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Original Research Article

Enhancement in growth and alkaloid content in trigonella foenumgraecum by non-rhizobial root nodule endophytes

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Abstract

Root nodule bacteria (RNB) recovered from wild medicinal legumes were inoculated to determine their effect on growth, nutrient uptake and alkaloid content of *Trigonella foenumgraecum.* Seven strains were selected based on their *in vitro* plant growth promoting (PGP) characteristics such as siderophore production, P solubilization and IAA production. The experiment was performed in non-sterile soil to depict natural field conditions and to determine the competence of these strains with native microbes of soil. Sterile and non-sterile controls were used for comparison of results. All seven bacterial strains were found to improve one and/or other growth parameters such as root and shoot length, fresh weight and dry weight, and alkaloid content. Treatment with the isolate BeTF5 outperformed other strains in terms of plant growth parameters (root and shoot dry weight), nutrient uptake and stimulated maximum alkaloid production (638.1 mg g^{-1} of plant material) in *T. foenumgraecum.* Analysis of 16S rDNA revealed that of these strains belonged to nonrhizobia. Based on the performance with bioassay and PGP properties, all the seven strains BeMI1 (*Pseudomonas fluorescens*), BeTF5 (*Enterobacter sp.*), BaIT1 (*Enterobacter asburiae*), BaAP5 (*Pseudomonas putida*) and BaAP2 (*Staphylococcus hominis*), BeTF1 (*Bacillus bataviensis*), BaAP4 (*Bacillus pumilus*) are the potential agents to improve growth and alkaloid content in *T. foenumgraecum*. The study also revealed that biased cultivation of rhizobia from root nodules may lead to loss of beneficial RNB diversity.

Key Words: Alkaloid, non rhizobia, plant growth promotion, 16Sr DNA sequence, root nodule bacteria, *Trigonella foenumgraecum*

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INTRODUCTION

The success of legumes is mainly due to their ability to form nitrogen fixing symbiosis with specific bacteria known as rhizobia manifested by the development of nodules on the plant roots, the sites where nitrogen fixation occurs. They form an important component of the economy because of nitrogen-fixing ability and medicinal propertie[s1]. Several of these legumes are potential sources of glycosides, biologics, antibiotics and alkaloids that are used in drug manufacturing. Madhya Pradesh harbours a diversity of medicinal legumes out of which several species grow wild and are used by local tribal community. Besides playing a major role in restoring soil fertility, reducing erosion, medicinal legumes are a rich source of alkaloids which are used in drug formulations to treat various diseases. Until recently, 1-2% of the medicinal legumes have been studied for their root nodule endophytes. In majority of cases, emphasis was laid on biased cultivation of rhizobia. Non rhizobial root nodule bacteria (RNB) have gained importance as a group of potential plant growth promoting (PGP) bacteria[2]. A total of 14 bacterial genera associated with nodules of wild legumes in Tunisia were studied by researchers[3]. Later,[4] reported over 24 non-rhizobial taxa isolated from legume tissues (primary and secondary root segments and stem) that included *Agrobacterium, Bacillus, Curtobacterium, Enterobacter, Erwinia, Mycobacterium, Paenibacillus, Pseudomonas, Phyllobacterium, Ochrabacterium* and *Sphingomonas.* However, *Phyllobacterium* and

Ochrabacterium are now placed in rhizobial taxa[5]. *Pseudoalteromonas*[6], *Pantoea*[7], *Acinetobacteria* [8], *Ralstonia*[9] and others like *Chelatococcus, Bosea* and *Balneomonas*[10] have also been reported as nodule endophytes of legumes. All the studies indicate improved plant yield, health and nodulation when co-inoculated with nodule endophytes, besides inoculation with rhizobia alone[11]. The RNB contribute to plant growth by enhancing nitrogen uptake, phytohormones production, mineral solubilization and chelation of iron via siderophores. Root nodule endophytes with these traits may account for better growth promotion in addition to enhanced active principles in medicinal legumes. Although the medicinal plants are studied for active principles, the PGP properties of microsymbionts from the root nodules of these plants have not been studied. Hence, the aim of the present study was to study and identify root nodule endophytes of *T. foenumgraecum* possessing PGP properties and to analyze the effect of their inoculation on the plant growth and alkaloid content.

