Isolation, Characterization and Phylogenetic Analysis of Nodule-Associated Bacteria from *Mimosa Pudica* L.

Maya Ravunni and Akkara Yusuf*

Interuniversity Centre for Plant Biotechnology, Department of Botany, University of Calicut, Malappuram, Kerala, India.

http://dx.doi.org/10.13005/bbra/3017

(Received: 10 April 2022; accepted: 17 September 2022)

The interaction between rhizobia and other nodule-associated bacteria assists to mitigate nutrient stress in leguminous plants by fixing atmospheric nitrogen and synthesizing plant growth regulators. The beneficial effects of microbial inoculants emphasize the need for further research and their use in modern agriculture. The present study describes the isolation, molecular identification, characterization, and phylogenetic analysis of nodule-associated bacteria from *Mimosa pudica* Linnaeus. Isolation and phenotypic characterization of nodule-associated bacteria were carried out according to standard procedures. Molecular characterization of the isolates was performed using 16S ribosomal RNA. Plant growth promoting the ability of selected isolates was analyzed by assessing indole acetic acid production, nitrogen-fixing ability and organic acid production. Evolutionary distance and relatedness were analyzed using the neighbor-joining method. Thirteen nodule-associated bacteria were isolated and identified using 16S rRNA gene sequencing. The selected isolates such as *Rhizobium* sp. CU8 and three other co-resident non-rhizobial nodule-associated bacteria (*Bacillus cereus* MY5, *Ralstonia pickettii* MY1 and *Lactococcus lactis* MY3) exhibited plant growth promotion and other potential microbial activities. Phylogenetic analysis revealed the genetic relatedness and evolutionary significance of all the thirteen isolates reside in the root nodule of *M. pudica*. The present study identified four isolates with plant growth promoting properties. *L. lactis* MY3 is the first report as a co-resident plant growth promoter from the root nodules of *M. pudica*.

**Keywords:** Nitrogen fixers; Phylogenetic analysis; Plant growth promoters; Root nodule; 16S rRNA.
nutrient uptake\(^1,\)\(^2\) and the production of substances like antibiotics\(^1\). In addition, the PGP microbes express abiotic stress tolerance like extreme temperature, drought, salinity, pH, heavy metal and pesticide pollution\(^3\).

PGPB has beneficial effects on legume growth and some strains enhance the nodulation and nitrogen fixation by effective interaction between plant and rhizobia\(^4\). Most of the nodulating bacteria are free-living rhizobacteria, however, some are intracellular or intercellular endophytes\(^5\) and gain advantage of being protected from environmental stresses and microbial competition\(^6\). The endophytes and epiphytes are the two different types of plant growth promoting rhizobia associated with host tissue. There are many endophytic and epiphytic bacteria which are directly or indirectly involved in plant growth and development. Endophytic bacteria live in plant tissues without affecting the normal metabolism of the host or gaining any benefit other than a noncompetitive environment inside the host. It has been demonstrated that bacterial endophytes play a beneficial role in host plants, such as growth promotion and biological control of pathogens\(^7,\)\(^8,\)\(^9\). The endophytes and epiphytes are the two different types of plant growth promoting rhizobia associated with host tissue. There are many endophytic and epiphytic bacteria which are directly or indirectly involved in plant growth and development. Endophytic bacteria live in plant tissues without affecting the normal metabolism of the host or gaining any benefit other than a noncompetitive environment inside the host. It has been demonstrated that bacterial endophytes play a beneficial role in host plants, such as growth promotion and biological control of pathogens\(^7,\)\(^8,\)\(^9\). Legume root nodules may contain microbes other than rhizobia\(^10,\)\(^11\), however, the function of these co-residents in the nodules is yet to be fully elucidated, and their main role might be to assist rhizobia during the nodule infection process and to promote plant growth\(^12,\)\(^13\).

Modern agriculture faces challenges, such as loss of soil fertility, fluctuating climatic factors, and increasing pathogen and pest attacks. The sustainability and environmental safety of agricultural production rely on eco-friendly approaches like the use of biofertilizers, biopesticides, and crop residue recycling. Increasing the food quality and quantity, without affecting sustainable plant productivity, and maintaining environmental quality is the principal aspect from the Agricultural and Ecological standpoint. The importance of nitrogen-fixing and plant growth promoting bacteria and their gene conservation can contribute better to sustain agriculture and 16S ribosomal RNA typing is used to identify microorganisms. The 16S rRNA based phylogenetic analysis revealed the relatedness of genus \textit{Rhizobium}, \textit{Bacillus}, \textit{Ralstonia}, \textit{Burkholderia}, \textit{cupriavidus} and \textit{Lactococcus} isolated from the root nodule of \textit{M. pudica}. Our understanding of microbial interactions in the rhizosphere must be complemented by combining the basic and applied studies.

