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Bacillus amyloliquefaciens T-5 may prevent *Ralstonia solanacearum* infection through competitive exclusion

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Abstract Investigation of the properties and mechanisms of the interactions of root-colonizing biocontrol bacteria and plant pathogens is necessary to optimize the biocontrol strategies. In the present study, the interaction of a biocontrol strain *Bacillus amyloliquefaciens* T-5 tagged with a green fluorescent protein marker and a bacterial wilt pathogen *Ralstonia solanacearum* QL-*Rs*1115 tagged with red fluorescent protein marker was studied on tomato roots using different inoculation methods. The results showed that in the co-culture experiment, the population of pathogen QL-RFP was decreased by increasing the initial inoculum concentration of biocontrol strain. In the greenhouse experiment, both strains T-5-GFP and QL-RFP colonized tomato roots (root tips, root hairs, primary roots, and root junctions) and formed a biofilm on the root surfaces as determined by dilution plating and confocal laser scanning microscopy (CLSM) techniques. However, the root colonization of pathogen strain QL-RFP was almost completely suppressed in the presence of biocontrol strain T-5-GFP when both soil and plant seedlings were treated with T-5-GFP. The results of this study revealed the effectiveness of strain *B. amyloliquefaciens* T-5 as a biocontrol agent against tomato wilt pathogen and the significance of inoculation method used to inoculate biocontrol strain.

Keywords Biological control · CLSM · Fluorescent protein · Root colonization · Tomato

Introduction

Bacterial wilt caused by the Gram-negative bacterium *Ralstonia solanacearum* is one of the most devastating soil-borne diseases, resulting in significant losses in many important economic crops in tropical and temperate regions (Hayward 1991). This bacterium usually invades plant roots via wounds or emerging secondary roots infecting the inner cortex and the vascular parenchyma and subsequently invades the protoxylem vessels by degrading cell walls, which results in chlorosis, stunting, wilting, and ultimately plant death (Vasse et al. 1995). *R. solanacearum* is a complex species with considerable diversity. It is subdivided into five races based on host range and six biovars according to physiological and biochemical characteristics (Huang et al. 2012). In addition, *R. solanacearum* can be easily disseminated via soil, irrigation water, surface water, farm equipment, and infected biological material and can survive for a long time (Janse 1996). Because of these factors, it is currently difficult to find effective control methods for this disease.

Bactericides are usually used to control plant diseases; however, their use has limited effectiveness and can pollute the environment and threaten human health (Al-Waili et al. 2012). Moreover, there are currently no effective chemicals

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to control bacterial wilt. Many studies have shown that the biological control of plant pathogens using beneficial microorganisms (BMs) is a promising strategy to protect plants from soil-borne diseases and to reduce the use of chemicals (Bhattacharyya and Jha 2012; Palaniyandi et al. 2013). Various antagonistic bacteria including *Acinetobacter*, *Enterobacter*, *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp. (Aliye et al. 2008; Takenaka et al. 2008; Xue et al. 2009; Ramesh and Phadke 2012; Tan et al. 2013b) have been reported to be effective against soil-borne plant pathogens. Antagonistic bacteria suppress plant diseases by various modes of action, such as antibiotics, volatile compounds, and siderophore production (D'Alvise et al. 2014; Raza et al. 2013; Yu et al. 2011), induction of plant systemic resistance (Kurabachew et al. 2013), and competition for space and nutrients (Takenaka et al. 2008). Moreover, the ability of biocontrol agents to colonize plant roots has been considered a prerequisite to control soil-borne pathogens (Compant et al. 2010).

As described in a previous study, an antagonistic strain (*Bacillus amyloliquefaciens* T-5) was isolated in our laboratory from the tomato rhizosphere using a three-cycle enrichment procedure as described by Kamilova et al. (2005). This antagonist (T-5) colonized tomato roots effectively and was effective against *R. solanacearum* in vitro and under greenhouse conditions (Tan et al. 2013a, b). In a greenhouse experiment, strain T-5 reduced the wilt symptoms caused by *R. solanacearum* QL-Rs1115 by 79.4 % compared to the control. Strain T-5 also produces antibiotics effective against *R. solanacearum* QL-Rs1115 (Tan et al. 2013b). Moreover, T-5 was reported to induce systemic resistance against bacterial wilt disease caused by *R. solanacearum* in tomato (Tan et al. 2013a). However, the mechanisms of the antagonistic effects of T-5, especially concerning its interaction with pathogens in the plant rhizosphere, were not completely explored.

Green fluorescent protein (GFP) and its derivatives are widely used to identify and quantify specific microbes and their activities in complex environments such as soil or plant tissues (Underwood et al. 2011). The presence of GFP, isolated from the jellyfish *Aequorea victoria*, within the bacterial cells and its gene expression and visualization do not require fixation or preparation protocols nor substrates or additional energy input (Errampalli et al. 1999). In addition, there are many important GFP derivatives that have diverse emission spectra (Heim and Tsien 1996; Goedhart et al. 2010). Many examples of antagonistic bacteria or pathogens tagged with GFP or its derivatives have been used to monitor their behavior in natural environments (Fan et al. 2011; Torres et al. 2013). However, there is still a need to better understand the interactions between the biocontrol agents and the plants to successfully and more predictably use those beneficial microbes. Moreover, only a few studies have been aimed using GFP and its derivatives to monitor interactions between

biocontrol agents and the pathogen *R. solanacearum*. This study was conducted to elucidate the interactions between *B. amyloliquefaciens* T-5 and *R. solanacearum* QL-Rs1115 on tomato roots as a preliminary step toward the possible commercial use of *B. amyloliquefaciens* T-5 as a biocontrol agent, which also requires the understanding of biocontrol mechanisms, the ecology of the biocontrol agent and the pathogen, their colonization patterns on tomato roots, and the capability of organisms to survive in the soil environment.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *B. amyloliquefaciens* T-5 (CGMCC accession No. 8547, China General Microbiology Culture Collection Center) was found to be highly effective against *R. solanacearum*, which causes tomato wilt disease (Tan et al. 2013a, b). *B. amyloliquefaciens* T-5 was grown in Luria-Bertani (LB) broth at 30 °C for 24–48 h with agitation (170 r min^{-1}). The pathogenic strain *R. solanacearum* QL-Rs1115 (CGMCC accession No. 9487, China General Microbiology Culture Collection Center), isolated from a wilted tomato plant, was grown in a nutrient medium (NA, glucose 10.0 g, peptone 5.0 g, yeast extract 0.5 g, beef extract 3.0 g, pH 7.0, H$_2$O 1 L) in a rotary shaker at 170 r min^{-1} at 30 °C (Wei et al. 2011). *Escherichia coli* top 10 was grown at 37 °C in LB medium. Whenever necessary, the following antibiotics were added to the growth media: gentamycin (Gm), 30 μg mL^{-1}; kanamycin (Km), 20 μg mL^{-1}; and ampicillin (Amp), 100 μg mL^{-1}.

