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REVIEW ARTICLE

Efficacy of *Bacillus* and *Trichoderma* on growth and anthracnose resistance in scallion

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Abstract

Biotic elicitors, including *Trichoderma* and *Bacillus* can improve plant growth and disease resistance. Current research evaluated the efficacy of 14 *Bacillus subtilis* strains, *Trichoderma viride*, *Trichoderma harzianum*, *Trichoderma* sp. on enhancing growth and managing anthracnose in scallion. The inhibition efficiency of *Bacillus* and *Trichoderma* on *Colletotrichum* growth was assessed *in vitro*. Besides, effective biotic treatments against anthracnose were evaluated at net house conditions. Then, defence mechanisms including endogenous SA accumulation, production of phenolic compounds, catalase, peroxidase, phenylalanine ammonia-lyase and b-1,3-glucanase were revealed. The results showed that four biotic elicitors, including *B. subtilis* strains CaSUT008-2, D604, SUNB1 and *T. harzianum* showed high antagonistic activity against *in vitro* growth of *Colletotrichum* colonies at 41-77 %. At 28 days after planting, scallion plants treated with *B. subtilis* strain CaSUT008-2 and SUNB1 gained stem length at 15.35-15.79 cm. These four biotic treatments had low disease severity in net-house conditions at 25.00-50.00 %. On mechanisms of anthracnose resistance, at 24 hours after inoculation (HAI), scallion plants treated with *T. harzianum* and *B. subtilis* strain CaSUT008-2 showed high content of salicylic acid at 2.17-2.20 µg/g fresh weight. At 48 HAI, scallion plants induced by *B. subtilis* strain SUNB1 increased phenolic compounds at 42.65 µg gallic acid/mg dry mass. Moreover, four biotic elicitors enhanced activities of catalase, peroxidase, phenylalanine ammonia-lyase and b-1,3-glucanase in treated scallion plants. This study suggests that *B. subtilis* strain CaSUT008-2 and SUNB1, *T. harzianum* stimulated growth, helped scallion plants against anthracnose disease. The combined *Trichoderma*-bacteria bio-inoculants may be a good strategy to develop biocontrol agent and plant growth promoter within green and sustainable production of scallion.

Keywords

Bacillus; *Colletotrichum gloeosporioides*; disease reduction; resistance mechanisms, scallion; *Trichoderma*

Introduction

Scallion, also known as green shallot (*Allium fistulosum*), is extensively grown in Southeast Asia, America and temperate regions of Europe (1). It is a valuable crop in Korea, China, Japan, Thailand and Vietnam (2). In 2021, the global cultivation area for scallions was approximately 1.22 million hectares, with a

total yield of 22.8 million tons (3). In Thailand, scallion production reached approximately 168.1 thousand metric tons in 2022 (4), while in Vietnam, the total cultivation area was around 15 thousand hectares (5).

However, scallion cultivation currently copes with significant challenges due to diseases (6). Anthracnose, with its pathogen *Colletotrichum gloeosporioides,* is a particularly severe disease in scallion crops (7). Leaf infection with anthracnose results in a huge loss of scallion yield (8). To manage anthracnose, recommended practices such as crop rotation, cultivating resistant cultivars, applying fungicides and controlling watering are recommended to reduce the severity of anthracnose in host plants (9-11). Previous studies have shown that fungicides like captan, difenoconazole, tebuconazole and propiconazole effectively inhibited the growth of *C. gloeosporioides* (12). Nonetheless, the use of the fungicide method has direct implications for farmer health and the environment. Excessive use of chemical fungicides leads to the development of resistance and outbreaks of *Colletotrichum* fungi. In addition, chemical toxins remaining in agricultural products cause poisoning and affect consumer health. Currently, sustainable agricultural and eco-friendly production tends to develop strongly. Biological measures are applied and highly effective in disease management, to promote safer scallion production.

Resistance mechanisms of plants could be activated or induced by abiotic elicitors, including salicylic acid (SA) and silicon (13-15), as well as biotic elicitors such as *Bacillus amyloliquefaciens, Bacillus subtilis*, *Bacillus pumilus, Pseudomonas putida, Trichoderma harzianum, Trichoderma asperellum* (16-19). When applied to the surface of plants, these elicitors can inhibit phytopathogens' growth by secreting lots of antimicrobial substances (20). Furthermore, an interaction between plants and biotic elicitors can increase the synthesis of phenolic (PN) compounds, SA, defence enzymes, and pathogenesis-related (PR) proteins, stimulating host plants' growth and activating resistance mechanisms. *Bacillus* sp. strains AN24, AN30, AN31 and AN35 stimulated germination and growth in rice seedlings (21). *Bacillus* sp. AZ6 increased growth, yield and quality in maize plants (22). Induced plants have been found to produce biochemical components more rapidly and at higher levels compared to non-induced plants, leading to lower disease severity. Previous studies have highlighted various aspects of plant resistance against phytopathogens (17, 23). In melon, biotic elicitor *B. subtilis* UMAF6639 enhanced activities of SA and defence enzymes against *P. fusca* (24). In a similar way, it was showed that *B. subtilis* BS16 enhanced activities of phenylalanine ammonia-lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) in chili plants to combat *Fusarium solani* (17). PAL plays a crucial role in synthesis of SA and activity of PN compounds (17). In onion plants, it was reported that biotic elicitor *Pseudomonas fluorescens* Pf1 exhibited *in vitro* antagonistic effects against *Alternaria palandui* and induced activities of chitinase, peroxidase, β-1,3-glucanase and PPO (25). Similarly, biotic elicitor *Bacillus* spp. have been found to enhance amount of PPO in shallots bulbs, enhancing resistance to *Xanthomonas axonopodis* pv. *allii* (26). In addition, *B. subtilis* has shown to trigger resistance mechanisms in melon against *Podosphaera fusca* (24), in chili against *C. acutatum* (27) and *F. solani* (17).

