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Research Article

Application of Silica Nanoparticles in Combination with *Bacillus velezensis* and *Bacillus thuringiensis* for Anthracnose Disease Control in Shallot

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Abstract

Background and Objective: Anthracnose in shallot contributes to significant losses. To solve this issue, silica nanoparticles, in combination with *Bacillus velezensis* and *Bacillus thuringiensis* were used together. **Materials and Methods:** *In vitro* antagonistic test of *Bacillus velezensis* B-27 with *Colletotrichum gloeosporioides* was carried out using dual culture and co-culture methods. Treatment in greenhouse experiments was carried out using single application of silica, *B. thuringiensis*, *B. velezensis*, a combination of *B. thuringiensis* and *B. velezensis* and a combination of *B. thuringiensis*, *B. velezensis* and silica. Detection of *B. velezensis* in the roots of shallot plants was carried out by PCR using a pair of specific primers. **Results:** *Bacillus velezensis* was able to inhibit the growth of *C. gloeosporioides* mycelium *in vitro*, both in the dual culture and co-culture methods, by 62.8 and 77.17%, respectively. Treatment of *B. thuringiensis* and *B. velezensis*, either individually or in combination with silica, could reduce the intensity of anthracnose disease by 20% each and stimulate the growth of shallot plants. The PCR detection using specific primers on the roots of shallot plants showed that *B. velezensis* was detected with a DNA band length of ± 576 bp. **Conclusion:** *Bacillus velezensis* can inhibit the growth of *C. gloeosporioides* mycelium *in vitro*. Applying *B. velezensis*, *B. thuringiensis* and silica can reduce the intensity of anthracnose disease, promote plant growth and increase plant productivity. Furthermore, *B. velezensis* was detected in the roots of shallot plants, revealing that the bacteria are well-established.

Key words: *Bacillus velezensis*, *Bacillus thuringiensis*, *Colletotrichum gloeosporioides*, shallot, silica

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Shallot cultivation sometimes experiences problems that cause losses with a decrease in shallot production due to plants infected by *Colletotrichum gloeosporioides*, the cause of anthracnose disease. The losses caused by anthracnose in shallots can be up to 100%¹. Symptoms of anthracnose on shallots are the appearance of small white lesions on the surface of the leaves. The lesion then expands and becomes dark, causing chlorosis and necrosis around the lesion, acervuli appear, which are orange in color and turn blackish brown. Then, when the symptoms are severe, lesions appear on the tubers, causing tuber rot and death in all parts of the plant². The symptoms are often found on the leaves because the pathogen spreads through spores carried by wind or water splashes, so the spread occurs quickly.

Control of anthracnose disease can be done using biological agents such as *Bacillus*, which belongs to the Plant Growth Promoting Rhizobacteria (PGPR) group. These bacteria can colonize in plant tissue and synthesize secondary metabolites such as siderophores, antibiotics, HCN and other enzyme synthesis. Apart from that, these bacteria can trigger plant growth and development with their ability to fix nitrogen, dissolve phosphate and produce indole-3-acetic acid (IAA) compounds. *Bacillus* also acts as an antagonist against pathogens because it produces antimicrobial components, including the lipopeptide iturin, surfactin, fengycin and bacteriocin, which function as fungal cell wall degradation enzymes³.

The species of *Bacillus* widely applied to plants are *B. velezensis* and *B. thuringiensis*. Used as PGPR, *B. velezensis* functions as a bio-fertilizer, bio-stimulant and bio-protectant⁴. The research by Chen *et al.*⁵ showed that *B. velezensis* LM2303 has the potential for biocontrol due to the presence of four main mechanisms in *Bacillus*, namely antibiosis by producing antibacterial metabolites and lipopeptides, activating the induction resistance system with the presence of toxins and lipopeptide surfactin, triggering the activation of plant growth hormones, thus helping in the absorption of nutrients and the ability of competition between *Bacillus* and pathogens.

The research results of Ye *et al.*⁶ showed that *B. velezensis* G341 can inhibit the appearance of symptoms of rice blast, bacterial leaf blight of rice, pepper anthracnose, tomato soft rot, wheat root rot and barley powdery mildew. Besides, Kim *et al.*⁷ also showed that *B. velezensis* TSA32-1 was able to inhibit the growth of pathogenic fungi, such as *Alternaria alternata*, *Colletotrichum acutatum*, *C. lymphaea*, *Cylindrocarpon destructans*, *Diaporthe actinidiae* and

Fusarium oxysporum f.sp. *radicis-lycopersici*. The ability of *B. velezensis* to initiate growth is due to its ability to produce the auxin hormone (IAA), cytokinin and gibberellin or jasmonic acid, which helps promote plant growth. This is shown by the application of *B. velezensis*, which can promote the growth of *Arabidopsis* with the presence of auxin hormone homeostasis⁸.

Bacillus thuringiensis produced parasporal protein or protein crystals consisting of δ -endotoxins. These crystal toxins can inhibit the growth of *Fusarium verticillioides* on maize⁹. The crystal proteins Cry1Ab, Cry1Ac and the chitinase enzyme produced by *B. thuringiensis* can also inhibit the growth of the mycelium of the pathogenic fungi *Rhizoctonia solani*, *Pyricularia grisea*, *Fusarium oxysporum* and *F. solani* due to the synergy between the cry protein and the chitinase enzyme, which can damage the pathogen cell wall¹⁰. de la Fuente-Salcido *et al.*¹¹ also mentioned the results of chitinase synthesis by *B. thuringiensis* subsp. *tenebrionis* DSM-2803, namely rChiA74, could inhibit mycelium growth and damage *C. gloeosporioides* hyphae. In addition, Yoshida *et al.*¹² reported that *B. thuringiensis* was able to induce plant resistance with the presence of genes encoding salicylic acids, such as PR-1 (P6), PR-5 and P4, thereby suppressing the development of disease in tomato seedlings¹³. With this ability, *B. thuringiensis* can also inhibit the symptoms of bacterial wilt caused by *Ralstonia solanacearum*.

