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Potentiality of Beneficial Microbe *Bacillus siamensis* GP-P8 for the Suppression of Anthracnose Pathogens and Pepper Plant Growth Promotion

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This study was carried out to screen the antifungal activity against *Colletotrichum acutatum*, *Colletotrichum dematium*, and *Colletotrichum coccodes*. Bacterial isolate GP-P8 from pepper soil was found to be effective against the tested pathogens with an average inhibition rate of 70.7% in *in vitro* dual culture assays. 16S rRNA gene sequencing analysis result showed that the effective bacterial isolate as *Bacillus siamensis*. Biochemical characterization of GP-P8 was also performed. According to the results, protease and cellulose, siderophore production, phosphate solubilization, starch hydrolysis, and indole-3-acetic acid production were shown by the GP-P8. Using specific primers, genes involved in the production of antibiotics, such as iturin, fengycin, difficidin, bacilysin, bacillibactin, surfactin, macrolactin, and bacillaene were also detected in *B. siamensis* GP-P8. Identification and analysis of volatile organic compounds through solid phase microextraction/gas chromatography-mass spectrometry (SPME/GC-MS) revealed that acetoin and 2,3-butanediol were produced by isolate GP-P8. *In vivo* tests showed that GP-P8 significantly reduced the anthracnose disease caused by *C. acutatum*, and enhanced the growth of pepper plant. Reverse transcription polymerase chain reaction analysis of pepper fruits revealed that GP-P8 treated pepper plants showed increased expression of immune genes such as *CaPR1*, *CaPR4*, *CaNPR1*, *CaMAPK4*, *CaJA2*, and *CaERF53*. These results strongly suggest that GP-P8 could be a promising biocontrol agent against pepper anthracnose disease and possibly a pepper plant growth-promoting agent.

Keywords **:** antifungal activity, beneficial microorganism, *Bacillus siamensis*, biological control, immune response system

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Capsicum annuum L., commonly known as chili pepper, plays an important role in the global economy. As of 2020, the worldwide cultivation area for pepper was approximately 3,68,130 ha (Barchenger and Khoury, 2022). However, in recent years, there has been a consistent decline in production, primarily owing to climate-related challenges, highlighting the need for increased productivity per unit area (Ro et al., 2021). Rising temperatures and increased precipitation levels disrupt the regular growth of pepper plants, making them more vulnerable to various infections, including anthracnose (Suprapta, 2022). *Colletotrichum* spp., the pathogen responsible for anthracnose in pepper fruit, is considered a significant factor in the occurrence of wounds in pepper fruit and the rapid increase in anthracnose cases in open-field pepper crops, particularly in humid and windy environments (Hong et al., 2015). In South Korea, reports have identified various fungi responsible for anthracnose in peppers, including *C. gloeosporioides*, *C. dematium*, *C. coccodes*, and *C. acutatum*, all belonging to

the *Colletotrichum* genus (Park and Kim, 1992; Ro et al., 2023). A recent genetic analysis of anthracnose in pepper fruits highlighted *C. acutatum* as the dominant species (Han et al., 2015a). Anthracnose-resistant pepper cultivars have been developed; however, chemical pesticides such as carbendazim, benomyl, and copper oxide are still heavily relied upon despite observed resistance in *C. acutatum* (Kwon et al., 2022). The continuous use of chemical fertilizers and pesticides has raised concerns about potentially harmful residues and toxic effects on both environmental and human health (Ons et al., 2020). Modern agriculture has focused on the advantages of chemical pesticides in terms of agricultural production and profits. However, the indiscriminate use of pesticides has a direct adverse effect on soil nutrient balance and ecosystem biodiversity, ultimately threatening the stability of agricultural products (Meena et al., 2020).

Amid these challenges and a rising demand for organic agricultural products, there is a growing focus on eco-friendly biological controls as alternatives to chemical inputs. Agricultural biological control predominantly involves microorganisms, with beneficial ones often isolated from plant roots and soil, serving as endophytes. Plant growth-promoting rhizobacteria (PGPR), beneficial bacteria influencing plant growth, are gaining attention as potential biological control agents for plant diseases (Zhou et al., 2016). For instance, *Bacillus* species have been widely studied as biological control agents due to their ability to produce a variety of antibiotics and enzymes that inhibit pathogen growth (Kloepper et al., 2004).

Rhizosphere bacteria consume amino acids and organic compounds present in plant root exudates for growth. In symbiotic relationships with plants, they produce plant growth-promoting metabolites and exhibit antagonistic activity against plant pathogens. This plant–microorganism interaction in the rhizosphere is a fundamental component of plant-microbe relationships (Ribeiro et al., 2012). Furthermore, the effectiveness of PGPR is associated with the enhancement of plant growth and activation of antagonistic effects. These effects are linked to siderophore production (Cherif-Silini et al., 2016), phosphate solubilization (Zaidi et al., 2009), nitrogen fixation (Vessey, 2003), and other mechanisms (Franche et al., 2009). Collectively, these activities indirectly contribute to both the biological control and promotion of plant growth (Goswami et al., 2014).

In this study, we ultimately selected the GP-P8 strain isolated from the rhizosphere of pepper soil with robust antagonistic activity through an antifungal test focused on anthracnose pathogens (*Colletotrichum acutatum*, *Colletotrichum dematium*, and *Colletotrichum coccodes*). The selected bacterial strain was examined to confirm the presence of antagonistic and secondary metabolites. Additionally, we assessed their inhibitory effects on anthracnose caused by *C. acutatum*, as well as their growth-promoting effects on pepper plants.

Materials and Methods

Isolation of bacterial strains from pepper soil. To identify strains with antifungal and plant growth-promoting activities, soil samples were collected from pepper fields in Gapyeong-gun, South Korea (latitude 37.8607, longitude 127.3557). Each soil sample, placed in a test tube with 9 ml of sterile distilled water, underwent serial dilution (using the serial dilution method) with the addition of 1 g of soil for bacteria isolation. Subsequently, 100 µl of each 10^{-4} and 10^{-5} dilution was evenly spread onto tryptic soy agar (TSA; KisanBio, Seoul, Korea) medium. Each single colony was isolated and then incubated on TSA at 30°C for 1 day. Stock cultures were stored in tryptic soy broth (TSB; KisanBio) medium supplemented with 10% skim milk at –80°C.

