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Potential Nitrogen Fixing Rhizobia Isolated from Some Wild Legumes of Nagaland Based on RAPD with *Nif*-directed Primer and Their Biochemical Activities

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Nodules

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RNB

RAPD

Symbiotic association

ABSTRACT

Wild legumes are widely dispersed and can survive in challenging environments as bacteria dwell in their nodules and help each other. Although Nagaland is home to many wild legume varieties, research on the microbial diversity that goes along with them is still in its infancy. This work aimed to characterize several wild legume root nodules and distinguish possible rhizobial isolates using RAPD and *nif*-directed RPO1 primer. Nodule bacteria were isolated in Yeast extract culture media. Based on their colony morphology, 150 isolates were selected for performing RAPD with *nif*-directed RPO1 primer. Eighty-four isolates were bonded with RPO1 primer, and a few biochemical tests were conducted on RPO1-positive isolates. Activities that promoted plant development were also investigated for these isolates. Of all the isolates, 18 exhibited phosphate solubilization capacity, while 38 isolates were found to produce IAA. This study entails a large variety of rhizobia in the nodules, which were able to promote growth. Hence, these isolates promise to be bio-fertilizers that could improve agricultural operations.

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1 Introduction

Microbes are cosmopolitan and found in almost every region of the world. Soil is considered one of the greatest reservoirs for microbial biodiversity, predominated by bacteria and fungi followed by Archaea and about 107-109 virus particles per gram (Banerjee and Van Der Heijden 2023). A single gram of soil is estimated to harbour up to 8 million microbial species, which are responsible for pollutant degradation, nutrient transformation, litter decomposition, and biosynthetic reactions (Jia et al. 2020; Rosselló-Móra and Stackebrandt 2021). Soil microbial diversity is prevalently studied because of its role in crop productivity and improving soil quality. Soil microbial communities represent probably the most known biological diversity reservoirs (Labouyrie et al. 2023). Microorganisms are quite inactive in bulk soil but show increased activities in the rhizosphere, a microbial soil hot spot, especially during the plant developmental stages (Schloter et al. 2018). Free-living soil bacteria beneficial for plant growth are typically called 'Plant Growth Promoting Rhizobacteria' (PGPR) as they can promote plant growth by colonizing the plant root (Tatung and Deb 2023). The PGPR is divided into two groups based on their residing hubs: (i) intracellular PGPR (iPGPR) (i.e., symbiotic bacteria), which live inside the plant cells and are localized inside the specialized structures called nodules and (ii) extracellular PGPR (ePGPR) (i.e., free-living rhizobacteria), which live outside the plant cells and do not form nodules, but they still prompt plant growth (Giannelli et al. 2023). Rhizobia that infects legumes produces nodules and develops a symbiotic relationship with the host, making legume-rhizobia interaction a prime example of iPGPR. Legume-*Rhizobium* symbiosis is the most economical and environment-friendly method and results in the incorporation of atmospheric N_2 into organic compounds, which is thought to account for around 200 Tg of organic N per year (Peoples et al. 2009). It is generally acknowledged that interactions between legumes and rhizobia fix more nitrogen than free-living bacteria. Typically, their connection fixes 25-60 kg of N_2 per year, whereas non-symbiotic species fix less than 5 kg ha^{-1} (Hopkins and Hüner 2014).

Over 17,000 legume species have been reported, of which 20% were found to form nodules (Hopkins and Hüner 2014). A nodule is an integral structure formed due to the exchange of signals between the host legume and compatible rhizobia. It is the site where rhizobia fixes nitrogen after differentiating into bacteriods. Nodules are morphologically categorized into two types: determinate and indeterminate. Determinate nodules are spherical because of their loss of meristematic activity. Indeterminate nodules have an active meristematic activity and are found in most legumes (Green et al. 2019). They are cylindrical and are branched. Rhizobia are one of the many PGP microbes that are vital for enriching soil to enhance plant growth and yield, which will help to cope with the growing demands of food of the increasing population in a sustainable way (Dabo et al. 2019; Ibny et al. 2019). Colony morphology and molecular techniques like Randomly Amplified Polymorphic DNA (RAPD) are generally applied to preliminary screening potential Rhizobial isolates. Williams et al. (1991) performed an initial study on Rhizobia with a gene-directed primer RPO1. Gene-directed RAPD primers are not random and bind to a specific sequence adjacent to a particular locus of the primer, such as RPO1 RAPD primer selectively binds to a sequence adjacent to the *nif* gene locus of bacteria if present. Based on the absence or presence of band(s), Rhizobial and non-rhizobial isolates can be distinguished. Different isolates exhibit different banding patterns; based on that, similar isolates of Rhizobia can be grouped. The homology in the banding patterns of the isolates indicates its primer annealing sites and similarity in the length of the amplified region. This provides a preliminary genetic fingerprint of the target gene and aids in distinguishing the genomes of a wide range of bacterial strains (Bassam et al. 1992), including *Rhizobium* (Richardson et al. 1995).

Nagaland is a part of the North-eastern region of India situated between 93°20″E - 95°5″E and 25°12′N-26°3′N. With 8,633,000 hectares of forest cover, the state occupies 16,579 Km^2 (Ritse et al. 2020). It is a state rich in biodiversity, with sub-tropical and tropical evergreen forests predominating (Achumi et al. 2014). Much research hasn't been done on Rhizobia in this state despite its diverse agroclimatic zones supporting enormous biodiversity, particularly in the flora (Chouhan et al. 2022; Megu et al. 2024). The fact that rhizospheric bacteria have been shown to support host survival in various environmental conditions is evidence of this. The present study aimed to isolate and differentiate potential rhizobial isolates from some wild legumes from Nagaland based on their RAPD fingerprints. The study also aimed to record the morphological structures of nodules and perform some biochemical tests on bacteria isolated from them.

2 Materials and Methods

2.1 Sampling sites

Nodules of wild legumes were collected from six sites in Zunheboto district, Nagaland, India: Alaphumi, Akuluto, Lumami, Sumi Setsii, Zaphumi and Zunhebhoto town. The details of the sites are given in Table 1.

2.2 Soil characterization

Basic soil properties were tested at different sampling sites (Table 1). One g of soil was suspended in 100ml distilled water, and the suspension was allowed to settle down for 3-4 h, and then *p*H was recorded. The widely used wet combustion method of rapid titration protocol by Walkley Blake (Anantha et al. 2020) was used with standard protocols for organic carbon estimation. To determine soil phosphorus, Bray's no 1 extraction method was followed by using a UV-Vis spectrophotometer (Bray and Kurtz 1945). Available potassium was estimated using the atomic absorption photometric

Table 1 Sampling sites and their soil characterization

S. N.	Collection Sites	*p*H	Phosphorus (Kg/ha)	Potassium (Kg/ha)	Organic Carbon (%)	N (Kg/Ha)
1	Sumi Setsu	6.66	11.854	106.265	6.18%	87.44
2	Alaphumi	6.23	10.612	103.110	6.03%	94.03
3	Zaphumi	5.92	12.158	172.102	7.20%	96.21
4	Lumami	6.00	13.251	149.430	8.32%	101.46
5	Akuluto	5.78	10.215	130.112	5.89%	95.71
6	Zunhebhoto	5.81	14.394	134.848	7.32%	97.056

method using a flame photometer (Trivedy and Goel 1984). The Kjeldahl Nitrogen Analyzer (Kelplus Nitrogen Estimation system) estimated soil nitrogen using the Kjeldahl method (Kjeldahl 1883).

2.3 Collection of legumes and root nodule characterization

The native wild legumes from the different collection sites were carefully uprooted, and nodules were collected. An average of 5-6 plants of each legume species were collected from the sites for nodule characterization. They were then taken to the laboratory, washed, and photographed. The number of nodules was counted and recorded for each plant. Nodulation types in collected legumes were recorded as well. Nodule characteristics were recorded and presented in Table 2.

