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Isolation and selection of nitrogen-fixing rhizosphere bacteria from vegetable crops

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Abstract. *Thuy NP, Tuu NT. 2025. Isolation and selection of nitrogen-fixing rhizosphere bacteria from vegetable crops. Biodiversitas 26: 4841-4850.* Intensive vegetable cultivation in Vietnam's Mekong Delta heavily relies on synthetic nitrogen fertilizers, causing significant environmental issues. The aim of this study was to identify indigenous nitrogen-fixing bacteria as sustainable biofertilizers, emphasizing their crucial role in enhancing soil health and agro-biodiversity. Rhizospheric soils were collected from 17 local vegetable species, isolating 36 distinct bacterial strains. Initial characterization revealed a numerical dominance of Gram-positive bacteria (>83%), reflecting adaptation to Tra Vinh's unique conditions, and included a filamentous *Actinobacteria* isolate. The functionally potent Gram-negative isolates exhibited morphological and biochemical characteristics consistent with genera, such as *Achromobacter*, *Stenotrophomonas*, and *Dyella*. Nitrogen fixation screening via micro-Kjeldahl showed activity from 5.89 mg/L to 24.59 mg/L NH_4^+. Five elite strains, identified through 16S rRNA gene sequencing as *Stenotrophomonas* sp. SM9.2, *Achromobacter* sp. IB1.2, *Enterobacter hormaechei* subsp. *xiangfangensis* SM18.2, *Achromobacter* sp. SM9.1, and *Dyella* sp. SM18.3, demonstrated the highest nitrogen fixation rates. Laboratory bioassays with water spinach (*Ipomoea aquatica*) confirmed significant plant growth promotion. Notably, *Stenotrophomonas* sp. SM9.2 increased fresh biomass by 27.7% and shoot length by 16.6%, while *Achromobacter* sp. IB1.2 improved seed germination by 9.9%. These findings highlight promising candidates for local biofertilizer production and underscore the importance of leveraging indigenous microbial biodiversity for sustainable farming solutions.

Keywords: *Achromobacter*, *Ipomoea aquatica*, nitrogen-fixing bacteria, rhizosphere, *Stenotrophomonas*

INTRODUCTION

Modern agriculture's success in meeting global food demands has come at a significant environmental cost, largely due to a profound dependency on synthetic nitrogen fertilizers. This dependency drives water pollution through nitrate runoff, increases greenhouse gas emissions (N_2O), and degrades soil health via acidification and organic matter loss (Bindraban et al. 2015). In agriculturally critical regions such as Vietnam's Mekong Delta, where intensive vegetable cultivation underpins both the local economy and national food security (Pham et al. 2023). These pressures create an urgent need for a paradigm shift towards more sustainable and ecologically resilient farming practices (Singh and Ryan 2015).

A promising solution to this challenge lies within the soil itself, specifically in the complex world of the plant microbiome. The rhizosphere, the thin layer of soil directly surrounding plant roots, is one of the most microbially dense and active habitats on the planet (Slaughter 2021). This zone hosts a diverse community of bacteria, fungi, and archaea that performs a suite of services essential for ecosystem function and plant vitality, including nutrient cycling, pathogen suppression, and the improvement of soil structure (Solanki et al. 2024). The relationship between a plant and its microbial community is so deeply integrated that they are often viewed as a single functional unit, or "holobiont," in which the microbiome acts as an extended genome, enhancing the host plant's ability to acquire nutrients and adapt to environmental stress (Averill et al. 2022).

The composition of beneficial rhizosphere communities is influenced by both the host plant and the local environment. Plants release root exudates, a mix of carbon-rich compounds, to attract specific microbes from the soil (Zhalnina et al. 2018; Santoyo 2022). This selective process results in a distinct microbial community uniquely adapted to the specific plant species as well as the local soil and climate conditions (Dwivedi et al. 2025; Johnson and Marín 2025). Evidence from crops like barley and maize confirms that while plant genetics play a role, environmental factors strongly influence the assembly of consistent and effective microbial communities in specific regions (He et al. 2024; Williams et al. 2024). This principle of habitat-driven specificity suggests that the most effective microbial inoculants for a given crop are likely to be indigenous strains already adapted to the target agricultural environment.

The rhizosphere microbiome plays a vital role in plant growth promotion and biological nitrogen fixation (BNF), both key for sustainable agriculture. Plant Growth-Promoting Rhizobacteria (PGPR) enhance crop yields through mechanisms like nutrient solubilization, phytohormone production, and improving stress tolerance (Souza et al. 2015; Igiehon and Babalola 2018; Shilev 2020). Diazotrophs, a group of bacteria, perform BNF, converting atmospheric nitrogen into ammonia for plant uptake (Ladha et al. 2016).

Utilizing native nitrogen-fixing bacteria (NFB) reduces dependence on synthetic fertilizers, lowering costs for farmers and minimizing environmental impacts (Sainju et al. 2019; Muratore et al. 2021).

