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Phyllospheric bacteria isolated from broccoli (*Brassica oleracea* L. var. italica) as biocontrol agents against *Ralstonia solanacearum* and as plant growth promoting bacteria

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Abstract

Importance of the work: Phyllospheric bacteria play a crucial role in shaping plant microenvironments and offer sustainable alternatives to synthetic fertilizers and pesticides. The increasing environmental concerns associated with agrochemicals highlight the need to explore plant growth-promoting bacteria (PGPB) for enhancing crop productivity and disease resistance. One major phytopathogen, *Ralstonia solanacearum*, causes bacterial wilt and greatly impacts a wide range of crops.

Objectives: 1) To characterize bacterial isolates from the broccoli phyllosphere; 2) to evaluate their antagonistic activity against *R. solanacearum*; 3) to assess their plant growth-promoting traits; and 4) to identify the most promising isolate using *16S rRNA* gene analysis.

Materials and Methods: Bacterial isolates were obtained from broccoli phyllosphere samples and screened for antagonistic activity against *R. solanacearum* using the disc diffusion method. Plant growth-promoting traits were evaluated based on nitrogen fixation, phosphate solubilization and indole-3-acetic acid (IAA) production assays. Molecular identification of the selected isolate was performed using *16S rRNA* sequencing.

Results: In total, 10 bacterial isolates were obtained, among which isolate FB5 had the highest antagonistic activity, with an inhibition zone diameter of 16.37 mm. FB5 tested negative for nitrogen fixation but was positive for phosphate solubilization and IAA production. Molecular identification revealed 91.43% similarity to *Bacillus velezensis*, indicating that the isolate belonged to the genus *Bacillus*.

Main finding: *Bacillus* sp. FB5 had strong antagonistic activity against *R. solanacearum* and displayed key plant growth-promoting traits, highlighting its potential as a biocontrol agent and biofertilizer for Solanaceae crops such as potato, tomato and chili.

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Introduction

After the soil, the phyllosphere is the second most densely populated habitat of microorganisms on Earth. With an estimated leaf surface area exceeding 6.4 x 10^8 m^2 for terrestrial plants (Lindow and Brandl, 2003), it encompasses a diverse community of microorganisms, including bacteria, viruses, fungi, algae, archaea and occasionally protozoa and nematodes. These organisms engage in symbiotic relationships with plants, predominantly residing on the leaves, stalks, buds and flowers, which are the aerial components of plants (Vorholt, 2012). For epiphytic microorganisms residing on plant surfaces, the phyllosphere represents an extreme and unstable habitat, with oligotrophic characteristics, such as limited carbon and/or nitrogen sources, together with the presence of several physicochemical barriers (light, ultraviolet radiation, temperature, desiccation) that fluctuate greatly (Bringel and Couée, 2015).

Plant growth-promoting bacteria (PGPB) can inhibit various plant pathogens in both the rhizosphere and above-ground areas by generating metabolites that counteract pathogens and enhance the natural immunity of a plant to pathogenic stresses (Berendsen et al., 2018). PGPB can trigger plant growth through various means, including soil improvement, synthesis of compounds that enhance growth, inhibition of pathogens and the fixation of nitrogen and phosphorus, as well as the mobilization of other nutrients, while additionally, they induce disease resistance and enhance stress tolerance in host plants (Rahman et al., 2018). The commercially available PGPB strains are*: Agrobacterium radiobacter*, *Paenobacillus macerans*, *Azospirillum brasilense*, *A. lipoferum*, *Bacillus subtilis* var. amyloliquefaciens, *Azotobacter chroococcum*, *Serratia entomophilia*, *Bacillus* spp., *B. fimus*, *B. licheniformis*, *B. megaterium*, *Streptomyces* spp, *S. lydicus*, *S. griseoviridis*, *B. mucilaginous*, *B. pumilus*, *Pantoea agglomerans*, *Pseudomonas* spp, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. solanacearum*, *P. syringae* and various *Rhizobia* spp.; however, only a small number of them are applied currently in agriculture (Glick, 2012).

