Application of Rhizobacteria for Plant Growth Promotion Effect and Biocontrol of Anthracnose Caused by *Colletotrichum acutatum* on Pepper

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In vitro and greenhouse screening of seven rhizobacterial isolates, AB05, AB10, AB11, AB12, AB14, AB15 and AB17, was conducted to investigate the plant growth promoting activities and inhibition against anthracnose caused by *Colletotrichum acutatum* in pepper. According to identification based on 16S rDNA sequencing, the majority of the isolates are members of *Bacillus* and a single isolate belongs to the genus *Paenibacillus*. All seven bacterial isolates were capable of inhibiting *C. acutatum* to various degrees. The results primarily showed that antibiotic substances produced by the selected bacteria were effective and resulted in strong antifungal activity against the fungi. However, isolate AB15 was the most effective bacterial strain, with the potential to suppress more than 50% mycelial growth of *C. acutatum* in vitro. Moreover, antibiotics from *Paenibacillus polymyxa* (AB15) and volatile compounds from *Bacillus subtilis* (AB14) exerted efficient antagonistic activity against the pathogens in a dual culture assay. In vivo suppression activity of selected bacteria was also analyzed in a greenhouse with the reference to their prominent *in vitro* antagonism efficacy. Induced systemic resistance in pepper against *C. acutatum* was also observed under greenhouse conditions. Where, isolate AB15 was found to be the most effective bacterial strain at suppressing pepper anthracnose under greenhouse conditions. Moreover, four isolates, AB10, AB12, AB15, and AB17, were identified as the most effective growth promoting bacteria under greenhouse conditions, with AB17 inducing the greatest enhancement of pepper growth.

KEYWORDS : Antagonistic activity, Biocontrol, *Colletotrichum acutatum*, Pepper (*Capsicum annuum*), Plant growth-promoting rhizobacteria

Introduction

Anthracnose diseases caused by the fungal species *Colletotrichum acutatum*, *C. gloeosporioides* and *C. coccodes* have been identified as fatal in pepper in Korea [1]. Among these species, *C. gloeosporioides* has been considered a major pathogen that causes disease; however, according to recent findings *C. acutatum* is a dominant species responsible for typical anthracnose lesions on pepper fruit in Korea and other countries [2-6]. Control of these fungal pathogens is largely based on genetic resistance in the host plant, management of the plant and its environment, and synthetic pesticides [7]. However, chemical control measures create imbalances in the microbial community, which may be unfavorable to the activity of beneficial organisms and could lead to the development of resistant strains of pathogens [8].

Bioculture using plant growth promoting rhizobacteria (PGPR) may represent a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops [9].

PGPR are generally considered a heterogeneous group of bacteria that live in the plant rhizosphere, where they contribute to plant growth and improve stands under stress conditions [10, 11]. PGPR can improve growth through various mechanisms and have been introduced to soil, seeds or roots to enhance plant growth and health [12]. Antagonistic activities in interspecies leading to biological control of plant pathogens have also been described [13]. In general, the relationship between two species involves antagonistic activity in which each adversely affects the other [14]. The present study was conducted to investigate seven rhizobacterial isolates belonging to species of *Bacillus* and *Paenibacillus* for their control efficacy against *C. acutatum* and for their multiple plant growth-promoting activities based on *in vitro* and *in vivo* screening. We also discussed the antagonistic activity of *Bacillus* spp. and *Paenibacillus* sp. against pepper anthracnose under greenhouse conditions as well as their efficacy against the mycelial growth of selected fungi with respect to controls. The data generated by this study have the potential to enable development of...
isolates as microbial pesticides.

**Materials and Methods**

**Soil sample collection.** Soil samples were randomly collected from various parts of Gangwon province, Korea. Several diverse habitats in different areas were selected for the soil sample collection, which included the rhizosphere of plants, agricultural soil, preserved areas and forest soils. The samples were collected from depths of up to 20 cm after removing approximately 3 cm of the soil surface. Samples were placed in sterilized polythene bags and then taken to the laboratory. Upon arrival in the laboratory, the samples were dried for four days, after which large roots and stones were removed and the soil was sieved through an autoclave-sterilized brass sieve with a 2 mm aperture size and stored at 4°C until further examination.

**Isolation of bacteria.** Soil dilutions were prepared by suspending 1 g of aliquots of each soil sample in 99 mL of sterile distilled water and shaking vigorously for 2 min. The soil suspensions were then serially diluted in sterile distilled water, after which 1 mL of each 10\(^{-1}\) and 10\(^{-6}\) dilution was plated on sterile tryptic soy agar (TSA) plates [15]. The plates were then incubated at 28°C for 12-14 days, after which colonies with different morphological appearances were selected from the countable plates and re-streaked onto a new plate of the same media to obtain pure colonies. For long term storage, bacterial strains were maintained at −80°C in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 20% glycerol.

