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Contributed Paper

Characterization and Evaluated Storage Viability of Strain, *Bacillus velezensis* PGA106 for Growth Promotion and Biocontrol of Fusarium Wilt in Tomato

Sirirat Siripornvisal [a,b], Thanasak Lomthong [c], Nantana Srisuk [a], Shinji Tokuyama [d] and Vichien Kitpreechavanich*[a,e]

[a] Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[b] Faculty of Science and Technology, Phranakhon Si Ayutthaya Rajabhat University, Phranakhon Si Ayutthaya 13000, Thailand.
[c] Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathumthani 12110, Thailand.
[d] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka, Japan.
[e] Center for advanced study in Tropical Natural Resources, National Research University- Kasetsart University (CASTNAR, NRU-KU), Kasetsart University, Bangkok 10900, Thailand.

*Author for correspondence; e-mail: fsciwck@ku.ac.th

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ABSTRACT

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fox*), is a serious disease affecting tomato cultivation worldwide. Recently, biological control using antagonistic microorganisms has widely been claimed as a sustainable way of controlling Fusarium wilt in crops. In order to discover an efficient *Bacillus* stain for biocontrol of Fusarium wilt disease and for improvment of tomato growth, 3 *Bacillus* strains designated as BMG4, KMS17 and PGA106, were demonstated for tomato plant growth promotion (PGP) and biocontrol activity against Fusarium wilt disease in pot condition for 102 days plantation. Among them, strain PGA106 showed the highest significantly increased tomato plant growth, yielding 22.9% increased shoot fresh weight, 53.9% increased shoot dry weight and 46.15% increased fruit yield compared to the control. In *Fox* infested condition, PGA106 treatment significantly lead to 66.7% reduction of wilt incidence while causing 21.2% increased shoot fresh weight, 42.8% incresed shoot dry weight and 194.6% increasd fruit yield as compared to the *Fox* infested control. Strain PGA106, identified as *Bacillus velezensi*s by the analysis of 16S rDNA sequences, showed several growth promoting traits as the ability on mineral phosphate solubilization and the production of IAA, siderophores, PGP volatiles, biofilm and γ-PGA. In addition, the properties that actively contribute antagonistic interaction with *FOX* by PGA106 were confirmed by synthesis of several fungal cell wall hydrolytic enzymes and lipopeptides that inhibited the germination and degradation of *Fox* conidia. Lipopeptide synthesis was indicated by the existence of lipopeptide genes on the genome. A talc-based bioformulation of PG106 was prepared to make it convenient to be use and extent storage time. Storage viability of the bioformulation at temperature between 4-55°C found that the specific rate of degradation increased linearly with the temperature up to 35°C which followed the Arrhenius equation.

Keywords: *Bacillus velezensis*, plant growth promotion, tomato, *Fusarium* wilt, storage viability

1. INTRODUCTION

Tomato (*Lycopersicon esculentum* L.) is an important horticultural crop grown in various regions of Thailand. Based on data from FAOSTAT in 2018, total tomato production in the country was estimated at 109,253 tons [1]. Although there is high yield, productivity of tomato cultivation in Thailand is below its economic potential due to various disturbing factors including physiological disorders and incidence of pests and diseases. Tomatoes are parasitized by a number of pathogens including *Fusarium oxysporum* f. sp. *lycopersici*, a soil-borne fungus causing *Fusarium* wilt disease [2]. Traditionally, *Fusarium* wilt can be controlled by a combined methods, including use of resistant cultivars and disinfecting fungal propagules in soil prior to planting that synthetic fungicides were simultaneously applied. However, the use of broad-spectrum fungicides (particularly methyl bromide) is environmentally damaging and creating imbalances the microbial community which lead to the development of resistant pathogen strains. As such, difficulties in controlling *Fusarium* wilt have stimulated tremendous efforts to develop alternative approaches for sustainable control of the disease.

Biological control of plant pathogens using microbial antagonists is now widely accepted as a safer and more sustainable alternative to the chemical approach [3]. Throughout the past decades, various groups of microorganisms including bacteria and filamentous fungi have been evaluated for their antagonistic activities against wilt pathogens and numerous effective antagonists were reported. Among them, aerobic spore-forming bacteria of *Bacillus* and related genera were found highly effective against *F. oxysporum* and used as biocontrol agents [4,5]. The most preferable antagonistic characteristic of *Bacillus* is the ability to produce diverse array of antimicrobial compounds which influence its biocontrol capability [6]. These compounds include predominant peptides, either those ribosomal origin or non-ribosomal origin [7]. In addition to these biocontrol properties, numerous strains of *Bacillus* or related genera are known to be able to promote plant growth through phytohormone production and soil mineral solubilization [8]. These features suggest that the bacteria are suitable for protection of food and commodity crops. Especially in the last few years, some reports have mentioned the fertilizer synergist effects of poly-γ-glutamic acid (γ-PGA) produced by *Bacillus* strains. Such synergism improved soil nutrient availability by adsorption and transfer of cationic metal fertilizers to plants, leading to the increased efficiency in fertilizer utilization of plants and hence reduced overall fertilizer cost [9]. Also, in some *Bacillus* strains, γ-PGA is essential for chemotactic motility, the formation of biofilm and colonization of plant root [10]. Therefore, the ability to produce γ-PGA could support the bacterial capability in colonizing plant roots and overcoming invasion of the pathogen. These features lead to the belief that the bacterium is suitable for use in the protection of food and commodity crops.

