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Phenotypic and Biochemical Characterization of Rhizobia Associated with *Medicago polymorpha* Growing in Rajasthan

Amit Yadav, Divya Solanki, Ghanshyam Sharma, Gunjan Dubey, Indu Singh Sankhla

Abstract: ***In present study, a total of 15 rhizobial isolates were isolated from the root nodules of Medicago polymorpha growing in Jaipur and were characterized for their phenotypic, biochemical and plant growth promoting activities. The root nodules of M. polymorpha were elongated, branched and indeterminate. All isolated rhizobia were highly diverse in their physiological traits. Based on colony morphology, all isolates were categorized into six groups. Major group containing 6 isolates (MP1, MP3, MP9, MP10, MP11 and MP13) showed white, opaque, raised, smooth edges, non-gummy, and mucilaginous characteristics. Four rhizobial strains MP3, MP4, MP9, and MP15 showed salt-tolerance up to 3% and were well adapted to high alkaline conditions and exhibited growth in extremely alkaline media (pH 10). Majority of strains showed positive result for IAA production, nitrate reductase and catalase activity. In addition, citrate utilization, ammonia production, phosphate solubilization and cellulase activity were observed in few isolates. In present study, some rhizobial isolates like MP3, and MP15 were very versatile rhizobia that showed high plant growth promoting activities (IAA production and phosphate solubilization) and were also tolerant to high pH and salt concentration. Such type of superior rhizobia can be part of biofertilizers to enhance legume crop productivity in an ecofriendly manner without application of chemical fertilizers. Our study suggested that rhizobial isolates associated with Medicago polymorpha were significantly diverse in their physiological and biochemical parameters.***

Keywords: Biochemical, Medicago Polymorpha, Phenotypic, Plant Growth Promoting Activities, Rhizobia

I. INTRODUCTION

The symbiotic relationship between legume plants and rhizobia improves soil fertility and health without harm to the environment.

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***** Correspondence Author (s)

Amit Yadav, Microbiology Laboratory, Department of Botany, University of Rajasthan, Jaipur (Rajasthan), India. Email: ay37736@gmail.com

Divya Solanki, Microbiology Laboratory, Department of Botany, University of Rajasthan, Jaipur (Rajasthan), India. Email: divusolanki2323@gmail.com

Ghanshyam Sharma, Microbiology Laboratory, Department of Botany, University of Rajasthan, Jaipur (Rajasthan), India. Email: ghanshyam9492sharma@gmail.com

Dr. Gunjan Dubey, Assistant professor, Microbiology Laboratory, Department of Botany, University of Rajasthan, Jaipur (Rajasthan), India. Email: gunjandubey22@gmail.com

Dr. Indu Singh Sankhla*, Assistant professor, Microbiology Laboratory, Department of Botany, University of Rajasthan, Jaipur (Rajasthan), India. Email: induiitkgp@gmail.com

Rhizobia is a heterogeneous group of gram negative, nitrogen-fixing, soil bacteria that elicit nodule formation on the root of the leguminous plants where they convert atmospheric nitrogen to ammonia for the benefit of the plant. Currently rhizobia group contains approximately 240 species in 21 genera belonging to the alpha- beta and gamma-proteobacteria, however, species from *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* and *Bradyrhizobium* genera are more prevalent [1]. Rhizobial diversity is highly dynamic, changing from one climatic region to the next. Identification of rhizobial strains with suitable trait is very important to develop effective bio-fertilizers to increase the productivity of the legume crops without application of chemical fertilizers. In addition of nitrogen fixation ability, rhizobia also possess several plant growth promoting activities such as phosphate solubilization, siderophore production, IAA production etc. that significantly contribute in growth enhancement of the plants. Therefore, effective indigenous rhizobia can be a substitute of chemical N fertilizers and can favor sustainable agriculture. India has a rich legume biodiversity, with 1,152 species found in various phytogeographic zones. Numerous researchers in India [2]-[5] have studied legumes and characterized their associated rhizobia, but still many legumes are unexplored and have to be characterized for their rhizobia.

