Applied Biological Research 26(4): 459-465; (2024)
DOI: 10.48165/abr.2024.26.01.53

ISOLATION OF ROOT NODULE ENDOPHYTES FROM COWPEA (*Vigna unguiculata* L.) GROWING IN JHUM FIELD IN NAGALAND (INDIA)

Bendangsenla Pongener, Asosii Paul*, and Chitta R. Deb

Department of Botany, Nagaland University, Lumami - 798 627, Nagaland (India)
*e-mail: *paulchachei@rediffmail.com/apaul@nagalanduniversity.ac.in*

(Received 26 April, 2024; accepted 16 November, 2024)

ABSTRACT

Legume root nodules are inhabited by numerous symbiotic endophytes which impact the plant growth and development. Cowpea (*Vigna unguiculata* L.) is one of the most widely grown legume crops in the Jhum fields of Nagaland (India). However, the information on root nodule endophytes of cowpea grown in Jhum (slash and burn) ecosystem is lacking. With the aim to decipher the root nodule micro-symbionts, in the present study, 17 endophytic bacteria were isolated from the root nodules of cowpea. These isolates were clustered into two major groups, constituting eight sub-groups based on RADP-fingerprinting using *nif*-directed RPO1 primer. Eight unique isolates representing each sub-group were selected for *16S rRNA* gene amplification and sequencing. Sequence analysis using BLASTn revealed that these isolates belonged to either *Enterobacter* or *Bacillus* species. The presence of these bacteria in root nodules suggests some vital role of non-rhizobial endophytes in the legume root nodule biology.

Keywords: Crop legume, root nodule endophytes, RAPD, *Vigna unguiculata*

INTRODUCTION

Legumes belong to the Leguminosae family and as a rich source of protein form an integral part of our diet (Giller *et al*., 2016). Additionally, legumes can form a symbiotic relationship with rhizobia, leading to a root nodule, which converts atmospheric nitrogen into usable reduced form (Ferguson *et al*., 2010). The nitrogen-fixing rhizobia belonged to α, β, and γ proteobacteria groups (Muktar *et al*., 2020), but as per recent reports, diverse non-rhizobial endophytes such as *Acinetobacter, Bacillus, Enterobacter, Paenibacillus, Pantoea, Mycobacterium, Micromonospora, Pseudomonas* and *Agrobacterium* are also found in the legume root nodules (Martínez-Hidalgo and Hirsch, 2017; Etesami, 2022). Though the functional role of non-rhizobial endophytes is not fully understood; the interaction between non-rhizobial bacteria and root nodulating rhizobia might potentially influence the root nodule formation and nitrogen fixation (Tariq *et al*., 2014; Martinez-Hidalgo and Hirsch, 2017; Etesami, 2022; Jesus *et al.*, 2023). Further, the endophytes are widely recognised for their ability to boost biotic and abiotic stress tolerance and improve the crop productivity (Gerding *et al.*, 2017; Watts *et al.*, 2023).

Cowpea (*Vigna unguiculata* L.) is a multi-season herbaceous legume widely grown in tropic and sub-tropic areas of the world (Odori *et al*., 2020). Cowpea is rich in nutrients and highly adaptable to abiotic stress (Odori *et al*., 2020; Jayawardhane *et al*., 2022). In Nagaland, a North Eastern state of India, cowpea occupies an area of 871 ha with annual production of 1,270 metric tonnes (Statistical Handbook of Nagaland, 2022-2023) and is widely grown in Jhum (slash and burn) fields as mix cropping with other legumes, cereals, spices and tuber crops. Although there are several studies on legume microsymbionts (Odori *et al.,* 2020; Jayawardhane *et al.,* 2022), but the

information on the root nodule endophytes of cowpea grown in the Jhum ecosystem is lacking. The present study was aimed to decipher the endophytes present in the root nodules of Jhum field-grown cowpea through *16S rRNA* sequence analysis using the Blast program.