Researches of biotic elicitors including *Bacillus* spp. and *Trichoderma* spp. on disease resistance in scallion against anthracnose have not many. Hence, the current research's general target was to assess *Bacillus* and *Trichoderma*'s efficacy on growth promotion and biocontrol mechanisms against anthracnose in scallion. First, dual culturing was conducted to evaluate whether *Bacillus subtilis* strains B04-1, BS008, CaSUT008-2, D604, SB2, SUNB1, 007, 007-1, 37-5, 37-6, 38-4, 111, 501, 501-1, *T. viride*, *T. harzianum*, *Trichoderma* sp. were antagonistic toward an aggressive *C. gloeosporioides* isolate. Then, on an assessment of interactions among scallion plants, effective biotic elicitors and the aggressive *Colletotrichum* isolate, severity and reduction of disease were assessed to evaluate whether the biotic elicitors could create induced resistance against scallion anthracnose. To further characterize defence mechanisms, amount of enzyme PAL, PO and chitinase of biotic elicitors were compared to those of the water control. Last objective was to determine if the biotic elicitors could increase amount of SA and PN compounds in induced scallion.

Materials and Methods

Preparation of microorganisms

The Plant Pathology and Biopesticide Laboratory at the Suranaree University of Technology, Thailand, provided biotic elicitors *Bacillus subtilis* strains B04-1, BS008, CaSUT008-2, D604, SB2, SUNB1, 007, 007-1, 37-5, 37-6, 38-4, 111, 501, 501-1, *T. viride*, *T. harzianum*, *Trichoderma* sp.; as well as aggressive fungi *C. gloeosporioides* isolate HH2.

On preparation of the fungal isolates, *Trichoderma* and *Colletotrichum* isolates were cultivated onto potato dextrose agar (PDA) medium containing 20 % potato, 2 % dextrose and 2 % agar. The plates were placed at room temperature (RT, 28±2°C) for 10-14 days (27). The density of spore suspensions used for the following experiments was approximately 10^8 conidia per mL.

On preparation of *Bacillus* strains, a single colony was streaked onto nutrient agar (NA) medium containing 0.03% beef extract, 0.5 % peptone, 0.5 % NaCl and 2 % agar. The plates were placed at RT for 3 days. Bacterial density used for the following experiments were approximately 10^8 colony-forming units (cfu) per mL.

Assessing inhibition efficiency of Bacillus and Trichoderma on hyphal growth of C. gloeosporioides isolate HH2

The experimental setup followed a completely randomized design (CRD) has 17 biotic treatments (biotic elicitors *Bacillus subtilis* strains B04-1, BS008, CaSUT008-2, D604, SB2, SUNB1, 007, 007-1, 37-5, 37-6, 38-4, 111, 501, 501-1, *T. viride*, *T. harzianum*, *Trichoderma* sp.) and a water control treatment, replicated three times. The dual culture (DC) assay was employed to evaluate the inhibition efficiency of *Bacillus* and *Trichoderma* under *in vitro* conditions (28). On DC plates, a *Colletotrichum* slice (8 mm diameter) was placed at 3 cm away from edge of plate. On the opposite side of the *Colletotrichum* slice, a *Trichoderma* slice (8 mm diameter) or

a vertical streak of *Bacillus* loof was positioned, also 3 cm away from the plate edge. Control Petri plates contained only a *Colletotrichum* slice at the center. Subsequently, all Petri plates were placed at room conditions (27±2°C, relative humidity of about 75-85 %) for 7 days.

The *Colletotrichum* radial growth in DC plates (L2) as well as in control plates (L1) were measured at 3, 5 and 7 days after placing the fungal slices (DPFS). The percentage inhibition of *Bacillus* and *Trichoderma* on the hyphal growth of *C. gloeosporioides* isolate HH2 was calculated using a formula: inhibition percentage = [(L1-L2)/L1]*100 %. From the experimental results, four effective biotic elicitors were chosen to do on the following experiments for disease reduction and biochemical activities.

Assessing efficacy of biotic elicitors Bacillus and Trichoderma against anthracnose in scallion plants

The experiment followed a CRD with six treatments, including a water control, chemical control (Prochloraz 0.05 %) and three strains of *B. subtilis* (D604, SUNB1, CaSUT008-2), as well as *T. harzianum*. Each treatment was replicated three times, with 10 plants per repetition. Uniformly shaped and sized scallion bulbs were used in the experiment. The scallion bulbs were soaked in the respective biotic treatment suspensions for approximately 20 min and grew in 40-cm pots. Then, scallion plants were further foliage-sprayed using the respective biotic treatment suspensions at 7, 14, 21 and 28 days after planting (DAP). At 30 DAP, the scallion plants were inoculated by a foliage spray using the *Colletotrichum* suspension (10^8 conidia per mL). Subsequently, the scallion plants were placed in an incubation chamber with a relative humidity of approximately 90±3% for one day. Following the incubation period, the scallions were placed at a net house. At the same time, measurements were taken for stem length and leaf quantity at 7, 14, 21 and 28 DAP to evaluate the efficacy of biotic elicitors *Bacillus* and *Trichoderma* on growth of scallion plants.

Additionally, at 14 days after inoculation (DAI), anthracnose lesions were measured using the scale (8). The scale ranges from 0 (no infection) to 10 (>61 % scallion leaf surface infected). Disease severity (DS) was calculated as a percentage using the following formula (1).

$$DS\ (\%) = \frac{\text{(summation of all ratings r 100)}}{\text{(total number of leaves evaluated r maximum disease scale)}}$$

Efficacy of biotic elicitors *Bacillus* and *Trichoderma* on scallion anthracnose was calculated as a percentage using the following formula: Reduction of DS (%) = [(DS of non treated - DS of elicitors treated)/DS of non treated] * 100.

