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Research article

Efficacy of *Bacillus subtilis* for controlling anthracnose in chilli

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Abstract

Importance of the work: Chilli cultivation in Thailand has a problem with anthracnose. Biological control is used to reduce such problems.

Objectives: To assess the biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum* spp. causing anthracnose in chilli.

Materials & Methods: The antagonistic effects were evaluated *in vitro* of *B. subtilis* on *Colletotrichum* spp. and the associated effects on chilli seed growth. In addition, the amount of salicylic acid (SA) and phenolic compounds in chilli plants and *B. subtilis* efficacy on anthracnose severity of leaves and fruits were assessed under net-house conditions.

Results: The results showed that the antagonistic percentage of *B. subtilis* was approximately 55% and it did not affect germination rates, shoot height or the root length of chilli seedlings. The *Bacillus* treatment reduction levels of anthracnose severity on chilli leaves and fruits were 86–100% and 49–74%, respectively. In the chilli plants, *B. subtilis* induced phenolic compound levels to approximately 22 mg per 100 g fresh weight (FW) and 19 mg per 100 g FW in leaves and fruits, respectively. Similarly, the amount of SA increased to 62–65 µg/g FW and 14–17 µg/g FW in chilli leaves and fruits, respectively.

Main finding: Application of *B. subtilis* inhibited anthracnose disease in chilli and could be a safe alternative to harmful chemical usage.

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Introduction

Chilli (*Capsicum annuum* L.) is an important spicy plant because of its widespread consumption, high vitamin content and high profitability in developed and developing countries (Oo and Oh, 2016). Chilli production is invariably affected by fungi, bacteria, viruses, nematodes and insects, leading to severe crop and fruit damage every year (Hussain and Abid, 2011; Daunde et al., 2018). Anthracnose, with its causal agent *Colletotrichum* spp., is the most important disease in chilli (Oanh et al., 2004; Than et al., 2008b). Severe anthracnose of chilli caused by *Colletotrichum* spp. can lead to yield losses of 50% (Pakdeevaraporn et al., 2005; Thumanu et al., 2017).

The chemical method of controlling anthracnose on chilli is highly effective; however, it has a major impact on human health and adverse environmental effects due to the chemical residues in chilli products (Buensanteai et al., 2009; Sahitya et al., 2014; Sasirekha and Srividya, 2016). The active ingredients of these chemicals for controlling anthracnose are azoxystrobin, propiconazole, pyraclostrobin and trifloxystrobin (Sharma et al., 2019). A novel approach to disease management using beneficial microorganisms would be critically important, contributing to safe agricultural production. Plant growth-promoting rhizobacteria, such as *Bacillus* species, could be prospective microorganisms for enhancing disease resistance and plant growth (Buensanteai et al., 2008). For example, *B. subtilis* activated the resistance of chilli plants to the soilborne pathogen *Fusarium solani* by elevating defense enzymes, including phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (PO) 7 d after challenge inoculation (Rajkumar et al., 2018). *Pseudomonas fluorescens*, another beneficial microorganism, was reported to protect chilli fruits against *C. capsici* and *Alternaria alternata*; in *Pseudomonas*-treated fruits, the concentrations of phenolic compounds and four defense enzymes (PO, PPO, PAL and catalase) started to increase on the first day and resistance activity remained for 2–3 d (Anand et al., 2007). Lanna-Filho et al. (2017) reported that *B. amyloliquefaciens* and *B. pumilis* stimulated resistance in tomato plants, reducing disease lesions by up to 62%. Mycorrhizal inocula including *Trichoderma harzianum*, *Funneliformis mosseae*, *B. subtilis*, *F. caledonius*, *P. fluorescens, F. coronatus*, *Agrobacterium radiobacter* and *Septoglomus viscosum* reduced the shoot density of triticale (Caruso et al., 2018). Oanh et al. (2006) showed that treatments with plant inducers of *Trichoderma harzianum,* salicylic acid (SA) and Bion® had no ability to increase polyphenol oxidase in chilli cotyledons. Under field conditions, the application of plant elicitors through foliar sprays decreased chilli anthracnose; application of 1×10^8 spores/mL of *T. harzianum* reduced it by 24.2–57.4%, 2 mM of SA by 19.8–36.6% and Bion® by 75.9–76.3%.

