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PHENOTYPIC AND METABOLIC DIVERSITY AMONG THE ROOT NODULE BACTERIA FROM THREE *Vigna* CROPS OF THE THAR DESERT, INDIA

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ABSTRACT

Root-nodule bacteria of *Vigna* species in the Indian arid zone remain poorly characterized relative to the wild legumes of the same region. Sixteen bacterial strains were isolated from determinate root nodules of three cultivated *Vigna* species (*V. unguiculata*, *V. aconitifolia* and *V. radiata*) grown in Jodhpur, Rajasthan. These strains were characterized for phenotypic traits, biochemical activities, and identified based on 16S rRNA gene sequence using the nucleotide Basic Local Alignment Search Tool (BLASTn). Every strain grew at pH 5.5 and up to 40°C, and 14 of 16 strains also grew at pH 10. Variation was seen in their salt tolerance all strains grew in 1% (w/v) NaCl, but only six of them grew in 5%. Carbon-source utilization varied, from seven of 21 sugars in VU9 to 19 in MEHA 5 and PDM 18; dextrose, maltose, salicin and trehalose were used by every strain tested. Chloramphenicol resistance was the most frequent antibiotic phenotype, and no strain resisted streptomycin or gentamicin. This collection of strains is phenotypically heterogeneous; salinity tolerance and carbon-source range differ widely among strains from the same soils. Sequencing of 16S rRNA gene placed eleven strains in *Sinorhizobium*, one each in *Rhizobium* and *Bradyrhizobium* and three in the Enterobacteriaceae. *Sinorhizobium*, rather than *Bradyrhizobium* dominated the *Vigna* root nodule isolates in present study and were metabolically diverse.

Key words : *Vigna*, Root-nodule bacteria, *Sinorhizobium*, Phenotype, Salinity, 16S rRNA.

Introduction

Mung bean (*Vigna radiata*), moth bean (*V. aconitifolia*) and cowpea (*V. unguiculata*) of tribe Phaseoleae of sub-family Papilionoideae are the principal pulse crops of arid zone of western Rajasthan. All three crop species form determinate "phaseoloid" type of root nodules, where the symbiotically fixed nitrogen by rhizobia is exported to plant in the form of ureides. The three crops were initially considered as one broad cowpea cross-inoculation miscellany nodulated by slow-growing *Bradyrhizobium* across much of Africa and Asia. Surveys across contrasting agro-ecological regions of India recovered *B. yuanmingense* as the principal symbiont of *V. mungo*, *V. radiata* and *V. unguiculata* (Appunu et al., 2009). Field-collected root nodules of mung bean in Pakistan had *B. yuanmingense* and fast-growing *Ensifer* (*Sinorhizobium*) *aridi* co-dominant, with *E. meliloti* specifically dominant in desert soils (Hakim *et al*., 2021). Several species have been described from *Vigna* nodules such as *B. manausense* from *V. unguiculata* in Brazil (Silva *et al*., 2014) and *B. kavangense* and *B. vignae* from cowpea, Bambara groundnut and peanut grown in the Kavango region of Namibia (Grönemeyer *et al*., 2015, 2016). Rhizobia reported from native legumes of western Rajasthan are genetically diverse and belong predominantly to *Ensifer*/*Sinorhizobium* rather than to *Bradyrhizobium* a pattern observed through multiple studies on mimosoid trees, herbaceous papilionoids and caesalpinioids alike (Gehlot *et al*., 2012; Tak *et al*., 2016a, 2016b; Sankhla *et al*., 2015, 2017, 2018; Rathi *et al*., 2017, 2018; Choudhary *et al*., 2017, 2018, 2020; Tak and Gehlot, 2019; Bissa *et al*.,

2024), even extended to invasive legumes (Chouhan *et al*., 2022, 2024; Bissa *et al*., 2024). Selection is not uniform across hosts: soil pH and other edaphic factors determine whether *Chamaecrista pumila* recruits *Bradyrhizobium* or *Ensifer* (Rathi *et al*., 2018). The *Vigna* crops that occupy most of the cropped area have not been studied except for wild *V. trilobata* (Tak *et al*., 2016a) and *Sinorhizobium saheli* was reported as predominant in moth bean nodules (Pancholy and Singh, 2021). Growth under salt, temperature and pH stress, together with biochemical profiles and carbon-source use, has long been the first screen of rhizobial collections (Somasegaran and Hoben, 1994). Therefore symbionts of *Vigna* in hot, saline, alkaline soils of Indian Thar desert needs to be identified and characterized. Present study identified sixteen bacterial strains isolated from root nodules of *V. radiata*, *V. aconitifolia* and *V. unguiculata* grown in arid Rajasthan, tested them for multiple phenotypic (tolerance of temperature, pH, and salinity), biochemical attributes, carbon-source range and intrinsic antibiotic resistance to analyze their metabolic diversity.