Quantification on amount of catalase, chitinase, peroxidase (PO) and phenylalanine ammonia-lyase (PAL) in induced scallion plants

The experiment was conducted in a CRD, six treatments, three repetitions, ten plants per one repetition. Six treatments of the experiment were water control, chemical control (prochloraz 0.05 %), *B. subtilis* strain CaSUT008-2, *B. subtilis* strain D604, *B. subtilis* strain SUNB1 and *T. harzianum*. The scallion plants were treated using the biotic treatment suspensions and inoculated using the *Colletotrichum* suspension similarly to the previous experiment.

Leaf sample preparation and enzyme extraction: scallion leaves were collected and immediately soaked in liquid nitrogen at 0, 3, 6, 12, 24 and 48 hours after inoculation (HAI) of the *Colletotrichum* suspension, then refrigerated at -800C. Extraction of crude enzymes was performed by finely cutting and weighing 2 g leaf samples into a chill mortar, adding 6-8 mL of liquid nitrogen and then grinding each sample with a chill pestle into fine powder. The powders were then transferred to an eppendorf tube. Next, to each eppendorf tube, 1.0 mL of 0.1 M borate buffer (pH 8.7) was added, then gently mixed for crude protein extraction. Next, the liquid mixture was centrifuged at a speed of 12000 rpm, 4°C for 5 min. Supernatant was then collected to investigate enzyme amount. Crude protein amount was quantified by Bradford method (29).

For investigation of PAL enzyme amount, a control sample (blank) was first measured at 290 nm and adjusted to zero. In the treatment sample, the mixture at each measurement included 0.5 µL of 0.1 M borate buffer (pH 8.7), 1 µL of 0.1 M L-Phe solution, 0.15 µL of distilled water and 0.2 µL of enzyme solution. Next, reactions were performed at 37˚C for 40 min, then stopped with 0.2 µL of 5.0 N HCl solution. The PAL enzyme was quantified based on transformation of L-phenylalanine to trans-cinnamic acid. Value of OD at 290 nm was recorded from the beginning of the reaction and then every 30 seconds until 2 min, using the Shimadzu UV - 1900 UV-VIS Spectrophotometer (Shimadzu Corporation, Japan). Furthermore, PAL enzyme amount was determined based on the standard curve between the OD value and trans-cinnamic acid concentrations. The PAL enzyme amount was presented in units/mg protein/min (29).

For investigation of chitinase enzyme activity, a control sample (blank) was first measured at 420 nm and adjusted to zero. In the treatment sample, the mixture at each measurement included 90 µL working solution (0.5 mg/mL solution 4-methylumbelliferone in DMSO) and 10 µL of enzyme solution. After that, reactions were conducted at 37˚C for 30 min, then stopped with 0.2 mL of 5.0 N HCl solution. Chitinase enxyme was quantified based on transformation of chitin to N-acetylglucosamine. Values of OD at 420 nm was recorded from the beginning of the reaction and then at 2 min, using the Shimadzu UV - 1900 UV-VIS Spectrophotometer (Shimadzu Corporation, Japan). Furthermore, the chitinase enzyme amount was determined based on the standard curve between the OD value and N-acetylglucosamine concentration. The chitinase enzyme content was expressed in units/mg protein/min (29).

A control sample (blank) was first measured at 240 nm and adjusted to zero to investigate catalase enzyme activity. In the treatment sample, reactions were set up by adding 25 µL of enzyme solution into 750 µL of phosphate buffer 0,1M (pH = 7) containing 475 µL of DMSO and 250 µL of H2O2 75mM. The catalase enzyme was quantified based on decreased concentration of H2O2 in the mixture (30). The

absorbance was measured at 240 nm for 1 min, using the Shimadzu UV - 1900 UV-VIS Spectrophotometer (Shimadzu Corporation, Japan). Catalase enzyme content was presented in units/mg protein/min.

For investigation of PO enzyme activity, a control sample (blank) was first measured at 470 nm and adjusted to zero. In the treatment sample, reactions were established by pipetting 20 µL of enzyme solution into 1.33 mL of 100 mM phosphate buffer (pH 7) containing 75 µL guaiacol 4 % and 75 µL H2O2 1 %. The PO enzyme was quantified based on transformation of guaiacol to tetra-guaiacol. Absorbance was measured at 470 nm for 1 min using the Shimadzu UV - 1900 UV-VIS Spectrophotometer (Shimadzu Corporation, Japan). PO enzyme content was presented in units/mg protein/min.

Quantification on amount of SA and PN compounds in induced scallion plants

The experiment was conducted in a CRD, similar treatments, repetitions, plants per one repetition to the experiment on enzyme quantification. The scallion plants were also treated using the biotic treatment suspensions and inoculated using the *Colletotrichum* suspension, similarly to the previous experiment. Scallion leaf samples were collected at 0, 24 and 48 HAI and stored at -80 °C for subsequent SA and PN compound assays.

A colorimetric method described by Warrier et al. (31) was used to quantify SA levels. In brief, 0.5 g of the scallion leaf sample was finely ground with 1 mL of 90% methanol. After centrifugation at 12000 rpm and 4°C for 5 min, 100 µL of supernatant was mixed with 100 µL of 0.02 M Ferric ammonium sulfate. Later, the mixture was measured at an absorbance of 530 nm for 5 min after 30-min incubation at RT.

PN compound content was determined using a modified method based on interaction between PN compounds and Folin-Ciocalteu's reagent (32). In this assay, 0.5 g of the scallion leaf sample was finely ground with 1 mL of 90 % methanol. After centrifugation at 12000 rpm and 4°C for 5 min, 20 µL of supernatant was mixed with 80 µL of 7 % sodium carbonate and 100 µL of 10 % Folin-Ciocalteu's reagent. Following a 30-min incubation at RT, the mixture was recorded at an absorbance of 760 nm.

Evaluating Bacillus density in tissues of induced scallion plants

Evaluation of *Bacillus* densities was conducted using a CRD with three repetitions of five treatments: water control, chemical control (prochloraz 0.05 %), *B. subtilis* strain CaSUT008-2, D604 and SUNB1. The scallion plants were induced using the biotic treatment suspensions, similar to the previous experiment. At 28 DAP, scallions plants were collected, carefully washed two times with tap water and ground with 10 mL of sterile distilled water using a set of sterile mortar and pestle. Next, the liquid solution was vortexed for 1 min and 50 µL of supernatant was spread on NA medium plates using an inox L-shape cell spreader. *Bacillus* colonies on NA medium plates were recorded at 24 hours after cultivation at RT.

