

Comparative Analysis of Rhizobacterial Isolates from Peanut (*Arachis hypogaea* L.) with Potential as Plant Growth-Promoting Rhizobacteria (PGPR) Based on 16S rRNA Identification

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Abstract: Plant Growth-Promoting Rhizobacteria (PGPR) are beneficial bacteria that colonize the rhizosphere and enhance plant growth through nutrient provision and phytohormone production. This study aimed to isolate bacteria from root nodules of peanut (*Arachis hypogaea* L.) and evaluate their potential as PGPR. Bacteria were isolated from peanut root nodules, followed by qualitative screening of PGPR traits, including nitrogen fixation, indole-3-acetic acid (IAA) production, phosphate solubilization, and protease activity. Selected isolates were further identified molecularly using the 16S rRNA gene. A total of 16 isolates were obtained, consisting of 12 Gram-negative and 4 Gram-positive bacteria with diverse colony morphologies. PGPR screening showed that 6 isolates were capable of nitrogen fixation, 8 produced IAA, all isolates solubilized phosphate on Pikovskaya medium, and 10 exhibited protease activity. Based on these traits, five representative isolates (AH2, AH5, AH9, AH11, and AH14) were selected for molecular identification. BLAST analysis revealed that the isolates were closely related to *Ralstonia* sp. (AH2 and AH14), *Ralstonia* sp. T21-1 (AH11), *Burkholderia stabilis* (AH5), and *Martinezella tropici* strain SARCC-715 (AH9). Phylogenetic analysis confirmed that isolate AH9 clustered within the *Rhizobium* clade, showing 97.28% sequence similarity and strong bootstrap support (100%). These results indicate that peanut root nodules harbor diverse bacteria with plant growth-promoting traits. Furthermore, the identification of *Ralstonia* and *Burkholderia* isolates raises biosafety concerns, rendering them unsuitable as biofertilizer candidates without extensive safety evaluations. Therefore, only isolate AH9 (closely related to *Martinezella tropici*) represents a promising and safer candidate for development as a biofertilizer for peanut cultivation. The *in planta* validation test shows that isolate AH9 is significantly effective in increasing peanut plant growth based on parameters such as number of leaves, plant height, and dry and fresh weight.

Keywords: *Arachis hypogaea*; IAA synthesis; nitrogen fixation; phosphate solubilization; *Martinezella tropici*

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1. Introduction

Plant growth–promoting rhizobacteria (PGPR) represent a diverse group of beneficial soil microbes that colonize the rhizosphere and enhance crop productivity through direct mechanisms such as phytohormone production, biological nitrogen fixation, and phosphate solubilization, as well as indirect mechanisms like pathogen suppression [1–3]. The application of PGPR as biofertilizers has gathered substantial momentum as a sustainable strategy to mitigate agricultural yield gaps. In leguminous crops like peanut (*Arachis hypogaea* L.), this interaction is particularly pronounced within root nodules, which are traditionally formed via mutualistic symbioses with nitrogen-fixing rhizobia belonging to genera such as *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, and *Sinorhizobium* [4–6]. Recent studies show that peanut root nodules harbor not only symbiotic rhizobia but also diverse non-rhizobial endophytes that support plant growth and stress tolerance. These nodule-associated bacteria may function as plant growth-promoting rhizobacteria (PGPR) through biological nitrogen fixation, phosphate solubilization, phytohormone production, siderophore secretion, and pathogen suppression. Consequently, peanut nodules represent a valuable ecological niche for the discovery of multifunctional bacterial inoculants with potential applications in sustainable agriculture. Furthermore, growing evidence indicates that microbial inoculants can improve crop productivity while reducing dependence on chemical fertilizers. However, the environmental safety and field performance of microbial inoculants are strain-dependent and should be supported by accurate taxonomic identification, functional characterization, and biosafety assessment prior to agricultural application [7, 8]. Groundnut (peanut) is an important agribusiness commodity with high economic value and is considered the second most important legume crop after soybean. Peanuts are nutritionally rich and serve as a valuable source of plant-based protein and edible oil. Peanut seeds contain approximately 40% fat, 30% protein, 12% carbohydrates, as well as essential minerals and vitamins [9, 10].

According to data from the USDA in the Center for Agricultural Data and Information Systems, Secretariat General of the Ministry of Agriculture of the Republic of Indonesia [11], global peanut consumption increased during the period 2017–2021. Indonesia ranks seventh worldwide, accounting for approximately 2.79% (1.4 million tons) of total global peanut consumption in 2021. Despite being a major agricultural country, Indonesia continues to face challenges in meeting domestic peanut demand and remains dependent on imports. Data from the Directorate General of Food Crops indicate that peanut import volumes increased from 287,095 tons in 2021 to 340,655 tons in 2022. One key factor contributing to low peanut production is declining soil fertility, largely attributed to the excessive and prolonged use of chemical fertilizers.

Therefore, applying PGPR as biofertilizers is a promising, environmentally friendly approach to improve soil fertility and enhance peanut productivity. This strategy is expected to support sustainable peanut production in Indonesia while reducing dependence on imports. This study aimed to molecularly identify bacteria isolated from Indonesian peanut root nodules, clarify their ecological roles within the nodule, and evaluate their multi-trait PGPR potential for developing localized, safe biofertilizers.

2. Materials and Methods

2.1. Sample Collection.

Peanut (*Arachis hypogaea* L.) root nodules were collected in September 2023 from a cultivated field located in West Ungaran Subdistrict, Semarang Regency, Central Java, Indonesia (7°06'38.8 "S and 110°21'39.4 "E) at an altitude of 500-600 meters above sea level. The soil collected from the peanut cultivation site had a neutral pH, with a pH (H₂O) of 7.02 and a pH (KCl) of 6.02. The soil contained 1.12% organic carbon (C-organic) and 0.12% total nitrogen (N-Kjeldahl). The concentration of available phosphorus (P₂O₅, Olsen method) was 16.71 ppm, while the available potassium (K) content, determined using the Morgan-Wolf extraction method, was 367.59 ppm. These results indicate that the soil provided moderate levels of essential nutrients required for peanut growth and rhizobial activity. A total of 10 healthy peanut plants were sampled, and root nodules were collected for bacterial isolation. Plants were carefully uprooted to preserve the root system, and adhering soil was gently removed. Healthy, well-developed nodules exhibiting a characteristic reddish internal coloration, indicative of leghemoglobin and active symbiosis with rhizobacteria, were selected for further analysis [12].

