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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF NATIVE *Bradyrhizobium* STRAINS ISOLATED FROM PIGEON PEA ROOT NODULES OF EASTERN INDIA

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ABSTRACT

Pigeon pea (*Cajanus cajan*) is a major legume in Eastern India, contributing to the nutritional security and soil fertility through symbiosis with *Bradyrhizobium* spp. However, the efficiency of native strains under local conditions remains poorly understood. This study aimed to isolate, characterize, and identify native isolates from pigeon pea root nodules, and to evaluate their symbiotic efficiency and plant growth-promoting traits. Fourteen bacterial isolates were obtained, of which 12 belonged to *Bradyrhizobium* spp., while isolates S5 and S15 were identified as *Pseudomonas azotoformans* and *Paenibacillus amylolyticus*, respectively. All *Bradyrhizobium* isolates tested positive for catalase, oxidase, nitrate reductase, and nitrogenase activity. Isolates S9, S3, S6, S13, and S1 showed significantly higher nitrogenase activity as compared to the other isolates. Plant growth-promoting assays revealed phosphate solubilisation, zinc solubilization, and potassium solubilization in ten, eight and five isolates, respectively. Eleven isolates produced siderophores and all of these synthesized indole-3-acetic acid (IAA). Notably, isolate S6 (*Bradyrhizobium yuanmingense*) exhibited all PGPR traits and high nitrogenase activity, identifying it as the most promising isolate. Isolates S3, S1, and S9 also demonstrated strong potential. These results demonstrated the value of efficient native isolates as region-specific bioinoculants for pigeon pea, reducing reliance on chemical fertilizers and promoting sustainable agriculture.

Keywords: Bioinoculants, *Bradyrhizobium,* Eastern India, PGPR, pigeon pea

INTRODUCTION

Pigeon pea (*Cajanus cajan*), a perennial legume native to Peninsular India, is a vital crop valued for nutritional security and sustainable agriculture in Eastern India and other developing regions. Valued for its protein-rich seeds, drought resilience, and role in enhancing soil fertility, pigeon pea contributes significantly to climate-resilient farming systems (Flores-Felix *et al*., 2023). A key attribute of this crop is its ability to improve soil nitrogen levels through biological nitrogen fixation (BNF), thereby

reducing the reliance on chemical fertilizers and supporting eco-friendly cultivation practices (Mhango *et al*., 2017).

The nitrogen-fixing potential of pigeon pea is mediated through its symbiotic associations with *Bradyrhizobium* spp., which inhabit root nodules and convert atmospheric nitrogen into plant-available forms (Jorrin *et al*., 2021). However, this symbiosis varies considerably across the regions, and is influenced by soil and climatic conditions. In Eastern India, recurrent droughts, erratic rainfall, and high temperatures often impair nodule function and nitrogen fixation efficiency, constraining the pigeon pea productivity (Fossou *et al*., 2020). Studies on pigeon pea symbionts reveal both potential and challenges in identifying effective *Bradyrhizobium* strains. Many nodule isolates are either non-symbiotic or lack essential nitrogen fixation genes such as *nifH*, underscoring the need for combined biochemical and molecular characterization (Jorrin *et al.,* 2021). In India, *B. yuanmingense* frequently predominates among pigeon pea endosymbionts across diverse agro-ecological zones (Jorrin *et al.,* 2021). Poor nodulation often results from the weak competitiveness of native *Bradyrhizobium* against non-nodulating *Rhizobium* spp., highlighting the necessity of evaluating both nitrogen-fixing efficiency and ecological competence when selecting inoculant strains (Chalasani *et al.*, 2021).

Globally, the diversity and adaptability of pigeon pea microsymbionts have widely been reported. In Cote d'Ivoire, *Bradyrhizobium ivorense* sp. nov. was identified as a novel lineage with variable symbiotic potential (Fossou *et al.*, 2020). In South Africa, nodules hosted diverse rhizobial populations, including *Bradyrhizobium*, *Rhizobium*, and non-rhizobial endophytes, thus necessitating the molecular confirmation of true symbionts (Bopape *et al.,* 2022). Given the limited characterization of native strains of Bihar and Eastern Uttar Pradesh, this study was aimed to identify efficient *Bradyrhizobium* isolates through biochemical and molecular analyses so as to develop region-specific inoculant candidates for pigeon pea.

