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Production of Surfactin and Iturin by *Bacillus licheniformis* N1 Responsible for Plant Disease Control Activity

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***Bacillus licheniformis* N1, previously developed as a biofungicide formulation N1E to control gray mold disease of plants, was investigated to study the bacterial traits that may be involved in its biological control activity. Two N1E based formulations, bacterial cell based formulation PN1E and culture supernatant based formulation SN1E, were evaluated for disease control activity against gray mold disease of tomato and strawberry plants. Neither PN1E nor SN1E was as effective as the original formulation N1E. Fractionation of antifungal compounds from the bacterial culture supernatant of *B. licheniformis* N1 indicated that two different cyclic lipopeptides were responsible for the antimicrobial activity of the N1 strain. These two purified compounds were identified as iturin A and surfactin by HPLC and LC-MS. The purified lipopeptides were evaluated for plant disease control activity against seven plant diseases. Crude extracts and purified compounds applied at 500 μg/ml concentration controlled tomato gray mold, tomato late blight and pepper anthracnose effectively with over 70% disease control value. While iturin showed broad spectrum activity against all tested plant diseases, the control activity by surfactin was limited to tomato gray mold, tomato late blight, and pepper anthracnose. Although antifungal compounds from *B. licheniformis* N1 exhibited disease control activity, our results suggested that bacterial cells present in the N1E formulation also contribute to the disease control activity together with the antifungal compounds.**

Keywords **:** antifungal activity, *Bacillus licheniformis*, biofungicide, iturin, surfactin

Successful development of biofungicides depends on the selection of effective biocontrol agents and development of efficient formulations. The selection of a good bacterial strain with diverse mechanisms to reduce plant disease incidence is one of the essential steps to develop effective biofungicides. Several mechanisms of biological control including antibiotics production, successful colonization and resistance induction on host plants have been reported (Emmert and Handelsman, 1999; Handelsman and Stabb, 1996). Especially, *Bacillus* species has been widely used to control multiple plant diseases with advantage of production of several antifungal compounds and long shelf-life as a result of endospore formation by the *Bacillus* species (Emmert and Handelsman, 1999).

Many strains of *Bacillus* species have been successfully developed as effective commercial biofungicides (Schisler et al., 2004). *Bacillus subtilis* GB03 has been used for seed treatment as a liquid formulation of endospores under the trade name of Kodiak® (Gustafson, USA) to control soilborne disease (Brannen and Kenney, 1997; Mahaffee and Backman, 1993). A wettable powder type formulation, Serenade®, was produced using *B. subtilis* QST-713 to control foliar diseases of plants (AgraQuest, Davis, USA). Other strains of *Bacillus* species have been also used to control plant diseases and are in the process of commercial development (Fravel et al., 1988; Gueldner et al., 1988; Touré et al., 2004).

Bacillus strains often produce a vast array of biologically active compounds, including antibiotics which are inhibitory to plant pathogens (Emmert and Handelsman, 1999; Stein, 2005). Among the antimicrobial compounds, some cyclic lipopeptides have been described frequently from several biocontrol *Bacillus* species and have been shown to exhibit inhibitory activity against a number of plant pathogens. The three families of cyclic lipopeptides are iturin, surfactin, and fengycin (Ongena and Jacques, 2007), which are produced by *B. subtilis*, *B. amyloliquefaciens*, *B. coagulans*, *B. pumilus*, *B. licheniformis*, *B. cereus*, and *B. thuringiensis* (Bonmatin et al., 2003; Huszcza and Burczyk, 2006; Jacques et al., 1999; Kim et al., 2004; Koumoutsi et al., 2004; Peypoux et al., 1999; Tsuge et al., 1999). These lipopeptides from *Bacillus* strains are structurally related compounds with different biological activities. The biocontrol strain *B.*

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subtilis QST-713 produces more than 30 different lipopeptide variants. Recent studies have revealed that the lipopeptides also play a role to enhance bacterial colonization on plant tissues and to induce plant resistance against pathogens (Ongena and Jacques, 2007).

Previously, we have shown that a specific formulation N1E developed by using bacterial fermentation culture of *B. licheniformis* N1 was effective to control gray mold diseases in tomato (Lee et al., 2006) and strawberry plants (Kim et al., 2007). Although a chitinase gene was found in the genome of *B. licheniformis* N1 strain, the gene was not functional due to the lack of expression (Lee et al., 2009). Our previous study using the cell-free formulation suggested that the production of antifungal compounds by the N1 strain may be involved in disease control activity on tomato plants (Lee et al., 2006). In this study, we have used biocontrol strain *B. licheniformis* N1 and its biofungicide formulation N1E to further investigate the biocontrol mechanism of N1 strain.