Antifungal assay and identification. An *in vitro* dual culture assay was conducted to screen for antagonistic bacteria against anthracnose pathogens—*C. acutatum*, *C. dematium*, and *C. coccode*. The fungi were cultured on potato dextrose agar (PDA; Difco, Detroit, MI, USA) medium at 30°C for 7 days. Medium was used to assess antifungal activity, including a mixture of PDA and TSA in a 1:1 ratio. Isolated bacterial species were cultured at a 1% concentration in 30 ml of TSB medium and incubated for 2 days in a shaking incubator at 30°C and 140 rpm. For the assay, each fungus and a paper disk (Advantec, Younjin, Seoul, Korea) were aligned on the same line on a mixture of PDA + TSA. Subsequently, 20 µl of a bacterial suspension with a concentration of 1×10^7 cfu/µl was inoculated onto each paper disk. A control group, where paper disks were devoid of bacteria, was also prepared. Following inoculation, the plates were incubated at 30°C for 1 week. Bacterial isolates with the highest antifungal activity were selected as effective antagonistic bacteria. The inhibition rate was calculated by comparing the fungal mycelium growth on the control plate to that on the plate inoculated with bacteria using the following formula:

$$\text{Inhibition rate (\%)} = [(C - d) - (T - d)]/(C - d) \times 100\%,$$

where *T* denotes fungal growth in the presence of bacteria, *C* denotes fungal growth in the control, and *d* denotes distance factors.

The identification of the selected antagonistic strain was sent to Macrogen (Seoul, Korea) for sequencing. The sequencing was performed using partial 16S rRNA gene sequencing with primers (universal primer 27F; 5′-AGAGTTTGATCTG GCTCA G-3′, 1492R; 5′-TACG-GYTACCTTGTTACGACTT-3′). The obtained sequences were submitted to NCBI (National Center for Biotechnology Information) to obtain accession numbers and to perform homology alignment analysis using BLAST (Basic Local Alignment Search Tool). A multigene phylogenetic tree was constructed in MEGA (Molecular Evolutionary Genetics Analysis) V.11, using the Neighbor-Joining method, on the concatenated alignments of 16S rRNA sequences of strain GP-P8.

Biological characteristics assay of *B. siamensis* GP-P8. The bacterial strain was screened for the production of antagonistic and plant growth promotion (PGP) enzymes. Protease medium was prepared using skim milk agar (skim milk powder 100.0 g, peptone 5.0 g, agar 15.0 g/l). And presence of clear zones was measured around the wells after 48 h. For detection of cellulose-degrading ability, the isolated strain was assessed using carboxymethylcellulose (CMC) agar (composed of potassium dihydrogen phosphate 2.0 g, magnesium sulfate heptahydrate 0.5 g, sodium chloride 0.5 g, ferrous sulfate heptahydrate 0.01 g, CMC 10.0 g, agar 12.0 g/l). The CAS blue solution of Chrome Azurol S (CAS) medium used in this study was prepared using an experimental method described previously (Schwyn and Neilands, 1987). Siderophore production was verified by observing the formation of a clear yellow-orange zone in the blue-colored medium. The ability of bacterial isolate to hydrolyze α-amylase was evaluated in starch agar (soluble starch 10.0 g, peptone 5.0 g, beef extract 3.0 g, bacterial agar 15.0 g/l). After 72 h of incubation, plates were stained with a 3% potassium iodide solution to observe the clear zone around the well. Pikovskaya's agar (PVK; $(NH_4)_2SO_4$ 0.5 g, $C_6H_{12}O_6$ 10.0 g, $Ca_3(PO_4)_2$ 25.0 g, yeast extract 0.5 g, KCl 0.2 g, magnesium sulfate 0.1 g, manganese (II) sulfate 0.0001 g, ferrous sulfate heptahydrate 0.0001 g, bacto agar 15.0 g/l) was used to create wells of equal size using a cork borer (Ø: 4 mm). Observing a clear zone around the well confirmed the ability to solubilize insoluble phosphate.

Indole acetic acid production. Pure bacterial isolates were grown in a 30 ml TSB medium supplemented with 0.1% L-tryptophan and without L-tryptophan at 28°C, 190 rpm for 72-120 h. After centrifugation, 1 ml of the supernatant was mixed with 500 µl Salkowski reagent ($FeCl_3$ [0.5 M]: distilled water: H_2SO_4 = 1:50:30 (v/v/v)), and the pink color change was measured at 530-540 nm using a spectrophotometer. A standard curve was created using indole-3-acetic acid (IAA) standard material (0-100 µg/ml), and the production amount of auxin was measured.

PCR of lipopeptide biosynthetic genes. *Bacillus* spp. have been shown to produce various secondary metabolites with antagonistic activities. In this study, eight different secondary metabolite lipopeptide biosynthetic genes, including *ItuA*, *FenA*, *DfnD*, *BacD*, *DhBe*, *srfA*, *mInA*, and *baeR*, were analyzed using a PCR-based method. Reference sequences were selected for screening (Hazarika et al., 2019), and *DhBe* and *FenA* nucleotide sequences were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/kegg/kegg2.html). The PCR primers used in this study are listed in Supplementary Table 1. Genomic DNA extracted from the four bacterial strains served as a template for the PCR amplification of secondary metabolite biosynthetic genes. The PCR reaction was conducted in a total volume of 50 µl, incorporating 10 pmol of each primer and 1 µl of genomic DNA. The PCR conditions consisted of an initial denaturation at 95°C for 3 min, 34 cycles of denaturation at 55°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. The amplified products were analyzed on 1.0% agarose gel.