Ralstonia solanacearum (Burkholderiaceae) is considered one of the most detrimental plant pathogens on a global scale due to its classification as a species that comprises a large group of strains with a wide range of geographic origins, host ranges and pathogenic behaviors. Specifically, the different species, as a whole, span many hosts, infecting up to 200 plant species from more than 50 families, causing brown rot in potatoes, bacterial wilt in tomatoes, tobacco and eggplant and Moko disease in bananas (Mansfield et al., 2012). Typically, *R. solanacearum* enters the host's roots through pre-existing wounds, which can occur either during the development of lateral roots or as the roots extend and grow within the soil; subsequently, the bacteria travel toward the vascular tissue, particularly the xylem, where they undergo multiplication before spreading further into the host's shoot (Caldwell et al., 2017). *R. solanacearum* will secrete exopolysaccharides that block the xylem tissue, causing wilting and eventually death (Caldwell et al., 2017).

Biological control using antagonistic microbes is emerging as an effective strategy in controlling soil-borne diseases, especially during post-harvest. In addition, these beneficial microbial agents increase crop yields without harming plants and the environment (Adnan et al., 2019). Biocontrol does not cause pollution, does not produce residues and is less likely to induce resistance (Hernández et al., 2018). Many of the microorganisms used as biocontrol agents are isolated from plants or other environments to enhance plant growth or suppress plant pathogens, or both (Booth et al., 2022). Concern over the use of synthetic fertilizers and pesticides has led to the exploration of PGPB as an excellent alternative to increase plant productivity in terms of growth and nutritional adequacy, as well as plant protection by suppressing disease-causing microbes (Majeed et al., 2018). Therefore, there is a need to investigate biofertilizer and biopesticide alternatives based on phyllospheric PGPB.

The current study aimed to characterize the macroscopic and microscopic morphologies of phyllospheric bacteria in broccoli and to screen the best bacterial isolate as a biocontrol agent against *R. solanacearum*, as well as its PGPB traits such as nitrogen fixation, indole-3-acetic acid (IAA) production and phosphate solubilization. Then, the potential strain was identified using *16S rRNA* barcoding.

Materials and Methods

Sampling

Broccoli *(Brassica oleracea* L. var. italica) leaves were taken from the Merbabu Organic Vegetables Farm in Kopeng Village, Semarang Regency, Central Java, Indonesia, in 2022. Fresh leaf samples were sampled randomly from three broccoli plants that had been selected carefully based on having no physical injuries or symptoms of malnutrition or disease.

The sampled leaves were placed immediately in a sterile bag in an ice box at 4°C and transported within 3 hr for further use in the laboratory.

Bacterial isolation

Bacterial isolation was carried out according to Wiraswati et al. (2019), with slight modifications. The broccoli leaves were washed with sterile distilled water to clean the leaf surfaces of debris. In total, 10 g of small leaf pieces were cut from the three broccoli plants. The leaf pieces were dissolved in 90 mL saline buffer solution (0.9% NaCl) and shaken at 150 rpm at 28°C for 1 hr using a shaking incubator (Labnet 311DS Shaking Incubator; USA) to obtain a homogeneous bacterial suspension. Serial dilutions were prepared by adding 1 mL of the homogenous suspension into 9 mL of the saline buffer in a test tube repeatedly until the 1×10^{-6} dilution level was reached. Then, each of the diluted cultures was added to solid nutrient agar (NA) (HiMedia Laboratories; India) in Petri dishes using the spread plate method and incubated at 28°C for 72 hr. Next, each phenotypically distinct colony was purified and grown on solid NA in separate Petri dishes under the same conditions and given a code starting with FB, followed by consecutive numbers depending on the total isolates.

Phenotypic characterization

The macroscopic characteristics of colony morphology, including margin, elevation, surface, texture and color based on Leboffe and Pierce (2006) were observed and documented. Microscopic cell morphology was characterized by observing bacteria after Gram's staining under a light microscope (Zeiss Axio Lab. A1; Germany) to determine the cell shape and type of bacteria.

