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Biological Control of Apple Anthracnose by *Paenibacillus polymyxa* APEC128, an Antagonistic Rhizobacterium

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The present study investigated the suppression of the disease development of anthracnose caused by *Colletotrichum gloeosporioides* and *C. acutatum* in harvested apples using an antagonistic rhizobacterium *Paenibacillus polymyxa* APEC128 (APEC128). Out of 30 bacterial isolates from apple rhizosphere screened for antagonistic activity, the most effective strain was APEC128 as inferred from the size of the inhibition zone. This strain showed a greater growth in brain-heart infusion (BHI) broth compared to other growth media. There was a reduction in anthracnose symptoms caused by the two fungal pathogens in harvested apples after their treatment with APEC128 in comparison with non-treated control. This effect is explained by the increased production of protease and amylase by APEC128, which might have inhibited mycelial growth. In apples treated with different APEC128 suspensions, the disease caused by *C. gloeosporioides* and *C. acutatum* was greatly suppressed (by 83.6% and 79%, respectively) in treatments with the concentration of 1×10^8 colony forming units (cfu)/ml compared to other lower dosages, suggesting that the suppression of anthracnose development on harvested apples is dose-dependent. These results indicated that APEC128 is one of the promising agents in the biocontrol of apple anthracnose, which might help to increase the shelf-life of apple fruit during the post-harvest period.

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Anthracnose is one of the important post-harvest diseases of apple fruit caused by *Colletotrichum gloeosporioides* and *C. acutatum*, resulting in serious damage to the fruits and considerable economic loss in various crops, including apple worldwide (Afanador-Kafuri et al., 2003; Bajpai et al., 2009; Lee et al., 2007; Víchová et al., 2012). The symptoms of apple anthracnose can be characterized as follows: the lesions begin as a small, light brown, circular lesion, and later gradually expands. As the lesion expands, conidiophores rupture the fruit epidermis, forming small tufts (Henz et al., 1992). The fruit decays rapidly as it ripens and eventually become mummified that may remain attached to the tree throughout the season. Under humid conditions, large numbers of spores are produced in a creamy mass, while under dry conditions, the spore mass appears crystalline. The rotted flesh beneath the surface of the lesion is watery, appearing in a V-shaped pattern in cross section that narrows toward the core (Onofre and Antoniazzi, 2014). They invade the plant tissues aggressively, killing the host cells to obtain nutrients (Deacon, 1997).

The occurrence of anthracnose and other post-harvest diseases of fruits have been controlled mostly by the use of chemical treatments. Biological control of plant diseases using microorganisms has been an effective alternative to chemical control for a long time, and many biocontrol agents have been introduced in the past several years to control fruit diseases, thus reducing the regular use of fungicides (Mari et al., 2009). Biocontrol of post-harvest diseases is dependent on a quantitative relationship between the antagonist and the biocontrol agent

(Nunes et al., 2002).

The antagonistic microorganisms, including both fungi and bacteria have been extensively studied as biocontrol agents against various soil-borne pathogens (Cook, 2000). Among the 20 genera of bacteria, *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp., are widely used as biocontrol agents. *Bacillus* spp., produced different kinds antibiotics (Ferreira et al., 1991). The genus *Paenibacillus* is comprised of more than 30 species of facultative anaerobes and belongs to endospore-forming gram-positive bacilli. *Paenibacillus polymyxa*, which was referred to as *Bacillus polymyxa* until 1993 (Ash et al., 1993), is a soil bacterium from the group of plant growth-promoting rhizobacteria, being used for the biocontrol of plant diseases (Dijksterhuis et al., 1999; Shishido et al., 1996). The activities associated with *P. polymyxa* treated plants in the earlier reports include nitrogen fixation (Heulin et al., 1994), soil phosphorus solubilization (Singh and Singh, 1993), and production of antibiotics (Rosado and Seldin, 1993), chitinase activity (Mavingui and Heulin, 1994), and the promotion of increased soil porosity (Gouzou et al., 1993). All these activities contributed to the promotion of plant growth at various times under various environmental conditions during the life cycle of a plant.

Until now, no information on the effectiveness of *P. polymyxa* strain on the control of post-harvest diseases in apple has been reported. Therefore, the main objective of this study was to investigate the efficacy of the strain *P. polymyxa* APEC128 in the control of anthracnose disease on apples after harvest.