MATERIALS AND METHODS

Study sites and medicinal legumes: Two study sites located in state Madhya Pradesh of central India were selected for the collection of wild medicinal legumes. Site 1: District Betul (21.58N, 77.59E); two legumes *Melilotus indica* and *Trigonella foenumgraecum* with root nodules were collected, site 2: District Balaghat (21.48N, 80.11E); two legumes *Abrus precatorious* and *Indigofera tinctoria* with root nodules were collected. All these legumes are known to possess medicinal properties. These plants were uprooted carefully along with root nodules, collected in sterile polythene bags and brought to the laboratory. Among these, *M. indica* and *T. foenumgraecum* are known for their medicinal properties related to infantile diarrhea and diabetes; *A. precatorious* is used as a nervous tonic and abortificant whereas *I. tinctoria* is useful for treatment of chronic myelocytic leukemia.

Isolation of bacteria from nodules: Following the standard procedure outlined by[12], isolation of bacteria from nodule samples was performed. The nodules were washed thoroughly in water, surface sterilized by exposing to 95% alcohol for 1 min and then immersing in 0.1% $HgCl_2$ solution acidified with HCl (1.0 g $HgCl_2$, 5 ml conc. HCl diluted to 1000 ml distilled water) for 5 min. The nodules were then thoroughly washed several times in single distilled water (SDW) to remove traces of $HgCl_2$. To ensure that the nodules were devoid of any microbial contamination, the surface sterilized nodules were placed on Yeast Extract Mannitol Agar (YEMA) plates and incubated at 28° C overnight. The nodules showing any microbial growth were discarded and only the clean nodules were selected for isolation of the microsymbiont. Individual nodules were crushed in 200µl of SDW in a sterile eppendorf tube; the resultant turbid bacteroid suspension was streaked on YEMA plates and incubated at 28° C for growth of bacteria. From these cultures, single cell colonies were picked up and re-streaked on YEMA plates to recover pure cultures.

Screening of bacterial isolates for PGP properties: The recovered bacterial cultures were screened for their PGP properties (P solubilization, IAA production and siderophore production) in qualitative plate assays (Table 1). P solubilization by bacterial cultures was assayed by spot inoculation on Pikovyskya agar plates followed by incubation at 28±2 °C for 48-72 h. A clear zone around the bacterial colony indicated a positive result for P solubilization[13]. Bacterial isolates were assayed for siderophore production by spot inoculating the isolates on Chrome Azurol Medium (Chrome Azurol Test); development of a yellow to orange halo around the bacterial colony after incubation at 28±2 °C for 48-72 h indicated a positive result for siderophore production[14]. AA production by bacterial cultures was assayed by using the qualitative method developed by Bric *et al.*[15] All the isolates were plated onto nutrient agar medium amended with tryptophan, overlaid with a cellulase membrane pre-soaked with Salkowsky reagent (2% vv^{-1}, 0.5 M $FeCl_3$ in 35% perchloric acid) and incubated at 28±2 °C for 48-72 h. Bacteria producing IAA were identified by the formation of a characteristic red halo immediately surrounding the colony.

Quantification of PGP Properties: Quantification of siderophore produced in culture broth was carried out in standard succinate medium using extinction coefficient of siderophore (Eλ= 16500, pH 7.2)[16]. Pure cultures of selected isolates were grown in standard succinate broth for 24 h. One ml active culture was inoculated in 100 ml standard succinate broth and flasks were incubated at 28±2 °C (120 rpm). After 72 h, 10 ml of culture were withdrawn and centrifuged at 10,000 rpm for 5 min. The O.D. of the supernatant was read at 400 nm and siderophore was quantified based on the extinction coefficient of pyoverdine as: siderophore (mg ml^{-1}) = A_{400}× Mol weight of pyoverdine (1500) / Eλ × 10^{-3}. For the quantitative measurement of P, 100 ml of Pikovyskaya broth containing tricalcium phosphate (TCP) were inoculated with 1 ml of fresh culture (10^8 cells ml^{-1}) and incubated for 5 d in a shaker (120 rpm) at 28±2 °C. The culture broth was centrifuged (12,000 rpm for 30 min) and the amount of water soluble phosphate released into the supernatant was estimated by the chlorostannous-reduced molybdophosphoric acid blue method[17]. The quantitative analysis of IAA was performed by the method suggested by[15]. Bacterial isolates were grown in

M9 broth (gl^{-1}: Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1.0; $MgSO_4$, 0.2; $CaCl_2$, 2 and pH 7.0) amended with tryptophan (2 mg ml^{-1}). After 24 h, 5 ml of culture were centrifuged (10,000 rpm for 15 min) and 2 ml of Salkowsky reagent were added to 2 ml of supernatant and incubated at 28 °C in the dark for 1 h. The IAA concentration was determined using a spectrophotometer (λ_{530} nm) against a standard curve.

