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ISOLATION AND CHARACTERIZATION OF RHIZOBIA ASSOCIATED WITH ROOT NODULES OF *BUTEA MONOSPERMA* (LAM.) TAUB. AND *PTEROCARPUS MARSUPIUM* ROXB.

SEEMA KASLIWAL, INDU SINGH SANKHLA and PRAVEEN MOHIL
Department of Botany, University of Rajasthan, Jaipur
***Corresponding Author's E-Mail:** induiitkgp@gmail.com

In present study ten root nodulating bacterial isolates associated with *Butea monosperma* (Lam.) Taub. and *Pterocarpus marsupium* Roxb. were isolated and characterized at phenotypic biochemical level. The root nodules of *Butea monosperma* were indeterminate, branched and fan shaped while nodules of *Pterocarpus marsupium* were determinate, globose and spherical in shape. All isolated ten nodule bacterial strains were characterized for their phenotypic and biochemical properties by using various methods. Most of the isolates (BM3, BM4, BM5, PM2, PM4 and PM5) showed similar colony colour and colony characteristics such as raised, light white, translucent, non-gummy, and non-mucilaginous while other (BM1, BM2, PM1 and PM3) were raised, white, opaque, gummy and mucilaginous on yeast extract mannitol agar media. Rhizobial isolates were diverse in BTB reaction; four rhizobial isolates (BM3, BM4, PM3 and PM4) in the current study were acid producers while remaining isolates were neutral. In addition, 60% isolates showed negative results for oxidase activity and 80% isolates were not able to utilize citrate. Isolated strains were significantly diverse in litmus milk reaction and were distributed into four groups. Many isolates were able to grow at pH from 6 to 9.5 and showed salinity tolerance up-to to 6% NaCl. Our study suggested that rhizobial isolates associated with *Butea monosperma* and *Pterocarpus marsupium* were significantly diverse in their physiological and biochemical parameters.

Keyword: *Butea monosperma,* Nodulating Bacteria, *Pterocarpus marsupium,* Rhizobia and Root nodule.

Introduction

Rhizobia are a specific group of gram negative bacteria that carried out symbiotic nitrogen fixation with legume plants. Rhizobia are able to establish symbiosis relationships with a wide range of legumes, such as trees[1], shrubs[2], herbs[3-4] and aquatic plants. Micro-symbionts associated with wild legumes attracted the attention of researchers because of their ability to tolerate extreme environmental conditions, such as high temperature, low precipitation, drought and high salinity[5].

Currently, rhizobia group contains 21 genera which are distributed in alpha-proteobacteria and beta-proteobacteria. *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* and *Bradyrhizobium* are the main genera in alpha-proteobacteria[6]. Further many studies confirmed that some other genera (*Blastobacter, Microvirga*, *Shinella, Ochrobactrum, Methylobacterium, Devosia*) in alpha-proteobacteria were also able to elicit nodule formation on roots of legumes and included in rhizobial taxonomy[7]. On other hand, *Burkholderia* and *Cupriavidus* are the main nodules forming and nitrogen-fixing genera in Betaproteobacteria[7-8]. Rhizobial taxonomy is growing very fast and every year new rhizobial species are reported.

Many legumes in Rajasthan such as species of *Acacia*, *Crotalaria*, *Rhynchosia, Tephrosia*, and *Vigna* species etc. have been characterized for their microsymbiont[9-13] but still some other plants including our study plants *Butea monosperma* (Lam.) Taub. and *Pterocarpus marsupium* Roxb. are not characterized for their micro-symbiont. *Butea monosperma* and *Pterocarpus marsupium* are widely distributed in the Central Arawali region and they are the multipurpose woody tree that is used as medicine, wood, soil enrichment, and fodder and timber production. Both plants are traditionally reported to possess an anticonvulsant, anti-gout, diuretic, anti-leprotic, anti-cancers, anti-microbial, anti-diabetic, antiviral anti-inflammatory and anti-hepatic properties[14-15]. The roots of these plants establish a symbiotic relationship with rhizobia and form nodules where atmospheric nitrogen is fixed and used for its growth and also for the enrichment of the rhizosphere[16].

Present study aimed to isolate and characterize rhizobia associated with *Butea monosperma* and *Pterocarpus marsupium*. The morphology and anatomy of nodules were studied and isolated rhizobia were characterized based on their phenotypic biochemical properties.

Material and Methods

Survey and collection

A survey was carried out in the Jhalana forest area to collect seeds, twigs and rhizospheric soil of *Butea monosperma* and *Pterocarpus marsupium* at depth of 20 to 40 cm from the soil surface. The collected soil samples were subjected for rhizobia trapping experiments.