The growth, development and health of shallot plants can not only be induced by the presence of PGPR but also by applying silica (Si) in the form of liquid fertilizer to the plants. Adding silica fertilizer to plants promotes plant growth, increases photosynthesis, reduces transpiration from the cuticle and protects plants from environmental stress. Apart from that, silica also helps improve root characteristics, inducing plant resistance to drought and the formation and thickening of cell walls so that it can also function in helping plants mechanical resistance to pathogen infections^{14,15}. Seebold *et al.*¹⁶ also reported that silica could suppress the intensity of rice blast disease caused by *P. grisea* due to the presence of phytoalexin and lignin compounds. Research by Ohri and Pannu¹⁷ on ginger plants showed that the application of silica affected phenol synthesis, where phenol is a secondary metabolite compound that can increase the phenylalanine ammonium lyase and synthesize chitinase, thus playing a role in plant resistance. Therefore, this research was conducted to determine the effect of applying a combination of *B. velezensis*, *B. thuringiensis* and silica fertilizer on the growth and development of shallot plants.

MATERIALS AND METHODS

Study area: The research was conducted at the Laboratory of Plant Pathology and the Greenhouse of the Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, from July 2022 to October 2023.

Culture of bacterial and fungal isolates: *Bacillus velezensis* B-27, *B. thuringiensis* and *C. gloeosporioides* were from the collection of the Laboratory of Plant Pathology, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta. The *Bacillus* spp. was cultured on Yeast Peptone Agar (0.5% yeast extract, 1% peptone and 1.5% agar). While *C. gloeosporioides* were cultured on Potato Dextrose Agar instant (Difco™). The bacteria were incubated at a temperature of 25°C for 24-48 hrs, while fungal isolates were incubated at 25°C for 3-4 days.

In vitro antagonistic test: The *in vitro* antagonistic test of *B. velezensis* B-27 against *C. gloeosporioides* was carried out using dual culture and co-culture methods following research by Jayanti and Joko¹⁸. The dual culture method was carried out by growing *B. velezensis* and *C. gloeosporioides* oppositely on potato dextrose agar (PDA) media. Meanwhile, the co-culture method was carried out by preparing PDA media in a petri dish and when it had solidified, 10 mL of 6% water agar was added to which 100 µL of bacterial suspension previously added, resulting in two layers of PDA media and water agar. The isolates of *C. gloeosporioides* were then grown on this medium. The antagonistic test was carried out to observe growth inhibition of *C. gloeosporioides* during incubation for 7 days and the inhibition rate was calculated using the following formula.

Dual culture:

$$\text{Inhibition rate (\%)} = \frac{R_1 - R_2}{R_1} \times 100$$

Where:

R1 = Growth diameter of *C. gloeosporioides* avoiding *B. velezensis* B-27

R2 = Growth diameter of *C. gloeosporioides* approaching *B. velezensis* B-27

Co-culture:

$$\text{Inhibition rate (\%)} = \frac{C - T}{C} \times 100$$

Where:

C = Growth diameter of *C. gloeosporioides* in control treatment

T = Growth diameter of *C. gloeosporioides* in the treatments of antagonistic bacteria (*B. velezensis* B-27)

Planting shallot in greenhouse: The experiment was carried out by planting bulbs of shallot cv. Tajuk in polybags measuring 35×35 cm containing planting media with a composition of loose soil, husk charcoal and manure (1:1:1). The treatments of inoculated and non-inoculated plants were carried out during 50-60 days until harvest. The treatments consisted of control (no treatment), silica spraying, *B. thuringiensis* spraying (spray B), *B. velezensis* spraying (spray Bv), *B. velezensis* dripping application (SC Bv), *B. velezensis* dripping application + *B. thuringiensis* spraying (Bv+Bt), *B. velezensis* dripping application + *B. thuringiensis* spraying + silica spraying (Bv+Bt+silica).

The dripping application treatment was carried out by dripping the shallot bulbs before planting in a suspension of *B. velezensis* for 30 min¹⁹. Spraying treatments of *B. velezensis* and *B. thuringiensis* were carried out at intervals of 7 days after planting. Silica spraying was performed using silica fertilizer (BIOMAX®) at 15, 30 and 45 days after planting (DAP). Four weeks after plating (WAP), inoculation was carried out by spraying *C. gloeosporioides* suspension to the shallot plant. To maintain humidity, cotton previously soaked in water was added to a polybag and covered for 24 hrs. After 24 hrs, the lid was opened to observe the incubation period of the pathogen, disease intensity and disease incidence.

