Original Research

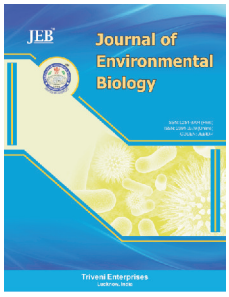
Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

DOI : http://doi.org/10.22438/jeb/39/5/MRN-709

JEB™

p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP



Identification and molecular characterization of root nodule microsymbiont of *Trigonella foenum-graecum* L. growing in different soils from Western Rajasthan, India

Authors Info

S. Gaur[1], N. Tak[1], S. Rathi[1], S. Choudhary[1,2] and H.S. Gehlot[1]*

[1]BNF and Microbial Genomics Lab., Department of Botany, UGC-Center of Advanced Study, Jai Narain Vyas University, Jodhpur - 342 001, India

[2]Forest Protection Division, Arid Forest Research Institute, Jodhpur - 342 005, India

*Corresponding Author Email : hsgehlot@gmail.com

Key words

Ensifer
Phylogeny
Semi-arid
Sym genes
Trigonella foenum-graecum

Publication Info

Paper received : 02.08.2017
Revised received : 06.12.2017
Re-revised received : 13.01.2018
Accepted : 06.02.2018

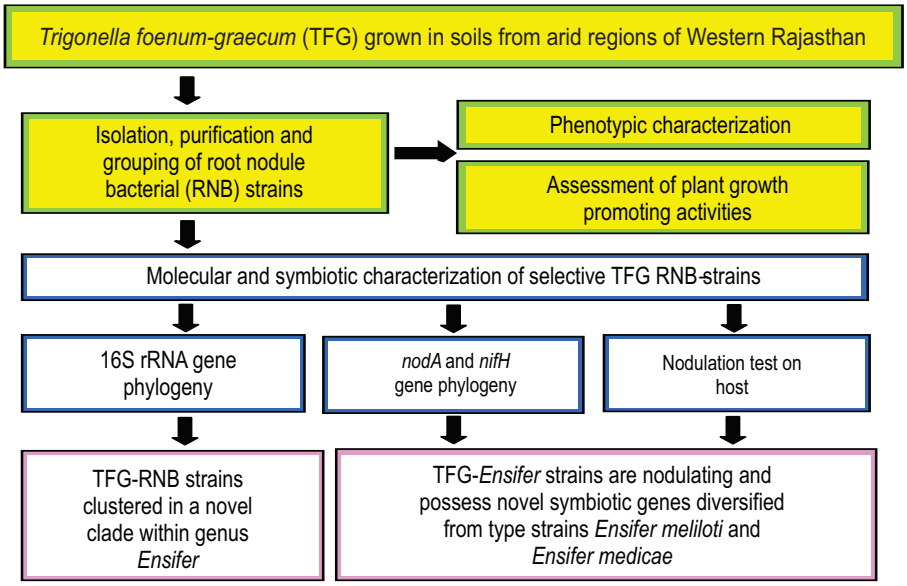
Abstract

Aim : To identify and characterize N fixing root nodule bacteria (RNB) of *Trigonella foenum-graecum* (TFG) growing in soils collected from different districts of Western Rajasthan.

Methodology : Selected RNB strains were characterised phenotypically and molecular phylogeny was studied using their 16S rRNA and symbiosis-related gene sequences.

Results : The 31 selected RNB strains formed three groups on the basis of colony characteristics. The TFG-RNB strains showed metabolic diversity in terms of carbon utilization and intrinsic antibiotic resistance, with few strains showing NaCl tolerance up to 4% and growth on a wide range of pH (4-11). In 16S rRNA gene phylogeny six (TFG22, TFG33, TFG53, TFG59, TFG64 and TFG66) selected strains clustered in a novel clade close to type strains *Ensifer medicae* and *Ensifer meliloti* forming two distinct 16S rRNA types. The symbiotic (*nodA* and *nifH*) and 16S rRNA phylogenies of TFG-*Ensifer* strains were in congruence. The *sym* genes of TFG strains have intermediate sequences diversified from closely related *E. meliloti* and *E. medicae* which indicates their evolution through horizontal gene transfer.

Interpretation : Nitrogen fixing TFG-*Ensifer* strains isolated from Western Rajasthan are potentially novel and showing plant growth promoting activity therefore can be used as inoculants in agricultural fields of semi-arid regions.



Introduction

Leguminosae is considered as the third largest family of flowering plants both in terms of number of genera as well as species and includes ca. 730 genera and ca. 19,400 species (Lewis *et al.,* 2005). Legumes are important source of protein rich food and nutritious fodder in addition to medicines. Legume-rhizobia symbiosis is well known for providing fixed nitrogen in the form of ammonia to the host plant by specialized bacteria commonly known as rhizobia, harbouring nodulation and nitrogen fixing genes. This accomplishment reduces plant's requirement for harmful nitrogenous fertilizers externally provided for their growth (Somasegaran and Hoben, 1994). *Trigonella, Medicago* and *Melilotus* are three related genera of tribe *Trifolieae* of legume sub-family Papilionoideae, whose natural distribution extends from Mediterranean basin to throughout West Asia where large number of species exists in these genera (Lesins and Lesins, 1979). The Nitrogen fixing bacteria associated with this group of plants are specific and known as *Trigonella-Medicago-Melilotus* group of microsymbionts such as *Ensifer* (*Sinorhizobium*) *meliloti* and *Ensifer medicae* are two species having significant economic importance nodulating forage crops (*Medicago sativa* and *Medicago truncatula*) in the world (Reeve *et al.,* 2010). Most of the work on symbiotic diversity associated with this group of plants has been done for *Medicago* species.

The *Medicago* microsymbionts are cross-inoculating *Trigonella* as well as *Melilotus*. Since the microsymbionts (*E. meliloti* and *E. medicae*) are unique for the *Trigonella-Medicago-Melilotus* group all over the world hence, it would be interesting to see if these microsymbionts show any geographical patterns. A study was carried out by Rajendran *et al.* (2012) on non-rhizobial nodule-associated bacteria (NAB) of *Trigonella foenum-graecum* which promoted plant growth when co-inoculated with *E. meliloti* strains. On the basis of ARDRA, the genetic diversity of few rhizobial strains associated with *T. foenum-graecum* was investigated by Pandey *et al.* (2004). Five species of *Trigonella* (*T. foenum-graecum, T. monantha, T. occulta, T. corniculata* and *T. hamosa*) have been reported from Western Rajasthan in Flora of Indian desert (Bhandari, 1990). In India, *Trigonella foenum-graecum* L. commonly called as Fenugreek (locally as Methi) is found growing in states such as Rajasthan, Gujarat, Uttar Pradesh and also in Punjab and Maharashtra as spice crop. The seeds and leaves of this annual aromatic herb plant are used for various medicinal purposes since ancient times. The present study aimed to characterize the root nodule microsymbionts of *T. foenum-graecum* grown in the alkaline desert soils of different regions of Western Rajasthan at molecular level.

