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PHENOTYPIC CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF N_2 FIXING SYMBIOTIC RHIZOBIA OF *DICHROSTACHYS CINEREA* FROM ARID AND SEMI-ARID SOILS OF RAJASTHAN, INDIA

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Abstract

Small tree form legume *Dichrostachys cinerea* is widely spread in western and southern states of India, but its N_2 fixing root nodule symbionts are not known so far. Here we report phenotypic and genotypic characterization of its rhizobia including their molecular identification. To trap its rhizobia seeds were grown in soils collected from eight sampling sites from arid and semi-arid regions of Rajasthan. Total twenty rhizobia were isolated which included both fast-growing mucilaginous EPS producing strains and gummy slow-growing strains. Six strains were phenotypically characterized of which the fast-growing rhizobia tolerated up to 1% NaCl and 48°C temperature, grew at pH range 6-11 and were acid producers. While the slow-growing rhizobia survived up to 0.5% NaCl, 48°C, pH range 5-11 and produced alkali. Fast-growing strains were resistant to antibiotic Erythromycin (E^{15}) while slow-growing were resistant to Tetracycline (TE^{30}) and Ciprofloxacin (CIP^{5}). Xylose and dextrose sugars were utilized by all tested strains. RPO1 primer based DNA fingerprinting of fourteen strains indicated significant genetic diversity among the *D. cinerea* rhizobia forming seven individual RAPD genotypes and three groups. 16S rRNA gene sequencing of five strains revealed that the fast-growing strains belonged to species of *Ensifer* and the slow-growing strains were identified as species of *Bradyrhizobium*. This suggests that *D. cinerea* is nodulated by both slow-growing (*Bradyrhizobium*) and fast-growing (*Ensifer*) alpha-rhizobia. Such phenotypically and genetically diverse rhizobia obtained from the arid environments of Rajasthan need further evaluation to screen them for their ability to nodulate other host plants for their potential use in agroforestry, reforestation and agriculture establishment programs specifically in desert regions to improve soil and plant health.

Key words: *Dichrostachys cinerea*, rhizobia, *Ensifer*, *Bradyrhizobium*, phenotypic and genetic characterization

Introduction

Fixation of the atmospheric nitrogen (N_2) to ammonia by the symbiotic rhizobia associated with legume hosts is important for the good health of soil and plants growing in nitrogen deprived poor soils. Legumes in the hot arid and semi-arid regions harbour diverse and promiscuous rhizobia as their symbionts (Sprent and Gehlot 2010). Rhizobia thriving in the adverse conditions of hot-arid regions encounters salt, drought and temperature stresses, such bacteria may have better tolerance to abiotic stresses and can be used as inoculums for crop legumes to enhance the productivity (Zahran 2001). Nodulation status and characterization of root nodule bacteria (RNB) associated with different native legumes found in arid and semi-arid regions of Thar Desert have been well documented (Gehlot *et al.*, 2012, 2014, 2018; Panwar *et al.*, 2014). Phenotypic characterization of RNB associated with few wild legumes has also been worked out for screening of temperature and salt tolerant strains (Sankhla *et al.*, 2015, 2018; Rathi *et al.*, 2017). Genetic diversity, symbiotic efficiency and molecular characterization of RNB associated with few mimosoid members including species of *Vachellia* and *Senegalia* have been reported in several recent papers (Sankhla *et al.*, 2017; Choudhary *et al.*, 2017, 2018, 2020). Genetic and genomic characterization showed that novel species of *Ensifer* and *Bradyrhizobium* are associated with various native legumes of Rajasthan (Tak *et al.*, 2013;

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Gehlot *et al.,* 2016; Le Quéré *et al.,* 2017; Sankhla *et al.,* 2017, 2018; Rathi *et al.,* 2018; Choudhary *et al.,* 2020). Microbes associated with legumes in fragile ecosystem of desert needs to be phenotypically characterized to screen drought and salinity tolerant strains for their sustainable utilization in agriculture system (Gehlot *et al.,* 2014).

