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PHENOTYPIC CHARACTERIZATION OF RHIZOBIAL STRAINS SYMBIOTICALLY ASSOCIATED WITH *PROSOPIS* SPECIES IN INDIA

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Abstract

With the increasing desertification, legume-rhizobia symbiosis has been found to be a considerable contributor of fixed nitrogen specifically in the arid and semi-arid soils. In the present investigation, root nodule bacteria were isolated by trapping two legumes, *Prosopis cineraria* and *Prosopis juliflora* in soils collected from five sampling sites in India. A total of 32 strains were purified, most of them were fast-growing and two were slow-growing. Ten strains were selected for phenotypic characterization including nine fast-growing and one slow-growing. Strain JNVU PJ1 tolerated up to 5% (w/v) NaCl while most of them could survive up to 2%. All the strains survived at pH range of 6–11 and temperature up to 45°C, while few could grow even at 50°C. Most of the strains were sensitive to antibiotics Ciprofloxacin, Gentamycin, Kanamycin, Neomycin, Streptomycin and Tetracycline; and resistant to Erythromycin. Strains were diverse in terms of utilizing different sugars as sole carbon source. Dextrose was utilized by maximum number of strains (6 strains) and trehalose by 5 strains of the 10 tested. None of them could utilize galactose and inositol. These phenotypic traits and metabolic capability play an important role in survival of rhizobia in stressed conditions. The *Prosopis*-rhizobial strains showing phenotypic and metabolic diversity can be used as inoculums in various agroforestry programs for improving nitrogen content of the depleting soils.

Key words: *Prosopis*, Legume, rhizobia, phenotypic traits

Introduction

The most important limiting factor for crop production is nitrogen. Biological Nitrogen Fixation (BNF) has been found to be a great contributor of fixed nitrogen in the terrestrial ecosystems, as it contributes at least 70 million tons of nitrogen every year (Peoples *et al.*, 1995). Increasing desertification is one of the major effects of global climate change, thus, studying legume-rhizobia combinations that are well adapted to survive in environmental stresses like drought, high temperature and salinity, would be helpful in selecting the inoculants that could survive and contribute to the fixed nitrogen in the arid and semi-arid deserts (Atieno and Lesueur, 2019). Both high and low temperature beyond the optimum range of 25-30°C that is suitable for rhizobia, affects the nodulation and nitrogen fixation in the legumes adversely (Zhang *et al.*, 1995). Thus, screening of rhizobia capable of enduring at high temperatures would be helpful in selecting tolerant strains to be used as inoculants especially in the dry arid regions. Antibiotics are another important marker for selection of efficient rhizobial inoculants as well as for characterization/identification of rhizobial strains at phenotypic level knowing that resistance and susceptibility to antibiotics is quite related with the symbiotic effectiveness (Schwinghamer and Dudman, 1973; Kremer and Peterson, 1982).

Tree legumes, like species of *Acacia* and *Prosopis* that are abundant in the arid and semi-arid deserts prove to be good agent for soil stabilization and nitrogen fixation in association with both fast-growing as well as slow-growing rhizobia (Zhang *et al.*, 1991). The genus *Prosopis* (Tribe, Mimoseae) comprises of approximately 44 known species (Burkart, 1976) and most of the genera belong to arid and semi-arid deserts of America, while very few belong to Africa and Asia (Catalano *et al.*, 2008). Basak and Goyal (1980) suggested 0.8–1.8% as optimum range of salt for survival of rhizobia isolated from tree legumes including *P. cineraria*, similarly temperature range in which rhizobia could best survive was 35–40°C; the pH values below 4 and above 10 was

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found to be unfavorable for rhizobial growth. Aronson *et al.,* (2002) suggested that neutral soils in the arid regions of Central Chile favored nodulation in both *Prosopis* and *Acacia* while nodulation reduced in the slightly acidic soils. Baker *et al.,* (1995) while studying the effect of sodium chloride on the growth and nitrogen fixation of *Prosopis juliflora* demonstrated that at high salinity rhizobia can't survive, thus, adversely affects the formation of nodules.

