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Non-nodulating *Enterobacter* strains with multiple Plant Growth Promoting (PGP) attributes dominantly co-exist with nodulating rhizobia as root nodule endophytes of wild perennial legumes, *Tephrosia* spp. of Indian Thar Desert

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Abstract

In the alkaline soils of the Indian Thar Desert, traditional rhizobial genera like *Sinorhizobium/Ensifer* and *Bradyrhizobium* nodulate wild legumes like *Tephrosia* species. Other root nodule bacterial endophytes isolated from *Tephrosia* spp. included members of *Agrobacterium*, *Enterobacter*, *Cronobacter*, *Pseudomonas*, *Bacillus* and *Phyllobacterium*. The predominant non-symbiotic endophytic bacteria were phenotypically and genetically diverse strains of *Enterobacter* (JNVU TF1, JNVU TF6, JNVU TF11, JNVU TF18, JNVU TF19, JNVU TW3, JNVU TW4 and JNVU TW6) which could tolerate high salt concentrations of up to 3%, grew at a broad pH range of 4.5–11, metabolized multiple sugars as sole carbon source and were resistant to multiple antibiotics demonstrating their robust metabolic profile. These fast-growing strains based on 16S rRNA gene shared similarities with *Enterobacter cloacae* sub sp. *cloacae* ATCC 13047T and *Enterobacter cloacae* sub sp. *dissolvens* LMG 2683T. Phylogenetically strains JNVU TW3 and JNVU TW4 formed a separate clade within *Enterobacter* species. These non-nodulating strains showed Plant Growth Promoting (PGP) traits such as ammonia production, solubilization of tricalcium phosphates and few could even efficiently mineralize phytate, the organic form of phosphate. Few strains showed chitinase activity and therefore such strains may exhibit antagonistic activity towards fungal plant pathogens. Our results demonstrate coexistence of non-rhizobia, *Enterobacter* strains showing good PGP attributes along with genetically diverse novel nodulating rhizobia within root nodules of *Tephrosia* spp.

Keywords: Non-nodulating, root-nodule endophytes, *Enterobacter*, Plant Growth Promoting (PGP)

Introduction

Endophytic bacteria typically enter plants by root hairs, epidermal junctions, and areas of damaged tissue. In addition to N-fixing rhizobia, a wide range of non-nodulating endophytic bacteria find an attractive niche in the root nodules of leguminous plants because they offer a confined protective environment for microbes and a plentiful source of nutrients. Large numbers of non-nodulating opportunistic root nodule endophytes such as species of *Burkholderia*, *Chryseobacterium*, *Chryseomonas*, *Cronobacter*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Flavimonas*, *Herbaspirillum*, *Arthrobacter*, *Acinetobacter*, *Achromobacter*, *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Kocuria*, *Lysinibacillus*, *Novosphingobium*, *PaeniBacillus*, *Mycobacterium*, *Microbacterium*, *Micrococcus*, *Paracoccus*, *Pseudomonas*, *Serratia*, *Rahnella*, *Staphylococcus*, *Stenotrophomonas*, *ThioBacillus*, *Xanthomonas*, *Variovorax*, *Sphingobacterium*, *Sphingomonas* and many more (De Meyer *et al.*, 2015; Hnini and Aurag 2024) [11, 22] have been isolated both from wild and crop legumes e.g. *Sphaerophysa salsula* (Deng *et al.*, 2011) [12]; species of *Crotalaria* and *Indigofera*, *Erythrina brucei* (Aserse *et al.*, 2013) [3]; *Colophospermum mopane* (Burbano *et al.*, 2015) [6]; *Abrus precatorius* (Ghosh *et al.*, 2015) [18]; *Vigna unguiculata* (Leite *et al.*, 2017; Muindi *et al.*, 2021) [31, 37]; *Glycine max* (Zhao *et al.*, 2018) [64]; *Mimosa pudica* (Sánchez-Cruz *et al.*, 2019) [49]; *Lupinus albus* (Ferchichi *et al.*, 2019) [16]; *Phaseolus lunatus* (Chibeba *et al.*, 2020) [10]; *Lens culinaris*

