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Biological Control of Strawberry *Fusarium* Wilt Caused by *Fusarium oxysporum* f. sp. *fragariae* Using *Bacillus velezensis* BS87 and RK1 Formulation

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Two isolates, *Bacillus* sp. BS87 and RK1, selected from soil in strawberry fields in Korea, showed high levels of antagonism towards *Fusarium oxysporum* f. sp. *fragariae* in vitro. The isolates were identified as *B. velezensis* based on the homology of their *gyrA* sequences to reference strains. BS87 and RK1 were evaluated for control of *Fusarium* wilt in strawberries in pot trials and field trials conducted in Nonsan, Korea. In the pot trials, the optimum applied concentration of BS87 and RK1 for pre-plant root-dip application to control *Fusarium* wilt was 10^5 and 10^6 colony-forming units (CFU)/ml, respectively. Meanwhile, in the 2003 and 2005 field trials, the biological control efficacies of formulations of RK1 were similar to that of a conventional fungicide (copper hydroxide) when compared with a non-treated control. The RK1 formulation was also more effective than BS87 in suppressing *Fusarium* wilt under field conditions. Therefore, the results indicated that formulations of *B. velezensis* BS87 and RK1 may have potential to control *Fusarium* wilt in strawberries.

Keywords: *Bacillus velezensis*, biological control, *Fusarium* wilt, strawberry

Strawberries (*Fragaria×ananassa* Duch.) are an important fruit in Korea, with an annual revenue of approximately US$680 million in 2006. The strawberry production area in Korea also ranks 8th in the world at 6,480 ha, while the fresh market production totals 201,995 M/T [2].

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *fragariae* Winks and Williams, is the most serious soil-borne disease affecting strawberries in Korea. Despite that outbreaks occurred in nursery fields of cv. Hokowase in the

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1970s and 1980s [3, 15], it was not subsequently observed after the introduction of cv. Nyoho from Japan. However, cvs. Maehyang and Tochiotome, recently released from Korea and Japan, respectively, are susceptible to *Fusarium* wilt, and transplant production losses can be as high as 30% in the case of a severe epidemic [23].

Fusarium can infect roots from the soil. In addition, transplants are infected through runners from infected mother plants [21]. Therefore, management strategies for *Fusarium* wilt epidemics rely on producing disease-free plants and applying soil fumigants. However, the production of disease-free plants is time- and labor-intensive.

The soil fungus *Trichoderma harzianum* has been reported to be effective for the biological control of *Fusarium* wilt in strawberries [22]; however, it is not yet commercially available in Korea. Furthermore, despite a long history of using biological control for *Fusarium* wilt, most control methods are either ineffective or difficult to apply [1]. As such, beneficial fungi and bacteria represent an attractive alternative to the use of fumigants, and are better for the environment [6]. *Fusarium* wilt suppression has been attempted using mycoparasitic fungi [22, 26], nonpathogenic *F. oxysporum* [11, 14, 33], vesicular-arbuscular mycorrhizal (VAM) fungi [8], and antagonistic bacteria [9, 30]. In addition, various commercial products have been evaluated, including Serenade (*Bacillus subtilis* QST 713) and BioYield (*B. amyloliquefaciens* GB 99+*B. subtilis* GB 122) [31].

B. velezensis sp. nov. was recently isolated during a research program focused on discovering novel bacterial strains capable of synthesizing new lipopeptides with surfactant and/or antimicrobial activity [27]. However, the ability of *B. velezensis* as a biological control for *Fusarium* wilt was not tested. Accordingly, the objective of the present research was to assess the ability of *B. velezensis* to suppress *Fusarium* wilt in strawberries in pot and field trials.

MATERIALS AND METHODS

Bacterial Strains

A total of 110 bacterial strains were isolated from soil in strawberry fields. Ten g of each soil sample was mixed with 50 ml of sterilized distilled water in a 250-ml flask. The bacterial colonies were isolated after 48 h of incubation of 100 µl of the suspension on nutrient agar (NA; Difco, Detroit, MI, U.S.A.) plates at 30°C. The bacterial strains were maintained on an NA plate. To evaluate their antagonistic activity against *F. oxysporum* f. sp. *fragariae*, 5-mm-diameter mycelial disks containing an isolate of the pathogen and bacterial isolate were placed 4 cm apart from each other on a potato dextrose agar (PDA; Difco, Detroit, MI, U.S.A.). The inhibition zones of mycelial growth of *F. oxysporum* f. sp. *fragariae* were measured after 7 days of incubation in an incubator at 25°C. The bacterial strains with inhibition zones >20 mm in diameter were selected for further evaluation and stored in 10% glycerol at -82°C.