The beneficial effects of microbial inoculants, particularly nitrogen-fixing and plant growth promoters (PGP), from the root nodule of \textit{M. pudica} accentuate the need for research and its application in modern agriculture. The present study is focused on the isolation, identification, characterization and comprehensive evaluation of phylogenetic relationship based on 16S rRNA gene of potential nitrogen fixers and plant growth promoters from root nodule of \textit{M. pudica}.

**MATERIALS AND METHODS**

**Isolation of nodule-associated bacteria**

Bacterial isolates were obtained from the root nodules of \textit{M. pudica} grown in different locations in the University of Calicut campus (11°08’01.0”N, 75°53’19.0”E; 11°08’00.4”N, 75°53’17.5”E). The nodule-associated bacteria (NAB), were isolated from healthy pink coloured root nodules, washed thoroughly using running tap water, surface sterilized using 70% (v/v) ethanol for 30s, 0.1% (w/v) HgCl\(_2\) for 2 min and washed thrice with sterile double distilled water under aseptic condition for one min\(^14\). Nodules were crushed using a sterile glass rod and the extracts were plated onto Yeast Mannitol agar medium pH 6.8, supplemented with Congo red dye (0.025 gl\(^{-1}\)). The cultures were incubated at 28±2°C for 24-48hrs. Single colony-forming units were checked for purity by repeated transferring on to nutrient agar medium having pH-7\(^15\). Pure cultures were maintained on a nutrient agar medium with regular subculturings and used for analysis.

**Phenotypic characterization**

Phenotypic characterization based on the morphological and biochemical characters were done on bacterial isolates grown in nutrient agar medium using Bergey’s manual of systematic bacteriology\(^16\). The morphological characters were listed using gram staining, motility test by hanging drop method and endospore staining by malachite green method using a phase contrast microscope. Biochemical analysis was performed using indole production, hydrolysis of urea, methyl red (MR) test, voges proskauer (VP), citrate utilization...
and nitrate reduction test. The intrinsic antibiotic resistance of the isolates was determined by the disc method with Ampicillin (Amp) (10 mcg/disc), Tetracycline (TE) (30 mcg/disc), and Penicillin G (PG) (10 IU/disc) 17.

**Molecular characterization**

**DNA extraction, 16S ribosomal RNA typing and sequencing**

Bacterial genomic DNA was extracted and purified using CTAB method 18. The purified DNA was quantified using a Nanodrop 2000 spectrometer (UV scanning Thermo scientific). PCR amplification of the 16S rRNA gene was carried out using the universal primers 1-27F (AGAGTTTGATCCTGGCTCAG) and 1495R (CTACGGCTACCTGTTACGA) 19. Amplification was performed in thermocycler with following PCR conditions: 30 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1.30 min with initial denaturation at 94°C for 3 min and final extension at 72°C for 10 min. The band size was verified using agarose gel electrophoresis. The PCR products were cleaned and sequenced from Agrigenome Lab Pvt Ltd, Cochin, Kerala. Cloned 16S rRNA sequences were minimally edited and manually aligned using Bioedit software. Species identification and homology of the sequences were identified using BLAST (https://www.ncbi.nlm.nih.gov/BLAST/). The cloned 16S rRNA sequences were submitted to GenBank, NCBI and accession numbers were obtained.

**Characterization of plant growth promoting potential of bacterial isolates**

The plant growth enhancement potential of the four isolates was verified using their potential to produce indole acetic acid, organic acid and capacity to fix atmospheric nitrogen in plants.

**Production of indole acetic acid (IAA)**

IAA production capacity of the isolates was identified using bacterial cultures grown in nutrient broth supplemented with 0.1% L-Tryptophan (w/v) incubated at 30°C for 48hrs. Indole acetic acid (IAA) production was analysed using the colorimetric method of Gordon and Weber 20. IAA in the culture was quantified using a standard calibration curve prepared using gradient concentrations of IAA.-

**Production of organic acid**

Assessed by growing bacterial culture in calcium carbonate agar [CaCO₃, 5 g L⁻¹, glucose 50 g L⁻¹, yeast extract 5 g L⁻¹, agar 15 g L⁻¹] medium and the clear zone around the colony confirmed the production of organic acid.

**Assessment of nitrogen fixation**

The N₂ fixation capacity was evaluated by growing the cultures in nitrogen-free malate containing bromothymol blue medium [DL-Malic acid 5 g L⁻¹, KOH 4 g L⁻¹, K₂HPO₄ 0.5 g L⁻¹, FeSO₄·7H₂O 0.05 g L⁻¹, MnSO₄·7H₂O 0.01 g L⁻¹, MgSO₄ 0.01 g L⁻¹, NaCl 0.02 g L⁻¹, CaCl₂ 0.01 g L⁻¹, Na₂MoO₄ 0.002 g L⁻¹, Yeast extract 0.05 g L⁻¹, Bromothymol Blue 2 mL L⁻¹] for 24-48 hrs at 30°C kept on a rotary shaker at 120 rpm 21. The change in colour of the medium from pale green to pale blue indicates the ability to fix atmospheric N₂.