A number of studies have been conducted on identification and characterization of stress tolerant rhizobia from various native and invasive legume species growing in Indian Thar Desert (Gehlot *et al.,* 2014; Panwar *et al.,* 2014; Tak *et al.,* 2016; Sankhla *et al.,* 2015, 2018; Rathi *et al.,* 2017, 2018; Gaur *et al.,* 2018; Choudhary *et al.,* 2017, 2018, 2020) and few novel Thar-*Ensifer* strains have been analyzed at genome level (Tak *et al.,* 2013; Gehlot *et al.,* 2016; Le Quéré *et al.,* 2017). The phylogenetic diversity of these Thar Desert rhizobia has been discussed in a recent review by Tak and Gehlot (2019).

In the present study we aimed to isolate root nodule bacteria (RNB) symbiotically associated with two species of tree legume, *Prosopis*; one is native to Asia i.e. *P. cineraria* while other is invasive *P. juliflora* (native to Central America) (Burkart, 1976). *Prosopis juliflora* has been introduced and naturalized in India since 1877 for restoration of the wastelands (Prasad and Tewari, 2016). Rhizobia from soils of semi-arid regions of India were trapped in root nodules of these two species of *Prosopis*. Strains were characterized at phenotypic level to screen rhizobia with tolerance to high salt concentration and temperatures as well as asses their metabolic capabilities. Such screening can help to identify efficient rhizobial inoculums for improving agricultural productivity in stressed environments.

Materials and Methods

Soil collection: Soil samples were collected from five sites table 1 in the semi-arid regions of Rajasthan, Haryana and Madhya Pradesh. Rhizospheric soils of *P. juliflora* were collected after digging up to approximately 20–30 cm depth. One month old seedlings of *P. cineraria* were also collected from Arid Forest Research Institute (AFRI) Jodhpur.

Seed germination: Healthy seeds of two legume species *P. cineraria* Fig. 1a and *P. juliflora* Fig. 1b, c growing in the field were collected and scarified with the help of sand paper. Surface sterilization was carried out with 90% (v/v) ethanol and 0.1% (w/v) fungicide Bavistin^R for 1 minute each, followed by 3–4 times rinsing with autoclaved distilled water (DW). Seeds were then transferred to 1% (v/v) sodium hypochlorite for 6 minutes followed by 6 times rinsing with sterile DW (Tak *et al.,* 2020). Post surface sterilization seeds were imbibed in hot water for 15 minutes. Imbibed seeds were placed on autoclaved moist filter paper in petri-plates and kept in dark at 28°C for 2–3 days until the seedlings appeared.

Set up for trapping experiment and excavation of nodules: Soils collected from various sampling sites were filled in small pots sterilized with 90% (v/v) alcohol. Germinated seedlings were planted into these pots and kept in sterilized and controlled conditions of the glass house. Plants were maintained for 8–10 weeks by watering with autoclaved tap water Fig. 1d. Root nodules were excavated from the mature plantlets and nodulation data was recorded Fig. 1e as described in Tak *et al.,* (2020).

Isolation of rhizobia: 3–4 root nodules per plant were surface sterilized with 90% (v/v) alcohol, 0.1% (w/v) Bavistin^R and 1% (v/v) sodium hypochlorite as described in Tak *et al.,* (2020). The surface sterilized nodules were cut into two halves and exudates were streaked onto YEMA (Yeast Extract Mannitol Agar) media supplemented with Congo red (CR) dye as an indicator (Howieson and Dilworth, 2016). White, round, convex, raised colonies with entire margins were picked up from the master plates and purified through quadrant streaking. Purified rhizobial strains were maintained on YEMA petri-plates at 28°C and stored on YEMA slants at 4°C.

Table 1: Nodulation status of *P. cineraria* and *P. juliflora* in different soils (Trap experiments).

