

## Biological Control Potential of *Bacillus amyloliquefaciens* KB3 Isolated from the Feces of *Allomyrina dichotoma* Larvae

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(Received on December 24, 2015; Revised on February 16, 2016; Accepted on February 20, 2016)

**Most biocontrol agents for plant diseases have been isolated from sources such as soils and plants. As an alternative source, we examined the feces of tertiary larvae of the herbivorous rhino beetle, *Allomyrina dichotoma* for presence of biocontrol-active microbes. The initial screen was performed to detect antifungal activity against two common fungal plant pathogens. The strain with strongest antifungal activity was identified as *Bacillus amyloliquefaciens* KB3. The inhibitory activity of this strain correlated with lipopeptide productions, including iturin A and surfactin. Production of these surfactants in the KB3 isolate varied with the culture phase and growth medium used. *In planta* biocontrol activities of cell-free culture filtrates of KB3 were similar to those of the commercial biocontrol agent, *B. subtilis* QST-713. These results support the presence of microbes with the potential to inhibit fungal growth, such as plant pathogens, in diverse ecological niches.**

**Keywords :** *Allomyrina dichotoma*, biosurfactants, cyclic lipopeptides, growth medium, insect feces

Biological control, in which antagonistic microorganisms such as bacteria, yeasts, and filamentous fungi, fight plant pathogens, is recognized as a safe and sustainable

alternative to chemical-based pesticides (Ongena and Jacques, 2008). Many biocontrol agents have been isolated from suppressive soils (Hornby, 1983; Schroth and Hancock, 1982). A microbiome analysis of the rhizosphere in disease-conducive and disease-suppressive soils indicates the importance of certain groups of root-colonizing *Pseudomonas* spp. (Mendes et al., 2011). *Bacillus* isolates from various sources have also demonstrated biocontrol capabilities (Choudhary and Johri, 2009; Siripornvisal, 2010), and *Bacillus* derived-products constitute about half of the commercially available biopesticides (Cawoy et al., 2011; Ongena and Jacques, 2008). Surfactant cyclic lipopeptides (CLP) produced by many *Bacillus* spp. are one class of metabolites implicated in biocontrol. The surfactants include surfactin, iturin A, and fengycin. These lipopeptides antagonize pathogen growth and induce systemic resistance against plant diseases (Ongena and Jacques, 2008). Additionally microbes have the potential to control insects. The entomopathogenic microbe, *B. thuringiensis*, is used globally to control various insects (Martin and Travers, 1989). *Pseudomonas* isolates also display insecticidal activities, in addition to properties that improve plant health (Kupferschmied et al., 2013; Olcott et al., 2010; Ruffner et al., 2013). Thus, insects act as hosts for biocontrol-active microbes (Nadarasah and Stavrinides, 2011; Sanchez-Contreras and Vlisidou, 2008).

In the present study, we screened for the feces of the larva of the herbivorous beetle, the Asian rhino beetle, *Allomyrina dichotoma*, for active strains of biocontrol agents. In nature, although the adults feed on fruit, nectar, and sap, the larvae consume decaying plant matter, thus, serving as primary decomposers in ecosystems. The enzymes produced by microorganisms in the guts of insects facilitate the breakdown of lignified woody plant