2.2. Isolation of Rhizobacteria from Nodules.

The isolation process began with surface sterilization of the healthy root nodules by immersion in 70% ethanol for 1 minute, followed by 2.5% sodium hypochlorite for 1 minute to eliminate surface microorganisms. Subsequently, the sterilized nodules were rinsed seven times with distilled water to ensure complete removal of residual disinfectant. The sterilized nodules were aseptically crushed to obtain a nodule suspension. The suspension was then spread onto Yeast Extract Mannitol Agar supplemented with Congo Red (YEMA+CR) medium using the spread plate technique and incubated at 28–30 °C for 3 - 7 days [13]. Bacterial growth was monitored daily. Colonies appearing white to transparent white (not absorbing Congo Red) and exhibiting distinct macroscopic characteristics were purified by repeated streak plating to obtain single colonies. The purified isolates were subsequently characterized both macroscopically and microscopically. Macroscopic characterization included observations of colony morphology, such as shape, color, elevation, margin, and texture. Microscopic characterization was performed by observing cell morphology and Gram staining reactions [14].

2.3. Nitrogen Fixation Activity.

The nitrogen-fixing ability of the bacterial isolates was evaluated qualitatively. Each isolate was inoculated onto semi-solid Nitrogen-Free bromothymol blue (NFb) medium and incubated at 28–30 °C for 10 days. Positive nitrogen fixation was indicated by a color change of the medium from greenish-yellow to blue, reflecting alkalization associated with diazotrophic activity.

2.4. Indole-Acetic-Acid (IAA) Production Ability.

Each isolate was cultured in Nutrient Broth supplemented with 1% L-tryptophan and incubated at 28–30 °C for 3 days in a rotary shaker at 120 rpm under dark conditions. After incubation, Salkowski reagent was added to the culture and the mixture was further incubated

in the dark for 30 minutes. The development of a pink coloration indicated a positive reaction for IAA production [15, 16].

2.5. Phosphate Solubilizing Activity.

Phosphate solubilization activity was evaluated by inoculating the isolates onto Pikovskaya (PK) agar and incubating for 7 days at 30°C. The formation of a clear halo zone surrounding the bacterial colony indicated a positive result. The phosphate solubilization index (PSI) was calculated according to Halimursyadah *et al.* [17] using equation (1).

$$PSI = \frac{\text{Clear Zone Diameter} - \text{Colony Diameter}}{\text{Colony Diameter}} \quad (1)$$

Where the colony diameter represents the diameter of bacterial growth and the halo zone diameter represents the total diameter of the clear zone formed around the colony. All measurements were recorded in millimeters.

PSI values were categorized according to the classification described by Halimursyadah & Rizva [18] (Table 1).

Table 1. Classification of PSI Values.

PSI Value	Category
<1.6	Low (+)
1.6-2.14	Medium (++)
2.15-2.59	High (+++)
>2.6	Very High (++++)

2.6. Protease Production Activity.

Protease activity was assessed by inoculating each bacterial isolate onto selective skim milk agar (SMA) medium, followed by incubation at 28–30 °C for 48–72 h. The presence of a clear zone surrounding the colony indicated casein hydrolysis and, consequently, the ability of the bacteria to produce extracellular protease enzymes [19]. The proteolytic index (PI) was calculated according to Lim *et al.* [20] using equation (2).

$$PI = \frac{\text{Clear Zone Diameter} - \text{Colony Diameter}}{\text{Colony Diameter}} \quad (2)$$

The obtained PI values were categorized based on the criteria presented in Table 2, following the classification proposed by Dewiyanti *et al.* [21].

Table 2. Classification of IP.

PI Value	Category
<1	Low (+)
1-2	Medium (++)
>2	High (+++)

2.7. BOX-PCRFingerprinting and Molecular Identification Based on 16S rRNA Genes.

Molecular identification of rhizobial isolates was performed through amplification and sequencing of the 16S rRNA gene. Each isolate was first cultured in Yeast Extract Mannitol Broth (YEMB) for 3 days, after which genomic DNA was extracted following the InstaGene Matrix (Bio-Rad Laboratories, USA) protocol. DNA concentration and purity were determined

using a NanoDrop-2000 Spectrophotometer (Thermo Fisher Scientific, USA) to ensure adequate template quality. High-concentration DNA samples were diluted to 200 ng/μL using ddH₂O.

Genetic fingerprinting of the rhizobia isolates obtained from active peanut root nodules was performed using BOX-PCR to assess genetic diversity among isolates. Amplification was carried out using the BOX-A1R primer (5'- CTACGGCAAGGCGACGCTGACG- 3') (Macrogen Inc., South Korea). The PCR program consisted of an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 8 min, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min. The amplified products were separated by 1.5% agarose gel electrophoresis for 60 min at 70 V and subsequently visualized under UV light [22]. PCR amplification was carried out using universal bacterial primers 27F (5'- AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT- 3') (Macrogen Inc., South Korea). The PCR mixture was prepared using a GoTaq[®] Green Master Mix (Promega, USA) according to the manufacturer's instructions. Amplification was performed using a thermocycler (Multigene Optimax–Labnet International Inc., USA) under the following conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 54 °C for 1 min, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 minutes. PCR products were separated by agarose gel electrophoresis and visualized under UV illumination using a GelDoc system (Uvidoc HD5-Uvitech, UK). Amplicons showing clear single bands of the expected size were selected and sent for Sanger sequencing.

2.8. Phylogenetic Analysis.

The obtained 16S rRNA gene sequences were compared with reference sequences available in the GenBank database using BLASTn (Basic Local Alignment Search Tool for nucleotide) through the National Center for Biotechnology Information (NCBI) website to determine sequence similarity and preliminary taxonomic affiliation. Phylogenetic relationships among isolates and closely related reference strains were subsequently inferred by constructing a phylogenetic tree using MEGA 11 software and the Neighbor-Joining method, with 1000 Bootstrap replications [23].

2.9. The effectiveness of Rhizobacteria on Peanut Growth.

The isolates identified as Rhizobia were applied to bean plants to observe their effects on plant growth. The experiment was conducted using a non-factorial completely randomized design with eight replications.