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(Jabborova *et al.*, 2021) ^[26]; *Trifolium repense* (Rahal and Chekireb 2021) ^[43] and *Arachis hypogaea* (Hossain *et al.*, 2023) ^[23]. It has been found that even human pathogens may live temporarily in plants as endophytes, and through a variety of direct or indirect methods, their interactions with the host plant can be either neutral or beneficial (Tyler and Triplett 2008) ^[60]. Free-living, commensal, and pathogenic bacteria belonging to the *Enterobacteriaceae* family are linked to host species that range from humans to plants. To date, a number of non-pathogenic *Enterobacteriaceae* members have been isolated from wild or cultivated plants (Markova *et al.*, 2005; Kan *et al.*, 2007; Torres *et al.*, 2008; Burbano *et al.*, 2015; Ghosh *et al.*, 2015; Hnini and Aurag 2024) ^[6, 33, 18, 28, 22]. Benhizia *et al.* (2004) ^[4] published the first report of Gamma-proteobacteria association to legume nodules. *Enterobacter cloacae* and *E. kobei* were isolated from root nodules of three species of *Hedysarum* (*H. carnosum*, *H. spinosissimum* and *H. pallidum*) growing in Algeria (Benhizia *et al.*, 2004) ^[4]. *Enterobacter agglomerans* was detected within nodules of *H. spinosissimum* by immunofluorescence microscopy (Muresu *et al.*, 2008) ^[38]. Role of endophytic *Enterobacteria* as alternative nitrogen fixing symbionts and inducers of nodule organogenesis however seems to be ruled out by the evidence such as the incapacity of pure cultures to nodulate the legume under axenic conditions (Muresu *et al.*, 2008) ^[38]. Two strains isolated from *H. pallidum* and *H. spinosissimum* were subjected to microarray analysis utilizing oligonucleotides for the *nodC* and *nifH* genes (Bontemps *et al.*, 2005) ^[5] in an attempt to find nodulation and nitrogen fixation genes in Gamma-proteobacteria. However, none of these genes were found. But in Tunisia, Zakhia *et al.* (2006) ^[63] discovered a variety of bacteria associated with legume root nodules and discovered a *nifH*-like gene in the genera *Microbacterium* and *Starkeya*.

Plant growth promoting (PGP) bacteria are thought to work by a number of methods, such as the solubilization of phosphate, production of phytohormones and siderophores, etc. Through the non-symbiotic fixation of nitrogen, these bacteria can also enhance plant growth. Certain bacteria that produce hydrolyzing enzymes, such as chitinases and glucanases, act as biocontrol agents. More than two or three PGP features, which either directly or indirectly or cooperatively support plant growth, may be displayed by the bacterial strains. For this reason, potential PGP bacteria are screened *in vitro* for multiple PGP traits. Such types of studies have not been done previously on bacterial endophytes in root nodules of wild leguminous plants of arid regions of Indian Thar Desert therefore an attempt was made to explore such possibility for bacterial endophytes of *Tephrosia* spp. Making consortium of such useful bacteria looks attractive for wide agricultural application in the future. However, the significance of these non-rhizobial root nodule bacterial endophytes has yet to be realized.

Materials and Methods

Isolation, phenotypic and biochemical characterization of bacterial root nodule endophytes

Isolation and purification of bacteria from root nodules of *Tephrosia* species was done as described in Somasegaran and Hoben (1994) ^[52]. To ensure that only endophytes and no surface bacteria are isolated the nodules were swapped on Congo-red (CR)-Yeast Extract Mannitol Agar (YEMA) plates before puncturing them. Pure bacterial strains were analyzed for phenotypic traits such as salt, pH and temperature tolerance as per Tak *et al.* (2020). HiMedia discs for carbohydrate fermentation were used to determine the bacteria's carbon

utilization patterns, and the Kirby Boyer disc diffusion test was used to determine the inherent antibiotic resistance profiles (Cappuccino and Sherman 2007) ^[7]. Numerous biochemical assays, including those for nitrate reductase activity, citrate utilization, methyl-red, Voges-Proskauer, gelatinase, catalase, and amylase synthesis, were conducted (Cappuccino and Sherman 2007) ^[7]. O-nitrophenyl- β -D-galactopyranoside (ONPG) test was carried using HiMedia ONPG discs; oxidase activity was tested with HiMedia oxidase discs and hydrogen sulfide production using HiMedia Lead acetate strips.

