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Characterization of antagonistic–potential of *Bacillus velezensis* SK71 against bacterial brown spot on a terrestrial orchid (*Habenaria lindleyana*)

Akarapisan, A.[1,2*] Khamtham, J.[1,2] and Kositratana, W.[2,3]

[1]Division of Plant Pathology, Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand; [2]Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand; [3]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand.

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Abstract *Bacillus velezensis* SK71 was isolated from the rhizosphere of a terrestrial orchid. The result showed that SK71 has strongly inhibited *Acidovorax avenae* subsp. *cattleyae*. Isolate SK71 produced a 22 mm inhibition zone against *A. avenae* subsp. *cattleyae* in paper disc culture plate confrontation on nutrient yeast dextrose agar plates. The antagonistic bacterial isolate was identified by using 16S rRNA sequence analysis. PCR detection of the *sfp* and *ituA* genes coding for iturin A and surfactin, respectively, indicated a potential for the production of these antibiotics. The production of hydrolytic enzyme and the plant growth–promotional attribute of SK71 confirmed this multifaceted potential. The mixture formulation of SK71 with diatomite powder as a carrier and carboxymethyl cellulose, K_2HPO_4 and glucose showed good effect in suppressing the pathogen *in vitro*. Bulb treatment and spraying with these formulation is an effective delivery system that can provide a conducive environment for *B. velezensis* SK71 to suppress brown spot disease on the terrestrial orchid. Greenhouse studies revealed that SK71 had a 53.34% efficacy in controlling brown spot disease. In this study, we consider that formulation of SK71 is a promising natural biocontrol product, with scale–up possibilities for health and growth promotion of terrestrial orchids.

Keyword: *Acidovorax avenae* subsp. *cattleyae*, *Habenaria lindleyana*, Biological control, *Bacillus velezensis*, 16S rRNA

Introduction

Orchids are the most important flowering ornamentals (Esposito–Polesi *et al*., 2017). Terrestrial orchids have the potential to become popular pot plants in the future. *Habenaria* Willd, including about 876 species, is one of the largest genera of terrestrial orchids, and is mainly distributed in tropical and subtropical areas (Cribb *et al*., 2003; Kurzwell, 2009). The genus *Habenaria* is

[*] **Corresponding Author:** Akarapisan, A.; **Email:** angsana.aka@gmail.com

a member of the subfamily Orchidoideae and occurs primarily in the forests of north and northeastern Thailand (Kurzwell, 2009; Pridgeon *et al*., 2001). The flowers of *H. lindleyana*, are large and white and this genus is horticulturally desirable and collected for conservation purposes (Kongsawad *et al*., 2013). Diseases affecting the family Orchidaceae include root diseases, stem and pseudobulb decays, leaf spots and flower blights (Keith *et al*., 2005). Bacterial brown spot is an important disease of family Orchidaceae. It caused by *Acidovorax avenae* subsp. *cattleyae* (Simone and Burnett, 2002).

Bacillus species have been shown to control a number of bacterial and fungal plant pathogens (Shrestha *et al*., 2016). Antibiotic compounds produced by *Bacillus* groups are classified as lipopeptide antibiotics (Falardeau *et al*., 2013; Jasim *et al*., 2016). These beneficial bacteria can form endospores that ensure survival under adverse environmental conditions making them ideal for formulation as biocontrol agents (Mart ńez–Álvarez *et al*., 2016). Spore–producing organisms, such as *Bacillus* are appropriate for commercialization because they can be easily stored under varying environmental conditions and periods with minimal loss of effectiveness (Errington, 2003). The formulation production process determines the optimal conditions for spore production and desiccation tolerance and their impact on biocontrol effectiveness (Mart ńez–Álvarez *et al*., 2016). Consequently, the materials to be used in the formulation must be selected to maintain product viability for long periods of time (Nakkeeran *et al*., 2006).

The objectives of this study were to develop a formulation of antagonistic bacteria isolated from the rhizosphere of a healthy terrestrial orchid and determine its efficacy against *A. avenae* subsp. *cattleyae in vitro* and *in vivo.*

Materials and methods

Isolation and identification of the pathogens

A. avenae subsp. *cattleyae* was isolated from brown leaf spots on *H. lindleyana*, collected from the Mae Hia Agricultural Research Demonstration and Training Center, Chiang Mai University, Thailand. Diseased tissue pieces were cut from leaf lesions, surface sterilized in 3% sodium hypochlorite for 2–3 min, rinsed in sterile distilled water and macerated in a sterile mortar with approximately 2 ml sterile water. The juice was streaked onto nutrient agar (NA) and were incubated at 28 ℃ for 2 days. Cultures were maintained on NA slants at 4 ℃. The isolates were identified using sequence analysis of 16S rRNA.