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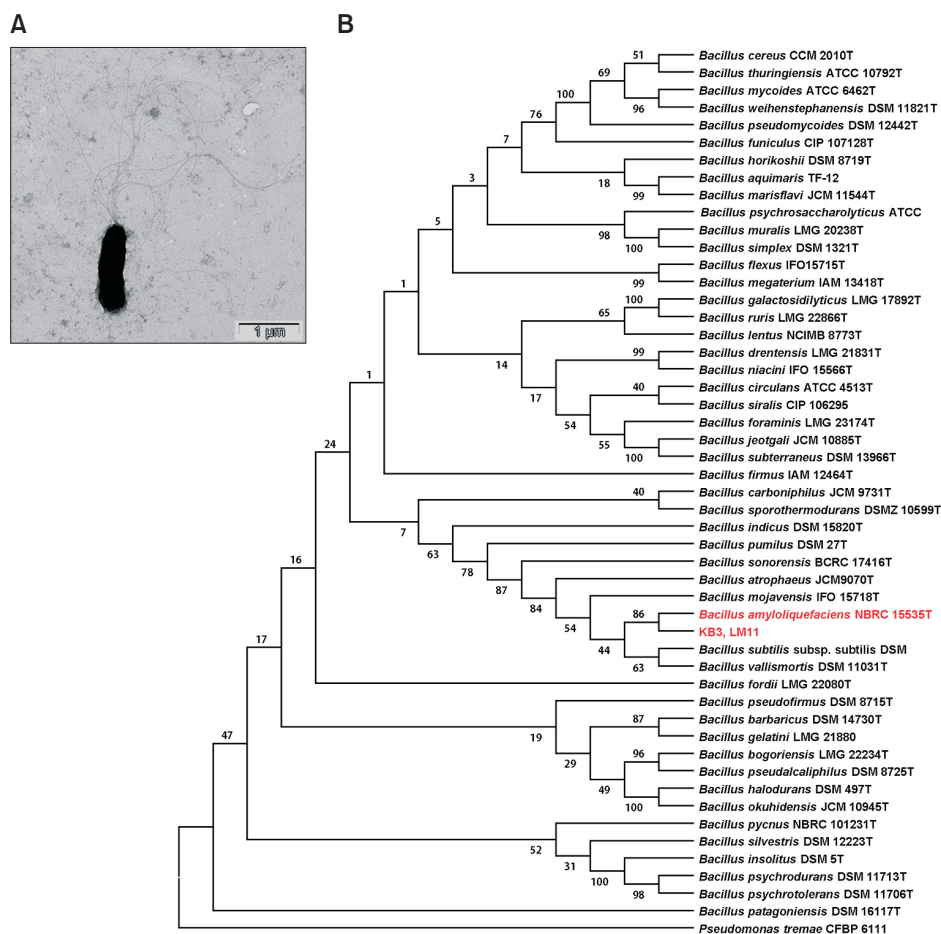
materials (Breznak and Brune, 1994). Consequently, in the present study, the larvae were grown on decomposing sawdust as a mimic of natural soil substrates. Additionally, gut microbes aid in insect nutrition, development, and reproduction, and contribute to pathogen resistance (Brune, 2003; Moran et al., 2005). For instance, *Bacillus* strains can activate immunity against plant diseases, as observed in foliar aphids and whiteflies feeding on pepper (Lee et al., 2012; Song et al., 2015).

Feces of *A. dichotoma* tertiary larvae were suspended in sterile phosphate buffer (pH 7.5) and serial dilutions were spread evenly on one-third strength King's B (KB) agar (King et al., 1954). Over 2,000 colonies were isolated from plates incubated at 28°C for 14 hours. Stocks of these isolates were maintained in full-strength KB broth (KBB) at -80°C. Cultures were revived by streaking suspension onto KB agar and incubating at 28°C ± 2°C for 48 hours. Other media used in this study were Luria-Bertani medium (LB; Difco, Sparks, MD, USA), M9 minimal agar (M9; Sambrook and Russell, 2001), Taylor and Francis medium (TF, containing 20 g peptone, 25 g sucrose, and 4.5 g/l yeast extract; Mezghanni et al., 2012), Ok Chun medium (OC, containing 6 g glucose, 8 g yeast

extract, 2.5 g K<sub>2</sub>HPO<sub>4</sub>, 1.5 g NaCl, 0.5 g Na<sub>2</sub>CO<sub>3</sub>, and 1 g/l MgSO<sub>4</sub>; Ha, 2013), nutrient broth medium (Difco), and potato dextrose broth (PDB; Difco). For growth on plates, liquid PDB was supplemented with agar for potato dextrose agar (PDA; Difco). Phytopathogenic fungal isolates were obtained from the Korean Agriculture Culture Center (Suwon, Korea) included *Phytophthora capsici* (KACC No. 40111), *Botrytis cinerea* (KACC No. 40573), *Colletotrichum coccodes* (KACC No.40010), *Rhizoctonia solani* (KACC No. 40101), and *Fusarium oxysporum* (KACC No. 40031). All fungi were cultured on PDA plates at room temperature.

Isolates were first screened for the inhibition of *B. cinerea* and *R. solani* growth using a radial diffusion assay (Bajpai and Kang, 2012). Isolates that inhibited these fungi were subsequently screened for biosurfactant production by an assay of their culture filtrates by observing the collapse of oil droplets (Bodour and Miller-Maier, 1998). One hundred and ninety isolates that were positive for both traits were screened further, and two isolates, KB3 and LM11, were selected for further study because they showed the strongest antifungal activity.

