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Characterization of nitrogen-fixing bacteria isolated from strawberry rhizosphere and their potency to increase seedling growth

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Abstract. *Fitriyani F, Hindersah R, Devnita R, Mubarok S, Nakayama M. 2025. Characterization of nitrogen-fixing bacteria isolated from strawberry rhizosphere and their potency to increase seedling growth. Biodiversitas 26: 1964-1974.* Nitrogen is vital for plant metabolism and strawberry production. Many farmers heavily rely on inorganic fertilizers to meet their nitrogen requirements, which can have adverse environmental impacts. Nitrogen-Fixing Bacteria (NFB) offer an alternative nitrogen source for sustainable strawberry cultivation. This study aimed to isolate and characterize NFB from potted soil and evaluate their potential to enhance strawberry growth and development. NFB was isolated using nitrogen-free media and characterized based on colony and cell morphology, biochemical properties, and growth-promoting abilities. A total of 21 NFB isolates were obtained from the rhizosphere and bulk soil of six-month-old potted strawberries. The isolates were predominantly Gram-negative and exhibited capsule formation. Eleven isolates underwent detailed biochemical characterization, and three were found to fix nitrogen, produce phytohormones, and synthesize exopolysaccharides. Among these, *P. megaterium* IA-7 exhibited the highest production of nitrogen (4.23 μmol $mL^{-1}g^{-1}h^{-1}$), IAA (31.29 μg mL^{-1}) and gibberellin (21.73 μg mL^{-1}), while *P. aeruginosa* 4A-5 demonstrated superior EPS (4.6 g L^{-1}), and cytokinin production (1.47 μg mL^{-1}). The in-planta experiment revealed that a 1% inoculant significantly increased leaf number (32.5%) and crown diameter (42.44%) compared to the control treatment. These results underscore that the NFB isolated from the strawberry rhizosphere and bulk soil have promising potential as biofertilizers, offering sustainable alternatives to chemical fertilizers that reduce environmental impact and support eco-friendly farming.

Keywords: Exopolysaccharides, nitrogen fixer bacteria, PGPR, phytohormones, strawberry

Abbreviations: C: Carbon; EPS: Exopolysaccharides; N: Nitrogen; NFB: Nitrogen-Fixing Bacteria; PGPR: Plant Growth-Promoting Rhizobacteria

INTRODUCTION

Strawberries are an important crop with global production reaching 10.48 million tons annually (FAO 2023). They are rich in nutrients and antioxidants (Giampieri et al. 2014). In tropical areas with high temperatures, high CO_2 concentrations, and favorable rainfall conditions, strawberry growth is less optimal, resulting in lower yields and fruit quality (Balasooriya et al. 2020). High daily temperatures affect the metabolism of strawberries, reducing flower bud differentiation and fruit development (Menzel 2021; Ullah et al. 2024). Indonesia's tropical climate leads to weathered soils with low carbon and nitrogen (Lyu et al. 2022), while heavy rains increase nitrogen leaching, reducing root biomass and fruit quality (Schattman et al. 2022).

Nitrogen deficiency limits growth, whereas adequate nitrogen enhances yield and fruit quality (Katel et al. 2023). Nitrogen is a pivotal nutrient for plant development and significantly influences strawberry yield and quality (Hassan 2015). In strawberries, nitrogen plays a crucial role in the photosynthesis process and enzymatic activity, thereby influencing the growth, yield, and quality of strawberries (Katel et al. 2023; Li et al. 2024). To address nutrient deficiencies, strawberry farmers often rely heavily on inorganic fertilizers. However, the majority of essential raw materials for fertilizer production are imported, making them costly and inaccessible for smallholder farmers (Wildayana et al. 2018).

Additionally, excessive fertilizer use disrupts nutrient balance and reduces soil microbial diversity (Kashyap et al. 2023; Kong et al. 2024). Long-term use without organic inputs leads to nitrogen buildup and greenhouse gas emissions (Jote 2023; Qiu et al. 2023), posing health risks (Yang et al. 2022). Exploring effective biofertilizers is critical to reducing reliance on inorganic fertilizers by enhancing nutrient use efficiency and increasing nutrient availability for strawberry growth.

Utilizing renewable and sustainable resources, such as soil-beneficial rhizobacteria, as biofertilizers has become a pivotal approach in regenerative agriculture. The Plant Growth-Promoting Rhizobacteria (PGPR) offer a viable alternative to chemical fertilizers, supporting strawberry production (Hindersah et al. 2021) through mechanisms such as nitrogen fixation, phosphate solubilization, and the production of plant growth hormones (Ruzzi and Aroca 2015; Chebotar et al. 2022; Paliwoda et al. 2022). Nitrogen-Fixing Bacteria (NFB), as PGPR enhance root growth via IAA production, improving nutrient uptake (Aasfar et al. 2021). Their nitrogen fixation potential is crucial in tropical soils (Pajares and Bohannan 2016). Some isolates belonging to the genus *Bacillus* from a tropical sanctuary determine variations in nitrogen fixation (Yousuf et al. 2017). NFB consists of *Azotobacter chroococcum, A. vinelandii, Azospirillum* sp., and *Acinetobacter* sp. improve strawberry development (Hindersah et al. 2021). *Priestia megaterium* help mitigate heat stress (Shaffique et al. 2024). Some produce EPS, supporting soil structure and drought survival (Sivapriya and Priya 2017; Khan and Bano 2019). High-EPS-producing NFB like *A. salinestris, P. megaterium,* and *P. aeruginosa* support strawberry growth under stress (Ali et al. 2023; Kurniati et al. 2024; Naseem et al. 2024). Therefore, utilizing *A. salinestris, P. megaterium,* and *P. aeruginosa* can be used as potential NFB for increasing strawberry growth and yield.