Growth observation and calculation of disease parameters:

The observed variables include plant height, number of leaves, disease intensity and disease incidence, which were observed every other week. Disease incidence and disease intensity were calculated using the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Number of observed plants}} \times 100$$

Disease intensity was determined using a scoring method on the percentage of damage due to disease symptoms on shallot plants²⁰ (Table 1) and the disease intensity value was calculated using the following formula:

$$\text{Disease intensity (\%)} = \frac{\sum (n \times v)}{N \times Z} \times 100$$

Where:

n = Number of diseased samples in each category

v = Score value in each category

Table 1: Scoring of anthracnose disease symptoms²⁰

Score	Damage (%)	Characteristics
0	0	No symptoms
1	1-10	Oval white spots on the leaves, curly leaves and chlorosis
2	11-20	Abnormal leaf elongation and acervuli appearing on the surface of the spots
3	21-40	Shallow, sunken necrotic spots on the acervuli
4	41-60	Elongated stems, small bulbs and the leaves showing symptoms of dieback
5	>61	Dieback, rot bulbs and changes in root color

N = Number of diseased sample plants observed

Z = Highest score value

Analysis of the Area Under Disease Progress Curve (AUDPC) value was calculated based on the development of disease intensity using the formula according to Rahma *et al.*¹⁹ as follows:

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{Y_{i+1} + Y_i}{2} \right) (t_{i+1} - t_i)$$

Where:

Y_{i+1} = ith observation data+1

Y_i = ith observation data

t_{i+1} = ith observation time+1

t_i = ith observation time

N = Total number of plants

Plants were harvested 60 days after planting and fresh weight, bulb weight, number of bulbs, root volume and root length were measured.

Detection of *Bacillus velezensis* in the roots of shallot plants:

Detection of *B. velezensis* in the roots of shallot plants was carried out by genomic DNA extraction using the ZymoBIOMICS® DNA extraction kit, followed by PCR analysis using a pair of specific primers UGM_Bv-F (5'-ACG GTT CAT CGT GAC GGA AA-3') and UGM_Bv-R (5'-AAT GGC AGT CAG CCC TTC TC-3') with an amplification target of ± 576 bp. The PCR was performed in a PCR machine (Bio-Rad T100, Germany) using 12.5 μ L MyTaq™ Red Mix (Bioline), 1 μ L forward primer, 1 μ L reverse primer, 1 μ L DNA and 9.5 μ L nuclease-free Water. The PCR conditions were carried out with a pre-denaturation at 95°C for 3 min, denaturation at 95°C for 1 min, annealing at 57°C for 30 sec and elongation at 72°C for 30 sec. The cycle was repeated 34 times and the final extension at 72°C was performed for 5 min²¹. The PCR products were analyzed by electrophoresis on a 1% agarose gel in 1×TBE. The DNA fragment was measured using a 1 kb DNA ladder (Promega). Electrophoresis was performed at 70 Volts DC for 50 min and visualized using a gel doc system (Bio-Rad).

Data analysis: The data obtained were analyzed using One-way Analysis of Variance (ANOVA) and continued with the Tukey's test at the 95% confident interval.

RESULTS AND DISCUSSION

In vitro antagonism of *B. velezensis* against

C. gloeosporioides. The dual culture antagonistic test between *B. velezensis* and *C. gloeosporioides* showed that the percentage of inhibition on the 1st day was 40% increasing to 62.8% on the 7th day, while in co-culture, the inhibition rate was 27% on the 1st day and on the 7th day increased to 77.17% (Table 2). The *B. velezensis* is a bacteria widely isolated and used as a bio-control and bio-fertilizer because it produces secondary metabolites. The presence of secondary metabolite and chitinase activity, such as bacillomycin D in *B. velezensis* in damaging fungal cell walls supports the growth and development of bacteria because they can obtain nutrients and act as an agent of parasitism from pathogens, thereby inhibiting the growth of the fungal cell. A clear zone on the PDA media indicates inhibition of fungal mycelium growth. The clear zone appears due to the decomposition of chitin by the chitinase enzyme, meaning that the clear zone is formed due to the presence of N-acetylglucosamine monomer produced from the chitin hydrolysis process by breaking the -1,4 bond of the N-acetylglucosamine homopolymer^{22,23}.

This research shows that inhibition of *B. velezensis* on pathogens *C. gloeosporioides* is more than 50% on PDA media. The *B. velezensis* grew along the streak line in the dual culture method, while *C. gloeosporioides* only grew in the opposite direction (Fig. 1a). When co-cultured, *B. velezensis* formed colonies that extended across the medium, whereas *C. gloeosporioides* couldn't grow in the medium (Fig. 1b). This occurred due to the production of antibiotics by *B. velezensis*, which inhibited the growth of *C. gloeosporioides*. In media without *B. velezensis* treatment, *C. gloeosporioides* grew normally (Fig. 1c). This is also demonstrated in the research of Li *et al.*²⁴, who reported that *B. velezensis* SYBCH47 was able to suppress the growth of *Fusarium oxysporum* mycelium by 20% in liquid media. The research of Torres *et al.*²⁵ reported

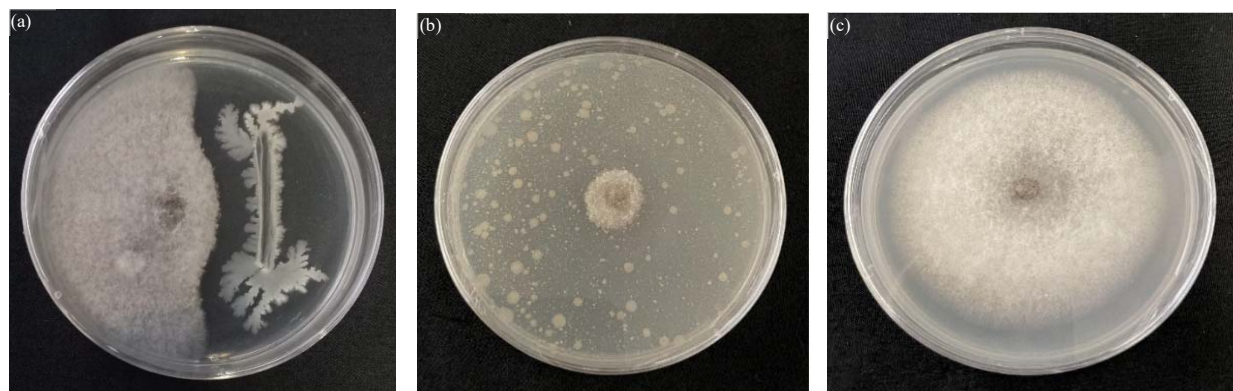


Fig. 1(a-c): Antagonistic test between *Bacillus velezensis* and *Colletotrichum gloeosporioides* using (a) Dual culture method, (b) Co-culture and (c) Without the addition of *B. velezensis*

