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Diffusible and Volatile Antifungal Compounds Produced by an Antagonistic *Bacillus velezensis* G341 against Various Phytopathogenic Fungi

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The aim of this study was to identify volatile and agar-diffusible antifungal metabolites produced by *Bacillus* sp. G341 with strong antifungal activity against various phytopathogenic fungi. Strain G341 isolated from four-year-old roots of Korean ginseng with rot symptoms was identified as *Bacillus velezensis* based on 16S rDNA and *gyrA* sequences. Strain G341 inhibited mycelial growth of all phytopathogenic fungi tested. *In vivo* experiment results revealed that *n*-butanol extract of fermentation broth effectively controlled the development of rice sheath blight, tomato gray mold, tomato late blight, wheat leaf rust, barley powdery mildew, and red pepper anthracnose. Two antifungal compounds were isolated from strain G341 and identified as bacillomycin L and fengycin A by MS/MS analysis. Moreover, volatile compounds emitted from strain G341 were found to be able to inhibit mycelial growth of various phytopathogenic fungi. Based on volatile compound profiles of strain G341 obtained through headspace collection and analysis on GC-MS, dimethylsulfoxide, 1-butanol, and 3-hydroxy-2-butanone (acetoin) were identified. Taken together, these results suggest that *B. valezensis* G341 can be used as a biocontrol agent for various plant diseases caused by phytopathogenic fungi.

Keywords **:** antifungal metabolite, *Bacillus velezensis*, biocontrol, phytopathogenic fungi

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Severe crop loss remains inevitable due to plant diseases, particularly those caused by pathogenic fungi. *Alternaria*, *Botrytis*, *Fusarium*, *Geotrichum*, *Phytophthora,* and *Rhizoctonia* are common and damaging plant pathogenic fungi (Liu et al., 2007). Phytopathogenic fungi reduce both yield and quality of crops. They are major restraints in sustainable agriculture production, especially in intensive cropping systems. Synthetic fungicides have been extensively used to control diseases caused by these pathogenic agents. Application of synthetic fungicides is one of the cheapest and the most effective approaches for the control of plant diseases (Fletcher et al., 2006). However, these chemicals may lead to toxic residues in treated products (Barnard et al., 1997; Isman, 2000). Synthetic pesticides can also cause environmental pollution due to their slow biodegradation (Kordali et al., 2008; Misra and Pavlostathis, 1997). Thus, many researchers have focused on the use of biological methods to protect crops from invasion and spread of pathogens. Indeed, management of pathogens by using antagonistic microorganisms or their secondary metabolites is now considered as a viable method for disease control (Han

et al., 2005; Liu et al., 2007).

As biocontrol agents, many antagonistic microorganisms have been shown to be effective against various pathogens (Cazorla et al., 2007; Stein, 2005). One representative candidate is *Bacillus* species belonging to Gram-positive bacteria. *Bacillus* species can produce various kinds of diffusible and volatile compounds with strong inhibitory activity against plant pathogens (Arrebola et al., 2010; Almenar et al., 2007; Hossain et al., 2016; Kim et al., 2015; Nam et al., 2016; Ongena and Jacques, 2008). Among various diffusible compounds, cyclolipopeptides have many advantages compared to chemical surfactants, including low toxicity, high biodegradability, environmentally friendly characteristics (Ongena and Jacques, 2008; Peypoux et al., 1999; Yu et al., 2002), and low foaming (Compant et al., 2005). Furthermore, cyclolipopeptides are produced by biocontrol agents to protect cells from attacks by microorganisms (Baehler et al., 2006; Cazorla et al., 2006; Fogliano et al., 2002; Shoda, 2000).

Moreover, volatile compounds can promote plant growth (Ryu et al., 2003) with antifungal activity (Arrebola et al., 2010). They can induce systemic resistance in crops (Farag et al., 2006). As for antifungal activity of volatile compounds produced by *Bacillus* species, they can inhibit mycelial growth of *Fusarium oxysporum* that causes *Fusarium* wilt of onion (Sharifi and Ramezani, 2003). They can also reduce postharvest decay in citrus (Arrebola et al., 2010). In order to facilitate multi-applications of diffusible and volatile compounds, a rapid and efficient approach to isolate and identify these useful compounds from *Bacillus* species needs to be established.

In view of this, the objectives of this study were: 1) to characterize bacterial antagonist with strong antifungal activity, 2) to identify volatile and agar-diffusible antifungal metabolites, and 3) to investigate *in vivo* antifungal activity of agar-diffusible metabolites against various plant diseases caused by fungal pathogens.

Materials and Methods

Identification of G341. Bacterial strain G341 was isolated from four-year-old roots of Korean ginseng with rot symptoms as described previously (Son et al., 2009). This strain was identified by sequence analyses of 16S rRNA and *gyrA* genes. Isolation of genomic DNA and PCR amplification of 16S rRNA gene sequence were performed using previously described methods (Park et al., 2005). PCR amplification for *gyrA* gene was performed using primer pairs p-*gyrA*-f/p-*gyrA*-r as described previously (Chun and Bae, 2000).

PCR fragments were purified using Wizard PCR prep Kit (Promega, Madison, WI, USA) and directly sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer's instruction. The same primers used for PCR amplification were used for sequencing. These sequences were compared to 16S rRNA and *gyrA* gene sequences available in public databases of GenBank. A neighbor-joining tree was inferred with Kimura's 2-parameter distance model (Kimura, 1980). The resultant neighbor-joining tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets. Alignment with representative sequence of *B. subtilis* complex and subsequent phylogenetic analyses were carried out using PHYDIT program available at http://plaza.snu.ac.kr/~jchun/phydit/ (Chun, 2001).

