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GENETICALLY DIVERSE ROOT NODULE BACTERIA ASSOCIATED WITH *ALYSICARPUS VAGINALIS* FROM ALKALINE SOIL OF RAJASTHAN, INDIA

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Abstract

About forty eight rhizobial strains were isolated and purified from the root nodules of *Alysicarpus vaginalis* growing in alkaline soil of eight different districts of Rajasthan. Rhizobial strains were initially grouped on the basis of colony characteristics as well as growth time and placed into three different colony groups comprising of both slow and fast growing rhizobia. Interestingly many of these strains showed some PGP traits like phosphate solubilization. On the basis of PCR-RFLP (ARDRA) pattern ten different genetic groups were formed showing significant genetic diversity among the strains. Sequencing of 16S rRNA gene of one strain from each of the three major phenotypic groups and the BLASTn results showed that the fast growing strains belong to the genera *Ensifer* (Colony group-I) and *Rhizobium* (Colony group- II) whereas the representative strain of third colony group (slow growing) belongs to the genus *Bradyrhizobium*. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain AV11 was identical to *Ensifer aridi* strains and showed close similarity with the type strains *E. kostiensis* and *E. saheli*. Fast growing strain AV21 showed similarity with *Rhizobium aegyptiacum*, *R. bangladeshense* and *R. binae*. The slow growing strain AV34 has close similarity with type strain *Bradyrhizobium vignae*. It is suggested that in the alkaline soil of Rajasthan, *Alysicarpus vaginalis* is nodulated with diverse type of fast and slow growing rhizobia's that needs further characterization.

Key words : *Alysicarpus*, Thar Desert, ARDRA, *Ensifer*, *Rhizobium*, *Bradyrhizobium*.

Introduction

The genera *Alysicarpus* Necker ex Desvaux consists of 25 to 30 species. It is a member of tribe Desmodieae and sub-tribe Desmodiinae under the sub-family Papilionoideae. It is distributed mainly in Africa, India, Malaysia, China, Japan and Australia. On the basis of certain molecular evidences tribe, Desmodieae is nested within the tribe Phaseoleae and both these tribes possess determinate types of root nodules (Sprent, 2009). In India, the genus *Alysicarpus* is represented by approximately 18 species, of which seven are endemic (Pokle, 2002). In Rajasthan approximately 12 different species of *Alysicarpus* are known (Shetty and Singh, 1987) whereas 5-6 species of *Alysicarpus* have been described from the Western Rajasthan in Flora of Indian desert (Bhandari, 1990).

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Occurrence of root nodules in *Alysicarpus vaginalis* has already been reported from India (Gehlot *et al*., 2012; Ojha *et al*., 2015) and other places of the world and almost all reported cases are showing association of slow growing strains supposed to be *Bradyrhizobium* (Doignon-Bourcier *et al*., 2000) except Leigh and Coplin (1992) who studied the tumor like structure formed by *Rhizobium* species on roots of *A. vaginalis*. Root nodule bacteria (RNB) are also known for possessing plant growth promoting (PGP) activity such as indole acetic acid (IAA) production, phosphate solubilization in addition to fixing nitrogen. Bhattacharyya and Pati (2000) isolated *Rhizobium* sp. from root nodules of *A. vaginalis* and described their growth behavior and IAA production. Prasuna (2014) also isolated *Rhizobium* species from *A. vaginalis* and characterized them on basis of antibiotic resistance. However, the latter two reports on *Rhizobium* are not supported with molecular identification of

symbiont.

The establishment of legume-rhizobia symbiosis in a unique stressed ecosystem of Indian Thar desert is ecologically and agriculturally important. The native desert-rhizobia associated with wild legumes may exhibit higher tolerance towards abiotic stresses (Sprent and Gehlot, 2010). Fast growing, broad host range *Ensifer* strains have been repeatedly isolated from number of native legumes belonging to various tribes in all the subfamilies of Leguminosae from the Indian Thar desert (Gehlot *et al*., 2012; Gehlot *et al*., 2013; Tak *et al*., 2013; Panwar *et al*., 2014; Ojha *et al*., 2015; Sankhla *et al*., 2015; Gehlot *et al*., 2016; Tak *et al*., 2016 (a); Tak *et al*., 2016 (b); Sankhla *et al*., 2017; Choudhary *et al*., 2017; Le Quere *et al*., 2017). Therefore, the aim of present study is to explore and characterize the rhizobial diversity associated with *A. vaginalis* growing in nutrient poor and alkaline soil of Thar Desert at phenotypic and genetic level and also to screen them for some PGP traits. It is also important to check if the dominant novel *Ensifer aridi* (Tak *et al*., 2016b; Le Quere *et al*., 2017) type of strains with broad host range nodulates the *A. vaginalis* belonging to the tribe Desmodieae (members of tribe are known to be nodulated mainly by *Bradyrhizobium*) in the alkaline soil of Thar Desert. The present study will add information about the diverse native N fixing rhizobia's associated with wild legume *A. vaginalis* in the alkaline and arid soil of Indian Thar Desert and their potential to be used as plant growth promoting bacteria.