The inoculum was prepared from potential PGPR isolates grown in 50 mL of Nutrient Broth medium, incubated for 24–48 hours. Cell density was measured using a UV-Vis spectrophotometer at OD₆₀₀ = 1.0, with an estimated cell density of 10⁸ [24].

The Leonard jars were assembled from two separate pots: the upper pot served as the container for sterile vermiculite, while the lower pot held the nutrient solution (sterile B&D nutrient). Peanut seeds were sterilized using 95% alcohol for 10 seconds, rinsed with sterile distilled water, soaked in 3% sodium hypochlorite for 20–30 seconds, and rewashed seven times using sterile distilled water. The seeds were then germinated in sterile vermiculite and grown for 48 hours under dark conditions. After sprouting, the seeds were transferred to the Leonard jars, and their growth was observed for up to 28 days [24, 25].

The parameters observed in the peanut plants included plant height, number of leaves, and fresh and dry weight. Data were analyzed using the ANOVA test, followed by Duncan's Multiple Range Test (DMRT) at a 5% significance level using Microsoft Excel and SPSS software

3. Results and Discussion

A total of 16 rhizobacteria isolates were successfully obtained from peanut root nodules collected from a farm located in Brangang Village, West Ungaran, Semarang. Isolation using Yeast Extract Mannitol Agar supplemented with Congo Red (YEMA+CR) produced well-separated colonies that were subsequently purified and coded AH1–AH16. The recovery of multiple isolates from nodules indicates the presence of diverse nodule-associated bacteria inhabiting the peanut rhizosphere and endosphere.

3.1. Isolation and Selection of Rhizobia.

The macroscopic and microscopic characteristics of the 16 isolates are summarized in Table 3. Colony morphology varied among isolates, including circular, punctiform, and irregular forms with entire or undulate margins. Similar morphological diversity among rhizobial isolates has been reported previously, where most colonies appeared circular but variations in size and edge structure were also observed [26]. These differences suggest heterogeneity among nodule-associated bacterial populations.

Gram staining revealed that 12 isolates were Gram-negative, whereas 4 isolates were Gram-positive, with cell morphologies consisting of bacilli, cocci, and coccobacilli. Gram-negative bacteria have a thin peptidoglycan layer and a relatively high lipid content in the outer membrane, causing them to lose the primary stain during decolorization and subsequently take up the safranin counterstain, appearing red or pink [27]. Rhizobia are typically Gram-negative bacteria; therefore, isolates that appear red or pink after Gram staining are consistent with expected rhizobial characteristics due to safranin uptake [28].

The presence of Gram-positive isolates suggests that peanut root nodules may also harbor non-rhizobial endophytic bacteria. Legume nodules are not exclusively colonized by rhizobia but may also contain other endophytic Gram-positive bacteria that coexist within nodule tissues and potentially contribute to plant growth promotion [29, 30]. Legume nodules harbor Gram-positive genera like *Bacillus*, *Paenibacillus*, and *Microbacterium*, as well as Gram-negative ones such as *Pseudomonas* and *Enterobacter*, which coexist within nodule tissues. Studies confirm opportunistic colonization, as seen in peanut nodules with Gammaproteobacteria (e.g., *Pseudomonas*, *Enterobacter*), but Gram-positive *Bacillus* strains promote nodule formation and plant growth when co-inoculated with *Bradyrhizobium* through synergistic interactions [29, 31].

Table 3. Macroscopic and Microscopic Characteristics of 16 Rhizobacteria Isolates.

Code	Shape	Margin	Elevation	Texture	Color	Gram	Cell Shape
AH 1	Irregular	Undulate	Flat	Smooth	Clear White	+	Bacilli
AH 2	Irregular	Undulate	Flat	Smooth	Yellow White	-	Cocobacilli
AH 3	Circular	Entire	Convex	Mucoid	White	-	Coccus
AH 4	Irregular	Entire	Raised	Mucoid	Clear White	+	Bacilli
AH 5	Circular	Entire	Convex	Mucoid	White	-	Bacilli
AH 6	Circular	Entire	Raised	Smooth	Light Pink	+	Bacilli
AH 7	Irregular	Undulate	Flat	Smooth	Clear White	+	Bacilli
AH 8	Irregular	Undulate	Umbonate	Smooth	Yellow White	-	Coccus

Code	Shape	Margin	Elevation	Texture	Color	Gram	Cell Shape
AH 9	Circular	Entire	Convex	Mucoid	White	-	Bacilli
AH 10	Irregular	Undulate	Raised	Smooth	White	-	Cocobacilli
AH 11	Irregular	Undulate	Raised	Smooth	White	-	Coccus
AH 12	Irregular	Undulate	Flat	Smooth	Yellow White	-	Coccus
AH 13	Irregular	Undulate	Flat	Smooth	Yellow White	-	Bacilli
AH 14	Irregular	Undulate	Flat	Smooth	Yellow White	-	Coccus
AH 15	Circular	Entire	Convex	Mucoid	White	-	Bacilli
AH 16	Irregular	Undulate	Umbonate	Dry	Yellow White	-	Bacilli

The occurrence of non-rhizobial bacteria in nodules can be attributed to their ability to colonize infection threads or enter through natural openings and subsequently persist within nodule tissues. These bacteria may exhibit plant growth-promoting traits such as phytohormone production, nutrient solubilization, or pathogen suppression, thereby supporting plant development alongside symbiotic rhizobia [30, 32]. Consequently, further molecular identification was required to accurately determine the taxonomic identity of each isolate.

3.2. Nitrogen Fixation Ability Test (N₂).

A total of 12 isolates that were confirmed as Gram-negative bacteria were further evaluated for their potential as Plant Growth-Promoting Rhizobacteria (PGPR). The screening was performed qualitatively and included tests for nitrogen fixation, indole-3-acetic acid (IAA) production, phosphate solubilization, and protease activity.

Among these traits, biological nitrogen fixation is a key mechanism supporting plant growth, as plants require relatively large amounts of nitrogen for protein and nucleic acid synthesis. Plants cannot directly utilize atmospheric nitrogen (N₂), so they depend on diazotrophic microorganisms capable of reducing N₂ to ammonia (NH₃), which can subsequently be assimilated by plants [33]. The results indicated that, of the 12 Gram-negative isolates, six isolates (AH2, AH5, AH9, AH10, AH11, and AH14) demonstrated nitrogen-fixing ability (Figure 1).