Table 2: Inhibition rate of *Bacillus velezensis* B-27 against *Colletotrichum gloeosporioides*

Isolate/ith day	Inhibition rate (%)						
	D1	D2	D3	D4	D5	D6	D7
Dual culture	40	17.8	51.6	53.8	61.2	62.4	62.8
Co-culture	27.45	33.40	52.38	62.67	68.85	73.74	77.17

Table 3: Intensity and AUDPC of anthracnose disease in shallot

Treatment	Intensity (%)	AUDPC
Control	44	10.42
Silica	38	8.56
Spray Bt	20	7.47
Spray Bv	20	7.00
SC Bv	24	7.22
Bv+Bt	20	7.16
Bv+Bt+silica	20	7.31

Control: Without any treatment, Silica: Silica spraying, Spray Bt: *Bacillus thuringiensis* spraying, Spray Bv: *Bacillus velezensis* spraying, SC Bv: *Bacillus velezensis* dripping application, Bv+Bt: *Bacillus velezensis* dripping application + *Bacillus thuringiensis* spraying and Bv+Bt+silica: *Bacillus velezensis* dripping application + *B. thuringiensis* spraying + silica spraying

that *B. velezensis* strain XT1 could inhibit the growth of *F. oxysporum* by 80%. *Bacillus velezensis* strain XT can also inhibit the growth of *Botrytis cinerea* due to the presence of siderophores, protease and lipopeptides, such as surfactin, bacillomycin and fengycin (10 g/L)²⁶. In a study by Huang *et al.*²⁷, *B. velezensis* HYE5-6 destroyed the cell wall of *Colletotrichum gloeosporioides* strain HYCG2-3 by secreting glucanases and proteases. As a result, the cell membrane of *C. gloeosporioides* swelled due to the low osmotic environment.

Effects of *Bacillus* spp. and silica on disease development of shallot plants: Single spray of *B. thuringiensis* and *B. velezensis*, a combination of *B. velezensis* dripping application and *B. thuringiensis* spraying and a combination of *B. velezensis*, *B. thuringiensis* and silica were able to reduce the intensity of anthracnose disease by 20% then with

dripping application of *B. velezensis*, disease intensity was 24 and 38% for silica spraying. Control treatment showed the highest disease intensity, reaching 44%. The highest Area Under Disease Progress Curve (AUDPC) value for anthracnose in shallot plants was seen in control plants, spraying silica, spraying *B. thuringiensis*, a combination of *B. velezensis*, *B. thuringiensis* and silica, *B. velezensis* dripping application a combination of *B. velezensis* dripping application and *B. thuringiensis* spraying with values 10.42, 8.56, 7.47, 7.31, 7.22, 7.16, respectively. The lowest was in plants treated with *B. velezensis* spraying application with a value of 7 (Table 3).

Penetration by *C. gloeosporioides*, a pathogen causing anthracnose disease in shallot plants, shows symptoms of lesions on the leaves. The infection of *C. gloeosporioides* is categorized into subcuticular or intramural necrotrophy, where infection occurs without a host defense mechanism so

Table 4: Effects of *Bacillus thuringiensis* and *Bacillus velezensis* on the growth of shallot

Treatment	Plant height (cm)	Number of leaves	Number of tillers
Control	33.58 ^b	18.3 ^b	3.3 ^d
Silica	37.48 ^{ab}	23.0 ^{ab}	6.7 ^{ab}
Spray Bt	36.43 ^{ab}	25.7 ^{ab}	7.0 ^a
Spray Bv	39.16 ^a	31.3 ^a	3.7 ^d
SC Bv	36.37 ^{ab}	24.3 ^{ab}	4.7 ^{cd}
Bv+Bt	37.76 ^{ab}	26.0 ^{ab}	5.3 ^{bc}
Bv+Bt+silica	36.71 ^{ab}	27.7 ^a	4.7 ^{cd}

Control: Without any treatment, Silica: Silica spraying, Spray Bt: *Bacillus thuringiensis* spraying, Spray Bv: *Bacillus velezensis* spraying, SC Bv: *Bacillus velezensis* dripping application, Bv+Bt: *Bacillus velezensis* dripping application+*Bacillus thuringiensis* spraying, Bv+Bt+silica: *Bacillus velezensis* dripping application+*B. thuringiensis* spraying+silica spraying, data were analyzed using Tukey's test and Numbers followed by letters in the same column shows results that are not significantly different at the 95% confidence interval

that the pathogen quickly spreads between cells and within tissues. Plant genetics, environmental variables, the isolate used and inoculation techniques can also contribute to the appearance of disease symptoms. In this research, all plants in the inoculation treatment died at 8 WAP or after inoculation.