The use of NFB as bio-inoculants for vegetable crops is well-established. For instance, *Paenibacillus polymyxa* WLY78 enhanced cucumber growth, providing nearly 26% of the plant's nitrogen (Li et al. 2022). Likewise, *Priestia*, *Ochrobactrum*, and *Enterobacter* have demonstrated the ability to improve the growth and yield of crops like cucumber, green chili, and wheat (Xu et al. 2015; Ghorai and Ghosh 2023; Buisset et al. 2025). However, microbial inoculants' success depends on their ability to survive and function in local conditions, highlighting the importance of bioprospecting indigenous strains. Indeed, research in Vietnam has already identified promising native NFB from *Azotobacter*, *Azospirillum*, and *Enterobacter* (Ha et al. 2018; Khuong et al. 2022; Thu et al. 2022). Despite this progress, a systematic investigation of NFB from the rhizospheres of the diverse and economically important vegetable crops of the Mekong Delta has not yet been conducted. The primary objectives of this study were to isolate and identify NFB from the root zones of selected vegetable species, characterize their nitrogen-fixing capabilities in vitro, and screen the most efficient nitrogen-fixers for additional plant growth-promoting traits. The identified specific native bacterial strains can be used to develop effective, locally adapted biofertilizers.

MATERIALS AND METHODS

Rhizosphere soil sampling

To obtain a representative sample of microbial diversity, rhizosphere soils were collected from 40 distinct vegetable fields across Tra Vinh Province, Vietnam. At each location, a composite sample was prepared by pooling five subsamples collected from the top 20 cm of soil directly adjacent to plant roots. 40 composite samples were placed in sterile polyethylene bags, transported on ice to the laboratory, and immediately processed. A portion of each sample was air-dried for 48 hours to select for desiccation-tolerant microorganisms, sieved (2 mm mesh) to remove debris, and stored at 4°C until analysis (Díaz et al. 2021).

Isolation of rhizobacteria

For each of 40 composite soil samples, a 10 g aliquot was suspended in 90 mL of sterile distilled water. The suspensions were agitated on a rotary shaker at 180 rpm for 30 minutes at 37°C to dislodge rhizobacteria. A ten-fold serial dilution series (10^{-4} to 10^{-7}) was prepared from each suspension. One hundred microliters (100 µL) of each dilution were spread-plated in triplicate onto Burk's N-free medium (Wilson and Knight 1952). Plates were incubated at 37°C for seven days. Colonies exhibiting distinct morphologies were selected and purified by repeated streaking on fresh Burk's N-free medium. Pure isolates were grown in Luria-Bertani (LB) broth and preserved at -80°C in 30% (v/v) sterile glycerol.

Phenotypic characterization of bacterial isolates

Initial characterization of pure isolates was based on colony morphology, color, shape, margin on LB agar, and Gram staining (Tripathi and Sapra 2020). To provide definitive identification, five elite isolates demonstrating high nitrogen-fixing capacity were selected for molecular analysis. Genomic DNA was extracted from overnight LB broth cultures using the DNeasy PowerLyzer Microbial Kit (Qiagen, Germany) following the manufacturer's protocol. DNA quality and concentration were verified using 0.8% agarose gel electrophoresis and NanoDrop spectrophotometer (Thermo Scientific, USA) (Kumbhare et al. 2015).

The 16S rRNA gene was amplified via polymerase chain reaction (PCR) using the universal primers 27F (5′-`AGA GTT TGA TCC TGG CTC AG`-3′) and 1492R (5′-`TAC GGT TAC CTT GTT ACG ACT T`-3′) (Lane 1991). PCR was performed in 50 µL reaction volumes containing 2 µL of template DNA, 1x Platinum™ SuperFi™ II PCR Master Mix (Invitrogen), and 0.4 µM of each primer. A negative control with nuclease-free water was included in each run. Thermocycling conditions were: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 10 min. Amplified PCR products were visualized on a 2% agarose gel, purified, and sequenced by Next Gen Scientific Co., Ltd (Ho Chi Minh City, Vietnam). The resulting sequences were analyzed using the NCBI BLASTn tool to determine species-level identity based on sequence similarity in the GenBank database (Shreshtha et al. 2024).

Quantitative assessment of nitrogen fixation

The nitrogen-fixing capacity of each isolate was quantified using the micro-Kjeldahl method to measure the increase in total nitrogen in a liquid N-free culture (Puri et al. 2018). Each isolate was inoculated into 50 mL of Döbereiner's nitrogen-free liquid medium and incubated at 37°C with shaking (180 rpm) for six days. Following incubation, cultures were centrifuged (10,000 × g, 15 min, 4°C), and the cell pellet was collected. The total nitrogen content of the pellet was determined using a micro-Kjeldahl digestion and distillation unit. The experiment was conducted in triplicate for each isolate (Zhang et al. 2022).

Evaluation of bacterial strains on the growth parameters of *Ipomoea aquatica*

To prepare inoculum suspensions, each bacterial strain was cultured in 250 mL Erlenmeyer flasks containing 50 mL of Tryptic Soy Broth (TSB) medium at 37°C with shaking at 180 rpm for 72 hours. Cultures were then centrifuged at 7000 rpm for 5 minutes, and the supernatant was discarded. The cell pellets were washed twice with sterile distilled water by centrifugation and resuspended in sterile distilled water to obtain an optical density at 600 nm (OD600) of approximately 1.0, which roughly corresponds to 10^8 colony-forming units CFU/mL, ensuring uniform bacterial concentrations across all strains (Garcia-Lemos et al. 2020).

Ipomoea aquatica seeds (East-West Seed Co. Ltd., Vietnam) were surface-sterilized by immersion in 0.5%

sodium hypochlorite solution for 2 minutes, followed by three washes with sterile distilled water. Seeds were then dried on sterile filter paper. Thirty *I. aquatica* seeds were soaked in 30 mL of each bacterial suspension for 4 hours. Control treatments were conducted using sterile distilled water instead of bacterial suspensions.