Materials and Methods

Microorganisms and culture conditions. *B. licheniformis* N1 was routinely grown in tryptic soy broth (TSB) or agar (TSA) medium at 30°C and cultured in the medium containing 5% dried soybean curd residue in distilled water (Biji meidum) for mass production. Soybean curd residue ('Biji' in Korean) was obtained from a Korean traditional tofu factory. Dried Biji was produced to make Biji medium as previously described (Lee at al., 2006). A fungal pathogen *Botrytis cinerea* LVF12, which causes gray mold on various plants, was routinely grown on potato dextrose agar (PDA) for 15 days at 25°C under a 12-hr light period condition to trigger conidia formation. The conidia were suspended in a 30% (v/v) tomato juice solution supplemented with 0.1 M KH_2PO_4 to a concentration of 1.6×10^6 conidia/ml. Commercial tomato juice was purchased from the Gaya tomato juice company in Korea.

Preparation of N1E formulations using *B. licheniformis*. To generate formulations of *B. licheniformis* N1 using Biji broth culture (Lee et al., 2006), the 400 ml pre-cultured bacterial cells were added directly to 4 liters of Biji broth in a 7-liter jar fermenter with 10 ml of 10-fold diluted antifoam emulsion (DB-110A, Dowcorning®) and grown for three days at 300 rpm, pH 7.0 (automatic control with 1 M HCl and 1 M NaOH), at 35°C. Approximately 4×10^9 cfu/ml bacterial cultures were divided into two parts. One part was directly used to generate N1E formulation by thoroughly mixing with various materials, as previously described (Lee et al., 2006). N1E contained 400 g of corn starch, 50 ml of olive oil, and 50 g of sucrose per one liter bacterial culture. The other part was used to separate bacterial cells from the culture broth. Bacterial cells were harvested by centrifugation at 4°C for 10 min at 7,000 rpm. Bacterial pellet and culture supernatant were separated to produce different formulations. Before mixing with formulation materials, bacterial pellet was washed once with phosphate-buffered saline (PBS) and resuspended in PBS to the equivalent volume of the original culture. Both resuspended bacterial solutions and culture supernatant were separately used to generate different formulations by mixing with materials for N1E formulation. The mixtures were used to generate, PN1E and SN1E formulations, as previously described (Lee et al., 2006). Formulations, PN1E and SN1E, represent the formulations generated by using bacterial pellet suspension and culture supernatant, respectively. The formulations were stored at 4°C until use. A control formulation lacking bacterial cells was prepared with Biji broth mixed with corn starch for wettable powder (WP) formulation. Only fungal pathogen inoculation and the control formulation lacking bacterial cells were used as controls for pot experiments in a growth chamber.

Plant growth and treatment. The tomato plants and strawberry plants used in this study were the cultivar Kwang-Myeong (Dongbu HiTek Co., Korea) and the cultivar Reiko, respectively. The plants were grown in 15-cm-diameter pots containing the commercial horticulture nursery media soil (Punong Co., Ltd, Korea) in a plastic house until the four to six-leaf stages. The N1E-based formulations were diluted 100-fold with tap water before being sprayed on the plants. The fungicides used for standard chemicals treatment on tomato and strawberry plants were mixture of diethofencarb (25%) and carbendazim (25%) (Dong-Bang Agro Co., Korea) and was iprodione (Rovral®) (Bayer CropScience Co., Korea), respectively. Both fungicides were used after 1,000-fold dilution with tap water.

Pathogen inoculation and assessment of disease control. Both tomato and strawberry plants were inoculated by spraying the conidial suspension of *Bo. cinerea* LVF12 prepared in the tomato juice with 0.1 M of KH_2PO_4 onto tomato and strawberry leaves until runoff one day after chemical fungicide or microbial fungicide treatment. The plants were maintained in a controlled growth room for one day (20 ± 2°C, 90% RH) and then transferred into a plastic house (25 ± 5°C). Each treatment included 10 pots per treatment with five replications. The disease severity on plant leaves was rated on various days after pathogen inoculation. The disease severity index of gray mold on the tomato and strawberry plants was defined as the percentage of diseased leaf area, where 0 = no disease symptoms, 1 = 0.1-5%, 2 = 5.1-20%, 3 = 20.1-40%, and 4 = 40.1-100%.