The use of *B. velezensis* AK-0 on apples carried out by Kim *et al.*²⁸ was able to suppress anthracnose disease caused by *C. gloeosporioides* APEC18-004, in which the lesion symptoms on apples treated with *B. velezensis* AK-0 had a diameter of 1.34 cm, while those on the fruits without treatment had a diameter of 7 cm. This study also showed that *B. velezensis* was able to suppress the development of root rot disease by 80.7% and it was able to suppress root rot disease by 54.9% five days after inoculation. This is due to the ability of *B. velezensis* AK-0 to inhibit spore germination and appressorium formation of *C. gloeosporioides* APEC18-004 after incubation for 16 hrs. In addition, research by Jiang *et al.*²⁹ showed that *B. velezensis* strains 5YN8 and DSN012 were also able to reduce the intensity of disease caused by *Botrytis cinerea* on pepper plants by around 50% due to the presence of antagonistic and hydrolase activity in *B. velezensis* strains 5YN8 and DSN012 by producing bacillomycin and fengycin, thereby being able to inhibit mycelium growth and sporulation of *B. cinerea* BC130.

Bacillus thuringiensis can be simultaneously utilized as a biocontrol agent for insect pests and plant diseases. A study by Choi *et al.*³⁰ showed *B. thuringiensis* effectively inhibited the progression of barley powdery mildew (*Blumeria graminis* f.sp. *hordei*), achieving a suppression rate of up to 70%; this is because these bacteria can produce zwittermicin A. Stabb *et al.*³¹ reported that *B. thuringiensis* was able to inhibit the development of disease caused by the pathogen *Phytophthora medicaginis* and in research by Kim *et al.*³², *B. thuringiensis* CMB26 was able to inhibit the growth of *C. gloeosporioides* *in vitro* by producing lipopeptides as toxic compounds for the pathogen.

Effects of *Bacillus* spp. and silica on the growth of shallot

plants: The treatment of *B. velezensis* dripping application was significantly able to promote plant height and number of leaves with values of 39.16 cm and 31.3, respectively, while those in the control treatment were only 33.58 cm and 18.3. Besides, the number of leaves in the combination *B. velezensis* dripping application+*B. thuringiensis* spraying+silica spraying treatment had a significantly different value than the control. Meanwhile, the number of tillers produced in the *B. thuringiensis* spraying, silica spraying and *B. velezensis*+*B. thuringiensis* dripping application treatments had significantly different values than in the control treatment (Table 4).

Table 5 shows that a single spray application of *B. velezensis* has the highest fresh weight and bulb weight with values of 21.33 and 9 g, respectively. Meanwhile, the highest value for the number of bulbs, root volume and root length was shown at the combination *B. velezensis* dripping application+*B. thuringiensis* spraying with the values of 10.78, 2.67 mL and 11.89 cm, respectively. The application of *Bacillus* showed a significant effect on the productivity of shallot plants, indicated by the fresh weight, bulb weight, number of bulbs, root volume and root length of shallot plants when *Bacillus* was applied both by spraying and dripping application, which had significantly different values compared to the control shallot plants.

In this study, the application of *B. velezensis* and *B. thuringiensis*, individually or in combination, shows an ability to enhance the growth and development of shallot plants compared to those without the application. Research by Meng *et al.*³³ showed that watering applications using *B. velezensis* BAC03 on carrots, cucumbers, beets, peppers, potatoes, radishes, squash, tomatoes and turnips were able to improve the growth and development of each plant by up to 200% of the control treatment or without *B. velezensis* BAC03. The BAC03 watering application showed optimal results at 10 DAP due to root development, producing exudates used as

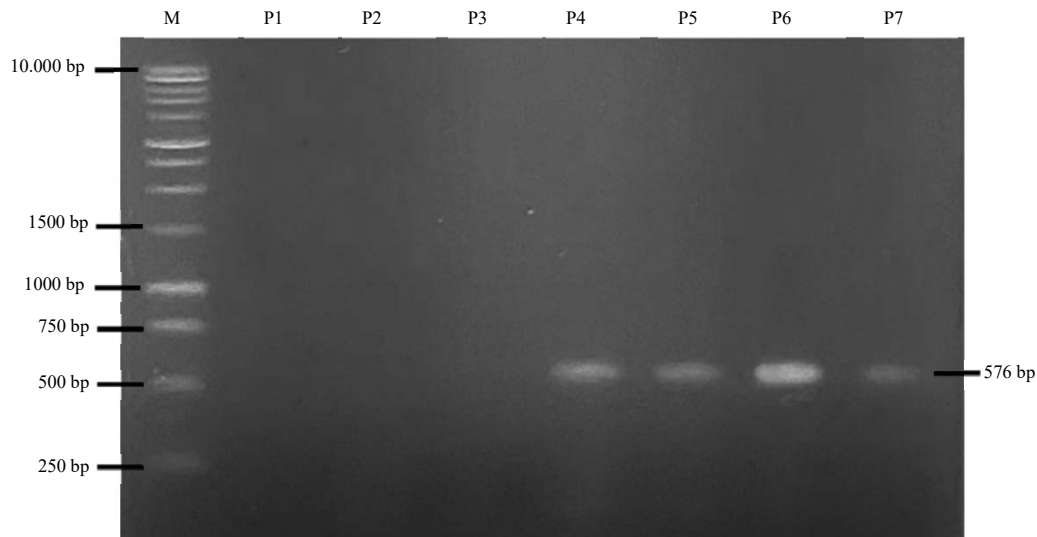


Fig. 2: Visualization of PCR product of shallot root using a pair of specific primers for detection of *Bacillus velezensis* on 1% agarose gel with target size ± 576 bp

