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Genetic Characterization and Diversity of Rhizobia Isolated from Root Nodules of Green Gram (*Vigna radiata* L.) found in Central Plateau of India

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ABSTRACT

Background: Plant growth promoting rhizobia play an important in agricultural ecosystem through symbiotic association with a wide range of leguminous plants. Legume-rhizobia association is a host specific symbiosis hence the need to identify the strains and the diversity of rhizobia associated with specific type of legume for better bio-prospecting of the associated benefits. A wide range of variation is present among the distribution of rhizobia and greatly affected by geographical locality, edaphic factors and environmental variation with time and space. Molecular techniques have been developed to aid the traditional phenotypic and morpho-cultural techniques in distinguishing the different microbial genera, species and strains. The present study aimed at isolation and morpho-molecular characterization of rhizobia from root nodule of green gram cultivated in central part of India.

Methods: In the field-laboratory investigation during 2018-2019, various locations of central India with contrasting agro climatic conditions were surveyed and rhizobia trapping done. A total of 40 rhizobia were retrieved from nodules of green gram and characterized in laboratory based on morphological, biochemical and molecular techniques and results validated for taxonomic identification.

Result: In our investigation all forty isolated rhizobia were found phosphate solubilizers, 38 IAA producers and 37 ammonia excretors and grew well at 28°C and 37°C. In carbohydrate fermentation test 34 isolates changed the broth colour from red to yellow with gas formation in durum tubes. Twenty diverse rhizobia isolates were selected with respect to their multifunctional properties and studied for molecular characterization. Based on 16S rRNA gene sequencing, these bacterial strains were identified under two genera: *Rhizobium* and *Brady Rhizobium*. Phylogenetic analysis divided 20 rhizobia isolates into two clusters. Major group included 12 strains and minor group included 8 strains.

Key words: Diversity, Green gram, Rhizobia, Root nodules.

INTRODUCTION

In agri-ecosystem, microorganisms play an important role, especially the group of rhizobacteria which are associated for promotion of plant growth and development. Rhizobia play a significant role in agricultural ecosystem through symbiotic association with a wide range of leguminous plant (Orrell and Bennet, 2013) and are commercially exploited. Several rhizobial strains have been identified for various objectives *viz*., to enhance the mineral uptake, production of phytohormones, nitrogen fixation, detoxification of metals and neutralizing biotic and abiotic stress, producing volatile organic compounds (VOCs) and enzymes to prevent diseases Koskey *et al*. (2018).

Legume-rhizobia association is a host specific symbiosis hence the need to identify the strains and the diversity of rhizobia associated with specific type of legume are essential for better bio-prospecting of the associated benefits Batista *et al*. (2015). A wide range of variation is present among the distribution of rhizobia and greatly affected by geographical locality, edaphic factors and environmental variation with time and space Martiny *et al*., (2006), Cao *et al*. (2014). Diverse heterogenous group of rhizobia consist of alpha and beta groups and have different geographical distributions. Currently, there are 40 recognized rhizobia species, within 7 genera, which belong to the class 'Alphaproteobacteria' (*AlloRhizobium, AzoRhizobium, BradyRhizobium, MesoRhizobium, Methylobacterium*, *Rhizobium* and *SinoRhizobium*) and two to the class 'Betaproteobacteria' (*Burkholderia* and *Ralstonia*) Kosaky *et al*., (2018).

Recently agriculture trend has become the use of sustainable farming practiced that are environmentally safe, eco-friendly, efficient, cost effective and reachable to farmers field. Demand of rhizobia as a bio-fertilizer in tropical and sub-tropical part of India has increased due to beneficial effect associated for crop yield as well as soil fertility Barman *et al*. (2017). However lack of awareness, unavailability of

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appropriate and efficient strains, erroneous government policies, lack of quality assurance, absence of supportive infrastructure and limited research the diverse and elite *rhizobia* strain associated with the plants is the constraints of the utilization of bio-fertilizers in green gram production Mahdi *et al*., (2010). Greengram (*Vigna radiata* L.) is a popular legume crop cultivated in the country and well fit into many cropping systems, including rice and sugarcane under both rainfed and irrigated conditions. In India, very limited work was conducted on rhizobia diversity associated with green gram in central plateau and hills with constricting environmental conditions, hence based on our present need we proposed this study. The results on distribution and molecular variation among the native rhizobia isolates will be used for selection of novel rhizobia strains for green gram crop in the country as well as and development of bio-fertilizer technology.

Now a days, molecular techniques have been developed to aid the traditional phenotypic and morpho-cultural techniques in distinguishing the different microbialgenera, species and strains (Koskey *et al*., 2018). In addition to determine the genetic diversity of native *rhizobia* the 16S rRNA which are highly conserved, have been sequenced to determine the taxonomic position of different rhizobia strains (Rahmani *et al*., 2011). 16S rRNA primer is used for phylogenetic taxonomy and identification of bacterial species. Therefore, the present study aimed at isolation and morpho-molecular characterization of rhizobia from root nodule of green gram cultivated in central part of India.

MATERIALS AND METHODS

Field trapping of rhizobia

The present study was performed at Biotechnology Centre, Jawaharlal Nehru Krishi Vishwa Vidhalya, Jabalpur, India. Field trapping of rhizobia was carried out during January to August 2018 from various locations of central India with contrasting agro climatic conditions. The rhizobia trapping were done from central part of India (Table 1). Sampling was done randomly by carefully uprooting five plants from middle of the field with the help of a sterile spade and trowel along with a soil block of ~20 cm diameter up to a depth of about 20 cm. The clump of soil and root were uplifted carefully, placed in sterile aluminum foil and keep in refrigerator at 4°C and next day used it the isolation of the Rhizobia. Further nodule were detached from the root and kept in onkrage tube and extraction was done.

