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ACC DEAMINASE PRODUCING *RHIZOBIUM LEGUMINOSARUM* RPN5 ISOLATED FROM ROOT NODULES OF *PHASEOLUS VULGARIS* L.

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Abstract

Seven root nodule bacteria of *Phaseolus vulgaris* L. were selected for detailed studies based on their morphological and biochemical characters. After the evaluation of their plant growth promoting (PGP) attributes, RPN5 was found to be the most potential strain for ACC deaminase activity and solubilization of various inorganic and organic phosphates and strongly inhibited the growth of plant pathogenic fungi including *Macrophomina phaseolina, Fusarium oxysporum, F. solani*, and *Sclerotinia sclerotiorum*. The taxonomic position based on 16S rRNA gene sequencing analysis and phylogenetic studies indicated that RPN5 strain showed maximum sequence similarity with *Rhizobium leguminosarum* strain SVD31 (100%), *R. leguminosarum* strain XGL175 (100%) and *R. leguminosarum* bv. viciae strain BIHB1160 (100%) and hence, recognized as *R. leguminosarum* RPN5 (Gene bank accession number: JN180926). These findings strongly recommend that root nodulating *R. leguminosarum* RPN5 could be successfully used as an efficient bioinoculant for plant growth promotion of common bean.

Introduction

Rhizobia are soil bacteria that fix nitrogen (diazotrophy) after becoming established inside the root nodules of legumes. There are several genera of rhizobia which belong to the Rhizobiales, a probably-monophyletic group of proteobacteria. Rhizobia are perhaps the best known beneficial plant-associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium*-legume symbiosis. There are so many evidences that reveal the growth and yield enhancement of *Phaseolus vulgaris* L. (common bean) by various plant growth promoting (PGP) mechanisms due to rhizobia in different climatic and soil conditions (Neila *et al.* 2014). Rhizobia can enhance plant growth in several ways *viz.*, IAA production, nitrogen fixation, solubilization of zinc, potassium and several inorganic and organic phosphate, as well as siderophore, and ACC (1-aminocyclopropane-1-carboxylate) deaminase production (Ahmad *et al.* 2013). Rhizobia are reported to inhibit several soil-borne phytopathogenic fungi and the antagonistic activities of rhizobia are mainly due to the production of secondary metabolites such as siderophores, HCN, competition for nutrients and phytoallexin production (Ji *et al.* 2014).

Leguminosae (Fabaceae), is the second largest family of flowering plants, comprising about 750 genera and more than 20,000 species and among them only 15% have been explored for rhizobial diversity. Legumes of economic importance are widely grown in India under various agro-climatic conditions, and the presence of native rhizobia has, therefore, been anticipated. Legumes are an important source of food and feed proteins (Duranti and Guis 1997). Amongst the legumes, the *Phaseolus vulgaris* (a member of sub-family Papilionaceae of Fabaceae, and considered to be an important legume for human nutrition and a major protein and calorie source in the world (Sharon 2003).

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The aim of the present study was to isolate ACC deaminase producing root nodulating bacteria from the rhizosphere of *Phaseolus vulgaris* and to evaluate their effect on plant growth promotion.

Materials and Methods

Root nodules were collected from *Phaseolus vulgaris* by collecting plant samples from different sites of Rajma growing farmer's field of Garhwal Himalaya (Uttarkashi and Tehri), India. For the isolation of root nodulating bacteria, standard microbiological techniques were followed (Dubey and Maheshwari 2012). On the basis of preliminary investigation, seven isolates (RPN1-RPN7) were selected, and maintained on YEM agar slants at 4^{o}C for further study. The cultures were identified by following Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) and compared against standard cultures procured from Microbial Type Culture Collection Center (MTCC), IMTECH, Chandigarh, India.

Samples of diseased root parts and seeds of common bean were randomly collected and fungal pathogens *Macrophomina phaseolina* and *Fusarium oxysporum* were isolated following the water agar and blotter techniques. Besides, *M. phaseolina* and *F. oxysporum*, procured, *F. solani*, and *Sclerotinia sclerotiorum* from departmental culture collection center were also used in this study. To determine the carbon source utilization, the isolates were tested for 35 carbon sources using Himedia Carbohydrate^{TM} kit.