MATERIALS AND METHODS

The study was conducted in the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, BHU, Varanasi (India) during the years 2018-2020. Root nodules were aseptically collected from 60-day-old pigeon pea (*Cajanus cajan*) plants grown in sandy loam soils (Inceptisols) under hot and humid conditions across the 14 representative sites in Bihar (Begusarai, Barauni, Kushamahaut, Bihat, Mahua, Samastipur, Hajipur) and Eastern Uttar Pradesh [Varanasi (BHU Agricultural Farm and Raja Talab), Mau, Mirzapur, and Ghazipur (Sadaat, Jakhaniyan, Parsonpur)]. These sites represented agro-ecological variability of the region. From each site, five plants were randomly uprooted, and healthy pink nodules excised, placed in sterile tubes, transported immediately in ice to the laboratory, and processed within 24 h for bacterial isolation. Fifteen isolates (S1-S15) were obtained and subjected to biochemical and molecular characterization. The isolate S14 was later excluded due to the contamination.

Isolation and purification of bacterial strains

The collected nodules were washed under running water to remove adhering soil particles, followed by surface sterilization through sequential immersion in 70% ethanol (30 sec) and 0.1% $HgCl_2$ (2 min), thorough rinsing (5-6 times) in sterile distilled water. Sterilized nodules were crushed aseptically in sterile water, and the suspension streaked onto yeast extract mannitol agar (YEMA) plates. The plates were incubated at 29±2°C for 3-5 days. The colonies with typical rhizobial morphology (gummy, opaque to creamy, raised) were repeatedly sub-cultured to obtain pure isolates. Cultures were preserved in 20% glycerol stocks at -80°C for long-term storage and on YEMA slants at 4°C for immediate use. For growth kinetics, each isolate was inoculated into 25 mL YEM broth in 100 mL Erlenmeyer flasks and incubated at 28°C on a rotary shaker at 125 rpm (Remi, India). Optical density (OD) was measured at 420 nm using a spectrophotometer (Genesys 20, Thermo Scientific, USA) on 3rd, 5th, and 7th day.

Morpho-biochemical and functional characterization

Morphological and biochemical tests were conducted to confirm the rhizobial identity included Gram reaction, catalase, oxidase, potassium hydroxide (KOH) solubility, and nitrate reductase assays (Coico, 2006). Nitrogenase activity was determined by acetylene reduction assay (Stewart *et al*., 1968). The plant growth-promoting traits were evaluated by assessing their phosphate solubilization (Vazquez *et al*., 2000), zinc solubilization (Ramesh *et al*., 2014), potassium solubilization (Parmar and Sindhu, 2013), siderophore production (Schwyn and Neilands, 1987), and indole-3-acetic acid (IAA) production potential (Gordon and Paleg, 1957).

Molecular characterization

DNA extraction: Pure cultures of each bacterial isolate were grown in 5 mL YEM broth (28°C, 130 rpm, 7 days). Cells were pelleted by centrifugation (12,000 rpm, 5 min), washed with 0.5 M NaCl, and genomic DNA was extracted using a phenol-chloroform protocol (Wright *et al.,* 2017), which employs enzymatic lysis (lysozyme, proteinase K) followed by phenol: chloroform: isoamyl alcohol purification and ethanol precipitation. The DNA pellets were resuspended in TE buffer and stored at -20°C. DNA quality were assessed using electrophoresis on 0.8% agarose gels.

PCR amplification of 16S rRNA gene: The 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTTGTTACGACTT-3′) (Lane, 1991). PCR was performed in 25 µL reactions containing 1 × Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 25 pmol of each primer, 50 ng template DNA, and 2 U Taq DNA polymerase. Thermal cycling (Veriti, Applied Biosystems, USA) included initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 45 sec, 56°C for 45 sec, and 72°C for 90 sec; followed by final extension at 72°C for 10 min. Amplicons (~1.3 kb) were checked on 1.2% agarose gels alongside a DNA ladder.

Purification and sequencing: The amplicons of expected size were excised and purified using the MinElute Gel Extraction Kit (Qiagen, Germany) as per the manufacturer's protocol. Purified products were sequenced bidirectionally using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

Sequence analysis: Consensus sequences were assembled using BioEdit v7.2.5 (Hall, 1999) and compared against the NCBI 16S rRNA sequence database using BLASTn. Taxonomic identity was assigned at ≥99% sequence similarity. The sequences obtained were submitted to GenBank, and accession numbers were assigned.