2.4 Isolation of root nodulating bacteria

About 8-10 pink and healthy nodules of each sample were surface sterilized and considered for bacteria isolation. The protocols followed were as given by Somasegaran and Hoben (1985). The nodules were washed with pure water followed by 70% (v/v) ethanol for 2-3 min. The nodules were washed with 1% (w/v) Bavistin (anti-fungal agent) for 2-3 minutes, followed by 0.1% $HgCl_2$ for 5-8 min. This was followed by washing with pure water 5-6 times to remove the traces of $HgCl_2$. The Nodules were then placed on a sterile glass Petri plate, and 2-3 drops of distilled water were added. The nodules were then crushed with a pair of sterile forceps and streaked on Yeast Extract Mannitol Agar (YEMA) media plates [Yeast extract ($0.5gL^{-1}$), Mannitol ($10gL^{-1}$), K_2HPO_4 ($0.5gL^{-1}$), $MgSO_4$ ($0.2gL^{-1}$), NaCl ($0.1gL^{-1}$) and agar ($15gL^{-1}$)]. Congo red dye was used as an indicator, as Rhizobia does not tend to take the dye during the initial stage of culture. The plates were then incubated at 28°C -30°C for 8-10 days. Repeated streaking of isolates was done to obtain pure cultures and for further study.

2.5 DNA extraction of bacterial isolates

Bulky, white, and translucent colonies were preliminary and phenotypically selected as Rhizobia. DNA of the selected isolates was extracted by following the protocol of Sambrook and Russell (2001). The quality of DNA was then checked by running it in 1% (w/v) agarose gel electrophoresis.

2.6 RAPD analysis

For nodule screening, 12 wild legumes were collected from the six sampling sites (Alaphumi, Akuluto, Lumami, Sumi Setsii, Zaphumi, and Zunhebhoto) of Zunheboto district, Nagaland. One hundred fifty bacterial strains in total were isolated from the root nodules. The RAPD analysis of the isolated bacterial isolates was carried out using *nif*-directed RPO1 primer 5'AATTTTCAACGCTCGTGCCA 3' (Richardson et al. 1995). The PCR mixture was made up of 2.5µl 10X TE buffer, 2.5µl of dNTPs, 5µl of RPO1 primer, 0.2µl of *Taq* polymerase, 2µl of template, and 13.8µl dH_2O. For the PCR cycle, the denaturation temperature was set at 94°C, annealing at 54°C, and elongation at 72°C for 30 cycles (BIO-RAD T100™ Thermal cycler). The PCR product was subjected to 1.5% (w/v) agarose gel electrophoresis, and the banding pattern was visualized in Bio-RadChemi Doc.

2.7 Biochemical characterization of root nodulating bacteria

Biochemical characterization of bacterial isolates was done based on their plant growth-promoting activities and biochemical characterization. Only fresh cultures (24-48 h old) were used to perform the tests.

2.7.1 Plant growth promoting (PGP) activities

2.7.1.1 Phosphate solubilization activity

For qualitative phosphate solubilizing activity assay, fresh culture was spot inoculated in Pikovskaya agar medium. The plates were then incubated at 28-30°C for 2-3 days. The occurrence of a halo zone around the spot indicates phosphate solubilizing activity.

2.7.1.2 IAA production

The protocol given by Gang et al. (2019) was used for the IAA test. Fresh cultures were inoculated in Tryptone-Yeast broth with added tryptophan as a precursor and incubated for 24-48 h. In a clean 2ml centrifuge tube, 500(l of Salkowski reagent and 50(l) fresh cultures were added. The tube was incubated in the dark for 30 min. The change of the reagent colour to pink indicates IAA production by the isolates.

Table 2 Wild legumes collected with their nodule characters and cultures isolated

Wild legumes	Nodule Characteristics				Total Isolates	RPO1 Positive	Unique Isolates
	Nodule Type	Avg. Nodule Size (l x w in mm)	Nodule colour	Number of nodules per plant			
A. americana L.	Determinate, stem and root, small sized and many nodules	3x2	Pink	20-30	11	AIS1, AIS3, AIS4, AIS5, AIS6, AIS7, AIS8, AIS9, AIS10, AIS12, AIR14 (Total positive isolates 11)	AIS1, AIS3, AIS5, AIS7, AIS8, AIS9, AIS10, IS12, AIR14 (Total Unique Isolates 9)
A. chinensis (Osbeck) Merr.	Determinate, primary and secondary root, small and medium-sized nodules, few nodules	3x1	Pink	3-6	13	LUMAC1, LUMAC2, LUMAC5, LUMAC6, LUMAC7, LUMAC8, LUMAC9, LUMAC10, LUMAC11, LUMAC12 (Total positive isolates 10)	LUMAC1, LUMAC5, LUMAC6, LUMAC9, LUMAC10, LUMAC11, LUMAC12 (Total Unique Isolates 7)
C. mysorensis Roth	Unbranched Indeterminate, primary roots, medium-sized nodules, high in number	3x2	Pink	15-20	8	LUMCF1, LUMCF2, LUMCF3, LUMCF5, LUMCF7, LUMCF10, LUMCF11, LUMCF12) (Total positive isolates 8)	LUMCF1, LUMCF2, LUMCF3, LUMCF7, LUMCF10, LUMCF11, LUMCF12 (Total Unique Isolates 7)
D. heterocarpum (L.) DC.	Determinate, small nodules, primary and secondary roots, many nodules	2x1	Pink	20-30	12	LUMDes1, LUMDes3, LUMDes4, LUMDes5, LUMDes6, LUMDes9, LUMDes10, LUMDes11) (Total positive isolates 8)	LUMDes1, LUMDes3, LUMDes5, LUMDes9, LUMDes11 (Total Unique Isolates 5)
D. triflorum (L.) DC.	Determinate, primary and secondary roots, small-sized nodules, few nodules	1x1	Pink	5-8	9	LUMDT2, LUMDT3, LUMDT6, LUMDT9, LUMDT11, LUMDT12, LUMDT16 (Total positive isolates 7)	LUMDT2, LUMDT3, LUMDT9, LUMDT11, LUMDT16 (Total Unique Isolates 5)
E. stricta Roxb.	Determinate, primary roots, medium-sized nodules, few nodules	4x3	Pink	6-8	7	LUMESA, LUMESB, LUMES1, LUMES3, LUMES4, LUMES5 (Total positive isolates 6)	LUMESA, LUMESB, LUMES4, LUMES5 (Total Unique Isolates 4)
L. leucocephala (Lam.) de Wit	Indeterminate and unbranched, large nodules, primary roots, few nodules.	3x2	Pink	2-4	15	LUMLL1, LUMLL2, LUMLL3, LUMLL6, LUMLL8, LUMLL9, LUMLL10, LUMLL11, LUMLL12, LUMLL13, LUMLL14, LUMLL15 (Total positive isolates 12)	LUMLL1, LIMLL2, LUMLL6, LUMLL8, LUMLL9, LUMLL11, LUMLL12, LUMLL13 (Total Unique Isolates 8)