Dichrostachys cinerea is a drought tolerant small tree legume naturalized in several states (western to southern part) of India (CABI 2020). To best of our knowledge rhizobia associated with *D. cinerea* have not been characterized so far from India. In the present study our aim is to isolate rhizobial symbionts from *D. cinerea* plants trapped in soils collected from different sampling areas in arid and semi-arid regions of Rajasthan, and to carry out phenotypic and genetic characterization of the microsymbionts. Phenotypic characterization gives us valuable information about morphological features, physiological and biochemical characters like sensitivity towards salt, high temperature, pH and antibiotics as well as gives idea about the metabolic capacity of strains in terms of carbon source utilization which ultimately helps in knowing the useful phenotypic traits of indigenous rhizobia for their utilization in agriculture and forestry.

Materials and Methods

***Soil sampling and seed germination*:** rhizospheric soils from around 15–20 cm depth were collected from eight sampling sites of arid and semi-arid regions of Rajasthan (India) and the geographical coordinates were recorded table 1. Seeds were collected from plants growing naturally in fields Fig. 1a, b. Surface sterilization was done by treating seeds with 90% (v/v) ethanol and 0.1% (w/v) fungicide Bavistin^R for 1 minute followed by 3-4 times rinsing with autoclaved distilled water (DW). Later the seeds were treated with 1% (v/v) sodium hypochlorite for about 6 minutes followed by 6 times washing with autoclaved DW (Tak *et al.,* 2020). Sterilized seeds were kept in hot water for about 15 minutes and then transferred to sterile moistened filter paper kept in petri dish. The plates with imbibed seeds were covered with aluminium foil and placed in dark at 28°C for about 2–3 days until the seedlings appeared Fig. 1c.

***Rhizobia trap experiment set-up*:** soils collected from various sampling sites were filled in plastic pots sanitized using 90% (v/v) ethanol. Soil in pots was moistened with autoclaved tap water and the germinated seedlings were transplanted in soil with the help of sterile forceps. Three seedlings were transferred in each pot and the pots were placed in a glass house under sterile and control conditions. Plants were watered regularly with autoclaved tap water. Mature plantlets were harvested after about 8 to 10 weeks Fig. 1d. Nodule(s) Fig. 1e were detached from the roots of plantlets for sterilization and isolation of bacteria.

***Isolation and purification of rhizobia*:** three to four root nodules were picked up from each plant, wrapped in a muslin cloth then initially sterilized in 90% (v/v) ethanol and also given an antifungal Bavistin^R 0.1% (w/v) treatment for about 1 minute followed by 2–3 times washing with sterile DW. Nodules were then treated with 4% (v/v) sodium hypochlorite for 3 minutes followed by 6 times washing with sterile DW. Surface sterilized root nodules were cut into two halves with a sterile scalpel and crushed with the help of sterile forceps in 50–100µl of autoclaved DW. Nodule exudates were streaked on Yeast Extract Mannitol Agar (YEMA) media containing 25 mgL^{-1} Congo Red (CR) as indicator for screening of the pure rhizobial colonies from contaminants. White or translucent or transparent, convex, raised and bacterial colonies with entire margins were picked up from the master plate and purified by four ways streaking on a YEMA-CR plate (Howieson and Dilworth 2016).

Table 1: Different soil sampling sites of Rajasthan with geographical coordinates and list of few strains isolated from Dichrostachys cinerea.

S. No.	Soil sampling sites	Geographical coordinates (https://www.latlong.net)	Total no. of strains purified per site	Strains selected for RPO1 based DNA fingerprinting
1	Pokhran, Jaisalmer	26°55'28.48"N 71°54'58.49"E	4	DC-RJ2, DC-RJ3, DC-RJ4
2	Mathania, Jodhpur	26°31'47.53?N 72°58'45.81?E	2	DC-RJ5
3	Devikot, Jaisalmer	26°41'51.75"N 71°11'54.06"E	2	DC-RJ7, DC-RJ8
4	Ladnun, Nagaur	27°38'23.50"N 74°23'44.95"E	2	DC-RJ10
5	Ramdevra, Jaisalmer	27°00'38.19"N 71°55'11.02"E	1	DC-RJ11
6	Samdari, Barmer	25°48'51.01"N 72°34'53.13"E	1	DC-RJ12
7	JNVU New Campus, Jodhpur	26°14'44.99"N 73°01'17.42"E	4	DC-RJ13, DC-RJ14
8	Lawan, Dausa	26°46'09.09"N 76°12'48.26"E	4	DC-RJ15, DC-RJ16, DC-RJ17