Rhizobia trapping experiment and Harvesting of nodules

Rhizobia trapping experiment was performed during the summer and monsoon season. Approximately 5 kg of collected rhizospheric soil samples were transferred to each earthen pot. Surface sterilized seeds of *Butea monosperma* and *Pterocarpus marsupium* were placed on prepared pots. Pots were kept under natural environmental conditions and irrigated manually using watering cans regularly. For morphological characterization of nodules ten replicates were used.

After three months of seed sowing, healthy plants of *Butea monosperma* and *Pterocarpus marsupium* were harvested at the Department of Botany, University of Rajasthan. The pots were placed under the running water tap and washed thoroughly which led gradual removal of soil particles from roots and mature nodules. Mature and fresh nodules preferably were selected for further morphological and ultra-structural studies of nodules and isolation of rhizobia by using standard method[17].

Preparation of microscopy

For light microscopy, the nodules with roots were fixed in FAA (formalin-acetic acid-ethanol) solution for 2 days and subsequently stored in 70% ethanol. Transverse sections of nodules were properly stained by using aqueous toluidine blue (in 1% borax, pH 4.4) and observed in a light microscope (Leica microscope).

Isolation and purification of root nodule bacteria

Selected fresh and healthy root nodules were surface sterilized by placing them in 70% ethanol for 30 seconds followed by dipping in 1% sodium hypochlorite solution (w/v) for 5 minutes. Subsequently, these nodules were washed six times with sterilized distilled water and finally transferred to sterile watch-glass containing 1-2 drops of sterile water. Gently nodules were crushed and a loop of suspensions was streaked on Yeast Extract Mannitol Agar plates containing Congo red. All Petri-plates were incubated at 28 ± 1°C for 24-72 h and observed regularly for growth of rhizobial colonies. The

observed colonies were picked up and further re-streaked on the YEMA plate for purification of rhizobial isolates.

Phenotypic and Biochemical characterization of rhizobial isolates

All root nodule bacterial strains were subjected to biochemical characterization by using various methods[17-18] such as Bromo-thymol blue (BTB) reaction, Oxidase activity, Citrate utilization ability, Litmus milk reaction, amylase activity, tolerance to pH and NaCl.

Bromothymol blue (BTB) reaction

Acid or alkali producing or neutral nature of rhizobial strains were determined by BTB test. YEM broth supplemented with 0.001% bromo-thymol blue indicator dye was used for BTB reaction. Prepared broth tubes were inoculated with the exponentially growing rhizobial cultures and incubated at 28°C. After 48-72 hrs., the change in colour of the media was observed. If colour of YEM broth changed from green to yellow and/or from green to blue, indicate the presence of acid or alkali producers, respectively. Neutral strain did not change the colour of growing media.

Oxidase activity

Oxidase activities of RNB isolates were accessed by touching and spreading the oxidase disc on the fresh growing colonies of the bacterial isolates. The results were observed immediately within 5-10 seconds at room temperature. A change in colour of the disc dark purple/blue was considered as positive reaction and no change at all considered as negative reaction.

Utilization of citrate

Simmon's citrate agar media in test tubes were used for the citrate utilization ability of isolates. A loop full of fresh bacterial colonies was streaked on the slant of Simmon's citrate agar media. Inoculated test tubes were kept at 28°C for up to 4-7 days and observed for colour changes. Growth with colour change from green to blue along the slant considered as positive result for citrate utilization and no growth and no colour change, slant remain green considered as negative result.

Litmus milk reaction

Litmus milk is an excellent medium that can differentiate microorganisms on the basis of differential metabolism of the substrates which are present in media. Lactose, casein and litmus are the main constituents of the litmus milk media. Litmus is a pH indicator that changed the colour of the media based on pH. The litmus milk broth was prepared by using standard method[18] and was inoculated with fresh rhizobial cultures. Subsequently inoculated broths were incubated at 28°C for 5 days and observed every day for any changes in media and results were recorded[18].

Amylase activity

Starch agar media plates were inoculated with fresh rhizobial cultures to analyse the amylase activity of the isolates. The inoculated plates were incubated at 28°C for 48 hr. After 48 hrs inoculated Petri-plates were flooded with iodine solution. The formation of clear a zone around the colonies confirms the amylase activity of the isolates.

pH and Salt (NaCl) tolerance

The pH tolerance of bacterial strains were determined by inoculating fresh bacterial culture to YEMA plates having pH value of 5-10. The inoculated plates were kept at 28°C for 3-5 days. Three replicate plates were prepared for each pH-isolate combination and growth was observed.