We constructed the shuttle plasmid pHAPII that contains a single copy of a constitutively expressed GFP gene under the control of the strong constitutive promoter *hap II* (Cao et al. 2011). The plasmid pTCV89 containing the *mCherry* (red fluorescent protein, RFP) gene and pYC12 containing constitutive promoter Ptac (Wang et al. 2008) were kindly provided by Associate Professor Zhong Zengtao of the Key Lab of Microbiological Engineering of Agricultural Environment, Nanjing Agricultural University.

Generation of fluorescent protein-tagged strains

The plasmid pHAPII was introduced into *B. amyloliquefaciens* T-5 by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA, USA) as described by Cao et al. (2011). Briefly, 200 μL of competent cells was mixed with 1.0 μg of plasmid pHAPII in a 0.2-cm-gap electroporation cuvette. After electroporation (2.5 kV, 200 Ω, 25 μF), the transformed cells were immediately transferred to 1 mL LB medium and incubated for 2 h at 30 °C with shaking at 100 r min^{-1} then plated on selective LBk

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference or source
E. coli top 10	F-mcrA Δ (mrr-hsdRMS-mcrBC) 880lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ (ara-leu) 7697 galE15 galK16 rpsL (StrR) endA1 λ-	Invitrogen Corporation (Shanghai, China)
B. amyloliquefaciens T-5	Wild type	Tan et al. (2013a, b)
R. solanacearum QL-*Rs*1115	Wild type	Wei et al. (2011)
Plasmid		
pTCV89	Amp^r; *mCherry* (*rfp*) report cassette	MOA[a]
pYC12	Gm^r; containing a constitutive Ptac Promoter	Wang et al. (2008)
pYC12-M	pYC12 containing *mCherry* fused to Ptac promoter	This work
pHAPII	pUBC19 containing *gfp* fused to *hap II* promoter	Cao et al. (2011)

[a] MOA: Key Lab of Microbiological Engineering of Agricultural Environment, Nanjing Agricultural University, Nanjing, China

[r] Resistance of the antibiotics

medium (LB medium containing 20 μg mL^{-1} Km). After the transformed cells were grown for 24–48 h at 30 °C and the colonies were observed under a fluorescence stereomicroscope (Olympus MVX10, Olympus, Hamburg, Germany). A colony on the plate that emitted green fluorescence was chosen for further study and named T-5-GFP.

Standard molecular techniques for DNA isolation, digestion with restriction enzymes, and construction of recombinant DNAs were carried out as previously described (Sambrook and Russell 2001). Restriction enzymes, Taq DNA polymerase, and DNA ligase were purchased from TaKaRa, China. Plasmids from *E. coli* were extracted using the Axyprep™ Plasmid Miniprep Kit (Axygen, Union City. USA). The *mCherry* gene bordered by *Nde*I and *Eco*RI sites was amplified from plasmid pTCV89 using F1 (5′-ATGGC TCATATGGTGAGCAAGGGCGAGGAG-3′) and R1 (5′-G TCGAATTCAGGGCGAATTGGAGCTCCTTAC-3′). After purification, the fragment was cloned into plasmid pYC12 to obtain the recombinant plasmid pYC12-M. Plasmid pYC12-M was introduced into cells of *R. solanacearum* by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. After electroporation (3.0 kV, 200 Ω, 25 μF), the transformed cells were immediately transferred to 1 mL NAg medium and incubated for 2 h at 30 °C with shaking at 100 r min^{-1} then plated on selective NAg (NA medium containing 30 μg mL^{-1} Gm) plates. After growth at 30 °C for 36 h on the selective plates, a colony that emitted red fluorescence under fluorescence stereomicroscope with red light filter was chosen and named QL-RFP.

Growth curves

The relative growth of GFP-tagged *B. amyloliquefaciens* strain T-5-GFP vs. the wild-type strain T-5 and the RFP-tagged *R. solanacearum* strain QL-RFP vs. QL-*Rs*1115 was

determined under aerobic conditions. Suspensions of overnight cultures of the strains were adjusted to OD_{600}=0.4. The suspensions of QL-RFP or QL-*Rs*1115 (0.5 mL) were used to inoculate 50 mL of fresh NA broth; 0.5 mL of suspensions of T-5-GFP or T-5 were used to inoculate 50 mL of fresh LB broth, and these cultures were then shaken at 30 °C for 48 h. OD_{600} was measured every 3 h during the growth process.

Virulence assays

The virulence of QL-RFP and the wild-type strain QL-*Rs*1115 in tomato plants was measured using both direct stem puncture and a natural soil soak inoculation method (Tans-Kersten et al. 2004). Briefly, for stem puncture inoculation, bacterial suspensions (10^8 colony-forming units (CFU) mL^{-1}) were injected with a needle into the second internode on the major stem of 21-day-old tomato plants (with 4 leaves). For the soil soak assay, unwounded tomato plants were inoculated after 21 days of growth by pouring a bacterial suspension into the soil to a final abundance of approximately 10^7 CFU g^{-1} dry weight of soil. After inoculation, the plants were cultured in a growth chamber at 28 °C with a 16-h light/8-h dark photoperiod and rated daily for symptoms on a disease index scale from 0 to 4 as described by Kempe and Sequeira (1983), where 0=no wilting, 1=1–25 % wilting, 2=26–50 %, 3= 51–75 %, and 4=76–100 % wilted or dead. The virulence assay included 15 plants per treatment and was repeated twice.