**16S rRNA-based phylogenetic analysis**

Phylogenetic analysis based on the 16S rRNA sequence was performed using MEGA 7.0 program 22 based on neighbor-joining statistical method 23 and the branching support of 1000 bootstrap 24. The phylogenetic tree construction based on 16S rRNA sequences from nodule-associated bacteria isolated from *M. pudica* was aligned using ClustalW. The model selection was performed using MEGA 7 22 based on the lowest Bayesian Information Criterion (BIC) value 25.

**Statistical analysis**

Using the SPSS software (27.0V, SPSS, Chicago, USA), one-way ANOVA was performed to analyze the concentration of IAA in the isolates after 48hrs. Statistical analysis was carried out according to Tukey’s test (Pd<0.05). The data were an average of 4 separate experimental observations with three independent replicates (n=3).

**RESULTS AND DISCUSSION**

**Isolation of root nodule associated bacteria**

A total of 13 root nodule-associated bacteria were isolated from *M. pudica*. All the isolates were purified and subcultured on nutrient agar medium (pH-7). The isolated pure bacterial strains were characterized using morphological, biochemical and molecular techniques.

The nodule surface sterilization was aimed to allow the obtention of nodule-associated bacteria 26 resulting in the isolation of thirteen nodule-associated bacteria from the root nodule of *M. pudica*. Out of the 13 NAB obtained, nine were non-rhizobial nodule associated
bacteria. According to the results of Rajendran\textsuperscript{14} about 10% of the surface sterilized nodules showed the presence of endophytic non-rhizobial flora and some nodules showed more than one morphologically distinct non-rhizobial colonies. In the past, bacteria isolated from the nodules with different growth and appearance to that of typical rhizobia were considered contaminants and discarded, however, recent studies convincingly demonstrated the occurrence of non-rhizobial bacteria in the nodules and their role on the host plants, rhizobial strains or the symbiosis are under investigation\textsuperscript{10}. It is now well recognized that non-rhizobial bacteria can promote plant growth by an array of mechanisms including solubilization and mobilization of nutrients\textsuperscript{27}, \textit{N\textsubscript{2}}-fixation\textsuperscript{28}, production of phytohormones\textsuperscript{29}, along with microbial processes. Nodule endophytes belonging

| Strain | Length (bp) | Accession number | Species | Accession number | Percentage of identity |
|--------|-------------|------------------|---------|------------------|------------------------|
| MY3    | 1487        | MW132401         | \textit{Lactococcus lactis} | MW429822 | 99.58% |
| MNMY3  | 1388        | MT039465         | \textit{Cupriavidus} sp. | MG798711 | 99.93% |
| CU8    | 1347        | MN744368         | \textit{Rhizobium} sp. | MT415399 | 99.85% |
| MY6    | 1428        | MN744356         | \textit{Burkholderia} sp. | KP744003 | 98.87% |
| CU3    | 1489        | MN744346         | \textit{Bacillus} sp. | MZ004949 | 90.32% |
| CU2    | 1500        | MN744342         | \textit{Bacillus} sp. | MT102910 | 90.62% |
| MY2    | 1406        | MK002738         | \textit{Bacillus} sp. | AB646981 | 100% |
| CUMY1  | 1406        | MK002737         | \textit{Bacillus thuringiensis} | KX977387 | 99.93% |
| MYB1   | 1401        | MK002734         | \textit{Bacillus cereus} | MT611946 | 100% |
| MY1    | 1397        | MH997486         | \textit{Ralstonia pickettii} | MT341804 | 99.93% |
| MYB5   | 1407        | MH997484         | \textit{Bacillus} sp. | MK847260 | 99.93% |
| MY5    | 1344        | MH997483         | \textit{Bacillus cereus} | DQ289077 | 99.18% |
| CUMY2  | 1407        | MH997482         | \textit{Bacillus cereus} | MK253249 | 99.93% |

**Table 1.** Accession numbers of 16S rRNA sequences obtained from the \textit{M. pudica} nodule associated bacteria

**Fig. 1.** The quantity of IAA produced in different bacterial spp. isolated during 48 hrs of culture. The different letters indicates the significantly difference at the P0\textless0.05 level. Values are given as mean\textpmSE for each sample
to the genera Bacillus, Burkholderia, Pseudomonas and Enterobacter have been isolated from different legumes. 

