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# A Potential of Biofilm Formation by Entomopathogenic *Bacillus thuringiensis* on Tomato Root Surface

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***Abstract:*** *Bacillus thuringiensis has been used as an effective bio-insecticide. But, recently B. thuringiensis was treated as a biological control agent which can suppress the plant disease. B. thuringiensis can suppress the growth of Ralstoniasolanacearum and the development of wilt symptoms in plants. Bacillus subtilis, which is ubiquitous in soil, can promote plant growth, protect against fungal pathogen attack as a biocontrol agent on plants. It is now widely recognized that Bacillus subtilis settings persist in association with surfaces by forming biofilm. In this study, we would like to determine if B. thuringiensis would colonize and form biofilms on tomato roots. We use 10 under test strains: B. thuringiensis sotto 15, israelensis 16, japonensis 17, kurstaki 18, roskildiensis 19, CR371-H 20, DF, GC-91, B.subtilis MBI600, B.simplex CGF2856. The microtiter plate assay was used to evaluate B. subtilis biofilm formation. Biofilm formation was quantified by measuring the $OD_{595}$ for each well using a spectrophotometer. Also the tomato root was soaked in each tested bacterial strains by co-culture method for 48h, and transplant tomato to soil condition. The result is B. thuringiensishas the ability of biofilm formation. Our results also indicate that B. thuringiensis formed biofilm can colonize plant root.*

***Keywords:*** *Biofilm, Bacillus thuringiensis, Bacillus subtilis, PGPR.*

## INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) promote plant growth and suppress plant disease by colonizing the plantroot surfaces and maintaining a stable relationship with the surface of plantroots. PGPR may increase plant yield by reducing plant pathogen populations in the soil, improving mineral nutrient uptake, phytohormone production, and maintaining a beneficial effect on plant growth [15, 20,21]. PGPR may also interact with a variety of soil microorganisms that are normally present in the rhizosphere, in some cases acting as a biocontrol agent against pathogenic bacteria [16]. Similarly, the sporulating gram-positive bacteria such as *Bacillus* spp. have also been used successfully as potential biological control agents (BCAs) to control plant disease [12, 14]. *Bacillus subtilis* is the best- characterized member of the Gram-positive bacteria, which is ubiquitous in soil, can protect against fungal pathogen attack, promote plant growth, and play a role in the degradation of organic polymers in the soil [1, 10]. Also, inoculate *B. subtilis* strains to Arabidopsis root which infected by *Pseudomonas syringae*, the mortality of Arabidopsis was reduced both in cultureand in soil was observed, because of formation of an antimicrobial-producing biofilm formed by *B. subtilis* was confirmed[4].

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Biofilms are widely found structures in which microorganisms are protected against various stresses, allowing them to persist in adverse environmental conditions.Bacterial biofilms are formed when unicellular organisms come together to form a community that is attached to a solid surface and encased in an exopolysaccharide matrix [22]. Furthermore, these microbial communities often interact with multiple species and their environment. The site of one such ecologically beneficial bacterial community is the root surfaces, where a rich microflora develops around the readily available nutrients released by roots [23]. Also, both *B. subtilis* and *B. thuringiensis* can form biofilms at air-liquid interfaces [11].

*Bacillus thuringiensis* has been used as an effective bioinsecticide [17, 18]. The specificity of *B. thuringiensis* is showed highly beneficial in agricultural biotechnology. Unlike most insecticides, *B. thuringiensis* insecticides are highly toxic against target insects and friendly towards beneficial insects, non-target organisms such as humans and wildlife [5]. It is also not harmful to the environment. *B. thuringiensis* has been used as an alternative to chemical pesticides for decades by organic farmers to control insects.

At present, *B. thuringiensis* is the only "microbial insecticide" in widespread use [8, 9]. Recently, *B. thuringiensis* has also attracted great attention as a biological control agent to suppress plant diseases [24]. Therefore, the new view is that the insecticide *B. thuringiensis* can be used as PGPR to control plant disease. Moreover, the activity of *B. thuringiensis* can suppress the growth of *Ralstoniasolanacearum* and the development of wilt symptoms has been examined in tomato plants [14].