Statistical analysis

The results from the DC experiment were presented as mean ± standard deviation of three repetitions. Data of other experiments were analyzed using an analysis of variance in the SPSS software version 20. The significance of treatments was determined by ANOVA F value with a significance level of P = 0.05.

Results

Inhibition efficiency of Bacillus and Trichoderma on hyphal growth of C. gloeosporioides isolate HH2

The inhibitory effect of biotic elicitors *Bacillus* and *Trichoderma* was evaluated using the DC assay. The antagonistic efficacy of these biotic treatments were 1-77 % (Table 1). Among biotic treatment group of *Trichoderma*, *T. harzianum* exhibited the highest inhibition percentage at approximately 63.78 % at 3 days post-fungal slice inoculation (DPFS), surpassing *T. viride* (58.31 %) and *Trichoderma* sp. (55.13 %). Furthermore, the inhibitory effect of *T. harzianum* increased to 77.01% at 5 DPFS, exceeding that of *Trichoderma* sp. (74.25 %) and *T. viride* (69.85 %) (Fig. 1A, 1B and 1C). Within the *Bacillus* elicitors group, *B. subtilis* strains CaSUT008-2, D604 and SUNB1 exhibited higher inhibition percentages at 7 DPFS, with values of 41.84 %, 43.25 %, and 45.26 %, respectively, compared to other *Bacillus* strains (Fig. 1D). Therefore, *B. subtilis* strains CaSUT008-2, D604 and SUNB1; *T. harzianum* were chosen as the effective biotic elicitors for further experiments.

Efficacy of effective biotic elicitors Bacillus and Trichoderma in scallion plants

The ability of *B. subtilis* strains CaSUT008-2, D604 and SUNB1; *T. harzianum* to activate plant resistance was evaluated by a bulb soak and four foliage sprays at 7, 14, 21, and 28 DAP. The results demonstrated that the biotic elicitor treatments significantly increased stem length and leaf quantity in scallion plants. At 28 DAP, the scallion plants treated with elicitors *B. subtilis* strains SUNB1 and CaSUT008-2 resulted in longer stem lengths of 15.35 and 15.79 cm, respectively, which were significantly higher

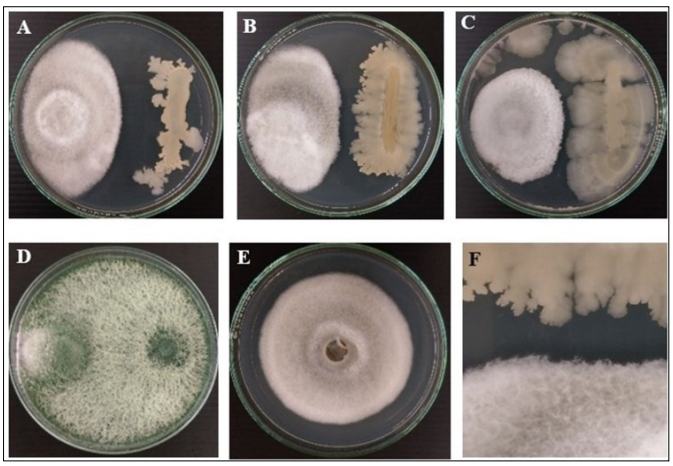

Fig. 1. The inhibitory effect of biotic elicitors against mycelial growth of *C. gloeosporioides* at *in vitro* conditions. A: dual culture (DC) of *B. subtilis* CaSUT008-2, B: DC of *B. subtilis* SUNB1, C: DC of *B. subtilis* D604, D: DC of *T. harzianum*, E: single culture of *C. gloeosporioides*, F: mycelium *C. gloeosporioides* grew toward the *B. subtilis* SUNB1 but its trend was toward up and sparse.

Table 1. The percentage inhibition of biotic elicitors against *C. gloeosporioides* at *in vitro* conditions

Biotic elicitors	Inhibition percentage[1] (%)		
	3 DPFS[2]	5 DPFS[2]	7 DPFS[2]
T. viride	58.31±8.72^{a}	69.85±3.60^{a}	-
T. harzianum	63.78±3.87^{a}	77.01±1.60^{a}	-
Trichoderma sp.	55.13±6.16^{a}	74.25±3.78^{a}	-
B. subtilis 37-6	4.10±9.86^{e}	11.56±12.51^{d}	14.45±11.71^{c}
B. subtilis 37-5	19.59±11.11^{b}	25.19±7.73^{b}	28.10±7.46^{b}
B. subtilis 111	3.42±0.79^{e}	5.90±2.93^{e}	9.24±10.18^{c}
B. subtilis D604	12.30±10.86^{cd}	28.39±10.90^{b}	43.25±6.76^{a}
B. subtilis BS008	14.12±16.85^{c}	18.59±12.84^{bcd}	33.54±11.17^{ab}
B. subtilis CaSUT008-2	19.13±4.18^{b}	28.02±3.99^{b}	41.84±3.40^{a}
B. subtilis B04-1	6.15±10.46^{d}	21.36±2.42^{bc}	34.80±1.70^{ab}
B. subtilis 38-4	12.53±8.54^{cd}	23.24±5.77^{bc}	39.21±3.90^{ab}
B. subtilis SUNB1	17.08±5.04^{bc}	28.77±2.29^{b}	45.26±3.45^{a}
B. subtilis SB2	14.58±11.00^{c}	18.09±10.23^{bcd}	24.30±7.36^{b}
B. subtilis 501	1.37±13.43^{e}	18.47±8.94^{bcd}	38.09±5.75^{ab}
B. subtilis 501-1	14.81±3.44^{c}	21.73±4.15^{bc}	35.83±6.62^{ab}
B. subtilis 007-1	12.30±0.39^{cd}	22.24±4.98^{bc}	34.01±2.77^{ab}
B. subtilis 007	11.85±8.94^{cd}	15.58±7.27^{d}	28.71±5.23^{b}
F test	*	*	*
CV (%)	24.78	25.15	16.31

Note: DPFS - days after putting fungal slices

[1]Mean ± standard deviation

[2]means followed by the same letter do not differ significantly according to DMRT at P ≤ 0.01 (**) or 0.01 < P ≤ 0.05 (*)

than those of the water control (12.46 cm) and the chemical control (10.69 cm) (Table 2). The average number of scallion leaves in the biotic elicitor treatments ranged from 6.65 to 7.85, showing no significant difference compared to the water control treatment (7.31) at 28 DAP (Table 2, Fig. 2).