***In vitro* antifungal activity.** Using *in vitro* dual-culture analysis, G341 was subjected to *in vitro* antifungal activity assay against the following nine phytopathogenic fungi: *A. panax* causing ginseng Alternaria blight, *B. cinerea* causing tomato gray mold, *Colletotrichum coccodes* causing red pepper anthracnose, *F. oxysporum* f. sp. *lycopersici* causing tomato Fusarium wilt, *Magnaporthe oryzae* causing rice blast, *P. infestans* causing tomato late blight, *Pythium ultimum* causing cucumber damping-off, *R. solani* causing rice sheath blight, and *Sclerotinia sclerotiorum* causing cucumber sclerotinia rot. Potato dextrose agar medium was used as basal medium. A plug (0.6-cm in diameter) containing mycelium was taken from 6-day-old target fungi and placed at the centre of PDA dual plates. Single G341 colonies were patched at a distance of about 3 cm from the fungus. After 3-7 days, the width of inhibition zone between bacterial colony and fungal pathogen was measured. Each treatment was repeated with five replicates. The experiment was also repeated twice.

Evaluation of *in vivo* control efficacy. One-day protective activities of the fermentation broth of G341 strain and purified substances were evaluated against seven plant pathogenic fungi on plants: *M. oryzae* on rice plants, *R. solani* on rice plants, *B. cinerea* on tomato plants, *P. infestans* on tomato plants, *Puccinia recondita* on wheat plants, *Blumeria graminis* f. sp. *hordei* on barley plants, and *C. coccodes* on red pepper plants. These *in vivo* antifungal bioassays were performed as described previously (Kim et al., 2001, 2004). The strain G341 was cultured in tryptic soy broth (TSB; BD, Sparks, MD, USA) medium at 37°C and 150 rpm for 3 days and then the fermentation broth was used for *in vivo* assay. Pots were arranged in a randomized complete block design (three replicates per treatment). All experiments for

in vivo antifungal activities of the purified substances were conducted twice. Six estimates for each treatment were converted into percentage (± standard deviation) compared to control treatments.

Blasticidin-S (50 μg mL^{-1}) for rice blast, validamycin (50 μg mL^{-1}) for rice sheath blight, fludioxonil (50 μg mL^{-1}) for tomato gray mold, dimethomorph (10 μg mL^{-1}) for wheat leaf rust, benomyl (100 μg mL^{-1}) for barley powdery mildew and dithianon (50 μg mL^{-1}) for red pepper anthracnose were applied as positive controls. Pots were arranged in a randomized complete block design (three replicates per treatment). All experiments were conducted twice. Six estimates for each treatment were converted into a percentage (± standard deviation) compared to control treatments using the following equation:

$$\% \text{ control} = 100\ [(A - B)/A]$$

Where A was area of infection (%) on leaves or sheaths sprayed with Tween-20 solution alone and B was area of infection (%) on treated leaves or sheaths.

Purification of antifungal antibiotics. In preliminary experiment, TSB medium was used as the optimal medium for the production of antifungal substances. Strain G341 was cultured in TSB medium (4 l) at 37°C and 150 rpm for 3 days. Bacterial cells were removed after centrifugation at 8,000 rpm for 6 min. The culture supernatant was successively partitioned twice with equal volumes of ethyl acetate (EtOAc) and *n*-butanol (BuOH). Each layer was concentrated by drying and then subjected to *in vitro* antifungal activity against *M. oryzae*. Among four organic fractions obtained from culture broth of strain G341, BuOH layer (17.2 g) was found to be the most active layer, followed by the EtOAc layer (10.2 g). The BuOH layer was applied onto a silica-gel column (3.6 (i.d.) × 60 cm, Kiesel gel 60, 400 g, 70-230 mesh; E. Merck, Darmstadt, Germany). It was then eluted with a mixture of chloroform-methanol-water (30:9:1 and 65:25:4, v/v/v). Active fractions showing inhibitory activity against *M. oryzae* were collected and applied onto a Sephadex LH-20 column (2.8 (i.d.) × 45 cm, 200 g; Sigma, MO, USA) after concentration. Active fractions eluted from the Sephadex LH-20 column with methanol were evaporated to dryness and loaded onto a Sep-Pak C_{18} cartridge (10 g) (Waters, Milford, MA, USA) with methanol-water. Using this protocol, 220 mg of compound **1** and 24 mg of compound **2** were obtained from 4 l of culture supernatant.

Electrospray ionization mass and tandem mass spectrometry. Purified antifungal substances were analyzed on a Hybrid Quadrupole-Time-Of-Flight (Q-TOF) mass spectrometer (QSTAR XL, AB Sciex Insturments, CA, USA) operated in electrospray ionization (ESI) positive ion mode. These samples were dissolved in 50% MeOH / 49% H_2O / 1% AcOH (v/v/v) and applied to nano-spray tip of Q-TOF MS. Compound **1** was analyzed by ESI-MS/MS without ring-opening. In contrast, compound **2** was ring-opened by cleavage of the lactone bond (Williams et al., 2002) and analyzed. Amino acid sequences were determined from series of b_n obtained from *de-novo* sequencing of lipopeptide. Amino acid compositions were obtained as described previously (Razafindralambo et al., 1998).