M: 1 kb DNA ladder, P1: No treatment, P2: Silica spraying, P3: *Bacillus thuringiensis* spraying, P4: *Bacillus velezensis* spraying, P5: *Bacillus velezensis* dripping application, P6: *Bacillus velezensis* dripping application+*Bacillus thuringiensis* spraying, P7: *Bacillus velezensis* dripping application+*Bacillus thuringiensis* spraying+silica spraying

Table 5: Effects of *Bacillus thuringiensis* and *Bacillus velezensis* on the productivity of shallot

Treatment	Fresh weight (g)	Bulb weight (g)	Number of bulbs	Root volume (mL)	Root length (cm)
Control	13.78 ^a	6.33 ^a	3.56 ^c	0.77 ^b	5.72 ^c
Silica	15.67 ^a	6.66 ^a	8.83 ^{ab}	1.50 ^{ab}	8.30 ^{abc}
Spray Bt	17.89 ^b	7.33 ^a	8.17 ^{ab}	1.50 ^{ab}	8.89 ^{abc}
Spray Bv	21.33 ^c	9.00 ^c	9.56 ^{ab}	1.83 ^{ab}	11.48 ^{ab}
SC Bv	16.56 ^b	7.28 ^a	8.67 ^{ab}	1.77 ^a	9.42 ^{abc}
Bv+Bt	16.78 ^b	7.56 ^b	10.78 ^a	2.67 ^a	11.89 ^a
Bv+Bt+silica	16.56 ^b	7.33 ^a	8.22 ^{ab}	1.40 ^{ab}	6.07 ^{bc}

Control: Without any treatment, Silica: Silica spraying, Spray Bt: *Bacillus thuringiensis* spraying, Spray Bv: *Bacillus velezensis* spraying, SC Bv: *Bacillus velezensis* dripping application, Bv+Bt: *Bacillus velezensis* dripping application+*Bacillus thuringiensis* spraying, Bv+Bt+silica: *Bacillus velezensis* dripping application+*B. thuringiensis* spraying+silica spraying, data were analyzed using Tukey's test and Numbers followed by letters in the same column shows results that are not significantly different at the 95% confidence interval

nutrition for bacteria. The use of *B. velezensis* XT1 in the research of Torres *et al.*²⁵ was able to increase plant height, number of leaves and fresh weight of various horticultural plants, such as pumpkin (9.8, 56.2 and 129.2%, respectively); cucumber (6.5, 100 and 100%, respectively); pepper (22.1, 16.7 and 63.6%, respectively) and tomato (12.1, 38.5 and 38.6%, respectively).

The application of a combination of *B. velezensis* and *B. thuringiensis* with silica also affects the number of leaves, fresh weight and number of bulbs shallot plants when compared to plants without application. Increased growth and development in pumpkin, cucumber, pepper and tomato plants occur because the *B. velezensis* strain XT1 can produce phosphatase, urease and ACC deaminase. Phosphatase functions in releasing phosphate from organic compounds and dissolving inorganic phosphate, making it

easily absorbed by plants. Urease functions in releasing ammonium as a source of nitrogen for plants. Meanwhile, ACC deaminase reduces ethylene levels in plants, which helps in stress conditions. Besides, siderophores, which can provide iron for plant growth, are also produced²⁵. The presence of phytohormones, such as IAA, gibberellin, cytokinin and ethylene, enzymes, such as phosphatase, urease and ACC deaminase and siderophores produced by *Bacillus* are capable of acting as PGPR that can promote plant growth³⁴. *Bacillus* can grow in various conditions by utilizing organic compounds as a carbon source to still carry out respiration and fermentation. *Bacillus* is also a facultative anaerobic, which can grow on the soil surface and in the rhizosphere²⁵. This ability causes bacteria to produce enzymes, phytohormones and siderophores that can promote growth and help plant development, thereby increasing productivity.

Detection of *Bacillus velezensis* in the roots of shallot plants:

Bacillus velezensis was detected in the roots of shallot plants treated either by spraying or dripping application in suspension. Positive results were indicated by the formation of a DNA band at ± 576 bp in treatments P4 (*B. velezensis* spraying), P5 (*B. velezensis* dripping application), P6 (*B. velezensis* dripping application + *B. thuringiensis* spraying) and P7 (*B. velezensis* immersion + *B. thuringiensis* spraying + silica spraying) (Fig. 2).

Bacillus velezensis is a rhizosphere bacterium or plant growth promoting rhizobacteria (PGPR) where this bacterium lives naturally in the rhizosphere of soil and plants so that it interacts with root exudates and forms a mutualistic symbiosis with plants³⁵. This symbiotic ability is why *B. velezensis* is widely used as a biofertilizer and biocontrol. This was shown in research by Chebotar *et al.*³⁶, reporting that *B. velezensis* BS89 was obtained from isolated roots of wheat plants and showed the ability to promote plant growth and produce auxin, hydrolytic enzymes and vitamins.

The research indicates that applying *B. velezensis* and *B. thuringiensis*, individually or in combination with silica, can improve plant growth, reduce disease intensity and increase productivity. This underscores the potential of *B. velezensis* and *B. thuringiensis* as biocontrol agents. The detection of *B. velezensis* in shallot roots confirms its presence and interaction with plants, supporting its role as a PGPR. Hopefully, this research can offer an environmentally safe alternative for controlling diseases in shallot. Nonetheless, adjusting the methods for each environmental condition and dosage used in specific regions is essential.