Methodology

Field survey, soil collection and rhizobia trapping

A field survey was carried out during monsoon and post monsoon season (2013-2016) to record the nodulation status of *A. vaginalis* and the soil samples were collected from 24 different sites of arid and semi-arid zones of Rajasthan (fig. 1). The rhizobia's were trapped (for detailed methods refer to Sankhla *et al*., 2017) inside the root nodules by growing *A. vaginalis* plants in different soils (table 1).

Nodule excavation, nodule characteristics and isolation of rhizobial strains

Plants of trap experiment were harvested after eight weeks with intact root system to record nodule numbers, shape, size, morphology and position on roots. Nodules were carefully detached with small intact root for preservation of nodules and isolation of rhizobia. Root nodule bacteria of *A. vaginalis* were isolated and purified under aseptic condition using standard protocol as described by Somasegaran and Hoben (1994). Purified bacterial strains were maintained on Tryptone Yeast (TY) agar plates or Yeast Extract Mannitol Agar (YEMA) plates supplemented with Congo red dye (CR) and incubated at 28ºC temperature in microbial chamber. YEM agar plates were checked regularly to record the growth and colony characteristics of isolates.

Phenotypic, biochemical and PGP characterization of rhizobial strains

Colony characteristics

The colonies were observed regularly on YEMA-CR plates after inoculation for colony growth time (fast or slow growing), shape (circular, convex, entire, raised, etc.), size (colony growth in mm), EPS production, pigmentation (if any), nature of colonies i.e. acid or alkali production, gummy, glistering, milky, opaque or translucent, etc. (Bergey's Manual of determinative bacteriology, 9th ed., 1994).

Salt tolerance, carbon utilization and IAR pattern

The rhizobial strains were evaluated for their competence to grow on YEMA media under various salt concentrations (0.5%, 1%, 2% and 3%). Carbon utilization profiles of selected strains were performed using HiMedia sugar discs following manufacturer's instructions. A total of 21 sugars were tested using Andrade's peptone water. The sensitivity or resistance of rhizobial strains to an antibiotic was determined using HiMedia antibiotic discs on YEM agar plates (Cappuccino and Sherman, 2007). The sugar and antibiotic discs used are given in tables 2 and 3.

Biochemical, bio-control and PGP activities

The isolates were also investigated for their various biochemical properties such as nitrate reduction (Cappuccino and Sherman, 2007), production of amylase enzyme that catalyzes the hydrolysis of starch into glucose (Cappuccino and Sherman, 2007) and production of other hydrolytic enzymes such as cellulase (Kasana *et al*., 2008), protease (Smibert and Krieg, 1994), pectinase (Ronald and James, 2006) and chitinase (Kim *et al*., 2003). Phosphate is one of the most essential elements for the growth of plants. Microbes have potential to degrade and solubilize the insoluble phosphates into soluble forms by secreting some organic acids or phosphatases. Phosphate solubilizing bacteria (PSB) were screened on Pikovskaya's (PVK) agar medium plates supplemented with calcium triphosphate as described by Pikovskaya (1948). Rhizobial strains were also tested for their ability to hydrolyze the phytic acid to myo-inositol and phosphoric acid. Bacterial strains were screened for phytase activity on Phytase agar medium supplemented with calcium

Fig. 1 : Google map view of various sampling sites studied in present investigation throughout Rajasthan.

phytate (Howson and Davis, 1983). Production of ammonia was also determined by growing pure activated rhizobial strains in peptone water and after incubation 1ml of Nessler's reagent was added to each tube (Cappuccino and Sherman, 2007).

Molecular characterization of rhizobial strains

Genomic DNA extraction

Genomic DNA of the rhizobial strains was extracted using the phenol-chloroform method described by Cheng and Jiang (2006). The purified DNA of concentration 100-1000 ng/µl was used as a template for amplification of small subunit ribosomal RNA (16S rRNA) gene.