Materials and Methods

Isolation of rhizosphere bacteria from apple orchard. Soil samples (50 g) were collected from several orchard apple trees free from disease symptoms in Andong, Gyeongbuk Province, South Korea to isolate antagonistic bacteria against apple pathogens. The soil samples were stored at 4°C in sterile plastic bags until their use. Soil samples (5 g) were diluted with 45 ml of sterile distilled water (SDW) and thoroughly dispersed by shaking at 150 rpm for 30 minutes at 28°C and from this 1 ml is taken and diluted 10^3- to 10^7-fold. Diluted samples (100 µl) were spread on nutrient agar (NA) medium (Kloepper, 1992), and incubated at 28°C for 24 hours. A single colony from each plate was picked up and streaked on freshly prepared NA plates. A total of 30 isolates were selected randomly from the culture plates, and the purified strains were stored on NA at 4°C. The isolated strains were maintained at –50°C in nutrient broth (NB) with glycerol (20%) for long-term storage. For preparing bacterial suspensions, a culture stored at –50°C was grown on NA for 24 hours at 28°C, and single colonies were transferred to NB and incubated for 24 hours at 28°C with shaking at 150 rpm. Bacteria were pelleted after centrifugation for 5 minutes at 8,000 rpm and resuspended in SDW to give a final concentration of 1×10^8 colony forming units (cfu)/ml (OD_{600} nm) before application.

***In vitro* antagonistic activity assay.** All the microbial isolates were tested *in vitro* for antagonistic effect against *C. gloeosporioides* (KACC 42390) and *C. acutatum* (KACC 40847) on potato dextrose agar (PDA; Difco, Detroit, MI, USA) including 0.5% peptone (PDAP) using a disc diffusion method (Park et al., 2013). The tested fungus (*C. gloeosporioides* or *C. acutatum*) was applied as mycelia plugs (6 mm in diameter) from the edge of a pre-cultured colony and placed onto the center of each PDAP plate (90 mm diameter). A sterile paper disk (8 mm diameter; Advantec Co., Tokyo, Japan) with a bacterial suspension (10^8 cfu/ml) was placed 30 mm from the pathogenic fungi. The inhibition of the mycelial growth of tested fungus was measured as a diameter (mm) 5 days after incubation at 25°C. The organism that produced the largest zone of inhibition during *in vitro* screening was selected for further study. Out of 30 strains screened, only one strain (APEC128) showed strong antagonistic activity and was selected for further experiments in this study. Each treatment contained five replications, and the assay was repeated three times, and results of one of the three experiments are shown here.

Isolation and preparation of fungal pathogen inocula. Fungal pathogens *C. gloeosporioides* (KACC 42390) and *C. acutatum* (KACC 40847) were obtained from Korean Agricultural Culture Collection. Formerly, the pathogens were isolated from symptomatic apple fruits as follows: Symptomatic tissues were surface sterilized in 1% sodium hypochlorite (NaOCl) solution for 1 minute and then placed in 70% ethanol for 30 seconds, followed by rinsing twice in SDW. After sterilization, the tissues were dried on sterile filter paper and transferred onto PDA plates. The plates were incubated at 25°C for 7 days. Single conidia were obtained by spreading PDA plates with conidia suspensions and incubating them at 25°C for 7 days. Suspension of conidia was prepared by suspending mycelia scraped from 7-day-old cultures of pathogenic fungi in PDA. The resulting suspensions were filtered through a double layer of cheesecloth. The concentration of spore suspensions was adjusted to 10^5 conidia/ml using a hemocytometer before application.

Molecular identification of *P. polymyxa* APEC128. The selected *P. polymyxa* strain APEC128 was subjected

to molecular identification using sequence homology of its 16S rRNA gene (Weisburg et al., 1991). Genomic DNA of APEC128 was isolated using a Genomic DNA Extraction Kit for bacteria (iNtRON Biotechnology, Seongnam, Korea) following manufacturer's instructions. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with Taq DNA polymerase and primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGYTACCTTGTTACGACTT-3'). The conditions for thermal cycling were as follows: denaturation at 94°C for 5 minutes followed by 30 cycles at 94°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 1 minute. At the end of the cycling, the reaction mixture was held at 72°C for 5 minutes and then cooled to 4°C. The obtained PCR products were sequenced with an automated sequencer (Genetic Analyzer 3130; Applied Biosystems, Foster City, CA, USA) using the same primers. The sequences were compared for similarity with the reference species of bacteria available in genomic database using the National Center for Biotechnology Information (NCBI)-BLAST tool. Sequence alignment and phylogenic tree construction were performed using a MEGA 4.0 program (Tamura et al., 2007).