Out of the numerous strains of *Bacillus* spp. described as potential biological control agents, only a few have been fully evaluated for their effectiveness at commercial scale, and especially for their ability to sustain and enhance overall crop yields [6]. Here, highly efficient *Bacillus* strain for *Fusarium* wilt biocontrol and plant growth promotion was investigated and characterized. A bioformulation of the strain was prepared for the farmer convenience. The viability of the selected *Bacillus* strain in the bioformulation at different storage temperatures was also determined in order to obtain data for suitable storage time prediction at desired temperature.

2. MATERIALS AND METHODS

2.1 Microorganisms and Cultivation Conditions

2.1.1 Bacteria

Three plant growth promoting *Bacillus* strains namely BMG4, KMS17 and PGA106, were used and kept at the Microbiology Program, Phranakhon

Si Ayutthaya Rajabhat University, Phranakhon Si Ayutthaya, Thailand. The bacteria had previously isolated from agricultural soil of Karnchanaburi and Nakhon Pathom provinces. According to our preliminary evaluations, the 3 strains were found to exhibit high antagonistic activity against *F. oxysporum* and high ability to promote tomato seedling growth [11]. For regular use, they were refreshed on glucose yeast extract peptone (GYP) agar (10 g/L Peptone, 5g/L Yeast extract, 20 g/L Dextrose and 15g/L Agar) slants and incubated at 30°C for 24 h. Bacterial suspensions were prepared by transferring fresh cultures to GYP broth (10 g/L Peptone, 5g/L Yeast extract and 20 g/L Dextrose) and incubating with constant shaking at 150 rpm, 30°C for 48 h. Bacterial cells were harvested by centrifugation at 10,000 ×g for 15 min, washed 2 times with sterile distilled water and resuspended in sterile normal saline solution to the required density.

2.1.2 Fungal pathogen

Fusarium oxysporum (*Fox*), a representative of *Fusarium* in nature, was isolated from wilted tomato plant collected from a tomato farm in Nakhon Pathom province [12]. The fungus was maintained on potato dextrose agar (PDA; 200 g/L Potatoes infusion, 20.0 g/L Dextrose and 20.0/L Agar) at 4°C and kept at the Microbiology Program, Phranakhon Si Ayutthaya Rajabhat University, Phranakhon Si Ayutthaya, Thailand. Conidia suspension was prepared according to Wu *et al.* [13] by transferring active growing mycelia into Bilay and Joffe's medium (1.0 g/L KH_2PO_4, 1.0 g/L KNO_3, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.2 g/L Soluble starch, 0.2 g/L Glucose, 0.2 g/L Sucrose and 4.0g/L Carboxymethylcellulose sodium salt) with incubation on a rotary shaker at 100 rpm for 7d. The culture suspension was filtered through sterile Whatman lens-cleaning tissue to eliminate mycelium. Conidia were counted using a haemocytometer, and the concentration was adjusted to 10^5 conidia/mL.

2.2 Evalution of the Efficiency of the Selected *Bacillus* strains on Growth Promoting and FOX Wilt Biocontrol of Tomato in Pot Experiment

Effectiveness of the bacteria on biocontrol of *Fusarium* wilt and promotion of tomato plant growth was evaluated by a pot experiment under net house condition. Eight treatments were established: (1) the control treatment without bacteria and *Fox* inoculation, (2) the treatment with BMG4 inoculation, (3) the treatment with KMS17 inoculation, (4) the treatment with PGA106 inoculation, (5) the treatment with *Fox* inoculation, (6) the treatment with BMG4 and *Fox* inoculation (7) the treatment with KMS17 and *Fox* inoculation (8) the treatment with PGA106 and *Fox* inoculation.

Tomato seeds of cultivar Tho (Chia Tai Co., Ltd., Thailand) were sown in seedling trays containing inoculated or uninoculated substrates. The inoculated substrate was peat moss mixed with the suspension (10^7 CFU/mL) of each strain at 1:1 ratio (v/v), whereas the uninoculated substrates was peat moss moisten with sterile water in the same proportions. The trays were maintained in a growth chamber under a 16:8 day-night regime at 25°C and 90% relative humidity (RH), for 20 d. For plant growth promotion treatments (treatment 1-4), the seedlings were planted into 20 cm pots each contained 100 g peatmoss. For *Fox* challenge treatments (treatment 5-8), the seedlings with roots were dipped in *Fox* spore suspension (10^5 spores/mL) for 8 h, and then were planted into 20 cm pots each contained 100 g peatmoss. All treatments were arranged in the net house with daily watering, 10 pots per block and 3 blocks per treatment. Ripening tomato fruits were harvested timely from each treatment up to 102 days after planting. Tomato fruit yields were recorded in terms of fruit weight. Plant growth parameters viz. shoot height, shoot fresh weight and dry weight, root fresh weight and root dry weight of each treatment were measured. Disease incidence and disease severity were also determined according to Eq. (1) and (2),

respectively. Disease incidence was the percentage of plants that visually displayed wilt symptoms. Disease severity was rated on a scale of 0 to 5, adapted from Moreno-Velandia *et al.* [14], where 0 is symptomless (normal growth), 1 is no browning but decreased growth, 2 is slight brown discoloration of the upper root system, 3 is moderate brown discoloration of two-thirds or less of the upper root system, 4 is extreme brown discoloration of the upper root system, and 5 is complete or almost complete plant death. Variations in plant growth and disease control parameters were determined by analysis of variance using SPSS 23 trial version (SPSS Inc., Chicago, IL, USA). Means were separated by Duncan's multiple range test for plant growth parameters and Tukey's test for disease control with a 95% confidence limit.