If the formationof biofilms by *B.thuringiensis*is greatly found in plantroot surfaces like *B.subtilis*,it may show that high bacteria densityand more stably*B.thuringiensis* can exist in tomato root surfaces. Also, the effect by using *B.thuringiensis*as a biological control agent to control plant soil disease was expected. However, there is little research to evaluate the formation of biofilm by *B. thuringiensis* on plant root surfaces. So the objectives of this study were to determine if *B. thuringiensis* strains have the ability to form biofilms on microtiterplates, to determine if *B. thuringiensis* strains could colonize and form biofilms on tomato roots by liquid and soil co-culture methods.

## MATERIALS AND METHODS

### Bacterial Strains and Tomato Seed Preparation

The tested *B. thuringiensis* strains (BT 15 ~ 20) used for the present investigation were obtained from Research Faculty of Agriculture, Applied Bioscience Applied Molecular Biology Laboratory, Hokkaido University. For inoculum preparation, six strains of *B. thuringiensis* were inoculated in Luria Bertani broth (LB) and grown for 1 week at 30°C. The other tested strains (*B. thuringiensis* DF, *B. thuringiensis GC-91*, *B. subtilis MBI600*, *B. simplex CGF2856*) were form commercial formulation. For the preparation of bacterial suspension, the ten strains of bacteria were inoculated in liquid LB broth and grown for 24 h with constant shaking (150 rpm) at 28 ± 2°C. Tomato seed (*Lycopersiconesculen-tum* Mill) was used in this experiment. Tomato seeds were surface-sterilized with 5% antiformin for 30 sec, 70%ethanol for 10 sec, and washed with sterilized water twice.

### Formation of Biofilms by *B. thuringiensis* in Microtiter Plates

The ability of the *B. thuringiensis* strains to form biofilms was tested. Pre-cultures in the exponential phase of growth were inoculated at an optical density at 600 nm($OD_{600}$) of 0.1 into fresh LB medium (10 g/liter bactopeptone,5 g/liter yeast extract, 5 g/liter NaCl) in 24-well microtiterplates. After 24, 48, 72 h of incubation at 25°C with 3 replicates, the biofilm density was measured as follows: the microtiter plate wells were washed once with phosphate-buffered saline, and bound cells were stained with a 1%(wt/vol) crystal violet solution at room temperature for 20 min.

The wells were then washed with phosphate-buffered saline three times, and the dye was solubilized with a 20%/80% acetone/ethanol mixture. The absorbance at 595 nm of the solubilized dye was subsequently determined [2].

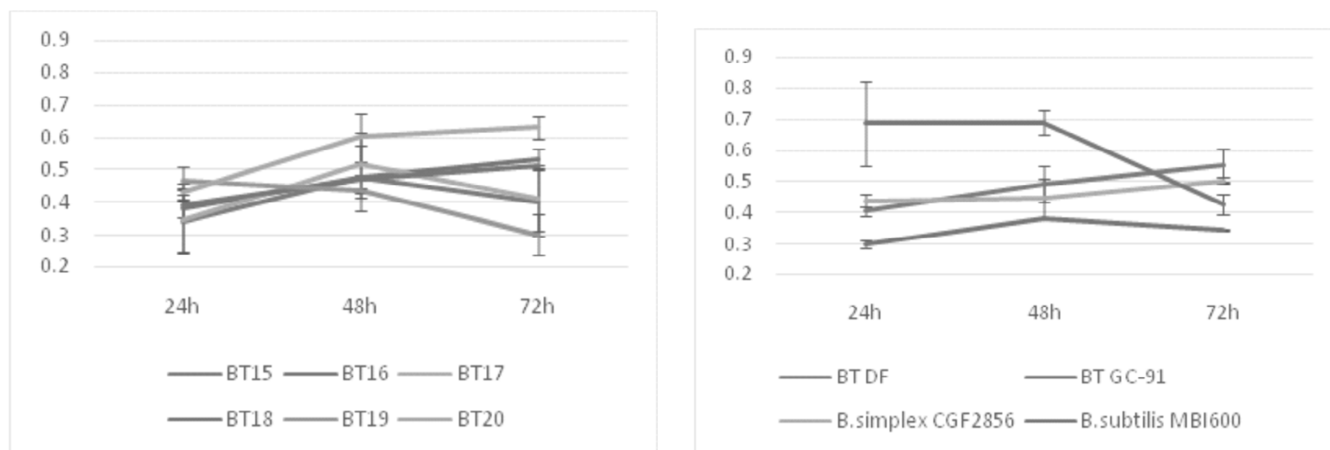


**Figure 1: OD595 of solubilized crystal violet from microtiter plate assay (filled circles). After 24h, 48h, 72h of incubation, biofilm density was measured as described in thetext. The data represent the means of three independent experiments. The error bars represent standard deviations.**