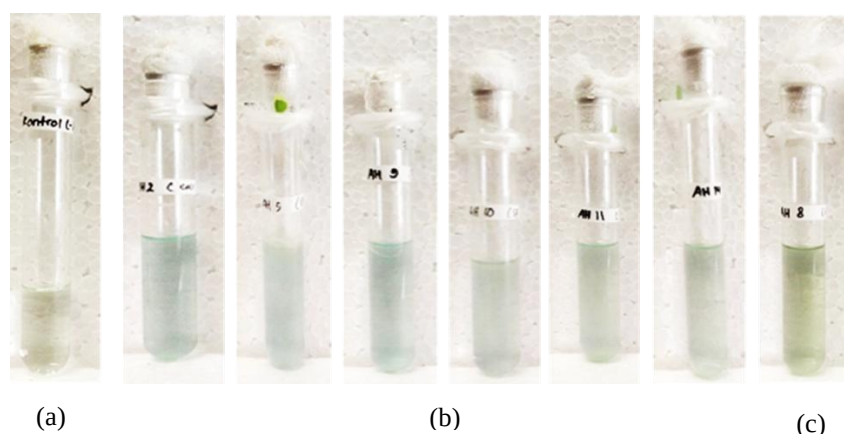


Figure 1. Nitrogen Fixation Test. (a) Control, (b) Positive, and (c) Negative.

Positive activity was observed on Nitrogen-Free Bromothymol Blue (NFB) medium after 7–10 days of incubation, indicated by a color change of the medium from green to bluish-blue. This change occurs because nitrogen fixation produces ammonium, which increases the medium's pH. Bromothymol blue acts as a pH indicator and shifts to blue under alkaline conditions. Therefore, blue coloration accompanied by bacterial growth suggests that the isolates can utilize atmospheric nitrogen as a nitrogen source. Similar observations using NFB medium as an indicator of diazotrophic activity have been reported previously [34, 35].

The presence of nitrogen-fixing isolates among nodule-associated bacteria suggests that some isolates may directly contribute to nitrogen availability in the rhizosphere and potentially enhance peanut growth. However, because the assay was qualitative, further quantitative evaluation (e.g., acetylene reduction or molecular detection of *nifH* genes) is needed to confirm and compare nitrogen-fixation capacity among isolates.

3.3. Indole Acetic Acid (IAA) Production Ability.

Screening for indole-3-acetic acid (IAA) production showed that 8 of the 12 tested isolates (AH2, AH8, AH9, AH10, AH12, AH14, AH15, and AH16) were positive (Figure 2). Positive results were indicated by the development of a red to pink coloration in NB medium supplemented with L-tryptophan after the addition of Salkowski reagent.

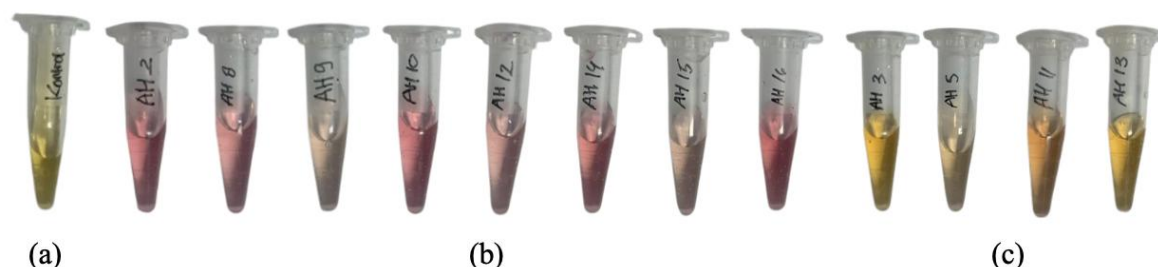


Figure 2. IAA Synthesis Test: (a) Control, (b) Positive, and (c) Negative.

The color change confirms the presence of indolic compounds produced by the isolates, which react with ferric ions in the Salkowski reagent to form a colored complex. Similar observations have been reported previously, in which IAA-producing bacteria develop a pink-to-red coloration after treatment with Salkowski reagent [36]. Supplementing the medium with L-tryptophan plays an important role in this process, as it serves as a precursor for bacterial IAA biosynthesis. Tryptophan availability has been shown to enhance IAA production compared with cultures grown without precursor addition. The relatively high proportion of positive isolates suggests that IAA production is a common trait among nodule-associated bacteria.

IAA is an important phytohormone involved in plant growth regulation, particularly in root initiation, cell elongation, and cell division [36]. Therefore, isolates capable of producing IAA may promote root system development and improve nutrient uptake in peanut plants. The presence of multiple IAA-producing isolates suggests their potential as plant growth-promoting inoculants, although quantitative determination of IAA levels is needed to evaluate their effectiveness.

3.4. Phosphate Solubilizing Activity.

Another PGPR trait evaluated was phosphate solubilization. All tested rhizobacterial isolates solubilized phosphate, as indicated by the formation of a clear halo around the colonies when grown on Pikovskaya agar (Figure 3). Halo formation results from the solubilization of insoluble tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) present in the medium, which is commonly used as an indicator of phosphate-solubilizing activity [37].

Quantitative assessment using the Phosphate Solubilization Index (PSI) showed that all isolates exhibited good solubilizing capacity, with PSI values greater than 2.15 (Table 4). The ability to convert insoluble phosphate into plant-available forms is a key characteristic of PGPR [37]. Through the production of organic acids and proton release, bacteria can lower the pH of

the surrounding medium, leading to the release of soluble orthophosphate ions (e.g., H_2PO_4^- and HPO_4^{2-}), which can be readily absorbed by plants.