The disease severity value was calculated using the following formula: Disease severity (%) = ((Σ (the number of diseased leaves × disease severity index))/(4 × the number of leaves rated)) × 100. The disease control value was calculated using the following formula: Disease control value (%) = ((A-B)/A) × 100, where A is the disease severity caused by pathogen inoculation alone and B is the disease severity after various treatments.

Data from the pot experiments were analyzed using analysis of variance (ANOVA) in a complete randomized design. Duncan's multiple range test was used to compare the means of the treatments in each experiment. All statistical analyses were conducted using SAS/STAT software (SAS Institute, 1989).

Purification of antifungal compounds. To isolate antifungal compounds from *B. licheniformis* N1, 5 liter culture of TSB was used. The N1 strain was grown in nutrient broth by shaking for 24 hr at 30°C and each 5 ml of the culture was inoculated in 500 ml of TSB and incubated at 150 rpm for 72 hr at 30°C. The culture supernatant (5 liter) was obtained by centrifugation of the bacterial culture for 20 min at 10,000 rpm and subsequently incubated overnight at 4°C after adjusting its pH to 2.0 by adding HCl. Precipitates formed in the acidified culture supernatant were harvested by centrifugation for 20 min at 10,000 rpm and subsequent washing with acidic distilled water (pH 2.0). The precipitates were dissolved in methanol and concentrated by a rotary vacuum evaporator. The overall process for the isolation of antifungal compounds is described in Fig. 1. Briefly, the residue was dissolved in methanol and subsequently applied to a silica gel column (5 cm × 60 cm; Kiesel gel 60, 500 g, 70-230 mesh) and the column was eluted with chloroform, methanol, and water (30:9:1, v/v/v) to obtain antifungal fractions from six fractions. The different fractions were further separated to isolate antifungal compounds by using another silica gel column (5 cm × 60 cm; Kiesel gel 60, 110 g, 230-400 mesh). The column was eluted with chloroform, methanol, water (30:9:1, v/v/v) and followed by a series of chromatography using C_{18} Sep-Pak columns eluted with series of diluted methanol (Fig. 1). To finally obtain purified compound 1, an additional fractionation was performed by using Sephadex LH-20 (50 g, 3.6 cm × 60 cm) gel permeation chromatography.

To analyze the acid precipitates and purified antifungal compounds, high performance liquid chromatography (HPLC) was performed with a Luna C_{18} column (Luna 5 μ C_{18}, 150 × 4.6 mm, Waters, USA) using a linear gradient for 60 min from 70% solvent A and 30% solvent B to 0%

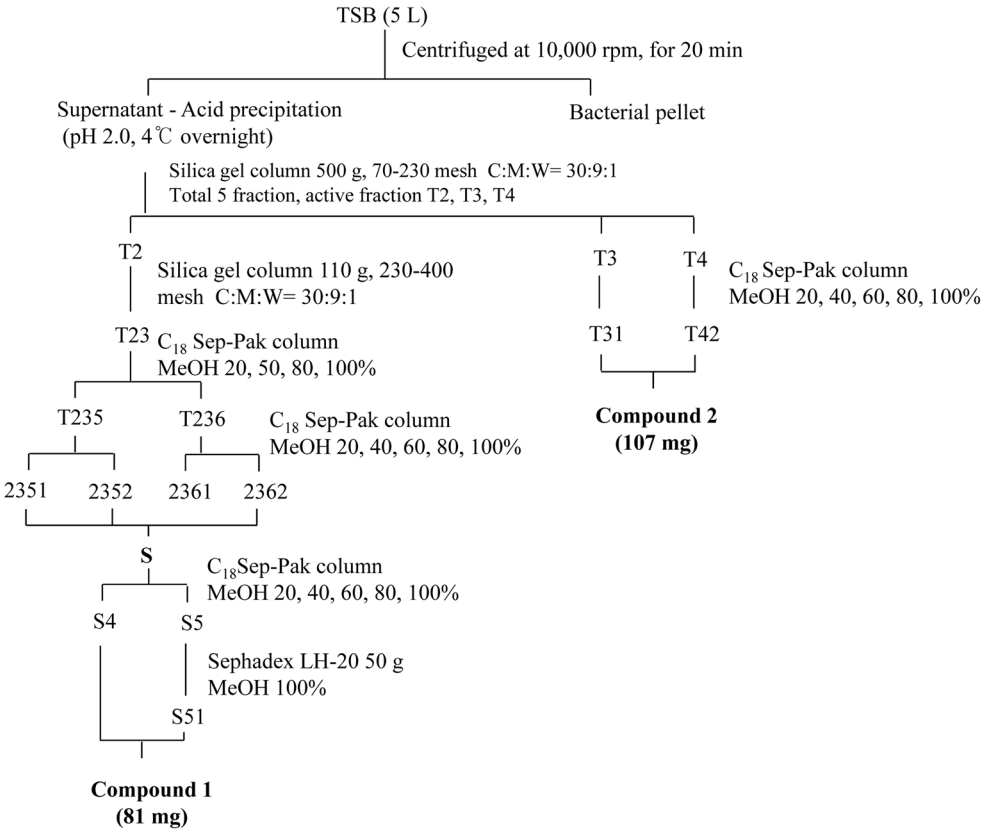

Fig. 1. Procedure for extraction and purification of compound 1 and compound 2 from tryptic soy broth cultures of *B. licheniformis* N1.