Experimental design for bioassay: The pot experiment was conducted to assess the potential of bacterial isolates with one or multiple plant growth promoting properties. Seven bacterial cultures (BeMI1, BeTF5, BaIT1, BaAP5, BaAP2, BeTF1 and BaAP4) possessing more than one PGP attributes were used (Table 2). Soil for pot experiment was collected from Barkatullah University campus and was analysed for various parameters. The soil pH was measured in a 1:5 (wv^{-1}) aqueous solution. Total nitrogen (N), available phosphorous (P) and potassium (K), and organic carbon (C) were analysed by National Fertilizer Limited, Bhopal, India. Non-sterile soil (4.0×10^4 CFU g^{-1}) was used for plant bioassay. Seeds of *T. foenumgraecum* were tested for their germination frequency prior to bioassay set up. Seeds were surface sterilized with 0.02% sodium hypochlorite (vv^{-1}) for 2 min and rinsed thoroughly with sterile water 3-5 times. Surface sterilized seeds were soaked in sterile distilled water for 1h prior to sowing. For plant bioassays non-sterile soil was air dried, mixed with sand in 1:1 ratio and filled in plastic bags (1kg bag^{-1}). Five seeds per bag were sown. After sowing, bags were inoculated by injecting 1 ml of log phase bacterial culture (3×10^7 CFU ml^{-1}) pregrown in tryptone soy broth for 24-48 h at 30 ºC near each seed. Two sets of control were used; one containing non-sterile soil and the other containing sterile soil. The treatments were T_0 non sterile control (non sterile soil (NS) without inoculum), TS_0 sterile control (sterile soil (SS) without inoculum), T1 (NS+ BeMI1), T2 (NS+ BeTF5), T3 (NS+ BaIT1), T4 (NS+ BaAP5), T5 (NS+ BaAP2), T6 (NS+ BeTF1) and T7 (NS+ BaAP4). One ml of TSB was added in the control treatment bags near each seed for comparison. Each treatment including both the controls was replicated ten times. The pots were kept under nursery conditions and watered on alternate days. Booster dose of inoculum (5ml of log phase culture having 3×10^7 CFU ml^{-1}) was given at fifteenth day of the experiment. Both the controls were supplemented with 5 ml TSB (without any inoculum). Harvesting was done after 60 days and growth parameters such as root and shoot length, fresh and dry weight were recorded. Shoot and leaf tissues were extracted in 10% (vv^{-1}) acetic acid in ethanol and alkaloid content was determined by the method of[18]. The soil sample from each treatment was also collected for analysis of nutrients as described earlier.

Sequencing of 16S rDNA region: Sequencing of 16S rDNA region of the bacterial isolates was performed by Vimta labs, Hyderabad, India and the obtained sequences were then analyzed by Blast tool and submitted to GeneBank- (http://www.ncbi.nlm.nih.gov).

Statstical Analysis: Fisher's least significance difference (LSD) test with 0.05% as level of significance followed by one-way ANOVA was performed to enumerate significant difference between control and treatments.