Isolation of rhizobia from root nodules

Healthy and pink nodules were selected for the isolation of rhizobia using standard protocol described by Somasegaran and Hoben (1994). A total 40 rhizobia were obtained and maintained on screw-capped YEMA slant bottles and preserved at 4°C.

Morphological characterization

Rhizobia isolates were examined for morphological characterization such as colony morphology, cell shape, border, muscocity, transparency, colour and gram staining using standard microbiological techniques described by Somasegaran and Hoben (1994).

Table 1: Sampling site and their geographical locations.

Name of strain	Sampling site	Geographical location	Name of strain	Sampling site	Geographical location
JRB 110	Jabalpur	23.1815°N 79.9864°E	JRB 130	Gonsa, Ujjain	23.2041°N 75.7548°E
JRB 111	Panagar, Jabalpur	23.2915°N 79.9964°E	JRB 131	Undasa, Ujjain	23.2252°N 75.8331°E
JRB 112	Bedaghat, Jabalpur	23.1306°N 79.8024°E	JRB 132	Datana, Ujjain	23.1024°N 75.8912°E
JRB 113	Powarkheda, Hoshangabad	22.7065°N 77.7398°E	JRB 133	Mindia, Ujjain	23.1765°N 75.7885°E
JRB 114	Itarshi, Hoshangabad	22.6615°N 77.7623°E	JRB 134	Kosmi, Betul	21.9008°N 77.8767°E
JRB 115	Balaghat	21.8129°N 80.1838°E	JRB 135	Badora, Betul	21.9008°N 77.8767°E
JRB 116	Waraseoni, Balaghat	21.7654°N 80.0591°E	JRB 136	Khaddar, Betual	21.5472°N 77.5428°E
JRB 117	Laalbarra, Balaghat	21.8759°N 80.0987°E	JRB 137	Sironja, Sargar	23.8124°N 78.8099°E
JRB 118	Bharghat, Seoni	22.0489°N 79.5767°E	JRB 138	Makroniya, Sagar	23.8554°N 78.7958°E
JRB 119	Bharghat, Seoni	22.0489°N 79.5767°E	JRB 139	Silera, Sagar	23.8449°N 78.6541°E
JRB 120	Khandwa	21.8314°N 76.3498°E	JRB 140	Bamora, Sagar	24.0598°N 78.0909°E
JRB 121	Borgaon, Khandwa	21.6181°N 76.3319°E	JRB 141	Surkhi, Sagar	23.6289°N 78.8298°E
JRB 122	Katni	23.8343°N 80.3894°E	JRB 142	Satna	24.6005°N 80.8322°E
JRB 123	Jhalwara, Katni	23.7702°N 80.4730°E	JRB 143	Satna	24.5362°N 81.3037°E
JRB 124	Pipraundh, Katni	23.7602°N 80.3352°E	JRB 144	Sohawal, Satna	24.5757°N 80.7630°E
JRB 125	Singrauli	24.1992°N 82.6645°E	JRB 145	Madhavgarh, Satna	24.5634°N 80.9118°E
JRB 126	Vidisha	23.5236°N 77.4140°E	JRB 146	Barbasa, Satna	24.5607°N 80.7293°E
JRB 127	Sanchi, Vidisha	23.5236°N 77.8140°E	JRB 147	Reewa	24.5362°N 81.3037°E
JRB 128	Jambar, Vidisha	23.5539°N 77.7652°E	JRB 148	Saman, Reewa	24.5499°N 81.3259°E
JRB 129	Ratlam	23.3315°N 75.0367°E	JRB 149	Karahiya, Reewa	24.5531°N 81.2712°E

Biochemical characterization

Biochemical test was done for all forty rhizobia isolates with standard techniques and its includes growth on glucose peptone agar, growth on YEMA with bromothymol blue, IAA production test, phosphate solubilisation test, ammonia production test, carbohydrate fermentation test, citrate utilization test, catalase test.

Gram staining

Gram reaction was carried out for bacterial isolates to classify them in two groups *i.e*., gram positive and gram negative as protocol given by Vincent, (1970). Young 2-3 days old pure cultures of YEMB were smeared on slides. The wet smear was air dried heat fixed and then gram stained. Prepared slides were observed under oil immersion on a compound microscope (Leica Microsystems).

Growth on glucose peptone agar

Growth on glucose peptone agar was done according to the protocol given by Kleczkowska *et al*. (1968). Isolated rhizobia were cultured in peptone glucose agar medium and plates were incubated at 28±2°C for 72-96 hours.

Growth on YEMA with bromo thymol blue

The production of acid or alkali was determined in YEMA medium containing bromo thymol blue according to the protocol given by Vincent, (1970). One ml of 1 per cent Bromothymol blue (BTB) was prepared and added to 10 ml of YEMA medium and poured in sterile petri plates then allowed to solidify. A loopful of *Rhizobia* isolates were inoculated on the media plates and incubated at 28±2°C for 7 days in dark. The isolates that changed the green colour of YEMA-BTB medium to blue were considered as alkaline producers and slow growers.

IAA production test

IAA production by the isolates was studied by the method of Hartmann *et al*. (1983).