These biotic elicitors could trigger many defense mechanisms, including the amount of SA, hypersensitive response (HR), callose, reactive oxygen species, defense-related genes, phenolic compounds, phytoalexins and defense enzymes. Consequently, defense activities could reinforce cell walls and kill phytopathogens when the plants are infected (Park et al., 2016; Daunde et al., 2018). The main defense components of disease resistance are SA and phenolic compounds. SA is a crucial signal during the triggering of systemic acquired resistance as well as other important defense events of the HR (Shanmugam and Narayanasamy, 2008; Tripathi et al., 2019). On induced systemic resistance to powdery mildew in cucurbits, *B. amyloliquefaciens* strain LJ02 markedly elevated the yield of salicylic acid (Li et al., 2015). Phenolic compound expression plays a crucial role in the formation of defense-related barriers by creating and enforcing defense structures in host cells against pathogen infection and invasion (Shafi et al., 2017). An accumulation of phenolics after treating with *Bacillus* contributed to the resistance of tomato plants against the fungus *A. solani* (Latha et al., 2009).

Beneficial *Bacillus*-based biological control agents and their induced resistance mechanisms are gaining commercial significance because they are safe and environmentally friendly (El-Saadony et al., 2022). Therefore, the general target of this research was to assess the biocontrol efficacy of *B. subtilis* against *Colletotrichum* spp. causing anthracnose in chilli. The primary objective was to collect chilli anthracnose samples and determine the pathogenicity of *Colletotrichum* isolates. DNA extraction, PCR steps and NCBI gene data bank were performed to identify the aggressive *Colletotrichum* isolate. Then, dual culturing was conducted to evaluate whether *B. subtilis* was antagonistic toward the aggressive *Colletotrichum* isolate. Another objective was to determine if the *B. subtilis* could affect the seed germination and seedling growth of chilli. On an assessment of interactions between the *B. subtilis* elicitor, chilli plants and the aggressive *Colletotrichum* isolate, disease severity and reductions in it were surveyed to determine if *B. subtilis* elicitor could induce resistance against anthracnose caused by the isolate. To further assess the induced resistance mechanism, the quantity of phenolic compounds and SA were compared with those from the water control treatment.

Materials and Methods

Elicitors and B. subtilis culture conditions

Stock cultures of *B. subtilis* strain CaSUT008 (CaSUT008) and strain CaSUT008-2 (CaSUT008-2), BIG® and COS® were obtained from the Plant Pathology and Biopesticide Laboratory of Suranaree University of Technology, Thailand. BIG® is a plant vaccine. COS® has a chitioligosaccharide component. Both BIG® and COS® are produced from crab and shrimp shells.

B. subtilis strains were cultured onto 500 mL shaking flasks (each flask contained 300 mL nutrient broth + 2% glucose), at 30 ± 2°C with constant shaking at 180 revolutions per minute for 48 hr. Next, the liquid *Bacillus* cultures were diluted with distilled water to achieve a *B. subtilis* suspension density of 1×10^8 colony forming units/mL (with optical density 0.2 at 600 nm).

Sample collection and Colletotrichum isolation

Samples of chilli anthracnose were collected from organic chilli farms in Nakhon Ratchasima, Thailand. The anthracnose samples were gently washed under tap water, then placed in a laminar flow hood and allowed to dry. After that, infected tissues of anthracnose lesions were cut into 3–4 mm squares, surface-sterilized in 1% sodium hypochloride (NaOCl) for 1 min, washed twice in sterile distilled water, dried on sterilized Whatman paper and plated on water agar. Subsequently, the Petri dishes were incubated at 25±2 °C, with a 12 hr photoperiod. After 1–2 d, single mycelial tips were transferred onto potato dextrose agar (PDA) medium. The pure fungal colonies were maintained in fresh PDA slants for further experiments (Sompong et al., 2012).

Determination of pathogenicity of Colletotrichum isolates

The experiment was carried out on ripe and unripe fruits of *Capsicum annuum* L. cv. Super Hot. The experiment was repeated three times with 10 chilli fruit per replication.

Each *Colletotrichum* strain was cultured on PDA medium. Spore suspensions were prepared with a density of 1×10^6 conidia/mL, which was sprayed onto chilli fruit until it ran off (Sompong et al., 2012). The ripe and unripe fruits were chosen of similar shape and color with no damage on their peels. A punch of four sterile needles was used to create a wound of approximately 1 mm depth at one position on the fruit peel. Then, 20 µL spore suspension was dropped on each wound. The chilli fruit were put inside plastic bags with wet cotton at 25 °C for 24 h in a dark inoculation room. After that, they were stored at room temperature. The lengths of anthracnose lesions were recorded at 5 d after inoculation. The pathogenicity experiment was performed three times with similar results. The most aggressive isolate of *Colletotrichum* spp. was identified using the following steps.