16S rRNA PCR amplification

PCR amplification of the nearly full-length 16S rRNA gene was performed using two universal primer pair: (i) 18F (AGAGTTTGATCCTGGCTCAG) and 1492R (CTACGGCTACCTTGTTACG) used for *Ensifer* and *Rhizobium* strains (ii) fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (CTTAAGGAGGTGATCCAGCC) was used for *Bradyrhizobium* strains (Weisburg *et al*., 1991). The amplification was carried out in 0.2 ml PCR tubes containing 25 µl of final reaction volume. The reaction mixture contained 15.25 µl of sterile Milli-Q water, 2.5 µl of Taq buffer (10X), 2 µl of $MgCl_2$ (25 mM), 1.5 µl of dNTP mix (2.5 mM each), 1.25 µl of DMSO (100%), 0.5 µl of each primer (50 µM), 0.25 µl (3U/µl) of *Taq* DNA polymerase (Genei Bangalore). After addition of 1.25 µl (approx. 100 ng) DNA template, PCR tubes were placed in thermocycler (BioRad T100). Thermal cycling conditions used for 18F/1492R primer is as follows: initial denaturation 95°C for 5 min followed by 35 cycles of 94ºC for 60s, 53ºC for 60s, 72ºC for 60s and a final extension at 72°C for 7 min. PCR cycling conditions used for fD1/rD1 primer is as follows: initial denaturation 95°C for 5 min followed by 30 cycles of 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min and a final extension at 72°C for 7 min. PCR amplified product (size approx. 1500 bp) along with 500bp DNA ladder (Genei Bangalore) were electrophoresed in a 0.8% agarose and visualized using ethidium bromide staining on BIO-RAD Gel Doc system.

DNA fingerprinting: Amplified rDNA Restriction Analysis (ARDRA)

Genetic diversity of a rhizobial strain was assessed by ARDRA (PCR-RFLP) (Laguerre *et al*., 1994) using tetracutter restriction endonuclease enzyme *Sau*3AI. A reaction mixture of 20µl was prepared constituting 7.45 µl of nuclease free water, 2.5U of enzyme (0.25 µl), 0.3 µl Bovine Serum Albumin (BSA), 2 µl of 1X buffer and10 µl of amplified PCR product (16S rRNA). The mixes were incubated for overnight at 37°C for restriction digestion. The digested PCR products were separated by running on 2% agarose gel at 80 V for 1-2 hrs and banding patterns were visualized using ethidium bromide staining on BIO-RAD Gel Doc system.

Table 1 : Origin and grouping of rhizobial strains isolated from *Alysicarpus vaginalis*.

Sampling site	Name of rhizobial strain	Growth time (days)	Grouping based on Colony characters	PCR-RFLP ARDRA) (Groups	NaCl Tolerance (%)
JNVU, Jodhpur	AV 2	3	**Group I** White, translucent, raised, convex, round, entire margin, non-gummy, highly mucilaginous, giving curdling appearance, less acid producing not turning color of YEMA-CR media to purplish.	I	2
Kaylana, Jodhpur	AV 3	3		II	2
Kaylana, Jodhpur	AV 4	3		II	2
Polytechnic college, Jodhpur	AV 5	3		I	3
Pal Balaji, Jodhpur	**AV 6**	3		I	3
Machia Biological Park, Jodhpur	AV 7	3		II	2
Osian, Jodhpur	AV 8	3		I	3
Phalodi, Jodhpur	AV 9	3		II	3
Salasar, Churu	AV 10	3		II	3
Bikaner	**AV 11**	3		II	2
Nokha, Bikaner	AV 12	3		III	1
Sujangarh, Churu	**AV 16**	3		II	2
Laxmangarh, Sikar	AV 17	3		II	1
Kuldhara, Jaisalmer	**AV 18**	3		I	2
Bhadrajun, Jalore	AV 23	3		IV	3
Baroodi, Jalore	**AV 26**	3		II	2
Chudiyas, Nagaur	AV27	2		IV	2
JNVU, Jodhpur	AV 1	2	**Group II** White, opaque, raised, convex, round, entire margin, non-gummy, highly mucilaginous, acid producing turns color of YEMA-CR media to purplish.	VII	1
Nokha, Bikaner	AV 13	2		VII	1
Bagra, Jalore	AV 14	2		VIII	1
Barmer	**AV 15**	2		VII	1
Khetolai, Jaisalmer	**AV 19**	2		VII	1
Khetolai, Jaisalmer	AV 20	2		VII	1
Jalore	**AV 21**	2		VII	2
Bagra, Jalore	AV 22	2		VII	1
Bhadrajun, Jalore	AV 24	2		VIII	1
Baroodi, Jalore	**AV 25**	2		VIII	1
Laxmangarh, Sikar	AV 28	2		VII	2
Osian, Jodhpur	AV 29	2		VII	2
Machia Biological Park, Jodhpur	AV 30	8	**Group III** White, opaque, raised, convex, round, entire margin with smooth edge, glistering, gummy, moderate mucilage producing.	VI	0.5
Pal Balaji, Jodhpur	AV 31	7		V	0.5
JNVU, Jodhpur	AV 32	8		V	0.5
Kaylana, Jodhpur	AV 33	8		V	0.5
Osian, Jodhpur	**AV 34**	8		V	0.5
Balotra, Barmer	AV 35	7		V	0.5
Salasar, Churu	AV 36	8		IX	0.5
Barmer	AV 37	8		V	0.5
Bagra, Jalore	AV 38	8		V	0.5
Bikaner	AV 39	7		V	0.5
Nokha, Bikaner	AV 40	8		VI	0.5
Ahore, Jalore	AV 41	8		V	0.5
Kuldhara, Jaisalmer	**AV 42**	8		VI	0.5
Sujangarh, Churu	AV 43	8		VI	0.5
Laxmangarh, Sikar	AV 44	8		V	0.5
Jhareli, Nagaur	AV 45	8		VI	0.5
Phalodi, Jodhpur	**AV 46**	8		VI	0.5
Jalore	AV 47	8		VI	0.5
Laxmangarh, Sikar	**AV 48**	7		X	0.5