Optimization of media, temperature, and pH conditions for culturing APEC128. To determine the growth of the strain APEC128 in different growth media, a single colony of APEC128 was inoculated into a test tube (20 mm diameter) containing 5 ml of growth medium and incubated for 24 hours at 28°C under shaking condition (250 rpm). The broth (5 ml) was transferred into a 500-ml baffled flask containing 100 ml growth medium and incubated at 28°C for 48 hours with shaking at 250 rpm. The various tested media included BHI media, potato dextrose broth, NB, tryptic soy broth (TSB), and Luria-Bertani broth. Samples were collected at 4-hour intervals. Cell growth was expressed as a measure of turbidity at 600 nm using a spectrophotometer (Ultrospec 4000 Spectrophotometer; Pharmacia Biotech Ltd., Little Chalfont, UK). For optimization of pH and temperature conditions, the isolate APEC128 was cultured at different incubation temperatures (15°C, 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C). All cultures were incubated on a rotary shaker at 180 rpm at different temperatures for 48 hours and absorbance was read at 600 nm. For optimization of pH, the broth was checked for different pH (3, 5, 7, and 9) before autoclaving. The culture broths were inoculated with the bacterial suspensions (10^8 cfu/ml), and the cultures were incubated under shaking condition (180 rpm) at 28°C for 48 hours and the absorbance was read at 600 nm.

Chitinase, amylase, cellulase, and protease activities of APEC128. Chitinase assay was conducted according to the method developed by Roberts and Selitrennikoff (1988). In brief, the bacterial culture was spotted on prepared minimal agar plates amended with 0.3% colloidal chitin and incubated at 30°C for 7 days. Development of a halo zone around the colony after addition of iodine was considered positive for chitinase enzyme activity. Amylolytic activity assay was performed according to the previously described method (Shaw et al., 1995). Twenty-four-hour-old bacterial culture spot inoculated on starch agar plate and incubated at 37°C for 24 to 48 hours. The petridishes were flooded with iodine solution and observed for starch hydrolysis, which was indicated by the formation of a clear zone (diameter, mm) around the bacterial spot. The cellulase activity assay was carried out qualitatively using carboxymethylcellulose (CMC) as the sole carbon and energy source (Hankin and Anagnostakis, 1977). CMC agar plates were inoculated with 24-hour-old grown bacterial cultures and incubated at 28°C for 48 hours. The halo zone due to cellulose hydrolysis around the colony was visualized by staining with 1% Congo red dye for 15 minutes, followed by destaining with 1 M NaCl solution for 20 minutes. The clear halo zone around the bacterial colony indicated the CMC hydrolyzing capacity. Proteolytic assay was performed according to the method described by Fleming et al. (1975). Briefly, proteolytic activity of APEC128 was determined by spot inoculation of bacterial culture (72-hour-old) on skim milk agar (100 ml of NA supplemented with sterilized skim milk) and incubation at 28°C for 24 to 48 hours. Formation of a clear zone (diameter, mm) around the bacterial spot was considered a positive test for proteolysis. Each treatment consisted of five replicates, and each experiment was repeated at least two times.

Suppression of anthracnose disease development by APEC128 suspension at various densities on harvested apples. Apple fruits cv. 'Fuji' of similar size were selected for our study. The fruits were surface-sterilized with 70% ethanol followed by 2% NaOCl for 5 minutes, and then washed three times with SDW and air-dried in a laminar air flow chamber. The effect of bacterial inoculum density on the development of anthracnose disease was tested by inoculating apple fruits with various concentrations of APEC128 suspensions (10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 cfu/ml). Surface-sterilized apple fruits were wounded by piercing them 1 to 2 mm deep with a sterile pin inoculated with 10 µl of inoculum and allowed to dry for 10 minutes. The spore suspensions (10 µl) of the pathogenic fungi were inoculated onto the wounds of the apple fruits. The diameter of the symptoms was observed 6 to 11 days

after incubation at 25°C and compared with the non-treated control (without bacterial suspension). Each treatment consisted of 18 replicates (fruits), and each experiment was repeated three times.

Statistical analysis. The data were subjected to ANOVA using SAS JMP software ver. 3 (SAS Institute, Cary, NC, USA; SAS Institute, 1995). Significant differences between the treatment means were determined using the least significant difference at $P < 0.05$. All the experiments were performed at least two times. For each experiment, the data were analyzed separately, and the results of one representative experiment are shown.

Results

Isolation, selection and identification of the biocontrol agent. The isolate APEC128 was selected based on the primary screening of antagonistic activity against *C. gloeosporioides* under *in vitro* conditions using a disc diffusion assay (Fig. 1). APEC128 was further characterized by 16S rRNA gene sequencing. Comparison of the specific sequence of the ribosomal gene with sequences deposited in GenBank suggested that the APEC128 isolate belonged to the *Paenibacillus* genus with the highest homology to *P. polymyxa* (99.8%). In the phylogenetic tree, APEC128 was clustered with other *Paenibacillus* spp., and it was sister to t *P. polymyxa* (bootstrap support of 42%) (Fig. 2). The comparative sequence analysis of 16S rRNA gene (1,480 bp) of APEC128 revealed that it was the member of the genus *Paenibacillus*.