Statistical analysis

All the experiments were conducted in triplicate in a completely randomised design. The data generated was analysed using R Studio (v3.1.2). The analysis of variance was performed, and mean separation carried out using Tukey's HSD at $p < 0.05$.

RESULTS AND DISCUSSION

Morphological and cultural diversity of bacterial isolates

Morphological characterization, a fundamental step in bacterial identification (Rai *et al*., 2014), exhibited common features such as a gummy and opaque appearance, yet notable variation was observed in their pigmentation, size, and margin patterns (Table 1). While most colonies were creamy in colour, except isolate S1 (slightly pinkish) and S5 (whitish), the colony size varied from small (e.g., S1, S8, S10, S12) to large (S3, S11), with rest showing medium-sized colonies. Shape was predominantly spherical, though isolate S2 displayed a wrinkled surface, isolates S7 and S13 showed irregular margins, and lobate or undulating edges as evident in isolate S8 and S10. All isolates, except S15, were Gram-negative, consistent with the general characteristics of *Rhizobium* spp. Despite these differences, all isolates exhibited a slow-growing habit, characteristic of *Bradyrhizobium* species

(Degefu *et al*., 2018). The morphological diversity observed among the isolates points to the potential underlying genetic variation. The colony morphology is influenced not only by genetic determinants but also by epigenetic regulation and protein sorting (Granek and Magwene, 2010). Such variation in colony traits among pigeon pea isolates may thus reflects adaptive mechanisms that confer ecological and functional diversity within the *Bradyrhizobium* population.

Table 1: Morphological and symbiotic performance of pigeon pea bacterial isolates under *in vitro* conditions

Bacterial isolates	Colony characteristics on YEMA	Nodules (No. plant^{-1})	Nitrogenase activity (nm C_2H_4 mg^{-1} protein h^{-1})
S1	Gummy, slightly pinkish, opaque, small, spherical, drop-like, smooth	6.00^{ab}	36.80^{b}
S2	Gummy, creamy, opaque, medium, wrinkled, spherical, raised, wavy	3.00^{de}	28.27^{f}
S3	Gummy, creamy, opaque, large, spherical, undulating, flat	6.67^{a}	36.94^{ab}
S4	Gummy, creamy, opaque, medium, spherical, raised and undulating	3.33^{cde}	29.41^{ef}
S5	Gummy, whitish, medium, circular, opaque	0.00^{f}	29.64^{e}
S6	Gummy, creamy, opaque, medium, spherical, raised	6.67^{a}	37.74^{ab}
S7	Gummy, creamy, opaque, medium, spherical, drop-like, irregular with spreading	3.33^{cde}	28.89^{ef}
S8	Gummy, creamy, opaque, small, spherical, convex, lobate	4.67^{bc}	32.65^{cd}
S9	Gummy, creamy, opaque, medium, spherical and smooth	7.00^{a}	38.27^{a}
S10	Gummy, slightly creamy, opaque, small, spherical, convex, lobate	4.33^{cd}	32.15^{d}
S11	Gummy, creamy, opaque, large, spherical, raised	4.67^{bc}	33.64^{c}
S12	Gummy, creamy, opaque, small, drop-like, smooth, spherical	2.33^{e}	26.84^{f}
S13	Gummy, creamy, opaque, medium, spherical, irregular, smooth	6.33^{a}	36.96^{ab}
S15	Gummy, creamy, opaque, medium, circular	0.00^{f}	25.83^{g}
Control	-	0.00^{f}	0.00^{h}
$LSD_{0.05}$		1.419	1.366
CV%		4.04	2.716

Values with same letters in same column are do not differ from each other

Biochemical characterization of bacterial isolates

Biochemical characterization confirmed that most of the isolates exhibited typical features of *Bradyrhizobium* spp. Gram staining classification was further supported by KOH solubility test, which was positive for all isolates, except S15. These results align with earlier findings that the members of *Rhizobium* and *Bradyrhizobium* (Alphaproteobacteria, Rhizobiales) are predominantly Gram-negative nitrogen-fixing bacteria (Upadhyay *et al.,* 2015). The positive KOH reaction in Gram-negative isolates arise from the disruption of thin peptidoglycan layer, leading to the cell lysis and formation of a viscous suspension.