$$\text{Disease incidence (\%)} = \frac{\text{number of wilted plants}}{\text{Total number of plants}} \times 100 \quad (1)$$

$$\text{Disease severity} = \frac{\sum \text{Disease grade} \times \text{Number of plants in each grade}}{\text{Total number of plants}} \quad (2)$$

2.3 Identification of Bacterial Strain

The most efficient strain was identified by the analysis of its 16S rDNA sequence. Genomic DNA was extracted using DNA extraction kit as per the manufacturer's protocol (Qiagen Inc., Valencia, USA). Amplification of 16s rDNA was carried out using two specific primers, 27F and 1492R (Supplemental Table S1). The PCR product was purified using QIAGEN PCR purification kit (Qiagen Inc., Valencia, USA) and sent to Macrogen Sequencing Service (Macrogen Inc., Seoul, Korea). The 16S rDNA sequence obtained was assembled and manually corrected using the BioEdit program version 7.0. Primary comparison of the obtained sequence with others in GenBank was performed using NCBI BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). Sequences were aligned with the corresponding sequence of the type strain of the closest species using the CLUSTAL_W program incorporated in MEGA-X software package. A neighbor-joining phylogenetic tree was constructed using MEGA-X software. Stability of the phylogenetic tree was assessed by bootstrap analyses based on 1,000 resamplings.

2.4 Characterization for Plant Growth Promoting Traits and Modes of Biocontrol Action of the Most Efficient Strain

2.4.1 Characterization of plant growth promoting traits

Phosphate solubilization was assessed by plate assay on Pikovskaya's agar (10.0 g/L Glucose, 5.0 g/L $Ca_3(PO_4)_2$, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L Yeast extract, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.2 g/L KCl, $MnSO_4$ (traces), $FeSO_4$ (traces) and 20.0 g/L Agar) as described by Zaidi *et al.* [15]. Inoculated plates were incubated at 30°C for 7d. The presence of transparent halo around the bacterium colony indicated phosphate solubilizing activity.

Indole acetic acid (IAA) production was assessed according to Prashanth and Mathivanan [16]. The bacterium was inoculated into GYP broth (10 g/L Peptone, 5g/L Yeast extract and 20 g/L Dextrose) supplemented with L-tryptophan (500 µg/mL), and then incubated at 30°C for 48 h with shaking. Cultured broth was collected at 10,000 × g centrifugation for 5 min. A 1 mL aliquot of supernatant was mixed with 2 mL of Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$) and allowed to react at 28°C for 20 min. The development of a pink color in the mixture indicated the presence of IAA.

Production of siderophores was detected by plate assay as described by Schwyn and Neilands [17]. The bacterium was spot-inoculated on chrome azurol sulfonate (CAS) agar (60.5 mg/ 50 mL d.w. Chrome Azurol Sulfonate, 72.9 mg/ 40mL d.w. Hexadecyltrimethyl ammonium bromide, 20.0 g/L Peptone, 1.5 g/L K_2HPO_4 1.5 g/L $MgSO_4 \cdot 7H_2O$, 20.0 g/L Agar) and incubated at 30°C for 7d. A yellowish halo emerged around the bacterial colony indicated the presence of siderophores.

Nitrogen fixing activity was detected according to Kumar and Narula [18], by streaking the bacterium strain on Jensen's nitrogen free medium (20.0 g/L Sucrose, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.5 g/L NaCl, 0.1 g/L $FeSO_4$, 0.005 g/L Na_2MoO_4, 2.0g/L $CaCO_3$ and 15.0g/L Agar) with incubation at 30°C for 7d. Nitrogen fixing capacity was indicated by the growth of the bacterium on the medium.

Plant growth promoting volatiles (PGP volatiles) production was assessed in gnotobiotic bottles as described by Castulo-Rubio *et al.* [19]. Tomato seeds were sown on Murashige and Skoog medium (1650 mg/L NH_4NO_3, 1900 mg/L KNO_3, 370 mg/L $MgSO_4$, 170 mg/L KH_2PO_4, 440 mg/L $CaCl_2 \cdot 2H_2O$, 0.83 mg/L KI, 6.20 mg/L H_3BO_3, 22.30 mg/L $MnSO_4 \cdot 4H_2O$, 8.60 mg/L $ZnSO_4 \cdot 7H_2O$, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 37.3 mg/L $Na_2EDTA \cdot 2H_2O$, 27.8 mg/L $FeSO_4 \cdot 7H_2O$, 0.5 mg/L Nicotinic acid, 0.5 mg/L Pyridoxin HCl, 0.1mg/L Thimine HCl, 2.0 mg/L Glycine, 100.0 mg/L Myo-Inositol and 9.0 g/L) in the gnotobiotic bottle. A vial containing 5 mL of the bacterium suspension (10^9 CFU/mL) in LB broth (10 g/L Tryptone, 10 g/L NaCl and 5 g/L Yeast extract) was placed inside the bottle to avoid physical contact between tomato seedling and the bacterium. For the control treatment vial containing sterile LB broth was used instead of bacterial suspension. The bottles were kept in a growth chamber at 25°C and 16:8 day-night regime. Three weeks after germination, tomato seedlings were harvested and measured for growth in terms of average dry biomass. The bacterium was assumed to produce PGP volatiles only when the seedlings of the bacterial treatment significantly grew higher than of the control.