Analysis of antifungal activities of volatile compounds. Strain G341 culture broth (50 μl) was spread onto half of a divided plate containing TSA. Following 24 h of incubation at 25°C, 5 mm mycelia plugs of *M. oryzae*, *R. solani*, *B. cinerea*, *P. infestans*, *F. oxysporum*, *S. sclerotiorum*, *P. capsici*, and *C. coccodes* were placed on the other half of the divided plate containing PDA. Plates were wrapped immediately in Parafilm to seal in volatiles. Measurements of radial mycelia growth were taken at 48 h to 72 h post incubation of the pathogen on both bacterial and control plates. The experiment was repeated twice.

Collection of volatile compounds. Headspace volatile compounds produced by strain G341 were collected using a setup recommended by DeMilo et al. (1996) with slight modifications. Briefly, strain G341 was cultured at 37°C with shaking (150 rpm) in 200 ml of TSB in 500 ml of Erlenmeyer flasks fitted by a two-way rubber cork with insertion of glass tubes. One of the inserted glass tubes was placed just 1 cm above the culture of strain G341. The other end of the tube was connected to a nitrogen supply system to remove headspace volatiles. One end of the second tube was placed near the neck of the flask while the other end was connected to a volatile trap made of glass tube (7 cm in length and 0.4 cm in diameter) containing 150 mg of activated charcoal (Darco, 20-40 mesh, Aldrich, Milwaukee, WI, USA). Before use, these traps were placed in 150 mm Petri plate wrapped in aluminum foil and sterilized in 350°C oven for 24-36 h. The neck of the conical flask was tightly sealed with Parafilm to prevent the escape of volatile compounds. TSB medium without bacteria was used as control. The stream of dry nitrogen flow was started at 12 h post inoculation of the bacterial strain and maintained at 300 ml/min for up to 48 h. Flasks were shaken throughout the collection process at 150 rpm. Volatile compounds in the activated charcoal trap were eluted into glass vials with 0.5 ml of methylene chloride.

GC-MS analysis of volatile compounds produced by G341. Volatile organic compounds produced by G341 strain were analyzed by GC-MS (Shimadzu GC-MS QP5050, Shimadzu co., Kyoto, Japan). A 1-µl aliquot of methylene chloride solution containing volatile compounds was injected into the injection port of the GC-MS. A capillary column SPB-5 (30 m × 0.25 mm in i.d., 0.25 µm in film thickness; PA, USA) was used. The initial temperature of the column was held at 30°C for 2 min and increased to 220°C at 5°C min^{-1}. The injection port and interface were set at 240°C and 200°C, respectively. Helium carrier gas was used at a flow rate of 2.2 ml min^{-1}. Mass spectra of unknown compounds were compared to those deposited in the NIST/EPA/NIH Mass Spec. Library (Version 2.0).

Statistical analysis. Analysis of variance was performed using PROC GLM procedure (SAS institute, Cary, NC, USA). If $P > F$ was less than 0.01, means were separated with Duncan's multiple range test at $P = 0.05$ level.

Results

Antifunagl activity. Strain G341 inhibited mycelial growth of all fungal pathogens tested. Mycelial growth of *A. panax*, *B. cinerea*, *C. coccodes*, *F. oxysporum*, *M. oryzae*, and *P. capsici* was significantly inhibited by G341. However, the antagonistic bacterium was weakly active to mycelial growth of *P. ultimum*, *R. solani* and *S. aclerotiorum* (Fig. 1).

Identification of G341 strain. Strain G341 exhibited phenotypic similarity with *Bacillus* spp. based on biochemical, morphological, and cultural characteristics (data not shown). Analysis of 16S rRNA gene sequence (MF167634) showed that strain G341 formed a monophyletic group with species of *B. subtilis* complex, sharing 99% sequence similarity (Fig. 2A) and the sequence analysis of *gyrA* genes revealed that strain G341 shared 96.6% similarity with type strain of *B. velezensis* LMG 22478^{T}. Based on the phylogenetic analysis of *gyrA* gene of G341 (MF167633)