Antagonistic activity assay

The antagonistic test was carried out using the paper disc diffusion assay. Each bacterial isolate and pathogenic bacteria *(R. solanacearum*) were added into nutrient broth (NB; HiMedia Laboratories; India) medium and incubated and shaken at 28°C and 150 revolutions per minute (rpm) for 24 hr. Then, the suspension of pathogenic bacteria was compared for turbidity according to the McFarland 0.5 standard solution (1.5 x 10^8/colony-forming units), applied onto the solid medium using a sterile cotton swab and allowed to dry. Paper discs were embedded on the surface of the agar medium for the diffusion of isolate suspensions, antibiotic and sterile distilled water, with 10 µL of each test bacterial suspension, antibiotic and distilled water being pipetted and dripped onto the paper discs. The antagonistic activity assay consisted of a positive control (ampicillin antibiotic at 400 parts per million, ppm) and a negative control (sterile distilled water) to compare the test results. All samples were incubated at 28°C for 48 hr prior to measuring the inhibition zone. According to Barry et al. (1979), the diameter of the inhibition zone was determined using a ruler with measurements to the nearest millimeter, including the diameter of the disc paper (6 mm) and if there were no inhibition, the zone was 6 mm. The endpoint of the zone measurement was taken at the boundary with the area not showing obvious growth that could be seen by the naked eye (without aids). Based on Greenwood's (1995) classification, a diameter of the inhibition zone exceeding 20 mm was considered a very strong inhibition of bacterial growth, in the range of 10–20 mm was strong, 5–10 mm was moderate and under 5 mm was weak. The bacterial isolate with the highest antagonistic activity was tested further for its PGPB traits. The assay was duplicated.

Nitrogen fixation assay

The qualitative assay of nitrogen fixation ability of bacterial isolate was assessed using nitrogen-free bromothymol blue (NFB) semi-solid medium, containing 2% glucose and bromothymol blue, following the method described by Baldani et al. (2014). The surface of the medium was implanted with the bacterial isolate using the spot inoculation method. Then, the bacterial culture was incubated at 28°C for 10 d and observed for any changes. A positive result of nitrogen fixation activity was indicated by a change in the color of the NFB medium from green to blue. The nitrogen fixation assay was duplicated.

Indole-3-acetic acid production assay

The bacterial isolate was inoculated on NB medium supplemented with 0.1% L-tryptophan as a precursor for IAA production (Gang et al., 2019). The bacterial isolate was incubated at 28°C and shaken at 200 rpm for 48 hr. After incubation, the bacterial culture was harvested based on centrifugation (LaboGene ScanSpeed 416 Low-Speed Centrifuge; South Korea)at 25°C and 3,000 rpm for 20 min. Then, 1 mL of the resulting supernatant was mixed with

2 mL of Salkowski reagent and stored in the dark at room temperature for 20 min. A positive result was indicated by a change in the color of the medium from clear to pink or red. The IAA production assay was duplicated.

Phosphate solubilization assay

The phosphate solubilization assay was conducted using Pikovskaya's agar medium, as described by Miladiarsi et al. (2017). The bacterial isolate was dripped onto the center of the growth medium and subsequently incubated for 7 d at 28°C. The potential of a bacterial isolate to solubilize phosphate was determined by the formation of a clear zone around the tested bacterial colonies after incubation. The phosphate solubilization assay was duplicated.