Strains in bold letters were studied for carbon (sugar) utilization and antibiotic resistance.

Table 2 : Carbon utilization pattern of selective rhizobial strains associated with *Alysicarpus vaginalis*.

Carbon (sugar) source	Group I (*Ensifer*)*					Group II (*Rhizobium*)*				Group III (*Bradyrhizobium*)*			
	AV6	AV11	AV16	AV18	AV26	AV15	AV19	AV21	AV25	AV34	AV42	AV46	AV48
Adonitol	+	+	+	+	+	-	-	-	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	-	-	-	+
Cellobiose	+	+	+	+	+	+	+	+	+	-	-	-	-
Dextrose	+	+	+	+	+	+	+	+	+	-	-	-	+
Dulcitol	+	+	+	+	+	-	-	-	-	-	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	-	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	-	+	+	-
Inositol	+	+	+	+	+	+	-	+	+	-	+	+	-
Inulin	+	+	+	-	+	-	-	-	+	-	+	+	+
Lactose	+	+	+	+	+	+	+	+	-	-	+	+	-
Maltose	+	+	+	+	+	+	+	+	+	-	+	+	-
Mannitol	+	+	+	+	+	+	+	+	-	+	-	-	-
Mannose	+	+	+	+	+	+	+	+	+	-	+	+	+
Melibiose	+	+	+	+	+	-	-	-	+	+	+	+	+
Raffinose	+	+	+	-	+	-	-	-	+	-	-	-	+
Rhamnose	+	+	+	+	+	-	-	-	+	-	-	-	+
Salicin	+	+	+	+	+	+	+	+	-	-	+	+	-
Sorbitol	+	+	+	+	+	-	-	-	+	-	+	+	+
Sucrose	+	+	+	+	+	-	-	-	+	-	+	+	-
Trehalose	-	+	+	+	+	-	-	-	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	-	+	+	+

(+) indicates sugar utilized and (-) with grey fill indicates sugar not utilized by strain.
* On the basis of 16S rRNA gene sequencing of representative strain from different colony groups.

Sequence analysis and phylogeny

Sequencing of the amplified PCR product (16S rRNA gene) was done by outsourcing through SciGenom Labs Private Ltd., Cochin, India. The sequences were analyzed using GeneTool Lite (version 1.0 Double Twist Inc., Oakland, CA, USA). The nucleotide sequences obtained after editing were used for sequence similarity search using nucleotide blast (BLASTn) [http://blast.ncbi.nlm.nih.gov]. After complete analysis the gene sequences were submitted to NCBI database using Sequin. The phylogenetic tree was constructed using software MEGA 7 (Kumar *et al.*, 2016) with the maximum likelihood method based on a GTR+G+I model. Confidence values for topologies were assessed using bootstrapping (1000 replicates).

Results and Discussion

Nodulation status and origin of strains

Alysicarpus vaginalis (L.) DC. plants are erect or spreading, perennial or annual herbaceous legume, leaflets broadly to narrowly oblong elliptic, sometimes few of them are lanceolate (fig. 2A), flowers are purplish in terminal raceme (fig. 2B), pods are 1.2-2.5 cm long (fig. 2C), seeds smooth yellowish to brownish red in color (fig. 2C). Nodules were distributed singly or in cluster of 2-5 on collar region, primary roots and few also found on secondary roots (fig. 2D). In *A. vaginalis* determinate desmodioid globular types of nodules were identified (Fig. 2D and 2E). The nodule morphology is in accordance with the tribe Desmodieae (Sprent, 2009). Sizes of nodules vary from 0.2 mm to 10 mm which is in accordance with Gehlot *et al.* (2012). In present investigation a total of forty eight rhizobial strains were isolated and purified from the root nodules of *A. vaginalis* from eight different districts of Rajasthan (table 1).