SPME/GC-MS analysis of bacterial volatile compounds. The strain GP-P8 was cultured in a 10 ml headspace vial containing TSB medium and incubated at 30°C with agitation at 140 rpm. Bacterial volatile compounds were collected from the headspace using an SPME fiber (Supelco, Bellefonte, PA, USA) at 60°C for 20 min. Subsequently, the extracts were analyzed by GC-MS (GC 7890A/5975C, Agilent Technologies, Santa Clara, CA, USA) with a DB-5 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Agilent Technologies). Helium served as the mobile phase, and the flow rate was maintained at 1.0 ml/min. The injector temperature was maintained at 250°C, and the column temperature was set at 60°C for 2 min, followed by an increase to 250°C at a rate of 10°C/min and then maintained at 250°C for 20 min. The mass spectrometer was operated at an ionization voltage of 70 eV in the positive ion mode, scanning the mass range from 50 to 400 m/z at 200°C. The obtained mass spectra were identified by comparing them with data from the WILEY8 library, and the content of each compound was expressed as the area ratio of the peak in the total ion chromatogram.

Efficacy assessment of rhizobacterial isolates in controlling anthracnose on pepper fruits. For the fruit test, green pepper fruits (*C. annuum* L. cv. Shinheung) were injured after harvesting. The surfaces of pepper fruits were first disinfected with 70% ethanol. Surface injuries were then created using a syringe needle, followed by the application of a bacterial suspension OD600. Subsequently, 20 µl of conidial suspension containing *C. acutatum* 1.0×10^6 conidia/ml. Inoculated fruits were moistened using two layers of wet paper towels and placed in plastic boxes. These boxes were then sealed to maintain humidity and incubated at 28°C. The percentage of infected areas was calculated to assess disease severity over an average of 7 days post-cultivation. Experiments were conducted in triplicate, and data are expressed as the mean of three samples with standard deviation.

Disease control efficacy against anthracnose under greenhouse conditions. This study used a pot test to evaluate the antifungal activity of pepper anthracnose *in vivo*. A 30 ml pellet of culture broth containing strains GP-P8 was diluted and used in the experiment. The pepper seeds (cv. Shinheung) were sterilized by soaking in a 70% ethanol solution for 3 min, followed by rinsing with sterile water. The sterilized seeds were then germinated on moist filter paper in Petri dishes at 28°C for 5 days. The pepper seedlings were transplanted into plastic pots (2 l, 17 cm × 13 cm), and 30 ml of the diluted pellets were irrigated into the rhizosphere four times at one-week intervals. Biological pesticides (Acadian29, Canada) were used as positive controls and applied using the same irrigation method. After pretreatment with antagonistic bacteria, pepper fruits were carefully injured using a syringe and inoculated with 10 µl of a conidial suspension containing 1.0×10^6 conidia/ml of *C. acutatum* to induce anthracnose. The greenhouse was consistently maintained at a temperature of 28°C. Seven days after *C. acutatum* treatment, fruit lesions were examined to confirm the immune response in both the control and treated plants. Various parameters, including fresh weight, shoot and root lengths, chlorophyll content (measured as SPAD value), and weight and number of peppers, were recorded. The disease index was assessed on a scale from 0 to 4 using the following scoring system: 0 = no lesion; 1 = lesion measuring 0-2 cm; 2 = lesion measuring 3-4 cm; 3 = lesion measuring 5-7 cm; and 4 = lesion measuring 8-10 cm. Experiments were conducted in triplicate, and data are expressed as the mean of three samples with standard deviation.

RNA extraction and real-time quantitative PCR of defense-related genes. To investigate plant immune response mechanisms to *C. actatum* in a pot test, pepper fruits were harvested from plants exposed to the conidial suspension for 1 week. Total RNA was extracted from the samples using the RNA TRIzol method (Connolly et al., 2006), and RNA quantity and quality were assessed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The samples included a negative control treated only with the pathogen *C. acutatum* spore suspension, a positive control treated with the known biological pesticide Acadian29, and a treatment group with the GP-P8 isolate. High-capacity cDNA was synthesized using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). Primers, designed using Beacon Designer software (version 8.0, Premier Biosoft, Palo Alto, CA, USA), were used to assess the relative expression levels of immune response-related genes (Supplementary Table 2). These genes included those associated with mitogen-activated protein kinase (*CaMAPK4*), pathogenesis-related defense (*CaPR1*, *CaPR4*), jasmonic acid (JA) (*CaJA2*), the salicylic acid (SA) signaling pathway (*CaNPR1*), ethylene-responsive factor (*CaERF53*). *CaActin* gene was used as a housekeeping gene. To ensure that the designed primers were amplified efficiently, real-time PCR, using Top real SYBR green qPCR Pre 2XMIX (Enzynomics, Daejeon, Korea), was used to confirm the detection limit and establish a standard curve. Each sample was evaluated in triplicate. The Bio-Rad CFX connected real-time system was used for analysis, with the following program settings: initial denatur-

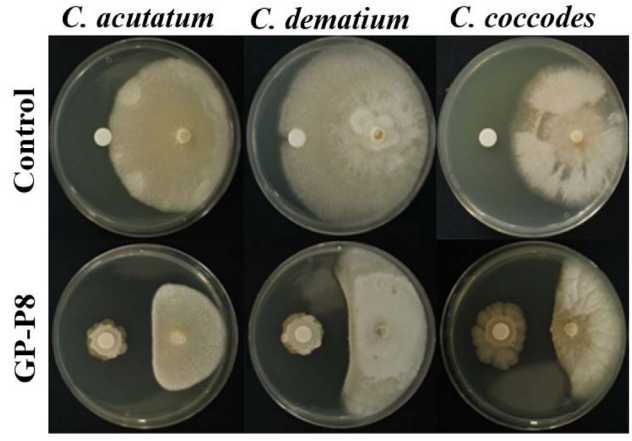

Fig. 1. *In vitro* antagonistic activities of GP-P8 against various fungal phytopathogens. Lowercase letters in Duncan's test using IBM SPSS Statistics 24 software signify that values sharing the same characters are not significantly different from each other at the $P < 0.05$.

Table 1. Inhibition rate (%) of isolate GP-P8 against various fungal pathogens in dual culture *in vitro* assays

	Fungal pathogens		
	Colletotrichum acutatum	*C. dematium*	*C. coccodes*
Inhibition rate (%)	78.47 ± 9.19 ab	71.03 ± 9.31 b	82.00 ± 1.00 a

Data represent the means ± standard deviation of replicates. Lowercase letters in Duncan's test using IBM SPSS Statistics 24 software signify that values sharing the same characteristics are not significantly different from each other at $P < 0.05$.

ation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s. The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) reactions for each RNA sample were performed triplicate times in this study. Finally, the relative expression level of each gene was calculated based on the $2^{-\Delta\Delta CT}$ method (Rao et al., 2013).