Identification of the aggressive Colletotrichum isolate

Ten-day-old *Colletotrichum* mycelia were collected and immediately put into liquid nitrogen. Next, approximately 1 g of frozen mycelia was gently ground into fine powder and put into a sterile 1.5 mL microcentrifuge tube with 500 µL of extraction buffer (25 mM ethylenediaminetetraacetic acid (EDTA), Tris-HCl 0.2 M, sodium dodecyl sulphate 2%, NaCl 0.25 M and pH 8.5). After that, a water bath (at 65 °C) was used to incubate the microcentrifuge tube containing fungal suspension for 30 min. Later, the tubes were centrifuged at 18,000×*g* for 10 min. The supernatant inside these tubes was transferred into new sterile 1.5 mL microcentrifuge tubes containing 200 µL of a solution of chloroform-to-isoamyl alcohol (24:1). These new tubes were vortexed for 30 s and centrifuged at 18,000×*g* for 10 min. Then, supernatant was transferred into a sterile 1.5 mL microcentrifuge tube, added with ice-cold absolute ethanol (at double the supernatant volume) and vortexed for around 30 s and then centrifuged at 9,500×*g* for 5 min. Finally, the liquid mixture was discarded and the remaining pellets were diluted and washed with 400 µL of 70% ethanol. The ethanol-diluted solution was air-dried under vacuum conditions for approximately 30–40 min. The air-dried pellets were suspended in 50 µL of TE buffer (EDTA 0.1 mM, Tris- HCl10 mM, pH 8.5) and stored at -20 °C in a freezer (Sangpueak et al., 2017).

During polymerase chain reaction (PCR) amplification, a 25 µL reaction volume (5 µL of buffer 10×, 0.2 µL of Taq DNA polymerase, 0.5 µL of 10 µM primers, 0.5 µL of 50 mM $MgCl_2$, 0.75 µL of 10 mM dNTP, 1 µL of genomic DNA and sterile distilled water) was processed with a thermal cycler (Bio- Rad PCR; CA, USA). The primers were ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS3 (5′-GCATCGATGAAGAACGCAGC-3′). The amplification procedure consisted of: an initial denaturation (94 °C, 5 min), 35 cycles of PCR as a denaturation step (94 °C, 30 s), an annealing step (59 °C, 30 s) and then extension (72 °C,

30 s) steps, with a final extension (72 °C, 7 min). An aliquot of 3 µL of PCR product was added to a well of agarose 1% (weight per volume) and run with buffer TBE 1× at 100 V for 25 min. Later, the gel was stained with buffer DNA Loading 6× Gene DireX's Novel Juice (USA) and captured with a GelDocXR (UVP GelDoc-It®2 Imager; Canada), according to Sangpueak et al. (2017).

Purified PCR product was sequenced by Pacific Scientific (Jakarta Barat, Indonesia), and analyzed using the Chromas Lite software (Technelysium Pty Ltd.; South Brisbane, Queensland, Australia) to determine the consensus sequence. Nucleotide sequence alignment was performed using the BLAST-N program and the MEGABLAST algorithm, based on NCBI gene bank data (National Center for Biotechnology Information; Bethesda, MD, USA, USA; http://www.ncbi.nlm.nih.gov).

Evaluation of antagonistic activity of B. subtilis elicitor

This experiment utilized a completely randomized design (CRD), with three treatments (CaSUT008, CaSUT008-2 and a water control) and five replications, using one Petri dish per replication. The experiment was repeated three times with similar results, following the dual culture methodology (Mojica-Marín et al., 2008). Radians of *Colletotrichum* colonies were recorded on days 2, 4, 6 and 8 after adding fungal slices. The inhibition percentage was calculated as Inhibition percentage = [1 – (Fungal growth of treated treatment / Fungal growth of control treatment)] × 100%.

Effect of B. subtilis elicitor on seed germination and seedling growth

The experiment was performed with a CRD consisting of five treatments (CaSUT008, CaSUT008-2, BIG®, COS® and a water control) with five replications consisting of 10 seeds each. Two abiotic commercial elicitors (BIG® and COS®) were surveyed as positive treatments. The chilli cv. SuperHot seeds were soaked in different elicitor suspensions for 3 hr, followed by shade-drying. Seeds from the control treatment were handled identically, whereas the elicitor solution was replaced with distilled water. Then, the seeds were incubated separately in Petri dishes containing two layers of wet Whatman papers. The percentage of germination, root length and shoot height were assessed on day 7 after treatment. The experiments were performed three times with similar results.