Medicago polymorpha commonly known as bur medic is a trifoliate annual herb that thrives in farms and gardens in the winter season. The bur medic originated from Mediterranean region and is now widely distributed throughout the world including Australia, Europe, North Africa, Middle East, and Central Asia including Indian subcontinent [6]. In Rajasthan it is a common weed in cultivated fields and gardens in Bhilwara, Ganganagar, Sirohi, Tonk, Jaipur etc. The most intriguing feature of this plant is the seed pod or bur that is spiraled-coiled, disk shaped with hooked prickles. It is widely cultivated in Australia for use as fodder and green manure. Physiological characteristics of rhizobial isolates associated *M. polymorpha* growing in Libya and Australian soil have been described by some researchers[7], [8]. However, the information about rhizobial isolates nodulating *M. polymorpha* growing in Indian soil is not available. While the characterization of indigenous rhizobia is primary need to improve crop production. Therefore the study was aimed to isolate and characterize the microsymbionts (rhizobia) associated with *M. polymorpha* growing in Rajasthan, India.

II. MATERIAL AND METHODS

A. Sampling area and Nodules Collection

In winter season three sampling sites in Jaipur; Rajasthan University Campus (coordinates 26°53'08.3"N 75°49'23.5"E), Maharaja College (26°54'41.8"N 75°49'03.4"E) and Amer hills (26°59'44.0"N 75°50'49.7"E) were visited to check the nodulation status of *Medicago polymorpha*. Five healthy plants from each sampling sites were excavated by using spade. Excavated plants were washed under running tap water to remove all soil particles adhered to the root system. Cleaned root systems of bur medic plants were carefully examined for the position, number, shape and size of the nodules and were documented. Nodules were kept in glass vial containing $CaCl_2$ beads for long term preservation.

B. Isolation of Root Nodule Bacteria

The fresh, healthy and pinkish root nodules were gently plucked from the intact root system and subsequently used to isolate rhizobial strains. The nodules were surface sterilized by sequential treatment of sterilizing agent 3% sodium hypochlorite for 3 min and 70% ethanol for 30s. Finally nodules were washed by sterile distilled water for 6 times to remove traces of sterilizing agent. Prepared nodules were crushed in a drop of sterile water and obtained suspension was streaked on Congo Red-Yeast Extract Mannitol Agar (CR-YEMA) plates as described by Somasegaran and Hoben [9]. Streaked plates were kept at 28°C for 2-5 days and checked regularly for the growth of rhizobial isolates. Observed colonies of isolates were purified by re-streaking them on YEMA plates.

C. Phenotypic and Biochemical Characterization of Rhizobial isolates

Purified colonies were examined for morphological characteristics such as colony size, shape, border, elevation, colour, mucosity, transparency. Further all isolated strains were tested for various phenotypic, biochemical and plant growth promoting activities.

BTB reaction: This test distinguishes between bacteria that produce acid and alkali. In order to observe an acidic or alkaline reaction, fresh rhizobial cultures were added to YEM broth in test tubes containing bromothymol blue (BTB) and incubated at 28°C for 48 hours. BTB is a pH indicator that changed the original green colour of media to yellow in acidic reaction (acid producing strains) and blue in alkali reaction (alkali producing strains). A neutral response is indicated by no colour change.

pH and NaCl Tolerance: The pH tolerance range of all rhizobial isolates were examined by growing them on YEMA plates having pH values from 5 to 10 [10]. Similarly NaCl tolerance range of all rhizobial isolates were examined by growing them on YEMA plates containing varied salt concentrations (1%, 2%, 3%, 4%, and 5%). All inoculated plates were incubated at 28°C for 48 hours and checked regularly for the growth of isolates.

Nitrate Reduction: It has long been known that nitrate reduces the action of nitrogenase and nodule organogenesis in plant nodules [11] however the rhizobia's enzyme nitrate reductase convert it to nitrite and favor the nitrogenase enzyme. The quantity of nitrite generated was used to evaluate the activity of nitrate reductase. The nitrate reduction potential of all isolates were determined by inoculating fresh rhizobial cultures to nitrate broth tubes and observation were recorded as described by Cappuccino and Sherman [10].

Catalase activity: The catalase activity of all rhizobial isolates were examined in vitro by adding few drops of 48 hours old rhizobial broth to 3% hydrogen peroxide. The appearance of oxygen bubbles infers a positive result [10].

Cellulase Activity: The identification of rhizobia that produce cellulase was done by inoculating them onto carboxymethyl cellulose (CMC) Congo red Agar media plates [10]. The inoculated plates were kept at 28°C for 3-4 days. A halo zone that appeared around the colony against a red background was evidence that isolates were using CMC as a carbon source because of its cellulase enzyme.