Statistical analysis. All statistical analyses were calculated as the mean or mean ± standard deviation of replicates using IBM SPSS Statistics 24 software (IBM Corp., Armonk, NY, USA). Statistically significant differences were assessed using one-way and two-way ANOVA tests in GraphPad Prism (v.8.4.3), with significance levels indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

Results

Screening of antagonistic effect against anthracnose pathogens: *in vitro* assay and identification. The antagonistic potentials of microorganisms isolated from pepper soil were tested against pathogens such as *C. acutatum*, *C. dematium*, and *C. coccode*. The beneficial microorganism was screened using a 1:1 co-culture method in a 1:1 mixture of PDA and TSA growth media (Fig. 1). In the antagonistic experiments against these anthracnose, the GP-P8 strain exhibited the highest antagonism against *C. coccodes* with an inhibition rate of 82.00% (Table 1). As a result, the GP-P8 isolate was identified as the strongest potential antagonistic microorganism. The phylogenetic tree represents a similarity level of 99% with *Bacillus siamensis*, and the accession number was registered as OR342274 in the GenBank database (Fig. 2).

Biological characterization of antagonistic GP-P8 effects. The biological traits of the GP-P8 strain, including proteases, cellulases, siderophores, α-amylase activity, phosphate solubilization, and auxin production, were analyzed. Strong enzyme activity was observed in all chemical media, and α-amylase activity exhibited large clear zones

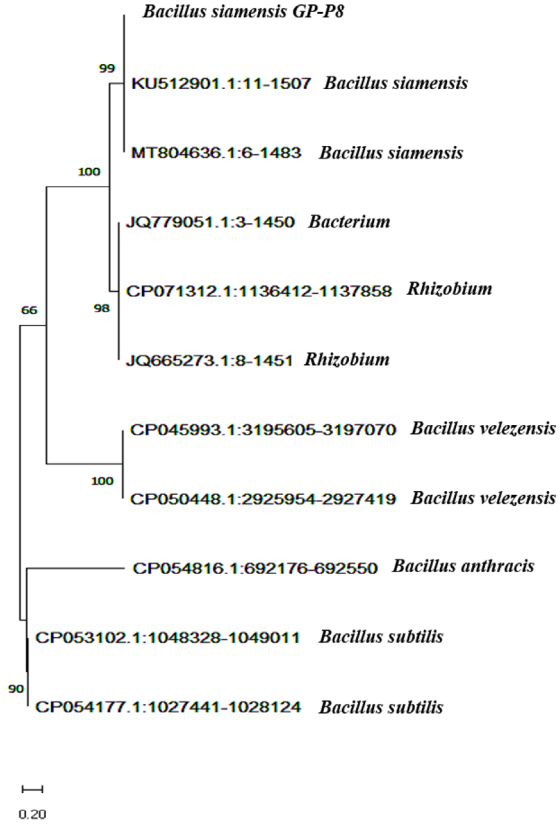

Fig. 2. Phylogenetic tree of the isolate GP-P8 showing a similarity level of 99% with *Bacillus siamensis*.

compared to the control. In order to analyze whether the presence of the precursor molecule L-tryptophan affected IAA production in the GP-P8 strain, standard values for IAA concentrations were established at 540 nm, and the production amounts were measured. Under controlled auxin conditions, the results confirmed that GP-P8 produced IAA, and the presence or absence of L-tryptophan did not significantly impact its production (Fig. 3A). The IAA production by GP-P8 was 7.76 μg/ml with L-tryptophan and 6.43 μg/ml without L-tryptophan (Supplementary Fig. 1).

In addition, the lipopeptide biosynthetic genes, such as Iturin, *ItuA*; Fengycin, *FenA*; Difficidin, *DfnD*; Bacilysin, *BacD*; Bacillibactin, *DhBe*; Surfactin, *srfA*; Macrolactin,