Antagonistic activity assays

The antagonistic effects of T-5-GFP and the wild-type strain T-5 against the pathogenic strain QL-*Rs*1115 were evaluated on NA medium using culture supernatants. Both T-5 and T-5-GFP were grown in LB at 30 °C on a rotary shaker for 48 h. Cultures were centrifuged at 10,000×g for 10 min, and the supernatants were collected. A cell suspension of QL-

Rs1115 (0.5 mL) at a concentration of 10^9 CFU mL^{-1} was spread onto NA plates. Four 7-mm-diameter wells were made, and each was filled with 50 µL of filtered bacterial supernatant. Plates were incubated at 30 °C for 48 h, and the extent of the inhibition zones was measured.

Co-culture experiment of QL-RFP and T-5-GFP

The ability of *B. amyloliquefaciens* T-5-GFP to inhibit the growth of *R. solanacearum* QL-RFP was evaluated in an exudate-like medium (ELM) as described by (Nihorimbere et al. 2012). QL-RFP and T-5-GFP were grown overnight in NAg and LBk at 30 °C, respectively. Bacterial cells were centrifuged (6000×g for 6 min) and washed twice with an equal volume of sterile saline solution (0.9 % NaCl). RE medium (2 mL) was co-inoculated with 20 µL of suspensions containing different abundances of T-5-GFP (0, 10^5, 10^6, 10^7, 10^8 CFU mL^{-1}) and 10^6 CFU mL^{-1} QL-RFP. Counts of both bacterial cells were determined by dilution plating after 12 h of incubation. T-5-GFP was grown on LBk and QL-RFP on NAg. Each experiment was repeated three times.

Greenhouse experiment

B. amyloliquefaciens T-5-GFP was plated and transferred into LBk broth and shaken at 180 r min^{-1} at 30 °C for 36 h. Cells of *B. amyloliquefaciens* T-5-GFP were collected by centrifugation (6000×g for 10 min at 4 °C), resuspended in sterile saline solution (10^9 CFU mL^{-1}), and stored at 4 °C until they were used. Suspensions of QL-RFP also were prepared as described above. Tomato seeds (*Lycopersicon esculentum*, cultivar '*Jiangshu*') were surface-sterilized in 2 % NaClO for 5 min, washed four times with sterile distilled water, and then germinated in 9-cm plates covered with sterile wet filter paper at 25 °C for 48 h. Germinated seeds were grown for 2 weeks in nursery pots containing vermiculite then transplanted into the greenhouse (at 20–31 °C and relative humidity of 63–80 %). Two tomato seedlings were transplanted into an 8-cm-diameter pot containing 400 g of sterile mixture (soil:vermiculite:sand (1:3:1)). The treatments were as follows: (1) neither seedlings nor soil were inoculated with bacterial cells (CK); (2) plant seedlings inoculated by soaking in a cell suspension (~10^8 CFU mL^{-1}) of T-5-GFP for approximately 30 min (P); (3) plant seedlings inoculated as described before, but also the soil was inoculated by drenching with cell suspension of T-5-GFP (10^8 CFU mL^{-1} dry weight soil) (S); (4) both plant seedlings and soil were inoculated with T-5-GFP (PS). To visualize the colonization patterns of both T-5-GFP and QL-RFP on tomato roots, soils in half of the pots were drenched with cell suspension of QL-RFP (10^5 CFU g^{-1} dry weight soil) before transplantation. The assays were conducted in a completely randomized design, using 40 plants per treatment.

For microscopical analysis, root samples were collected 7 days after inoculation, cut in pieces 1–2 cm long, and were observed by a confocal laser scanning microscopy (CLSM, Leica Model TCS SP2, Heidelberg, Germany). To visualize the plant cells, a 405-nm (excitation) ultraviolet laser with a 450-nm filter (emission) was used. For excitation of the GFP and the RFP in bacterial cells, a 495-nm laser with a 505-nm emission filter and a 532-nm laser with a 610-nm emission filter were used, respectively. Photographs were obtained using Leica confocal software, version 2.61.

Four roots from each treatment were collected 10 and 20 days after inoculation with QL-RFP. The tomato seedlings were uprooted from the soil and gently shaken to remove all of the loose, adhering bulk soil and then soaked in distilled water and shaken to obtain the root samples. The roots were cut into 1-cm segments, and 5 g of these segments was placed into 45 mL sterile saline and vortexed (Scientific industries, Inc., Bohemia, NY, USA) for 15 min at maximum speed. Both of QL-RFP and T-5-GFP were immediately enumerated on each sampling day by a standard dilution-plate-count method using a selective medium with 30 µg mL^{-1} Gm added to a modified SMSA medium (Elphinstone et al. 1996) or to a 20 µg mL^{-1} Km selective medium (Turner and Backman 1991), respectively. After incubating the cells in an incubator at 30 °C for 36–48 h, the bacterial colonies were examined by fluorescence microscopy (Olympus MVX10), and those emitting red or green fluorescence were counted.

Statistical analyses

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

Results and discussion

Construction of marker strains tagged with GFP or RFP

The fluorescent proteins have been used to study the interactions of biocontrol bacteria and pathogens on the plant roots (Rochat et al. 2010; Kato et al. 2012). In this study, we marked the biocontrol strain *B. amyloliquefaciens* T-5 and the pathogen strain *R. solanacearum* QL-Rs1115 with the plasmids pHAPII and pYC12, respectively, to study their interactions on tomato roots. Transformation of

B. amyloliquefaciens T-5 with pHAPII plasmid resulted in 56 transformants, which could be easily distinguished by GFP fluorescence being visualized by fluorescence microscopy (Fig. S1), and the colony with the highest fluorescence was selected for the further study. The growth rate of T-5-GFP on LB medium (data not shown) and its antagonistic ability against *R. solanacearum* on NA plates were similar to those of the wild-type strain T-5 (Fig. 1), indicating that the presence of the plasmid did not interfere with the growth and antagonistic ability of strain T-5. Similar results were reported by Cao et al. (2011) when *Bacillus subtilis* SQR-9 was transformed with plasmid pHAPII.