Ammonia Production: The NH_3 production ability of strains was examined by growing them in peptone broth at 28°C for 2-3 days. After incubation 0.5 ml of Nessler's reagent was added to each sample. The alteration in broth colour from brown to yellow recorded as positive result for NH_3 production.

Citrate Utilization: The test was performed by inoculating fresh bacterial cultures onto Simmon's citrate agar slants to determine the ability of isolates to use citrate as a carbon source [10]. The inoculated slants were kept at 28°C in incubator for 48 hrs and observed regularly for the growth and change in colour. Utilization of citrate leads to the conversion of ammonium salts into ammonia, which raising media alkalinity and changing medium colour from green to blue.

Indole Production: The indole test approves whether a bacterial species have the capability to convert tryptophan to indole through their biochemical process. Fresh cultures were used and added to tubes containing tryptone broth and incubated at 28°C for 2-3 days. After incubation few drops of Kovac's reagent were added to each tube. Appearance of a pink layer on top of the tryptone broth denotes a successful outcome for indole production.

D. Plant growth promoting (PGP) activities of Rhizobial isolates

IAA Production: A method developed by Gorden and Weber [12] was used to determine Indole acetic acid production by rhizobial isolates. The rhizobial cultures were grown in LB broth having 1mM tryptophan for 10 days in the dark condition at 28°C and 120 rpm. After 10 days the rhizobial cultures were harvested by centrifugation at 10000 rpm for 10 min. Further 2ml of the supernatant were taken and combined with 4ml of Salkowski's reagent and few droplets of ortho-phospheric acid. The appearance of a pink colour denotes positive results for IAA production. Each sample's optical densities were measured at 535 nm by using a spectrophotometer.

Phosphate solubilization: The ability of rhizobial strains to solubilize phosphate was assessed by spotting them on Pikovskaya's agar media plates. All inoculated plates were kept at 28°C for 2-3 days.

The formation of a clear zone around the colonies recorded as positive outcome for the test.

III. RESULTS AND DISCUSSION

A. Nodulation Status and Morphology of Nodules

In order to explore nodulation status of *Medicago polymorpha*, a survey was conducted during winter season in Jaipur, Rajasthan. The well flourished bur medic plants with pods were found at all sampling sites (Fig 1a & b). A total of five plants of *Medicago polymorpha* aged between 5-6 weeks were excavated form each sampling sites (Fig 1c). All of the excavated plants had nodules however the numbers of nodules were varied. The highest (20) and lowest (13) average numbers of nodules per plant was observed at Rajasthan University Campus and Amer hills sites, respectively. The biggest difference between the two locations is the amount of soil moisture content. The Rajasthan University Campus site is situated in a garden that receives regular irrigation, whereas the Amer Hills experience water scarcity in the winter. Our findings were supported by the study of Adjetey and Nxumalo [13] that water scarcity causes a decline in the symbiotic interaction between legumes and rhizobia and decreases nodule formation. In addition 17 nodules per plant were observed in Maharaja's college site.

The morphology of nodules of *Medicago polymorpha* was carefully examined and it was found that nodules in early developmental stages were tiny and globular however they became elongated and branched in later stages (Fig 1d, e & f). Such types of indeterminate nodules were also observed in other *Medicago* species (*M. truncatula*, *M. sativa*) by researchers [14], [15]. In addition nodules were distributed throughout root system although the primary root had the most nodules (Fig 1d, e).

B. Phenotypic Characteristics of isolates

In present study a total of 15 rhizobial isolates were isolated form root nodules of the *M. polymorpha* and characterized for their physiological and biochemical lcharacteristics. Isolated rhizobial strains showed significant variation in their colony characteristics. Based on colony morphology all isolates were categorized into six groups. Major group containing 6 isolates (MP1, MP3, MP9, MP10, MP11 and MP13) were showed white, opaque, raised, smooth edges, non-gummy and mucilaginous characteristics (Fig 2a; Table I). Some isolates showed reddish (MP5, MP7, MP12 and MP15) and pinkish (MP2 and MP8) patches on their colonies. The colony characteristics of all isolates were showed in table I. Similar colony traits were also seen in rhizobial isolates taken from *M. ciliaris*, *M. minima* and *M. sativa* [16], [17] and subsequently these were identified as strains of *Ensifer* sp. and *Rhizobium* sp. on the basis of molecular characteristics. Similar to MP2 and MP8 isolates, Sebbane et al. [18] were isolated pink pigmented strain from *M. polymorpha* in the Algeria. The potential of isolates to produce acid or base in the medium was assessed through BTB reaction. Five Isolates from study (MP1, MP2, MP5, MP6 and MP13) were showed alkali reaction (media turned blue) while three isolates (MP8, MP8 and MP15) showed acidic reaction (media turned yellow) (Fig 2b; Table I). All remaining isolates were neutral as they did not change the colour of medium.