***In vitro* screening antagonism test.** The antagonistic activity of APEC128 against *C. gloeosporioides* was assessed using the disc diffusion assay. Growth inhibition of phytopathogenic mycelia in culture plates was observed 5 days after inoculation. The strain producing the largest zone of fungal growth inhibition (18.5 mm) during the screening under *in vitro* conditions was identified as *P. polymyxa* APEC128. The antagonist strongly inhibited the development of anthracnose caused by *C. gloeosporioides* and *C. acutatum* (Fig. 3) compared to other strains tested, suggesting that strain APEC128 could significantly inhibit the growth of the two fungal pathogens.

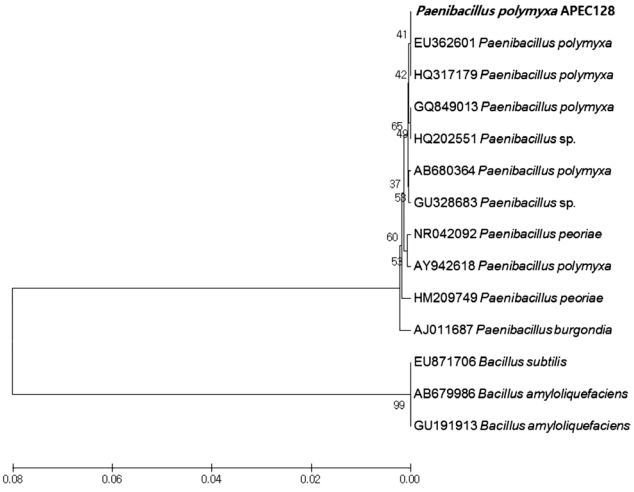


Fig. 2. Phylogenetic dendrogram inferred from the 16S rRNA gene sequences showing the relationships between *Paenibacillus polymyxa* strain APEC128 (APEC128) and related *Paenibacillus* species. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch points and the species names are preceded by the National Center for Biotechnology Information GenBank accession numbers. Phylogenetic tree was generated using maximum parsimony implemented in MEGA 4.0 program. The scale bar indicates 0.02 substitutions per nucleotide position.

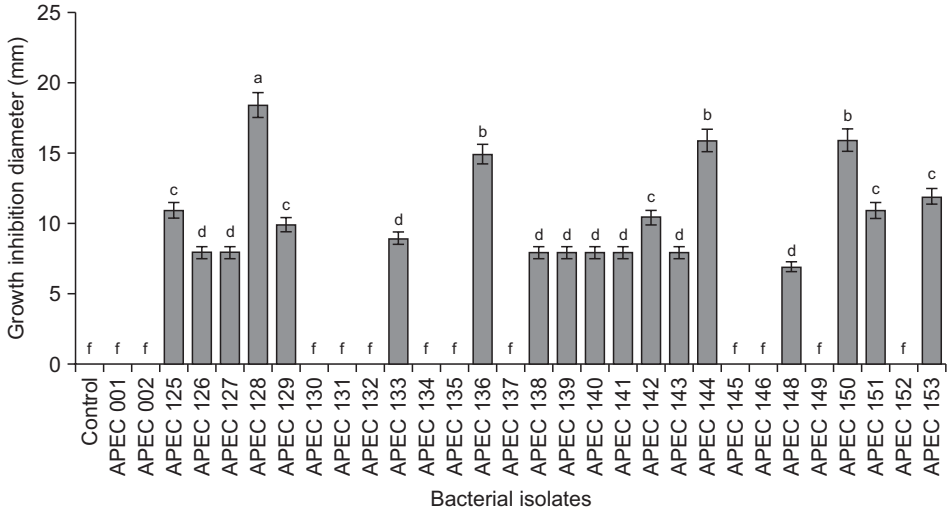
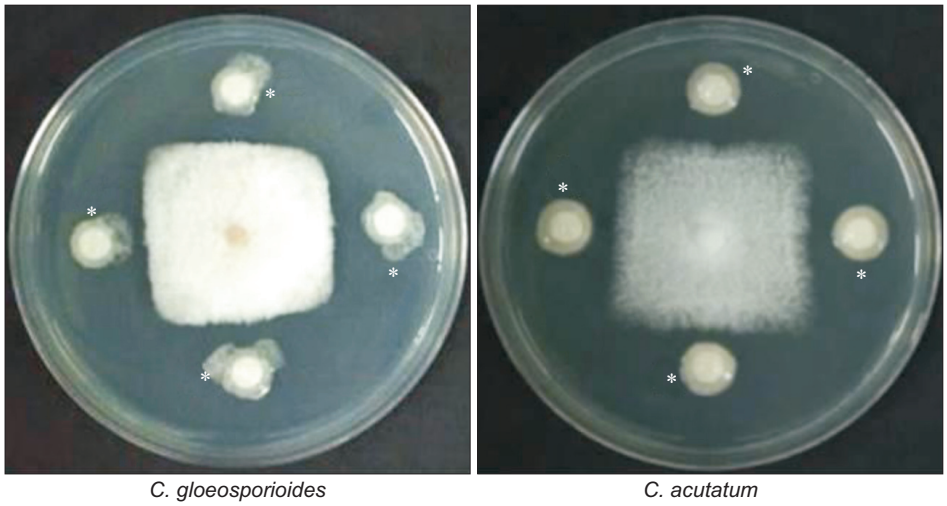