Screening of bacterial strains for hydrolytic enzyme production and characteristics that promote plant growth

The standard procedures for ammonia production (Cappuccino and Sherman 2007) ^[7], IAA production (Gordon and Weber 1951) ^[19], siderophore production (Schwyn and Neilands 1987) ^[50], HCN production (Bakker and Schippers 1987), phosphate solubilization (Pikovskaya 1948) ^[42], and phytase activity (Howson and Davis 1983) ^[24] were used to screen the root nodule bacterial endophytes for a variety of PGP traits. Ability of strains to produce some hydrolytic enzymes was also tested using the methods as described by Smibert and Krieg (1994) ^[51] for protease activity; Ramesh *et al.* (2008) ^[44] for cellulase activity; Kim *et al.* (2003) ^[29] for chitinase activity; and Ronald and James (2006) ^[47] for pectinase activity.

Analyzing the genetic diversity, amplification and sequencing of 16S ribosomal RNA gene of root nodule endophytes

To analyze the genetic diversity among the strains the RPO1 primer, as described by Richardson *et al.* (1995) ^[46] was used, the PCR mix and thermal cycling conditions used were as stated by Tak *et al.* (2020). Using the primers 18F (AGAGTTTGATCCTGGCTCAG) and 1492R (CTACGGCTACCTTGTACG) described by Weisburg *et al.* (1991) ^[61] the intragenic segment of 1475 bp from the 16S rRNA gene was amplified in selective bacterial strains. PCR thermal cycling conditions were 5 min. initial denaturation at 95 °C followed by 35 cycles (1 min. 94 °C, 1 min. 53 °C, 1 min. 72 °C) and final extension of 7 min. at 72 °C. A 0.8% (w/v) agarose gel prepared in pH 8.0 1X TAE buffer was then used for gel electrophoresis of the amplified 16S rRNA products. Sybr Safe was used to pre-stain the gels (8 μ l/100 ml). The amplified products were run with a 1 Kb DNA ladder (Promega). For one hour, electrophoresis was performed at 80 V. A UV transilluminator was used to see the gels, and a gel documentation system was used to take digital pictures of the gels. The QIAGEN- QIAquick PCR Purification Kit was used to purify the amplified PCR products. Both 18F and 1492R primers were used for sequencing of 16S rRNA gene using the Applied Biosystems Big Dye version 3.1 terminator.

Analysis of sequences and submission to NCBI GenBank

The GENETOOL software [GeneTool Lite version 1.0 Double Twist Inc., Oakland, CA, USA] was used to evaluate the ABI files that were acquired from the sequencing company. EzBioCloud (Chalita *et al.*, 2024) ^[8], a platform for analyzing the taxonomic hierarchy of bacteria through quality controlled sequences of the 16S rRNA gene, and the NCBI- BLASTN tool (Altschul *et al.*, 1990) ^[1] were used for sequence similarity searches after the sequences had been edited, trimmed, and assembled. Using the SEQUIN software (www.ncbi.nlm.nih.gov/Sequin), sequences were uploaded to NCBI GenBank in order to receive accession numbers.

Phylogenetic analysis

Molecular Evolutionary Genetics Analysis (MEGA7) software was used for phylogenetic analysis (Kumar *et al.*, 2016). The FASTA sequences of the *Enterobacter* strains from this investigation and bacteria that shared similarities according to EzBioCloud were aligned using ClustalW in MEGA7 for molecular phylogenetic analysis based on 16S rRNA gene sequences. Every position with lacking information or gaps was removed. The Maximum Likelihood approach, which is based on the General Time Reversible model, was used to infer the evolutionary history.

Results and Discussion

Diversity of bacterial root nodule endophytes of *Tephrosia* spp.