Efficacy of B. subtilis elicitor against anthracnose in chilli plants under net house conditions

The experiment was performed with a CRD consisting of five treatments (CaSUT008, CaSUT008-2, BIG® and COS® and a water control), with five replications of 10 plants each. The elicitors were treated by seed soaking once. Elicitors were further induced with three applications of foliar spray at 1 d after planting (DAP), 29 DAP and 59 DAP. Later, at 60 DAP, the chilli plants were inoculated using a *Colletotrichum* suspension with a density of 1×10^6 spore mL^{-1}. The chilli plants were placed in an inoculation room at 25 ± 2°C and a relative humidity of approximately 98% for 24 hr. After that, the plants were moved to a net house.

The level of anthracnose severity in the leaves and fruit were assessed at 5 d after inoculation (DAI) using an anthracnose scale based on the total disease area on leaves (Thumanu et al., 2017), where 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100%. The percentage of anthracnose severity (AS) was determined as AS (%) = [Sum of all numerical ratings / (Quantity of leaves counted × 4)] × 100%. Next, efficacy of treatments against anthracnose was determined based on a reduction of anthracnose severity as AS reduction (%) = $[(AS_{control} – AS_{treatment}) / AS_{control}] \times 100\%$ (Thumanu et al., 2017). The experiments were performed three times with similar results.

Quantification of phenolic compounds and salicylic acid in induced chilli plants

This experiment was performed using a CRD, with similar treatments to the previous experiment with three replications. Fresh *B. subtilis* elicitors were treated once with seed soaking and then three times with foliar sprays at 1 DAP, 29 DAP and 59 DAP. At 60 DAP, the chilli plants were inoculated in the same manner as for the previous experiment. The chilli leaves and fruits were sampled 1 d before and 1 d after treatment with the *B. subtilis* elicitors (Anand et al., 2007), and stored immediately at -80 °C in a freezer. The samples were used to perform assays of phenolic compounds and SA.

The quantification of phenolic compounds was conducted using the method of Blainski et al. (2013). Briefly, 0.5 g of the chilli sample was carefully ground with 1 mL 90% methanol. The mixture was centrifuged at approximately 12,000×*g* and 4°C for 5 min. After that, 20 µL of supernatant were added

to a solution of 80 µL 7% sodium carbonate and 100 µL 10% Folin-Ciocalteau reagent. Next, the mixture was incubated at room temperature for 30 min and its absorbance was measured at 760 nm. The absorbance of the treatment and a subsequent reference standard were compared and the quantity of phenolic compounds was calculated.

The SA amount was evaluated using the procedure of Warrier et al. (2013). First, 0.5 g of chilli sample was ground in 1 mL of 90% methanol. The mixture was centrifuged at 12,000×*g* and 4 °C for 5 min. Then, 100 µL supernatant was mixed with 100 µL 0.02 M ferric ammonium sulphate. After incubation (room temperature, 5 min), this mixture was measured at 530 nm using an absorbance spectrophotometer. The measured absorbances of the treatment and subsequent reference were used to calculate the amount of SA.

The experiments were performed three times with similar results.

Statistical analyses

The data were analyzed using analysis of variance in the SPSS20 software package (SPSS Inc.; Chicago, Il, USA). Duncan's multiple range test and a t test were applied to determine significant differences among means. The tests were considered significant when $p < 0.05$. Prior to the analyses, a homogeneity test and normality test were conducted.

Results

Pathogenicity of Colletotrichum isolates

In total, 47 samples of chilli anthracnose were collected. The pathogenicity of pure colonies of eight *Colletotrichum* isolates were quickly checked on red (ripe) and green (unripe) chilli fruits. On the red chilli fruits, *Colletotrichum* spp. isolates ChilliCole. 3, 4, 6 and 7 were the most damaging treatments, with lesions having average diameters of approximately 16.04 mm, 15.08 mm, 16.25 mm and 14.90 mm, respectively. On the green chilli fruits, *Colletotrichum* spp. isolates ChilliCole. 1, ChilliCole. 4 and ChilliCole.5 had anthracnose lesions with average diameters of approximately 10.40 mm, 10.45 mm and 11.07 mm, respectively, confirming that they were aggressive isolates (Table 1). Therefore, the *Colletotrichum* sp. isolate ChilliCole. 4 was chosen to study further because it caused severe damage to both stages of chilli fruit.

Identification of aggressive Colletotrichum sp. isolate ChilliCole. 4

DNA fragments of around 490 bps were successfully obtained from the PCR reaction (Fig. 1). The resulting identification indicated that the pathogen from isolate ChilliCole. 4 was *C. acutatum*.

