



Characterization of Plant Growth-Promoting Potential of Root Nodule Endophytes from *Albizia lebbek* (L.) Benth

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Authors' contributions

Methodology, investigation and validation done by authors AM, SJ and RS. Investigation done by author AM. Formal analyzed by authors AM, ISS and AT. Writing - original draft prepared by author AM. Writing - review & editing done by authors ISS and AT. Supervised by authors ISS and AT. All authors read and approved the final manuscript.

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Abstract

Albizia lebbek (L.) Benth. is a leguminous tree with significant potential in agroforestry. Root nodules are known to harbour rhizobia as well as non-rhizobial endophytes with plant growth-promoting (PGP) traits. The present study was undertaken to isolate and characterize endophytic bacteria from root nodules of *A. lebbek*. A total 48 bacterial isolates were isolated and purified from root nodules of *A. lebbek* and were screened qualitatively for four PGP traits; IAA production, ammonia production, phosphate solubilization and chitinase activity. Identification of the effective PGP bacterial strains were done by sequencing of 16S rRNA gene. Results revealed that 52.08% of bacterial isolates exhibited IAA production, 50% of bacterial isolates exhibited ammonia production, while 31.25% isolates showed phosphate solubilization. Chitinase activity was

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present in only one bacterial isolate (Al-DOB7). *Pseudomonas* sp. Al-DOB7 were identified as multifunctional PGP bacteria and showed all four PGP activity screened. Three bacterial isolates; *Rhizobium* sp. Al-Am1, *Rhizobium* sp Al-Kh2 and *Ensifer* sp. Al-Rs5 exhibited IAA production, ammonia production and phosphate solubilization. Further quantitative screening and greenhouse validation of these nodule-associated bacteria are essential for their sustainable application in agricultural and agroforestry systems.

Keywords: *Albizia lebbbeck*; endophytes; PGPR; IAA; phosphate solubilization; chitinase.

1. Introduction

Albizia lebbbeck (L.) Benth., commonly known as Siris, is a deciduous, fast-growing multipurpose tree. It is distributed in tropical and subtropical regions of the world. It is valued for its timber, fodder, soil improvement properties and traditional medicinal uses. *A. lebbbeck* forms symbiotic associations with nitrogen-fixing bacteria for nitrogen fixation. Besides rhizobia, root nodules of legumes harbour a diverse community of non-rhizobial endophytic bacteria that do not form nodules but colonize the nodule interior (Martínez-Hidalgo & Hirsch, 2017; Wang et al., 2022). These endophytes, often referred to as “opportunistic” or “co-resident” bacteria, can include species of *Bacillus*, *Pseudomonas*, *Enterobacter*, *Paenibacillus* and others (Pandya et al., 2013; Saini et al., 2015). These PGP bacteria contribute to plant growth through multiple mechanisms including production of phytohormones such as indole-3-acetic acid (IAA), solubilization of insoluble phosphate, production of siderophores and antimicrobial compounds and enhancement of stress tolerance (Pandya et al., 2013; Tariq et al., 2021; Miljaković et al., 2022).

The diversity and plant growth-promoting (PGP) potential of rhizobial and non-rhizobial endophytes associated with *A. lebbbeck* nodules remain largely uncharacterized. The present study was undertaken to isolate and characterize both rhizobial and non-rhizobial endophytic bacteria from root nodules of *A. lebbbeck* and to evaluate their PGP activities, including IAA production, phosphate solubilization, ammonia production and chitinase activity. These PGP isolates may provide functional diversity of nodule endophytes for designing effective microbial inoculants that can enhance the growth of *A. lebbbeck* and other legumes.

2. Material and Methods

2.1 Isolation of Bacterial isolates

Bacteria were isolated from excavated root nodules of *A. lebbbeck*. Nodules were surface sterilized through immersing and rinsing periodically 90% ethanol for about 1 min, 0.1% (w/v) and antifungal agent BavistinR for about 30 sec followed by 1% sodium hypochlorite (NaOCl) for 4 minutes. Nodules were crushed and exudate was streaked on a plate containing YEMA-CR media. Plates were incubated at 30-35 °C, after 3-4 days colonies with different morphologies were picked and streaked subsequently until pure colonies were obtained. Purified rhizobial strains were maintained on YEMA petri plates at 28°C.