Biofilm formation was determined using a microtiter plate method as described by Hamon and Lazazzera [20]. The bacterium was cultured in a 24-well polystyrene microtiter plate filled with Luria–Bertani (LB) medium plus 0.15 M ammonium sulphate, 100 mM potassium phosphate, 34 mM sodium citrate, 1 mM MgSO4 and 0.1% glucose. The plates were statically incubated at 37°C for 48 h and the presence of biofilm pellicle was observed directly.

γ-PGA production was assessed according to Zeng *et al.* [21] with slight modification. The bacterium was spread onto a minimal medium containing L-glutamic acid (30 g/L Glucose, 2.5 g/L Yeast extract, 20 g/L L-glutamate, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$) and incubated at 37°C for 24 h. The development of mucoid coloies indicating the ability of the bacterium to produce γ-PGA.

2.4.2 Determination of modes of biocontrol action

An assay method adapted from that of Ko *et al.* [22] was used to investigate the bacterium synthesis of extracellular hydrolytic enzymes during antagonistic interaction with the fungal pathogen. Firstly, dual-culture plates of the most efficient strain and *Fox* on PDA were prepared and incubated at 30°C until the inhibitory zone appeared clearly. Agar piece was aseptically cut from the inhibition zone in between the bacterial and fungal colonies, then placed separately on barley glucans agar (BGA; 3.0 g/L Beef extract, 5.0 g/L Peptone, 5.0 g/L NaCl, Barley glucan 5 g/L and 20.0 g/L Agar), carboxymethylcellulose agar (CMCA ; Yeast extract 2.0 g/L, KH_2PO_4 1.0 g/L, $MgSO_4$ 5.0 g/L, Carboxymethylcellulose sodium salt 5.0 g/L Agar 15.0 g/L), colloidal chitin agar (CCA; 3.0 g/L Beef extract, 5.0 g/L Peptone, 5.0 g/L NaCl, 5 g/L, Colloidal chitin 10 g/L and 20.0 g/L Agar) or skimmed milk agar (SMA; 3.0 g/L Beef extract, 5.0 g/L Peptone, 5.0 g/L NaCl, 5 g/L, Skimmed milk 10 g/L and 20.0 g/L Agar) and incubated at room temperature for 24 h. For β-glucanase and cellulase, the clear zone could be visualized by flooding BGA and CMCA plates with 0.1% congo red for 15 min and washed with 1 M NaCl. Clear hydrolytic zone developed around agar pieces on the 4 media indicated the existence of β-glucanase, cellulase, chitinase, and

proteinase, respectively.

The capability of the most efficient strain to produce antifungal lipopeptides was determined based on the PCR amplification of four lipopeptide biosynthetic genes: *srfAA*, *fenD*, *bmyB* and *ituC*. Specific primers used are listed in supplementary table (Table S1). PCR amplification of each gene was conducted following the method of Saechow *et al.* [23]. PCR products were detected by 1% agarose gel electrophoresis.

The effect of cell-free supernatant of the most efficient strain on the germination *Fox* conidia was determined. The bacterium was cultivated in flask containing 50 mL GYP broth, incubated at 30°C and shaken at 150 rpm for 24 h. Cell-free supernatant was obtained by centrifugation at 10,000 × g for 10 min and filtered through 0.22 μm membrane filter. To a well of sterile microtiter plate, a solution of 100 μL the cell-free supernatant was added. The control well was filled with sterile GYP medium instead of the supernatant. A 100 μL aliquot of *Fox* conidia suspension (10^5 conidia/mL in PDB) was added to each well. The microtiter plate was incubated at 30°C for 48 h with continuous lighting. Conidial germination was observed by light microscopy at magnification of 400X for at least 200 conidia per each treatment. Each treatment was performed in triplicate.

2.5 Storage and Viability of the Selected Efficient *Bacillus* Strain

2.5.1 Bioformulation of the most efficient strain

To make it convenient for use and handling, a talc formulation of the most efficient strain was prepared according to the process described by Jorjani *et al.* [24], the most efficient strain was cultivated in the medium, for co-production of biomass and γ- PGA (35.5 g/L Glycerol, 2.5 g/L Yeast extract, 38.1 g/L L-glutamic acid, 0.1 g/L $K_2HPO_4 \cdot 3H_2O$, 0.5 g/L KH_2PO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$ and 500 mg/L L-tryptophan and pH 7.2, obtained from preliminary experiment) with shaking incubation at 150 rpm, 30°C for 60h. Thereafter, the bacterial cells with the culture medium was adjusted to 10^{11} CFU/mL with 59.86 g/L γ-PGA. A 400 mL aliquot of the bacterial suspension was added to the sterile mixture of talc (1,000 g), calcium carbonate (15 g) and carboxymethyl cellulose (10 g). The formulation was dried in an oven at 50°C to reduce moisture content to ≤ 20% (wet basis). Approximate 50 g portions of the dried bioformulation were transferred into polypropylene bags.