**Molecular characterization**

**DNA extraction, 16S ribosomal RNA typing and sequencing**

Genomic DNA from the thirteen isolates was extracted using CTAB method. The 260/280 ratio of the DNA samples was 1.8-2 indicating the purity of the samples. Molecular characterization and homology search using the 16S rRNA sequences confirmed that the thirteen isolates are strain CU8, strain MY5, strain MY1, strain MY3, strain MNMY3, strain MY6, strain CU3, strain CU2, strain MY2, strain CUMY1, strain MYB1, strain MYB5 and strain CUMY2 which shows higher similarity to *Rhizobium* sp., *Bacillus cereus*, *Ralstonia pickettii*, *Lactococcus lactis*, *Cupriavidus sp.*, *Burkholderia sp.*, *Bacillus sp.*., *Bacillus thuringiensis*, *B. cereus*, *B. cereus* MY1, *B. cereus* MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. thuringiensis* CUMY1, *Burkholderia* sp. MY6 and *Cupriavidus* sp. MNMY3 were possessed 99-100% homology with the nucleotide sequence of other species available in NCBL. *L. lactis* MY3 from the root nodules of *M. pudica* is not reported earlier. GenBank accession numbers provided for the 16S rRNA gene sequence of thirteen nodule-associated bacteria are given in Table 1.

*Rhizobia* are a functional class of soil bacteria having a nitrogen-fixing symbiosis with legumes, also termed legume nodulating bacteria (or LNB). The ability to nodulate legumes is spread among the alpha and beta- subclasses of Proteobacteria. Beta-rhizobia was originally described in 2001 in two parallel studies: the first study identified *Burkholderia tuberum* and *B. phymatum* from *Aspalathus carnosa* and *Machaerium lunatum* plant respectively which were belongs to the family Papilionoideae and the second study isolated *R. taiwanensis* from two *Mimosa* species which was later named as *Cupriavidus taiwanensis* 31. Verma 32 has

---

Fig. 2. Neighbor joining tree constructed using 16S rRNA gene sequences of 13 nodule associated bacteria isolated from *M. pudica*. Numbers beneath nodes are Bootstrap support (BS) indices and branch length
demonstrated the widespread occurrence of beta rhizobia as symbionts in Indian *Mimosa* species.

It has previously been documented that many non-rhizobial endophytes are often associated with root nodules of a variety of legumes \(^{30,15,10}\) and the genetic diversity of these endophytes is often high \(^{13,10}\). Among these, *Bacillus* and *Pseudomonas* are particularly common \(^{15,10}\) and these genera are well-recognized for their roles in plant growth promotion and biocontrol over soilborne pathogens. \(^{34}\) These two genera are also prominent among rhizoplane bacteria of a variety of plants. Thus, the high diversity of root nodule-associated bacteria in *Mimosa* and the predominance of *Bacillus* and *Pseudomonas* was not unexpected inside the *M. pudica* nodule, as seen in previous studies. \(^{4}\)

**Phenotypic characterization**

Phenotypic characteristics such as shape, gram’s reaction, motility and spore formation and biochemical characterization like indole production, hydrolysis of urea, MR-VP, citrate utilization, nitrate reduction and antibiotic sensitivity are presented in supplementary Table 1. All the twelve isolates except *L. lactis* MY3 were rod-shaped and motile and *L. lactis* MY3 are spherical and non-motile. *Rhizobium* sp. CUMY1, *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *Ralstonia pickettii* MY1 were gram negative and *Bacillus cereus* CUMY2, *Bacillus cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CUMY, *Bacillus* sp. CU3, *Bacillus thuringensis* CUMY1 and *L. lactis* MY3 were gram-positive reactions. Except for *Rhizobium* sp. CUMY1, *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *R. pickettii* MY1 all the isolates showed sporulation. In MR-VP biochemical characteristics, except *L. lactis* MY3 all the isolates were negative in MR test and except *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *L. lactis* MY3 showed a positive response to VP test. Nitrate reduction *Cupriavidus* sp. MNMY3, *Rhizobium* sp. CUMY, *B. cereus* MY5 and *B. thuringensis* CUMY1 were positive to nitrate reduction and all other isolates were negative. In urease characteristics, all the cultures showed positive urease test. The isolates *Bacillus* sp. CUMY1, *B. cereus* MYB1 and *Bacillus* sp. MYB5 showed delayed positive urease activity. *Rhizobium* sp. CUMY, *B. cereus* MY5 and *R. pickettii* MY1 were positive to indole and others were negative. Except *B. cereus* CUMY2 were negative to citrate utilization. Of the thirteen bacterial isolates tested for antibiotic such as tetracycline (30 µg/disc), penicillin-G (10 IU/disc), ampicillin (10 mcg/disc) and erythromycin (15 µg/disc) showed atleast sensitive to one antibiotic.

There were many reports on the diversity of microorganisms in the rhizosphere, the present study revealed nodule bacterial diversity exists even among the organisms associated with the nodules. According to Rajendran \(^{14}\) probably all the organisms whose presence has a beneficial relation might get associated with the root nodules. The isolated NAB showed 80% similarity in the biochemical features examined. The morphological and microscopic features of the isolates were in congruence with the earlier reports of the species. In agriculture, the use of PGPB as inoculants is widely applied but only limited studies addressed their antibiotic resistance. Thus, the best practice is to do that systematically, to limit antibiotic resistance gene (ARG) distribution into the environment \(^{35}\) and also the use of high quality, effective rhizobia on agriculture have contributed significantly to the economy of farming systems through the biological nitrogen fixation in the rhizosphere. However, the rhizosphere comprises large populations of antibiotic-producing microorganisms, which affect susceptible rhizobia. \(^{36}\) Thus, antibiotic resistance is an extremely valuable and positive selection marker to select symbiotically effective bacteria. Our findings show that all the isolates were sensitive to at least one standard antibiotics and can be used as a safe biofertilizer candidate.