The potassium hydroxide test (Gregersen, 1978) and



**Fig. 1.** (A) Transmission electron microscopic image of the cell of isolate KB3 harvested at logarithmic growth phase from nutrient broth. (B) The phylogenetic tree of 16S rRNA sequences of isolates KB3 and LM11 showing 100% identity over the 1.4 kb sequence with those reported for reference *Bacillus* strains. The phylogenetic tree was constructed using the neighbor-joining method and Kimura's two-parameter model. The numbers at nodes indicate consensus bootstrap values based on 1,000 replications. Scale bar = 0.002 substitutions per nucleotide position. *Pseudomonas tremae* was used as the out group.

scanning electron microscopy indicated that KB3 and LM11 were both rod-shaped gram-positive bacteria possessing lophotrichous flagella (Fig. 1). Biochemical analysis with the Vitek2 Compact BCL card system (bioMérieux, Hazelwood, MO, USA) showed a 90% probability match with *Bacillus amyloliquefaciens*. Both strains produced beta-xylosidase, Ala-Phe-Pro arylamidase, L-pyrrolydonyl-arylamidase, alpha-galactosidase, phenylalanine arylamidase, and beta-glucosidase, and utilized D-mannose, D-trehalose, D-mannitol, pyruvate, palatinose, and myo-inositol. In addition, both strains exhibited esculin hydrolysis, methyl- $\alpha$ -D-glucopyranoside acidification, tetrazolium red production, and polymyxin B and kanamycin resistance. The isolates tolerated high salinity, growing at 6.5% NaCl.

The identification of KB3 and LM11 as *B. amyloliquefaciens* isolates was confirmed by their 16S rRNA sequences. The gene was amplified by PCR using universal primers, forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-ACG GCT ACC TTG TTA CGA CTT-3' (Weisburg et al., 1991) from KB3 and LM11 genomic DNAs extracted from log-phase cultures in LB broth using an ExiPrep™ bacteria genomic DNA Kit (Bioneer Inc., Daejeon, Korea). The PCR reaction was performed with a Bioneer premix (Bioneer Inc.), and the 1.4 kb 16S rRNA sequence was obtained by Solgent ASSA service (Solgent, Daejeon, Korea). Nucleotide sequences were analyzed using BLASTN and the National Center for Biotechnology Information database. A phylogenetic comparison of the 16S rDNA sequence with related *Bacillus* spp. was performed. First, the sequence was aligned using CLUSTAL X and edited using BioEdit version 5. Then, a phylogenetic tree was constructed with MEGA6 software (Tamura et al., 2013) using the neighbor-joining method with the Kimura two-parameter model (Kimura, 1980) and bootstrap values based on 1,000 replications. The phylogenetic analysis showed that the 16S rDNA sequences of both isolates were 99.99% identical to the *B. amyloliquefaciens* subsp. *plantarum* CAU B946 sequence (Fig. 1). Consequently, the KB3 and LM11 isolates were identified as isolates of *B. amyloliquefaciens*.

*B. amyloliquefaciens* strains from other sources are used commercially in agriculture as biopesticides and biofertilizers (Li et al., 2015; Pérez-García et al., 2011). Interestingly, endophytic *B. amyloliquefaciens* isolated from the seeds of ornamental hostas induced host resistance against plant diseases and insects (Li et al., 2015). However, there are limited reports on the isolation of *Bacillus* strains from insects (Sreerag et al., 2014).