In the Indian Thar Desert perennial wild legumes of genus *Tephrosia* (sub-family Papilionoideae) is represented by nodulating species such as *T. wallichii*, *T. purpurea*, *T. villosa*, *T. falciformis*, *T. leptostachya*, *T. subtriflora* and *T. uniflora* (Panwar *et al.*, 2014)^[40]. Detailed sampling, nodulation studies, characterization and molecular identification of rhizobia associated with root nodules were done for *T. leptostachya*, *T. purpurea*, *T. villosa*, *T. falciformis*, and *T. wallichii* (Tak 2012). The rhizobial microsymbionts associated with these species were identified at molecular level and characterized (Tak 2012; Gehlot *et al.*, 2012; Tak *et al.* 2013, 2016; Ojha *et al.*, 2015; Tak and Gehlot 2019)^[17, 54, 39]. The dominant nodulating endophytes isolated from these species of *Tephrosia* were fast-growing strains of *Sinorhizobium/Ensifer* (Gehlot *et al.*, 2012; Tak 2012; Tak *et al.* 2013, 2016)^[17]. In addition fast-growing *Sinorhizobium/Ensifer*, slow-growing *Bradyrhizobium* strains were also isolated from *Tephrosia* spp. (Tak 2012; Gehlot *et al.*, 2012; Tak and Gehlot 2019)^[17, 54]. The other bacterial strains isolated from root nodules of *Tephrosia* spp. belonged to *Rhizobium/Agrobacterium* sp. (JN593343, JN560161, JN560162, JN560163, JN560164, JN638039, and JN560165); *Microvirga* sp. (HM042680.1); *Enterobacter* sp. (JN228093, JN228092, JN832584, JN228094, JN228095); *Cronobacter* sp. (MN453618); *Pseudomonas* sp. (MN453621); *Bacillus* sp. (MN453616) and *Phyllobacterium* sp. (MN453617). The predominant non-symbiotic nodule endophytic bacteria in *Tephrosia* spp. were *Enterobacter* sp. (Tak 2012). Non-nodulating endophytic bacteria (*Bacillus*, *PaeniBacillus*, *Pseudomonas*, *Agrobacterium* and *Enterobacter*) are commonly found in the root nodules of crop legumes such as *Glycine max*, *Vigna radiata*, *Lens culinaris* and *Phaseolus vulgaris*. Multiple studies confirm these genera as the most prevalent non-rhizobial endophytic bacteria. This suggests that even if these non-rhizobial populations do not cause nodule formation, their frequency is linked to a number of characteristics that promote plant growth (Hnini and Aurag 2024)^[22].

Biochemical and physiological characteristics of *Enterobacter* strains

Eight (JNVU TF1, JNVU TF6, JNVU TF11, JNVU TF18, JNVU TF19, JNVU TW3, JNVU TW4 and JNVU TW6) fast growing *Enterobacter* strains isolated from *T. falciformis* and *T. wallichii* were characterised at phenotypic (NaCl, pH and temperature tolerance) level (Table 1). Up to 3% (w/v) NaCl, these *Enterobacter* strains can withstand, two strains JNVU TF1, and JNVU TF11 could grow up to 4%. Strains JNVU TF1, JNVU TF11, JNVU TW3, JNVU TW4 and JNVU TW6 were capable of growing in a broad pH range of 4.5–11. *Rhizobium tropici* CIAT899 was isolated from bean nodules by Graham *et*

al. (1994)^[29] and was able to thrive on YEMA medium with a pH of 4.0. Tested eight strains turned the color of the Bromo Thymol Blue (BTB) media yellow due to production of acids. These eight *Enterobacter* strains utilized most of the sugars except adonitol, dulcitol, inulin and salicin that were metabolized by only few strains (Fig.1). These *Enterobacter* strains were sensitive to most of the antibiotics tested and resistant to different concentrations of Nitrofurantoin, Ampicillin, Augmentin, Cephoxitin and Cephalothin (Table 2). In bacteria, horizontal gene transfer plays a significant role in evolution and is a potent driver of phenotypic, biochemical, and genetic variety. Several biochemical tests were performed on the eight strains from this study (Table 1). It was observed that all tested strains displayed color changes (to red precipitate) upon the addition of reagents alpha-naphthylamine and sulfanilic acid, indicating positive nitrate reductase activity. (Table 1). Five of tested strains could hydrolyze the protein polymer, gelatin, to amino acids (Table 1). These results are comparable with results of Farmer *et al.* (1980)^[14]. Further all strains were found to *in vitro* utilize citrate as the sole source of carbon. Members of genera belonging to the *Enterobacteriaceae* family are well known to use citrate in the medium (Farmer *et al.* 1980, 1985)^[14, 15]. However all tested *Enterobacter* strains could enzymatically liberate sulfur from sulfur-containing compounds and turned the lead acetate strip black which is contrary with results obtained by Farmer *et al.* (1980, 1985)^[14, 15]. Most of the characters/traits mentioned above are related to catabolism and are mostly present on plasmids and are prone to horizontal transfer and therefore can be added or lost in the course of evolution at particular place.

