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Antagonistic Potential and Plant Growth Enhancement by Endophytic *Bacillus* Isolated from Citrus Plants

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ABSTRACT

Citrus is a horticultural commodity with high economic value. However, citrus production is constrained by various plant diseases caused by infectious pathogens. Endophytic bacteria that live in plant tissues can function as plant growth promoters and biological control agents by producing growth hormones and encoding antibacterial and antifungal genes. This study aimed to isolate endophytic *Bacillus* from citrus plants as plant growth-promoting bacteria. Endophytic bacteria were initially isolated from citrus leaf tissue, followed by morphological characterization and KOH tests and the detection of growth-encoding (*ipdC*, *acdS*, *pqqE*, and *nifH*), antibacterial (*aiiA* and *sfp*), and antifungal (*fenD*, *bamC*, and *ituA*) genes with specific primers. Thereafter, antagonistic tests against *Colletotrichum* sp. were performed, and the *Bacillus* isolates were applied to citrus seedlings. Ten *Bacillus* isolates were obtained from different locations. Detection of the plant-beneficial traits encoding genes showed that the isolate BYL-4 had all the genes encoding for growth, antibacterial, and antifungal properties. Antagonist testing was performed using the dual culture and coculture methods, which revealed that the SH-1, SH-2, SH-3, BYL-1, BYL-2, BYL-3, B2B, M2, and P4 isolates were able to inhibit the growth of *Colletotrichum* sp. Based on the application of the *Bacillus* isolates to seedlings, the *Bacillus* BYL-3 isolate significantly increased the height, fresh weight, dry weight, and root weight of the citrus plant seedlings. Molecular identification using conventional PCR was performed with a pair of universal 16S rRNA primers, 27F/1492R, with an amplification target of ±1500 bp, followed by Sanger sequencing. This showed that the isolated endophytic bacteria were closely related to two *Bacillus* species, namely *Bacillus subtilis* subsp. *subtilis* and *Bacillus tropicus*.

Keywords: Antibacterial, antifungal, plant-beneficial traits, PCR, 16S rDNA

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INTRODUCTION

Citrus is a horticultural commodity that has been extensively developed worldwide. Brazil is the largest citrus producer in the world, with an estimated annual citrus production of 17.3 million tons (Dala-Paula et al., 2019). In 2016, global citrus production reached approximately 131 million tons of fresh fruit, comprising 52% oranges, 29% mandarins, 12% limes and lemons, and 7% grapefruits. Meanwhile, in the Asian region, China is one of the largest citrus producers, with fruit production reaching 7.3 million tons per year, followed by countries in the European region, which is the third largest citrus producer, with total production reaching 11,497 million tons per year (Sariasih et al., 2024). Moreover, in the Middle East region, as well as in countries such as India, Pakistan, Brazil, Argentina, and Mexico, citrus fruit production contributes to approximately 5% of the country's total agricultural economic income (Donkersley et al., 2018). As a tropical and subtropical crop, citrus is host to numerous pests and diseases. This plant is highly vulnerable to various destructive diseases that continue to emerge, thus potentially hindering or even completely wiping out production. Various bacterial, fungal, and viral diseases pose an ongoing threat to citrus cultivation, which leads to a substantial reduction in yield across all growing regions worldwide (Poveda et al., 2021). Fungi account for 25%-75% of citrus leaf disease (Asharo et al., 2024). One of the fungal pathogens that infects citrus plants is *Colletotrichum*. This fungus causes anthracnose disease in citrus plants and is characterized by leaf and flower necrosis as well as premature fruit fall (Munoz-Guerrero et al., 2021; Silva et al., 2014).

Colletotrichum was recently recognized as a major plant pathogen causing anthracnose, a plant disease that affects various hosts from trees to grasses (Gautam, 2014). *Colletotrichum* species can infect over 30 plant genera, which leads to anthracnose disease and postharvest decay in a diverse range of crops, including tropical, subtropical, and temperate fruits, grasses, vegetables, and ornamental plants. The agricultural losses caused by *Colletotrichum* infection are particularly severe in staple food crops cultivated in developing regions across the tropics and subtropics. Moreover, many *Colletotrichum* species function as latent pathogens, endophytes, epiphytes, or saprobes and shift to a pathogenic state when the host plants experience stress or are stored postharvest. Several *Colletotrichum* species have been associated with citrus, belonging to four species complexes: the *C. boninense* species complex (including *C. boninense*, *C. citricola*, *C. constrictum*, *C. karstii*, and *C. novae-zelandiae)*; the *C. acutatum* species complex (including *C. abscissum*, *C. acutatum*, *C. citri*, *C. godetiae*, *C. johnstonii*, *C. limetticola,* and *C. simmondsii*); and the *C. gloeosporioides* species complex (including *C. fructicola*, *C. gloeosporioides*, *C. kahawae* subsp. *ciggaro,* and *C. siamense*) (Guarnaccia et al., 2017).

In general, pest and disease control techniques in citrus plants are performed by managing pests and diseases in an integrated manner, namely using disease-free citrus seeds, utilizing biological agents, conducting environmental sanitation and good cultivation

practices, maintaining plants optimally, and monitoring them regularly (Paudyal, 2016; Widyaningsih et al., 2017). One effort that is currently being considered for development is using biological agents to induce plant resistance, one of which is endophytic antagonistic bacteria (Navitasari et al., 2020). Endophytic *Bacillus* are often used as biological control agents in agriculture for plant diseases (Chen et al., 2018). These bacteria have high antibiosis capabilities and can inhibit competitors with parasitism. *Bacillus* species are reported to form endospores and produce various beneficial metabolites, such as antibiotics and enzymes, and secondary metabolites that are antimicrobial and plant growth promoters.

Munir et al. (2022) reported that endophytic *B. subtilis* L1-21 isolated from healthy citrus plants presented an innovative approach to disease management in citrus plants. Furthermore, research by Nan et al. (2021) showed that *B. velezensis* was able to produce antimicrobial compounds, such as surfactin, fengycin, iturin A, macrolactin, difficidin, bacillaene, bacilysin, and bacillibactin. In addition, the application of *B. amyloliquefaciens* to two-year-old citrus plants infected with *Candidatus* Liberibacter asiaticus via root irrigation resulted in higher photosynthesis parameters, chlorophyll content, resistance-related enzyme content, and defense-related gene expression compared with those of the control plants (Lestiyani et al., 2024). Moreover, research using the antagonism test on endophytic bacteria against the anthracnose disease-causing pathogenic fungus *C. scovillei* in large chilies showed that the endophytic bacteria tested had excellent potential growth inhibitory activity against *C. scovillei* with an inhibition value of up to 80% (Wei et al., 2023). Other research related to the testing of the inhibitory effect of endophytic bacteria on the fungus *Colletotrichum*, which also causes anthracnose disease in strawberries, showed that the endophytic bacteria tested had a growth inhibitory activity of 60%–75% (Murtado et al., 2020). Based on the results of previous studies about the abilities of endophytic bacteria, this study aimed to isolate and identify endophytic bacteria from citrus plants and assess their potential as biological control agents.