Table 1. Solvent elution program for liquid chromatography

Time (min)*	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)
0	70	30	1
50	0	100	1
60	0	100	1
63	70	30	1

*Elution was by a linear gradient from 0 to 50 min and from 60 to 63 min.

solvent A and 100% solvent B. Solvent A and solvent B consisted of mobile phases with acetonitrile : water : trifluoroacetic acid (TFA, 10 : 89.9 : 0.1) and acetonitrile : TFA (99.95 : 0.5), respectively. The crude extracts, purified compounds and standard compounds (iturin and surfactin) were dissolved in methanol to a final concentration of 1 mg/ml for HPLC application. The solvent elution program is described in Table 1 and the compounds were detected by using a charged aerosol detector (ESA Inc, UK).

***In vivo* disease control using purified compounds.** Acid precipitates of bacterial culture supernatant from *B. licheniformis* N1 and purified antifungal compounds were tested for their disease control activity against seven plant diseases such as rice blast (RCB), rice sheath blight (RSB), tomato gray mold (TGM), tomato late blight (TLB), wheat leaf rust (WLR), barley powdery mildew (BPM) and pepper anthracnose (PAN). Host plants were grown for 1 to 4 weeks in a controlled greenhouse at 25 ± 5°C and were sprayed with chemicals prepared in the solution of Tween 20 (250 μg/ml) until run-off. Chemical fungicides were treated in two concentrations to compare disease control activity as follows: Blasticidin-S for RCB, 1 and 50 μg/ml; Validamycin for RSB, 5 and 50 μg/ml; Fludioxonil for TGM, 5 and 50 μg/ml; Dimethomorph for TLB, 2 and 10 μg/ml; Flusilazole for WLR, 2 and 10 μg/ml; Flusilazole for BPM, 0.5 and 10 μg/ml; Dithianon for PAN, 10 and 50 μg/ml. The treated plants were maintained for 24 hr before pathogen inoculation and subsequently inoculated with respective pathogens for seven diseases as previously described (Cho et al., 2006; Kim et al., 2001). Pots were arranged in a completely randomized design with three replicates per treatment. Disease severity was assessed 4-7 days after pathogen inoculation as previously described (Kim et al., 2001). The disease control value was calculated using the following formula: Disease control value (%) = ((A–B)/A) × 100, where A is the disease severity triggered by Tween 20 treatment alone and B is the disease severity after various treatments, including chemical fungicides or purified antifungal compounds.

Results

Biological control activity of cell free culture supernatant. Here, we tested if the bacterial cells itself or bacterial metabolites were responsible for the biocontrol activity against gray mold disease of tomato and strawberry plants using N1E-based formulations consisting of either bacterial cells or bacterial culture supernatant. N1E itself containing both bacterial cells and metabolites exhibited the highest disease control value of 92.3% and 91.2% against