Sampling sites for collection of soils	Number of plants trapped	Average number of nodules per plant	
		P. cineraria	*P. juliflora*
Lavan, Dausa, Rajasthan	3	4	2
JNVU New Campus, Jodhpur, Rajasthan	3	1	3
AFRI, Jodhpur, Rajasthan	3	4	Not tested
Narnaual, Haryana	3	17	2
Semariya, Madhya Pradesh	3	9	4

Phenotypic characterization: 10 selected rhizobial strains (listed in table 2) were characterized phenotypically for the following traits:

***Salt and temperature tolerance*:** Strains were streaked on YEMA media supplemented with different concentrations (0.5, 1, 2, 3, 4 and 5) % (w/v) of NaCl and kept in the BOD incubator at 28°C for 3–4 days. While

the range of temperature tolerance was determined by streaking strains onto YEMA media and incubating the streaked plates at different temperatures (35°C, 40°C, 42°C, 48°C, 50°C and 28°C as control) for appearance of bacterial growth.

pH tolerance and BTB (Bromo Thymol Blue) reaction: pH tolerance of the strains was tested by inoculating 10 µl of the activated culture into YEM broth adjusted at pH 4 and spotting on YEM agar plates adjusted at pH 5–11 using 1N HCl, 1N NaOH and different buffers (Howieson and Dilworth, 2016). YEM broth with Bromo thymol blue (BTB) as a pH indicator was used for assessing the ability of strains to produce acid or alkali (Somasegaran and Hoben, 1994). Inoculated YEM-BTB broths were kept at 28°C in an incubator shaker and observed for change in the color to yellow (acidic reaction) or blue (alkaline reaction).

***Carbon (sugar) utilization test*:** Andrade's peptone water (HiMedia Laboratories) was prepared as per the manufacturer's instructions, autoclaved and allowed to cool. The pale straw colored solution was dispensed in sterile 24-well plates and inoculated with activated broth culture of pure rhizobial strains. Twenty-one different HiMedia sugar discs (as listed in table 3) were aseptically added to each well and incubated at 28°C (Tak *et al.*, 2020). Observations were made after every 24 hours. Change of the color from pale straw to pink due to decrease in pH, indicated the metabolism of certain sugars Fig. 1f.

***Intrinsic Antibiotic Resistance (IAR) test*:** IAR was determined with the help of Kirby Boyer's disc diffusion method. The pure rhizobial strains were swabbed on YEMA plates and then HiMedia antibiotic discs of different concentrations (as listed in table 4) were placed aseptically. Plates were incubated at 28°C and the zones of inhibition(s) Fig. 1g were recorded using the scale in mm after 2–3 days.

Results and discussion

Nodulation was observed in the two *Prosopis* species (*P. cineraria* and *P. juliflora*) trapped in soils collected from the semi-arid regions of India. Data for the number of nodules formed per plant in different sampling sites is presented in table 1. Higher nodulation was observed in *P. cineraria* trapped in soils of Haryana and Madhya Pradesh table 1. Comparatively in the soils of Rajasthan,