Sterilized filter paper was placed in sterile plastic containers, and 30 seeds (treated with either bacterial suspension or sterile water) were placed on each filter paper. 10 mL of sterile distilled water were added to each container. The containers were sealed and incubated in the dark at 28±2°C. The experiment was replicated three times, with each replicate containing 30 seeds. After 6 days of incubation, the following parameters were recorded: (i) Plant height: Measured from the base of the stem to the tip of the longest leaf. (ii) Root length: Measured from the base of the stem to the tip of the longest root. (iii) Fresh biomass: Measured by weighing the entire plant after gently removing excess water.

Germination percentage: Germination percentage (%) = (Number of germinated seeds / Total number of seeds) × 100.

Statistical analysis

Data were statistically analyzed using SPSS 25.0. Mean values were compared using Duncan's New Multiple Range Test at a 5% level of significance ($p<0.05$) to determine significant differences between treatments.

RESULTS AND DISCUSSION

Isolation and phenotypic diversity of putative nitrogen-fixing bacteria

From the rhizospheres of 17 distinct vegetable crop species cultivated in Tra Vinh Province, a total of 36 bacterial isolates were successfully obtained using the selective pressure of Burk’s nitrogen-free medium. These isolates represent a phenotypically diverse collection from a wide range of host plants, including leafy greens, fruit-bearing vegetables, and herbs (Table 1).

Table 1. Source and morphological characteristics of 36 bacteria isolated from vegetable rhizosphere soils

Isolates code	Host plants	Colony morphology					Bacteria morphology	
		Form	Color	Size	Elevation	Margin	Gram staining	Shape
IB1.2	*Ipomoea batatas*	Round	Opaque white	Large	Flat	Entire	Negative	Rod
LS2.5	*Lactuca sativa*	Round	Transparent white	Small	Flat	Entire	Positive	Rod
LS2.7	*Lactuca sativa*	Round	Transparent white	Small	Convex	Entire	Positive	Rod
LS2.8	*Lagenaria siceraria*	Round	Opaque white	Small	Flat	Entire	Positive	Rod
BJ3.1	*Brassica juncea*	Round	Transparent white	Large	Convex	Entire	Positive	Rod
HC4.1	*Houttuynia cordata*	Round	Transparent white	Large	Raised	Entire	Positive	Cocci
CABE5.1	*Capsicum annuum*	Round	Creamy white	Small	Flat	Entire	Positive	Cocci
CA6.1	*Centella asiatica*	Round	Opaque white	Small	Flat	Entire	Positive	Cocci
CA6.2	*Centella asiatica*	Round	Transparent white	Large	Convex	Entire	Positive	Cocci
CA6.3	*Centella asiatica*	Round	Pale yellow	Large	Convex	Entire	Negative	Rod
AE7.1	*Abelmoschus esculentus*	Round	Transparent white	Small	Raised	Entire	Positive	Cocci
OJ8.1	*Oenanthe javanica*	Round	Transparent white	Small	Convex	Entire	Positive	Rod
OJ8.2	*Oenanthe javanica*	Round	Opaque white	Small	Raised	Entire	Positive	Rod
SM9.1	*Solanum melongena*	Round	Transparent white	Large	Convex	Entire	Positive	Cocci
SM9.2	*Solanum melongena*	Round	Transparent white	Small	Convex	Entire	Negative	Rod
SM9.3	*Solanum melongena*	Round	Opaque white	Large	Flat	Entire	Negative	Rod
CF10.2	*Capsicum frutescens*	Round	Transparent white	Small	Flat	Entire	Positive	Rod
MCL11.1	*Momordica charantia*	Round	Opaque white	Small	Raised	Entire	Positive	Cocci
BH12.1	*Benincasa hispida*	Round	Transparent white	Large	Convex	Entire	Positive	Cocci
IA13.1	*Ipomoea aquatica*	Round	Transparent white	Small	Convex	Entire	Positive	Cocci
CSL14.2	*Cucumis sativus*	Round	Transparent white	Small	Raised	Entire	Positive	Cocci
SL15.1	*Solanum lycopersicum*	Round	Transparent white	Large	Convex	Entire	Positive	Rod
MCL16.1	*Momordica charantia*	Round	Transparent white	Small	Raised	Entire	Positive	Cocci
CSL17.1	*Cucumis sativus*	Round	Transparent white	Small	Raised	Entire	Positive	Rod
CSL17.2	*Cucumis sativus*	Round	Transparent white	Small	Raised	Entire	Positive	Rod
CSL17.3	*Cucumis sativus*	Round	Transparent white	Small	Raised	Entire	Positive	Cocci
CSL17.4	*Cucumis sativus*	Round	Transparent white	Small	Raised	Entire	Positive	Cocci
SM18.1	*Solanum melongena*	Round	Creamy white	Small	Convex	Entire	Positive	Cocci
SM18.2	*Solanum melongena*	Round	Creamy white	Large	Convex	Entire	Negative	Rod
SM18.3	*Solanum melongena*	Round	Transparent white	Large	Raised	Entire	Negative	Rod
SM18.4	*Solanum melongena*	Round	Transparent white	Small	Raised	Entire	Positive	Cocci
SL19.1	*Solanum lycopersicum*	Round	Transparent white	Large	Convex	Entire	Positive	Rod
CMD20.1	*Cucurbita moschata*	Round	Transparent white	Small	Raised	Entire	Positive	Filamentous
CMD20.2	*Cucurbita moschata*	Round	Transparent white	Large	Convex	Entire	Positive	Cocci
CMD20.3	*Cucurbita moschata*	Round	Creamy white	Small	Raised	Entire	Positive	Cocci
CMD20.4	*Cucurbita moschata*	Round	Opaque white	Small	Convex	Entire	Positive	Cocci

