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RESEARCH ARTICLE

Isolation and Physico-chemical Characterization of *Rhizobium* Strains Isolated from *Vigna* Fields of Gird Region of Madhya Bharat

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Abstract

The present study was aimed to isolate the beneficial nitrogen fixing *Rhizobium* from root nodules of *Cowpea* (*Vigna*) plant. Total 155 nodules samples were collected from 15 different fields of Gird region of Madhya Bharat and were subjected to culture on Yeast Extract Mannitol Agar (YEMA). Initially 11 *Rhizobium* strains was isolated from *Cowpea* and characterized on the basis of colony morphology, rod shape, gram negative staining and mucous producing. After 45 d observation in pot nodulation experiments, 4 *Rhizobium* isolates were considered as true *Rhizobium* (R1, R2, R3 and R4) and used for temperature, pH, drought and salt tolerance.

Keywords: *Rhizobium* strains, pot nodulation, temperature, pH, drought, salt tolerance.

Introduction

Rhizobia are symbiotic nitrogen-fixing bacteria and are the most important contributors of fixed nitrogen in soil. Legume inoculation with *Rhizobium* is an age old practice that has been carried out for more than a century in agricultural systems (Qureshi *et al.,* 2009). *Rhizobium* species are well known group of bacteria that act as the primary symbiotic fixer of nitrogen. The bacteria form nodules on the roots or rarely on the stem of legume hosts and by fixing atmospheric nitrogen into ammonia; they provide an easy and inexpensive way to enhance soil fertility and agricultural productivity. Food legumes also constitute an important part of the diet of a large section of the population in the developing world, as good inoculation of forage and grain legumes with *Rhizobia* is an important process to maximize biological N_2 fixation capacity in these crops. Inoculation has also has the potential of increasing dry matter yield, N yield and residual N level (Rathore *et al*., 2009). Madhya Pradesh comprising Gwalior, Morena, Bhind, Shivpuri and Guna districts, is known as Gird sub-zone. This region is rich in alluvial soils but has a large proportion of ravines. About 37% of geographical area is cultivated and out of which only 36% is irrigated. Rainfall is limited to only 670 mm per annum in this area. In India, *Cowpea (Vigna unguiculata* L. Walp) is cultivated by commercial and subsistence farmers. They provide a valuable source of protein and thereby sustaining the nutritional balances of low income populations (Singh *et al*., 1997). In the present study, an effort has been made to isolate *Rhizobium* sp. available in the rhizosphere of non-traditional legume plants grown in the Gird region and study their physico-chemical characterization, which can be further used for the improvement of crop of these non-traditional legumes for efficient nodulation in the field. Further, it can be used in biofertilizer industry to manufacture inoculants for the above crops. Therefore, such plant is thought to be given priorities in the work and the current investigation will be useful for improving plant performance.

Materials and methods

Collection of root nodules: During Sep to Oct of 2012 and 2013, we have extensively visited Gwalior, Morena, Bhind, Shivpuri and Guna districts of Gird regions to collect 155 nodules of *Cowpea* along with roots for field cultivating non-traditional leguminous *Cowpea.* From these fields, we randomly selected about 5 plants per field (Fig. 1)**.** Fifteen root nodule samples of *Cowpea* were collected from different places of Madhya Bharat and numbers of root nodules were also counted (Table 1).

Fig. 1. Effective pink nodule samples on the roots of *Cowpea.*

Table 1. Details of sample, place of *Cowpea* and number of nodules.

S. No.	Crop	Place of sample collection	No. of nodules	Type of nodules
1.	*Cowpea*	Ghati Gaon, Gwalior, MP, India	11	#About 50% nodules were globular large and pink rest was small white
2.	*Cowpea*	Mohna Gaon, Gwalior, MP, India	12	#Almost 75% nodule were globular and pink
3.	*Cowpea*	Baroley Gaon, Gwalior, MP, India	10	#50% were true globular large and pink nodules
4.	*Cowpea*	Chinor Gaon, Dabra, MP, India	11	#50% were true globular large and pink nodules
5.	*Cowpea*	Chhimak Gaon, Dabra, MP, India	5	Very poor small white nodules
6.	*Cowpea*	Amrol Gaon, Dabra, MP, India	11	7 were true globular large and pink nodules
7.	*Cowpea*	Magroni Gaon, Shivpuri, MP, India	12	#50% were true globular large and pink nodules
8.	*Cowpea*	Deegod Gaon, Shivpuri, MP, India	13	#All were true globular large and pink nodules
9.	*Cowpea*	Payaga Gaon, Shivpuri, MP, India	10	Very poor small white nodules
10.	*Cowpea*	Piprauda Gaon, Guna, MP, India	13	7 were true globular large and pink nodules
11.	*Cowpea*	Vinayak khedi Gaon, Guna, MP, India	8	Only 3 were globular medium and pink nodules
12.	*Cowpea*	Aron District, Guna, MP, India	10	#60% were true globular large and pink nodules
13.	*Cowpea*	Saktpur, Guna, MP, India	7	Only 3 were globular medium and pink nodules
14.	*Cowpea*	Roopsahakapura Gaon, Bhind, MP, India	13	#All were true globular large and pink nodules
15.	*Cowpea*	Saroda Dist., Ashoknagar, MP, India	9	50% were true globular large and pink nodules