Fig. 1. *In vitro* screening of antagonistic activity of 30 bacterial isolates, including APEC128 against mycelial growth of anthracnose caused by *Colletotrichum gloeosporioides* by disc diffusion assay. The inhibition zone was measured 5 days after incubating at 25°C. The experiment was conducted at least twice with eight replications per treatment with similar results. Bars indicate the standard error of the mean, and bars with the same letters do not differ significantly between each other according to the least significant difference ($P < 0.05$).



C. gloeosporioides *C. acutatum*

Fig. 3. *In vitro* antagonistic activity of *Paenibacillus polymyxa* strain APEC128 (asterisks) against phytopathogens, *Colletotrichum gloeosporioides* and *C. acutatum*, assessed with the disc diffusion assay. Photographs were taken after 5 days of incubation at 25°C. The experiment was repeated at least two times.

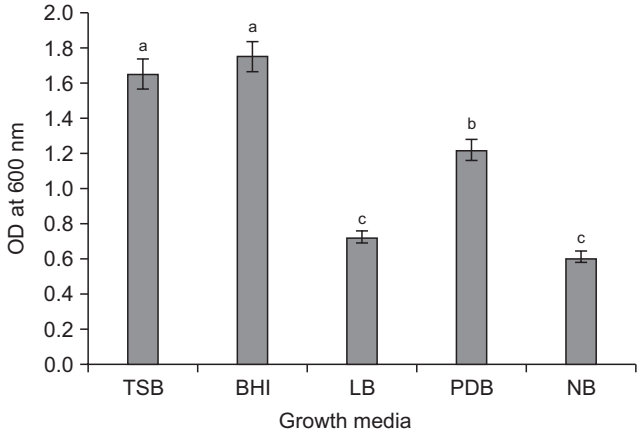


Fig. 4. Effect of various growth media on APEC128. Optical density (OD) was read at 600 nm 48 hours after incubation at 28°C. The experiment was repeated three times. Bars with the same letters do not differ significantly according to the least significant difference ($P < 0.05$). TSB, tryptic soy broth; BHI, brain-heart infusion; LB, Luria-Bertani broth; PDB, potato dextrose broth; NB, nutrient broth.

Optimization of growth media, pH, and temperature conditions for culturing of APEC128. The APEC128 cell numbers were relatively higher when cultured in BHI broth as compared to other media 48 hours after incubating at 28°C (Fig. 4). The next optimum growth medium was TSB. The smallest number of bacterial cells of APEC128 was observed when the cells were cultured in NB medium. The results clearly showed that BHI was the optimal medium to support the most efficient proliferation of APEC128 cells. The optimal pH and temperature conditions for growth of APEC128 were determined for the BHI medium. Thus a greater bacterial cell growth was observed at pH 8 (Fig. 5A) and a temperature of 30°C (Fig. 5B). The optimum temperature was 30°C for better bacterial cell growth in comparison with other temperature conditions (Fig. 5B). Interestingly, bacterial growth at 25°C was identical to that at 35°C, but it was drastically diminished at temperatures above 35°C, as measured by turbidity after incubation.

Chitinase, amylase, cellulase, and protease activities of the antagonistic microorganism. The strain APEC128 was evaluated for its potential to produce various enzymes *in vitro*. APEC128 produced amylase and protease at a greater level, compared to chitinase and cellulase, which were secreted only at a minor level, as indicated by the zone of hydrolysis around the bacterial colonies (Fig. 6). These results suggested that the strain APEC128 had a potential for production of various enzymes *in vitro*, which contribute to various biological activities.