Initial characterization revealed considerable morphological diversity among the isolates. While all isolates formed circular colonies with entire margins, there was significant variation in elevation, color, and size. Convex (41.7%) and raised (38.9%) colony elevations were most common. The majority of isolates were transparent white (66.7%), with a smaller proportion appearing opaque white (19.4%) or creamy white (11.1%). A single isolate, CA6.3, was distinguished by a pale-yellow pigmentation. Microscopic analysis showed a strong predominance of Gram-positive bacteria, which accounted for 83.3% (n=30) of the collection. The remaining 16.7% (n=6) were Gram-negative. Cellular morphology was evenly distributed between coccoid (50.0%) and short-rod shapes (47.2%), with one isolate (CMD20.1) exhibiting a distinct filamentous structure, characteristic of Actinobacteria.

Isolate distribution and richness across host plants

The distribution and richness of culturable isolates varied considerably among the different host vegetable species, indicating a potential influence of the host plant on the diazotrophic community (Table 2). The highest isolate richness was recovered from the rhizosphere of eggplant (*Solanum melongena*), which yielded seven distinct isolates, followed by cucumber (*Cucumis sativus*) with five isolates and pumpkin (*Cucurbita moschata*) with four isolates. In contrast, several crops, including mustard greens (*Brassica juncea*) and water spinach (*Ipomoea aquatica*), yielded only a single isolate each. Gram-positive bacteria were ubiquitous, being isolated from 15 of the 17 host species, whereas Gram-negative isolates were found in only five species, suggesting a widespread prevalence of Gram-positive diazotrophs in these agricultural soils.

Evaluation of nitrogen fixation capacity of isolated strains

To directly assess their primary functional trait, all 36 purified isolates were screened for their ability to fix atmospheric nitrogen. This was quantified by measuring the accumulation of ammonium (NH_4^+) in the supernatant of cultures grown in Döbereiner's nitrogen-free liquid medium over a six-day incubation period. The micro-Kjeldahl method was employed for ammonium quantification. The isolates displayed a broad spectrum of diazotrophic activity, with measured ammonium concentrations ranging from a low of 5.89 mg/L to a high of 24.59 mg/L (Figure 1). Based on this functional screening, five strains were selected for further analysis due to their exceptionally high performance. The most effective isolate was strain SM9.2, which produced 24.59±7.44 mg/L of NH_4^+. The other four elite strains also demonstrated robust activity: IB1.2 (19.72±0.84 mg/L), SM18.2 (19.51±1.17 mg/L), SM9.1 (18.21±0.63 mg/L), and SM18.3 (16.33±1.55 mg/L). In contrast, the majority of the remaining isolates showed moderate to low but still detectable levels of nitrogen fixation, typically falling within the 6-12 mg/L range. These five top-performing isolates were chosen as the primary candidates for subsequent molecular identification and plant growth promotion assays.

Table 2. Distribution and phenotypic richness of bacterial isolates across different vegetable crop rhizospheres

Host plants	Number of isolates	Gram-positive	Gram-negative
Solanum melongena	7	4	3
Cucumis sativus	5	5	0
Cucurbita moschata	4	4	0
Centella asiatica	3	2	1
Lactuca sativa	2	2	0
Oenanthe javanica	2	2	0
Momordica charantia	2	2	0
Solanum lycopersicum	2	2	0
Lagenaria siceraria	1	1	0
Brassica juncea	1	1	0
Houttuynia cordata	1	1	0
Capsicum annuum	1	1	0
Abelmoschus esculentus	1	1	0
Benincasa hispida	1	1	0
Ipomoea aquatica	1	1	0
Ipomoea batatas	1	0	1
Capsicum frutescens	1	1	0
Total	36	30	6