Phosphates solubilisation test

Phosphate solubilisation activity was done by prepared plated with Pikovskaya's media and isolates were spot inoculated on the centre of agar plate aseptically. All the plates were incubated at 28 ± 2°C for 5-6 days. A clear zone around a growing colony indicated phosphate solubilisation (Pikovaskya, 1948).

Ammonia production test

Ammonia production of different isolates was done by methods of Laslo *et al*. (2012).

Carbohydrate fermentation and citrate utilization test

Both the tests were performed by methods of manual (Aneja, 2003), (APHA, 1998) and Bergey's manual (1964). While catalase test was performed to study the presence of catalase enzyme in bacterial colonies. Pure isolates (24 h old) were taken on glass slides and one drop of H_2O_2 (30 per cent) was added. Appearance of gas bubble indicated the presence of catalase enzyme (Rangaswami and Bagyaraj, 1993). The effect of temperature was performed in terms of growth. Rhizobia were grown in YEMA medium on petriplates and incubated at three different temperatures *viz*., 28°C, 37°C and 48°C for 3 days and the growth was noted.

Genotype characterization

Molecular study was carried out selected twenty rhizobia isolates. The analysis of genetic relatedness of the native rhizobia isolates from Central Plateau of India was carried out using sequence analysis of amplified 16s rDNA genes

Genomic DNA isolation

Total genomic DNA extraction of twenty rhizobia isolates was carried with minor modification of a method outlined by Wilson (2001). PCR amplification was carried out in 20 µl reaction with 12.6 µl nuclease free water, 2 µl buffer (Fermentas), 0.4 µl dNTP (10mm), 1 µl of each 27F and 1492F primers, 1.6 µl $MgCl_2$ (10mm), 0.4 µl Taq DNA Polymerase (Fermentas) and 2 µl of DNA template. 16S rRNA primers 27F-5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R-5′ TACCTTGTTTTACGACTT-3′ sequenced were used.

DNA sequencing using 27F and 1492R forward and reverse primers

PCR amplicon of the 20 selected rhizobia isolates were sent for sequencing to Agri genome labs Pvt Ltd. Kochi, Kerala, India. Sequence results obtained from the sequencing lab were used to assess the genetic relatedness. Sequences were blast at National Centre on Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast) for similarity search. Phylogenetic relationship analysis was performed using neighbor joining method with 1000 bootstrap value using DARwin softw are.

RESULTS AND DISCUSSION

Isolation and morpho-cultural characteristics of isolates

A total of 40 bacterial isolates were isolated from various agro-climatic zones of Madhya Pradesh. The colony colour varied with creamy, white, milky, yellow, watery, mucoid with round shaped, colonies being observed with firm gummy or smooth muscoid texture to the margin of the colonies (Plate 1). These morphological characteristics approaches were closer to the genus *Rhizobium* as described by Jordan and Allen (1974). Thirty six isolates were found as rod shaped bacteria and considered as gram negative bacteria whereas four isolates *viz.,* JRB 118 JRB 120, JRB 130 and JRB 144 were gram positive bacteria (Table 2). Similarly, all the isolates were grown on YEMA with congored and incubated in dark for 48-72 hours and observed that 35 isolates did not absorb red colour and considered as gram negative *Rhizobium* while JRB 111, JRB 118, JRB 130, JRB 134 and JRB 144 absorb red colour on YEMA plate and considered as gram positive (Plate 1) (Table 2). Previously, Shetta *et al.* (2011), Gauri *et al*. (2012), Kang *et al*. (2013) and Hamza and Albejo, (2017) identified *Rhizobium* isolates

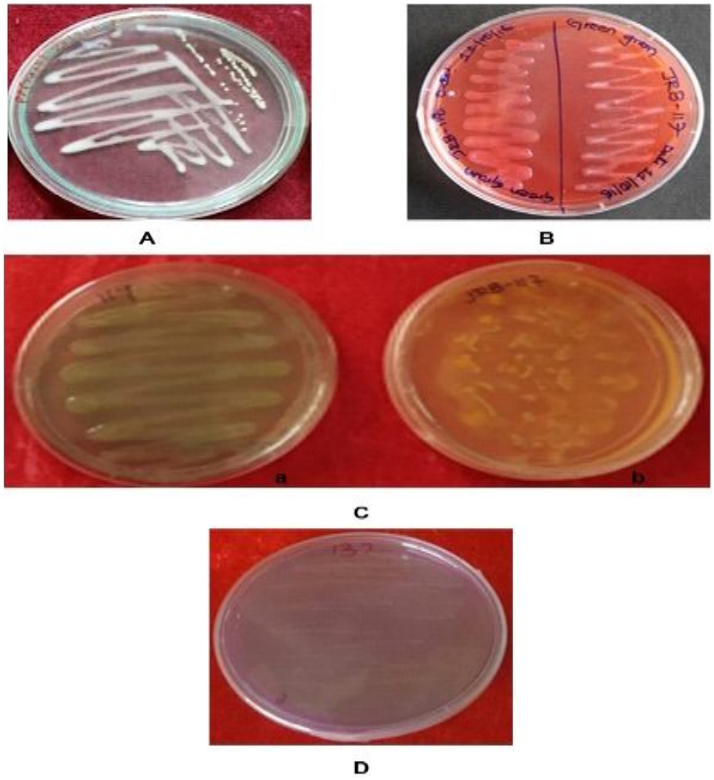

Plate 1: Morphological characterization of *Rhizobial* isolates.
A- Growth of Rhizobia bacteria on YEMA media.
B- Growth of Rhizobia bacteria on YEMA media with congored.
C- Growth of Rhizobia bacteria on YEMA media with Bromothymol blue dey.
D- Growth of Rhizobia glucosepeptone agar media.

on YEMA media with congored dye. Notably 32 isolates showed very weak growth on glucose peptone agar media and whereas, eight isolates, JRB 111, JRB118, JRB 120, JRB126, JRB 130, JRB 142, JRB 144 and JRB 146 showned strong growth and concluded as they did not belong to *Rhizobium* species. Previously, Tyagi *et al*. (2017) and Timmusk *et al*. (2017) also worked on green gram rhizobia and reported that all the rhizobial isolates showed positive results.