CONCLUSION

Bacillus velezensis can inhibit the growth of *Colletotrichum gloeosporioides* mycelium *in vitro*. Applying *B. velezensis* and *B. thuringiensis*, individually or in combination with silica, can reduce the intensity of anthracnose disease, promote plant growth and increase shallot productivity. Positive results for detecting *B. velezensis* in the roots of shallot plants were indicated with a DNA band measuring ± 576 bp. Accordingly, using *Bacillus* and silica can offer an opportunity to reduce the use of chemicals that may pose risks to human health and the environment.

SIGNIFICANCE STATEMENT

Bacillus velezensis as PGPR has functioned as a biofertilizer, bio-stimulant and bio-protectant. While

B. velezensis is commonly used to control plant diseases, *B. thuringiensis* is widely used as a biopesticide. This research aims to control anthracnose disease in shallot using a combination of *B. velezensis*, *B. thuringiensis* and silica application. In this study, the efficacy of *B. velezensis* in controlling anthracnose disease is evidenced by its ability to inhibit the growth of *Colletotrichum gloeosporioides* mycelium *in vitro*. Combining *B. velezensis* and *B. thuringiensis* with silica reduced the intensity of anthracnose disease in shallots. This combination also significantly promotes shallot growth and productivity.

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REFERENCES

- Herlina, L., Reflinur, Sobir, A. Maharijaya and S. Wiyono, 2019. The genetic diversity and population structure of shallots (*Allium cepa* var. *aggregatum*) in Indonesia based on R gene-derived markers. Biodiversitas J. Biol. Divers., 20: 696-703.
- Dutta, R., K. Jayalakshmi, S.M. Nadig, D.C. Manjunathagowda, V.S. Gurav and M. Singh, 2022. Anthracnose of onion (*Allium cepa* L.): A twister disease. Pathogens, Vol. 11. 10.3390/pathogens11080884.
- Myo, E.M., B. Liu, J. Ma, L. Shi, M. Jiang, K. Zhang and B. Ge, 2019. Evaluation of *Bacillus velezensis* NKG-2 for bio-control activities against fungal diseases and potential plant growth promotion. Biol. Control, 134: 23-31.
- Hafidhotul Ilmiah, H., E. Sulistyaningsih and T. Joko, 2021. Fruit morphology, antioxidant activity, total phenolic and flavonoid contents of *Salacca zalacca* (Gaertner) Voss by applications of goat manures and *Bacillus velezensis* B-27. Caraka Tani: J. Sustainable Agric., 36: 270-282.
- Chen, L., J. Heng, S. Qin and K. Bian, 2018. A comprehensive understanding of the biocontrol potential of *Bacillus velezensis* LM2303 against *Fusarium* head blight. PLoS ONE, Vol. 13. 10.1371/journal.pone.0198560.
- Ye, M., X. Tang, R. Yang, H. Zhang and F. Li *et al.*, 2018. Characteristics and application of a novel species of *Bacillus*: *Bacillus velezensis*. ACS Chem. Biol., 13: 500-505.
- Kim, J.A., J.S. Song, P.I. Kim, D.H. Kim and Y. Kim, 2022. *Bacillus velezensis* TSA32-1 as a promising agent for biocontrol of plant pathogenic fungi. J. Fungi, Vol. 8. 10.3390/jof8101053.