The isolation of genomic DNA was made according to Sambrook and Russel (2001). Amplification of 16S rRNA gene of RPN5 was carried out by PCR (PTC 100, M.J. Research, USA) using universal eubacterial primers FD1 5' CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TC AG 3' and RD1 5' CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC 3' (Weisburg *et al.* 1991). Similarity of 16S rRNA gene sequence was aligned using BLAST programme of GenBank database (NCBI).

Following Dubey and Maheshwari (2012), biochemical activities *viz.*, IAA production, phosphate solubilization, phytase activity, siderophore production, chitinase production, glucanase activities and production of oxalate oxidase etc. of the isolates were determined. All rhizobial isolates were tested for their antifungal activities against *Macrophomina phaseolina*, *Fusarium oxysporum*, *F. solani* and *S. sclerotiorum*, on agar medium following the method of Kumar *et al.* (2011).

Results and Discussion

On the basis of preliminary investigations and good survivability on YEMA plates, seven isolates were selected for detailed study. All the rhizobial isolates utilized glucose except RPN2. Only RPN4 and RPN5 utilized lactose, whereas RPN2, RPN3, RPN4 and RPN5 utilized glycerol. RPN3, RPN4 and RPN6 utilized sucrose. None of the isolates utilized inulin, salicin, glucosamine, dulcitol, melezitose, α-methyl D-mannoside, xylitol, D-arabinose, and sorbose (Data not shown). A neighbour-joining dendogram was generated using the sequence (1342 bp) of *Rhizobium* sp. RPN5 (NCBI gene bank accession number JN180926) and representative *Rhizobium leguminosarum* sequences from Gene Bank databases (NCBI). It was noticed that RPN5 aligned to the same cluster (100% bootstrap value) with *R. leguminosarum* strain SVD 31 (KC462449), *R. leguminosarum* strain XGL175 (JQ041735), *R. leguminosarum* bv. *viciae* strain BIHB (EU730590) (Fig. 1).

All the isolates of *Rhizobium* spp. (RPN1 to RPN7) were positive for IAA production except RPN6 (Table 1). All the isolates of *Rhizobium* spp. (except RPN1), formed clear halos around their spot inoculations on the Pikovskya's agar. Ability of bacteria to solubilize inorganic

phosphate may cause a decrease in pH, this resulted in the color change from blue to yellow on Pikovskaya's medium having bromothymol blue (BTB) due to acid pH. The same experiment was performed by replacing TCP in Pikovskaya's agar medium with DCP (dicalcium phosphate) and ZP. None of the isolates solubilized DCP, while three rhizobial isolates (RPN1, RPN5 and RPN7) solubilized ZP as indicated by formation of clear halo around spot inoculation (Table 1). To compare the results, in NBRIP, only three rhizobial isolates (RPN3, RPN5 and RPN6), solubilized P of TCP, while only two (RPN4 and RPN5) solubilized P of DCP. In addition, RPN3, RPN5 and RPN6 solubilized P of ZP as visible by halo zone. In rhizospheric PSM, only three rhizobial isolates (RPN2, RPN4 and RPN5) solubilized P of TCP, only two (RPN4 and RPN5) solubilized P of DCP, and only three isolate solubilized P of ZP. Production profile of phosphate solubilization was estimated with different inorganic P substrates in NBRIP broth, and it was found that RPN5 showed maximum production of phosphate solubilization with NBRIP broth having different inorganic phosphate. There was a variation in level of phosphate solubilization with variation of inorganic phosphate sources, which was 20 mg/ml with TCP, 14 mg/ml with DCP and 17 mg/ml with ZP after 7th day of incubation (Table 1). Only *R. leguminosarum* RPN5 solubilized calcium phytate by forming a halo around its spot indicating the release of free P, while none of the isolates solubilized sodium phytate (Table 1). Data has been given only as + (for presence) and – (for absence) for phosphate solubilization ability of test bacteria.