Phenotypic characterization

A) Salt tolerance and temperature

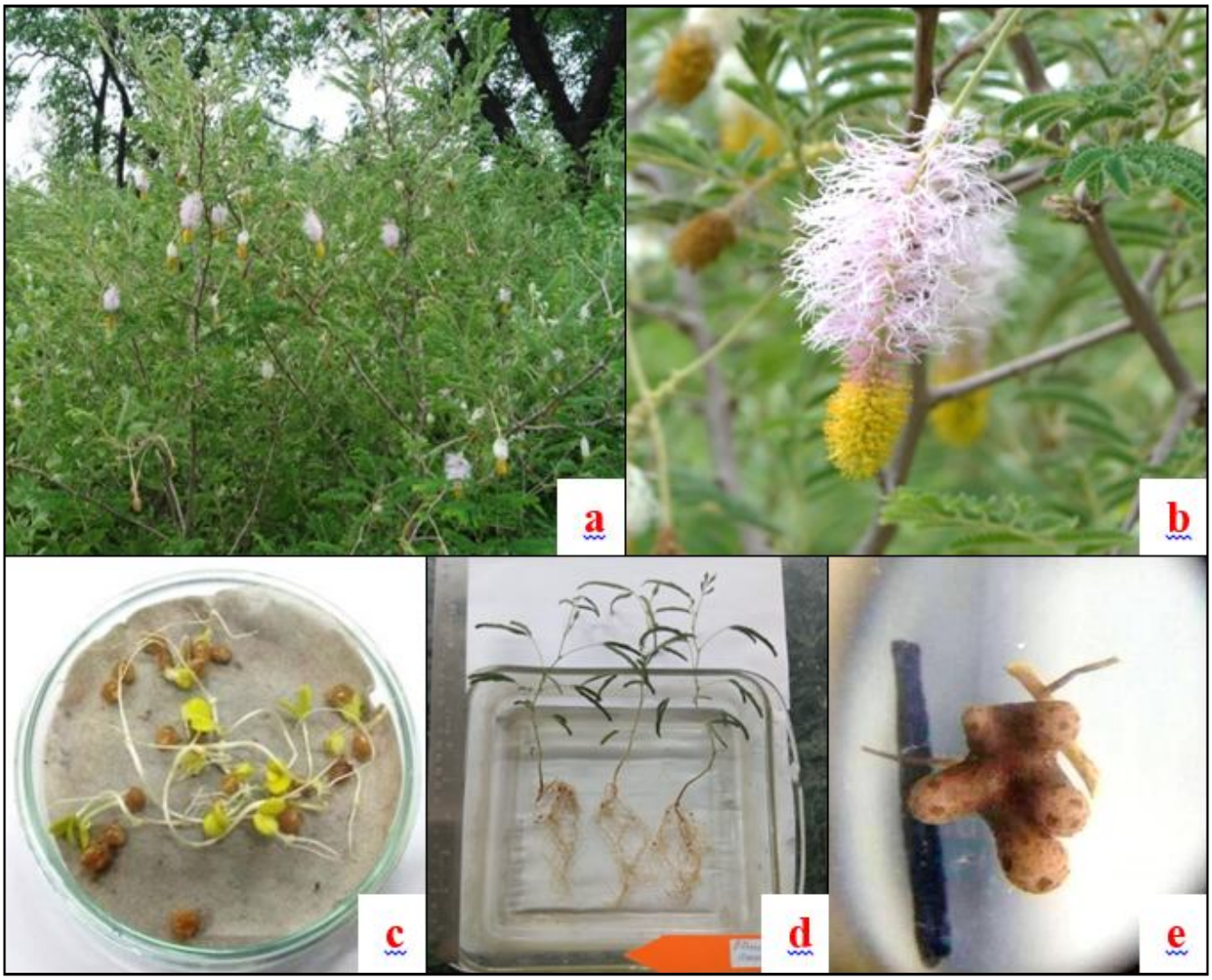

Fig. 1: *Dichrostachys cinerea* growing in field (a); Inflorescence (b); Germinated seeds (c); Excavated plantlet(s) with nodules attached to the roots (d); Branched indeterminate nodule under dissecting microscope (e).

