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Effects of *Bacillus cereus* F-6 on Promoting Vanilla (*Vanilla planifolia* Andrews.) Plant Growth and Controlling Stem and Root Rot Disease

Qingyun Zhao, Hui Wang, Zihui Zhu, Yinghui Song, Huan Yu*

Spice and Beverage Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wanning, China
Email: *727251431@qq.com

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Abstract

A lipopeptide-producing bacterium, *Bacillus cereus* (F-6), was isolated from the rhizosphere soil of a healthy vanilla (*Vanilla planifolia*) plant cultivated on a plantation under 21 years of continuous cropping with vanilla. A pot experiment was conducted to investigate the effects of the green fluorescent protein-tagged F-6 (F-6-gfp) and its bio-organic fertilizer (BIO) on vanilla plant growth and stem and root rot disease, using the same plantation soil. The application of BIO significantly increased the vanilla plant root, stem and leave dry weights; however, there was not a significant difference between the F-6-gfp-inoculated treatment and the control. Meanwhile, the BIO application also significantly reduced the severity of stem and root rot disease compared to the control. The rhizosphere soil population of *Fusarium* was approximately 10-fold smaller in the BIO treatment compared to the control treatment at 150 days after transplantation. The number of *B. cereus* F-6-gfp in the rhizosphere soil of the BIO treatment remained significantly higher than that of the F-6-gfp-inoculated treatment throughout the experiment. In conclusion, F-6-gfp successfully colonized the rhizosphere soil in the BIO treatment, promoting vanilla plant growth, reducing the disease severity index, and decreasing the *Fusarium* population number, helping to remove barriers to the continuous cropping of vanilla.

Keywords

Bacillus cereus*, Bio-Organic Fertilizer, Continuous Cropping Barriers, Lipopeptide-Producing Bacterium, *Vanilla planifolia

*Corresponding author.

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1. Introduction

Vanilla (*Vanilla planifolia* Andrews.) is a perennial tropical liana of high economic value. Vanilla can be grown in tropical and subtropical areas from sea level to an altitude of 1500 m, and the optimum temperature range is from 21˚C - 32˚C [1]. Vanilla is known as "the king of food spices" and is widely used in the food, beverage, and cosmetic industries [2]. The consumption of natural vanillin (the most important active ingredient in the vanilla bean) has increased by approximately 10% per annum in the international market.

However, vanilla plants are typically slow growing and suffer from successive cropping obstacles when they are replanted after a previous crop is removed [3], similar to apple [4], cotton [5], melon [6]. A number of soil-borne diseases can cause successive cropping obstacles, and stem and root rot disease, which is caused by the fungal pathogen *Fusarium oxysporum* f. sp. *vanilla*, causes heavy economic losses in numerous vanilla-planting regions [7]. On long-term monoculture vanilla plantations, this disease has spread widely; the plant symptoms have worsened and the soil microbial community structure is out of balance [3]. In addition, the intensive growth of vanilla requires a very high consumption of chemical fertilizers and pesticides, resulting in serious environmental and food safety problems. Planting problems associated with stem and root rot disease and high inputs of agrochemicals have seriously inhibited the successful development of vanilla, and thus, there is an urgent need to take steps to alleviate these problems.

Lipopeptide-producing bacteria are widely emphasized for their potential for plant growth promotion and for biological control of plant diseases [8] [9]. These bacteria exhibit strong antifungal activity against a plethora of yeast and fungi [10] and thus have attracted biotechnological interests [11]. *Bacillus* lipopeptides can facilitate the persistence of biocontrol agents and promote plant growth [12] [13]. These lipopeptides can also act as chemical signals for motile bacteria to move to the root surface [13]. Their colonization ability is a prerequisite to imparting beneficial effects [14]. Therefore, a reliable technique is required to enumerate the number of the targeted bacteria.

Organic fertilizers fortified with beneficial microbes have shown efficacy in plant growth promotion and disease control [15]-[17]. To the best of our knowledge, the effects of applications of lipopeptide-producing bacteria on promoting vanilla plant growth and suppressing stem and root rot disease have not yet been evaluated. The objectives of this study were 1) to develop a bio-organic fertilizer for both the promotion of vanilla plant growth and the control of stem and root rot disease, 2) to test the efficacy of the bio-organic fertilizer in pot experiments, and 3) to investigate the colonization ability of the inocula in rhizosphere soil.