MATERIALS AND METHODS

The research was performed at the Laboratory of Plant Pathology, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Isolation of Endophytic *Bacillus*

Healthy Citrus Siamese cultivar leaf samples, approximately two weeks old on stages V5 (Ribeiro et al., 2021), were collected from four locations: Purworejo, Yogyakarta, Magelang, and Boyolali. The leaf samples were disinfected with 70% alcohol for 1 min, 2% NaOCl for 3 min, absolute alcohol for 30 s, and sterile distilled water for 5 min, with three repetitions of rinsing with sterile distilled water. The leaf veins were taken and cut into small pieces until the sample weight reached 2 g. The sample was then ground using a

mortar and pestle with a mixture of 1 g of fresh leaf vein sample per 3 mL of 0.85% NaCl until smooth. The resulting suspension was then diluted to a 10^{-10} dilution series with 0.85% NaCl, and each dilution series was homogenized. The suspension was then heated at 60°C for 15 min using a water bath. Heat treatment was specifically applied for the isolation of *Bacillus* from leaf tissues to minimize the growth of non-*Bacillus* bacteria because *Bacillus* is classified as a thermophilic bacterium capable of surviving at temperatures up to 70°C (Abdollahi et al., 2021). This approach is based on the fact that most vegetative bacterial cells cannot withstand prolonged exposure to high temperatures, whereas *Bacillus* spores can remain viable. The endospores produced by *Bacillus* are formed in response to high cell density or nutritional stress, typically under carbon and nitrogen starvation conditions. Mature *Bacillus* and related genera endospores exhibit resistance to heat, UV radiation, and γ-radiation (Logan & Vos, 2015). The appropriate dilution was then spread on yeast peptone agar (YPA) media containing 0.5% yeast extract, 1% polypeptone, and 1.5% agar and incubated under aerobic conditions at 37°C for 24 h (Unban et al., 2020). YPA medium was used to culture *Bacillus* because of its rich nutrient content that supports both aerobic and facultative anaerobic metabolism, as well as spore germination. Yeast extract provides essential vitamins and amino acids, while peptone serves as a nitrogen source for protein synthesis and cellular functions (Davami et al., 2015). YPA has proven effective for promoting *Bacillus* growth and enzyme production, as shown in studies using similar media (Ismail et al., 2018), and is suitable for culturing various *Bacillus* species. Endophytic bacterial isolates were then purified by taking bacterial colonies with different characteristics from each Petri dish. The selected colonies were streaked on YPA media and incubated at 37°C for 24–48 h.

Morphological Observation and the KOH Test

The morphological characteristics of the endophytic bacterial colonies were observed based on their shape, edges, elevation, size, gloss, and texture (Lata et al., 2024). The bacterial isolate was first placed on a glass surface, and then 3% KOH was dripped onto it to perform the Gram test. The bacterial isolate and 3% KOH were mixed until smooth and then observed. The bacterial isolate being tested was determined to be a gram-negative bacterial group if mucus forms (sticky) or a gram-positive bacterial group if no mucus forms (not sticky) (Afriani et al., 2018).

Identification Based on 16S rRNA Gene Fragments

A total of 10 endophytic *Bacillus* isolates were subjected to PCR analysis using a pair of universal 16S rRNA primers, namely 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492 R (5′ GGT TAC CTT GTT ACG ACTT 3′), with an amplification target of ±1500 bp. PCR was performed on a standard thermocycler (Bio-Rad T100, Germany) using 25

µL of master mix (GoTaq, Promega), 4 µL each of the forward and reverse primers, 8 µL of DNA template, and 9 µL of nuclease-free water to a total volume of 50 µL. Next, amplification was performed according to the following PCR protocol, with an initial denaturation program of 94°C for 2 min followed by 34 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were analyzed using electrophoresis on a 1.5% agarose gel in 1× TBE buffer, with 2 µL of RedSafe added (Intron Biotechnology, Korea). DNA-size comparison was made using a 100-bp DNA ladder marker (Promega). Electrophoresis was performed using an electrophoresis machine (Bio-Rad) at 50 V for 50 min. Gel visualization was performed under a UV transilluminator (Trianom et al., 2019).

The PCR products were then purified and sent to a sequencing service company (1st Base, Malaysia). The sequencing results were analyzed using the basic local alignment search tool (BLAST), which is available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/Blast.cgi), to determine the homology percentage. The nucleotide sequence of the endophytic *Bacillus* isolates was checked using the BLAST-N program, and phylogenetic analysis was performed using the Maximum Likelihood method with 1,000 bootstrap iterations and the Kimura-2 model.

Detection of Plant-Beneficial Trait (PBT) Encoding Genes

PBT-encoding gene detection was performed on the 10 identified endophytic *Bacillus*. The isolates were grown on YPA media and incubated for 48 h. Bacterial DNA was extracted using the G-spin™ Genomic DNA Extraction Kit (for Bacteria) (Intron Biotechnology, Korea) using a previously reported protocol (Kim et al., 2022). The identified PBT-encoding genes included growth promotion, antibacterial, and antifungal encoding genes, which were detected using the standard PCR method. The PCR reagents used for each reaction were 5 µL of master mix (GoTaq, Promega), 1 µL of each forward and reverse primer, 1 µL of bacterial DNA, and 2 µL of nuclease-free water to a total volume of 10 µL. The specific gene primers used are presented in Table 1. Furthermore, amplification was performed on a thermocycler (Bio-Rad T100 Thermal Cyclers) with a PCR protocol of an initial denaturation program of 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 30 s (see Table 1 for specific annealing temperatures), extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min and a final hold at 12°C for 1 min (Handiyanti et al., 2018).

The amplified DNA products were visualized using electrophoresis on a 1% agarose gel and 3 µL RedSafe (Intron Biotechnology, Korea) in 1× TBE buffer with a 100-bp and 1-kb DNA ladder (Promega) for size comparison. Electrophoresis was performed using an electrophoresis machine (Bio-Rad DNA Electrophoresis Cell) at 50 V for 50 min. The gel was then observed under a UV transilluminator.