All tested isolates showed significant variation in their salt and pH tolerance level. The isolates MP1, MP2, MP6, MP7, MP8, MP10 and MP11 were sensitive to high salt concentration therefore did not showed growth beyond 1% salt concentration. Four isolates MP3, MP4, MP9 and MP15 were showed growth up to 3% salt concentration while remaining MP5, MP12, MP13 and MP14 were able to tolerant upto 2% salt concentration (Fig 2j; Table I). No Growth Was Observed at 4% and 5% salt concentration in all isolates. In Tunisia, Djedidi et al. [16] were observed similar salt tolerance pattern in rhizobia isolated from *M. polymorpha*, *M. ciliaris*, *M. sativa* and *M. minima*. Salt-tolerant rhizobia can successfully colonise the roots of legume plants and boost output in saline situations by adjusting their osmotic potential. The pH tolerance patterns of isolated bacterial strains varied significantly and were divided into three groups. Group I isolates (MP3, MP4, MP5, MP9 and MP15) were showed growth at pH range 5-10 whereas isolates from groups II (MP1, MP2, MP6, MP7, MP10, MP11 and MP13) and III (MP8, MP12 and MP14) could grow at range of pH 6-9 and pH 5-9, respectively (Table I). It was found that the rhizobial strains MP3, MP4, MP9, and MP15, which showed salt-tolerant up to 3%, were also well adapted to high alkaline conditions and exhibited growth in extremely alkaline media (pH 10). Similar correlation was also observed by Shamseldin and Werner [19].

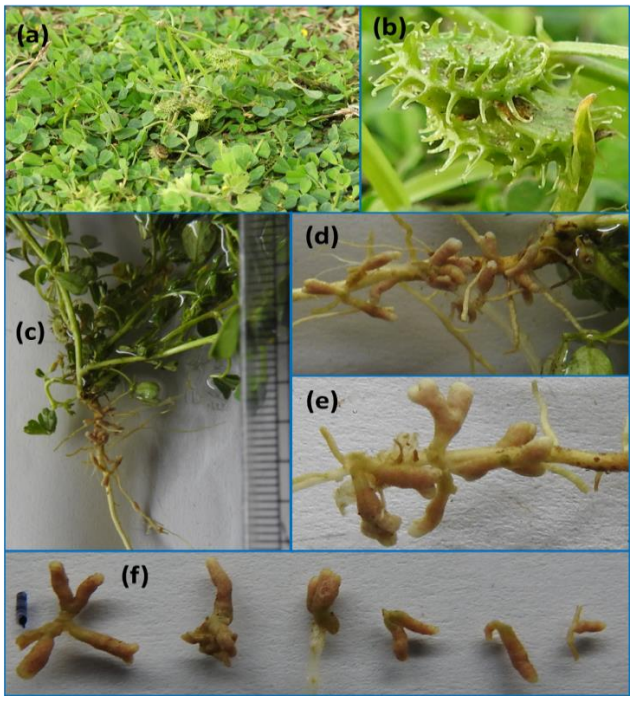

Figure 1: Plant system *Medicago Polymorpha*: (a) field view of plant, (b) pods, (c) excavated plant with root system, (d-e) close up view of root with nodules and (f) different developmental stages of nodules with 5mm scale sign

Table I: Colony morphology and phenotypic characteristics of rhizobial strains isolated from root nodules of *Medicago polymorpha*