Materials and Methods

Origin of bacterial strains : Sixteen bacterial strains isolated from root nodules of different cultivars of three *Vigna* species: *V. radiata* (cultivars MEHA and PDM-139), and *V. aconitifolia* (cultivars RMO-435 and RMB-44) sampled from field of the Agriculture University, Jodhpur (AUJ) and plants of *V. unguiculata* (cultivars DC-15 and PL-3) sampled from ICAR-National Bureau of Plant Genetic Resources (NBPGR), following the standard procedure of Somasegaran and Hoben (1994) and Tak *et al*. (2020). These selective purified bacteria were used in present study for phenotypic and biochemical characterization: four from *V. unguiculata* (VU1, VU5, VU9 and VU12), four from *V. aconitifolia* (VA3, VA4, VA12 and VA14) and eight from *V. radiata* (MEHA 4, MEHA 5, MEHA 10, PDM 5, PDM 11, PDM 14, PDM 15 and PDM 18).

Phenotypic characterization : Tolerance of these sixteen bacterial strains to abiotic stress was assessed on yeast extract mannitol agar (YEMA) as per Somasegaran and Hoben (1994), Tak *et al*. (2020). Activated bacterial broth cultures were used to spot onto plates and growth was recorded after incubation at 28°C for 4-5 days, unless stated otherwise. Temperature tolerance was determined by incubating inoculated plates at 28°C, 35°C and 40°C; pH tolerance was determined on YEMA adjusted to pH 5.5, 6.5 and 10.0 before autoclaving; and salt tolerance was determined on YEMA amended with 1%, 2%, 3%, 4% and 5% (w/v) NaCl (Somasegaran and Hoben, 1994; Tak *et al*., 2020). For intrinsic antibiotic resistance (IAR) test strains activated in YEM broth were swabbed uniformly onto YEMA plates, and sterile antibiotic discs of kanamycin (30 µg), chloramphenicol (30 µg), tetracycline (30 µg), streptomycin (300 µg), gentamicin (10 µg) and ciprofloxacin (5 µg) were placed aseptically on the inoculated plates followed by incubation at 28°C for 2-4 days and the diameter of the zone of inhibition around each disc was measured in millimeters (Cappuccino and Sherman, 2007; Tak *et al*., 2020). Carbon source utilization was evaluated in Andrade peptone water (HiMedia) with sterile discs (HiMedia) of 21 sugars added as the sole carbon source. Wells were examined every 24 h for up to 3-4 days, a change from pale straw to light or dark pink indicating utilization of the corresponding sugar (Tak *et al*., 2020).

Biochemical characterization : A panel of biochemical tests were done following the standard procedures by Cappuccino and Sherman (2007) for catalase (effervescence on adding 3% hydrogen peroxide on colony); oxidase (disc with 1% [w/v] tetramethyl-p-phenylenediamine dihydrochloride turning deep purplish in color); o-nitrophenyl-β-D-galactopyranoside (ONPG) disc to assay β-Galactosidase activity; Methyl red and Voges–Proskauer reactions in MR-VP broth confirmed with methyl red indicator and Barritt's reagents, respectively; citrate utilization was tested by development of a blue color by strains on Simmons citrate agar slants; hydrogen sulphide (H_2S) production was detected by blackening along the stab-inoculated triple sugar iron (TSI) agar slants; gelatin hydrolysis was assessed by liquefaction of nutrient gelatin at chilling temperature (4°C) and Lipase activity was detected through formation of clear zone on tributyrin agar. Starch hydrolysis was assessed by streaking strains onto starch agar and incubating at 28°C for 24 h. Plates were then flooded with a few drops of iodine solution; a blue-black color indicated that starch had not been utilized, whereas a clear halo around the growth indicated starch hydrolysis (Cappuccino and Sherman, 2007). In addition to these routine biochemical assays the strains were tested for nitrate reduction ability in nitrate broth that was incubated at 28°C for 48 h after inoculation. Five drops each of sulphanilic acid and α-naphthylamine solution were added to each tube; development of a pink to red color indicated the presence of nitrite and was scored as positive. Colorless tubes were confirmed by the addition of zinc dust: development of a pink color after zinc addition indicated that residual nitrate was still present and the strain was scored as nitrate-reduction negative, whereas

the absence of color indicated that nitrate had been reduced beyond nitrite (Cappuccino and Sherman, 2007).