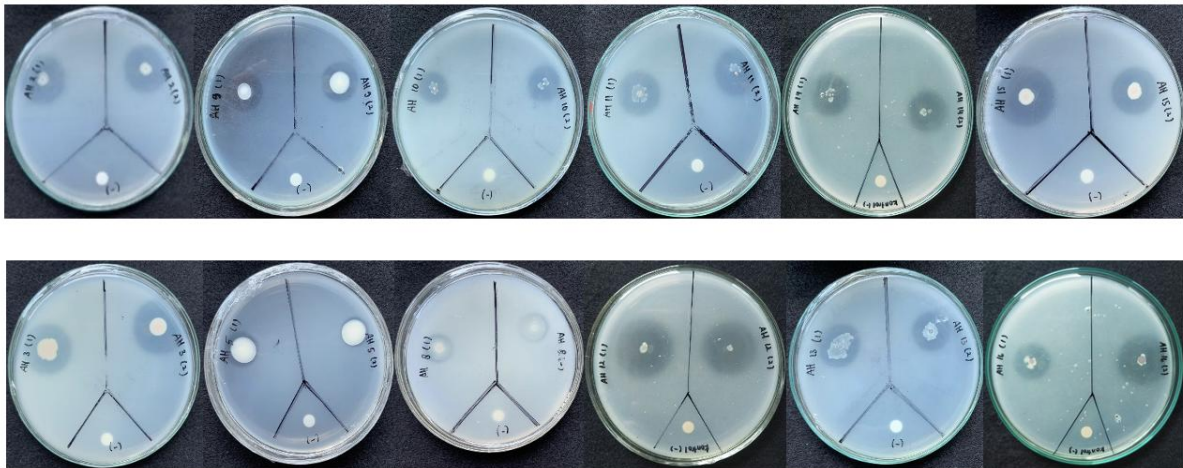


Figure 3. Phosphate Solubility Test.

The presence of phosphate-solubilizing activity in all isolates suggests that nodule-associated bacteria may contribute not only to nitrogen nutrition but also to phosphorus availability in the rhizosphere. This dual nutrient-supporting function may enhance plant growth and nutrient uptake efficiency in peanut cultivation, particularly in soils where phosphorus is present predominantly in insoluble forms.

3.5. Protease Production Activity.

Protease activity screening showed that 10 of the 12 tested isolates (AH3, AH8, AH9, AH10, AH11, AH12, AH13, AH14, AH15, and AH16) were positive (Figure 4). Positive activity was indicated by a clear halo surrounding colonies grown on skim milk agar (SMA). Halo formation reflects casein hydrolysis in the medium, demonstrating the isolates' ability to secrete extracellular protease enzymes [38].

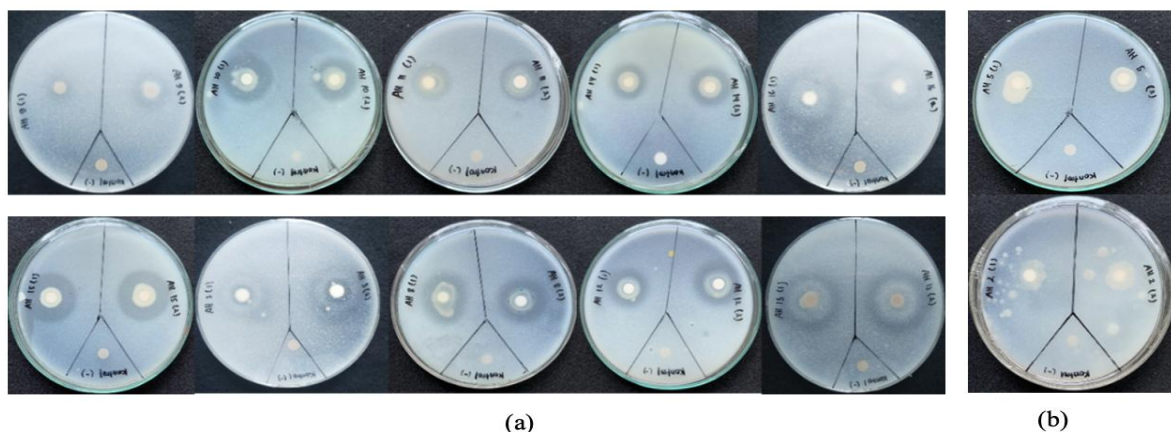


Figure 4. Protease Enzyme Synthesis Test: (a) Positive; (b) Negative.

Quantitative evaluation using the Proteolytic Index (PI) showed that all positive isolates exhibited strong proteolytic activity, with PI values >1.00 (Table 4). The degradation of casein into smaller peptides and amino acids confirms the enzymatic breakdown of protein substrates in the medium.

Protease production is an important indirect plant growth-promoting trait because extracellular enzymes can help suppress phytopathogens. Proteolytic enzymes may degrade

structural proteins and other components associated with pathogen cell integrity, thereby inhibiting pathogen colonization in the rhizosphere. Consequently, isolates with protease activity may contribute to plant protection in addition to their nutritional and hormonal roles [39].

The relatively high proportion of protease-producing isolates suggests that nodule-associated bacteria may function not only as symbionts or nutrient providers but also as potential biocontrol agents. However, further antagonistic assays against specific plant pathogens would be required to confirm their biocontrol effectiveness.

3.6. Selection of Superior Isolates as PGPR.

Based on the screening of PGPR traits (Table 4), the 12 isolates exhibited different functional characteristics across the tested parameters. Six isolates (AH2, AH5, AH9, AH10, AH11, and AH14) demonstrated nitrogen-fixing ability. Because biological nitrogen fixation is a principal characteristic of rhizobia and a key mechanism supporting plant nutrition, these isolates were considered the most promising candidates. Rhizobia are known to infect legume roots, form nodules, and convert atmospheric nitrogen into plant-available forms, thereby directly contributing to plant growth [33]. Therefore, we selected the six nitrogen-fixing isolates for further molecular identification using 16S rRNA gene analysis.

Table 4. Screening Results of Isolates' Ability Test as PGPR Qualitatively.

Code	Nitrogen Fixation	IAA Production	Phosphate Solubilization (PSI)	Protease Activity (PI)	Gram Staining
AH 2	++	+++	5,91	-	Negative
AH 3	-	-	4,08	2.33	Negative
AH 5	+	-	2,33	-	Negative
AH 8	-	++	8,98	1.60	Negative
AH 9	++	+	3,11	2.64	Negative
AH 10	+	+++	5,38	2.00	Negative
AH 11	+	-	4,62	1.31	Negative
AH 12	-	++	13,24	1.60	Negative
AH 13	-	-	3,28	1.36	Negative
AH 14	+	+++	6,54	1.67	Negative
AH 15	-	+	4,91	2.38	Negative
AH 16	-	+++	7,67	1.37	Negative

Description: “-” : Negative; “+” : Low; “++” : Medium; “+++” : High; “++++” : Very High

3.7. BOX PCR and Molecular Identification Based on 16S rRNA.

Genomic DNA extracted from the selected isolates showed acceptable concentration and purity (Table 5). DNA purity was evaluated based on the A260/A280 ratio, with values between 1.8 and 2.0 indicating minimal protein contamination [40, 41]. The obtained ratios confirmed that the extracted DNA was suitable for PCR amplification.