Plant growth-promoting (PGP) traits of *Enterobacter* strains

Plant growth-promoting endophytic, diazotrophic non-rhizobial bacteria such as *Enterobacter cloacae*, *Enterobacter ludwigii*, *Klebsiella pneumoniae*, *Klebsiella variicola* and *Pseudomonas aeruginosa* are commonly isolated from leguminous crops such as *Vigna* spp. (*V. radiata*, *V. mungo*, and *V. unguiculata*), *Glycine max* and *Arachis hypogaea* (Zhao *et al.*, 2018; Hossain *et al.*, 2023; Dhole *et al.*, 2016; Hnini and Aurag 2024)^[64, 22, 23]. We tested the eight *Enterobacter* strains from present study for multiple PGP traits to screen best strains (Table 1). It was interesting finding that the all tested strains could produce ammonia by hydrolysis of peptones (Table 1). In the present study only one (JNVU TF1) of eight tested *Enterobacter* strains showed moderate IAA activity. Mehnaz *et al.* (2001)^[34] observed PGP activity of *Enterobacter* strains in rice and Mirza *et al.* (2001)^[35] showed PGP activity of *Enterobacter* strains in sugarcane due to production of IAA. It has been demonstrated that *Enterobacter cloacae* produces auxins that aid in promoting plant growth (Patten and Glick 1996)^[41]. Seven of eight tested *Enterobacter* strains could form yellow halo zone against dark blue background indicating production of siderophores. Siderophores have been discovered to be produced by gram-negative bacteria such as *Enterobacter* and *Pseudomonas* (Tian *et al.*, 2009)^[58]. All of the *Enterobacter* strains included in the study formed a clear halo zone around the colony on Pikovskaya medium by solubilizing tricalcium phosphate (TCP) present in it. Solubilization index (SI) was 3-6 for these *Enterobacter* strains. Four (JNVU TF19, JNVU TW3, JNVU TW4 and JNVU TW6) of eight tested *Enterobacter* strains could produce phytase and efficiently mineralize phytate organic form of phosphate. Few studies have identified rhizobacteria that can effectively mineralize phytate. According to reports, the rhizobacteria that produce phytase include *Bacillus*,

Enterobacter, *Burkholderia*, *Serratia*, *Pseudomonas*, and *Staphylococcus* (Hussin *et al.*, 2007) [25]. All tested strains showed protease activity with strain JNVU TF6 showing maximum activity. Morales *et al.* (2003) [36] reported proteolytic activity by *Enterobacteriaceae* strains. Only three of the tested eight *Enterobacterial* strains showed slight cellulase activity. Similarly, Sami *et al.* (2008) [48] reported the production of cellulases by *Enterobacter cloacae*. Cellulose has been shown to be hydrolyzed by *Enterobacter* sp. from *Bombyx mori* digestive system (Anand *et al.*, 2010) [2]. All tested eight *Enterobacterial* strains showed positive pectinase activity. Contrary to our results *Enterobacter* sp. from *B. mori* did not hydrolyze pectin (Anand *et al.*, 2010) [2]. Five of eight tested *Enterobacterial* strains showed slight chitinase activity. In earlier studies by Chernin *et al.* (1995) [9] chitinolytic *Enterobacter agglomerans* has been found antagonistic towards fungal plant pathogens.

Genetic diversity and 16S rRNA phylogeny of *Enterobacter* strains

RPO1 DNA-fingerprinting profile (Fig. 2) of eight (JNVU TF1, JNVU TF6, JNVU TF11, JNVU TF18, JNVU TF19, JNVU TW3, JNVU TW4 and JNVU TW6) fast growing *Enterobacterial* strains isolated from *T. falciiformis* and *T. wallichii* revealed genetic diversity among bacteria. However strains JNVU TF11, JNVU TF18 and JNVU TF19 had similarity in few bands. Using the 16S rRNA gene sequences and a BLASTN sequence similarity search, non-nodulating root nodule bacterial strains (JNVU TF1, JNVU TF6, JNVU TW3, JNVU TW4 and JNVU TW6) were identified as species of *Enterobacter* (Table 3). These strains showed high similarities with type strain *Enterobacter cloacae* sub sp. *cloacae* strain ATCC 13047T (isolated from human cerebrospinal fluid) (Ren *et al.*, 2010) and *Enterobacter cloacae* sub sp. *dissolvens* LMG