RESULTS

Table 1: Source, location and plant growth promoting traits of root nodule bacterial strains used in study

Strain	Source	Location	PGP traits
BeMI1	Root nodules of *Melilotus indica*	Betul, Madhya Pradesh, India	Siderophore, P solubilization, IAA production
BeTF5	Root nodules of *Trigonella foenumgraecum*	Betul, Madhya Pradesh, India	Siderophore, P solubilization, IAA production
BaIT1	Root nodules of *Indigofera tinctoria*	Balaghat, Madhya Pradesh, India	Siderophore, P solubilization, IAA production
BaAP5	Root nodules of *Abrus precatorious*	Balaghat, Madhya Pradesh, India	Siderophore, P solubilization, IAA production
BaAP2	Root nodules of *Abrus precatorious*	Balaghat, Madhya Pradesh, India	Siderophore, P solubilization
BeTF1	Root nodules of *Trigonella foenumgraecum*	Betul, Madhya Pradesh, India	Siderophore, P solubilization, IAA production
BaAP4	Root nodules of *Abrus precatorious*	Balaghat, Madhya Pradesh, India	Siderophore, IAA production

Table 2: In vitro quantification of plant growth promoting traits of selected root nodule bacterial strains

Strain	Siderophore production (mg ml^{-1})	Phosphate solubilization (µg ml^{-1})	IAA production (µg ml^{-1})
BeMI1	45.5±0.01	535.6±0.8	55.5±0.8
BeTF5	38.1±0.04	480.0±0.5	7.35±0.3
BaIT1	36.6±0.50	536.0±0.5	11.2±0.1

BaAP5	60.0±0.06	537.0±0.01	1.1±0.04	BaAP4	3.54±0.02	53.6±0.04	5±0.01
BaAP2	37.3±0.6	48.4±0.08	ND				
BeTF1	36.2±0.05	584.0±0.8	5±0.02				

ND- No activity detected. Results are expressed as mean ± SE (n=3)

Table 3: Effect of root nodule bacterial treatments on root and shoot growth of 60-day-old *T. foenumgraecum* plants

Treatments	Root			Shoot		
	Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)
T_0	0.12±0.10[b]	0.05±0.05[b]	21.25±0.69	1.52±0.19[b]	0.28±0.09	22.95±0.59
Ts_0	0.08±0.15[a]	0.03±0.04[a]	18.6±0.57	0.90±0.20[a]	0.24±0.08	18.7±0.56
T_1	0.27±0.11[a]	0.08±0.03[a]	27.9±0.74[ab]	1.55±0.19[a]	0.28±0.08	23.8±0.59[b]
T_2	0.28±0.14[a]	0.17±0.16	23.5±0.81	1.44±0.18[a]	0.27±0.08	21.35±0.58
T_3	0.12±0.08[b]	0.06±0.04[b]	20±0.74	1.36±0.16	0.26±0.09	24.05±0.56[b]
T_4	0.09±0.05[b]	0.05±0.04[b]	25.2±0.74[b]	0.99±0.15[ab]	0.20±0.07[a]	20.95±0.61
T_5	0.12±0.08[b]	0.06±0.05	23.9±0.82	1.46±0.24	0.28±0.08	23.2±0.56
T_6	0.11±0.06[b]	0.06±0.04	21.4±0.68	1.26±0.16	0.30±0.08[b]	24.75±0.28[b]
T_7	0.23±0.16	0.06±0.03[b]	20.9±0.64	1.29±0.17	0.25±0.08	23.6±0.64

Results are expressed as mean±SE (n=10). Fisher's least significance difference (LSD) test with 0.05% as level of significance followed by one-way ANOVA was performed for each time.

- significantly different with non sterile control ($p \leq 0.05$)
- significantly different with sterile control ($p \leq 0.05$)

The treatments were T_0 Non sterile control (non sterile soil (NS) without inoculum), TS_0 Sterile control (sterile soil (SS) without inoculum), T_1 (NS+ BeMI1), T_2 (NS+ BeTF5), T_3 (NS+ BaIT1), T_4 (NS+ BaAP5), T_5 (NS+ BaAP2), T_6 (NS+ BeTF1), T_7 (NS+ BaAP4).

Table 4: Effect of root nodule bacterial treatments on the nutrient uptake by plants in a bioassay after 60-days of inoculation

Treatments	Nutrients uptake by plants		
	N %	P %	K %
T_0	4.82±0.02	0.07±0.002	2.78±0.03
Ts_0	0.62±0.01	0.012±0.002	0.50±0.01
T_1	4.98±0.07	0.078±0.003	3.88±0.01[a]
T_2	5.67±0.06[ab]	0.052±0.004[ab]	3.68±0.02[a]
T_3	3.90±0.06[b]	0.048±0.002[b]	1.82±0.04
T_4	3.82±0.04[b]	0.065±0.005[ab]	2.12±0.03
T_5	4.28±0.02	0.02±0.001[ab]	3.08±0.02
T_6	3.44±0.08[b]	0.082±0.009	3.28±0.04
T_7	4.82±0.03	0.042±0.002[ab]	2.58±0.03

Results are expressed as mean±SE (n=10). Fisher's least significance difference (LSD) test with 0.05% as level of significance followed by one-way ANOVA was performed for each time.