2.2 IAA Production

Method given by Gordon and Weber (1951) was used for screening of IAA producing microbes. YEM Broth supplemented with 1 mM L-tryptophan were inoculated with bacterial strains and incubated at 28 °C for 10 days in complete dark. After incubation, cultures were centrifuge at 10,000 rpm for 15 min to separate bacterial cells. 2ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of the Salkowski reagent (1 ml 0.5 FeCl₃ solution in 50 ml of 35% of perchloric acid). Development of pink color was recorded as positive results for IAA production.

2.3 Ammonia Production

Release of ammonia by microbes is a result of deamination of amino acids. Peptone water was prepared for testing of ammonia production by RNB strains. Bacterial strains were incubated at 28 °C for 48-72 hours. After incubation 1 ml of Nessler’s reagent was added to each tube. Presence of yellow color indicates small amount of

ammonia production, while deep orange to brownish color indicates maximum production of ammonia (Cappuccino & Sherman, 2013).

2.4 Phosphate Solubilization

Phosphate is one of the vital nutrient, which is not available freely. Several microbes are known to secrete some organic acids which solubilize the insoluble phosphates into available form. PVK agar medium was used to determine phosphate solubilization. Spot inoculation of bacterial strain was done on PVK agar medium and incubated at 28 °C for 48-72 hours. Formation of clear zone around the colonies was considered as positive result.

2.5 Chitinase Activity

Chitinase are the group of enzyme that decomposes chitin, a homo polysaccharide made up of monomers of N-acetyl glucosamine composed by glycoside β -(1-4) bonds. To screen chitinase activity colloidal chitin agar medium was used (Kim et al., 2003). Bacterial isolates were spot inoculated and kept for 48-72 hours at 28 °C. To check results plates were flooded with iodine solution. Formation of clear zone around the colonies was considered as positive result.

2.6 Identification of Bacterial Isolates

DNA was extracted using the method described by Cheng and Jiang, (2006). The 16S rRNA gene was amplified using the universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTACGACTT-3'), as described by Weisburg et al. (1991). The resulting sequences were analyzed using the BLASTn algorithm against the National Center for Biotechnology Information (NCBI) database to identify the bacterial isolates.

3. Results and Discussion

Plant growth-promoting (PGP) bacteria promote plant growth through multiple mechanisms. These mechanisms include biological nitrogen fixation (BNF), phosphate solubilization and mineralization, siderophore production, IAA synthesis, ACC deaminase activity, chitinases enzymes Production (Khatoun et al., 2020; Grover et al., 2021; De Andrade et al., 2023). Besides rhizobia, a large number of studies have shown the presence of diverse non-nodulating bacteria—such as *Pseudomonas* spp., *Bacillus* spp., *Azospirillum* spp., *Enterobacter* spp. within legume nodules (Pandya et al., 2013; Martínez-Hidalgo & Hirsch, 2017; Wang et al., 2022, Saini et al., 2015). The root nodules of *A. lebeck* harbour nitrogen-fixing rhizobia as well as endophytic bacteria with distinct plant growth-promoting potential. The present study focuses on a detailed characterization of PGP activities of endophytes from *A. lebeck* nodules.