All isolates tested positive for catalase, oxidase, and nitrate reductase activity. Catalase activity reflects the ability to detoxify H_2O_2, an important defense mechanism during host colonization and symbiosis, since *Rhizobium*-legume interactions often involve reactive oxygen species (ROS) (Puppo *et al*., 2013). Catalase activity is linked to improved nodulation and nitrogen fixation efficiency, and *Bradyrhizobium japonicum* is reported to possess multiple catalase genes, including *katG* encoding catalase-peroxidase (Panek and O'Brian, 2004). Similarly, oxidase activity observed in all isolates suggests the presence of cytochrome *C* oxidase, enabling aerobic respiration via electron transport chain. Positive nitrate reductase activity across the strains, highlights their ability to reduce nitrate to nitrite in the initial step of nitrate assimilation. The results suggest that 13 of the 14 isolates (S1-S13) possess the characteristic biochemical attributes of *Bradyrhizobium* spp., while isolate S15 was Gram-positive and negative in KOH test, providing preliminary taxonomic confirmation before molecular identification.

Nitrogenase activity

Significant variation in nitrogenase activity was recorded among the 14 bacterial isolates (Table 1).

The highest activity occurred in isolates S9, S6, S13, and S3, which were at par with each other, while S1 also showed comparable performance. These five isolates consistently outperformed the remaining isolates. In contrast, isolates S2, S7, S4, and S12 recorded lower activity, and the non-nodulating isolates S5 and S15 showed minimal values (29.64 and 25.83, respectively). As expected, control plants exhibited neither nodulation nor nitrogenase activity. The variation in enzyme activity reflects considerable functional diversity among pigeon pea nodule-associated *Bradyrhizobium* isolates. The superior performance of isolates S9, S6, S13, S3, and S1 suggests their high efficiency in nitrogen fixation, likely due to effective symbiotic interaction, optimal electron transfer within nitrogenase complex, and greater nodule occupancy, leading to enhanced ammonia production. Conversely, the low activity of isolate S12 and poor performance of isolates S5 and S15 may result from host incompatibility or limited expression of nitrogenase-related genes. These results are consistent with earlier reports where strain-dependent differences in nitrogenase activity were documented among *Bradyrhizobium* spp, isolated from pigeon pea and other legumes (Jorrin *et al.*, 2021; Chalasani *et al.,* 2021).

Plant growth promoting activities

Phosphate solubilisation*:* Phosphorus is an essential macronutrient for plant growth but often remains unavailable due to precipitation and fixation in soil, causing widespread P deficiency and yield loss. Microbial phosphate solubilization is an ecologically sustainable means to improve P availability in the rhizosphere. In this study, out of the 14 isolates tested, ten produced visible halo zones after 96 h, indicating solubilization activity (Table 2). The solubilization index (SI) varied from 2.1 (isolate S6) to 3.4 (isolates S4 and S11). Isolates S8, S9, S11, and S13 were particularly effective, producing larger halo zones (21–32 mm) and higher SI, whereas isolates S2, S5, S7, and S15 failed to solubilize phosphate, showing strain-specific variability.

Quantitative estimation in Pikovskaya broth supported these results (Table 3). Significant differences ($p < 0.001$) were observed among isolates at all intervals (24-96 h). The most efficient solubilizers (isolates S11, S4, S9, and S13) recorded consistently higher OD values at 72 and 96 h. Isolate S11 exhibited highest solubilization (1.98 OD at 96 h), followed by isolates S4 (1.82), S13 (1.76), and S9 (1.74), indicating the superior and stable efficiency of these isolates over time. Phosphate solubilization by rhizobia occurs through the secretion of organic and inorganic acids and phosphatases, converting insoluble phosphates into plant-available forms (Walpola *et al.,* 2013).