Phenotypic, biochemical and PGP characteristics of rhizobial strains

Both slow-growing (*Bradyrhizobium* type of strains) and fast-growing (*Ensifer* and *Rhizobium* type of strains) rhizobial colonies were identified as root nodule bacteria of *A. vaginalis*. Slow growing colonies appearance were whitish, opaque, raised, convex, round in shape with entire

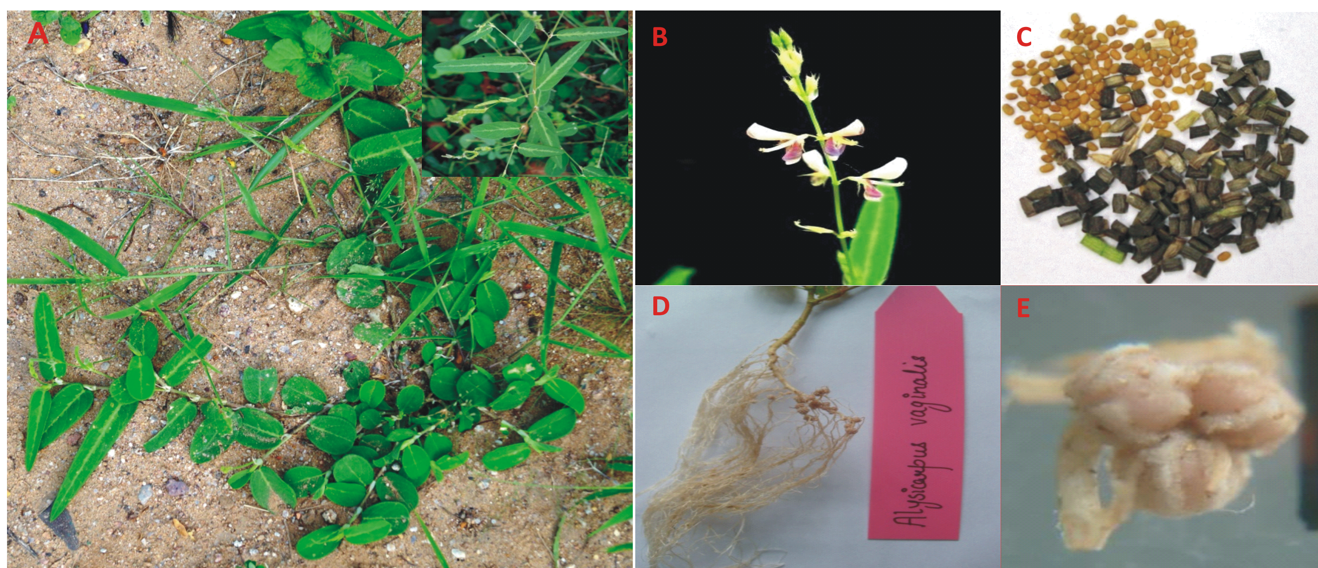

Fig. 2 : *Alysicarpus vaginalis* plant growing in open field with immature pods focused in inset image (A), flowers (B) collected mature pods and seeds (C) excavated plant roots with nodules (D) typical desmodioid root nodules (E).

Table 3 : Intrinsic antibiotic resistance (IAR) pattern of selective rhizobial strains associated with *Alysicarpus vaginalis* (Zone of inhibition in mm)

Antibiotics (concentration of antibiotic in µg)	Group I (*Ensifer*)					Group II (*Rhizobium*)				Group III (*Bradyrhizobium*)			
	AV6	**AV11**	**AV16**	**AV18**	**AV26**	**AV15**	**AV19**	**AV21**	**AV25**	**AV34**	**AV42**	**AV46**	**AV48**
Carbenicillin (CB^{100})	22	22	0	0	24	30	13	30	21	0	0	12	0
Ciprofloxacin (CIP^{10})	36	36	22	40	36	40	32	40	0	25	23	31	0
Co-trimazine (CM^{25})	37	37	0	36	37	0	12	0	0	0	0	0	0
Kanamycin (K^{30})	35	35	24	22	34	35	25	35	13	11	14	23	0
Nitrofurantoin (NIT^{300})	0	0	0	0	0	0	0	0	0	0	0	21	0
Streptomycin (S^{10})	30	40	22	28	40	30	33	30	35	21	<40	24	28
Tetracycline (TE^{30})	40	<40	30	40	40	<40	<40	<40	30	0	0	25	0
Amikacin (AK^{10})	28	28	20	26	28	29	26	29	34	35	33	22	24
Gentamicin (GEN^{10})	25	25	35	25	22	21	24	21	32	0	0	25	24
Co-trimoxazole (COT^{25})	21	21	22	29	21	0	0	0	0	0	0	0	0
Levofloxacin (LE^{5})	27	27	24	32	26	36	35	36	28	0	28	32	0
Netillin (NET^{30})	22	22	0	26	22	20	24	20	30	0	0	19	0
Amoxyclav (AMC^{30})	0	0	0	0	0	0	0	0	0	0	0	24	0
Ofloxacin (OF^{5})	0	0	0	22	0	22	0	23	23	0	18	30	0
Ceftriaxone (CTR^{30})	15	15	0	0	15	29	0	29	24	0	0	21	0