**Characterization of plant growth promoting activities**

**IAA production potential of the isolates**

IAA production during the 48hr of growth was quantified in *Rhizobium* sp. CUMY, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 using Salkowski reagent. *R. pickettii* MY1 and *Rhizobium* sp. CUMY developed colour immediately after the addition of reagents indicating the formation of IAA and better IAA production was observed when the cultures were incubated for 25 min in dark. The highest quantity of IAA was produced in *R. pickettii* MY1 (49.8630±0.1779 µg/ml) followed by *B. cereus* MY5 (13.5159±0.2416 µg/ml), *Rhizobium* sp. CUMY (11.6895±0.1837 µg/ml) and *L. lactis* MY3 (4.9315±0.0790 µg/ml) (Fig. 1) after 48hrs
of incubation, which was significant at P<0.05.

A diverse group of microbes, including free-living, epiphytotic and tissue colonizing bacteria synthesizes IAA. The four strains produced a considerable quantity of IAA, which is comparable with earlier studies on various bacteria including *Rhizobium* sp., *B. cereus*, *R. pickettii* and *L. lactis*. According to Datta and Basu, most of the studies reported that IAA-producing organisms are gram-negative, however, few *Bacillus* are known to produce IAA which is gram-positive strains. The present study showed that *B. cereus* MY5 is IAA-producing gram-positive bacteria.

**Production of organic acid**

Among the four isolates, *L. lactis* MY3 showed a clear zone after 24hrs of incubation due to the degradation of calcium carbonate leading to the production of organic acid. The other three isolates don’t show any clear zone around the colony.

*L. lactis* is a rare observation from the root nodule of *M. pudica* and can be used as an agent for plant growth promotion. *L. lactis* develop organic acid indicating the interactions between PGPR and plants can enhance the secretion of organic acids, which play an important role in the process of the activation and absorption of insoluble nutrients by plants.

**Nitrogen fixing potential of the isolates**

The four isolates, *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 exhibited N₂ fixation ability grown in nitrogen-free malate medium containing bromothymol blue as an indicator. The *Rhizobium* sp. CU8, *B. cereus* MY5 and *R. pickettii* MY1 showed a significant colour change from pale green to pale blue indicating N₂ fixing ability within 24hrs. However, *L. lactis* MY3 developed the colour change only after 48hrs.

The interaction between rhizobia and other nodule-associated bacteria is of high relevance due to the N₂ fixation and other plant growth promotion capacities in leguminous plants. Zhao reported endophytic non-rhizobial *Bacillus cereus* and *Ralstonia* spp. are potent N₂ fixers. The genus *Rhizobium* is the first bacteria participating in nitrogen fixation in legumes. According to Higdon, *Lactococcus* bacteria exist as a diazotroph in maize without *nifHDKENB* homologs and hypothesized that *L. lactis* isolates from the mucilage microbiota of *Sierra Mixe* maize possess genes enabling BNF activity and elucidated that all the important genes for the BNF trait in *L. lactis* underpinning the ability to fix atmospheric nitrogen present in the mucilage-derived *Lactococci*, which supports the hypothesis that *Lactococci* can exist as diazotrophs.

**Phylogeny based on 16S rRNA gene**

The cloned 16S rRNA sequences were used to construct the phylogenetic tree using neighbor-joining (NJ) method with 1000 bootstraps. Models with the lowest BIC scores were considered to describe the best nucleotide substitution pattern. Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) are the best-fit nucleotide-substitution models determined using MEGA 7.0. Models with the lowest BIC scores (Bayesian Information Criterion) are depicted as the best substitution pattern with 16S rRNA sequence of the 13 nodule-associated bacteria isolated from *M. pudica* provided TN93+1 (Tamura 3-parameter model), with the lowest BIC score (11977.858), and lowest AIC score (11755.020).

The TN93+1 (Tamura Nei Model) displayed the lowest BIC scores (11977.858-supplementary data 1) to construct a consensus NJ tree from the aligned sequences. In NJ tree, Group I consist of *B. cereus* MYB1, *Bacillus* sp. MY2, *B. cereus* MY5, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *L. lactis* MY3, *B. cereus* CUMY2, *B. thuringiensis* CUMY1 and *Bacillus* sp. MYB5 and Group II consist of *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6, *R. pickettii* MY1 and *Rhizobium* sp. CU8 (Fig. 2). The optimal tree with the sum of branch length = 0.3866 is shown in Fig. 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of transitional substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1482 positions in the final dataset. In NJ tree, the isolates in the first groups includes in the phylum Firmicutes and the Group II isolates belongs to the phylum Proteobacteria. The branching where started from phylum to genus level.