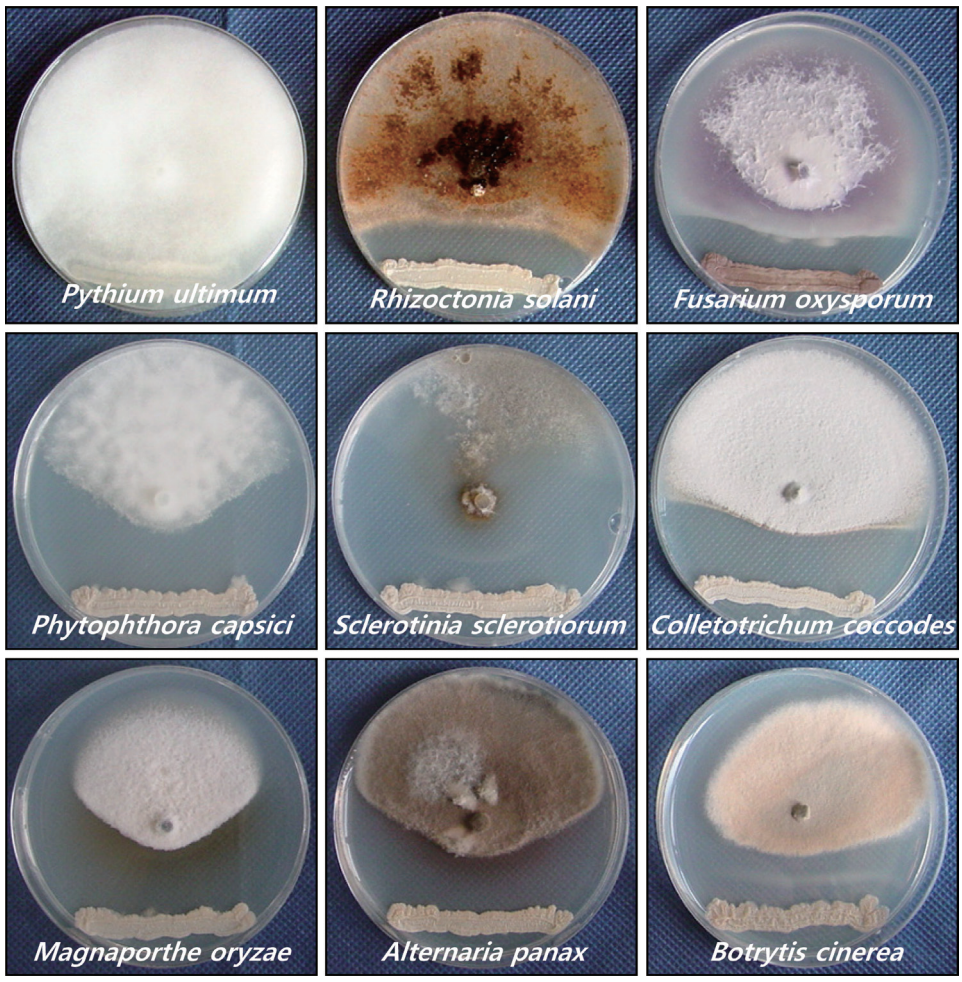

Fig. 1. *In vitro* antifungal activity of *Bacillus* sp. G341 against various phytopathogenic fungi in a dual culture assay.

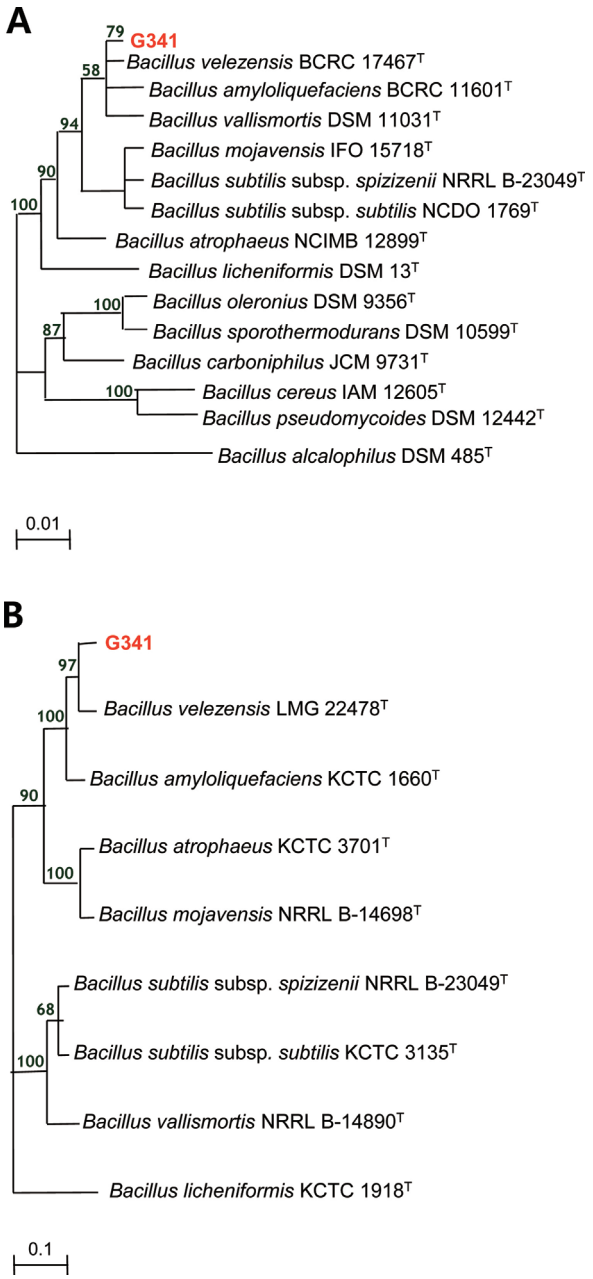

Fig. 2. Neighbour-joining phylogenetic trees showing relationships between strain G341 and several other strains of *Bacillus* based on their 16S rDNA (A) and *gyrA* gene (B) sequences.

with *Bacillus* spp. belonging to the monophyletic group, strain G341 was identified as *B. velezensis* (Fig. 2B).