A new RFP plasmid was constructed by introducing a *mCherry* gene fragment into plasmid pYC12, which contains a constitutive Ptac promoter, to produce the plasmid pYC12-M (Fig. S2). The transformation of *R. solanacearum* with plasmid pYC12-M resulted in 72 transformants, which could be easily distinguished by RFP fluorescence when visualized by fluorescence microscopy (Fig. S3). The strain QL-RFP displayed similar growth characteristics as the wild-type strain QL-*Rs*1115 in the liquid media, indicating that the growth of the strain QL-*Rs*1115 was not affected either by the presence of the pYC12-M plasmid or by the expression of red fluorescent protein (data not shown).

A previous study showed that the introduction of exogenous plasmid, which carried the reporter gene and antibiotic resistance genes, into bacteria required energy for functioning and affected the growth and metabolism of the host (Falk et al. 1995). We used recombinant plasmid pHAP II, which has been used by other researchers and is considered a useful tool for tagging *Bacillus* strains to monitor their behavior in the natural environment (Cao et al. 2011; Ling et al. 2012). The recombinant plasmid pYC12-M was derived from the broad host range expression vector pYC12, used in many bacterial strains (Wang et al. 2008). In this study, both plasmids (pHAP II and pYC12-M) did not affect the growth and

Fig. 1 Antagonistic effects of T-5 and T-5-GFP supernatants against *R. solanacearum*

antagonistic activity of T-5-GFP (Fig. 1) and the growth and pathogenicity of QL-RFP (Fig. 2), respectively. Different results have been reported about the stability of plasmids in transformants, like Unge et al. (1999) and Njoloma et al. (2006) reported that the plasmids can be maintained for several months in the transformants without antibiotic selection, while Oliveira et al. (2009) reported that plasmid was lost within several days in the absence of antibiotic selection.

Colonization patterns of labeled strains on tomato roots

The GFP-tagged biocontrol agent T-5-GFP and RPF-tagged pathogen QL-RFP were monitored in situ during their colonization of tomato roots using different application methods. Seven days after the inoculation with T-5-GFP, the root systems were examined by CLSM for the presence of bacterial cells. The GFP-tagged cells could be distinguished easily from the background fluorescence of the root and showed different colonization patterns with different inoculation methods (Fig. S4). The non-inoculated roots did not show any fluorescent cells (Fig. 3a), while GFP signals were found on the root tips (Fig. 3b), primary roots (Fig. 3c), and lateral root junctions (Fig. 3d) in the PS treatment. In addition, the CLSM images showed that the T-5-GFP could also colonize root hairs and even form biofilm and a semitransparent extracellular matrix on the root surface of tomato in both S and PS treatments (Fig. 3e, f). It has been shown that some bacterial strains colonized plant roots and even the interior of plant tissues by forming aggregates or micro-colonies (Fan et al. 2011; Zhang et al. 2011). From the GFP fluorescence images, we observed that T-5-GFP colonized tomato roots rapidly and efficiently after inoculation. In addition, the colonization of T-5-GFP occurred as a biofilm, which is an important trait of biocontrol agents against soil-borne pathogens (Bais et al. 2004). Similar to our results, biofilm formation by *B. subtilis*, *B. amyloliquefaciens*, and *Paenibacillus polymyxa* strains was also detected after their colonization on the roots of *Arabidopsis thaliana* or *Lemna* (Bais et al. 2004; Timmusk et al. 2005; Fan et al. 2011).

Seven days after the inoculation of the pathogenic strain QL-RFP, tomato roots were examined by CLSM for the presence of QL-RFP. The results showed that QL-RFP could invade all parts of the roots and even form biofilms on the root (Fig. 4). These results indicated that the pathogen QL-RFP could strongly colonize the roots and possibly invade the interior tissues of the tomato plant, as it has been reported that the tomato wilt pathogen colonizes the root surface and then invades xylem vessels by degrading the cell wall and producing large amounts of exopolysaccharides that block water flow causing chlorosis, wilting, and ultimately plant death (Swanson et al. 2005). To evaluate
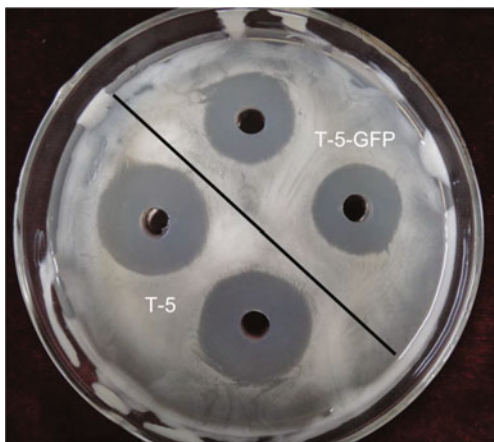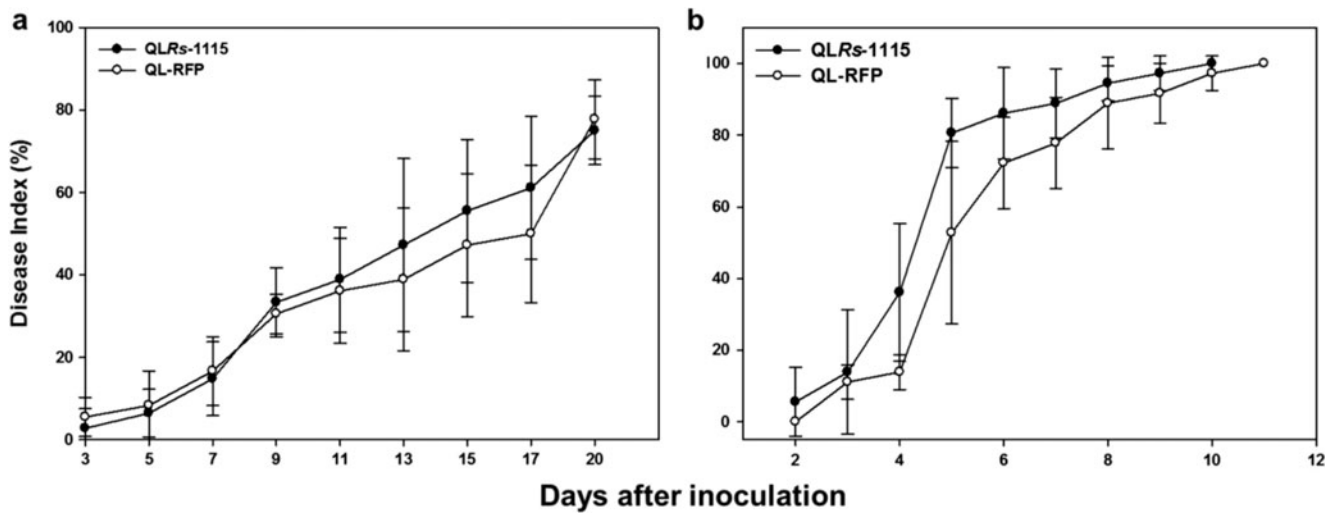
Fig. 2 Virulence of QL-Rs1115 and QL-RFP in tomato plants. **a** Soil soaking inoculation, **b** stem puncture inoculation. The data were collected from 15 replicates of each treatment and are expressed as the mean±standard deviation