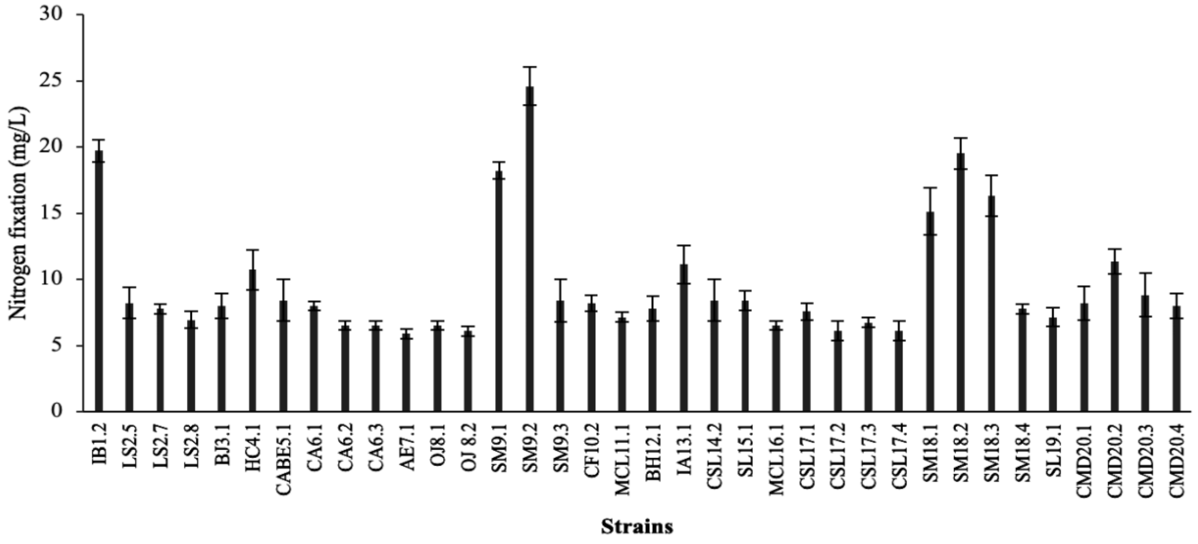

Figure 1. Nitrogen fixation in a nitrogen-free liquid medium (mg/L of NH_4^+) for all 36 isolates

Molecular identification of elite nitrogen-fixing strains using 16S rRNA gene sequencing

To determine the taxonomic identities of the five most potent nitrogen-fixing isolates, we performed molecular analysis based on partial sequencing of the 16S rRNA gene, a conserved molecular marker widely used for bacterial phylogeny. Genomic DNA was extracted from pure cultures of each of the five selected strains (SM9.2, IB1.2, SM18.2, SM9.1, and SM18.3). A significant fragment of the 16S rRNA gene (approximately 1,465 bp) was amplified via PCR using the universal bacterial primers 27F and 1492R. The resulting PCR amplicons were purified and sequenced. The obtained nucleotide sequences were then subjected to a BLASTn analysis against the National Center for Biotechnology Information (NCBI) GenBank database to identify the closest known relatives.

The sequence analysis provided robust genus-level or species-level identifications for all five elite strains (Table 3). The results of molecular analysis revealed that the five elite isolates represent a taxonomically broad and valuable collection, spanning four distinct genera (*Stenotrophomonas*, *Achromobacter*, *Enterobacter*, and *Dyella*) across at least three different bacterial families: Xanthomonadaceae, Alcaligenaceae, and Enterobacteriaceae.

The specific identifications were as follows: SM9.2, the top-performing nitrogen fixer, showed 100% sequence identity to *Stenotrophomonas* sp. pho (CP029759.1), unequivocally placing it within genus *Stenotrophomonas*. IB1.2 and SM9.1, both strong nitrogen fixers, were identified as belonging to the genus *Achromobacter*. Strain IB1.2 displayed 93% sequence similarity to *Achromobacter* sp. (MH773279.1), while strain SM9.1 showed a very high 99% similarity to *Achromobacter* sp. (MZ768702.1). SM18.2 was identified as *Enterobacter hormaechei* subsp. *xiangfangensis*, with a sequence similarity of 87% (accession OQ786924.1). This place is firmly within the well-known plant-associated family Enterobacteriaceae. SM18.3 exhibited 98% sequence similarity to *Dyella* sp. RD51540026 (KT368943.1), indicating its affiliation with the genus *Dyella*.

In vitro evaluation of plant growth-promoting activities

Five elite nitrogen-fixing isolates were selected to evaluate PGP potential through a controlled bioassay using water spinach (*I. aquatica*), a fast-growing and regionally important model crop was conducted. The effect of seed inoculation with each of the five strains (T1: SM9.1, T2: SM18.3, T3: SM18.2, T4: SM9.2, and T5: IB1.2) was compared against an uninoculated control. After six days of incubation, several key growth parameters of control and treated plants were observed (Figure 2).

Effect on seed germination: Inoculation positively affected seed germination. The control group exhibited a germination rate of 71.00±1.73%. Strain IB1.2 (T5) induced the highest germination percentage at 78.00±1.73%, which was statistically significantly higher than the control. Strain SM9.2 (T4) also significantly improved germination to 74.00±2.30%. The other three strains (T1, T2, T3) produced germination rates that were not statistically different from the control.

Effect on seedling elongation: All bacterial treatments, with the exception of T2, led to an increase in shoot length, though not all were statistically significant. Strain SM9.2 (T4) produced the longest shoots, averaging 12.19±0.08 cm, a highly significant increase over the control's 10.45±0.10 cm and all other treatments. Strain IB1.2 (T5) also resulted in significantly longer shoots (11.76±0.06 cm), as did strain SM18.2 (T3) (11.33±0.09 cm). Root development was even more broadly stimulated. Inoculation with four of the five strains SM9.1 (T1), SM18.3 (T2), SM9.2 (T4), and IB1.2 (T5) all resulted in root lengths that were statistically significantly longer than those of the control seedlings.

Effect on fresh biomass: Overall plant vigor, as measured by fresh biomass, was significantly enhanced by several bacterial treatments. The control plants had an average fresh biomass of 7.83±0.76 g. Plants inoculated with strain SM9.2 (T4) achieved the highest fresh biomass of 10.00±0.20 g, which was significantly greater than the control. Strains SM18.2 (T3) and IB1.2 (T5) also produced significantly higher biomass than the control plants, at 9.43±0.40 g and 9.33±0.29 g, respectively.