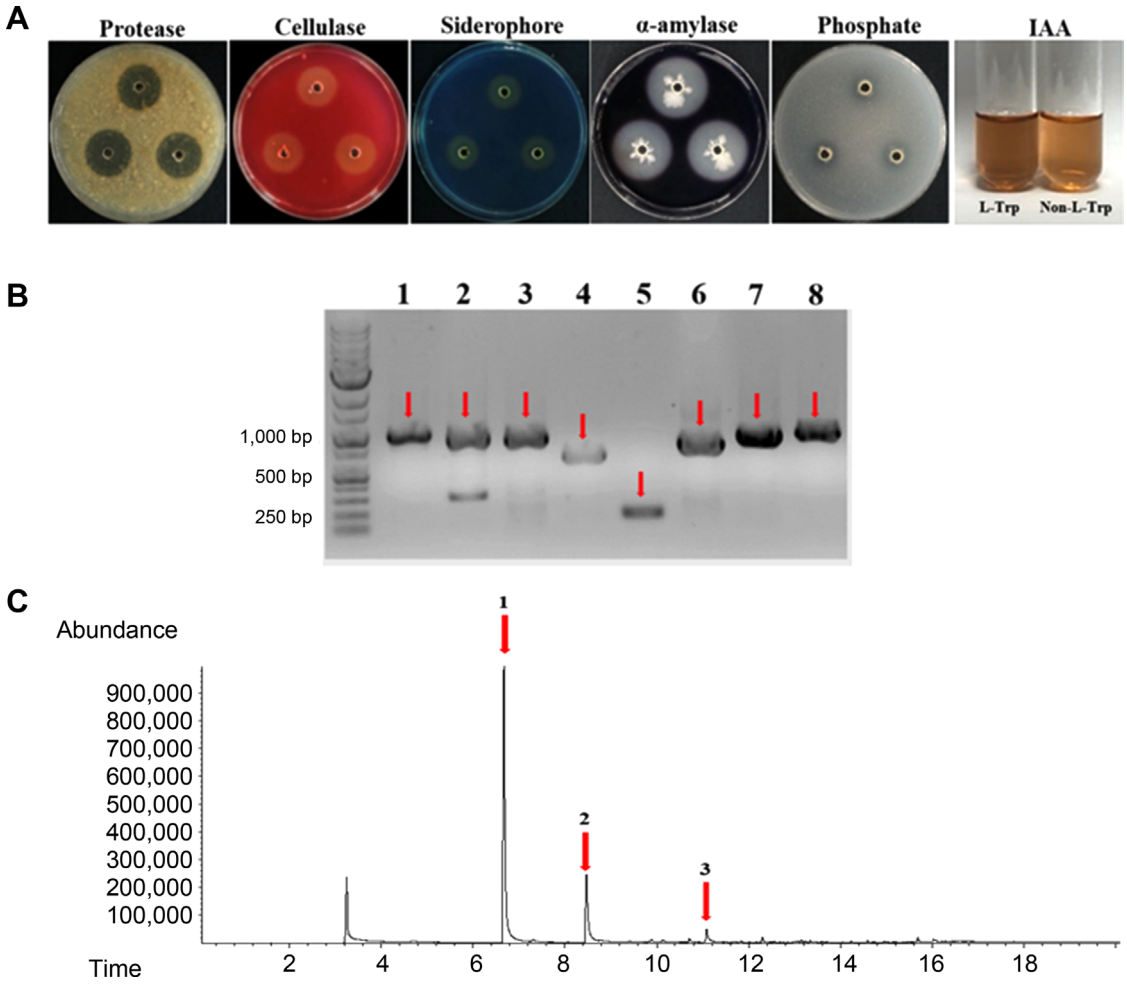

Fig. 3. Screening for the antagonistic traits of the isolate GP-P8. (A) Various antagonistic biochemical tests and indole-3-acetic acid (IAA) production assay of the bacterial isolate GP-P8. (B) PCR-amplified products of the eight secondary metabolite biosynthetic genes on 1% agarose gel. 1, Iturin, *ItuA*; 2, Fengycin, *FenA*; 3, Difficidin, *DfnD*; 4, Bacilysin, *BacD*; 5, Bacillibactin, *DhBe*; 6, Surfactin, *srfA*; 7, Macrolactin, *mInA*; 8, Bacillaene, *baeR*. (C) GC/MS analysis of volatile compounds from bacterial isolate GP-P8. 1, acetoin; 2, 2,3-butanediol; 3, pyrazine.

mInA; and Bacillaene, *baeR* related to secondary metabolites which are crucial for inhibiting pathogen growth, were detected. PCR amplification of GP-P8 genomic DNA using primers designed to amplify biosynthetic genes resulted in the presence of lipopeptide gene bands in the GP-P8 strain, suggesting the contribution to antifungal effects (Fig. 3B). Lastly, SPME/GC-MS analysis was performed to identify the volatile organic compounds (VOCs) produced by GP-P8. The obtained mass spectra were identified by comparing them with NIST and WILEY 8 library data. The analysis revealed that GP-P8 predominantly produces acetoin as the highest amounts of volatile compound, as shown in Fig. 3C.

Suppression of anthracnose development in detached pepper fruits using antagonistic bacterial suspension. We created five wounds using a syringe to evaluate the potential of antifungal suspension treatments in preventing anthracnose caused by *C. acutatum*. Subsequently, the antagonist bacteria GP-P8 were inoculated, followed by the injection of *C. acutatum* at a concentration of 1.0×10^6 conidia/ml. After 7 days, lesion size was measured to calculate the inhibition rate. The control group exhibited an average lesion size of 0.83 ± 0.13 cm, indicating a high disease incidence rate. However, the GP-P8 treatment resulted in a slight decrease to 0.35 ± 0.08 cm, demonstrating a considerable reduction in lesion size of nearly 3.00-fold (Fig. 4). Upon assessing the infection inhibition

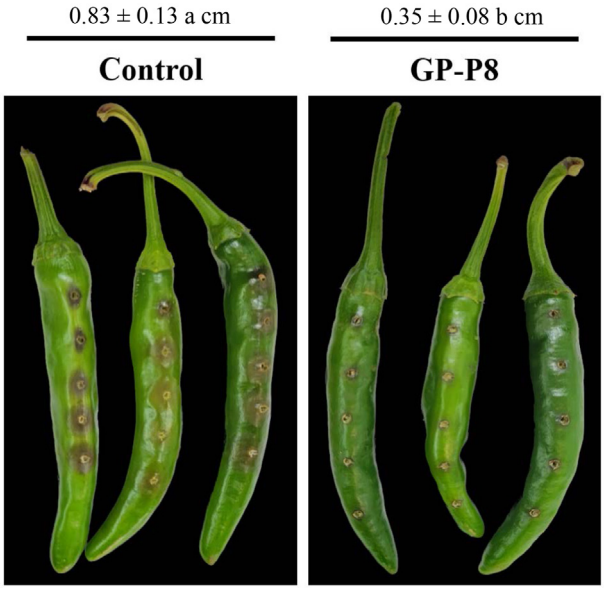

Fig. 4. Suppression of anthracnose on pepper fruit by antagonistic bacteria GP-P8. Disease lesion size (cm) data represent the means ± standard deviation of replicates. Lowercase letters in Duncan's test using IBM SPSS Statistics 24 software signify that values sharing the different characteristics significantly differ at $P < 0.05$.

rate of *C. acutatum*, we observed that the GP-P8 treatment exhibited a 56% inhibition rate. This suggests that pretreatment with the antagonist GP-P8 strain effectively prevented and suppressed disease severity in *C. acutatum* infections.