Amplification and sequencing of 16S rRNA gene : Total genomic DNA of bacterial strains was extracted from 48-72 h YEM broth cultures by phenol-chloroform method. The 16S rRNA gene was amplified with primers 18F (5′ -AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′ -CTACGGCTACCTTGTTACG-3′) (Weisburg *et al*., 1991). Cycling comprised initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 45 s, 60°C for 1 min and 72°C for 1.5 min, and a final extension at 72°C for 7 min. PCR products were resolved by electrophoresis in pre-stained (ethidium bromide) 1% (w/v) agarose gels alongside a DNA ladder and visualized under BioRad Gel documentation system. Purified amplicons of the 16S rRNA were Sanger sequenced through outsourcing. The edited sequences in FASTA format were used for similarity searches against the GenBank database of the National Center for Biotechnology Information (NCBI) using nucleotide BLAST (megablast with default parameters).

Results and Discussion

Tolerance to pH, temperature and salinity : Sixteen bacterial strains isolated from root nodules of different cultivars of three *Vigna* species sampled from field of the AUJ and ICAR- NBPGR were profiled for various phenotypic traits. All sixteen strains grew at pH 5.5 and 6.5, and fourteen also grew at pH 10; except VU12 and VA12 that failed at the alkaline extreme (Fig. 1). Every strain from this study grew at 40°C thus are thermo tolerant. Salinity was the trait that separated the strains most clearly with growth in 1% (w/v) NaCl was universal, at 2% strains PDM 15 and PDM 18 failed to grow, and at 4% only nine strains grew. Six strains (VA3, VA4, VA14, MEHA 4, MEHA 5 and MEHA 10) grew in 5% NaCl. The phenotypic profile of the strains from present study is broadly consistent with the *S. aridi* species description: all sixteen strains from this study grew at 40°C and fourteen at pH 10, and the six strains that grew in 5% (w/v) NaCl, a tolerance exceeding the 300 mM (~1.75%) reported for the type material (Rocha *et al*., 2020). Wide variation in salt and physiological tolerance within a single collection of *Vigna*-nodulating rhizobia is not unusual, the indigenous *V. unguiculata* rhizobia from Zimbabwean soils showed comparably wide physiological variation between isolates of the same collection (Mpepereki *et al*., 1997).

Intrinsic antibiotic resistance : Zones of inhibition against different antibiotics tested are shown in Fig. 2. No strain was resistant to streptomycin (300 µg) or gentamicin (10 µg); in contrast chloramphenicol was the