All the isolates were turned BTB indicator from deep green to yellow when grown on YEMA-BTB. Twenty five isolates were shown yellow colour and indicate acidic in nature and the 15 isolates were showed green color which resulted alkaline in nature. Based on the results obtained in regard to extent and growth period on YEMA with Bromothymol blue the bacterial isolates were placed into two broad groups.

a) Isolates which showned growth within 3-5 days and the color change on YEMA-BTB from blue to yellow suggested that all isolates were fast growers and fall under the genus *Rhizobium* species Mamun *et al.* (2013).

b) Isolates which were showed slow growth after one week and the color change on YEMA-BTB from blue to green were regarded as slow growers *i.e., BradyRhizobium* species. Previously Sharma *et al.* (2011) and Hamza and Alebejo, (2017) also defined the nature of bacterial species from different sources.

Bio-chemical characterization

Fourty isolates were tested for IAA assay in the presence of L- tryptophan in the medium and 38 isolates were shown pink colour in the medium and identifies IAA producer strains, while JRB 111 and JRB 118 had shown negative results in the medium (Table 3). Our results are supported by the work of Elazanty *et al.* (2015) in *Vicia faba* and *Vigna mungo*. For phosphate solubilization all the 40 strains were grown on pikovskaya's medium and shown an evident of different size of halo zone (Table 2). Our results are in close agreement with the findings of Sridevi *et al*. (2007) in crotalaria and Mondal and Gena (2020) in cluster bean. All the rhizobia isolates were tested and were found as ammonia excretors. Thirty seven isolates were moderate ammonia excretors as they showed deep yellow colour to brownish colour whereas JRB 111 and JRB 118 isolates does not produced any colour and concluded as non ammonia excretors (Table 3). Similar to our work, Manasa *et al.* (2017) revealed in his studies 15 *rhizobial* isolates for positive for ammonia production.

Various carbon sources like, phenol red glucose, phenol red lactose and phenol red sucrose broth *etc*. were used by the bacteria. In our study out 40 Rhizobia, 34 isolates were shown carbohydrate degradation activity by changing the broth colour from red to yellow colour and gas

formation in durum tubes while six isolates JRB 111, JRB 118, JRB 120, JRB 130, JRB 144 and JRB 146 showed negative results (Table 3). Earlier, Kucuk *et al.,* (2006) and Wadhwa *et al.,* (2017) were also found positive for utilization of glucose as carbon source among studied *Rhizobium* strains. In citrate utilization test, only 10 Rhizobia isolates *viz*., JRB 115, JRB 117, JRB 119, JRB 120, JRB 123, JRB 125, JRB 126, JRB 144, JRB 147 and JRB 148 were shown citrate utilizing activity in the medium by changed colour from green to blue while remaining 30 Rhizobia isolates remained as green in colour (Table 3). Earlier, Hamza and Alebejo, (2017); Deshwal *et al.,* (2014) and Girija *et al*., (2020) were isolated rhizobia and categorized *Rhizobium* isolates for citrate utilization activity. Catalase test was performed in 40 strains and all strains formed a bubble on glass slide by adding 2-3 drops of hydrogen peroxide on freshly grown cultures (Table 3). Our results are in accordance with earlier findings of Gachande and Khansole, (2011) and Naz *et al.* (2009) found positive rhizobia isolates for catalase test. Further, all the isolates were evaluated for

Table 2: Results of morphological characterization of *Rhizobial* isolates.