The severity of anthracnose disease and its reduction were assessed on scallion leaves at 14 DAI. All biotic treatments exhibited DS ranging from 25.00 % to 58.33 %, lower than water control treatment (83.33 %). Among them, *B. subtilis* strain SUNB1 showed the lowest DS at 25.00 % and the highest reduction of DS at 70.00 % (Table 2).

Amount of catalase, chitinase, peroxidase (PO) and phenylalanine ammonia-lyase (PAL) in induced scallion plants

Next, the enzyme content of catalase, chitinase, PAL and PO were investigated, showing the changes in these

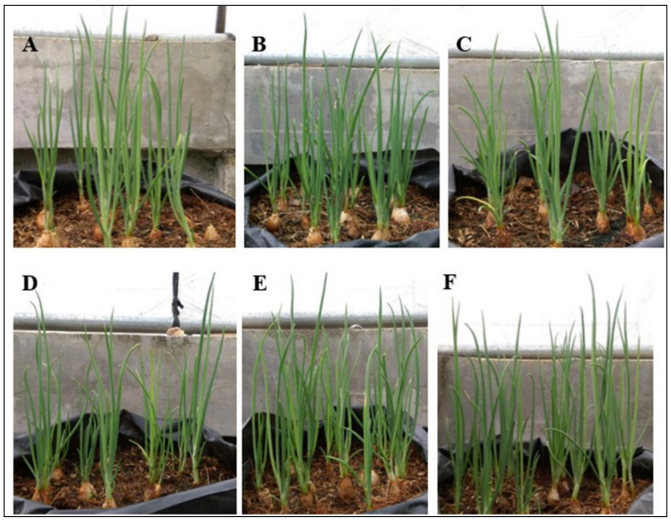

Fig. 2. The efficacy of fresh biotic elicitors against anthracnose disease in scallion plants at 28 days after plating in net-house conditions. A: water control, B: chemical control, C: *B. subtilis* D604, D: *B. subtilis* SUNB1, E: *B. subtilis* CaSUT008-2, F: *T. harzianum.*

Table 2. The efficacy of fresh biotic elicitors on stem length of scallion plants in net-house conditions

Treatments	Stem length[1] (cm)				The number of leaves[1]				Disease severity[1](%)	Reduction of disease severity[2] (%)
	7 DAP	14 DAP	21 DAP	28 DAP	7 DAP	14 DAP	21 DAP	28 DAP		
Water control	0.75±0.06^{b}	3.59±0.18^{b}	7.34±0.24^{bc}	12.46±0.32^{cd}	1.25±0.09^{c}	4.04±0.17^{ab}	5.72±0.23^{bc}	7.31±0.15$^{a}_{b}$	83.33±10.15^{a}	-
Chemical control	1.41±0.11^{a}	3.97±0.13^{b}	6.44±0.23^{c}	10.69±0.31^{d}	2.00±0.12^{b}	3.51±0.17^{bc}	5.32±0.22^{c}	5.77±0.18^{c}	58.33±7.37^{a}	30.00
B. subtilis D604	1.58±0.09^{a}	3.95±0.14^{b}	6.35±0.22^{c}	12.07±0.17^{cd}	2.38±0.13^{b}	2.60±0.25^{c}	5.48±0.30^{bc}	7.63±0.21$^{a}_{b}$	50.00±5.99^{bc}	40.00
B. subtilis SUNB1	0.86±0.06^{b}	5.42±0.22^{a}	11.35±0.36^{a}	15.35±0.19^{ab}	2.53±0.14^{b}	3.14±0.21^{bc}	4.85±0.17^{c}	6.65±0.20$^{b}_{c}$	25.00±3.21^{c}	70.00
B. subtilis CaSUT008-2	0.97±0.05^{b}	3.40±0.19^{b}	8.92±0.27^{abc}	15.79±0.28^{a}	1.92±0.17^{b}	3.76±0.24^{bc}	6.30±0.26^{ab}	7.85±0.14^{a}	41.67±5.32^{bc}	50.00
T. harzianum	1.52±0.12^{a}	6.09±0.26^{a}	9.36±0.35^{ab}	13.76±0.32^{bc}	3.48±0.22^{a}	5.13±0.32^{a}	7.00±3.12^{a}	7.80±0.18^{a}	50.00±5.11^{bc}	40.00
F test	*	**	*	**	**	*	**	*	**	
CV (%)	15.17	9.27	12.22	5.92	15.41	12.6	6.24	6.03	28.09	

Note: DAP - days after planting

[1]means followed by the same letter do not differ significantly according to DMRT at P ≤ 0.01 (**) or 0.01 < P ≤ 0.05 (*)

[2]The reduction of disease severity - DS on treatments, was calculated using the formula as follows: Reduction of DS (%) = [(DS of control - DS of each other treatment)/(DS of control)]*100%