- significantly different with non sterile control ($p \leq 0.05$)
- significantly different with sterile control ($p \leq 0.05$)

Table 5: Closest relatives of root nodule bacterial strains used in this study as analysed through 16S sequencing and BLAST search

Isolate	*BLAST similarity closest relative*	*% Similarity*	*Groupof bacteria*	*Accession no.*
BeMI1(T_1)	*Pseudomonas fluorescens*	97	*γ-proteobacteria*	JF309197
BeTF5(T_2)	*Enterobacter sp.*	93	*γ proteobacteria*	NS
BaIT1(T_3)	*Enterobacter asburiae*	80	*γ proteobacteria*	JQ734774
BaAP5(T_4)	*Pseudomonas putida*	88	*γ proteobacteria*	JQ734771
BaAP2(T_5)	*Staphylococcus hominis*	95	*Bacilli*	JQ734768
BeTF1(T_6)	*Bacillus bataviensis*	98	*Bacilli*	JQ734772
BaAP4(T_7)	*Bacillus pumilus*	99	*Bacilli*	JF904523

NS- not submitted to Genebank (under process)

Figure legends: Fig. 1 Effect of root nodule bacterial treatments on alkaloid content of plant parts (leaves and shoot) of 60-days-old *T. foenumgraecum* plants. Error bars indicate standard error of the mean (n=3). a significantly different with non sterile control ($p \leq 0.05$) b significantly different with sterile control ($p \leq 0.05$) The treatments were T_0 Non sterile control (non sterile soil (NS) without inoculum), T_{S0} Sterile control (sterile soil (SS) without inoculum), T_1 (NS+ BeMI1), T_2 (NS+ BeTF5), T_3 (NS+ BaIT1), T_4 (NS+ BaAP5), T_5 (NS+ BaAP2), T_6 (NS+ BeTF1), T_7 (NS+ BaAP4).

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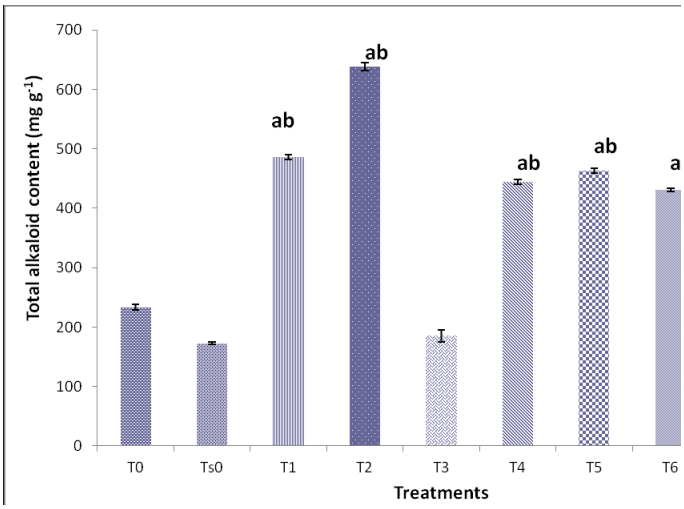

Figure 1:

