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Diversity in a promiscuous group of rhizobia from three *Sesbania* spp. colonizing ecologically distinct habitats of the semi-arid Delhi region

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Abstract

Sesbania-rhizobia associations have immense significance in soil amelioration programs for diverse habitats. Diversity in symbiotic properties, LPS profiles, *Sym* plasmid and rhizobiophage sensitivity of 28 root- and stem-nodulating bacterial isolates of three *Sesbania* species (*S. sesban*, *S. aegyptica* and *S. rostrata*) inhabiting six ecologically distinct sites of semi-arid Delhi region was analyzed. The isolates were highly promiscuous among the symbiotic partners (*Sesbania* spp.). The root nodules formed by all the isolates were morphologically similar but they differed in their symbiotic efficiency and effectiveness. 16S rDNA sequence analyses revealed that root nodule isolates of sesbanias belong to diverse rhizobial taxa (*Sinorhizobium saheli, S. meliloti, Rhizobium huautlense*) whereas stem-nodule isolates were strictly *Azorhizobium caulinodans*. *Sinorhizobium* spp. seem to dominate as microsymbiont partner of *Sesbania* in the Delhi region. The genetic diversity revealed by cluster analyses based on NPC-PCR reflects sorting of isolates across the ecological gradient. Parallel diversity was also observed in the grouping based on LPS profiles and *sym* plasmid (NPC-PCR). Segregation of different rhizobial taxa into distinct types/clusters based on LPS and NPC-PCR analyses suggest its significance in the circumscription of the taxa. However, subtypes and subclusters showed their sorting across the ecological gradients. *Sesbania* rhizobia showed extremely high specificity to rhizobiophages. Enormous diversity in LPS profiles and high specificity of rhizobiophages might be the result of environmental selection pressures operating in ecologically distinct habitats. The ability of sesbanias to enter into effective symbioses with different rhizobial taxa and colonize diverse habitats with various biotic and abiotic stresses appears to contribute to its wide ecological amplitude.

Keywords: *Sesbania* spp.; Diversity in root- and stem-nodulating bacteria; Promiscuity; LPS; Phage sensitivity; NPC-PCR; Ecological niches

1. Introduction

Legume-nodulating bacteria belong to at least 11 genera and 47 species. Of these, 41 species belonging to five genera of the family *Rhizobiaceae* (*Rhizobium*, *Mesorhizobium*, *Azorhizobium*, *Sinorhizobium*, *Bradyrhizobium*) are commonly known as rhizobia [26,35,37]. The rhizobia form nitrogen-fixing nodules on roots and occasionally at stem-located root primordia of leguminous species. Successful symbiosis between the legume and rhizobial partner contributes to the ecological success of a leguminous species. Major steps in symbiosis include bacterium-plant recognition, nodule formation and nitrogen fixation [31]. These processes are partner-specific and generally, a particular rhi-

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zobial species can only nodulate a limited and defined range of legumes [7].

The root infection and development of the nodule begins with the recognition between plant root lectin and outer membrane lipopolysaccharides (LPS) of rhizobia [11]. The LPS also leads to successful suppression of the host defense reaction and helps rhizobia to colonize the legume root [8]. In fact, LPS mutants of *R. phaseoli* were found to be defective in infection thread development. Ability of rhizobia to evolve strategies to combat biotic stress, and particularly rhizobiophags (soil-borne *Rhizobium* phage), is important for their colonization of the legume rhizosphere [27]. Symbiotic genes are plasmid-borne in rhizobia, except for *Azo*(*Brady*)*rhizobium,* where they are located on the chromosome [17]. The intimate association of symbionts with many partners (promiscuity) and the development of effective symbioses (nodulation, nitrogen fixation and biomass enhancement) have been proposed to determine the nitrogen input into various ecological niches [21]. However, promiscuity between the two partners and diversification of rhizobia in functional adaptive traits across the ecological gradients is poorly understood.

Sesbanias are a unique group of legumes native to the tropics and subtropics. They inhabit a wide range of ecologically diversified habitats from lowland, wet tropical ecosystems to semi-arid ecosystems and from seacoasts to freshwater swamps. These habitats vary in soil type, nutrient status and abiotic and biotic stresses. Sesbanias are excellent legume green manure (LGM) plants cultivated in rice paddies and alley cropping. In fact, the value of N accumulated in a root nodulating LGM crop of *Sesbania* ranges from 146 to 267 kg N per hectare [25]. They also yield firewood and fodder, in addition to improving soil fertility in arid and semi-arid regions. [15]. *S. sesban, S. aegyptica* and *S. rostrata* are among the major agroforestry species in the Delhi region which are used to ameliorate salinity and habitat processing in rehabilitation programs [10]. Soil amelioration and habitat processing properties of sesbanias are associated with its nitrogen-fixing symbiotic partner, i.e., rhizobia.

Diverse rhizobial taxa are known to nodulate *Sesbania* spp. in different parts of the world; however, the diversity among rhizobial taxa nodulating sesbanias in the semi-arid Delhi region has not been investigated [2,3]. Since sesbanias occupy a wide range of ecological niches, the study on symbiotic properties and functional traits of associated rhizobia would help in understanding the role of microsymbiont attributed to their wide ecological amplitude. Assessment of diversity in these traits will also help in the development of strategies to effectively utilize *Sesbania*-rhizobia symbioses in various soil amelioration programs. Therefore, in the present study symbiotic properties, surface polysaccharides, plasmid genotype and rhizobiophage sensitivity of 28 isolates from three *Sesbania* sp. (*S. sesban, S. aegyptica* and *S. rostrata*) inhabiting six ecologically distinct sites were examined.

2. Materials and methods

2.1. Study area and its ecological characteristics

Six ecologically distinct sites were selected from the Delhi region for the present investigation (Fig. 1). The soil characteristics were investigated by determining the pH, NPK and organic matter [1]. Observations were also recorded on the landscape features, land use pattern, anthropogenic activities and the vegetation types.

2.2. Legume-nodulating bacterial strains and their culture conditions

Legume-nodulating bacterial isolates were taken from root and stem nodules of different species of *Sesbania* (*S. sesban, S. rostrata* and *S. aegyptica*) collected from the Delhi region (Table 1). ORS571 and WE7 [20] were obtained from the *Rhizobium* Germplasm Collection (RGC) maintained at the University of Delhi. The isolates have already been characterized using standard microbiological methods (Sharma, R.S., 1999, Ph.D. thesis). The cultures for all the isolates were raised in YM/YL broth at $28 \pm 1\,^{\circ}C$, 130 ± 5 rpm [32].