Identification of Antagonistic Bacteria in Pot Trials

Isolates BS87, RK1, and BS36, which showed potential as biological control agents, were identified based on their phenotypic characteristics, as previously described by the Bacteriology Committee of the American Phytopathological Society [29], and a sequence analysis of their 16S rRNA, *gyrA*, and *recA* genes. The DNA extraction and purification were conducted according to a previously established protocol [16]. The 16S rRNA genes of strains BS87, RK1, and BS36 were amplified using universal primers 27f/1492r [19], as described previously [16]. The *gyrA* genes were amplified, as previously described, using the primer pairs p-gyrA-f (5'-CAG TCA GGA AAT GCG TAC GTC CTT-3') and p-gyrA-r (5'-CAA GGT AAT GCT CCA GGC ATT GCT-3') to identify the *Bacillus* spp. [5]. Isolate BS36 was also analyzed based on its *recA* sequence (BCR1 primer 5'-TGA CCG CCG AGA AGA GCA A-3' and BCR2 primer 5'-CTC TTC TTC GTC CAT CGC CTC-3') to identify the *Burkholderia* spp. [20]. The resulting amplicons were purified using Wizard PCR Prep Kits (Promega, Madison, WI, U.S.A.), and sequenced in both directions using an ABI 310 automated DNA sequencer and BIG-dye cyclic sequencing kits (PE Biosystems, Foster City, CA, U.S.A.), following the manufacturer's instructions. The same primers were used for the sequencing reactions. The resulting sequences were aligned manually with representative sequences of *B. subtilis* and related taxa obtained from the GenBank database. A phylogenetic tree based on the *gyrA* gene sequences was inferred using the neighbor-joining method [28]. The evolutionary distance matrices for the neighbor-joining method were generated according to the Kimura's 2-parameter distance model [17]. The resultant neighbor-joining tree topology was evaluated by a bootstrap analysis [10] based on 1,000 resampled data sets. The alignment and phylogenetic analyses were carried out using the PHYDIT program, version 3.1 [4].

Application of Antagonistic Bacteria

The antagonistic bacteria were applied using the root-dip method as follows. A pathogenic isolate of *F. oxysporum* f. sp. *fragariae* Fo47 [23] was cultured in a 200 rpm shaking incubator at 28°C for 1 week in 50 ml of a basal liquid medium in a 250-ml flask [7], and then 1×10⁶ conidia/ml was used to inoculate a commercial horticulture nursery substrate (Seoul Agricultural Materials Co., Ltd.; cocopeat: peatmoss:vermiculite:perlite:zeolite=68−73:10−14:6−8:7−10:2−4).

The commercial horticulture nursery substrate was inoculated by applying 50 ml of the conidial suspension per plastic pot (16× 13 cm) as a drench. Tissue-cultured plantings of strawberry cv. Tochiotome, which is highly susceptible to *Fusarium* wilt, were used in the experiment. The roots of the transplants were dipped for 30 min in a bacterial suspension of *B. velezensis* strains BS87 and RK1 at a concentration of 10⁵, 10⁶, or 10⁷ colony-forming units (CFU)/ml, and then the plants were planted in pots containing infested substrates. A copper hydroxide fungicide (Kocide 77WP, Dongbu HiTek, Seoul, Korea) at 1 g/l, which is registered for use with strawberries in Korea, was applied to the control pots as a 100-ml drench per plant.

The experimental design was completely randomized: the pots were located on a greenhouse bench, with 12 plants per treatment. The temperature in the greenhouse ranged from 15 to 20°C at night and 25 to 30°C during the day. After 55 days of treatment, the disease development was rated using the following disease index: 0=no symptoms, 1= 1–2 leaves rolled and yellowed leaves, 2=all leaves rolled and deformed, 3=chlorosis and early plant wilting, 4= necrosis and entire plant wilting, and 5=dead [23].

Formulation of Antagonistic Bacteria

Two or three days before use, the *B. velezensis* isolates BS87 and RK1 were recovered from storage by streaking partially thawed glycerol onto a 1/5 strength tryptic soy broth agar (TSBA/5; Difco, Detroit, MI, U.S.A.). The cells were then restreaked onto TSBA/5, incubated at 28°C for 18 h, removed from the agar surface using sterile cotton swabs, and suspended in a 0.1 M PO₄ (potassium phosphate) buffer. Thereafter, 50 ml of 24-h-old washed cells in a 125-ml flask was used to inoculate 200 ml of a tryptic soy broth (TSB/5) in a 500-ml flask [32], followed by incubation at 25°C for 96 h on a 180 rpm shaker incubator (Model HB-201SF; Hanbaek Scientific Technology, Bucheon, Korea). The *B. velezensis* cells were then pelleted by centrifugation (Model Supra22K; Hanil Science Industrial, Incheon, Korea) of the culture broth at 15,000 ×g for 10 min and washed once in a 0.1 M PO₄ buffer. Prior to use, the slightly turbid suspensions of antagonist cells were adjusted to an optical density (3×10⁸ CFU/ml) of 0.170 at a 620 nm wavelength.