Growth curve of strain G341 and its production of antifungal metabolites. As shown in Fig. 3, strain G341 could grow relatively quickly. It reached stationary phase at 12 h after inoculation. There was almost no lag phase. The antifungal activity of its culture broth sampled at different time

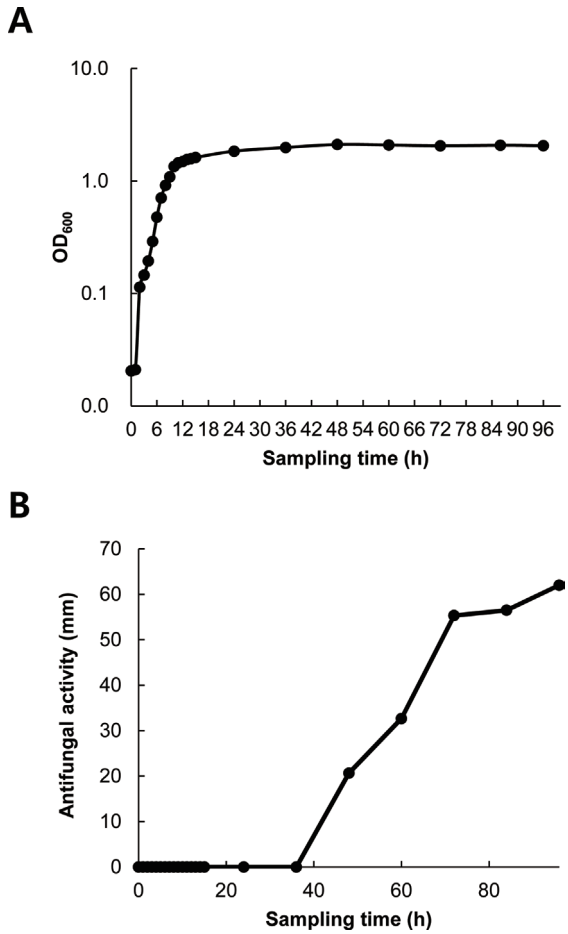

Fig. 3. Correlation between cell growth (A) and antifungal activity (B) of *Bacillus velezensis* G341 culture filtrate.

intervals was significantly correlated with cell growth over the four-day growth period. The strongest antifungal activity against *M. oryzae* was obtained at 96 h after incubation. Therefore, the optimal harvest time for antifungal metabolites of strain G341 might be at 96 h after inoculation under the culture conditions used in this study.

***In vivo* antifungal activity and structure determination.** The fermentation broth of G341 showed potent antifungal activity against *M. oryzae* and *R. solani* on rice plants, *B. cinerea* on tomato plants, and *C. coccodes* on red pepper plants. It reduced the development of the four diseases by more than 50% even at a 9-fold dilution (Table 1).

Among four organic fractions obtained from culture broth of strain G341, BuOH fraction showed the strongest *in vivo* antifungal activity. The BuOH fraction also exhibited strong *in vivo* antifungal activity against rice blast, rice sheath blight, tomato gray mold, and red pepper anthracnose (data not shown). Because the BuOH fraction

Table 1. Control efficacy of *Bacillus velezensis* G341 liquid culture filtrate against seven plant diseases caused by fungal pathogens[a]

Dilution	Control value (%)[b]						
	RCB[c]	RSB	TGM	TLB	WLR	BPM	RPA
1-fold	79 ± 5.4	100 ± 0.0	82 ± 2.7	77 ± 3.2	83 ± 1.3	50 ± 10	91 ± 1.2
3-fold	85 ± 3.3	100 ± 0.0	61 ± 10	47 ± 4.1	20 ± 5.6	33 ± 2.3	69 ± 7.2
9-fold	56 ± 12	53 ± 5.7	58 ± 15	17 ± 5.7	13 ± 7.2	0 ± 0.0	55 ± 4.3
27-fold	0 ± 0.0	16 ± 13	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	45 ± 8.1

[a]Seedlings were inoculated with spores or mycelial suspensions of the test organism at 1 day after spraying with the liquid culture filtrates.
[b]Each value represents the mean ± standard deviation of two runs with three replicates per run.
The synthetic fungicides used as positive controls showed high control values over 85% as previously reported by Yoon et al (2010).
[c]RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mold; TLB, tomato late blight; WLR, wheat leaf rust; BPM, barley powdery mildew; RPA, red pepper anthracnose.

was the most active one, its constituents were separated by bioassay-lead fractionation. Two antifungal substances were isolated from the BuOH fraction by repeated column chromatography such as silica-gel, Sephadex LH-20, and Sep-Pack C18. Finally, two active compounds (**1** and **2**) were isolated.

In order to identify chemical structures of the two isolated metabolites, their mass spectra were recorded by ESI-TOF mass spectrometry. The mass spectra of compound **1** showed a series of mass number (*m/z* 1021.5, 1035.5, and 1049.5). It revealed differences of 14 Da, suggesting that the purified compound had a different carbon chain length (-CH_2-). Based on tandem mass spectrometry, the amino acid composition of compound **1** was determined to be Asp, Tyr, Ser, Glu, and Thr in a ratio of 2:1:2:1:1. The sequence deduced from MS/MS spectrum obtained from *m/z* 1035.5 ion peak of compound **1** was identical to that of bacillomycin L (Fig. 4A). Thus, compound **1** was identified as bacillomycin L with fatty acid moieties of C_{14}-C_{16}.

ESI-TOF mass spectrum of compound **2** showed $[M+H]^+$ ion peaks at *m/z* 1449.7, 1463.7, 1477.7, 1491.7, and 1505.7. Amino acid analysis revealed that compound **2** comprised Glu, Orn, Tyr, Thr, Ala, Pro, and Ile in a ratio of 3:1:2:1:1:1:1. ESI-MS of hydrolysate of compound **2** showed a protonated peak at *m/z* 1481.7. The mass gain of 18 Da from *m/z* 1463.7 of compound **2** could be due to hydrolysis of a lactone bond. MS/MS spectrum of *m/z* 1481.7 ion obtained from hydrolysate of compound **2** revealed that the amino acid sequence of compound **2** was identical to that of fengycin A (Fig. 4B). Thus, compound **2** was identified as fengycin A with fatty acid moieties of C_{15}-C_{19}. Based on the above results, chemicals of lipopeptides produced by G341 were found to be bacillomycin L and fengycin A (Fig. 5).