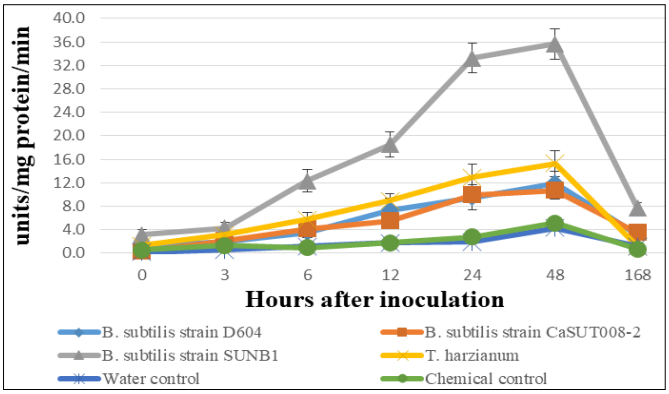

Fig. 3. Induction of Phenylalanine ammonia lyase (PAL) activity in treated scallion plants.

enzyme content in leaf tissues for 0, 3, 6, 12, 24 and 48 HAI (Fig. 3, 4, 5 and 6). The concentrations of PAL in the elicitor treatments showed high increase over the hours after inoculation and were different from the chemical treatment and the control. The concentration of PAL of treatments including *B. subtilis* strain CaSUT008-2, D604, SUNB1 and *T. harzianum* were high at 48h (10.72, 11.91 35.64 and 15.30 units/mg protein/min, respectively) and lowest at 0h when pathogens began to appear (approximately 0.15-3.14 units/mg protein/min). The increased PAL amount of the treatment *B. subtilis* strain SUNB1 was significantly different to those of other three elicitors as *T. harzianum*, *B. subtilis* strain CaSUT008-2 and D604 during all observation time points (Fig. 3).

Results revealed the increased activities of the PO enzyme. The maximum PO activities of all treatments were observed on the 48 HAI and thereafter a decrease was observed. Among the treatments, treatment of elicitor *B. subtilis* strain SUNB1 recorded higher PO activity (4547.22 units/mg protein/min) at 48 HAI, compared to the other treatments. This was followed by treatments *B. subtilis* CaSUT008-2 (3775.00 units/mg protein/min), *T. harzianum* (3069.44 units/mg protein/min) and *B. subtilis* strain D604 (2544.44 units/mg protein/min), in decreasing order of merit. The PO activities of water and chemical control were low, achieving 1641.67 and 1672.22 units/mg protein/min, respectively, at 48 HAI (Fig. 4).

In the catalase activity, the plants inoculated with Colletotrichum spores also demonstrated gradual increase from 0 HAI, up to the 48 HAI and then showed a rapidly decline. The high catalase activity was observed in treatment *B. subtilis* strain SUNB1 (120.06 units/mg protein/

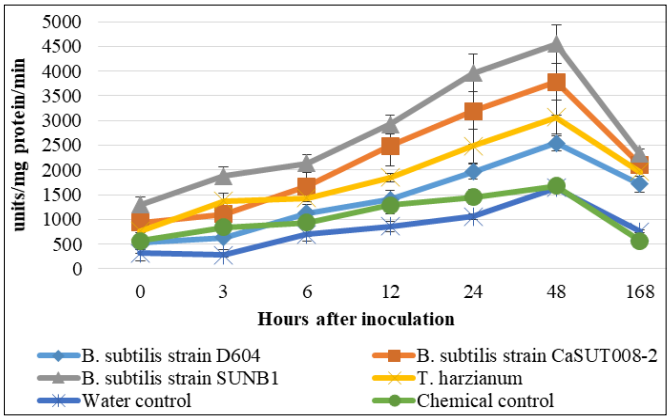

Fig. 4. Induction of peroxidase (PO) activity in treated scallion plants.

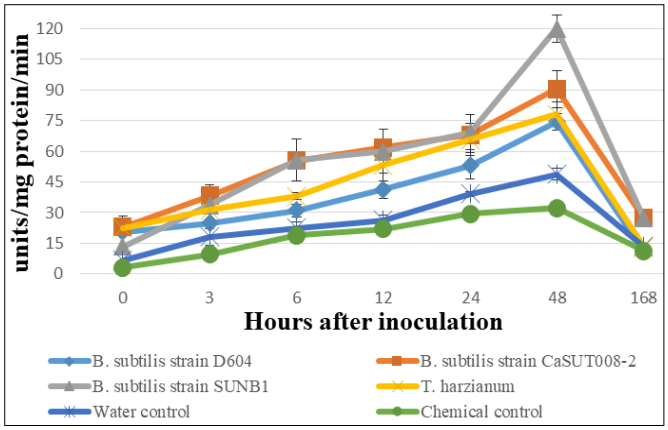

Fig. 5. Induction of catalase activity in treated scallion plants.

min), treatment *B. subtilis* CaSUT008-2 (90.42 units/mg protein/min), *T. harzianum* (78.05 units/mg protein/min) and *B. subtilis* strain D604 (74.52 units/mg protein/min), all significantly higher than those of the water control (48.78 units/mg protein/min) and the chemical control (32.38 units/mg protein/min) at 48 HAI (Fig. 5).

A rise in β-1,3-glucanase activity (Fig. 6) was observed in all treatments including controls and continued

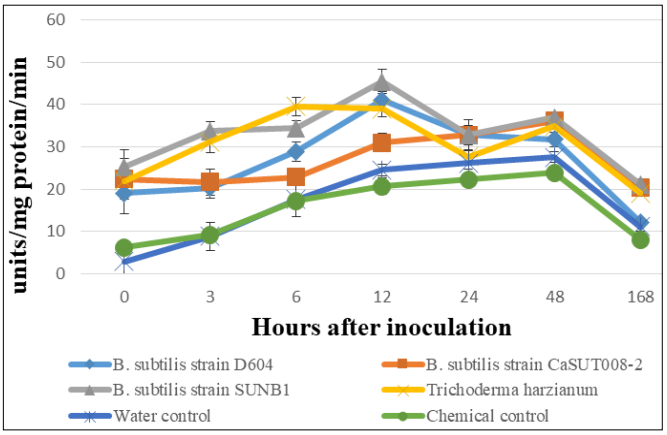

Fig. 6. Induction of b-1,3-glucanase activity in treated.

to increase till 12 HAI; then it followed a downward trend. Significant maximum rise in β-1,3-glucanase amount was observed in treatment of *B. subtilis* strain SUNB1 (45.42 units/mg protein/min at 12 HAI), followed by treatment *B. subtilis* strain D604 (41.02 units/mg protein/min at 12 HAI) and treatment *T. harzianum* (39.55 and 38.96 units/mg protein/min at 6 and 12 HAI, respectively).