In summary, when considering the combined effects across all measured growth parameters, strains SM9.2 (*Stenotrophomonas* sp.) and IB1.2 (*Achromobacter* sp.) consistently demonstrated the most potent and comprehensive PGP activities in this laboratory assay. They significantly improved germination, shoot length, root length, and fresh biomass, highlighting their strong potential as effective biofertilizers for *I. aquatica* and likely other vegetable crops.

Table 3. Distribution and phenotypic richness of bacterial isolates across different vegetable crop rhizospheres

Isolates code	Host plants	Species identification	Identity value (%)	NCBI accession number
SM9.2	*Solanum melongena*	*Stenotrophomonas* sp. pho	100	CP029759.1
IB1.2	*Ipomoea batatas*	*Achromobacter* sp.	93	MH773279.1
SM18.2	*Solanum melongena*	*Enterobacter hormaechei* subsp. *xiangfangensis*	87	OQ786924.1
SM9.1	*Solanum melongena*	*Achromobacter* sp.	99	MZ768702.1
SM18.3	*Solanum melongena*	*Dyella* sp. RD51540026	98	KT368943.1

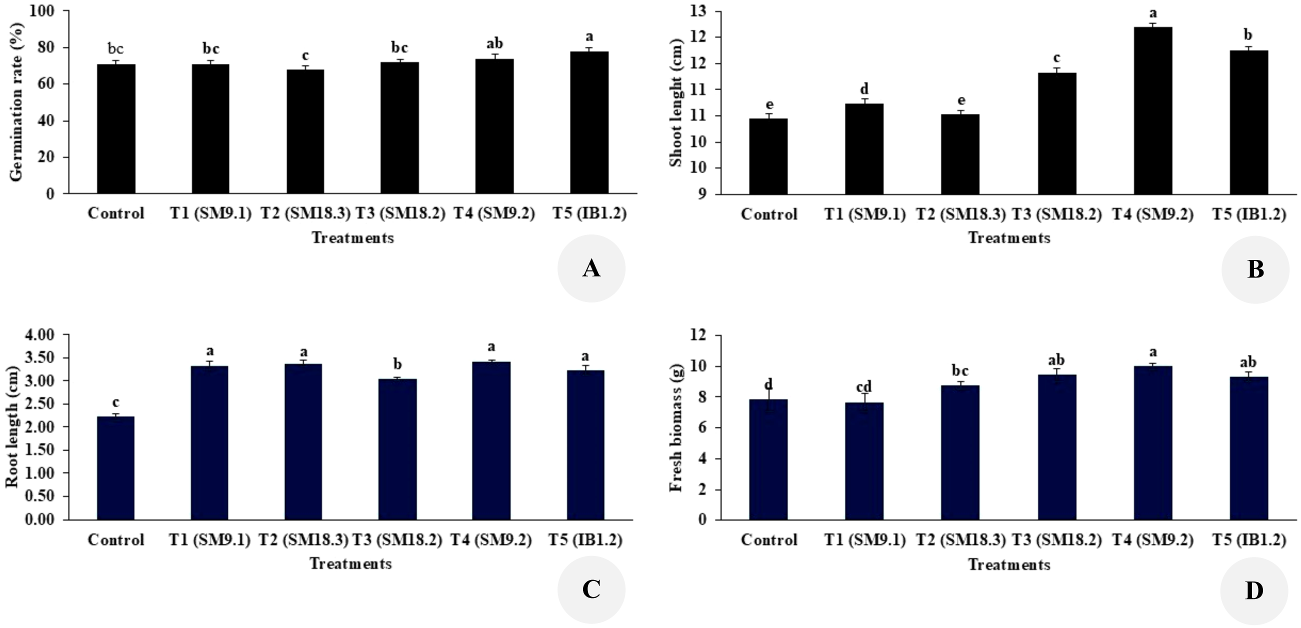

Figure 2. Plant growth-promoting effects of five elite bacterial strains on *Ipomoea aquatica* after six days. The charts show the impact of inoculation on: A. germination rate, B. shoot length, C. root length, and D. fresh biomass. Treatments were control (uninoculated), T1 (SM9.1), T2 (SM18.3), T3 (SM18.2), T4 (SM9.2), and T5 (IB1.2). Different letters above the bars indicate statistically significant differences between treatments (Duncan's test, p<0.05)

Discussion

Intensive agriculture in Vietnam's Mekong Delta has led to an over-reliance on synthetic fertilizers, causing significant environmental and economic strain (Atieno et al. 2020). A key strategy for achieving this is to leverage the indigenous soil microbiome, which is known to be a source of beneficial microbes shaped by local agroecological conditions (Bindraban et al. 2015; Shivay et al. 2020). This research contributes new, localized evidence to this field by specifically demonstrating that the rhizospheres of vegetable crops in this region are a significant and untapped reservoir of nitrogen-fixing bacteria.