***In vivo* antifungal activity.** Results of *in vivo* antifungal

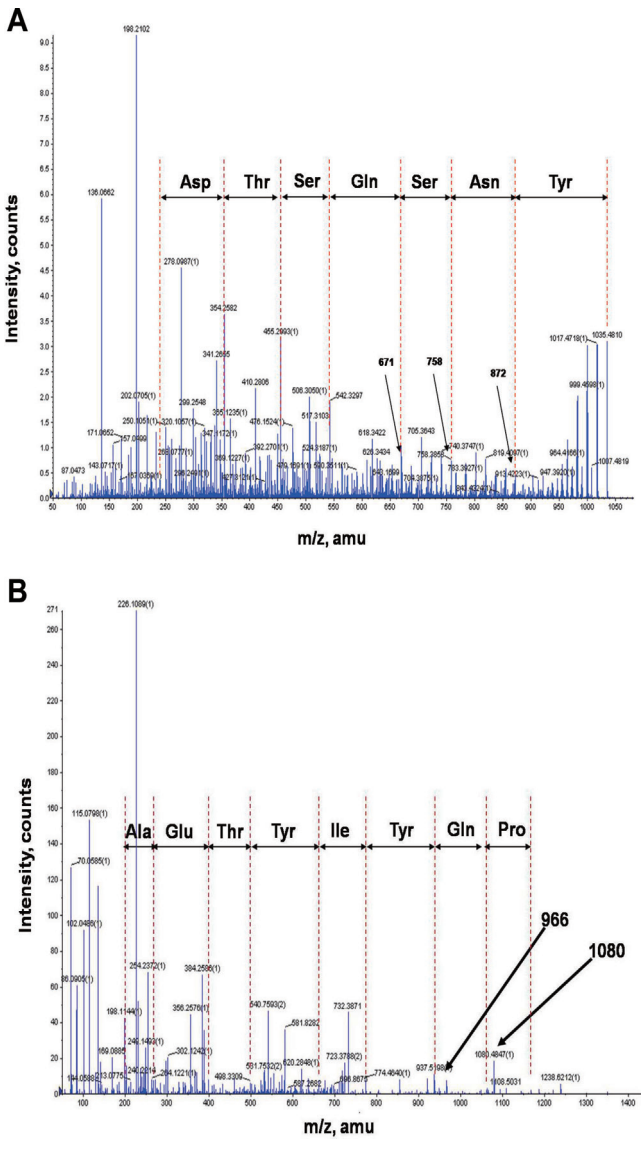

Fig. 4. Electrospray ionization (ESI)-tandem mass spectrometry spectra of bacillomycin L (A) and fengycin A (B) isolated from liquid culture of *Bacillus velezensis* G341.