**BT15(*sotto* 15), BT16(*israelensis* 16), BT17(*japonensis* 17), BT18(*kurstaki* 18), BT19(*roskildiensis* 19), BT20(*CR371-H* 20), BT DF,BT *GC-91*, *B. simplex CGF2856*, *B. subtilis MBI600***

### Formation of Biofilms by *B. thuringiensis* with Liquid and Soil co-culture Methods

The ability *B. thuringiensis* biofilm formation on tomato roots was tested by liquid and soil co-culture methods(Bais *et al.*,2004). The tested bacterial suspensions were prepared as described before.In liquid co-culture, the tomato seeds were placed in a culture dish for germination in 8 days. Elongated tomato roots were soaked in a glass bottle (4.5 cm in height, 2 cm in width), which filled with 5ml bacterial suspension for 3 days.In soil co-cultivation, the tomato seeds were placed in a culture dish for germination in 4 or 5 days. After 4 or 5 days, germinated tomato seedlings were plant in 15 ml of tubes added with 2.5 g sterilization soil, filled with sterilized water. 1 ml/day tested bacterial suspensions were inoculated to the tomato seedlings, inoculation was taken three days in total.Root of the tomato seedlings treated with liquid and soil co-culture methods were rinsed with a phosphate-buffered saline twice and soaked with 70% methanol for 10 min. The tomato seedlings were dyed with 5% trypan blue for 10 min. An optical microscope was used to observe the tomato root surfaces in a field of vision of 1,000 times. Cut 2 cm from the tip of taproot and evaluated the biofilm formation of the tomato root surfaces.

Bacterial concentration in liquid co-culture experiment: *B. thuringiensis sotto* 15: 2.08 × 10*x* cfu/ml; *israelensis* 16: 8.16 × 10w cfu/ml; *japonensis* 17: 7.6 × 10w cfu/ml: *kurstaki* 18: 1.97 × 10*x* cfu/ml; *roskildiensis* 19: 9.34 × 10w cfu/ml; *CR371-H* 20: 9.26 × 10w cfu/ml; DF: 1.68 × 10*x* cfu/ml; *GC-91*: 1.64 × 10*x* cfu/ml; *B.subtilis MBI600*: 1.47 × 10w cfu/ml; *B. simplex CGF2856*: 1.78 × 10*x* cfu/ml.

Bacterial concentration in soil co-cultivation experiment: *B. thuringiensis sotto* 15: 1.95 × 10*x* cfu/ml; *israelensis* 16: 1.04 × 10*x* cfu/ml; *japonensis* 17: 1.10 × 10*x* cfu/ml; *kurstaki* 18: 2.0 × 10*x* cfu/ml; *roskildiensis* 19: 1.13 × 10*x* cfu/ml; *CR371-H* 20: 1.17 × 10*x* cfu/ml; DF: 1.81 × 10*x* cfu/ml; *GC-91*: 1.79 × 10*x* cfu/ml; *B. subtilis MBI600*: 9.51 × 10w cfu/ml; *B. simplex CGF2856*: 1.74 × 10*x* cfu/ml.

## RESULTS

### Formation of Biofilms by *B. thuringiensis* in Microtiter Plates

The result of formation of biofilms by *B. thuringiensis* in microtiter plates was all tested strains could form biofilms in microtiter plates (Figure 1). All tested strains showed a rise in absorbance from the first day to the second day. From second day to third day, *B. thuringiensis israelensis* 16, *kurstaki* 18, *CR371-H*20, DF, *B.simplexCGF2856* showed a rising trend in absorbance, *B. thuringiensis sotto* 15, *japonensis* 17, *roskildiensis* 19, *B.thuringiensis GC-91*, *B. subtilis MBI600* showed adecrease trend in absorbance (Figure 1). To determine the kinetics of biofilm formation, a microtiterplate was inoculated