Materials and Methods

Rhizobia trapping experiment and isolation of root nodule bacterial (RNB) strains : To trap rhizobia, the soils collected from different arid and semi-arid regions of Western Rajasthan were used to grow the plants of *T. foenum-graecum*. The trapping experiments were conducted following the procedure of Gehlot *et al.* (2013) and Sankhla *et al.* (2017). Excavated nodules were studied to observe nodule morphology including nodule size, shape, color, number and position of nodules on roots. Root nodules were surface sterilized and streaked on Congo red-Yeast Extract Mannitol Agar (CR-YEMA) medium (Somasegaran and Hoben, 1994). The plates after streaking were incubated at 28°C for about one week and regularly checked for growth of bacterial strains. Bacterial cultures were purified by four-way streaking and pure single cell colonies were sub-cultured regularly and stored at 4°C.

Colony characteristics : The pure bacterial colonies cultured on YEMA medium were observed for colony characters such as colony shape, growth duration, color, EPS (exopolysaccharide) production, visibility across colonies (opaque, transparent or translucent), shiny, margins, curdling and acid production.

Tolerance to salt, pH and temperature; and reaction to BTB : The capacity of bacterial strains to tolerate NaCl {1 to 4% NaCl (w/v)} and grow at range of pH (acidic to alkaline) was determined using the methods described in Somasegaran and Hoben (1994). The temperature tolerance of RNB strains was determined by streaking YEMA plates with activated bacterial cultures and incubating them at different temperatures (28 to 42ºC) in a BOD incubator for 4-5 days. The production of acid or alkali by RNB strains was tested as described by Somasegaran and Hoben (1994). The list of strains on which phenotypic tests were performed is given in Table 1.

Intrinsic antibiotic resistance (IAR) and carbon (sugar) utilization pattern of RNB strains : Sensitivity or resistance of bacterial strains to various antibiotics (Table 2) was determined using ready to use HiMedia Octa antibiotic discs with different concentrations of antibiotic. Activated bacterial broths were spread with help of sterile spreader on the YEM agar plates before placing the Hi-Media Octa antibiotic discs aseptically. Plates were incubated for 48 hrs at 28±2ºC. The capability of the strains to utilize 21 different carbon (sugar) sources (Table 2) was tested using HiMedia Discs for Carbohydrate fermentation tests and Andrade's peptone water as per manufacturer's instruction. The change in color of Andrade indicator from straw coloured to pink due to production of acids was recorded as positive.

Plant growth promoting (PGP) activity of root nodule bacteria : Production of indole acetic acid (IAA) by RNB strains was tested using the method described by Gordon and Weber (1951). The YEM broth supplemented with 1mM L-tryptophan was inoculated using activated bacterial broth and incubated at 30ºC and 100 rpm in a BOD incubator shaker for 5 days in dark. After incubation, cultures were centrifuged at 10,000 rpm for 10 min and 2 ml of supernatant of each strain was taken in another test tube and treated with 2-3 drops of orthophosphoric acid and 4 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ solution in 50 ml, 35% of perchloric acid). After vortexing, the test tubes were kept in dark for 30 min. Appearance of pink color reveals the production of IAA and the amount of production is directly proportional to the

intensity of color. Pikovskaya's agar media (PVK) supplemented with calcium triphosphate was used for screening of phosphate solubilizing bacteria. Phytate agar medium containing calcium phytate was used for screening of strains with phytase activity. Activated bacterial cultures were spotted on PVK and phytase agar plates and were incubated at 28±2ºC for 5-7 days. The activity was recorded in terms of clear halo zone (measured in mm) formed around a colony in an otherwise opaque medium. The solubilization index (SI) was calculated by the following formula :

(colony diameter + diameter of clear halo zone)/colony diameter.

Molecular characterization : Standard phenol-chloroform method was used for genomic DNA isolation. The purified DNA of concentration 100-200 ng μl^{-1} was used as a template for PCR amplification of genes. Primers of broad specificity (Universal) 18F and 1492R were used for amplifying nearly full-length fragment (1500 bp) of 16S rRNA gene. The amplification and gel electrophoresis was carried out as described by Sankhla *et al.* (2017). The amplified PCR products (100 ng μl^{-1}) were sent for sequencing using the corresponding forward and reverse primers through outsourcing company Agri Genome Labs Pvt. Ltd., Cochin. GeneTool Lite software (version 1.0

Table 1 : Phenotypic traits and plant growth promoting activities shown by *Trigonella foenum-graecum*-RNB strains and comparison with selective closely related *Ensifer* type strains

	Phenotypic test				Plant growth promoting activity		
Strain	**NaCl tolerance (%)**	**Temperature range (ºC)**	**pH range**	**BTB reaction**	**IAA production**	**Phytase activity (SI)**	**Phosphate solubilization (SI)**
TFG1	1	28-42	6-11	Acidic	-	8	8
TFG6	1-3	28-42	5-11	Acidic	++	8	11
TFG10	1-4	28-42	5-11	Acidic	-	7	9
TFG11	1-2	28-42	6-11	Acidic	-	7	7.5
TFG12	1-3	28-42	5-11	Acidic	-	7.5	10.5
TFG13	<1	28-42	6-11	Acidic	-	7.5	8
TFG18	1-3	28-42	5-11	Acidic	-	7.5	8
TFG19	1-3	28-42	5-11	Acidic	-	8	9.5
TFG20	1-3	28-42	6-11	Acidic	-	7.5	9
TFG22	1-4	28-42	6-11	Acidic	-	7.5	9
TFG30	1-3	28-42	6-11	Neutral	-	7.5	7
TFG33	1-3	28-42	5-11	Acidic	-	9	6
TFG34	1-3	28-42	5-11	Acidic	++	7	8
TFG37	1-3	28-42	5-11	Acidic	-	8.5	6
TFG40	1	28-42	6-11	Neutral	-	7	9
TFG44	1	28-42	6-11	Acidic	-	8	9
TFG47	1-3	28-42	6-11	Acidic	+++	8	9
TFG48	1-3	28-42	5-11	Acidic	-	8	7
TFG50	1	28-42	6-11	Acidic	+	7	8
TFG53	1-2	28-42	5-11	Acidic	-	-	9
TFG55	1-4	28-42	5-11	Acidic	-	5.5	8
TFG59	1-3	28-42	6-11	Acidic	-	7	7
TFG60	1-2	28-42	5-11	Acidic	-	7	6
TFG62	1-2	28-42	6-11	Acidic	-	-	8
TFG63	1-4	28-42	4-11	Acidic	-	6	6
TFG64	1-4	28-42	4-11	Acidic	-	9	9
TFG65	1-4	28-42	4-11	Acidic	+	15	6
TFG66	1-4	28-42	5-11	Acidic	-	8	7
TFG67	1-4	28-42	5-11	Acidic	-	7	6
TFG68	1-4	28-42	5-11	Acidic	-	7	7
TFG69	1-4	28-42	5-11	Acidic	-	7	6
Ensifer meliloti USDA 1002^T	1-2	28-42	6-9	ND	ND	ND	ND
Ensifer medicae USDA 1037^T	1-2	28-37	5-10	ND	ND	ND	ND
Ensifer kummerowiae CCBAU 71714^T	1-2	28-39	6-10	ND	ND	ND	ND