2. Materials and Methods

2.1. Isolation of Lipopeptide-Producing Bacteria

In blood-plate hemolysis, a strain is identified as a biosurfactant-producing bacterium if it exhibits the hemolysis phenomenon on a sheep blood-plate [18]. A rhizosphere soil was sampled from healthy vanilla plants, 10-fold serially diluted, and spread onto blood-plates. The composition of this medium has been described by Carrillo *et al*. [19]. The plates were incubated at 28˚C for 3 - 4 days. A strain that produces a wide hemolysis circle was purified and designated as F-6.

2.2. *In-Vitro* Antagonistic Assay

The isolated strain (F-6) was inoculated into an optimized Landy medium [20] [21] and then shaken at a speed of 170 rpm at 28˚C for 24 h. Lipopeptides were extracted by following the method of Pradhan *et al*. [22] with minor modifications. The pH of the supernatant was adjusted to 2 with concentrated HCl. The acidic mixture was stored at 4˚C for 7 h to precipitate completely and then centrifuged at 12,000 ×*g* for 10 min at 4˚C. The precipitates were added to HPLC grade methanol and the pH was adjusted to 7. The samples were vortexed thoroughly and stored at 4˚C for 5 h and then centrifuged using the above-described conditions. The supernatant was collected and recognized as crude lipopeptides. The crude lipopeptides were filtered through a 0.22 μm membrane and stored at −20˚C for further use.

An agar diffusion assay was performed by following the method of Li *et al*. [23] with modifications. The *Fusarium oxysporum* f. sp. *vanilla* (FOV) phytopathogen was incubated on potato dextrose agar (PDA) in a Petri plate for 5 days. An 8-mm plug from the leading edge of a 5-day-old culture of FOV was placed onto the center of a new PDA plate. Wells (8-mm in diameter) were produced using a sterilized pontil-borer. A 30 μl aliquot of

crude lipopeptide was added to each well. The plate was incubated at 28˚C for the antagonistic test. An aliquot of HPLC grade methanol was analyzed as the control. The experiment was repeated three times.

2.3. Identification of the Isolate (F-6) by the 16S rDNA Sequence

The isolated strain (F-6), which produced the largest hemolysis circle in the *in-vitro* tests, was identified based on its 16S rRNA gene sequence. The F-6 strain was cultured in 10 ml of nutrient broth medium at 28˚C for 24 h. Cell suspensions were centrifuged at 3000 ×*g* at 4˚C for 5 min. Genomic DNA was extracted, and the 16S ribosomal RNA gene was amplified [24] using the B27 F (5'-AGA GTT TGA TCC TGG CTC AG-3') and U1492 R (5'-GGT TAC CTT GTT ACG ACT T-3') primers. The PCR product was purified and sequenced. A comparison of nucleotide sequences was performed against the BLAST database (http://blast.ncbi.nlm.nih.gov) of the National Center for Biotechnology Information.

2.4. Construction of GFP-Tagged F-6

The green fluorescent protein (gfp) plasmid HapII (GenBank accession number HM151400) that was used to tag F-6 was kindly provided by Dr. Zhenhua Zhang of Life Sciences College of Nanjing Agricultural University, China. The plasmid was introduced into F-6 by electroporation as described by Turgeon *et al*. [25]. In brief, a cell suspension was washed three times with a cold electroporation buffer that contained 272 mM sucrose, 1 mM $MgCl_2$, and 7 mM KH_2PO_4 (pH 7.4) and was then concentrated to 1 ml. The HapII plasmid was added to this mixture, transferred to an electroporation cuvette, and electroporated (2.4 kV, 200 Ω, 25 μF). After electroporation, cell suspensions were immediately added to 0.8 ml of Luria-Bertani (LB) medium and were shaken at 120 rpm at 37˚C for 3 h. Aliquots were spread onto LB agar plates supplemented with kanamycin (30 $\mu g \cdot ml^{-1}$). Fluorescence was visualized by epifluorescence stereo microscopy (Eclipse 80i; Nikon Corporation, Japan). Gfp-tagged F-6 (F-6-gfp) was stored at −80˚C before use.