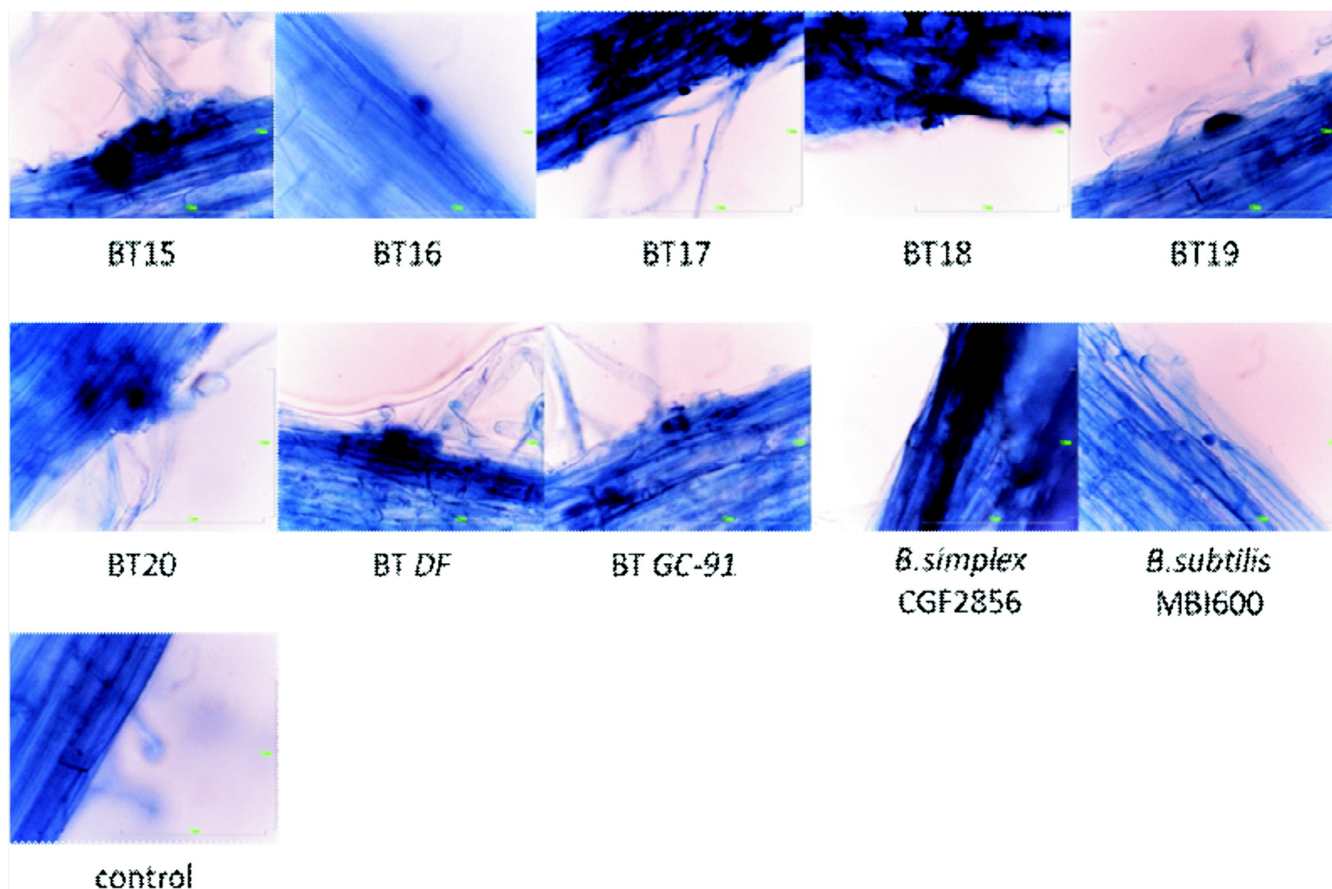


**Figure 2: In liquid co-culture, the tomato roots cultured with liquid bacterial suspension for 3 days. After 3 days culture, the tomato seedlings were dyed with 5% trypan blue. An optical microscope was used to observe the colony formed by bacteria on root surfaces.**

**BT15 (*sotto* 15), BT16 (*israelensis* 16), BT17 (*japonensis* 17), BT18 (*kurstaki* 18), BT19 (*roskildiensis* 19), BT20 (*CR371-H* 20), DF, *B.thuringien-sis GC-91*, *B. simplex CGF2856*, *B. subtilis MBI600***

with the bacterial strain as described above. A measurable amount of biofilm was detected after 24 h of inoculation (Figure 1). The number of viable cells in thebiofilm rings was determined as follows. The biofilm was manually scraped from the sides of the wells using a pipette tip and re-suspended in LB medium. After serial dilutions, cells were plated onto LB medium. The increase in crystal violet staining with time of incubation was proportional to the increase in the number of viable cells in the biofilm (Figure 1).