Wild legumes	Nodule Characteristics				Total Isolates	RPO1 Positive	Unique Isolates
	Nodule Type	Avg. Nodule Size (l x w in mm)	Nodule colour	Number of nodules per plant			
M. diplotricha C. Wright	Branched Indeterminate, primary and secondary root, medium-sized nodules, several nodules	2x1	Pink	7-10	15	LUMMD2, LUMMD3, LUMMD5, LUMMD6, LUMMD9, LUMMD12, LUMMD13, LUMMD15, LUMMD16, LUMMD18, LUMMDa, LUMMDb, LUMMDc, LUMMDd, LUMMDG, LUMMDm (Total positive isolates 14)	LUMMD2, LUMMD3, LUMMD5, LUMMD6, LUMMD9, LUMMD12 LUMMD13, LUMMD15, LUMMD16, LUMMDb, LUMMDc, LUMMDd, LUMMDm (Total Unique Isolates 13)
M. pudica L.	Indeterminate, primary and secondary roots, medium-sized, several nodules	3x1	Pink	10-15	15	LUMMP1, LUMMP2, LUMMP4, LUMMP10, LUMMP16, LUMMP19, LUMMPF, LUMMPO (Total positive isolates 8)	LUMMP1, LUMMP2, LUMMP4, LUMMP10, LUMMPF (Total Unique Isolates 5)
T. candida DC.	Indeterminate globular nodules, primary roots, few nodules	4x2	Pink	3-5	16	LUMTC1, LUMTC3, LUMTC4, LUMTC7, LUMTC8, LUMTC9, LUMTC10, LUMTC11, LUMTC12, LUMTC13, LUMTC14, LUMTC15, LUMTCA (Total positive isolates 13)	LUMTC1, LUMTC3, LUMTC11, LUMTC15, LUMTCA (Total Unique Isolates 5)
V. nepalensis Tateishi & Maxted	Determinate, small-sized nodules, primary and secondary roots, many nodules	2x1	Pink	30-50	18	LUMVRW1, LUMVRW2, LUMVRW3, LUMVRW5, LUMVRW6, LUMVRW7, LUMVRW8, LUMVRW9, LUMVRW10, LUMVRW11, LUMVRW12, LUMVRW13) (Total positive isolates 12)	LUMVRW1, LUMVRW2, LUMVRW8, LUMVRW9, LUMVRW10, LUMVRW11, LUMVRW12 (Total Unique Isolates 7)
V. vexillata (L.) A. Rich	Determinate, medium-sized, globular nodules, primary roots, several nodules	3x2	Pink	10-12	11	LUMVV1, LUMVV2, LUMVV4, LUMVV9, LUMVV10, LUMVV11, LUMVV13, LUMVVO, LUMVVx) (Total positive isolates 9)	LUMVV1, LUMVV2, LUMVV4, LUMVV9, LUMVV10, LUMVV11, LUMVV13, LUMVVO, LUMVVx (Total Unique Isolates 9)
B. variegate L.	Non-nodulating	-	-	-	-		
C. pulcherrima (L.) Sw.	Non-nodulating	-	-	-	-		
P. speciosa Hassk.	Non-nodulating	-	-	-	-		

2.7.2 Biochemical characterization

2.7.2.1 Catalase test

A colony from fresh culture was picked and then smeared on a glass slide. 2-3 drops of 0.5% (v/v) H2O2 were added to the smear. The appearance of bubbles on the smear marks positive catalase activity (Shoaib et al. 2020).

2.7.2.2 Citrate test

The test was performed by streaking the pure and fresh colony on Simmon's citrate agar slants and incubated for 24-48 h at 28°C - 30°C. Positive results showed a change of colour from green to blue (Shoaib et al. 2020).

2.7.2.3 Carbon utilization test

Carbon utilization or fermentation tests of six sugars, namely dextrose, fructose, glucose, maltose, mannitol, and sucrose were performed. Respective sugar broths (peptone, sugar, NaCl, water) were made with phenol red as an indicator, and a fresh colony was inoculated in each sugar broth. Broths were then inoculated with fresh cultures and incubated at 28°C for 18-24 h. All of the tests were performed in triplicates. The carbon broth is usually pink in colour because of the phenol indicator. The broth changes colour from pink to yellow as a sign of positive fermentation of the respective sugar (Shoaib et al. 2020).

2.7.2.4 Starch hydrolysis

Starch hydrolysis was done to assay the amylase production activity by isolates. The bacterial isolates were spot inoculated on starch agar medium [Beef extract (3 gL^{-1}), peptone (5 gL^{-1}), soluble starch (2 gL^{-1}), agar (15 gL^{-1})] plates and incubated at 30°C for 48 h. The plates were flooded with freshly prepared grams of iodine solution, kept for a minute and then poured off the excess iodine solution. Iodine reacts with starch to form a blue colour compound. This blue colour fades rapidly. Hence, the colourless zone resembling a halo surrounding colonies indicates amylase production (Shoaib et al. 2020).

2.7.3 Stress tolerant properties

2.7.3.1 *p*H tolerance test

Tolerance to acidic or alkaline media was assessed by spot inoculating fresh cultures in YEMA plates with varying *p*H 3, 5, 9, and 11. The *p*H of the media was taken using a *p*H meter before adding agar. The media was then boiled, autoclaved for 15 minutes, and poured into Petri plates in the laminar hood. These experiments were performed in triplicates for each isolate for the respective *p*H. The plates were then incubated at 28-30°C, and the growth of cultures was checked after 3 days (Bissa et al. 2020).

2.7.3.2 Salinity tolerance test

For the salt tolerance study, YEMA media without salt was prepared, and varying salt concentrations (w/v), i.e., 1-4%, were added to each medium and autoclaved for 15 min. Media was poured into Petri plates in the laminar chamber, cooled, and treated with UV for 15 min. Salt tolerance of the bacterial isolates was determined by spot-inoculating fresh cultures in YEMA plates supplemented with different concentrations of NaCl. The experiment was performed in triplicates for each isolate in each NaCl concentration. The inoculated plates were incubated at 28°C for 3 days, and growth was checked (Bissa et al. 2020).

2.7.3.3 Temperature tolerance

The ability of bacterial isolates to survive in different temperatures was assessed by inoculating fresh cultures and incubating them in different temperatures (10, 20, 30, 40, and 50°C) for 3 days. Fresh YEMA media was prepared, and in the laminar hood, the media plates were spot inoculated with isolates in triplicates for each temperature at which they would be incubated. The growth chambers were adjusted at different temperatures, and the plates were incubated (Bissa et al. 2020). The results were then recorded for their survivability.

3 Results

3.1 Collection of legumes and their nodule characteristics

A total of 15 legume species were identified and collected (Figure 1). Wild legumes are notably diverse, and some of them, such as *Mimosa pudica*, *Desmodium heterocarpum*, *Tephrosia candida*, *Albizia chinensis*, *Mimosa diplotricha*, *Leucaena leucocephala*, were found to be extensively cosmopolitan and are found in almost all the sites like higher altitude, temperature, and other abiotic conditions. *Crotalaria mysorensis*, recorded as native to India (POWO 2024), was found in the Lumami of Zunhebhoto district. Further, *Desmodium* species, *D. heterocarpum* and *D. triflorum* were of sub-shrub habit and commonly found in all the sites. *Erythrina stricta*, a deciduous tree legume, was also recorded in Lumami. *Vigna* species, *V. vexillata* and *V. nepalensis* were commonly found in all surveyed sites. Further, determinate and indeterminate nodule types commonly occur in wild legumes (Figure 2). Seven legumes formed determinate nodules, while five were formed indeterminate type (Table 2). The nodules of some leguminous species like *Albizia chinensis* (Figure 2b) and *Vigna vexillata* (Figure 2i) had nodules with rough outer texture, which may protect against microbes or pests.

3.2 Isolation of root nodulating bacteria

A total of 150 bacteria were isolated from root nodules of different legumes. The isolates took 3-8 days to grow in the YEM medium,