Similarly, NaCl tolerance of bacterial strains were determined by inoculating fresh bacterial culture to YEMA plates having NaCl value of 0.5-8%. The inoculated plates were kept at 28°C for 3-5 days. Three replicate plates were prepared for each NaCl-isolate combination and growth was observed.

Result and Discussion

- *Plant identification and collection of nodules:*

Butea monosperma and *Pterocarpus marsupium* were identified by submitting the herbarium sheet of each plant to the Department of Botany, University of Rajasthan, Jaipur with RUBL numbers RUBL 21256 and RUBL 21257 respectively. The summer season (March to May) was appropriate for seed germination of *Butea monosperma* and rainy season (July to September) for *Pterocarpus marsupium.* The root nodules were formed within 80 days in both plants at the vegetative stage. The fresh nodules of both plants were collected by harvesting of 3 months of plants grown in pots in rhizobia trapping experiments.

- *Nodule morphology and anatomy:*

Nodules of *Butea monosperma* were light brown to dark brown, rough and slightly soft in texture with irregular scars and distributed on the main tuberous root as well as lateral roots (Fig. 1a and 1b). They were present singly as well as in clusters. Similarly, the root nodules of *Albizia lebbeck* developed singly and in clusters on the primary and secondary roots[19]. The nodules of *Butea monosperma* were showed variation in their shape. Initially, the nodules were oval and spherical but at maturity, they become elongated, branched and fan shaped (Fig. 1c) therefore they can be classified as indeterminate type of nodules[20]. Similarly branched, fan-shaped, cylindrical and globose shaped nodules were observed in some members of the tribe Trifolieae[21]. In the Thar Desert, many species of *Acacia* and *Indigofera* formed such type of indeterminate root nodule[9,11,22]. The nodules of *Pterocarpus marsupium* were light brown, rough and hard in texture with surface lenticels and distributed on the main roots as well as lateral roots (Fig. 1d and 1e). Morphologically nodules of *Pterocarpus marsupium* were spherical and globose in shape throughout the lifecycle (Fig. 1f) therefore they can be classified as determinate type of nodules[20]. Similar types of root nodules were observed in *Pterocarpus indicus* in Malaysia[23]. Such types of root nodules were also observed in species of *Rhynchosia* and *Vigna* in the Thar Desert[9].

General structure of root nodules of *Pterocarpus marsupium* were observed, which containing four regions such as nodule epidermal tissue (NET), nodule cortex (NC), vascular tissues (VT) and bacteroid region (BR). The cortex was surrounded by a thick epidermal tissue. The centre of nodules was observed with both infected and uninfected cells (Fig. 1g). Similarly, *Pterocarpus indicus* showed such type of root nodule anatomy[23].

- *Phenotypic and Biochemical characteristics of isolates:*

A total of ten bacterial strains (five from each plant species) were isolated from fresh and healthy root nodules of *Butea monosperma* and *Pterocarpus marsupium* and were designated as BM1 to BM5 and PM1 to PM5, respectively. On the basis of colony characteristics all isolates were formed two groups. Colony characteristics of isolates in group I (BM1, BM2, PM1, and PM3) were raised, white, opaque, gummy and mucilaginous while in group II (BM3, BM4, BM5, PM2, PM4 and PM5) were raised, light white, translucent, non-gummy, and non-mucilaginous (Table 1; Fig. 2a and 2b). Such types of colony characteristics of rhizobia were also observed by many researchers. Isolated root nodules bacterial strains associated with *Glycine max* showed similar type of colony characteristics[24]. In addition, the *Rhizobium* sp. formed gummy, and mucilaginous colonies on yeast extract agar media[25]. Similar to present results, it was observed that the isolates of *Pterocarpus indicum* also produced white and gummy colonies on YEMA-CR[26].