MATERIALS AND METHODS

The root nodules were collected from cowpea (*Vigna unguiculata*) grown in a Jhum field located at E 94°24'57.96" and N 26°09'43.90" with an altitude of 827 m masl under Zunheboto district, Nagaland (India). The collected nodules were carefully washed to remove the adhering soil particles. The root nodules were examined for morphological characteristics as per Vincent (1970). A few root nodules were cut open to check the presence of leghemoglobin. For soil physiochemical analysis, rhizospheric soil was collected from the sampling site, air-dried and passed through a sieve to retain fine soil particles. The soil type was determined by using the pipette method (Piper, 1942). Electric conductivity (EC) was measured by using an EC meter (LMCM-20, Labman Scientific Instrument, India) and water holding capacity (WHC) was determined as per the method of Keen and Raczkowski (1921). The soil pH was measured using a digital pH meter (pH System 361, Systronics India). The soil physiochemical properties like organic carbon, nitrogen, phosphorus, and potassium contents were determined by the standard methods of Walkley and Black (1934), Barbano *et al.* (1990); Bray and Kurtz (1945) and Hanway and Heidal (1952), respectively. Transverse sections of root nodule were prepared as per the method of van Spronsen *et al.* (2001). Briefly, the root nodules were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 12 h. The samples were then dehydrated in a series of graded ethanol, embedded in paraffin wax, and thin transverse sections were cut using a microtome. The sections were dewaxed using xylene and then stained with 1% toluidine blue and observed under a light microscope (CXL microscope, Cxl 300, India) under 40X magnification.

The nodule-endophytes were isolated as per the methods of Somasegaran and Hoben (1994) with slight modification. Briefly, healthy and pink nodules were selected and surface sterilized with 90% ethanol for 5 min, followed by washing three times with sterile water. The nodules were dipped in 0.1% Bavistin (Bio Stadt India Limited, India) for 5 min, rinsed in sterile water, then treated with 0.1% mercuric chloride for 5 min and again washed 3 times with sterile water. The nodules were crushed and the suspension streaked on a yeast extract mannitol agar (YEMA) plate containing Congo red and incubated at 28 ± 2ºC for 3-8 days. Well separated single colonies were picked and streaked on Congo red YEMA plate to obtain pure cultures. The bacterial colony characteristics such as growth rate, colour and appearance were noted (Somasegaran and Hoben, 1994).

Screening through RAPD-fingerprinting

To obtain unique isolates, rapid amplification of polymorphic DNA (RAPD)-fingerprint for 17 isolates was generated using *nif*-directed RPO1 primer (5'-AATTTTCAAGCGTCGTGCCA-3') as described by Richardson *et al.* (1995) with slight modification. The *nif*-directed RPO1 primer was shown to differentiate diverse *Rhizobium* species (Richardson *et al*., 1995). Briefly, a single colony was picked with a sterilized toothpick and then dipped in 60 µL colony lysate buffer containing 150 mM NaCl, 50 mM tris-Cl (pH 7.4), and 1% (v/v) triton X 100. The mixture was then heated in boiling water for 10 min, and the lysate kept in cool ice bath for 2 min. The sample was centrifuged at 10,000 rpm for 2 min and 2 µL supernatant used as DNA template for polymerase chain reaction (PCR) amplification on a BIO-RAD T100 thermal cycler (BIO-RAD, USA), with the conditions as: initial denaturation at 94ºC for 3 min followed by 30 cycles of 94ºC for 30 sec, 47ºC for 30 sec, 72ºC for 1 min and a final extension at 72ºC for 5 min. The PCR product was run on 1.5% agarose gel and visualized using a documentation system (Transilluminator Bio View, USA). The RAPD

fingerprint was scored for the presence or absence of a band, and then a dendrogram prepared by using NTSYSpc version 2.02 (Applied Biostatistics Inc., New York, USA).