Antagonistic efficacy of B. subtilis for control of pathogen

The antagonistic abilities of CaSUT008 and CaSUT008-2 against the pathogenic *C. acutatum* were assessed at 2 d, 4 d, 6 d and 8 d after adding the fungal slices. At all time points, the radii of the *Colletotrichum* colonies in both treatments of CaSUT were significantly different to those of the control treatment. At 8 d after adding the fungal slices, the radii of

Table 1 Average length mean ± SD of anthracnose caused by *Colletotrichum* spp. on chilli fruits

Colletotrichum isolate	Average anthracnose length (mm) at 5 d after inoculation	
	Green chilli fruits	Red chilli fruits
ChilliCole. 1	10.40 ± 0.97^{cd}	13.89 ± 1.05^{ab}
ChilliCole. 2	8.84 ± 0.84^{abc}	11.46 ± 0.95^{a}
ChilliCole. 3	9.69 ± 0.87^{bcd}	16.40 ± 1.07^{b}
ChilliCole. 4	10.45 ± 0.95^{cd}	15.08 ± 1.00^{b}
ChilliCole. 5	11.07 ± 1.02^{d}	12.99 ± 1.00^{ab}
ChilliCole. 6	8.55 ± 0.84^{ab}	16.25 ± 1.12^{b}
ChilliCole. 7	9.52 ± 0.97^{bcd}	14.90 ± 1.10^{ab}
ChilliCole. 8	7.24 ± 0.81^{a}	12.80 ± 0.89^{ab}
F test	*	*
CV (%)	9.30	13.03

Mean±SD in each column superscripted by different lowercase letters are significantly different ($p<0.05$).

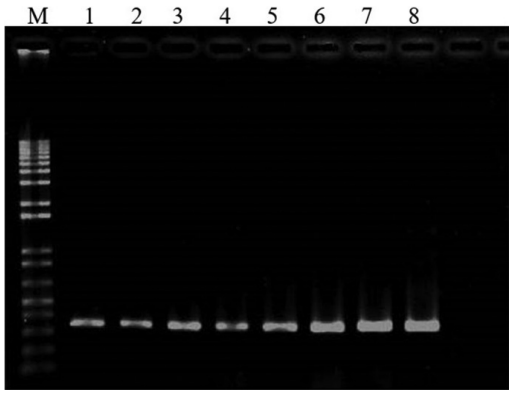

Fig. 1 Amplification of *Colletotrichum* DNAs samples using primer pairs ITS3 and ITS4, where lane M = PCR ladder of 1 Kb; lane 1 = ChilliCole. 1; lane 2 = ChilliCole. 2; lane 3 = ChilliCole. 3; lane 4 = ChilliCole. 4; lane 5 = ChilliCole. 5, lane 6 = ChilliCole. 6; lane 7 = ChilliCole. 7; lane 8 = ChilliCole. 8

the *Colletotrichum* colonies CaSUT008 and CaSUT008-2 were 20.40 mm and 20.50 mm, respectively (Table 2). However, their inhibition percentages were not significantly different at any time point. The antagonistic percentages of CaSUT008 and CaSUT008-2 were 55.22% and 55.52%, respectively (Table 3, Fig. 2). Therefore, both *Bacillus* treatments were chosen for research on chilli seeds and plants.

Effect of B. subtilis elicitor on germination and seedling growth

The germination rate, root length and shoot height of *Bacillus* and other treatments were not significantly different (Table 4). The germination rate, root length and shoot height of the treatments and of the control were in the ranges of approximately 83–92%, 6–7 mm and 5–6 mm, respectively.

Table 2 Average sterile ring radius (mm) of *B. subtilis* with *Colletotrichum acutatum*

Treatment	Days after putting fungal slices			
	2	4	6	8
Control	26.92±1.32[a]	16.76±1.41[a]	9.60±1.32[a]	2.40±0.45[a]
CaSUT008	27.70±1.58[b]	20.80±1.41[b]	20.40±1.41[b]	20.40±1.32[b]
CaSUT008-2	27.72±1.58[b]	21.46±1.50[c]	20.50±1.32[b]	20.50±1.41[b]
F test	*	*	*	*
CV (%)	0.38	1.38	2.66	2.37

* = significant ($p < 0.05$); CV = coefficient of variation
Mean±SD in each column superscripted by different lowercase letters are significantly different ($p<0.05$).