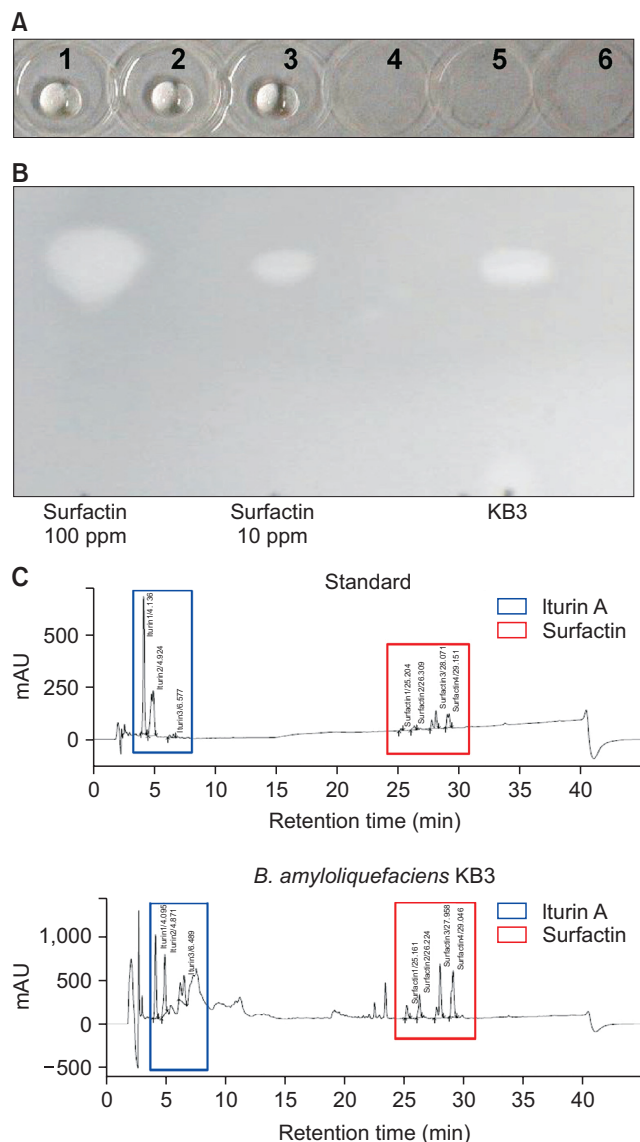
Biocontrol-active isolates of *B. amyloliquefaciens* and *B. subtilis* produce various antimicrobial CLP, including

surfactin, iturin A, and fengycin (Chowdhury et al., 2015; Ongena and Jacques, 2008; Pérez-García et al., 2011). These metabolites have antifungal and antibacterial activities, affect bacterial motility and biofilm formation, and act as effectors to induce systemic resistance (Pérez-García et al., 2011).

Isolates KB3 and LM11 produced surfactants that caused oil drops to collapse in the procedures to identify changes in surface tension as described by Bodour and Miller-Maier (1998). The method involved adding 2  $\mu$ l of 10W-40 Pennzoil (Pennzoil, Houston, TX, USA) to wells of a 4-well cell culture plate (SPL Lifesciences, Pocheon, Korea) followed by the application of 5  $\mu$ l of the cell-free cultures of KB3 and LM11. Cells were removed from 2-day cultures, grown in KBB with shaking at 200 rpm at 30°C, by centrifugation for 20 minutes at 10,000g (Vision, Bucheon, Korea). The supernatants were filtered through 0.2- $\mu$ m filters to remove all cells. Cell-free culture filtrates from biocontrol bacteria, *Lysobacter antibioticus* HS124 (Gardener et al., 2014) and *Pseudomonas* sp. NJ134 (Kang, 2012), which do not produce CLPs, were used as negative controls. *B. subtilis* QST-713, the main microbial ingredient of the biocontrol product, Serenade (AgraQuest, Davis, CA, USA), was used as a positive control. The collapse of an oil droplet in a well indicated surfactant activity; cell-free filtrates of *B. amyloliquefaciens* KB3 and LM11, and *B. subtilis* QST-713, caused droplet collapse (Fig. 2A).

The chemistry of the surfactants from isolate KB3 was investigated by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Standards for surfactin and iturin A were used (Sigma-Aldrich, St. Louis, MO, USA). *B. amyloliquefaciens* KB3 was grown for 2 days at 30°C in a shaking incubator at 180 rpm in KBB. The culture filtrate was adjusted to pH 2.0 using 6 N HCl centrifuged for 20 minutes at 10,000g. After incubation at 4°C for 24 hours, the suspension was centrifuged and the supernatant collected and dried by rotary vacuum concentration. The dry materials were dissolved in chloroform and methanol (65:15, v/v) and spotted onto TLC plates (HX246144; Merck KGaA, Darmstadt, Germany) with chloroform:methanol:water (65:25:1, v/v/v) as the mobile phase solvent. Surfactin at 100 and 10 ppm (Sigma-Aldrich) was applied as a standard. Plates were developed and compounds detected as previously described (Nitschke and Pastore, 2006). Both the surfactin standard and the KB3 sample produced a white spot with a retention factor of 0.55 (Fig. 2B). The concentration of the KB3 culture extract was estimated to be approximately 20 ppm.