Siderophore production was maximum in isolate BaAP5 (60.0 mg ml^{-1}). However, all seven bacterial strains were able to produce siderophore in the range between 3.5-60.0 mg ml^{-1}. Phosphate solubilization in bacterial isolates ranged between 480-48.4 µg ml^{-1}; isolates BaAP2 and BaAP4 showed poor phosphate solubilization. Amount of IAA produced by these strains ranged from 1.1-55.5 µg ml^{-1} with isolate BeMI1 producing the maximum amount; no IAA production was recorded in BaAP2. Among the seven bacterial isolates selected, six showed all the three tested PGP traits. Isolates BaAP2 possessed only two PGP traits (Table 2). Significant increase in root fresh weight (RFW) was observed in treatments T1 and T2 (Table 3) which improved the fresh weight by 125% and 133%, respectively, compared to non sterile control (T_0). However, no significant improvement in RFW was observed in all the treatments when compared to sterile control (T_{S0}). Treatment T_1 increased the root dry weight (RDW) by 60.0% and 14.0% over treatments T_0 and T_{S0}, respectively. Shoot fresh weight (SFW) was significantly enhanced by treatments T_2 and T_5 compared to Ts_0. All the treatments enhanced the SDW (Shoot dry weight) over the sterile control T_{S0}, however maximum increase in SDW was observed in treatment T_6. Treatment T_1 showed maximum increase in root length (RL) compared to both the controls. However, rest of the treatments also enhanced the RL of the inoculated plants. Among the treatments, T_6 enhanced the shoot length (SL) of the inoculated plants by 19.6% and 7.84% compared to T_{S0} and T_0 respectively (Table 3). All the treatments increased the N content in the plants as compared to Ts_0 (Table 4), however the increase in N uptake was significant in treatments T2, T3, T4 and T6. All the treatments enhanced the uptake of phosphorous in plants as compared to sterile control Ts0 however significant increase was observed in treatments T2, T3, T4, T5 and T7. Significant increase in uptake of K content was recorded in treatments T_1, T_2, T_5 and T_6 compared to T_0. All the treatments had a stimulating effect on the alkaloid content in plant parts except treatment T_3. Significant increase in alkaloid content was also recorded in treatment T_0 when compared with T_{S0}, however it was lowest among other treatments. Treatment T_2 showed maximum enhancement in the alkaloid content in plant parts i.e. 638.1 mg g^{-1} of plant material (Fig. 1). Other treatments also showed enhanced alkaloid levels compared to treatments T_0 and Ts_0.

16S rDNA sequencing of the bacterial isolates used as bioinoculants in the study were performed and the closest affiliation according to sequencing is shown in Table 5. BLAST similarity search analysis of the available sequences identified the isolates BeMI1, BeTF5, BaIT1, BaAP5, BaAP2, BeTF1 and BaAP4 as *Pseudomonas fluorescens, Enterobacter sp., Enterobacter asburiae, Pseudomonas putida., Staphylococcus hominis, Bacillus bataviensis* and *Bacillus pumilus,* respectively.

DISCUSSION

Effects of PGP bacteria on different crops are studied by many researchers. Inoculation with such potential PGP strains could compensate for nutrient deficiency in soils, improve plant growth and development by production of plant growth regulators which stimulate root development of plants which leads to better absorption of nutrients from soil[19]. Inoculation of *T. foenumgraecum* with root nodule bacterial inoculants had in general, had a stimulating effect on plant growth. Similarly, promotion in growth parameters was reported by several workers in various plants on inoculation with PGP bacteria[20]. Auxins produced by bacteria can improve root development which enhances the nutrient uptake leading to increased plant growth[21]. The enhancing effect of seed inoculation with rhizobacteria on SDW and yield of maize were reported by earlier researchers[22]. P one of the most essential plant macronutrient is unavailable to the plants as it remains in insoluble form. Many rhizospheric bacteria have the ability of transforming immobilized soil P into plant available form by releasing phosphates. Inoculation of plants with P solubilizing micro-organisms has been reported to promote plant growth by increasing phosphorous uptake[23]. Maximum IAA production by the isolates BeMI1, BeTF5 is positively related with all plant traits with the exception of correlation with SL which was not significant with non- sterile control ($P \leq 0.05$) probably due to the variable effect of IAA on root elongation. A low level of IAA produced by bacteria promotes primary root elongation whereas high level increases lateral and adventitious root formation but inhibits the primary root growth[24]. This seems to play an