Sampling Site	Purified rhizobial isolates	Colony characteristic	BTB Reaction	Salt tolerance (up to %)	pH tolerance range
Rajasthan University Campus	MP1	White, opaque, raised, smooth edges, non-gummy, mucilaginous	Basic	1	6-9
	MP2	Pinkish-white, opaque, raised, smooth edges, gummy, mucilaginous	Basic	1	6-9
	MP3	White, opaque, raised, smooth edges, non-gummy, mucilaginous	Neutral	3	5-10
	MP4	White, opaque, raised, smooth edges, non-gummy, non-mucilaginous	Neutral	3	5-10
	MP5	Reddish-white, opaque, raised, smooth edges, non-gummy, non-mucilaginous	Basic	2	5-10
	MP6	White, opaque, raised, smooth edges, gummy, non-mucilaginous	Basic	1	6-9
Maharaja's College	MP7	Reddish-white, opaque, raised, smooth edges, non-gummy, non-mucilaginous	Neutral	1	6-9
	MP8	Pinkish-white, translucent, raised, raised, smooth edges, gummy, on-mucilaginous	Acidic	1	5-9
	MP9	White, opaque, raised, smooth edges, non-gummy, mucilaginous	Acidic	3	5-10
	MP10	White, opaque, raised, smooth edges, non-gummy, mucilaginous	Neutral	1	6-9
	MP11	White, opaque, raised, smooth edges, non-gummy, mucilaginous	Neutral	1	6-9
	MP12	Reddish-white, opaque, raised, smooth edges, non-gummy, non-mucilaginous	Neutral	2	5-9
Amer Hills	MP13	White, opaque, raised, smooth edges, non-gummy, mucilaginous	Basic	2	6-9
	MP14	White, opaque, raised, smooth edges, non-gummy, non-mucilaginous	Neutral	2	5-9
	MP15	Reddish-white, opaque, raised, smooth edges, non-gummy, non-mucilaginous	Acidic	3	5-10

C. Biochemical Characteristics of isolates

All of the rhizobial isolates in the current investigation shown significant diversity in their biochemical activity, indicating the presence of more than one rhizobial species.

Nitrate reduction: Out of 15 isolates 10 isolates were showed positive results in terms of nitrate reduction (Fig 2c; Table II). Similar results were also observed for rhizobial strains isolated from *Cajanus cajan* and *Cicer arietinum* [20], [21]. Nitrate reductase activity promotes nodule organogenesis by overcoming nitrate's inhibitory effect on cytokinin biosynthesis [11].

Catalase activity: In the current study ten out of fifteen isolates (MP2, MP3, MP4, MP5, MP7, MP10, MP11, MP12, MP13 and MP15) were tested positive for catalase activity (Fig 2d; Table II). Rhizobial isolates with high catalase activity may be useful in improving N-fixing ability of nodules by lowering hydrogen peroxide content [22]. Previously Shahzad et al. [23] reported catalase producing rhizobia from *Medicago sativa* in Pakistan.

Cellulase Activity: Fungi are the most common producers of cellulase however some bacteria including rhizobia are also able to produce it. Only four bacterial isolates (MP2, MP3, MP11 and MP12) were showed cellulolytic activity on CMC agar plates (Fig 2e; Table II). The rhizobial isolates from *Trigonella foenumgraecum* [24] showed positive outcome for cellulase activity. Similarly only one cellulase producing rhizobial isolate, RhW1, was screened by Teresa et al. [25] after studying 75 RNB strains isolated from native legumes of the Congo (Africa). Cellulase activity in microorganisms allows them to use cellulose as a carbon source.

Ammonia Production: All isolates were tested for ammonia production in peptone broth and only three isolates (MP2, MP10, MP11 and MP13) were able to generate ammonia (Fig 2f; Table II). In study of Manasa et al. [26] all rhizobial isolates from rhizospeicc soil in Telangana showed ammonia production. In addition many researchers found ammonia producing rhizobial strains from different part of the world [20], [21].

Citrate Utilization and indole production: All isolates except MP4 and MP13 were unable to utilize citrate (Fig 2g; Table II). Similarly out of seven only one rhizobial isolates AIQ-1 isolated from Chickpea showed utilization of citrate [21]. The Singha et al. [27] reported negative result for citrate utilization for rhizobia isolated from *Cajanus cajan* and *Lablab purpureus* in Asam. In present study all tested strains were showed negative results for indole production.

