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MOLECULAR CHARACTERIZATION OF SOIL BACTERIA FOR IMPROVING CROP YIELD IN PAKISTAN

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Abstract

Nine Gram - positive bacterial strains designated as RH-1 to RH-9 were isolated from legumes rhizospheric soil and characterized for plant growth promoting (PGP) activities in legume and cereal crops as well as detailed morphological, phenotypic and biochemical studies. The strains were identified using 16S rRNA gene sequencing and chemotaxonomic traits. Based on comparative analysis of 16S rRNA gene sequencing, the strains showed highest similarity (97.9–99.8%) to the genus *Bacillus*. *In vitro* growth promoting assay indicated that the strain RH-5 proved potential PGPR by solubilizing highest amount of inorganic P (141 µg mL^{-1}), positive for *nif*H gene (+) and produced indole acetic acid (IAA; 0.82 µg mL^{-1}) followed by strain RH-2. Phosphorus solubilization in broth culture was associated with significant drop in pH by the strain RH-5 from 7.0 to 4.8. These two strains were further evaluated for plant growth promotion in beans (mung bean; *Vigna radiate*, mash bean; *Vigna mungo*) and wheat (*Triticum aestivium*) under axenic condition. The strain RH-5 proved the best PGPR by increasing crops biomass and grains (wheat; 40-45%, beans; 50%) over un-inoculated control. The chemotaxonomic data (pre dominant menaquinone system: MK-7, DNA G + C content: 40-41 mol%, major cellular fatty acid: anteiso-$C_{15:0}$) also supported the affiliation of both strains to the genus *Bacillus*. Phenotypic characterization showed that the strain RH-5 tolerated temperature: 50°C, pH: 10 and NaCl: 9%. Phylogenic and genotypic analysis showed the association of strains RH-5 and RH-2 with genus *Bacillus* having single phylitic lines to *Bacilus gibsonii and Bacillus subtilis*.

Introduction

Soil bacteria play important role in crop production and have been used for sustainable agricultural development. Their functions involve different direct and indirect mechanisms by synthesizing growth promoting hormones, facilitating the uptake of mineral nutrients from crop rhizosphere and preventing crops from pathogens and diseases (Döbbelaere *et al.,* 2003; Hayat *et al.,* 2010; Çakmakçi *et al.,* 2006). These beneficial soil bacteria are classified as plant growth promoting rhizobacteria (PGPR) or plant health promoting rhizobacteria (PHPR) or nodule promoting rhizobacteria (NPR) (Hayat *et al.,* 2010). Soil bacteria enhance resistance to variety of agriculture stresses including drought (Al Varez *et al.,* 1996), water logging and salinity (Saleem *et al.,* 2007), and oxidative stress (Stajner *et al.,* 1997; Khan *et al.,* 2013). Soil bacteria are considered important biological agents to improve soil physical conditions, retaining more essential nutrient elements in crop rhizosphere and increasing their availability through reducing the external inorganic inputs (Kennedy *et al.,* 2004).

Bacillus, a Gram positive, aerobic or facultative endospore forming motile bacteria belongs to family *Bacillaceae* (Claus & Berkeley 1986). Bacillus strains have wide genetic heterogeneity in term of DNA G + C composition which ranges from 32-69% of known *Bacillus* spp. Bacillus can resist and survive in a variety of environmental stresses and adverse conditions and considered as very important microbiota due to its diverse ecophysiology, direct and indirect functions such as N_2 fixer (*Bacillus megaterium*; Liu *et al.,* 2006), denitrifiers (*Bacillus azotoformans*; Suharti *et al.,* 2004), P-solubolizers (*Bacillus polymyxa;* Rodríguez & Fraga 1999), insect pathogens (*Bacillus thuringiensis*; Roh *et al.,* 2007), antibiotic (*Bacillus mojavensis*; Bacon & Hinton, 2002) and phytohormones (*Bacillus subtilis*; Araujo *et al.,* 2005) producers. Thus they are considered as potential candidate for biofertilizer biotechnology (Hayat *et al*., 2013). The genus *Bacillus* contained 250 species and 7 subspecies till date (Euzéby: www.bacterio.cict.fr/b/bacillus.htm) with a variety of distinctive characteristics.

Variety of molecular techniques like whole cell protein analysis; detection of specific DNA sequences by hybridization restriction digestion and sequence comparison (Yap *et al.,* 1996) have been used for assessing the diversity among soil bacteria (Head *et al.,* 1998). The rRNA genes are allied component of DNA of all organisms and 16S rRNA is specifically highly conserved in bacteria and being used as biological marker for identification of species, genus and families. Using 16S rDNA sequence analysis, soil bacteria can be phylogenetically classified and reclassified into new genera (Olsen & Woese 1993). Next to molecular markers, chemotaxonomic features are considered essential for correct identification at genus level. Chemotaxonomic assay deals with low molecular weight components of bacterial cell involving cell wall composition, fatty acid, menaquinone and polar lipids etc. These traits may be variable within the genus and family (Kämpfer, 2011).

Pakistani soils are deficient in plant available nutrients (Rashid *et al.,* 1997). Inorganic nutrients (N, P, K, Zn etc) represent a major input cost for crop

production in the country. There is a need and interest in using soil bacteria as bio-inoculants to mobilize the indigenous nutrients from soil. The potential for development of bacterial inoculants exist in Pakistan but their country wide application remains limited as no single biofertilizer is registered in the country. This reflects the poor understanding and knowledge of bacterial validation from rich ecology of Pakistan. Efforts are needed to validate the soil beneficial bacteria based upon molecular tagging (DNA sequence) and use as biofertilizers for sustainable crop production. The objective of this study was to characterize soil bacteria based upon 16SrRNA gene sequencing and chemotaxonomic traits for enhancing crops yield.