Table 3 Growth inhibition (mean±SD, %) of *B. subtilis* in dual culture against *C. acutatum*

Treatment	Days after placing with fungal slices			
	2	4	6	8
CaSUT008	9.65±0.50	22.15±1.32	42.52±2.12	55.22±2.18
CaSUT008-2	9.90±0.71	25.77±1.22	42.91±2.18	55.52±2.06
F test	ns	ns	ns	ns
CV (%)	16.00	6.44	3.57	2.15

ns = non-significant ($p \geq 0.05$); CV = coefficient of variation

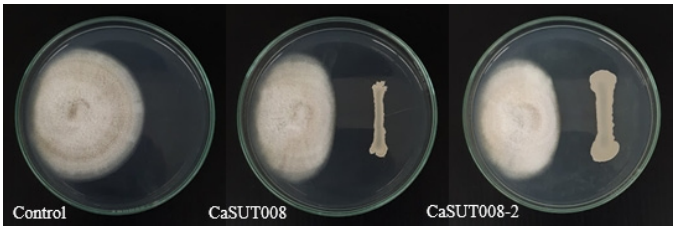

Fig. 2 Antagonistic effect of *Bacillus subtilis* on *Colletotrichum acutatum* at 8 d after placing fungal slice with *in vitro* conditions, where experiment was repeated thrice, with each treatment carried out with five replicates

Table 4 Efficacy of elicitors on germination and growth of chilli seeds (values shown as mean±SD) at 8 days after treatment

Treatment	Germination[a,b]	Plant growth[a,b,c]	
		Shoot height (mm)	Root length (mm)
CaSUT008	86.67±2.29	5.06±0.50	6.69±0.87
CaSUT008-2	91.67±2.74	6.04±0.50	6.99±0.84
BIG®	91.67±2.78	4.98±0.71	6.49±0.77
COS®	90.00±2.60	5.27±0.87	6.49±0.87
Control	83.33±2.83	5.02±0.71	6.36±0.71
F test	ns	ns	ns
CV (%)	7.70	22.31	16.34

ns = non-significant ($p \geq 0.05$)

Efficacy of B. subtilis on anthracnose severity of chilli leaves and fruit

The leaf and fruit disease severity levels of the treatments CaSUT008 and CaSUT008-2 as well as the abiotic treatments BIG® and COS® were significantly lower than those of the control treatment, confirming that resistance had been induced (Figs. 3 and 4). On chilli leaves, the anthracnose severity of the treatments CaSUT008 and CaSUT008-2 were 6.44% and 3.45%, respectively, significantly lower than those of BIG® and COS®. The disease severity levels of the anthracnose lesions on fruits were similar to those on leaves. Treatment with CaSUT008-2 had the lowest disease severity at 0%, followed by treatment with CaSUT008, with disease severity of approximately 19.56%. Reductions in disease severity on chilli leaves and fruits following the treatments CaSUT008-2 and CaSUT008 were 86–100% and 49–74%, respectively, (Table 5).

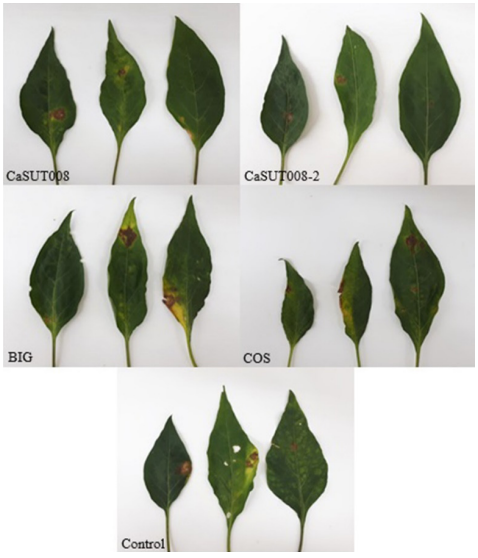

Fig. 3 Efficacy of *Bacillus* elicitors on inhibiting anthracnose on chilli leaves at 7 d after inoculation

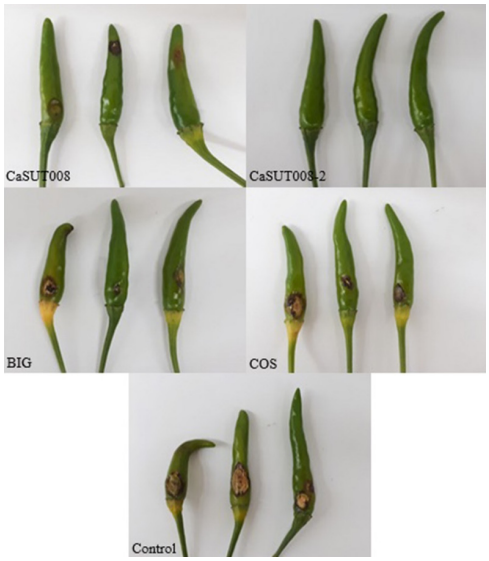

Fig. 4 Efficacy of *Bacillus* elicitors on inhibiting anthracnose on chilli fruits at 7 d after inoculation

