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Morphophysiological responses of black soybean to nitrogen-fixing bacterial inoculants from diverse agroecosystems

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Abstract. *Hibatullah FH, Yaseen U, Ahmad F, Khumairah FH, Karuniawan A, Sofyan ET, Nurbaity A, Simarmata T. 2026. Morphophysiological responses of black soybean to nitrogen-fixing bacterial inoculants from diverse agroecosystems. Biodiversitas 27 (1): d270133. https://doi.org/10.13057/biodiv/d270133.* Black soybean (*Glycine max*) is a legume crop of high nutritional and economic value, especially in sustainable agricultural systems. Its productivity, however, is often limited by nutrient-deficient soils. We hypothesized that inoculation with Symbiotic Nitrogen-Fixing Bacteria (SNFB) sourced from diverse agroecosystems could enhance morphophysiological performance under controlled conditions. This study aimed to evaluate the morphophysiological responses of black soybean to inoculation with SNFB isolated from diverse agroecosystems. The study comprised (i) isolation and phenotypic characterization of SNFB strains and (ii) greenhouse evaluation using a randomized complete block design with six bacterial treatments (BJ-H1, BJ-H2, BJ-H3, BJ-H4, BJ-H5, and BJ-H6) and an uninoculated control, each with four replicates. Parameters measured included plant height, chlorophyll content, fresh and dry biomass, and nodulation. Analysis of Variance (ANOVA) followed by post-hoc comparisons ($p<0.05$) revealed that BJ-H5 significantly increased plant height (2.41%), chlorophyll content (14.7%), fresh shoot biomass (59.33%), and dry shoot biomass (21.11%) relative to the control, BJ-H2 and BJ-H3 induced the highest nodulation, indicative of enhanced nitrogen fixation. Correlation and principal component analyses demonstrated robust positive associations between inoculation treatments and growth traits. These findings provide statistically substantiated evidence that targeted SNFB inoculation, particularly with strain BJ-H5, represents a viable biofertilizer strategy for improving black soybean productivity on marginal or degraded soils, thereby reducing dependence on synthetic nitrogen inputs and supporting sustainable crop intensification.

Keywords: *Bacillus megaterium*, low-input farming, microbial interaction, sustainable agriculture, symbiotic bacteria

INTRODUCTION

Soybean is a versatile and highly beneficial crop, particularly the black soybean variety (*Glycine max* (L.) Merrill), which stands out as a functional food due to its elevated levels of bioactive compounds. In many regions, particularly in Asia and parts of Africa and South America, black soybean contributes significantly to food security, traditional diets, and emerging health-oriented markets. Black soybean is gaining attention owing to its superior nutritional properties and associated health benefits, such as high protein and anthocyanin content and excellent antioxidant effects, compared to yellow soybean (Bhartiya et al. 2020; Khosravi and Razavi 2021; Nirmal et al. 2023). Black soybean plays a critical role in human nutrition as it constitutes a rich source of proteins (41.38%-44.32%), carbohydrates (30.35%-32.8%), Dietary Fibers (DFs; 27.61%-30.47%), lipids (10.37%-18.56%), vitamins (such as VB, VC, and VE), and minerals (such assodium (Na), potassium (K), phosphorus (P), and iron (Fe)), as well as bioactive compounds including saponins, phenolic acids, isoflavones, and anthocyanins (Cho et al. 2013; Zhou et al. 2017; Kan et al. 2018; Koriyama et al. 2023). Black soybean seed coats contain nine distinct anthocyanin compounds, which not only confer their characteristic dark pigmentation but also contribute to their classification as functional foods with superior health-promoting properties (Cho et al. 2013). These attributes position black soybean as a strategic crop for addressing nutritional security while reducing reliance on imported plant-based protein sources. In Indonesia, demand for soybeans has increased sharply; consumption rose from 5.95 kg capita^{-1} in 2016 to 8.78 kg capita^{-1} in 2017, a 47.5% increase (MOA 2021). However, domestic productivity has remained stagnant at 13-16 kg ha^{-1} over the last five years (Central Bureau of Statistics 2021), largely due to reduced cultivation area and low yields on nutrient-depleted soils. To meet national demand, the government implemented a soybean import policy (MOA 2021).

Improving productivity in challenging soils depends largely on soil microbial diversity and function. Effective SNFB are key to optimizing Biological Nitrogen Fixation (BNF) and plant performance (Nguyen et al. 2020). Incorporating beneficial microbes into organic and regenerative farming systems offers a smart, sustainable solution for enhancing soil fertility and plant resilience (Kiprotich et al. 2025). SNFB improves root development, nutrient uptake, and tolerance to environmental stresses (Jarecki et al. 2024), while bacterial inoculation promotes microbial activity, nutrient cycling, and overall crop productivity (Verma et al. 2022). *Bradyrhizobium* inoculation enhances biological soil fertility by facilitating symbiotic nitrogen fixation, which improves soybean growth, yield, and grain quality. Studies have shown that inoculation can increase soybean yields by 20-60%, depending on soil type, organic matter content, and native microbial populations (Gitonga et al. 2021). On degraded soils, yield increases may exceed 50%, and inoculation can reduce synthetic nitrogen use by 50-100 kg N/ha without sacrificing yield (Youseif et al. 2017). Despite these advances, little research has explored the potential of SNFB isolates from different agroecosystems, which may harbor distinct adaptive traits to enhance black soybean performance under nutrient-deficient conditions. This represents a critical knowledge gap in developing location-specific, climate-resilient bioinoculants.

We hypothesize that inoculation with SNFB isolates sourced from diverse agroecosystems will significantly enhance the morphophysiological performance and nodulation of black soybean under nutrient-deficient soil conditions compared to non-inoculated controls. The objectives of this study were to: (i) evaluate the effectiveness of selected SNFB isolates in improving black soybean growth, biomass accumulation, chlorophyll content, and nodulation; (ii) compare the performance of isolates originating from different agroecosystems; and (iii) identify the most promising strains for development as bioinoculants in sustainable crop production systems. By investigating the responses of black soybean to multiple SNFB isolates under controlled greenhouse conditions, this research aims to identify superior strains with the capacity to restore soil fertility, improve productivity on marginal lands, and contribute to climate-resilient, low-input agricultural systems.

MATERIALS AND METHODS

Location of sampling

Screening and characterization of SNFB were conducted using soybean nodules collected from two distinct ecosystems: The Ciparanje soybean fields (-6551.896 S, 1074614.724 E) and fields previously used as rice paddies (-6551.284 S, 1074615.894 E). Nodules were sampled from healthy black soybean (*Glycine max*) plants, with eight representative plants selected from each variety. Nodules chosen for isolation were large and displayed a pink coloration when bisected, indicating active nitrogen fixation. The experimental phase of the study was carried out from May to July 2024, and laboratory analyses were performed in the Soil Fertility and Plant Nutrition Laboratory and the Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran, Indonesia.

Procedures

Physico-chemical analysis of soil samples

Soil sampling was conducted on Ciparanje soybean fields and fields previously used as rice paddies (Figure 1). Soil sampling was done at a depth of 0-20 cm from the top of the soil surface using a shovel. Samples from five collection points were then composited and put into zip bags to be used as soil physico-chemical testing samples.

Soil chemical properties and soil health

Soil sampling was done at a depth of 0-20 cm from the top of the soil surface using a shovel. Samples from two collection points were then composited and put into a zip bag to be used as soil chemical testing samples. Soil chemical properties of soil samples (collected from ciparanje soybean field and soybean field ex-paddy) analyzed during the present study are summarized in Table 1. Soil fertility is a fundamental aspect of agricultural productivity, particularly in soybean cultivation, which is sensitive to nutrient availability and soil health.

