*BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA*, September 2022. Vol. **19**(3), p. 645-655

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

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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 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.

The members of Leguminosae, are associated with endophytic, root nodule-associated bacteria (NAB) which ameliorates nutrient stress by fixing atmospheric nitrogen ($N_2$) and producing plant growth promoters. The genus *Mimosa* received considerable attention in recent years because of its potential to fix atmospheric nitrogen. Biological nitrogen fixation is ecologically important, contributing ~100-290 million tons of nitrogen annually to the natural ecosystem and enhancing the growth of agronomically important forage and crop plants. Biological nitrogen fixation (both symbiotic fixers and non-symbiotic fixers) reduces the use of synthetic nitrogen fertilizers.

Plant growth promoting bacteria (PGPB) enhance plant growth by fixing atmospheric nitrogen and its assimilation to the plant, production of siderophores to chelate iron and its absorption, solubilization of minerals such as phosphorus, synthesis of phytohormones, augmenting plant

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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]. 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 *Rhizobium*, *Bacillus*, *Ralstonia, Burkholderia, cupriavidus* and *Lactococcus* isolated from the root nodule of *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 *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 *M. pudica*.

## MATERIALS AND METHODS

### Isolation of nodule-associated bacteria

Bacterial isolates were obtained from the root nodules of *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±2ºC 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 subculturing 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 ºC for 45 s, 50 ºC for 1 min, and 72 ºC for 1.30 min with initial denaturation at 94 ºC for 3 min and final extension at 72 ºC 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 ºC 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 [$CaCO_3$ 5 $gl^{-1}$, glucose 50 $gl^{-1}$, yeast extract 5 $gl^{-1}$, agar 15 $gl^{-1}$] medium and the clear zone around the colony confirmed the production of organic acid.

**Assessment of nitrogen fixation**

The $N_2$ fixation capacity was evaluated by growing the cultures in nitrogen-free malate containing bromothymol blue medium [DL- Malic acid 5 $gl^{-1}$, KOH 4 $gl^{-1}$, $K_2HPO_4$ 0.5 $gl^{-1}$, $FeSO_4.7H_2O$ 0.05 $gl^{-1}$, $MnSO_4.7H_2O$ 0.01 $gl^{-1}$, $MgSO_4$ 0.01 $gl^{-1}$, NaCl 0.02 $gl^{-1}$, $CaCl_2$ 0.01 $gl^{-1}$, $Na_2MoO_4$ 0.002 $gl^{-1}$, Yeast extract 0.05 $gl^{-1}$, Bromothymol Blue 2 $mLl^{-1}$ ] for 24-48 hrs at 30ºC 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 $N_2$.

**16S rRNA-based phylogenetic analysis**

Phylogenetic analysis based on the16S 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 ($P \leq 0.05$). 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[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 [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 [27], $N_2$-fixation [28], production of phytohormones [29], along with microbial processes. Nodule endophytes belonging

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

| Isolated strain sequences deposited in GenBank | | | Closest match among bacteria (16S rRNA) (GenBank) | | |
|---|---|---|---|---|---|
| Strain | Length (bp) | Accession number | Species | Accession number | Percentage of identity |
| MY3 | 1487 | MW132401 | *Lactococcus lactis* | MW429822 | 99.58% |
| MNMY3 | 1388 | MT039465 | *Cupriavidus* sp. | MG798711 | 99.93% |
| CU8 | 1347 | MN744368 | *Rhizobium* sp. | MT415399 | 99.85% |
| MY6 | 1428 | MN744356 | *Burkholderia* sp. | KP744003 | 98.87% |
| CU3 | 1489 | MN744346 | *Bacillus* sp. | MZ004949 | 90.32% |
| CU2 | 1500 | MN744342 | *Bacillus* sp. | MT102910 | 90.62% |
| MY2 | 1406 | MK002738 | *Bacillus* sp. | AB646981 | 100% |
| CUMYI | 1406 | MK002737 | *Bacillus thuringiensis* | KX977387 | 99.93% |
| MYB1 | 1401 | MK002734 | *Bacillus cereus* | MT611946 | 100% |
| MY1 | 1397 | MH997486 | *Ralstonia pickettii* | MT341804 | 99.93% |
| MYB5 | 1407 | MH997484 | *Bacillus* sp. | MK847260 | 99.93% |
| MY5 | 1344 | MH997483 | *Bacillus cereus* | DQ289077 | 99.18% |
| CUMY2 | 1407 | MH997482 | *Bacillus cereus* | MK253249 | 99.93% |

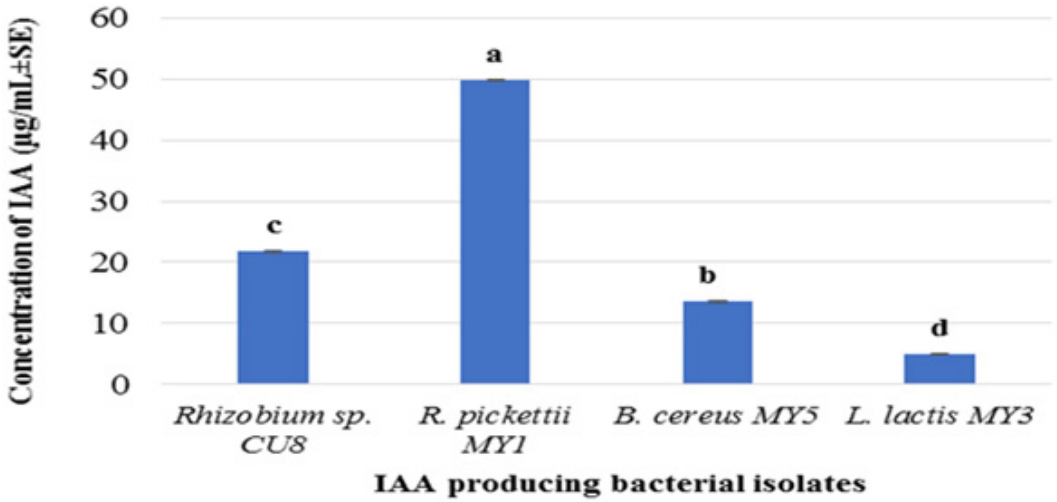


**Fig. 1.** The quantity of IAA produced in different bacterial spp. isolated during 48 hrs of culture. The different letters indicates the significantly difference at the $P \leq 0.05$ level. Values are given as mean±SE for each sample