**Fungi and cultural conditions.** The fungal pathogen *C. acutatum* used in this study was obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Korea. Fungal isolates were maintained on potato dextrose agar (PDA; Difco) plates and incubated at 28°C. After several days, actively growing mycelia were selected from plates, again sub-cultured in their respective freshly prepared agar media and then incubated at their optimum growth temperature for further use.

**In vitro inhibition assay.** A dual culture inhibition assay was conducted on PDA-TSA (1:1 v/v) medium. Agar plugs containing fungi from the margin of young colonies and 20 µL of bacteria were placed on dual medium plates about 4.5 cm apart from each other and assessed for about 15 days during incubation at 28°C to observe the zone of inhibition between the pathogens and bacteria. After incubation, suppression of fungal growth was measured as the distance of the clear zone between the bacterial colony and each fungus. Zones of inhibition indicated antifungal activity and the strains were ranked according to the inhibition zone. The inhibition rate was expressed relative to a control strain on the same plate. Five replicates were run for each pairing. The isolates showing the greatest inhibitory activity were then selected as bacteria with a potential antagonistic effect against the fungi. The radii of the fungal colonies toward and away from the bacterial colonies were also noted. The percentage growth inhibition was defined using the following equation:

\[
\text{Inhibition (\%) = \left( \frac{R - r}{R} \right) \times 100}
\]

where, \(r\) is the radius of the fungal colony opposite the bacterial colony and \(R\) is the maximum radius of the fungal colony away from the bacterial colony.

**Identification of bacterial isolates based on 16S rDNA homology.** DNA was extracted from the bacterial cells by harvesting 10 mL of cells following overnight culture and lysing them in 1 mL lysis buffer (25% sucrose, 20 mM ethylenediaminetetraacetic acid, 50 mM Tris-HCl and 5 mg/mL of lysozyme). Chromosomal DNA was extracted according to the procedure described by Weisburg et al. [16]. The 16S rDNA was amplified using PCR with the universal primers 27F and 1492R [17]. PCR was performed in a thermocycler by subjecting the samples to 35 cycles of 94°C (45 sec), 55°C (60 sec), and 72°C (60 sec) followed by final extension at 72°C for 7 min. The PCR products were then removed using a Montage PCR Clean up kit (Millipore, Billerica, MA, USA). Purified PCR products of approximately 1,400 bp were subsequently sequenced using universal primers 518F and 800R (Macrogen Inc., Seoul, Korea) and a big dye terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA). Sequencing products were resolved on an Applied Biosystems 3730XL automated DNA sequencing system (Applied Biosystems) at Macrogen Inc. The sequences of isolates were identified using the BLAST program (http://www.ncbi.nlm.nih.gov/Blast/).

**Greenhouse experiments: plant growth, inoculation and disease assessment.** Pepper (*Capsicum annuum* c.v. Manata) seeds were surface sterilized for 20 min in 20% sodium hypochlorite followed by a brief rinse with 70% methanol, after which they were air dried on the sieve for 24 hr at room temperature. The seeds were then sown in a plastic tray (55 cm × 35 cm × 15 cm) containing commercial soil (Baroker; Seoul Bio Co., Ltd., Eumseong, Korea) with 10% perlite (Parat; Sam Son, Ansan, Korea). Seedlings at the two-leaf stage were transplanted to plastic pots (5 cm × 15 cm × 10 cm) containing the same commercial soil. Pepper plants were raised in a greenhouse under 16 hr day illuminations at 28 ± 2°C and subsequently used for the disease inoculum and biocontrol assay.

*C. acutatum* (KACC 40042) was cultured on PDA (Difco) at 28°C for 10 days. A conidial suspension of *C. acutatum* was then prepared as follows. Fungal isolates...
were grown on Petri plates (90 mm diameter) containing 15 mL of PDA under constant fluorescent light. After 10 days of incubation at 28°C, the conidial suspensions were collected by scraping the colony surface with a sterile scalpel and 10 mL of sterile distilled water, after which they were filtered through four layers of cheesecloth to remove any mycelial debris, and spores were counted with a hemocytometer and adjusted to 2.1 × 10⁶ conidia/mL. The pepper plants were then inoculated according to the method described by Oh et al. [1], with minor modifications. The control plants were inoculated with sterilized water.