Figure 1. Soil sampling location in Ciparanje, West Java, Indonesia. A. Soybean field, B. Soybean field previously cultivated as rice paddies

Soil pH is commonly measured by preparing a suspension of soil in either distilled water (pH_2O) or a 1 M potassium chloride solution (pH KCl), typically at a soil-to-solution ratio of 1:2.5 or 1:5. The mixture is stirred and allowed to equilibrate before the pH is determined using a glass electrode. This method provides insight into soil acidity and the potential availability of nutrients and toxic elements, with pH in KCl often being lower than in water due to displacement of exchangeable hydrogen and aluminum ions (Nel et al. 2023).

Organic carbon in soil is frequently determined using the Walkley-Black wet oxidation method or by dry combustion using an elemental analyzer, which provides a direct measurement of carbon content. Total nitrogen is typically measured by the Kjeldahl method. The C/N ratio is then calculated from these values, offering important information about soil fertility and organic matter decomposition (Räty et al. 2021).

Available phosphorus can be extracted using different chemical solutions depending on soil type. The Bray I method, suitable for acidic soils, uses a mixture of ammonium fluoride and hydrochloric acid to extract labile phosphorus, which is then measured colorimetrically. Alternatively, extraction with 25% HCl is used in some contexts, followed by colorimetric determination of phosphorus concentration. These methods help estimate the pool of phosphorus available for plant uptake (Hartono et al. 2021).

Exchangeable potassium is often extracted with 25% HCl or, more commonly, with 1 M ammonium acetate (NH_4OAc) at pH 7.0, which is also used for extracting other exchangeable cations such as sodium, calcium, and magnesium. The extracted cations are quantified using flame photometry, atomic absorption spectroscopy, or Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). These measurements are essential for assessing soil fertility and nutrient management (Dunne et al. 2021; Nel et al. 2023).

CEC is determined by saturating the soil with a cation such as ammonium (NH_4^+) or cobalt(III)hexamine, followed by displacement and quantification of the adsorbed cations. The NH_4OAc method at pH 7.0 is widely used, but other methods such as Cohex and $BaCl_2$ extraction are also applied, especially for variable charge soils. Base saturation is calculated as the proportion of the CEC occupied by basic cations (Ca^{2+}, Mg^{2+}, K^+, Na^+), providing an index of soil fertility (Jain and Taylor 2023).

Exchangeable aluminum (Al-dd) is typically extracted with 1 M KCl and measured by ICP-OES or colorimetry. Exchangeable hydrogen (H-dd) is determined by titration after extraction with unbuffered salt solutions. Aluminum saturation is calculated as the percentage of CEC occupied by exchangeable aluminum, which is important for assessing potential toxicity in acidic soils (Souza et al. 2023).

Isolation of SNFB

Isolation, selection, and characterization of SNFB were conducted at the Soil Biology Laboratory of Padjadjaran University from May to July 2024. Isolation was carried out to obtain isolates of SNFB from 2 different ecosystems, namely Ciparanje dryland soybean fields and former rice field soybean fields. Isolation of bacteria using selective media YEMA (Yeast Extract Mannitol Agar). Surface sterilization of soybean root nodules was performed using distilled water, 0.1% $HgCl_2$, and 70% alcohol. Large, round nodules were selected, then cut and separated from the roots. The nodules were split using sterile tweezers in the petri dish until the nodule fluid emerged. This fluid was taken using a sterile ose and scratched in a zigzag manner on the YEMA media in the petri dish, starting from quadrant 1 to quadrant 2 and 3. After that, it was incubated for 2-3 days at the appropriate temperature until bacterial colonies were seen growing. After the bacterial colony grows, purification is done by selecting separate colonies and transferring them to new YEMA media in sterile petri dishes. This process aims to ensure that the isolated bacteria are truly pure and ready to be used for further research (Sari et al. 2019).

Gram staining

A clean glass slide is prepared by wiping it with 70% ethanol to remove any grease or contaminants, then drying with cotton wool or tissue paper. A sterile inoculating loop, previously heat-sterilized by flaming, transfers a small amount of bacterial suspension onto the slide. The suspension is spread evenly to form a thin smear and left to air dry. Once dry, the smear is heat-fixed by passing the slide briefly through a flame, which ensures the bacteria adhere to the slide and are killed. The slide is then flooded with crystal violet, the primary stain, and left for one minute. After rinsing with distilled water, Gram's iodine (Lugol's solution) is applied for one minute to act as a mordant, forming a complex with the crystal violet retained in Gram-positive cell walls. The slide is then decolorized with 95% ethanol, applied dropwise for approximately 15-30 seconds or until the runoff is clear, followed by an immediate rinse with distilled water to halt the decolorization. Next, the smear is counterstained with safranin for one minute, which imparts a pink-red color to Gram-negative bacteria. A final rinse is performed with distilled water, and the slide is gently blotted dry with absorbent paper. A drop of immersion oil is placed directly onto the smear, and the slide is examined under a compound microscope at 1000× magnification using a 100× oil immersion objective and a 10× eyepiece. Under the microscope, Gram-positive bacteria appear purple due to the retention of the crystal violet-iodine complex, while Gram-negative bacteria appear pink or red from the safranin counterstain (Paray et al. 2023).

Nitrogenase enzyme activity test of SNFB

Nitrogenase activity was determined using the Acetylene Reduction Assay (ARA) method as an indirect measure of biological nitrogen fixation (Weaver and Danso 2018). Bacterial isolates were cultured on Okon's slant agar, sealed with rubber caps, and injected with 10% (v/v) acetylene gas into the headspace. After 1 hour of incubation at 28°C, 1 mL of the gas phase was analyzed using a gas chromatograph equipped with a flame ionization detector. Nitrogenase activity was calculated from the amount of ethylene (C_2H_4)

produced, using an ethylene standard curve (0-50 µmol C_2H_4 mL^{-1}) for quantification. Results were normalized per Gram of bacterial dry biomass per hour and expressed as µmol C_2H_4 g^{-1} h^{-1}, allowing comparison across isolates (Susilowati and Setyowati 2016; Sapalina et al. 2022).

Phytohormone indole acetic acid test

The ability of rhizobacteria to produce Indole Acetic Acid (IAA) was assessed following Fatimah et al. (2022) with minor modifications. Each bacterial isolate was rejuvenated on selective media and prepared as a 10 mL suspension with a cell density of approximately 10^7 CFU mL^{-1}, standardized using the MacFarland method. Cultures were incubated in Yeast Mannitol Broth supplemented with 0.1% L-tryptophan for 48 h at 28°C under shaking (120 rpm). After incubation, 5 mL of culture was centrifuged at 5,500 rpm for 10 min, and 1 mL of the resulting supernatant was mixed with 4 mL of Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$) at a ratio of 1:4. The mixture was incubated in the dark for 20 min, and absorbance was measured at 535 nm using a spectrophotometer. IAA concentration was determined using an analytical-grade IAA standard curve (0-100 ppm; $R^2>0.99$), and isolates were categorized as low (<10 ppm), moderate (10-30 ppm), or high (>30 ppm) producers (Spaepen and Vanderleyden 2014).