Effect of inoculum concentration and *in vivo* biological control of apple anthracnose disease. Optimal inoculum concentration suppressed the disease development at a maximum level. Fig. 7 shows that in apples treated with APEC128 cells at the concentration of 1×10^8 cfu/ml, anthracnose caused by *C. gloeosporioides* and *C. acutatum* was suppressed by 83.6% (Fig. 7A) and 79% (Fig. 7B), respectively, 10 days after inoculation. Apples treated with strain APEC128 at the concentration of either 1×10^6 or 1×10^7 cfu/ml showed rot symptoms, and the disease was suppressed by < 50% on the 6th day after inoculation. These results confirmed the biocontrol activity of the strain APEC128 against *C. gloeosporioides* and *C. acutatum in vivo*, which activity was positively correlated with the concentrations used. To investigate the potential for biological control activity of APEC128 against these two pathogens *in vivo*; the apples were treated with a bacterial suspension prior to fungal pathogen exposure.

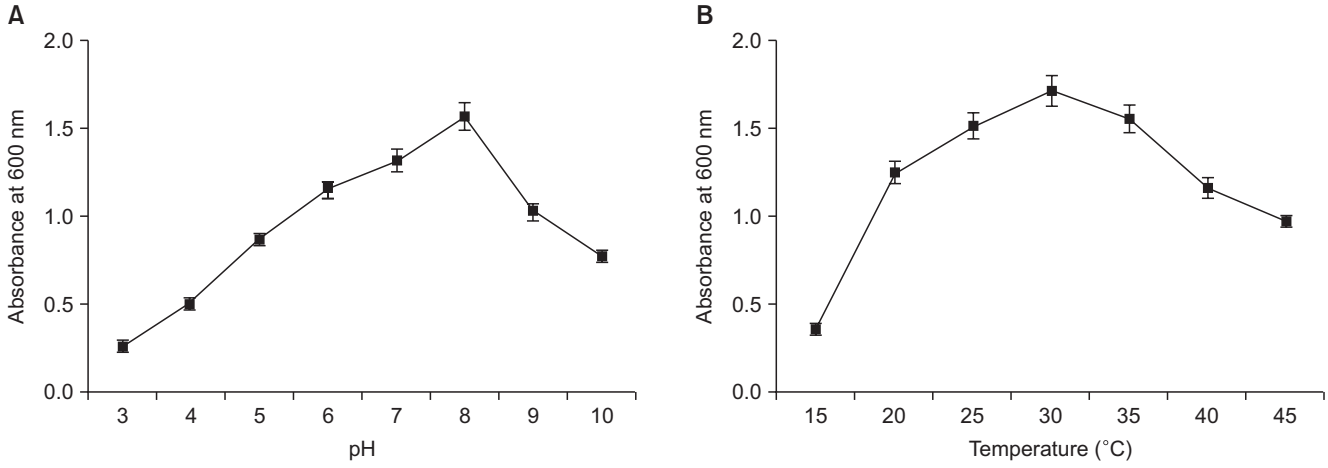


Fig. 5. Effect of pH (A) and temperature (B) on the growth of APEC128 cells. The experiment was repeated at least two times. Values are mean ± standard error.

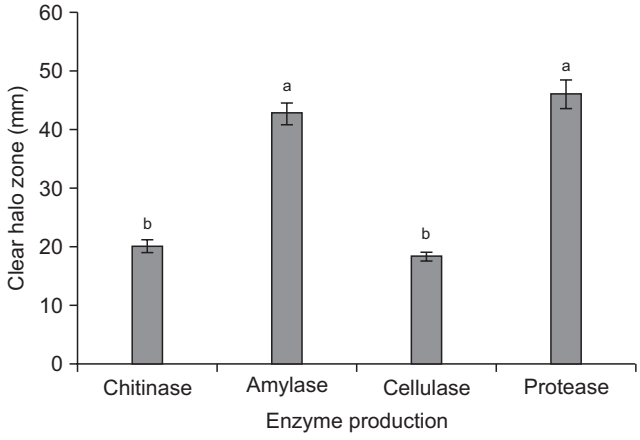


Fig. 6. The level of chitinase, amylase, cellulase, and protease activity *in vitro* by APEC128 inferred from the size of the halo zones. The experiment was repeated at two times. Bars with the same letters do not differ significantly according to the least significant difference ($P < 0.05$).

The symptoms started to appear 7 days post-inoculation when apples were soaked in a bacterial suspension at a concentration of 1×10^8 cfu/ml prior to inoculation with spore suspensions. There was a greater suppression of disease development of *C. gloeosporioides* and *C. acutatum* in APEC128-treated apple fruits when compared to non-treated control. The lesion areas caused by *C. gloeosporioides* and *C. acutatum* were 5.2 mm (Fig. 8A) and 4.4 mm (Fig. 8B) in diameter, respectively, where those non-treated apples were about 3-fold greater compared to lesions in APEC128-treated apples.

