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Exploring the *Bacillus* from vegetable rhizosphere for plant growth

Rara Rahmatika Risanti[1], Reginawanti Hindersah[2*], Betty Natalie Fitriatin[2], Pujawati Suryatmana[2], Iman Permana Maksum[3], Mieke Rochimi Setiawati[2], Fasa Aditya Hanindipto[4], Gita Bina Nugraha[4]

[1] Student Doctoral Program, Faculty of Agriculture, Universitas Padjadjaran, Sumedang 45363, Indonesia
[2] Department of Soil Science, Faculty of Agriculture, Universitas Padjadjaran, Sumedang 45363, Indonesia
[3] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang 45363, Indonesia
[4] Indonesian Fertilizer Research Institute, PT. Pupuk Indonesia, Jl. Taman Anggrek No. 2, Kemanggisan Jakarta Barat 11480, Indonesia
* Corresponding author's e-mail: reginawanti@unpad.ac.id

ABSTRACT

Bacillus is a ubiquitous soil bacterium for its plant growth-promoting properties, and it is widely used as a biofertilizer. This research aimed to isolate and determine the morphology and biochemical properties associated with plant-growth promotion ability characterizing *Bacillus* strains. The bacteria were isolated from the rhizosphere of various highland vegetables grown in Andisols soil in Indonesia. *Bacillus* isolation was conducted using the serial dilution plate method by means of tryptic soy agar. Characterization of the isolated bacteria included Gram staining, biochemical characteristics, pathogenicity testing, and the production of metabolites, including organic acids, phytohormones, and exopolysaccharides. Four bacterial isolates were identified as *Bacillus* based on colony and cell morphology, the presence of endospore, as well as biochemical properties and metabolite production. The species determination by PCR amplification and 16S rRNA gene sequence analysis revealed that the four *Bacillus* were identified as *Bacillus safensis* strain MDL5, *Bacillus altitudinis* strain RPW2, and *Bacillus* sp. strain SZ057. The study presents the occurrence of *Bacillus* within the rhizosphere of vegetables and the understanding of their properties being considered for biofertilizer development.

Keywords: biochemical properties, *Bacillus*, exopolysaccharides, organic acids, phosphatase, phytohormones, plant growth-promoting rhizobacteria.

INTRODUCTION

Numerous dietary guidelines for maintaining human health and preventing disease have emphasized eating a diet high in fresh vegetables and low in simple carbohydrates, sodium, and saturated fats. As a result, the demand for fresh vegetables has significantly increased, driving up their global production. Nowadays, global vegetable production mainly depends on chemical fertilizers to provide plant nutrients, particularly in low-fertility soil. However, long-term and excessive use of chemical fertilizers has been shown to deplete soil (Qaswar et al., 2020), raising concerns regarding sustainable agriculture techniques. In response to these concerns, biofertilizer applications are gaining popularity as a viable alternative to reduce the use of chemical fertilizers. Biofertilizers, which contain beneficial bacteria, improve soil fertility and encourage sustainable farming by including beneficial bacteria that support plant development and health.

On the basis of its beneficial features, *Bacillus* is widely utilized as a biofertilizer for various food crops. Species of the genus *Bacillus* are well-known beneficial soil bacteria present in relatively high populations in soil (Liu et al., 2022). *Bacillus* are widely distributed in soil and

have been identified for their ability to produce endospores, which allow them to survive under stressful conditions. They are essential in nutrient cycling and are used in various industrial applications (Sulistiyani et al., 2021). The benefits of *Bacillus* are improving soil quality, supporting agricultural productivity, and promoting soil health (Radhakrishnan and Lee, 2016). Moreover, *Bacillus* can produce phytohormones (gibberellin, zeatin, and kinetin), improve nutrient availability, protect against plant diseases, and engage in biochemical activities that promote plant growth (Bandopadhyay, 2020; Masood et al., 2020; Poveda and González-Andrés, 2021). Therefore, *Bacillus* is a crucial bioactive ingredient used in biofertilizer formulation.

Gram-positive *Bacillus* enable to form dormant endospores that preserve material genetics under extreme conditions during its life cycle; it is crucial in the formulation of biofertilizers due to the endospore's resistance to drought stress (Chaudhary et al., 2022). Researchers report that *Bacillus* decomposes organic matter and converts organic compounds in the soil into available nutrients for plants, such as nitrogen and phosphorus (Rawat et al., 2021; Sun et al., 2020). The ability of *bacillus* to fix the nitrogen (N) and to solubilize the unavailable phosphorus (P) are the keys to increasing the N and P availability in soil (Silva et al., 2023). It has been reported elsewhere that *Bacillus* produces phytohormones that are crucial to plant growth (Soni and Keharia, 2021). Recent research demonstrated that *Bacillus* produces exopolysaccharides (EPS) that have a role in improving soil porosity (Bhagat et al., 2021). Therefore, the multifunction of *Bacillus* benefits plant growth and soil quality.

