae* mycelia, resulting in rupture of the

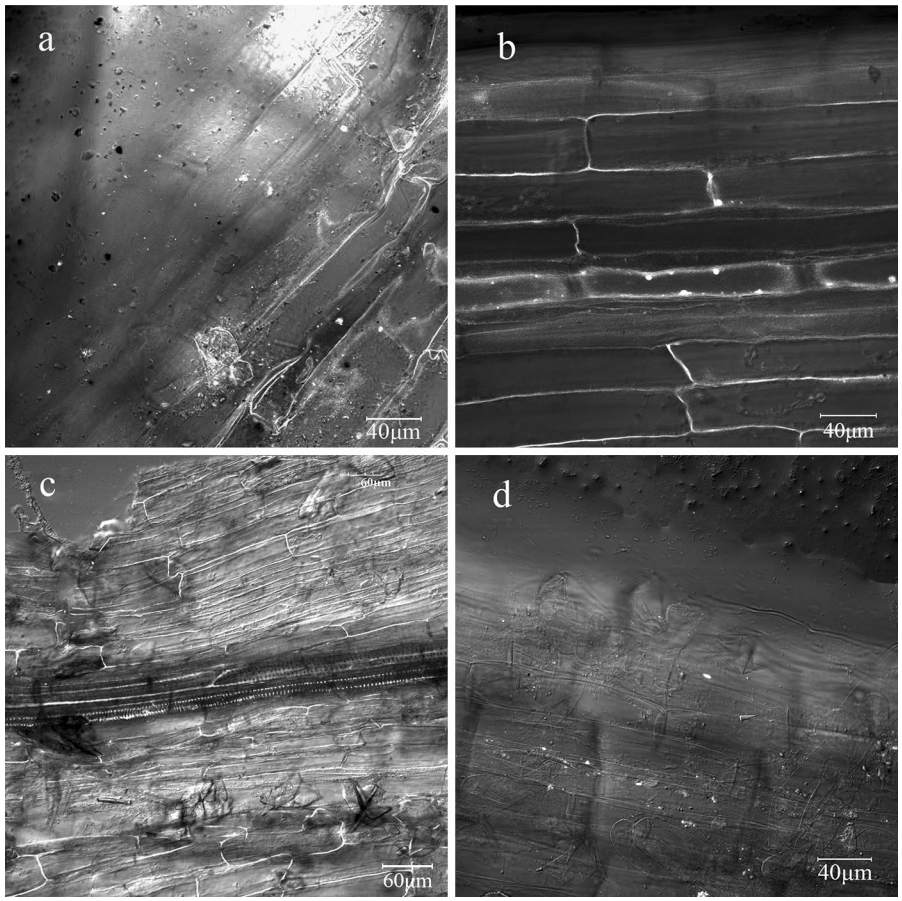

Fig. 4 Colonization of Tpb55-GFP in the tobacco root. **a** 24 h after inoculation, partial formation of biofilms; **b** four days after inoculation, large numbers of Tpb55-GFP colonies in the vascular space of tobacco roots; **c** four days after inoculation, large numbers of Tpb55-GFP colonies in the intercellular spaces in tobacco roots; **d** 30 days after inoculation, small numbers of Tpb55-GFP colonies in tobacco roots

Table 1 Dynamics of Tpb55-GFP colonization in tobacco rhizosphere

Time	Population density (10^5 cfu g^{-1})[a]
2 h	144.54 ± 15.05a
1 day	124.00 ± 19.08b
2 days	10.80 ± 1.17d
4 days	151.00 ± 22.89a
8 days	54.20 ± 4.53c
12 days	11.00 ± 0.79d
20 days	8.87 ± 1.69d
30 days	6.72 ± 0.58d

[a] Values are the means of three replicates ± SD, population density ($F = 97.34$, $df = 7, 16$, $P < 0.05$). Values followed by the same lower case letters are not significantly different ($P < 0.05$)

mycelium and leakage of the protoplasm. This showed that the Tpb55 strain not only has antifungal effects but also a fungicidal effect. Its metabolites species should be analyzed and studied.

The abilities of biocontrol bacteria to colonize, proliferate, and survive around plant roots are also considered important to stable, long-lasting disease prevention (Perez-Garcia et al. 2011). In order to study the ability of Tpb55 strain to colonize on tobacco roots, it was labeled with a GFP tag. The Tpb55-GFP strain produced in this study did not show significant differences in growth rates from its unlabeled counterpart. These results are consistent with previous works (Ren et al. 2012; Li et al. 2013). The Tpb55-GFP strain was also found to be relatively stable. After ten generations, the plasmid stability was 88 %. After

30 generations, it was 65 %. It was here considered suitable for use in the observation of the colonization of *B. subtilis* Tpb55 at tobacco roots.

Previous studies have shown that different strains of biocontrol bacterial differ in the way they colonize plant surfaces (Ramos et al. 2000; Neveu et al. 2007; Cao et al. 2011b; Sang and Kim 2014). *B. subtilis* HJ5 strains mainly colonize the cotton root elongation zone (Li et al. 2013). *B. subtilis* SQR9 strain mainly colonizes the cucumber root's meristematic zone, elongation zone, and root hair zone (Cao et al. 2011b). The results show that after inoculation Tpb55-GFP on tobacco, Tpb55-GFP can successfully colonize on the surfaces of tobacco roots. The site of colonization showed a diffuse distribution, though the root meristem and elongation zone were colonized to a greater extent than other areas. Many beneficial bacteria form biofilms on the surface of plant roots (Ren et al. 2012; Li et al. 2013). This plays an important role in the prevention and control of disease (Eberl et al. 2007; Chen et al. 2013). In the present study, Tpb55-GFP could gather on the surfaces of tobacco roots to form microcolonies and biofilm-like structures. Furthermore, in the current work, the Tpb55 was found to mainly colonize the intercellular spaces on the root surfaces, and the vascular bundles also showed some bacterial colonization, indicating that this strain has endophytic behavior.

Dynamic quantitative detection of Tpb55-GFP showed the density of Tpb55-GFP at tobacco rhizosphere to be about 10^5–10^6 cfu g^{-1}. These results are consistent with previous reports, and they indicate that the bacterium reaches sufficient concentrations for antagonism to take place (Timmusk et al. 2005; Zhang et al. 2011). As time after exposure continued, Tpb55-GFP colony density decreases, this phenomenon has also been reported (Hiddink et al. 2005; Zhang et al. 2011). This may be due to competition with indigenous microorganisms or to loss of plasmid. This is also the main reason why biocontrol bacteria cannot be directly applied to the soil. At 8–12 days after Tpb55-GFP inoculation, the number of colonies declined rapidly at the surfaces of the roots; whereby, in order to ensure biocontrol effect, it is necessary to strengthen the effect with a booster inoculation of 8–13 days (slow recession stage), to maintain a certain population.

Pot experiments and field experiment results show that the Tpb55strain of *Bacillus subtilis* has strong ability to control the effects on tobacco black shanks, disease prevention at 50–60 %, and is close to previous report of biocontrol of tobacco black shank (Ren et al. 2012; Chen et al. 2013). This study showed that the control effect of Tpb55 on tobacco black shank is related to its ability to inhibit fungal growth and it can successfully colonize tobacco roots. In order to more effectively take advantage of the biocontrol activity of Tpb55 in practice, a better understanding of the various environmental factors that affect its colonization must be developed.

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