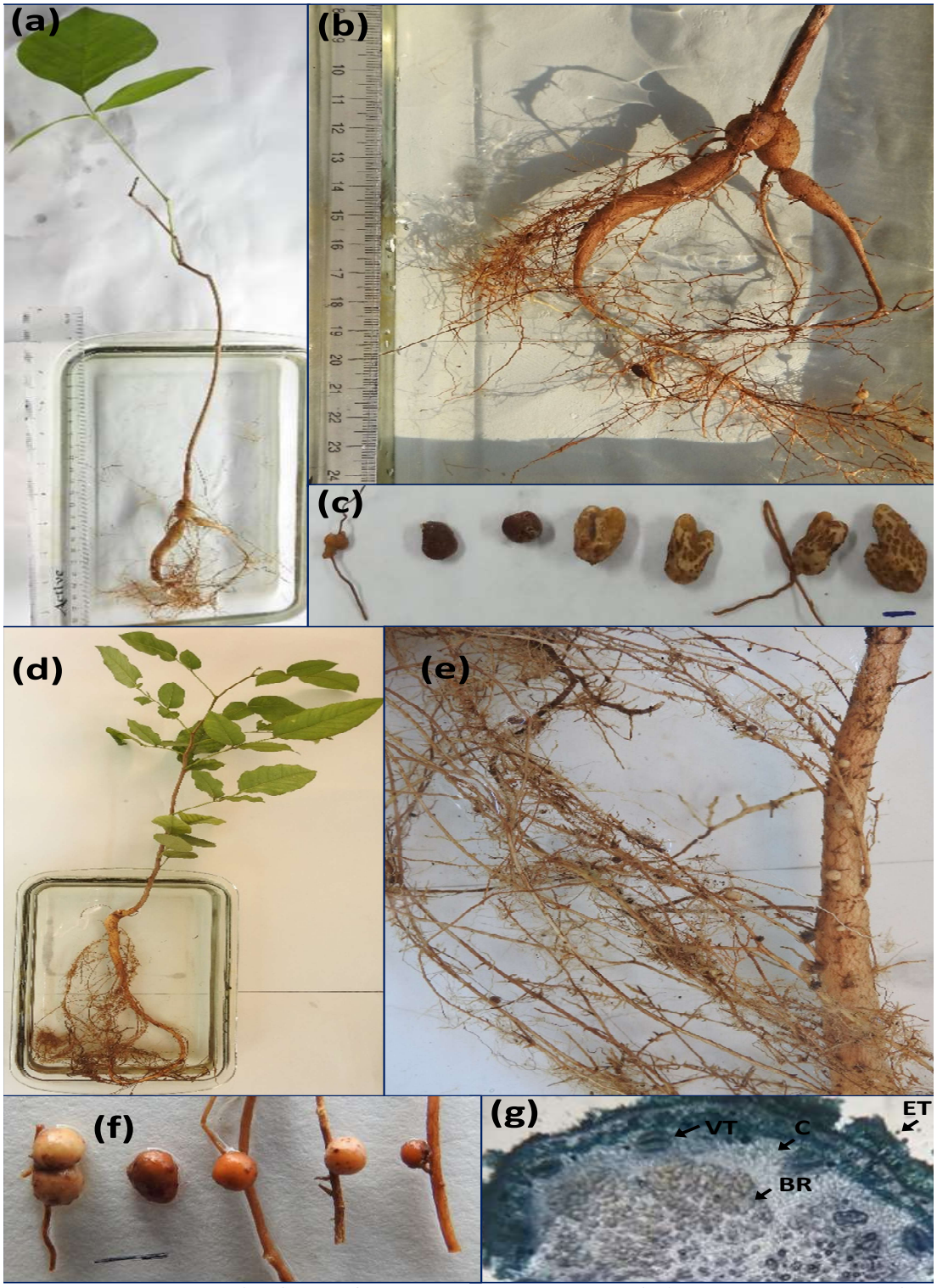

Figure 1.: Excavated plant of *Butea monosperma* and its roots system (a-b), developmental stages of root nodules of *Butea monosperma* (c), Excavated plant of *Pterocarpus marsupium* and its roots system (d-e), developmental stages of root nodules of *Pterocarpus marsupium* (f), Transverse section of root nodules of *Pterocarpus marsupium* (g).