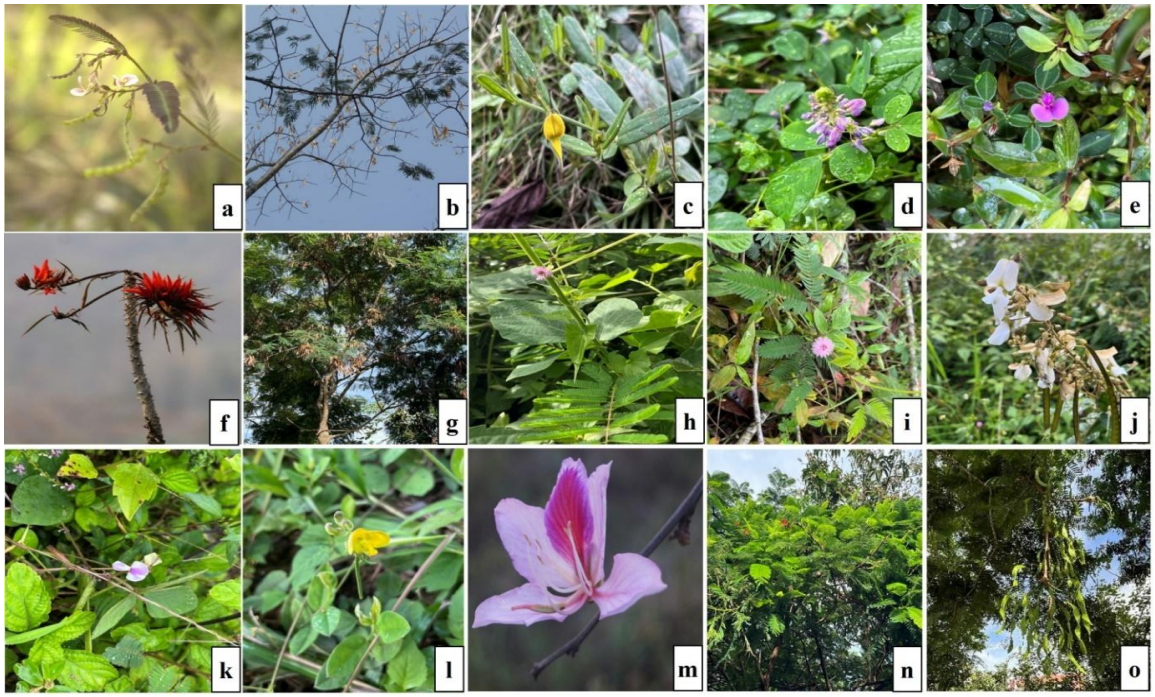

Figure 1 Wild legumes collected from Zunheboto district, a. *Aeschynomene indica*, b. *Albizia chinensis*, c. *Crotalaria mysorensis*, d. *Desmodium heterocarpum*, e. *Desmodium triflorum*, f. *Erythrina stricta*, g. *Leucaena leucocephala*, h. *Mimosa diplotricha*, i. *Mimosa pudica*, j. *Tephrosia candida*, k. *Vigna vexillata*, l *Vigna nepalensis*, m. *Bauhinia variegata*, n. *Caesalpinia pulcherrima*, o. *Parkia speciosa*.

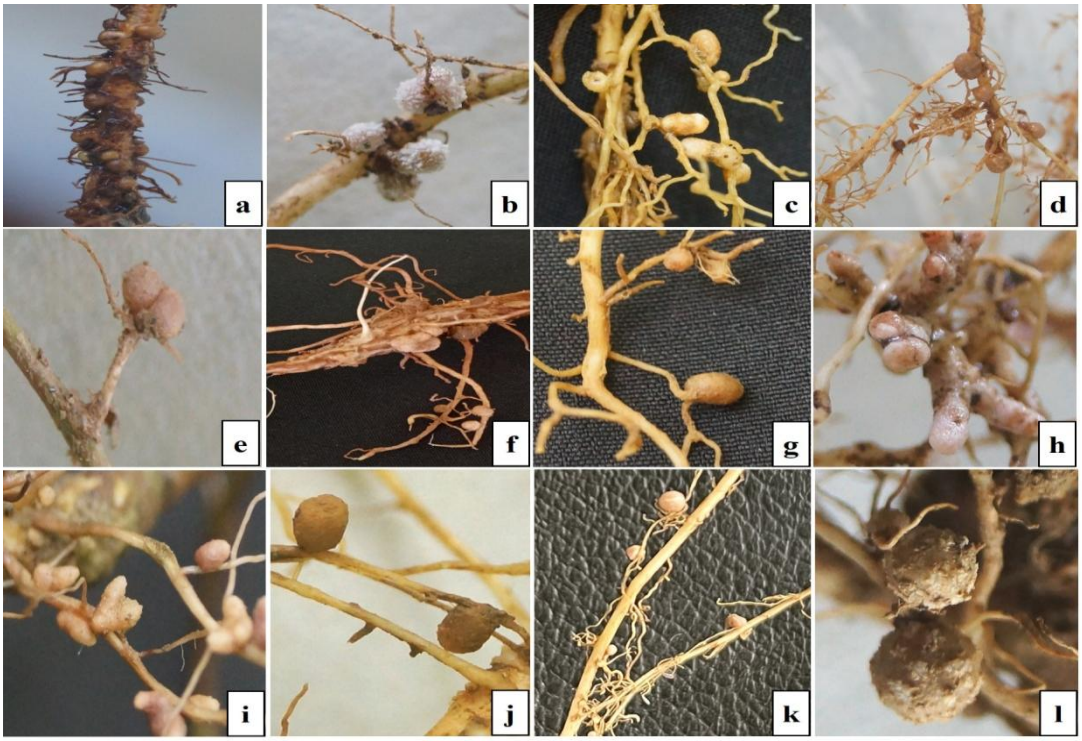

Figure 2 Nodule morphology of collected wild legumes, a. *Aeschynomene indica*, b. *Albizia chinensis*, c. *Crotalaria mysorensis*, d. *Desmodium heterocarpum*, e. *Desmodium triflorum*, f. *Erytherina stricta*, g. *Leucaena leucocephala*, h. *Mimosa diplotricha*, i. *Mimosa pudica*, j. *Tephrosia candida*, k. *Vigna nepalensis*, l. *Vigna vexillata*.

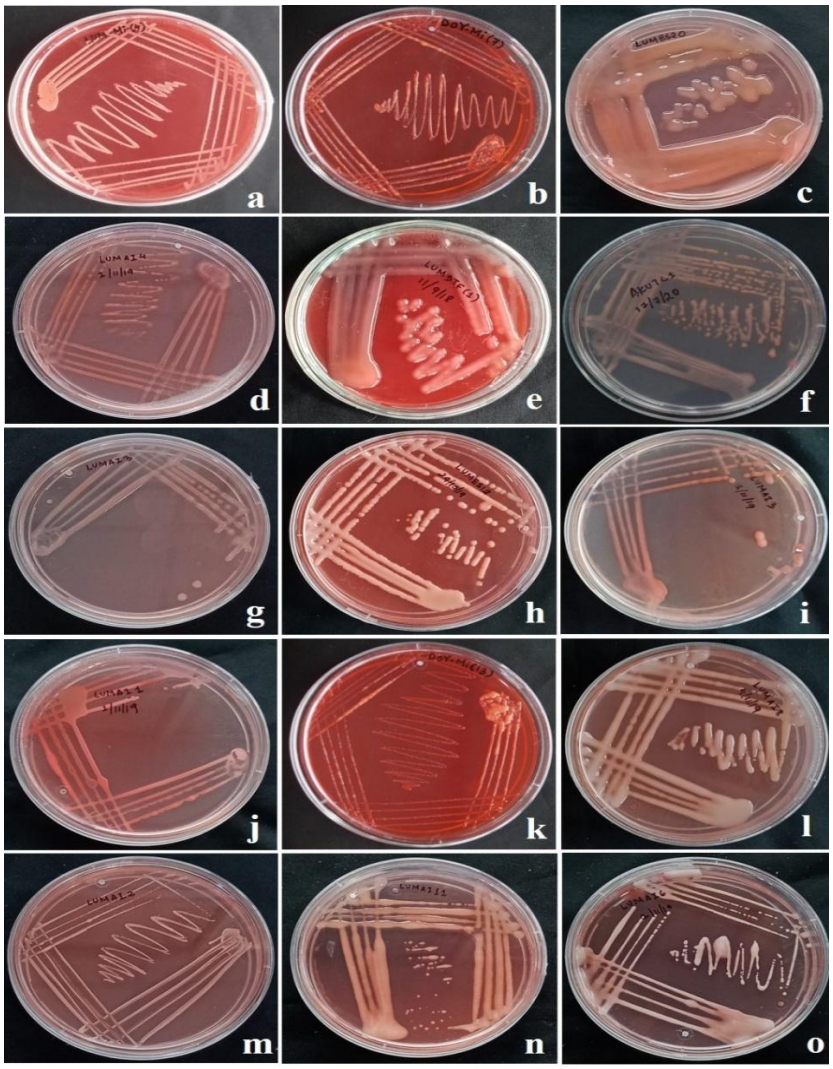

Figure 3 Some root nodulating bacterial endophytes isolated from wild legumes in YEMA-CR medium. A. LUMMi, b. DOYMi7, c. LUMES20, d. LUMAI4, e. LUMDTF1, f. AKUTC1, g. LUMAI3, h. LUMES12, i. LUMAI5, j. LUMAI1, k. DOYMi13, l. LUMAI8, m. AIS9, n. LUMAI1, o. LUMAI6.

showing that some were fast and slow-growing. Some pure cultures obtained were observed to be translucent white, while some were opaque. The cultures were predominantly white, but some tended to be pink after absorbing the Congo red dye and incubating for longer days. Bacterial colonies were also observed to be bulky, raised, and creamy. Excretion of exopolysaccharides was also recorded from some bacteria due to their mucus colony and sticky nature. The colonies were commonly observed to have irregular and continuous borders (Figure 3).