Amplification of 16S rRNA and sequence analysis

To identify the isolates, *16S rRNA* gene was amplified as per Weisberg *et al.* (1991) by using 18F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACG-3') primers. The amplicons were analyzed in 1.5% agarose gel, and the images captured using a gel documentation system (Transilluminator Bio view, USA). The amplicons were sequenced using the Sanger sequencing method at Eurofins Genomics India Pvt Ltd, Bangalore, India. The sequences were then analyzed using BLASTn tool (Altschul *et al*., 1990) available at the National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA. The phylogenetic tree was constructed using ClustalX 2.1 software, and the tree was generated using FigTree™ version 1.4.4 (*http://tree.bio.ed.ac.uk/software/figtree/*).

RESULTS AND DISCUSSION

The Jhum soil was sandy clay-loam type, moderately acidic with a pH 5.6, EC value 20.54 dsm^{-1}, and water-holding capacity 75%. The organic carbon, available nitrogen, potassium, and phosphorus levels were 2.70%, 292.00 kg ha^{-1}, 8.44 kg ha^{-1}, and 106.18 kg ha^{-1}, respectively. Similar to our observations Sentimenla (2020) also reported moderately acidic soil with a pH between 6.0-6.4, EC with a value of 0.22-0.44 dsm^{-1}, available nitrogen between 229-293 kg ha^{-1}, potassium ranges from 187.23-257.81 kg ha^{-1}, and phosphorus between 8.89-21.21 kg ha^{-1} in jhum fields of 16 villages of the same Zunheboto district.

The genetics of host plant determines the root nodule morphology, including its size and shape (Sadowsky and Graham, 2006). Morphologically, the legume root nodules are of two types, the determinate spherical shape with limited growth, and indeterminate cylindrical shape with continuous growth (Hirsch, 1992). In present study the root nodules of *V. unguiculata* belonged to determinate type with spherical shape having lenticels (Fig. 1A), with a diameter of 4.27 ± 0.649 mm. When cut open, the nodules were pink indicating the presence of leghaemoglobin (Fig. 1B). The transverse section stained with toluidine blue showed deeply stained infection zones (Fig. 1C). Root nodule N-fixing ability is proportional to the size and number of infection zones (Weisz and Sinclair, 1988; Maróti and Kondorosi, 2014). Jesus *et al*. (2023) reported that any increase in the number of infected cells is accompanied by a change in microbial community composition and diversity in root nodule which increases stress tolerance and improves seedling survivability of *Acacia longifolia* after fire.

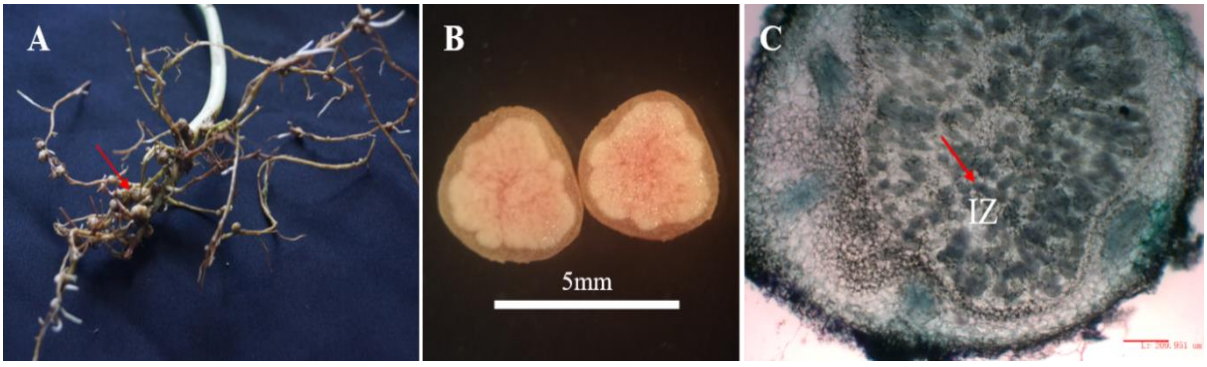

Fig. 1: *Vigna unguiculata* root nodule characteristics; A) Root nodules, B) Cut root nodule with pink color indicating the presence of leghemoglobin; C) The 5 µm transverse section of root nodule stained with toluidine blue. The deeply stained infected zones (IZ) are indicated by a red arrow. The scale bar equals 209.95 µm