Biomolecular test of bacterial species identification using 16S rRNA sequencing

Genomic DNA was extracted using the CTAB method, and the 16S rRNA gene was amplified by PCR using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR reactions (25 µL) contained 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 1 U Taq polymerase, and 20 ng template DNA. The thermal profile was: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 90 s; and a final extension at 72°C for 7 min. PCR products (~1,500 bp) were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide and purified using a commercial PCR clean-up kit (Qiagen). Sequencing was performed bidirectionally using the same primers (Genetic Science Indonesia Laboratory). Resulting sequences were edited and aligned using BioEdit v7.0.5.3 (Hall 1999) and compared with GenBank entries via BLASTn for taxonomic identification. Phylogenetic trees were constructed using MEGA X (Kumar et al. 2018) with the Neighbor-Joining algorithm. Bootstrap analysis (500 replications) was applied to assess branch confidence. The sequences with ≥97% identity to reference strains were assigned to the corresponding species following Janda and Abbott (2007).

Biological assay of SNFB on black soybean

The biological assay test for SNFB used a Randomized Complete Block Design (RCBD) with seven treatments (Control, BJ-H1, BJ-H2, BJ-H3, BJ-H4, BJ-H5, BJ-H6) and four replicates, resulting in 28 polybags. SNFB isolates were grown on Yeast Mannitol Broth medium and then shaken and incubated until their population reached 10^8 CFU mL^{-1}. Inoculum was applied as 10 mL liquid culture per polybag at sowing and again at 14 Days After Planting (DAP). The biological activity test of the selected SNFB isolates was conducted using 20 × 25 cm poly bags filled with 1 kg of soil. The experimental soil was taken from Ciparanje (Inceptisol, neutral pH, medium C-organic, Table 1). Before use, soil was air-dried, sieved (2 mm), homogenized and not sterilized. Black soybean seeds of the BS 114 cultivar were cleaned by rinsing with distilled water and then germinated for 4-5 days at room temperature. Fifty seeds were planted in each roll. After observation, the germination rate of black soybean seeds was 95%. Liquid inoculant application was performed at planting and 14 DAP by adding 10 mL of bacterial liquid culture to each polybag according to the treatment. Plant samples were collected after the plants were 28 DAP. Harvesting was carried out by pulling the plants out of the soil, then cleaning them with running water to remove adhering soil. Next, the aboveground and root parts were separated and placed in brown envelopes. Drying was performed by oven-drying the plants at 70°C for 48 hours, followed by weighing the plants (Handoko et al. 2010).

Ethics, permits, and biosafety compliance

This study was conducted in accordance with the biosafety and ethical research guidelines of Universitas Padjadjaran, Indonesia. Isolates obtained during this study have been preserved in the Soil Biology Laboratory culture collection, Faculty of Agriculture, Universitas Padjadjaran, under long-term cryopreservation for future research and reference. The work did not involve pathogenic strains, endangered species, or genetically modified organisms. All procedures complied with standard microbiological safety protocols and national regulations governing microbial resource use.

Data observed and statistical analysis

The greenhouse experiment was conducted from May-July 2024 at Universitas Padjadjaran, Indonesia, using a Randomized Complete Block Design (RCBD) with seven treatments (Control, BJ-H1, BJ-H2, BJ-H3, BJ-H4, BJ-H5, BJ-H6). Each treatment had four replicates, totaling 28 experimental units. Black soybean (*Glycine max* cv. BS 114) was germinated for 4-5 days before transplanting. Plants were grown in 20 × 25 cm polybags placed in a greenhouse under natural light conditions, at 28 DAP, plants were harvested. Measured parameters included: crown height, root length, crown wet weight, root wet weight, crown dry weight, root dry weight, and number of root nodules. Data were analyzed using the Statistical Product, OriginLab, and Service Solutions (SPSS) software. The impacts of various treatments on the measured variables were assessed using ANOVA with SPSS (Statistical Package for the Social Sciences) software. Significant differences were identified through Duncan's test, with significance established at $p<0.05$. Correlation analysis was conducted using Pearson correlation testing, and Principal Component Analysis (PCA) was also performed. Pearson's correlation test and Principal Component Analysis (PCA) were performed to analyze the relationships and patterns among multiple measured variables related to the growth and physiological responses of black soybean plants under different bacterial inoculation treatments. Data visualization of the research findings was accomplished using Prism 9 software.

RESULTS AND DISCUSSION

Soil chemical properties and soil health

Soil sampling was done at a depth of 0-20 cm from the top of the soil surface using a shovel. Samples from two collection points were then composited and put into a zip bag to be used as soil chemical testing samples. Soil chemical properties of soil samples (collected from ciparanje soybean field and soybean field ex-paddy) analyzed during the present study are summarized in Table 1.

Ciparanje soybean field analysis reveals a neutral pH (7.00) and slightly acidic conditions (pH KCl 6.34), indicating generally favorable conditions for plant growth Multazam (2023), Organic carbon (2.49%) and total nitrogen (0.35%) levels are moderate, supporting nutrient cycling, while the C:N ratio of 7.15 suggests balanced decomposition rates Yanti and Kusuma (2021). Phosphorus availability is a concern, with medium levels of P_2O_5 (35.09 mg/100g) but low bioavailable phosphorus (8.83 ppm), indicating a need for supplemental fertilization. Potassium levels are low (16.88 mg/100g), which could hinder plant development, necessitating potassium-rich amendments. High calcium (10.61 cmol/kg) and magnesium (3.94 cmol/kg) levels contribute positively to soil health, while a medium cation exchange capacity (24.81 cmol/kg) and base saturation (60.76%) suggest reasonable nutrient retention capabilities.

The soil analysis of Ciparanje ex-paddy soils shows a neutral pH of 6.51 in water and slightly acidic conditions with a pH of 5.98 in KCl, which is generally favorable for plant growth (Kabała 2016). Organic carbon content is moderate at 2.22%, while total nitrogen is low at 0.18%. The C: N ratio of 12 is medium, indicating a balanced decomposition process, but the low nitrogen level may limit plant productivity Shivanna and Nagendrappa (2019). Phosphorus levels are very high P_2O_5 at 98.27 mg/100g) with excellent bioavailability (42.36 ppm), ensuring ample phosphorus supply for crops. Potassium levels are high (K_2O at 45.95 mg/100g), providing sufficient potassium for plant health. Calcium (13.74 cmol/kg) and magnesium (4.06 cmol/kg) levels are also high, contributing positively to soil structure and nutrient availability. The Cation Exchange Capacity (CEC) is moderate at 24.93 cmol/kg, indicating reasonable nutrient retention capabilities, while base saturation is high at 76.28%, reflecting a favorable balance of basic cations.

Characteristics of SNFB isolates from different ecosystem

The results of the characterization of SNFB isolates from 2 different ecosystems (ciparanje soybean field and soybean field ex-paddy soil) revealed that total six isolates were successfully identified (Table 2). The morphology of SNFB isolates is shown in Figure 2. All the isolates isolated in the present study displayed Gram-negative characteristics after staining. Bacteria that have a red colour are Gram-negative bacteria. The cell wall of Gram-negative bacteria consists of lipopolysaccharides. Gram-negative bacterial cells cannot bind gentian violet colour because the cell wall of Gram-negative bacteria has a thinner layer than the cell wall of Gram-positive bacteria, which has a peptide cross-link (Hamidah et al. 2019). The diversity of morphological characters of bacterial isolates indicates different potentials in their ability to fix nitrogen. Bacteria with red pigments, such as *Rhizobium* spp., are often associated with high nitrogen fixation efficiency in legume crops, including black soybean (Zahran 1999).