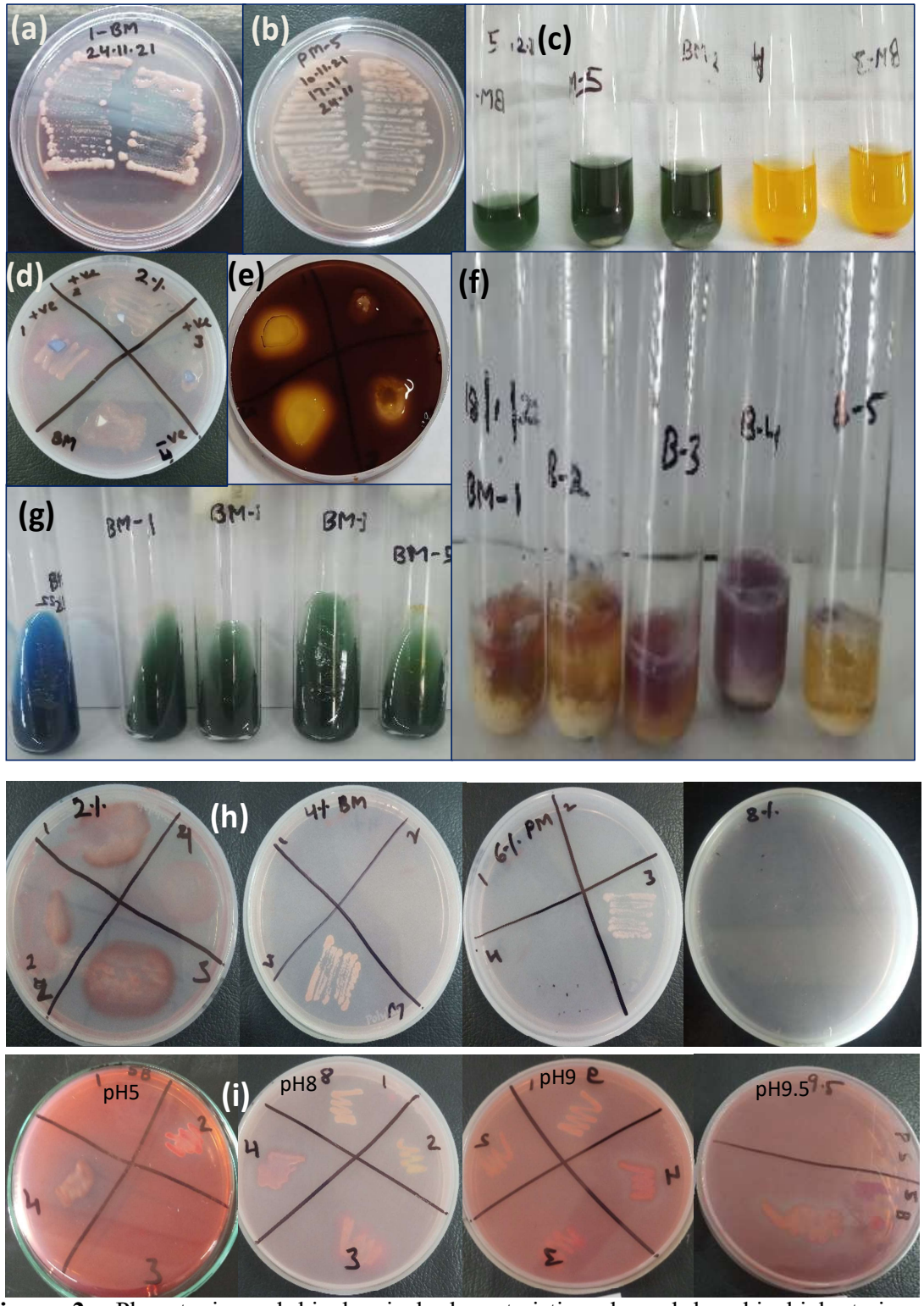

Figure 2.: Phenotypic and biochemical characteristics showed by rhizobial strains isolated from *Butea monosperma* and *Pterocarpus marsupium*: Colonies of pure cultures (a-b), BTB reaction (c), Oxidase activity (d), Amylase activity (e), Litmus milk reaction (f), Citrate utilization (g), NaCl tolerance range (h) and pH tolerance range (i).

Further purified isolates were classified as alkali and acid-producing rhizobia by growing them in YEM broth supplemented with BTB. The four rhizobial isolates (BM3, BM4, PM3 and PM4) in the current study were acid-producing as they changed the colour of the broth from green to yellow. Whereas, remaining isolates (BM1, BM2, BM5, PM1, PM2 and PM5) did not change the green colour of the broth, indicated that they were neutral (Table 1; Fig. 2c). Many researchers[17,27] suggested that on the basis of BTB reaction the rhizobial isolates can be assumed as fast (performing acidic reaction) and slow growers (Performing alkali or neutral reaction).

In the current study four bacterial isolates (BM4, PM1, PM2 and PM3) were showed positive responses with oxidase disc and the remaining six were negative (BM1, BM2, BM3, BM5, PM4 and PM5) (Table 1; Fig. 2d). Similarly, rhizobial isolates RhBC and NRA1 isolated from root nodules of ground nut showed oxidase activity[28]. Amylase activity was observed in three isolates (BM5, PM1 and PM2) (Table 1; Fig. 2e). These isolates were capable to hydrolyze starch present in the medium. The remaining all isolates were showed negative results for amylase activity. Similar results were found for rhizobia associated with nine cultivated legumes[29]. In addition, it was observed that root nodule bacteria isolated from various sources by soil trapping experiment had amylase activity and were able to utilize starch[30].

All tested root nodule bacterial strains were showed various types of litmus milk reactions (Table 1; Fig. 2f). Three isolates (BM2, PM2 and PM4) were showed lactose fermentation while other three (BM1, BM5 and PM5) were showed acid and gas production followed by reduction and curdling formation. In addition BM3, PM3 showed proteolysis and alkaline reaction. The remaining two isolates BM4 and PM1 did not show any reaction. Citrate utilization test was found negative in eight strains (BM1, BM2, BM3, BM5, PM1, PM2, PM3 and PM5) and positive in two strains (BM4 and PM4) (Table 1; Fig. 2g). Results of present study was accordance to findings of other researchers in which they found rhizobial strains associated with root nodules of some cultivated legume crops showed negative results for citrate utilization[29].