2.5. Preparation of Bio-Organic Fertilizer

The organic fertilizer that was applied in the present study has been described previously [15] [26] [27]. It is composed of a mixture of an amino-acid fertilizer and pig manure compost (1:1, w/w). The amino acid fertilizer was made from rapeseed meal by performing solid-state fermentation for seven days, with added proteinase-producing bacteria. The pig manure compost was purchased commercially from Jiangsu Tianniang Ltd., China, and contained 30.4% organic matter, 2.0% total N, 1.6% P, and 0.9% K.

F-6-gfp was cultured in nutrient broth (supplemented with 30$\mu g \cdot ml^{-1}$ kanamycin) for 24 h at 30˚C and 170 rpm on a rotary shaker. Cultures were centrifuged at 8000 ×*g* at 4˚C for 10 min, and the cell pellets were resuspended in sterile, distilled water (SDW). The suspension was sprayed onto the organic fertilizer at a concentration of 200 ml suspension per 1 kg organic fertilizer. These mixtures were fermented at 30˚C - 45˚C and 40% relative humidity for five days to increase bacterial numbers and remixed every day to maintain the temperature below 50˚C. After incubation, the number of F-6-gfp was measured by the plate-count method using a *Bacillus*-selective medium [28]. The final cell density was 10^9 cfu of F-6-gfp per gram of fertilizer. The product this process produced is termed bio-organic fertilizer (BIO).

2.6. Pot Experimental Design

A sandy soil was collected from vanilla fields under 21 years of continuous vanilla cropping. Vanilla growth had been almost entirely decimated in this field [3]. The field was located in the Spice and Beverage Research Institute of the Chinese Academy of Tropical Agricultural Sciences, Xinglong, Hainan Province (110˚20'E, 18˚73'N); the soil contained 35.9 $g \cdot kg^{-1}$ of organic matter, 82.52 $mg \cdot kg^{-1}$ of available N, 336.36 $mg \cdot kg^{-1}$ of available P (Olsen), and 98.85 $mg \cdot kg^{-1}$ of available K (NH_4OAc). The *F. oxysporum* population was 1.7×10^5 cfu g^{-1} soil. The soil was passed through a 6-mm sieve before the addition to pots.

The uniform vanilla seedlings, each containing 5 nodes, were obtained from the nursery seedling plantation. The selected seedlings were left in shady location to lose water for 3 days before transplantation.

Each pot (590 × 405 × 175 mm in length × width × height) contained 12 kg of soil. The pot experiment was designed with four treatments: 1) CK (control, the pot soil amended with nothing); 2) S+BIO (the pot soil amended with bio-organic fertilizer); 3) S+M (the pot soil amended with organic fertilizer); 4) S+F6 (the pot soil

amended with F-6-gfp). Three independent replicate sets of pot experiments, each containing the same four treatments, were included and treated as blocks. Each treatment was replicated 15 times (15 pots). Each pot contained one plant.

Three plants per treatment were sampled each at 30, 90, and 150 days after transplantation. The sampled plants were carefully shaken by hand to remove bulk soil. The soil tightly adhering to the roots was considered rhizosphere soil [28]. Soil samples were stored at 4˚C until analysis of F-6-gfp and *F. oxysporum* populations. The remaining six plants per treatment were used to assess the disease severity index and the plant dry weight at 150 days after transplantation. The disease severity index was determined by following the method of Liu *et al.* [15]. The *F. oxysporum* and F-6-gfp populations were measured by the standard dilution plate-count method [29] [30]. Plates were incubated at 28˚C for 4 - 6 days for *F. oxysporum* or 24˚C for 3 days for F-6-gfp.