Isolation of antagonistic bacteria

The antagonistic bacteria were isolated from the rhizosphere of different varieties of healthy terrestrial orchids: *Habenaria lindleyana*, *Habenaria rhodocheila* and *Pecteilis sagarikii* from a greenhouse in the Mae Hia Agricultural Research Demonstration and Training Center, Chiang Mai University Thailand (Figure 1b). For the isolation of the antagonistic bacteria, 2–month–old roots were rinsed three times with tap water and cut into small pieces (2–3 cm long) then dropped into 100 ml sterile distilled water and shaken at 150 rpm for 1 h. After performing 10–fold serial dilutions up to 10^{-3}, 100 µl aliquots were plated on NA and incubated at 28°C for 2 days before subculturing (Johnson and Curl, 1972). Pure cultures were selected for further studies by streaking three to four times on the NA. Twentytwo bacterial isolates were firstly screened for their ability to inhibit the growth of the pathogens. The best of antagonistic bacteria, which showed high inhibition efficacy was selected for further studies and was stored on NA slants at 4 ℃.

Evalulation of antagonistic bacteria against Acidovorax avenae subsp. Cattleyae

Paper disc culture of rhizosphere antagonistic bacteria against *Acidovorax avenae* subsp. *Cattleyae* was done. Exactly 1 ml suspension of 1.0×10^7 cfu/ml of *A. avenae* subsp. *cattleyae* was added to 100 ml melted nutrient yeast dextrose agar (NYDA). The temperature should be lower than 45 ℃. The medium containing the pathogen was poured into 90 mm–diameter Petri dishes. Paper disks (0.5 cm–diameter) were dipped into a suspension of bacteria, placed onto the plates and incubated at 28 ℃ for 3 days. The inhibition zone was measured (Jiang *et al*., 2015).

Morphological and biochemical characteristics of the antagonistic bacteria

The rhizosphere bacteria were grown on NA for 24 h and colony morphological features: size, form, margin, elevation and texture were collected. The various staining techniques (gram reactions and spore formation) were determined according to standard microbiological procedures (Gerhardt *et al*. 1994). Biochemical/physiological tests included starch hydrolysis, cellulose hydrolysis, growth on 6.5% NaCl, the methyl red–Vogues Proskauer (VP) test, citrate utilization, motility test, NH_3 production, phosphate solubilization and IAA production. The test results were correlated with the identification flow chart of Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994).

Indole-3-acetic-acid (IAA) production

Indole–3–acetic acid (IAA), produced by the antagonistic bacteria cultures was estimated by growing the isolates in liquid Czapek medium supplemented with L–tryptophan as precursor of IAA as described by Rashid *et al*. (2012). Selected bacterial cultures were grown for 48 h in liquid Czapek medium. A loopful of bacterial inoculum was transferred into 50 ml flasks containing 20 ml liquid Czapek medium, supplemented with 200 mg/ml of L–tryptophan (Sigma, USA). Cultivation was performed in the dark at 30 ℃ on a shaker (180 rpm) for 14 days. One mL aliquot was removed from the medium and centrifuged at 4 ℃ for 15 min at 8000 rpm. Then, the supernatant was mixed with Salkowski coloring reagent (50 ml 35% perchloric acid, 1 ml 0.5 M $FeCl_3$) in the ratio of 2:1 and incubated in the dark for 30 min (Libbert and Risch, 1969). After the reaction, red color formation was determined at 530 nm in a Nanodrop 2000C spectrophotometer (Thermo scientific). The IAA concentration in the culture was estimated based on the IAA standard curve (Lee *et al*., 2005).

DNA extraction, PCR amplification and sequence analysis of the of 16S rRNA gene

DNA of actively growing cultures was extracted by using the phenol–chloroform extraction methods of Cheng and Jiang (2006) and Borshchevskaya *et al*. (2013). The 16S rRNA gene (rDNA) fragments were amplified by PCR using universal primers. Two microliters of DNA template were used in a 50µl of PCR reaction volume which contained: 2 µl 20mM forward primer (16SF: 5'–AGAGTTTGATCCTGGCTCAG–3'), 2 µl 20mM reverse primer (16SR: 5'–AAGGAGGTGATCCAGCCGCA–3') (Liu *et al*., 2009), 5µl 10X PCR buffer, 2µl 50 mM MgCl, 0.2µl 5U Taq DNA polymerase (Invitrogen), 1 µl 10mM dNTP mix and 35.8µl sterile distilled water. The conditions for amplification were as follows: initial denaturation for 5 min at 95 ℃, followed by 35 cycles of denaturation at 94 ℃ for 30s, annealing at 60 ℃ for 30s and extension at 72 ℃ for 1 min with a further 10 min of final extension at 72 ℃. The product of PCR amplification of the 16S rRNA of the bacterial isolate was analyzed using 1% agarose gel electrophoresis with a target band of 1500 bp, which was reported by Klijn *et al*. (1991). The PCR product was purified and directly sequenced by fluorescent dye–terminator sequencing using an ABI PrismTM 3730xl DNA Sequencer (Bio Basic Inc).