The evolutionary history was derived using the neighbor-joining method and maximum
Likelihood method based on the Tamura-Nei model\(^9\). The bootstrap consensus tree developed from 1000 replicates represented the evolutionary history of the taxa analyzed\(^{24}\). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches\(^{24}\). The development of the bacterial taxonomy can be traced through earlier reviews by Jordan\(^{51}\), Graham\(^{52}\), Young\(^{53}\), Elkan\(^{54}\), and Martinez-Romero\(^{55}\), and the 13 nodule-associated bacterial isolates showed evolutionary relatedness and grouping in congruence with the bacterial taxonomic classification.

**CONCLUSIONS**

This study reports the isolation, molecular identification, characterization and phylogenetic relationship of the thirteen root nodule-associated bacteria of *M. pudica*. The biochemical analysis confirms the nitrogen-fixing potential, plant growth promotion and other potential microbial activities of the *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3. The bacteria with N\(_2\) fixing capacity act as plant growth promoters and hence can be used as biofertilizers. *L. lactis* strain MY3 is a new report from the root nodule of *M. pudica* with plant growth promotion and N\(_2\) fixation capacity. Phylogenetic analysis using neighbor-joining method showed the relatedness and evolutionary position of the isolates. The analysis showed that non-rhizobial bacteria, *B. cereus* MY5, *B. cereus* CUMY2, *B. cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. thuringensis* CUMY1, and *L. lactis* MY3 may co-exist with *Rhizobium* sp.CU8, *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *R. pickettii* MY1 in the root nodule of *M. pudica*. However, it requires further studies to assess the role of these isolates in N\(_2\) fixation and plant growth promotion under pot culture as well as in field condition and these can be used as a potential biofertilizer.

**ACKNOWLEDGEMENT**

The author acknowledge the facilities provided by the Director, Interuniversity Centre for Plant Biotechnology, University of Calicut, for providing facilities.

**Conflicts of interest**

The authors declare that they have no conflict of interest.

**Funding source**

This work was supported by the University of Calicut, Government of Kerala for the research grants (Grant numbers 11347/2016/Admin).

**Statement of informed consent**

Authors declares that they have consented to participate in the manuscript and publish it.

**Ethical statement**

This article does not contain any studies with human participants and/or animals performed by any authors. Formal consent is not required in this study.

**REFERENCES**

1. Glick B. R. The enhancement of plant growth by free living bacteria. *Can J Microbiol*. 1995; 41: 109-117. https://doi.org/10.1139/m95-015
2. Kloepper J. W. Plant growth-promoting rhizobacteria as biological control agents. *Soil microbial ecology: applications in agricultural and environmental management*. 1992; 255-274.
3. Gopalakrishnan S., Sathya A., Vijayabharathi R., Varshney R. K., Gowda C. L., Krishnamurthy L. Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech*. 2015; 5(4):355-377. https://doi.org/10.1007/s13205-014-0241-x
4. Parmar N and Dadarwal K. R. Stimulation of nitrogen fixation and induction of flavonoid-like compounds by rhizobacteria. *J Appl Microbiol*. 1999; 86: 36-64. https://doi.org/10.1046/j.1365-2672.1999.00634.x
5. Stucz A.V and Nowak J. Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. *Appl. Soil Ecol*. 2000; 15: 183-190. https://doi.org/10.1016/S0929-1393(00)00094-9
6. Kobayashi D.Y and Palumbo J. D. Bacterial endophytes and their effects on plants and uses in agriculture. In *Microbial endophytes*, CRC Press. 2000; pp. 213-250. eBook ISBN9780429179334
7. Downing K. J and Thomson J. A. Introduction of the *Serratia marcescens* chiA gene into an endophytic *Pseudomonas fluorescens* for the biocontrol of phytopathogenic fungi. *Can. J. Microbiol*. 2000; 46: 363-369. https://doi.org/10.1139/w99-147
8. Ryu C. M., Kim J. W., Choi O. H., Park S. Y.
Park S. H., Park C. S. Nature of a root associated *Paenibacillus polymyxa* from field-grown winter barley in Korea. *J Microbiol Biotechnol*. 2005; 15: 984-991.