3.3 RAPD analysis of the isolates

Potential rhizobia at the molecular level was identified through RAPD analysis. RAPD was performed on 150 isolates, and among these, 119 isolates confirmed the presence of *nif*-gene by binding to the RPO1 primer. The gel image showed various banding patterns, indicating that some isolates have the *nif* gene (Figure 4). Some isolates from the same host showed similar banding patterns in RAPD when visualizing the gel image. For instance, when RAPD was performed on isolates from *Aeschynomene indica*, band patterns in Lane 4 (L4) and Lane 5 (L5) were similar, possibly due to the genetic similarities between these two strains. Hence, they were considered the same species, and one was chosen to represent that group. It was helpful to group similar bacterial isolates and chose a representative to minimize the possibility of attaining repeated strains. A similar situation has been reported for *Desmodium triflorum*, where the prominent bands in L3, L5, and L9 are similar. A total of 11 isolates from *Aeschynomene indica* bound with RPO1 primer, and on excluding the occurrence of common bands in the banding pattern of isolates, nine of them

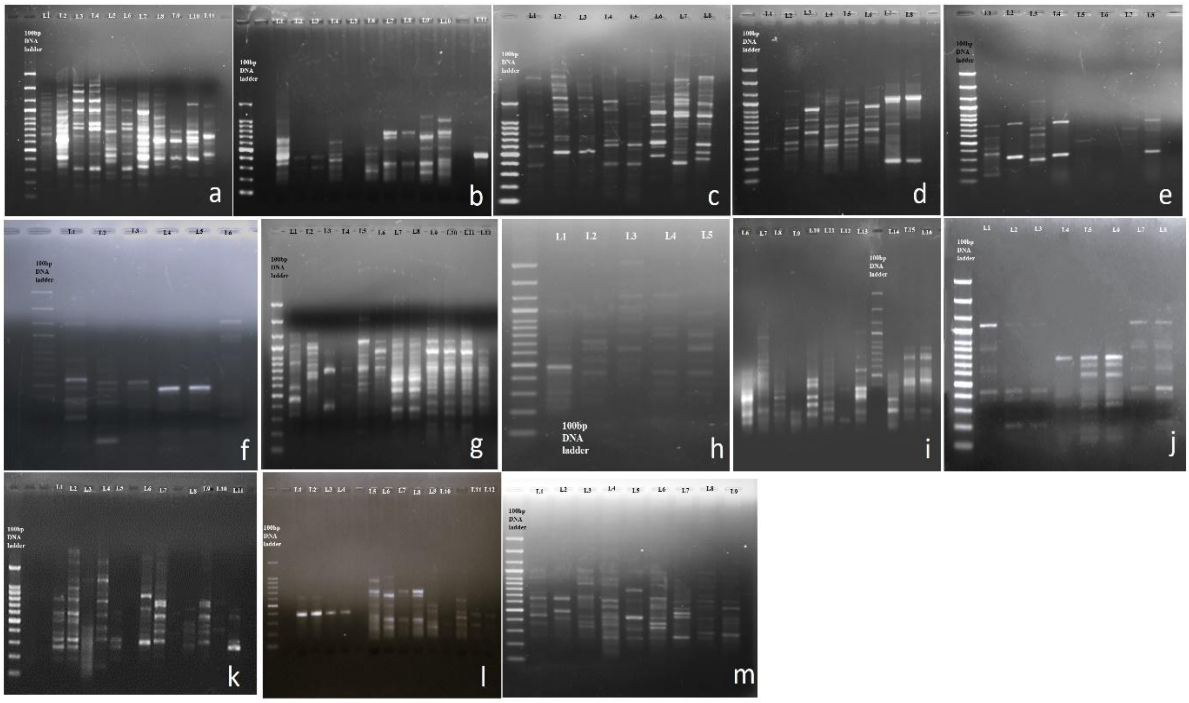

Figure 4 Gel images of RAPD of isolates with RPO1*nif*- directed primer, a. *Aeschynomene indica*, b. *Albizia chinensis*, c. *Crotalaria mysorensis*, d. *Desmodium heterocarpum*, e. *Desmodium triflorum*, f. *Erytherina stricta*, g. *Leucaena leucocephala*, h-i. *Mimosa diplotricha*, j. *Mimosa pudica*, k. *Tephrosia candida*, l. *Vigna nepalensis*, m. *Vigna vexillata*.

were found to be unique, which were L2, L3, L4, L6, L7, L8, L9, L10 and L11 (Figure 4a). From the nodules of *Albizia chinensis*, a total of 13 isolates were cultured, of which 10 were RPO1 positive and seven lanes, namely L2, L3, L5, L6, L7, L9, and L11 showed unique banding patterns (Figure 4b). *Crotalaria mysorensis* also showed unique banding patterns in seven of its isolates. L2, L3 and L4 were considered similar, while L5, L6, L7, L8 and L9 were observed to be unique (Figure 4c). *Desmodium heterocarpum* showed unique banding patterns in five isolates (L2, L3, L4, L5 and L8) out of eight **(**Figure 4d) and in *Desmodium triflorum* L2, L3 (similar to L5 and L9), L5, L6 and L8 were unique (Figure 4e). A total of six isolates were RPO1 positive from *Erythrina stricta*, of which four were unique (Figure 4f), namely L2, L3, L5, and L7. In the case of *Leucaena leucocephala*, 12 isolates bound with RPO1 primer, of which eight unique banding patterns in lanes L2, L3, L4, L5, L6, L7, L8, and L10 were observed in the gel images (Figure 4g). *Mimosa diplotrica* showed the highest Rhizobial diversity in isolates, with 13 out of 16 isolates showing unique RAPD banding patterns (Figure 4h-i). Lanes 5 and 6 had the same bandings; similarly, Lanes 11 and 12 showed similar patterns, as did Lanes 15 and 16. Five isolates were unique in *Mimosa pudica* (Figure 4j), which were L2, L4, L5, L6, and L8 out of eight. In *Tephrosia candida*, 7 isolates in lanes L2, L5, L6, L7, L8, L9 and L11 were deemed unique from 11 RPO1 positive bacterial isolates (Figure 4k). From wild *Vigna nepalensis* legume species, 13 RPO1 positive isolates were cultured, out of which seven unique band patterns (L1, L5, L6, L7, L9, L10, and L11) were observed (Figure 4l). Nine RPO1 positive isolates were isolated from *Vigna vexillata*, and all nine had unique bands (Figure 4m). On excluding similar banding patterns in the isolates, 84 unique Rhizobial isolates were deduced and considered diverse.

3.4 Biochemical characterization

3.4.1 Catalase test

A catalase test is done to screen the catalase enzyme. On adding drops of 0.5% (v/v) H_2O_2 to the bacterial smear, bubbles were formed for 28 isolates isolated from *A. americana, D. heterocarpum*, *A. chinensis*, *Crotalaria*, *Erytherina*, *Leucaena*, *Mimosa* species, *Tephrosia* and *Vigna* species confirming their ability to produce catalase enzyme (Table 3).

3.4.2 Citrate test

When pure cultures were streaked on Simon's citrate agar slants, out of 84 isolates tested, 23 were able to change the colour of media from green to blue, which included 1 each from *A. americana* and *A. chinensis*, 3 from *D. heterocarpum*, 5 from *D. triflorum*, 2 from *L. leucocephala*, 8 and 2 isolated from *M. diplotricha* and *M. pudica* respectively, 5 from *T. candida* and 6 from *V. nepalensis*. Colour change confirmed the activity of the citrate enzyme in those isolates (Table 3).