+ indicates less activity, ++ indicates moderate activity, +++ indicates high activity, - indicates no activity, SI is solubilization index and ND, data not available

Double Twist Inc., Oakland, CA, USA) was used for sequence editing, alignment and assembly. The assembled nucleotide sequences were used to identify strains using NCBI blastn (Basic Local Alignment Search Tool) suite (https://blast.ncbi.nlm.nih.gov). The phylogenetic trees were constructed using software MEGA7 (Kumar *et al.,* 2016) with maximum likelihood method based on a GTR+G+I model using bootstrap value of 1000 replicates. All the sequences after annotation were submitted to NCBI GenBank database and accession numbers were obtained.

Amplification and sequencing of symbiotic genes : Symbiosis related *nodA* and *nifH* genes coding for the *N*-acyltransferase nodulation protein (650 bp) and dinitrogenase reductase (750 bp) respectively were amplified and sequenced using primers nodA1 and nodA2 for *nodA* and primers nifHF and nifHI were used for

Table 2 : Intrinsic antibiotic resistance (IAR) and carbon utilization pattern shown by selective *Trigonella foenum-graecum-Ensifer* strains compared with selective closely related *Ensifer* type/reference strains

Name of strain	*Ensifer meliloti* LMG 6133^T	*Ensifer medicae* USDA 1037^T	*Ensifer kummerowiae* CCBAU 71714^T	*Ensifer meliloti* 1021	*Ensifer* sp. TFG22	*Ensifer* sp. TFG33	*Ensifer* sp. TFG64	*Ensifer* sp. TFG53	*Ensifer* sp. TFG59	*Ensifer* sp. TFG66
Antibiotic (concentration in µg)										
Carbenicillin (CB^{100})	S	ND	ND	S	S	S	S	S	S	S
Ciprofloxacin (CIP^{10})	ND	ND	ND	R	R	R	R	R	R	R
Co-trimazine (CM^{25})	ND	ND	ND	S	R	R	R	S	R	R
Kanamycin (K^{30})	S	S	S	R	R	R	R	R	R	R
Nitrofurantoin (NIT^{300})	ND	ND	ND	S	S	S	S	R	R	R
Streptomycin (S^{10})	S	R	R	R	R	R	R	R	R	R
Tetracycline (TE^{30})	ND	ND	ND	R	R	R	R	R	R	R
Amikacin (AK^{10})	ND	ND	ND	R	R	R	R	R	R	R
Gentamicin (GEN^{10})	S	S	S	R	R	R	R	R	R	R
Cotrimoxazole (COT^{25})	ND	ND	ND	R	S	S	S	S	R	R
Levofloxacin (LE^{5})	ND	ND	ND	R	R	R	R	R	R	R
Netillin (NET^{30})	ND	ND	ND	S	R	R	R	R	R	R
Amoxyclav (AMC^{30})	ND	ND	ND	R	R	S	S	S	S	S
Ofloxacin (OF^{5})	ND	ND	ND	R	R	R	R	R	R	R
Ciftriaxone (CTR^{30})	ND	ND	ND	S	R	R	S	S	R	R
Carbon (Sugar)										
Adonitol	+	V	ND	+	+	+	+	-	+	+
Arabinose	+	+	ND	+	+	+	+	-	+	+
Cellibiose	+	+	ND	+	+	+	+	+	+	+
Dextrose	ND	ND	ND	-	+	+	+	+	+	+
Dulcitol	+	ND	ND	-	+	-	+	+	-	+
Fructose	+	+	-	-	+	-	+	+	+	+
Galactose	+	+	+	+	+	-	+	+	+	+
Inositol	+	+	ND	+	+	+	+	+	-	+
Inulin	-	ND	ND	+	-	-	+	+	+	+
Lactose	+	ND	-	+	+	+	+	-	+	+
Maltose	+	+	+	-	+	-	+	+	-	+
Mannitol	+	+	-	-	-	-	+	+	-	+
Mannose	+	+	ND	+	+	+	+	+	+	+
Melibiose	+	-	+	-	+	-	+	+	+	+
Raffinose	+	-	+	-	-	+	+	+	-	+
Rhamnose	+	ND	+	-	+	+	+	+	+	+
Salicin	+	ND	ND	+	-	+	+	-	+	+
Sorbitol	+	+	+	+	+	-	+	-	-	+
Sucrose	+	+	+	+	+	+	+	-	-	+
Trehlose	+	+	ND	+	-	-	+	+	+	+
Xylose	+	ND	ND	+	+	+	+	+	+	+

S, sensitive; R, resistant; ND, data not available; V, variable, (+) with grey fill indicates sugar utilized and (-) indicates sugar not utilized by strain

nifH. Reaction mixture and PCR thermal cycler conditions as described by Sankhla *et al.* (2017) were used. Agarose gel electrophoresis, sequencing and phylogenetic analysis of symbiotic genes was performed as described for 16S rRNA gene.

Authentication of root nodule bacteria : Few strains were inoculated on their host in glasshouse conditions for authentication. The nodulation experiments were conducted as described earlier by Tak *et al.* (2016). After 8-9 weeks, the inoculated and control plants were harvested and observed for their nodulation status, as well as growth/biomass of inoculated plants was compared to N^+ and N^- controls. Bacteria were re-isolated from root nodules of inoculated plants and the colonies of re-isolates were compared with the parent colonies.