Isolates	Colony shape	Cell shape	Colony color	Gram reaction	CG	BTB	GPA
JRB 110	Circular	Rod	White	-ve	+	Acid	+
JRB 111	Circular	Rod	White	-ve	-	Acid	-
JRB 112	Circular	Rod	White	-ve	+	Alkali	+
JRB 113	Circular	Rod	White	-ve	+	Acid	+
JRB 114	Circular	Rod	White	-ve	+	Acid	+
JRB 115	Circular	Rod	Milky white	-ve	+	Alkali	+
JRB 116	Circular	Rod	Milky white	-ve	+	Acid	+
JRB 117	Circular	Rod	Milky white	-ve	+	Acid	+
JRB 118	Circular	Rod	Milky white	+ve	-	Acid	-
JRB 119	Circular	Rod	White	-ve	+	Alkali	+
JRB 120	Circular	Rod	Milky white	+ve	+	Acid	-
JRB 121	Circular	Rod	White	-ve	+	Alkali	+
JRB 122	Circular	Rod	White	-ve	+	Acid	+
JRB 123	Circular	Rod	White	-ve	+	Alkali	+
JRB 124	Circular	Rod	Creamy white	-ve	+	Acid	+
JRB 125	Circular	Rod	Creamy white	-ve	+	Acid	+
JRB 126	Circular	Rod	Creamy white	-ve	+	Acid	-
JRB 127	Circular	Rod	Creamy white	-ve	+	Alkali	+
JRB 128	Circular	Rod	Creamy white	-ve	+	Acid	+
JRB 129	Circular	Rod	Creamy white	-ve	+	Alkali	+
JRB 130	Circular	Rod	Creamy white	+ve	-	Acid	-
JRB 131	Circular	Rod	Creamy white	-ve	+	Alkali	+
JRB 132	Circular	Rod	White	-ve	+	Acid	+
JRB 133	Circular	Rod	White	-ve	+	Alkali	+
JRB 134	Circular	Rod	White	-ve	-	Acid	+
JRB 135	Circular	Rod	White	-ve	+	Acid	+
JRB 136	Circular	Rod	White	-ve	+	Alkali	+
JRB 137	Circular	Rod	White	-ve	+	Acid	+
JRB 138	Circular	Rod	White	-ve	+	Alkali	+
JRB 139	Circular	Rod	White	-ve	+	Acid	+
JRB 140	Circular	Rod	Creamy white	-ve	+	Acid	+
JRB 141	Circular	Rod	Creamy white	-ve	+	Acid	+
JRB 142	Circular	Rod	Creamy white	-ve	+	Alkali	-
JRB 143	Circular	Rod	Creamy white	-ve	+	Acid	+
JRB 144	Circular	Rod	Creamy white	+ve	-	Acid	-
JRB 145	Circular	Rod	Creamy white	-ve	+	Alkali	+
JRB 146	Circular	Rod	White	-ve	+	Alkali	-
JRB 147	Circular	Rod	White	-ve	+	Acid	+
JRB 148	Circular	Rod	Creamy white	-ve	+	Acid	+
JRB 149	Circular	Rod	Creamy white	-ve	+	Alkali	+

Where, CG= Congored, BTB= Bromothymol blue, GPA= Glucose peptone agar.

tolerance to three temperature regimes *viz.,* 28°C, 37°C and 48°C. At 28°C all isolates shows excellent growth where as 37°C moderate growth was observed. At 48°C, thirty seven isolates were shown poor growth and five isolates *viz.,* JRB112, JRB121, JRB131, JRB143 and JRB149 were shown good growth and this resulted that the isolates can survive at extreme environmental conditions. Results of Naz *et al.* (2009) and Ruiz-Diez *et al.* (2012) were supported our findings.

PCR amplification of 16S rRNA region

PCR amplification was performed for 20 diverse selected rhizobia isolates by using 27F and 1492R primers (Biorad Thermal Cycler). A single sharp band of ~1500 bp was amplified and visualized in 1.2 per cent agarose gel (Plate 2). As 16S rRNA region is much conserved and no diversity was seen and amplicon size corresponds to the expected size earlier reported by Naz *et al.* (2009), Rajendran and Gunasekaran, (2011) and Tyagi *et al.* (2017).

Table 3: Results of bio-chemical test of *Rhizobia* isolates.

Isolates	IAA test	Phosphate solubilizing test	Ammonia production	Citrate utilizing	Catalase test	Carbohydrate Fermentation Test
JRB 110	+	+	+	-	+	+
JRB 111	-	+	-	-	+	-
JRB 112	+	+	+	-	+	+
JRB 113	+	+	+	-	+	+
JRB 114	+	+	+	-	+	+
JRB 115	+	+	+	+	+	+
JRB 116	+	+	+	-	+	+
JRB 117	+	+	+	+	+	+
JRB 118	-	+	-	-	+	-
JRB 119	+	+	+	+	+	+
JRB 120	+	+	+	+	+	-
JRB 121	+	+	+	-	+	+
JRB 122	+	+	+	-	+	+
JRB 123	+	+	+	+	+	+
JRB 124	+	+	+	-	+	+
JRB 125	+	+	+	+	+	+
JRB 126	+	+	+	+	+	+
JRB 127	+	+	+	-	+	+
JRB 128	+	+	+	-	+	+
JRB 129	+	+	+	-	+	+
JRB 130	+	+	+	-	+	-
JRB 131	+	+	+	-	+	+
JRB 132	+	+	+	-	+	+
JRB 133	+	+	+	-	+	+
JRB 134	+	+	+	-	+	+
JRB 135	+	+	+	-	+	+
JRB 136	+	+	+	-	+	+
JRB 137	+	+	+	-	+	+
JRB 138	+	+	+	-	+	+
JRB 139	+	+	+	-	+	+
JRB 140	+	+	+	-	+	+
JRB 141	+	+	+	-	+	+
JRB 142	+	+	+	-	+	+
JRB 143	+	+	+	-	+	+
JRB 144	+	+	+	+	+	-
JRB 145	+	+	+	-	+	+
JRB 146	+	+	+	-	+	-
JRB 147	+	+	+	+	+	+
JRB 148	+	+	+	+	+	+
JRB 149	+	+	+	-	+	+

Where '+' = Positive result, '-' = Negative result.

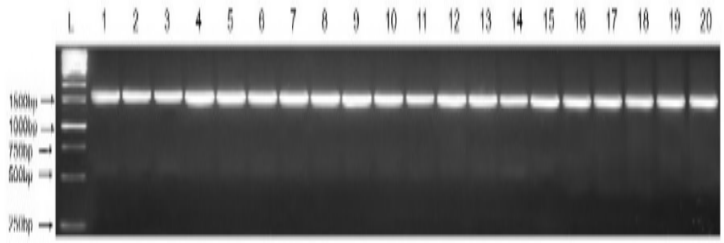

Plate 2: Pattern of gel electrophoresis of PCR amplified product of 20 bacterial DNA using 16S rRNA primer.