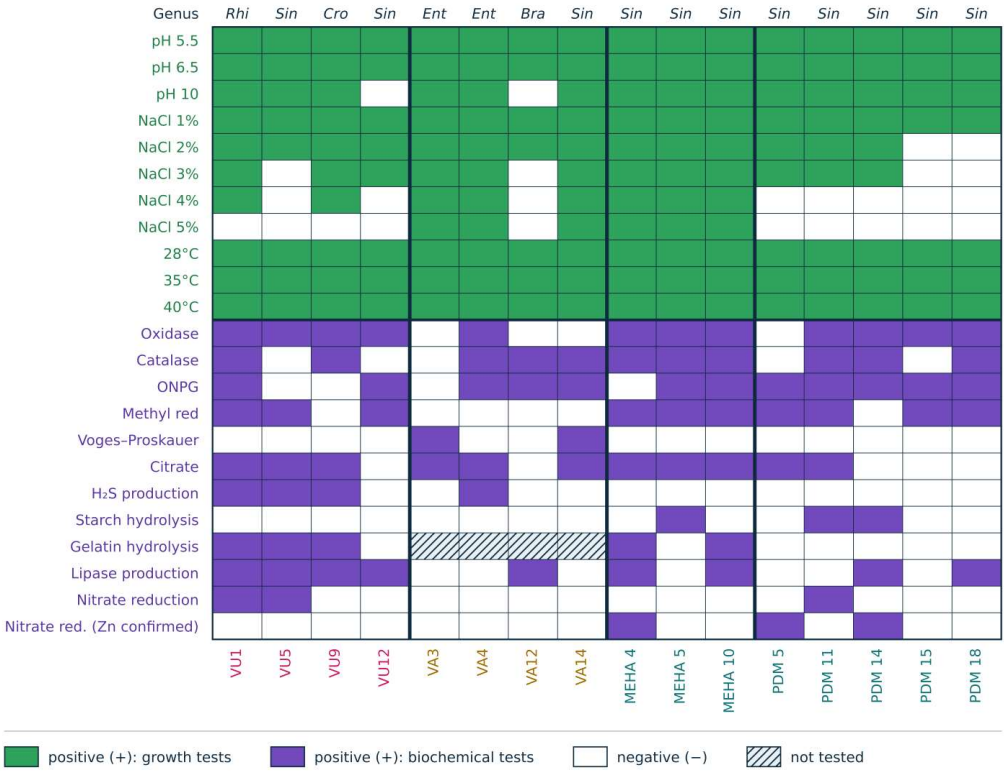


Fig. 1 : Phenotypic and biochemical profile of sixteen bacterial strains isolated from root nodules of *Vigna radiata*, *V. aconitifolia* and *V. unguiculata*. (Abbreviations: *Rhi, Rhizobium; Sin, Sinorhizobium* (*Ensifer*); *Cro, Cronobacter*; *Ent, Enterobacter*; *Bra, Bradyrhizobium*).

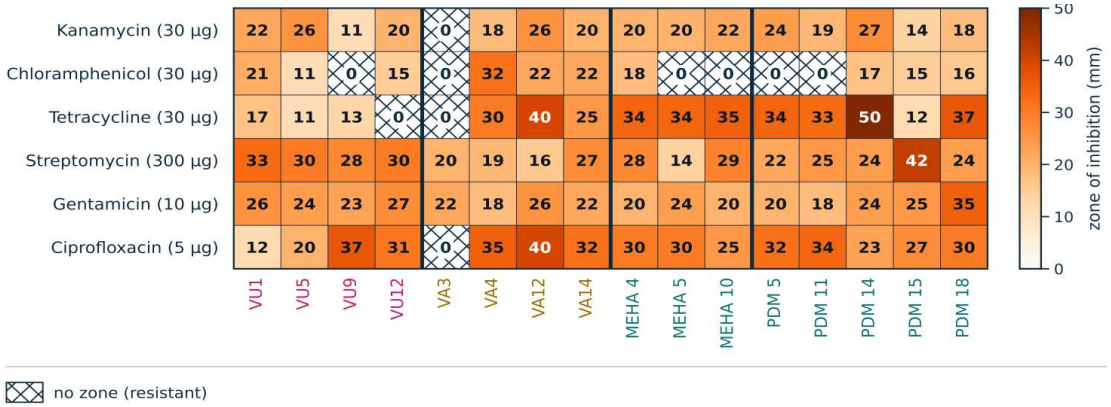


Fig. 2 : Intrinsic antibiotic resistance of the sixteen *Vigna* bacterial strains.

most frequently resisted antibiotic, giving no zone at all in six (VU9, VA3, MEHA 5, MEHA 10, PDM 5 and PDM 11) strains. VA3 was the most resistant strain showing no inhibition to kanamycin, chloramphenicol, tetracycline or ciprofloxacin. Even within one species the profiles can differ greatly: among strains of *S. aridi*, the Indian strain TP6 grew in the presence of chloramphenicol, kanamycin, streptomycin, nalidixic acid and spectinomycin, whereas Moroccan and Mexican strains resisted only nalidixic acid and spectinomycin (Le Quéré *et al*., 2017).

Metabolic profiling : Across the 21 sugars tested, the number of sugars utilized as sole carbon source per strain ranged from 7 by VU9 to nineteen by strains MEHA 5 and PDM 18 (Fig. 3). Four sugars dextrose, maltose, salicin and trehalose were utilized by every strain tested. Followed by fructose and mannose, only two strains VU9 and VA14 respectively failed to break them down. At the other hand, adonitol was used by strain MEHA 10 alone; dulcitol and inositol by three strains each; and rhamnose by five tested strains (Fig. 3). Sugars cellobiose, melibiose and raffinose were unused by VU1, VU5 and VU9 but used by almost every other strain tested. Salicin sugar was utilized by all sixteen strains, whereas it is negative or variable across *S. meliloti*, *S. saheli*, *S. terangae*, *S. sojae*, *S. mexicanum* and *S. kostiense* type strains (Kuykendall *et al*., 2005; Li *et al*., 2011), hence it is a collection-level character that distinguishes these *Vigna* root-nodule symbionts from established type strains. Rocha *et al*. (2020) also noted a lack of correlation between metabolic activity and taxonomy of *Ensifer* strains.