Table 5 Efficacy of elicitors on anthracnose lesions in chilli cv. Super Hot (values shown as mean±SD)

Treatments	Anthracnose severity (%)[a,b,c]		Disease reduction (%)	
	Leaves	Fruits	Leaves	Fruits
CaSUT008	6.44±0.71[d]	19.56±1.22[c]	74.11	49.29
CaSUT008-2	3.45±0.50[e]	0.00[d]	86.16	100.00
BIG®	21.00±1.32[b]	23.56±1.50[b]	15.63	38.91
COS®	18.00±1.41[c]	25.33±1.50[b]	27.68	34.30
Control	24.89±1.58[a]	38.56±2.00[a]	0	0
F test	*	*		
CV (%)	9.37	5.91		

* = significant ($p < 0.05$); CV = coefficient of variation

[a] = Mean ± SD in each column superscripted by different lowercase letters are significantly ($p<0.05$) different.

Quantity of phenolic compounds and salicylic acid in induced chilli plants

The quantities of phenolic compounds in both the leaves and fruits of all treatments were not significantly different before inoculation with *Colletotrichum*. However, after pathogen inoculation, all induced treatments had significantly higher levels of phenolic compounds than the control. The quantities of phenolic compounds in the induced treatments of CaSUT008 and CaSUT008-2 were approximately 22.32–22.56 mg/100 g FW and 18.74–19.03 mg/100 g FW in leaves and fruits, respectively (Table 6).

The chilli plants were treated with biotic and abiotic elicitors at 1 DAP, 29 DAP and 59 DAP. On pre-inoculation chilli leaves, the three treatments of CaSUT008, CaSUT008-2 and COS had approximately 62.52 µg/g FW of SA, 65.77 µg/g FW of SA and 65.87 µg/g FW of SA, respectively, which were significantly higher than for the control treatment (56.66 µg/g FW). After the inoculation, the concentration of SA was still high following the treatments BIG® (109.58 µg/g FW) and COS® (101.20 µg/g FW), which were both significantly different to the control. On the chilli fruits before the inoculation, the two treatments of CaSUT008-2 and COS® had high concentrations of SA, at approximately 9.00 µg/g FW and 8.95 µg/g FW. However, after the inoculation, the SA concentrations of the CaSUT008, CaSUT008-2 and BIG® treatments were 14.70 µg/g FW, 17.14 µg/g FW and 13.58 µg/g FW, respectively, which were higher than for the control (11.33 µg/g FW), as shown in Table 7.

Table 6 Quantity of total phenolic compounds in chilli leaves and fruits treated by elicitors (values shown as mean±SD)

Treatment	Phenolic (GAE/100 g FW)			
	Chilli leaves		Chilli fruits	
	Before inoculation	After inoculation	Before inoculation	After inoculation
CaSUT008	13.59±1.10[b]	22.56±1.45[b]	5.37±0.55[a]	19.03±1.18[b]
CaSUT008-2	14.10±1.07[bc]	22.32±1.41[b]	6.27±0.59[b]	18.74±1.20[b]
BIG®	10.52±1.05[a]	22.49±1.45[b]	4.71±0.50[a]	19.01±1.24[b]
COS®	14.35±1.12[bc]	22.84±1.43[b]	4.88±0.50[a]	20.07±1.41[c]
Control	14.81±1.20[c]	21.52±1.41[a]	4.75±0.59[a]	15.25±1.12[a]
F test	*	*	*	*
CV (%)	4.47	1.63	6.52	2.35

* = significant ($p < 0.05$); CV = coefficient of variation

Mean ± SD in each column superscripted by different lowercase letters are significantly ($p<0.05$) different.