9. Sturz A. V., Christie B. R., Matheson B. G., Arsenault W. J., Buchanan N. A. Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. *Plant Pathol*. 1999; 48(3):360-369. https://doi.org/10.1046/j.1365-3059.1999.00351.x

10. Martinez-Hidalgo P and Hirsch A. M. The nodule microbiome: N-fixing rhizobia do not live alone. *Phytobiomes*. 2017; 1(6): 7082. https://doi.org/10.1094/PBIOMES-12-16-0019-RVW

11. Zgadzaj R, James E. K., Kelly S., Kawaharada Y, de Jonge N., Jensen D. B., Madsen L. H., Radutoiu S. A legume genetic framework controls infection of nodules by symbiotic and endophytic bacteria. *PLoS Genet*. 2015; 11(6): e1005280. https://doi.org/10.1371/journal.pgen.1005280

12. Chibeba A. M., Pereira C. S., Antunes J. E. L., Ribeiro R. A., de Almeida Lopes A. C., Gomes R. L. F., Hungaria M, Araujo A. S. F. Polyphasic characterisation of nitrogen-fixing and co-resident bacteria in nodules of *Phaseolus lunatus* inoculated with soils from Piauí State, Northeast Brazil. *Symbiosis*. 2020; 80(3): 279-292. https://doi.org/10.1007/s13199-020-00672-1

13. Peix A., Ramírez-Bahena M. H., Velázquez E., Bedmar E. J. Bacterial associations with legumes. *CRC Crit Rev Plant Sci*. 2015; 34(1-3): 17-42. https://doi.org/10.1080/07352689.2014.897899

14. Rajendran G., Patel M. H., Joshi S. J. Isolation and characterisation of nodule-associated *Exiguobacterium* sp. from the root nodules of fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion. *Int J Microbiol*. 2012. https://doi.org/10.1155/2012/693982

15. Vincent J. M. A manual for the practical study of the root-nodule bacteria. Black well scientific publication, Oxford. 1970. https://doi.org/10.1002/jobm.19720120524

16. Boone D. R., and Castenholz R. W.: *Bergey’s Manual for the Practical Identification of Bacteria*. Williams & Wilkins, Baltimore. 1974.

17. Cappuccino J. G and Sherman N; *Microbiology: A laboratory manual*, 10th edn Addison-1999. 1983.

18. Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Seidman J. G., Smith J. A., Struhl K. (ed): *Short Protocols in Molecular Biology*, 3rd edn. New York: John Wiley and Sons Inc. 1995; pp 2.11- 2.12.99

19. Lane D. J. 16S/23S rRNA Sequencing. In: Stackebrandt, E. and Good fellow, M., Eds., Nucleic Acid Techniques in Bacterial Systematic, John Wiley and Sons, New York. 1991; pp. 115-175.

20. Gordon S. A and Weber R. P. Colorimetric estimation of indole acetic acid. *Plant physiol*. 1951; 26(1): 192. https://dx.doi.org/10.1104%2Fpp.26.1.klo92

21. Döbereiner J and Day J. M. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. In Proceedings of the 1st international symposium on nitrogen fixation, Washington State University Press, Pullman, 1976; 2:518-538

22. Kumar S., Stecher G., Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol*. 2016; 33(7):1870-1874. https://doi.org/10.1093/molbev/msw054

23. Felsenstein J. Evolutionary trees from gene frequencies and quantitative characters: finding maximum likelihood estimates. *Evol*. 1981: 1299-1242. https://doi.org/10.2307/2408134

24. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. *Evol*. 1985; 39: 783-791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

25. Schwarz G. Estimating the dimension of a model. *Ann Stat*. 1978; 6(2): 461-464. https://doi.org/10.1214/aos/1176344136

26. Rajendran G., Sing F., Desai A. J., Archana G. Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. *Bioresearch Technol*. 2008; 99(11):4544-4550. https://doi.org/10.1016/j.biortech.2007.06.057

27. Srivastwa P. K., Kanhaiyaji V., Nishi K. Growth promotion of plant by nutrient mobilizing PGPR of salt-affected soil. *Asian J Soil Sci*. 2014; 9(1):126-129

28. Castellano-Hinojosa A., Correa-Galeote D., Palau J., Bedmar E. J. Isolation of N$_2$-fixing rhizobacteria from *Lolium perenne* and evaluating their plant growth promoting traits. *J Basic Microbiol*. 2016; 56(1):85-91. https://doi.org/10.1002/jobm.201500247

29. ChinnaSwamy A., Coba de la Peña T., Stoll A., de la Peña R. D., Bravo J., Rincón A., Lucas M., Pueyo J. A nodule endophytic *Bacillus megaterium* strain isolated from *Medicago polymorpha* enhances growth, promotes nodulation by *Ensifer medicae* and alleviates salt stress in alfalfa plants. *Ann Appl Biol*. 2018; 172(3):295-308. https://doi.org/10.1111/
30. Dudeja S., Giri R., Saini R., Sunee-J-Madan P., Kothe E. Interaction of endophytic microbes with legumes. *J Basic Microbiol.* 2012; 52(3): 248-260. https://doi.org/10.1002/jobm.201100063

31. Mishra R. P., Tisseyre P., Melkonian R., Chaintreuil C., Miche L., Klonsowska A., Moulin L. Genetic diversity of *Mimosa pudica* rhizobial symbionts in soils of French Guiana: investigating the origin and diversity of *Burkholderia phymatum* and other beta-rhizobia. *FEMS microbiology ecology.* 2012; 79(2): 487-503.