Table 3 Biochemical activities of isolates from root nodules of wild legumes

Host Species	Isolates	PGP Activities*		Biocontrol and Biochemical Analysis*			Sugar Fermentation*					
		IAA	PSB	Catalase	Citrate	Starch	Dextrose	Fructose	Glucose	Maltose	Mannitol	Sucrose
A. americana L.	AIS1	+	+	+	+	-	+	+	+	+	+	+
	AIS3	+	-	-	-	-	+	+	+	+	+	-
	AIS5	-	-	-	-	-	-	-	-	-	-	-
	AIS7	-	-	-	-	-	+	+	+	+	+	+
	AIS8	-	-	-	-	-	+	+	+	+	+	+
	AIS9	+	-	+	-	-	+	+	+	+	+	+
	AIS10	-	-	-	-	-	+	+	+	+	+	+
	AIS12	+	-	-	-	-	-	-	+	+	-	-
	AIR14	+	-	-	-	-	+	+	+	+	-	+
A. chinensis (Osbeck) Merr.	LUMAC1	+	-	-	-	-	+	+	+	+	-	+
	LUMAC5	-	-	-	-	-	+	+	+	+	-	+
	LUMAC6	-	-	+	-	+	+	+	+	+	+	+
	LUMAC9	+	-	-	-	-	+	+	+	+	-	-
	LUMAC10	-	+	+	+	-	+	+	+	+	+	+
	LUMAC11	-	-	-	-	-	+	+	+	+	-	-
	LUMAC12	-	-	-	-	-	+	+	-	-	-	-
C. mysorensis Roth	LUMCF1	-	+	-	-	-	+	+	+	+	+	+
	LUMCF2	+	+	-	-	-	+	+	+	+	+	+
	LUMCF3	-	-	-	-	+	-	+	+	+	+	+
	LUMCF7	+	-	+	-	-	+	+	+	+	+	+
	LUMCF10	-	+	+	-	-	+	+	+	+	+	+
	LUMCF11	-	-	-	-	-	-	-	-	-	-	-
	LUMCF12	-	+	-	-	-	+	+	-	-	+	+
D. heterocarpum (L.) DC.	LUMDes1	+	+	-	-	+	+	+	+	+	+	+
	LUMDes3	+	+	-	+	+	+	+	+	+	+	+
	LUMDes5	+	+	-	+	-	+	+	+	+	+	+
	LUMDes9	-	-	-	+	-	+	+	+	+	+	+
	LUMDes11	+	-	-	-	-	+	+	+	+	+	+
D. triflorum (L.) DC.	LUMDT2	-	+	-	+	-	+	+	+	+	+	+
	LUMDT3	+	+	+	+	-	+	+	+	+	+	+
	LUMDT9	+	+	+	+	-	+	+	+	+	+	+
	LUMDT11	-	-	-	+	+	+	+	+	+	+	+
	LUMDT16	+	+	+	+	-	-	+	+	+	+	-
E. stricta Roxb	LUMESA	-	-	+	-	-	+	+	+	+	-	-
	LUMESB	+	-	-	-		-	-	-	-	-	-
	LUMES4	+	-	+	-	-	+	+	+	+	-	-
	LUMES5	+	-	+	-	-	+	+	+	+	-	-

Host Species	Isolates	PGP Activities*		Biocontrol and Biochemical Analysis*			Sugar Fermentation*					
		IAA	PSB	Catalase	Citrate	Starch	Dextrose	Fructose	Glucose	Maltose	Mannitol	Sucrose
L. leucocephala (Lam.) de Wit	LUMLL1	-	-	-	-	-	-	-	-	-	-	-
	LIMLL2	-	-	-	-	-	-	-	-	-	-	-
	LUMLL6	-	+	-	-	-	+	+	+	+	+	+
	LUMLL8	-		-	-	-	+	+	+	+	+	+
	LUMLL9	+	-	+	+	-	+	+	+	+	+	+
	LUMLL11	+	-	-	+	-	+	+	+	+	+	+
	LUMLL12	+	-	-	-	-	+	+	+	-	-	-
	LUMLL13	+	-	-	-	-	+	+	+	+	+	+
M. diplotricha C. Wright	LUMMD2	-	-	-	-	-	+	-	+	+	+	-
	LUMMD3	+	+	+	+		+	-	+	+	-	+
	LUMMD5	+	+	+	+	+	-	-	+	-	-	-
	LUMMD6	-	-	-	+	-	-	+	+	-	-	-
	LUMMD9	-	+	-	-	-	+	+	-	+	+	+
	LUMMD12	+	-	-	+	-	-	-	+	+	+	+
	LUMMD13	+	-	+	+	+	+	+	+	+	+	+
	LUMMD15	-	-	-	-	-	-	-	-	-	-	-
	LUMMD16	+	-	-	+	+	-	+	+	-	-	+
	LUMMDb	-	-	-	-	-	+	+	+	+	-	+
	LUMMDc	-	-	-	-	+	+	-	+	+	+	-
	LUMMDd	-	-	-	+	+	+	-	+	+	+	-
	LUMMDm	-	-	-	+	-	+	+	+	+	-	+
M. pudica L.	LUMMP1	-	-	-	-	+	-	+	+	+	-	-
	LUMMP2	-	-	-	-	-	-	+	+	-	-	-
	LUMMP4	+	+	+	-	-	+	+	+	+	+	+
	LUMMP10	-	-	-	+	-	+	+	+	+	+	+
	LUMMPF	-	-	-	+	-	-	+	+	+	-	-
T. candida DC.	LUMTC1	+	-	+	+	+	+	+	-	+	-	+
	LUMTC3	+	-	+	+	-	+	-	+	+	-	+
	LUMTC11	+	-	+	+	-	+	-	-	+	-	+
	LUMTC15	-	-	+	+	-	+	+	+	+	-	+
	LUMTCA	+	-	+	+	-	+	+	+	+	-	+
V. nepalensis Tateishi & Maxted	LUMVRW1	+	-	+	-	-	-	+	-	+	-	-
	LUMVRW2	+	-	+	+	-	-	-	-	+	-	-
	LUMVRW8	-	-	-	+	-	-	-	+		-	-
	LUMVRW9	-	-	-	+	-	-	-	-	-	-	-
	LUMVRW10	-	-	-	+	-	-	-	-	-	-	-
	LUMVRW11	+	-	+	+	-	-	-	-	-	-	-
	LUMVRW12	-	-	-	+	-	-	-	-	-	-	-

Host Species	Isolates	PGP Activities*		Biocontrol and Biochemical Analysis*			Sugar Fermentation*					
		IAA	PSB	Catalase	Citrate	Starch	Dextrose	Fructose	Glucose	Maltose	Mannitol	Sucrose
V. vexillata (L.) A. Rich	LUMVV1	-	-	-	-	+	+	+	+	+	-	-
	LUMVV2	-	-	-	-	-	+	+	+	+	-	-
	LUMVV4	-	-	-	-	+	+	+	+	+	-	+
	LUMVV9	-	-	+	-	+	+	+	+	+	+	+
	LUMVV10	+	-	-	-	-	-	-	-	-	-	-
	LUMVV11	+	-	+	-	-	-	-	-	-	-	-
	LUMVV13	-	-	+	-	-	-	-	-	-	-	-
	LUMVVO	-	-	-	-	+	-	+	-	-	-	-
	LUMVVx	-	-	-	-	+	-	+	+	+	-	-

* Note: +: Positive activity; -: Negative activity.

3.4.3 Starch hydrolysis tests

For screening the biocontrol properties of the 84 isolates, an amylase production or starch hydrolysis test was done. A halo zone was formed by 19 isolates, of which 1 was from *A. chinensis*, *D. triflorum*, *M. pudica* and *T. candida*, 2 from *C. mysorensis* and *D. heterocarpum*, 5 from *M. diplotricha*, and 5 from *V. vexillata* confirming amylase production by them (Table 3).