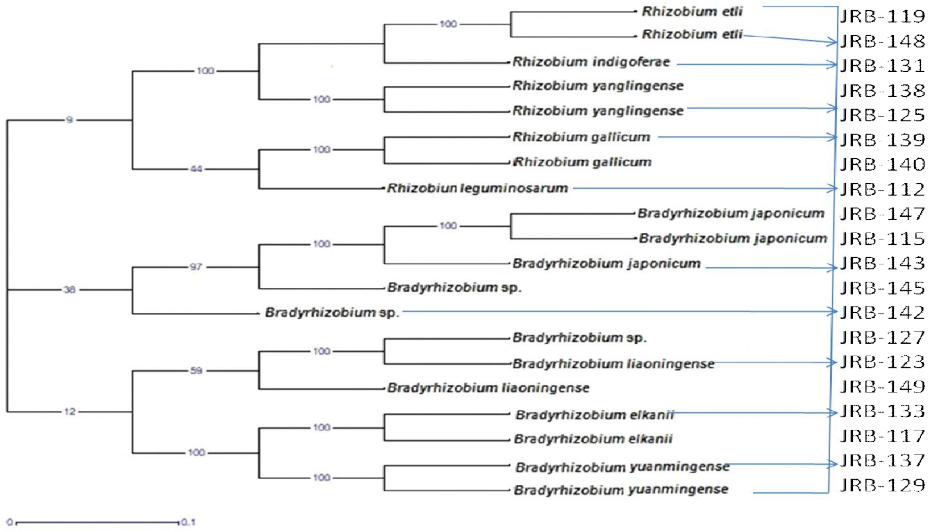

Fig 1: Dendogram of 20 *Rhizobial* isolates generated by using Neighbour-joining method of 1000 Bootstrap value by DARwin software.

Sequencing of 16S rRNA region using 27F forward 1492R reverse primer

PCR amplified product 20 µl with 16S rRNA primer was send for sequencing to Agri genome Pvt, Ltd, Kochi, Kerala, India. The identity of 16S rRNA sequences was performed a similarity search in the gene bank database of NCBI (http:// www.ncbi.nih.gov.BLAST). The sequencing results were matched as positive that all the strains were shown similarity to *Rhizobia* sp and identified as *Rhizobium* and *BradyRhizobium spp.* Similar results were observed by Kang *et al.* (2013) and Gilbert *et al.* (2018).

Phylogenetic analysis

Phylogenetic relationship analysis was performed using neighbor joining method with 1000 bootstrap value by DARwin method phylogenetic analysis inferred from 16S rRNA gene sequences showed the evolutionary relationship of bacterial strains with the respective genera. Phylogenetic analysis revealed that *rhizobia* under study were divided into two groups (Fig 1). Major group 'A' and minor group 'B'. Major group 'A' is further divided into two subgroups 'a1' and 'a2'. Subgroup 'a1' have five species of bacteria *viz*., *BradyRhizobium japonicum, BradyRhizobium japonicum, BradyRhizobium japonicum, BradyRhizobium sp, BradyRhizobium sp*. Subgroup 'a2' have seven bacteria spp *viz*., *BradyRhizobium sp, BradyRhizobium liaoningense, BradyRhizobium liaoningense, BradyRhizobium elkanii, BradyRhizobium elkanii, BradyRhizobium yuanmingense,* and *BradyRhizobium yuanmingense*. Minor group 'B' divided into two subgroups 'b1' and 'b2'. Subgroup 'b1' contained five bacteria spp *viz*., *Rhizobium etli, Rhizobium etli, Rhizobium indigoferae, Rhizobium yanglingense, Rhizobium yanglingense* and subgroup 'b'2 contained three bacteria sp *viz*., *Rhizobium gallicum, Rhizobium gallicum and Rhizobium leguminosarum* (Fig 1). The maximum distance was observed between JRB-119 and JRB-129 and it is due to huge variation among the sampling site *viz*., Bharghat, Seoni and Ratlam district and they fall in different agroclimatic zones of the state. Similarly, the variation in other strains is due to broad area of soil sampling with huge variation of agro-ecological practices. Previously, Nahar *et al.* (2012) revealed that the *rhizobia* isolates of genetic relatedness was assessed by comparing the sequences of 16S rRNA and two distinct clusters were seen in the dendrogram constructed by the complete linkage method.

CONCLUSION

In the present investigation 40 bacterial isolated were isolated from root nodule of green gram growing region of

Madhya Pradesh. Isolated species are fast and slow growers and belong to genus *Rhizobium* and *BradyRhizobium*. Morphological characterization results were shown resemblance to *Rhizobial* strains due to failure in absorbance of Congored on YEMA medium, weak growth on glucose peptone agar media. Biochemical results were shown positive results in IAA production, phosphate solubilization, ammonia production, catalase test, citrate utilization test, carbohydrate degradation test and five isolates were shown good survival at extreme temperature conditions *i.e*., 48°C. Further 20 diverse *Rhizobial* strains were selected for PCR amplification using 27F forward and 1492R reverse primers and an amplicon of ~1500bp were observed and sequenced. DNA sequencing results revealed that all the 20 isolates were belonging to genera *Rhizobium* and *BradyRhizobium* spp.