2683T isolated from *Zea mays*, United Kingdom (Hauben *et al.*, 1998). Similarly, in 16S rRNA based maximum likelihood (ML) phylogenetic tree, strain JNVU TF1 clustered with LMG 2683T whereas strains JNVU TF6 and JNVU TW6 clustered close to ATCC 13047T (Fig. 3). Two strains JNVU TW3 and JNVU TW4 from *T. wallichii* grouped together to form a separate clade within *Enterobacter* species. These non nodulating strains showed good PGP activity and were found dominant root nodule endophytic strains in *falciiformis* and *T. wallichii* plants in this investigation. Similar reports have been reported from Coimbatore, Tamil Nadu, India, about *Enterobacter arachidis* Ah-143T (EU672801), a diazotrophic bacterium that promotes plant growth and was isolated from groundnut rhizosphere soil (Madhaiyan *et al.*, 2010) [32]. Strains JNVU TW3 and JNVU TW4 also exhibited similarities to the type strain *Enterobacter radicincitans* D5/23T (NR_042982), which was isolated from the winter wheat phyllosphere and is capable of fixing atmospheric nitrogen and producing auxins and cytokinins (Kämpfer *et al.*, 2005) [27]. According to Benhizia *et al.* (2004) [4], nodules in *Hedysarum* species are not caused by a single species of rhizobia. Surprisingly, they contain a large number of gamma proteobacteria, including *Enterobacter kobei*, *Enterobacter cloacae*, *Leclercia adedecarboxylata*, *Pantoea agglomerans*, *Escherichia vulneris*, and *Pseudomonas* sp. This suggests that the only organisms that live in *Hedysarum* nodules are either members of the *Enterobacteriales* or *Pseudomonadales* order. Presence of symbiotic genes if any and their divergence in members of *Enterobacteriaceae* need to be examined to include them as nodulating. The presence of endophytic *Enterobacteria* within perfectly healthy plants shows that other positive interactions may be at work, despite the failure of attempts to test Koch's postulates, which calls for determining whether the bacteria can re-nodulate their hosts.

Table 1: Phenotypic and biochemical traits; and plant growth promoting (PGP) activities shown by *Enterobacterial* root nodule bacterial endophytes

Activity/Test	TF1	TF6	TF11	TF18	TF19	TW3	TW4	TW6
NaCl (Salt) tolerance up to	4%	3%	4%	3%	3%	3%	3%	3%
pH range	4.5-11	5-11	4.5-11	5-11	5-11	4.5-11	4.5-11	4.5-11
Temperature tolerance range (in oC)	28-36	28-38	28-38	28-36	28-36	28-38	28-38	28-38
Nitrate reductase	+++	+++	+++	+++	+++	+++	+++	++
Citrate utilization	+	+	+	+	+	+	+	+
Methyl red test	-	-	-	-	-	-	-	-
Voges-Proskauer	++	++	++	++	++	+	+	+
ONPG test	+	+	+	++	+	+	+	+
Catalase activity	+	+	+	+	+	++	+	++
Oxidase activity	-	+	+	-	-	-	-	-
Gelatinase activity	++	+	+++	+++	+++	-	-	-
Amylase production	-	-	-	-	-	-	-	-
H ₂ S production	+	+	+	+++	+++	+	+	+++
Ammonia production	+	++	+	++	++	++	++	++
IAA production	++	-	-	-	-	-	-	-
Siderophore production	+	++	-	+	+	+	+	+
HCN production	-	-	-	-	-	-	-	-
Phosphate solubilization	+++	+++	+++	+++	+++	++	+++	+
Phytase activity	-	-	-	-	+	+	+	+
Protease activity	++	+++	+	+	+	++	++	++
Cellulase activity	-	-	-	-	-	+	+	+
Chitinase activity	+	+	-	-	-	+	++	+
Pectinase activity	+	+	+	+	+	+	++	+