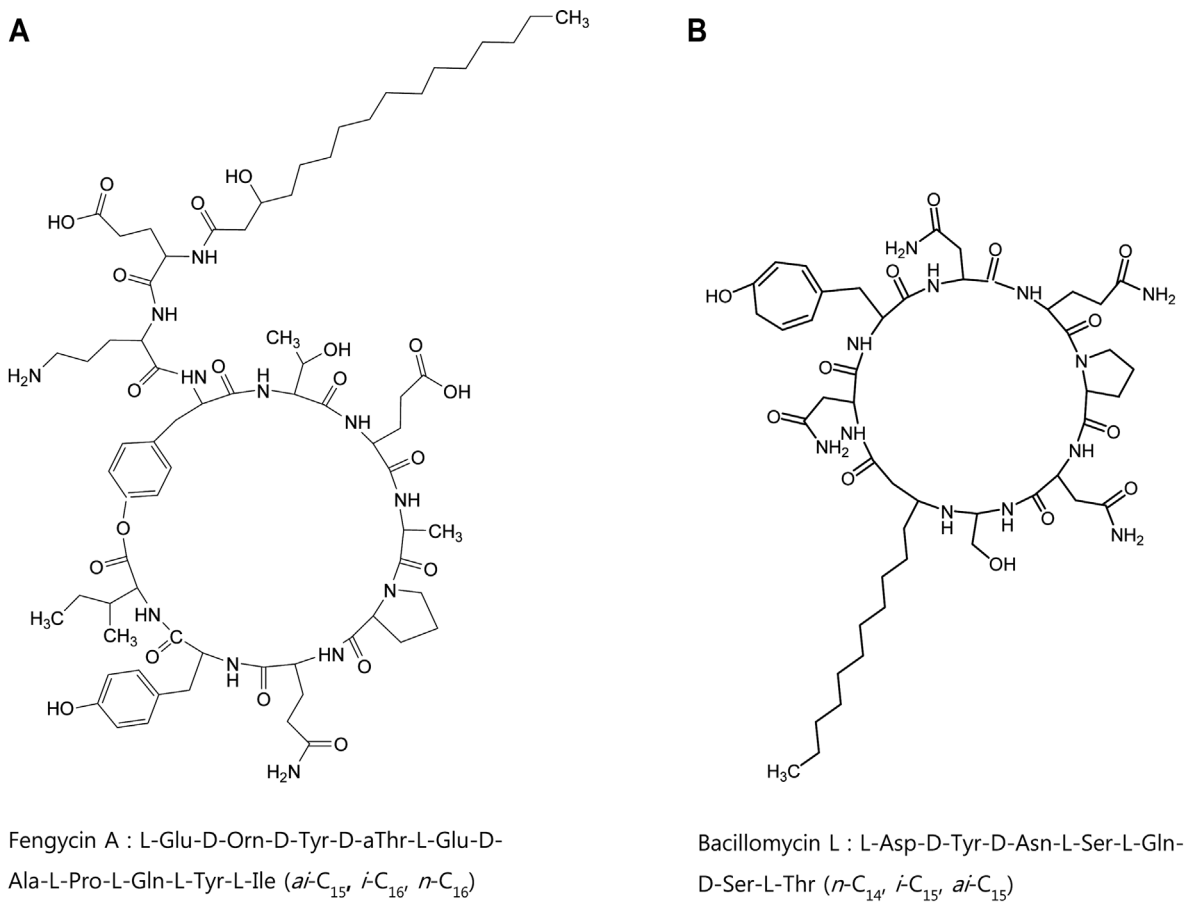

Fig. 5. Chemical structures of bacillomycin L (A) and fengycin A (B) isolated from liquid culture of *Bacillus velezensis* G341.

activities of the two lipopeptides isolated from strain G341 are summarized in Table 2. Out of four plant diseases tested, the two lipopeptides effectively suppressed the development of rice sheath blight. The *in vivo* antifungal spectra of the two lipopeptides were found to be different from each other. Bacillomycin L was active against rice blast, rice sheath blight, and red pepper anthracnose. However, it was virtually inactive against tomato gray mold. In comparison, fengycin A showed *in vivo* antifungal activity against rice sheath blight and tomato gray mold, but not against rice blast or red pepper anthracnose.

***In vitro* antifungal activity and analysis of volatile compounds of G341.** Strain G341 produced antifungal

Table 2. Control efficacy of compounds **1** and **2** isolated from *Bacillus velezensis* G341 against four plant diseases caused by fungal pathogens[a]

Chemical	Conc. (μg/ml)	Control value (%)[b]			
		RCB[c]	RSB	TGM	RPA
Compound **1**	500	56 ± 12	74 ± 5.7	33 ± 2.3	50 ± 12
	250	38 ± 5.2	74 ± 5.7	8 ± 4.2	19 ± 13
Compound **2**	500	0 ± 0.0	89 ± 2.3	67 ± 5.9	0 ± 0.0
	250	0 ± 0.0	74 ± 4.2	33 ± 5.2	0 ± 0.0

[a]Seedlings were inoculated with spores or mycelial suspensions of the test organism at 1 day after spraying with liquid culture filtrates.
[b]Each value represents the mean ± standard deviation of two runs with three replicates per run.
The synthetic fungicides used as positive controls showed high control values over 85% as previously reported by Yoon et al (2010).
[c]RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mold; RPA, red pepper anthracnose.

Table 3. Mycelia growth of phytopathogenic fungi inhibited by volatile compounds produced by *Bacillus velezensis* G341

Pathogen	Mycelial growth inhibition (%)
Rhizoctonia solani	63
Magnaporthe oryzae	15
Botrytis cinerea	58
Colletotrichum coccodes	21
Fusarium oxysporum f. sp. *lycopersici*	0
Sclerotinia sclerotiorum	78
Phytophthora capsici	28
Phytophthora infestans	31

Table 4. GC-MS volatile profile of *Bacillus velezensis* G341

Retention time (second)	Relative peak area (%)	Compound
497.71	4.06	Dimethylsulfoxide
601.01	5.91	1-Butanol
744.31	82.88	3-Hydroxy-2-butanone (acetoin)

volatiles and inhibited mycelial growth of various fungal pathogens. It significantly inhibited mycelial growth of *S. sclerotiorum*, *R. solani*, and *B. cinerea* (Table 3). *P. infestans*, *P. capsici*, *C. coccodes*, and *M. oryzae* were relatively resistant to these volatile compounds produced by G341. Volatile profiles indicated that strain G341 produced 3 volatile compound: dimethylsulfoxide, 1-butanol, and 3-hydroxy-2-butanone (acetoin). Among them, acetoin was found to be the major compound (Table 4, Supplementary Fig. 1).

Discussion

One of the biggest ecological challenges facing microbiologists and plant pathologists in the near future is the development of environmentally friendly alternatives to chemical pesticides for combating crop diseases. The use of beneficial microorganisms is considered as one of the most promising methods for more rational and safe crop-management practices (Ongena and Jacques, 2008). In this respect, we attempted to isolate antagonistic bacteria with strong antifungal activity against various phytopathogenic fungi. As a result, we found that G341 strain inhibited mycelial growth of various plant pathogenic fungi tested. These preliminary results suggested that *B. velezensis* G341 strain could produce antifungals. It is well-known that bacteria can commonly produce cell wall-degrading enzymes and secondary metabolites to hinder the growth of other microorganisms (Shoda, 2000).

Identification of *B. subtilis* complex has been difficult due to their almost identical phenotypic characteristics and 16S rRNA gene sequences. Recently, partial *gyrA* sequences coding for DNA gyrase subunit A have been found to be able to provide rapid and accurate classification and identification for *B. subtilis* and closely related taxa (Chun and Bae, 2000). In this study, strain G341 was found to share 99% sequence similarity with species of the *B. subtilis* complex based on sequence analysis of 16S rRNA gene. It shared 96.6% of sequence similarity with type strain *B. velezensis* LMG 22478^T based on sequence analysis of partial *gyrA* gene (Fig. 1). Thus, it is reasonable to identify the strain G341 as *B. velezensis*.