Table 2: Biochemical characterization of pigeon pea bacterial isolates

Bacterial isolates	Phosphate solubilisation			Zinc solubilisation				KSA	SP
	DHZ	Col. Dia.	SI	DHZ	Col. Dia.	SE	QE		
S1	18^{d}	12^{c}	2.5	32^{d}	16^{e}	2.00	35.12^{cd}	-	32^{a}
S2	-	-	-	38^{b}	18^{d}	2.11	34.56^{cde}	-	28^{cd}
S3	12^{e}	10^{d}	2.2	38^{b}	14^{f}	2.71	38.62^{a}	-	27^{d}
S4	12^{e}	5^{f}	3.4	18^{e}	14^{f}	1.29	32.28^{f}	24^{b}	31^{b}
S5	-	-	-	-	-	-	-	-	-
S6	4^{g}	3.5^{g}	2.1	48^{a}	22^{b}	2.18	36.97^{ab}	22^{c}	32^{a}
S7	-	-	-	32^{d}	14^{f}	2.29	36.25^{bc}	-	-
S8	29^{b}	24^{a}	2.2	-	-	-	-	-	30^{b}
S9	32^{a}	16^{b}	3.0	-	-	-	-	-	29^{bc}
S10	6^{f}	3^{g}	3.0	16^{f}	14^{f}	1.14	33.65^{def}	-	28^{cd}
S11	22^{c}	9^{e}	3.4	48^{a}	32^{a}	1.50	32.51^{f}	20^{d}	22^{f}
S12	6^{f}	3^{g}	3.0	-	-	-	-	22^{c}	25^{e}
S13	21^{c}	10^{d}	3.1	-	-	-	-	38^{a}	20^{g}
S15	-	-	-	36^{c}	20^{c}	1.80	33.26^{ef}	-	28^{cd}
$LSD_{0.05}$	1.45	0.76		3.22	2.71		2.88	5.45	2.71
CV%	4.42	3.98		1.87	0.84		1.72	0.82	1.07

Values with same letters are statistically at par; DHZ: Diameter of halo zone (mm); Col. Dia.: Colony diameter (mm); SI: Solubilisation index; SE: Solubilization efficiency; QE: Quantitative estimation (μg Zn mL^{-1}); KSA: Potash solubilisation; SP: Siderophore production

Zinc solubilisation assay

Zinc (Zn) is an essential micronutrient required in trace amounts (5-100 mg kg^{-1}) for plant growth, yet its deficiency disrupts auxin and carbohydrate synthesis, nucleotide metabolism, and membrane stability. Zinc-solubilizing bacteria (ZSB), therefore, provide an eco-friendly strategy to mobilize Zn into plant-available forms (Saravanan *et al*., 2007). In this study, nine isolates (S1, S2, S3, S4, S6, S7, S10, S11, & S15) solubilized ZnO *in vitro* on mineral salts medium (Table 2). Solubilization zones ranged from 16 mm (S10) to 48 mm (S6, S11). While isolates S6 and S11 produced the largest halos (48 mm), solubilization efficiency (SE) was highest in isolate S3 (2.71), followed by S7 (2.29) and S6 (2.18). Quantitative Zn estimation confirmed the isolate S3 (38.62 µg mL^{-1}) as the most efficient strain, followed by isolates S6 (36.97) and S7 (36.25). Despite its large halo, isolate S11 showed lower SE (32.51 µg mL^{-1}), likely due to larger colony size. These differences show strain-specific mechanisms, commonly involving secretion of organic acids, siderophores, and other metabolites.

Table 3: Phosphate solubilisation of bacterial strains on Pikovskaya broth

Strains	24 h	48 h	72 h	96 h
S1	0.826^{c}	0.924^{d}	1.122^{c}	1.401^{de}
S3	0.651^{ef}	0.895^{de}	0.983^{de}	1.366^{de}
S4	0.912^{b}	1.462^{a}	1.539^{a}	1.824^{b}
S6	0.754^{d}	0.869^{ef}	0.947^{e}	1.246^{f}
S8	0.658^{e}	0.839^{f}	1.026^{d}	1.332^{e}
S9	0.825^{c}	0.978^{c}	1.346^{b}	1.747^{b}
S10	0.621^{f}	0.865^{ef}	1.112^{c}	1.554^{c}
S11	0.954^{a}	1.254^{b}	1.548^{a}	1.984^{a}
S12	0.542^{g}	0.698^{g}	0.973^{e}	1.421^{d}
S13	0.841^{c}	0.982^{c}	1.385^{b}	1.759^{b}
$LSD_{0.05}$	0.0335	0.0465	0.0472	0.0838
CV%	2.578	2.78	2.299	3.125

Values with same letters are statistically at par

The strong solubilization ability of isolates S3, S6, and S7 positions them as promising ZSB candidates for bioinoculant development in Zn-deficient soils of Eastern India. Their dual capacity for nitrogen fixation and micronutrient mobilization further enhances their agronomic relevance. These findings align with earlier reports that rhizobia and other PGPR are also involved in multiple growth-promoting traits including mineral solubilization, IAA production, EPS synthesis, and siderophore release (Verma *et al*., 2020). Overall, isolates S3, S6, and S7 emerge as superior ZSB, combining effective Zn solubilization with BNF capacity.