Isolation, phenotypic diversity, and distribution of rhizobacteria

The results showed that 36 morphologically distinct bacterial isolates were associated with the vegetable rhizospheres in Tra Vinh Province, providing a first look into the culturable diazotrophic communities of this vital agricultural region. A striking and ecologically significant finding was the pronounced dominance of Gram-positive bacteria, which constituted 83.3% of all successful isolates. This prevalence is not a random occurrence but rather a direct reflection of the intense selective pressures inherent to the Tra Vinh agroecosystem. The region's alluvial soils, characterized by high clay content, are subject to extreme seasonal fluctuations, from waterlogged, anaerobic conditions during the monsoon season to periods of intense heat and desiccation in the dry season (Aasfar et al. 2021). Gram-positive bacteria, particularly those from genera known to form highly resilient endospores like *Bacillus* and *Paenibacillus*, possess a decisive adaptive advantage in such environments (Raimi et al. 2023; Chen et al. 2024). The endospore state allows them to enter a dormant, protected phase, conferring exceptional tolerance to environmental stresses, including osmotic shock, nutrient limitation, and exposure to agrochemicals This dormant state confers exceptional tolerance to the fluctuating and severe environmental stresses common in agricultural fields, such as drought, waterlogging from seasonal floods, abrupt changes in soil chemistry, and agrochemical exposure (Aasfar et al. 2021). This survival strategy ensures their persistence through unfavorable periods, allowing them to rapidly resume metabolic activity and colonize plant roots when conditions improve (Sun et al. 2025). This finding sharply contrasts with studies conducted in more stable, temperate agricultural systems, where Gram-negative bacteria are often found to be more abundant in the rhizosphere (Abedinzadeh et al. 2019), underscoring the principle that local environmental conditions are a primary determinant of microbial community structure. The isolation of a filamentous Actinobacterium (CMD20.1) further supports this conclusion, as its mycelial growth habit is particularly advantageous for exploring and colonizing the complex pore structure of heavy clay soils, enhancing its access to nutrients and water (Barka et al. 2016).

Furthermore, the findings demonstrated that diversity and distribution of these isolates were significantly influenced by the host plant species. The rhizospheres of eggplant (*Solanum melongena*) and cucumber (*Cucumis sativus*) yielded the highest number of distinct isolates (seven and five, respectively), while other crops like mustard greens (*Brassica juncea*) yielded only one. This observation strongly supports the well-established ecological principle that plants are not passive inhabitants of the soil but actively architect their own rhizosphere microbiomes

(Kumar et al. 2021; Santoyo 2022). This process, known as rhizodeposition, involves the secretion of a complex cocktail of carbon-rich compounds, including sugars, organic acids, amino acids, and species-specific secondary metabolites, collectively known as root exudates (Hu et al. 2018; Zhalnina et al. 2018; Backer et al. 2018; Qin et al. 2019; Zhao et al. 2025). These exudates create unique biochemical niches that function as both signals and nutritional substrates, selectively recruiting and fostering specific microbial partners from the bulk soil. The diverse plant families sampled in our study, from Solanaceae and Cucurbitaceae to Brassicaceae, are known to produce distinct exudate profiles. Solanaceae plants often exude specific alkaloids, while cucurbits release different sets of organic acids, each selecting for a different microbial consortium capable of metabolizing these specific compounds (Wen et al. 2020; Bouwmeester et al. 2025). This host-driven selection underscores a critical point for agricultural application: the concept of a universal, "one-size-fits-all" biofertilizer is inherently flawed. An inoculant's success its ability to survive, compete, colonize roots, and perform its intended function is intrinsically linked to its ecological compatibility with the host plant's physiology and exudate chemistry (Fourneau et al. 2024). Consequently, our findings robustly validate the strategy of bioprospecting directly from local, adapted plant-soil systems. This approach is the most rational and promising method for identifying microbial strains that are not only functionally potent but also pre-adapted to both the target crop and the challenging local environment, paving the way for the development of tailored and highly effective biofertilizer solutions for the Mekong Delta.

Therefore, alongside soil type and geography (Qin et al. 2019), host-specificity is a paramount factor. The findings of current study validate the strategy of bioprospecting directly from local, adapted plant-soil systems. This approach is the most rational and promising method for identifying microbial partners that are not only functionally potent but also ecologically compatible with the target crops, paving the way for tailored and effective biofertilizer solutions in the Mekong Delta.

Functional fixation capacity and molecular identity of elite strains

The isolated strains displayed a broad spectrum of nitrogen-fixing capabilities, with ammonium accumulation ranging from 5.89 to 24.59 mg/L. This functional diversity suggests a resilient native diazotrophic community, where different members are likely to contribute to the ecosystem's nitrogen budget under various conditions. The exceptional performance of the top isolate, *Stenotrophomonas* sp. SM9.2 (24.59 mg/L), establishes a new regional benchmark, significantly surpassing rates reported in other studies from Vietnam (Ha et al. 2018) and for endophytic bacteria from other globally important crops (Zhang et al. 2022).

Biological nitrogen fixation is an energy-intensive process tightly regulated by environmental factors, particularly the availability of carbon from root exudates and the absence of inhibitory oxygen levels (Coskun et al. 2017; Timofeeva et al. 2023). The superior performance of our elite isolates strongly implies they possess sophisticated adaptations to the local environment. These strains are likely highly efficient at metabolizing the specific carbon sources supplied by local vegetable crops while employing robust mechanisms to protect their oxygen-sensitive nitrogenase enzyme. This latter trait is critical for survival in the fluctuating redox conditions of Tra Vinh's alluvial soils (Roper and Halsall 1986).