Biochemical profiling : The biochemical results (Fig. 1) were heterogeneous in this collection of sixteen strains. Oxidase was positive in twelve and catalase in eleven strains tested. Starch was not hydrolyzed by thirteen of the sixteen strains, the exceptions being MEHA 5, PDM 11 and PDM 14. Citrate was utilized by eleven strains; gelatin was hydrolyzed by five (VU1, VU5, VU9, MEHA 4 and MEHA 10) strains and lipase activity was detected in nine strains with clear zone of solubilization. Nitrate reduction was positive in three strains VU1, VU5 and PDM 11 on adding the reagents. From the thirteen colorless tubes MEHA 4, PDM 5 and PDM 14 gave a pink color after zinc addition, confirming that nitrate had not been reduced in those tubes while the other ten strains gave no color, which indicates reduction beyond nitrite. Methyl red was recorded as positive in ten of the sixteen strains, although Voges-Proskauer was positive in only VA3 and VA14 and H_2S production was seen in TSI medium only four (VU1, VU5, VU9 and VA4). Starch hydrolysis was negative in thirteen of sixteen strains, matching the genus-level pattern (Li *et al*., 2011), while catalase activity (positive in eleven of sixteen) showed the same species-level variability documented between *S. psoraleae* and the closely related *E. sesbaniae* (Wang *et al*., 2013; Li *et al*., 2016).

Identification by 16S rRNA gene sequence similarity: Of the sixteen strains studied, based on 16S rRNA gene sequences eleven were identified as *Sinorhizobium*, one as *Rhizobium* (VU1) and one as *Bradyrhizobium* (VA12); while the remaining three VU9, VA3 and VA4 belonged to the Enterobacteriaceae (*Cronobacter* and *Enterobacter*, respectively). In the BLAST search against the NCBI nt database strains VA3 and VA4 were 100% identical to *Enterobacter cloacae* JNVU TF1 and JNVU TW3, respectively (Tak and Gehlot, 2025). Strain VU9 was 98% identical to *Cronobacter sakazakii* Cs-YZU01 (CP193903). The three non-rhizobial strains VA3 and VA4 (*Enterobacter cloacae*) and VU9 (*Cronobacter sakazakii*) are a well-documented feature of surface-sterilized nodule isolations; non-rhizobial bacteria enter the root nodules through rhizobial infection threads (Pandya *et al*., 2013). The halo-tolerance and high antibiotic resistance of VA3 in particular, are consistent with an Enterobacteriaceae background,