Materials and Methods

Isolation, screening and phenotypic characterization of soil bacteria: Nine bacterial strains RH-1 to RH-9 were isolated from the rhizospheric soil of legumes [lentil (*Lens culinaris*), mash bean (*Vigna mungo*) and groundnut (*Arachis hypogaea*)] grown at research farm of the department of Soil Science & SWC, PMAS-Arid Agriculture University Rawalpindi, Pakistan. The soil of the experimental field (33° 38′ 48″ N, 73° 04′ 59″ E) was sandy clay loam and belonged to Rawalpindi soil series (week medium and coarse sun angular blocky with nearly continuous thin cutans, Typic Ustocrepts, Eutric Cambisols). The pH of the soil was 7.5 with 3.92 mg kg^{-1} NO_3-N and 6.60 mg kg^{-1} of P. The strains were isolated by using dilution plate technique where phosphate buffered saline (PBS) was used as saline solution. The bacteria were grown on Tryptic Soya Agar (TSA; Difco) medium and incubated at 28°C for at least 72 hours. After bacterial growth, individual colonies were picked and streaked on plates containing TSA medium for purification and screening. Single colonies were re-streaked on TSA medium until purified cultures were obtained and preserved in 35% glycerol (w/v) at -80°C and subjected to characterization. Light microscope (Olympus BX 60) was used to examine cell shape, size and motility. The range and optimum pH required for bacterial growth were determined by inoculating strains in Tryptic Soya Broth (TSB; Difco) adjusted to a range of pH 4.0 to 10.0 at an increment of 1 pH. For NaCl tolerance, the strains were inoculated in TSB with different NaCl concentrations from 0 to 10% (w/v) at optimum pH. During pH and NaCl optimization, the growth was assayed by measuring the absorbance on spectrophotometer at 600 nm after 12 and 24 h. Temperature optimization was carried out by streaking bacterial strains on TSA plates adjusted with optimum pH and NaCl (%) and incubated at different temperatures from 4, 10, 16, 22, 28, 32, 37, 45 and 50°C. The ability of the isolates to utilize particular substrate was assessed using AP 20E galleries (bioMérieux, France). Oxidase and catalase activities were determined using Oxidase Kit (bioMérieux) and following the procedure described by Cowan & Steel (2004), respectively. All of these commercial kits were used according to the manufacturer's protocol.

Plant growth promoting assay: Bacterial strains were evaluated for their capacity of indole acetic acid **(**IAA) production, phosphorous solubilzation and *nif*H gene amplification (nitrogen fixation). For IAA production, bacterial cultures were grown for 24 h on tryptic soya broth (TSB) at 28±2°C. Bacterial suspension (100 µL each) was inoculated in 5ml Luria Broth (LB medium) with (500 µg ml^{-1}) or without adding tryptophan. Bacterial cultures were placed for 48 h on incubating shaker at 28±2°C. The cultures were harvested by centrifuge at 10000 rpm for 10 min. Two drops of Orthophosphoric acid and 4ml of the Salkowski reagents (50ml, 35% of perchloric acid, 1ml 0.5M $FeCl_3$ solution) were added in 2 ml supernatant. Development of pink color indicated IAA production. Optical density was recorded using spectrophotometer at 530 nm. Concentrations of IAA produced by strains were measured with the help of standard curve graph and standards range was up to 10µg ml^{-1} (Brick *et al.,* 1991). The potential of the strains phosphorus solubility was determined both qualitatively as well as quantitatively. Qualitative analysis of P-solubility by strains was performed by measuring the halo zone around bacterial growth on Pikovskaya (PKV) agar medium (Pikovskaya, 1948; Gaur, 1990). To determine the quantitative P-solubilization capacity of bacteria, the broth or liquid PKV medium was prepared and 100 ml was dispensed in each 250 ml conical flask. To each flask, 5 g L^{-1} insoluble phosphate in the form of tricalcium phosphate was added. The pH of medium was recorded and the medium were sterilized at 121°C for 15 min. The medium was inoculated with 500 µL bacterial suspension and placed on shaker for 8 days at 30°C. The pH of the medium was recorded after 8 days. Each culture was harvested by centrifuge at 8500 rpm for 25 min. The available phosphorus in supernatant of each culture was determined by method of Watanabe & Olsen (1965). Optical density was taken at 700 nm and concentration of P-solubilized by strains was measured with the help of standard curve graph and standards range was up to 1 µg ml^{-1}. The *nif H* gene was amplified to check the nitrogen fixing ability of bacterial strains after PCR amplification of the genes using universal forward $PolF^b$(TGC GAY CCS AAR GCB GAC TC) and reverse primer $PolR^b$ (ATS GCC ATC ATY TCR CCG GA).

Identification of bacterial strains using 16SrRNA gene sequencing: The bacterial strains were identified using standard method of 16S rRNA gene sequencing. DNA template was prepared by picking individual colony of each strain and amplification of 16S rRNA gene was carried out by PCR. PCR amplification of DNA was done using universal primers: 9F (5́-GAGTTTGATCCTGGCTCAG-3́) and 1510R (5́-GGCTACCTTGTTACGA-3́). Reaction mixture (25 µL), prepared for full-length 16S rRNA gene amplification was initially denatured at 94°C for 2 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min; primer annealing at 52°C for 1:30 min. and primer extension at 72°C for 2 min and finally extension at 72°C for 10 min in a thermocycler. Amplified PCR products of 16S ribosomal gene were separated on 1% agarose gel in 0.5X TE (Tris-EDTA) buffer containing 2 µL ethidium bromide (20 mg/mL). λ Hind-III ladder was used as a size marker. The gel was viewed under UV light and photographed using gel documentation system. Amplified PCR products of full-

length 16S rRNA genes were purified using PCR purification kit (QIAGEN) according to the standard protocol recommended by the manufacturer. The purified PCR product samples were sequenced using DNA sequencing service of MACROGEN, Korea (http://dna.macrogen.com/eng) using universal 16Sr RNA gene sequencing primers. The sequence results were blast through NCBI/Eztaxon (Chun *et al.,* 2007) and sequence of all the related species were retrieved to get the exact nomenclature of the isolates. Phylogenetic analyses were performed using bioinformatics software MEGA-5 (Tamura *et al.,* 2007). Other software used for sequence alignment and comparisons were CLUSTAL X and BioEdit. DNA accession numbers of each strain were obtained from DNA Data Bank of Japan (DDBJ).