Bacterial consortia improve effectiveness through synergy (Al Mamun et al. 2024). Isolates from the strawberry rhizosphere increases their adaptability during inoculation and already accustomed to root exudates and rhizosphere conditions. Root exudates act as chemical signals, enhancing colonization and nutrient uptake (Chen and Liu 2024). Root exudates, which serve as a primary carbon source, significantly influence the microbial composition in the rhizosphere, highlighting the importance of isolating strains directly from strawberries. This gap underscores the need for region-specific studies to assess the effectiveness of biofertilizers under diverse environmental conditions. Studies have demonstrated that NFB inoculation enhances strawberry yield in subtropical regions (de Andrade et al. 2019), limited data exist for the tropics. Furthermore, understanding environmental conditions and plant-microbe interactions plays a significant role in optimally increasing strawberry production. Based on the above rationale, our research focused on strategies to isolate the potential NFB as PGPR for increasing strawberry growth, particularly in tropical regions.

MATERIALS AND METHODS

Study area

Soil samples were obtained from potted strawberries cultivated at the "Sweetberry Agrowisata" field in Pacet, Cianjur, West Java (6°42'24" S and 107°02'48" E), situated at an altitude of 959 m above sea level with an annual rainfall of 733 mm. The pot experiment was conducted in a greenhouse belonging to the Faculty of Agriculture, Universitas Padjadjaran (6°55'12.1" S and 107°46'22.9" E). During soil sampling, the mean daily temperature and humidity were recorded at 28°C and 39%, respectively

Procedures

Soil sampling

Permissions for soil sample collection were obtained from the landowners prior to the sampling being conducted. Soil samples were collected during the dry season in October 2022. Sampling focused on the rhizosphere at 0-20 cm (upper portion of potted soil) of six-month-old strawberry plants and the bulk soil at 20-50 cm (lower portion of potted soil) with three pots and for each condition and composite for each rhizosphere and bulk soil condition, as illustrated in Figure 1. Samples were collected in duplicate from both the rhizosphere and the bulk soil.

Figure 1. A. Strawberry potting in the Pacet district; B. Size of six-month-old potted strawberries; C. Depth of the upper part of the potted strawberries

Isolation of nitrogen-fixing bacteria

The Nitrogen-Fixing Bacteria (NFB) were isolated using N-free Ashby's Mannitol selective broth composed of 10 g L^{-1} mannitol, 0.2 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} $CaSO_4 \cdot 2H_2O$, 0.2 g L^{-1} NaCl, and 5 g L^{-1} $CaCO_3$. Soil samples from the rhizosphere and bulk soil were added to 50 mL of Ashby's broth in 100 mL vial bottles, each containing 5 g of soil. Cultures were incubated at room temperature (25-28°C) for 3-5 days until pellicles formed on the surface of the broth. A loop of pellicle was sub-cultured onto Ashby plate agar and incubated at 30°C for 3 days. Colonies were selected based on morphology and purified through streaking on Ashby plate agar, with three zones, repeated three times. Pure cultures of the isolates were maintained on Ashby slant agar at 4°C and sub-cultured monthly to maintain their viability. Total plate counts with serial dilution were conducted to estimate viable NFB populations in the samples (Ben-David and Davidson 2014).

Morphological and biochemical tests

Colony morphology was assessed following Borkar's method (2017). Colonies were sub-cultured on Ashby slants before performing morphological and biochemical analyses. Morphological tests included evaluating colony morphology, cell morphology, Gram staining, and capsule staining. Additional cellular morphology was determined using procedures outlined by Seltmann and Holst (2013). Capsulated NFB isolates underwent biochemical characterization, including glucose fermentation, catalase activity, motility, oxidase activity, citrate utilization, indole production, triple sugar iron test, and nitrate reduction, as per Bhumbla (2018). The three highest-ranked biochemical tests, based on multiple traits, were selected. The three isolates with the highest populations in Ashby broth were selected for bioassays to validate their plant growth-promoting mechanisms.

Extraction of isolate DNA

The identification of bacterial isolates was conducted by determining and analyzing the 16S rRNA gene sequences. Total genomic DNA was extracted from the bacterial strains using the Presto Mini Genomic DNA Bacteria kit, following the kit protocol. Bacterial isolates were grown in Ashby's broth until a concentration of 1 × 10^9 CFU mL^{-1} was reached. Approximately 1.5 mL of culture was transferred to a microcentrifuge tube, spun for 1 minute, and then the supernatant was discarded. Add 180 µL GT buffer, then resuspend the cell pellet (for Gram-positive isolates). Transfer 200 µL Gram+ Buffer and add lysozyme (0.8 mg/200 µL) to a 15 mL centrifuge tube. Then, vortex to dissolve the lysozyme. Transfer 200 µL of Gram+ Buffer and lysozyme to the sample in the 1.5 mL microcentrifuge tube and resuspend the pellet. Incubated at 37°C for 30 minutes and inverted the tube every 10 minutes (for Gram+ isolate). Add 20 µL of proteinase K, then mix by vortexing. During incubation, invert the tube every 3 minutes. Add 200 µL of GB Buffer mix for 10 seconds and incubate at 70°C for 10 minutes until the lysate clears. Then, preheat the Elution Buffer to 70°C. Add 200 µL of ethanol and mix thoroughly. Transfer the mixture to the GD Column in a 2 mL collection tube. Centrifuge at 16,000 rpm for 2 minutes, then discard the flow-through. Add 400 µL of W1 Buffer to the GD column centrifuge and centrifuge at 16,000 rpm for 30 seconds to wash the pellet. Then, discard the flow-through. Add 600 µL of wash buffer, centrifuge for 30 seconds, and discard the flow-through. DNA was dissolved in TE buffer.