To catalogue the micro-symbiont associated with cowpea crop, the root nodules of Jhum field-grown plants were collected and a total of 17 endophytic bacterial isolates cultured. Unique isolates were selected using RPO1 (*nif*-directed) primer-based RAPD fingerprint (Fig. 2). Richardson *et al.* (1995) reported the effective differentiation of several *Rhizobium* strains using *nif*-directed RPO1 primer. According to the RAPD-fingerprint derived dendrogram, the 17 isolates were clustered into 2 major groups, namely group I and II with subsequent 8 sub-groups (Fig. 2B). Eight unique isolates representing the 8 subgroups were further selected for *16S rRNA* gene amplification and sequencing (Fig. 3). However, BLASTn sequence analysis confirmed that 4 isolates belonged to *Enterobacter* sp., 2 isolates to *Bacillus* sp., and for the isolates 4 and 9, the sequencing failed. The *16S rRNA* sequences were submitted to NCBI GenBank with their accession numbers given in Table 1. Similarly, *nif*-directed RPO1 primer was shown to produce RAPD fingerprints with both rhizobial and non-rhizobial taxa suggesting that RPO1 primer can bind to *nif* promoter gene and other regions (Gerding *et al*., 2017; Pongener *et al*., 2024). Based on the growth, all 8 isolates were fast growers and the colony characteristics are given in Table 1.