Wheat and beans inoculation: Pot experiments were carried out in the glasshouse to investigate the beneficial effects of two potential bacterial strains (on the basis of PGP tests) in wheat (*Triticum aestivium*) and beans (mung bean; *Vigna radiate*, mash bean; *Vigna mungo*) regarding growth and yield during summer and winter seasons of 2010. Prior to sowing, pots were filled with 5 Kg of autoclaved soil. Seeds of each crop were sown in each pot. After germination, the plants were thinned to 2 seedlings per pot. The crops were treated with inoculums of two different strains. The control was considered un-inoculated plant. The experiment was laid down in completely randomized design with five replications. To evaluate the response of bacterial strains, growth parameters (plant height, spike length (wheat only), shoot dry weight and grain weight per plant) were observed at the time of maturity. Data were statistically analyzed through ANOVA and means were compared by LSD with significance levels of ≤0.05 (Steel *et al.,* 1997).

Chemotaxonomic characterization: Two potential strains were also characterized by chemotaxonomic traits i.e., fatty acid profile, menaquinones system and DNA G + C contents. Cellular fatty acid composition was determined with Sherlock Microbial Identification system (MIDI). Fatty acid methyl esters were prepared from biomass grown at 28°C on TSA (Difco) for 72 h. Extraction was performed according to Sasser, (1990). Fatty acid methyl esters were analyzed by a Hewlett Packard 5890 series II gas chromatograph equipped with Ultra2 capillary column. Cellular fatty acid were identified by comparing the equivalent chain length (ECL) of each compound to a peak naming table that contains over 115 known standards. The quantity of each compound was determined as a percentage of fatty acid compounds present within bacterium. For isoprenoid quinones, dried cells (200 mg) were added in 20 ml chloroform-methanol (2:1 v/v) and stirred with stirrer bar for overnight at room temperature. The suspension was filtered and evaporated in vacuo. Quinones were extracted with 5 ml of acetone and evaporated in vacuo. The acetone fraction solved in 200 µl of ethyl alcohol. The extraction was developed with TLC plates (silica gel 60 F254, Merck, Darmstadt, Germany) in toluene. Quinone spots were detected under UV light at 275 nm. The spots were scrapped off and extracted with acetone. After the extraction was dried with a flow of N_2 gas, 100 µl of ethyl alcohol was added. The quinone solution was analyzed with HPLC, (model LC-10AD HPLC apparatus; Shimadzu, Japan) equipped with Cosmosil 5Ci8R column (Nacalin). Moreover, LC-MS (LCMS-QP-8000a, Shimadzu) was performed to detect saturation side chain. Cultures of two strains were grown at 28°C on TSA (Difco) for 72 hours for the determination of DNA G + C contents. DNA was extracted using QIAGEN Genomic tips as described by manufacturer (Qiagen, Germany). DNA G+C content was analyzed according to the method described by Mesbah *et al.,* (1989) using HPLC, (model LC-10Ad VP; Shimadzu, Japan) under the following condition: column, Cosmil 5C18R (Nacalai); mobile phase. 0.2 M ammonium phosphate: acetonitorile (40:1); column temperature, 40°C.

Results

Plant growth promoting activities: The results of PGP experiments of all bacterial strains are shown in Table 1. IAA produced by strains in broth culture ranged from 0.40 to 0.82 µg ml^{-1} with and without tryptophan. The inorganic P-solubilization by the strains ranged from 26.85 to 141.00 µg ml^{-1}. Significant drop in pH of broth medium was observed at harvest of the strains in P-solubilization experiment by different strains. Results indicated that strain RH-5 possess three tested PGP traits i.e. production of IAA, solubilization of insoluble tricalcium phosphate and *nif*H positive. All strains solubilized substantial quantity of inorganic phosphorus, however, maximum phosphate solubilization (141 µg ml^{-1}) was observed by the inoculation of RH-5 followed by RH-2 (117.19 µg ml^{-1}). The maximum pH (upto 4.83 from an initial pH level of 7.0 during eight days of incubation) was dropped by strain RH-5 with highest amount of tri-calcium phosphate solubilization. Interestingly, strong negative correlation (r = -0.655, p≥0.05) was observed between phosphorus solubilization and decrease in pH. Two strains produced IAA with and without the addition of tryptophan in a similar manner and the quantity produced were lower and ranged from 0.67- 0.90 µg ml^{-1}. Nearly 400bp *nif*H gene amplification was only observed in strain RH-5 while the *nif*H gene was absent in all others strains. Based on PGP test results, two strains RH-2 and RH-5were selected as potential bacterial strains for further characterization.

Biochemical and phenotypic characterization: Biochemically all strains were motile rods, Gram positive but variable in oxidase, catalase, urease, indole and H_2S production etc (Table 2). Morphologically both potential strains RH-2 and RH-5 were different species and phase contrast microscopy confirmed short chain motile rods, and central ellipsoidal spores (Fig. 1). Phenotypic characteristics like pH, salinity and temperature of both bacterial strains were also observed (Table 3). Optimum temperature for growth of RH-2 was 32°C with a range of 10-37°C. Strain RH-2 tolerated NaCl up to 8% (optimum 2-4%) with a pH range of 6-10 (optimum 8). Strain RH-5 was found thermo tolerant in nature as it can grow between 16-50°C with a wide optimum temperature range of 28-37°C. Strain RH-5 was also able to grow in pH ranging from 5-10 (optimum pH 7).

Table 1. Plant growth promoting traits of bacterial strains.

Strains ID	Source (Rhizospheric soil)	P-solubilization		IAA mg L^{-1} with tryptophan	IAA mg L^{-1} without tryptophan	*nif*H gene
		(µg mL^{-1}) ± S.E	pH*	(µg mL^{-1}) ± S.E	(µg mL^{-1}) ± S.E	
Control	(Un-inoculated)	12.06 ± 2.2	6.46			
RH-1	Lentil (*Lens culinaris*)	31.95 ± 2.5	4.37	0.80 ± 0.92	0.62 ± 0.42	-
RH-2	Lentil (*Lens culinaris*)	117.19 ± 2.8	6.43	0.90 ± 1.04	0.67 ± 0.64	-
RH-3	Mash bean (*Vigna mungo*)	26.85 ± 1.7	4.89	0.58 ± 0.86	0.49 ± 0.04	-
RH-4	Mash bean (*Vigna mungo*)	28.52 ± 1.5	4.95	0.54 ± 0.21	0.45 ± 0.08	-
RH-5	Mash bean (*Vigna mungo*)	141.00 ± 1.7	4.83	0.82 ± 0.04	0.75 ± 0.40	+
RH-6	Groundnut (*Arachis hypogaea*)	24.23 ± 4.7	6.64	0.76 ± 0.82	0.64 ± 0.92	-
RH-7	Groundnut (*Arachis hypogaea*)	85.30 ± 3.6	6.32	0.56 ± 1.03	0.44 ± 1.02	-
RH-8	Groundnut (*Arachis hypogaea*)	22.46 ± 1.8	6.84	0.48 ± 1.22	0.40 ± 0.96	-
RH-9	Groundnut (*Arachis hypogaea*)	37.64 ± 1.7	5.34	0.64 ± 1.24	0.54 ± 0.98	-