2.7. Data Analysis

Three independent experiments were carried out to check the reproducibility of results. The data were statistically analyzed using the statistical program SPSS for Windows, version 19 (SPSS, Inc., Chicago, IL, USA). Data were subjected to multiple comparisons under one-way ANOVA program by choosing 0.05 as the threshold significance level for Duncan's test. The rhizosphere colonization data were converted to log10 values before the statistical analysis.

3. Results

3.1. *In-Vitro* Assay of Lipopeptide-Producing Bacteria

Twelve effective strains were isolated that exhibited hemolysis on a sheep blood-plate. One potential lipopeptide-producing strain, F-6, was selected for its consistent production of a wide hemolysis zone on blood-plates (**Figure 1**). The cell-free filtrate of the F-6 culture suppressed FOV growth and produced a clear inhibition zone in the in vitro test (**Figure 2**).

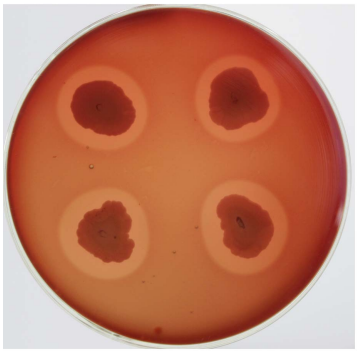

Figure 1. Hemolysis phenomenon exhibited by *B. cereus* F-6 on a sheep blood-plate.

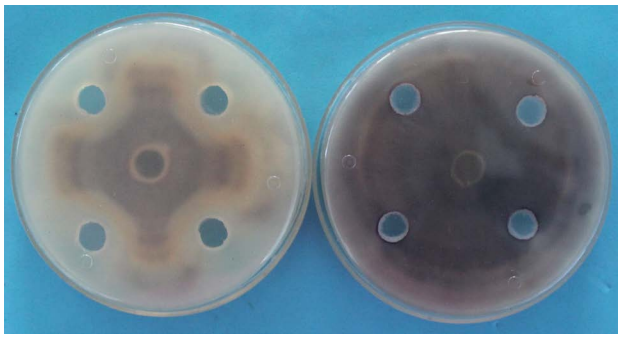

Figure 2. The antagonistic activity of *B. cereus* F-6 culture filtrate was detected in an *in-vitro* assay. Left, aliquots of 30 µl of culture filtrate were added to wells. Right, control, aliquots of 30 µl of Landy medium were added to wells.

3.2. Identification of Strain F-6

The 16S rRNA gene sequences of F-6 produced a 100% similarity to *Bacillus cereus* strain S000628319 in the BLAST search against all of the nucleotide sequences in the NCBI database (**Figure 3**). The F-6 16S rRNA gene sequence was deposited into GenBank under accession number KF137573. The F-6 strain was deposited at the China General Microbiological Culture Collection Center under accession no. 7979.

3.3. *B. cereus* F-6-gfp

B. cereus F-6 was tagged with gfp to facilitate the assay of colonization ability. The HapII plasmid was successfully transformed into *B. cereus* F-6 using electroporation. Green-colored colonies and the emission of green fluorescence were visualized under UV light. The gfp-tagged *B. cereus* F-6 strain was named *B. cereus* F-6-gfp. Green fluorescence could still be visualized under UV light after multiple sub-culturing of *B. cereus* F-6-gfp for thirty days, confirming that the vector was stable in terms of expressing gfp genes for the length of the present experiment.

3.4. Effects of *B. cereus* F-6-gfp on Vanilla Plant Growth and Disease Control

The application of the bio-organic fertilizer to the soil (S+BIO) promoted the highest biomass production among all of the treatments, while the control treatment produced the lowest yield (**Figure 4(a)**). The dry weights of the vanilla roots, stems and leaves in the S+BIO treatment were 52.4%, 55.8% and 89% greater, respectively, compared to the non-treated control. While the dry weights of the plant roots, shoots and leaves in the S+F6 treatment were also greater than those of the control, the differences were not significant. As for the plant roots and shoots, there were no significant differences among the S+M, S+F6 and non-treated control treatments. This result suggests that applications of the bio-organic fertilizer could significantly promote vanilla plant growth. In addition, the application of F-6-gfp or organic fertilizer individually only produced minor growth promotion effects.