Amount of SA and PN compounds in induced scallion plants

SA content in scallion leaves showed no significant differences among treatments at 0 and 48 HAI. However, at 24 HAI, scallion plants induced with *B. subtilis* strain CaSUT008-2 and *T. harzianum* exhibited SA levels of 2.197 and 2.165 µg/g fresh weight, respectively, which were significantly higher than those of the water control (2.15 µg/g fresh weight) and the chemical control (2.20 µg/g fresh weight) treatments (Table 3).

Regarding PN compounds, there were no significant differences among treatments at 0 and 24 HAI. However, at 48 HAI, scallion plants treated with *B. subtilis* strain SUNB1 showed upward of PN compounds at 42.65 µg gallic acid equivalent/mg dry mass, significantly higher than those of

Table 3. The effect of fresh biotic elicitors on amount of salicylic acid in scallion plants

Treatments	Salicylic acid (µg g^{-1} fresh weight)			Phenolic compounds (µg gallic acid equivalent mg^{-1} dry mass)		
	0 HAI	24 HAI	48 HAI	0 HAI	24 HAI	48 HAI
Water control	2.13±0.03	2.11±0.09^{c}	2.15±0.05	42.50±5.93	42.43±7.22	42.56±5.11^{b}
Chemical control	2.18±0.07	2.14±0.08^{c}	2.20±0.09	42.48±2.17	42.45±6.17	42.57±7.22^{b}
B. subtilis D604	2.11±0.05	2.10±0.09^{c}	2.13±0.08	42.47±4.99	42.43±3.76	42.51±6.05^{b}
B. subtilis SUNB1	2.12±0.11	2.14±0.11^{bc}	2.12±0.06	42.62±6.02	42.46±6.43	42.65±6.94^{a}
B. subtilis CaSUT008-2	2.13±0.06	2.20±0.13^{a}	2.14±0.05	42.58±3.85	42.49±5.28	42.56±7.22^{ab}
T. harzianum	2.16±0.13	2.17±0.14^{ab}	2.16±0.13	42.52±5.36	42.43±7.32	42.50±6.17^{b}
F test	ns	**	ns	ns	ns	*
CV (%)	1.48	1.23	2.09	0.17	0.07	0.12

Note: HAI - hours after inoculation

[1]mean followed by the same letter do not differ significantly according to DMRT at P ≤ 0.01 (**). ns: non-significantly different

the water control (42.56 µg gallic acid equivalent/mg dry mass) and the chemical control (42.57 µg gallic acid equivalent/mg dry mass) (Table 3).

Bacillus density in tissues of induced scallion plants

An essential trait of biotic elicitors compared to abiotic ones is their ability to reside within plant tissues and then activate resistance mechanisms, leading to prolonged efficacy of induced resistance in host plants. Presence of the biotic elicitor of Bacillus in scallion plants showed that the number of Bacillus colonies per 1 g of scallion plant in the treatments treated with *B. subtilis* strains CaSUT008-2, D604 and SUNB1 were 8.4x102, 6x102 and 0.4x102 cfu, respectively, which were higher than those of the water control and chemical control, both at 0 cfu (Table 4).

Table 4. Colonization of fresh bacterial elicitors in scallion tissues at 30 days after planting

Treatments	The number of single colonies (CFU g-1 fresh plant) 1
Water control	0.00 ^{c}
Chemical control	0.00 ^{c}
B. subtilis D604	6x10^{2} ^{a}
B. subtilis SUNB1	0.4x10^{2} ^{b}
B. subtilis CaSUT008-2	8.4x10^{2} ^{a}
F test	*
CV (%)	28.26

Note: [1]means followed by the same letter do not differ significantly according to DMRT at P ≤ 0.01 (**) or 0.01 < P ≤ 0.05 (*)

Discussion

The present study assessed the efficacy of biotic elicitors *Bacillus* and *Trichoderma* in promoting plant growth and inducing resistance against anthracnose disease in scallions. The antagonistic effects of *Bacillus* and *Trichoderma* were initially evaluated using a *in vitro* DC assay. Among the *B. subtilis* strains tested, CaSUT008-2, D604 and SUNB1 exhibited high inhibition percentages, exceeding 40 % at 7 DPFS. These findings are consistent with several studies on various plants infected with anthracnose fungi. For instance, *B. subtilis* limited growth of *C. gloeosporioides* strains isolated from pear, apple, sour cherry and chili with inhibition percentages ranging from 25 % to 57 % at different DPFS (33, 34). In pea, three strains of *Bacillus* spp. LBF- 02, LBF- 03 and LBF- 05 high antimicrobial activity against root rot pathogen *Fusarium oxysporum* L. in a dual culture assay (35). In the present study, the mycelium of *Colletotrichum* displayed sparse growth towards *B. subtilis*, possibly due to anti-fungal compounds produced by *B. subtilis* (34). *Trichoderma* isolates, specifically *T. viridae* and *T. harzianum*, demonstrated inhibition percentages of 69.85 % to 77.01 % at 5 DPFS. The hyphae of parasitic *T. harzianum* were observed to coil around the mycelium of *C. gloeosporioides* and *Trichoderma* species produce antibiotics that inhibit *Colletotrichum* growth (33). Based on these findings, *B. subtilis* strains CaSUT008-2, D604 and SUNB1, as well as *T. harzianum*, were selected for further evaluation of their growth induction and disease resistance in scallion plants.