Isolation of Rhizobium from root nodules: Only globular and pink root nodules of 3-8 mm size were selected from each plant and sterilized initially by immersing in 0.1% (w/v) sodium hypochlorite solution for 5 min followed by 95% (v/v) ethanol for 10 sec and finally washed 6-7 times with sterile water. In order to confirm nodule surface sterility, the tubes were gently shaken for 2 min in sterile water and then an amount of 0.1 mL of the surface-wash water was spread on YEMA plates. The plates were incubated at 29±1°C for 4 d and observed for the growth of any microorganism. After confirmation of the sterility, four nodules from each plant per field were placed in separate sterile Eppendorf tube with 1 mL of sterile H_2O and crushed with sterile rods. The supernatant was drawn and serially diluted up to 10^{-8} dilution. *Rhizobium* cultures were prepared from all dilutions on YEM agar medium containing 0.0025% (w/v) Congo red using spread plate method (Vincent, 1970). The plates were incubated in inverted position for the growth of targeted *Rhizobial* species

Morphological study: The colony morphology of isolates was examined on YEM agar plates. After incubation for 2-3 d at 29±1°C, individual colonies were characterized based on their color, shape, Gram stain reaction, and capacity to produce exo-polysaccharide gum (Jordan, 1984). Microscopic studies were performed using Gram staining for morphology of bacteria as suggested by Graham and Parker (1964). All tests were carried out in triplicates. Before inoculation, strains were grown in YEM broth to log phase (10^8 cell mL^{-1}). According to the procedure developed by Hungia *et al*. (2001), 3 mL of sterile normal saline was added to a slant of pure culture tube of *Rhizobium.* Then scrapped with the help of sterilized (red hot and then cooled) loop and transferred 2 mL to 60 mL sterile YEM broth medium in a 100 mL conical flask inside a laminar air flow cabinet. The cultures were left in incubator shaker (Remi) at 150 rpm and continuous fluorescent light for 5 d. About 30 µL of inoculums was used to score the cells.

Pot nodulation test: All isolates were confirmed by pot nodulation in specific crop for their ability to nodulation (Cregan and Keyser, 1986). For this purpose, plant assay was performed in sterilized soil in plastic pots (*Rhizobium* Inoculants–specification IS 8268:2001).

Surface sterilization of seeds: The seeds were immersed in 95% alcohol followed by surface sterilization in freshly prepared chlorine water or sodium hypochlorite solution (for 15 to 20 min) and then rinsed with several changes of sterile water (at least 10 times) to get rid of the sterilant.

Preparation of sterile pots: Earthen ware or autoclavable plastic pots were filled with soil mixture-two part soil and one part of washed coarse sand (pH 6-7) and autoclaved for 2 h at 120°C. After 2 d incubation at room temperature, autoclaving was repeated to ensure complete sterility of soil.

Inoculation of surface sterilized seeds to sterile pots: Surface-sterilized seeds were inoculated with log phase (10^8 cell mL^{-1}) broth culture of the isolated strains at the rate of 100 g seeds per 5 mL of culture and then sown on the soil in sterilized pots.

A set of pots with un-inoculated seeds as control were also kept alongside and incubated them in a pot culture house during appropriate season. Care was taken to separate the inoculated pots from the control pots.

Effect of strain on nodulation and maintaining the inoculated and control pots: In pot nodulation experiments, 11 isolates of *Cowpea* was tested. The isolates were inoculated on the same crops. About 8-10 seeds were sown in each pot at equal distance. Now each pot was irrigated once to moisture holding capacity with sterilized and cooled plant nutrient solution, prepared earlier. Subsequently, irrigated the seedlings periodically with sterile cooled water preferably through a plastic tube. Care was taken to prevent splashing of water from inoculated pots to un-inoculated one. At the end of 45 d, one set of pots of control and inoculated series was separated and the plants were carefully removed from the soil and washed under slow running water. The data of the number, color and mass of nodules were recorded according to the procedure of Kosslak *et al.* (1983) and McDermott *et al.* (1987).