Phylogenetic inference

The 16S rRNA sequence of antagonistic bacterial was compared with other fourteen sequences from species available in the GenBank database (Table 1). The sequences were aligned using the CLUSTALW program. The phylogenetic relationships between antagonistic bacterial and representative species of the genus *Bacillus* spp. were defined by the neighbor–joining method (Tamura *et al*., 2004; Kumar *et al*., 2016), using 1,000 bootstrap replicates with the program MEGA version 7. The sequence of *Bacillus thuringiensis* ATCC10792 was used as the out–group in the phylogenetic analyses.

Table 1. *Bacillaceae* species used in this study, and GenBank accession numbers for 16S rDNA

***Bacillus* species**	**GenBank Accn. no.**	**References**
Bacillus amyloliquefaciens BCRC11601	NR116022	Wang *et al*. (2007)
Bacillus amyloliquefaciens NBRC15535	NR112685	Meerak *et al*. (2008)
Bacillus velezensis FZB42	NR075005	Chen *et al*. (2007)
Bacillus atrophaeus	AB021181	Goto *et al*. (2000)
Bacillus atrophaeus JMC9070	NR024689	Goto *et al*. (2000)
Bacillus licheniformis BCRC1702	NR116023	Wang *et al*. (2007)
Bacillus licheniformis ATCC14580	NR074923	Rey *et al*. (2004)
Bacillus mojavensis	AB021191	Goto *et al*. (2000)
Bacillus mojavensis IFO15718	NR024693	Goto *et al*. (2000)
Bacillus sonorensis NRRLB23154	AF302118	Palmisano *et al*. (2001)
Bacillus subtilis	X60646	Ash *et al*. (1991)
Bacillus subtilis supsp. *spizizenii*	AF074970	Nakamura *et al*. (1999)
Bacillus vallismortis	AB021198	Goto *et al*. (2000)
Bacillus vallismortis DSN11031	NR024696	Goto *et al*. (2000)
Bacillus thuringiensis ATCC10792	NR114581	Ticknor *et al*. (2001)

PCR detection of lipopeptide biosynthesis genes

Previously published primers were used in the PCR amplifications of lipopeptide biosynthesis genes (Chung *et al*., 2008). One microliter of template was used in a 25µl PCR reaction which contained: 1 µl o20 mM each forward and reverse primers, 2.5µl 10x PCR buffer, 1µl 50mM MgCl, 0.25µl 5U Taq DNA polymerase (Invitrogen), 0.5µl 10 mol/L dNTP mix and 17.75µl sterile distilled water. The PCR programs were as follows: (1) the *sfp* gene (surfactin) used SFP-F/SFP-R (Table 2) –initial denaturation at 94 ℃ for 30s; 35 cycles of denaturation at 94 ℃ for 1 min, annealing at 43 ℃ for 30s and extension at 72 ℃ for 1 min; and final extension at 72 ℃ for 10 min; (2) the *ItuA* gene (Iturin A) was amplified from bacterial genomic DNA by using oligonucleotide primers ITUD1F/ITUD1R (Table 2) –initial denaturation at 94 ℃ for 30 s; 35 cycles of

denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 1 min; and final extension at 72 ℃ for 10 min. The amplified products were detected by agarose gel electrophoresis (1% (w/v) agarose, 100 V, 30 min) followed by RedSafeTM (iNtRON biotechnology) staining and documented under an UltraSlim LED Illuminator. PCR was scored positive when a band of the appropriate size was visualized.

Table 2. Characteristics of specific primers developed for the detection of genes of iturin A and surfactin synthetases (Chung *et al*., 2008)

Target	Genes	Primer name	Primer sequence	Product length (bp)
Surfactin	*sfP*	SFP-F1 SFP-R1	5'-ATGAAGATTTACGGAATTTA-3' 5'-TTATAAAAGCTCTTCGTACG-3'	675
Iturin A	*ituA*	ITUD1F ITUD1R	5'-GATGCGATCTCCTTGGATGT-3' 5'-ATCGTCATGTGCTGCTTGAG-3'	647

Production and formulation of antagonistic bacterial inoculum

A 3 ml initial bacterial suspension of antagonistic bacteria was inoculated and cultured using 300 ml LB medium, in 500 ml Erlenmeyer flasks and incubated in an orbital shaker at 180 rev/min at 30 ℃ for 5 days until a final optical density close to 3.0 was obtained (7.66×10^{10} cfu/ml). The cell suspension was centrifuged at 5,000 rpm for 10 min and the cells were subsequently washed in 0.1 M NaCl buffer and re-centrifuged at 3500 rpm for 5 min. To produce 25 g of the bacterial formulation, 10ml of the bacterial suspension was added under sterile conditions to a mixture containing 19.38 g purified diatomite powder, 5g carboxymethyl cellulose, 0.5g K_2HPO_4 and 0.12g glucose. Subsequently, they were mixed, dried at 35 ℃ for 12 h, packed in polypropylene bags, sealed and stored at room temperature until use. The number of viable spores was determined through serial dilutions using the plate count technique (Chung *et al*., 2010).