Polymerase chain reaction amplification of isolate DNA

The 16S rDNA gene was amplified via Polymerase Chain Reaction (PCR) using universal primers F 16F27/Sequence (`AGA GTT TGA TCM TGC CTC AG`) and primer R 16R 1492/Sequence (`TAC GGY TAC CTT GTT ACG ACT T`), with the KOD FX Neo PCR Kit (Toyobo Co., Ltd.) with the KOD FX Neo PCR Kit (Toyobo Co., Ltd.), following the kit protocol (Bangun et al. 2023). The PCR conditions included an initial denaturation at 94°C for 2 minutes, followed by 34 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 30 seconds, concluding with a final extension at 68°C for 5 minutes. The amplified 16S rRNA sequences were compared against known sequences in the NCBI database using the BLAST (Basic Local Alignment Search Tool) algorithm to identify the closest matches and confirm bacterial species. The phylogenetic tree was constructed using MEGA11 software.

Determination of rhizobacteria plant growth-promoting test

The plant growth-promoting traits of the three Nitrogen-Fixing Bacteria (NFB) isolates were evaluated based on their nitrogenase activity, Exopolysaccharide (EPS) production, and phytohormone synthesis (IAA, cytokinin, and gibberellin). Each experiment was repeated twice and conducted with three replicates per treatment. The mean values were calculated and analyzed statistically. Nitrogenase activity was determined using the Acetylene Reduction Assay (ARA) with an uninoculated growing medium serving as the control (Montes-Luz et al. 2023). EPS production was quantified using the gravimetric method described by Hindersah et al. (2017), with the control consisting of an uninoculated medium. The phytohormone procedure was followed as described by Amaresan et al. (2022). Indole-3-Acetic Acid (IAA) production was measured using a colorimetric assay with Salkowski's reagent, and both negative and positive controls were included for comparative analysis. Gibberellin and cytokinin production were analyzed using acetonitrile extraction and quantified through High-Performance Liquid Chromatography (HPLC). A standard curve was constructed using standard Gibberellic Acid (GA3) and zeatin solutions at concentrations ranging from 0 to 100 µg mL^{-1}.

Evaluation of rhizobacteria on strawberry growth

The bacterial liquid inoculant used in this study consisted of a balanced mixture of the *Prestia megaterium* strain IA-7, *Azotobacter salinestris* strain 3A-1, and *Pseudomonas aeruginosa* strain 4A-5. The experiment was conducted on the strawberry (*Fragaria* × *ananassa*) 'Benihoppe,' initially introduced in Japan.

A Completely Randomized Design (CRD) was employed with three treatments and six replications. The treatments included a control (without bacterial inoculation) and Nitrogen-Fixing Bacterial (NFB) liquid inoculants applied at concentrations of 1% and 2% (% w/w). Six-month-old strawberry seedlings, cultivated in a controlled nursery, were transplanted into a growth medium consisting of field soil, poultry manure, and charcoal paddy husk in a 1:1:3 ratio, using 20 cm-high black polyethylene bags.

The liquid inoculant was prepared by suspending bacterial isolates grown on slate agar in a 10 mL sterilized physiological salt solution (0.85% NaCl). The initial Optical Density (OD) of each isolate suspension at the wavelength 600 nm gave approximately cell density of *A. salinestris* (OD = 1.79, 6.35×10^{10} CFU mL^{-1}), *P. megaterium* (OD = 0.659, 6.65×10^{10} CFU mL^{-1}), *P. aeruginosa* (OD = 0.29, 6.62×10^{10} CFU mL^{-1}), therefore mixed into 1:1:1 (v/v) ratio. Inoculant application of 16 mL (1%) and 32 mL (2%) was administered to the soil surface surrounding the plant roots on the 7th and 28th days post-planting. After dilution into 1%, the mixed density was 6×10^8, while the 2% was 1.3×10^9 CFU mL^{-1}.

Consequently, the plants were given a supplemental nutrient solution containing 1.2 g calcium nitrate, 0.4 g potassium nitrate, 0.4 g monopotassium phosphate, 0.8 g magnesium sulfate, 0.08 g Fe (13%), 0.016 g boric acid, 0.016 g manganese sulfate, 0.008 g zinc sulfate, 0.016 g copper sulfate, 0.0016 g ammonium molybdate, and 1 L distilled water with a conductivity of 1000 µS cm^{-1}. Fertilization was integrated into irrigation, with 50 mL applied per pot daily for the first two weeks and increased to twice daily until six weeks after transplanting.