All values are average of three replicates. * Initial pH 7.0

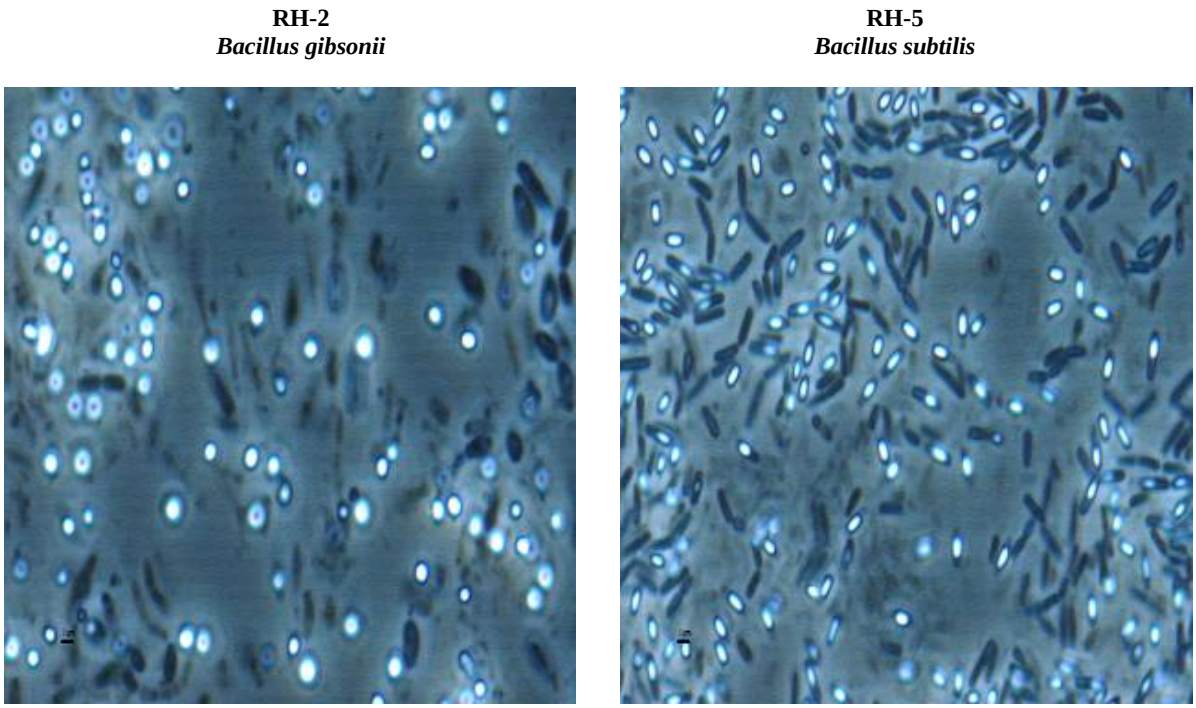

Fig. 1. Phase-contrast micrograph showing endospores of strains. Bar, 10 mm.

Molecular identification based on 16S rRNA gene sequence: Identification of bacterial strains based on 16S rRNA gene sequences is shown in Table 3. The sequence analysis of 16S rRNA gene placed the strains in genus *Bacillus*. BLAST search results through NCBI and Eztaxon server showed highest (99.8%) similarity of RH-2 with *Bacillus gibsonii* X76446. Other closely related species of RH-2 identified by BLAST search are *Bacillus plakortidies* AJ880003 (99.4%), *Bacillus murimartini* AJ316316 (99.2%) and *Bacillus clausii* AP006627 (99.2%) etc. Similarly, the highest sequence similarity of RH-5 matched with *Bacillus subtilis* subsp. *inaquosorum* EU138467 (99.6%), followed by *Brevibacterium halotolerans* AJ620368 (98.9%), *Bacillus subtilis* subsp. *spizizenii* AF074970 (98.8%), *Bacillus mojavensis* AB021191 (98.7%), and *Bacillus tequilensis* HQ223107 (98.603%) etc. The gene sequences of all bacterial strains were submitted to DNA Data Bank of Japan (DDBJ GenBank) for accession numbers (Table 3). Figure 2 reveals a phylogenic tree among isolated strains and sequences of some closely related species obtained from Eztaxon. In phylogenetic tree, RH-2 and RH-5 are closely homologous having single phylitic line to *Bacillus gibsonii* and *Bacillus subtilis,* respectively. The sequence similarity of RH-1 (97.97%) with closely related species retrieved from databases and its phylogenetic analysis showed the strain as a candidate novel species which will be validated by further studies in adopting the minimal standard required for the validation of new taxa (Logan *et al.,* 2009).

Table 2. Morphological, biochemical and chemotaxonomic comparisons of bacterial strains.