2.3. Plant nodulation assay

Seeds of *Sesbania* spp. were surface-sterilized (A. Mohmmed, Ph.D. thesis), germinated on 1.5% water agar plates and transferred to acid (0.1 M HCl) washed sterile quartz sand in pots (15 cm diameter) filled up to 10 cm depth. The pots were maintained for 14 weeks in a growth chamber with a temperature of 28/20 °C (day/night) and 14 h photoperiod. The plants were watered with sterile distilled water every 2 days, and sterile 1:4 diluted Jensen N-free nutrient media [32] once a week.

The host range of 28 isolates of rhizobia was tested by the root nodulation assay on *S. sesban, S. aegyptica and S. rostrata* in all possible combinations. The root and stem of plantlets were inoculated with 5×10^{10} bacterial cells on the 7th and 21st day after sowing. In the case of *S. rostrata*, the stem nodulation assay was considered positive if nodules were observed within 4–5 weeks. Surface-sterilized seeds without inoculation were sown as negative control. Plants were uprooted after 45 days and checked for nodulation. The location, number and shape of nodules were also recorded.

2.4. Symbiotic efficiency and effectiveness

Symbiotic efficiency and effectiveness of the isolates were assessed on the homologous hosts. The symbiotic efficiency was measured in terms of nitrogenase activity tested by the acetylene reduction assay [29]. The healthy intact nodules were weighed, surface-dried and kept in an airtight vial (15 ml). 10% air of the vial was replaced with pure acetylene and the vial was incubated for 1 h at 28 °C. The

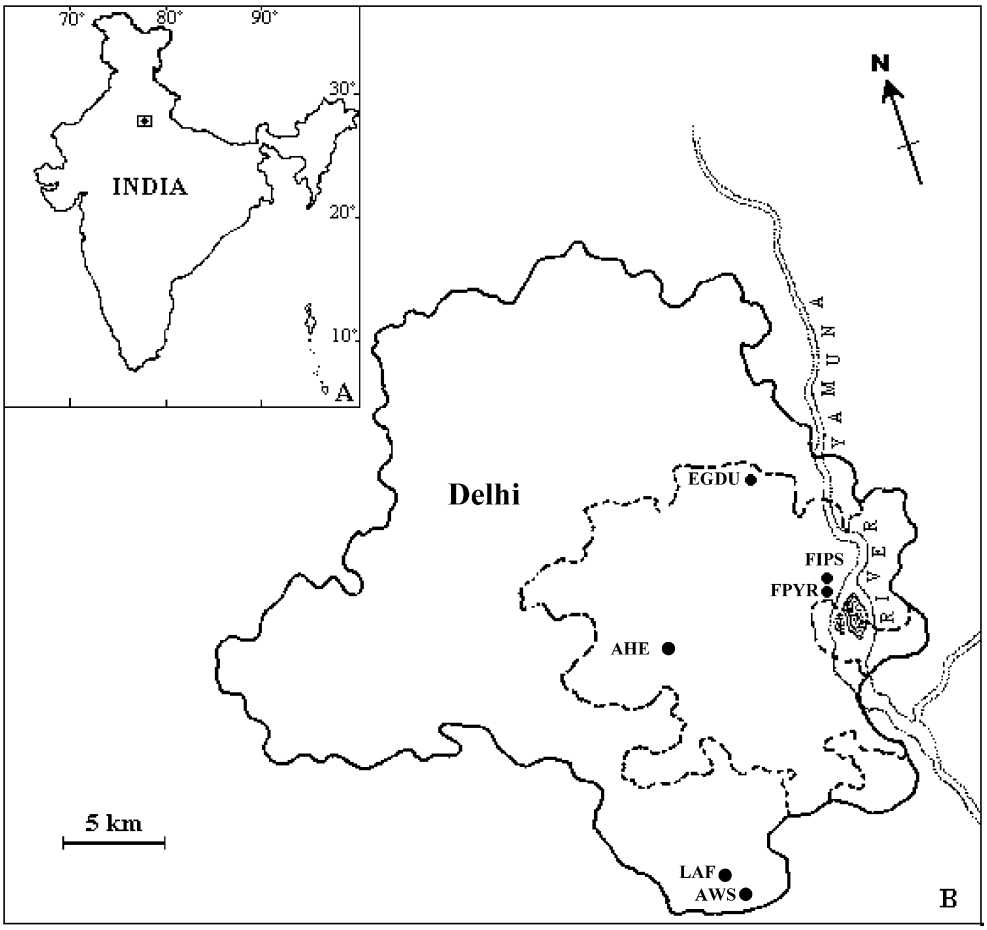

Fig. 1. Map showing location of the Delhi region in India (A), and locations of sampling sites (∗) in the Delhi region (B). (EGDU: Experimental garden, Delhi University; FIPS: Fly-ash dumps Indraprastha power station; FPYR: Flood plains of Yamuna river; AHE: Aravalli hill extensions; LAF: Low-lying agriculture field; and AWS: Asola wildlife sanctuary.)

ethylene gas formed was estimated by gas chromatography by comparison with the standard and ethylene formed per h per mg of nodule fresh weight was calculated [29]. Symbiotic effectiveness was expressed as percent of dry weight of the aerial biomass of the test plant to that of nitrogen control plants, which were maintained with Jensen's medium containing 0.1 M KNO_3.

2.5. Preparation of LPS samples

LPS fraction was purified by the standard protocol [6]. Bacterial biomass was harvested at late-log phase and the cell pellet was washed with phosphate buffer saline (10 mM phosphate buffer, 0.9% NaCl, pH 7.4). The pellet was suspended in 5 ml of 50 mM Tris–HCl, pH 8.5 (1 g/5 ml) and cells were lysed by sonication (Heat System Ultrasonics, W-3851) for 3 min as previously described [19]. Cell lysate was centrifuged at 1000 *g* for 20 min and supernatant was treated with 0.2 mg/ml lysozyme for 30 min at room temperature. KCl solution was added to make a final concentration of 0.2 M. The cell envelope was pelleted down by centrifuging at 12 000 *g* for 60 min and washed with sterile distilled water. The pellet was suspended in 2 mM Tris–HCl (pH 7.8), heated for 10 min at 95 °C, and cooled to 60 °C. It was treated with proteinase K (0.2 mg/ml) and incubated at 60 °C for 60 min.