In vitro antagonistic tests with the powder formulation

In vitro assays were performed to measure how the formulation process and storage for 360 days affected the antagonistic activity of antagonistic bacterial against *A. avenae* subsp. *cattleyae*. The isolate of *A. avenae* subsp. *cattleyae* was grown on nutrient yeast dextrose agar (NYDA) and antagonistic test was performed using the paper disc method. The plates were incubated at 28 ℃ for 2 days and the zone of inhibition was measured.

Effect of the application of antagonistic bacteria on incidence of brown spot disease caused by A. avenae subsp. cattleyae under greenhouse

For greenhouse experiments *H. lindleyana*, *H. rhodocheila* and *P. sagarikii* was used as the test plants. Bulb of terrestrial orchids were inoculated before planting time with diatomite base powder formulation of the antagonistic bacterium. Two weeks after planting, terrestrial orchid leaves were inoculated by wounding the leaf margin using a suspension with 10^8 cfu/ml of the bacterial isolate. The plants were placed into a dew chamber at 28 ℃ for 1 day (Schaad *et al*., 2008). Diatomite base powder formulation dissolved in water (1% w/v) were sprayed every 2 weeks for 1 month.

The 7 treatments used in the experiment were: (T1) distilled water (positive control); (T2) control–pathogen inoculated (negative control); (T3) bulb treatment and pathogen inoculated; (T4) bulb treatment and spraying at 1 hour before pathogen inoculation; (T5) bulb treatment and spraying at 24 hours before pathogen inoculation; (T6) bulb treatment and spraying at 1 hour after pathogen inoculation and (T7) bulb treatment and spraying at 24 hours after pathogen inoculation.

The disease index (DI) was determined using a scale of 0–5 depending on the percentage of leaves that were symptomatic: 0 = no disease symptoms of the leaves; 1 = 1–20% or diameter of wound 0.1–0.5 cm, green leaves; 2 = 21–40% or diameter of wound 0.1–0.5 cm, green leaves and infected leaf vein ; 3 = 41–60% or diameter of wound 0.5–1.0 cm, rather yellow leaves, infected leaf vein and stem; 4 = 61–80% or diameter of wound 1.5–2.0 cm, yellow leaves and damping off; 5 = 80–100% or leaves dropping and dead. The experiment was set up in a randomized block design with 12 replications (pot).

Disease severity and biocontrol efficacy were calculated as follows:

$$\text{Disease severity (\%)} = \frac{\text{Sum of all diseased leaves in each grade x 100}}{\text{Total number of leaves investigated x the highest disease index grade}}$$

Results

Isolation and identification of the pathogen

The terrestrial orchid pathogen, *A. avenae* subsp. *cattleyae* was isolated from *H. lindleyana* tissues showing spot symptom on leaf (Figure 1b), leaf spot lesions were observed on the leaves inoculated with the isolate (Figure 1c).

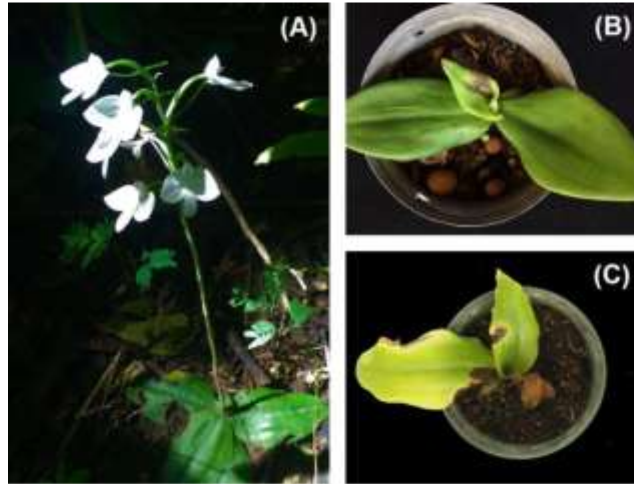

Figure 1. Bacterial brown spot symptom on terrestrial orchid plants. (A): Healthy *Habenaria lindleyana* in deciduous dipterocarp forest, (B): Bacterial brown spot of *Habenaria lindleyana* caused by *Acidovorax avenae* subsp. *cattleyae* and (C): Symptom on *H. lindleyana* after inoculation with *A. avenae* subsp. *Cattleyae* at 14 days

Isolation and evaluation of antagonistic bacteria

Twenty two bacterial isolates were isolated from the rhizospheres of healthy *H. lindleyana*, *H. rhodocheila* and *P. sagarikii*. Testing was carried out on isolates from rhizosphere which were found to be antagonistic to *A. avenae* subsp. *cattleyae*. Bacterial isolate SK71, isolated from the roots of *P. sagarikii*, showed the highest antagonistic inhibitory activity against the pathogen; SK71 inhibited the radial growth of exhibited a 22 mm-wide zone of inhibition against *A. avenae* subsp. *cattleyae* (Figure 2).