Table 1. Soil chemical properties from two ecosystems

Parameters	Ciparanje soybean field	Soybean fields ex-paddy soil
pH H_2O	7.00	6.51
pH KCl	6.34	5.98
C-Organic (%)	2.49	2.22
N-Total (%)	0.35	0.18
C/N	7.15	12
P_2O_5 HCl 25% (mg/100g)	35.09	98.27
P_2O_5 (Bray) (ppm P)	8.83	42.36
K_2O HCl 25% (mg/100g)	16.88	45.95
K-dd (cmol.kg^{-1})	0.34	0.89
Na-dd (cmol.kg^{-1})	0.18	0.33
Ca-dd (cmol.kg^{-1})	10.61	13.74
Mg-dd (cmol.kg^{-1})	3.94	4.06
CEC (cmol.kg^{-1})	24.81	24.93
Base saturation (%)	60.76	76.28
Al-dd (cmol.kg^{-1})	0.19	0.06
H-dd (cmol.kg^{-1})	0.34	0.20
Al-saturation (%)	1.23	0.33

Table 2. Morphological characteristics of SNFB isolates on yeast mannitol agar media

Isolates code	Colony shape	Colony colour	Edge	Consistency	Elevation	Gram staining	Cell shape
BJ-H1	Circular	Light red	Entire	Pliable	Flat	Gram negative	Bacilli
BJ-H2	Circular	Light red	Entire	Pliable	Flat	Gram negative	Bacilli
BJ-H3	Irregular	Milky white	Lobate	Pliable	Umbonate	Gram negative	Bacilli
BJ-H4	Circular	Light red	Entire	Pliable	Flat	Gram negative	Bacilli
BJ-H5	Circular	Light red	Entire	Pliable	Raised	Gram negative	Bacilli
BJ-H6	Circular	Light red	Entire	Pliable	Raised	Gram negative	Bacilli

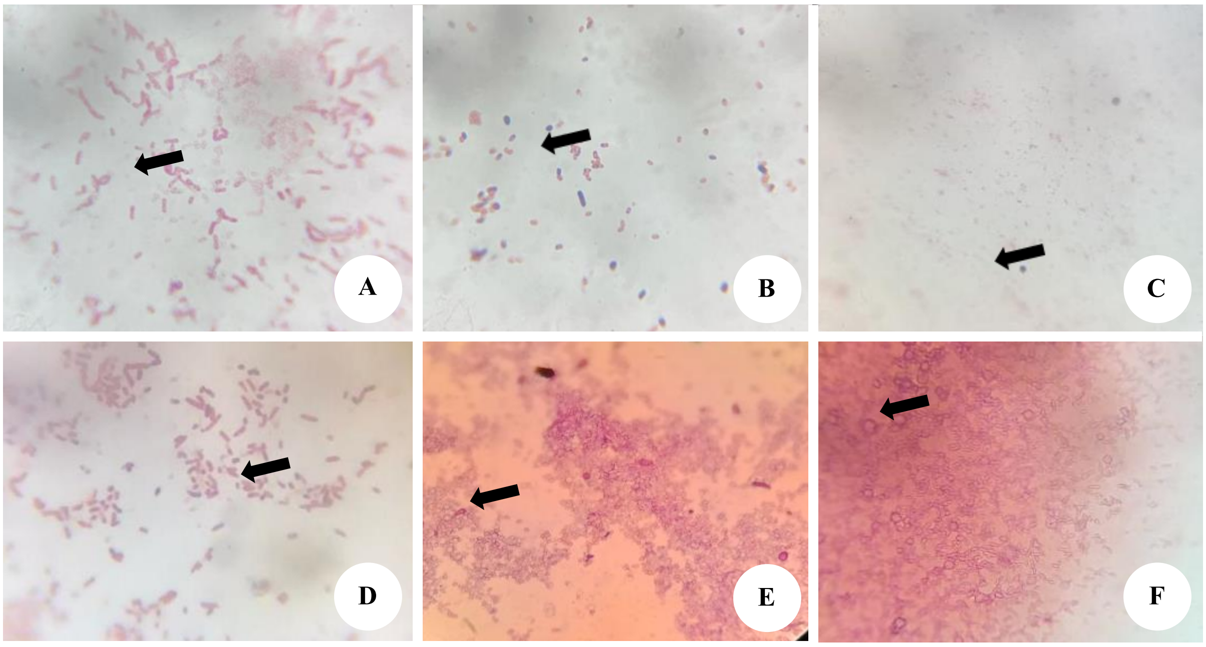

Figure 2. Morphology of with isolates of SNFB using the Gram Staining method with 1000× Magnification. A. Isolate BJ-H1, B. Isolate BJ-H2, C. Isolate BJ-H3, D. Isolate BJ-H4, E. Isolate BJ-H5, F. Isolate BJ-H6. SNFB exhibits cells with bacilli and a red Gram stain, indicating that they are Gram-negative (Li et al. 2020)

Nitrogenase activity of SNFB

Nitrogenase is a key indicator of the capacity of bacteria to fix nitrogen and release nitrogen into the soil, which can then be transformed by Nitrosomonas and Nitrobacter bacteria into a form available to plants. The ability of bacteria to fix nitrogen using the ARA method is shown in Table 3.

Among the tested isolates, BJ-H5 exhibited the highest nitrogenase activity, reaching 24.906 µM ml^{-1} g^{-1} h^{-1}, which was more than twice the activity observed in BJ-H1 (11.108 µM mL^{-1} g^{-1} h^{-1}), and significantly higher than the near-negligible levels in BJ-H6 (0.685 µM ml^{-1} g^{-1} h^{-1}) and the control (0.00 µM mL^{-1} g^{-1} h^{-1}) (Table 3). These findings suggest that BJ-H5 is a highly efficient nitrogen-fixing strain and a strong candidate for bioinoculant development in sustainable agriculture. The higher effectiveness of BJ-H5 compared to BJ-H1 is likely due to higher nitrogenase enzyme efficiency, which may be influenced by the genetic composition of the bacteria and their ability to maintain enzyme stability in an oxidative environment (Takimoto et al. 2022). The superior nitrogenase activity of BJ-H5 may be attributed to its effective adaptation to local soil conditions and its genetic potential to fix atmospheric nitrogen under stress conditions.

The variation in nitrogenase activity among isolates aligns with previous studies that emphasize the influence of the origin of bacterial strain, rhizosphere conditions, and host compatibility on enzyme activity and nitrogen fixation rates (Rilling et al. 2019; Santos et al. 2019). The indigenous strains often outperform commercial inoculants because of their co-evolution with native plant varieties and local soil microbiomes, allowing for better colonization and symbiosis (Omari et al. 2022). The marked difference between BJ-H5 and BJ-H6 further indicates that not all native isolates possess equal nitrogen-fixing potential, underlining the importance of rigorous strain selection and screening in biofertilizer development. Moreover, the nitrogenase activity observed in BJ-H5 correlates with its enhanced performance in plant growth parameters, as shown in related experiments.

IAA production from selected bacterial

Bacteria can produce phytohormones, particularly Indole Acetic Acid (IAA), which can be beneficial for plant growth and development. The results of testing IAA phytohormones produced by SNFB are presented in Table 4. IAA phytohormone testing shows that isolate BJ-H5 produces the highest IAA phytohormone of 6.292 ppm. The isolate with the second-highest IAA concentration value is BJ-H6 of 5.122 ppm, and the lowest is BJ-H1 of 4.304 ppm. This shows that BJ-H5 has high potential in increasing plant growth because it can combine the ability of nitrogen fixation with the production of growth hormones that support the development of the root system. Higher IAA production in BJ-H5 compared to BJ-H1 and BJ-H6 can be caused by more efficient genetic regulation in IAA biosynthesis and its ability to colonize plant roots. IAA is an auxin phytohormone that is vital in stimulating plant growth. This hormone involves various physiological processes such as cell elongation, differentiation, and root formation.