tolerance test: purified rhizobial strains were streaked on YEMA media supplemented with different concentrations [0.5%, 1%, 2% and 3% (w/v)] of NaCl salt and incubated at 28°C for 2 to 6 days. To determine the tolerance of strains to grow at different temperatures the rhizobial strains were streaked on YEMA media and incubated at different temperatures [32°C, 35°C, 40°C, 45°C and 48°C] for about 2 to 6 days. The growth of rhizobial colonies at different concentrations of salt and at range of temperatures was recorded.

B) pH tolerance and BTB (Bromo Thymol Blue) test: ability of strains to grow at a range of pH was tested by spot inoculating 10µl of activated YEM broth on YEMA media adjusted at different pH (5, 6, 8, 9, 10, 11) and the plates were incubated at 28°C for 2-6 days. The pH of YEMA media was adjusted by adding 1N HCl or 1N NaOH and different buffers (Sankhla *et al.*, 2017). Bromo Thymol Blue (BTB) was used in YEM broth as an indicator of acid or alkali producing bacteria. Inoculated YEM-BTB broth was kept on incubator shaker at 28°C for 2 to 6 days and observed for change in colour. The BTB remains green at neutral pH (used as control), the colour changes to yellow on acid production and blue on alkali production (Somasegaran and Hoben 1994) Fig. 2a.

C) Intrinsic antibiotic resistance (IAR) test: disc diffusion method was used to determine the IAR ability of strains. Pure rhizobial strains were activated in YEM broth and swabbed on the YEMA plates using sterile cotton buds. Different HiMedia antibiotic discs of varied concentrations were aseptically placed on the inoculated plates and incubated at 28°C for 2–6 days. The plates were observed for zone of inhibition Fig. 2b and the zones formed were measured in mm using the scale (Cappuccino and Sherman 2007).

D) Sugar utilization test: Andrade's peptone water

was prepared as per the manufacturers (HiMedia Company) instructions. Twenty one different sugar (HiMedia) discs were aseptically placed in a sterilized 24-well plate. After autoclaving the Andrade's peptone water was cooled (colour was pale straw) and was dispensed in the wells. The wells in plate were inoculated with the activated broth of rhizobial strains and incubated at 28°C. After every 24 hours the plates were observed for change of colour from pale straw to light or deep pinkish indicating the utilization of sugars Fig. 2c.

Genetic diversity and characterization

A) DNA isolation and RPO1 primer based DNA fingerprinting: genomic DNA was extracted from the pure bacterial cultures using the phenol-chloroform method as described by Cheng and Jiang (2006). Genetic diversity of rhizobia was determined using a 20-base (AATTTTCAAGCGTCGTGCCA) RPO1 primer (Richardson *et al.,* 1995). It is complimentary to the *nifH* promotor region and is used to differentiate between groups of rhizobia as it amplifies a unique profile for each rhizobium. The process was carried out in thermal cycler (Bio-Rad) with 20 µl reaction mixture. The PCR mixture of 20 µl was prepared by adding 10.25 µl nuclease free water, 2.0 µl Taq buffer (10X), 3.0 µl of $MgCl_2$ (25 mM), 1.2 µl of dNTP mix (2.5 mM each), 1.0 µl of DMSO (100%), 1.2 µl of RPO1 primer (50 mM), 0.35 µl (3 U/ml) of Taq DNA polymerase and 1 µl DNA template (100-1000 ng). Following thermal cycling conditions were used for RPO1 profiling: initial denaturation at 94°C for 5 min followed by 5 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1.5 min; 30 cycles of 94°C for 30 s, 58°C for 25 s, 72°C for 30 s and a final extension at 72°C for 7 min. Amplified PCR product(s) along with a 100 bp DNA ladder were run on 2% agarose gel (pre-stained with ethidium bromide) at 80 V for 2 hrs. Gels were visualized under UV rays in Bio-Rad Gel Doc system.