Molecular identification of the five most potent nitrogen-fixers revealed their distribution across four distinct genera: *Stenotrophomonas*, *Achromobacter*, *Enterobacter*, and *Dyella*. This taxonomic breadth is a significant finding, as it demonstrates that the vital ecosystem function of nitrogen fixation is not confined to a single bacterial lineage but is a widespread trait among phylogenetically diverse members of the rhizosphere community. Such functional redundancy is a hallmark of a resilient agroecosystem.

Although some species within the *Stenotrophomonas* genus are known as opportunistic pathogens, plant-associated strains like SM9.2 are increasingly recognized for their profound agricultural benefits (Kumar et al. 2023). Their metabolic versatility is a key asset, often including a full suite of plant growth-promoting traits beyond nitrogen fixation (Bashandy et al. 2020; Huda et al. 2022; Singh et al. 2020). Similarly, the genus *Achromobacter* was well-represented by two potent isolates, IB1.2 and SM9.1. Members of this genus are common soil inhabitants and rhizospheric partners of various crops, where they are known to enhance nutrient uptake and mitigate plant stress (Rimi et al. 2024). Finding two highly effective strains suggests that *Achromobacter* may be a functionally important group in the local agricultural soils.

The identification of strain SM18.2 as *Enterobacter hormaechei* aligns with extensive literature establishing *Enterobacter* as a cornerstone genus in plant growth-promoting rhizobacteria (PGPR) research (Rodrigues et al. 2016; Khuong et al. 2022). Notably, the 87% 16S rRNA gene sequence similarity of strain SM18.2 to its closest match suggests it could represent a novel strain or subspecies, warranting further genomic investigation. Finally, the isolation of a potent nitrogen-fixing *Dyella* strain (SM18.3) is a valuable discovery, as this genus is less commonly highlighted in PGPR literature. While known to possess PGP traits (Sembiring and Sabrina 2021), this finding indicates *Dyella* may play a more significant role in soil nitrogen dynamics than previously appreciated, marking it as an intriguing candidate for future study.

In vitro plant growth promotion on Ipomoea aquatica

A critical validation for any potential biofertilizer is demonstrating that its biochemical capabilities translate into tangible plant growth benefits. Our *in vitro* bioassay using water spinach (*I. aquatica*) provided clear evidence of this translation, with our elite isolates delivering substantial improvements to key growth parameters. Specifically, inoculation with *Stenotrophomonas* sp. SM9.2 led to a 27.7% increase in fresh biomass and a 16.6% increase in shoot length, while *Achromobacter* sp. IB1.2 improved seed germination by 9.9%.

In the present study, about 10% improvement in the germination rate was observed with *Achromobacter* sp. IB1.2. Nhu and Riddech (2016) reported that various rhizobacteria increased the germination rate of *I. aquatica* by approximately 5-7%. Results showed that nitrogen-fixing bacteria (*Stenotrophomonas* sp. SM9.2) increased shoot length by 16.6%, representating strong promotional effect, highlighting its superior potential. Similarly, Xa et al. (2020) also demonstrated that nitrogen-fixing bacteria could boost the shoot length of water spinach seedlings. It was also observed that the strong correlation between the top nitrogen-fixers and the most potent growth promoters in the present study strongly suggests that direct nitrogen supply is a primary mechanism driving these benefits.

However, it is highly probable that nitrogen fixation is not the sole mechanism responsible for the observed improvements. The genera identified in our study, *Stenotrophomonas*, *Achromobacter*, and *Enterobacter,* are known to be functionally versatile, often possessing a suite of synergistic PGP traits (Souza et al. 2015; Timofeeva et al. 2023). These can include the production of phytohormones like indole-3-acetic acid (IAA) to modify root architecture, the solubilization of mineral phosphate, or the synthesis of iron-chelating siderophores, all of which address major nutrient limitations in agricultural soils. Therefore, the comprehensive growth enhancements documented in our study are likely the result of multiple, complementary actions characteristic of elite PGPR. This work successfully identifies potent, locally adapted strains that are promising candidates for the development of effective bio-inoculants for sustainable vegetable production in the Mekong Delta.

In conclusion, this study successfully prospected the rhizospheres of local vegetable crops in Trà Vinh, Vietnam, leading to the isolation of 36 distinct nitrogen-fixing bacterial strains. Morphological analysis revealed a phenotypically diverse collection, characterized by a significant dominance (83.3%) of Gram-positive bacteria, reflecting a community well-adapted to the region's specific environmental stresses. Subsequent functional screening and molecular identification via 16S rRNA gene sequencing pinpointed five elite isolates belonging to the genera *Stenotrophomonas*, *Achromobacter*, *Enterobacter*, and *Dyella*. Among these, *Stenotrophomonas* sp. SM9.2 and *Achromobacter* sp. IB1.2 emerged as the most promising candidate, demonstrating superior nitrogen-fixing capabilities and delivering the most significant plant growth-promoting effects on *I. aquatica*, notably enhancing biomass and seed germination, respectively. The discovery of these effective, locally-adapted strains demonstrates that the agricultural soils of the Mekong Delta are a rich source of unique microbial life. This biodiversity represents a valuable natural resource for developing next-generation, sustainable agricultural technologies. Harnessing these indigenous microbes offers an ecologically sound strategy that supports the native soil ecosystem and avoids the potential risks associated with introducing non-native organisms. Furthermore, field trials should be conducted to validate the efficacy of these species, leading to the development of a resilient and sustainable agricultural future for Vietnam, built on the foundation of its natural biodiversity.

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