the interactions between the biocontrol agent and the pathogen, the tomato seedlings (P), soil (S), or both (PS) were inoculated with T-5-GFP, and the soil was then inoculated with QL-RFP by drenching. The CLSM images showed that there was a significant influence of the biocontrol agent on the colonization pattern of the pathogen. In the PS+QL-RFP treatment, almost all the root tips were occupied by the T-5-GFP (Fig. 5i), while in the P+QL-RFP treatment, the pathogen QL-RFP occupied the root tips (Fig. 5a). In the S+QL-RFP treatment, both T-5-GFP and QL-RFP strains almost equally colonized the root tips (Fig. 5e). A similar phenomenon was also found for the other root parts. QL-RFP formed a biofilm on the root surface and possibly invaded the interior of the roots in the P+QL-RFP
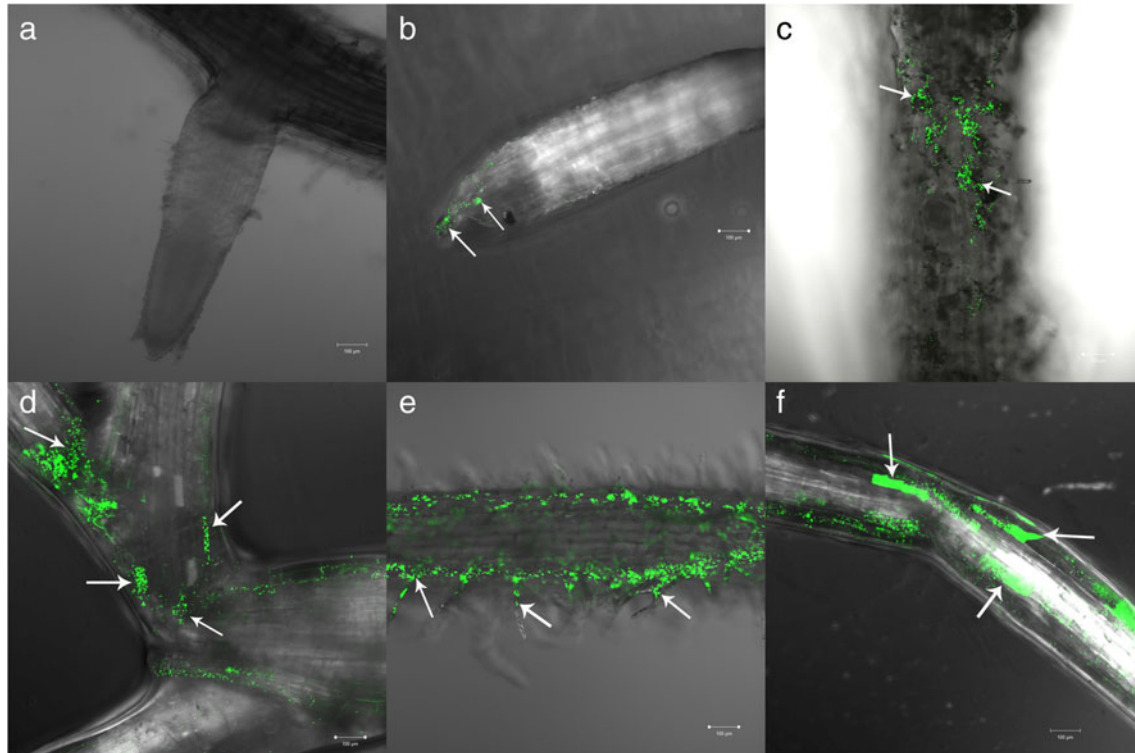

Fig. 3 CLSM images of T-5-GFP cells colonization on roots of tomato. **a** Control. **b** T-5-GFP colonized on tomato root tip. **c** T-5-GFP colonized on tomato root surface. **d** T-5-GFP colonized on junctions between the roots of tomato. **e** T-5-GFP colonized on root hairs. **f** T-5-GFP formed biofilms on tomato root surfaces. Bacterial cells are indicated by *white arrows*