Qualitative and quantitative analyses of iturin A and surfactin in the lipopeptide extracts were carried out by



**Fig. 2.** Detection of surfactants produced by *Bacillus amyloliquefaciens* KB3. (A) The wells of six plates containing oil and surfactants in the drop collapse assay. The lanes contained: 1, distilled water; 2, *Lysobacter antibioticus* HS124; 3, *B. thuringiensis* KACC12072; 4, *B. amyloliquefaciens* KB3; 5, *B. amyloliquefaciens* LM11; 6, *B. subtilis* QST-713. (B) Detection of surfactant activity by thin layer chromatography. The lanes show results from with standard surfactin (100 ppm), standard surfactin (10 ppm), and *B. amyloliquefaciens* KB3. (C) High-performance liquid chromatograms for the metabolites eluted from KB3 versus the iturin A and surfactin standards.

comparing HPLC chromatogram peaks of iturin A and surfactin standards with those observed from the extracts (Fig. 2C). The supernatant was mixed 1:1 (v/v) with ethyl acetate, and the upper layer removed and filtered with a filter paper (Cat. No. 1006-110, Whatman; GE Healthcare, Buckinghamshire, UK). The sample was concen-

trated under nitrogen gas, weighed, and then re-dissolved in methanol. The methanol extract was passed through an Isolute C-18 CE type cartridge (International Sorbent Technology Ltd., Hengoed, UK) and injected onto a reverse-phase C18 column (Shimadzu Shim-pack VP-ODS, 250  $1 \times 4.6$  mm; Shimadzu, Kyoto, Japan) using an HPLC instrument (Shimadzu Prominence LC-20AD; Shimadzu). The column oven temperature was set at 40°C, and different ratios of distilled water, tetrahydrofuran, and acetonitrile were used as mobile solvents. The biosurfactants were detected at 214 nm. Iturin A and surfactin (Sigma-Aldrich) were dissolved in methanol to a final concentration of 1 mg/ml and used as standards (Kim et al., 2010). Retention times of the iturin A standard were 4.1, 4.9, and 6.5 minutes. Chromatograms of the culture filtrates from KB3 showed these same three peaks (Fig. 2C). Retention times of the surfactin standard were 25.2, 26.3, 28.0, and 29.1 minutes, and peaks at these times were detected from the KB3 extracts (Fig. 2C). The concentrations of the surfactants, calculated by peak areas, suggest that iturin A and surfactin were present at  $1,064 \pm 236$   $\mu\text{g/ml}$  and  $3,779 \pm 899$   $\mu\text{g/ml}$ , respectively.

PCR was used to identify genes that might be involved in the biosynthesis of surfactin and iturin A by strain KB3. Biocontrol lipopeptides are non-ribosomally synthesized by modularly organized mega-enzymes called, peptide synthases (NRPS). NRPS genes were clustered in an operon (Arguelles-Arias et al., 2009; Chen et al., 2007; Chowdhury et al., 2015). Primers used to amplify the genes encoding the lipopeptides were based on sequences from *B. amyloliquefaciens* FZB42 (Arguelles-Arias et al., 2009; Chen et al., 2007). Primers specific for the gene *sfp* involved in surfactin production were: forward, 5'- ATG AAG ATT TAC GGA ATT TA -3' and reverse 5'- TTA TAA AAG CTC TTC GTA CG -3'. The primers for the *ituD* gene involved in iturin A production were: forward, 5'- ATG AAC AAT CTT GCC TTT TTA -3' and reverse, 5'- TTA TTT TAA AAT CCG CAA TT -3'. The expected PCR had sizes of 600 bp for *sfp* and 1,203 bp for *ituD*. PCR reactions were performed in a thermocycler (Bioneer) in a 50  $\mu\text{l}$  reaction volume containing KB3 genomic DNA with HelixAM™ Premium-Taq Polymerase Kit (NanoHelix Co., Daejeon, Korea). Negative controls without DNA and with the genomic DNA of *Bacillus thuringiensis* KACC12072 isolate that does not produce these surfactants were included in the assay. The amplified products were visualized in a 1.5% agarose gel, stained by EcoDye (SolGent). The expected PCR products were efficiently amplified from KB3, but not from *B. thuringiensis* KACC12072 (data not shown).