The high microbiological quality of commercial biofertilizer formulations, in terms of viability or microbial count and their plant-promoting ability, is required. An initial step in developing a biofertilizer is isolating the target bacteria from the rhizosphere, the soil region adjacent to plant roots, which hosts a denser microbial community than bulk soil (Tahir et al., 2013). The prolific vegetable fields are the source of the isolating for this study. The isolation and identification of *Bacillus* species from the rhizosphere of vegetable plants are necessary to develop biofertilizers for agriculture and soil research. Higher microbial communities and denser microbial populations inhabit the rhizosphere than bulk soil, including *Bacillus* bacteria (Ling et al., 2022). *Bacillus* inoculation benefits the plants, including nutrient availability and disease protection, their isolation and application in biofertilizers are prominent for vegetable cultivation. Studies demonstrate the efficacy of *Bacillus* in enhancing vegetable growth and disease resistance. Ortega-García et al. (2021) found that inoculating asparagus plants with *B. amyloliquefaciens* significantly increased the fresh weight, root weight, and crown size of plants. Similarly, the application of *B. cereus* and *B. thuringiensis* on chili plants improved pepper growth in seedbeds and pots and protected against the bacterial pathogen *Xanthomonas euvesicatoria* (Hernández-Huerta et al., 2023). Furthermore, inoculation of *B. subtilis* on cabbage plants increased plant height, root number, crop weight, yield per plot, and potential yield per hectare (Suwarto and Hilmi, 2023). These studies underscore the potential of *Bacillus* species as effective biofertilizers that can enhance growth and yield while providing disease resistance in various vegetable crops.

A better understanding of the function of *Bacillus* bacteria in the rhizosphere of vegetables may help design more effective biofertilizers. This research aimed to isolate *Bacillus* from the rhizosphere of vegetables grown in Andisol in Indonesia and determine its plant-growth-related characteristics. The findings of this study will contribute to ongoing research and assist farmers in expanding the usage of biofertilizers based on *Bacillus*.

MATERIAL AND METHOD

The *Bacillus* were isolated from the rhizospheres of tomato, lettuce, pak choy, broccoli, and strawberries grown in the tropical mountains in the Lembang District, West Bandung Regency, Indonesia (Figure 1a). The altitude of the study area is 1.242 m above the sea level, with annual temperature and humidity of 18 and 91%, respectively. Rhizosphere soil was collected by carefully removing the soil around the roots by gentle shaking to expose the soil firmly attached to the roots. Then, the rhizosphere soil was taken by using a small brush (Figure 1b). The geographical position of sampling locations is indicated in Table 1.

Isolation of *Bacillus*

Bacillus were isolated by using the serial dilution plat method on tryptic soy agar (TSA)

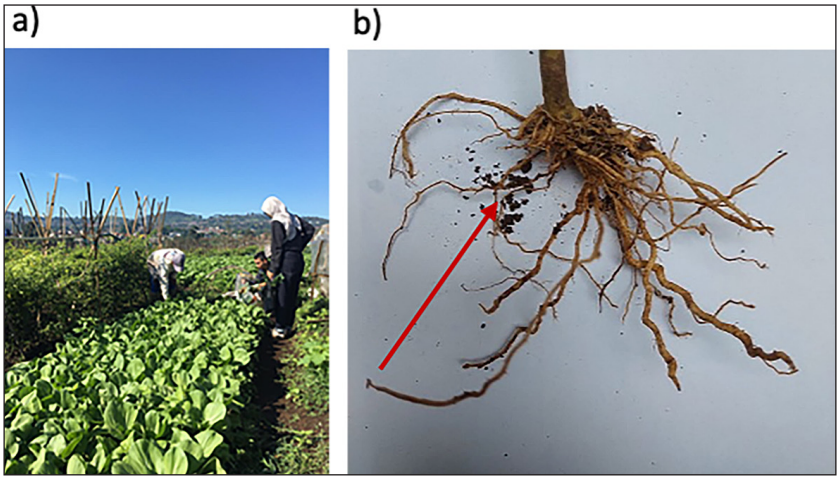

Figure 1. (a) Soil sampling area in Lembang for isolating the *Bacillus*, (b) the rhizosphere of lettuce