Data analysis

Growth parameters, including plant height, leaf number, and crown diameter, were measured every two weeks at 2, 4, and 6 weeks after transplanting. Data were expressed as mean±Standard Deviation (SD) with six replications. Statistical analysis of data from six-week-old plants was performed to test for normality and homogeneity of variances using Analysis of Variance (ANOVA), with significance determined at $p<0.05$. Post hoc comparisons were conducted using Duncan's Multiple Range tests to identify significant differences between treatments, as analyzed using IBM SPSS statistical software version 27.0.

RESULTS AND DISCUSSION

Morphological characteristics of bacterial isolates

The presence of a pellicle on the surface of the media (Figure 2.A) confirmed the successful isolation of NFB. The pellicle exhibited a brown coloration, indicating pigment production by the NFB. The thickness of the pellicle in Ashby broth containing rhizosphere soil was comparable to that observed in broth containing bulk soil. Twenty-one isolates were obtained, with 12 colonies from the rhizosphere and nine from the bulk soil (Table 1). Following a week of growth on Ashby's media, all isolates produced black and brown pigments except for isolates 2A-1, 2A-2, and 2A-5. The colony properties of the isolates on Ashby's plate agar were predominantly round and convex (Table 1). Colony colors varied, appearing cream, white, or brown, and they exhibited similar margins, forms, and elevations. The colonies grown on Ashby's agar medium were generally round, convex, and mucoid in texture (Figure 2.B).

The cell morphology characteristics of the isolates are detailed in Table 1. Most isolates exhibited a coccus cell shape, while isolates IA-3, 1A-5, 1A-6, 2A-2, 4A-3, 4A-4, and 4A-5 were identified as bacilli (Table 1). Most isolates were Gram-negative, although 1A-1, 1A-6, and 4A-3 were Gram-positive (Figure 3). Additionally, 11 isolates were observed to form capsules surrounding their cells. The presence of a capsule suggests that these NFB produce EPS. Consequently, isolates exhibiting capsule formation were selected for further biochemical testing.

A B

Figure 2. Pellicle formation on Ashby's medium broth with brown pigment: A. Indicating successful isolation of NFB; B. Pure culture isolation using the streak plate method

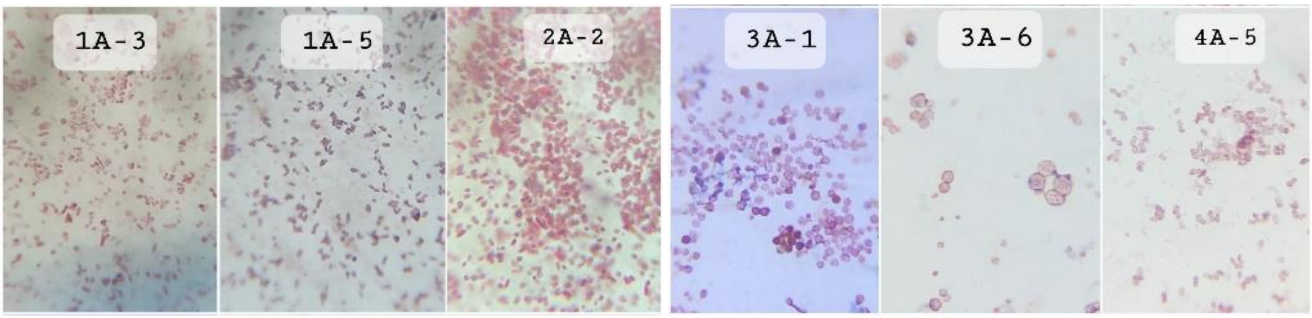

Figure 3. Results of Gram staining of nitrogen-fixing bacterial isolates from: A. The rhizosphere; and B. The bulk soil of strawberry plants, indicating the characteristics of each isolate

Table 1. Morphological traits of nitrogen-fixing bacteria isolated from strawberry plants' rhizosphere and bulk soil

Source of isolates	Code	Morphology of colony			Gram	Capsule	Cell shape
		Color	Form	Elevation			
Rhizosphere	1A-1	Dark-brown	Round	Convex	+	-	Coccus
	1A-2	Brown	Round	Convex	-	-	Coccus
	1A-3	Brown	Round	Convex	-	-	Bacilli
	1A-4	Brown	Round	Convex	-	-	Coccus
	1A-5	Brown	Round	Convex	-	-	Bacilli
	1A-6	Brown	Round	Convex	+	-	Bacilli
	1A-7	Dark brown	Round	Convex	+	+	Bacilli
	2A-1	Cream	Round	Convex	-	+	Coccus
	2A-2	Cream	Round	Convex	-	+	Bacilli
	2A-3	Brown	Round	Convex	-	+	Coccus
	2A-4	Brown	Round	Convex	-	-	Coccus
	2A-5	Cream	Round	Convex	-	+	Coccus
Bulk soil	3A-1	Brown	Round	Convex	-	+	Coccus
	3A-2	Brown	Round	Convex	-	-	Coccus
	3A-3	Brown	Round	Convex	-	+	Coccus
	3A-4	Brown	Round	Convex	-	+	Coccus
	3A-6	Brown	Round	Convex	-	+	Coccus
	4A-3	Brown	Round	Convex	+	-	Bacilli
	4A-4	Cream	Round	Convex	-	+	Bacilli
	4A-5	Cream	Round	Convex	-	+	Bacilli
	4A-6	Cream	Round	Convex	-	-	Coccus

Biochemical characteristics of bacterial isolates

Before molecular identification using Polymerase Chain Reaction (PCR) amplification and 16S rRNA gene sequence, a biochemical test was conducted to compare the selected isolates to reference strains of *Azotobacter*, a widely recognized NFB used in biofertilizer formulations. Biochemical identification involves analyzing metabolic characteristics, oxygen relationships, and fermentation reactions to select the highest potential NFB for molecular identification. Eleven isolates exhibiting capsule formation were selected for biochemical characterization (Table 2). The carbohydrate fermentation test, employed to assess the ability of bacteria to ferment specific carbohydrates, showed that all isolates tested positive for fermentation with glucose, maltose, sucrose, mannitol, fructose, rhamnose, raffinose, and sorbitol. Most isolates demonstrated the ability to hydrolyze urea, except isolates 1A-7, 2A-2, 3A-4, and 3A-6.