Molecular identification based on 16S rRNA

The DNA extraction was performed following the InstaGene Protocol using an InstaGene Matrix Kit (Bio-Rad; USA) for bacterial DNA extraction (Gray et al., 2014), with slight modifications. The DNA concentration was checked using a nanodrop spectrophotometer (Thermo Fisher Scientific Nanodrop 2000 Spectrophotometer; USA). The DNA was amplified using a polymerase chain reaction (PCR) thermocycler (Labnet MultiGene OptiMax Thermal Cycler; USA) with the universal primers 27F (forward) and 1492R (reverse), according to Oo et al. (2020). The PCR master mix of 50 µL consisted of 25 µL MyTaq PCR Kit (Bioline; USA), 2 µL forward primer, 2 µL reverse primer, 19 µL double- distilled water and 2 µL sample Then, the PCR result was checked using gel electrophoresis (Mupid-EXu Electrophoresis; Japan). A 1% agarose gel was diluted in 1X TAE Buffer, stained with FloroSafe Stain (1st BASE; Singapore), placed in the gel electrophoresis chamber and run at 100 V for 30 min. The size of the band was compared with a DNA 1 kb (kilobase) ladder (Promega; USA) and was visualized using the Gel Documentation tool (Uvitec Uvidoc Hd2; UK). The PCR product was sequenced at PT Genetika Science Indonesia. The sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov) to obtain results from similarity checking of the sequences. The sequences were aligned and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis 11 (MEGA11; https://www.megasoftware.net, The MEGA Software Development Team) software. The phylogenetic tree was constructed as a neighbor-joining tree using Tamura-Nei model parameters with 1,000 times bootstrap replication. Later, the *16S rRNA* gene sequence of the isolate was submitted to the NCBI gene database (accession number PQ774858).

Data analysis

Data from the study were analyzed descriptively using tables and figures for bacterial isolation, characterization, antagonistic activity and plant growth promotion traits. Molecular identification data were analyzed with the relevant software and database described in the methods above.

Results

Isolation and characterization of phyllospheric bacteria

The isolation of phyllospheric bacteria from broccoli leaves resulted in different phenotypes, as depicted in Fig. 1 that were subsequently purified to obtain 10 isolates, coded as FB1–FB10. The Gram staining procedure revealed various morphologies of the bacteria (Table 1). Out of these, five bacteria were Gram-positive, while the remaining were identified as Gram-negative. Additionally, six bacterial isolates had a rod cell shape, one had an ovoid coccus shape and the three others were cocci-shaped (Table 2).

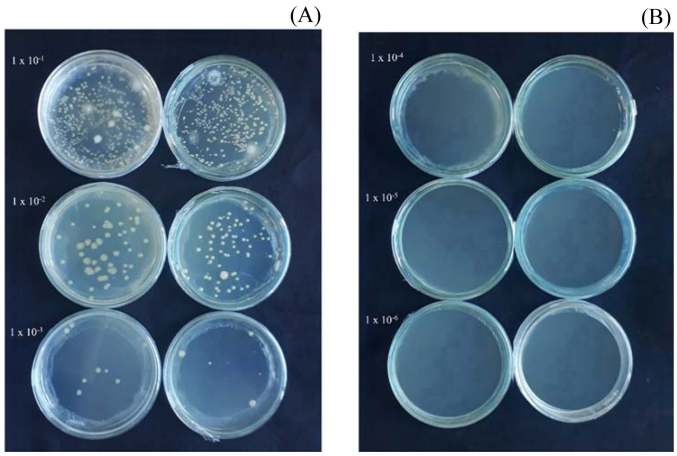

Fig. 1 Isolation results at 72 hr of phyllospheric bacteria from broccoli leaves after serial dilutions in duplicate tests: (A) ($1 \times 10^{-1} - 1 \times 10^{-3}$); (B) ($1 \times 10^{-4} - 1 \times 10^{-6}$).

Table 1 Macroscopic characterization of isolated bacteria

Isolate code	Whole colony	Margin	Elevation	Surface	Texture	Color
FB1	Irregular	Irregular	Flat	Dull	Moist	White
FB2	Round	Smooth	Convex	Glistening	Moist	Whitish yellow
FB3	Round	Lobate	Raised	Wrinkled	Moist	Yellowish white
FB4	Round	Smooth	Convex	Glistening	Moist	Orange red
FB5	Round	Irregular	Flat	Wrinkled	Dry	Pale white
FB6	Round	Smooth	Convex	Glistening	Moist	Yellow
FB7	Round	Smooth	Convex	Glistening	Moist	Light yellow
FB8	Round	Smooth	Convex	Glistening	Moist	Light orange
FB9	Irregular	Irregular	Raised	Glistening	Moist	White
FB10	Round	Smooth	Raised	Wrinkled	Dry	Yellowish white