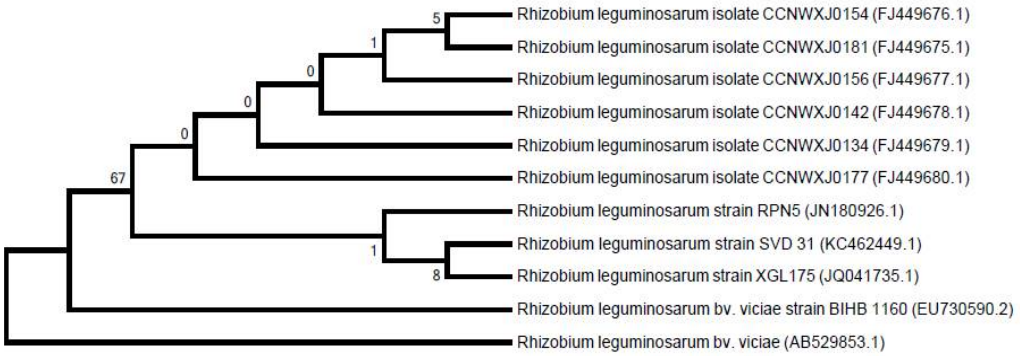

Fig. 1. Phylogenetic analysis of *R. leguminosarum* RPN5 isolate showing the relationship with other closely related bacteria based on 16S rRNA gene sequences.

When TCP was used as a substrate, almost all rhizobial isolates (except RPN3 and RPN7), exhibited a clear zone around the bacterial colonies with a shift of pink/orange colored background to white one indicating production of organic acids.

When DCP was used as a substrate, similar background changes were observed only in four rhizobial isolates (RPN1, RPN2, RPN3 and RPN5) while in case of ZP, rhizobial spp. RPN2, RPN3, RPN4 and RPN5 showed similar results. Only two of rhizobial isolates (RPN3 and RPN5) produced a visible zone on Aleksandrov agar due to solubilization of potassium along with a change in color of pH indicator on Aleksandrov agar plates. Only RPN4 and RPN5 found to solubilize zinc phosphate with a visible change in pH as indicated by change in color around bacterial spots on the medium. None of the isolates solubilized zinc carbonate (Table 2). All the isolate of *Rhizobium* spp. except (RPN1 and RPN4) produced siderophore as indicated by the formation of orange halos around their spots on the CAS agar. *R. leguminosarum* RPN5 produced 30 µg/ml siderophore. None of the isolate produced hydrogen cyanide (HCN).

Table 1. Plant growth-promoting attributes of root nodulating *Rhizobium* spp. isolated from *Phaseolus vulgaris*.

Isolates	IAA[A]	HCN[B]	Sidero-phore[C]	Phosphate solubilization[D]										
				Inorganic phosphate						Organic phosphate				
				Pikovskaya's medium			NBRI medium			Rhizospheric PS medium			Phytase production	
				TCP	DCP	ZP	TCP	DCP	ZP	TCP	DCP	ZP	Ca-Phy	Na-Phy
RPN1	+	-	-	-	-	+	-	-	-	-	-	-	-	-
RPN2	++	-	+	+	-	-	-	-	-	+	-	-	-	-
RPN3	++	-	+	+	-	-	++	-	+	-	-	-	-	-
RPN4	++	-	-	+	-	-	-	+	-	+	+	-	-	-
RPN5	+++	-	++	+++	-	++	++	++	++	++	+	++	+	-
RPN6	-	-	+	+	-	-	+	-	+	-	-	+	-	-
RPN7	++	-	+	+	-	+	-	-	-	-	-	-	-	-
Standard culture														
MTCC 99	+	-	-	+	-	-	+	-	-	+	-	-	-	-
MTCC 100	-	-	-	+	-	-	+	-	-	+	-	-	-	-
MTCC 2378	+	-	-	+	-	-	+	-	-	+	-	-	-	-

[A] -, IAA negative; +, IAA positive; [B] -, HCN negative; +, HCN positive; [C] - siderophore production negative; +, siderophore production positive; [D] -, phosphate solubilization negative; +, phosphate solubilization positive, -, absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0 cm wide surrounding colonies; NBRI-BPB medium as pH indicator; Rhizospheric BCG medium as pH indicator; TCT (Tri-calcium phosphate), DCT (Di-calcium phosphate), ZP (Zinc phosphate); *Rhizobium leguminosarum* MTCC-99; *Sinorhizobium meliloti* MTCC-100; *Mesorhizobium loti* MTCC-2378; All experiments were done in triplicate with three independent trials.