Potassium solubilisation assay

Potassium (K) is the 3rd most important macronutrient in plants, essential for enzyme activation, osmoregulation, and stress tolerance. Yet, over 90% of soil K occurs in insoluble mineral-bound forms, leaving only a small fraction plant-available. Potassium-solubilizing bacteria (KSB) offer a biological means to mobilize insoluble K, reducing dependence on costly potash fertilizers (Sindhu *et al*., 2010). In this study, five isolates (S4, S6, S11, S12, & S13) produced clear solubilization zones (Table 2). Isolate S13 was most efficient (38 mm), significantly larger than other isolates, followed by isolates S4 (24 mm), S6 (22 mm), and S12 (22 mm), while isolate S11 showed the smallest positive zone (20 mm). Statistical analysis confirmed the isolate S13 as the superior solubilizer.

K solubilization is mediated by bacterial secretion of organic acids that dissolve aluminosilicate minerals, releasing K along with Al and Si. This trait has been documented in diverse genera such as *Acidothiobacillus ferrooxidans, Pseudomonas* sp.*, Paenibacillus* sp.*, Burkholderia* sp.*, Bacillus mucilaginosus, B. edaphicus,* and *B. circulans* (Liu *et al*., 2012). Our findings indicate that certain *Bradyrhizobium* strains also possess this ability, broadening the diversity of KSB relevant for biofertilizer use. Egamberdieva *et al*. (2010) reported that *Bradyrhizobium* inoculation enhanced K uptake and drought tolerance compared to uninoculated controls.

Siderophore production

Iron (Fe) is a vital micronutrient in plants, serving as a cofactor in chlorophyll biosynthesis, photosynthesis, respiration, and biological nitrogen fixation. Yet, in many soils Fe is locked in insoluble forms, limiting its availability. To overcome this, bacteria secrete siderophores, a low molecular weight chelators that solubilize Fe and thus facilitate its uptake by plants and microbes. Siderophores also indirectly promote plant health by restricting phytopathogens through competitive

Fe sequestration (Jasim *et al*., 2013). In present study, 11 isolates formed distinct orange/yellow halos, confirming siderophore secretion (Table 2). Halo sizes differed significantly, ranging from 20 to 32 mm. Isolates S1 and S6 showed the highest activity (32 mm), significantly outperforming most isolates, followed by isolates S4 (31 mm), S8 (30 mm), and S9 (29 mm). Moderate production was observed in isolates S2, S10, S15 (28 mm each) and S3 (27 mm), whereas isolates S11 (22 mm) and S13 (20 mm) were the lowest producers. The observed variability reflects strain-specific differences in siderophore biosynthesis and secretion. Importantly, the high activity of isolates S1 and S6 suggests a dual advantage *i.e.,* enhancing Fe availability for pigeon pea and limiting pathogenic competition. Reportedly, *Bradyrhizobium* strains from groundnut also exhibited strong siderophore production (van Rossum *et al*., 1994). Our findings indicate that pigeon pea–associated *Bradyrhizobium* isolates, particularly S1 and S6, combine biological nitrogen fixation with efficient Fe mobilization.

Indole acetic acid (IAA) production

IAA is a key phytohormone regulating plant growth through cell elongation, division, root initiation, shoot growth, and seedling vigor. Tryptophan serves as its principal microbial precursor, influencing IAA biosynthesis in both plants and their symbionts (Zaidi *et al*., 2009). In legume–rhizobia symbiosis, IAA production is particularly important as it directly affects root architecture and nodule initiation. In present study, IAA levels (after 48 h) ranged from 20.21 µg mL^{-1} (S5) to 72.67 µg mL^{-1} (S6). By 72 h, production increased further, ranging from 31.08 µg mL^{-1} (S11) to 82.78 µg mL^{-1} (S6). The most efficient producers at 72 h were isolate S6 (82.78 µg mL^{-1}), followed by isolates S15 (76.04), S9 (75.66), S3 (72.67), S12 (70.04), S1 (69.67), and S13 (64.42) (Table 4). Thus, several isolates consistently maintained high IAA production, with isolate S6 emerging as the most potent isolate. Although IAA levels increased between 48 and 72 h for all the isolates, the relative rise was below 20% for highest producers (isolates S6, S9, S3, & S12), suggesting stabilization. This pattern is in agreement with Datta and Basu (2000) who reported IAA peak near 72 h before declining, likely due to microbial per-oxidases and IAA oxidase. Ghosh *et al*. (2013) highlighted its role in root elongation and branching, enhancing nutrient uptake and nodulation.