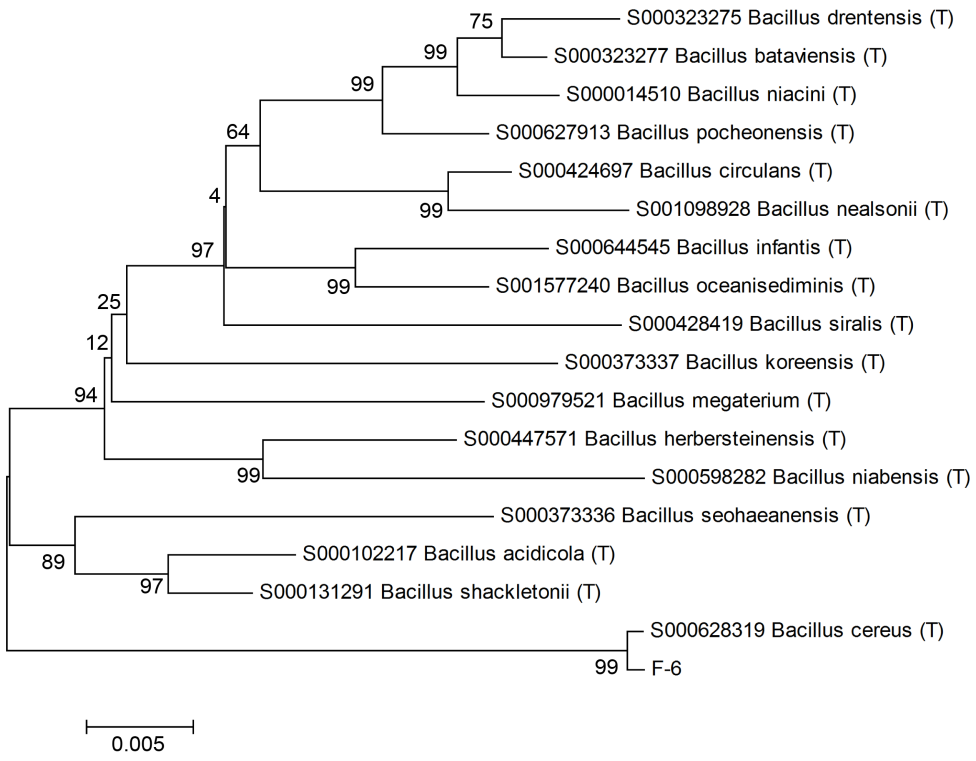

Figure 3. Phylogenetic tree based on the 16S rDNA sequences of *Bacillus cereus* strain F-6 and related bacteria, produced using the neighbor-joining method.