B) 16S rRNA gene amplification and sequencing: conserved housekeeping 16S rRNA gene was amplified in few rhizobial strains using following pair of primer: 18F (AGAGTTTGATCCTGGCTCAG) and 1492R (CTACGGCTACCTTGTTACG) (Weisburg *et al.,* 1991). PCR mixture of 25 µl included following components: 15.05 µl nuclease free water, 2.5 µl Taq buffer (10X), 2.0 µl of $MgCl_2$ (25 mM), 1.5 µl of dNTP mix (2.5 mM each), 1.25 µl of DMSO (100%), 0.6 µl of each primer (50 mM), 0.25 µl (3 U/ml) of Taq DNA

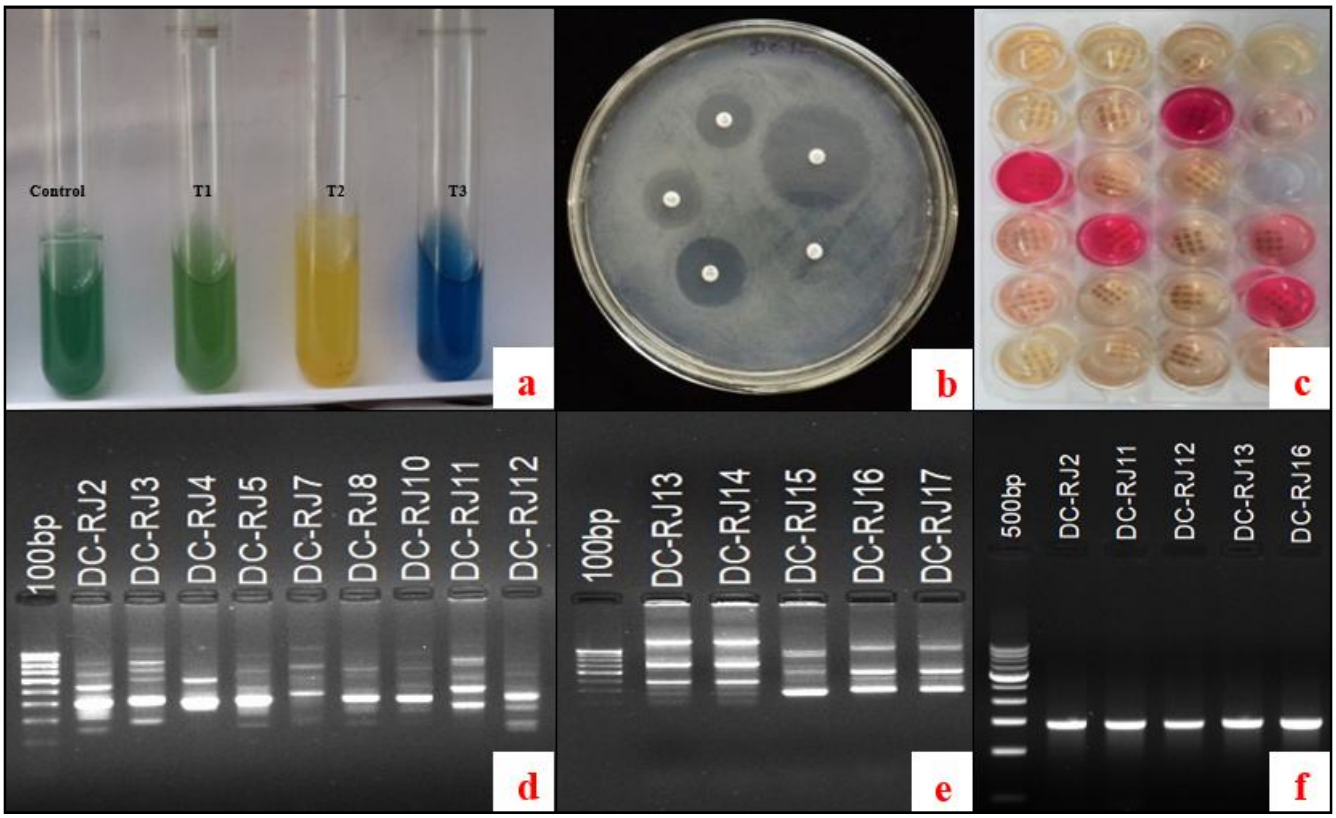

Fig. 2: Phenotypic characterization: BTB response of tested strains compared to control (a); Zone(s) of inhibition formed in IAR test (b); Carbon utilization test: dark pink color in wells indicating sole sugar utilized by tested strains (c); Genetic characterization: RPO1 primer based genetic profile of *Dichrostachys cinerea* strains (d, e); Amplification of 16S rRNA gene (f).