Results and Discussion

The *T. foenum-graecum* plants nodulated in all the soil samples collected from different regions of Western Rajasthan and more than 60 fast growing strains were isolated. Colonies were white, raised, round with entire margins, highly mucilaginous and translucent or opaque. The TFG-RNB strains were divided into three major groups on the basis of colony characters such as translucent or opaque, curdling, acid producing and turning the media purple. Among these thirty one strains were selected for phenotypic characterization and to test their PGP activities (Table 1). Most of the strains tested for BTB reaction were acid producers, which is a common character of fast growing bacterial strains. Ten strains showed high NaCl tolerance up to 4% (w/v) among which seven strains are those which were isolated from Agriculture University Mandore (Jodhpur). These results are similar to the earlier reports for fast growing *Ensifer* (Choudhary *et al.,* 2017; Sankhla *et al.,* 2017). Most of the tested strains were able to tolerate a wide range of pH (5 to 11). The strains TFG63, TFG64 and TFG65 isolated from Agriculture University, Jodhpur could grow up to pH 4 which is in accordance with the report of *E. medicae* strains isolated from acidic soils in Australia (Garau *et al.,* 2005). These results

Table 3 : Evolutionary percentage sequence similarities of *Trigonella foenum-graecum-Ensifer* strains with selective type strains based on 16S rRNA gene and symbiotic *(nodA* and *nifH)* gene sequences computed using Maximum Composite Likelihood model in MEGA7

Name of strain	Biological host and geographical origin	16S rRNA gene						*nodA gene*			*nifH gene*		
		TFG 22	TFG 33	TFG 64	TFG 53	TFG 59	TFG 66	TFG 22	TFG 33	TFG 64	TFG 22	TFG 33	TFG 64
Bradyrhizobium japonicum USDA 6^T	*Glycine max,* Japan	87.7	87.7	87.6	87.6	87.7	87.6	49.6	50.1	51.3	65.9	68.6	68.5
E. americanus CFNEI 156^T	*Mariosousa acatlensis,* Mexico	98.7	98.7	98.6	98.6	98.7	98.6	59.5	60.7	60.4	85.8	86.3	85.8
E. fredii USDA 205^T	*Glycine max,* China	98.7	98.7	98.6	98.6	98.7	98.6	58.5	59.2	59.9	85.7	86.5	85.9
E. glycinis CCBAU 23380^T	*Astragalus mongholicus,* China	98.1	98.1	98.0	98.0	98.1	98.0	58.5	59.2	59.9	85.7	86.5	85.9
E. kostiensis HAMBI 1489^T	*Senegalia senegal,* Sudan	98.2	98.2	98.3	98.3	98.2	98.3	53.3	54.6	54.3	83.9	85.2	84.6
E. kummerowiae CCBAU 71714^T	*Kummerowia stipulacea,* China	98.6	98.6	98.7	98.7	98.6	98.7				**99.1**	95.4	95.1
E. medicae $A321^T$	*Medicago truncatula,* France	**99.6**	**99.6**	**99.7**	**99.7**	**99.6**	**99.7**	98.2	98.4	97.4	95.6	**98.8**	**98.1**
E. meliloti ATCC 9930^T	*Medicago sativa,* USA	99.5	99.5	99.6	99.6	99.5	99.6	**99.2**	**98.6**	**97.6**	**99.1**	95.4	95.1
E. mexicanus ITTG-$R7^T$	*Acaciella angustissima,* Mexico	97.8	97.8	97.7	97.7	97.8	97.7	60.4	61.9	61.9	88.0	88.5	88.0
E. saheli LMG 7837^T	*Sesbania cannabina,* Senegal	98.5	98.5	98.4	98.4	98.5	98.4	57.8	57.8	58.1	85.4	86.3	85.7
E. shofinae CCBAU 251167^T	*Glycine max,* China	98.5	98.5	98.4	98.4	98.5	98.4	58.1	58.8	59.5	85.1	85.9	85.9
E. sojae CCBAU 05684^T	*Glycine max,* China	98.3	98.3	98.1	98.1	98.3	98.1	58.5	59.2	59.9	85.7	86.5	85.9
E. terangae ORS 1009^T	*Senegalia laeta,* Senegal	97.8	97.8	97.7	97.7	97.8	97.7	58.7	59.9	59.6	85.7	86.5	86.0
E. xinjiangensis CCBAU 110^T	*Glycine max,* China	98.6	98.6	98.5	98.5	98.6	98.5	58.5	59.2	59.9	85.7	86.5	85.9
Ensifer sp. JNVU TW10	*Tephrosia wallichii,* India	98.5	98.5	98.6	98.6	98.5	98.6	59.1	59.8	59.8	86.3	87.7	87.2
Ensifer sp. JNVU AJ31	*Vachellia jacquemontii,* India	98.4	98.4	98.3	98.3	98.4	98.3	54.8	56.1	55.8	84.8	85.1	84.5
Ensifer sp. JNVU AJ32	*Vachellia jacquemontii,* India	98.5	98.5	98.4	98.4	98.5	98.4	54.6	55.9	55.5	83.7	85.2	84.6
Ensifer sp. JNVU AL5	*Vachellia leucophloea,* India	98.4	98.4	98.3	98.3	98.4	98.3	54.6	55.9	55.5	83.4	84.3	83.7
Ensifer sp. JNVU MH40	*Mimosa hamata,* India	98.4	98.4	98.3	98.3	98.4	98.3	54.6	55.9	55.5	84.8	85.1	84.5
Ensifer sp. TFG22	*Trigonella foenum-graecum,* India	100	100	99.9	99.9	100	99.9	100	99.0	98.0	100	95.6	95.4
Ensifer sp. TFG33	*Trigonella foenum-graecum,* India	100	100	99.9	99.9	100	99.9	99.0	100	99.0	95.6	100	99.3
Ensifer sp. TFG64	*Trigonella foenum-graecum,* India	99.9	99.9	100	100	99.9	100	98.0	99.0	100	95.4	99.3	100
Ensifer sp. TFG53	*Trigonella foenum-graecum,* India	99.9	99.9	100	100	99.9	100						
Ensifer sp. TFG59	*Trigonella foenum-graecum,* India	100	100	99.9	99.9	100	99.9						
Ensifer sp. TFG66	*Trigonella foenum-graecum,* India	99.9	99.9	100	100	99.9	100						