Table 2 Microscopic characterization of isolated bacteria

Isolate code	Gram stain result	Color	Cell shape
FB1	+	Purple	Rod
FB2	-	Red	Rod
FB3	-	Red	Rod
FB4	+	Purple	Rod
FB5	+	Purple	Rod
FB6	-	Red	Cocci ovoid
FB7	+	Purple	Cocci
FB8	-	Red	Cocci
FB9	+	Purple	Cocci
FB10	-	Red	Rod

Antagonistic activity against Ralstonia solanacearum

The isolated bacteria were tested for their antagonistic potential against the pathogen *R. solanacearum* based on producing a clear zone of inhibition, with the results shown in Fig. 2. The positive control used was ampicillin (at 400 ppm), with distilled water as the negative control. Based on the results of the 10 phyllospheric bacteria, isolate FB5 showed strong potential against *R. solanacearum*, with an average inhibition zone of 16.37 mm.

Fig. 2 Antagonistic activity results of FB5 testing (duplicates) against *Ralstonia solanacearum*.

Plant growth-promoting traits of isolate FB5

The outcomes of the PGPB qualitative assay suggested that FB5 had the capacity to generate IAA and solubilize the phosphate; however, there was no nitrogen fixation activity. The nitrogen fixation ability test of FB5 produced negative results on semi-solid NFB medium, where the medium remained unchanged (no shift in color from green to blue), as shown in Fig. 3B in the duplicates and was not different from its uninoculated medium (Fig. 3A). The results for the IAA production test from FB5 showed a positive result, as shown in Figs. 4B and 4C, marked by a change in color to red after the addition of the Salkowski reagent, while the negative control showed no color change, as shown in Fig. 4A. The phosphate solubilization test produced a clear zone around the FB5 isolate on Pikovskaya's medium after 7 d (Figs. 5A and 5B).

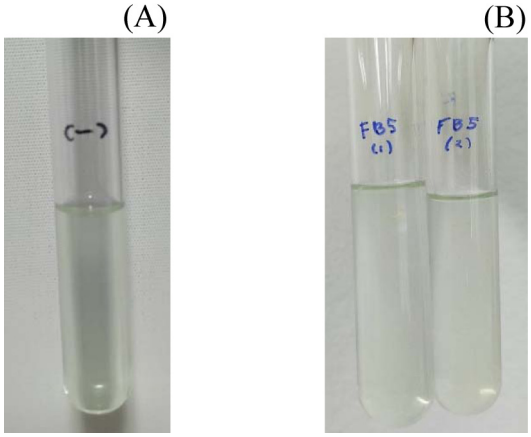

Fig. 3 FB5 nitrogen fixation test results in nitrogen-free bromothymol blue medium for: (A) uninoculated medium; (B) inoculated medium (in duplicate).

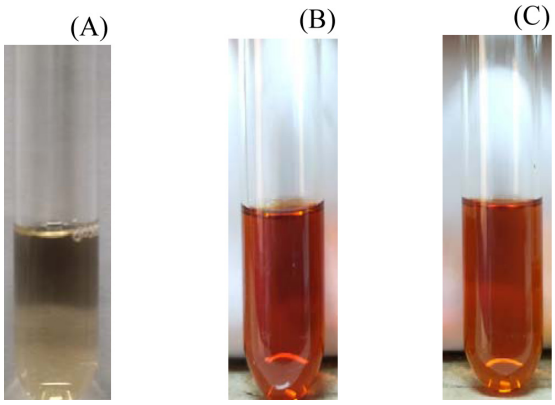

Fig. 4 FB5 indole-3-acetic acid production test results: (A) uninoculated medium; (B and C) inoculated medium in duplicate tests.