All isolates exhibited growth at 35°C, with three isolates (2A-2, 3A-1, and 4A-4) identified as thermophilic, capable of growing at 60°C when incubated on a slant for 48 h. The citrate test indicated that isolates 1A-7, 2A-2, 3A-1, 3A-4, and 3A-6 were positive, suggesting their ability to utilize citrate via the enzyme citrate lyase, which decomposes citrate into oxaloacetate and acetate. Catalase activity was observed in six isolates (1A-7, 2A-2, 3A-1, 3A-6, 4A-4, and 4A-5), while the remaining five isolates tested negative. Indole test results showed positive outcomes for isolates 1A-7 and 2A-3, indicating their ability to produce the enzyme tryptophanase, which hydrolyzes tryptophan into indole, pyruvic acid, and ammonia.

The Triple Sugar Iron Agar (TSIA) test is employed for bacterial differentiation based on the fermentation of dextrose (glucose), lactose, and sucrose, as well as the production of hydrogen sulfide (H_2S). Results are interpreted through color changes, with the slant turning from red to yellow and the appearance of a black butt (Saimin et al. 2020). Most isolates exhibited an A/A reaction, except 2A-1, 2A-5, and 4A-5. An A/A result indicates fermentation of lactose or sucrose, while K/K suggests no fermentation of sugars. The development of black color on the butt after 24 h of incubation indicates H_2S production. None of the isolates showed gas production, suggesting the absence of H_2S production. Therefore, no isolates produced H_2S.

Biochemical profiling revealed that isolate 1A-7 was positive for all tests except urease, 3A-1 was positive except for indole, and 4A-5 was positive except for indole, citrate, and urease. Compared to the other eight isolates, which also showed negative results for these three tests but had additional negative reactions, 4A-5 demonstrated superior overall biochemical characteristics. These isolates (1A-7, 3A-1, and 4A-5) exhibited the most favorable biochemical characteristics and showed the highest similarity to *Azotobacter* sp., the target, and were therefore selected for further analysis.

Table 2. Biochemical properties for the identification of nitrogen-fixing bacterial species

Isolate code	Temp. 35°C	Temp. 60°C	Glucose	Maltose	Sucrose	Mannitol	Fructose	Ramnose	Raffi-nose	Sorbitol	Indole	Catalase	Citrate	Urease	Oxidase	TSI
IA-7	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	A/A
2A-1	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	K/K
2A-2	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	A/A
2A-3	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-	A/A
2A-5	+	-	+	+	+	+	+	+	+	+	-	-	-	+	-	K/K
3A-1	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	A/A
3A-3	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	A/A
3A-4	+	-	+	+	+	+	+	+	+	+	-	-	+	-	+	A/A
3A-6	+	-	+	+	+	+	+	+	+	+	-	+	+	-	-	A/A
4A-4	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	A/A
4A-5	+	-	+	+	+	+	+	+	+	+	-	+	-	+	-	K/K

Note: TSI: Triple Sugar Iron; K/A: Alkaline/Acidic; K/K: Alkaline/Alkaline; A/A: Acidic/Acidic

Table 3. Plant growth-promoting activities of nitrogen-fixing bacterial isolates

Isolate strain	Nitrogen fixation (µmol mL^{-1} g^{-1} h^{-1})	EPS production (g L^{-1})	IAA (µg mL^{-1})	Cytokinin (µg mL^{-1})	Gibberellin (µg mL^{-1})
P. megaterium strain IA-7	4.23 ± 0.08	4.4 ± 0.00	31.29 ± 3.72	1.39 ± 0.01	21.73 ± 0.13
A. salinestris strain 3A-1	1.54 ± 0.02	4.4 ± 0.03	15.98 ± 1.91	1.38 ± 0.03	12.41 ± 0.10
P. aeruginosa strain 4A-5	0.48 ± 0.00	4.6 ± 0.03	12.41 ± 0.17	1.47 ± 0.01	13.71 ± 0.26

Notes: Each value represents the means of three replicates±standard deviation (n = 3); Values in the same column marked with identical letter(s) are not significantly different at p<0.05