to the genera *Bacillus*, *Burkholderia, Pseudomonas* and *Enterobacter* have been isolated from different legumes [10, 30].

**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* sp*., Bacillus thuringiensis, Bacillus cereus, Bacillus* sp. and *Bacillus cereus* respectively. The 16S rRNA gene from the strain *Rhizobium* sp. CU8*, B. cereus* MY5*, R. pickettii* MY1, *L. lactis* MY3, *B. cereus* CUMY2, *B. cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. thuringensis* CUMY1, *Burkholderia* sp. MY6 and *Cupriavidus* sp. MNMY3 were possessed 99-100% homology with the nucleotide sequence of other species available in NCBI. *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

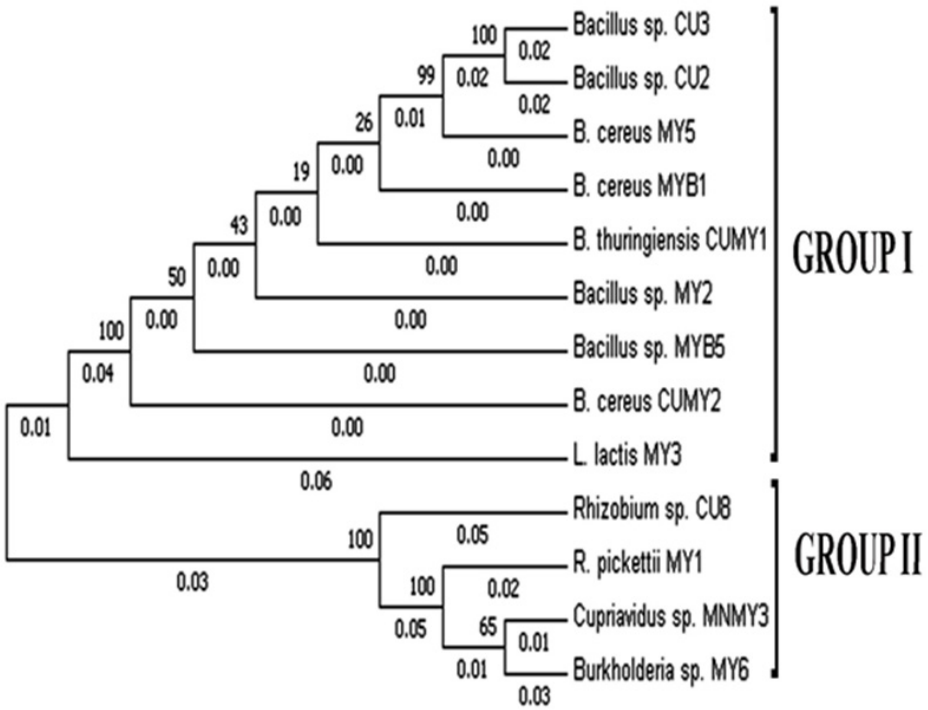


**Fig. 2.** Neighbor joining tree constructed using 16S rRNA gene sequences of 13 nodule associated bacteria isolated from *M. pudica*. Numbers beneath nodes are Bootstrap support (BS) indices and branch length

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 [13, 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.CU8, *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *Ralstonia pickettii* MY1 were gram negative and *Bacillus cereus* MY5, *Bacillus cereus* CUMY2, *Bacillus cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *Bacillus thuringensis* CUMY1, and *L. lactis* MY3 were gram-positive reactions. Except for *Rhizobium* sp.CU8, *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. CU8, *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. CU3, *B. cereus* MYB1 and *Bacillus* sp. MYB5 showed delayed positive urease activity. *Rhizobium* sp. CU8, *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. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 using Salkowski reagent. *R. pickettii* MY1 and *Rhizobium* sp. CU8 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. CU8 (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, epiphytic and tissue colonizing bacteria synthesizes IAA [37]. 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* [38, 39, 40, 41]. According to Datta and Basu [42], 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 [43]. 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[44]. *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 [45].

**Nitrogen fixing potential of the isolates**

The four isolates**,** *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 exhibited $N_2$ fixing 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 $N_2$ 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 $N_2$ fixation and other plant growth promotion capacities in leguminous plants [46, 26] Zhao [47] reported endophytic non-rhizobial *Bacillus cereus* and *Ralstonia* spp. are potent $N_2$ fixers. The genus *Rhizobium* is the first bacteria participating in nitrogen fixation in legumes [48]. According to Higdon [49], *Lactococcal* 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+I (Tamura 3-parameter model), with the lowest BIC score (11977.858), and lowest AIC score (11755.020).

The TN93+I (Tamura Nei Model) displayed the lowest BIC scores (11977.858-supplementary data 2) 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 $1^{st}+2^{nd}+3^{rd}+$ 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 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[50]. 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/Admn).

**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.

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