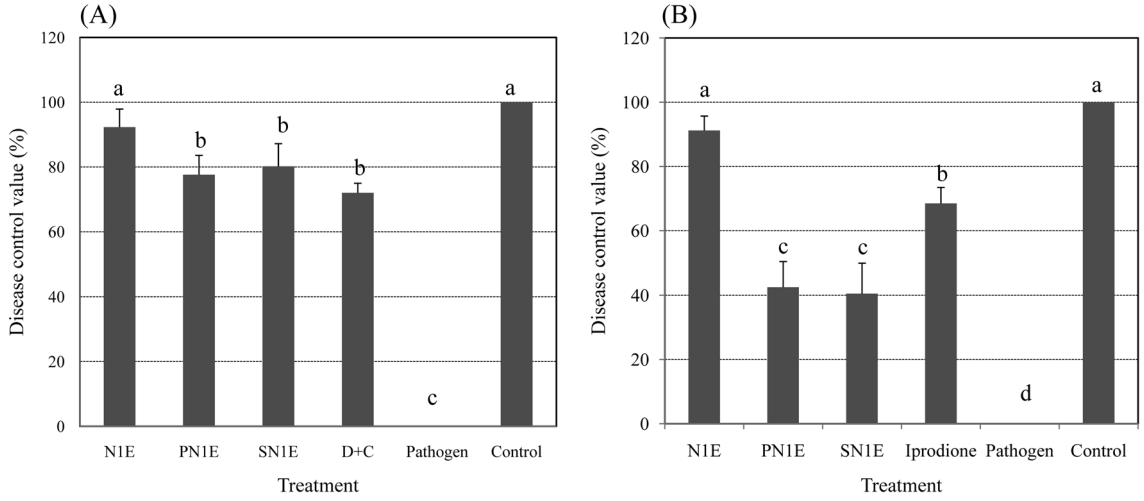

Fig. 2. Effect of N1E formulations of *Bacillus licheniformis* N1 on the control of gray mold *Botrytis cinerea* on tomato (A) and strawberry plants (B) in pots in a plastic house. PN1E and SN1E represent bacterial pellet formulation and bacterial culture supernatant formulation, respectively. D+C represents a mix of the chemical fungicides diethofencarb and carbendazim. Pathogen and control indicate artificial inoculation of the fungal pathogen and no treatment, respectively. Means with the same letter are not significantly different at 5% level by Duncan's multiple range test. Error bar represents the standard deviation of five replications.

gray mold on tomato plant and strawberry plant at the 100-fold diluted application, respectively (Fig. 2). The disease control activity was higher than chemical fungicides treated by 1000-fold dilution. Disease control value was lower when either bacterial cell formulation (PN1E) (77.6%) or culture supernatant formulation (SN1E) (80.2%) in comparison to the N1E treatment used against gray mold disease on tomato plants. Similary, disease control values of 42.5% (PN1E) or 40.5% (SN1E) were significantly lower than N1E treatment on strawberry plants. Both PN1E and SN1E exhibited the same disease control activity with chemical fungicide mixture consisting of diethofencarb and carbendazim, which was used for the treatment on tomato plants (Fig. 2A). On the other hand, disease control activity by PN1E and SN1E was significantly lower than that by iprodione treated on strawberry plants (Fig. 2B). In any case, the disease control activity by PN1E and SN1E was not significantly different from each other. The negative control formulations prepared without bacterial cells did not suppress disease development or affect plant growth.

Purification of iturin and surfactin. Antifungal components of *B. licheniformis* N1 were purified by a series of column chromatography using acid precipitates of culture supernatant. Two pure fractions appeared to be active against plant pathogenic fungi. Fractionation of antifungal compounds with ethylacetate or hexane was not successful, while acid precipitates of the culture supernatant retained antifungal activity against several plant pathogenic fungi (data not shown). Both compound 1 and compound 2 were partitioned after acid precipitation steps in the bacterial culture supernatant. We first compared the retention time of our purified compounds with standard lipopeptide compounds using HPLC. Among several lipopeptides, purified compound 1 shared the same retention time with that of surfactin standard and compound 2 shared the same retention time with that of iturin A (Fig. 3). Our further analysis of the purified compounds with LC-MS showed that both compounds had the same mass profiles as that of standard iturin A and surfactin (data not shown). In addition, HPLC chromatogram of the acid precipitates (Fig. 3A) appeared to contain major peaks corresponding to surfactin (Fig. 3B) and those corresponding to iturin (Fig. 3C). Therefore, the

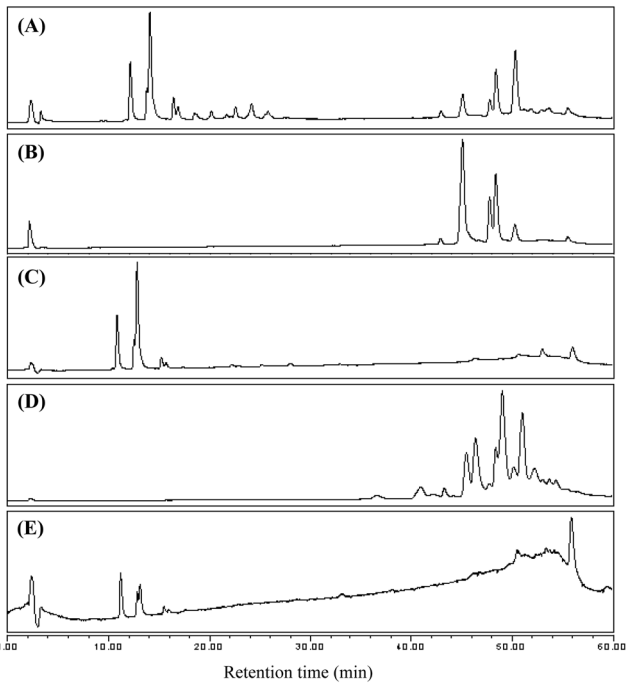

Fig. 3. HPLC chromatogram of standard compounds, crude extracts and purified compounds of *B. licheniformis* N1. (A) Acid precipitation extract obtained from liquid cultures of *B. licheniformis* N1; (B) Purified compound 1; (C) Purified compound 2; (D) Surfactin standard (Sigma); (E) Iturin A standard (Sigma).