Discussion

Biological control of various plant diseases using microorganisms is an effective approach to control diseases in an eco-friendly manner. The first step of this process is to screen for potential biological control agents, and the main screening method under *in vitro* conditions is based on antagonistic activity. Spores are the major inoculants both in the field and in the post-harvest period for many plant diseases, including apple anthracnose. Our results confirmed a strong inhibitory activity of bacterial cells of APEC128 on the mycelial growth of fungal pathogens *C. gloeosporioides* and *C. acutatum* (Fig. 3). These results are consistent with previous reports (Lee et al., 2012; Li et al., 2009) and indicated that spore germination of several pathogens is inhibited under *in vitro* condition. At present, the use of post-harvest biocontrol agents is constrained by the lack of consistent efficacy and the high level of control required during post-harvest conditions. Recently, there has been an increased interest in further development of commercial products. The strain APEC128 isolated in this study protected apples from the post-harvest disease anthracnose at the required concentration of antagonistic bacterial cells.

Saravanakumar et al. (2008) reported that yeast *Metschnikowia pulcherrima* is an effective biocontrol agent that inhibited several fungal pathogens on harvested apples in low temperatures under controlled conditions. The yeast out-competes fungal pathogens for iron available in the rhizosphere soil, thus restricting fungal growth since iron is essential for their fungal growth and pathogenesis. Previously, the microorganism *Bacillus subtilis* has been reported to control diseases in harvested fruits (Yánez-

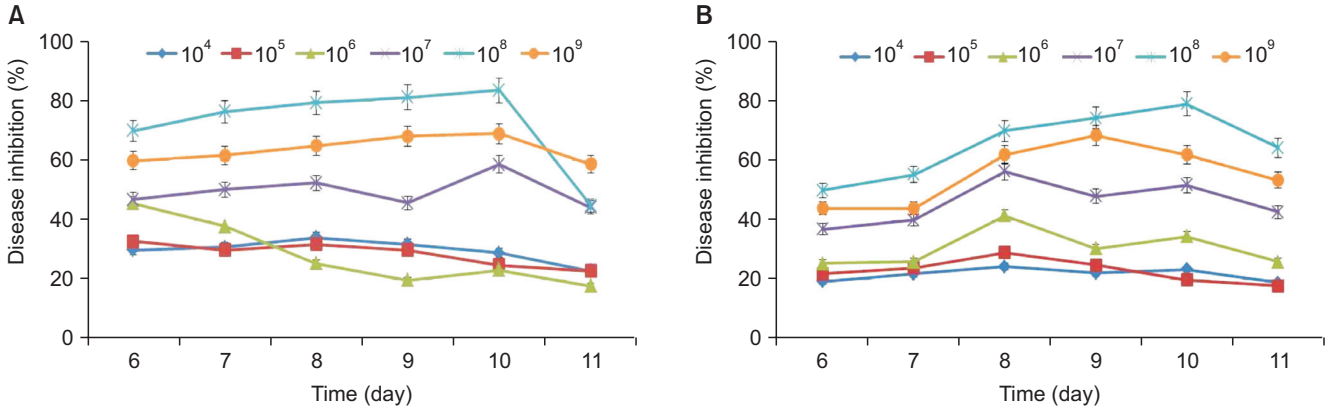


Fig. 7. Suppression of anthracnose caused by *Colletotrichum gloeosporioides* (A) and *C. acutatum* (B) at different inoculation concentrations of APEC128 suspension. The experiment was repeated at least two times. Values are mean ± standard error.