While IAA concentrations were categorized as low, it's important to note that even low levels of IAA can significantly influence root development, especially when sustained over time or in synergy with other microbial functions (Spaepen and Vanderleyden 2014). Moreover, IAA production is often influenced by environmental factors, substrate availability, and the presence of plant root exudates, suggesting that in soil or rhizosphere environments, these isolates may express higher activity levels than observed in vitro (Etesami and Glick 2024).

Biomolecular identification of selected isolates

Biomolecular analysis and species identification of SNFB for the three selected isolates are presented in Table 5. SNFB isolates were obtained from soybean nodules collected from soybean fields in Ciparanje and former rice fields in West Java, Indonesia. After purification and morphological as well as biochemical characterization, bioassays were conducted using soybean plants. The 16S rRNA gene sequencing, performed at the Genetic Science Indonesia Testing Laboratory, identified three isolates: BJ-H1, BJ-H5, and BJ-H6, while BJ-H2, BJ-H3, and BJ-H4 remained unidentified (Figure 3). Based on BLAST analysis, BJ-H1 showed 98.86% similarity with *Priestia aryabhattai* strain IRHB1-52, BJ-H5 showed 100.00% similarity with *Bacillus megaterium* strain HT-Z56, and BJ-H6 showed 100.00% similarity with *Rhizobium* sp. Mp10. According to Janda and Abbott (2007), isolates with ≥97% sequence similarity are considered the same species, whereas those with <97% may represent new species. The phylogenetic tree visualization for each bacterial species is shown in Figure 4.

Morphological response

The table compares results from seven treatments, including a control group. Each treatment labelled BJ-H1, BJ-H2, BJ-H3, BJ-H4, BJ-H5, and BJ-H6 most likely reflects a distinct bioinoculant, fertilizer mix, or growth-promoting chemical. The measured variables include morphological features (shoot height, root length), physiological measures (leaf chlorophyll), and biomass accumulation (wet and dry weights), including nodulation (Table 6). The growth of roots and crowns of black soybean plants compared to the control group on 28 DAP is shown in Figure 5.

The values for all treatments differ slightly, with BJ-H5 (15.7 cm) having the maximum height and BJ-H3 having the lowest (14.38). While most treatments persist around the control (15.33 cm), no significant shifts indicate that shoot height remains consistent throughout treatments. BJ-H5 may indicate the minimum activation. Root length data indicate considerable fluctuation: BJ-H2 greatly increases root length to 37.25 cm compared to the control (24.25 cm), whereas BJ-H3 and BJ-H4 significantly lower it (21.25 cm and 21.88 cm). Longer roots, notably in BJ-H2 and BJ-H6 (32.00 cm), may increase soil exploration capacity and drought resilience. Leaf chlorophyll indicates photosynthetic efficiency. BJ-H5 has the highest value (24.792), a significant improvement above the control (21.61). BJ-H1 also marginally increases chlorophyll levels (22.70), whereas BJ-H4 decreases significantly to 17.017. These findings imply that BJ-H5 may increase photosynthetic activity and general vigor, perhaps leading to improved growth and production. The number of nodules differs sharply from the control (0.1), with BJ-H3 (3.25) and BJ-H2 (2) having the highest nodule counts. This is especially relevant to nitrogen-fixing legumes. Increased nodulation indicates symbiotic enhancement, possibly due to rhizobial inoculation or bio stimulants promoting nodule development. BJ-H5 leads again at 487.5 mg, followed by BJ-H1 at 482.5 mg. The constancy of wet and dry measures shows actual biomass accumulation, not merely water content. The control value is astonishingly high (1130 mg), but BJ-H6 also performs well (860 mg), indicating strong root growth. BJ-H5, despite its good shoot metrics, has the smallest root dry weight (480 mg), implying that resource allocation trade-offs favor aerial growth.

Table 3. Ability of nitrogen-fixing bacteria to produce nitrogenase enzymes

Isolates code	Nitrogenase activity µM mL^{-1} g^{-1} h^{-1}
Control	0,00
BJ-H1	11,108
BJ-H5	24,906
BJ-H6	0,685

Table 4. Indole Acetic Acid (IAA) production of selected SNFB isolates

Isolates code	IAA concentration (ppm)	Category
BJ-H1	4,304	Low
BJ-H5	6,292	Low
BJ-H6	5,122	Low

Table 5. Determining isolated bacterial species through analysis of 16S rRNA sequences

Code	Description	Per. identity	Accession
BJ-H1	*Priestia aryabhattai* strain IRHB1-52	98,86%	OP364538.1
BJ-H5	*Bacillus megaterium* strain HT-Z56	100,00%	KJ526880.1
BJ-H6	*Rhizobium* sp. Mp10	100,00%	GQ355325.1

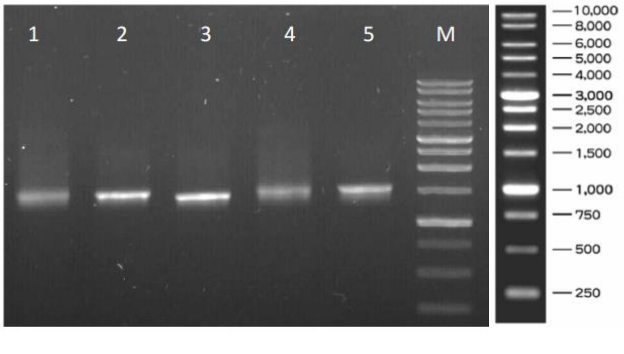

Figure 3. Visualisation of 16S rRNA sequences of isolates BJ-H1, BJ-H5, and BJ-H6 showing PCR reaction products of approximately 1500 bp in length. 1: BJ-H1, 2: BJ-H5, 3: BJ-H6 M: 1Kbp DNA ladder

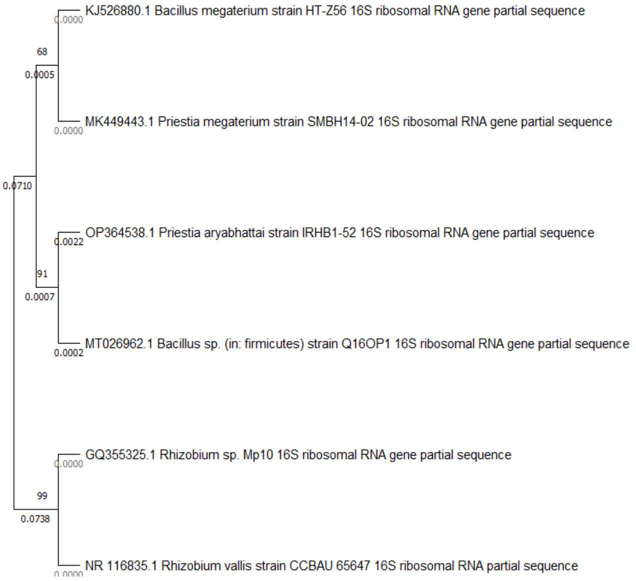

Figure 4. The phylogenetic tree

Correlations and comparative traits expression

This correlation matrix depicts the correlations between seven variables correlated to plant shape and biomass. Red hues suggest positive connections, while blue hues indicate negative ones, with color intensity signifying strength. Wet weight of the shoot vs. dry weight of the crown (r = 0.89): Heavier fresh biomass equates to higher crown dry matter, indicating persistent biomass accumulation (Figure 6). Shoot height and dry weight of the crown (r = 0.66): Taller plants acquire drier biomass in the crown, which indicates vigor. Shoot height and leaf chlorophyll content (r = -0.79): Taller plants may have lower chlorophyll density due to dilution effects or resource allocation trade-offs. Wet weight of the shoot and dry weight of the roots (r = -0.63): Plants with higher mass above ground may have smaller root systems, indicating a biomass partitioning trade-off.