Table 1. Geographical position of sampling point

Sample code	Vegetable crops	Geographical position
A	Tomato	6.80042° S, 107.64986° E
B	Lettuce	6.82042° S, 107.61616° E
C	Pak choy	6.82030° S, 107.61622° E
D	Broccoli	6.82038° S, 107.61610° E
E	Strawberries	6.79973° S, 107.56812° E

medium containing casein peptone 15 g L^{-1}, soya peptone 5 g L^{-1}, natrium chloride 5 g L^{-1}, agar-agar 15 g L^{-1}, and 1 L distilled water. All soil samples were diluted to 10^{-7} in 0.85% sodium chloride solution. A total of 1 mL of all soil suspension was grown in TSA using pour method; the cultures were incubated at 37 °C for 24 h. The colonies were screened for the morphology of a typical *Bacillus* colony, which is rough, opaque, fuzzy white, or slightly yellow with jagged edges (Bai et al., 2013; Ming et al., 2008). This study found bacterial colonies with a circular or irregular form and crateriform elevation with undulate margins. Thirteen *Bacillus* isolates were obtained. Pure cultures of all isolates were maintained on tryptic soy broth (TSB) slants at 4 °C for 24 hours before staining and biochemical characterization. All suspected *Bacillus* colonies were then subjected to Gram, Endospore, Capsule, and Acid-fast Staining, according to Bisen (2014).

Biochemical characterization of *Bacillus*

Each *Bacillus* pure culture loop was inoculated unto TSB at room temperature 25–27 °C for 18 h with 115 rpm shaking. Biochemical characteristics included motility in semi-solid nutrient agar (NA), catalase and oxidase in NA, starch hydrolysis in starch agar plate, nitrate reduction in nitrate medium, and Voges-Proskauer and methyl red tests on GPB broth medium (Zerin, 2020). Salinity resistance testing was conducted using 3.4% NaCl, four times the concentration of physiological NaCl (Hindersah et al., 2019). The ability of *Bacillus* to ferment sugar was indicated by a change in the suspension color from red to yellow, which indicated the pH reduction and the presence of gas in the Durham tube.

Pathogenicity determination

Pathogenic tests are essential to ensure that the selected *Bacillus* strains are not pathogenic to plants. The test was conducted on tobacco leaves using the method described by Lelliot and Stead (1987). Each inoculum was injected with 1 mL using a sterile syringe with a needle into the lower surface of the healthy tobacco leaf, specifically into the mesophyll tissue between the leaf veins. A positive reaction was indicated by a change in the color of the inoculated leaf tissue, turning from green to brown due to necrosis, which is the drying out of the tissue.

Determination of metabolite production

Organic acid analysis

Organic acid was measured using high-performance liquid chromatography (HPLC) at specified time points using the series Waters e2695 HPLC system. The *Bacillus* suspension was prepared by inoculating a loopful of *Bacillus* pure culture to 100 mL TSB and incubated for 14 h at 30 °C. Afterwards, 2 ml suspension was poured into a microcentrifuge bottle and then centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was collected and filtered using a filter syringe and injected into the HPLC with column C18 with a wavelength of 210 nm; the mobile phase solution was KH_2PO_4 (0.76%) with pH 4. The organic acid standards include lactate, malate, oxalate, citrate, and tartrate. The concentrations of organic acid for the calibration curve are 5, 10, 15, and 20 ppm. The results of these standards are analyzed based on the retention time observed in the standard curve using HPLC.

Exopolysaccharides analysis

Firstly, 20 mL of *Bacillus* liquid culture was centrifuged at 9000 rpm at 4 °C for 20 min. The supernatant and 2 volumes of cold acetone were collected and left overnight at 4 °C before centrifugation at 9000 rpm at 4 °C for 20 minutes. The supernatant was removed, and EPS on the bottom of the tube was collected onto the Whatman No. 1 filter paper. The dry weight of EPS was determined using gravimetric method at 35 °C for 30 minutes. The dry weight of EPS is the different weight of filter paper with EPS and without EPS (Hindersah and Sudirja, 2010).

Phosphatase analysis

Phosphatase enzymes were analyzed using a spectrophotometer. Samples of bacterial isolates were diluted using physiological NaCl to 10^{-4}, then 4 mL buffer phosphate and 1 mL of p-nitrophenylphosphatase were added as well as mixed homogenized by vortex, and incubated for 1 hour at 37 °C. After incubation, 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added. Then, the solution was diluted 10 times with distilled water, shaken, and filtered by Whatman No.1. After that, the analysis used a spectrophotometer with a wavelength of 400 nm (Schinner et al., 1996).