Table 1
List of primers for genes encoding plant-beneficial traits in Bacillus *species*

Gene Expression	Gene	Primer name	Primer Sequence (5'-3')	Annealing (^{0}C)	Reference
Indolepyruvate	*ipdC*	F-ipdC	CAYTTGAAAACKCAMTATACTG	50	Raddadi et al., 2008
decarboxylase		R-ipdC	AAGAATTTGYWKGCCGAATCT		
ACC deaminase	*acdS*	105F-adS	TGCCAAGCGTGAAGACTGC	58	Jaya et al., 2019
		224R-acdS	GGGTCTGGTTCGACTGGAT		
Phosphatase solubilization	*pqqE*	pqqE-F	GARCTGACYTAYCGCTGYCC	55	Ding et al., 2005
		pqqE-R	TSAGSAKRARSGCCTGR		
Nitrogenase	*nifH*	nifH-F	GGCTGCGATCCVAAGGCCGAYTCVACCCG	55	Suleman et al., 2018
		nifH-R	CTGVGCCTTGTTYTCGCGGATSGGCATGGC		
Surfactin	*sfp*	P17	ATGAAGATTTACGGAATTTA	46	Lahlali et al., 2020
		P18	TTATAAAAGCTCTTCGTACG		
Anti quorum- sensing	*aiiA*	aiiA240B1	ATGGGATCCATGACGTAAAGAAGCTTTAT	55	Dong et al., 2002
		aiiACOT1	GTCGAATTCCTCAACAAGATACTCCTAATG		
Fengycin	*fenD*	FENDF	GGCCCGTTCTCTAAATCCAT	62	Mora et al., 2011
		FENDR	GTCATGCTGACGAGAGCAAA		
Bacillomycin	*bamC*	BACC1F	GAAGGACACGGCAGAGAGTC	60	Ramarathnam et al., 2007
		BACC1R	CGCTGATGACTGTTCATGCT		
iturinA	*ituA*	ITUD1F	GATGCGATCTCCTTGGATGT	60	Athukorala et al., 2009
		ITUD1R	ATCGTCATGTGCTGCTTGAG		

In Vitro Antagonistic Test of Endophytic *Bacillus* against *Colletotrichum* sp.

An antagonistic test of endophytic *Bacillus* against *Colletotrichum* sp. isolated from citrus plants was conducted *in vitro* using the dual culture (Wisanggeni et al., 2023) and coculture (Jayanti & Joko, 2020) methods. The dual culture method was performed by growing *Colletotrichum* sp. in the middle of potato dextrose agar (PDA) medium. Furthermore, the endophytic bacterial isolate was scratched approximately 4 cm long on the edge of the Petri dish, which was 2 cm from the pathogenic fungus. Observations were made by measuring the diameter of the fungal colony for 7 days at 25°C-28°C. The ability of the antagonistic bacteria to inhibit fungal growth was calculated using the following formula (Abdullah et al., 2024):

$$P = \frac{(R1 - R2)}{R1} x\ 100$$

Remarks:
P = Percentage of Inhibition of Radial Growth (PIRG) (%)
R1 = Average radius of plant pathogenic fungal colonies without bacteria (cm)
R2 = Average radius of fungal colonies approaching the bacteria (cm)

Meanwhile, the coculture method was conducted by adding 10 mL of 0.7% water agar (WA) at 50°C with 100 µL of bacterial suspension at a density of 10^8 CFU/mL onto PDA medium in a Petri dish. Furthermore, the *Colletotrichum* isolate was placed in the middle of the WA medium. The culture was incubated for 7 days at room temperature. The inhibitory ability of the endophytic bacterial isolates was measured by calculating the mycelium growth of *Colletotrichum* sp. for 7 days using the following formula:

$$P = \frac{C - T}{C} x\ 100$$

Remarks:
P = Percentage of Inhibition of Radial Growth (PIRG) (%)
C = Diameter of pathogenic fungal growth in the control treatment (cm)
T = Diameter of pathogenic fungal growth in the antagonistic bacteria treatment (cm)

Application of Endophytic *Bacillus* to Citrus Seedlings

Endophytic *Bacillus* was applied to Citrus Siamese cultivar seedlings by preparing the plants and suspensions of all 10 endophytic *Bacillus* isolates. A total of 55 citrus seedlings were used, with five replicates of each endophytic *Bacillus* isolate application and five control seedlings. The application was performed by watering the roots of the seedlings

once a week with a *Bacillus* isolate at a density of 10^8 CFU/mL (Marisna et al., 2024; Riera et al., 2017) and an application volume of 20 mL per tray hole.

Observation of the Effects of Endophytic *Bacillus* on the Growth of Citrus Seedlings

Observation of the citrus seedlings growth was performed for 60 days after planting. Plant height was measured using a meter ruler in centimeters (cm) from the ground surface to the tip of the last growing point once a week for 8 weeks. The fresh weight of the plant was measured using an analytical scale, while the dry weight was measured using an analytical scale after being oven-dried at 60°C for 72 h. The roots were cleaned and then measured with a ruler from the base to the tip in cm to determine the length. The root volume was determined by measuring the difference between the water volume after the roots were put into the measuring beaker and the initial water volume.

Statistical Analysis

Statistical analysis was performed using an analysis of variance with Tukey's test and a 95% confidence level in SPSS software.

RESULTS

Isolation and Characterization of Endophytic *Bacillus* Isolates

The bacterial isolates were characterized morphologically based on color, size, shape, edge, and elevation (Figure 1). A total of 10 isolates with different morphologies were successfully isolated from four regions: Boyolali (isolates BYL-1, BYL-2, BYL-3, and BYL-4), Yogyakarta (isolates SH-1, SH-2, SH-3, and P4), Purworejo (isolate B2B), and Magelang (isolate M2). All of the isolates collected were gram-positive bacteria based on the Gram test, which was indicated by the absence of mucus formation after 3% KOH dripping.

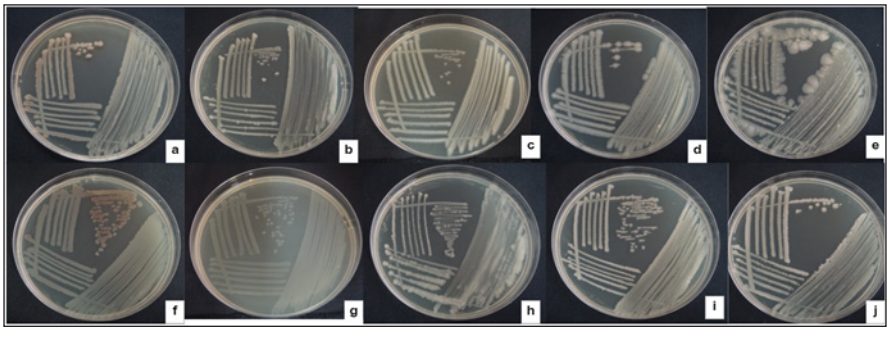

Figure 1. Bacterial growth on YPA media after 48 h. a: isolate BYL-1; b: isolate BYL-2; c: isolate BYL-3; d: isolate BYL-4; e: isolate SH-1; f: isolate SH-2; g: isolate SH-3; h: isolate P4; i: isolate M2; and j: isolate B2B

The BYL-1 isolate (Figure 1a) exhibited morphological characteristics on YPA medium, forming a reddish-white colony with a circular shape, irregular edges, convex elevation, and a shiny appearance. This aligns with the report by Flori et al. (2020), which mentions that *Bacillus* colonies are generally white to yellow, although some exhibit black, brown, orange, or pink pigments, with a convex to raised elevation. Similarly, the SH-2 isolate (Figure 1f) displayed a reddish-white colony with a circular shape, irregular edges, and a flat elevation. The BYL-2 and SH-3 isolates (Figures 1b and g) exhibited morphological similarities, characterized by cream-white colonies with a circular shape, irregular edges, and a non-shiny surface. The BYL-3 and BYL-4 isolates (Figures 1c and d) also shared similar morphological characteristics, exhibiting cream-white, non-shiny colonies with a convex elevation. Meanwhile, the SH-1, P4, M2, and B2B isolates (Figures 1e, h, i, and j) displayed comparable morphologies, forming cream-white, non-shiny, and powdery colonies with a circular shape, irregular edges, and flat elevation.