2.6. Electrophoretic analyses and preferential staining of LPS

The LPS sample was appropriately diluted with sample buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 4% SDS and 30% sucrose) and loaded (∼10 μg) on the gel. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in the presence of 0.2% SDS [6]. Gels were fixed overnight in the fixing solution (25% isopropanol in 7% acetic acid) and LPS resolved on the gels were localized by Ag-LPS preferential staining method [9]. Stained gels were photographed and used for analyses [16].

2.7. Rhizobiophage isolation and phage typing

Rhizobiophages were isolated, multiplied and maintained as previously described [27]. Based on the plaque characteristics and morphological features (examined by transmission electron microscopy) of the rhizobiophages,

Table 1
Details of ecological characteristics of sampling sites, host legume and rhizobial isolates nodulating sesbanias

Sampling site	Ecological characteristics		Host legume species	Source of nodule	*Sesbania*-nodulating rhizobial isolates
	Vegetation type and land use	Physico-chemical properties of soil			
I. Experimental garden, Department of Botany, University of Delhi (EGDU)	Man-managed site, introduction of microbes and plants is common	Sandy loam, pH 6.4, OM 9.0%, PO_4–P 94.9 ppm, NO_3–N 72.9 ppm, K 110.2 ppm	*S. sesban* *S. aegyptica* *S. rostrata*	Root Root Root Stem	SSR301, 302, 304, 305, 312 SAR608, 610, 611 SRR915 ORS571, SRS1, SRS2
II. Aravalli hill extension (AHE)	Open degraded land dominated by invasive weeds	Skeletal, pH 6.0, OM 0.8%, PO_4–P 24.2 ppm, NO_3–N 20.2 ppm, K 52.2 ppm	*S. sesban*	Root	SSR353, 354, 355, 356, 357, 361, 362
III. Asola wildlife sanctuary (AWS)	Desertified and xeric flat land with loose soil inhabited by xerophytic vegetation; grazing is common	Skeletal, pH 7.8, OM 0.8%, PO_4–P 5.2 ppm, NO_3–N 18.2 ppm, K 40.0 ppm	*S. aegyptica*	Root	SAR615, 621
IV. Low-land agriculture field (LAF)	Man-managed cultivated field used for cereals and vegetables, frequently irrigated	Sandy loam, pH 5.4, OM 1.2%, PO_4–P 6.0 ppm, NO_3–N 24.0 ppm, K 20.2 ppm	*S. sesban*	Root	SSR327, 328
V. Flood plain of Yamuna river (FPYR)	Inundated for 4 months, used for seasonal agriculture	Sandy, pH 6.0, OM 2.1%, PO_4–P 12.5 ppm, NO_3–N 74.0 ppm, K 58.0 ppm	*S. sesban*	Root	SSR341, 342
VI. Fly-ash dumps of Indraprastha power station (FIPS)	Wet fly-ash dumps, usually barren except for cultivation of sesbanias to stabilize the dump	Fine ash, pH 7.3, OM 2.5%, PO_4–P 32.4 ppm, NO_3–N 69.9 ppm, K 31.0 ppm	*S. sesban*	Root	SSR335, 337

different plaque morphotypes (PM) were identified. The sensitivity pattern of the bacterial isolates to the rhizobiophages (plaque-morphotypes) was assessed by the plaque assay.

2.8. Diversity in the sym plasmid

Variations in the *sym* plasmid were assessed by a PCR-amplified DNA product pattern using an *nif*-directed primer containing the nitrogen fixation promoter consensus element (NPC-PCR) [18]. The sequence of the 20 nucleotide length *nif* promoter consensus primer (NPC) used was 5′-AAT TTT CAA GCG TCG TGC CA-3′. PCR amplification was done in a 10 µl reaction mix and the amplified products were separated on 1.5% agarose gel [24]. The profiles were used for grouping of rhizobia by UPGMA (unweighted pair group method using arithmetic averages) cluster analysis.

2.9. 16S Ribosomal DNA (rDNA) sequence analyses

Nine isolates from *S. sesban* and *S. aegyptica* root nodules (SSR301, 302, 312, 321,335, 354 and SAR610, 611, 617) and 3 isolates from *S. rostrata* root (SRR915) and stem nodules (SRS1, SRS2) were selected for 16S rDNA sequence analyses. Partial 16S rDNA sequences, corresponding to positions 44–337 in *Escherichia coli* were amplified by primers Y1 (5′-TGG CTC AGA ACG AAC GCT GGC GGC-3′) and Y2 (5′-CCC ACT GCT GCC TCC CGT AGG AGT-3′) [36]. PCRs were performed in a 25 µl reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each nucleotide, 15 pM of each primer, 1 U of *Taq* polymerase (Perkin–Elmer Cetus) and 50 ng of genomic DNA. PCR consisted of initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 62 °C for 40 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Purified PCR products were cloned in pGEM-T vector (Promega) and sequenced using an automated DNA sequencer (Applied Biosystems 310). The sequences have been submitted to the GenBank (AF469897–AF469905, AY723751–AY723755). The genera and species were identified by comparing with the 16S rDNA sequences of standard isolates available in the GenBank.

3. Results

3.1. Ecological characteristics of the study area and details of legume host and rhizobial isolates

The six study sites selected were distributed within an area of ∼1500 km^2 of the Delhi region (Fig. 1). Human-

induced habitat changes were common to all sites, but the sites were ecologically distinct with respect to the landscape features and physicochemical properties of the soil (Table 1). The experimental garden at Delhi University (EGDU) and the low-lying agriculture field (LAF) were man-managed sites characterized by high anthropogenic activity. At both sites, soil was acidic sandy loam, but LAF was poor in NO_3, PO_4–P, K content and organic matter. The flood plain of the Yamuna River (FPYR) is a natural habitat characterized by periodic flooding, and is used for low-input seasonal agriculture. However, the fly-ash dump at the Indraprastha power station (FIPS) was a man-made barren site developed due to dumping of fine ash left after burning of coal. Both sites having low organic matter (2.1–2.5%) differed in soil pH, PO_4–P and K content (Table 1). The Aravalli hill extension (AHE) and the Asola wildlife sanctuary (AWS) were degraded lands. At AHE, developmental activities were common; however, at AWS, overgrazing was the major stress. The soil type was skeletal at both sites; however, AHE was slightly acidic and AWS was slightly basic. They also differed in the vegetation characteristics, as a weedy community was found at AHE and xerophytic scrub vegetation at AWS.