Only *R. leguminosarum* (RPN5) found to produce intracellular 1-aminocyclopropane-1-carboxylate deaminase (ACCD) whereas other isolates failed to utilize 1-aminocyclopropane-1-carboxylate (ACC) as a sole source of N in minimal medium showed their inability to produce ACCD. Quantitatively, the ACC deaminase activity was estimated for *R. leguminosarum* (RPN5) as 58 nm-α-ketobutyrate/mg/h, where **o**ne unit of enzyme activity was defined as amount of enzyme which liberates 1 µg of α-ketobutyrate per min (Table 2). All rhizobial isolates showed growth over seven successive generations on Ashby`s nitrogen free medium which indicate their ability to fix atmospheric nitrogen. All isolates were found positive for NR and NiR activities. Quantitatively, *R. leguminosarum* RPN5 was able to reduce nitrate and nitrite by 1.21 and 0.58 µM/ml/min/mg protein, respectively after 120 hrs of incubation time. Apart from indicative of presumptive N-fixation, NR and NiR activities also indicate ability of these isolates to participate in N cycle.

Table 2. Plant growth-promoting attributes of root nodulating *Rhizobium* spp. isolated from *Phaseolus vulgaris*.

Isolates	ACCD[A]	Potassium solubilization[B] (Pot. aluminium silicate)	Organic acid production[C]			Zinc solubilization[D]	
			TCP	DCP	ZP	ZP	ZC
RPN1	-	-	+	+	-	-	-
RPN2	-	-	+	+	+	-	-
RPN3	-	+	-	+	+	-	-
RPN4	-	-	+	-	+	+	-
RPN5	+	+	++	+	+	+	-
RPN6	-	-	+	-	-	-	-
RPN7	-	-	-	-	-	-	-
Standard culture							
MTCC 99	-	-	+	+	+	-	-
MTCC 100	-	-	+	+	+	-	-
MTCC 2378	-	-	+	+	+	-	-

Abbreviations: [A] -, ACCD negative, + ACCD positive; [B] - potassium solubilization negative, +, potassium solubilization positive with a drop in pH; [C] - organic acid production negative, + organic acid production positive; [D] - Zinc solubilization negative, + zinc solubilization positive with drop in pH; -, absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0 cm wide surrounding colonies; TCT (tri-calcium phosphate), DCT (di-calcium phosphate), ZP (zinc phosphate), ZC (zinc carbonate); tris-minimal zinc solubilization media and Aleksandrov's media for potassium solubilization were amended with bromo phenol blue (0.025%) as pH indicator; *Rhizobium leguminosarum* MTCC-99; *Sinorhizobium meliloti* MTCC-100; *Mesorhizobium loti* MTCC-2378. All experiments were done in triplicate with three independent trials.

Plant growth promoting attributes of root nodulating *Rhizobium* spp. are shown in Table 3. Ammonia production itself was tested and it was observed that only two isolates (RPN2 and RPN5) produced ammonia by forming yellowish precipitates in peptone broth. None of *Rhizobium* spp. showed chitinase activity. *Rhizobium* spp. RPN1, RPN2 and RPN5 were able to grow on laminarin azure amended minimal medium plates indicated β-1, 3-glucanase production. Only RPN2 and RPN5 utilized cellulose in minimal medium indicative of β-1, 4-glucanase production.

Only *R. leguminosarum* RPN5 was found to degrade oxalic acid by producing oxalate-oxidase enzyme as indicated by formation of clear halo around bacterial spot on plates containing oxalic acid degradation selective medium.

Table 3. Plant growth-promoting attributes of root nodulating (*Rhizobium* spp.) isolated from *Phaseolus vulgaris*.

Isolates	Growth on Ashby's N-free medium[A]	NiR[B]	NR[C]	Ammonia production[D]	Chitinase[E]	β-1,3-glucanase[F] (Lami-narinase)	β-1,4-glucanase[G]	Oxalate-oxidase[H]
RPN1	+	+	+	-	-	+	-	-
RPN2	-	-	-	+	-	+	+	-
RPN3	+	+	+	-	-	-	-	-
RPN4	+	-	-	-	-	-	-	-
RPN5	+	+	+	+	-	+	+	+
RPN6	+	+	+	-	-	-	-	-
RPN7	+	+	+	-	-	-	-	-
Standard culture								
MTCC 99	+	+	+	-	-	-	-	-
MTCC 100	+	+	+	-	-	-	-	-
MTCC 2378	+	-	-	-	-	-	-	-