Molecular Detection of PBT-Encoding Genes

In this study, specific primer pairs were used to detect growth-promoting (indole pyruvate decarboxylase, ACC deaminase, phosphatase solubilization, and nitrogenase), antibacterial (antiquorum sensing and surfactin), and antifungal (fengycin, bacillomycin D, and iturin A) encoding genes. The results showed that among the 10 *Bacillus* isolates, six isolates (SH-2, BYL-1, BYL-3, BYL-4, B2B, and M2) had the growth promotion-encoding gene indole pyruvate decarboxylase (*ipdC*), with an amplicon size of 1850 bp (Figure 2a). Six isolates (BYL-1, SH-1, SH-2, SH-3, BYL-4, and B2B) had the ACC deaminase (*acdS*) encoding gene, with an amplicon size of 1,017 bp (Figure 2b). Six isolates (SH-2, SH-3, BYL-2, BYL-4, B2B, and M2) had the phosphatase solubilization (*pqqE*) encoding gene, as evidenced by a 451 bp amplicon size (Figure 2c). Five isolates (SH-2, SH-3, BYL-4, B2B, and M2) had the nitrogenase (*nifH*) encoding gene, with an amplicon size of 323 bp (Figure 2d). Two isolates (BYL-3 and BYL-4) had the antiquorum sensing (*aiiA*) encoding gene (antibacterial), with an amplicon size of 900 bp (Figure 2e). All isolates had the surfactin (*sfp*) encoding gene with an amplicon size of 675 bp (Figure 2f), fengycin (f*enD*) encoding gene with an amplicon size of 269 bp (Figure 2g), bacillomycin D (*bamC*) encoding gene with an amplicon size of 875 bp (Figure 2h), and iturin A (*ituA*) encoding gene with an amplicon size of 647 bp (Figure 2i).

Antagonistic Test of Endophytic *Bacillus* Against *Colletotrichum* sp.

The antagonist test using the dual culture method revealed that 70% of the endophytic *Bacillus* isolates could inhibit *Colletotrichum* grown on the same media. Of the 10 endophytic bacterial isolates tested using the dual culture method against the pathogenic fungus *Colletotrichum* sp., the endophytic bacterial isolate SH-1 exhibited the smallest mycelial diameter of *Colletotrichum* sp. compared with the control (Figure 3), with an

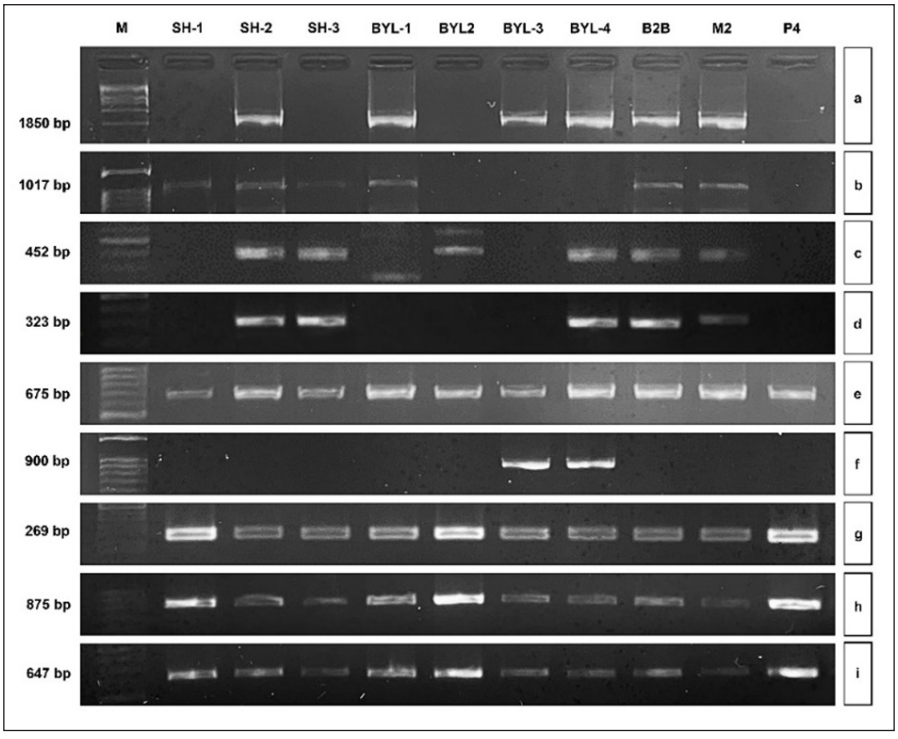

Figure 2. Amplification of plant growth-promoting bacteria (PGPB) coding genes in endophytic *Bacillu*s on a 1% agarose gel. (a) *ipdC*; (b) *acdS*; (c) *pqqE*; (d) *nifH*; (e) *sfp*; (f) *aiiA*; (g) *fenD*; (h) *bamC*; and (i) i*tuA*. The formation of a DNA band indicates a positive result