Temperature tolerance: Test tubes containing 5 mL of YEMB was inoculated with *Rhizobial* isolates. The tubes were then incubated at 10, 15, 30 and 40^{0}C in rotary shaker operated at 180 rpm. The survival ability of each isolates was recorded on 1st, 3rd, 5th, 7th, 10th d by spotting 10 µL on YEMA plate (Aneja, 2003).

pH tolerance: According to Rodriguez *et al.* (2000), the test tubes containing 5 mL of YEMB with pH 3, 7 and 11 were inoculated with *Rhizobial* isolates. The tubes were then incubated at 29±1^{0}C, in incubator shaker at 180 rpm and survival was recorded on 1st, 3rd, 5th, 7th, 10th d by spotting 10 µL on YEMA plate.

Drought tolerance: According to Arrese-Igor *et al.* (2011), the test tubes containing 5 mL of YEMB with 45% Poly Ethlene Glycol (PEG) were inoculated with *Rhizobial* isolates. The tubes were then incubated in incubator shaker at 29±1^{0}C, 180 rpm and survival was recorded on 1st, 3rd, 5th, 7th, 10th d by spotting 10 µL on YEMA plate.

Salt tolerance: Test tubes containing 5 mL of YEMB with 6% NaCl were inoculated with *Rhizobial* isolates. The tubes were then incubated at 29±1^{0}C, in incubator shaker at 180 rpm and survival was recorded on 1st, 3rd, 5th, 7th, 10th d by spotting 10 µL on YEMA plate (Sheik and Wood, 1989).

Results and discussion

In the present study, survey of the native *Rhizobium* sp. population was undertaken in the Gird region of Madhya Bharat, where non–traditional legume crops plant *Cowpea (Vigna unguiculata)* were cultivated in kharif season. Systematically, we have visited the field of Gwalior, Morena, Bhitarwar, Shivpurl, Guna, Bhind and Dabra Gird region of Madhya Pradesh in the month of Sep-Oct 2012 and 2013. Fifteen villages were selected for *Cowpea* (Table 1). During survey, it was observed that most of the samples of root nodules of *Cowpea* were globular, pink and big in size indicating the presence of effective *Rhizobium.* However, we also found few samples with either absence of nodules or presence of only small size white nodules indicating either absence or very poor population of *Rhizobium.* Out of 155 samples of root nodules from *Vigna* plant, initially, 11 strains (CP1-11) were found positive for presence of *Rhizobium.* After pot nodulation experiments, 4 strains were considered as true *Rhizobium* (R1, R2, R3 and R4) and used for temperature, pH, drought and salt tolerance. Similarly Jain *et al.* (2012) also studied the nodulation pattern in soya bean plant. Colonies of *Rhizobium* were obtained on YEMA media after incubation at 29±1^{o}C for 2-3 d. The colonies were having white sticky appearance showing the production of mucous though at lower levels. All isolates were Gram negative and rod shaped as seen under microscope (Fig. 2 and 3). Colony characters include colony size, surface, margin, elevation, optical features and Gram staining (Table 2). In pot nodulation experiments, a total of 11 strains from *Cowpea* were tested for nodulation ability. Observations made after 45 d, showed that all isolates were not able to form nodules. No nodulation was observed in control plant. *Cowpea Rhizobium* isolates CP-2, 5, 6 and 10 successfully produced large pink nodules while isolates 1, 3, 4, 7, 8, 9 and 11 failed to do so. The isolated 4 strains of *Cowpea* (R1, R2, R3 and R4), were considered as true *Rhizobium* and used for temperature, pH, drought and salt tolerance.

Fig. 2. Colony morphology of *Rhizobium Cowpea.*

Fig. 3. Gram staining slide of *Rhizobium Cowpea.*

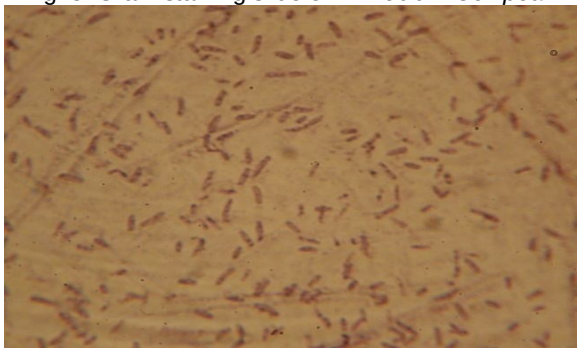

Table 2. Colony characteristics of *Rhizobial* strains under study.