E, Ensifer; superscript ^{T} indicates type strain and grey fill with numbers in bold indicates first closest type strain

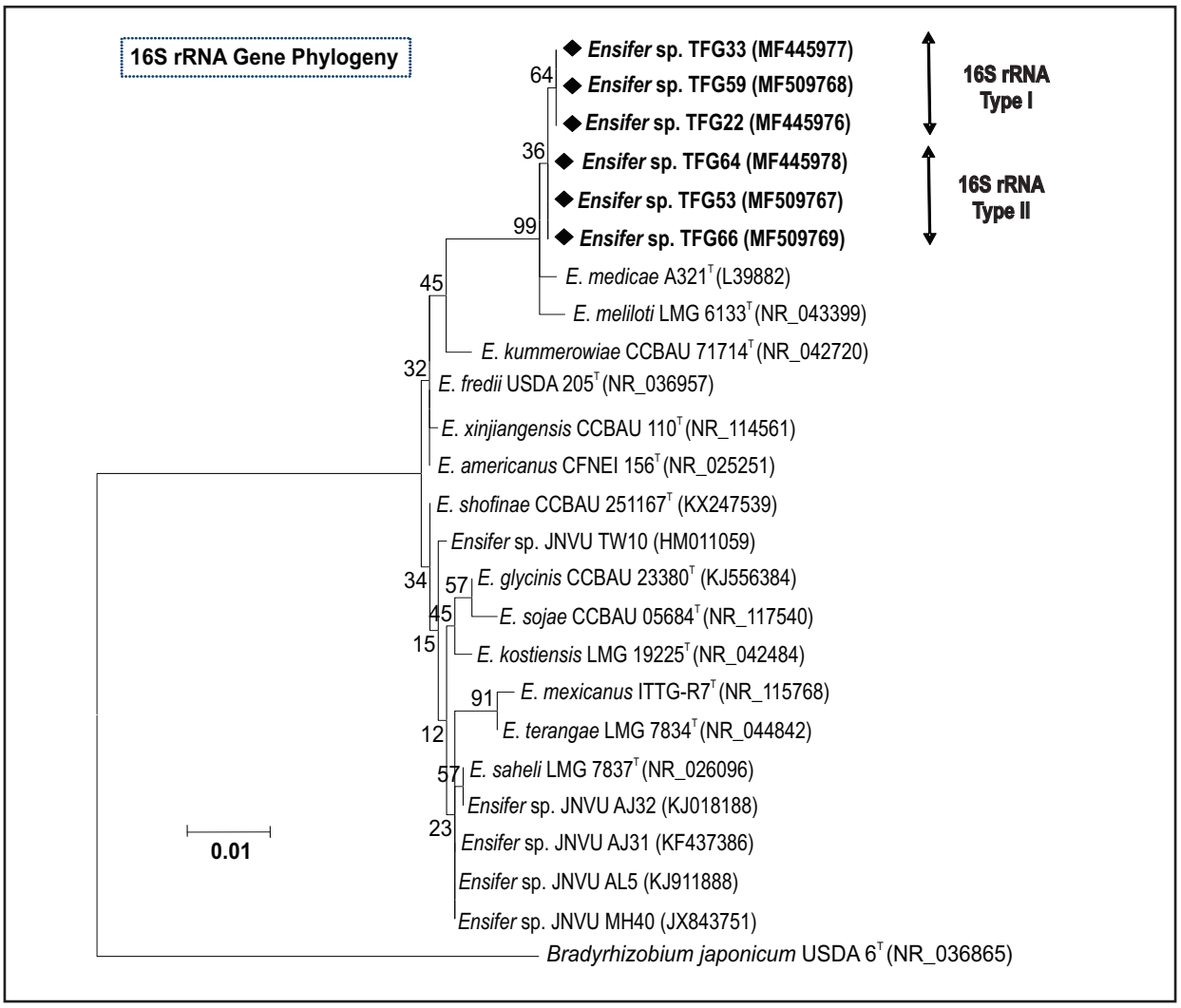


Fig. 1 : Phylogenetic tree constructed using 16S rRNA gene sequences of selected type strains of *Ensifer* along with Root Nodule Bacterial (RNB) strains of *Trigonella foenum-graecum* growing in Western Rajasthan. (Abbreviations: *E, Ensifer*; NR, NCBI Reference sequence and superscript ^{T} indicates type strain)

suggest that *Ensifer* strains nodulating legume group *"Trigonella-Medicago-Melilotus"* have no particular pH preference and are adapted to wide range of pH (5 to 11). In addition to this, all the tested TFG strains were able to grow up to 42ºC showing their adaptability to high temperature prevailing in arid and semi-arid conditions of Indian Thar Desert.

Six (TFG22, TFG33, TFG53, TFG59, TFG64 and TFG66) selective TFG strains were tested for utilization of carbon and IAR pattern. The strains TFG64 and TFG66 utilized all the 21 sugars tested. Sugars such as cellibiose, dextrose, mannose, rhamnose and xylose were utilized by all tested six strains (Table 2). The utilization of wide range of carbon source by these six strains is in accordance with the previous reports on diverse desert-*Ensifer* strains isolated from various native legumes such as species of *Tephrosia, Acacia, Crotalaria* and *Alysicarpus* (Gehlot *et al.,* 2012; Tak *et al.,* 2016; Sankhla *et al.,* 2017; Le Queré *et al.,* 2017; Choudhary *et al.,* 2017; Rathi *et al.,* 2017) growing in arid and semi-arid regions of W. Rajasthan and confirms that range of carbohydrate utilization is wider in fast growing strains as compared to slow growing strains. These six TFG strains also showed variation in their intrinsic antibiotic resistance pattern (Table 2). The results of present investigation are in contrast to the closely related *Ensifer* type strains (*Ensifer kummerowiae*, *E. meliloti* and *E. medicae*) which are sensitive to Kanamycin and Gentamicin (Table 2). These variations in the phenotypic characters shown by the *Ensifer* strains might be due to horizontal gene transfer (HGT) of the plasmid based metabolic genes (Sankhla *et al.,* 2017). These selected six TFG strains were inoculated on their host and were found nodulating. The inoculated host plants were greener and healthy in comparison to N^{+} and N^{-} controls. The re-isolates were similar to parental-TFG strains on the basis of colony characters.