Characters	RH-1	RH-2	RH-3	RH-4	RH-5	RH-6	RH-7	RH-8	RH-9
Form	punctiform	circular	filamentous	filamentous	filamentous	circular	irregular	filamentous	circular
Margin	entire	entire	filamentous	filamentous	erose	entire	entire	erose	entire
Surface	smooth/dull	smooth/ shiny	rough/ dull	rough/ dull	rough/dull	smooth/ shiny	smooth	rough/dull	smooth
Elevation	flat	raised	umbonate	umbonate	umbonate	flat	raised	umbonate	flat
Capacity	opaque	opaque	translucent	translucent	opaque	opaque	opaque	opaque	opaque
Color	off white	yellow	light yellow	light yellow	light yellow	off white	white	Off white	yellow
Gram reactions	+	+	+	+	+	+	+	+	+
Oxidase	w	-	+	-	+		+	+	+
Catalase	+	+	-	-	+		+	+	
2-nitrophenyl-ß Dgalactopyranoside	-	+	-	-	+	-	-	-	-
L-arginine	-	-	-	+	-	+	+	-	+
L-lysine	-	-	+	+	-	+	-	-	+
L-ornithine	-	-	+	+	-	+	-	-	+
Trisodium citrate	-	-	+	+	-	+	+	+	+
H_2S production	-	-	+	+	-	-	-	-	-
Urease	-	-	+	+	-	+	+	-	-
Tryptophane Deaminase	-	-	-	-	-	-	-	-	+
Indole production	-	-	-	-	-	-	-	-	+
Acetoin production	-	-	-	-	+	-	-	-	-
Gelatinase	-	-	+	+	+	-	+	+	+
D-glucose	+	+	-	+	+	-	+	-	-
D-mannitol	-	+	+	-	-	-	+	-	+
Inositol	+	+	+	-	-	+	+	-	+
D-sorbitol	+	+	+	+	+	-	+	-	+
L-rhamnose	+	-	-	+	-	-	+	-	-
D-sucrose	-	-	+	+	-	+	+	+	+
D-melibiose	+	-	+	+	-	-	-	+	-
Amygdalin	-	-	-	-	-	+	-	-	-
L-arabinose	-	+	+	+	+	+	-	+	+
NO_2 production	-	+	-	-	-	+	+	-	-
Reduction to N_2 gas	+	-	+	+	+	+	_	_	+
Menaquinone system	nd	MK-7	nd	nd	MK-7	nd	nd	nd	nd
Major fatty acid	nd	anteiso-$c_{15:0}$	nd	nd	anteiso-$c_{15:0}$	nd	nd	nd	nd
G+C content (mol%)	nd	40	nd	nd	41	nd	nd	nd	nd

Effect of inoculation with RH-2 and RH-5 on growth of beans and wheat: Inoculation with bacterial strains in pots under axenic conditions improved wheat plant height, dry shoot weight, spike length and grain weight per plant as compared with un-inoculated control plants (Fig. 3). Strain RH-5 proved the best among two by producing maximum wheat plant height (77 cm), spike length (8.86 cm), shoot dry weight (4.42 g plant^{-1}) and grain weight (6.17 g plant^{-1}). Significantly higher growth and grains were observed by inoculating the beans in sterile soil with RH-5 as compared to non-inoculated sterile control (Fig. 4).

Chemotaxonomic identification: The cellular fatty acid composition of the two strains was analyzed by MIDI system equipped with gas chromatograph. The major fatty acid observed were anteiso-$c_{15:0}$ 42.53±0.01 in RH-2 and 39.14±0.01 in RH-5 followed by iso-$c_{15:0}$ 27.54±0.00 in RH-2 and 17.04±0.02 in RH-5, respectively (Table 4). Others minor components were anteiso-$c_{17:0}$ (6.30±0.01; 14.85±0.01) and iso-$c_{17:0}$ (5.28±0.00; 12.38±0.03) in RH-2 and RH-5, respectively. Respiratory quinone system analyzed in both bacterial strains was MK-7 (>90%). The G+C contents determined by HPLC were 40 and 41 mol% in RH-2 and RH-5, respectively.

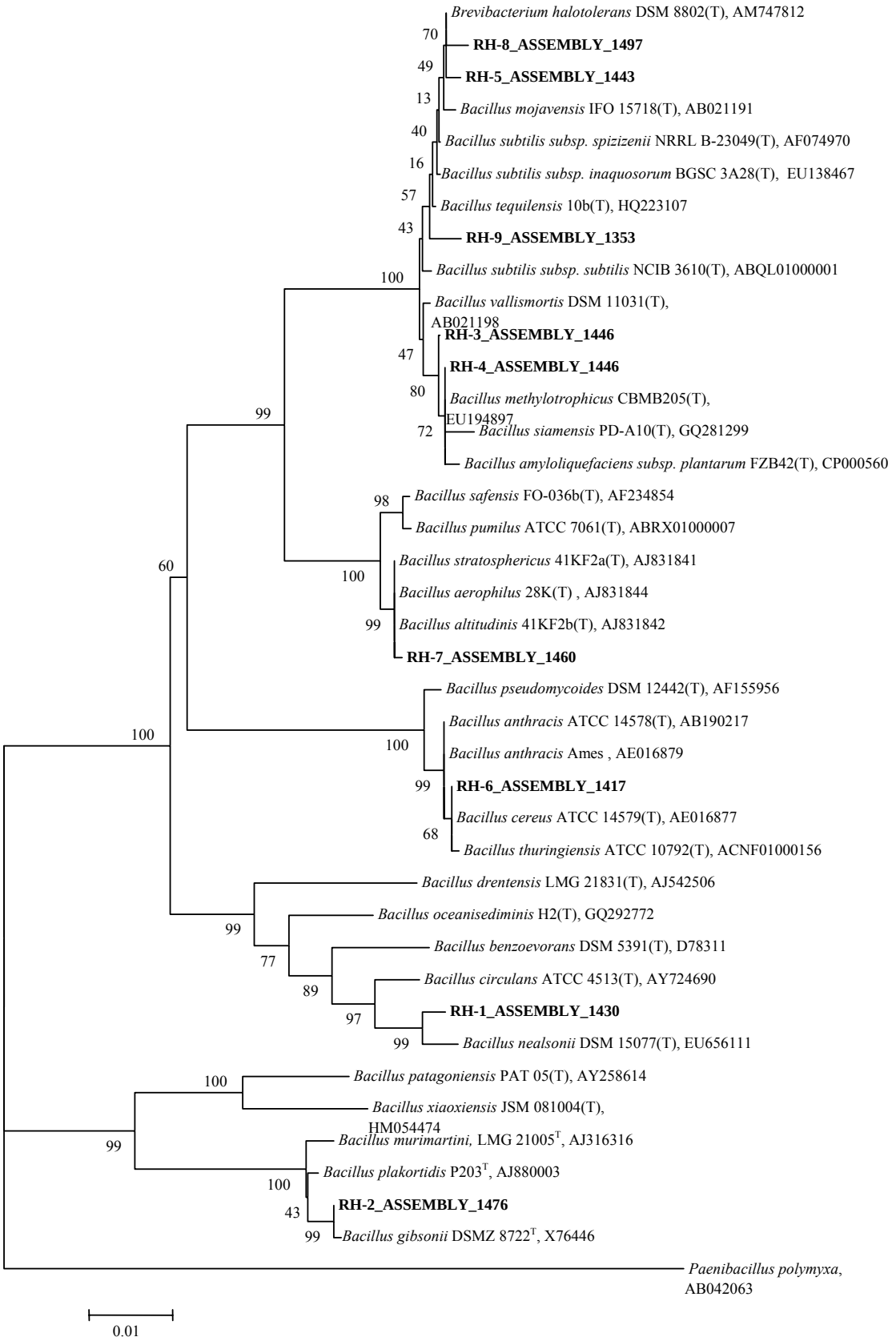

Fig 2. Neighbor-joining phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequences of the gram-positive representative isolates and some of their closest phylogenetic taxa.