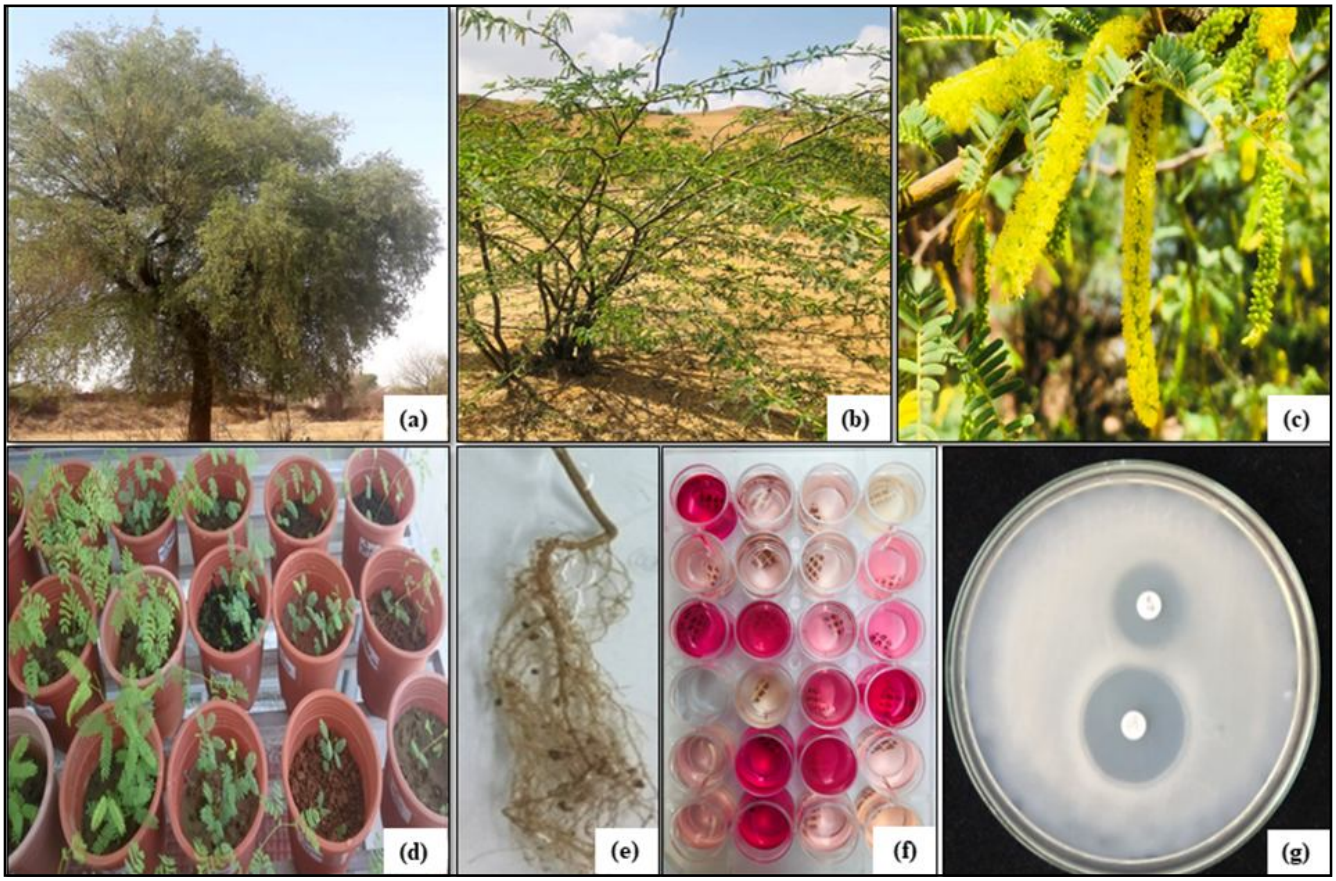

Fig. 1: Field view of *P. cineraria* (a) and *P. juliflora* (b); Inflorescence of *P. juliflora* (c); Trap experiment set-up (d); Roots of *P. juliflora* with nodules attached (e); Pink color observed for sugars utilized by rhizobia in carbon utilization test (f); Zone of inhibition(s) observed in Intrinsic Antibiotic Resistance (IAR) test (g).

Table 2: Few phenotypic traits studied in selective *Prosopis* rhizobial strains.