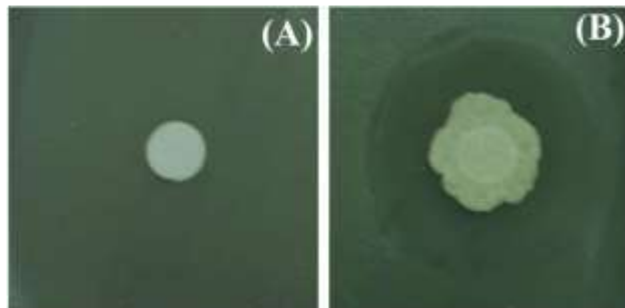

Figure 2. Inhibitory activity of antagonistic bacteria recovered from a healthy terrestrial orchid against *Acidovorax avenae* subsp. *cattleyae* (A): Distilled water and (B): Antagonistic bacterial isolate SK71

Morphological and biochemical characteristics of the antagonistic bacterium SK71

Isolate SK71 was first identified using morphological, biochemical and physiological techniques (Table 3). The colony morphology of SK71 was white, irregular, and round with an undulate margin. Isolate SK71 was gram-positive and had a central endospore position. The following reactions were positive: starch, motility, VP test, catalase, oxidase and utilization of citrate. The isolate was characterized morphologically and biochemically following the Bergey's Manual of Determinative Bacteriology. Isolate SK71 showed features of the members of genus *Bacillus*.

Table 3. Morphological, physiological and biochemical characteristics of *Bacillus velezensis* SK71

Characteristics	**Result**
GenBank accession	*Bacillus velezensis* SK71 (MF989445)
6.5% NaCl	–
Growth on 55 ℃	–
Citrate utilization	+
Motility test	+
Starch hydrolysis	+
Voges–Proskauer test	+
Cellulase production	+
IAA production	+
NH_3 production	+
Phosphate solubilization	+

+= positive; – = negative

Indole-3-acetic-acid (IAA) production

IAA production of *Bacillus* sp. SK71 was estimated and found that maximum production of IAA was usually produced in the stationary phase with a concentrations of 163.21µg/ml after culturing in NB supplemented with tryptophan for 48 h.

PCR amplification, sequencing and phylogenetic analysis of the 16S rRNA gene

The sequence of the 16S rRNA gene was used to identify isolate SK71 down to the species level. PCR amplification of the 16S rRNA of the bacterial isolate produced a typical 1500bp band using agarose gel electrophoresis, which was further purified and sequenced. A BLAST search of 16S rRNA sequence supported the morphological, physiological and biochemical data

indicating that SK71 was *Bacillus* sp. The result of BLAST analysis was confirmed using phylogenetic tree analysis of the sequence along with the other sequences retrieved from the NCBI database. The phylogenetic analysis of the sequences was conducted as previously described (Tamura *et al*., 2004; Kumar *et al*., 2016) using MEGA 7 software. This analysis showed a distinct clustering of isolate SK71 with *B. velezensis* strain FZB42 (NR075005) at 98% of homology (Figure 3). Therefore, isolate SK71 was identified as *B. velezensis.*

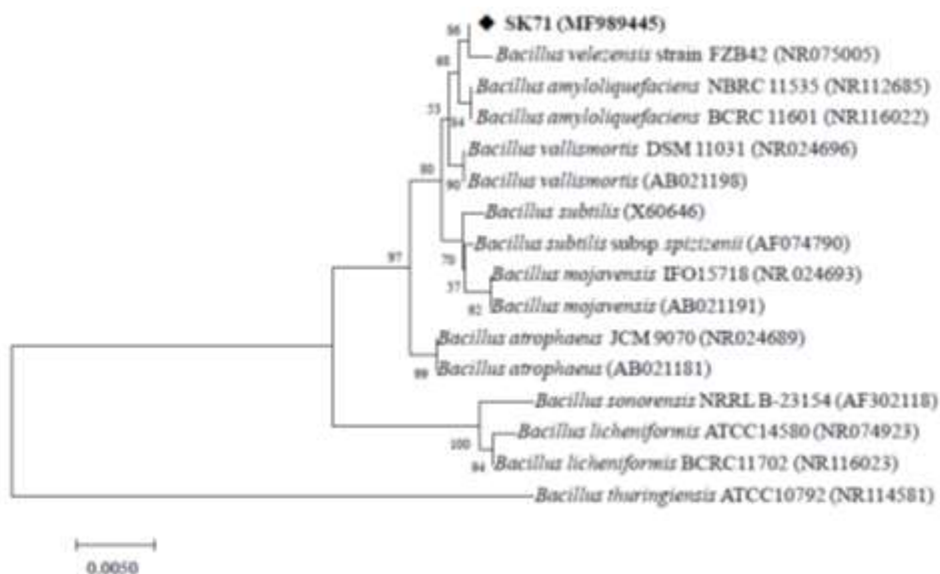

Figure 3. Phylogenetic tree based on comparative 16S rRNA gene of isolate SK71 on available reference sequences from GenBank. A bootstrap neighbor–joining phylogenetic tree was constructed using MEGA 7 and 1,000 replicates