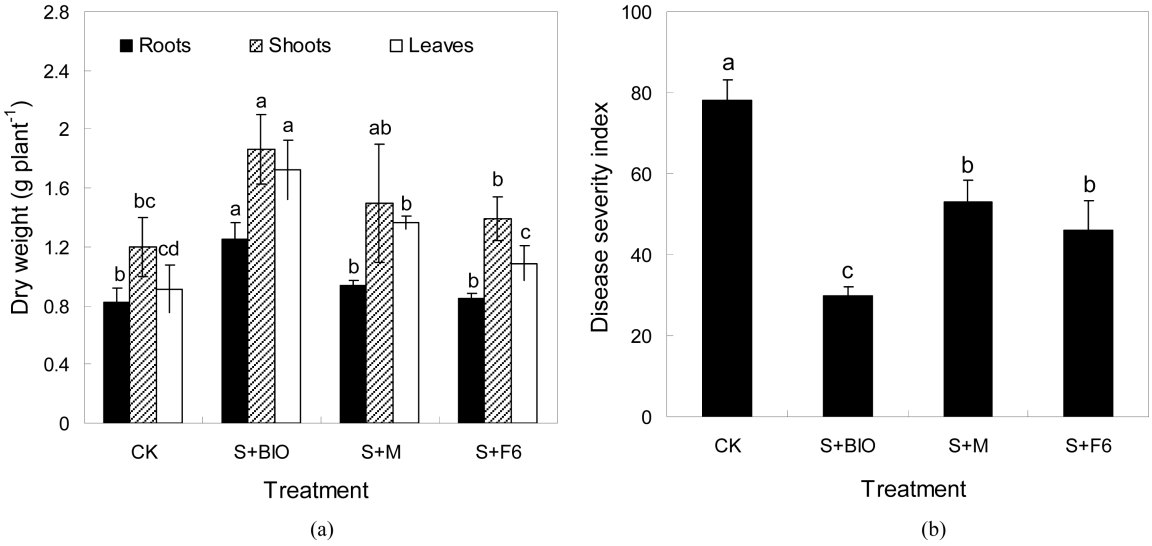

Figure 4. Effects of *B. cereus* F-6-gfp application on (a) plant dry weight and (b) disease severity index. CK: control, the pot soil amended with nothing; S+BIO: the pot soil amended with bio-organic fertilizer; S+M: the pot soil amended with organic fertilizer; S+F6: the pot soil amended with F-6-gfp. Vertical boxes ± bars are the mean ± standard deviation of three independent experiments with six replicates. Bars with different letters differ significantly according to the Duncan test ($P \leq 0.05$).

There are significant differences among treatments for the disease severity index (**Figure 4(b)**). The disease severity index of the BIO treatment was 30, which was significantly lower than all of the other treatments. Significant differences in disease severity were not observed between the S+F6 and S+M treatments, but these treatments were also significantly lower than the control.

3.5. Population Dynamics of *F. oxysporum* in Rhizosphere Soil

The rhizosphere soil *F. oxysporum* numbers exhibited an increasing temporal trend for all of the treatments, but these increases differed in magnitude (**Figure 5**). In the S+BIO treatment, the *Fusarium* number enumerated 150 days after transplantation was 1.0 log cfu g^{-1} soil greater than at 30 days after transplantation. In contrast, the corresponding values for the other treatments were 1.3 - 1.5 log cfu g^{-1} soil greater than at 30 days after transplantation. The *Fusarium* population in S+BIO treatment was 0.6 - 1.3 log cfu g^{-1} soil lower than those of the S+M, S+F6 and control treatments at 150 days after transplantation.

3.6. Population Dynamics of *B. cereus* F-6-gfp in Rhizosphere Soil

B. cereus F-6-gfp was not detected in the control and S+M treatments, but was detected in the S+BIO and S+F6 treatments (**Figure 6**). The number of F-6-gfp tended to decrease over time in the rhizosphere soil in the latter two treatments; however, the rate of decrease differed. The F-6-gfp number enumerated in the S+BIO treatment 150 days after transplantation was approximately 2.7 log cfu g^{-1} soil lower than at 30 days after transplantation. However, the corresponding value was approximately 3.1 log cfu g^{-1} soil lower for the S+F6 treatment. In addition, the population of F-6-gfp in the rhizosphere soil of the S+BIO treatment was significantly greater than that of the S+F6 treatment at 150 days after transplantation.

4. Discussion

In our study, the lipopeptide-producing bacterium *B. cereus* F-6, when fortified with organic fertilizer (BIO), significantly promoted vanilla plant growth and suppressed stem and root rot disease. We have, for the first time, developed a bio-organic fertilizer and investigated its effects on vanilla plant growth and disease control under the continuous cropping of soil with vanilla. The results have provided a potential method for alleviating barriers to the continuous cropping of vanilla and other tropical perennial crops.

Bacillus lipopeptides have mainly been studied for their antagonistic activity against a wide range of phytopathogens [13]. For example, *B. cereus* and *B. subtilis* have been extensively used for controlling *Fusarium*