Bacterial identification based on 16S rRNA analysis

Analysis of 16S rRNA for selected isolates was conducted at the Laboratory of the Indonesia Centre for Biodiversity and Biotechnology in Bogor, West Java. DNA isolation from bacterial colonies and PCR amplification were carried out simultaneously using a direct PCR Kit (KOD FX Neo, Toyobo) following the Kit protocol, the PCR machine used was a personal master cycler brand Eppendorf using universal primers Primer F: 16F27 / Sequence: AGA GTT TGA TCM TGC CTC AG and Primer R: 16R 1492 / Sequence: TAC GGY TAC CTT GTT ACG ACT T. The raw data from the sequencing is then edited using the BioEdit program. The sequence data has been edited further in Blast with genomic data that has been registered with NCBI/National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to determine the taxon/species that has the largest homology/similarity and is molecularly closest.

RESULTS

Twenty-two isolates were obtained from the rhizosphere of tomatoes, lettuce, pak choy, broccoli, and strawberries (Table 2). Each isolate was re-cultured in tryptic soy plate agar and given an initial code according to its plant origin. On the basis of their cell purity, 13 pure cultures were obtained, which were subjected to cell-morphology characterization.

Table 2. The number of *Bacillus* isolated from the rhizosphere of vegetable plants in Lembang

Code	Rhizosphere	Number of isolates	Isolates code
A	Tomato	4	A2, A4
B	Lettuce	5	B1, B1-4, B1-8
C	Pak choy	5	C4, C5
D	Broccoli	4	D1, D2, D6
E	Strawberry	4	E2, E3, E6

Cell-morphology characteristics of *Bacillus*

Thirteen pure colonies were subjected to Gram, endospore, capsule, and acid-fast staining. Only one Gram-negative isolate formed the capsule, and one Gram-positive isolate was acid-resistant. Confirmation through Gram staining showed that the nine isolates are Gram-positive and rod-shaped (Table 3), which fit the morphological characteristics of the genus *Bacillus*. Thus, the nine isolates have proceeded to the biochemical test.

Biochemical characteristics of *Bacillus*

Generally, the biochemical characteristics of *Bacillus* are positive in motility, catalase, starch hydrolase, nitrate reduction, Voges-Proskauer, Methyl red, and NaCl reaction as well as negative in the indole test (Holt et al., 1994; Lay, 1994; Logan et al., 2009). The results of the biochemical tests presented in Table 4 show the diverse characteristics of presumed *Bacillus* isolates. However, all isolates were motile and positive in catalase tests. Most isolates are also positive in oxidase, starch hydrolysis, and nitrate reduction tests, although there are variations in some isolates. The Voges-Proskauer test and growth at 3.4% NaCl demonstrated different results between isolates. The indole test of all isolates was negative. A comparison of biochemical profiles showed that B1 and D1 isolates had the highest similarity (100%) with reference *Bacillus* isolates, while D2 and E2 showed 88.8% similarity. Although there are variations in some biochemical tests, the isolates showed typical characteristics of the genus *Bacillus*.

Table 5 presents the results of the Methyl Red test, which shows the variation in sugar fermentation ability in *Bacillus* B1, D1, D2, and E2 isolates. B1 isolation can ferment sucrose, while D1, D2, and E2 isolation can only ferment glucose. None of the isolates can ferment maltose, mannitol, or lactose. These results indicate a different specificity in utilizing carbon sources by each isolate, which changed the color of methyl red to orange/yellow. Glucose was the most common carbon source used by *Bacillus* isolates in this study. These differences in fermentation profiles can be the basis for differentiating different *Bacillus* species (Marista et al., 2013). The B1, D1, D2, and E2 isolates (Figures 2 and 3) were selected based on similarities to *Bacillus* and further subjected to analysis of plant-growth-related function.

The four isolates, B1, D1, D2, and E2 were Gram-positive bacteria, indicating the presence of a thick cell wall capable of maintaining the violet-iodine crystal complex. The shape of the cells seen in these four isolates aligns with the morphology of the genus *Bacillus*. The results of this Gram staining provide visual confirmation of biochemical data showing that the four isolates have characteristics consistent with the genus *Bacillus* (Figure 2).

The *Bacillus* colonies maintained in the TSA slant during 48 h showed a very distinctive morphology based on the characteristics of the genus *Bacillus*. The colonies formed are white to yellowish, a common feature of *Bacillus*. The texture of the colony looks rough, and the edges are jagged, which characterizes an uneven morphology (Figure 3).

Table 3. The cell morphological characteristics of *Bacillus* isolates

No	Isolates	Shape	Gram	Endospora	Capsule	Acid resistant
1	A2	Rod	-	+	+	-
2	A4	Cylinder	-	+	-	-
3	B1*	Rod	+	+	-	-
4	B1-4	Rod	+	+	-	-
5	B1-8	Rod	+	+	-	-
6	C4	Rod	-	+	-	-
7	C5	Rod	-	+	-	-
8	D1	Rod	+	+	-	-
9	D2*	Rod	+	+	-	-
10	D6	Rod	+	+	-	-
11	E2*	Rod	+	+	-	-
12	E3	Rod	+	+	-	+
13	E6	Rod	+	+	-	-

Note: * Isolates with cell morphology fit with *Bacillus* properties.