polymerase and 1.25 µl DNA template (100-1000 ng). Reaction mixture tubes were kept in a PCR machine at following thermal cycling conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of 94ºC for 1 min, 53ºC for 1 min, 72ºC for 1 min and then final extension at 72°C for 7 min. Amplified PCR product(s) along with 500 bp DNA ladder were run on 0.89% agarose gel at 80 V for 1 hr. Amplified 16S rRNA gene band of size 1.5 kb was visualized under UV rays in Bio-Rad Gel Doc system. The amplified PCR products were pooled up to 60 µl and sent to AgriGenome Labs Pvt. Ltd., Kerala for the purification of amplified product and Sanger sequencing.

C) Identification of bacteria using nucleotide Basic Local Alignment Search Tool (BLASTn): The chromatographs obtained from the outsourcing company were analyzed using the Gene Tool software. The 16S rRNA gene sequences in FASTA format were used for BLASTn analysis at the NCBI (National Center for Biotechnology Information) site for identification of bacterial strains. The nucleotide BLAST percentage similarity results of query sequences with the type strains were recorded.

Results and Discussion

Rhizobia isolated from root nodules and their phenotypic characte*rization*: *D. cinerea* plants grown in soil samples collected from arid and semi-arid regions of Rajasthan nodulated well Fig. 1d. The root nodules were branched and indeterminate type Fig. 1e. A total of twenty rhizobial strains were purified from the eight sampling sites table 1. Purified rhizobial strains were observed to have different colony morphology like white opaque or translucent or transparent and showed varied levels (low to high) of exopoly saccharide (EPS) production. Both slow-growing and fast-growing strains were isolated in present study which takes duration of about 6–7 days and 2–3 days respectively for growth at optimum temperature (28°C). Slow-growing strains isolated from soils of Jaisalmer (DC-RJ11) and Jodhpur (DC-RJ13) formed gummy and mild mucilaginous colonies. Six strains (DC-RJ2, DC-RJ8, DC-RJ11, DC-RJ12, DC-RJ13 and DC-RJ16) were selected for phenotypic characterization based on following physiological properties: tolerance to different concentrations of salt; ability to grow at high temperature(s) and wide range of pH; acid or alkaline producing and resistance or sensitivity to various antibiotics table 2. In the present study fast-growing rhizobia could tolerate up to 1% (w/v) NaCl while the slow-growing strains survived up to 0.5% (w/v) NaCl. *Ensifer* strains isolated from root nodules of native tree legumes such as *Vachellia jacquemontii*, *Vachellia leucophloea* and *Senegalia senegal* from the Thar Desert have been reported to tolerate high salt concentrations up to 3% (Sankhla *et al.,* 2017; Choudhary *et al.*, 2017, 2018) whereas the slow-growing *Bradyrhizobium* strains isolated from herb *Alysicarpus vaginalis* could survive maximum at 0.5% salt (Rathi *et al.,* 2017) as observed in the present study. Both fast- and slow-growing strains isolated from *D. cinerea* could survive at temperature range 32–48°C. Heat-tolerant rhizobia are likely to exist in the environments influenced by high temperature stress. Zahran *et al.,* (1994) reported high temperature tolerance in rhizobia isolated from the tree legumes found in hot and dry regions. Generally the fast-growing rhizobia are less tolerant to acidic pH in comparison to the slow-growing rhizobia (Graham *et al.,* 1994). Similar pH tolerance pattern was observed in this study where the slow-growing strains survived at a low pH 5 whereas the fast-growing strains showed pH tolerance range of 6 to 11. The fast-growing strains showed acidic reaction in BTB test and the slow-growing strains showed alkaline or neutral reaction. Variable IAR pattern was shown by *D. cinerea* strains table 2 however, all were found to be sensitive to Streptomycin (HLS^{300}). The fast-growing strains showed resistance or slight sensitivity (DC-RJ12) to Erythromycin (E^{15}) while the slow-growing strains were resistant to Ciprofloxacin (CIP^{5}) and Tetracycline (TE^{30}). Resistance or

Table 2: Phenotypic characterization of selective *Dichrostachys cinerea* rhizobial strains.