In the net-house experiments, treatments with *B. subtilis* CaSUT008-2 and SUNB1 significantly boosted stem length of scallion plants at 28 days after planting (DAP). Additionally, *B. subtilis* CaSUT008-2 and *T. harzianum* treatments significantly increased leaf quantity at 21 DAP, although no significant difference was observed at 28 DAP. *B. subtilis* CaSUT008-2 exhibited a higher density of cells in scallion leaves compared to *B. subtilis* SUNB1, which may have contributed to the greater increase in stem length at 28 DAP. These results are consistent with several studies reporting positive effects of *B. subtilis* on stem height, root length, fresh root weight and stem weight in scallion and other plants (36, 37). In ginger, *B. subtilis* L2 significantly increased plant height (20 %), raised leaf length, increased the number of leaves per plant (30 %) and leaf width (21 %) at 8 weeks after planting (38). The mechanisms underlying the growth induction by *B. subtilis* involve in enhanced photosynthesis, increased plant growth hormones and volatile organic compounds (37, 39).

Furthermore, the efficacy of biotic elicitors *B. subtilis* strains CaSUT008-2, D604, and SUNB1, as well as *T. harzianum*, on enhancing anthracnose resistance was evident through the decrease in DS and expression of defense components. In the current study, treatments with these biotic elicitors resulted in a significant decrease in anthracnose severity, ranging from 40 % to 70 % at 14 days after pathogen inoculation (DAI), confirming the occurrence of disease resistance. Comparatively, in a study using *Pseudomonas fluorescens* strain Pfl, DS of biotic-treated scallion plants was 19-27 % lower than the

results in the present study (25). In addition, it was showed that *B. subtilis* strain D604 also reduced disease severity by around 31 % in anthracnose-infected chili plants (27). Previous research reported that *B. subtilis* limited lesions of powdery mildew through induced resistance in melon plants, resulting in a 50 % reduction in DS (24). Reduction in anthracnose severity observed in this study is attributable to the activation of defense components associated with disease resistance.

After treatments with biotic elicitors, disease resistance mechanisms in scallion plants was induced. In these resistance mechanisms, SA plays as a systemic signal in induced plants. SA also has anti-fungal ability to affect the hyphal growth of pathogens inside induced plants directly. In the present study, SA amount of biotic treatments including *T. harzianum* and *B. subtilis* strain CaSUT008-2 were 2.165 and 2.197 µg/g fresh weight, respectively; while the SA amount were low at the water control (2.15 µg/g fresh weight) and the chemical control (2.20 µg/g fresh weight). Activity of SA signal could result in other defense components such as PAL, PO, catalase, b-1,3 -glucanase and PN compounds.

In a general process of infection and invation, *Colletotrichum* spores begin to germinate after landing on the leaf surface. Phenomenon of spore germination occurred at 3 HAI and appressoria formation happened at 6 HAI (40). Next, *Colletotrichum*'s infection directly occurred via penetration pegs under *Colletotrichum*'s appressoria, resulted in penetration phenomenon at the plant cuticle at 48 HAI (40, 41). These events of *Colletotrichum* spores are by enzyme activities of the present research. Enzyme content of PAL, PO and catalase began to increase at 3 HAI, peaked at 48 HAI, and then leveled off. Enzyme b-1,3-glucanase had a similar increase at 3 HAI but showed the highest point at 6-12 HAI. PAL plays a crucial role in disease resistance.

PAL enzyme involves in the biosynthesis of systemic signal SA during systemic resistance in induced plants (42). Increased PAL amount is seen as a chemical barrier in the induced plants against infection and invasion pathogens (43). In addition, enzymes PAL and PO as well as PN compounds play important roles in cell wall fortification (lignin, suberin and callose) providing a physical barrier to a spread of fungal hyphae from the first infection site to the adjacent cells and also to an additional infection of pathogens at plant surface (44). Catalases are highly expressed enzymes and thus an intermediary compound of the plant antioxidative system, involving disease resistance (45). For the last enzyme surveyed in the present study, β-1,3-glucanases is crucial to slow down fungal growth and limit leaf lesions (46). Another response of plants to fungal pathogen is the accumulation of PN compounds. PN compounds, as secondary plant metabolites, play crucial roles in the defense mechanisms of host plants. They possess antibiotic and antifungal activities and serve as building blocks for synthesis of suberin and lignin, which reinforce cell walls in induced plants (47, 48). In the present study, *B. subtilis* SUNB1 significantly enhanced amount of PN compounds at 48 hours after inoculation (HAI). This finding aligns with the previous research which demonstrated a correlation between the buildup of PN compounds and the disease resistance in induced cassava plants against *C. gloeosporioides* (49). Phenol, a lignin precursor, functions as a crucial signaling substance, leading to systemic acquired resistance.

Conclusion

B. subtilis strains CaSUT008-2, D604 and SUNB1, as well as *T. harzianum*, exhibited high inhibition efficacy in inhibiting the hyphal growth of *C. gloeosporioides*. Moreover, *B. subtilis* strain CaSUT008-2 enhanced stem length, increased the amount of SA, at a high *Bacillus* density and effectively suppressed anthracnose severity. *B. subtilis* strain D604 demonstrated effectiveness in reducing disease severity. *B. subtilis* strain SUNB1 promoted stem length and amount of PN compounds, reducing anthracnose severity. *T. harzianum* enhanced SA production and effectively limited anthracnose severity. Additionally, all these biotic elicitors increased the content of enzymes, including catalase, PAL, β-1,3-glucanase and PO in treated scallion plants at different levels. Therefore, these biotic elicitors can be considered effective and environmentally friendly strategies for scallion cultivation and anthracnose control. Among them, *B. subtilis* SUNB1 stood out as a prominent biotic elicitor.

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Not applicable.

Authors' contributions

TLT and TPTP conceived the project. TLT, NB designed experiments. TLT, NKP, THH, NHH carried out experiments. TLT, HN analyzed data and prepared figures and tables. TLT, NKP, NHH wrote the manuscript. TLT, TPTP, HN and NB revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors have no conflicts of interest to declare.

Ethical issues: None

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