Table 4. Biochemical characteristics of isolates for the determination of *Bacillus* genus

Isolate	Motility	Catalase	Oxidase	Starch hydrolysis	Nitrate Reduction	VP**	Indole test	NaCl 3.4 %	Red methyl	Similarities (%)
B1*	+	+	+	+	+	+	-	+	+	9/9 (100)
B1-4	-	+	-	+	+	-	-	+	-	5/9 (55.5)
B1-8	-	+	+	+	+	-	-	+	-	6/9 (66.6)
D1*	+	+	+	+	+	+	-	+	+	9/9 (100)
D2*	+	+	+	+	+	+	-	-	+	8/9 (88.8)
D6	+	+	+	+	+	+	-	-	-	7/9 (77.7)
E2*	+	+	+	-	+	+	-	+	+	8/9 (88.8)
E3	+	+	+	-	-	+	-	-	+	6/9 (66.6)
E6	+	+	+	+	-	-	-	-	-	4/9 (44.4)

Note: *isolates with cell morphology fit with *Bacillus* properties; **VP Voges-Proskauer

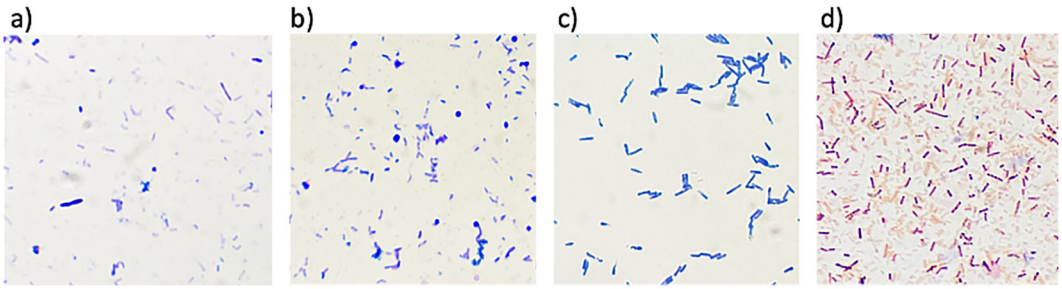

Figure 2. Gram-positive *Bacillus* isolated from the rhizosphere of vegetable (a) B1, (b) D1, (c) D2, (d) E2

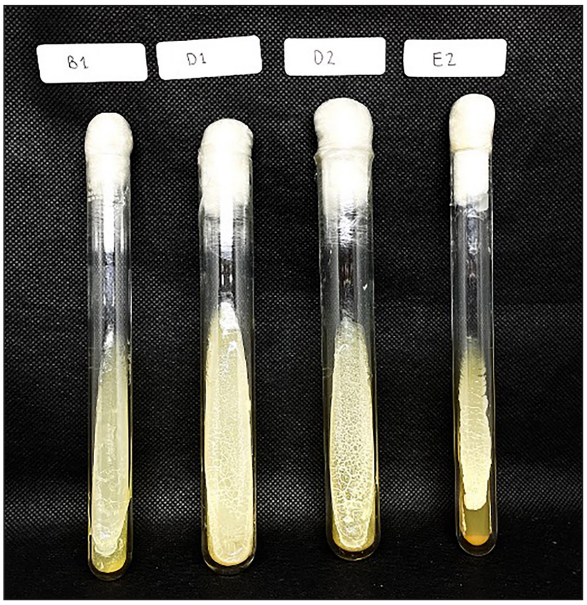

Figure 3. The colony of *Bacillus* B1, D1, D2, and E2 in TSA slant

Pathogenicity

Tobacco leaves were tested with five selected bacillus isolates, bacillus B1, D1, D2, E2, and A2, as a comparison for the four selected isolates. On the basis of the test results, the four selected isolates did not show necrosis in the leaf tissue. Isolate A2 is the control *Bacillus* that caused the presence of brown spots and induced necrosis on the tobacco leaf tissue (Figure 4).

The A2 isolate induced necrosis on the tissue of the first leaf. However, the remaining four isolates did not exhibit any necrotic symptoms. These findings indicate that the four *bacillus* strains pose no phytopathogenic risk and thus have the potential to be developed as a biofertilizer.