In the present study the results of most of the phenotypic and biochemical tests were similar as observed for rhizobial spp. in literature[25,31-33].

- *Salt (NaCl) and pH tolerance:*

Salt stress can cause a reduction in nitrogen fixation and nodulation ability of the plants. On the basis of NaCl tolerance range all isolates were classified into three groups. The isolates of group I (BM3, PM2, PM3, and PM5) were highly NaCl tolerant and were able to grow upto6 % NaCl, while isolates of group II (BM4, PM1 and PM4) and group III (BM1, BM2 and BM5) were able to grow upto 4% and 2% NaCl, respectively (Table 1; Fig. 2h). No one isolate showed growth on YEMA media containing more than 6% NaCl. The results were consistent with the study of other researcher[29] who also reported diverse nature of the rhizobial strains for their NaCl tolerance ability. Similar results were obtained for 81 rhizobial strains isolated from the root nodules of *Pisum sativum* and reported that 16% isolates showed only upto 2% NaCl tolerance[34].

Soil pH is an important parameter and significantly affects the growth and development of rhizobia. Similarly, small variations in the pH of media can alter the growth of bacteria[24]. On the basis of pH tolerance range, all rhizobial strains were categorized into three groups. The isolates of group I (BM1, BM5, PM1and PM4) were able to grow at pH 6-9.5 while isolates of

group II (BM3, PM2,PM3 and PM5) and group III (BM2 and BM4) were showed significant growth at pH 6-9 and pH 5-9.5 respectively. No one isolates were able to grow at pH below 5 (Table 1; Fig. 2i). All isolated strains were showed prime growth at pH 7 to pH 8.0. It can be concluded that isolated strains were more tolerant to alkalinity. Notably in *Bradyrhizobium* sp., maximum growth was found at pH 7 followed by pH 6 and 8 and very poor growth was observed at pH 4, 5, and 10^{35}.

Table 1.: Phenotypic and biochemical characteristic of rhizobial isolates.

Isolates	Colony characteristics	BTB reaction	Oxidase activity	Citrate utilization	Amylase activity	Litmus milk reaction	NaCl tolerance (in %)	pH tolerance
BM1	Raised, White, gummy, mucilaginous and translucent	Neutral	-	-	-	Acid, gas, reduction, curd with proteolysis	0.5-2	6-9.5
BM2	Raised, White, gummy, mucilaginous and translucent	Neutral	-	-	-	Lactose fermentation	0.5-2	5-9.5
BM3	Raised, light White, nongummy, nonmucilagenous and transparent	Acid Production	-	-	-	Alkaline reaction	0.5-6	6-9
BM4	Raised, light white, nongummy, nonmucilagenous and transparent	Acid Production	+	+	-	Lactose not fermented	0.5-4	5-9.5
BM5	Raised, light white, nongummy, nonmucilagenous and transparent	Neutral	-	-	+	Acid, gas, reduction, curd with proteolysis	0.5-2	6 - 9.5
PM1	Raised, White, gummy, mucilaginous and translucent	Neutral	+	-	+	Lactose not fermented	0.5-4	6-9.5
PM2	Raised, light white, nongummy, nonmucilagenous and transparent	Neutral	+	-	+	Lactose fermentation	0.5-6	6-9
PM3	Raised, White, gummy, mucilaginous and translucent	Acid Production	+	-	-	Alkaline reaction	0.5-6	6-9
PM4	Raised, light white, nongummy, nonmucilagenous and transparent	Acid Production	-	-	-	Lactose fermentation	0.5-4	6-9.5
PM5	Raised, light white, nongummy, nonmucilagenous and transparent	Neutral	-	+	-	Acid, gas, reduction, curd with proteolysis	0.5-6	6-9

Conclusion

Rhizobia-legume interaction is a highly specific category of symbiosis which converts atmospheric nitrogen into nitrate. In the present study, indeterminate and determinate types of nodules were observed on the main root as well as lateral root system of *Butea monosperma* and *Pterocarpus marsupium* respectively. Anatomically nodules were differentiated into four regions thick epidermal tissue, cortex, vascular bundle and bacteroid infected region. A total of ten rhizobial isolates (five from each plant) were isolated and characterized for their phenotypic and biochemical activities. In present study, rhizobial isolates from both plants were showed significant variation in their phenotypic and biochemical activities which indicate that both plants are promiscuous host and nodulated by more than one species of rhizobia. Both plants are well adapted to xerophytic conditions, therefore can be used to improve soil quality by symbiotic nitrogen fixation in Rajasthan. Effective rhizobial strains from the present study may be used for the development of bio-fertilizers, which can be used as substitutes for synthetic chemical fertilizers to enhance sustainable agriculture practices.