Table 3. Phenotypic characterization and identification of bacterial strains.

Strain ID	Source of isolation Rhizospheric soil	Strain Name/ Genus	16S rRNA gene (ntd)	DDBJ Accession number for 16S rRNA gene sequence of the isolated strains	pH range (optimum) for growth	Temp. range (optimum) for growth (C°)	NaCl tolerance (%)	Closely related taxa identified by BLAST search using EzTaxon Server (http://147.47.212.35:8080/)		
								Species/ Strain	DDBJ accession of 16S rRNA gene sequence	Highest similarity of 16SrRNA gene sequence (%)
RH-1	Lentil (*Lens culinaris*)	*Bacillus*	1430	AB547220	6-10 (6-7)	20-60 (35)	0-5 (0)	*Bacillus nealsonii* DSM 15077(T)	EU656111	97.971
RH-2	Lentil (*Lens culinaris*)	*Bacillus*	1476	AB665727	6-10 (8)	10-37 (32)	0-8 (2-4)	*Bacillus gibsonii* DSMZ 8722^T	X76446	99.79
RH-3	Mash bean (*Vigna mungo*)	*Bacillus*	**1446**	AB547228	5-10 (7)	16-50 (28-37)	0-9 (0)	*Bacillus subtilis* subsp. *inaquosorum* BGSC 3A28(T)	EU138467	99.572
RH-4	Mash bean (*Vigna mungo*)	*Bacillus*	**1446**	AB547229	6-10 (7)	10-40 (30)	0-8 (0)	*Bacillus methylotrophicus* CBMB205(T)	EU194897	99.791
RH-5	Mash bean (*Vigna mungo*)	*Bacillus*	**1443**	AB665172	5-10 (7)	16-50 (28-37)	0-9 (0)	*Bacillus subtilis* subsp. *inaquosorum* BGSC 3A28(T)	EU138467	99.658
RH-6	Groundnut (*Arachis hypogaea*)	*Bacillus*	1417	AB678448	6-10 (7)	10-30 (28)	0-5 (0)	*Bacillus anthracis* ATCC 14578(T)	AB190217	98.391
RH-7	Groundnut (*Arachis hypogaea*)	*Bacillus*	1460	AB678449	5-10 (7)	10-30 (28)	0-10 (0-5)	*Bacillus stratosphericus* 41KF2a(T)	AJ831841	99.587
RH-8	Groundnut (*Arachis hypogaea*)	*Bacillus*	1497	AB678450	6-10 (7)	10-30 (28)	0-5 (0)	*Bacillus subtilis* subsp. *spizizenii* NRRL B-23049(T)	AF074970	99.716
RH-9	Groundnut (*Arachis hypogaea*)	*Bacillus*	1353	AB678451	6-10 (7)	10-30 (28)	0-5 (0)	*Bacillus tequilensis* 10b(T)	HQ223107	99.627

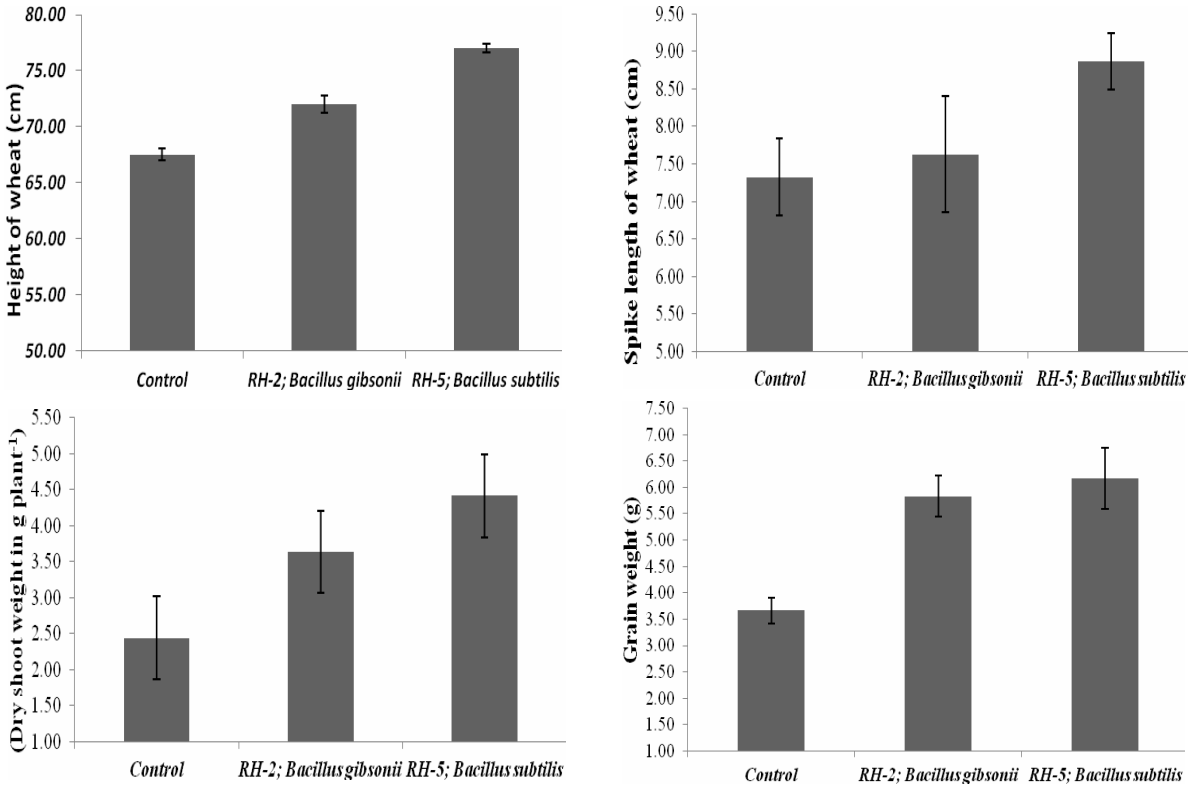

Fig. 3. Effect of bacterial strains on wheat yield.