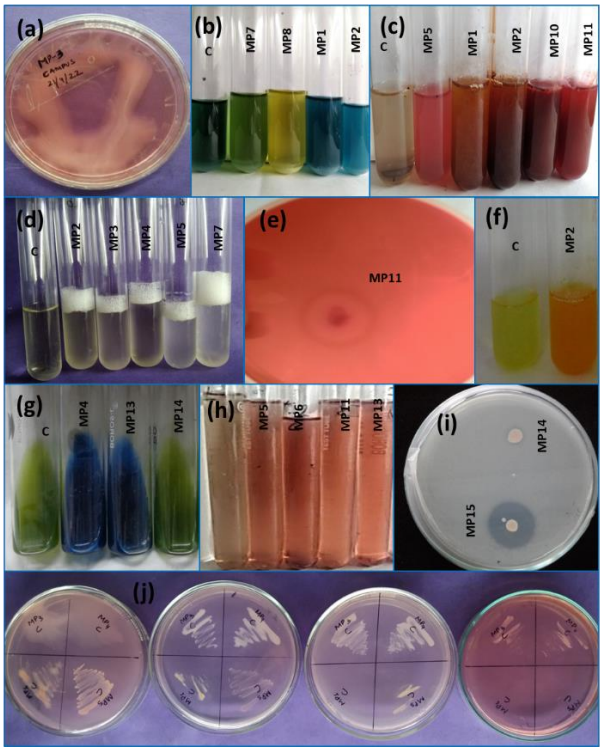

Figure2: Phenotypic, biochemical and plant growth promoting activity of rhizobial isolates: (a) purified isolate, (b) BTB reaction, (c) Nitrate reduction, (d) Catalase activity, (e) Cellulase activity, (f) Ammonia production, (g) citrate utilization, (h) IAA Production, (i) phosphate solubilization and (g) NaCl tolerance

D. Plant growth promoting activates of isolates

IAA Production: The ability of each rhizobial isolate to stimulate plant growth was examined in term of IAA production and phosphate solubilization. Indole-3-acetic acid, or IAA, is the main auxin present in plants. The IAA production was observed in 10 isolates (MP3, MP5, MP6, and MP9 to MP15) however intensity of developed pink colour was varied (Fig 2h; Table II). In present study the most intense pink colour was produced by two rhizobial isolates MP3 and MP15 indicated that these isolates were the most effective at producing IAA. Similar pattern of IAA production was also observed in several rhizobial strains isolated from *Medicago sativa* [17], *Cajanus cajan* [20] *Cicer arietinum* [21], *Phaseolus vulgaris* [28] and rhizospheric soil [26]. IAA stimulates development in plants by boosting root biomass, which encourages the movement of nutrients and water toward the plants.

Phosphate solubilization: Phosphorus is a crucial nutrient for the growth and development of plants; it is structural component of macromolecules and energy currency ATP and also actively participates in major physiological process of plants. Phosphates, both organic and inorganic, are poorly accessible to plants in soil due to their severe complexation and insoluble nature. Several bacteria including rhizobia are able to transform the insoluble form of phosphate into the soluble form. In present study two rhizobial isolates MP3 and MP15 showed phosphate solubilization (Fig 2i; Table II). These strains are very important because they also produced IAA with the greatest efficiency. Similar results were also observed by several researchers throughout the world [17], [20], [21].

Table II: Biochemical traits and plant growth promoting activities of rhizobial strains isolated from root nodules of *Medicago polymorpha*

Isolates	Biochemical activities							
	Nitrate Reduction	Ammoniaproduction	Catalase activity	Cellulase activity	Citrate Utilisation	Indole production	IAA Production	Phosphate solubilization
MP1	+	-	-	-	-	-	-	-
MP2	+	+	+	+	-	-	-	-
MP3	-	-	+	+	-	-	+++	+
MP4	-	-	+	-	+	-	-	-
MP5	+	-	+	-	-	-	+	-
MP6	-	-	-	-	-	-	++	-
MP7	-	-	+	-	-	-	-	-
MP8	+	-	-	-	-	-	-	-
MP9	-	-	-	-	-	-	++	-
MP10	+	+	+	-	-	-	++	-
MP11	+	+	+	+	-	-	++	-
MP12	+	-	+	+	-	-	+	-
MP13	+	+	+	-	+	-	+	-
MP14	+	-	-	-	-	-	++	-
MP15	+	-	+	-	-	-	+++	+