Author contributions

RSS and MS conceived and designed the research and sample collection and participated in drafting the manuscript. SM, RSR and AKS participated in data analyses and wrote the manuscript. All authors read and approved the final manuscript.

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REFERENCES

American Public Health Association (APHA), (1998). Standard Methods for the Examination of Water and Waste Water; 20th edition, Washington.

Aneja, K.R. (2003). Experiments in microbiology, Plant Pathology and Biotechnology, 4th edition. New Age International Publishers, New Delhi pp 275.

Barman, M., Paul. S., Choudhury, A., Roy, P. and Sen, J. (2017). Biofertilizer as prospective input for sustainable agriculture in India. International Journal of Current Microbiology and Applied Sciences. 6(11): 1177-1186. https://doi.org/10.20546/ijcmas.2017.611.141.

Batista, L. Irisarri, P., Rebuffo, M., Cuitiño, M.J., Sanjuán, J., Monza, J. (2015). Nodulation competitiveness as a requisite for improved *rhizobial* inoculants of *Trifolium pratense.* Biology and Fertility of Soils. 51: 11-20. https://doi.org/10.1007/s00374-014-0946-3.

Bergey's Manual of Determinative Bacteriology (7th ed.) (1964). American Journal Public Health Nations Health. 54(3): 544.

Cao Ying, En-Tao Wang., Liang Zhao, Wei-Min Chen Ge-Hong Wei. (2014). Diversity and distribution of rhizobia nodulated with *Phaseolus vulgaris* in two ecoregions of China. Soil Biology and Biochemistry. 78: 128-137. DOI: 10.1016/j.soilbio.2014.07.026.

Deshwal, V.K. Dubey, R.C. and Maheshwari, D.K. (2014). Isolation of plant growth-promoting *Brady rhizobium* sp with biocontrol potential against *Macrophomina phaseolina* causing charcoal rot of peanut. Journal of Current Science. 84(3): 443-448.

Elzanaty, A.M. Hewedy, O.A. Nagaty, H.H. and Abd-Elbary, M.I. (2015). Molecular and biochemical characterization of some Egyptian genotypes *Rhizobium* (*Vicia faba*) isolates. Journal of Bioengineering and Biomedical Sciences. 5: 145. DOI: 10.4172/2155-9538.1000145.

Gachande, B.D. and Khansole, G.S. (2011). Morphological, cultural and biochemical characteristics of *Rhizobium japonicum* syn and *BradyRhizobium japonicum* of soybean. Bioscience Discovery. 2(1):1-4.

Gauri, Singh A.K. and Bamania, M. (2012). Characterization of *MesoRhizobium* sp. Isolated from root nodules of *Cicer arietinum*. International Journal of Agricultural Science. 2(3): 142-154.

Gilbert, K., Mburu, W.S., Kimiti, J.M., Omwoyo, O., Maingi, M.M. and Ezekiel, M.N. (2018). Genetic characterization and diversity of *Rhizobium* isolated from root nodules of mid-altitude Climbing Bean (*Phaseolus vulgaris* L.) varieties. Frontiers Microbiology Research. 9:968. doi: 10.3389 fmicb. 2018. 00968.

Girija, D., Panchami, P.S., Jose, P.E., Saeed T. and Nair, S.S. (2020). Isolation and Characterization of native cowpea rhizobia from Wayanad India. Legume Research-An International Journal. 43(1): 126-133. DOI: 10.18805/LR-3951.

Hartmann, A. Singh, M. Klingmüller, W. (1983). Isolation and characterization of *Azospirillum* mutants excreting high amounts of indole acetic acid. Canadian Journal Microbiology. 29: 916-923.

Hamza, T.A. and Alebejo, A.L. (2017). Isolation and characterization of *rhizobia* from rhizosphere and root nodule of cowpea, elephant and lab lab plants. International Journal of Novel Research in Interdisciplinary Studies. 4(4):1-7.

Jordan, D.C. and Allen, O N. (1974). Family III. Rhizobiaceae Conn, 1938. In Bergey's Manual of Determinative Bacteriology, 8th edn., (Edited byR.E.Buchanan and N.E. Gibbons). Baltimore, MD: Williams and Wilkins. pp. 261-264.

Kang, J.W. Song, J. Doty, S.L and Lee, D.K. (2013). Diversity of rhizobia associated with leguminous trees growing in South Korea. Journal of Basic Microbiology. 53(3): 291-8. doi: 10.1002/jobm.201100504.

Kleczkowska, J. Nutman, P.S. Skinner, F.A. and Vincent, J.M. (1968). The identification and classification of *Rhizobium*. In: Identification Methods for Microbiologists, Part B. [Gibbs, B.M., Shapton, D.A. (eds)]. Academic Press, London.

Koskey, G. Mburu, S.W. Kimiti, J.M. Ombori, O, Maingi, J.M. and Njeru, E.M. (2018). Genetic characterization and diversity of *Rhizobium* isolated from root nodules of mid-altitude climbing bean (*Phaseolus vulgaris* L.) varieties. Frontiers in Microbiology. 9: 968. Doi:10.3389/fmicb.2018.00968.

Kucuk, C. Kivanc, M. and Kinaci, E. (2006). Characterization of *Rhizobium* Sp. isolated from bean. Turkish Journal of Biology. 30: 127-132.