Table 7 Total salicylic acid (mean ± SD) in chilli leaves and fruits treated by elicitors

Treatment	Salicylic acid (µg/g FW)			
	Chilli leaves		Chilli fruits	
	Before inoculation	After inoculation	Before inoculation	After inoculation
CaSUT008	62.52±2.18[c]	95.95±2.78[b]	7.19±0.74[ab]	14.70±0.87[b]
CaSUT008-2	65.77±2.29[c]	88.24±2.69[a]	9.00±0.77[c]	17.14±0.92[c]
BIG®	48.46±2.12[a]	109.58±2.83[d]	6.94±0.67[a]	13.58±0.95[b]
COS®	65.87±2.45[c]	101.20±2.96[c]	8.95±0.74[c]	11.62±0.71[a]
Control	56.66±2.50[b]	91.81±3.00[ab]	7.70±0.59[b]	11.33±0.77[a]
F test	*	*	*	*
CV	3.89	2.49	4.35	6.17

* = significant ($p < 0.05$); FW = fresh weight; CV = coefficient of variation

Mean ± SD in each column superscripted by different lowercase letters are significantly ($p<0.05$) different.

Discussion

C. acutatum is the most aggressive fungal species causing chilli anthracnose. In the current research, the hyphae of *C. acutatum* had a pale pink, thick, cottony texture and its mycelia formed an entire margin in the PDA medium. The *C. acutatum* colonies had a pink phenotype with an average growth of approximately 4.73 mm/d. The conidia of *C. acutatum* were hyaline and cylindrical with round ends and a size of approximately 9.85 µm × 2.83 µm. These traits were similar to those reported in the characterization of *C. acutatum* by Than et al. (2008a). The fungal pathogen *C. acutatum* affects chilli quality, causes serious yield loss and leads to economic consequences in many chilli cultivation areas, including Korea (Kim et al., 2007), Thailand (Than et al., 2008a), Taiwan (Liao et al., 2012) and the USA (Harp et al., 2014). Therefore, the current research would be useful in managing chilli anthracnose caused by *C. acutatum* in both Thailand and Vietnam.

Considering the interaction of *B. subtilis* and *C. acutatum*, the antagonistic efficacies of the two strains of *B. subtilis* (CaSUT008 and CaSUT008-2) were 55.22% and 55.52%, respectively, 8 d after adding the fungal slices. The antagonistic results were consistent with the results reported by Kwon et al. (2022) who reported that *Bacillus tequilensis* GYUN-300 had good inhibition of the growth of *C. acutatum* KACC42403 based on dual culture plate assay at 7 d after placing with fungal slices.

On the interaction of the *B. subtilis* and chilli hosts, the elicitors did not affect seed germination, shoot length or root height. These results were different to those reported by Kwon et al. (2022), who applied another *Bacillus* species (*B. tequilensis*) which slightly stimulated seed germination and seedling growth.

On the interaction of the *B. subtilis*–host–*C. acutatum*, the elicitors triggered the production of SA and elevated the quantity of phenolic compounds, decreasing anthracnose severity on both the leaves and fruits of the chilli plants. The reductions in disease severity in the treatments CaSUT008 and CaSUT008-2 were approximately 40–100% in the leaves and fruit. The most important trait of induced systemic resistance is that the resistance mechanisms of the induced host plants are activated. After an attack of pathogens, the resistance mechanisms respond more strongly and quickly than in non-induced plants. The resistance mechanisms may consist of structural barriers and chemical defense compounds.

Phenolic compounds are one of the most crucial defense compounds of induced plants. The elevated phenolic compounds in the current research were similar to those reported by Anand et al. (2007) who indicated that a treatment of *P. fluorescens* could activate production of phenolic compounds. The accumulation of phenols began after an inoculation of *Colletotrichum* and decreased after 3 d following the inoculation. Phenolic compounds are fungitoxic in nature, affecting *Colletotrichum* growth inside the host tissues (Roy et al., 2018). In addition, these compounds help the plant host to increase the physical and mechanical strength of its cell walls, inhibiting the *Colletotrichum* invasion (Anand et al., 2007). Furthermore, SA plays a crucial role as a systemic defense signal triggering the resistance mechanism of the induced plants. SA is a systemic defense signal of induced chilli plants (Shafique et al., 2018). In the induced plants, the induced signal was triggered after treatment with elicitors. The results of testing on phenolic compounds and SA revealed and confirmed the systemic resistance mechanism of induced chilli plants after an attack of *C. acutatum*.

In general, the results of the current study indicated that *B. subtilis* was an eco-friendly biocontrol agent against *C. acutatum*-caused anthracnose in chilli. *B. subtilis* had an antagonistic percentage to *C. acutatum* of approximately 55%. *Bacillus* treatment decreased the anthracnose severity on the chilli leaves and fruits by 86–100% and 49–74%, respectively. In addition, the amount of phenolic compounds and SA increased in induced chilli plants. Further research should investigate phenylalanine ammonia lyase, peroxidase and callose involvement in the induced resistance mechanism against *C. acutatum*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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