Strain Name	Salt tolerance Upto (%)	Temperature tolerance range (°C)	pH tolerance range	Bromo Thymol Blue reaction
PC-RJ6	2	32-45	6-11	Acidic
PC-RJ44	2	32-50	6-11	Acidic
PJ-RJ23	2	32-45	6-11	Acidic
PJ-RJ24	2	32-50	6-11	Acidic
JNVU PJ1	5	32-45	6-11	Acidic
JNVU PJ11	2	32-50	6-11	Acidic
JNVU PJ18	2	32-45	6-11	Acidic
PC-HR9	2	32-45	6-11	Acidic
PC-MP25	1	32-50	6-11	Acidic
PJ-MP30	1	32-45	6-11	Neutral

the average number of nodules per plant was less. Plant wise comparison indicates that nodulation in native *P. cineraria* is relatively more than invasive *P. juliflora*. This could be further compared and analyzed by characterizing the compatible rhizobial strains associated with these native and invasive species. Several reasons including soil nutrients, adaptability of the native and exotic plant species, plant genetics and availability of compatible rhizobia in soils can be responsible for the difference in number of nodules per plant in the present investigation.

Thirty two strains were purified from root nodules of both the *Prosopis* species, of which ten strains were selected for phenotypic characterization. Colonies of rhizobial strains purified from both the legume hosts were white, opaque, mucilaginous and exopolysaccharide producing. Out of 32 strains, 30 were fast-growing with high EPS production, while two strains isolated from *P. juliflora* trapped in the soil of Madhya Pradesh were slow growing and showed growth after 4–7 days, with gummy and thick EPS.

Phenotypic traits, majorly expressed by the accessory genome, can be transferred from one strain to another through horizontal gene transfer. Such phenomena are responsible for conferring the important adaptations for ecological success of rhizobia in a particular environment (Remigi *et al.*, 2016). Ten rhizobial strains including nine fast-growing and one slow-growing (PJ-MP30) were characterized for phenotypic traits such as assessing their ability to produce acid or alkali through BTB test; tolerance to survive at different pH, high salt (NaCl) concentrations and high temperatures table 2; ability to utilize various sugars table 3 and Intrinsic Antibiotic Resistance table 4.

Table 3: Sugar (Carbon) utilization pattern of selected *Prosopis* rhizobial strains.

Sugars	PC-RJ6	PC-RJ44	PJ-RJ23	PJ-RJ24	JNVU PJ1	JNVU PJ11	JNVU PJ18	PC-HR9	PC-MP25	PJ-MP30
Adonitol	-	-	-	-	-	+	-	-	+	+
Arabinose	-	-	+	-	+	-	-	+	-	-
Cellobiose	+	-	-	-	-	-	-	+	-	+
Dextrose	-	-	+	-	+	+	-	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	+
Fructose	-	-	+	-	+	-	-	-	-	+
Galactose	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	+	-	-
Lactose	-	-	-	-	-	-	+	+	-	-
Maltose	-	-	+	-	-	-	+	+	-	-
Mannitol	-	-	-	-	-	-	-	+	-	-
Mannose	+	-	-	-	+	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	+	-	+
Raffinose	-	-	-	-	+	+	-	+	-	-
Rhamnose	-	-	-	-	-	+	-	+	-	-
Salicin	-	-	-	-	-	-	-	+	-	+
Sorbitol	-	-	-	-	-	-	-	+	-	-
Sucrose	-	+	-	-	-	-	-	+	-	+
Trehlose	-	-	+	-	-	+	+	+	-	+
Xylose	-	-	+	-	+	-	-	+	-	+

Note: (+) is sugar utilized and (-) is not utilized.

Most of the fast-growing strains tested could survive at 2% NaCl while the slow-growing strain PJ-MP30 tolerated up to 1% only table 2. Interestingly one fast-growing strain JNVU PJ1 could tolerate up to 5% NaCl. Six of the tested strains could best survive between 32-

Table 4: Intrinsic Antibiotic Resistance (IAR) profile of selected *Prosopis* rhizobial strains (Zones of Inhibition measured in mm).