2.5.2 Survival of PGA106 in the bioformulation

The PGA106 bioformulation was stored at different temperatures (4, 25, 35 and 55°C). Viability of PGA106 in the formulation was checked at 0, 7, 14, 21, and 30d by standard plate count technique.

Stability of the bioformulation was determined and the slope of the regression of the log viability and time at each temperature from Eq. (3) was the specific rate of degradation per day (k), where N_0 is the initial viable number of PGA106, N is the viable number of PGA106 at the observed times, and t is the time in day.

The correlation between the temperature (Kelvin) and the k values can be described by Arrhenius Eq. (4), where R is the universal gas constant (8.32 j/mol·K), E_a is the apparent activation energy in kilojoules per mol, T is the absolute temperature in Kelvin, and k_0 is the pre-exponential constant [25].

$$\text{Log } N = \log N_0 - kt \quad (3)$$

$$k = k_0 e^{(-E_a/RT)} \quad (4)$$

3. Results and Discussion

3.1 Evalution of the Efficiency of the Selected *Bacillus* Strains on Growth Promoting and FOX Wilt Biocontrol of Tomato (in Pot Experiment)

In terms of growth promotion under non pathogen condition, only strain PGA106 significantly increased tomato plant growth. Almost growth

parameters (272.54 g shoot fresh weight, 37.15 g shoot dry weight, 68.87 g root fresh weight and 28.28 g root dry weight) were higher than those of the control (221.74 g shoot fresh weight, 24.14 g shoot dry weight, 70.70 g root fresh weight and 24.60 g root dry weight). By contrast, inoculation of BMG4 and KMS17 showed non significant effects of tomato plants in almost all the parameters observed ($p > 0.05$). However, inoculation of KMS17 and PGA106 significantly increased total fruit yield from 2.59 kg/block in the control to 3.15 and 3.76 kg/block, respectively (Table 1).

Under pathogen infested condition, the highest degree of promotion was found in treatment 8 followed by treatment 6 and 7, respectively (Table 1). Inoculation of PGA106 with *Fox* effectively prevented tomato plant from *Fox* infestation since almost all growth parameters (221.6 g shoot fresh weight, 25.9 g shoot dry weight, 66.9 g root fresh weight and 25.40 g root dry weight) were equivalent to treatment 1 (221.7 g shoot fresh weight, 24.1 g shoot dry weight, 70.7 g root fresh weight and 24.60 g root dry weight). Treatment 6, 7 and 8 significantly alleviated the degree of yield loss up to 2.69, 1.39 and 2.95 folds of the pathogen infested control (Table 1).

In terms of disease control, treatments of 6, 7 and 8 significantly decrease incidence and severity of wilt symptoms ($P<0.05$). Disease incidence estimated for the treatments of 5, 6, 7 and 8 were 40.0, 26.7, 16.7 and 13.3%, respectively and disease severity indexes for these treatments were

Table 1. Tomato plant growth promoting and biocontrol efficacies of the bacterial strians BMG4, KMS17 and PGA106 under non-FOX and FOX-infested conditions.

	Parameters	Treatment			
		1	2	3	4
PGP efficacy under non-FOX condition	Shoot height (cm)	225.70±65.28^{ab}	201.10±59.29^{a}	191.70±46.37^{a}	244.60±86.44^{b}
	Shoot FW(g)	221.74±31.31^{b}	218.48±20.97^{ab}	242.98±39.00^{c}	272.54±43.36^{c}
	Shoot DW(g)	24.14±4.04^{ab}	29.21±5.42^{b}	28.31±7.60^{b}	37.15±17.14^{c}
	Root FW (g)	70.70±11.36^{b}	63.96±15.55^{b}	73.86±7.06^{b}	68.58±24.79^{b}
	Root DW (g)	24.60±6.06^{b}	24.17±4.74^{b}	25.68±4.43^{b}	28.82±4.92^{b}
	Fruit yield (kg/block)	2.59±0.58^{b}	2.40±0.767^{b}	3.15±0.24^{c}	3.76±0.39^{c}
		5	6	7	8
PGP and biocontrol efficacy under FOX infested condition	Shoot height (cm)	149.80±44.59^{ab}	150.70±28.88^{ab}	118.00±37.75^{a}	165.70±43.96^{b}
	Shoot FW (g)	182.66±39.01^{a}	181.87±30.88^{a}	185.37±53.29^{ab}	221.61±42.91^{b}
	Shoot DW (g)	18.17±3.63^{a}	24.10±3.47^{ab}	17.21±5.81^{a}	25.94±5.61^{b}
	Root FW (g)	50.04±7.64^{a}	69.72±9.64^{b}	63.97±16.21^{b}	66.86±16.00^{b}
	Root DW (g)	16.20±2.70^{a}	26.21±5.91b^{b}	21.37±2.77^{a}	25.40±8.22b^{b}
	Fruit yield (kg/block)	0.54±0.432^{a}	1.44±0.66^{b}	0.75±0.11^{a}	1.58±0.480^{b}
	Disease incidence(%)	40.00 ± 10.00^{c}	26.67 ± 5.80^{b}	16.67 ± 5.8^{a}	13.33 ± 5.8^{a}
	Disease severity	2.12± 0.17^{c}	1.53± 0.33^{b}	0.95± 0.27^{a}	0.78± 0.67^{a}