Bacterial colonies purified were white, opaque, mucilaginous and exopolysaccharide producing. Maximum strains were found to be fast growing. Forty-eight bacterial isolates were isolated and purified and were further screened for multiple plant growth-promoting (PGP) activities, including ammonia production, phosphate solubilization, indole-3-acetic acid (IAA) production and chitinase activity (Fig. 1). Indole 3-acetic acid (IAA) production is considered as one of the key mechanisms of PGP that stimulates root elongation and lateral root formation (Duca et al., 2014). IAA production by rhizobia strains was reported by many workers (Arora et al., 2001; Ghosh & Basu, 2002; Mandal et al., 2007; Sridevi & Mallaiiah, 2007; Gallarato et al., 2015; Ahmed et al., 2021). In our study, IAA production was observed in 25 isolates out of forty-eight (Fig. 2); although the amount of IAA produced was varied from low, intermediate to high production (Table 1). Five bacterial isolates (Al-Am1, Al-Am18, Al-Barr2, Al-Kh2, Al-DOB7) showed high IAA production. Ammonia production by bacteria results in the accumulation of ammonia in the rhizosphere. Ammonia production was observed in the many isolates, around 50% of strains were able to produce ammonia (Figs. 2, 3). Four isolates (Al-DOB7, Al-RS5, Al-Za3, Al-Za12) exhibited strong positive responses, while twenty isolates showed medium to low-level ammonia production (Table 1).

Phosphate solubilization is also important PGP trait in phosphorus limiting soils (Khan et al., 2014). The P solubilizing bacteria produces low molecular weight organic acids which helps to dissolve the unavailable soil P (Deubel et al., 2000). Phosphate solubilization was comparatively restricted, with only 31.25% efficient

solubilizers strains (Figs. 2, 3). Bacterial strains Al-Aj13, Al-Am4, Al-Bi2, Al-Dept4, Al-Kh4, Al-PC5, Al-RS2, Al-Rs4, Al-Rs5, Al-Shiv2, Al-Am1, Al-Kh2, Al-Barr2, Al-DOB7 and Al-Za12 showed low to medium phosphate solubilization activity. Alikhani et al. (2006) also reported 44% of isolated strains including *Bradyrhizobium*, *Mesorhizobium* and *Ensifer* were able to solubilize phosphate. Pandya et al., (2015) also reported non-rhizobial endophytes as efficient phosphate solubilizers.

Table 1. Plant growth promoting activities of nodule endophytes isolated from *A. lebeck*

PGP activity	Low	Medium	High
Ammonia production	Al-Aj11, Al-Am18, Al-Am8, Al-Bi2, Al-Dept4, Al-Gm2, Al-Jh1, Al-Jh4, Al-Jh7, Al-Kh4, Al-Kh5, Al-RS1, Al-RS14, Al-Za9	Al-Jh 8, Al-Am4, Al-Am1, Al-Kh2, Al-RS13, Al-RS3	Al-DOB7, Al-RS5, Al-Za3, Al-Za12
Phosphate solubilization	Al-Aj13, Al-Am4, Al-Bi2, Al-Dept4, Al-Kh4, Al-PC5, Al-RS2, Al-Rs4, Al-Rs5, Al-Shiv2	Al-Am1, Al-Kh2, Al-Barr2, Al-DOB7, Al-Za12	-
IAA production	Al-Bi4, Al-Dept9, Al-Jh 8, Al-Jh1, Al-Jh7, Al-Kh6, Al-Kis11, Al-PC4, Al-RS14, Al-RS3, Al-Rs5, Al-Shiv2, Al-Za9	Al-Aj13, Al-Am3, Al-Am5, Al-Gm2, Al-Gm4, Al-Za3, Al-Za7	Al-Am1, Al-Am18, Al-Barr2, Al-Kh2, Al-DOB7
Chitinase activity	-	Al-DOB7	-