Note: + Positive (low), ++ (moderate), +++ (high), – negative or no activity

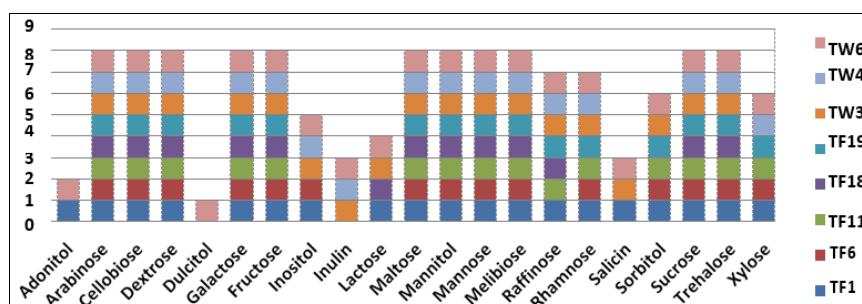
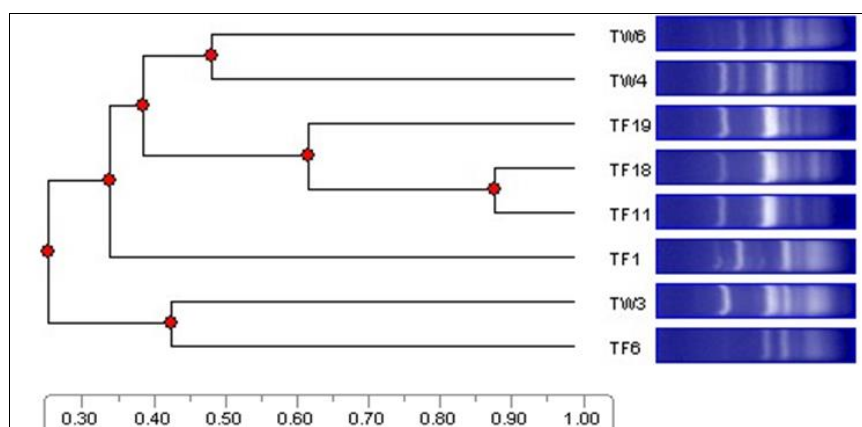
Table 2: Intrinsic antibiotic resistance (IAR) of non-nodulating *Enterobacterial* strains (Zone of inhibition in mm)

Antibiotic (concentration in µg)	TF1	TF6	TF11	TF18	TF19	TW3	TW4	TW6
Ceftazidime (30)	20	25	25	26	25	26	30	R
Ciprofloxacin (5)	35	40	30	38	35	32	30	R
Amikacin (30)	25	28	25	23	25	21	20	23
Nitrofurantoin (300)	15	R	15	R	R	R	R	R
Netillin (30)	18	25	15	R	R	20	20	22
Nalidixic acid (30)	21	25	30	25	28	29	30	R
CoTrimoxazole (25)	22	28	20	30	32	29	15	R
Amoxycylav (30)	10	R	20	R	R	18	R	R
Gentamicin (10)	20	30	25	25	20	20	R	21
Tetracycline (30)	28	41	30	20	22	21	20	35
Ofloxacin (5)	30	30	32	35	35	26	27	32
Cefuroxime (30)	20	20	15	15	15	27	30	21
Ampicillin (10)	R	R	R	R	R	21	R	R
Augmentin (30)	20	R	R	R	R	10	R	R
Cephoxitin (30)	R	R	R	R	R	15	30	R
Cephalothin (30)	R	R	R	R	R	R	R	R
Aztreonam (30)	20	R	22	12	15	21	23	R
Piperacillin (100)	20	15	15	28	30	26	26	19
Imipenam (10)	20	20	30	30	30	27	34	35

Note: R stands for resistant

Table 3: Identification of non-nodulating bacterial strains based on BLASTN sequence similarity search using the 16S rRNA gene sequences

Legume host	Strain name (GenBank accession number)	Sampling site	Closest match with type strain (NCBI Reference Sequence)	Sequence similarity
<i>Tephrosia falciiformis</i>	JNVU TF1 (JN228093)	Sikar, Rajasthan	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> strain LMG 2683T	99.73%
<i>Tephrosia falciiformis</i>	JNVU TF6 (JN228092)	New campus, JNVU, Jodhpur, Rajasthan	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> strain LMG 2683T	98.4%
<i>Tephrosia wallichii</i>	JNVU TW3 (JN832584)	New campus, JNVU, Jodhpur, Rajasthan	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> strain ATCC 23373T	98.79%
<i>Tephrosia wallichii</i>	JNVU TW4 (JN228094)	New campus, JNVU, Jodhpur, Rajasthan	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> strain LMG 2683T	99.27%
<i>Tephrosia wallichii</i>	JNVU TW6 (JN228095)	New campus, JNVU, Jodhpur, Rajasthan	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> strain LMG 2683T	99.47%

**Fig 1:** Utilization of various sugars as carbon sources by eight *Enterobacter* strains isolated from *Tephrosia* spp**Fig 2:** UPGMA dendrogram of RPO1 profile of eight *Enterobacter* strains obtained from the similarity matrix using Phoretix 1D