Note: Different letters in each row indicate significant difference at $p < 0.05$.
Treatment 1: the control without any inoculation, Treatment 2: inoculation with BMG4, Treatment 3: inoculation with KMS17, Treatment 4: inoculation with PGA106, Treatment 5: inoculation with *Fox*, Treatment 6: inoculation with BMG4 and *Fox*, Treatment 7: inoculation with KMS17 and *Fox* and Treatment 8 is inoculation with PGA106 and *Fox*.
FW and DW were abbrevation of fresh weight and dry weight, respectively

2.12, 1.53, 0.95 and 0.78, respectively (Table 1).

From the above results it is obvious that PGA106 is the best of the three *Bacillus* strains tested because of its high activity in promoting tomato growth and enhance tomato fruit yield in non *Fox* condition. While in *Fox* infested condition, strain PGA106 showed the best results for control wilt disease, promote tomato plant growth and alleveate yield loss due to *Fox* infestration. Therefore it was selected for further characterization and formulation study.

3.2 Identification of the Most Efficient Strain

Strain PGA106 that showed the best results for wilt disease control and tomato plant growth promotion was selected for identification. The strain was observed to be a gram positive, rod-shaped, endospore-forming and catalase positive. Thus, it was expected to be a member of the *Bacillus*. The analysis of the 16S rRNA gene sequence (GenBank accession NR075005.2) indicated that PGA106 was closely related to *B. velezensis* CR502^T with 99% similarity (Figure 1).

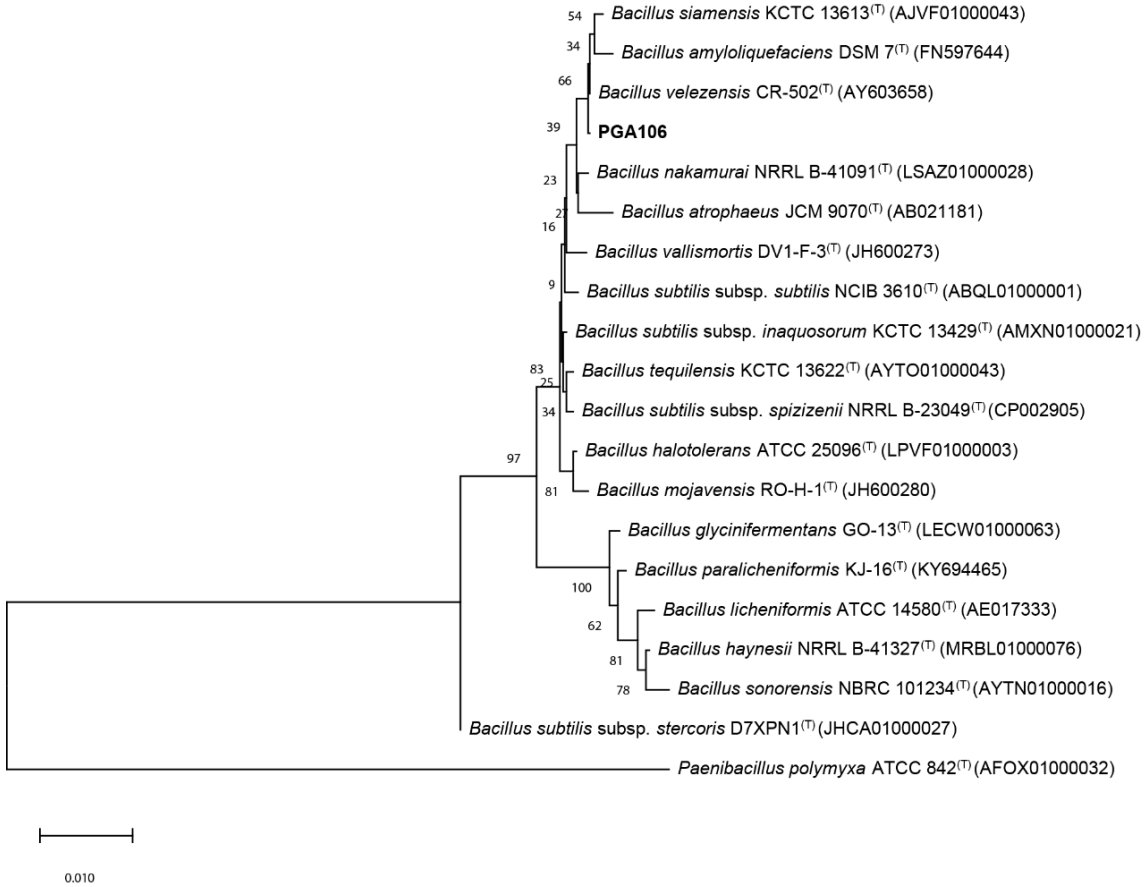

Figure 1. Neighbor-joining phylogenetic tree showing the phylogenetic positions of *B. velezensis* PGA106 and other related taxa based on 16S rDNA sequence. Bootstrap values (expressed as percentages of 1,000 replicates) are shown at branch points. Bar, 0.01 nt substitution rate (*Knuc*).