The heatmap compares plant morpho-physiological features among seven samples (BJ-H1, BJ-H2, BJ-H3, BJ-H4, BJ-H5 and BJ-H6 plus a control) (Figure 7). Each trait, shoot height, nodulation, root length, biomass metrics, and chlorophyll content has a color coding, with blue suggesting higher values and black indicating lower ones. The dendrograms group variables and samples based on similarity, providing insights into connected outcomes (Figure 8). BJ-H5 is the top-performing sample, with peak values for root length, crown and root biomass, and shoot wet weight. This indicates a strong overall vigor and the potential for significant productivity. In contrast, BJ-H2 had uniformly low values, which could indicate stress, poor adaptation, or a genetic disadvantage under testing conditions. BJ-H1, BJ-H4, and BJ-H6 cluster, indicating moderate trait expression across variables. Their intermediate profiles may signify balanced growth with no significant weaknesses or distinguishing characteristics. The control sample exhibits a range of responses in mid-level chlorophyll and shoot height, but lower biomass, implying that it may fall short of hybrid performance, particularly under resource-intensive conditions.

Principal Component Analysis (PCA) of bioassay SNFB on black soybean

The Principal Component Analysis (PCA) provided a clear multivariate overview of soybean responses to inoculation with SNFB (Figure 9). The first two principal components explained 71.2% of the total variance (PC1 = 47.6%, PC2 = 23.6%), indicating that most treatment differences were effectively captured by the biplot. PC1 was strongly associated with root dry weight, number of nodules, and shoot wet weight, while PC2 reflected variation in plant architecture traits such as plant height and root length. The distribution of isolates in the PCA biplot revealed functional differentiation: soybean-field isolates BJ-H2 and BJ-H3 clustered with nodulation and root biomass traits, confirming their effectiveness in promoting symbiotic nitrogen fixation. In contrast, ex-paddy isolate BJ-H5 (*B. megaterium*) aligned with shoot biomass and chlorophyll content, highlighting its strength in enhancing photosynthetic activity and vegetative growth. These findings underscore that the most impactful strains were BJ-H2 and BJ-H3 for nodulation, and BJ-H5 for aboveground biomass, demonstrating complementary functional roles that could be exploited in tailored biofertilizer applications.

Inoculation with SNFB can increase plant height by up to 20% compared to uninoculated controls, primarily due to enhanced BNF, improved photosynthetic efficiency, and increased biomass accumulation (Szpunar-Krok et al. 2023). SNFB significantly promotes root elongation by enhancing nutrient acquisition and synthesizing phytohormones, particularly auxins, which stimulate root cell expansion and lateral root development (Hiltenbrand et al. 2016). A 25-30% increase in root length in plants inoculated with SNFB compared to uninoculated controls, primarily due to improved nitrogen availability and the synthesis of growth-promoting metabolites such as Indole-3-Acetic Acid (IAA) (Egamberdieva et al. 2018). Experimenting with non-sterile soil represents a closer approximation to field conditions, since inoculated isolates must compete and interact with indigenous microbial populations. The positive effects of BJ-H5 (*B. megaterium*) and other isolates under these conditions suggest strong ecological competitiveness and functional robustness. However, because native microbial communities may have influenced nodulation and plant responses, variability among isolates could partly reflect differences in ecological adaptation. This reinforces the importance of testing inoculants under conditions that simulate real agricultural soils.

Table 6. Response of black soybean plants to nitrogen-fixing bacterial inoculants

Treatments	Plant height (cm)	Root length (cm)	Leaf chlorophyll (CCI)	Number of nodules	Shoot dry weight (mg)	Root dry weight (mg)
Control	15.33±1.12	24.25±4.67	21.61±7.54	0±0.0	402.5±0.1	1130±0.19
BJ-H1	15.55±1.05	26.25±8.84	22.70±7.71	0.75±0.5	482.5±0.04	747.5±0.11
BJ-H2	15.13±0.98	37.25±10.44	19.50±8.71	2±2.45	400±0.08	595±0.2
BJ-H3	14.38±2.06	21.25±8.70	19.93±6.03	3.25±2.22	402.5±0.1	737.5±0.43
BJ-H4	15.08±1.56	21.88±9.17	17.017±8.07	0.5±0.58	435±0.03	772.5±0.1
BJ-H5	15.70±1.78	3063±2.69	24.792±2.67	0.75±0.98	487.5±0.09	480±0.2
BJ-H6	14.88±1.65	32±13.42	1818±5.93	1.5±1.00	442.5±0.09	860±0.1

Note: Each value represents the means of three replicates±standard deviation (n = 4)

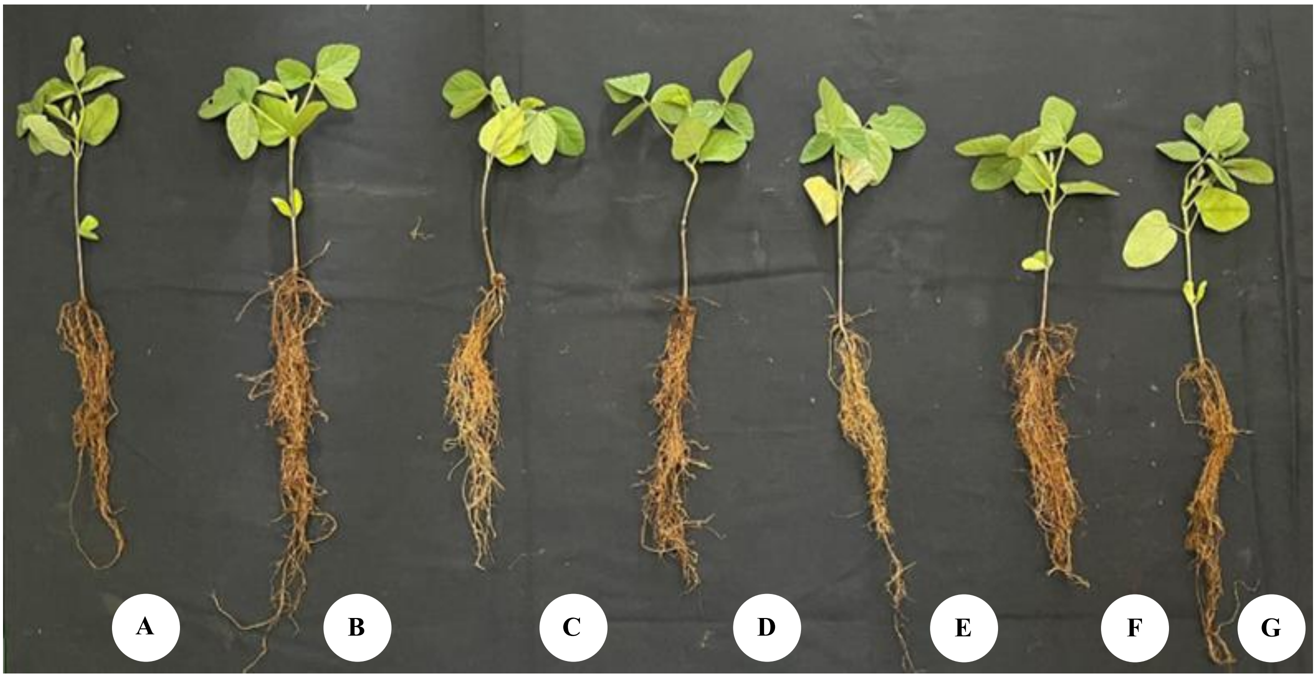

Figure 5. Root and crown growth of black soybean plants compared to control at 28 days, a marked increase in root biomass and crown expansion reflects the beneficial physiological response induced by microbial or nutritional intervention. A. Control, B. BJ-H1, C. BJ-H2, D. BJ-H3, E. BJ-H4, F. BJ-H5, G. BJ-H6. These morphological differences suggest improved nutrient uptake efficiency and early-stage vigor in treated plants relative to controls