3.4.4 Sugar fermentation tests

Sugar fermentation tests were done to screen the isolates based on their ability to form organic compounds after metabolizing the carbohydrates. Six sugars were used as carbon sources: dextrose, fructose, glucose, maltose, mannitol, and sucrose. Positive sugar fermentation was confirmed after the colour change of media from pink to yellow. On testing 119 strains, dextrose was fermented by 56 isolates, fructose by 60 isolates, 63 isolates fermented glucose, 62 maltose, 38 fermented mannitol, and 46 isolates that could ferment sucrose. It was found that the isolates from *E. stricta* could not ferment mannitol and sucrose, bacterial isolates from *T. candida* failed to ferment mannitol and isolates from *V. nepalensis* could not hydrolyze dextrose, mannitol and sucrose sugars (Table 3).

3.5 PGPR activities

The *nif* gene-positive isolates were screened for plant growth-promoting traits like phosphate solubilization and IAA production. Out of 84 bacterial strains tested, 38 isolates showed IAA production activity. These isolates include 5 from *A. americana* and *M. diplotricha*, 2 each from *A. chinensis*, *C. mysorensis*, and *V. vexillata*, 3 each from *D. triflorum*, *E. stricta* and *V. nepalensis*, 4 from *D. heterocarpum*, *L. leucocephala* and *T. candida*. Further, 18 isolates showed phosphate solubilizing activities forming a halo zone a, which included 1 each from *A. americana*, *A. chinensis*, *L. leucocephala* and *M. pudica*, 3 from *D. heterocarpum* and *M. diplotricha* and 4 from *C. mysorensis* and *D. triflorum* (Table 3).

3.6 Abiotic stress tolerance

The abiotic stress tolerant ability of the 84 isolates was based on *p*H, salt, and temperature tolerance (Table 4). To check for acidic

Table 4 Stress tolerance tests of bacteria isolated from root nodules of wild legumes.

Host Species	Isolates	*p*H Tolerance				Temperature Tolerance (°C)				Salinity Tolerance (NaCl, %)			
		3	5	9	11	10	20	40	50	1	2	3	4
A. americana L.	AIS1	+	+	+	+	+	+	+	+	+	+	+	-
	AIS3	-	-	+	+	-	-	+	+	+	+	+	-
	AIS5	-	+	+	+	+	+	+	+	+	+	+	+
	AIS7	-	-	+	+	-	-	+	+	+	+	+	+
	AIS8	-	-	+	-	-	+	+	+	+	+	+	+
	AIS9	-	-	+	+	-	+	+	+	+	+	+	+
	AIS10	-	-	+	-	-	-	+	+	+	+	+	+
	AIS12	-	-	+	-	-	+	+	+	+	+	+	+
	AIR14	-	-	+	+	-	+	+	+	+	+	+	+

Host Species	Isolates	*p*H Tolerance				Temperature Tolerance (°C)				Salinity Tolerance (NaCl, %)			
		3	5	9	11	10	20	40	50	1	2	3	4
A. chinensis (Osbeck) Merr.	LUMAC1	-	-	+	-	-	-	+	+	+	+	+	+
	LUMAC5	-	-	+	-	+	-	+	+	+	+	+	+
	LUMAC6	-	-	+	-	+	+	+	+	+	+	+	+
	LUMAC9	+	+	+	-	+	+	+	+	+	+	+	+
	LUMAC10	-	-	+	-	+	-	+	+	+	+	+	+
	LUMAC11	-	-	+	-	-	-	+	+	+	+	+	+
	LUMAC12	-	-	+	-	-	-	+	+	+	+	+	+
C .mysorensis Roth	LUMCF1	-	-	+	-	-	-	+	+	+	+	-	-
	LUMCF2	-	-	+	-	-	+	+	+	+	+	+	+
	LUMCF3	-	-	+	+	+	+	+	+	+	+	+	+
	LUMCF7	-	-	+	+	-	-	+	+	+	+	-	-
	LUMCF10	-	-	+	-	+	+	+	+	+	+	-	-
	LUMCF11	-	-	+	-	-	+	+	+	+	+	+	+
	LUMCF12	-	-	+	-	-	+	+	+	+	+	+	+
D. heterocarpum (L.) DC.	LUMDes1	-	-	+	-	-	+	+	+	+	+	+	+
	LUMDes3	-	-	+	-	+	+	+	+	+	+	+	+
	LUMDes5	-	-	+	-	+	-	+	+	+	+	+	+
	LUMDes9	-	-	+	-	-		+	+	+	+	+	+
	LUMDes11	-	-	+	-	-	+	+	+	+	+	+	+
D. triflorum (L.) DC.	LUMDT2	-	-	+	+	-	+	+	+	+	+	+	+
	LUMDT3	-	-	+	+		+	+	+	+	+	+	+
	LUMDT9	-	-	+	+	+	+	+	+	+	+	+	+
	LUMDT11	-	-	+	+	+	-	+	+	+	+	-	+
	LUMDT16		+	+	+	-	+	+	+	+	+	-	+
E. stricta Roxb	LUMESA	-	+	+	-	-	-	+	+	+	+	+	+
	LUMESB	-	-	+	-	-	+	+	+	+	+	+	+
	LUMES4	-	-	+	-	+	-	+	+	+	+	+	+
	LUMES5	-	-	+	-	+	-	+	+	+	+	+	+
L. leucocephala (Lam.) de Wit	LUMLL1	-	-	+	-	-	+	+	+	+	+	-	-
	LIMLL2	-	-	+	-	-	+	+	+	+	+	+	+
	LUMLL6	-	-	+	+	-	+	+	+	+	+	+	+
	LUMLL8	-	-	+	+	-	-	+	+	+	+	+	+
	LUMLL9	-	-	+	+	+	-	+	+	+	+	+	+
	LUMLL11	-	-	+	+	-	+	+	+	+	+	+	+
	LUMLL12	-	-	+	+	-	+	+	+	+	+	+	+
	LUMLL13	-	-	+	+	-	+	+	+	+	+	+	+

Host Species	Isolates	*p*H Tolerance				Temperature Tolerance (°C)				Salinity Tolerance (NaCl, %)			
		3	5	9	11	10	20	40	50	1	2	3	4
M. diplotricha C. Wright	LUMMD2	-	-	+	-	+	+	+	+	+	+	+	-
	LUMMD3	-	-	+	+	+	+	+	+	+	+	+	-
	LUMMD5	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD6	-	-	+	-	-	-	+	+	+	+	+	+
	LUMMD9	-	-	+	-	-	-	+	+	+	+	+	+
	LUMMD12	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD13	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD15	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD16	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMDb	-	-	+	-	-	-	+	+	+	+	-	+
	LUMMDc	-	-	+	-	-	+	+	+	+	+	+	+
	LUMMDd	-	-	+	-	-	-	+	+	+	+	+	+
	LUMMDm	-	-	+	-	-	+	+	+	+	+	+	-
M. pudica L.	LUMMP1	+	-	+	-	+	-	+	+	+	+	+	-
	LUMMP2	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMP4	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMP10	-	-	+	+	+	+	+	+	+	+		+
	LUMMPF	-	-	+	+	-	+	+	+	+	+	+	+
T. candida DC.	LUMTC1	-	-	+	-	-	+	+	+	+	+	-	-
	LUMTC3	-	-	+	-	+		+	+	+	+	+	+
	LUMTC11	-	-	+	-	-	+	+	+	+	+	+	-
	LUMTC15	-	-	+	-	+	+	+	+	+	+	+	+
	LUMTCA	-	-	+	-	-	+	+	+	+	+	+	+
V. nepalensis Tateishi & Maxted	LUMVRW1	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVRW2	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVRW8	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVRW9	-	-	+	-	-	+	+	+	+	+	+	+
	LUMVRW10	-	-	+	-	-		+	+	+	+	+	+
	LUMVRW11	-	-	+	-	-	+	+	+	+	+	+	+
	LUMVRW12	-	-	+	-	-	+	+	+	+	+		-
V. vexillata (L.) A. Rich	LUMVV1	-	-	+	-	+	+	+	+	+	+	+	-
	LUMVV2	-	-	+	-	+	+	+	+	+	+	+	-
	LUMVV4	-	-	+	-	+	+	+	+	+	+	-	-
	LUMVV9	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVV10	-	-	+	-	-	+	+	+	+	+	-	-
	LUMVV11	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVV13	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVVO	-	-	+	-	+	+	+	+	+	+	+	+

tolerance, isolates were grown in media with *p*H 3 and 5, and for alkaline tolerance, they were incubated in media adjusted with *p*H 9 and 11. Of all the strains tested, 17 survived at pH 3 media, except 2 isolates survived in acidic *p*H of 5. All the isolates could thrive in media with an alkaline pH of 9, while 22 could grow in a *p*H11 medium. At lower temperatures 10°C, 36 isolates could grow and at 20°C growth, 60 isolates could survive. All the isolates survived at 40°C and 50°C, except 6 isolates that did not grow at 50 °C. All the isolates thrived at NaCl concentrations of 1% and 2%. In the salt concentration of 3%, 12 were unable to thrive, and in 4% salt concentration, 17 could not grow.