One kg of kaolin powder (Duck Yu Ceramics, Seongnam, Korea) was spread on a sterilized metal tray and its pH adjusted to neutral by adding CaCO₃ at 15 g/kg. Ten g of carboxy methyl cellulose (CMC; Zhengzhou Bestchem Imp. & Exp. Co., Ltd., Zhengzhou, China) was then added to the 1 kg of kaolin, mixed well, and the mixture autoclaved for 30 min on two consecutive days. Next, 400 ml of the 96-h-old bacterial suspension containing 9×10⁸ CFU/ml was thoroughly and aseptically mixed by hand with the carrier-cellulose mixture. After drying to a 35% moisture content overnight under aseptic conditions, the product was packed in polypropylene bags, and then sealed and stored at room temperature (25±2°C). At the time of application, the populations of BS87 and RK1 bacteria in the kaolin powder formulation were 1×10⁵ and 10⁶ CFU/g, respectively.

Effect of Bacterial Formulation on *Fusarium* Wilt in Field Trials

The formulations of antagonistic bacteria were tested against *Fusarium* wilt in strawberries in a loam soil (clay 16%; sand 38%; silt 46%) at the Nonsan Strawberry Experiment Station, Korea, in 2003 and 2005. The soil was artificially infested with the *F. oxysporum* f. sp. *fragariae* Fo47 isolate to obtain a final density of 1×10⁵ conidia/g of soil by thoroughly mixing with a shovel. The roots of tissue-cultured transplants of cv. Tochiotome at the 3-leaf

stage were dipped for 30 min in a 1,000-fold dilution of a formulation of antagonistic bacteria before transplanting on 20 September 2003 and 9 July 2005, plus the same formulations were used to drench the soil twice at 100 ml per plant at 7-day intervals. Each bed contained two rows of plants spaced 20 cm apart within a row, and each treatment subplot was 10 m long. The experiment was conducted using a completely randomized block design with three replications. The treatments consisted of two bacteria formulations, a fungicide (copper hydroxide), and non-treated control. The incidence of diseased plants was assessed as the number of diseased plants/the number of total plants × 100, on 25 November 2003 and 10 September 2005.

Effect of Bacterial Formulation on Plant Growth

Strawberry plugs (cv. Maehyang) were planted in raised beds on 27 September 2003 and 2004 in a plastic house. Each bed contained two rows of plants spaced 20 cm apart within a row, and each treatment subplot was 4 m long. The formulations of antagonistic bacteria were applied 3 times at 10-day intervals, starting on 8 January 2004 and 1 December 2005. The plant height, leaf length, crown diameter, chlorophyll content (with SPAD; Milota Co. Ltd., Osaka, Japan), and fresh weight were all examined on 11 February 2004 and 4 January 2005. The experiment was conducted using a completely randomized block design with three replications.

Data Analysis

The data on the *Fusarium* wilt incidence and plant growth were subjected to an analysis of variance (ANOVA), and the means were separated using Fisher's protected least significant difference (LSD) test at $P=0.05$ (SAS Institute, Inc., Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

All three bacterial isolates (BS36, BS87, and RK1) inhibited the *in vitro* mycelial growth of *F. oxysporum* f. sp. *fragariae*, creating inhibition zones of more than 20 mm after 7 days of incubation at 25°C. Isolates RK1 and BS87 showed an inhibition zone of 36 mm and 22 mm, respectively (data not shown). As such, isolate RK1 exhibited the highest level of *F. oxysporum* f. sp. *fragariae* growth inhibition.