PCR detection of lipopeptide biosynthesis genes

PCR for detection of biosynthesis genes using appropriate primers was employed to determine whether the *B. velezensis* isolate SK71 can produce different types of antimicrobial lipopeptides. The primer pairs were specific for the genes involved in biosynthesis of individual antibiotics. Amplicons of the expected size were obtained with primer pairs for detection of genes involved in biosynthesis of surfactin and iturin A. Sequence analysis of a 675bp of PCR product using the SFP–F1/R1 primer pair corresponded with the surfactin biosynthesis gene cluster (Figure 4A). Analysis of 468bp from a partial sequence of the 647 bp of PCR product from reactions with the ITUA-F1/R1 primer pair corresponded with the iturin biosynthesis gene cluster (Figure 4B).

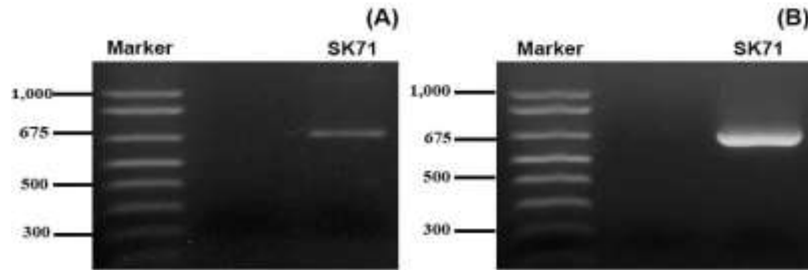

Figure 4. PCR screening of biosynthetic potential of antagonistic bacterial isolate *Bacillus velezensis* SK71. (A): Gel image of PCR product of the surfactin (*srf*) biosynthesis gene and (B): Gel image of PCR product of the iturin (*ItuA*) biosynthesis gene (Marker: 1 Kb plus DNA ladder, control: distilled water and SK71: *B.velezensis* SK71)

Production and formulation of antagonistic bacterial

The viability of *B. velezensis* SK71 in the diatomite-based powder formulation remained relatively high. Cell counts decreased from 9.35 $\log_{10}$ cfu/g on the day of preparation to 8.06 $\log_{10}$ cfu/g after 360 days of storage at room temperature (Figure 5A).

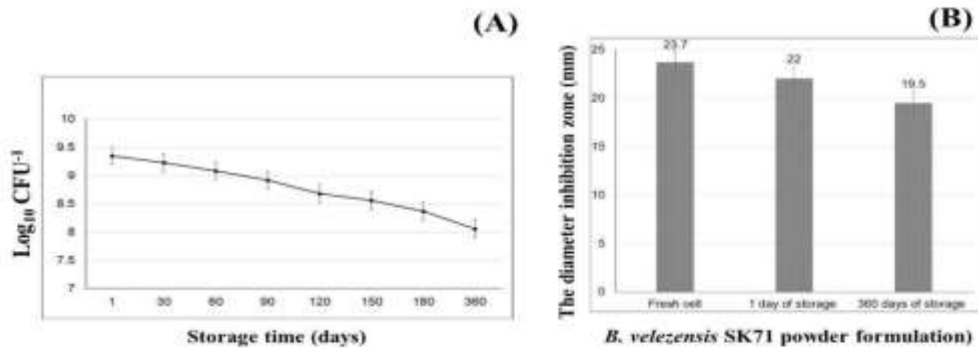

Figure 5. Effect of diatomite–based powder formulation of *Bacillus velezensis* SK71. (A): Viability of *B. velezensis* SK71 formulated with over 360 days of storage at room temperature and (B): The diameter of inhibition zone of *Acidovorax avenae* subsp. *cattleyae*

In vitro antagonistic tests with the powder formulation

The diatomite-based powder formulation inhibited the growth of *A. avenae* subsp. *cattleyae*. This demonstrates that the antagonistic activity of the bacteria against *A. avenae* subsp. *cattleyae* remained stable even after the formulation process and storage for about one year. The bacterium also

demonstrated antagonistic activity against *A. avenae* subsp. *cattleyae* by producing zone of inhibition of 23.7, 22.0 and 19.5 mm at fresh cell, 1 day after formulation and after 360 days of storage, respectively (Figure 5B).