Table 2. *In vivo* disease control activity by surfactin and iturin isolated from *B. licheniformis* N1 against seven plant fungal diseases

Treatment[a] (μg/ml)	Disease control value (%)[b]						
	RCB	RSB	TGM	TLB	WLR	BPM	PAN
Acid precipitate (500)	84±1.2	80±0	88±3.0	88±5.9	53±0.0	50±23.6	76±12.9
Acid precipitate (250)	79±5.9	40±14.1	68±15.2	42±11.8	53±0.0	58±11.8	72±8.3
Iturin (500)	96±1.2	75±7.1	94±2.0	93±2.3	43±14.1	8±11.8	94±1.2
Iturin (250)	90±0	75±7.1	91±2.1	83±0.0	33±0.0	17±23.6	66±18.9
Surfactin (500)	30±5.9	0	90±0.0	93±2.3	33±0.0	0	64±24.8
Surfactin (250)	0	5±7.1	82±5.0	75±0.0	0	8±11.8	40±11.8
Standard-high	100±0	100±0	100±0	100±0	100±0	100±0	83±10.6
Standard-low	84±1.2	75±7.1	78.6±0	93±2.3	73±9.4	83±0	25±35.4

[a] Standard-high and Standard-low represent the control chemical fungicides for respective disease sprayed at high concentration and low concentration, respectively. Each concentration is indicated in the Materials and methods section.

[b] RCB, rice blight; RSB, rice sheath blight; TGM, tomato gray mold; TLB, tomato late blight; WLR, wheat leaf rust; BPM, barly powdery mildew; PAN, pepper anthracnose. Each disease control value represents the mean ± standard deviations of three replicates.

major antifungal compounds produced by *B. licheniformis* N1 were identified as iturin A and surfactin.

Disease control activity of iturin and surfactin. Since major antifungal compounds from *B. licheniformis* N1 were identified as iturin and surfactin, we further tested if these two compounds would be effective to control major plant fungal disease *in vivo*. Acid precipitate of bacterial culture supernatants exhibited over 70% disease control value against five plant diseases such as RCB, RSB, TGM, TLB and PAN at 500 μg/ml concentration (Table 2). Application of the acid precipitate at 250 μg/ml concentration was also effective against RCB, TGM and PAN. When purified iturin and surfactin were tested for disease control activity against these seven plant disease, iturin exhibited the stable disease control activity on five plant diseases, except for WLR and BPM. However, surfactin exhibited effective disease control only against TGM, TLB, and PAN, while disease control activity against RCB, RSB, WLR and BPM was not recognized by surfactin application. Acid precipitates containing both iturin and surfactin, showed over 50% disease control activity against WLR and BPM at 250 μg/ml concentration. In contrast, individual treatment of iturin and surfactin showed decreased activity against WLR and BPM at the same concentration.

Discussion

Our previous studies have shown that N1E, a wettable powder formulation, using *B. licheniformis* N1 was the most effective to control gray mold disease on tomato and strawberry in comparison to the other tested formulations (Kim et al., 2007; Lee et al., 2006). In this study, we investigated the bacterial traits involved in biocontrol activity using an antagonistic bacterium *B. licheniformis* N1. Two formulations, PN1E and SN1E, derived from N1E were tested for disease control activity against gray mold disease on tomato and strawberry plants. Best disease control activity was observed when N1E formulation was used. The disease control activity by SN1E using bacterial culture supernatant was significantly lower than that by N1E (Fig. 1). Since PN1E formulation was as effective as SN1E to control gray mold disease, bacterial cells of N1 strain present in the formulation are probably involved in the disease control activity. Therefore, disease control activity by N1E is not solely due to antifungal compounds present in the culture supernatant and the effective disease control activity by N1E will be the consequence of synergistic effects of bacterial cells and antifungal compounds present in the culture supernatant.