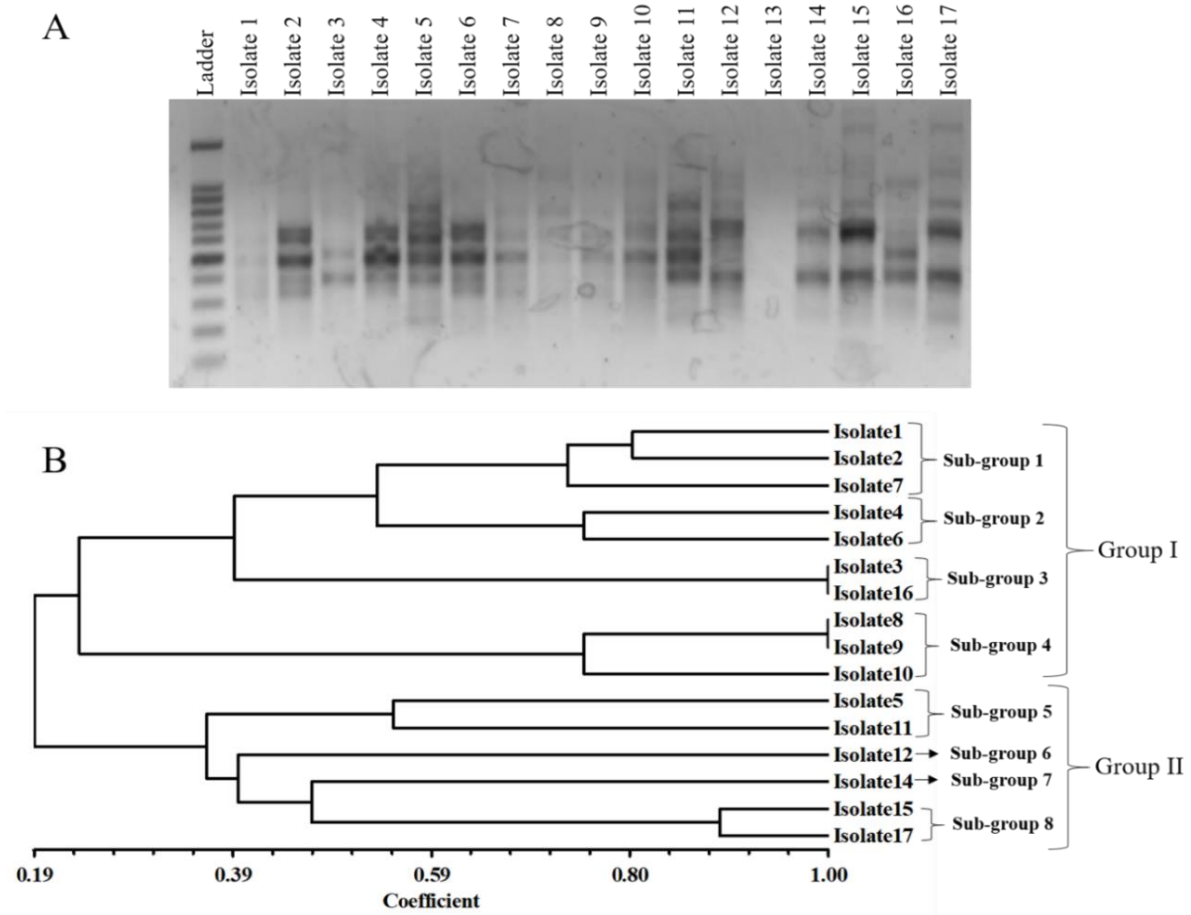

Fig. 2: Screening for unique isolates using *nif*-directed RPO1 primer; A) RAPD-fingerprint of 17 isolates obtained from *V. unguiculata*. Ladder with 100 bp; B) Unweighted pair group method with arithmetic mean (UPGMA) dendrogram categorized the 17 isolates into 8 sub-groups. Representative isolate from each sub-group was selected for *16S rRNA* amplification. The pair-wise similarity coefficient was estimated using Jaccard's similarity index.

The phylogenetic tree, constructed using *16S rRNA* sequences, further supports the grouping of 4 *Enterobacter* sp. and 2 *Bacillus* sp. into groups 1 and 2, like that obtained through RAPD-dendrogram cluster analysis (Fig. 2B; 3B). Despite the reported occurrence of rhizobial species in root

Table 1: *16S rRNA* sequence analysis of the unique bacterial isolates representing each sub-group based on the RAPD-fingerprint cluster analysis. Isolate 4 and 9 sequencing failed.

Isolate code	Strain ID	Accession No.	Best Blastn annotation (reference Acc. No)	Percent identity	E-value	Colony characteristics
Isolate 2	VUNVK7	PP422803	*Enterobacter sp.* (KR190183.1)	100.00%	0.0	Round, white or pink, and opaque
Isolate 3	VUNVK8	PP422815	*Enterobacter mori* (MW965524.1)	99.89%	0.0	Round, white or pink, and opaque
Isolate 5	VUNVK1	PP422898	*Enterobacter sp.* (MH669189.1)	100.00%	5e-136	Round, mucoid, and translucent with low exopolysaccharide
Isolate 12	VUNVK3	PP422901	*Enterobacter ludwigii* (MT180566.1)	99.91%	0.0	Round, mucoid, and translucent with low exopolysaccharide
Isolate 14	VUNVK5	PP422902	*Bacillus aryabhattai* (KJ009537.1)	99.78%	0.0	Round, mucoid, and translucent with low exopolysaccharide
Isolate 17	VUNVK2	PP422903	*Bacillus stratosphericus* (MT379479.1)	100.00%	8e-77	Round, white or pink, and opaque