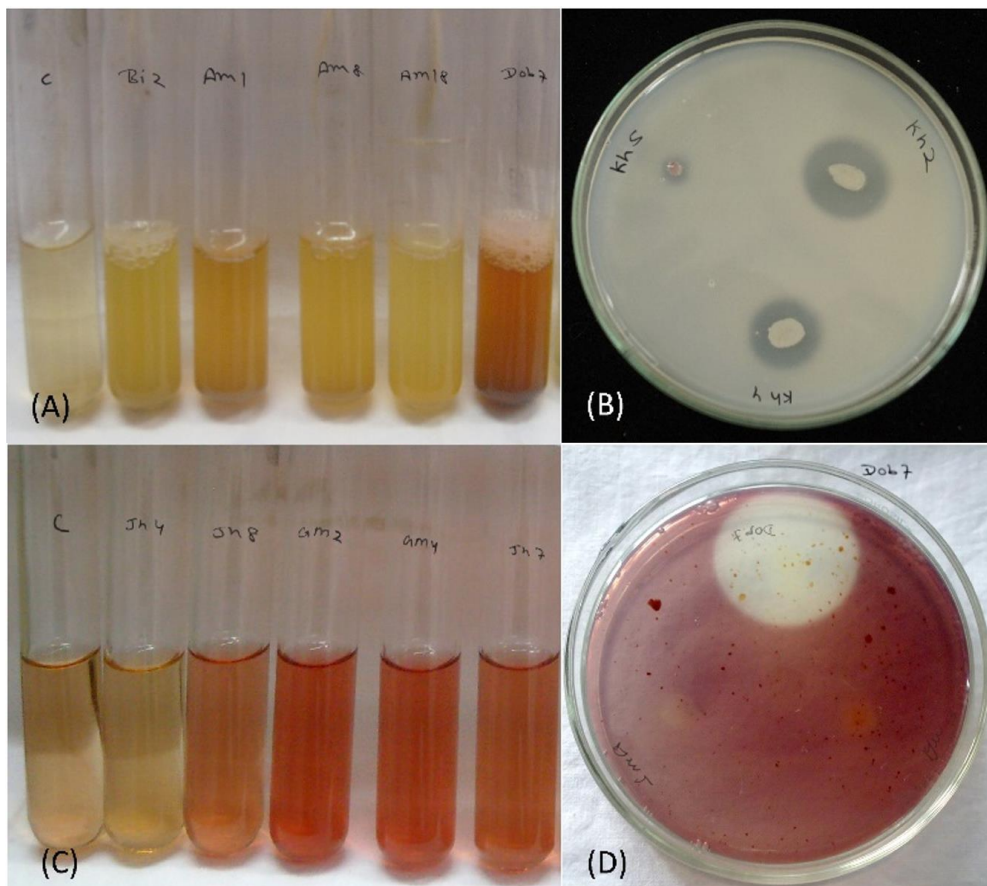


Fig. 1. Screening of PGP activity of nodule endophyte isolated from *A. lebeck* (A) Ammonia production (B) Phosphate solubilization (C) IAA production (D) Chitinase activity

Chitinase enzyme is a cell wall hydrolytic enzyme that have role in controlling fungi possessing chitin as cell wall component. Mehboob et al. (2011) found that strains of *Mesorhizobium* and *Rhizobium* isolated from root nodules of chickpea, mung bean and lentil have chitinase activity. In our study, chitinase activity was detected only in Al-DOB7 (Fig. 2, Table 1).