The formulation PN1E using bacterial cells probably contained bacterial endospores rather than the vegetative cells. This is because we cultured the N1 strain for 3 days and subsequently proceeded to formulations steps, including drying the formulation mixture at 55°C and grinding the dried formulation (Lee et al., 2006). Therefore, most of the live bacteria would be present as endospores in the PN1E formulation. The production of endospores is one the unique characteristics in *Bacillus* species, which are advantageous for surviving in harsh environmental conditions (Emmert and Handelsman, 1999). Although it was not clear if the endospores would be effective to exhibit biological activity when applied on plant surfaces, our study suggests that endospores of *B. licheniformis* N1 present in N1E formulation might be involved in disease control by *Bo. cinerea*. Since endospores are in a dormant stage, it may be necessary that the endospores should be germinated on plant surface to exhibit its biocontrol activity. We speculate that the formulation medium provides endospores with nutrients for germination, promotes endospores to attach to plant surfaces, and protects the germinating endospores from harsh condition.

Two antifungal compounds from the N1 strain were partitioned after acid precipitation steps in the bacterial culture supernatant, suggesting that they might be lipopeptides-like antimicrobial compounds. Several antimicrobial lipopeptides have been described from *Bacillus* species, such as surfactin, iturin and fengycin (Ongena and Jacques, 2007). Our analysis of bacterial culture supernatant indicated that two cyclic lipopeptides, iturin and surfactin, are produced from the N1 strain and contribute to the biocontrol activity directly and indirectly. Production of cyclic lipopoptides in *Bacillus* species has been well described and three familes, iturin, surfactin and fengycin, have been recognized for their potential uses in biotechnology and biopharmaceutical applications (Ongena and Jacques, 2007). Production of two lipopeptides seems to confer important bacterial trait, such as efficient plant tissue colonization and direct antifungal activity, on *B. licheniformis* N1 which is effective for plant disease control. Direct antifungal activity by iturin from *B. subtilis* RB14 has been shown to suppress damping off of tomato by *Rhizoctonia solani* (Asaka and Shoda, 1996) and from other *Bacillus* species (Ongena and Jacques, 2007). Surfactin production is also beneficial to *Bacillus* species for bacterial motility and attachment on plant surfaces (Kinsinger et al., 2003; Leclère et al., 2006). Interestingly, it has been reported that the simultaneous production of surfactin and iturin exhibits synergistic effect and enhances antimicrobial activity (Maget-Dana et al., 1992). It is likely that the production of iturin and surfacin from our N1 strain confers not only antifungal activity but also advantage on ecological fitness of the N1E formulation. This speculation might explain the reason why N1E is more effective to control gray mold on tomato and straw-

berry plants than PN1E or SN1E. However, purified lipopeptides were not effective enough to explain the disease control value of acid precipitates against BPM or WLR. There may be a differential effect of lipopeptides on wheat or barley diseases. High disease control values obtained by using purified lipopeptides and crude extracts against tomato gray mold and tomato late blight suggested that our N1E formulation would be applicable to use to control major tomato fungal diseases.

Recent studies indicated that lipopeptide production in *Bacillus* species is also involved in plant induced resistance (Ongena et al., 2005: Ongena et al., 2007). It will be interesting to investigate if N1E application induces plant resistance to plant diseases. Our previous study on tomato demonstrated that N1E application promoted tomato growth and increased tomato yield (Lee et al., 2006). In conclusion, both lipopeptides produced from *B. licheniformis* N1 culture and bacterial cells, probably present as endospores in the formulation, might be responsible for biocontrol activity of N1E formulation against plant diseases.

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References

Asaka, O. and Shoda, M. 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.* 62:4081-4085.

Bonmatin, J-M., Laprevote, O. and Peypoux, F. 2003. Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. *Comb. Chem. High Throughput Screen.* 6:541-556.

Brannen, P. M. and Kenney, D. S. 1997. Kodiak®-a successful biological-control product for suppression of soil-borne plant pathogens of cotton. *J. Ind. Microbiol. Biot.* 19:169-171.

Cho, J-Y., Choi, G. J., Lee, S-W., Jang, K. S., Lim, H. K., Lim, C. H., Lee, S. O., Cho, K. Y. and Kim, J-C. 2006. Antifungal activity against *Collectotrichum* spp. of curcuminoids isolated from *Curcuma longa* L. rhizomes. *J. Microbiol. Biotechnol.* 16:280-285.

Emmert, E. A. B. and Handelsman, J. 1999. Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiol. Lett.* 171:1-9.