S. sesban was common to all sites except AWS; *S. aegyptica* was sampled from EGDU and AWS and *S. rostrata*, which is an introduced species, was restricted to EGDU only. Root nodules from all *Sesbania* species and stem nodules from *S. rostrata* were used for isolation of *Rhizobium*. Twenty-eight isolates varying in their culture characteristics and growth parameters were selected for the study.

3.2. Symbiotic properties (host range, nodulation characteristics, symbiotic efficiency and effectiveness) of root and stem nodulating isolates of sesbanias

All isolates produced globose nodules located at the crown region as well as the primary roots (Fig. 2). The range in the mean number of nodules produced per plant was 4–13 (Table 2). Nodulation was not observed in the control experimental set-up involving non-inoculated seeds even after 3 months. Cross-inoculation studies involving all three *Sesbania* spp. and 28 isolates were carried out to assess the host range. The root-nodulating isolate of *S. rostrata* (SRR915) nodulated roots of all *Sesbania* species tested but did not nodulate the stem of *S. rostrata* itself. ORS571 nodulated the stem of *S. rostrata* and the roots of all three *Sesbania* spp. In addition to the homologous hosts, all isolates formed functionally effective nodules with copious leghemoglobin on the roots of heterologous hosts in all combinations. Interestingly, the shape and location of nodules formed on the heterologous host were similar to those of the homologous host. No attempt was made to assess the symbiotic efficiency and effectiveness of the nodules formed on heterologous hosts.

The isolates showing high symbiotic efficiency showed high symbiotic effectiveness in general (Table 2). *S. sesban* isolates from LAF, FPYR and AHE showed moderately higher values of mean nodule number, symbiotic efficiency and symbiotic effectiveness, whereas EGDU and FIPS isolates were relatively poor in these properties. *S. aegyptica* isolates from EGDU showed moderately higher values while those from AWS were poor in symbiotic properties (Table 2). Interestingly, low symbiotic efficiency of *S. rostrata* isolates from EGDU was coupled with high mean nodule number and symbiotic effectiveness.

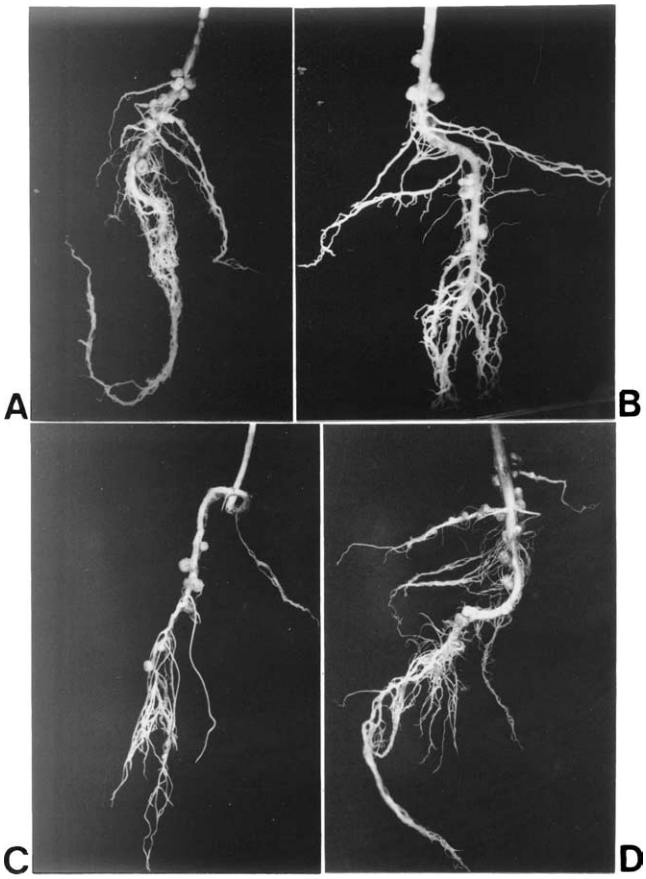

Fig. 2. Nodulation pattern on the intact root system of plants belonging to different species of *Sesbania* inoculated with their rhizobia. (A) *S. sesban* inoculated with SSR327 (B) *S. rostrata* inoculated with SRR915, (C) *S. aegyptica* inoculated with SAR610, (D) *S. sesban* inoculated with SAR611.

3.3. Heterogeneity in LPS profiles

LPS is an integral component of the outer membrane of rhizobia, which generally display two regions differing in electrophoretic mobility [13]. The LPS I region with lower mobility contains complete LPS molecules with lipid A, core oligosaccharide (core) and O-antigenic polysaccharide; however, the LPS II region with higher mobility contains incomplete LPS molecules with lipid A and core region only. LPS profiles of rhizobia-nodulating sesbanias were extremely heterogeneous (Fig. 3) particularly with respect to the LPS I and O-antigen (>20 kDa). LPS I was further divided into two zones based on mobility. The slow-moving

Table 2
Details of rhizobial isolates of *Sesbania* spp., their sampling sites, LPS types, symbiotic properties, phage types, and NPC–PCR cluster and exact generic and specific identification based on 16S rDNA sequence analysis