32. Verma S. C., Chowdhury S. P., and Tripathi A. K. Phylogeny based on 16S rDNA and nifH sequences of *Ralstonia taiwanensis* strains isolated from nitrogen-fixing nodules of *Mimosa pudica*, in India. *Can. J. Microbiol.* 2004; 50(5):313-322.

33. De Meyer S. E., De Beuf K., Vekeman B and De Meyer S. E., De Beuf K., Vekeman B and Verma S. C., Chowdhury S. P., and Tripathi V. *Plant Growth-Promoting Rhizobacteria* *A. J. Microbiol.* 2012; 22(8): 855-872.

34. Fahi N., Mahdi I., Mesfioui A., Biskri L., Allaoui R. Isolation and characterization of soybean associated bacteria and their potential for plant growth promotion. *Soil Sci Plant Nutr.* 2013; 59(3): 638-649. http://dx.doi.org/10.4067/S0718-95162013050000051

35. Strafella S., Simpson D. J., Yaghoubi Khandhari M., De Angelis M., Gänzl E., Minervini F., Crecchio C. Comparative Genomics and In Vitro Plant Growth Promotion and Biocontrol Traits of Lactic Acid Bacteria from the Wheat Rhizosphere. *Microorganisms.* 2021; 9(1):78. https://doi.org/10.3390/microorganisms9010078

36. Datta C and Basu P. S. Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, *Cajanus cajan*. *Microbiol Res.* 2000; 155(2):123-127. https://doi.org/10.1016/S0944-5013(00)80047-6

37. Wahyudi A. T., Astdi R. P., Widiyawati A., Mery A., Nawangshih A. A. Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. *J Microbiol. Antimicrob.* 2011; 3(2):34-40. https://doi.org/10.5897/JMA.9000020

38. Lamont J. R., Wilkins O., Bywater-Ekegärd M., Smith D. L. From yogurt to yield: Potential applications of lactic acid bacteria in plant production. *Soil Biol. Biochem.* 2017; 111:1-9. https://doi.org/10.1016/j.soilbio.2017.03.015

39. Pii Y., Penn A., Terzano R., Crecchio C., Mimo T., Cesco S. Plant-microorganism-soil interactions influence the Fe availability in the rhizosphere of cucumber plants. *Plant Physiol. Biochem.* 2015; 87:45-52. https://doi.org/10.1016/j.plaphy.2014.12.014

40. Barea J. M., Pozo M. J., Azco’ n R., Azco’n-Aguilar C. Microbial co-operation in the rhizosphere. *J Exp. Bot.* 2005; 56:1761-1778. https://doi.org/10.1093/jxb/eri197

41. Zhao L., Xu Y., Sun R., Deng Z., Yang W., Wei G. Identification and characterization of the endophytic plant growth promoter *Bacillus cereus* strain MQ23 isolated from *Sophora alopecuroides* root nodules. *Braz J Microbiol.* 2011;42(2):567-575. https://dx.doi.org/10.1590%2FS1517-83822011000200022

42. Lindström K and Mousavi S. A. Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotechnol.* 2020; 13(5):1314-1335. https://doi.org/10.1111/1751-7915.13517

43. Higdon S. M., Pozzo T., Kong N., Huang B. C., Yang M. L., Jeannette R., Weiner BC. Genomic characterization of a diazotrophic microbiota associated with maize aerial root mucilage. *PLoS one.* 2020; 15(9):e0239677. https://doi.org/10.1371/journal.pone.0239677

44. Tamura K and Nei M. Estimation of the number of nucleotide substitutions in the control region of rhizospheric soil and its effect on plant growth. *Soil Sci Plant Nutr.* 2013; 59(3), 638-649. http://dx.doi.org/10.4067/S0718-95162013050000051
mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol.* 1993; 10:512-526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

51. Jordan D. C.: Rhizobiaceae. *Bergey's manual of systematic bacteriology*, 1984; 1: 234-256

52. Graham P. H., Sadowsky M. J., Keyser H. H., Barnet Y. M., Bradley R. S., Cooper J. E., Young J. P. W. Proposed minimal standards for the description of new genera and species of root-and stem-nodulating bacteria. *International Journal of Systematic and Evolutionary Microbiology*. 1991; 41(4): 582-587.

53. Young J. P. W. Phylogenetic classification of nitrogen-fixing organisms. *Biological nitrogen fixation*. 1992; 1544:43-86.

54. Elkan G. H. *Taxonomy of the rhizobia*. *Can. J. Microbiol.* 1992; 38(6): 446-450.

55. Martinez-Romero E.: Recent developments in Rhizobium taxonomy. *In Symbiotic Nitrogen Fixation*. Springer, Dordrecht. 1994; pp. 11-20.