Production of plant-growth related metabolites

Table 6 shows that all *Bacillus* produced lactic, malic, oxalic, citric, and tartaric acid. However, the concentration of organic acids in their liquid culture differed (Table 6). *Bacillus* B1 produced only approximately 8% and 10% less of malic acid and oxalic acid, respectively, compared to another isolate. Meanwhile, the liquid culture of *Bacillus* D2 contained the highest concentration of citric acid but the lowest lactic and tartaric acid. The highest tartaric acid content was found in the liquid culture of E2.

All *Bacillus* cultures contained EPS and phosphatase in different concentrations. *Bacillus*

Table 5. Methyl Red Test for fermentation of various simple sugars by four isolates of *Bacillus* bacteria

Isolate/speciеS	Maltose	Sucrose	Glucose	Mannitol	Lactose
Bacillus B1	-	+	+++	-	-
Bacillus D1	-	-	+++	-	-
Bacillus D2	-	-	+++	-	-
Bacillus E2	-	-	+++	-	-

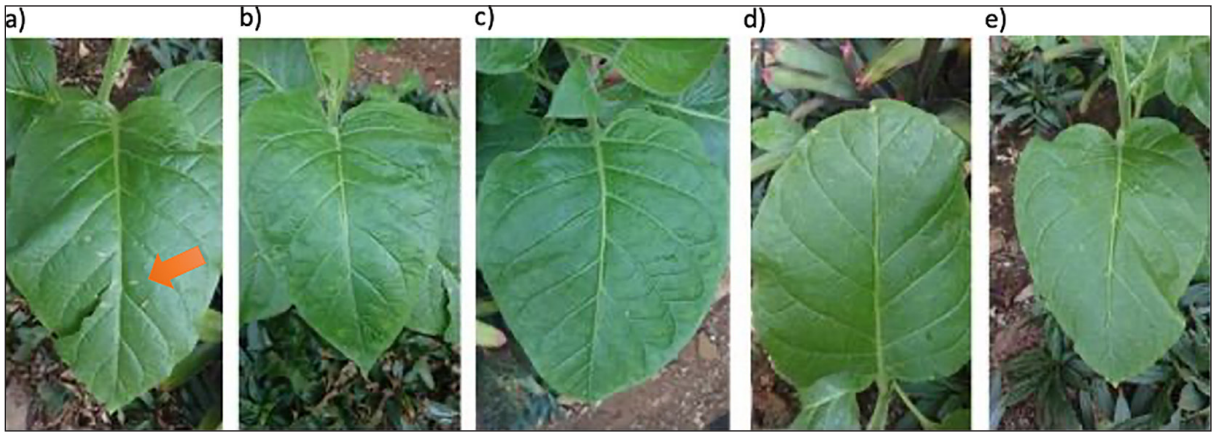

Figure 4. Bacterial pathogenicity test on tobacco leaves (a) A2 (control), (b) B1, (c) D1, (d) D2, (e) E2

Table 6. Organic acids were detected in liquid cultures of some isolates and species of *Bacillus*

Isolate/species	Organic acid (mg/L)*				
	Lactic	Malic	Oxalic	Citrate	Tartaric
Bacillus B1	33.66	3.84	13.94	5.16	38.36
Bacillus D1	31.51	38.54	135.76	41.09	21.17
Bacillus D2	14.15	39.24	134.31	183.55	14.83
Bacillus E2	37.89	39.39	132.64	17.53	71.97

Note: *Mean values are from two replications.

B1 produced approximately 50% more EPS than other isolates, but the culture of *Bacillus* D2 contained a higher phosphatase content (Table 7). *Bacillus* E2 only produced 0.05 mg/L phosphatase, the lowest among other isolates.

Bacterial characteristics based on 16S rRNA analysis

The results of the 16S rRNA analysis verified that all isolates belong to the genus *bacillus* (Table 8). The *Bacillus* isolates B1 has 100% homology with *Bacillus safenesis* strain MDL5, while the homology of *Bacillus* D1 with *Bacillus* safensis strain MDL5, was 99.93%. *Bacillus* D2 and E2 have less than 100% homology with *Bacillus altitudinis* strain RPW2 and *Bacillus* sp. strain SZ057, respectively.

DISCUSSION

One promising approach to utilizing plant growth-promoting rhizobacteria (PGPR) is to develop an effective biofertilizer. Therefore, collecting and identifying the PGPR prior to biofertilizer formulation is a necessity. Beneficial microbes in the rhizosphere, the region around plant roots, can promote plant growth and protect plants from pathogens (Ahmed et al., 2014). Various studies have shown that plants can selectively enrich beneficial microbes in their rhizosphere, an evolutionary adaptation important for establishing terrestrial environments (Andreote et al., 2014). Rhizobacteria, which promote plant growth, can improve plant health through various mechanisms, such as nutrient mobilization, phytohormone production, and pathogen suppression (Spaepen et al., 2009).