margin and smooth edges, glistering, gummy, moderate mucilage producing and takes about 5-8 days to grow on YEM agar plates while fast growing colonies were white, translucent/opaque, raised, convex, round, entire margin, non-gummy, highly mucilaginous and colonies appeared in 2-3 days. Few strains giving curdling appearance and some of them turned color of YEMA-CR media from red to purplish. Grouping of strains on the basis of colony morphology and the growth time (in days) of each strain is presented in table 1. The strains were highly variable in terms of tolerance to NaCl (table 1). Graham and Parker (1964) reported that fast-growing rhizobia were generally more salt tolerant than the slow-growing rhizobia. In the present work six fast growing rhizobial strain showed tolerance upto 3% NaCl (w/v) while remaining fast growing strain were able to tolerate salt upto 2% whereas slow growing rhizobial strain did not tolerate even 1% of salt.

Graham and Parker (1964) and many others had reported that the fast-growing rhizobia tend to use a wider variety of carbohydrates than the slow growers. In accordance with the above mentioned literature the rhizobial strains of colony group type I (fast growers) like AV11, AV16 and AV26 were able to utilize all the 21 sugars tested while AV6 and AV18 of the same colony group were not able to utilize trehalose and inulin and raffinose respectively. The fast growing strains AV15, AV19 and AV21of colony group-II showed similar C utilization pattern while strain AV25 showed different utilization pattern (table 2). Strains AV42 and AV46 of slow growing colony group-III were similar to each other in C utilization trait while other two strains AV34 and AV48 showed variable pattern. Strain AV34 utilized only four sugar out of twenty one tested. Our results suggest that few strains have wider range of C utilization for their survival under local soil and environmental conditions.

Tested rhizobial strains showed variable intrinsic antibiotic resistance pattern as shown in table 3. Madrzak *et al*. (1995) reported that the fast growing strains were more sensitive to antibiotics than slow growing rhizobia. The present study results also support same contention where all the tested slow growing strains showed resistance to maximum antibiotic tested except AV46 whereas fast growers showed sensitivity towards maximum antibiotics. All the tested strains were resistant to nitrofurantoin (NIT^{300}) and amoxyclav (AMC^{30}) except one slow growing strain AV46. Strain AV48 showed resistance to maximum antibiotic tested and was sensitive for three antibiotic namely streptomycin (S^{10}), amikacin (AK^{10}) and gentamicin (GEN^{10}).

All the tested forty eight rhizobial strains showed variation in their biochemical traits and plant growth

Table 4 : Biochemical test and plant growth promoting activities shown by rhizobial strains associated with *Alysicarpus vaginalis*.

Test	Strain showing high activity	Strain showing moderate activity	Strain showing slight activity
Nitrate reduction	AV5, AV13, AV14, AV15, AV19, AV27, AV30, AV34, AV35, AV37, AV40, AV41, AV42, AV43, AV46, AV47	AV1, AV8, AV11, AV17, AV18, AV28	AV2, AV4, AV9, AV10, AV12, AV16, AV21, AV23, AV24, AV25, AV26, AV29, AV32, AV36, AV44
Cellulase activity	AV36, AV38, AV39, AV41, AV44, AV47	AV18, AV19, AV24, AV30, AV31, AV32, AV34, AV35, AV37, AV40, AV42, AV43, AV45, AV48	AV23, AV25, AV27, AV28
Phosphate solubilization	AV5, AV15	AV6, AV8, AV11, AV16, AV18, AV23, AV36, AV37	AV1, AV2, AV3, AV4, AV10, AV13, AV14, AV15, AV17, AV19, AV20, AV21, AV22, AV24, AV25, AV28, AV29, AV34
Phytase activity	AV6, AV8, AV10, AV16, AV17, AV19, AV23, AV24, AV27, AV29	AV2, AV3, AV4, AV5, AV11, AV14, AV26, AV31	AV1, AV13, AV18, AV20, AV21

High (+++), moderate (++) and slight (+).

Table 5 : Percentage sequence similarity of rhizobial strains isolated from root nodules of *Alysicarpus vaginalis* with closest type strains based on 16S rRNA gene.