### Formation of Biofilms by *B. thuringiensis* with Liquid and Soil co-culture Methods

3-day after liquid and soil co-cultivation,tomato root surfaces was washed, fixed and dyed,then observed the formation of biofilms on tomato root surfaces by an optical microscope. As a result, in the liquid bacteria co-culture experiment, except *B. simplex CGF2856* and control treatment, all of bacterial strains could form biofilms on tomato roots rhizo plane were confirmed (Figure 2). Also, in the soil co-cultivation experiment, except *B. thuringiensis roskildiensis 19, CR371-H 20,* and *B. simplex CGF2856*, all of bacterial strains could form biofilms on tomato root surfaces were confirmed (Figure 3).

## DISCUSSION

The ability of biofilms formation by bacterial strains was evaluated in 24-well microtiter plates as described before. As a result, we identified all tested bacterial strains could form biofilm (an annular formed in suspension in the well and the interface with the gas) after 2 days static culture. In addition, because of the interaction between the quantity of CV dyeing and the number of colony forming unit of attached cells, the increase and decrease of

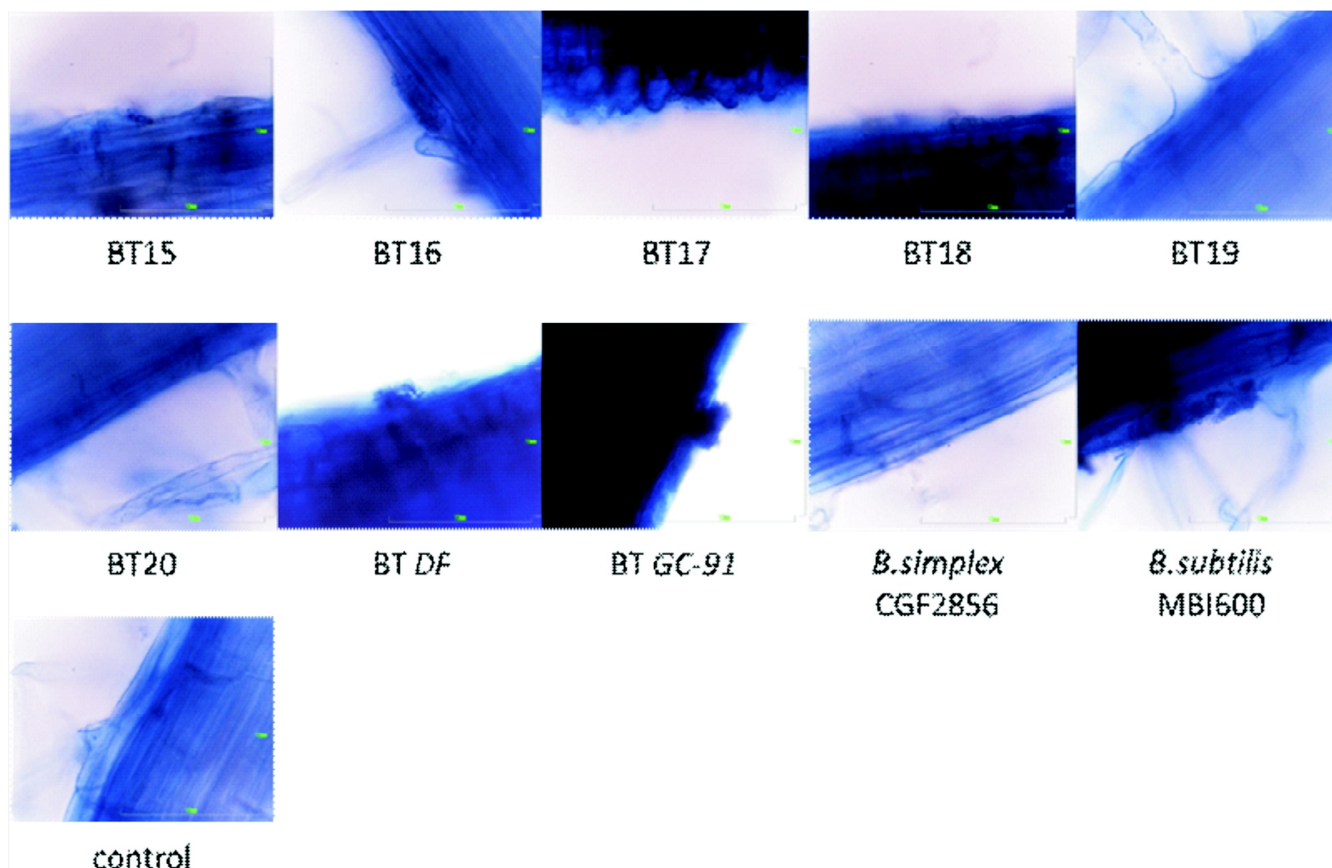


**Figure3. In soil co-cultivation, the tomato roots were plantin soil conditionand inoculated bacterial suspension. After 5 days culture, the tomato seedlings were dyed with 5% trypan blue. An optical microscope was used to observe the colony formed by bacteria on root surfaces (Bars=10mm).**