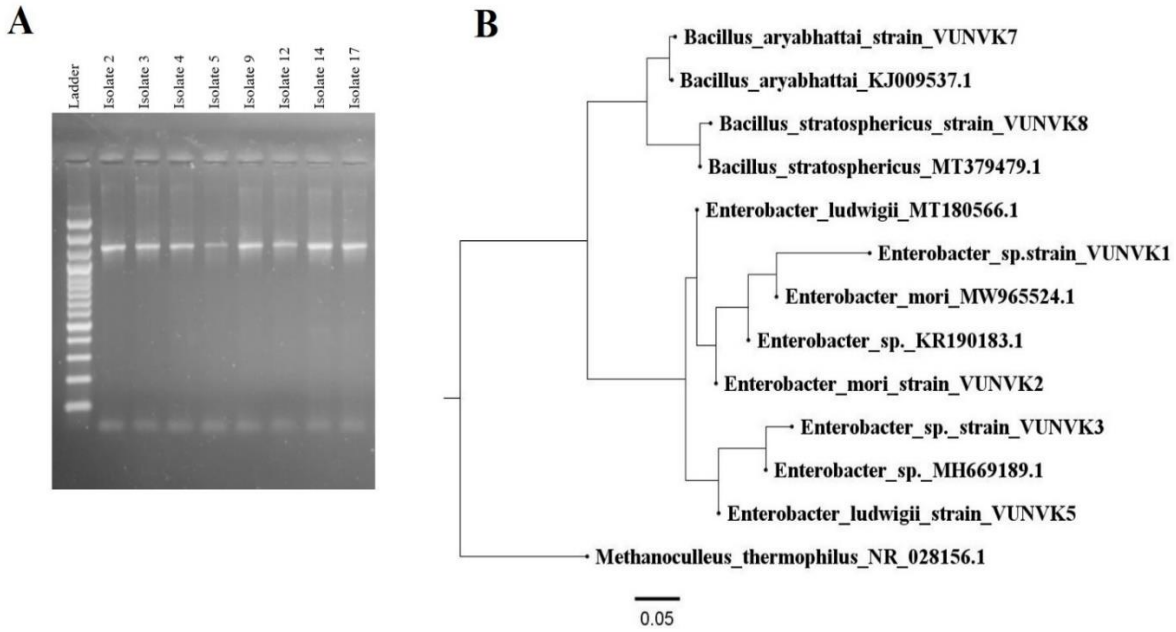

Fig. 3: *16S rRNA* gene amplification and sequence analysis; A) *16S rRNA* gene amplificons with a 100 bp ladder. The isolate code is mentioned above each well. B) Neighbor-joining phylogenetic tree constructed using ClustalX 2.1. The GenBank accession numbers of reference sequences are mentioned. *Methanoculleus thermophilus* was used as an outgroup. The vertical line represents the genetic distance.

nodules of *V. unguiculata* (Odori *et al*., 2020), in present study no rhizobial species was isolated. Previously, Muresu *et al.* (2008) reported the occurrence of a large number of non-culturable rhizobial populations in root nodules of legumes growing in wild. From 100 field-collected nodules, the culturable rhizobial isolates were hardly ever found, whereas over 24 other non-rhizobial bacterial *taxa* were isolated. Interestingly, a direct *16S rRNA* gene metagenomic analysis of the same root nodules suspension revealed that rhizobia were the predominant population in most of the nodules examined (Muresu *et al*., 2008). Similarly, the absence of rhizobia in present study might be due to

the unculturable rhizobia existing in Jhum (slash and burn) ecosystem where wild legumes might be growing previously. In conclusion, the study found non-rhizobial species belonging to *Enterobacter* sp. and *Bacillus* sp. to be endophytic in the root nodules of jhum field grown *V. unguiculata*, suggesting they might play an important role in the root nodule biology.

Acknowledgment: Bendangsenla Pongener (BP) is grateful to the University Grants Commission, Govt. of India, New Delhi for the award of fellowship.

Conflict of interest: The authors declare that there is no conflict of interest.

Authors contribution: BP performed the research work. Asosii Paul (AP) contributed to data analysis and supervised the work. The first draft of the manuscript was written by BP, which was then improved by edits and suggestions from AP. Dr. Chitta R. Deb contributes to supervision. All the authors read and approved the final manuscript.

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