4 Discussions

Nagaland, home to numerous legume types, has long understood the value of legumes. Wild legumes have been traditionally utilized as medicines in several Nagaland districts due to their widespread prevalence. In the six sites explored, the distribution of legumes was mostly similar. It is to the findings by Rathi et al. (2018) and Pires et al. (2018) that the soil *p*H and other ecological factors play a significant role in the biogeography of rhizobial microsymbionts and influence the selection of them by the host in a particular ecological region hence deciding the diversity of legumes in that area.

Trap experiments are performed when nodules are not collected at the site. It was observed that some legumes did not form nodules in greenhouse conditions, although they formed nodules naturally. This might imply that even if the growth conditions are favourable for the host, it might not be true for rhizobia, hence the lack of nodules. It illustrates that environmental conditions play an important role in both the growth and efficient nodulation of legumes, and the present findings are in agreement with Choudhary et al. (2020), who argued that the biogeography of rhizobia is a result of interactions among the host legumes, the genomic backgrounds of compatible bacterial partners and their environments leading to co-evolution of the legume hosts and compatible rhizobia.

The nodules uprooted were found to be mainly of two common types, determinate and indeterminate, e.g., *Mimosa* species. The size of the nodules also varied from very small, like that of *Desmodium triflorum*, to large, like that of *Tephrosia candida*. When dissected and observed, the nodules were mostly pink due to leghaemoglobin indicating active nitrogen fixation, as described by Aung and Oo (2020).

Streaking of the root exudates of healthy and pink nodules produced cultures with varying phenotypic characteristics. Most of them were regular, raised, and bulky. Some isolates were slow growing and took 5-7 days to grow; these were probably *Bradyrhizobia* (Dinkwar et al. 2020; Padukkage et al. 2021). The slow-growing character of *Bradyrhizobium* was attributed to its distinct cell physiology that includes the display of cell aggregation rather than cell division during the lag and initial exponential growth phase, hindrance in their growth due to an increase in specific nutrient concentration (Medici et al. 2024). The culture obtained had varying morphological characteristics. Cultures obtained were pink, white, creamy, translucent, and sometimes yellow. Since wild legumes are infected by various symbionts with varying degrees of nodulation and nitrogen fixation efficiency, DNA fingerprinting could be an essential method for studying their microbial diversity. Further, performing RAPD is a vital method to assess their genetic diversity. A similar opinion was also expressed by Aoki et al. (2010), as the similarity in the banding patterns obtained after amplification indicated homogenous isolates.

Biochemical tests are performed when it is difficult to distinguish bacteria morphologically; hence, they are identified by differences in their metabolic activities. Various biochemical tests were performed to determine their biocontrol, enzyme activities, plant growth promoting, sugar fermentation, and stress-tolerant activities. Biochemical characterization of isolates suggested that starch hydrolysis was negative for most of the isolates, similar to the results obtained by Singha et al. (2015).

In the present study, out of 84 isolates, 18 showed phosphate solubilizing activity, and 38 were able to produce IAA. Sijilmassi et al. (2020) and Oparah et al. (2024) reported that *Rhizobium* was good for the solubilization of phosphate. Similar findings have been recorded by Sijilmassi et al. (2020) and Sharma and Goswami (2020) in Rhizobial species to produce IAA.

Growth of rhizobia in soils is sensitive to pH, and it has been shown to limit survival and persistence in soils (Kapembwa et al. 2016). The isolates obtained were all able to grow in an acidic medium (pH 3 to 5), who reported that the tested isolates exhibited very poor growth at acidic pH. Rhizobial cultures isolated in this study showed normal growth at pH 6.8. These results are similar to those of Jain et al. (2020), who reported the best rhizobial growth in media with a pH around neutral. LUMMP4, LUMTC4, LUMCF10, LUMAC1, AIS6, and LUMDes5 were some isolates out of 22 that could thrive in extremely alkaline pH11.

Several studies reported that most rhizobia are salt tolerant (Bissa et al. 2020; Oparah et al. 2024). In the present study, some isolates like LUMMD3, LUMESA, and LUMDes11 were able to grow profusely after 24h of spot inoculation, while colonies of other isolates like AIS9, LUMCF3, LUMCF11, LUMMDa, LUMMDc, LUMMPO were seen after 32h of inoculation in the varying salt concentrations. The isolates differed in their growth rate but were able to thrive in all salt concentrations irrespective of salt

concentration, which is in contrast to the reports of Ali et al. (2009) and Thrall et al. (2008), who reported that with increasing salt concentration there was a decrease in growth of rhizobial isolates. It has been stated that Salinization/ alkalization is known to limit nodulation and nitrogen fixation. Response of legumes varies greatly; some legumes, e.g., *Vicia faba* and *Phaseolus vulgaris*, are more salt tolerant than others, such as *Pisum sativum*. Other legumes like *Prosopis, Acacia,* and *Medicago sativa* are sensitive to high salt, but their rhizobia is more salt tolerant than the host plants (Zahran 1999).

Sugar fermentation tests are performed to investigate if the bacterial isolates can ferment different carbohydrates and form organic compounds. Six sugars were used: Dextrose, Fructose, Glucose, Maltose, Mannitol, and Sucrose. There was diversity in the ability of isolates to ferment different carbon sources because both fast and slow-growing Rhizobia were isolated. This corroborates with the study by Kapembwa et al. (2016), which found that fast-growing Rhizobia has a wider range for carbon utilization while slow-growing Rhizobia has a narrow range. Further, 63 isolates were able to ferment glucose, which is a confirmatory test for *Rhizobium*, as given by Rai and Sen (2015). Twenty-eight isolates were able to ferment all the carbohydrate sources, assuring their identity to be Rhizobia, as per Hossain et al. (2019).

Conclusions

This study uncovered the vast microbial diversity present in the nodules of wild legumes, positively asserting the wide scope of bacterial diversity study possible from legumes of Nagaland. Out of 150 isolates, 119 were bound with RPO1 primer preliminary, confirming their identity as nitrogen-fixing rhizobia. RAPD band polymorphisms among the isolates were observed, which indicated the huge bacterial diversity in the nodules. It affirms that nif-directed RAPD is beneficial in giving a Head Start for subsequent molecular works on the isolates. The present work also observed 11 isolates that could solubilize phosphate and produce IAA, certifying their ability to benefit plants. Remarkably, most of the isolates were also tolerant towards abiotic stress, which was considered in our study. Thus, the current research findings offer the initial examination of the phylogenetic diversity of native Rhizobia that nodulates several significant wild legumes in Nagaland. Research on native rhizobia in the wild without a history of rhizobial inoculation could be crucial for the selection of novel strains suitable for the regional environment. Also, since the rhizobial diversity of Nagaland is vast and still unexplored, there is a possibility of obtaining novel strains since PCR methods are designed to amplify only the target regions, and the known primers might not be applicable for the novel strains. Further molecular work with nitrogen fixation genes and other housekeeping genes is recommended to solidify the identity of rhizobia.

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Conflicts of Interest

Authors declare that no conflicts exist.

Consent of authorship

All authors consented to a part of the paper. Both CRD and MM have an equal share of authorship.

Authors Contributions

Fund arrangement, concept development, experimental design, supervision of the research work, data analysis and manuscript correction done by CRD, MM executed the research work, data analysis, manuscript draft preparation as a per of her Ph. D. research, AP is the Co-Supervisor of MM and partially supervised initial part of the research work.

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