Table 4: IAA production by various *Bradyrhizobium* isolates from root nodules of pigeon pea

Bacterial isolates	IAA production (µg mL^{-1})		Rate of increase (%)
	48 h	72 h	
S1	62.55^{d}	69.67^{d}	11.38
S2	27.70^{f}	40.82^{f}	47.37
S3	67.05^{b}	72.67^{c}	8.38
S4	23.59^{h}	37.82^{g}	60.36
S5	20.21^{i}	34.45^{hi}	70.43
S6	72.67^{a}	82.78^{a}	13.92
S7	25.08^{gh}	32.95^{hij}	31.37
S8	56.93^{e}	65.17^{e}	14.48
S9	68.55^{b}	75.66^{b}	10.39
S10	21.71^{i}	32.20^{ij}	48.32
S11	26.21^{fg}	31.08^{j}	18.58
S12	65.18^{c}	70.04^{d}	7.47
S13	55.43^{e}	64.42^{e}	16.22
S15	67.42^{b}	76.04^{b}	12.78
CV%	2.12	2.84	-
$LSD_{0.05}$	1.62	2.60	-

Values with same letters are statistically at par

Taken together, the multi-trait performance of these isolates highlights their potential as elite bioinoculants for pigeon pea cultivation in Eastern India. Isolate S6 emerged as the most consistent performer, combining high nitrogenase activity with strong Zn solubilization, siderophore production, and the highest IAA output, along with moderate K solubilization. Isolate S9 also demonstrated superior efficiency, excelling in nitrogen fixation, phosphate solubilization, and IAA production. Isolate S13 combined strong N_2 fixation with the highest K solubilization, confirming the reports that KSB such as *Bradyrhizobium* spp. can enhance plant stress tolerance and yield (Egamberdieva *et al*., 2010). Isolate S1 contributed through robust siderophore production and high IAA levels, making it valuable trait in Fe-limited soils. Although isolate S15 lacked nodulation ability, its high IAA production suggests utility as a co-inoculant to stimulate root growth, in agreement with earlier findings that rhizobial auxins enhance root elongation and nodulation sites (Datta and Basu, 2000). The complementary trait profiles of these isolates indicate that either individually or as microbial consortia, they could reduce reliance on chemical fertilizers by mobilizing N, P, K, Zn, and Fe to

improve pigeon pea productivity under the edaphic and climatic conditions of Eastern India (Sindhu *et al*., 2010; Verma *et al*., 2020).

Molecular characterization

Amplification of 16S rRNA gene using universal primers 27F and 1492R yielded ~1300 bp products from all the isolates. The purified amplicons were sequenced bidirectionally, and consensus sequences were subjected to BLASTn analysis against the NCBI 16S rRNA database. Sequence similarity of ≥ 99% confirmed that the majority of isolates belonged to *Bradyrhizobium* spp. (12 isolates), with additional representatives of *Pseudomonas* (S5) and *Paenibacillus* (S15) (Table 5). The isolates were identified as *Bradyrhizobium japonicum* (S12), *B. subterraneum* (S1, S7, S8, S10, & S13), *B. yuanmingense* (S2, S3, S4, S6, S9, & S11), *Pseudomonas azotoformans* (S5) and *Paenibacillus seodonensis* (S15) (Kumar *et al*., 2023). Phylogenetic tree grouped the isolates into distinct clades related to their BLASTn-based identities (Fig. 1). *Bradyrhizobium* isolates were clustered into three major groups representing *B. yuanmingense*, *B. subterraneum*, and *B. japonicum*, while *Pseudomonas* (S5) and *Paenibacillus* (S15) formed separate outgroups, indicating clear taxonomic distinction. The small branch lengths observed in *Bradyrhizobium* clusters denote high sequence homology among isolates.