3.3 Plant Growth Promoting Traits of *B. velezensis* PGA106

B. velezensis PGA106 exhibited almost the traits assessed. It can solubilized mineral phosphate and was found to produce IAA, siderophores, PGP volatiles, biofilm and γ-PGA, but failed to grow in N-free medium. These results were strongly correlated with the results of pot experiment presented above. As it is believed that bacterial strains that hold multiple PGP traits tend to show higher plant growth promotion activities.

3.4 Characterization for the Modes of Biocontrol Action of *B. velezensis* PGA106

The detection of hydrolytic enzyme activities in agar pieces cut from inhibition zone from the dual culture between *B. velezensis* PGA106 and *Fox* confirmed the existence of 4 active hydrolytic enzymes: β-glucanase, cellulase, chitinase and proteinase in the agar pieces. This indicated that those 4 enzymes were involved in *B. velezensis* PGA106 antagonism against *Fox* (Supplemental Figure S1A).

Strains of *B. velezensis* have been reported to synthesize multiple hydrolytic enzymes, including chitinase, β-glucanase, cellulase and proteinase [26, 27]. Such enzymes are known to degrade fungal cell wall, and thus contribute to the biocontrol capacity of biocontrol agents. The detection of chitinase, β-glucanase, cellulase and proteinase from the agar inhibition zone of bacterium-fungus antagonism can be assumed to induce responses triggered by the fungal derived molecules. This phenomena confirmed that the synthesis and secretion of hydrolytic enzymes by *B. velezensis* PGA106 was one of the important role on biological control of *Fox*.

Lipopeptides are well-recognized antimicrobial compounds which contribute antagonistic properties of bacteria [6]. In *Bacillus*, 3 families of lipopeptides as surfactins, iturins and fengycin were identified as key components of their biocontrol capacity. In this study, four bands of the PCR products which sizes were 201, 269, 423 and 370 bp correspoding to the sizes of *srfAA*, *fenD*, ituC and *bmyB* genes, respectively were found (Figure 2A). This may hint that the strain may produce the respective lipopeptides surfactin, bacillomycin L, fengycin and iturin A during its growth.

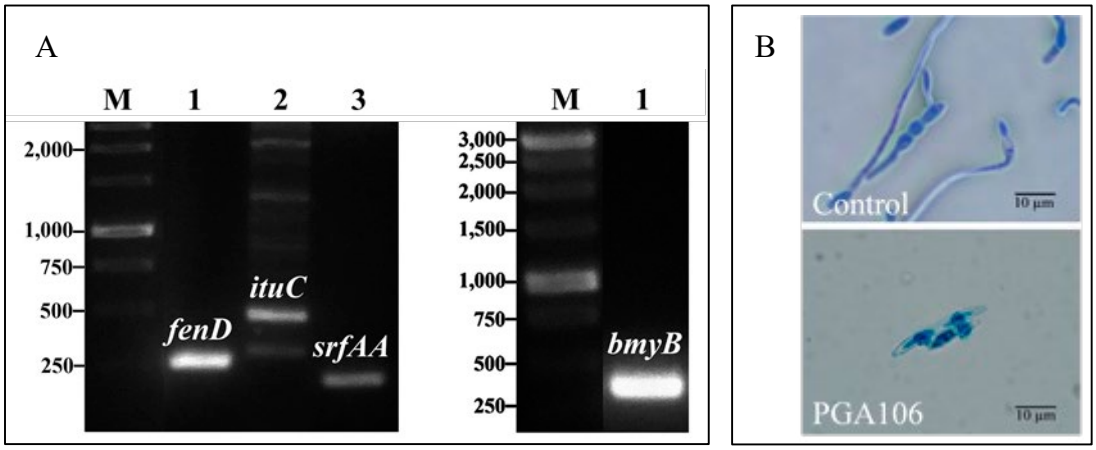

Figure 2. Evidence related to biocontrol trait of *B. velezensis* PGA106. Amplified products of antimicrobial lipopeptide genes (A). Inhibitory effect of cell-free supernatant from *B. velezensis* PGA106 culture on *Fox* conidia germination (B).