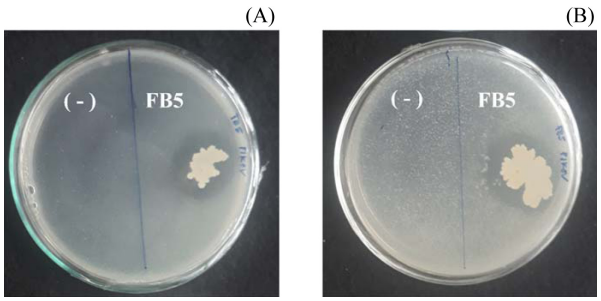

Fig. 5 FB5 phosphate solubilization test results in Pikovskaya's agar medium: (A and B) duplicate tests.

Molecular identification of isolate FB5

FB5 was sequenced and analyzed, showing the highest similarity percentage to a bacterial strain from the genus *Bacillus*. The phylogenetic tree is shown in Fig. 6, where the separation of FB5 and *B. velezensis* FI383 showed a very strong confidence level with a bootstrap value of 100.

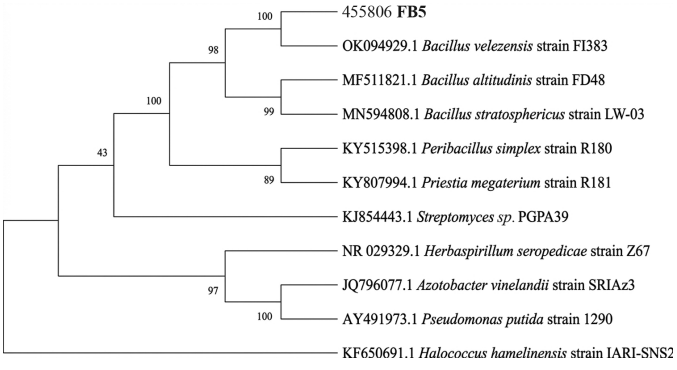

Fig. 6 Phylogenetic tree construction using MEGA11 software and based on *16S rRNA* gene using neighbor-joining method with 1,000 bootstrap replicates, showing relationship of FB5 isolate (in bold) with other plant growth-promoting microbes.

Discussion

The number of bacterial isolates from broccoli leaves is strongly influenced by environmental factors, since bacteria need to adapt for their survival on leaf surfaces. According to Aragón et al. (2017), bacteria represent the predominant microorganisms that inhabit the phyllosphere in large numbers. Bacteria need to cope with the hydrophobic conditions of leaves and the limited availability of nutrients, including multiple carbon sources and various volatile organic compounds, as well as the metabolism of the host plant. Consequently, only bacteria that are well adapted to the phyllosphere's specific conditions can thrive and survive in this environment. The bacterial screenings from the phyllosphere of plants vary in number. For example, Ali et al. (2021) reported 10 bacteria from the phyllosphere of *Avena fatua*, while Sharath et al. (2021) reported 31 isolates of cotton phyllospheric bacteria.

According to Legein et al. (2020), phyllospheric microbes have diverse adaptation and biocontrol mechanisms, enabling them to thrive under in the phyllosphere environment. For example, Mazinani et al. (2017) reported that the phyllospheric bacterium, *B. amyloliquefaciens*, had antimicrobial activities against various bacterial and fungal pathogens. Although *R. solanacearum* is not a pathogen of broccoli, it has been studied intensively, because, according to Cai et al. (2021), it is one of the most destructive pathogens in several important crops. In the current study, FB5 was classified as a strong inhibitor with an inhibition zone of 16.37 mm against *R. solanacearum*. Hossain et al. (2021) reported that *B. subtilis* inhibited *R. solanacearum* (17 ± 0.9 mm diameter zone of inhibition (DZI)), while one of the methanolic extracts from the medicinal plant *Cymbopogon citratus*, had DZI of 20 ± 0.06 mm. Furthermore, that same study showed that ampicillin at 10 µg/disc had the highest antibacterial activity among several antibiotics with a DZI of 24.66 ± 0.09 mm. In another study by Fauzaan et al. (2023), *B. subtilis* isolated from broccoli's rhizosphere had a DZI of 6 mm. Cao et al. (2018) reported that the two PGPB strains *B. velezensis* Y6 and Y7 showed strong levels of antagonistic activity against *R. solanacearum* and *Fusarium oxysporum* and they identified three lipopeptide compounds (surfactin, iturin, fengycin) that were responsible for the antimicrobial activity of these strains against pathogens. Based on the current findings, FB5 is a potential antagonist and an antimicrobial producer against *R. solanacearum*; however, the inhibition mechanism of the pathogen in plants needs to be further explored.