Fig. 4 CLSM images of QL-RFP cells colonizing the roots of tomato. **a** Control, **b** root tips, **c** root surface, **d** junctions of roots. Bacterial cells are indicated by *white arrows*
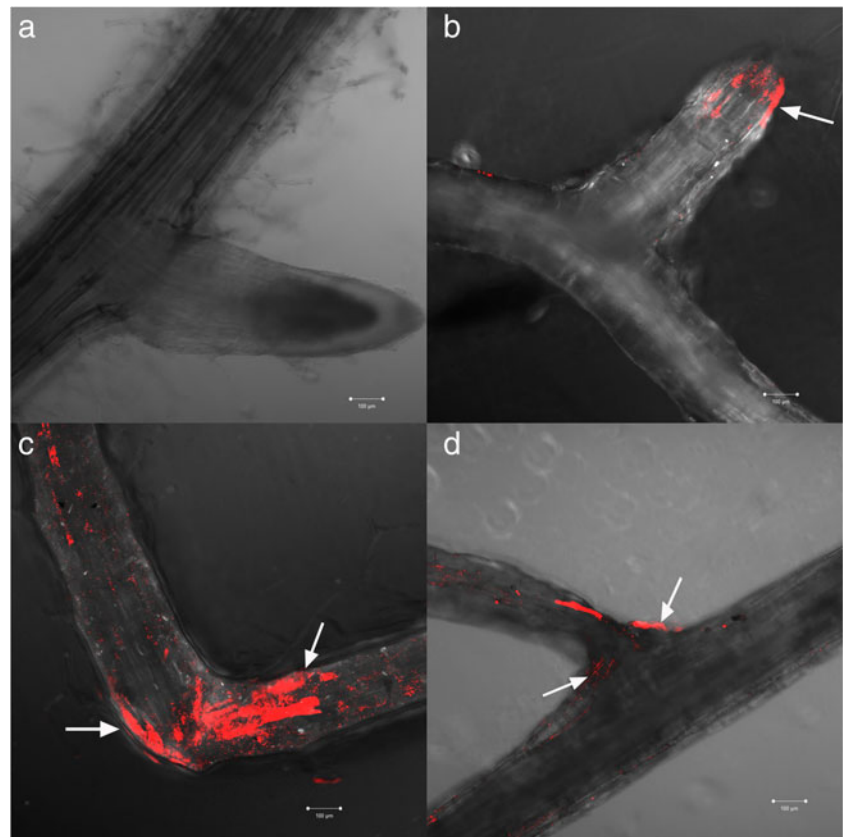
treatment (Fig. 5b, c). However, in the PS+QL-RFP treatment, this phenomenon did not occur and the biocontrol agent T-5-GFP occupied the root surfaces and formed a biofilm (Fig. 5j, k). In S+QL-RFP treatment, both the biocontrol agent T-5-GFP and the pathogenic strain QL-RFP equally colonized the root surfaces (Fig. 5f, g). The images obtained from the root junctions showed that in the P+QL-RFP treatment, QL-RFP had higher abundance and formed a biofilm (Fig. 5d), but in the S+QL-RFP and PS+QL-RFP treatments, T-5-GFP colonized most of the space and formed a biofilm (Fig. 5h, l). These results revealed that the dual inoculation method (PS; seedling soaking and soil drenching) was the most effective in preventing the tomato root colonization by pathogen QL-RFP, while soil drenching method showed better results compared to seedling soaking method. Our results were similar to the previously reported studies of biocontrol agent and pathogen interactions on plant roots. Like the biocontrol agent, *Pythium oligandrum* was observed to suppress the colonization of *R. solanacearum* at the surface of taproots, junctions between taproots and lateral roots, and in the middle sections of the lateral roots of tomato (Masunaka et al. 2009). Similarly, the GFP-tagged biocontrol agent *Serratia plymuthica* A30 colonized root surfaces and suppressed the invasion of the pathogenic strain *Dickeya* sp. (Czajkowski et al. 2012).

Inhibitory effect of T-5-GFP on QL-RFP in the co-culture experiment and on the root surface

The inhibitory effect of biocontrol agent T-5-GFP on pathogen QL-RFP was determined in the exudate-like medium (ELM) and on the root surface. The results showed that in the co-culture experiment in ELM, the presence of pathogen QL-RFP did not affect the growth of the biocontrol agent T-5-GFP at any initial inoculum concentration of T-5-GFP (Fig. 6). The biocontrol agent T-5-GFP grew faster at the initial inoculum concentrations of 10^5, 10^6, and 10^7 CFU mL^{-1}, but not at 10^8 CFU mL^{-1}; however, the population of T-5-GFP was similar in all treatments at the end of the incubation. On the other hand, the population of pathogen QL-RFP was increased from 10^6 to 10^8 CFU mL^{-1} when inoculated alone; however, the numbers of QL-RFP cells were decreased rapidly in the ELM when co-cultured with biocontrol agent T-5-GFP. The decrease in QL-RFP cells was increased by increasing the initial inoculum concentration of T-5-GFP (Fig. 6).