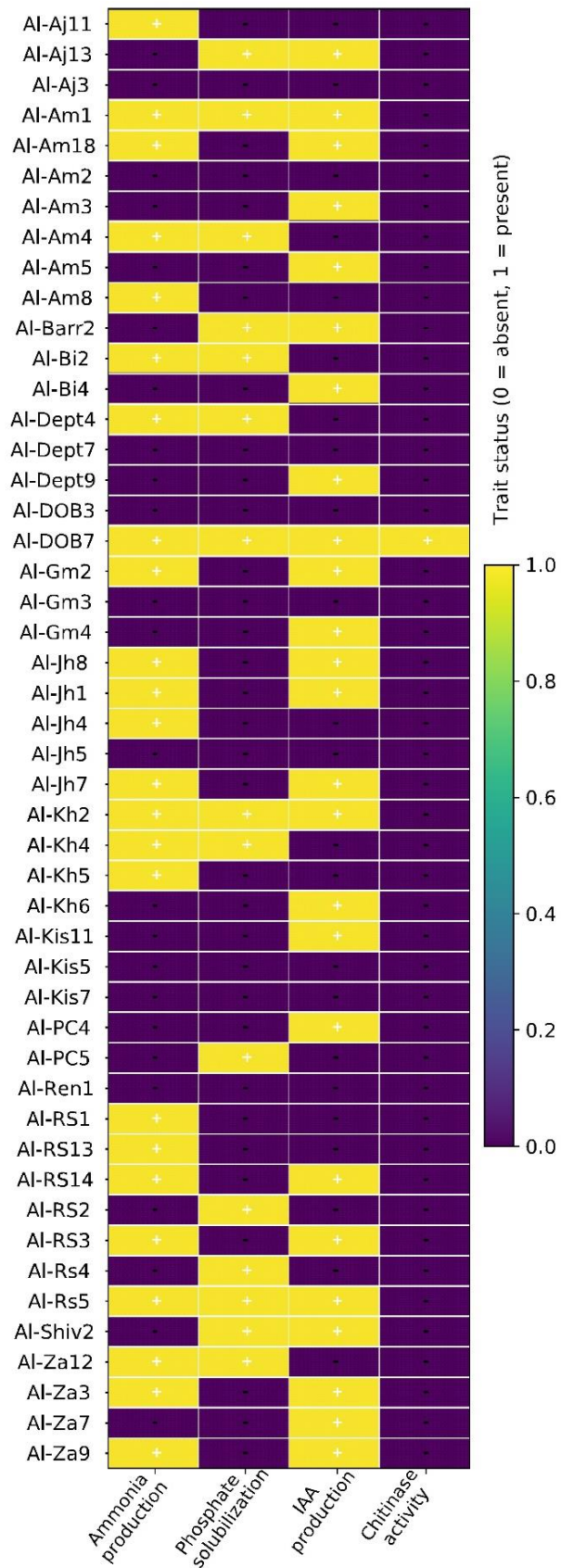


Fig. 2. Heatmap of PGPR traits of endophytic bacteria isolated from *A. lebeck*

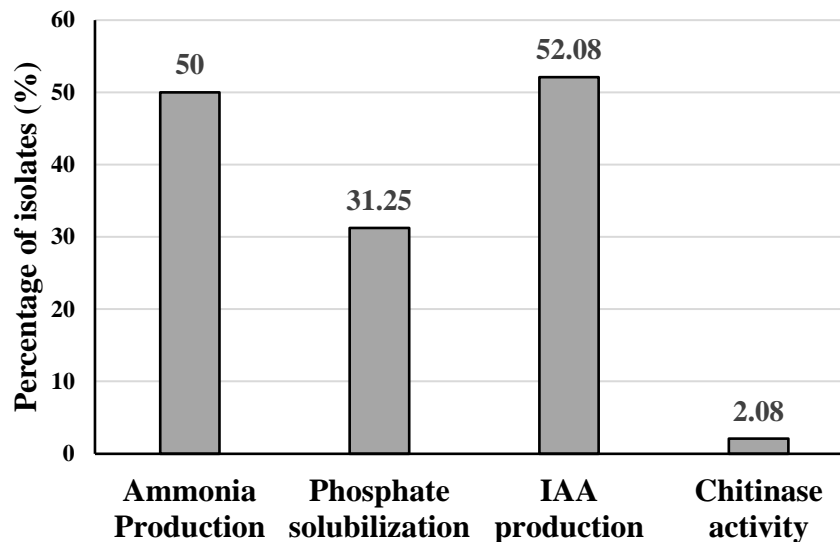


Fig. 3. PGP trait frequency (%) of bacterial isolates isolated from *A. lebeck*

Four bacterial strains (Al-Am1, Al-Kh2, Al-Rs5 and Al-DOB7) were selected for sequencing of the 16S rRNA gene for identification. Closest BLASTn match of these sequences along with sequence similarity has been showed in Table 2. Strain Al-Am1 and Al-Kh2 showed 100% and 99.90% of sequence similarity with type strain *Rhizobium aegyptiacum* 1010 respectively. Bacterial strain Al-Rs5 showed 99.55% sequence similarity to closest type strain *Ensifer mexicanum* ITTG-R7. Bacterial strain Al-DOB7 with maximum PGP traits was identified as *Pseudomonas* sp. and showed 96.96% of sequence similarity with type strain *Pseudomonas juntendi* BML3.