Strain	NCBI GenBank accession number	Closest type strain	Geographical origin of type strain	Host plant	Sequence Similarity (%)
AV11	KT781665	*E. kostiensis* LMG 19225^T (NR_042484)	Sudan	*Prosopis chilensis*,	100
		E. saheli LMG 7837^T (NR_026096)	Senegal	*Sesbania cannabina*	
AV21	KT781670	*R. aegyptiacum* 1010^T (NR_137399)	Egypt	*Trifolium alexandrinum*	
		R. bangladeshense BLR175^T (NR_137241)	Bangladesh	*Lens culinaris*	
		R. binae BLR195^T (NR_137242)	Bangladesh	*Lens culinaris*	100
AV34	KR071001	*B. vignae* 7-2^T (KP899563)	Namibia	*Vigna unguiculata*	100

Abbreviations: *B*., *Bradyrhizobium*; *E*., *Ensifer*; *R. Rhizobium*, NR, NCBI reference and ^{T}, type strain.

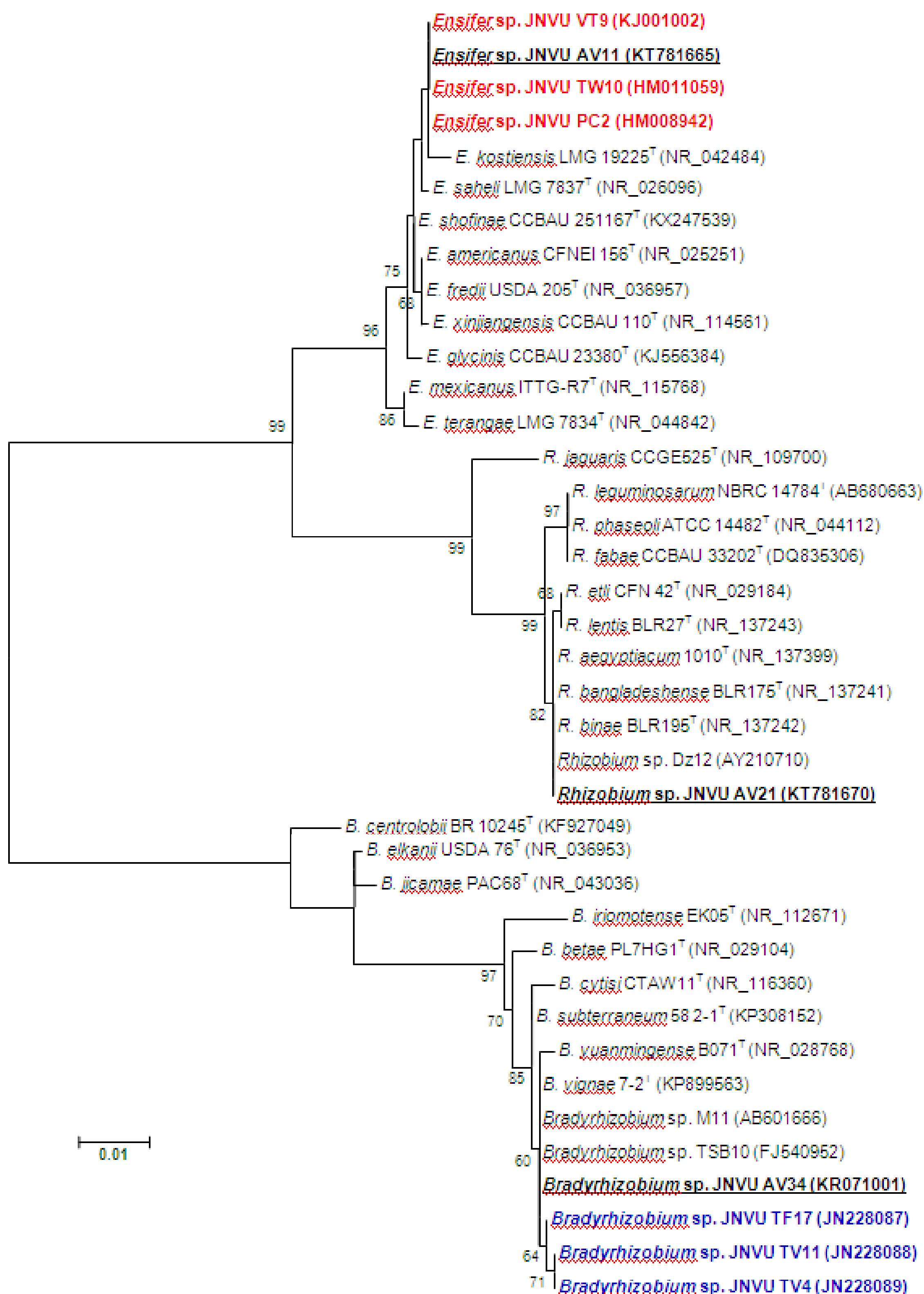

Fig. 3 : Maximum Likelihood tree using 16S rRNA gene sequences of selective type strains of *Bradyrhizobium*, *Ensifer* and *Rhizobium* along with RNB strains associated with *Alysicarpus vaginalis* (AV) growing in Thar Desert. The scale bar indicates 1% nucleotide substitution per site. GenBank accession numbers are given in parenthesis. (Abbreviations: *B*., *Bradyrhizobium*; *E*., *Ensifer*; *R*. *Rhizobium*, NR, NCBI reference and ^{T}, type strain).