Table 5: Molecular identification of bacterial isolates from pigeon pea root nodules based on 16S rRNA gene sequencing

Bacterial species	Isolates (length in bp)	Closest match with NCBI Accession No. (99-100% similarity)	Submitted Accession No.
Bradyrhizobium subterraneum	S1 (1386), S7 (1386), S8 (1386), S10 (1386), S13 (1386)	MT501100.1	MW547460, MW566706, MW566707, MW566709, MW566712
Bradyrhizobium yuanmingense	S2 (1360), S3 (1350), S4 (1350), S6 (1350), S9 (1370), S11 (1342)	MT533796.1 (S2) / MT533802.1 (S3, S4, S6) / MT501098.1 (S9) / MT544597.1 (S11)	MW566702, MW566703, MW566704, MW566705, MW566708, MW566710, MW566715
Bradyrhizobium japonicum	S12 (1379)	MK559521.1	MW566711
Pseudomonas azotoformans	S5 (1172)	MG757959.1	OR016148
Paenibacillus seodonensis	S15 (1475)	MH169324.1	MW566713

Molecular characterization of pigeon pea nodule isolates in this study revealed *Bradyrhizobium* spp. as the predominant microsymbionts, with *B. yuanmingense*, *B. subterraneum*, and *B. japonicum* collectively representing the majority of the isolates. This predominance of *Bradyrhizobium* is consistent with earlier reports from India and abroad, which identify members of this genus as the primary symbionts of pigeon pea (Jorrin *et al*., 2021). In particular, *B. yuanmingense* has frequently been described as dominant species nodulating pigeon pea in Indian agro-ecosystems (Jorrin *et al*., 2021). The occurrence of *B. subterraneum* and *B. japonicum* among the isolates

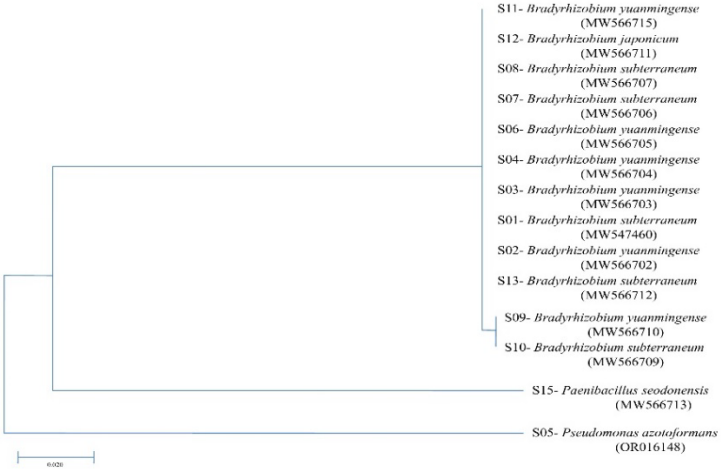

Fig. 1: Phylogenetic tree based on 16S rRNA gene sequences of bacterial isolates

further indicates intra-genus diversity (Fossou *et al*., 2020). Interestingly, the identification of *Pseudomonas azotoformans* and *Paenibacillus seodonensis* suggests the presence of non-rhizobial endophytes (NREs) within pigeon pea nodules. Similar findings have been reported in South Africa, where pigeon pea nodules were shown to harbour diverse bacterial taxa including *Pseudomonas*, *Bacillus*, and *Paenibacillus* alongside canonical rhizobia (Bopape *et al*., 2022). Their co-existence with *Bradyrhizobium* in nodules could therefore enhance pigeon pea performance through complementary mechanisms beyond nitrogen fixation.

Conclusion: This study demonstrates that native *Bradyrhizobium* isolates from pigeon pea nodules in Eastern India possess significant functional diversity, with several strains exhibiting strong nitrogen-fixing ability along with multiple plant growth-promoting traits. Among them, isolates S6, S9, and S13 emerged as elite multi-trait performers, supported by contributions from isolates S1 and S15. Their combined capacities for nitrogen fixation, phosphate, zinc, and potassium solubilization, siderophore production, and IAA synthesis underscore their potential as region-specific bioinoculants. Harnessing these isolates could reduce dependence on chemical fertilizers, enhance pigeon pea productivity, and promote sustainable, low-input agriculture in nutrient-deficient soils.

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Author's contributions: SK, RKS: Formulated and designed the research work; SK, PS, SCK, UB, AKP: Conducted research work, generated data; NRP: Analysed the data; SK, PS: Prepared the manuscript; VS, MN: Reviewed and improved the manuscript.

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