Table 5. Results of DNA Concentration and Purity of Rhizobacteria Isolates.

Code	Nucleic Acid Concentration (ng/μL)	Absorbance		A260/A280	Sample Type
		A260	A280		
AH2	2695,5	54,577	26,479	2,03	DNA
AH5	1322	26,440	13,251	1,99	DNA
AH9	680,2	13,603	6,305	2,15	DNA
AH10	2840,9	55,763	27,912	1,99	DNA
AH11	3002	60,040	29,999	2,00	DNA
AH14	2121,5	42,431	21,126	2,01	DNA

BOX-PCR fingerprinting was performed to evaluate the genetic diversity among candidate isolates and to avoid sequencing genetically identical strains. This approach has been widely used to assess heterogeneity among rhizobial isolates [22]. The amplification patterns (Figure 5) revealed distinct banding profiles for most isolates. However, two isolates (AH10 and AH14) displayed highly similar band patterns, indicating that they were likely closely related or genetically identical strains. Consequently, AH14 was selected for sequencing as representative because its PGPR traits were greater than AH10 (Table 4). Five isolates (AH2, AH5, AH9, AH11, and AH14) were therefore subjected to 16S rRNA gene analysis for molecular identification.

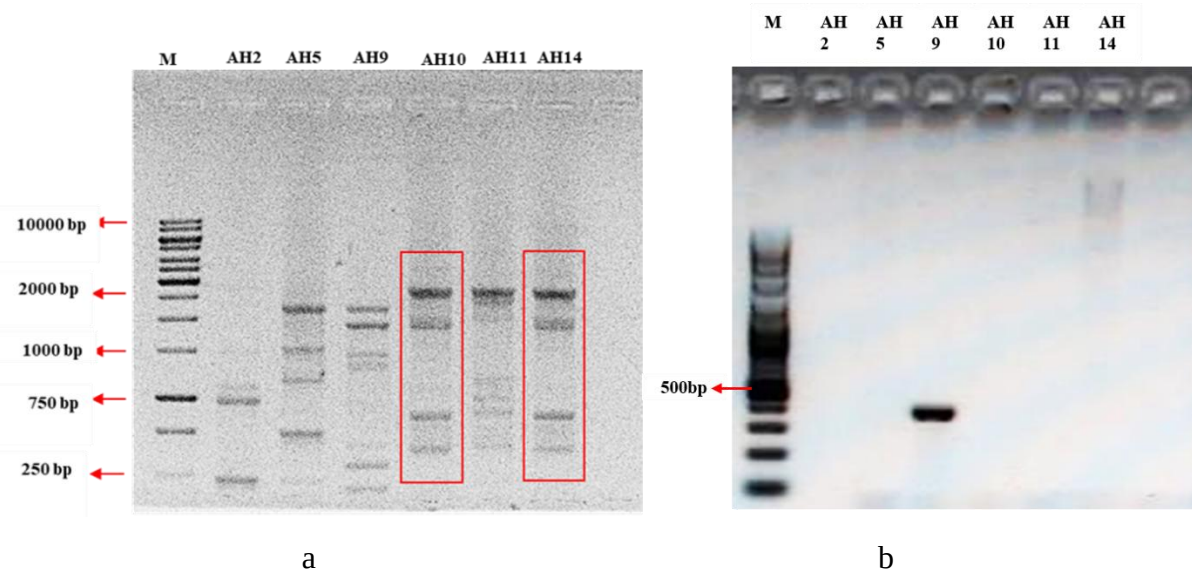


Figure 5. Molecular characterization of rhizobial isolates. **(a)** BOX-PCR fingerprinting profiles of isolates AH2, AH5, AH9, AH10, AH11, and AH14, showing genetic diversity among the isolates. Lane M: marker. The distinct banding patterns indicate genomic variability, while similar profiles observed in AH10 and AH14 suggest a close genetic relationship. **(b)** PCR amplification of the *nodA* gene, a key nodulation gene involved in the establishment of symbiosis between rhizobia and leguminous plants. A specific amplicon of approximately 400 bp was detected exclusively in isolate AH9, whereas no amplification was observed in the other isolates. The presence of the *nodA* gene in AH9 supports its potential role in root nodulation and biological nitrogen fixation in peanut plants.

PCR amplification of the 16S rRNA gene was successfully performed for five isolates (AH2, AH5, AH9, AH11, and AH14) using universal primers 27F and 1492R. Agarose gel electrophoresis (1%) revealed clear single bands of approximately 1,500 bp for all amplified samples (Figure 6), corresponding to the expected size of the bacterial 16S rRNA gene. The presence of distinct bands without smearing indicated good DNA quality and successful amplification. The 16S rRNA gene is widely used for bacterial identification because it is conserved among bacteria but contains variable regions that allow differentiation at the genus and, in many cases, species level [42].

The obtained sequences were analyzed using BLASTn through the NCBI database to determine their closest relatives in GenBank. The BLAST results (Table 6) were interpreted based on three main parameters: percent identity, query coverage, and e-value. Sequences showing high percent identity and query coverage, together with an e-value approaching zero, indicate a strong match with reference sequences [43].

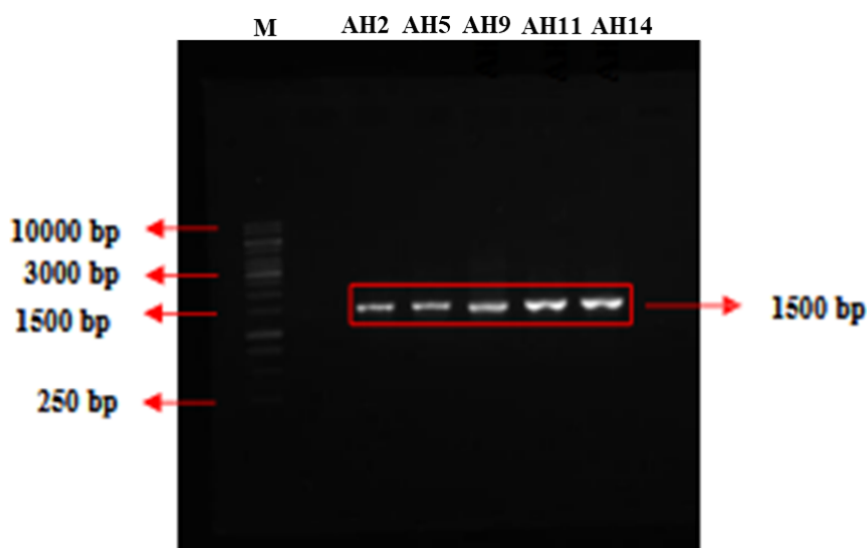


Figure 6. Visualization results of 16S gene amplification with 1% agarose gel

Table 6. BLASTN 16S rRNA results of bacteria.