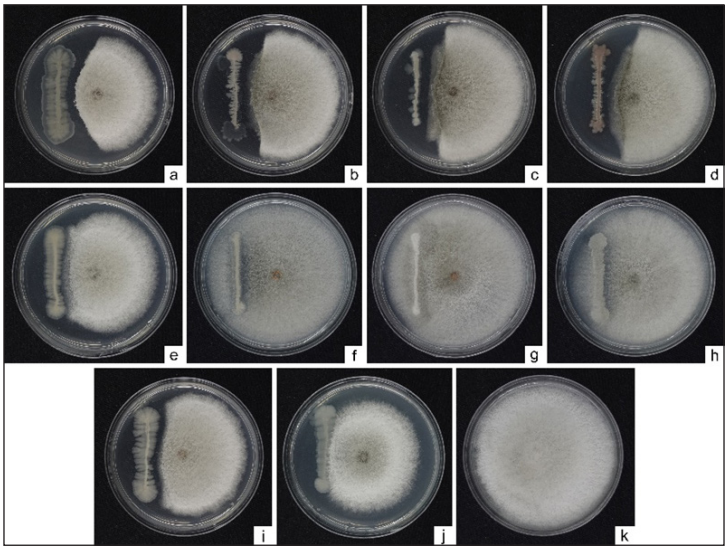

Figure 3. Antagonistic test of endophytic *Bacillus* isolates against *Colletotrichum* sp. on day 7 using the dual culture method. (A) SH-1; (B) SH-2; (C) SH-3; (D) BYL-1; (E) BYL-2; (F) BYL-3; (G) BYL-4; (H) B2B; (I) M2; (J) P4; and (K) Control

inhibitory rate of 64.81%. Seven out of the ten isolates showed significantly different results from the control, namely isolates SH-1, SH-2, SH-3, BYL-1, BYL-2, M2, and P4, while isolates BYL-3, BYL-4, and B2B did not differ significantly from the control (Table 2).

Table 2
Endophytic Bacillus *isolates' ability to inhibit the growth of* Colletotrichum *sp. on day 7 using the dual culture method*

Isolate	Mycelial diameter (cm)	Percentage of Inhibition of Radial Growth (PIRG) (%)
SH-1	5.27	64.81 a
SH-2	5.60	59.93 ab
SH-3	5.40	63.81 a
BYL-1	5.67	54.37 ab
BYL-2	5.73	46.37 b
BYL-3	7.47	5.36 d
BYL-4	7.50	9.43 d
B2B	7.87	3.33 d
M2	5.47	60.96 ab
P4	6.07	28.95 c
Control	8.13	0 d

The antagonistic tests using the coculture method on 10 endophytic *Bacillus* isolates grown on the same media, namely PDA and WA media, showed that all isolates could inhibit the growth of *Colletotrichum* sp., but had different inhibition rates. The endophytic bacterial isolates SH-1, SH-2, SH-3, and BYL-1 had the smallest mycelial diameter of *Colletotrichum* sp. compared to the control (Figure 4), with an inhibitory rate of 100% (Table 3).

Table 3
Endophytic Bacillus *isolates' ability to inhibit the growth of* Colletotrichum *sp. on day 7 using the coculture method*

Isolate	Mycelial diameter (cm)	Percentage of Inhibition of Radial Growth (PIRG) (%)
SH-1	0	100 a
SH-2	0	100 a
SH-3	0	100 a
BYL-1	0	100 a
BYL-2	3.6	55 c
BYL-3	5.42	32.25 d
BYL-4	6.9	13.75 e
B2B	4.48	44 cd
M2	3.74	78.25 c
P4	1.74	53.25 b
Control	8	0 e

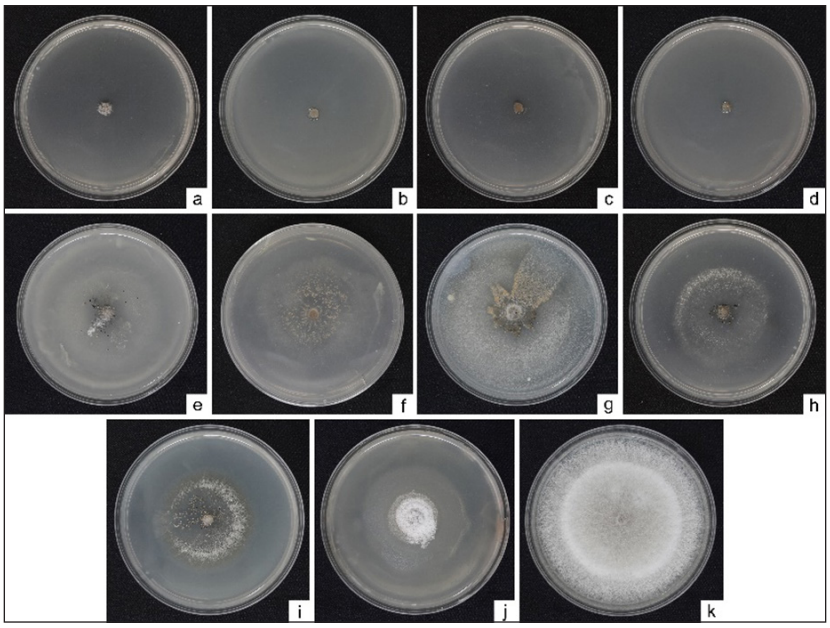

Figure 4. Antagonistic test of endophytic bacterial isolates with *Colletotrichum* sp. on day 7 using the coculture method; (A) SH-1; (B) SH-2; (C) SH-3; (D) BYL-1; (E) BYL-2; (F) BYL-3; (G) BYL-4; (H) B2B; (I) M2; (J) P4; and (K) Control

Effects of Endophytic *Bacillus* on the Growth of Citrus Seedlings

The efficiency of endophytic *Bacillus* in improving the citrus seedlings' growth was assessed in a greenhouse experiment. Citrus seedlings that were treated with endophytic *Bacillus* exhibited better agronomic characteristics compared with the control plants (Figure 5). Treatment with the BYL-3 isolate provided the best results and was significantly different from the control in terms of fresh weight, dry weight, and root weight. All treatments, except for isolate BYL-1, significantly affected the fresh weight. Meanwhile, all treatments, except for isolate BYL-2, did not significantly affect seedling height (Table 4).

Table 4
Effects of endophytic Bacillus *isolate treatment on citrus seedling height, fresh weight, dry weight, root weight, and root volume at 8 weeks after planting (mean ± standard error (SE)*

Isolate	SH (cm)	FW (gram)	DW (gram)	RW (gram)	RV (mL)
BYL-1	6.42 ± 0.92 ab	0.37 ± 0.02 cd	0.12 ± 0.01 c	0.15 ± 0.02 d	0.83 ± 0.17 a
BYL-2	4.21 ± 1.10 b	0.66 ± 0.05 abc	0.13 ± 0.04 c	0.17 ± 0.04 cd	0.63 ± 0.19 a
BYL-3	7.65 ± 0.53 a	0.98 ± 0.07 a	0.27 ± 0.04 a	0.42 ± 0.03 a	1.33 ± 0.33 a
BYL-4	7.45 ± 0. 36 a	0.76 ± 0.06 ab	0.23 ± 0.02 abc	0.30 ± 0.05 abcd	1.33 ± 0.33 a
SH-1	5.80 ± 0,68 ab	0.71 ± 0.03 ab	0.20 ± 0.01 abc	0.32 ± 0.01 abc	1.33 ± 0.33 a
SH-2	5.35 ± 0.59 ab	0.77 ± 0.04 ab	0.23 ± 0,02 abc	0.30 ± 0.02 abcd	1.00 ± 0.00 a