LPS profile type	LPS profile subtype	Rhizobial isolates	Sampling site	Symbiotic properties			Phage type	NPC–PCR cluster subgroup	Rhizobial taxa
				Mean nodule number	Symbiotic efficiency (nM/g fresh wt/h)	Symbiotic effectiveness (%)			
A	A1	SSR327	LAF	13.0	297.5	81.5	R	I^b	*Rhizobium*
		SSR328	LAF	12.5	330.0	81.0	R	I^b	*huautlense*
	A2	SSR353	AHE	6.0	260.0	66.5	R	I^a	–
		SSR354	AHE	10.0	485.0	81.7	R	I^a	*R. huautlense*
		SSR355	AHE	9.3	402.5	85.5	R	I^a	–
		SSR356	AHE	9.5	435.0	80.5	R	I^a	–
		SSR357	AHE	9.8	382.5	78.1	R	I^a	–
B	B1	SSR335	FIPS	5.3	82.5	54.0	R	II^b	*Sinorhizobium*
		SSR337	FIPS	5.6	110.7	66.0	R	II^b	*meliloti*
	B2	SSR341	FPYR	12.5	372.5	79.5	R	II^a	–
		SSR342	FPYR	9.3	270.0	55.5	R	I^c	–
	B3	SSR361	AHE	5.0	305.0	78.5	R	I^d	–
		SSR362	AHE	5.0	330.0	74.5	R	I^d	–
	B4	SAR608	EGDU	8.6	247.5	85.5	PM14	III^d	–
		SAR610	EGDU	8.3	232.0	65.0	PM14	III^d	*S. saheli*
		SAR611	EGDU	10.0	180.0	83.0	R	III^c	*S. saheli*
C	C1	SAR621	AWS	8.5	71.25	67.0	R	III^a	–
		SAR615	AWS	9.0	75.25	68.0	R	III^a	–
	C2	SSR302	EGDU	5.0	127.5	55.5	PM12	II^c	*S. meliloti*
	C3	SRR915	EGDU	10.8	72.5	82.5	R	III^b	*S. saheli*
	C4	SSR304	EGDU	8.5	78.5	71.5	R	III^f	–
		SSR305	EGDU	7.5	74.5	58.0	R	III^f	–
		SSR312	EGDU	12.6	78.7	70.5	PM13	III^e	*S. saheli*
	C5	SSR301	EGDU	7.1	360.0	74.8	R	III^g	*S. saheli*
	C6	WE7	RGC*	–	–	–	PM10	–	–
D	D1	ORS571	RGC	–	–	–	PM1–9, 15–16	–	*Azorhizobium caulinodans*
		SRS1	EGDU	–	–	–	PM1–9, 15–16	–	*A. caulinodans*
		SRS2	EGDU	–	–	–	PM1–9, 15–16	–	*A. caulinodans*
		SRS3	EGDU	–	–	–	PM1–9, 15–16	–	*A. caulinodans*

* RGC: *Rhizobium* Germplasm Collection Center, University of Delhi; '–': not investigated
[a–g] Represent subclusters formed based on NPC-PCR profiles: see also Fig. 4.

(>95 kDa) and the fast-moving (23–95 kDa) zones were designated as zone I and zone II, respectively (Fig. 3). Zone I was always characterized by distinct bands; however, in some of the profiles, zone II showed distinct (SSR 304, 305, 335, 337) or diffuse bands (SSR 361, 362) and in others it was a darkly stained diffused region (SAR608, 610 etc.) (Fig. 3). A similar pattern of variation in LPS I was also observed in rhizobia that nodulate *Acacia senegal* and *Prosopis chilensis* [13].

Four major LPS types referred to as A, B, C and D were recognized based on: (i) the presence or absence of zones I and II, (ii) the length of zone I, and (iii) the continuity of zones I and II (Fig. 3). Type A was characterized by a very short inconspicuous zone II, while in type B zone II was quite distinct. Type C was represented by profiles showing distinct and widely separated discontinuous zones I and II. In contrast, type D was characterized by profiles having continuous zones I and II forming a ladder like pattern (Fig. 3). The four major LPS types, A, B, C and D, were not strictly host-specific. For example, all isolates representing LPS types A and D were obtained from *S. sesban* and *S. rostrata*, respectively, but other isolates from the same species were also included in LPS types B and C. Within each LPS type, microvariations were detected and the four LPS types were further classified into 13 subtypes (Fig. 3, Table 2). Profiles having a similar nature for zone II (distinct/diffuse bands or highly diffuse region) and more than 80% similarity in the bands were grouped under the same subtype. A type was categorized into two subtypes having less than 80% similarity in bands. Type B was classified into four subtypes. B1 and B2, with distinct bands, showed only 75% similarity; however, B3 showed diffuse bands and B4 showed an intensely stained highly diffuse region representing zone II. Type C was the largest group with high heterogeneity comprising six subtypes. These subtypes showed widely separated zone I and zone II, although they were different in appearance and