Strains Phenotypictraits	**DC-RJ2**	**DC-RJ8**	**DC-RJ11**	**DC-RJ12**	**DC-RJ13**	**DC-RJ16**
Growth period (in days)	3	3	6-7	3	6-7	2
Salt tolerance up to (%)	1	1	0.5	1	0.5	1
Temperature tolerance range (°C)	32-48	32-48	32-48	32-48	32-48	32-48
pH tolerance range	6-11	6-11	5-11	6-11	5-11	6-11
BTB reaction	Acidic	Acidic	Neutral	Acidic	Alkaline	Acidic
Antibiotics (µg)	Zone of inhibition (measured in mm)					
Erythromycin (E^{15})	0	0	21	10	22	0
Neomycin (N^{30})	20	20	34	25	25	18
Kanamycin (K^{30})	27	27	10	27	SS	21
Gentamicin (GEN^{10})	22	28	24	28	18	21
Streptomycin (HLS^{300})	42	44	45	43	40	43
Tetracycline (TE^{30})	44	42	0	42	0	40
Ciprofloxacin (CIP^{5})	19	19	0	18	0	21

Note: SS- indicates slight sensitivity against antibiotic

sensitivity of strains to particular antibiotics has been used as a parameter to group the strains as in this study we can see a distinct IAR pattern of the fast- and slow-growing strains.

Five strains (DC-RJ2, DC-RJ11, DC-RJ12, DC-RJ13 and DC-RJ16) were tested for carbon (sugar) source utilization and a variable metabolic pattern was observed among them table 3. Out of the 21 sugars tested adonitol, dulcitol, inulin and sorbitol were not utilized by any of the tested strains while the dextrose and xylose were utilized by all. The fast-growing rhizobial strains are reported to utilize comparatively more diverse carbon sources than the slow-growing *Bradyrhizobium* like strains (Stowers 1985; Rathi *et al.,* 2017) but in the present study the slow-growing strain DC-RJ11 utilized maximum (17) sugars and was an exception, however more such strains have to be studied for further confirmation of such unusual trait.

Table 3: Sugar utilization pattern shown by selective *Dichrostachys cinerea* rhizobial strains.

Sugars (carbon) source	DC-RJ2	DC-RJ11	DC-RJ12	DC-RJ13	DC-RJ16
Adonitol	-	-	-	-	-
Arabinose	+	+	+	-	+
Cellobiose	+	+	-	-	-
Dextrose	+	+	+	+	+
Dulcitol	-	-	-	-	-
Fructose	-	+	-	-	-
Galactose	+	+	+	-	-
Inositol	-	+	-	-	-
Inulin	-	-	-	-	-
Lactose	-	+	-	-	-
Maltose	+	+	+	-	+
Mannitol	-	+	-	+	-
Mannose	+	+	+	-	-
Melibiose	-	+	-	-	-
Raffinose	-	+	-	-	-
Rhamnose	-	+	-	-	-
Salicin	+	+	-	+	-
Sorbitol	-	-	-	-	-
Sucrose	+	+	-	-	-
Trehalose	-	+	-	-	-
Xylose	+	+	+	+	+

Note- "+" indicates sugar utilized and "-" indicates sugar not utilized by the tested strain.

***Genetic diversity and identification of rhizobia*:** RPO1 primer based DNA fingerprinting of fourteen *D. cinerea* strains table 1 and Fig. 2d, e showed a significant genetic diversity among them. Seven strains (DC-RJ2, DC-RJ3, DC-RJ4, DC-RJ7, DC-RJ11, DC-RJ12 and DC-RJ15) formed individual RPO1-genotypes (I to VII) and the remaining strains clustered to form three genetic groups (I–III) table 4. These results based on limited sampling indicate that genetically diverse rhizobial strains are root nodule microsymbionts of *D. cinerea* in the arid and semi-arid regions of Rajasthan. The conserved housekeeping 16S rRNA gene was amplified and sequenced in five strains (DC-RJ2, DC-RJ11, DC-RJ12, DC-RJ13 and DC-RJ16) Fig. 2f to identify the rhizobia