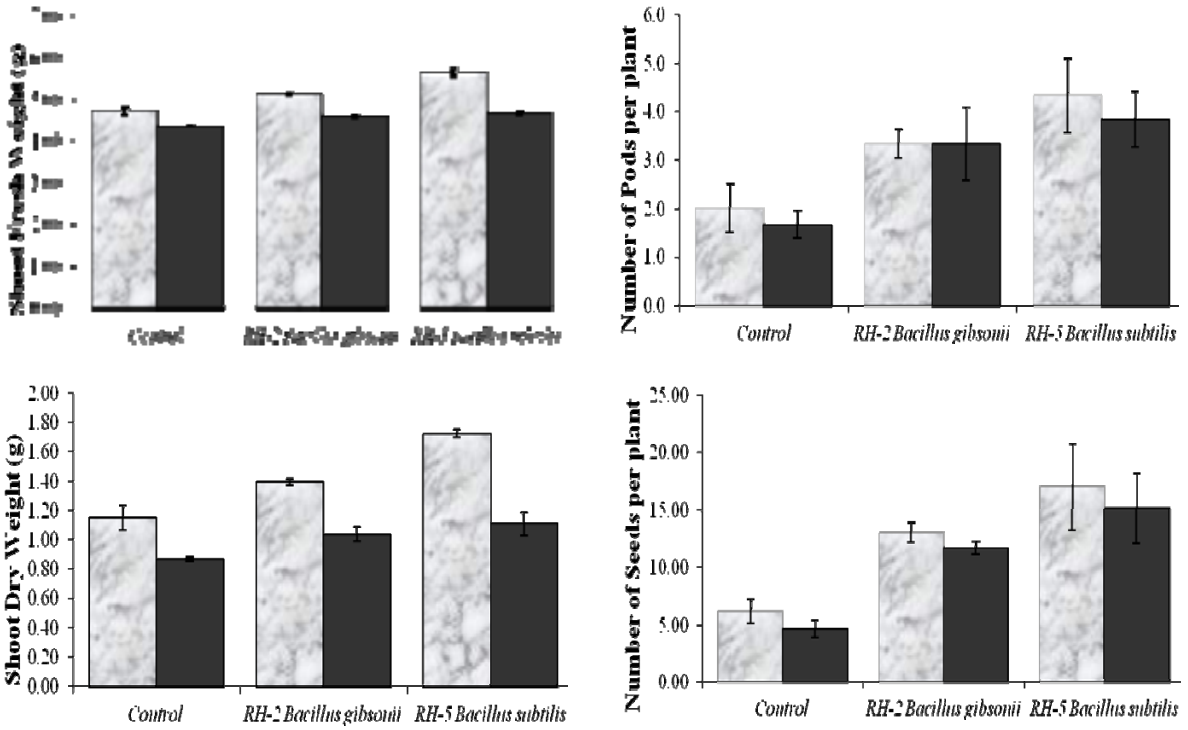

Fig 4. Effect of bacterial strains on mung bean and mash bean yield.

Table 4. Cellular fatty acid composition of bacterial strains.

Fatty acids	RH-2	RH-5
12:0	0.67 ± 0.01	0.00 ± 0.00
iso-$_{14:0}$	1.69 ± 0.00	1.20 ± 0.00
14:0	1.58 ± 0.01	0.00 ± 0.00
iso-$_{15:0}$	27.54 ± 0.00	17.04 ± 0.02
anteiso-$_{15:0}$	42.53 ± 0.01	39.14 ± 0.01
15:0	1.08 ± 0.00	0.00 ± 0.00
iso-E$_{16:1}$	0.00 ± 0.00	1.25 ± 0.01
iso-$_{16:0}$	3.43 ± 0.01	5.44 ± 0.01
16:0	7.60 ± 0.00	1.78 ± 0.01
A $_{16:1}$	0.00 ± 0.00	0.00 ± 0.00
anteiso-$_{17:0}$	6.30 ± 0.01	14.85 ± 0.01
iso-E$_{17:1}$	0.00 ± 0.00	4.59 ± 0.02
iso-$_{17:0}$	5.28 ± 0.00	12.38 ± 0.03
18:0	1.78 ± 0.01	0.00 ± 0.00
CIS 9 $_{18:1}$	0.51 ± 0.00	0.00 ± 0.00
Sum in feature 5	0.00 ± 0.00	2.37 ± 0.00
Summed feature 5	0.00 ± 0.00	2.37 ± 0.00

All values are average of three replicates

Discussion

It is interesting to note that all *Bacillus* strains characterized in this study solubilized tricalcium phosphate (20.47- 141 µg mL^{-1}) along with sharp decreased of pH in broth culture. RH-2 and RH-5 appeared as potential PGPRs by solubilizing inorganic P. Strain RH-5 also positive for *nif*H gene indicating its N_2-fixing ability. When applied as inoculants using sterile soil, these two strains significantly enhanced wheat and beans growth under axenic environment as compared to un-inoculated control plants. The genus *Bacillus* were previously characterized as P-solubilizers in different part of world and when applied to soil not only increased P-uptake in crops but also enhanced plant available P by solubilizing fixed phosphates in soil and utilizing rock phosphates (deFreitas *et al.,* 1997). Mechanisms involved in phosphate solubilization by *Bacillus* sp. include biosynthesis of different kind of organic acids (gluconic acid, lactic, acetic, citric and propionic acid etc) thus creating acidification conditions in the medium (Illmer & Schinner 1992; Chen *et al.,* 2006, Goldstein & Krishnaraj 2007; Delvasto *et al.,* 2008). Phosphate solubilizaton is also enhancing through gene modification followed by their expression in specific bacterial strains (Rodríguez *et al.,* 2006). Quantitative trait loci (QTL) and gene characterization and identification involved in phosphate solubilization and transport have also been studied extensively (Goldstein & Liu, 1987). Phytase genes have been cloned from number of *Bacillus* species (Tye *et al.,* 2002). The efficiency of PSB depends on bacterial sp., host crop, soil and environmental conditions (Çakmakçi *et al.,* 2006). In our studies, strain RH-5 solubilized tri-calcium phosphate (141 µg mL^{-1}) in broth culture with significant drop in pH from 7.0 to 4.83. Strong negative correlation between phosphate solubilization and broth pH was observed, which indicated the acidic condition required for phosphate solubilization. Similar pH drop in broth medium had been reported by Illmer & Shinner (1992) and Yu *et al.,* (2011). The quantity of IAA produced by *Bacillus* sp. varied widely. All isolates