For the disease assessment, suspensions of conidia and microbial cells of C. acutatum in 0.04% Tween 80 were sprayed onto six seedlings of pepper plants until it ran off (5 mL/seedling). Plants were kept in the greenhouse for 12 hr before and 12 hr after inoculation to maintain high humidity and facilitate infection. Plants were scored as 1) uninfected; 2) 1/3 of the leaves infected; 3) 2/3 of the leaves infected; or 4) dead. Water treated plants with or without pathogen inoculation were collected at the same intervals. Eight wk later, plants were removed from the soil and the roots washed with sterile distilled water, excised from the plant and analyzed. Data collected included root and crown rot severity assessed on a rating scale of 0–4 [18], where 0, no infection; 1, 1–25% infection; 2, 26–50% infection; 3, 51–75% infection; and 4, 76–100% infection in the root and crown regions. The percentage suppression of root and crown rot was then calculated based on the disease severity index [19].

Suppression (%) = [(A - B)/A] × 100

where, A, disease severity exhibited in the leaves and stem region due to the pathogen alone and; B, disease severity exhibited on the leaf and stem region after inoculation with both the pathogen and bacterial antagonists. Pepper fruits from six plants per treatment were excised and the disease severity and disease incidence rate were also calculated.

Evaluation of plant growth promotion by rhizobacteria.

For the greenhouse pot experiment, pepper seeds were surface sterilized by submersion for 20 min in 20% sodium hypochlorite, after which they were briefly rinsed with 70% methanol and then planted in sterile soil. Seeds were sown in a plastic pot (50 holes, 4 cm diameter each) filled with commercial soil (Baroker; Seoul Bio Co., Ltd.) containing 10% perlite (Parat; Sam Son). Seedlings were grown in a greenhouse maintained at 28°C during the day and 25°C at night. Plants received about 30 mL of water per plant once a day. After 2 wk, individual pepper seedlings were planted in plastic pots (15 cm in diameter) filled with the same commercial soil and 10% perlite as described above. Each treatment consisted of one replicate and three replications in a randomized block design. The bacterial inoculum (10⁸–10⁹ cfu/mL) was applied in the form of soil drenching twice with a wk in between treatments. The temperature of the greenhouse was maintained at 28 ± 2°C and watering was conducted twice daily. The plants were harvested 3 wk after the last inoculation, and the shoot and root length as well as fresh and dry weights were compared with the un-inoculated control. Chlorophyll content was also measured using a SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc., Osaka, Japan).

**Scanning electron microscopy.** Scanning electron microscopy was conducted according to the method described by Skerlavaj et al. [20]. Briefly, the subcultured fungal pathogen was incubated at 30°C for 1–3 days, after which fungal specimens in dual culture plates were fixed with an equal volume of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2) at 30°C for 3 hr. The samples were then centrifuged at 1,500 xg and washed with the same buffer and the fungal cells were dehydrated in graded ethanol. Following lyophilization and gold coating, the fungal cells were examined on a scanning electron microscope (S-3500N; Hitachi Co., Tokyo, Japan) at an accelerating voltage of 10 kV.

**Statistical analysis.** Data from in vitro experiments were analyzed using analysis of variance (ANOVA) to evaluate the efficiency of bacterial isolates against phytopathogens. Duncan’s multiple range test was used to compare the means of the treatments in each experiment. All statistical analyses were conducted using the SAS software ver. 9.2. (SAS Institute Inc., Cary, NC, USA).

**Results**

**Screening and identification of bacterial isolates.** Among 125 bacterial strains isolated from rhizospheric soil, seven were capable of inhibiting C. acutatum to various degrees during in vitro dual culture screening. Overall, six of seven potential bacterial isolates were identified as species belonging to Bacillus and one was identified as Paenibacillus polymyxa based on 16S rDNA sequencing (Table 1). Among the isolates, AB10, AB12 and AB14 were identified as Bacillus subtilis, AB05 was characterized as Bacillus amyloliquefaciens, and AB15 and AB11 were identified as P. polymyxa and Bacillus sp., respectively. The GenBank accession numbers of the isolates most similar to the selected bacteria used in this study are presented in Table 1.