Table 4: Genetic diversity types of strains isolated from *Dichrostachys cinerea.*

RPO1 based diversity types	Strains
Group-I	DC-RJ5, DC-RJ8, DC-RJ10
Group-II	DC-RJ13, DC-RJ14
Group-III	DC-RJ16, DC-RJ17
Individual types: I-VII	DC-RJ2, DC-RJ3, DC-RJ4, DC-RJ7, DC-RJ11, DC-RJ12, DC-RJ15

Note: Strains in bold were identified using the 16S rRNA gene sequencing

Table 5: BLASTn sequence similarity search results based on the 16S rRNA gene of rhizobia isolated from *Dichrostachys cinerea* with the closest type strain(s).

Strain	Closest type strain(s) (NCBI reference number)	Geographical origin	Biological origin/ Host	Sequence similarity (%)
DC-RJ2	*Ensifer kostiensis* NBRC 100382^T (NR_113889)	Sudan	*Acacia senegal*	100
DC-RJ11	*Bradyrhizobium yuanmingense* NBRC 100594^T (NR_112928)	China	*Lespedeza* sp.	99.93
DC-RJ12	*Ensifer fredii* NBRC 14780^T (NR_113669)	China	*Glycine max*	99.88
	Ensifer xinjiangensis LMG 17930^T (NR_114987)	China	*Glycine max*	99.88
	Ensifer americanum CFNEI 156^T (NR_025251)	Mexico	*Acacia acatlensis*	99.88
DC-RJ13	*Bradyrhizobium yuanmingense* NBRC 100594^T (NR_112928)	China	*Lespedeza* sp.	100
	Bradyrhizobium subterraneum 58 2-1^T (NR_137331)	Namibia	*Arachis hypogaea*	100
DC-RJ16	*Ensifer terangae* NBRC 100385^T(NR_113891)	Senegal	*Acacia laeta*	99.35
	Ensifer mexicanus ITTG-R7^T(NR_115768)	Mexico	*Acaciella angustissima*	99.35

of *D. cinerea*. The maximum percentage nucleotide similarity for these five strains with the closest type strain(s) based on the BLASTn search results are presented in table 5. Of the five strains sequenced three were identified as species of *Ensifer* and two as species of *Bradyrhizobium*. The fast-growing strain DC-RJ2 showed 100% similarity with the type strain *E. kostiensis* isolated from other mimosoid tree, *Acacia senegal*. Likewise the slow-growing strain DC-RJ13 was identical with type strains *B. yuanmingense* and *B. subterraneum*. The remaining three strains (DC-RJ11, DC-RJ12 and DC-RJ16) showed little divergence from the closest type strain(s). The other slow-growing strain DC-RJ11 also shared similarity (99.93%) with *B. yuanmingense* originally isolated from *Lespedeza* sp. in China. The two fast-growing strains (DC-RJ12 and DC-RJ16) identified as species of *Ensifer* shared maximum similarities with multiple type strains reflecting the poor resolution of 16S rRNA gene sequences in differentiating among strains at species level. Strain DC-RJ12 shared similarities (99.88%) with *E. fredii*, *E. americanum* and *E. xinjiangensis*. Strain DC-RJ16 showed maximum similarities (99.35%) with type strains *E. mexicanus* and *E. terangae*.

To conclude *D. cinerea* is nodulated by both slow-growing (species of *Bradyrhizobium*) and fast-growing (species of *Ensifer*) alpha-rhizobia in arid and semi-arid regions of Rajasthan. Molecular characterization of strains helped in identifying the type of rhizobia preferred as root nodule microsymbiont by *D. cinerea* and also the diversity of rhizobia prevailing in particular soils. Phenotypic characterization of selective strains presented their adaptability to different limiting factors such as salt concentration, pH, temperature, etc. Genetic characterization demonstrated the significant diversity among the strains. Such phenotypically and genetically diverse native tree-rhizobia of the arid and semi-arid regions are an important bio-resource and could be the potential rhizobial candidates for use in agriculture sector in near future.

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