Our chemical analyses and the genetic information indicate that Isolate KB3 produces surfactants similar to

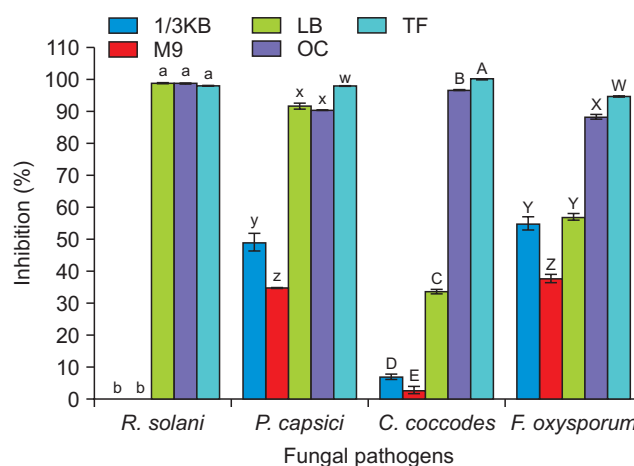
surfactin and iturin A. These findings suggest that isolate KB3 expresses biocontrol activity in plants through several mechanisms, including inhibition of fungal pathogen growth, induction of systemic resistance in plants, and prevention of adherence of pathogens to plant surfaces (Chen et al., 2013; Chowdhury et al., 2015; Dietel et al., 2013; Kim et al., 2010; Ongena and Jacques, 2008; Wang et al., 2010). Surfactins from *B. amyloliquefaciens* S499 have been detected in a gnotobiotic rhizosphere (Nihorimbere et al., 2012), showing that nutrition from plants is adequate for their production. Whether lipopeptides increase plant growth requires further investigation (Buensanteai et al., 2008; Ernst et al., 1971). Surfactants are insecticidal in the larval midgut of the leaf miner *Tuta absoluta* (Ben Khedher et al., 2015) and the aphid *Myzus persicae* (Yun et al., 2013). The insecticidal activity for isolate KB3 is under investigation, and whether rhino beetle larvae are resistant has been considered. However, these findings illustrate that insects are valuable sources of microbes with the potential for agricultural significance in improving plant health.

For an organism to be commercially viable, methods for growth must be cost-effective. Consequently, antifungal activity was compared after growth of KB3 for 5 days at 30°C in a shaking incubator at 180 rpm on different growth media, including; LB, M9 containing glucose as the sole carbon source, TF, OC, and one-third strength KB. Cell-free culture filtrates were prepared by centrifugation at 13,000 rpm for 20 minutes at 4°C and filtration through a sterile membrane with a 0.2 µm pore size (Minisart, 0.2 µm; Sartorius Stedim Biotech, Melsungen, Germany). The cell-free filtrates were added to PDA at final concentrations between 0% and 10% (v/v). PDA was amended with the same amounts of sterile medium to allow measurement of control growth. Mycelial plugs from 7-day-old cultures of the fungal plant pathogens, *R. solani*, *P. capsici*, *C. coccodes*, and *F. oxysporum* were placed in the center of the PDA plates and incubated at 28°C in the dark. Mycelial growth was measured 1 week post incubation. Growth inhibition was determined using the following formula. Growth inhibition percentage =  $(R - r) / R \times 100$ ; where R is the radius of the fungal growth in the control plates, and r is the radius of the fungal growth in the plates containing the bacterial culture filtrates. Each experiment was repeated at least two times with three replicates per experiment.

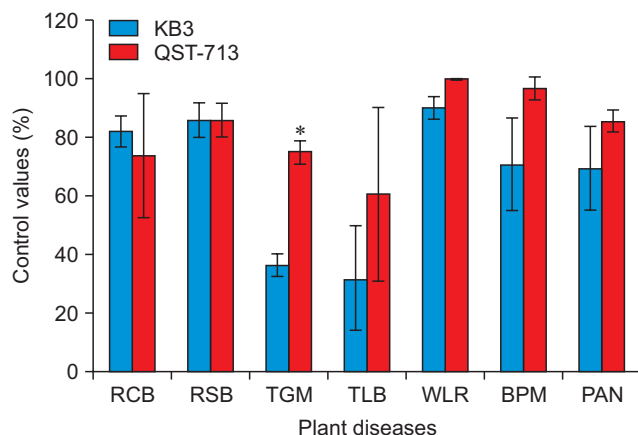
The antifungal activities of KB3 significantly differed with the growth media (Fig. 3). The cell-free bacterial culture of KB3 grown in TF broth showed the highest *in vitro* antifungal activity for all fungal pathogens. The differences in antifungal activity were not related to bacterial cell number; the number of culturable cells in one-