Based on the current results, FB5 produced a positive result for IAA production, which according to Spaepen et al. (2007), although IAA in bacterial cells does not function the same as a plant hormone, the ability to produce the substance is important in the plant-microbe relationship. Phytohormone production is an example of increasing plant growth through a direct mechanism based on PGPB. The study by Park et al. (2021) demonstrated that the bacterial culture supernatant used increased the root mass by twofold compared to the control, with root number and length values being 46% and 18% higher than for the control. Based on the research of Nutaratat et al. (2017), the presence of L-tryptophan was very important in the production of IAA by the rice phyllospheric bacterium *Enterobacter* sp. DMKU-RP206. Furthermore, this was supported by the research of Bharucha et al. (2013), who demonstrated that *P. putida* UB1 had PGPB ability by increasing the shoot length, root length and the number of adventitious roots on mustard *(Brassica nigra*).

Zimmerman et al. (2014) explained that phosphorus (P) plays a critical role in the storage and transfer of biological information, energy metabolism and membrane integrity. Often, a P limitation in bacteria results in ribosome deficiency and slow growth. For example, Zhu et al. (2011) explained that using a P-solubilizing microorganism (PSM) can be used to increase the availability of soil-insoluble P for plants. In the current study, the positive result of the phosphate solubilization test of FB5 was consistent with other reports of clear zone formation in Pikovskaya's solid medium by PSMs (Miladiarsi et al., 2017; Thapa et al., 2017; Sharath et al., 2021; Widawati et al., 2022). Furthermore, application of phosphate-solubilizing bacteria can be beneficial, especially in P-deficient soils, as reported in the study of Pereira and Castro (2014).

B. velezensis has been reported to promote plant growth, as well as acting as a biocontrol. For example, Yin et al. (2022) showed that *B. velezensis* produced IAA and siderophore, which solubilized phosphate, as well as inhibiting the mycelial growth and spore germination of *Coniella vitis*. Based on the research of Choi et al., (2023), the biocontrol ability of *B. velezensis* CE100 resulted in degrading the cuticle of *Dasineura jujubifolia* larvae, as well as having PGPB ability in phosphate solubilization and IAA production, resulting in increased root development. In addition, Sui et al. (2022) reported that *B. velezenzis* inhibited the growth and colonization of *R. solanacearum* in tobacco roots. Additionally, its role as a biocontrol agent was supported by the research of Chen et al. (2020), reporting on the synthesis by *B. velezensis* FJAT-46737 of antimicrobial compounds with a broad spectrum against bacteria and fungi. Its crude lipopetides (iturins, fengycins, surfactins) had considerable antagonistic activity, particularly fengycin, and were the major antibacterial components of the lipopetides against *R. solanacearum in vitro*. However, the results of the current study were primarily qualitative with limited scope and replication. Nonetheless, the current study might provide insights into how *Bacillus* sp. FB5 could be further explored based on *in vitro* assays or plant field tests to gain more understanding on this isolate as well as its ability as a PGPB.

Conclusion

In total, 10 bacteria were isolated successfully from the broccoli phyllosphere and showed FB5 isolate as a promising candidate for controlling *R. solanacearum*. In addition, FB5 could solubilize phosphate and produce IAA, although it did not produce any nitrogen-fixing activity. Nevertheless, the findings still suggest that FB5 could be a potential isolate for further studies in promoting plant growth and disease suppression. Based on the *16S rRNA* analysis, FB5 was identified as *Bacillus* sp. Although the study was preliminary and has its limitations, the current results should provide valuable insights into *Bacillus* sp. FB5 for further studies on its plant growth-promoting ability, including quantitative assay and field applications with crops.

Conflict of Interest

The authors declare that there are no conflicts of interest

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