Evaluations of antagonistic bacterial strain for anthracnose suppression in pepper plants under greenhouse conditions. A greenhouse pot test was conducted to assess the disease control effects of GP-P8 against the anthracnose fungus *C. acutatum*. Cell suspensions of antagonistic bacteria were applied as treatments four times at one-week intervals, and after the pepper fruits had grown, *C. acutatum* was inoculated into the fruits. Pathogenic test results revealed that strain GP-P8 treated plants exhibited the highest fresh weight (47.23 g/plant) and chlorophyll content (28.93 SPAD value/plant). In contrast, the control recorded values for shoot length (45.66 cm/plant) and root length (31.52 cm/plant). Similarly, data on pepper fruit indicated that the number and weight of fruits were the highest in the GP-P8 strain. All growth attribute results surpassed those observed in the untreated control group and the positive control group treated with biological pesticides (Table 2). However, after inoculation, *C. acutatum* was detected in all treatment groups, confirming the disease incidence rate. Inoculated solely with *C. acutatum*, the negative control exhibited disease severity at five levels. However, all treatment groups demonstrated a reduction in symptoms compared to the negative control. Notably, the GP-P8 strain displayed the lowest disease severity (Table 2, Fig. 5). These results indicate that the selected antagonistic bacteria GP-P8 has promising disease control and plant growth potential.

Relative expression of plant defense-related genes in pepper. In the greenhouse pot test, pepper fruits were infected with anthracnose fungi, which induced the expression of immune genes in pepper fruits by pretreatment with antagonistic microorganisms against *C. acutatum*. Total mRNA was extracted from the pepper fruits inoculated with *C. acutatum*. The qRT-PCR results revealed the overexpression of the JA and SA pathways along with immune-associated marker genes (*CaPR1*, *CaPR4*, *CaNPR1*, *CaJA2*), and the ethylene-responsive factor *CaERF53* in response to *C. actatum* infection (Fig. 6). These findings were in contrast to pepper fruits treated solely with anthracnose (negative control). For the *CaPR1* genes, the expression increased by 3.50-fold in GP-P8, compared with the

Table 2. Disease control efficacy of antagonistic isolate GP-P8 against *Colletotrichum acutatum* under greenhouse conditions

	Fresh weight (g/plant)	SPAD value	Plant length (cm)		No. of fruits	Fresh fruit yield (g/plant)	Disease severity (0-5)[a]
			Shoot	Root			
Control	32.90 ± 0.61 bc	21.41 ± 3.80 b	45.66 ± 3.69 b	31.52 ± 1.82 a	2.33 ± 1.53 b	6.08 ± 1.58 ab	0.00 ± 0.00 d
NC	37.46 ± 5.71 bc	22.47 ± 0.87 ab	50.93 ± 2.70 ab	31.62 ± 1.81 a	2.00 ± 1.00 b	2.94 ± 1.39 b	5.00 ± 0.00 a
PC	42.33 ± 7.35 bc	22.67 ± 4.86 ab	53.84 ± 7.11 ab	31.47 ± 1.06 a	2.67 ± 1.53 b	6.41 ± 4.37 ab	4.75 ± 0.50 ab
GP-P8	47.23 ± 6.14 a	28.93 ± 1.19 a	53.37 ± 7.71 ab	32.51 ± 1.23 a	4.00 ± 1.00 a	10.61 ± 2.38 a	2.25 ± 0.50 c

Data represent the means ± standard deviation of replicates. Lowercase letters in Duncan's test using IBM SPSS Statistics 24 software signify that values sharing the same characteristics are not significantly different from each other at $P < 0.05$.
NC, negative control; PC, positive control: biological pesticides (Acadian29, Canada).
[a]Disease severity (0-5): 0 (0%), 1 (1-20%), 2 (20-40%), 3 (40-60%), 4 (60-80%), 5 (80-100%).