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References

1. Khbaya BM, Neyra P, Normand K, Zerhari and Filali-Maltouf A 1998, Genetic diversity and phylogeny of rhizobia that nodulate *Acacia* spp. in Morocco assessed by analysis of rRNA genes. *Appl Environ Microbiol*. **64** 4912–4917.
2. Donate-Correa J, Leon-Barrios M, Hernandez M, Perez-Galdona, R and Del Arco-Aguilar M 2007, Different *Mesorhizobium* species sharing the same symbiotic genes nodulate the shrub legume *Anagyris latifolia*. *Syst Appl Microbiol.* **30** 615–623.
3. Giongo A, Ambrosini A, Vargas LK, Freire JRJ, Bodanese ZMH and Passaglia LMP 2008, Evaluation of genetic diversity of *Bradyrhizobia* strains nodulating soybean *Glycine max* (L.) Merrill isolated from South Brazilian fields. *Appl Soil Ecol.* **38** 261–269.
4. Odee DW, Sutherland JM, Makatiani TMc, Inroy SG and Sprent JI 1997, Phenotypic characteristics and composition of Rhizobia associated with woody legumes growing in diverse Kenyan conditions. *Plant Soil*. **188** 65–75.
5. Zahran HH 2001, Rhizobia from wild legumes: Diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *J Biotechnol*. **91** 143-153.
6. Okazaki S, Nukui, N, Sugawara M and Minamisawa.K 2004, Rhizobial strategies to enhance symbiotic. Interactions: *Rhizobiotoxine* and 1-Aminocyclopropane-1-Carboxylate deaminase. *Microbes Environ*. **19** 99-111.
7. Weir BS 2016, The Current Taxonomy of rhizobia. *New Zealand rhizobia website.* Acessed on: Nov. 22, 2023. http://www.rhizobia.co.nz/taxonomy/rhizobia.htlm.
8. Benhizia Y, Benhizia H, Benguedouar A, Muresu RA, Giacomini and Squartini 2004, A Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. *Syst Appl Microbiol*. **27** 462-468.
9. Gehlot HS, Panwar D, Tak N, Tak A, Sankhla IS, Poonar N, Parihar R, Shekhawat NS, Kumar M, Tiwari R, Ardley J, James EK and Sprent JI 2012, Nodulation of legumes from the Thar desert of India and molecular characterization of their rhizobia. *Plant Soil*. **357** 227–243.
10. Sankhla IS, Meghwal RR, Tak N, Tak A and Gehlot HS 2015, Phenotypic and molecular characterization of microsymbionts associated with *Crotalaria medicagenia:* a native legume of the Indian Thar Desert. *Plant Arch*. **15** 1003.
11. Sankhla IS, Tak N, Meghwal RR, Choudhary S, Tak A, Rathi S, Sprent JI, James EK and Gehlot HS 2017, Molecular characterization of nitrogen fixing microsymbionts from root nodules of *Vachellia* (*Acacia*) *jacquemontii*, a native legume from the Thar Desert of India. *Plant Soil*. **410** 21–40.
12. Sankhla IS, Meghwal RR, Choudhary S, Rathi S, Tak N, Tak A and Gehlot HS 2018. Molecular characterization of microsymbionts associated with root nodules of *Crotalaria burhia* Buch.-Ham. ex Benth., a native keystone legume species from Thar Desert of India. *Indian J Exp Biol*. **56** 373-385.
13. Tak N, Gehlot HS, Kaushik M, Choudhary S, Tiwari R, Tian R, Hill Y, Bräu L, Goodwin L, Han J, Liolios K, Huntemann M, Palaniappan K, Pati A, Mavromatis K, Ivanova N, Markowitz V, Woyke T, Kyrpides N and Reeve W 2013,

Genome sequence of *Ensifer* sp. TW10; a *Tephrosia wallichii* (Biyani) microsymbiont native to the Indian Thar Desert. *Stand Genomic Sci*. **9** 304-314.