Table 4 *(continue)*

Isolate	SH (cm)	FW (gram)	DW (gram)	RW (gram)	RV (mL)
SH-3	6.85 ± 0.50 ab	0.61 ± 0.04 bc	0.16 ± 0.02 abc	0.26 ± 0.02 bcd	0.83 ± 0.17 a
P4	5.67 ± 0.50 ab	0.69 ± 0.06 abc	0.19 ± 0.02 abc	0.34 ± 0.03 ab	0.83 ± 0.17 a
M2	6.48 ± 0.59 ab	0.83 ± 0.12 ab	0.24 ± 0.03 ab	0.31 ± 0.04 abc	1.33 ± 0.35 a
B2B	6.82 ± 0.71 ab	0.75 ± 0.01 ab	0.18 ± 0.03 abc	0.28 ± 0.03 abcd	1.17 ± 0.44 a
Control	6.07 ± 0.87 ab	0.26 ± 0.11 d	0.13 ± 0.02 bc	0.22 ± 0.03 bcd	0.67 ± 0.17 a

Note. SH: seedling height; FW: fresh weight; DW: dry weight; RW: root weight; RV: root volume. Means followed by the same letters in the same column are not significantly different based on the Tukey test with a 95% confidence level

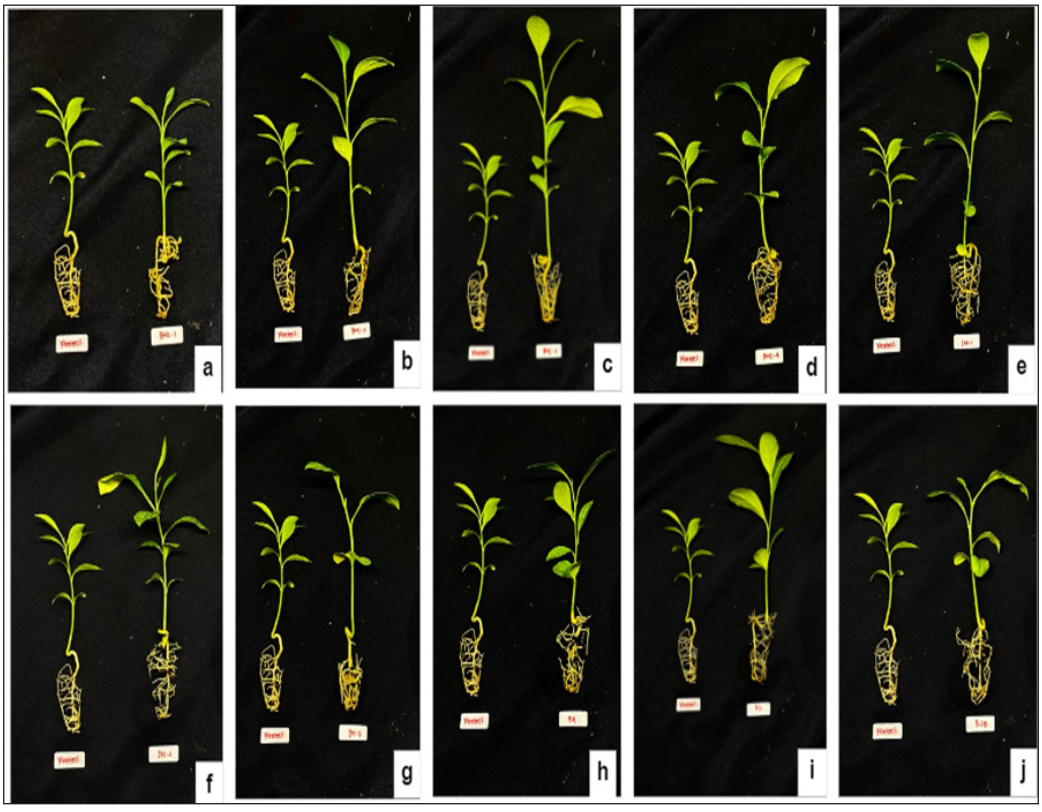

Figure 5. Comparison between citrus seedlings treated with endophytic *Bacillus* isolate and the control. (a) control vs. BYL-1; (b) control vs. BYL-2; (c) control vs. BYL-3; (d) control vs. BYL-4; (e) control vs. SH-1; (f) control vs. SH-2; (g) control vs. SH-3; (h) control vs. P4; (i) control vs. M2; and (j) control vs. B2B

Note. Right side: citrus seedlings with isolate treatment; Left side: control

Identification Based on 16S rRNA Gene Fragments

Molecular identification of the endophytic *Bacillus* isolates was performed using a pair of 16S universal primers. The amplicons of all endophytic bacterial isolates using PCR with a pair of primers 27F/1492R were ±1500 bp in size and observed using a UV transilluminator (Figure 6). Furthermore, Sanger sequencing was performed to determine the nucleotide sequences of the DNA amplicon. Phylogenetic analysis (Figure 7) revealed that isolates SH-1, SH-2, B2B, M2, BYL-1, SH-3, P4, and BYL-2 were most closely related to *B. subtilis* subsp. *subtilis*, whereas isolates BYL-3 and BYL-4 were most closely related to *B. tropicus*.

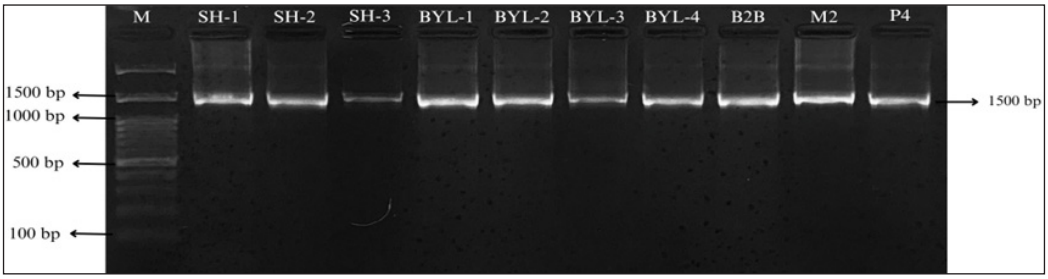

Figure 6. PCR amplification products of the 16S rRNA gene markers from 10 endophytic *Bacillus* isolates were verified on a 1.5% agarose gel
Note. M = 1 kb DNA ladder marker