Table 7. Exopolysaccharides (EPS) and phosphatase enzymes in liquid cultures of four *Bacillus* isolates

Isolate/species	EPS (g/L)*	Phosphatase (mg/L)*
Bacillus B1	8.66	0.27
Bacillus D1	4.16	0.24
Bacillus D2	4.50	0.49
Bacillus E2	4.50	0.05

Note: *Mean values are from two replications.

Table 8. Homology of isolates with *Bacillus* species based on partial sequence of 16S ribosomal RNA gene

Isolate	Species and strains	Homology
Bacillus B1	*Bacillus safensis* strain MDL5	Homology 99.72%. Query Cover 100%.
Bacillus D1	*Bacillus safensis* strain MDL5	Homology 99.93%. Query Cover 100%.
Bacillus D2	*Bacillus altitudinis* strain RPW2	Homology 99.64%. Query Cover 100%.
Bacillus E2	*Bacillus* sp. strain SZ057	Homology 99.86%. Query Cover 100%.

The rhizosphere of vegetable crops grown in productive Andisols soils in mountainous regions is the niche of a wide variety of beneficial bacteria, including the genus *Bacillus*, which is well-known for its ability to promote plant growth and also produce antimicrobial compounds (del Barrio-Duque et al., 2019; Kesaulya et al., 2021). The *bacillus* were isolated from specific soil and niches; they can be used in other tropical area due to their adaptability and resilience under various soil conditions, including temperature extremes, pH variations, and nutrient availability (Radhakrishnan et al., 2017). *Bacillus* are also able to produce bioactive compounds, such as phytohormones and enzymes for enhancing plant growth in various agroecological zones (Hinsinger et al., 2009; Nwachukwu et al., 2021). Therefore, isolating and characterizing *Bacillus* from the rhizosphere of these vegetable can discover new strains with certain properties related to plant growth promotion.

Morphological and biochemical characterization were essential in identifying *Bacillus*. *Bacillus* is Gram-negative with a thick cell wall (Wyrick' And and Rogers, 1973), and forms endospores (Borriss, 2020). This dormant endospore is resistant to heat conditions, nutritional deficiencies, ultraviolet radiation, or toxic chemicals. The endospore-forming PGPR is an important characteristic for withstanding high temperatures and limited soil nutrient content in tropical agriculture. In this study, *Bacillus* bacteria do not exhibit acid-resistant properties, because they do not have a waxy layer of mycolic acid on their cell walls. The absence of this wax layer makes *Bacillus* unable to withstand the exposure to strong acids and special staining for resistant acid bacteria (Mukhtar et al., 2023).

The biochemical properties of suspected *bacillus* in this study agree with *Bacillus* in hot springs (Abdelkrim et al., 2021), which produce catalase and motile. The *Bacillus* sp. from strawberry rhizosphere is also positive in the catalase, biochemical such as organic acid (lactic, malic, oxalic, citrate, tartaric), exopolysaccharides, and phosphatase enzymes, and then fermentation tests such as maltose, sucrose, glucose, mannitol, and lactose (Putra et al., 2020). The selection of general biochemical characteristics with some of these tests can determine the characteristics of *Bacillus* isolates. In this study, the negative reaction of the indole test was shown by all isolates since bacillus does not have the tryptophanase enzyme (Lay, 1994).

Determination of metabolites, such as organic acids, exopolysaccharides, and phosphatase enzymes in *bacillus* bacteria is an essential step in evaluating their potential as biofertilizers. Characterization of the ability of isolates to produce these metabolites is essential to understanding the mechanism of increasing plant growth. The organic acids of *Bacillus* dissolve insoluble phosphate compounds and increase phosphorus availability in soil (Patel et al., 2008; Setiawati et al., 2022). The results agree with the ability of other *Bacillus* strains to synthesize organic acid. The *B. altitudinis* isolated from chickpeas produce oxalic acid of 120 mg/L (Kushwaha et al., 2021), while *Bacillus* sp. isolated from corn produces lactic acid in the range of 48-932 mg/L and citric acid of 3.84 mg/L (Mumtaz et al., 2019).

The concentrations of lactic acid and citric acid reported in the study, were comparable to the results of the presented research (Table 6). Meanwhile, the enzyme phosphatase mineralizes organic phosphorus into an inorganic form that the roots can absorb (Tian et al., 2021). The exopolysaccharides produced are an extracellular matrix that protects bacterial cells and increases water and nutrient retention around the rhizosphere, indirectly supporting plant growth (Morcillo and Manzanera, 2021).