14. Sutariya BK and Saraf MN 2015, A Comprehensive review on Pharmacological Profile of *Butea monosperma* (Lam.) Taub. *J. Appl Pharm Sci.* **5** 159-166.
15. Rahman S, Mujahid M, Siddiqui A, Rahman A, Arif M,Eram S, Khan A and Azeemuddin M 2018, Ethnobotanical Uses, Phyto-chemistry and Pharmacological Activities of *Pterocarpus marsupium*: A Review. *Pharmacogn J*. **10** s1-s8.
16. Danger TK and Basu PS 1984, Studies on Root Nodules of Leguminous Trees: Seasonal Variation of Plant Hormones and IAA Metabolism with Reference to Nitrogen Fixation in *Pterocarpus mursupium* ROXB. *Biochem Physiol Pflanzen*. **179** 359-368.
17. Somasegaran P and Hoben HJ 1985, *Methods in Legume - Rhizobium Technology,* University of Hawaii, NIFTAL Project and Micren, Dept. of Agronomy and Soil. pp. 1-300.
18. Cappuccino JG, Sherman N 2014, *Microbiology: A Laboratory Manual* (10thed.). Pearson education inc.
19. Qadri R and Mahmood A 2005, Ultra structure studies on root nodules of *Albizia lebbeck* (L.) Benth. *Pak J Bot*. **37** 815-821,
20. Corby HDL 1981, The systematic value of leguminous root nodules. In: Palhill RM and Raven PH (Eds.), *Advances in legume systematics*. Royal Botanical Garden Kew, Kew Publisher. pp. 657–669.
21. Shah GL and Rao GM 1982, Initiation, development and structure of root nodules in some members of the tribe *Trifolieae* (Papillionaceae). *Plant Sci*. **91** 309-318.
22. Choudhary S, Meghwal RR, Sankhla IS, Tak and Gehlot HS 2017, Molecular characterization and phylogeny of novel diverse nitrogen fixing microsymbionts associated with *Vachellia leucophloea* in arid and semi-arid regions of Rajasthan. *Indian For*. **143** 266-278.
23. Lok EH, O'Hara G, Dell B 2006, Nodulation of the legume *Pterocarpus indicus* by diverse strains of rhizobia. *J Trop For Sci*. 188-194.
24. Singh B, Kaur R and Singh K 2008, Characterization of *Rhizobium* strains isolated from roots of *Trigonella foenum-graecum*. *Afr J Biotechnol*. **7** 3067-3076.
25. Deora GS and Singhal K 2010, Isolation, biochemical characterization and preparation of biofertilizers using *Rhizobium* strains for commercial use. *Biotech Res Comm*. **3** 132-136.
26. Malisorn K and Prasarn C 2014, Isolation and characterization of *Rhizobium* spp. from root of legume plants species. *Khon Kaen Agr J*. **42** 157-160.
27. Hungaria M, Compo RJ, Chueire LMO, Grange L and Megias M 2001, Symbiotic effectiveness of fast growing rhizobial strains isolated from soybean nodules in Brazil. *BiolFertil Soils*. **33** 387-394.
28. Hossain A, Gunri SK, Barman M, Sabagh AE, da Silva JAT 2019, Isolation, characterization and purification of *Rhizobium* strain to enrich the productivity of groundnut (*Arachis hypogaea* L.). *Open Agric*. **4** 400-409.
29. Paudyal P, Kunwar V, Paudel N and Das BD 2021, Isolation and characterization of rhizobia from the root nodule of some cultivated legume crops. *Eur J Biol Res*. **11** 294-306.

30. Oliveira AN, Oliveira LA, Andrade JS, Chagas JAF 2007, Rhizobia amylase production using various starchy substance as carbon substrates. *Braz J Microbiol* **38** 208-216.
31. Upadhayay SP, Pareek N and Mishra G 2015, Isolation and biochemical characterization of *Rhizobium* strains from nodules of lentil and pea in Tarai agro-ecosystem, Pantnagar, India. *Nusantara Biosci*.**7** 73-76.
32. Dhiman M, Dhiman VK, Rana N and Dipt B 2019, Isolation and Characterization of Rhizobium Associated with Root Nodules of *Dalbergia sissoo*. *Int J Curr Microbiol Appl Sci*. **8** 1910-1918.
33. Islam R, Imran MAS and Mahmud SN 2020, Isolation, identification and characterization of *Rhizobium* species from soil of *Cicerarietinum* field of Faridpur in Bangladesh. *IntJ Curr Res*. **12** 10322-10325.
34. Deshwal VK and Chaubey A 2014, Isolation and Characterization of *Rhizobium leguminosarum* from Root nodule of *Pisum sativum* L. *J Acad Ind Res*. **2** 464-467.
35. Dinkwar GT, Thakur KD, Bramhankar SB, Pillai TS, Isokar SS, Ravali T. and Kharat. VM 2020, Biochemical and physiological characterization of *Brady rhizobiumj aponicum*. Int JChem Stud. 8 1589-1594.