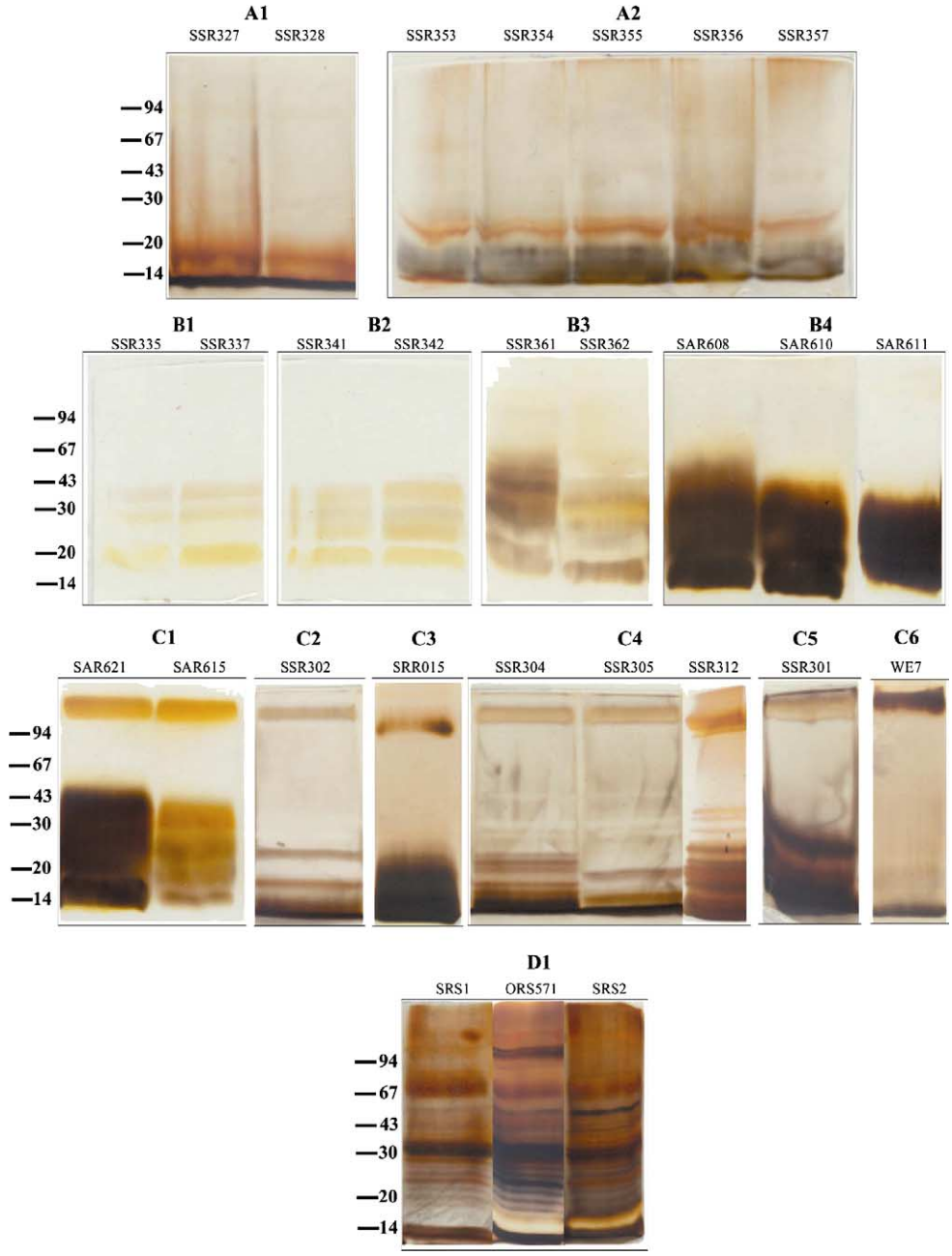

Fig. 3. Silver-stained SDS–PAGE profiles of purified lipopolysaccharides of twenty-eight isolates of rhizobia-nodulating sesbanias showing heterogeneity in LPS I (O-antigen) and forming 4 distinct LPS types with 13 subtypes. LPS type A with inconspicuous zone II (LPS I); type B with distinct zone II (LPS I); type C with distinct and discontinuous zones I and II (LPS I); and type D with continuous zones I and II (LPS I).

the number of bands in zone II. C1 and C3 subtypes showed a diffuse zone II, although they varied in the length of zone II and the mobility of zone I. C5 showed diffuse bands in zone II; however, C2 and C4 showed distinct bands having less than 80% similarity. C6 was represented by an insignificant zone II and a distinct band in zone I.

S. sesban isolates colonizing two different habitats, LAF and AHE, were segregated into two LPS subtypes, A1 and A2. Nineteen isolates of *S. sesban* from different habitats were segregated into 8 subtypes (A1, A2, B1, B2, B3, C2, C4, C5) but all the isolates under a single subtype were derived from a single host individual and habitat (Table 2). All the isolates of a single legume host from different habitats (SAR608, 610 and 611 from EGDU-B4 subtype; SAR621 and 615 from AWS-C1 subtype) and different legume hosts from the same habitat (from EGDU, SSR304, 305-C4 subtype and SAR608, 610 and 611-B4 subtype) were included in different LPS subtypes (Table 2). Isolates from individuals of the same species sampled from different microhabitats within the same habitat also showed distinct LPS subtypes. For example, five isolates obtained from legume phenotypes sampled from different microhabi-

tats within EGDU formed three different subtypes (SSR302-C2; SSR304, 305, 312-C4; SSR 301-C5).

3.4. Diversity in the plasmid genotype

Diversity in the *sym* plasmid was assessed by analyzing variations in NPC-PCR-amplified products using a primer corresponding to the conserved sequence in the *nif* H gene forming a part of the repeated sequence in the *sym* plasmid [34]. Based on the number and length of the amplified fragments, the 28 isolates were segregated into three major groups by cluster analyses (Fig. 4). Within each major cluster, the isolates having more than 80% similarity were considered as one subcluster. Ten isolates from *S. sesban* roots (SSR327, 328, 342, 353, 354, 355, 356, 357, 361 and 362) represented cluster I and formed 4 distinct subclusters, four isolates from *S. sesban* roots (SSR302, 335, 337, 341) represented cluster II and segregated into 3 subclusters, whereas ten isolates from *S. sesban*, *S. aegyptica* and *S. rostrata* roots (SSR301, 304, 305, 312, SAR608, 610, 611, 615, 621, SRR915) represented cluster III and segregated into 7 subclusters. The genetic diversity revealed by cluster analysis based on NPC-PCR reflects sorting of isolates across ecological diversity (Fig. 4). For example, isolates of *S. aegyptica* from EGDU (SAR608 610, 611) and AWS (SAR615, 621) formed markedly distinct clusters with a low percent of similarity (Fig. 3). Similarly, *S. sesban* isolates from fly ash (SSR335, 337) and a dry habitat (AHE) (SSR353, 354, 355, 357, and 361, 362) showed distinct clusters having a low percent of similarity. Interestingly, *S. aegyptica* isolates from EGDU showed higher similarity (62–72%) with profiles of *S. sesban* isolates from the same site and formed one compound cluster with them, but they showed low similarity (13–33%) with profiles of other isolates of *S. aegyptica* from AWS.

The grouping of isolates based on LPS types was reflected to a great extent in that observed based on the sym plasmids. All the isolates representing LPS type A were grouped together and formed cluster I, while isolates representing the C type were grouped under cluster III (Table 2). LPS type B was mainly represented by the isolates forming cluster II, although a few isolates of cluster I (SSR361, 362) and cluster III (SAR608, 610, 611) were also included in the B type (Table 2, Fig. 4).

3.5. Diversity in the host range of rhizobiophages

Rhizobiophages of rhizobia-nodulating *Sesbania* were segregated into 15 different plaque morphotypes (PM1-PM16) [27] and distributed among 2 different families, *Myoviridae* (PM1-9 and PM15-16) and *Siphoviridae* (PM10, 12, 13 and 14). These phages were obtained from 6 indicator rhizobial hosts (ORS571, WE7, SSR302, SSR312, SAR610, SRR910) representing all three species (*S. ses-*