Code	Description	Max Score	Total Score	Query Cover	E-value	Percent Identity	Accession Number
AH2	<i>Ralstonia</i> sp. strain 22TCJT01-2	2041	2041	99%	0,0	97,82%	OP404239.1
AH5	<i>Burkholderia cepacia</i> strain CM1	1941	1941	97%	0,0	97,24%	KP340218.1
AH9	<i>Martinezella tropici</i> strain SARCC-715	1862	1862	95%	0,0	97,28%	MZ149921.1
AH11	<i>Ralstonia</i> sp. strain T21-1	2028	2028	96%	0,0	99,12%	MG254771.1
AH14	<i>Ralstonia</i> sp. strain 22TCJT01-2	2002	2002	96%	0,0	98,68%	OP404239.1

Percent identity reflects the degree of similarity between the query and reference sequences, with higher values indicating closer taxonomic relationships [44]. Generally, a similarity value $\geq 97\%$ suggests identification at the genus level, whereas values around 99% or higher may indicate species-level similarity [45]. Query coverage represents the proportion of the sequence aligned with the database entry, while the e-value indicates the statistical significance of the match; lower e-values indicate more reliable identifications [43, 44]. Overall, the BLAST analysis enabled taxonomic assignment of the candidate isolates and confirmed that the selected nitrogen-fixing bacteria belong to rhizobial or closely related groups, supporting the phenotypic screening results obtained during PGPR characterization. The AH9 isolate sequence was deposited in the NCBI database with accession number PZ477852.

3.8. Phylogenetic Analysis.

Among the sequenced isolates, one isolate (AH9) was confirmed as a member of the rhizobial group. BLAST analysis of the 16S rRNA gene sequence of isolate AH9 showed 97.28% similarity to the closely related species *Martinezella tropici* strain SARCC-715. To further determine its taxonomic position, phylogenetic analysis was conducted using MEGA 11 software. A phylogenetic tree was constructed using the Neighbor-Joining method with 1.000 bootstrap replications.

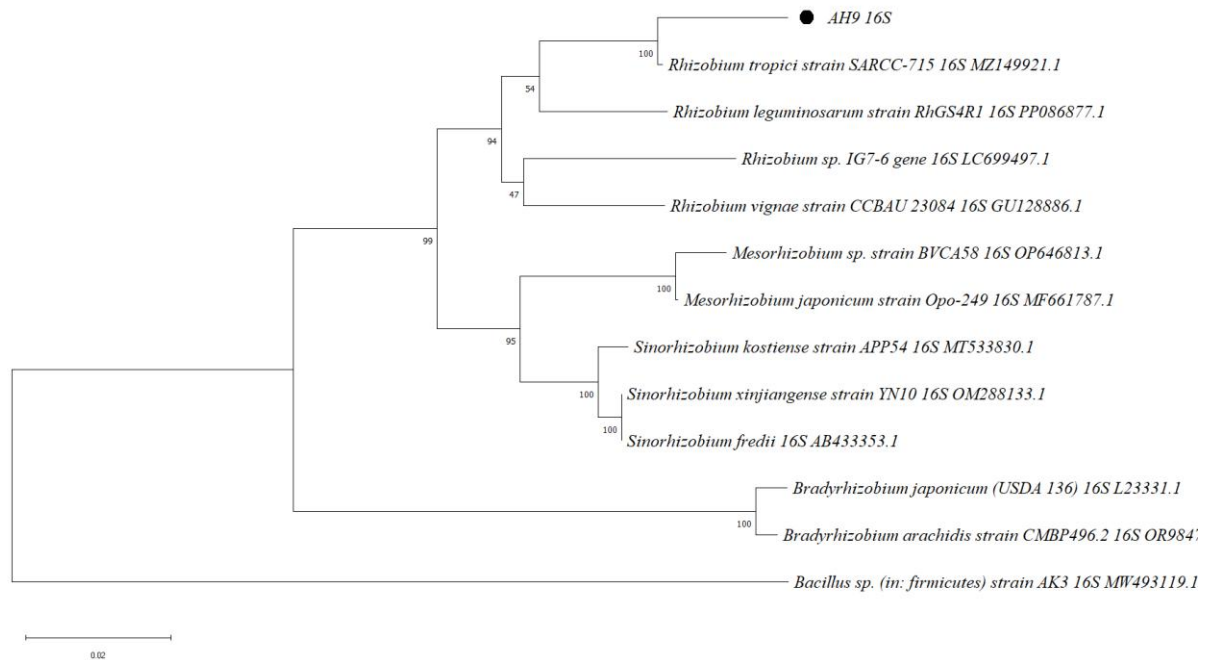


Figure 7. Phylogenetic Tree of Isolate AH9. The phylogenetic tree was constructed using MEGA XI software with the Neighbor-Joining method and 1000 bootstrap replications. Several Rhizobia isolates are included as ingroups and *Bacillus* sp. as an outgroup.

The analysis included several related species as ingroups (*Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium* species), while *Bacillus* sp. strain AK3 (phylum Firmicutes) was used as an outgroup to root the tree. The phylogenetic tree (Figure 7) showed that isolate AH9 clustered within the *Rhizobium* clade and formed a close relationship with *M. tropici* strain SARCC-715, supported by a bootstrap value of 100%, indicating strong phylogenetic confidence ($\geq 70\%$ is generally considered reliable) [38].