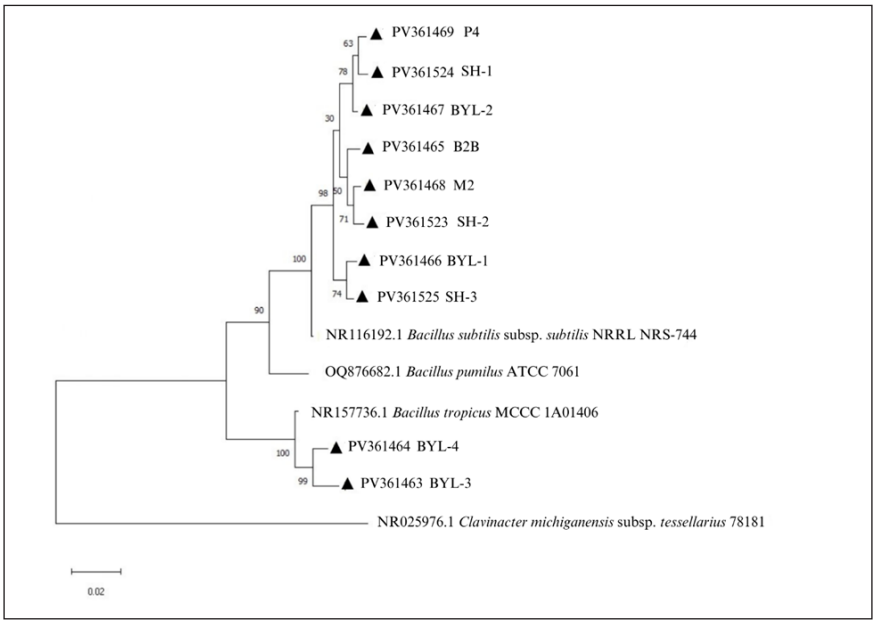

Figure 7. Phylogenetic analysis of 16S rRNA sequences of 10 endophytic *Bacillus* isolates with reference sequences from NCBI using the MEGA Maximum Likelihood method based on the Kimura-2 model with 1,000 bootstrap iterations. *Clavibacter michiganensis* subsp. *tessellarius* 78181 was used as an outgroup

DISCUSSION

This study examined the potential of endophytic *Bacillus* as a beneficial bacterium that can increase plant growth and encode several genes related to antifungal and antibacterial activities. The morphological characteristics of the 10 successfully isolated endophytic bacteria isolates revealed that they had a similar morphology to that of *Bacillus*, namely cream-colored colonies with uneven, non-slimy edges, convex or flat elevations, dry and powdery surfaces, and shiny or non-shiny appearance. This finding is supported by Astuti et al. (2017), who mentioned that *Bacillus* has round colonies with wavy edges, is white in color, and has flat or raised elevations. The collected bacterial isolates did not form mucus when reacted with 3% KOH, indicating that the bacteria were gram-positive. Gram-positive bacteria have a simpler and thicker cell wall structure (approximately 30–100 nm) compared with that of gram-negative bacteria (approximately < 10 nm), which is composed of peptidoglycan (Rohde, 2019).

The systemic induction of plant growth and antibacterial and antifungal genes by beneficial bacteria has the potential to contribute to pathogen control and plant growth enhancement. This study detected genes encoding beneficial traits in endophytic *Bacillus* isolates using PCR. Most endophytic *Bacillus* isolates used in this study demonstrated the ability to synthesize growth-promoting, antibacterial, and antifungal encoding genes. Most isolates were able to encode plant growth-encoding genes, such as indole pyruvate decarboxylase (*ipdC*), ACC deaminase (*acdS*), phosphatase solubilization (*pqqE*), and nitrogenase (*nifH*), and all isolates contained genes that are potentially involved in the synthesis of surfactin, bacillomycin D, iturin A, and fengycin. Indole pyruvate decarboxylase is an enzyme involved in the biosynthesis of indole-3-acetic acid (IAA) from tryptophan via the indole pyruvate (IpyA) pathway, which is then converted into indole 3-acetaldehyde (IAAId) (Shah et al., 2022). Batista et al. (2021) reported the presence of the *ipdC* gene in *B. thuringiensis* RZ2MS9, which encodes indole pyruvate decarboxylase. However, the presence of the *ipdC* gene in other *Bacillus* sp. strains is rare, especially in the *B. amyloliquefaciens* strain, which is known as the best auxin stimulator. ACC deaminase produced by endophytic bacteria in plants protects plants from abiotic stresses, such as salinity, drought, and heavy metal stress (Naing et al., 2021). *B. amyloliquefaciens* and *B. cereus* have been reported as being able to encode the ACC deaminase (*acdS*) gene (Shahid et al., 2023; Tian et al., 2022). Most *Bacillus* species have been reported to be able to dissolve phosphate in the environment, thereby making it available to plants. The phosphorus content in plants is important for the process of cell division and development of new tissues. In general, the availability of phosphate in the soil is abundant, but only 0.1% of the total phosphate is available to plants (Mei et al., 2021). Yahya et al. (2022) reported that *Bacillus* species, such as *B. subtilis*, *B. cereus*, *B. polymyxa*, *B. circulans*, and *B. megaterium*, can dissolve organic phosphate hydrolyzed by acid phosphatase,

alkaline phosphatase, and phytase encoded by the phosphatase gene. *B. cereus* is reported to play a role as a strong phosphate solubilizer and is resistant to soil salinity (Kulkova et al., 2023). In addition, *B. megaterium* MJ1212 plays a role in dissolving phosphate and regulating the plants' carbohydrate and amino acid content to support plant growth (Mohamed et al., 2018). Yao and Xiaomei (2020) also reported that *B. mycoides* Gnyt1 could encode phosphatase solubilization encoding genes, such as *pqqA*, *pqqB*, *pqqC*, and *pqqE*. Nitrogenase is an enzyme that catalyzes nitrogen fixation. Several genes are involved in the nitrogen fixation process, including the *nif* gene, which codes for the primary nitrogenase components (Fernandes et al., 2014). The nitrogenase gene in plant growth-promoting bacteria increases N_2 fixation from the atmosphere, thus making it available to plants. Several species of *Bacillus*, such as *B. cereus*, *B. subtilis*, and *B. licheniformis,* can encode nitrogenase-encoding genes.