Strains BS87 and RK1 exhibited a phenotypic similarity with *Bacillus* spp., whereas strain BS36 exhibited a phenotypic similarity with *Burkholderia* spp., based on their biochemical, morphological, and cultural characteristics (Park and Nam, unpublished data). In the 16S rDNA sequence analysis, strains BS87 and RK1 formed a monophyletic group with species of the *B. subtilis* complex group, and shared a 99% sequence similarity with the same group (Park and Nam, unpublished data). In addition, the sequence analysis of the *gyrA* genes revealed that strains BS87 and RK1 shared a 99.9% sequence similarity with each other. These strains also shared similarity values of 98.9–99.0% with the *B. velezensis* type strains, and similarity values of between 81.4% and 95.8% with other validly described strains from the *B. subtilis* complex group (Fig. 1). Strain BS36 shared a similarity value of 99% with the *B. cepacia* complex group based on the *recA* sequences (Park, unpublished data).
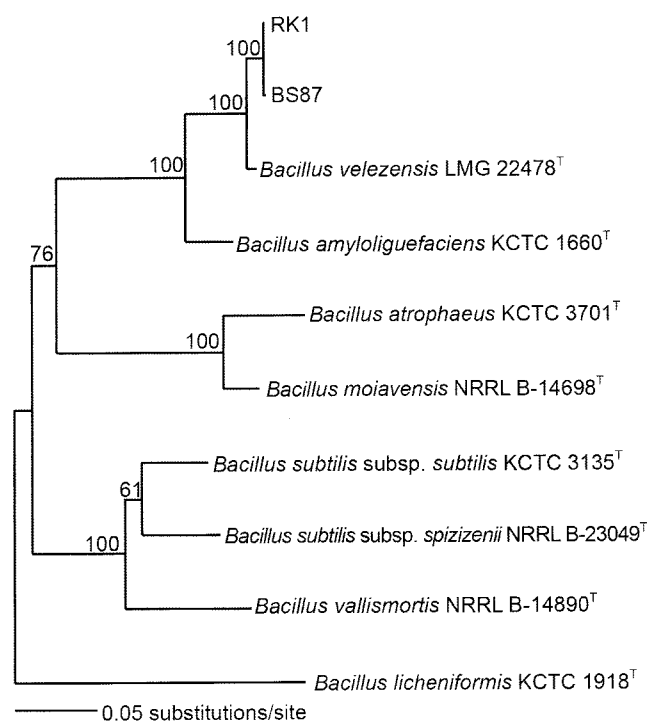

Fig. 1. Neighbor-joining tree based on nearly partial *gyrA* gene sequences showing relationships among strains BS87 and RK1 and species of the *Bacillus subtilis* complex group.

The percentage numbers above each branch indicate the levels of bootstrap support (>50%) for the branch point based on 1,000 resamplings. The bar represents 0.05 substitutions per site.

Thus, based on the sequence analysis of *gyrA* and *recA*, strains BS87 and RK1 were identified as *B. velezensis*, whereas strain BS36 was identified as *B. cepacia*. Recently, partial *gyrA* sequences, coding for DNA gyrase subunit A, was found to provide a firm framework for the rapid and accurate classification and identification of *B. subtilis* and related taxa [5]. *B. cepacia* has been reported to be an important pathogen for humans, especially in hospitals with cystic fibrosis patients [25], plus previous experiences with *B. cepacia* have demonstrated the danger of its application [13]. Consequently, BS36 was rejected for commercialization as a biocontrol fungicide. Thus, based on the results of the present study, *B. velezensis* strains BS87 and RK1 would appear to have potential as microbial antagonists against *Fusarium* wilt in strawberries.

In pot trials, BS87 and RK1 reduced the disease index of *Fusarium* wilt when compared with the non-treated control (Fig. 2). When using BS87, the disease index increased when increasing the inoculum density of this bacterium, although the disparity was not significantly different. In contrast, RK1 produced the lowest disease index at the highest feasible concentration, 10^6 CFU/ml. Thus, to increase the control of *Fusarium* wilt in strawberries, it is suggested that antagonistic bacteria should be applied based on an optimal application concentration.
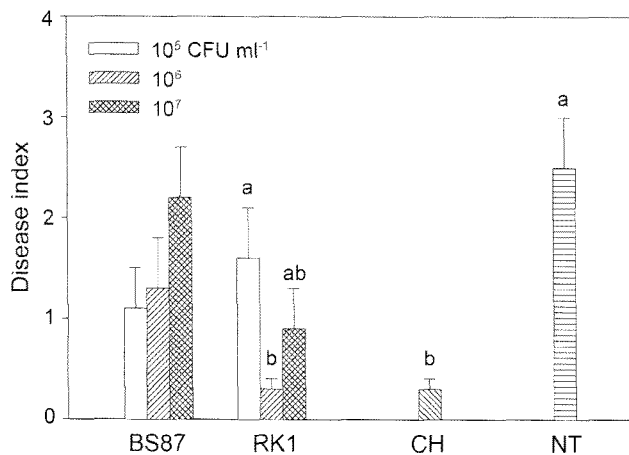
Fig. 2. *Fusarium* wilt severity resulting from treatment with different concentrations of *Bacillus velezensis* BS87 and RK1 in the greenhouse.