8. Zhang, H., M.S. Kim, V. Krishnamachari, P. Payton and Y. Sun *et al.*, 2007. Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. *Planta*, 226: 839-851.
9. Rocha, L.O., S.M. Tralamazza, G.M. Reis, L. Rabinovitch, C.B. Barbosa and B. Corrêa, 2014. Multi-method approach for characterizing the interaction between *Fusarium verticillioides* and *Bacillus thuringiensis* subsp. *Kurstaki*. *PLoS ONE*, Vol. 14. 10.1371/journal.pone.0092189.
10. Knaak, N., A.A. Rohr and L.M. Fiuza, 2007. *In vitro* effect of *Bacillus thuringiensis* strains and cry proteins in phytopathogenic fungi of paddy rice-field. *Braz. J. Microbiol.*, 38: 526-530.
11. de la Fuente-Salcido, N.M., L.E. Casados-Vázquez, A.P. García-Pérez, U.E. Barboza-Pérez and D.K. Bideshi *et al.*, 2016. The endochitinase ChiA Btt of *Bacillus thuringiensis* subsp. *tenebrionis* DSM-2803 and its potential use to control the phytopathogen *Colletotrichum gloeosporioides*. *MicrobiologyOpen*, 5: 819-829.
12. Yoshida, S., M. Koitabashi, D. Yaginuma, M. Anzai and M. Fukuda, 2019. Potential of bioinsecticidal *Bacillus thuringiensis* inoculum to suppress gray mold in tomato based on induced systemic resistance. *J. Phytopathol.*, 167: 679-685.
13. Hyakumachi, M., M. Nishimura, T. Arakawa, S. Asano, S. Yoshida, S. Tsushim and H. Takahashi, 2013. *Bacillus thuringiensis* suppresses bacterial wilt disease caused by *Ralstonia solanacearum* with systemic induction of defense-related gene expression in tomato. *Microbes Environ.*, 28: 128-134.
14. Tayade, R., A. Ghimire, W. Khan, L. Lay, J.Q. Attipoe and Y. Kim, 2022. Silicon as a smart fertilizer for sustainability and crop improvement. *Biomolecules*, Vol. 12. 10.3390/biom12081027.
15. Pavlovic, J., L. Kostic, P. Bosnic, E.A. Kirkby and M. Nikolic, 2021. Interactions of silicon with essential and beneficial elements in plants. *Front. Plant Sci.*, Vol. 12. 10.3389/fpls.2021.697592.
16. Seebold, K.W., L.E. Datnoff, F.J. Correa-Victoria, T.A. Kucharek and G.H. Snyder, 2000. Effect of silicon rate and host resistance on blast, scald, and yield of upland rice. *Plant Dis.*, 84: 871-876.
17. Ohri, P. and S.K. Pannu, 2010. Effect of phenolic compounds on nematodes-A review. *J. Appl. Nat. Sci.*, 2: 344-350.
18. Jayanti, R.M. and T. Joko, 2020. Plant growth promoting and antagonistic potential of endophytic bacteria isolated from melon in Indonesia. *Plant Pathol. J.*, 19: 200-210.
19. Rahma, A.A., Suryanti, S. Somowiyarjo and T. Joko, 2020. Induced disease resistance and promotion of shallot growth by *Bacillus velezensis* B-27. *Pak. J. Biol. Sci.*, 23: 1113-1121.
20. Amallia, R., Suryanti and T. Joko, 2023. The potential of *Rhizophagus intraradices*, *Bacillus thuringiensis* Bt BMKP and silica for anthracnose disease control in shallot. *Caraka Tani: J. Sustainable Agric.*, 38: 433-446.
21. Trianom, B., T. Arwiyanto and T. Joko, 2019. Morphological and molecular characterization of Sumatra disease of clove in Central Java, Indonesia. *Trop. Life Sci. Res.*, 30: 107-118.
22. Jin, P., H. Wang, Z. Tan, Z. Xuan and G.Y. Dahar *et al.*, 2020. Antifungal mechanism of bacillomycin D from *Bacillus velezensis* HN-2 against *Colletotrichum gloeosporioides* Penz. *Pestic. Biochem. Physiol.*, 163: 102-107.
23. Joko, T., M. Umehara, T. Murata, H. Etoh, K. Izumori and S. Tsuyumu, 2018. Hyperinduction of pectate lyase in *Dickeya chrysanthemi* EC16 by plant-derived sugars. *J. Plant Interact.*, 13: 141-150.
24. Li, X., Y. Zhang, Z. Wei, Z. Guan, Y. Cai and X. Liao, 2016. Antifungal activity of isolated *Bacillus amyloliquefaciens* SYBC H47 for the biocontrol of peach gummosis. *PLoS ONE*, Vol. 11. 10.1371/journal.pone.0162125.
25. Torres, M., I. Llamas, B. Torres, L. Toral, I. Sampedro and V. Béjar, 2020. Growth promotion on horticultural crops and antifungal activity of *Bacillus velezensis* XT1. *Appl. Soil Ecol.*, Vol. 150. 10.1016/j.apsoil.2019.103453.
26. Toral, L., M. Rodríguez, V. Béjar and I. Sampedro, 2018. Antifungal activity of lipopeptides from *Bacillus* XT1 CECT 8661 against *Botrytis cinerea*. *Front. Microbiol.*, Vol. 9. 10.3389/fmicb.2018.01315.
27. Huang, L., Q.C. Li, Y. Hou, G.Q. Li, J.Y. Yang, D.W. Li and J.R. Ye, 2017. *Bacillus velezensis* strain HYEB5-6 as a potential biocontrol agent against anthracnose on *Euonymus japonicus*. *Biocontrol Sci. Technol.*, 27: 636-653.
28. Kim, Y.S., Y. Lee, W. Cheon, J. Park and H.T. Kwon *et al.*, 2021. Characterization of *Bacillus velezensis* AK-0 as a biocontrol agent against apple bitter rot caused by *Colletotrichum gloeosporioides*. *Sci. Rep.*, Vol. 11. 10.1038/s41598-020-80231-2.
29. Jiang, C.H., M.J. Liao, H.K. Wang, M.Z. Zheng, J.J. Xu and J.H. Guo, 2018. *Bacillus velezensis*, a potential and efficient biocontrol agent in control of pepper gray mold caused by *Botrytis cinerea*. *Biol. Control*, 126: 147-157.
30. Choi, G.J., J.C. Kim, K.S. Jang and D.H. Lee, 2007. Antifungal activities of *Bacillus thuringiensis* isolates on barley and cucumber powdery mildews. *J. Microbiol. Biotechnol.*, 17: 2071-2075.
31. Stabb, E.V., L.M. Jacobson and J. Handelsman, 1994. Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. *Appl. Environ. Microbiol.*, 60: 4404-4412.
32. Kim, P.I., H. Bai, D. Bai, H. Chae and S. Chung *et al.*, 2004. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *J. Appl. Microbiol.*, 97: 942-949.

33. Meng, Q., H. Jiang and J.J. Hao, 2016. Effects of *Bacillus velezensis* strain BAC03 in promoting plant growth. *Biol. Control*, 98: 18-26.
34. Sharma, A. and B.N. Johri, 2003. Growth promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS₉ in maize (*Zea mays* L.) under iron limiting conditions. *Microbiol. Res.*, 158: 243-248.
35. Navitasari, L., T. Joko, R.H. Murti and T. Arwiyanto, 2020. Rhizobacterial community structure in grafted tomato plants infected by *Ralstonia solanacearum*. *Biodiversitas*, 21: 4888-4895.
36. Chebotar', V.K., N.M. Makarova, A.I. Shaposhnikov and L.V. Kravchenko, 2009. Antifungal and phytostimulating characteristics of *Bacillus subtilis* Ch-13 rhizospheric strain, producer of biopreparations. *Appl. Biochem. Microbiol.*, 45: 419-423.