Effect of the application of antagonistic bacteria on incidence of brown spot disease caused by A. avenae subsp. cattleyae under greenhouse

We next examined the efficacy of the *B. velezensis* SK71 diatomite based powder formulation on the suppression of brown spot disease of *H. lindleyana, H. rhodocheila* and *P. sagarikii* for 30 days under glasshouse conditions (Table 4 and Figure 6). Brown spot of terrestrial orchids were first visible at 15 days after being inoculated with *A. avenae* subsp. *cattleyae.* The bulb treatment and spraying at 1 hour before pathogen inoculation (T4) and bulb treatment and spraying at 24 hours before pathogen inoculation (T5) with *B. velezensis* SK71 powder formulation decreased the brown spot incidence (28.33 and 36.67%) as compared to the negative control.

Nevertheless, bulb treatment and spraying of the *B. velezensis* SK71 powder formulation at 1 hour and 24 hours after pathogen inoculation (T6, T7) reduced brown spot disease less than the bulb treatment and spraying at 1 hour and 24 hours before pathogen inoculation (T4, T5). This is probably due to the increase in the *B. velezensis* SK71 population in the rhizosphere coming from the powder formulation. Furthermore, the results indicate that bulb treatment and spraying with the *B. velezensis* SK71 powder formulation were effective in controlling *A. avenae* subsp. *cattleyae* in terrestrial orchids.

Table 4. Effect of *Bacillus velezensis* SK71 powder formulation on leaf spot incidence of terrestrial orchid caused *Acidovorax avenae* subsp. *cattleyae* at grown under greenhouse conditions

Treatment	Disease incidence
T1 Control–distilled water	0.00[d 1/]
T2 Control–pathogen inoculation	81.67[a]
T3 Bulb treatment and pathogen inoculation	50.00[b]
T4 Bulb treatment and spraying 1 hour before pathogen inoculation	28.33[c]
T5 Bulb treatment and spraying 24 hours before pathogen inoculation	36.67[c]
T6 Bulb treatment and spraying 1 hour after pathogen inoculation	53.33[b]
T7 Bulb treatment and spraying 24 hours after pathogen inoculation	52.50[b]

1/: Different letters indicate significant differences (P = 0.05) according to RCBD. Values indicate the average of twelve replicates repeated

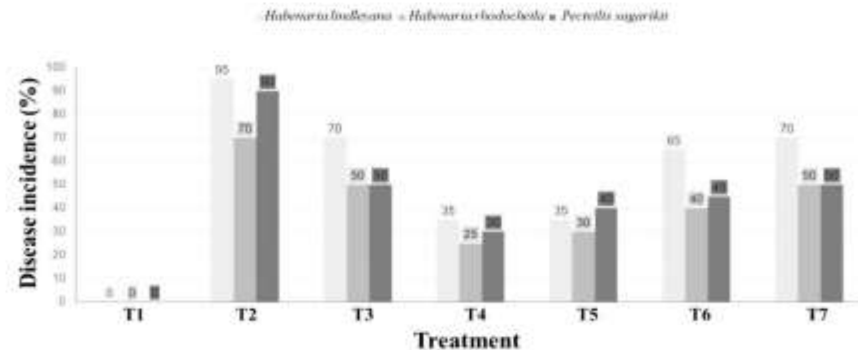

Figure 6. Effect of the powder formulation on brown spot disease symptom caused by *Acidovorax avenae* subsp. *cattleyae*. T1: Control – distilled water (positive control), T2: Control – pathogen inoculation (negative control), T3: Bulb treatment and pathogen inoculation, T4: Bulb treatment and spraying 1 hour before pathogen inoculation, T5: Bulb treatment and spraying 24 hours before pathogen inoculation, T6: Bulb treatment and spraying 1 hour after pathogen inoculation and T7: Bulb treatment and spraying 24 hours after pathogen inoculation

Discussion

In the rainy season of 2016, outbreaks of bacterial brown spot of terrestrial orchid, *H. lindleyana* were found in a greenhouse in the Mae Hia Agricultural Research Demonstration and Training Center, Chiang Mai University, Thailand. Common diseases of terrestrial orchid, include tuber rot and leaf drop caused by *A. avenae* subsp. *cattleyae*. Bacterial pathogens of orchids caused critical crop damage worldwide. Similar investigations were reported by Miller (1990) and Simone and Burnett (2002), who showed that *A. avenae* subsp. *cattleyae* was found on Phalaenopsis orchids and caused death of seedlings in community pots.