third KB and M9 media was  $2.5 \pm 1.4 \times 10^7$  and  $3.5 \pm 2.5 \times 10^5$  cfu/ml, respectively, whereas, in other media, the means were  $5.8 \pm 4.6 \times 10^8$  cfu/ml. Differential effects of media have been observed with other bacteria. The biocontrol activity of *Pseudomonas chlororaphis* O6 was influenced by the presence of glucose (Park et al., 2011), and biocontrol-active *B. subtilis* has produced high yields of biosurfactants with glucose as a carbon source, urea or ammonium chloride as a nitrogen source, and oxygen-limited conditions (Ghribi and Ellouze-Chaabouni, 2011).

The biocontrol activity of the culture filtrates of *B. amyloliquefaciens* KB3 and *B. subtilis* QST-713 as a positive control, grown on KB, was tested *in planta* against six different plant pathogens causing rice blast, rice sheath blight, tomato gray mold, tomato late blight, wheat leaf rust, barley powdery mildew, and pepper anthracnose (Kim et al., 2001). Briefly, plants were grown in vinyl pots in a green house at 25°C ± 5°C for 1–4 weeks. The plants were sprayed with 1:3 diluted cell-free 5-day-old culture filtrates grown in one-third KB. Tween 20 was used as a wetting agent. Distilled water with Tween 20 was used as the negative control. The treated plant seedlings were inoculated with spores or mycelial suspensions from one of the six plant pathogens 24 hours after exposure to culture filtrate, as described previously (Kim et al.,



**Fig. 3.** Effects of growth media for isolate KB3 on *in vitro* antifungal activity. Data are shown for growth on potato dextrose agar amended with 10% cell-free filtrates from each of the five media. Different letters indicate significant differences between different growth media according to a one-way ANOVA ( $P < 0.05$ ). Data are expressed as the means of three replicates in each of three plates, and error bars show the standard deviations. 1/3KB, one-third strength King's B medium; M9, M9 minimal medium; LB, Luria-Bertani medium; OC, Ok Chun medium; TF, Taylor and Francis medium; *R. solani*, *Rhizoctonia solani*; *P. capsici*, *Phytophthora capsici*; *C. coccodes*, *Colletotrichum coccodes*; *F. oxysporum*, *Fusarium oxysporum*.



**Fig. 4.** *In planta* biocontrol efficacies of the cell-free filtrates from *Bacillus amyloliquefaciens* KB3 and *B. subtilis* QST-713. Intact plants were sprayed with cell-free filtrates from *B. amyloliquefaciens* KB3 and *B. subtilis* QST-713 grown in one-third King's B (KB) at 30°C for 2 days (1:3 dilution). Water was used as the negative control. The disease index for each pathogen was based on the areas lesions on infected plants. Data are presented as the means  $\pm$  standard deviations of three independent experiments with nine plants per treatment. Bars labeled with \* for each pathosystem show significantly difference ( $P < 0.05$ ) in disease based on Student's t-test. RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mold; TLB, tomato late blight; WLR, wheat leaf rust; BPM; barley powdery mildew; PAN, red pepper anthracnose.

2001). Disease symptoms were assessed 3–7 days after inoculation, depending on the pathogen. The pots were arranged in a randomized complete-block design, with three replicates per treatment, and each replicate consisted of nine plants. The disease index was determined by measuring the percentage of the leaf area or plants that was infected. The experiment was conducted three times.

Foliar application of the KB3 bacterial culture filtrates significantly reduced disease incidence in four pathosystems, similar to the control achieved with *B. subtilis* QST-713 (Fig. 4). The preparations were least effective against tomato gray mold and tomato late blight (Fig. 4).

In conclusion our studies show that the feces of the tertiary larvae of the rhino beetle, *A. dichotoma* possess microbes with the potential for biocontrol of plant pests. Thus, these studies expand potential sources for such microbes. The KB3 strain isolated in this study showed broad-spectrum antifungal activity and disease control. Surfactin and iturin A, metabolites of importance in biocontrol, were detected. We are currently investigating the roles of CLPs in plant disease and insect control and the molecular regulatory mechanisms involved in the expression of CLP genes in the rhizosphere and insect guts.

## Acknowledgments

This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the Agri-Bio Industry Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (grant 314084-3).

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