produced minute quantity of IAA with and without tryptophan. Qualitative assay using AP-20E kits also showed negative reaction for indole production in most bacterial strains. Vessey, (2003) claimed very poor production of IAA by *Bacillus* sp. whereas Park *et al*., (2005); Çakmakçi *et al.,* (2007) and Beneduzi *et al.,* (2008) observed that *Bacillus* sp. are efficient in IAA production and enhanced wheat, spinach and rice growth.

Two bacterial strains characterized as potential PGPR and identified by 16SrRNA gene sequencing belonged to genus *Bacillus* having more than 99% sequence similarity with *Bacillus gibsonii* and *Bacillus subtilis*. Morphological characters also resembled with those of genus *Bacillus* and clearly distinguished both strains. Oxidase activities were positive in RH-5 while observed negative in RH-2. Optimum temperature required for the growth of both bacterial strains was 28-37^{o}C. However, strain RH-5 tolerate extreme conditions of temperature (as high as 50^{o}C), pH (up to 10) and NaCl concentration (0-9%) *In vitro* indicating its capability to survive in extreme soil environments. These in vitro temperature ranges cannot be compared and considered a tool for field inoculation (Hungria & Vergas 2000) but may be useful for isolating beneficial bacteria that can adjust easily according to field environment. Similarly, NaCl tolerance limit indicates the presence and survival of bacteria to a range of salinity and salt tolerance strain may be considered potential bioalternative for saline soils. Soil pH also acts as limiting factor for bacteria in soil (Brockwell *et al.,* 1991). Tolerance to lower and higher pH ranges confirmed the bacterial candidature to survive and improve alkaline and acidic soils.

The results of this study showed that two bacterial strains inoculated to soil significantly enhanced beans and wheat growth. This increase in crop growth indicated the PGP and PHP traits of these strains (Yasmeen *et al*., 2012). Bacillus spp. are used as PGPR with plant growth promoting traits like phosphate solubilization; N_2-fixation and auxin IAA production (Vessey, 2003; Liu *et al*., 2006; Beneduzi *et al.,* 2008) and are also being used as bioinoculants for crop production. The *Bacillus* species are reported to increase the yield in wheat (de Freits, 2000; Çakmakçi *et al.,* 2007), maize (Pal, 1998), sugar beet (Çakmakçi *et al.,* 2006), and spinach (Çakmakçi *et al.,* 2007). Lee *et al.,* (2005) observed increase in growth and yield of beans by co-inoculating *Bacillus* strains with *Rhizobium leguminosarum* bv. *phaseoli*. Gram positive spore forming *Bacillus* strains were also isolated from soybean root nodules by Bai *et al.,* (2002) for PGP activities. N_2-fixation by *Bacillus megaterium* was reported previously by Liu *et al.,* (2006). He found *Bacillus megaterium* as diazotroph with colonization pattern similar to those of gram negative N_2-fixer *Azospirillum brasilense*. Bacillus can be found in a diverse soil environment and behaved as PGPR even in acidic soils (Yadav *et al*., 2011) and are widely used as plant health promoting rhizobacteria (PHPR) by reducing diseases and producing antibiotic (Herman *et al.,* 2008; Dutta *et al.,* 2008). Bacon & Hinton, (2002) also inoculated *Bacillus mojavensis* to maize crop for testing its PHP biocontrol characterization and found it as antagonistic to endophytic mycotoxin producing fungi (*fusarium moniliforme*). Mena-Violante & Vector (2007) inoculated *Bacillus subtilis* on tomato and observed increased in yield and tomato quality. Similar positive response of tomato to *Bacillus subtilis* were also investigated by Adesemoye *et al.,* (2008).

Results of chemotaxonomic analyses of both bacterial strains in this study confirmed identification of RH-2 and RH-5 and placed in genus Bacillus. Chemotaxonomic features along with 16S rRNA gene sequencing are considered essential for correct identification at species level. The predominant menaquinone in strains RH-2 and RH-5 was MK-7. The results of this study correlate the findings of a great deal of previous work in this field. Genus *Bacillus* is characterized by quinone system-MK-7 (Claus & Berkeley, 1986) and DNA G + C composition differentiates species with in genus but *Bacillus* had a wide range of DNA G + C contents. This genetic heterogeneity in DNA G + C contents that often ranged from 32-69% was also present with in strains of *Bacillus* spp., (Claus & Berkeley, 1986). Chemotaxonomic characterization of RH-2 and RH-5 (major cellular fatty acid anteiso-$c_{15:0}$; quinine MK-7 and DNA G + C contents 40-41 mol%) was similar to those in closely related *B. gibsonii* (Nielsen *et al.,* 1995) and *B. subtilis* subsp. *inaquosorum* (Rooney *et al.,* 2009). Quinone analysis, G + C contents along with cellular fatty acid profile justify the nomenclature by placing both bacterial strains in genus *Bacillus*.

This study has given an account of characterization of different bacterial strains and suggested that bacterial strain RH-5 isolated from mash bean (*Vigna mungo*) rhizospheric soil and identified as *Bacillus* using 16S rRNA gene sequencing and chemotaxonomic assays. Keeping in view its capacity to solubilize tricalcium phosphate and a carrier of *nif*H gene differentiate this strain from others and confirmed as PGPR under invitro and glass house experiments. When applied as biofertilizer, it increased wheat and beans growth, biomass and grains under controlled conditions. Phenotypic characterization of this strain in term of pH, temperature and NaCl tolerance also indicated its environmental adoptability. To make it patent as potential PGPR, further inoculation experiments under field conditions for testing on variety of crops may be required.

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