Nodule endophytes and symbionts show various PGP activities such as production of indole acetic acid (IAA) and siderophores; solubilization of phosphates and production of hydrolytic enzymes involved in anti-fungal biocontrol mechanism (Singha *et al.,* 2017). In the present study all tested strains were found positive for solubilization of tri-calcium phosphate and most of them also showed phytase activity. Whereas only five strain were found positive for production of indole acetic acid (Table 1). The screening of effective rhizobial strains showing PGP traits would help in the co-inoculation studies involving rhizobia and known PGPR for improvement in agricultural production.

The six (TFG22, TFG33, TFG53, TFG59, TFG64 and TFG66) selected strains representing three colony groups were identified as species of *Ensifer,* based on 16S rRNA gene sequence similarity search in BLASTn. On the basis of 16S rRNA gene phylogeny (Fig. 1) the six TFG strains clustered into a separate clade within genus *Ensifer* close to type strains *E. medicae* A321^T (=NBRC 100384^T or USDA 1037^T) (Rome *et al.,* 1996) and *E. meliloti* LMG 6133^T (=ATCC 9930^T or USDA 1002^T) (de Lajudie *et al.,* 1994). In 16S rRNA gene phylogeny the six strains clustering in a novel clade within genus *Ensifer* were further sub-divided into two 16S rRNA types. Three strains (TFG22, TFG33 and TFG59) of 16S rRNA type-I showed maximum percentage similarity (99.6%) with *E. medicae* A321^T isolated from *M. truncatula,* France (Rome *et al.,* 1996). These strains showed second close similarity (99.5%) with *E. meliloti* LMG 6133^T isolated from root nodules of *M. sativa* in USA and 98.6% similarity with the closely related species of *E. kummerowiae* CCBAU 71714^T isolated from *Kummerowia stipulacea* in China (Wei *et al.,* 2002). Similarly, the three strains (TFG53, TFG64 and TFG66) belonging to 16S rRNA type-II showed maximum close similarity (99.7%) with *E. medicae* followed by 99.6% with *E. meliloti* and 98.7% similarity with *E. kummerowiae.* TFG-*Ensifer* strains from Western Rajasthan, India are close to the known group of Medics-Melilots and *Trigonella* microsymbionts such as *E. meliloti* and *E. medicae.* In present study, it was observed that TFG-*Ensifer* strains had an

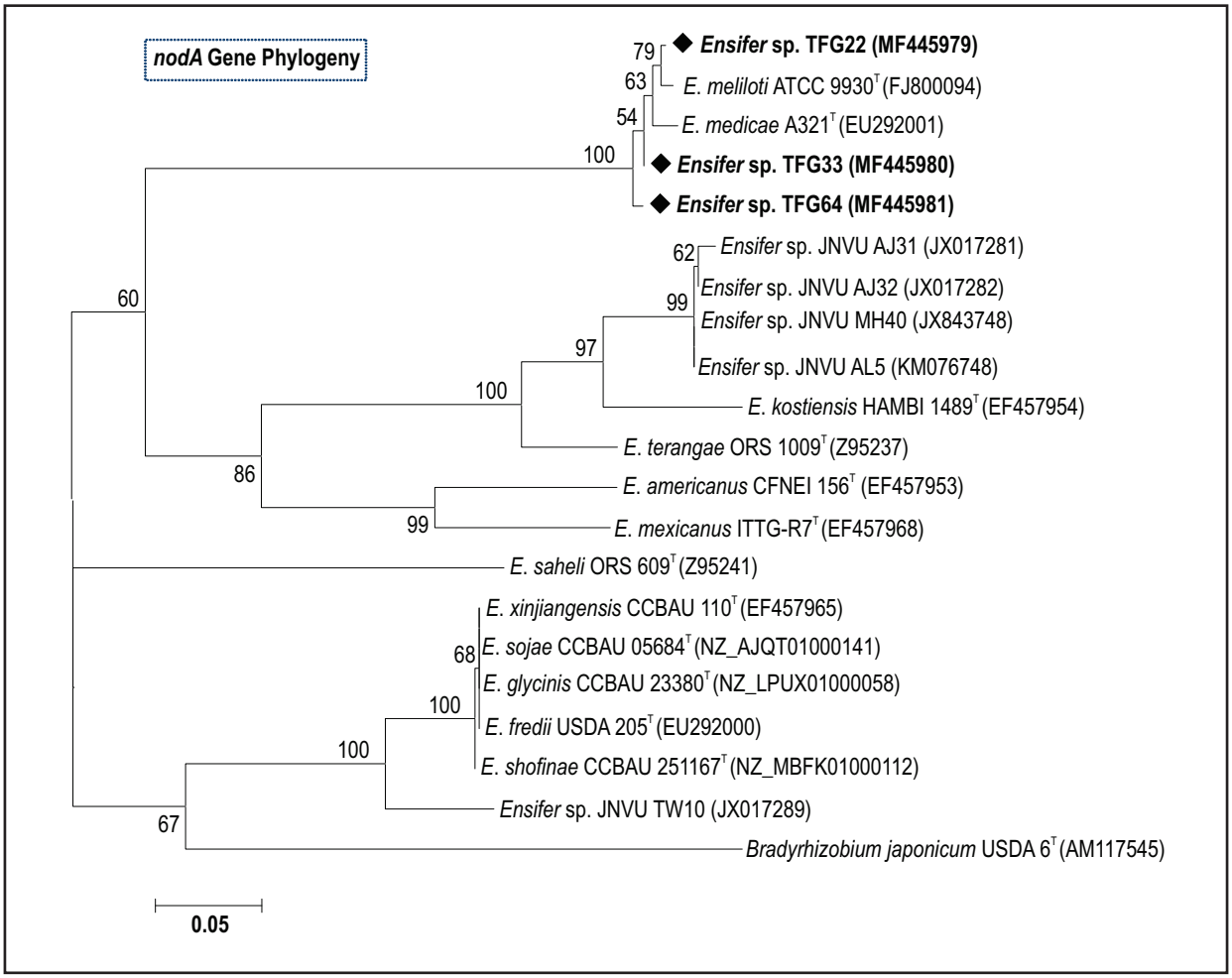


Fig. 2 : Phylogenetic tree constructed using *nodA* gene sequences of selected type strains of *Ensifer* along with Root Nodule Bacterial (RNB) strains of *Trigonella foenum-graecum* growing in Western Rajasthan. (Abbreviations: *E, Ensifer* and superscript ^{T} indicates type strain)