B. velezensis sp. nov. was recently isolated during a research focusing on discovering novel bacterial strains capable of synthesizing new lipopeptides with surfactant and/or antimicrobial activity (Ruiz-Garcia et al., 2005). Several research groups have reported that *B. velezensis* have potential to control Fusarium wilt in strawberries (Nam et al., 2009), wheat powdery mildew (Cai et al., 2017), Fusarium head blight (Palazzini et al., 2016). Recently, Gao et al. (2017) reported that one endophytic *B. velezensis* ZSY-1 strain produces volatile compounds having antifungal activity against phytopathogenic fungi. However, to the best of our knowledge, there has been no report that *B. velezensis* can produce both diffusible and volatile antifungal compounds. Accordingly, we investigated the control efficacy of diffusible and volatile compounds produced by strain G341 against plant diseases caused by phytopathogenic fungi.

In *in vivo* experiment, the fermentation broth of strain G341effectively controlled the development of rice blast, sheath blight, tomato gray mold, and red pepper anthracnose. In order to identify antifungal principles produced by this strain, we isolated two antifungal compounds by partitioning with BuOH, silica-gel column chromatography, Sephadex LH-20 column chromatography, and C_{18} column chromatography. The two compounds were identified as bacillomycin L and fengycin A based on MS/MS analyses (Fig. 3, 4). Bacillomycin L is a member of the iturin group produced by *B. subtilis*. It is one of the extensively studied peptide antibiotics. Bacillomycin D, F, and L have been reported as antifungal peptides produced by *B. subtilis* (Besson et al., 1978; Mhammedi et al., 1982; Moyne et al. 2001; Qian et al., 2016). Fengycin A is a biologically active lipopeptide produced by several *B. subtilis* (Vanittanakom et al., 1986). Its structure is composed of a β-hydroxyl fatty acid linked to a peptide part comprising 10 amino

acids, with 8 of them being organized in a cyclic structure. Loeffler et al. (1986) have also proved that fengycin is less toxic to tested plants. It protected these plants from some filamentous pathogenic fungi better than several peptide antibiotics such as iturin (Loeffler et al., 1986). In this study, bacillomycin L was found to be active against rice blast, rice sheath blight, and red pepper anthracnose while fengycin was found to be active against rice sheath blight and tomato gray mold (Table 2). Up to date, various types of lipopeptides isolated from *B. subtilis* have been reported to possess antifungal activity against plant diseases (Ongena and Jacques, 2008). Among iturin group, iturin A produced by *B. subtilis* RB14 can reduce the development of damping-off of tomato (a seedling disease) caused by *R. solasni* (Asaka and Shoda, 1996). Fengycin is known to possess antifungal activity against filamentous fungi (Kulimushi et al., 2017). Its hemolytic activity is 40-fold lower than that of surfactin (Schneider et al., 1999; Vanittanakom et al., 1986). Cazorla et al. (2007) have reported that iturin, fengycin A, and surfactin isolated from *B. subtilis* strains have antifungal activity against soil-borne phytopathogenic fungi from avocado rhizoplane.

Strain G341 also produced volatile antifungal compounds that could inhibit mycelial growth of phytopathogenic fungi in sealed plates (Table 3). Antifungal volatile compounds have been demonstrated previously in several pathogen systems. For example, trimethylamine has been shown to be able to inhibit hyphal extension and formation of arthrospore in *Geotrichum candidum* (Robinson et al., 1989). Allyl alcohol an also inhibit carpogenic germination of sclerotia of *S. sclerotiorum* in bean (Huang et al., 1997). Hydrogen cyanide produced by *Pseudomonas* has been used to control root rot of tobacco (Voisard et al., 1989). Our data indicate that strain G341 could inhibit mycelial growth of various phytopathogenic fungi by producing dimethylsulfoxide, 1-butanol, and acetoin (Table 4). Among them, acetoin was found to be a major volatile metabolite. Fernando et al. (2005) have reported that sulfur-based compounds benzothiazole and dimethyl trisulfide possess high fungicidal activity. Many commercially used fungicides and soil fumigants are sulfur-based. Alcohols such as 1-hexanol also have antifungal activity which can be used to prevent diseases (Archibold et al., 1997). Acetoin can significantly reduce symptomatic leaves inoculated with soft rot causing pathogen *Erwinia carotovora* by inducing systemic resistance (Ryu et al., 2003). Arrebola et al. (2010) have reported that *B. amyloliquefaciens* PPCB004 can produce acetoin as a major volatile compound. Radial growth of several *Penicillium* species has been found to be inhibited *in vitro* in the presence of volatile compound of PPCB004. They have also reported that antagonist PPCB004 could significantly reduce decay incidence and severity in Valencia inoculated with *P. crustosum* (Arrebola et al., 2010). Gao et al. (2017) reported that *B. velezensis* ZSY-1 strain can produce various volatile and antifungal metabolites such as 2-tridecanone, pyrazine (2,5-dimethyl), benzothiazole, and phenol (4-chloro-3-methyl).

Taken together, our results suggest that antagonist *B. velezensis* G341 can be used as a good biocontrol agent candidate, although how effective this antagonist would be under field conditions is currently unclear. Diffusible and volatile compounds produced by *B. velezensis* G341 might be used in agriculture as a direct contact biofungicide and biofumigant.

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