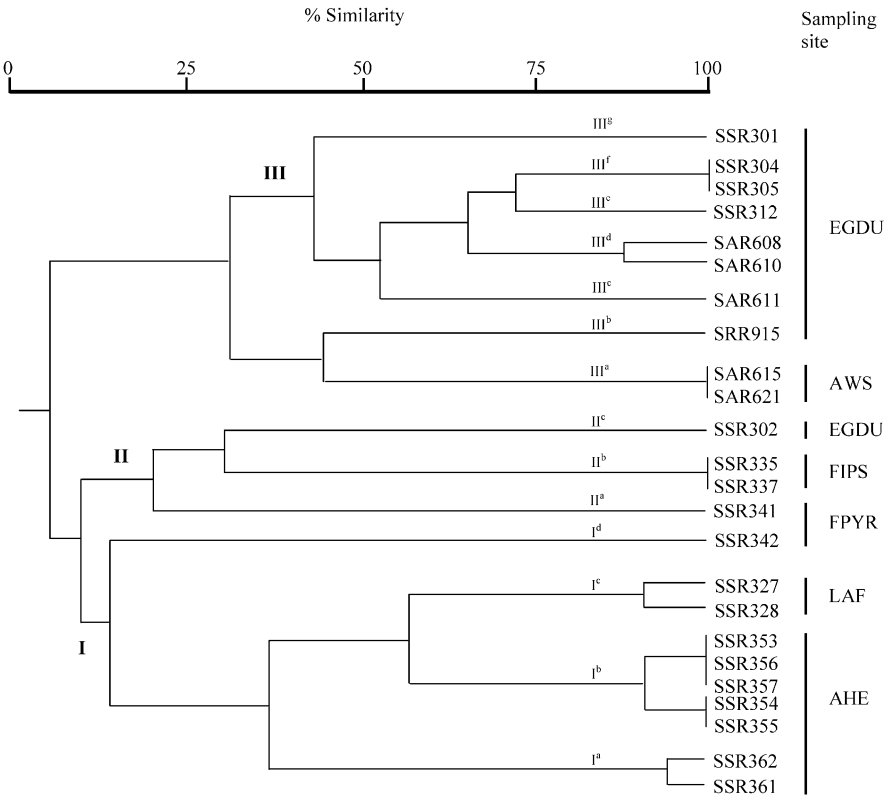

Fig. 4. Dendrogram showing the relationships among the root-nodulating bacteria of sesbanias at different levels of similarity. The dendrogram was constructed using UPGMA based on similarity matrix constructed using profiles of NPC-PCR products.

ban, *S. aegyptica* and *S. rostrata*). Of all the isolates, 35% of rhizobial species showed susceptibility to the rhizobiophages (Table 2). The rhizobiophages were highly specialized and showed a narrow host range. Interestingly, all phage-susceptible isolates were originally isolated from EGDU only. ORS571 and SRS1, 2, 3 showed susceptibility to PM1-9 and PM15-16; WE7 to PM10; SSR 302 to PM12, SSR 312 to PM13, and SAR 608 and 610 to PM14 phages.

3.6. 16S rDNA sequences of root- and stem-nodulating isolates from sesbanias

Representative isolates from all species inhabiting different habitats were used for 16S rDNA sequence analyses. The isolates represented all groups formed on the basis of LPS profiles and NPC-PCR (Table 2). SRS1 and SRS2 showed 100% sequence identity with *Azorhizobium caulinodans*; SSR301, SAR610 and SAR611 showed 100% sequence identity with *Sinorhizobium* sp. S005 and 99% with *S. saheli*; SSR312, SRR915 showed >99% identity with *Sinorhizobium* sp. S005 and were also close to *S. saheli*; SSR302 and SSR336 showed >99% sequence identity with *Sinorhizobium* sp. S002 and 99% with *S. meliloti*, and were close to *S. fredii* and *S. xingiangensis*. On the other hand, SSR327 and SSR354 showed >99% sequence identity with *R. huautlense* and 99% with *R. galgae*.

4. Discussion

All isolates successfully nodulated the homologous legume host (*Sesbania* sp.), suggesting that they belong to the rhizobia complex. 16S rDNA sequence analyses revealed that root nodule isolates of *S. sesban*, *S. aegyptica* and *S. rostrata* belong to *Sinorhizobium saheli*, *S. meliloti*, *Rhizobium huautlense*, and stem-nodule isolates of *Sesbania rostrata* are *Azorhizobium caulinodans*. Different rhizobial taxa (*Sinorhizobium*, *Rhizobium*, *Mesorhizobium*, *Azorhizobium*) from sesbanias have been reported from different geographical regions [2,3,5]; however, this is the first report of such distinct rhizobial species from the same host plant (*S. sesban*) and geographical region. *R. huautlense* has been reported from *S. herbacea* (Mexico) and *S. aculeata* (India and Japan). The limited diversity in *R. huautlense* from Mexico was proposed to be due to restricted geographical distribution of the legume host in flooded soils [33]. In contrast, it nodulated ecologically distinct sites—a dry and stressed habitat (AHE) and a low-lying, seasonally flooded habitat (LAF) of the semi-arid region of Delhi (Table 1). The five root-nodulating isolates (SSR301, 312, SAR610, 611, SRR915) from all three species represented *Sinorhizobium saheli* (Table 2). Interestingly, all three species of *Sesbania* were collected from a single habitat, i.e., EGDU. Trapping of specific rhizobial taxa by a legume host at a particular habitat seems to be influenced by the associated plant species and the history of the land use pattern at the sampling site. The community composition of the legume host has been shown to determine the rhizobial taxa nodulating *S. sesban* in African soils [2]. In the Delhi region diverse rhizobial taxa were found to nodulate the native population of *S. sesban*, and effective nodules were formed at ecologically diverse habitats varying in soil edaphic conditions (Table 1), whereas the distribution of compatible rhizobia was restricted to soils with at least 10% clay from low-lying areas in Africa [2]. It is noteworthy that different species of *Sinorhizobium* seem to dominate in the semi-arid Delhi region (Table 2), whereas *Mesorhizobuim* is dominant in African soils.