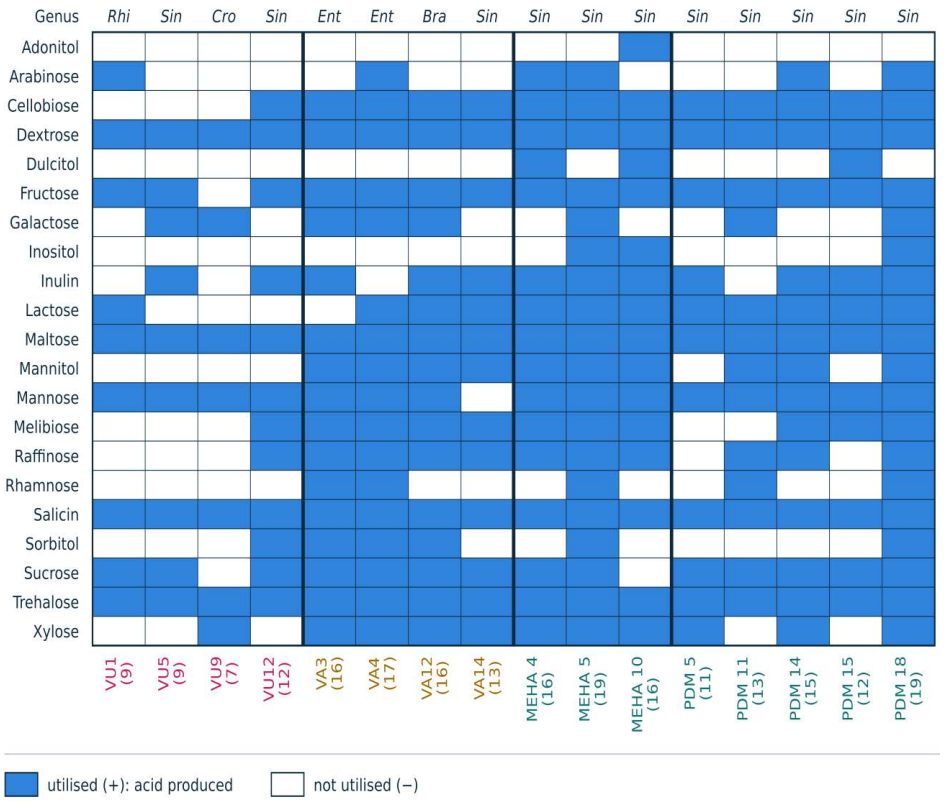


Fig. 3 : Utilization of 21 sugars as sole carbon source by the sixteen strains in Andrade peptone water. The number of sugars utilized by each strain is given in parentheses beneath the strain name.

confirming that it is not a functional symbiont regardless of its nodule origin.

Strain VU1 was identified as *Rhizobium*, sharing 100% similarity with *Rhizobium fabae* AS8 (PQ151672). The single *Bradyrhizobium* strain, VA12 from this study shared similarities with *B. yuanmingense* CCBAU 010071^{T} (isolated from *Lespedeza cuneata*, China) and many other strains isolated from legumes of Indian Thar desert. Remaining eleven strains shared similarities with *Sinorhizobium* strains particularly with Indian Thar desert *S. aridi* clade (Tak *et al*., 2013, 2016b; Le Quéré *et al*., 2017); strains adapted to stressed environments LMR001^{T} (isolated from *Acacia gummifera*), LMR003 (from *Acacia tortilis* ssp. *raddiana*) and RMT3 (isolated from *Retama monosperma*) from Morocco; additionally with strains isolated from root nodules of *Tephrosia strigosa* (TS40, TS54 and TS64) and *Indigofera linnaei* (RUIL19, RUIL30) from Aravalli Region in Rajasthan; strain RCAM05007 from *Cyamopsis tetragonoloba*, Nagaur; and several other Indian *Ensifer*/*Sinorhizobium* strains isolated from Indian Thar desert-legume hosts. The most striking point of this study is the dominance of fast-growing *Sinorhizobium* strains, with *Bradyrhizobium* represented by only a single isolate, VA12, from *V. aconitifolia*. This contrast with the prevailing picture of *Vigna* symbiosis, in which *Bradyrhizobium* is the usual microsymbiont: *B. yuanmingense* was the principal symbiont of *V. mungo*, *V. radiata* and *V. unguiculata* across several Indian agro-ecological regions (Appunu *et al*., 2009). In profiling of mung bean nodules across four Pakistani habitats *Bradyrhizobium* was found dominant at three sites and *Ensifer* at the fourth (Hakim *et al*., 2021). Multiple studies from Western Rajasthan, India has consistently recovered *Sinorhizobium*/*Ensifer* as the preferred symbionts of legumes in Thar Desert soils (Gehlot *et al*., 2012; Tak *et al*., 2016a, 2016b; Sankhla *et al*., 2015, 2017, 2018; Rathi *et al*., 2017, 2018; Choudhary *et al*., 2017, 2018, 2020; Tak and Gehlot, 2019; Chouhan *et al.*, 2022, 2024; Bissa *et al*., 2024). Rathi *et al*. (2018) study demonstrated that soil pH and edaphic factors, rather than host identity alone, determine which rhizobial genus is recruited as symbiont. Therefore the present result also confirms the selection of preferred *Sinorhizobium* symbiont in arid and alkaline conditions.

Conclusion

This study establishes that fast-growing *S. aridi*, is a dominant microsymbiont of *Vigna* species in the arid alkaline soils of western Rajasthan, this differs from the *Bradyrhizobium*-centered picture of *Vigna* symbionts established elsewhere in India and Asia. The reason for

dominance of this *S. aridi*-type in arid regions of Thar Desert and also extended to other agroclimatic regions of India might be the arid alkaline saline soils, high temperature and low humidity. For strains that might eventually serve as inoculants for dry-land ecosystem, what matters is whether they can survive under salinity, high temperature and alkalinity. Therefore, selecting stress-tolerant indigenous desert rhizobia rather than importing commercial inoculants will prove effective. *Sinorhizobium* strains from this study meet out these criteria as they are stress tolerant making them potential candidates for inoculant development.

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Conflict of interest : The authors declare that they have no conflicts of interest.

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