The abundance of the biocontrol agent T-5-GFP and pathogen QL-RFP on the root surface was measured on selective antibiotic plates. The results showed that the pathogen could grow and strongly colonize tomato roots. The abundance of QL-RFP was 6.79 and 7.15 log CFU g^{-1} after 10 and 20 days of transplantation, respectively, when the soil was treated with
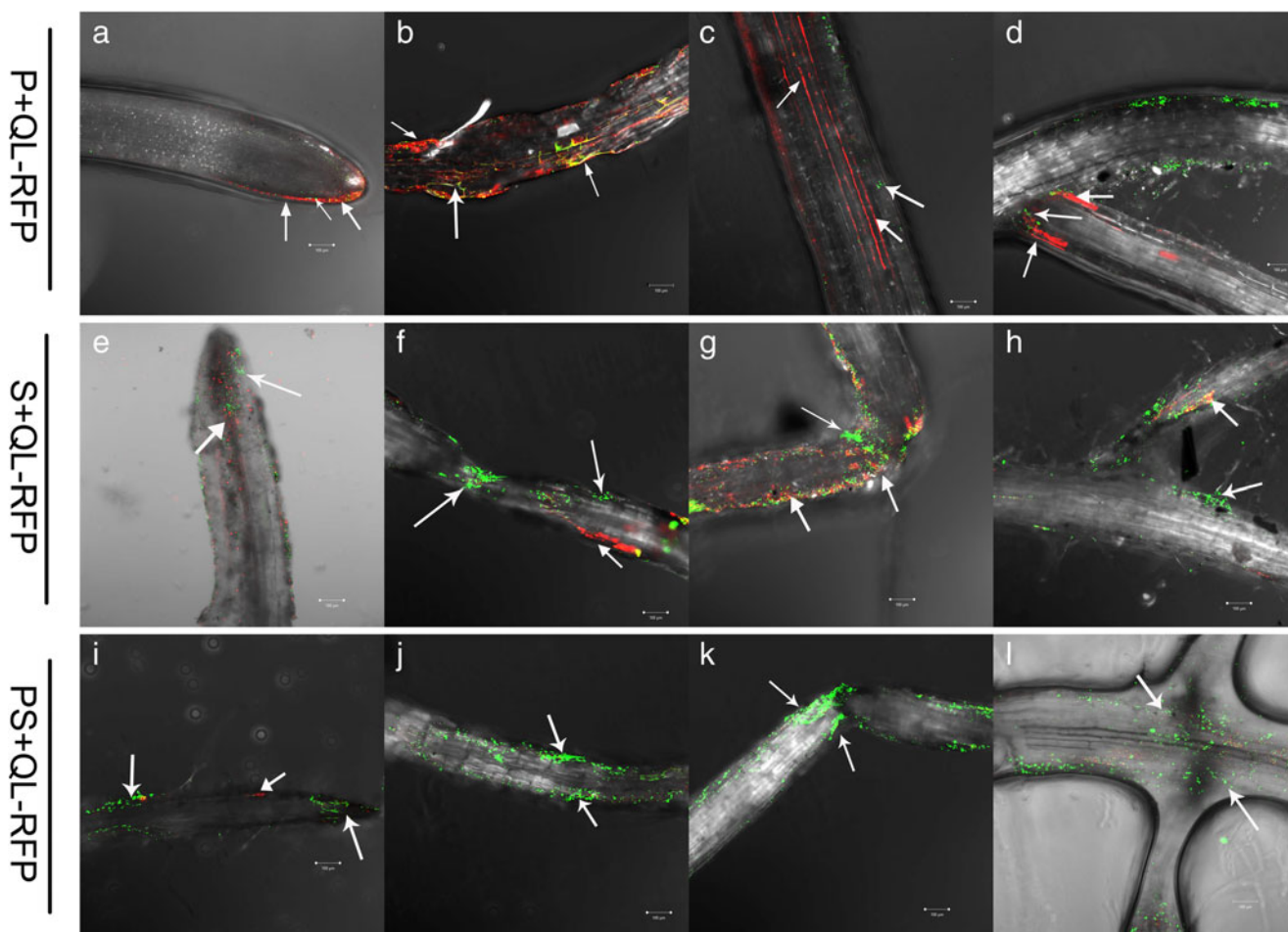
Fig. 5 CLSM images of T-5-GFP and QL-RFP cells colonizing on tomato roots. **a** Bacterial cells colonized on tomato root tip in the P+QL-RFP treatment; **b**, **c** bacterial cells colonized on tomato root surfaces in the P+QL-RFP treatment; **d** bacterial cells colonized on junctions between the roots of tomato in the P+QL-RFP treatment; **e** bacterial cells colonized on tomato root tip in the S+QL-RFP treatment; **f**, **g** bacterial cells colonized on tomato root surfaces in the S+QL-RFP treatment; **h** bacterial cells colonized on junctions between the roots of tomato in the S+QL-RFP treatment; **i** bacterial cells colonized on tomato root tip in the PS+QL-RFP treatment; **j**, **k** bacterial cells colonized on tomato root surfaces in the PS+QL-RFP treatment; **l** bacterial cells colonized on junctions between the roots of tomato in the PS+QL-RFP treatment. T-5-GFP bacterial cells were indicated by *white arrows*, and QL-RFP were indicated by *white arrowheads*

the pathogen QL-RFP alone (Table 2). The growth of QL-RFP was inhibited by the presence of the biocontrol agent T-5-GFP and was also influenced by the inoculation method of T-5-GFP. Maximum inhibition of QL-RFP was found in the PS+QL-RFP treatment where both tomato seedlings and soil were treated with the biocontrol agent T-5-GFP followed by the S+QL-RFP treatment where the soil alone was treated with biocontrol agent T-5-GFP (Table 2). The abundance of T-5-GFP on the root surface was also significantly influenced by the inoculation methods (Table 2). The highest abundance (approximately 7.0 log CFU g^{-1}) of T-5-GFP on the root surface was found in the PS+QL-RFP treatment followed by the S+QL-RFP treatment. The soil infected by the pathogen QL-RFP did not have a significant effect on the growth of T-5-GFP (Table 2). These results are in agreement with the previous reports (Bais et al. 2004; Ren et al. 2012). The abundance of *B. amyloliquefaciens* T-5 on the root surface protected

the tomato from soil-borne pathogen, as the root colonization by biocontrol agents has been reported to be critical for the effective control of phytopathogens (Kamilova et al. 2005; Fan et al. 2011; Zhang et al. 2011).