On the basis of the results of this study, it can be concluded that four isolates, *Bacillus* B1, D1, D2, and E2, three species, *B. safensis, B. altitudinis*, and *Bacillus* sp. have great potential to be developed as biofertilizers. These isolates do not show pathogenicity; *Bacillus* do have not strong virulence factors such as toxins or specialized surface structures that can exterminate host cells (Popoff, 2024). The four *Bacillus* species produce the metabolites related to the plant growth promotion. The *B. safensis* isolate obtained in this study showed a significantly higher production of secondary metabolites, such as organic acids, exopolysaccharides, and phosphatase enzymes, which were significantly higher than *bacillus* sp. and *B. altitudinis*. These findings are in line with the research by Chebotar et al., (2024) and Mukhtar et al., (2023), which shows that the *B. safensis* isolate from tomato plants can produce organic acids, phosphatase enzymes, and exopolysaccharides. These metabolites increase plant growth and resistance to salinity stress and temperature extremes. In comparison, the research by Zhao et al. (2022), reported that the *B. altitudinis* isolated from *Lycium barbarum* produced a variety of organic acids and phosphatase enzymes. In addition, the research by Sun et al., (2021) showed that the *B. altitudinis* isolated from Ginkgo biloba can produce exopolysaccharides that effectively inhibit the growth of plant pathogens, such as *Alternaria alternata* in apple plants.

Analysis of 16S rRNA revealed that bacterial communities in the rhizosphere of vegetable crops are very diverse (Hu et al., 2020). More than 100 different species of bacteria were identified from soil samples. *Bacillus* is the most dominant phylum of bacteria, followed by *Pseudomonas* and *Rhizobium*. The high bacterial diversity in the rhizosphere of vegetable crops is most likely due to various factors, including habitat diversity, resource abundance, and interactions between species. While *Bacillus* isolates show great potential as plant growth-promoting agents, their introduction into agricultural systems requires careful consideration of possible ecological impacts. To date, the research on *Bacillus*-based biofertilizer used in long-term agriculture has not yet been found. Exogenous *Bacillus* strains may disrupt native microbial communities, leading to ecological shifts. Therefore, long-term monitoring of microbial community dynamics after *bacillus* inoculation is essential to maintain ecological balance. Such monitoring is critical for the sustainable development of biofertilizers. The structure of bacterial communities in the rhizosphere of vegetable crops is influenced by factors such as soil pH, produced root exudate, and human activities. Isolation carried out from the rhizosphere of vegetable plants obtained four pure isolates that are known to be *bacillus* and have the ability to produce metabolites in the form of organic acids, exopolysaccharides, and phosphatase enzymes (Tables 5 and 6). All metabolites have direct or indirect role in the availability of nutrients and then plant growth,

The findings of this study provide strong evidence that the *Bacillus* isolates from the rhizosphere of highland vegetables have excellent potential as a plant growth-promoting agent. Introducing *Bacillus*-based biofertilizer offers a sustainable way to increase plant productivity and better soil management. With further research and field trials, *Bacillus* becomes important in sustainable agriculture strategies in Indonesia and other tropical regions. Future research should focus on formulating *Bacillus*-based biofertilizers following with bioassay techniques to ensure their effectiveness under actual agricultural conditions and compare them with commercial biofertilizers regarding ease of application and improved yield. Further research should focus on stable, easy-to-apply, and effective formulations of *Bacillus* biofertilizers to ensure widespread acceptance by farmers and their central role in sustainable agriculture strategies.

CONCLUSIONS

Microbes isolated from vegetable crops of tomatoes, lettuce, broccoli, pak choy, and strawberries obtained 13 isolates. The tests carried out for *Bacillus* isolate selection were gram staining, biochemical characteristics test, pathogenicity test, production of metabolites, and 16S rRNA

analysis. After the test, four *Bacillus* isolates were obtained from the rhizosphere of lettuce, broccoli, and strawberry plants. On the basis of 16S rRNA, B1 is *Bacillus safensis* strain MDL5, D1 is *Bacillus safensis* strain MDL5, D2 is *Bacillus altitudinis* strain RPW2, and E2 is *Bacillus* sp. strain SZ057. All isolates produced metabolites in the form of organic acids, exopolysaccharides, and various phosphatase enzymes. It shows potential as a biofertilizer to enhance plant productivity and reduce the reliance on inorganic fertilizers.

The findings of this study provide strong evidence that the *Bacillus* isolates from the rhizosphere of highland vegetables have excellent potential as a plant growth-promoting agent. Implementing *bacillus* as a biofertilizer can offer a sustainable solution for increasing plant productivity and better soil management. With further research and field trials, *Bacillus* can become significant in sustainable agriculture strategies in Indonesia and other regions.

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