Remarkable uniformity in the shape and position of nodules formed by all isolates belonging to different taxa of rhizobia suggests a narrow genetic basis of these phenotypic traits. *S. sesban* isolates at AHE and LAF with moderately high symbiotic properties represented *R. huautlense*, while poor symbiotic efficiency and effectiveness of the isolates from EGDU and FIPS were contributed by different strains of *Sinorhizobium saheli* and *S. meliloti*. Different strains *of S. meliloti* nodulating *S. aegyptica* at EGDU and AWS showed significantly different symbiotic properties. Interestingly, *S. saheli* nodulating roots of *S. rostrata* showed low symbiotic efficiency coupled with a high mean nodule number and symbiotic effectiveness (Table 2). It may be noted that no significant difference was observed in symbiotic properties of *S. saheli* and *S. terangae* nodulating *S. rostrata* [3]. The symbiotic properties of root- and stem-nodule isolates of *S. rostrata* seem to be significantly different from those reported earlier [3]. The stem-nodulating isolates ORS571, SRS1, SRS2 (*Azorhizobium caulinodans*) nodulated the root and stem of *S. rostrata*, whereas the root-nodulating SRR915 (*S. saheli*) (from the semi-arid Delhi region) failed to nodulate the stem. However, *S. saheli*, *S. terangae* (root nodulating isolates from Africa) and *Azorhizobium* sp. were equally effective in nodulating the stem of *S. rostrata* [3]. Irrespective of the host legume and sampling site, the root nodule isolates lacked host specificity and nodulated all three *Sesbania* spp. (*S. sesban*, *S. aegyptica* and *S. rostrata*), suggesting the highly promiscuous nature of these rhizobia (*Sinorhizobium* spp. and *R. huautlense*). Most of the genes responsible for nodulation and nitrogen fixation are located on plasmids (except in *Azo*(*Brady*)*rhizobium*), and a certain degree of promiscuity can be expected due to transfer and recombination of symbiotic plasmids [18]. Hybridization studies of the *sym* plasmid of the isolates also suggested that recombination and rearrangement of sequences is common and the gene pool of different rhizobial taxa nodulating sesbanias is in a state of flux [18]. Nodulation of these rhizobia to different *Sesbania* spp. supports the hypothesis of a common inoculation group within the genus *Sesbania* [15]. The high promiscuity among sesbanias and its rhizobia seems to be responsible for the wide ecological amplitude of this plant

genus. Indeed, other legumes occupying a wide range of habitats also displayed high promiscuity and symbiotic effectiveness [30].

Lack of host-specific LPS profiles can be attributed to the fact that *Sesbania* spp. and the nodulating rhizobia are highly promiscuous in nature. Recognition of LPS subtypes led to sorting of isolates across diversity in host species and the habitat (Table 2). Therefore, each subtype was represented by isolates derived from a single legume host species sampled from the same habitat. The above observations suggest that between the *Sesbania* species, it is the habitat and host species together which might influence the diversity in LPS profiles. Isolates showing an A type LPS were represented by *R. huautlense*, which is phylogenetically close to *R. galegae*. Remarkable homogeneity in the LPS profiles of different strains of *R. huautlense* (LPS type A) (Fig. 3) corroborates observations made by other workers [33]. Isolates with B and C types were represented by *Sinorhizobium* spp. (*S. saheli*, *S. meliloti*), while the D type was exclusively represented by *Azorhizobium caulinodans* (Table 2). The B and C type profiles were found to be highly diverse in their O-antigen and strain specificity (Fig. 3), which is in contrast to the LPS profiles of *S. meliloti* and *S. fredii* from *Glycine max* and *Medicago sativa* [23]. Such heterogeneity might serve the adaptational needs of rhizobia to the local soil environment. LPS have been proposed to play an important role in colonization of bacteria to specific ecological niches, and heterogeneity was shown to be associated with different plant microhabitats and soil environments [22]. LPS diversity has also been correlated with enhanced survival of rhizobia under different ecological conditions [23,28]. Therefore, the diversity observed in LPS subtypes may be taken as a measure of the degree of adaptation of *Sesbania* rhizobia toward colonizing ecologically diverse habitats in the semi-arid Delhi region. Variability observed in the O-antigen chain (LPS I) seems to have significance in strain identification. Such variations have also been used for circumscription of rhizobial taxa [12,14].

Genetically diverse rhizobia showed a distinct pattern of rhizobiophage sensitivity. Indeed, different strains of *S. saheli* also varied in their phage susceptibility (Table 2), for example, SSR301 and SAR611 were phage-resistant, while SSR312 and SAR610 were susceptible to PM13 and PM14, respectively. These isolates belonging to the B_4, C_2 and C_5 subtype also showed extensive diversity in their LPS profiles. The diversity observed in LPS profiles might also be associated with the pattern of sensitivity to rhizobiophages. LPS has been shown to be involved in the phage sensitivity trait in rhizobia [4].

Grouping of isolates from different host plants in the same cluster based on similarity in NPC-PCR profiles (Fig. 4) seems to be associated with promiscuity; for example, *S. aegyptica* isolates from EGDU (SAR610, SAR611) joined together with *S. sesban* isolates (SSR301, SSR312) from the same habitat and grouped under cluster III, while the other isolates of *S. sesban* (SSR302) from the same habitat were grouped with SSR335 under cluster II. It may be noted that SAR610, SAR611, SSR301, and SSR312 belong to *S. saheli*, while SSR302 and SSR335 belong to *S. meliloti*. The isolates representing strains of *R. huautlense* were grouped under cluster I, while the other isolates representing *S. meliloti* were grouped into cluster II (Table 2). All five isolates representing strains of *S. saheli* were grouped together under cluster III. All isolates grouped together under different LPS subtypes also formed distinct subclusters based on NPC-PCR. Therefore, parallel diversity was observed in the grouping of the isolates based on the *sym* plasmid and LPS profiles. Segregation of different rhizobial species into distinct types/clusters based on LPS and NPC-PCR analyses suggests their significance in the circumscription of the rhizobial taxa. However, subtypes and subclusters showed their sorting across the ecological gradients.

The enormous diversity in LPS profiles and the extremely high specificity of rhizobiophages among the strains of different rhizobial taxa might result from environmental selection pressures operating in ecologically distinct habitats and leading to strain differentiation. The wild populations of rhizobia are subjected to a number of stresses and interactions in nature, and therefore, extensive investigations of these populations from the same legume host occurring across the ecological gradients are essential for improving legume-*Rhizobium* symbiotic technology.

In conclusion, the ability of sesbanias to enter into effective symbioses with different rhizobial taxa and colonize diverse habitats with various biotic and abiotic stresses appears to be associated with its wide ecological amplitude. Diversity among rhizobia in their functional traits reflects their competence in surviving and colonizing the rhizosphere of different species of *Sesbania* in diverse habitats. Consequently, microsymbionts associated with sesbanias contribute to their ability to colonize a maximum of the possible ecological niches available. Therefore, promiscuity among rhizobia as well as *Sesbania* has ecological significance. The present study provides a scientific basis for the successful use of sesbanias in soil rehabilitation programs. It also provides baseline information for the improvement of strategies for using sesbanias and the selection of appropriate rhizobial taxa as inoculants in ecologically diverse habitats.

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