Antibiotics (Concentration in µg)	PC-RJ6	PC-RJ44	PJ-RJ23	PJ-RJ24	JNVU PJ1	JNVU PJ11	JNVU PJ18	PC-HR9	PC-MP25	PJ-MP30
Kanamycin (K^{30})	29	29	30	14	33	21	22	31	20	24
Neomycin (N^{30})	29	40	29	21	28	31	23	40	16	30
Streptomycin (HLS^{300})	33	10	38	34	31	34	35	18	40	40
Gentamycin (Gen^{10})	21	40	21	25	30	22	31	40	22	34
Ciprofloxacin (CIP^{5})	22	35	32	27	23	36	31	28	22	37
Tetracycline (TE^{30})	34	10	37	33	32	34	40	20	29	30
Erythromycin (E^{15})	0	19	0	0	0	0	0	21	0	16

45°C, while few fast-growing strains could grow even at 50°C table 2. All ten strains could grow optimally at pH 6-11, while none of the strains could survive below pH 6. These results are in accordance with previous reports from Basak and Goyal (1980) who had observed that the range of salt tolerance for the strains isolated from *P. cineraria* was between 0.8–1.8%, while Zhang *et al*., (1991) reported up to 3% salt tolerance in the strains isolated from *Prosopis chilensis*. Kulkarni and Nautiyal (1999) have reported the highest temperature tolerance as 60°C, salt tolerance up to 10% and pH up to 12 while testing the *Rhizobium* strains of *P. juliflora* in the alkaline soils. The strains of *Prosopis farcta* showed salt tolerance up to 3% and temperature tolerance up to 40°C (Fterich *et al.,* 2011). The tolerance to high temperature by the strains in this investigation is significant looking to the available reports.

All the fast-growing strains were found to be acid producing, while the one slow-growing strain (PJ-MP30) showed neutral reaction in BTB test table 2. These results are in accordance with the earlier reports for the fast-growing bacteria obtained from Indian Thar desert which were mostly acid producers (Sankhla *et al.,* 2015; Tak *et al.,* 2016; Rathi *et al.,* 2017; Gaur *et al.,* 2018).

Response of different rhizobial strains towards utilization of various sugars as sole source of carbon was variable, with dextrose being utilized by maximum number of strains, while galactose and inositol were not utilized by any of them table 3. Inulin, mannitol and sorbitol were utilized by only one strain PC-HR9 and dulcitol by only PJ-MP30. Strain PC-HR9 utilized 15 of the 21 sugars tested and is most efficient strain in terms of utilization of various sugars and may have better opportunity to grow in the presence of varied sources of carbon present in the environment. Similarly, variability was observed in the intrinsic antibiotic resistance (IAR) profile of the ten strains. All the strains were sensitive towards antibiotics Ciprofloxacin, Gentamycin, Kanamycin, Neomycin, Streptomycin and Tetracycline. Most of the strains were found to be resistant to Erythromycin with the absence of zone of inhibition table 4. Similar results with variable IAR and carbon utilization pattern were found in several RNBs isolated from various wild legumes from the Indian Thar Desert (Sankhla *et al.,* 2015; Tak *et al.,* 2016; Rathi *et al.,* 2017; Choudhary *et al.,* 2017, 2018; Gaur *et al.,* 2018).

The dominance of fast-growing *Ensifer* strains as primary microsymbiont(s) of various native legumes have been reported in several studies from the Thar Desert (see commentary by Ardley, 2017 on Legumes of the Thar Desert and their nitrogen fixing *Ensifer* symbionts). The harsh climatic conditions of desert add various abiotic stresses both on host and microsymbiont resulting in a long-term co-evolution between the two symbiotic partners leading to selection of a specific type of symbionts (Tak *et al.,* 2016; Rathi *et al.,* 2018; Gehlot *et al.,* 2018; Tak and Gehlot, 2019). Such rhizobial strains with wide range of tolerance for the various parameters studied in this investigation needs to be further characterized at molecular level and can be cross-inoculated on the crop plants for inferring range of hosts that can be nodulated by these tree-rhizobia. These stress tolerant tree-rhizobia could be used as potential inoculants for improving the agricultural productivity and nitrogen content of soils in the stressed arid environments of deserts.

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