Disease index: 0=no symptoms, 1=1–2 leaves rolled and yellowed leaves, 2=all leaves rolled and deformed, 3=chlorosis and early plant wilting, 4=necrosis and entire plant wilting, 5=death. The disease index was rated 55 days after inoculation with *Fusarium oxsporum* f. sp. *fragariae*. CH=copper hydroxide (×1,000), and NT=non-treated control. Treatments with the same letters are not significantly different according to Fisher's protected LSD test ($P \leq 0.05$). Error bars represent ±1 SEM.

Fig. 3. Effect of *Bacillus velezensis* BS87 and RK1 on *Fusarium* wilt in strawberries in 2003 and 2005 field trials in Nonsan District, Korea.

The disease incidence was calculated as the number of diseased plants/total number of plants ×100. Treatments with the same letters are not significantly different according to Fisher's protected LSD test ($P \leq 0.05$). NT=non-treated control. Error bars represent ±1 SEM.

In the 2003 and 2005 field trials, the RK1 and BS87 formulations reduced the disease severity compared with that in the non-treated control, and the differences were significant according to Fisher's protected LSD test ($P \leq 0.05$) (Fig. 3). In addition, in the plant growth trials, the aboveground growth rates with the bacteria-amended treatments were similar to those for the non-treated control plants, although the fresh weights with the BS87 and RK1 treatment were greater than those for the non-treated control plants in 2003–2004 and 2004–2005 ($P \leq 0.05$) (Table 1). It is possible that BS87 may include plant-growth-promoting substances affecting the plant yields [18, 24]. Such substances have been reported to promote plant growth through the fixation of atmospheric nitrogen, production of siderophores, solubilization of minerals,
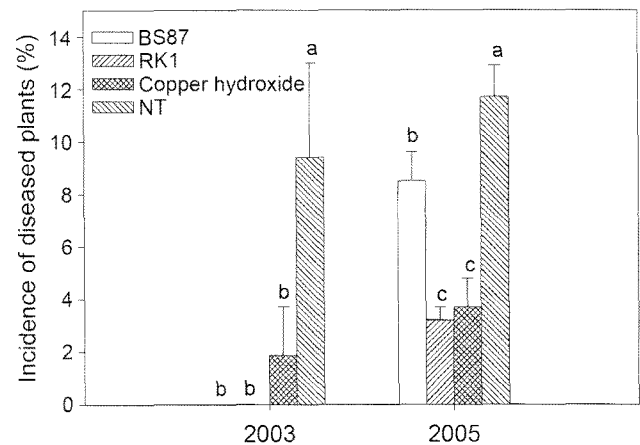
and synthesis of phytohormones [12]. Thus, further research is needed to understand the interaction of biocontrol agents and plant growth in the field.

In conclusion, this study demonstrated that the formulations of *B. velezensis* BS87 and RK1 reduced the incidence of *Fusarium* wilt in field trials. Consequently, these formulations have potential commercial use for reducing *Fusarium* wilt in strawberries. In addition, these biofungicides also need to be tested in conjunction with other management tactics, including conventional fungicides, disease-free plants, and soil solarization.

Acknowledgment

Table 1. Effect of antagonistic bacteria on growth characteristics of strawberry "Maehyang" in 2003–2004 and 2004–2005.[a]

Treatment	Plant height (cm)	Leaf width (cm)	Number of leaves	Crown diameter (mm)	Chlorophyll (SPAD[d])	Fresh weight (g/plant)
2003-2004						
BS87	21.9 a[c]	6.1 a	6.2 a	12.3 a	51.8 a	26.0 a
RK1	18.9 a	5.2 a	5.1 a	11.7 a	53.3 a	22.2 ab
Control[b]	18.6 a	5.3 a	5.4 a	12.8 a	54.1 a	20.6 b
2004-2005						
BS87	20.7 a	5.6 a	4.5 a	10.7 a	50.1 a	23.2 a
RK1	18.3 a	4.9 a	4.2 a	9.7 a	52.2 a	20.6 a
Control	17.7 a	4.8 a	4.6 a	9.2 a	51.2 a	19.0 a

[a]Plant parameters were measured on 11 February 2004 and 4 January 2005 after 3 treatments with antagonistic bacterial formulations.
[b]Control=untreated control.
[c]Numbers followed by the same letter within each column are not significantly different according to Fisher's protected LSD ($P \leq 0.05$).
[d]SPAD=Minolta SPAD-502 chlorophyll meter values.

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