IV. CONCLUSION

Medicago polymorpha is a good fodder that helps to prevent soil erosion and increase soil fertility by fixing nitrogen. In the current study, *Medicago polymorpha* was found well nodulated at all sampling locations in Jaipur, Rajasthan, however, the number of nodules exhibited an increasing tendency with increase in soil moisture content. Mature nodules were branched and indeterminate and distributed throughout root system although most of the nodules were present on the primary root. All 15 isolates were diverse in their phenotypic, biochemical and plant growth promoting activities. All rhizobial strains were fast growing and showed *Ensifer* type of colony characteristics. In present study, some rhizobial isolates (MP3, MP9, MP15 etc.) had notable physiological traits, in terms of high salt and pH tolerance, IAA production and phosphate solubilization. Such elite rhizobia and PGP strains could be used to make bio-fertilizers that would replace synthetic chemical fertilizers and could prove to be a sustainable method for increasing the productivity of legumes. Further suitable molecular methods can be applied to identify and discuss phylogeny of isolates to comprehend its evolution. It is crucial to educate local farmers on the value of growing specific leguminous feedstocks because doing so will boost both the country economy and farmers' socioeconomic standards.

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AUTHORS PROFILE

Amit Yadav, M.Sc. (Botany), doctoral students of Botany Department, University of Rajasthan, Jaipur and exploring rhizobial diversity associated with native legumes of the Aravalli Region. He completed his master degree in 2019 from Rajasthan University, Jaipur. Since master degree he is continuously characterizing the indigenous plant growth promoting bacteria by using polyphasic methods. For his doctoral research work, he is now receiving a senior research fellowship (SRF) from Council of Scientific and Industrial Research (CSIR), New Delhi. He is proficient in a number of molecular methods, such as 2D electrophoresis, real-time PCR, RAPD, and ARDRA. He presented his research works in 2 national conferences. He is a very good learner and has good writing skills.

Divya Solanki, M.Sc. (Biotechnology), recently completed her dissertation at Botany Department, University of Rajasthan, Jaipur on Isolation and characterization of rhizobia associated with *Medicago* species in Rajasthan. She completed her master degree in 2022 from Lachoo Memorial College of Science and Technology, Jodhpur. She is an expert in the isolation and biochemical characterization of root nodule bacteria. She also expertise in handling of bioinformatics tools and software's Bioedit, Gene Tool lite, NTSYS etc. She is currently preparing for the Council of Scientific and Industrial Research's (CSIR) NET-JRF examination and willing to join as Ph. D. research scholar in reputed institutes.

Ghanshyam Sharma, M.Sc. (Botany), doctoral students of Botany Department, University of Rajasthan, Jaipur and working on molecular characterization and MLSA (Multi locus sequence analysis) study of microsymbionts associated with native legumes of the Central Aravalli Region in Rajasthan. He completed his master degree in 2016 from Rajasthan University, Jaipur. He is a highly experienced researcher with a background in molecular biology and bioinformatics. He is currently receiving a senior research fellowship (SRF) from the Council of Scientific and Industrial Research (CSIR), New Delhi, for his doctoral research work. Recently he published a good article on 'Fungal degradation of bioplastics: An overview'.

Dr. Gunjan Dubey, M.Sc., Ph. D. working as assistant professor at Department of Botany, University of Rajasthan, Jaipur. Her broad research area is investigation on the regulation of physiological, biochemical and molecular responses in higher plants and cyanobacteria under abiotic stress such as heavy metal toxicity, nanoparticles, salinity, UV-B, etc. with reference to involvement of reactive oxygen species, phytohormonal signaling. She published more than 10 research papers and book chapters in various journals of national and international repute. In addition she also presented her research works and findings in many national and international conferences. Currently she is guiding four doctoral students in Rajasthan University.

Dr. Indu Singh Sankhla, M.Sc., M. Tech. (IIT Kharagpur), Ph. D., working as assistant professor at Department of Botany, University of Rajasthan, Jaipur. His broad research area is molecular biology, rhizobia technology and bioinformatics. He has investigated molecular diversity of rhizobia associated with native legumes of Thar Desert as well as Aravalli region in Rajasthan and published more than 10 research paper in various journals of national and international repute. He also published 6 book chapters in national and international books. He acted as resource person in many national and international conferences. More than 220 gene sequences of housekeeping and symbiotic genes have been submitted in NCBI database by him. He also submitted elite rhizobial culture to MMC Pune under accession no MCC 3322, MCC 3323, MCC 3194 and MCC 3197.