Laslo, E. Gyorgy, G. Mara, E. Tamas, B. Abraham, S. and Lanyi. (2012). Screening of plant growth promoting rhizobacteria as potential microbial inoculants. Crop Protection. 40: 43-48.

Mahdi S., Sheraz, G. I., Samoon S. A., Rather H. A., Showkat A., and Zehra B. (2010). Bio-fertilizers in organic agriculture. Journal of Physiology. 2(10): 42-54.

Mamun, A. Mehed, M. Hassan, M. Rahman, M. Jakaria, S. and Mujahidy, A. (2013). Isolation and charaterization of *Rhizobium* Spp. and determination of their potency for growth factor production. International Research Journal of Biotechnology. 4(7):117-123.

Manasa, K. Subhash, R. Reddy, S. Triveni, B. Kumar, K. and Gowri Priya N. (2017). Characterization of Rhizobium isolates and their potential PGPR characteristics of different rhizosphere soils of Telangana Region, India. International Journal of Current Microbiology Applied Science. 6(5): 2808-2813.

Martiny, B.J.M. Bohannan, J.H. Brown, R.K. Colwell, J.A. Fuhrman, J.L. Green, M.C. *et al.* (2006). Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology. 4: 102-112. doi: 10.1038/nrmicro1341.

Mondal, H.K and Gera, R. (2020). Molecular diversity of stress-tolerant PGPR rhizobia nodulating cluster bean (*Cynopsis tetragonoloba* L.) grown in hyper-arid zone of Rajasthan. Legume Research- An International Journal. 42(1): 134-139.

Nahar, M. Mahal, Z. Zahid, H.M. Zaman, K. Jahan, F. Rahman, M.M. and Noor, R. (2012). Effects of plasmid curing on *Rhizobium* spp. Stamford Journal Microbiology. 2:1-4.

Naz. I., Bano, A. and UI-Hassan, T. (2009). Morphological, biochemical and molecular characterization of rhizobia from halophytes of khewra salt range and attock. Pakistan Journal of Biotechnology. 41(6): 3159-3168.

Orrell, P. and Bennet, A.E. (2013). How can we exploit above below ground interactions to assist in addressing the challenges of food security. Frontiers in Plant Sciences. 4: 432. doi: 10.3389/fpls.2013.00432.

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Mikrobiologiya. 17: 362-370.

Rahmani H.A., Räsänen L.A., Afshari M. and Lindström K. (2011). Genetic diversity and symbiotic effectiveness of *rhizobia* isolated from root nodules of *Phaseolus vulgaris* L. grown in soils of Iran. Applied Soil Ecology. 48:287-293. Doi:10. 1016/j.apsoil.2011.04.010.

Rajendhran, J. and Gunasekaran, P. (2011). Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence doi: 10.1016/j.micres.2010.02.003.

Rangaswami, G. and Bagayarajm D.J. (1993). Microbial Biotechnology, Agricultural Microbiology. Prentice Hall of India Pvt Ltd, New Delhi. pp 389-405.

Ruiz-Díez, B., Fajardo, S., Felipe, M.R. and Fernandez, P.M. (2012). Characterization of *rhizobia* from legumes of agronomic interest grown in semi-arid areas of Central Spain relates genetic differences to soil properties. Journal of Basic Microbiology. 52(1): 66-78. doi: 10.1002/jobm.201100058.

Sharma, P., Sardana, V. and Kandola, S.S. (2011). Response of groundnut (*Arachis hypogaea* L.) to *Rhizobium* inoculation. Libyan Agriculture Research Center Journal International. 2: 101-104.

Shetta, N.D., Al-Shaharani, T.S. and Abdel-Aal, M. (2011). Identification and characterization of *Rhizobium* associated with woody legume trees grown under Saudi Arabia condition. American-Eurasian Journal of Agricultural and Environ--mental Sciences. 10(3): 410-418.

Somasegaran, P. and Hoben, H.J. (1994). Handbook for *Rhizobia*. Springer-Verlag, New York.

Sridevi, M., Mallaiah, K.V. and Yadav, N.C.S. (2007). Phosphate solubilization by *Rhizobium* isolates from *Crotalaria* Species. Journal of Plant Sciences. 2(6): 635-639.

Timmusk, S., Behers, L., Muthoni, J., Muraya, A. and Aronsson, A.C. (2017). Perspectives and challenges of microbial application for crop improvement. Frontiers in Plant Sciences. 8: 49-56. doi: 10.3389/fpls.2017.00049.

Tyagi, A. Kumar, V. Purushottam, and Tomar, A. (2017). Isolation, identification, biochemical and antibiotic sensitivity characterization of *Rhizobium* strains from *Vigna mungo, Cicer arietinum* and *Vigna radiata*. India. International Journal of Current Microbiology and Applied Sciences. 6(12): 2024-2035.

Vincent, J.M. (1970). A Manual for the Practical Study of Root Nodule Bacteria. In: I.B.P. Handbook No. 15, Blackwell Scientific Publications, Oxford, England. pp 73-97.

Wadhwa, Z. Srivastava, V. Tanvi, R.R. Makkar, K. and Jangra, S. (2017). Isolation and characterization of *Rhizobium* from Chickpea (*Cicer arietinum*). International Journal of Current Microbiology and Applied Sciences. 6(11): 2880-2893. DOI: 10.20546/ijcmas.2017.611.340.

Wilson, K. (2001). Preparation of Genomic DNA from Bacteria. In (Ausubel FM and Brent reds.).