Fravel, D. R., Connick Jr., W. J. and Lewis, J. A. 1998. Formulation of microorganisms to control plant diseases. In: *Formulation of microbial pesticides: Beneficial microorganisms, nematodes and seed treatments,* eds by H. D. Burges, pp. 187-202. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Gueldner, R. C., Reilly, C. C., Pusey, P. L., Costello, C. E., Arrendale, R. F., Cox, R. H., Himmelsbach, D. S., Crumley, F. G. and Cutler, G. 1988. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. *J. Agric. Food Chem.* 36:366-370.

Handelsman, J. and Stabb, E. V. 1996. Biocontrol of soilborne plant pathogens. *Plant Cell* 8:1855-1869.

Huszcz, E. and Burczyk, B. 2006. Surfactin isoforms from *Bacillus coagulans*. *Z. Naturforsch. [C]* 61:727-733.

Jacques, P., Hbid, C., Destain, J., Razafindralambo, H., Paquot, M., De Pauw, E. and Thonart, P. 1999. Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett-Burman design. *Appl. Biochem. Biotechnol.* 77:223-233.

Kim, H. J., Lee, S. H., Kim, C. S., Lim, E. K., Choi, K. H., Kong, H. G., Kim, D. W., Lee, S-W. and Moon, B. J. 2007. Biological control of strawberry gray mold caused by *Botrytis cinerea* using *Bacillus licheniformis* N1 formulation. *J. Microbiol. Biotechnol.* 17:438-444.

Kim, J-C., Choi, G. J., Kim, H-J., Kim, H. T., Ahn, J. W. and Cho, K. Y. 2001. Activity against plant pathogenic fungi of phomalactone isolated from *Nigrospora sphaerica*. *Pest. Manag. Sci.* 60:803-808.

Kim, P. I., Bai, H., Bai, D., Chae, H., Chung, S., Kim, Y., Park, R. M. and Chi, Y.-T. 2004. Purification and characterizatio of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *J. Appl. Microbiol.* 97:942-949.

Kinsinger, R. F., Shirk, M. C. and Fall, R. 2003. Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. *J. Bacteriol.* 185:5627-5631.

Koumoutsi, A., Chen, X-H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J. and Borriss, R. 2004. Structural and functional characterization of gene clusters directing nonribiosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *J. Bacteriol.* 186:1084-1096.

Leclère, V., Marti, R., Béchet, M., Fickers, P. and Jacques, P. 2006. The lipopeptides mycosubtilin and surfactin enhance spreading of *Bacillus subtilis* strains by their surface-active properties. *Arch. Microbiol.* 186:475-483.

Lee, K. Y., Heo, K. R., Choi, K. H., Kong, H. G, Nam, J., Yi, Y. B., Park, S. H., Lee, S-W. and Moon, B. J. 2009. Characterization of a chitinase gene exhibiting antifungal activity from a biocontrol bacterium *Bacillus licheniformis* N1. *Plant Pathol. J.* 25:344-351.

Lee, J. P., Lee, S.-W., Kim, C. S., Son, J. H., Song, J. H., Lee, K. W., Kim, H. J., Jung, S. J. and Moon, B. J. 2006. Evalution of formulations of *Baillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. *Biol. Control* 37: 329-337.

Maget-Dana, R., Thimon, L., Peypoux, F. and Ptak, M. 1992. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. *Biochimie* 74:1047-1051.

Mahaffee, W. F. and Backman, P. A. 1993. Effects of seed factors on spermosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. *Phytopathology* 83:1120-1125.

Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J. and Thonart, P. 2005. *Bacillus subtilis* M4 decreases

plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. *Appl. Microbiol. Biotechnol.* 67:692-698.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J.-L. and Thonart, P. 2007. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environ. Microbiol.* 9:1084-1090.

Ongena, M. and Jacques, P. 2007. *Bacillus* lipopeptides: versatile weapons for plant disease control. *Trends Microbiol.* 16:115-125.

Peypoux, F., Bonmatin, J.-M. and Wallach, J. 1999. Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol.* 51:553-563.

Schisler, D. A., Slininger, P. J., Behle, R. W. and Jackson, M. A. 2004. Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology* 94:1267-1271.

Stein, T. 2005. *Bacillus subtilis* an antibiotics: structure, syntheses and specific functions. *Mol. Microbiol.* 56:845-857.

Touré, Y., Ongena, M., Jacques, P., Guiro, A. and Thonart, P. 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. *J. Appl. Microbiol.* 96:1151-1160.

Tsuge, K., Ano, T., Hirai, M., Nakamura, Y. and Shoda, K. 1999. The genes *degQ*, *pps*, and *lpa-8 (sfp)* are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. *Antimicrob. Agents Chemother.* 43:2183-2192.