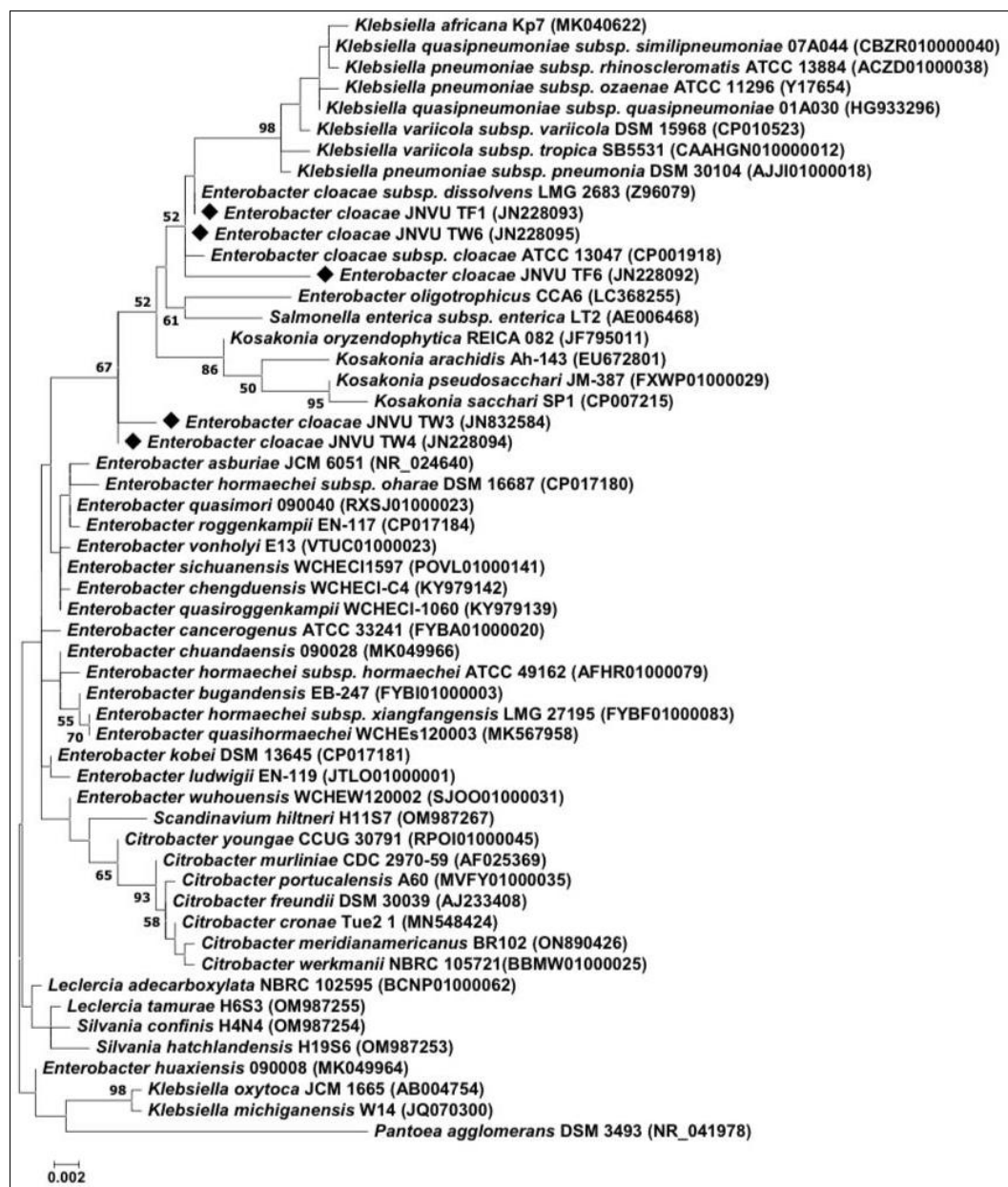


Fig 3: Maximum Likelihood phylogenetic analysis based on 16S rRNA gene sequences of *Enterobacter* strains from present study. Bootstrap values calculated for 1,000 replications greater than 50% are indicated. The tree is drawn to scale with branch lengths measured in the number of nucleotide substitutions per site. Accession numbers from GenBank are in parenthesis.

Conclusion

Our findings show that a variety of symbiotic and nodulating bacterial taxa coexist with primarily non-rhizobia in the nodules of wild legumes. The genetic diversity of nodule endophytic bacteria served as a valuable pool for discovering PGP bacteria. The fact that *Enterobacter* is frequently found in the root nodules of many plants indicates the need for additional molecular research on the signal exchange, colonization of the root nodules, and importance of this gamma-proteobacteria as endophytes in legumes.

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