Discussion

Priestia is a type of plant growth-promoting bacterium found in the rhizosphere or within plants. Research has shown that the genus *Priestia* can enhance soil fertility, increase soil microbial diversity, and promote plant growth (Shahid et al. 2022; Jo et al. 2023). The species *Priestia aryabhattai* can solubilize potassium, solubilize phosphorus, solubilize zinc, and produce iron carriers. This can enhance the growth of plants such as cowpea, ryegrass, or corn (Abiala et al. 2023; Moturu et al. 2023; Srithaworn et al. 2023; Wang et al. 2023). *B. megaterium*, known for its ability to solubilize phosphate, has been extensively studied to determine its effectiveness in remediating soil contaminated with heavy metals such as boron (B), lead (Pb), and cadmium (Cd) (Esringü et al. 2014; Deng et al. 2020). Nascimento et al. (2019) demonstrated the ability of *B. megaterium* to enhance plant growth under optimal and stress conditions. *Rhizobium* sp. is renowned for their ability to form nodules and fix atmospheric nitrogen, highlighting the importance of the *Rhizobium* sp. SL42 isolate (Ilangumaran et al. 2024). Additionally, *Rhizobium* sp. SL42 is capable of forming small nodules on soybean plants. Genome analysis has revealed various genes associated with various biological functions in *Rhizobium* sp. SL42 which plays an important role in plant-microbe interactions and enhanced plant growth (Nelson and Sadowsky 2015).

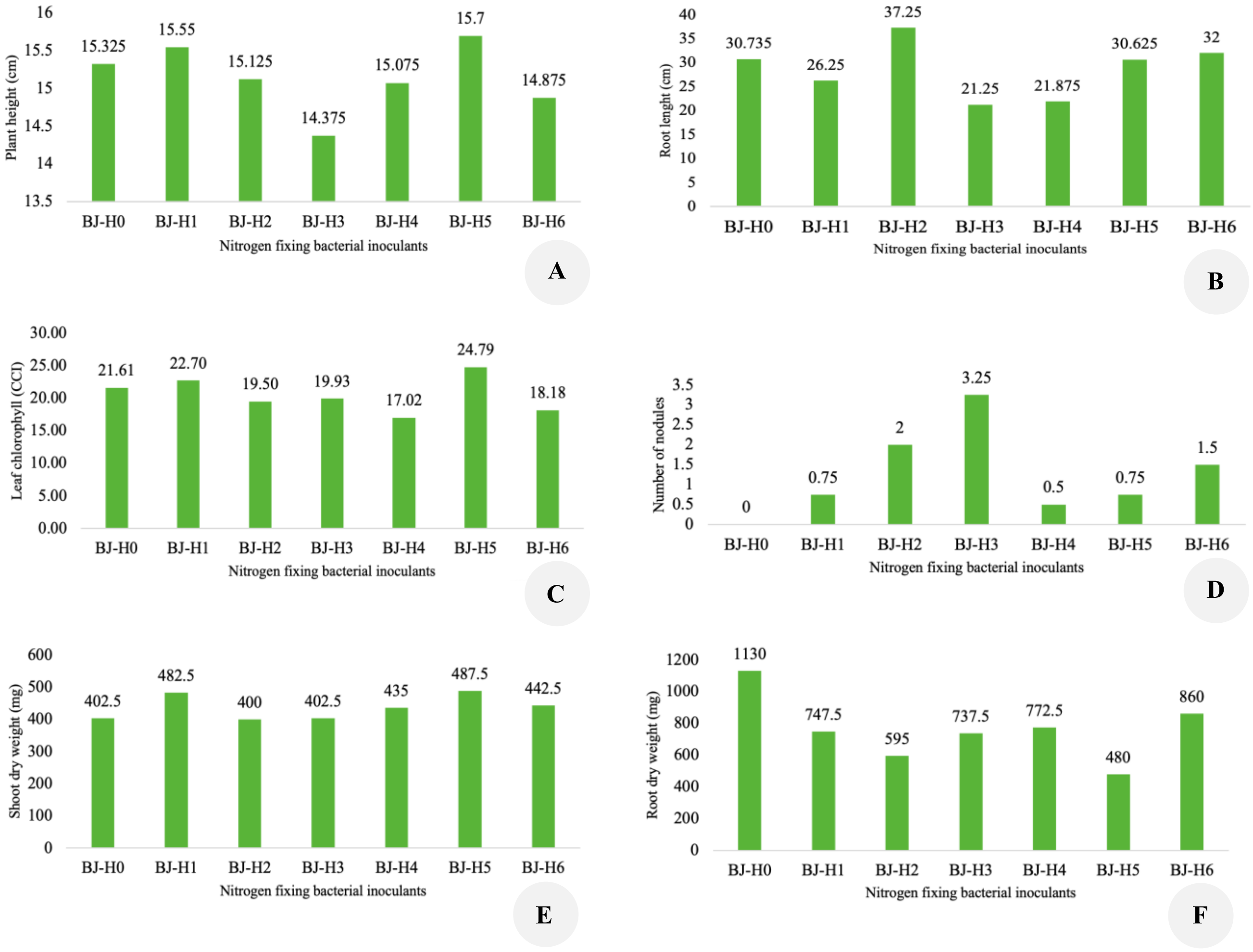

Figure 6. Response of black soybean plants to nitrogen-fixing bacterial inoculants on several plant parameters (plant height, root length, leaf chlorophyll, number of nodules, shoot dry weight, and root dry weight)

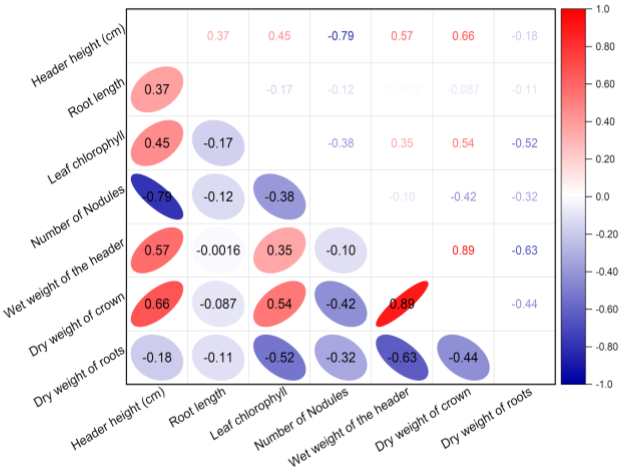

Figure 7. Correlation matrix showing the Pearson correlation coefficients among seven morphophysiological traits of black soybean, including parameters related to plant structure and biomass. Strong positive and negative correlations are highlighted, indicating interrelationships between growth attributes and their potential influence on overall plant performance under SNFB inoculation treatments