**BT15 (*sotto* 15), BT16 (*israelensis* 16), BT17 (*japonensis* 17), BT18 (*kurstaki* 18), BT19 (*roskildiensis* 19), BT20(*CR371-H* 20), DF, *B. thuringien-sis GC-91*, *B. simplex CGF2856*, *B. subtilis MBI600***

absorbance corresponded to quantity of biofilm formation in the well. Furthermore, from the first day to the second day, all tested strains showed an increase in the quantity of biofilms formation, from second day to third day, biofilm quantities showed both increase and decrease for every each strain were confirmed. In biofilm formative process, the biofilm would flow away from direction, which prevent biofilm growth and the structure, composition, and function of biofilm are changing dynamically, during biofilm formation [7]. So the disintegration of the biofilm or the falling off from a well wall surface may lead to decrease of already developed, thick biofilm.

The results of biofilm formation evaluated by liquid and the soil co-cultivation were that, except some strains colony biofilm were confirmed in all tested strains treated tomato root surfaces under both culture conditions. Under a liquid culture condition, all of the *B. thuringiensis* strains and *B. subtilis MBI600* biofilm formation ability were confirmed, but there was no biofilm formed by *B. simplex CGF2856*. On the other hand, under soil co-cultivation, except *B. thuringiensis roskildiensis 19*, *CR371-H 20*, and *B. simplex CGF2856*, all of bacterial strains could form biofilms on tomato root surfaces were confirmed.

For *B. simplex* the reason might be the ability for biofilm formation to plant root surfaces was lacked; for *B. thuringiensis* the reason might be a soil particle became the inhibition under soil culture, so there was no biofilm formation in rhizoplane. In this study, we documented *B. thuringiensis* strains have the ability to colonize and form biofilms on plant root surfaces.

Biofilms share an important structural feature: their constituent cells are bound together by an extracellular matrix that mainly consists of macro-

molecules, including proteins, polysaccharides, and nucleic acids, that are producedby the cells themselves [6]. Bacterial biofilms are found in most natural and man-made environments where bacteria are associated predominantly with surfaces rather than in a free-floating state[3]. *B. subtilis* has been a model organism for the studyof Gram-positive bacterial physiology. Recently, it has been reported that *B. subtilis* forms adhering biofilms on inert surfaces under the control of avariety of transcription factors [13, 19]. Further more, *B. subtilis* 6051 could reduce mortality of Arabidopsis, which root infected by*Pseudomonas syringae*, because of an antimicrobial-producing biofilm formed by *B. subtilis* 6051. So the ability of *B. subtilis* 6051 to control *P. syringae* infectivity of Arabidops is was directly proportional to its ability to colonize and form biofilms on plant root surfaces was documented[4].

*B. thuringiensis* is a naturally abundant Gram-positive bacterium and a well-known, effective bio-insecticide [18]. *B. thuringiensis* can product crystal proteins, which are highly toxic to insects, but not to mammals, and are not harmful to the environment. It hasbeen widely used as an alternative to chemical pesticides or genetically engineered into crops to provide constant protection [5]. Moreover, the activity of *B. thuringiensis* can suppress the growth of *Ralstoniasolanacearum* and the development of wilt symptoms has been examined in tomato plants [14].

In this study, although we documented *B. thuringiensis* strains have the ability to colonize and form biofilms on plant root surfaces, in the future, using the fluorescence flagella antibody to observe the three-dimensional biofilm and quantify biofilm formed by*B. thuringiensis* will be necessary. Recently, we proved that *B. thuringiensis* strains suppressed the development of wilt symptoms caused by FOL in tomato plants. And *B. thuringiensis* strains are plant growth promoting rhizobacteria (PGPR) that can promote plant growth, seed germination and shoot elongation (unpublished). Furthermore, we would like to determine if the biofilm formation and colonization by *B. thuringiensis* strains could provide protection to the plant and control plant disease.

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