In this study, the strain T-5-GFP colonized sites of root tips, root hairs, primary roots, and root junctions and even formed a biofilm on the root surface (Fig. 3) when inoculated by seedling soaking and soil drenching methods. It has been reported that the application methods of biocontrol agents could significantly affect the biocontrol efficiency; both the initial concentration of biocontrol agent and pathogen would play an important role in the biocontrol of the pathogen. For example, Tan et al. (2013b) reported that the application methods significantly affected the disease incidence and the biocontrol efficacy of *B. amyloliquefaciens* T-5 against tomato wilt pathogen *R. solanacearum*. The different inoculations of *Paenibacillus alvei* K-165 showed different biocontrol
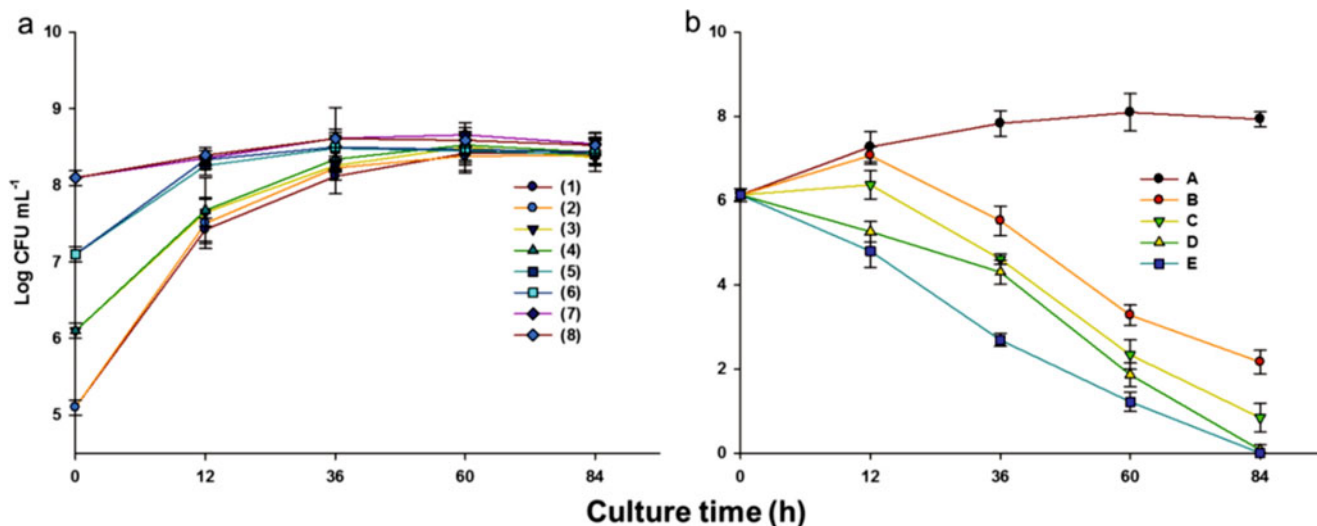
Fig. 6 Co-culture T-5-GFP and QL-RFP in exudate-like medium (ELM). The ELM medium was co-inoculated with different initial concentrations of T-5-GFP (0, 10^5, 10^6, 10^7, 10^8 CFU mL^{-1}) and 10^6 CFU mL^{-1} QL-RFP. **a** Growth of T-5-GFP, (1) T-5-GFP with abundance of 10^5 CFU mL^{-1}; (2) T-5-GFP with abundance of 10^5 CFU mL^{-1} and co-culture with QL-RFP; (3) T-5-GFP with abundance of 10^6 CFU mL^{-1}; (4) T-5-GFP with abundance of 10^6 CFU mL^{-1} and co-culture with QL-RFP; (5) T-5-GFP with abundance of 10^7 CFU mL^{-1}; (6) T-5-GFP with abundance of 10^7 CFU mL^{-1} and co-culture with QL-RFP; (7) T-5-GFP with abundance of 10^8 CFU mL^{-1}; (8) T-5-GFP with abundance of 10^8 CFU mL^{-1} and co-culture with QL-RFP. **b** Growth of QL-RFP, A, ELM only cultured with QL-RFP; B, ELM co-cultured with QL-RFP and T-5-GFP with abundance of 10^5 CFU mL^{-1}; C, ELM co-cultured QL-RFP and T-5-GFP with abundance of 10^6 CFU mL^{-1}; D, ELM co-cultured QL-RFP and T-5-GFP with abundance of 10^7 CFU mL^{-1}; E, ELM co-cultured QL-RFP and T-5-GFP with abundance of 10^8 CFU mL^{-1}. The data were collected from three replicates of each treatment and are expressed as the mean±standard deviation

efficiencies to control black root rot disease in cotton (Schoina et al. 2011). Xue et al. (2009) reported that the soil drenching method showed better root colonization and biocontrol efficiency of tomato bacterial wilt by biocontrol agents under greenhouse and field conditions. Qiu et al. (2014) also suggested that decoupling of root-microbiome associations followed by antagonist inoculation can improve rhizosphere soil suppressiveness. The suitable application methods ensure the biocontrol agent occupation of niches on roots by competing with the pathogen for food and niche; it will also ensure the suppression of pathogen in the rhizosphere by other biocontrol mechanisms like the production of antibiotics, hydrolytic enzymes, and volatile organic compounds.

Table 2 Abundance of T-5-GFP and QL-RFP on the root surface

Treatment	Bacterial numbers (log CFU g^{-1})			
	T-5-GFP		QL-RFP	
	10 days	20 days	10 days	20 days
P	5.13±0.23 c	4.57±0.27 c	–	–
S	6.42±0.37 b	5.76±0.15 b	–	–
PS	7.05±0.11 a	6.78±0.13 a	–	–
QL-RFP	–	–	6.79±0.21 a	7.15±0.15 a
P+ QL-RFP	4.97±0.32 c	4.46±0.35 c	6.68±0.09 a	6.49±0.17 b
S+ QL-RFP	6.35±0.21 b	5.68±0.14 b	6.02±0.07 b	5.97±0.36 bc
PS+ QL-RFP	6.97±0.15 b	6.59±0.16 a	5.62±0.11 c	5.16±0.14 c

Note: P, tomato seedlings treated with T-5-GFP; S, the pot soil was treated with T-5-GFP; PS, both tomato seedlings and soil were treated with T-5-GFP; QL-RFP, the soil was treated with QL-RFP; P+ QL-RFP, tomato seedlings were treated with T-5-GFP and the soil was treated with QL-RFP; S+ QL-RFP, the soil was treated with both T-5-GFP and QL-RFP; PS+ QL-RFP, both tomato seedlings and soil were treated with T-5-GFP, and the soil was only treated with QL-RFP. Treatments were replicated four times, and each replicate had one plant. Data are expressed as the mean±standard error. The data in columns with different small letters differ significantly at $P < 0.05$ by Duncan's test

Conclusion

This study describes the potential of spatial and temporal distribution of a biocontrol strain *B. amyloliquefaciens* T-5 and a pathogenic strain *R. solanacearum* QL-Rs1115 on the roots of tomato. The biocontrol strain *B. amyloliquefaciens* T-5 not only colonized the tomato roots effectively but also inhibited the colonization of pathogenic strain *R. solanacearum* QL-Rs1115, which might be a mechanism to control bacterial wilt disease. Furthermore, the importance of the application method for the biocontrol agent, which is essential to enhance the survival and root colonization, was also highlighted. These results support the commercial use of *B. amyloliquefaciens* T-5 as a biocontrol agent for controlling bacterial wilt disease of tomato.

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