Strain No.	Colony size	Surface	Margin	Elevation	Optical features	Gram staining
R-1	Spreaded	Creamy	Smooth	Raised	Opaque	Negative
R-2	Medium	Shiny	Smooth	Raised	Opaque	Negative
R-3	Spreaded	Shiny	Smooth	Raised	Opaque	Negative
R-4	Medium	Shiny	Smooth	Raised	Translucent	Negative

Table 3. Temperature tolerance of different *Rhizobial* strains.

Strain No.	10°C					15°C					30°C					40°C				
	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d
R-1	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-
R-2	+++	++	++	++	++	+++	++	++	++	++	+++	++	++	++	++	+++	++	++	-	-
R-3	+++	++	++	++	++	+++	+++	+++	+++	++	+++	+++	+++	+++	++	+++	++	++	+	-
R-4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

+= Poor growth; ++= Moderate growth; +++= Better growth; – = No growth.

Table 4. pH tolerance of different *Rhizobial* strains.

Strain No.	pH 3					pH 7					pH 11				
	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d
R-1	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
R-2	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
R-3	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
R-4	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Table 5. Salt tolerance of different *Rhizobial* strains.

Strain No.	100 mM					250 mM					500 mM					1 M				
	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d
R-1	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	++	-	-	-
R-2	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+	-	-	-
R-3	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	-
R-4	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	-

Table 6. Drought tolerance of different *Rhizobial* strains.

Strain No.	30% PEG					45% PEG					60% PEG				
	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d
R-1	+++	++	++	++	++	+++	++	++	-	-	+++	++	-	-	-
R-2	+++	++	++	++	++	+++	+	-	-	-	++	+	-	-	-
R-3	++	++	++	++	++	+++	++	++	++	++	++	++	-	-	-
R-4	+++	++	++	++	++	+++	++	++	++	++	+++	++	+	-	-

Temperature and pH are important parameters for the growth of the organism. Slight variations in pH of medium might have enormous effect on the growth of organism. Our results indicated that all *Rhizobial* isolates (R1-R4) exhibited growth at temperature 10, 15 and 30^{0}C while only one strain R4 was found resistant up to 40^{o}C (Table 3). Superior growth of *Rhizobium* has been reported at neutral pH. Results showed that cells were able to grow only at pH 7 at 30°C. No growth was observed in medium with pH 3 and temperature more than 40°C. Similar observation was made by Sheikh and Wood (1989) and Arrese-Igor *et al.* (2011). Our *Rhizobial* isolates (R1-R4) showed growth at pH 7 and 11 up to 10th d of culture but failed to grow at acid pH (3) which is in accordance with Mensah *et al.* (2006) (Table 4). Further, all *Rhizobial* isolates exhibited growth on YEM medium containing 250 and 500 mM sodium chloride concentration and very poor growth only on 1 M sodium chloride concentration up to 7th d. Our study, therefore reported the isolation of strains highly tolerant to high salt concentrations. Salt tolerant *Rhizobia* may have the potential to improve yield of legumes under salinity stress (Table 5). Drought is the major environmental factor limiting crop production and has a particularly negative impact on symbiotic nitrogen fixation (SNF) (Zahran, 1999; Serraj, 2003; Arrese-Igor *et al*., 2011). In the present study, drought tolerance of *Rhizobium* isolates was performed by culturing in PEG supplement YEM broth medium. All isolates (R1-R4) were tolerant to 30% PEG concentration. While R3 and R4 were sensitive to 45% PEG concentration while some strains showed resistance to 45% PEG and 60%concentration (Table 6). Our findings are in close agreement with Sheikh and Wood (1989) who also characterized the *Rhizobium* from soil and sunflower root nodules with same positive test. The presence of the strains growing under stressed laboratory conditions in our study indicates their significance in contributing biologically fixed nitrogen to stressful ecosystems. This shows the possibility of screening tolerant strains from the soil where they are naturally selected (O'Hara *et al*., 2002). The presence of tolerant strains becomes more interesting since the selected strains are good for nodulation and plant growth.

Conclusion

It can be concluded from the present study that if effort can be made, this type of plants can be grown in Gird region to find out the broad host range of *Rhizobium,* as applicable as agricultural, industrial crops and for large scale inoculant preparation.

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