promoting activities (table 4). About 77% rhizobial strains were found positive for nitrate reductase test, 50% strains were positive for cellulase activity, 58% strains could solubilize tri-calcium phosphate and formed halo zones and 48% strains showed positive phytase activity. All tested rhizobial strains showed negative results for production of various hydrolytic enzymes such as amylase, protease, pectinase and chitinase and also for ammonia production. The significant variation in phenotypic, biochemical and PGP traits of strains may explain that these traits are plasmid borne and the variation among these features is more often affected by horizontal gene transfer (HGT) among the variety of soil bacteria. The various stressed environmental condition may results in such frequent horizontal gene transfer (Amina and Amin, 2010).

Assessment of genetic diversity

The strains belonging to three phenotypic groups (based on colony characteristics) were assessed for their genetic diversity. In present study DNA fingerprinting based on PCR-RFLP (ARDRA) of total 48 rhizobial strains was carried out. The ARDRA of bacterial strains resulted in total 10 groups (I - X) as given in table 1. Colony group-I and colony group-II comprising of fast growing rhizobial strains formed four (I to IV) and two ARDRA groups (VII and VIII) respectively. Colony group-III comprising of slow-growing rhizobial strains formed four ARDRA groups (V, VI, IX and X). ARDRA grouping of these 48 purified strains clearly reflected that the RNB strains associated with *A. vaginalis* are genetically diverse and hence it will be interesting to carry out further phylogenetic studies on these strains.

Identification and phylogeny of root nodule bacteria

In the present investigation, one representative strain (AV11, AV21 and AV34) from each (three) major colony group were identified as strains of *Ensifer* (colony group-I), *Rhizobium* (colony group- II) and *Bradyrhizobium* (colony group-III) respectively on the basis of 16S rRNA gene sequencing and BLASTn search results (table 5). The results confirmed that *A. vaginalis* is nodulated by both fast as well as slow growing rhizobia. In the phylogenetic tree constructed using 16S rRNA gene the three strains (AV11, AV21 and AV34) clustered within *Ensifer*, *Rhizobium* and *Bradyrhizobium* type strains respectively supported by strong bootstrap values (fig. 3).

The strain AV11 was identical to *Ensifer aridi* type of strains like TW10 (isolated from *Tephrosia wallichii*) (Tak *et al*., 2016b) and VT9 (isolated from *Vigna trilobata*) (Tak *et al*., 2016a). It also showed close similarity with the type strains *E. kostiensis* LMG 19225^T (isolated from *Prosopis chilensis,* Sudan) and *E. saheli* LMG 7837^T (isolated from *Sesbania cannabina,* Senegal). Occurrence of *Ensifer* type of strains (colony group-I) from the root nodules of *A. vaginalis* confirms that the native novel *Ensifer aridi* type (Le Quere *et al*., 2017) of strains from alkaline soil of Thar Desert of India has broad host range and do nodulates members of tribe Desmodieae, although study of symbiotic genes may throw light about acquisition of *sym* genes. The fast growing strain AV21 (colony group-II) showed close similarity with newly described type strains *Rhizobium bangladeshense* BLR175^T and *R. binae* BLR195^T isolated from root nodules of lentil (*Lens culinaris*) from Bangladesh (Rashid *et al*., 2015) and *R. aegyptiacum* 1010^T isolated from Egyptian clover (*Trifolium alexandrinum*) nodules from Egypt (Shamseldin *et al*., 2016). The slow growing strain AV34 (colony group-III) isolated from *A. vaginalis* showed close similarity with the type strain *B. vignae* 7-2^T isolated from root nodules of *Vigna unguiculata* growing in field Kavango region of Namibia (Gronemeyer *et al*., 2016). Strain AV34 is different from the previously reported *Bradyrhizobium* strains (TF17, TV4 and TV11) from species of *Tephrosia* from Thar Desert (Gehlot *et al*., 2012).

Detailed multilocus sequence analysis as well as symbiotic phylogenetic studies is needed to differentiate Thar Desert strains from closely related strains within genera *Ensifer*, *Rhizobium* and *Bradyrhizobium*. Some of the strains showing phosphate solubilization as well as phytase activity suggest that the root nodule bacteria associated with native legumes of the Indian Thar Desert in alkaline soil are useful as their rhizospheric presence could help in reclamation of the fragile soil by increasing available phosphate for the plants.

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