**In vitro inhibition of C. acutatum.** The inhibition activities of bacterial isolates against C. acutatum were evaluated
The results showed that all bacterial isolates had greater than 50% potential to inhibit radial growth of *C. acutatum* on dual culture plates (Fig. 1). The highest inhibition of mycelial growth against the fungi was exerted by isolate AB15 (69.22%); however, AB05, AB11, AB12, and AB17 also induced more than 60% inhibition of the radial growth of *C. acutatum*. Bacterial isolate AB10 showed 54.90% inhibition and AB14 showed 54.48% inhibition of the radial growth of *C. acutatum* (Fig. 1). The zone of inhibition induced by the bacterial isolates was highest for AB15, which produced a 15 mm wide zone (Fig. 1B). These results showed that the zone of inhibition is directly related to mycelial suppression in dual culture plates. Bacterial strains AB05, AB12, AB14, and AB17 produced moderate zones of inhibition with average antagonistic activity, whereas AB10 and AB11 had lower antagonism against *C. acutatum* with a zone of inhibition.

### Table 1. Identification of bacterial isolates to species level based on 16S rDNA sequences

| Bacterial isolates | GenBank accession No. | Closest GenBank library strain          | Gram reaction | Similarity (%) |
|--------------------|-----------------------|----------------------------------------|---------------|----------------|
| AB05               | AB301002.1            | *Bacillus amyloliquefaciens*           | +             | 99             |
| AB10               | HM101166.1            | *Bacillus subtilis*                    | +             | 99             |
| AB11               | HM032893.1            | *Bacillus sp.*                         | +             | 99             |
| AB12               | HM101166.1            | *Bacillus subtilis*                    | +             | 99             |
| AB14               | AJ880761.1            | *Bacillus subtilis*                    | +             | 98             |
| AB15               | CP000154.1            | *Paenibacillus polymyxa*               | +             | 99             |
| AB17               | GQ375229.1            | *Bacillus subtilis subsp. subtilis*    | +             | 98             |
of less than 5 mm (Fig. 1B).

**Scanning electron microscopy.** Morphological changes in fungal mycelia due to the effects of bacterial isolates were examined by scanning electron microscopy (Fig. 2). The results showed that hyphal septa of *C. acutatum* were abnormally degraded after two days and then burst due to the antibiosis of isolate AB15 (Fig. 2B). However, control cultures showed no morphological changes in mycelia (Fig. 2A). Fungal spores were also degraded by the antagonistic effect of bacterial isolate AB15, which induced shrinkage and lysis of the conidial surface (Fig. 2B). Based on these results, antibiosis caused by the bacterial isolate disrupted the fungal cell wall and conidial germination.

**In vivo inhibition of pepper anthracnose** (*C. acutatum*). The results showed that anthracnose occurrence and fruit yield in pepper were affected by root treatment of rhizobacteria in the greenhouse. All bacterial strains reduced anthracnose disease symptoms on foliage and fruits of pepper plants when compared to pathogen-inoculated controls (Figs. 3 and 4). Isolate AB15 showed the greatest disease suppression rate of approximately 40% when compared to pathogen-inoculated (control) plants. Moreover, isolates AB05, AB12, and AB17 showed more than 30% disease reduction (Fig. 3). Other isolates also showed

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**Fig. 3.** Efficacy of bacterial isolates at reduction of anthracnose disease in pepper caused by *Colletotrichum acutatum* in greenhouse trials. Control plant indicates healthy plants not inoculated with *C. acutatum* or bacteria. Bars with the same letters indicate no significant differences between means as determined by the least significant difference test (*p* ≤ 0.05). Error bars indicates ± SD.

**Fig. 4.** Effect of rhizobacterial isolates on reduction of anthracnose symptoms in pepper fruits caused by *Colletotrichum acutatum* in greenhouse trials. Control indicates fruit obtained from plants inoculated with pathogen (*C. acutatum*) only. Bars with the same letters indicate no significant differences between means as determined by the least significant difference test (*p* ≤ 0.05). Error bars indicate ± SD.

**Fig. 5.** Symptoms of anthracnose disease in pepper fruits collected from a greenhouse assay. A, Fruit collected from bacteria inoculated plants without fungal infection; B, Fruit infected by anthracnose disease in plants inoculated with pathogen (*Colletotrichum acutatum*) only.
disease inhibition activity when compared to the controls, but the results were not significant. Fruit yield was also increased in plants treated with both pathogen and bacterial isolates when compared to plants inoculated with pathogen alone. In the test, strain AB15 showed an increased number of non-infected fruits when compared with the pathogen inoculated controls (Fig. 4). However, in the case of the weight of pepper fruit, strains AB12, AB05, AB17, and AB15 induced a significant increase in the weight of green, red, or combined fruit when compared with the controls (data not shown). Overall, strain AB15 consistently inhibited the symptoms of anthracnose disease in foliar parts and fruits of pepper. However, some other isolates also showed high numbers of red and combined non-infected pepper fruits (Fig. 5). Furthermore, rhizobacteria treated plants showed an increase in fresh weight of red and combined fruit when compared with the pathogen-inoculated controls (Figs. 4 and 5).