Martinezella tropici belongs to the family Rhizobiaceae within the class Alphaproteobacteria and is a fast-growing rhizobial species that typically forms visible colonies within 3-5 days. The colony morphology of isolate AH9-circular, convex, mucoid, entire margin, and white-was consistent with previously reported characteristics of *M. tropici* [46]. The physiological traits of isolate AH9 further supported this identification. The isolate demonstrated nitrogen fixation, IAA production, phosphate solubilization, and protease activity, which are recognized PGPR characteristics. Previous studies have similarly reported that *M. tropici* strains possess multiple plant growth-promoting functions, including biological nitrogen fixation, phytohormone production, phosphate solubilization, and extracellular enzyme activity [47–49].

3.10. Effectiveness on Peanut Plant Growth.

Isolate AH9 (closely related to *M. tropici*) was selected for further *in planta* validation on peanut plants. The other isolates (AH2, AH5, AH11, AH14) are closely related to pathogen species and raised biosafety concerns.

Based on growth parameters after 28 days, isolate AH9 significantly affected plant height, fresh weight, dry weight, and leaf number (Figure 8). For plant height, plants inoculated with AH9 grew to 25.12 cm, taller than the control plants at 18 cm. The fresh and dry weight parameters for the AH9 inoculation treatment were 7.73 g and 2.71 g, respectively, compared to 6.95 g and 2.43 g in the control. Isolate AH9 possesses the ability to produce growth

hormones, such as indole-acetic acid (IAA) and cytokinins, which stimulate cell division and nutrient uptake by roots, thereby impacting stem elongation. According to Sugiyanti and Octafiani [50], bacteria of the genus *Rhizobium* sp. Are capable of enhancing plant growth through phytohormone synthesis. Furthermore, research by Irfan [51] demonstrated that PGPR can improve the growth and yield of peanut plants—specifically regarding growth parameters like stem height, number of leaves, and dry weight—through synergistic interactions with the rhizosphere environment.

The number of leaves in the peanut plants treated with AH9 was 23, whereas the control had 16. The number of leaves is closely related to the nitrogen fixation capacity of PGPR bacteria. PGPR can fix atmospheric nitrogen and increase plant nutrient availability by solubilizing bound phosphorus (P). Consequently, the availability of N and P nutrients in the growing medium increases, making them accessible for plant uptake. These N and P elements can enhance the photosynthetic process and chlorophyll production, which in turn influences leaf blade development, leading to an increased number of leaves. According to Mu *et al.* [52], the increase in leaf area and number is influenced by the presence of nitrogen, which stimulates overall growth. Nitrogen plays a vital role in the formation of chlorophyll, which is essential for photosynthesis—the process wherein starch is produced for the plant.

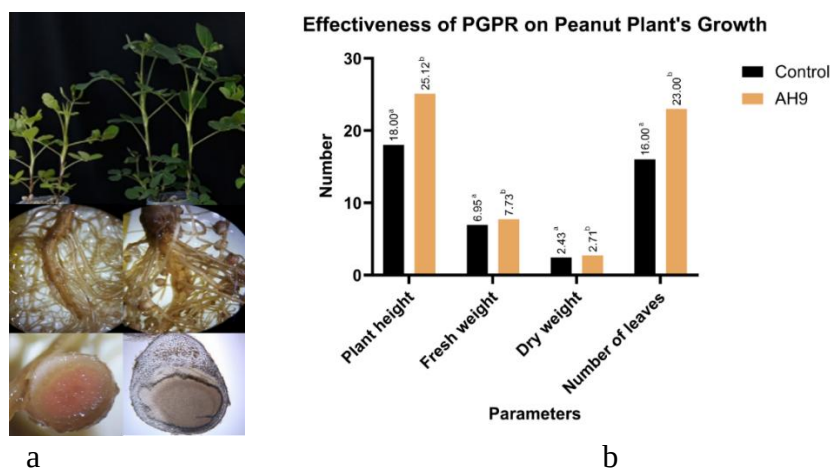


Figure 8. In planta validation of PGPR isolate AH9 on peanut (*Arachis hypogaea* L.) growth. **(a)** Representative images showing the phenotypic response of peanut plants inoculated with AH9, including enhanced shoot growth, root development, and root nodulation compared with the uninoculated control. **(b)** Effect of AH9 inoculation on plant growth parameters, including plant height (cm), fresh weight (g), dry weight (g), and number of leaves. Values represent the mean $\pm$ standard deviation of biological replicates. Different superscript letters above bars indicate significant differences between treatments according to Duncan's Multiple Range Test (DMRT) at $p < 0.05$. AH9 treatment significantly increased all measured growth parameters relative to the control, demonstrating its potential as a plant growth-promoting rhizobacterium (PGPR).

4. Conclusions

Sixteen bacterial isolates were successfully obtained from peanut (*Arachis hypogaea* L.) root nodules and exhibited diverse macroscopic and microscopic characteristics. Among them, 12 isolates were Gram-negative, and 4 were Gram-positive. Screening for plant growth-promoting traits identified six isolates (AH2, AH5, AH9, AH10, AH11, and AH14) as potential PGPR candidates because they demonstrated nitrogen fixation, indole-3-acetic acid (IAA) production, phosphate solubilization, and protease activity. Based on BOX-PCR fingerprinting, five representative isolates (AH2, AH5, AH9, AH11, and AH14) were selected for molecular identification using 16S rRNA gene sequencing. Phylogenetic analysis revealed

that isolate AH9 was closely related to putative *Martinezella tropici*, showing 97.28% sequence similarity and strong phylogenetic support (bootstrap value 100%). Isolate AH9 significantly affected peanut plant growth based on in planta validation results. These findings indicate that peanut root nodules harbor multifunctional bacteria with potential application as biofertilizers.

Author Contributions

Conceptualization, D.W.; data curation, D.W.; investigation, A.A.K.P. and M.J.D.P.; writing—original draft preparation, D.W.; writing—review and editing, D.W., A.B., I.S., F.A.P. and I.D.N. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

Abbreviation	Definition
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic Acid
IAA	Indole-3-acetic-acid
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
NFb	Nitrogen-free Bromothymol Blue
PCR	Polymerase Chains Reactions
PGPR	Plant Growth–Promoting Rhizobacteria
PI	Proteolytic Index
PSI	Phosphate Solubilization Index
rRNA	Ribosomal Ribonucleic Acid
SMA	Skim Milk Agar
USDA	United States Department of Agriculture
YEMA	Yeast Extract Mannitol Agar
YEMB	Yeast Extract Mannitol Broth

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