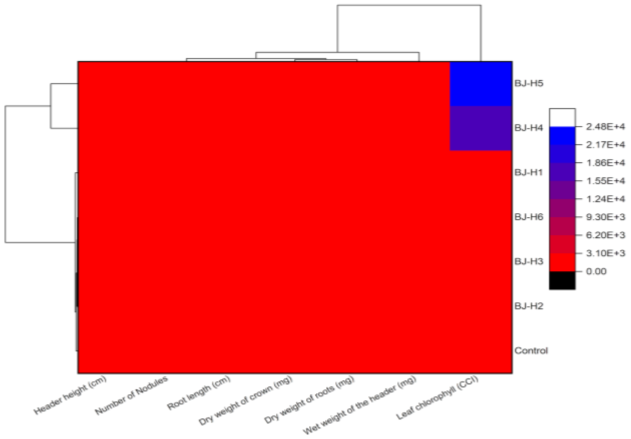

Figure 8. Clustering of the observed traits presented by Dendrogram Heatmap Analysis. Notably, features such as the crown's dry weight and the shoot's wet weight are constantly co-cluster, confirming their positive link as seen in the correlation matrix

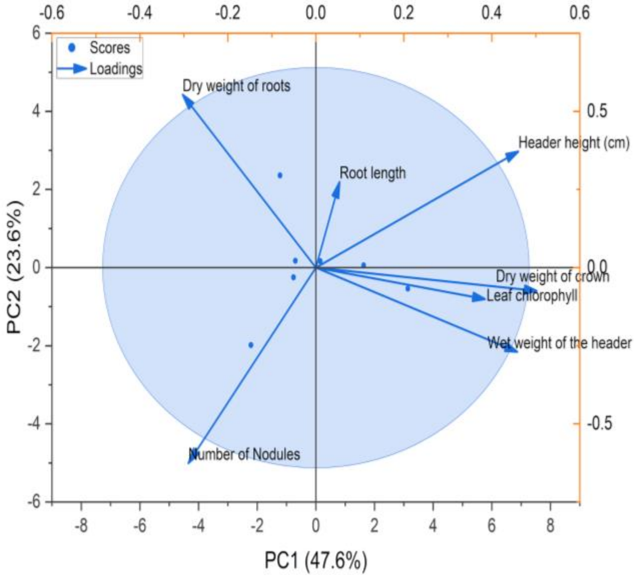

Figure 9. Principal Component Analysis (PCA) biplot of the traits showing the distribution of Principal Component scores (PC scores) and loadings on the morphological traits of soybean plants. The relationships between these variables are described by the direction and length of the loading vectors, where variables with the same loading vector direction have a high positive correlation

This study demonstrates that inoculation with selected SNFB from diverse agroecosystems enhances the morphophysiological performance of black soybean under nutrient-deficient conditions. The observed improvements in plant height, chlorophyll content, nodulation, and biomass accumulation indicate that certain native strains, particularly BJ-H5, BJ-H2, and BJ-H3 possess traits beneficial to crop productivity and soil health (Szpunar-Krok et al. 2023). The magnitude of response was consistent with the measured nitrogenase activity (Table 3) and IAA production (Table 4), showing that these physiological mechanisms plausibly contributed to plant growth promotion

The superior performance of BJ-H5 (*B. megaterium*) aligns with its high nitrogenase activity (24.906 µM ml^{-1} g^{-1} h^{-1}) and measurable IAA production (6.292 ppm). These characteristics are consistent with its capacity to fix atmospheric nitrogen and to synthesize growth-promoting metabolites that influence shoot vigor and chlorophyll accumulation (Egamberdieva et al. 2018; Etesami and Glick 2024). While direct causation cannot be inferred, the positive association between these biochemical traits and shoot biomass (Table 6) suggests that nitrogen fixation and low-level auxin production may jointly enhance nutrient uptake efficiency and photosynthetic potential. Similar interactions have been reported in soybean-PGPR systems where BNF and phytohormone synthesis co-occur (de Castilho et al. 2021; Fahde et al. 2023). In contrast, isolates BJ-H2 and BJ-H3, both originating from the soybean-field ecosystem, produced the highest nodule numbers and increased root biomass. These outcomes are consistent with the higher organic C and N content but lower available P and K observed in the soybean-field soil (Table 1), conditions that typically favor symbiotic nitrogen fixation over shoot biomass allocation (Nguyen et al. 2020). The adaptation of these isolates to legume-associated nutrient regimes may explain their effectiveness in nodulation and nitrogen assimilation (Omari et al. 2022).

By comparison, BJ-H5 isolated from ex-paddy soil enriched in available P (42.36 ppm) and K (45.95 mg 100 g^{-1}) was associated with higher chlorophyll content and shoot dry weight. These traits are often supported by greater P and K availability, nutrients known to enhance photosynthetic capacity and carbon allocation (Shivanna and Nagendrappa 2019). Thus, the functional differentiation among isolates plausibly reflects their ecosystem origin: ex-paddy isolates adapted to P/K-rich soils promote above-ground biomass, while soybean-field isolates adapted to lower-P environments favor nodulation and N-fixation. This ecosystem-origin linkage provides an important ecological interpretation of the dataset. It demonstrates that microbial functional specialization corresponds to the soil nutrient status of the source environment, a finding consistent with studies showing that native PGPR perform best in soils matching their origin (Rilling et al. 2019; Kiprotich et al. 2025). While the current study was conducted under greenhouse conditions, the results highlight the need for field validation to determine whether these associations persist under natural soil variability and microbial competition.

Beyond individual plant responses, the use of native SNFB inoculants has broader agronomic and ecological significance. Effective inoculation can reduce synthetic nitrogen fertilizer inputs by 50-100 kg N ha^{-1} without yield penalties (Santos et al. 2019; Gitonga et al. 2021). The positive correlations between nitrogenase activity, chlorophyll content, and biomass found here support the potential of SNFB to contribute to sustainable intensification. Nevertheless, strain performance is likely context-specific, depending on soil fertility, climate, and crop genotype. Therefore, continued research integrating strain selection, soil nutrient profiling, and multi-location trials will be critical for the development of reliable, ecosystem-tailored biofertilizers.

In conclusion, this study demonstrates that Symbiotic Nitrogen-Fixing Bacteria (SNFB) from different agroecosystems distinctly influenced black soybean growth under nutrient-limited conditions. *B. megaterium* (BJ-H5) from the ex-paddy ecosystem, with high nitrogenase and IAA activities, was associated with increased plant height, chlorophyll content, and shoot biomass, while soybean-field isolates BJ-H2 and BJ-H3 enhanced nodulation. These outcomes reflect functional differentiation linked to ecosystem origin, where nutrient-rich ex-paddy soils (high P and K) favoured shoot traits and nutrient-poor soybean-field soils promoted symbiotic nitrogen fixation. The findings emphasize the eco-functional importance of native microbial diversity in Indonesia's agroecosystems as a valuable biological resource for sustainable intensification and nutrient cycling. Harnessing such indigenous microbes offers an ecologically sound strategy to improve productivity while reducing chemical fertilizer dependence. The BJ-H5 strain represents a promising bioinoculant for enhancing shoot vigor, whereas BJ-H2 and BJ-H3 are suited for nodulation improvement. Future research should focus on field-scale validation, formulation stability, and multi-site trials to confirm efficacy and adaptability across varying agroecological conditions.

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