Bacillus species can produce three types of antibiotics (Ramarathnam et al., 2007; Ye et al., 2012). Surfactin, bacillomycin, and iturin are the most common lipopeptide antibiotics produced by *Bacillus* species. Several antibiotic genes reported to be produced by *B. subtilis* and *B. amyloliquefaciens* include bacillomycin D (*bamC*), fengycin (*fenD*), iturin (*ituA*), surfactin (*sfp*), and zwittermicin (*zmaR*) (Olanrewaju et al., 2017). Surfactin, iturin, and bacillomycin exhibit high antifungal activity. In addition, surfactin has the highest biosurfactant ability (Arthukorala et al., 2009) and is known to induce the formation of biofilms in bacteria. Meanwhile, the mechanism of fengycin as an antifungal agent against pathogens occurs via the destruction of the plasma membrane, cell wall, hyphae, and fungal conidia. According to Hanif et al. (2019), this can result in reduced virulence, cell death, damage to cell membranes and organs, and the inhibition of pathogen DNA synthesis. Bacillomycin D causes morphological changes in the plasma membrane and cell walls of hyphae and conidia, which ultimately cause pathogenic cell death (Gu et al., 2017). Iturin has limited antibacterial activity but has a wide range of antifungal activity (Ye et al., 2012). In addition to having a high antibiotic activity, iturin can increase swarming motility in bacteria (Joko et al., 2007). This is in agreement with the findings of this study, which showed that several endophytic *Bacillus* isolates demonstrated strong inhibitory effects against *Colletotrichum* sp., with some achieving up to 100% inhibition in the coculture method. Meanwhile, the results of the dual culture method did not reveal better outcomes compared to the coculture method. This is because the dual culture method involves positioning the bacterial isolate and fungal pathogen on opposite sides of the growth medium to observe the inhibition zones. In contrast, the coculture method allows bacterial and fungal cultures to grow together within the same medium, thus enabling their direct interactions. This method closely mimics natural conditions, which facilitates the expression of antagonistic mechanisms such as competition for nutrients and space, production of lytic enzymes, and secretion of antimicrobial compounds (Selegato & Castro-Gamboa, 2023).

In addition, the antiquorum sensing gene (*aiiA*) in some isolates exhibited inhibitory activity against quorum-sensing formation in pathogenic bacteria. In general, the *aiiA* gene can deactivate N-acyl-homoserine lactones (AHLs) or quorum sensing from gram-negative bacteria. Disruption of the pathogen quorum-sensing system with quorum quenching is an effective control strategy, where there are three enzymatic mechanisms of quorum quenching in inhibiting the formation of quorum sensing in pathogens, namely AHL lactonase, AHL acylase, and AHL oxidase and reductase. Among these mechanisms, AHL lactonase encoded by the *aiiA* gene is widely found in *Bacillus* (Rafaat et al., 2019).

Bacillus is known to be able to improve plant growth, especially during the seedling phase. This occurs because *Bacillus* produces phytohormones, such as IAA, cytokinins, gibberellins, ethylene, and abscisic acid. In general, IAA is an active form of the auxin hormone found in plants, where the hormone plays a role in improving the plant quality and yield. In addition, IAA functions in increasing cell development, stimulating new roots, accelerating flowering, increasing enzyme activity, and even being able to stimulate gene expression signals of several antagonistic bacteria with plants; thus, the direct application of *Bacillus* can increase the fresh weight of plants (Ilmiah et al., 2021; Oleńska et al., 2020). However, in this study, not all endophytic *Bacillus* isolate treatments significantly affected plant growth compared with the control treatment. This depends on the plant's response to the hormones that *Bacillus* can produce, where the plant's response to these hormones usually does not depend on the absolute quantity of hormones but rather on their relative concentration compared to that of other hormones. This influences the hormonal effects that *Bacillus* produces, meaning that even if the application of *Bacillus* concentration is increased to a certain point, the hormonal effects may not be significant. This is supported by the research of Giassi et al. (2016), who reported that the application of endophytic *Bacillus* can increase the height, fresh weight, dry weight, and root weight of citrus plants because endophytic *Bacillus* can produce IAA and fix nitrogen. However, this ability depends on the concentration of bacteria during the application. In general, low application concentrations can stimulate root growth, whereas applying bacteria at high concentrations will inhibit plant growth. In addition, another report from Giassi et al. (2016) mentioned that the application of *Bacillus* to citrus seedlings did not affect the development of the stem diameter but could increase the root weight.

In this study, the application of *Bacillus tropicus* BYL-3 to citrus plants was associated with an increase in the height, fresh weight, dry weight, and root weight of the citrus seedlings under controlled conditions. Meng et al. (2016) reported that *Bacillus* increases plant growth via root colonization. *B. velezensis* has been reported to be able to produce IAA, and *B. megaterium* produces cytokinin hormones during the root colonization process (Dunlap et al., 2015). In this process, *Bacillus* plays a role in increasing plant growth directly through the secretion of cytokinins and other volatile compounds, which later

affect the plant hormone tissues (Tsotetsi et al., 2022). Although this experiment was only conducted *in vitro*, the effect of *Bacillus* on plants supports the idea that citrus plants can develop disease resistance through the application of endophytic *Bacillus*. Marisna et al. (2024) reported the potential of *B. cereus* RC76 and *B. velezensis* B-27 in suppressing the feeding behavior of *Diaphorina citri*, the vector of *Candidatus* Liberibacter asiaticus. The study found that *D. citri* feeding on citrus seedlings treated with *B. cereus* RC76 exhibited a prolonged total duration of Np and C waveform activity, which indicates a possible disruption in feeding efficiency. In addition, citrus seedlings treated with *B. velezensis* B-27 revealed an extended total duration of the Np waveform in *D. citri* compared with the control, with the longest total duration observed for waveform G. These findings suggest that the application of *Bacillus* can interfere with *D. citri* feeding behavior, thereby potentially reducing pathogen transmission.

The molecular identification of the endophytic *Bacillus* isolates was conducted based on 16S rRNA gene analysis. The phylogenetic analysis results revealed that the isolates SH-1 (PV361524), SH-2 (PV361523), SH-3 (PV361525), BYL-1 (PV361466), BYL-2 (PV361467), B2B (PV361465), M2 (PV361468), and P4 (PV361469) were closely related to *B. subtilis* subsp. *subtilis*. Meanwhile, isolates BYL-3 (PV361463) and BYL-4 (PV361464) were closely related to *B. tropicus*. The type strain of *B. subtilis* subsp. *subtilis* NRL NRS-744 has a similarity with isolate SH-1 of 96.315%, SH-2 of 96.897%, SH-3 of 93.572%, BYL-1 of 96.983%, BYL-2 of 96.034%, B2B of 97.672%, M2 of 97.432%, and P4 of 96.466%. Meanwhile, the *B. tropicus* MCCC 1A01406 type strain has a similarity with isolate BYL-3 of 93.309% and BYL-4 of 94.989%.

CONCLUSION

Ten endophytic *Bacillus* isolates were isolated from citrus plants, with four of the isolates having the best ability as PGPB, namely isolates BYL-3, SH-1, SH-2, and SH-3. The detection of PBT genes showed that isolate BYL-3 encodes the *ipdC*, *nifH*, *aiiA*, *Sfp*, *ituA*, *bamC*, and *fenD* genes. Meanwhile, isolates SH-1, SH-2, and SH-3 encode the *acdS*, *sfp*, *ituA*, *bamC*, and *fenD* genes. Isolate BYL-3, which is closely related to *B. tropicus*, can significantly improve the growth of citrus seedlings, whereas isolates SH-1, SH-2, and SH-3, which are closely associated with *B. subtilis* subsp. *subtilis*, significantly suppresses the mycelium growth of *Colletotrichum* sp.

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