[A] -, growth on Ashby's N-free medium negative; +, growth on Ashby's N-free medium positive; [B] -, NiR negative; +, NiR positive; [C] -, NR negative; +, NR positive; [D] -, ammonia production negative; +, Ammonia production positive; [E] -, chitinase negative; +, chitinase positive; F -, β-1,3-glucanase negative; + β-1,3-glucanase positive; G -, β-1,4-glucanase negative; + β-1,4-glucanase positive; [H] -, absence of clearing zone around bacterial; +, presence of clearing zone around bacterial spot on oxalic acid degrading agar media; *Rhizobium leguminosarum* MTCC-99; *Sinorhizobium meliloti* MTCC-100; *Mesorhizobium loti* MTCC-2378. All experiments were done in triplicate with three independent trials.

The maximum percentage radial growth inhibition was caused by *R. leguminosarum* RPN5 against *M. phaseolina* (54.00 ± 0.63, 44.11 ± 0.66), *F. oxysporum* (53.96 ± 0.65, 51.00 ± 0.51), *F. solani* (55.17 ± 0.58, 43.27 ± 0.61) and *S. sclerotiorum* (50.39 ± 0.64, 45.59 ± 0.53) after 7 days of incubation in both dual culture and cell-free culture filtrate (Table 4).

Table 4. Antagonistic activities of *R. leguminosarum* RPN5 against fungal phytopathogens.

Fungal pathogens	Growth inhibition (%) by *R. leguminosarum* RPN5	
	Dual culture	Cell free culture filtrate
M. phaseolina	54.00 ± 0.63	44.11 ± 0.66
F. oxysporum	53.96 ± 0.65	51.00 ± 0.51
F. solani	55.17 ± 0.58	43.27 ± 0.61
S. sclerotiorum	50.39 ± 0.64	45.59 ± 0.53

On the basis of phenotypic characters and 16S rRNA gene sequencing RPN5 was identified as *R. leguminosarum* RPN5. In our study, all the isolates (except RPN6) produced IAA either in presence or absence of tryptophan, a precursor of IAA, where, RPN5 showed maximum IAA

production in both condition i.e. either in presence or absence of tryptophan. IAA production is more common in rhizobia. Previously several workers have observed direct involvement of rhizobial IAA in plant growth-promotion (Ji *et al.* 2014, Kaur *et al*. 2015).

The precipitated inorganic phosphate is solubilized by the action of mineral and organic acids produced by bacteria (Henri *et al*. 2008). Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition (Khan *et al.* 2009).

The liquid medium of *R. leguminosarum* RPN5 showed decrease in the pH with enhancement of phosphate solubilization by producing organic acid. There are several findings that reveal the phosphate solubilization properties of rhizobia (Ji *et al.* 2014). The mineralization of calcium phytate by RPN5 was carried out by means of the action of phytase. Alikhani *et al* (2006) also isolated several rhizobia from Iranian soils having ability to dissolve inorganic and organic phosphates, confirmed by the drop in pH of the culture filtrate with the release of soluble phosphate which indicates the importance of organic acid production. Recently, Kumar *et al.* (2011) also isolated three phytase producing bacteria and reported for their P solubilization abilities on solid media and NBRIP broth (193-642 µg/ml), which was similar to present findings.

Seventy one percent *Rhizobium* spp., calculated by the formula (100 × number of siderophore producing bacteria divided by total number of selected rhizobial isolates) produced siderophore. Various strains of rhizobia have been reported to produce a wide range of siderophores such as rhizobactin, citrate, catechol, and anthranalate under iron-deficient conditions (Ji *et al.* 2014). In this study, *R. leguminosarum* RPN5 produced hydroxamate type of siderophore. Only RPN4 and RPN5 of *Rhizobium* spp. solubilized zinc phosphate. Iqbal *et al.* (2010) observed that some plant growth promoting rhizobacteria (PGPR) have the ability to enhance mung bean plant growth in the presence of water insoluble zinc phosphate, and could be utilized to improve the growth of economically important cash crops. ACC deaminase containing PGPR usually give very consistent plant growth and yield, and thus are considered good candidates for bio-fertilizer formulation (Duan *et al.* 2009). In our study, only *R. leguminosarum* RPN5 produced significant amount of ACC deaminase enzyme and improved the growth and yield of common bean. In our study, only 28% *Rhizobium* spp., produced ammonia by forming yellowish precipitate in peptone broth (Joseph *et al.* 2007). *R. leguminosarum* RPN5, potentially inhibited radial growth of *M. phaseolina, F. oxysporum, F. solani*, and *S. sclerotiorum* which might be due to production of antimicrobial substances, such as chitinolytic enzymes, laminarinase, cellulase, oxalate-oxidase enzyme HCN, antibiotics, siderophore and nutrient competition (Kumar *et al.* 2011, Ji *et al.* 2014).