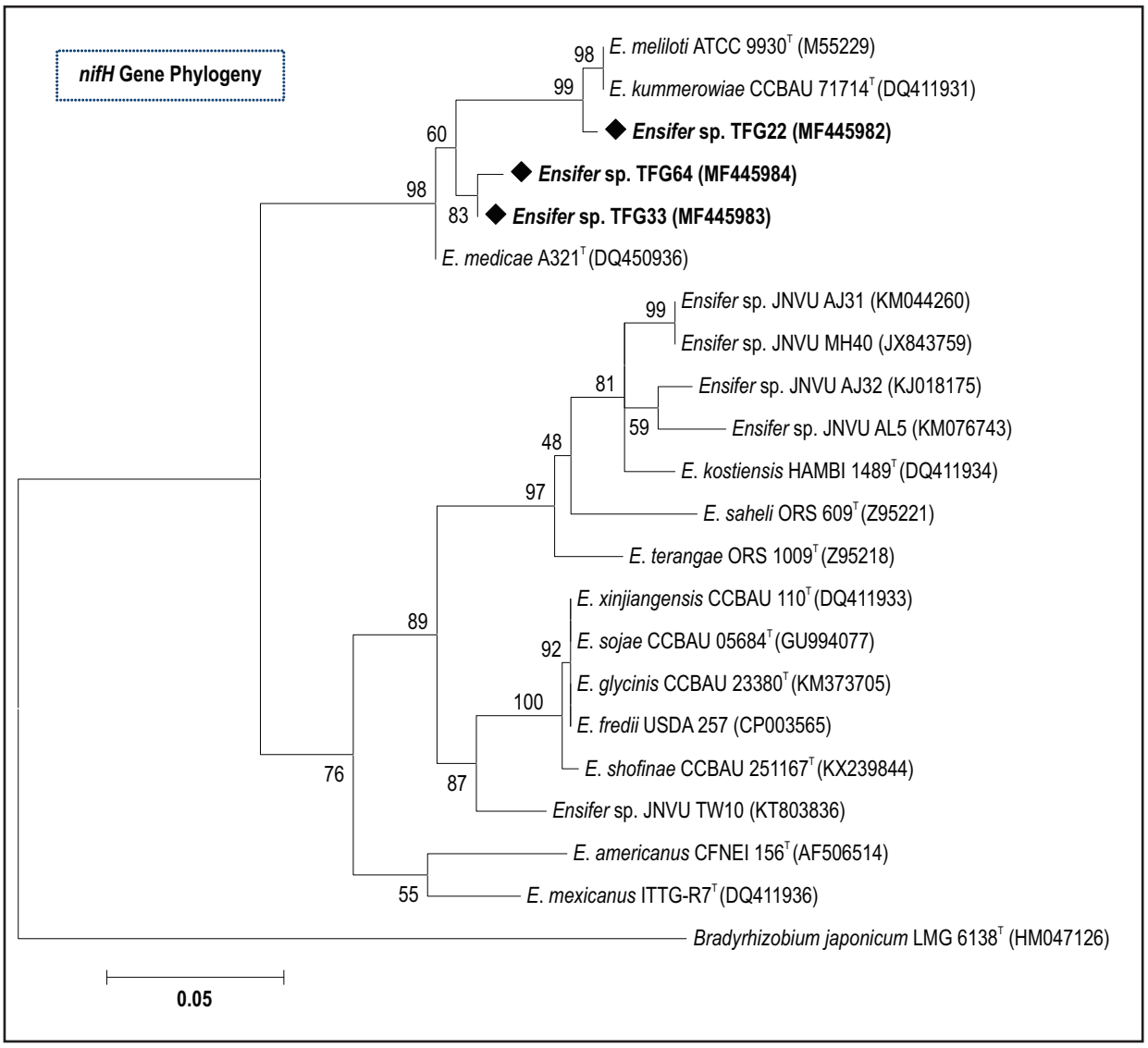


Fig. 3 : Phylogenetic tree constructed using *nifH* gene sequences of selected type/reference strains of *Ensifer* along with Root Nodule Bacterial (RNB) strains of *Trigonella foenum-graecum* growing in Western Rajasthan. (Abbreviations : *E, Ensifer* and superscript ^{T} indicates type strain)

intermediate sequences showing close similarity both with *E. medicae* and *E. meliloti* strains on the basis of 16S rRNA gene.

The analysis of symbiosis related genes (*nodA* and *nifH*) and their phylogeny provides information about origin and evolution of *sym* genes as well as if these are being transferred vertically or through HGT in the investigated strains. The host range studies and *sym* gene analysis helps in identifying the symbiovar of a particular species in global context (El Batanony *et al.,* 2015). In the present investigation, the phylogenetic tree reconstructed using *nodA* gene sequences of selective three (TFG22, TFG33 and TFG64) strains representing both the 16S rRNA types suggests that TFG-*Ensifer* strains are closer to *E. meliloti* ATCC 9930^T (de Lajudie *et al.,* 1994) with 97.6 to 99.2% similarity in contrast to their 16S rRNA phylogeny results (Table 3 and Fig. 2). In *nifH* phylogenetic tree, three selected TFG-*Ensifer* strains formed two *nifH* types. Two strains (TFG33 and TFG64) formed a separate cluster and showed maximum sequence similarity (98.8% and 98.1% respectively) with *E. medicae* A321^T (Rome *et al.,* 1996). Whereas *nifH* gene sequence of strain TFG22 was divergent from other TFG strains forming a separate novel lineage and showing 99.1% similarity with *E. meliloti* ATCC 9930^T (de Lajudie *et al.,* 1994) and *E. kummerowiae* CCBAU 71714^T (Wei *et al.,* 2002) (Table 3 and Fig. 3). Hence, it is interesting to note that *sym* genes in TFG-*Ensifer* strains have originated from two closely related species of *Ensifer* (*E. meliloti* and *E. medicae*) isolated from different species of *Medicago* (*M. sativa* and *M. truncatula*) which specifies acquisition of these mega-plasmid based *sym* genes through HGT. The host authentication test results and *sym* gene

phylogeny reveals that TFG-*Ensifer* strains belong to symbiovar meliloti of *E. meliloti* (El Batanony *et al.,* 2015).

The TFG-*Ensifer* strains characterised in present study are diverse from the *Ensifer* type strains reported to nodulate legumes of *Trigonella-Medicago-Melilotus* group. The geographical and edaphic factors prevailing in the Indian Thar Desert affected diversity of strains. More sampling from other regions of India is needed to conclude total genetic diversity in TFG strains for preparing indigenous inoculums.