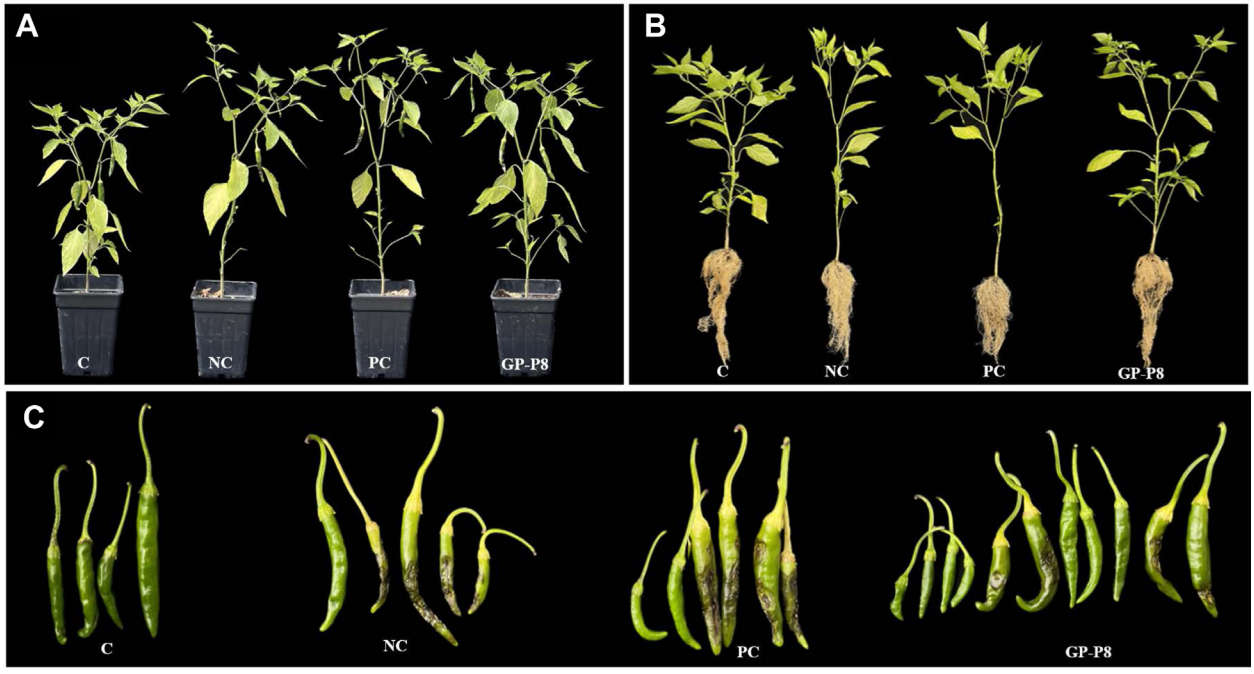

Fig. 5. Disease control efficacy of antagonistic bacterial isolate GP-P8 for suppression of *Colletotrichum acutatum* under greenhouse conditions. A 30 ml bacterial suspension (OD 600 of 0.1) was applied four times at 1-week intervals to pots containing 1-week-old pepper seedlings (cv. Shinheung). Subsequently, pepper fruits were carefully injured using a syringe, and 10 µl of a conidial suspension containing 1.0×10^6 conidia/ml of *C. acutatum* was inoculated to induce anthracnose. Photographs were taken 1 week after the inoculation with *C. acutatum.* (A) Effect of antagonistic bacteria on pepper plant growth against *C. acutatum* and roots (B). (C) Phenotype of pepper fruit following pretreatment with antagonistic bacterial isolate and inoculation with *C. acutatum* 7 days after the pretreatment. C, control (untreated group); NC, negative control (inoculated only with the pathogen *C. acutatum* spore suspension); PC, positive control (biological pesticides; Acadian29, Canada).

negative control treatment. *CaPR4* expression increased by 1.37-, and 1.26-fold in the positive control, and GP-P8 treatments, respectively. The *CaNPR1* gene showed the highest expression (7.28-fold) with the GP-P8 treatment. In the case of *CaMAPK4*, *CaJA2*, and *CaERF53* GP-P8 treatment resulted in the highest expression levels of 1.23- and 18.38-fold, respectively, compared to the negative control treatment. These data indicate that the antagonistic bacterial GP-P8 isolate plays a significant role in the immune response to anthracnose in peppers.

Discussion

In this study, GP-P8 strain demonstrated antagonistic effects against fungal pathogens that cause anthracnose (*C. acutatum*, *C. dematium*, and *C. coccode*.) (Table 1, Fig. 1). The GP-P8 isolate was identified using 16S rRNA-based sequencing as *B. siamensis* (Fig. 2), comprising gram-positive bacteria known to form endogenous spores, have demonstrated efficacy as beneficial microorganisms promoting plant growth, particularly in water-deprived environments. This characteristic enhances their value in research and development, including the formulation of biological agents (Yashaswini et al., 2021). Ongoing studies focus on *Bacillus* spp.'s potential in anthracnose suppression (Han et al., 2015b). PGPR exhibits various direct and indirect mechanisms that contribute to the suppression of plant pathogens. The direct mechanisms utilized to suppress plant pathogens involved biochemical tests on the GP-P8 strain to assess the production of siderophore, cellulase, and proteases. *B. siamensis* GP-P8, formed cellulase and protease, and analysis of siderophore production revealed clear zones in the selection medium, indicative of enzyme secretion (Fig. 3A). Microorganisms that produce proteases play a vital role in plant defense against pests and pathogens (Clemente et al., 2019). Furthermore, siderophores are low-molecular-weight secondary metabolites that can chelate iron and help meet iron requirements in the soil. Siderophores produced by PGPR protect plants from pathogens and contribute to plant growth (Arora and Verma, 2017). Producing enzymes involved in phosphate solubilization, starch hydrolysis, and plant hormones such as IAA represents a PGP trait. These

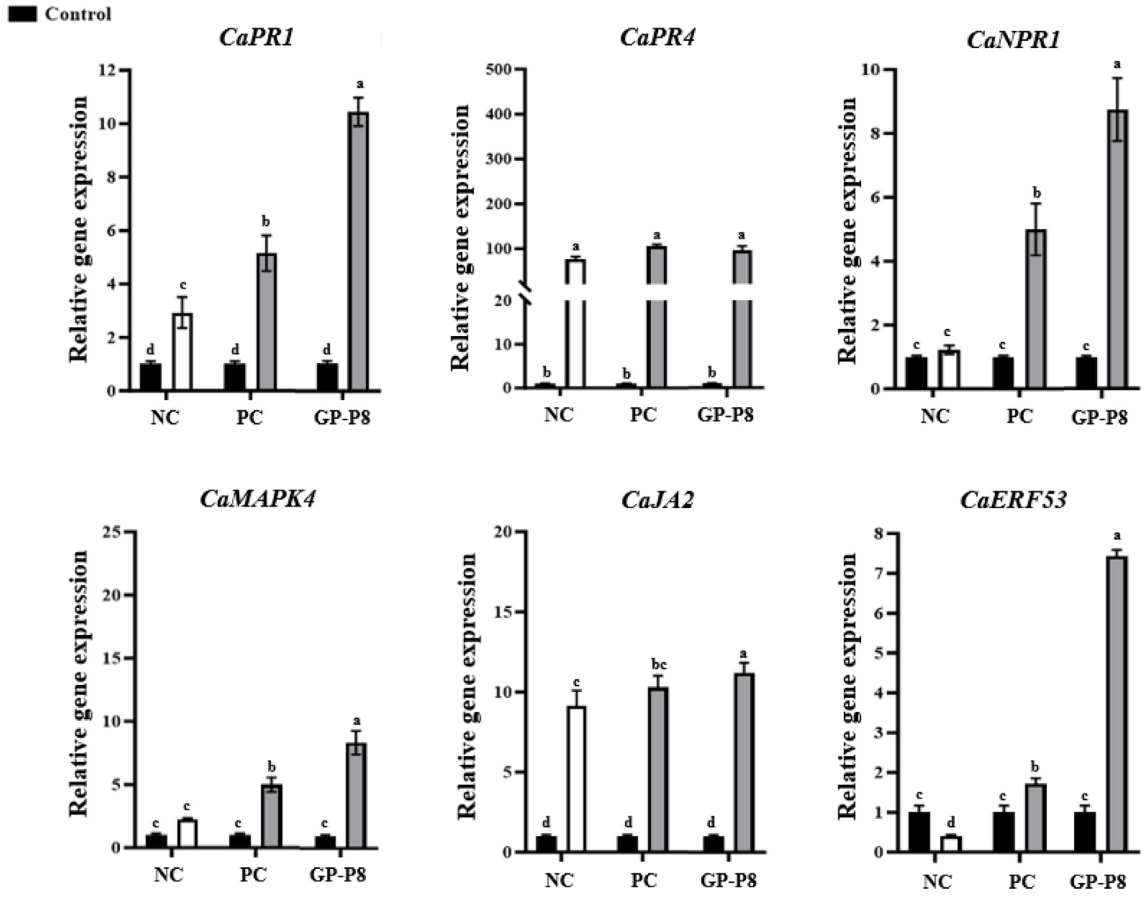

Fig. 6. Immune genes (*CaPR1*, *CaPR4*, *CaNPR1*, *CaMAPK4*, *CaJA2*, and *CaERF53*) expressed on pepper fruits with treatment of GP-P8 7 days after artificial inoculation of *C. acutatum*. NC, negative control; PC, positive control. Lowercase letters in Duncan's test using IBM SPSS Statistics 24 software signify that values sharing the same characters are not significantly different from each other at the $P < 0.05$.