Rhizobium leguminosarum RPN5 showed varied plant growth promoting properties and induced fungal resistance against soil pathogenic fungi confirming that RPN5 could be well utilized as an efficient bioinoculant for sustainable agro-productivity of common bean.

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References

Ahmad E, Khan MS and Zaidi A 2013. ACC deaminase producing *Pseudomonas putida* strain PSE3 and *Rhizobium leguminosarum* strain RP2 in synergism improves growth, nodulation and yield of pea grown in alluvial soils. Symbiosis **61**: 93-104.

Alikhani HA, Saleh-Rastin N and Antoun H 2006. Phosphate solubilization activity of rhizobia native to Iranian soils. Plant Soil **287**: 3-41.

Duan J, Muller KM, Charles TC, Vesely S and Glick BR 2009. 1-Aminocyclopropane-1-carboxylate (ACC) deaminase genes in Rhizobia from southern Saskatchewan. Microb. Ecol. **57**: 423-436.

Dubey RC and Maheshwari DK 2012. Practical Microbiology. S. Chand and Co., New Delhi, India.

Duranti M and Guis C 1997. Legume seeds: protein content and nutritional value. Field Crops Res. **53**: 31-45.

Henri F, Laurette NN, Annette D, John Q, Wolfgang M, François-Xavier E and Dieudonné N 2008. Solubilization of inorganic phosphates and plant growth promotion by strains of Pseudomonas fluorescens isolated from acidic soils of Cameroon. African J. Microbiol. Res. **2**: 171-178.

Holt JG, Krieg NR, Sneath PHA, Staley JT and Williams ST 1994. In: Bergey's Manual of Determinative Bacteriology. Williams and Wilkins Press, Baltimore, USA.

Iqbal U, Jamil N, Ali I and Hasnain S 2010. Effect of zinc-phosphate-solubilizing bacterial isolates on growth of *Vigna radiata*. Ann. Microbiol. **60**: 243-248.

Ji SH, Gururani MA and Chun SC 2014. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol. Res. **169**: 83-98.

Joseph B, Patra R and Lawrence R 2007. Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). Int. J. Plant Prod. **1**: 141-151.

Kaur J, Khanna V Kumari P and Sharma R 2015. Influence of psychrotolerant plant growth promoting rhizobacteria (PGPR) as coinoculants with Rhizobium on growth parameters and yield of lentil (*Lens culinaris* Medikus). African J. Microbiol. Res. **9**: 258-264.

Khan AA, Jilani G, Akhtar MS, Muhammad S, Naqvi S and Rasheed M 2009. Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. J. Agri. Biol. Sci. **1**: 48-58.

Kumar H, Dubey RC and Maheshwari DK 2011. Effect of plant growth promoting rhizobia on seed germination, growth promotion and suppression of *Fusarium* wilt of fenugreek (*Trigonella foenum-graecum* L.) Crop Prot. **30**: 1396-1403.

Neila A, Adnane B, Mustapha F, Manel B, Imen H, Boulbaba L, Cherki G and Bouaziz S 2014. *Phaseolus vulgaris*-rhizobia symbiosis increases the phosphorus uptake and symbiotic N_2 fixation under insoluble phosphorus. J. Plant Nutri. **37**: 643-65.

Sambrook J, Russel D 2001. Molecular cloning: A laboratory manual. Cold spring harbor laboratory press, cold Spring Harbor NY, USA.

Schwyn B and Neilands J 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**: 47-56.

Sharon DA 2003. Dry bean production guide. Ist Edn, North Dakota State University, Fargo North Dakota.

Weisburg WG, Barns SM, Pelletier DA and Lane DJ 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**: 697-703.

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