Acknowldgments

Sushma Gaur is thankful to Jai Narain Vyas University, Jodhpur for providing University Fellowship. Authors acknowledge University Grants Commission, New Delhi, (UGC-SAPII-CAS-I and UGC-MRP F.No.43-129/2014[SR]) and Department of Science and Technology (DST-FIST and DST-SERB F.No.SB/YS/LS-369/2013) for financial assistance. Authors are thankful to Santosh Choudhary and Bagda Ram Choudhary for their help in sampling at the Agriculture University, Mandore, Jodhpur.

References

Bhandari, M.M.: Flora of the Indian Desert. Scientific Publisher Jodhpur (1990).

Choudhary, S., R.R. Meghwal, I.S. Sankhla, N. Tak and H.S. Gehlot : Molecular characterization and phylogeny of novel diverse nitrogen fixing microsymbionts associated with *Vachellia* (*Acacia*) *leucophloea* in arid and semi arid regions of Rajasthan. *Indian Forester*, **143**, 266-278 (2017).

de Lajudie, P., A. Willems, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M.D. Collins, B. Dreyfus, K. Kersters and M. Gillis: Polyphasic taxonomy of Rhizobia : Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int. J. Syst. Bacteriol.,* **44**, 715-733 (1994).

El Batanony, N.H., A. Castellano-Hinojosa, D. Correa-Galeote and E.J. Bedmar : The diversity of rhizobia nodulating the *Medicago, Melilotus* and *Trigonella* inoculation group in Egypt is marked by the dominance of two genetic types. *Symbiosis*, **67**, 3-10 (2015).

Garau, G., W.G. Reeve, L. Brau, P. Deiana, R.J. Yates, D. James, R. Tiwari, G.W. O'Hara and J.G. Howieson : The symbiotic requirements of different *Medicago* spp. suggest the evolution of *Sinorhizobium meliloti* and *S. medicae* with hosts differentially adapted to soil pH. *Plant Soil,* **176**, 263-277 (2005).

Gehlot, H.S., D. Panwar, N. Tak, A. Tak, I.S. Sankhla, N. Poonar, R. Parihar, N.S. Shekhawat, M. Kumar, R. Tiwari, J. Ardley, E.K. James and J.I. Sprent : Nodulation of legumes from the Thar Desert of India and molecular characterization of their rhizobia. *Plant Soil,* **357**, 227-243 (2012).

Gehlot, H.S., N. Tak, M. Kaushik, S. Mitra, W.M. Chen, N. Poweleit, D. Panwar, N. Poonar, R. Parihar, A. Tak, I.S. Sankhla, A. Ojha, S.R. Rao, M.F. Simon, F.B. Jr. dos Reis, N. Perigolo, A.K. Tripathi, J.I. Sprent, J.W.P. Young, E.K. James and P. Gyaneshwar : An invasive *Mimosa* in India does not adopt the symbionts of its native relatives. *Ann. Bot.,* **112**, 179-196 (2013).

Gordon, S.A. and R.P. Weber : Colorimetric estimation of indole acetic acid. *Plant Physiol.,* **26**, 192-195 (1951).

Kumar, S., G. Stecher and K. Tamura : MEGA 7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.,* **33**, 1870-1874 (2016).

Le Queré, A., N. Tak, H.S. Gehlot, C. Lavire, T. Meyer, D. Chapulliot, S. Rathi, I. Sakrouhi, G. Rocha, M. Rohmer, D. Severac, A. Filali-Maltouf and J.A. Munive : Genomic characterization of *Ensifer aridi,* a proposed new species of nitrogen-fixing rhizobium recovered from Asian, African and American deserts. *BMC Genomics,* **18**, 85 (2017).

Lesins, K.A. and I. Lesins: Genus : Medicago (Leguminosae), a taxogenatic study. Junk, The Hague (1979).

Lewis, G.P., B. Schrire, B. Mackinder and M. Lock : Legumes of the world. Royal Botanic Gardens Kew (2005).

Pandey, P., M. Sahgal, D.K. Maheshwari and B.N. Johri : Genetic diversity of rhizobia isolated from medicinal legumes growing in the sub-Himalayan region of Uttaranchal. *Curr. Sci.,* **86**, 202-207 (2004).

Rajendran, R., M.H. Patel and S.J. Joshi: Isolation and characterization of nodule-associated *Exiguobacterium* sp. from the root nodules of fenugreek *(Trigonella foenum-graecum)* and their possible role in plant growth promotion. *Int. J. Microbiol.,* **2012**, 693982 (2012).

Rathi, S., S. Gaur, N. Tak, A. Tak and H.S. Gehlot : Genetically diverse root nodule bacteria associated with *Alysicarpus vaginalis* from alkaline soil of Rajasthan, India. *Plant Archives,* **17**, 495-505 (2017).

Reeve, W., P. Chain, G.W. O'Hara, J. Ardley, K. Nandesena, L. Brau, R. Tiwari, S. Malfatti, H. Kiss and A. Lapidus : Complete genome sequence of the *Medicago* microsymbiont *Ensifer (Sinorhizobium) medicae* strain WSM419. *Stand. Genomic Sci.,* **2**, 77-86 (2010).

Rome, S., M.P. Fernandez, B. Brunel, P. Normand and J.C. Cleyet-Marel: *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int. J. Syst. Bacteriol.,* **46**, 972-980 (1996).

Sankhla, I.S., N. Tak, R.R. Meghwal, S. Choudhary, A. Tak, S. Rathi, J.I. Sprent, E.K. James and H.S. Gehlot : Molecular characterization of nitrogen fixing microsymbionts from root nodules of *Vachellia (Acacia) jacquemontii,* a native legume from the Thar Desert of India. *Plant Soil,* **410**, 21-40 (2017).

Singha, B., P.B. Mazumder and P. Pandey : Characterization of plant growth promoting rhizobia from root nodule of *Mimosa pudica* grown in Assam, *India. J. Env. Biol.,* **38**, 441-447 (2017).

Somasegaran, P. and H.J. Hoben : Handbook for rhizobia. Springer New York (1994).

Tak, N., E. Awasthi, G. Bissa, R.R. Meghwal, E.K. James, J.I. Sprent and H.S. Gehlot: Multi locus sequence analysis and symbiotic characterization of novel *Ensifer* strains nodulating *Tephrosia* spp. in the Indian Thar Desert. *Syst. Appl. Microbiol.,* **39**, 534-545 (2016).

Wei, G.H., E.T. Wang, Z.Y. Tan, M.E. Zhu and W.X. Chen : *Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov., respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea. Int. J. Syst. Evol. Microbiol.,* **52**, 2231-2239 (2002).