Table 2. Identification of bacterial strains isolated from *A. lebeck* on the basis of 16S rRNA gene sequence

S. No.	Bacterial strains	Genbank accession no.	Closest type strain	% Similarity with closest type strain
1	<i>Rhizobium</i> sp. Al-Am1	PZ291824	<i>Rhizobium aegyptiacum</i> strain 1010	100
2	<i>Rhizobium</i> sp. Al-Kh2	PZ291825	<i>Rhizobium aegyptiacum</i> strain 1010	99.90
3	<i>Ensifer</i> sp. Al-Rs5	PZ291826	<i>Ensifer mexicanum</i> ITTG-R7	99.55
4	<i>Pseudomonas</i> sp. Al-DOB7	PZ291827	<i>Pseudomonas juntendi</i> strain BML3	96.96

The present study of the Plant Growth Promoting Rhizobacteria (PGPR) showed IAA production (52.08%) and ammonia production (50.00%) as the most common traits, followed by phosphate solubilization (31.25%), whereas chitinase activity was observed in only one isolate Al-DOB7 (2.08%). Species of *Pseudomonas* genus is widely reported to have multiple PGP activities such as nutrient-mobilizing, phytohormone production and antagonistic activity against plant pathogens (Caulier et al., 2018; Khalifa et al., 2022; Singh et al., 2022; Msaddak et al., 2023). In our study, bacterial isolate *Pseudomonas* sp Al-DOB7 showed all four PGP activities. *Pseudomonas* strains have been reported as opportunists nodule endophytes from nodules of different legumes (Zhao et al., 2018; Ferchichi et al., 2019; Hnini et al., 2023; Msaddak et al., 2023). Similarly, numerous studies have reported the isolation of other opportunists non-nodulating rhizobacteria from leguminous hosts exhibiting diverse plant growth-promoting (PGPR) traits (Hnini & Aurag, 2024). *Acinetobacter* spp. from *Medicago sativa* L. (Tafaraji et al., 2022); *Bacillus* spp. PSB10 and *Enterobacter asburiae* from *Cicer arietinum* L. (Wani et al., 2007; Saikia et al., 2023) showed positive indole-3-acetic acid (IAA) production, phosphate solubilization and siderophore activity.

Rhizobial species from genera such as *Rhizobium*, *Bradyrhizobium*, *Ensifer* and *Mesorhizobium* has been reported to exhibit additional plant growth-promoting activities beyond nodulation (Charest et al., 2005; García-Pérez et al., 2024; Korir et al., 2017; Yadav et al., 2022). In this study, three bacterial isolates, *Rhizobium* sp. Al-Am1, *Rhizobium* sp. Al-Kh2 and *Ensifer* sp. Al-Rs5 showed three PGP activities i.e. IAA production, phosphate solubilization and ammonia production. Several *Rhizobium* species also demonstrated strong PGPR potential including *Rhizobium* sp. from *C. arietinum* L. and *Lens culinaris* (Alinia et al., 2022; Banjare et al., 2023).

4. Conclusion

The PGP ability of rhizospheric and endophytic bacteria have diverse functional potential. Their ability to enhance plant growth makes them valuable components of sustainable agricultural practices. In the present study, *Pseudomonas* sp. Al-DOB7, *Rhizobium* sp. Al-Am1, *Rhizobium* sp. Al-Kh2 and *Ensifer* sp. Al-Rs5 were identified as a potent PGPR. These nodules associated bacteria could further investigated for quantitative screening, greenhouse validation and formulation studies for biocontrol and biofertilizer development.

Disclaimer (Artificial Intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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Competing Interests

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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