traits contribute to enhancing plant growth, yield, and nutrient uptake (Singh et al., 2019). Phosphate is a crucial element for plant growth (Yazdani et al., 2009), whereas starch is an energy source for plant metabolism (Fincher, 1989). IAA production is an important mechanism in plant growth and development (Pal et al., 2019). IAA promotes various processes, such as cell division, cell extension, cell differentiation, flowering, and lateral root formation, directly contributing to enhanced plant growth and improved nutrient absorption in the surrounding soil. The results of this study demonstrate the specific biochemical activities of the GP-P8 strain before its application to crops. Such insights enable the determination of strain characteristics to enhance PGP effects. We also strained for the presence of secondary metabolite biosynthetic genes in the bacterial genome polyketides and lipopeptides using gene-specific primers (Supplementary Table 1). *Bacillus* strains produce various antibacterial and antifungal compounds, including non-ribosomal peptides (surfactin, fengycin, bacillomycin-D, bacilysin, and bacillibactin) (Ngalimat et al., 2021) and polyketides (bacillaene, macrolactin, and difficidin) (Palazzini et al., 2016) that can be correlated with the biocontrol potential of bacteria. Among the eight tested genes, eight genes viz. *ituA* (Iturin), *FenA* (Fengycin), *DfnD* (Difficidin), *BacD* (Bacilysin), *DhBe* (Bacillibactin), *srfA* (Surfactin), *mInA* (Macrolactin), and *baeR* (Bacillaene) were detected in the GP-P8 genomic DNA (Fig. 3B). SPME/GC-MS analysis identified four compounds from the GP-P8 strain based on retention time and peak area percentage: carbon dioxide, 2-butanone, 3-hydroxy (acetoin), 2,3-butanediol, and pyrazine (Fig. 3C). *Bacillus* spp. produce secondary metabolites, particularly antibiotics (Raaijmakers and Mazzola, 2012). Four *Bacillus amyloliquefaciens plantarum* strains (UCMB5033, UCMB5036, UCMB5113, and FZB42) have antagonistic activity against fungal strains *B.cinerea*, *A. brassicicola*, and *S. sclerotiorum*, which are

phytopathogens. Various VOCs were produced, including 2,3-butanedione, acetoin, 5-methyl-heptanone, 2-methylpyridine, 2-pentanone, 2-heptanone, and 3-methylbutanol (Asari et al., 2016). Many studies have reported the production of pyrazine from *Bacillus* spp. to exhibit antifungal (Caulier et al., 2019; Chaves-López et al., 2015; Haidar et al., 2016). The secondary metabolites of biosynthesis genes and volatile compounds detected in this work show the crucial role of the antagonistic effect against phytopathogens and their protective capability against pepper diseases. *In vivo* greenhouse experiments were conducted using the bacterial strains that exhibited antagonistic and plant growth effects, as observed in *in vitro* tests. *B. velesensis* BS1 inhibits red pepper anthracnose disease and promotes growth when inoculated with a *B. velesensis* BS1 suspension (Shin et al., 2021). Suspensions of the GP-P8 strain were pretreated in the rhizosphere of pepper soil, and anthracnose was induced by wounding the red pepper fruit and inoculating it with *C. acutatum*. Pretreatment with antagonists induced systemic resistance (ISR), leading to an average 46% reduction in disease severity when treated with the GP-P8 strain, as opposed to the negative control treatment using only water. In the PGP analysis, the GP-P8 strain significantly promoted growth and positively impacted fruit yield compared with the control (Table 2, Fig. 5). Acadian29, a seaweed extract, was used as a positive control in this study. Seaweed extracts contain many bioactive substances that induce plant growth and immune responses (Khan et al., 2009). These extracts enhance plant resistance against fungal and bacterial pathogens in the soil, protecting crops from significant economic damage (Du Jardin, 2015; Fei et al., 2017). However, while seaweed extracts elicit various responses to biotic and abiotic stresses in water, most of the genetic, physiological, and biochemical mechanisms remain largely unexplored and require systematic research (Ali et al., 2021). Overall, defense mechanisms can be classified into two types: systemic acquired resistance and ISR (Van Loon, 2007; Van Loon et al., 1998). ISR, which PGPR potentiates, depends on the pathways regulated by JAs, SA, and ethylene (Son et al., 2014; Van Loon, 2007). The production of pathogenesis-related (PR) proteins is vital because they enhance the overall resistance of plants to various pathogens (Borad and Sriram, 2008). Pretreatment of anthracnose-infected pepper fruit with the GP-P8 bacterial strain induced the expression of PR genes and activated genes associated with the immune and defense systems. After confirming the molecular immunization mechanism, the effects of the more deeply isolated strain were investigated (Fig. 6). These results support the beneficial effects of microorganisms on anthracnose control and the promotion of pepper plant growth.

This study established the antifungal efficacy potential of isolate *B. siamensis* GP-P8. Through *in vitro* tests, we validated antagonistic activity against anthracnose pathogens, including the *C. acutatum*, *C. dematium*, and *C. coccode*. The antagonistic effects are attributed to its biochemical characteristics, the presence of a lipopeptide biosynthetic gene, and the production of volatile compounds. These characteristics can be utilized as effective means for controlling infections caused by a range of pathogens. Moreover, through *in vivo* greenhouse tests, the biocontrol efficacy of the bacterial isolate GP-P8 against *C. acutatum* causing anthracnose has applicability in the field condition.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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