important role in P acquisition by plants. The phosphorous uptake was enhanced in all the treatments; this might be due to the P solubilizing trait of the inoculants which might have resulted in the increase in P uptake in the soil. However the uptake of the solubilized P could have taken place in higher amount in plants with increased RL and DW. This may be due to the altered morphology of the roots by IAA production which might have enhanced the uptake of P, thereby reducing the P in the soil. In our study the increase in RL and RDW was maximum in plants with treatments T_1, T_2 and T_4; this might be due to the increased uptake of nutrients by roots of these plants. The difference in the uptake of nutrients may be due to variation in the growth of root system due to difference in the levels of IAA produced. Effect of K on various growth parameters has been reported by earlier researches[25] which proves the role of K as an important nutrient in plant metabolism. Heterologous iron uptake mechanisms are possessed by many plants due to presence of native soil microbes.[26] found that plants cultivated under non-sterile conditions show no iron deficiency symptoms as compared to plant grown in sterile condition, this depicts the role of soil microbiota in iron acquisition via iron-bacterial-siderophore complex formation. All the isolates used for the bioassay possessed siderophore producing capability however the growth promotion may be due to the combination of mechanisms that increase the nutrient availability, suppress pathogens or affect root growth via phytohormone production. Many PGP strains are likely function by more than one mechanism.[27] have reviewed the potential of P solubilizing microorganisms that provide phytopathogen biocontrol as well as affect plant growth via production of siderophores, hydrolytic enzymes and IAA. Alkaloids are the secondary metabolites with basic character, containing heterocyclic N, and are synthesized from amino acids or their immediate derivatives[28]. Since N is an important constituent of alkaloids, it is positively regulated by N content. Thus enhanced uptake of N may positively affect the alkaloid content in the plants. It has been reported that application of N and P supplements increased the alkaloid content in *Cathranthus roseus*[29,30] reported that soil nutrient deficiency may affect the total alkaloid concentration. Thus, it can be concluded that multiple effect of PGP traits, availability of sufficient nutrients and their uptake by the plants might have contributed to increased alkaloid production in the medicinal plant *T. foenumgraecum.* This study was conducted in non-sterile soil to assess the competitive ability of bacterial inoculants to survive and affect the growth of inoculated plants in the presence of indigenous microflora. All the plant growth parameters were recorded to be lower in sterile control compared to non-sterile control. It could be due to the presence of other native microbes in the non-sterile soil which might have affected the growth whereas the sterile soil does not contain any native microbes and it does not depict the natural field conditions. The difference in plant growth promotion potential among the isolates is attributed to their individual competencies[31,32] have suggested that inocula of effective PGPR strains can shift the bacterial community equilibrium at early stages of plant growth and can favour growth of beneficial bacterial populations which enhance the performance of inoculated bacteria via multiple mechanisms. Root nodules of leguminous plants were found to shelter large population of endophytic bacteria of diverse genera and species which are unrelated to rhizobial symbiotic nitrogen fixing bacteria[3,4,33]. These non-rhizobial root nodule endophytes improved plant growth as well as nodulation when co-inoculated with *Rhizobium*[11,34]. Based on 16S rDNA sequencing, RNB used in this study belonged to nonrhizobia. The existence of non symbiotic endophytic bacteria in leguminous root nodules is reported as a universal phenomenon by several researchers[35]. The finding of *Bacillus*, *Pseudomonas* and *Enterobacterial* species as common nodule endophytes has been reported earlier by several research[3,33]. In *Sphaerophysa salsula*, non rhizobial genera including *Staphylococcus*, *Bacillus* and *Pseudomonas* have been reported as root nodule endophytes[36]. *Bacillus thuringiensis*, *Enterobacter asburiae* and *Serratia marcescens* were also isolated as RNB from *Kudzu.* These RNB were shown to promote growth and positively influenced the nutrient uptake parameters of wheat seedlings[31].

CONCLUSION

The present study showed the presence of non rhizobia as RNB of wild legume plants and emphasizes the need of non-biased cultivation of RNB to ensure not to lose any potential nodule endophyte. A positive effect of the seven strains on the growth, nutrient uptake and alkaloid content under nursery conditions was demonstrated in non sterile soil to ensure that the effects of isolates on plant growth was not masked by native microflora, other soil and climatic factors. Enhancement in alkaloid content of *T. foenumgraecum* is beneficial for pharma industry. However, future prospects of investigation include the application of these selected RNB isolates to other medicinal legumes as well, in order to assess their potential for plant growth and enhancement of alkaloid content in plant parts. The results of this study suggest that the simultaneous screening of bacteria for growth parameters under pot conditions using non sterile soil is a good tool for assessing the potential of inoculants. This helps in assessing the survivability and competition with

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the indigenous microflora which may be helpful in selecting the effective PGP bacteria for biofertilizer development.

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