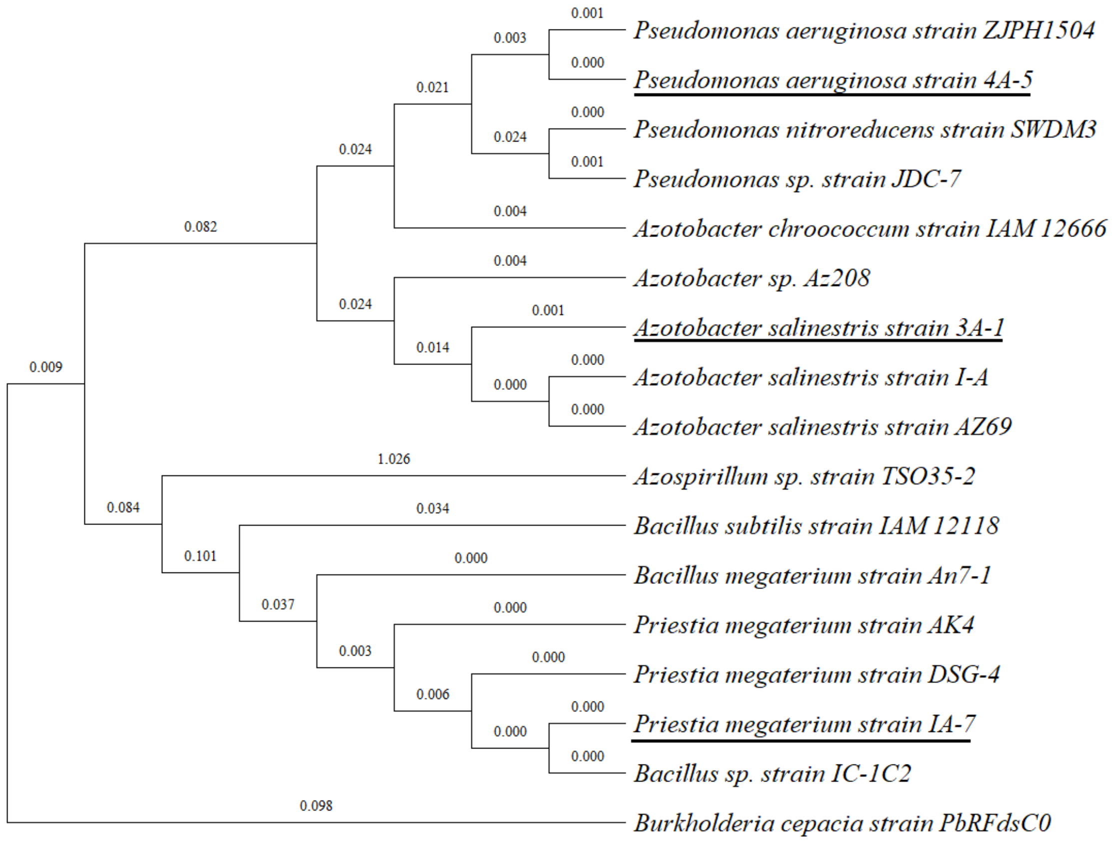

Figure 4. Phylogenetic analysis based on 16S rRNA gene sequences from the European Molecular Biology Laboratory (NCBI) database

Molecular characteristics of bacterial isolates

The 16S rRNA sequence analysis of the isolates (Figure 4) revealed high genetic similarity with known bacterial strains. Isolate 1A-7 showed 99.85% identity with *Priestia megaterium* strain AK4 and was closely related to *P. megaterium* strain DSG-4. Isolate 3A-1 exhibited 99.93% identity with *Azotobacter salinestris*. Similarly, isolate 4A-5 demonstrated 99.78% identity with the *P. aeruginosa* strain ZJPH1504 and clustered with other *Pseudomonas* species.

Plant growth-promoting rhizobacteria are characteristic of bacterial isolates

The three isolates of NFB exhibited nitrogenase activity and the ability to produce EPS and phytohormones, including IAA, cytokinin, and gibberellin (GA_3) (Table 3). The highest nitrogenase activity was observed in *Priestia megaterium* strain IA-7 (4.23 µmol mL^{-1}g^{-1}h^{-1}), followed by *Azotobacter salinestris* strain 3A-1 (1.54 µmol mL^{-1}g^{-1}h^{-1}) and *P. aeruginosa* strain 4A-5 (0.48 µmol mL^{-1}g^{-1}h^{-1}). Regarding EPS production, *P. aeruginosa* strain 4A-5 exhibited the highest level (4.6 g L^{-1}), while *P. megaterium* strain IA-7 and *A. salinestris* strain 3A-1 produced comparable amounts (4.4 g L^{-1}). Among the phytohormones, *P. megaterium* strain IA-7 produced the highest IAA (31.29 µg mL^{-1}) and GA_3 (21.73 µg mL^{-1}). In contrast, *P. aeruginosa* strain 4A-5 exhibited the highest cytokinin production (1.47 µg mL^{-1}).

Effect of N-fixer bacteria on strawberry growth

The efficacy of *Priestia megaterium* strain IA-7, *Azotobacter salinestris* strain 3A-1, and *P. aeruginosa* strain 4A-5 as an NFB consortium for promoting strawberry growth was evaluated by assessing plant growth, crown diameter, and leaves numbers (Figure 5). Two Weeks After Planting (WAP), a higher concentration of NFB did not affect plant height, the number of leaves, or crown diameter; however, it showed a slight increase in the number of leaves by four WAPs compared to the uninoculated plants. By six WAP, NFB inoculation significantly enhanced the number of leaves and diameter of the crown, although plant height remained unchanged. The number of leaves in these treatments ranged from 10.6 to 11.6 per plant, whereas the control had only 8.6 leaves per plant. It increased up to 32.5%.