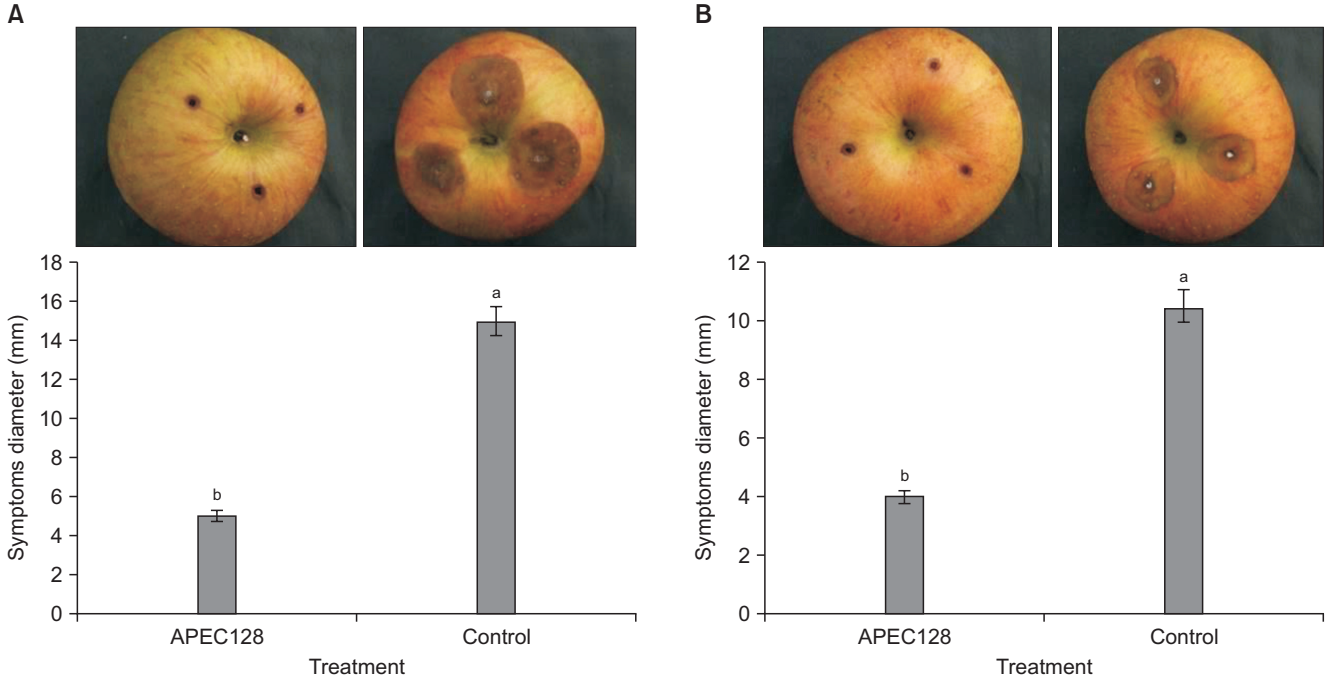


Fig. 8. *In planta* assay of suppression of spore development of *Colletotrichum gloeosporioides* (A) and *C. acutatum* (B) on harvested apples treated with APEC128 (1×10^8 cfu/ml) suspension. The experiment was repeated at least two times. The photographs were taken 10 days after incubation at 25°C.

Mendizábal et al., 2011). Its biocontrol activity is mainly due to the production of antifungal compounds such as antibiotics, surfactin, iturin and gramicidin (Cho et al., 2003). In addition, a previous study by Sadhana and Silvia (2009) has demonstrated that *P. polymyxa* strain P13 isolated from fermented sauce secretes a compound referred to polyxin exhibited the antagonistic activity against *Lactobacillus* strains; and Xu et al. (2014) showed that *Paenibacillus kribbensis* exhibited antagonistic activity against fungal pathogens by secreting lytic enzymes, which has the ability to break down fungal cell walls. Similarly, another report by Kim et al. (2009) stated that *Paenibacillus* spp., produce a variety of secondary metabolites such as antibiotics, siderophores, hydrogen cyanide, and a variety of enzymes. Herein, we show that pH and temperature were important factors for the optimum growth of the strain APEC128. On the other hand, chitinase produced by various microbial source is involved in biological control of fungal diseases since it degrades chitin, a major component of the fungal cell wall, thus

inactivating pathogenic fungi (Agrawal and Kotasthane, 2012). Furthermore, production of other enzymes by bacteria, such as amylase (Fossi et al., 2009), protease and cellulase by *Paenibacillus* spp. may be involved in suppressing diseases caused by pathogenic fungi (Alvarez et al., 2006; Liang et al., 2014), and these results are further supported by a recent study (Han et al., 2015), stated that protease and cellulase production by *B. amyloliquefaciens* responsible for the antagonistic activity.

Multiple modes of action of *Paenibacillus* spp. were assumed to contribute to the successful biocontrol activity, including competition for nutrients, induced resistance, and production of antibiotics and cell wall-degrading enzymes such as chitinases and β-1,3-glucanase (Droby et al., 2009). The results presented herein indicate similar modes of action by the strain APEC128 against anthracnose-causing pathogens. Many studies have reported the different antifungal compounds produced by *Bacillus* spp. and their importance in biocontrol activity (Lee et al., 2009; Liu et al., 2011). The strain APEC128 strongly inhibited the growth of fungal pathogens *in vitro*, probably due to its production of antifungal compounds. Previously, Janisiewicz and Korsten (2002) reported that the lytic enzymes produced by *Bacillus* spp. are involved in the biological control activity.

In conclusion, this study demonstrates that APEC128 is an effective biocontrol agent against apple anthracnose *in vitro* and *in vivo*. Moreover, the post-harvest treatment of apples with a bacterial suspension of APEC128 significantly suppressed the disease development during the storage period. Our results indicate that APEC128 is one of the promising agents for the biocontrol of apple anthracnose both in the field and after harvest. Further investigation will elucidate its mechanism of biocontrol ability on apple fruits.

Acknowledgments

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