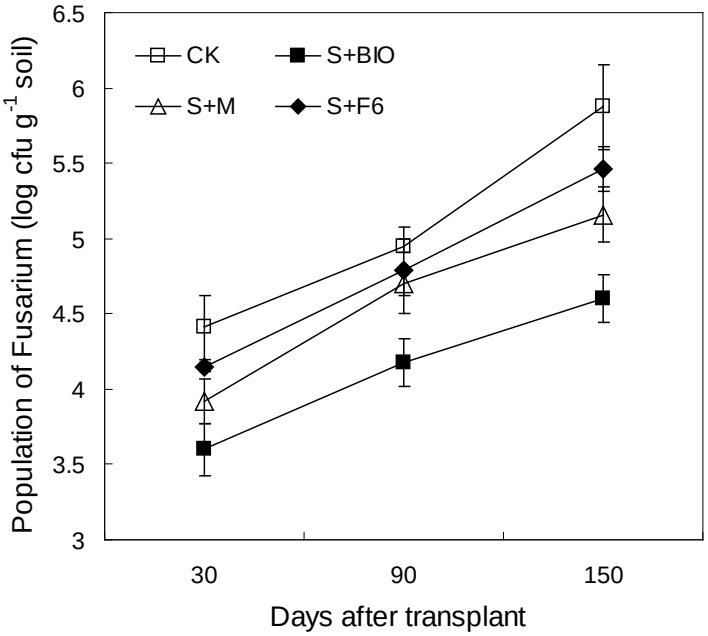

Figure 5. Effect of *B. cereus* F-6-gfp application on *Fusarium oxysporum* population dynamics in rhizosphere soil. CK: control, the pot soil amended with nothing; S+BIO: the pot soil amended with bio-organic fertilizer; S+M: the pot soil amended with organic fertilizer; S+F6: the pot soil amended with F-6-gfp. Data were $\log_{10}$-transformed before being analyzed by Duncan's test. Bars represent the standard deviations of three replicates.

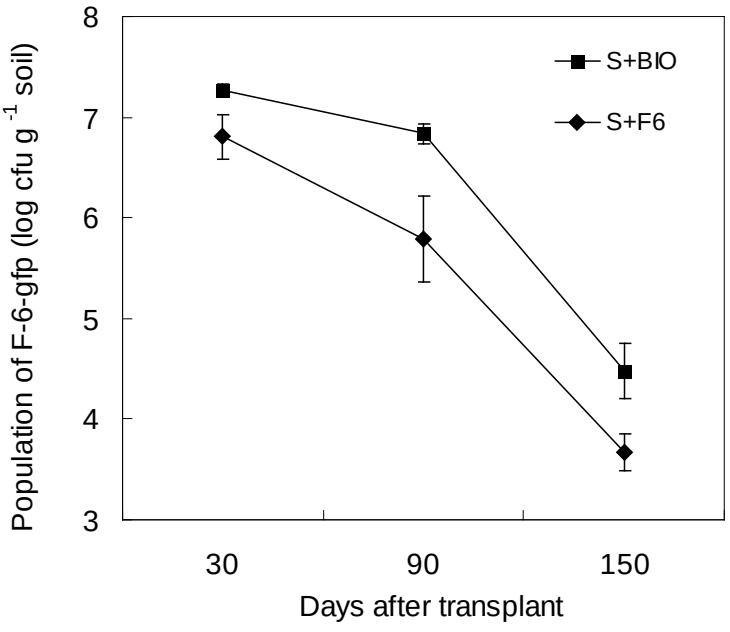

Figure 6. *B. cereus* F-6-gfp population dynamics in rhizosphere soil. S+BIO: the pot soil amended with bio-organic fertilizer; S+F6: the pot soil amended with F-6-gfp. Data were $\log_{10}$-transformed before being analyzed by Duncan's test. Bars represent the standard deviations of three replicates.

disease [26] [31]. In the present study, a novel lipopeptide-producing strain designated F-6 has been isolated from vanilla rhizosphere soil and identified as *Bacillus cereus*, and antifungal compounds that exhibit antagonistic activities against FOV in an agar-diffusion assay (**Figure 2**) have been successfully extracted from the F-6 culture filtrate by HCl precipitation. Lipopeptide production has been previously noted to play an important role in the successful suppression of phytopathogens [12] [32]. Chan *et al*. [33] have reported that the antifungal activity of the *B. subtilis* D1/2 against *Fusarium graminearum* in maize and wheat is attributable to lipopeptides of fengycins. Cao *et al*. [8] have demonstrated that the protection of cucumber plants from attack by *Fusarium* imparted by *B. subtilis* SQR 9 is mainly attributed to the antibiotic production of bacillomycin and fengycin. Various groups of lipopeptides can confer an advantage to lipopeptide-producing *Bacillus* in specific ecological niches [34].