Additionally, the crown diameter in inoculated treatments was significantly higher, 42.44% (16.48 mm) on concentration 1% and 46.53% (16.68 mm) on concentration 2%, compared to the un-inoculated treatment (15.15 mm). The plant height of strawberries on these treatments ranged between 21.58 and 22.22 cm. The number of leaves and diameter of crown strawberries treated with 1% and 2% NFB inoculants were notably more remarkable than those of the control group, with a slightly higher leaf number observed in plants receiving the 2% concentration.

Discussion

The potential of rhizobacteria isolated from the rhizosphere and bulk soil of strawberries grown in potted soil was examined and characterized through morphological, biochemical, and PGPR traits. The results indicate that rhizosphere bacteria promote beneficial interactions with plant-associated microbes. The significantly higher number of isolates in the rhizosphere compared to bulk soil reflects the rhizosphere's favorable conditions for the proliferation and activity of Nitrogen-Fixing Bacteria (NFB) due to the root exudates rich in sugars and amino acids. These components provide the energy and nutrients essential for NFB to transform atmospheric nitrogen (N_2) into ammonia as NH_3 (Rilling et al. 2018; Vats et al. 2021). The rhizosphere was rich in exudates, whereas the bulk soil also had abundant nutrients to support the proliferation of microbes. Soil properties significantly influence the rhizosphere microbiome, with a stronger correlation between soil characteristics and bacterial diversity in the rhizosphere than in bulk soil (Wei et al. 2023).

The colony morphology of selected isolates was generally round, convex, and pigmented (Table 1). It suggests that it is influenced by nutrient availability and growth conditions. Mostly, selected isolates had brown-like pigment. It was associated with melanin, a bacterial strategy to mitigate environmental stress, and can aid in identifying bacterial genera (Sajjad et al. 2020; Agarwal et al. 2023). This study investigated the formation of brown pigment in *A. salinestris* and *P. megaterium*. A study also found that brown pigment was formed in *P. aeruginosa* (Kimura et al. 2015). For the morphologies, the isolates were Gram-negative with coccus shapes, although Gram-positive bacilli were also observed. They were also selected for capsule formation. The capsule, as the bacterial cell's outermost layer, protects against desiccation, allowing the bacteria to survive under drought conditions. Some bacterial genera, including *Bacillus*, form polypeptide capsules around their cells for protection (St John et al. 2023). The formation of capsules in bacteria such as *Azotobacter* sp. is associated with EPS content (Hindersah et al. 2017).

As a nitrogen-free medium, Ashby's medium contains mannitol as the primary carbon source, which is predominantly utilized by the selected isolates (Table 2). It was indicated that all the selected isolates used mannitol. *Azotobacter* sp., *Pseudomonas* sp., and *Bacillus* sp. have also been found to utilize mannitol as a carbon source (Hoffmann and Altenbuchner 2015; Chen et al. 2018; Guzmán-Moreno et al. 2022). Unexpectedly, these bacteria also utilized glucose, maltose, fructose, rhamnose, raffinose, and sorbitol. The ability to metabolize various carbon sources is a characteristic feature of bacterial strains. Larrainzar et al. (2020) indicated that catalase activity is associated with enhanced nitrogenase and hemoglobin in symbiotic NFB. This is linked to oxidative protection enzymes that increase cellular tolerance to harmful oxygen species. Some non-symbiotic NFB, such as *Azotobacter*, are catalase-positive, which protects against toxic levels of H_2O_2. Furthermore, certain bacteria can survive at temperatures as high as 60°C, demonstrating the isolates' heat tolerance. *Bacillus* sp. forms spores to endure high-temperature environments (Berendsen et al. 2016).

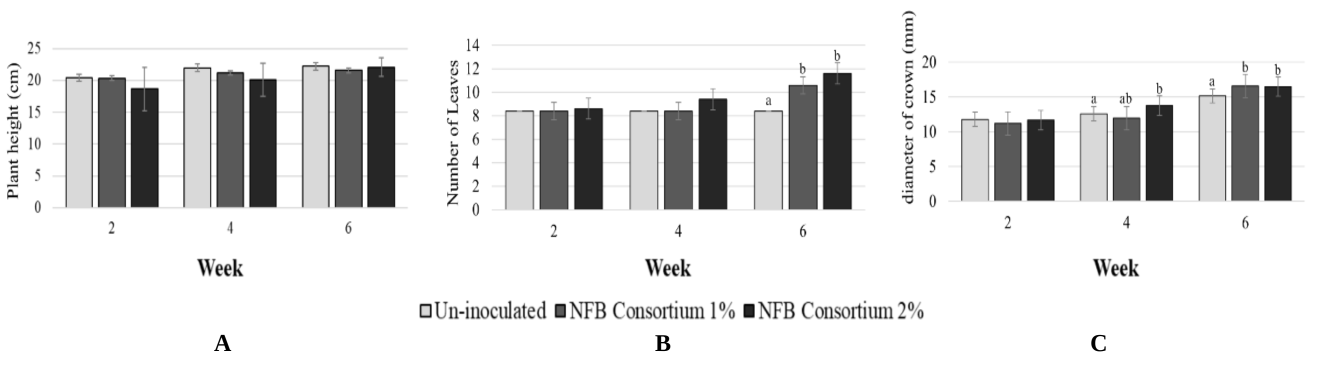

Figure 5. Impact of NFB inoculation on: A. Plant height; B. Number of leaves; C. Crown diameter of strawberries after six weeks of planting. Values represent means±standard error (n = 6). Identical letters indicate no significant difference between treatments, as determined by Duncan's test at the 5% significance level

Ashby's medium was selected for NFB because it is N-free and is commonly used to target *Azotobacter* sp. (Roa et al. 2016; Hindersah et al. 2019; Wakarera et al. 2022; Prawan et al. 2023), although other genera, such as *B. megatherium* and *B. mojavensis,* also show good growth in this medium (Jia et al. 2024; Ze et al. 2024). This experiment shows that the NFB was varied, not only *Azotobacter* sp. Consequently, Ashby's medium is not exclusive to *Azotobacter* sp. isolates. Therefore, selected isolates should be examined using morphological and biochemical tests to determine bacterial or strain characteristics and metabolic properties.