Biological control with beneficial microbes is becoming more common in agriculture as a way to address some of the concerns about food security, effectiveness, ecological compatibility and sustainability for plant pathogen control (Adesemoye and Egamberdieva, 2013; Chen *et al*., 2007). Bacterial rhizosphere isolate SK71 is a potential biological control agent that may contribute to the protection of terrestrial orchids against damaging pathogens, as indicated by its suppression of *A. avenae* subsp. *Cattleyae.* Such antagonists may secrete metabolites with antibiotic activity to suppress the growth of pathogens (Jiang *et al*., 2015). Isolate SK71 from the roots of *P. sagarikii* was identified as *B. velezensis* using morphological, physiological, biochemical and molecular techniques. Esposito-Polesi *et al*. (2017) reported the presence of

Bacillus sp. as an endophyte of orchid *Oncidium lexuosum*. The endophytic *B. amyloliquefaciens* was studied in detail by Jiang *et al*. (2015) for its antagonistic properties against the bacterial pathogen of watermelon, *A. avenae* subsp. *citrulli*. However, Fan *et al*. (2017a) have reported that *B. amyloliquefaciens* should be considered as a taxonomic unit above of species level. It was found to be "operational group *B. amyloliquefaciens*", including *B. amyloliquefaciens*, *B. siamensis* and *B. velezensis* whose members are closely related on the genomic sequence.

The results of the present study demonstrated that *B. velezensis* SK71 has biosynthetic genes encoding for antimicrobial antibiotic substances *in vitro* that may have inhibited the tested pathogens of *H. lindleyana*. In the current PCR screening, the *Bacillus* group studied was positive for the antibiotics surfactin and iturin A, similar to the results of Ongena *et al.* (2005) and Montesinos (2007). The study of Han *et al*. (2015) suggested the role of iturin A produced by *B. amyloliquefaciens* in suppression of *Fusarium oxysporum* and other soil–borne fungal plant pathogens. White *et al*. (2014) detected the gene for phosphopantetheinyl transferase (*sfp*) in antagonistic endophytic bacterial isolates from vanilla orchids (*Vanilla phaeantha*) and the ability of surfactin to inhibit fungi and reduce seedling necrosis in plant tissues were also reported. Palazzini *et al*. (2016) presented that *B. velezensis* produced ericin and a diversity of secondary metabolites from *B. velezensis* clade and ifferent strains produce different antibacterial products.

The present work reports on an improved powder formulation of a bacterial biocontrol agent and its ability to suppress and bacterial brown spot disease. The diatomite–based powder formulations of *B. velezensis* SK71 had a shelf–life of viable spores for up to about 12 months at room temperature (approximately 28±3 ℃) which would be acceptable for most commercial applications. The objective of formulating materials for optimum biocontrol effectiveness is the survival of the antagonist (Martı´nez–A lvarez *et al*., 2016). Moreover, formulation components and carriers should not cause any damage to the host plant and do not affect the viability and effectiveness of the biocontrol agent (Shaikh and Sayyed, 2015). The diatomite carrier may have improved the stability of *B. velezensis* SK71 spores by protecting the bacterium against environmental stresses during storage. The isolate used in this study presented IAA production in the *in vitro* test that used tryptophan as a precursor for IAA biosynthesis with a production of 163.21 μg/ml. IAA is a phytohormone that influence many cellular functions in plants (Glick *et al*., 1999). Several *Bacillus* species such as *B. amyloliquefaciens* populations increase the bioavailability of essential compounds to the host rhizosphere and stimulate root proliferation and nutrient uptake (Rahman *et al*., 2016). The

diatomite based formulation of *Bacillus velezensis* SK71 (spraying at 1 and 24 hours before pathogen inoculation) demonstrated strong efficacy in reducing the disease incidence of brown spot disease on treated terrestrial orchid plants to 28.33–36.67%, compared to 81.67% of pathogen inoculated in the greenhouse test within a 1 month period. These results are in agreement with the studies of Al-Ali *et al*. (2017) which demonstrated that *B. velezensis* FZB42 from rhizosphere of tomato produced surfactin, and form biofilm from roots exudates. When strain FZB42 was compared with *B. amyloliquefaciens* S499, the result showed similar capability to colonize tomato rhizosphere. It is known that beneficial bacterial strains can positively influence metabolites with surfactin antibiotic activity and existence of other mechanisms are playing some roles during the suppression of *A. avenae* subsp. *cattleyae* growth (Jiang *et al*., 2015; Fan *et al*., 2017b)

In summary, we screened a bacterium which is highly inhibitory to *A. avenae* subsp. *cattleyae*, an important pathogens of terrestrial orchids. We identified the bacterium SK71 as *B. velezensis*. We also found that SK71 could produce antimicrobial compounds including surfactin and iturin A which have been shown to protect plants against bacterial and fungal pathogens. This result indicates that the diatomite–based powder formulation of *B. velezensis* SK71 has a reasonably long shelf life and therefore it has potential for commercialization. The *B. velezensis* SK71 powder formulation, even when applied in greenhouse, effectively controlled *A. avenae* subsp. *Cattleyae.*

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