**Efficacy of PGPR on pepper.** All bacterial isolates induced a significant increase in observed growth parameters except for root dry weight. Among them, AB17 induced the greatest increase in shoot/root length and shoot/root biomass. Specifically, AB17 induced increases of 39% in plant height, 40.44% in root length, 42% in shoot fresh weight and 47% in root biomass. All other treatments also induced significant increases in plant height and root length (Table 2). The highest level of chlorophyll was observed in plants treated with isolate AB10, which led to levels 48% higher than those of control plants. Control plants were observed to have physically retarded growth and pale color when compared to other treatments (Fig. 6).

**Discussion**

The best sources of antagonistic microorganisms are their natural environments, where they compete with naturally colonized microflora that includes pathogen or spoilage microorganisms. This study was conducted to screen bacterial strains isolated from the rhizospheric soil for the ability to control pepper anthracnose caused by *C. acutatum*. The selected isolates were identified as *Bacillus* spp. and one isolate belonging to *Paenibacillus* sp. based on 16S rDNA sequencing. An *in vitro* antagonism assay showed that these seven bacterial isolates are capable of antagonizing *C. acutatum*. However, some bacterial isolates were found to be highly inhibitory of fungal growth, whereas others showed only minor activity. These findings suggest that the mode of action exerted and or the type of antifungal metabolite produced by the isolates varies and that the bacterial isolates are taxonomically different from each other [21]. The isolates that most effectively inhibited fungal growth in the dual culture experiment resulted in an inhibition zone large enough to prevent physical contact with the pathogens, suggesting that the rhizobacteria could produce certain antifungal metabolites [22]. Moreover, scanning microscopic analysis revealed that the bacterial isolate *B. subtilis* (AB14) produced volatile metabolites *in vitro* that inhibited growth and spore germination of *C. acutatum*.
The observed mycelial malformation was probably due to the toxic effects of antibiotic substances interfering with normal growth processes [23]. Several studies have shown that P. polymyxa produces many antagonistic substances and controls a wide variety of soil and foliar pathogens [24, 25]. Compant et al. [26] reported that B. subtilis produced some volatile compounds that activated an induction of systemic resistance pathway in Arabidopsis. In addition, Bacillus species isolated from rhizosphere soil have been reported to be effective at controlling a variety of soil-borne plant pathogens [21]. Choudhary and Johri [27] elucidated the mechanisms and role of Bacillus species as inducers of systemic resistance in relation to plant-microbe interactions and identified the pathways involved in their regulation. Moreover, available reports suggest that specific strains of the species B. amyloliquefaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides, and B. sphaericus elicit significant reductions in the incidence or severity of various diseases on a variety of hosts including greenhouse studies or field trials on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, Arabidopsis species, cucumber, loblolly pine, and tropical crops [28]. In the present study, we found that the selected bacterial antagonists were capable of producing phytohormone indole-3-acetic acid and phosphate solubilizing capacity (data not shown). Both factors are regarded as systemic acquired resistance inducers in various plants and these bacterial isolates will likely be regarded as potential biocontrol agents.

Overall, an in vitro assay and greenhouse studies indicated that bacterial isolates belonging to Bacillus sp. and Psenibacillus sp. exert antagonistic activity against C. acutatum. However, the study clearly showed that P. polymyxa (AB15) was the most promising biocontrol agent. The present study demonstrated that the selected bacterial isolates enhance shoot and root length, fresh biomass and total dry matter, as well as chlorophyll in inoculated plants. This might be due to the higher N accumulation by bacterial N2 fixation and better root growth, which promoted the greater uptake of water and nutrients. The higher N incorporation apparently increased the formation of protein and enzyme, which improved physiological activities. The higher N also contributed to the formation of chlorophyll, which consequently increased the photosynthetic activity. Therefore, PGPR inoculation resulted in enhancement of the photosynthetic rate in plants [29].

Based on the growth promotion activity of selected bacteria and their abilities to produce siderophores, phytohormones and phosphate solubilizing activity, the results of the present study suggest that the rhizobacterial isolates, P. polymyxa AB15 and other Bacillus spp. have great potential to increase the yield, growth and nutrition of various vegetable crops under greenhouse and field conditions. Therefore, these isolates can be utilized as biofertilizer and biological control agents for fruit and vegetable production in sustainable and ecological agricultural systems.

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