In the experiment, *P. megaterium* 1A-7 demonstrated the highest nitrogen activity compared to *A. salinestris* and *P. aeruginosa*. NFB also reduced acetylene during nitrogen fixation, and the ARA method was utilized to measure nitrogen activity. The nitrogen-fixing ability of NFB varied depending on the activation of the nitrogenase enzyme, which can differ between strains. Upadhyay et al. (2015) reported several conditions, such as oxygen level, that can affect the activation of nitrogenase enzymes. Additionally, the EPS can help to protect the nitrogenase by limiting oxygen exposure (He et al. 2021). In this case, *P. megaterium* 1A-7 was a Gram-positive bacterium of the *Bacillus* species characterized by capsular EPS. Therefore, EPS content was important in screening for potential NFB in this research. Additionally, the microbial population may influence nitrogen fixation rates and alter the nitrogen content.

The NFB must demonstrate other key traits, such as phytohormone production, to portray the potential bacterial strain as PGPR. Some rhizobacteria are known to produce IAA, cytokinin, and GA-like substances, which directly improve plant growth. Previous reports show that NFB produces auxins, cytokinins, and GA-like compounds associated with enhanced plant growth (Li et al. 2017; Li et al. 2022). *Priestia megaterium* strain IA-7 had higher production of IAA (31.29 $\mu g\ mL^{-1}$) and GA_3 (21.73 $\mu g\ mL^{-1}$), followed by *A. salinestris* strain 3A-1 and *P. aeruginosa* strain 4A-5. Therefore, *P. megaterium* strain 1A-7, *A. salinestris* 3A-1, and *P. aeruginosa* strain 4A-5 were selected as potential PGPR for strawberries.

In this study, the NFB strains *P. megaterium* strain IA-7, *A. salinestris* strain 3A-1, and *P. aeruginosa* strain 4A-5 demonstrated plant growth-promoting properties, significantly improving the number of leaves and diameter of the crown in strawberries under greenhouse conditions. The inoculation of NFB on strawberry seedlings was applied twice, at 7 and 28 days after transplanting, with concentrations of 1% and 2% (w/w). The treatments led to a significant increase in the number of leaves and diameter of the crown compared to the control group after six weeks, while plant height remained unaffected (Figure 5). This study suggests that the NFB with phytohormone traits may have a regulatory impact on strawberry development. This effect is likely due to the bacteria's ability to produce IAA, GA3, and cytokinins. Previous studies suggest that IAA promotes aboveground growth and development in strawberries (de Moura et al. 2022). Other studies indicated a positive correlation between the diameter of the crown and phytohormone production. Similar findings also reported that the inoculation of NFB, such as *Azospirillum* sp. and *Azotobacter* sp., enhances strawberry plant height, leaf count, and branch number (Kasa et al. 2015; Rueda et al. 2016; de Andrade et al. 2019).

However, the minimal impact of NFB inoculation on some growth parameters may be attributed to the greenhouse's elevated temperatures and low humidity conditions. Additionally, inorganic fertilizer was still applied to supplement the nutrient supply, ensuring that plants had access to essential nutrients despite the suboptimal conditions. High temperatures can affect the physiological and metabolic processes of Benihoppe strawberry cultivar's growth, which is related to photosynthesis, water, and nutrient uptake. Consequently, the growth was not optimal. Despite these challenges, the results demonstrate that NFB inoculation effectively promotes strawberry growth under the experimental conditions.

The present study focused on the characterization of Nitrogen-Fixing Bacteria (NFB) in enhancing strawberry growth by providing nutrients and hormones. However, NFB also have potential roles in disease suppression and plant resistance, which were not explored in this study. The short-term bioassay examined a limited set of growth parameters; further research is therefore needed to evaluate the long-term impacts of NFB on microbial communities and their mechanisms. Furthermore, the performance of NFB is subject to variation based on environmental conditions. To enhance the applicability of these findings to diverse farming systems, localized trials are necessary.

In conclusion, the study identified 21 NFB isolates from the strawberry rhizosphere and bulk soil based on colony features, with most being Gram-negative and 11 forming capsules. Capsule-forming isolates exhibited distinct biochemical traits, including dark brown and brown colonies, as well as bacillus or coccus shapes. Of these, three isolates—*P. megaterium* strain IA-7, *A. salinestris* strain 3A-1, and *P. aeruginosa* strain 4A-5—were confirmed to fix nitrogen and produce phytohormones like IAA, cytokinin, and gibberellin (GA_3). Inoculating strawberries with a 1% NFB liquid culture significantly increased leaf count and crown diameter after six weeks, though plant height remained unchanged. Future research should focus on enhancing nitrogen fixation, EPS production, and phytohormone synthesis while optimizing the composition of microbial consortia to improve strawberry growth, particularly in tropical environments. Localized trials are needed to improve applicability across farming systems.

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