Various bacteria, for example, *Bacillus*, have been widely employed for biocontrol and for plant growth promotion for numerous crops [35]. Ding *et al*. [36] have reported that *B. amyloliquefaciens* LH23-fortified or *B. subtilis* LH36-fortified bio-organic fertilizer can significantly reduce the incidence of potato bacterial wilt disease, mainly through the alternation of soil microbial community. Tan *et al*. [16] have demonstrated that inoculations of *B. amyloliquefaciens* CM-2 and T-5 in both seedlings and soils can effectively control tomato bacterial wilt and promote plant growth. Our results have demonstrated that applications of organic fertilizer, F-6 or bio-organic fertilizer reduce the disease severity index and enhance the plant growth for vanilla. Moreover, a sound ability for plant growth promotion and the efficacy for disease control have been demonstrated for the bio-organic fertilizer treatment (**Figure 4**). Zhang *et al*. [37] reported that both *Trichoderma harzianum* T-E5-fortified and wild-type SQR-T037-fortified bio-organic fertilizers can promote cucumber growth because of their capacities for indole acetic acid production and plant colonization. The plant growth promotion hormones and lipopeptides produced by F-6 are currently being analyzed in our laboratory to understand the growth promotion and biocontrol mechanisms.

The ability of special strains to colonize the rhizosphere is a prerequisite to achieving beneficial effects [14]. Green fluorescent protein technology has been developed in combination with the dilution plate-counting method to detect and quantify specific microorganisms [38]. *B. cereus* F-6-gfp was not detected in either the control or organic fertilizer treatments, confirming the reliability of the combined method employed in this study. The number of F-6-gfp in the rhizosphere soil of the S+BIO treatment remained higher than that in the S+F6 treatment (**Figure 6**), suggesting that the organic fertilizer supplied a source of carbon and nutrients that facilitated the survival of and colonization by the microbes [39]. The strong colonization ability of F-6-gfp in rhizosphere soil may play an important role in plant growth promotion and disease control for vanilla. We speculate that F-6-gfp can form a biofilm, creating a hostile environment for the growth of pathogenic *Fusarium*. Cao *et al*. [32] reported that *B. subtilis* SQR 9 could promote plant growth and control *Fusarium* wilt in cucumber by colonizing the plant roots. Ren *et al*. [27] have suggested that the colonization of tobacco roots by *Paenibacillus polymyxa* C5 provides a mechanism to support the protection of tobacco plants from infection by pathogens.

The number of *F. oxysporum* in the rhizosphere soil remained significantly lower in the bio-organic fertilizer treatment compared to other treatments for the duration of the experiment (**Figure 5**). This result indicates that the BIO application inhibits the growth of *F. oxysporum*, which supports the report by Liu *et al*. [15] that applications of bio-organic fertilizer fortified with *Brevibacillus brevis* L-25 and *Streptomyces rochei* L-9 significantly decrease the *Ralstonia solanacearum* population in tobacco rhizosphere soil. Zhang *et al*. [40] documented that the application of *B. subtilis* SQR-5-fortified bio-organic fertilizer at the cucumber seedling stage significantly reduced the *F. oxysporum* population in the rhizosphere soil.

This is the first report to investigate the biocontrol of vanilla stem and root rot disease and the promotion of plant growth through the application of a novel *B. cereus*-fortified bio-organic fertilizer. The results indicate that the application of an organic fertilizer fortified with beneficial microbes could provide a new approach to alleviate problems associated with successive obstacles to the continuous cropping of vanilla and to reduce chemical fertilizer applications. The strong ability of *B. cereus* F-6 to colonize the rhizosphere soil may be one of the mechanisms related to the provision of beneficial effects. Further study should focus on the isolation and identification of the plant growth promotion and antagonistic substances produced by F-6 and the soil-microbe-plant interactions to understand the mechanisms underlying these beneficial effects.

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