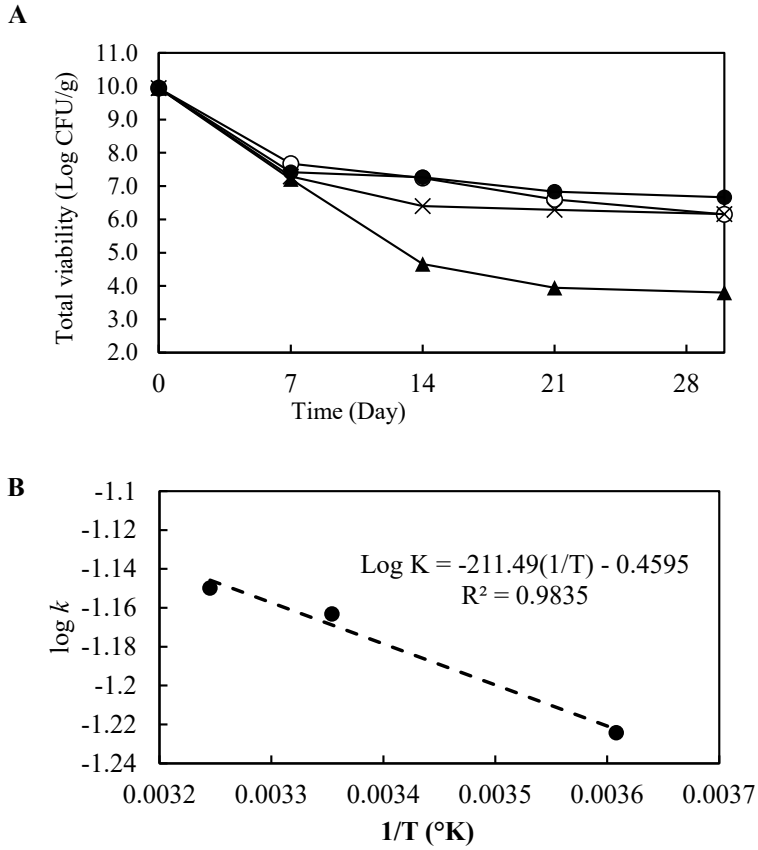

Figure 3. Effect of storage temperatures on *B. velezensis* PGA106 viability in talc formulation, total viability (A), Arrhenius plots on the thermal degradation of the PGA106 in the bioformulation during storage (B).

The cell-free supernatant from the bacterial culture caused inhibitory effect on *Fox* conidia germination because Only 0.25% of conidia were germinated when it was treated with the supernatant, compared to 76.77% germinated conidia those found when treated with the sterile medium. The cell-free supernatant from *B. velezensis* PGA106 culture caused severe damaging effects on *Fox* conidia as cell lysis (Figure 2B). We suspected that the four lipopeptides genes of *B. velezensis* PGA106 may contribute to such damages. Therefore, we assumed that *B. velezensis* PGA106 constitutively produced the extracelluar lipopeptides in GYP broth used in this study like those have been reported in other *B. velezensis* strains that produce lipopeptides in common media such as LB broth [30].

Since conidia of the *Fusarium* in soil are important inocula of wilt disease. Elimination of these propagule at an early stage of planting is crucial to alleviate the development and consequent spread of the disease. Therefore, the ability to produce cell wall degrading enzymes and the highly activity in inhibiting of *Fox* conidia are important features that supports PGA106 to effectively control wilt disease at the pot scale. As shown in Table 1 treatment with PGA106 in *Fox*-infested condition (treatment 8) can dramatically reduce

wilt incidence (13.33%) and severity (0.78) as compared to the control (treatment 5), which has 40.00 % disease incidence and 2.12 disease severity.

3.5 Storage and Viability of the Selected Efficient *Bacillus* Strain

Bacterial biocontrol agents should be produced in form of finished products that are convenient to be used and stored with an extended shelf-life. In this study, *B. velezensis* PGA106 was formulated in a dry solid form using talc powder as the main carrier material.

Viability of *B. velezensis* PGA106 dried solid was evaluated when at different storage temperatures (Figure 3A). The viability of *B. velezensis* PGA106 in bioformulation decreased continunously after a long storage time. At high temperature, 55 °C, the bacterial population sharply declined during the first 14 d and became stable thereafter. Whereas at lower temperatures (4, 25 and 35°C), the bacterial population rapidly declined during the first 7 d and then found stable. The average specific rate of degradation per day (k) derived from the monitoring data indicated that the stabilty of the bioformulation depended directly on change in temperature (Supplemental Table S2). The obtained k values of target storage temperature, 4-35°C, decreased linearly with the increase of temperature as shown in Figure 3B. The effect of storage temperature (4-35°C) on the average specific rate of degradation per day (k) followed Arrhenius equation. The k values at each storage temperature in a range of 4-35°C were calculated from equation (4) in Figure 3B. Storage time of PGA106 bioformulation at the target storage temperature in a range of 4-35°C can be predicted by placing the specific rate of degradation (k) of each storage temperature, a minimum effective cell number that against *Fox* (N) and in the initial cell number in the bioformulation 8.87×10^9 CFU/g (N_0) in equation (3).

4. Conclusions

Overall, *B. velezensis* PGA106 showed the best results for *Fusarium* wilt control and tomato plant growth promotion. The bacterium was highly effective in increasing tomato plant growth and fruit yield in non-*Fox* condition, highly efficient in reducing wilt incidence, and also alleviated tomato yield loss in pathogen infested conditions. The stain attributes on PGP and biocontrol traits enabled it to effectively promote plant growth and control Fusarium wilt disease. Dried biofomulation of *B. velezensis* PGA106 using talc as the carrier was found to maintain the strain survival for an extended storage time and make it convenient for use. The data obtained from the evaluation of the bacterium viability is useful for prediction of suitable storage time of the bioformulation at a desired temperature in a range of 4-35°C.

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