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Characterization of Phosphate-solubilizing Isolates of *Bacillus* from Cumin Rhizosphere

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Abstract: *This study focus on characterization of plant growth promoting isolates of Bacillus from rhizosphere of cumin plants. Ten efficient phosphate-solubilizing isolates of Bacillus were selected for detailed study, of which, the isolate BCRh10 proved most efficient in solubilization of inorganic phosphates (37.84 and 96.76 µg/mL after 2 and 10 days, respectively). Numerical analysis of the carbon utilization profiles revealed wide diversity among the isolates and placed them into two main groups at a similarity coefficient of 0.71. Although, the isolate BCRh10 was positive for most of the carbon sources, it stood as an outlier in the dendrogram. These isolates were also tested for PGPR (plant growth promoting rhizobacteria) activities like, ability to produce enzymes (ACC deaminase, protease, cellulase, pectinase, and chitinase) necessary for catalyzing reactions for nutrient mobilization; which in addition to certain metabolite (siderophore and HCN production) promote inhibition of fungal plant pathogens. The isolate BCRh10 produced highest quantity of IAA (94.82 µg/mL), was positive for most of the metabolites was most efficient in suppressing Alternaria burnsii, the causative agent of cumin blight. The 16rRNA gene sequence similarity was used to confirm the phylogenetic positions of these isolates. The evolutionary history inferred using the UPGMA method and evolutionary distances computed using Maximum Composite Likelihood method using MEGA5 based on 16S-rRNA gene sequences grouped these isolates in four groups with high bootstrap values (83-100, n = 500), which provided high degree of confidence for molecular characterization of* Bacillus *isolates.*

Keywords: Alternaria burnsii, *hydrolytic enzymes; MEGA5, PGPR, siderophore.*

INTRODUCTION

Phosphorous is one of the most fundamental macronutrient for promotion of plant growth. Large portion of P from fertilizers, after being applied in the field, bind to calcium ions in calcareous soils or with iron or aluminum in acidic soils, and so, becomes insoluble in water and get fixed in soil layers, and thus, turn unavailable to plants (Richardson *et al*., 2001).

However, numerous microorganisms present in soil possess the ability to improve P-availability by transforming the fixed/insoluble forms of phosphorus into soluble forms (Richardson, 2001), and thus, contribute in maintaining the soil health and quality. The beneficial bacteria that effectively colonize the root zone and favor plant growth directly or indirectly are known as plant growth promoting rhizobacteria (PGPR) (Singh, 2013). These PGPR are assumed to be one of the most important input for reducing chemical fertilizers and pesticides consumption in agriculture. These are known to stimulate plant growth either by production of plant growth regulators (IAA, gibberellins, cytokinins), enhancement of plant nutrients uptake, production of metabolites such as antibiotics, HCN, or siderophores or by promoting induce systemic resistance (Bloemberg and Lugtenberg, 2001), and hence, are important in sustaining the agroecosystems.

India is the largest producer, consumer and exporter of cumin seed in the world and contributes about 73% to the global production (Indianspices.com,

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2012). The North-Western states of Gujarat and Rajasthan are the major cumin producing states in India which mainly falls in the semi-arid regions and is marked by scanty rainfall, lack of natural resources, extreme diurnal and seasonal fluctuation of temperature, poor farming community, marginal lands, and lack of adoption of effective technologies for sustaining agricultural productivity. However, such agroecosystems may be explored for efficient strains of rhizobacteria which may be used as biofertilizers and/or antagonists against major phytopathogens and may provide a promising alternative to chemical fertilizers and pesticides in such disadvantaged areas.

Bacteria belonging to the genus *Bacillus* are generally Gram-positive, rod-shaped, spore-forming, aerobe or facultative anaerobe. This genus includes a large number of species having quite different phenotypes (Holt *et al.* 1994). The asymbiotic nature and spore forming ability of this group of bacteria make them a potential PGPR due to increased adaptation (Liu and Sinclair, 1993). The use of glycerol as carbon source by these bacteria may play an important role in microbiological processes like, production of PHB, biofilm formation, increased sporulation, and inducible effect on enzyme production. *Bacillus* is one of the most predominant bacteria frequently encountered in the rhizosphere and many of them may present inside the plants as endophytes. Application of *Bacillus* based bioinoculants in agricultural fields cause alteration in the nutrient and microflora composition of rhizosphere and result in increased plant growth and yield (Vessey and Buss, 2002). Additionally, a number of *Bacillus* isolates increase the vitality and ability of the plant to cope with pests and diseases, and favor plant growth (Wahyudi *et al.*, 2011). Due to the ability of these bacteria for rapid colonization of rhizosphere and stimulation of plant growth, there exists considerable interest in exploiting these rhizosphere bacteria for qualitative and quantitative improvement of crop production. Therefore, the present research work aims to isolate and characterize *Bacillus* sp. from the rhizosphere of cumin plants growing in the semi-arid region for development of microbial inoculants and to screen them for their plant growth promoting attributes.

MATERIALS AND METHODS

Isolation of *Bacillus* from the Cumin Rhizosphere

Cumin (*Cuminum cyminum* L.) rhizospheric soil was collected from the experimental plots of the S.D. Agricultural University, Gujarat, India. The soil was characterized as loamy sand (sand 83.54%, silt 5.96%, clay 10.45%) having pH 7.8. Ten grams of soil was heat-treated at 80°C for 10 minutes in order to stimulate endospore formation by *Bacillus*. The heat-treated soil was transferred in a flask, fairly dissolved in small amount of sterile distilled water, and adjusted the volume to 100 ml.

The contents were further mixed by placing the flask on a rotary shaker for 5 minutes. The soil suspension was put to serial dilution and 0.1 ml suspension of 10^5 dilutions was spread over pre-sterilized and cooled down petriplates containing nutrient agar medium following standard plating method. The plates were then incubated at 30±1°C for 18-24 hours. Fifty rough abundant colonies showing waxy growth (1-4 mm dia) and irregular spreading edge were selected and made into pure culture. The *Bacillus* species were determined based on morphology and physiology as per the Bergeys' Manual of Determinative Bacteriology (Holt *et al.*, 1994). The isolates were maintained on nutrient agar slants at 4°C.

Solubilization of Insoluble Phosphate

Solubilization of water insoluble tri-calcium phosphate by *Bacillus* isolates was detected in the Pikovskaya's agar medium (composition/L: 0.5 g yeast extract, 10 g dextrose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.0001 g $MnSO_4.H_2O$, 0.0001 g $FeSO_4.7H_2O$ and 15 g agar). The isolate was streaked on the surface of this medium, incubated at 28°C for three days, and phosphate solubilization activity was determined by the development of clear zone around bacterial colony (Katznelson and Bose, 1959). Quantification of phosphate solubilization was done by growing the bacteria in broth medium (pH 7.0) at 28°C up to 10 days. 5 ml aliquot was collected at two days interval till 10 days. The suspension was centrifugation at 9,000 *g* for 20 min and the soluble free phosphate in culture supernatant was estimated

from the absorbance values obtained using Phospho-molybdate method. Finally, we selected ten isolates that showed good growth on Pikovskaya's agar medium and were efficient solubilisers of inorganic phosphate for further study.

Biochemical Characterization of Phosphate-solubilizing Bacteria

Biochemical characterization of these phosphate-solubilizing bacteria was done based on carbon utilization pattern. The carbon utilization profiles were generated using Hicarbohydrate™ kit (KB009, Himedia Laboratories, Mumbai, India) following manufacturer protocol. Single colony was inoculated into 5 ml Brain Heart Infusion Broth and incubated at 35-37°C for 4-6 hrs until the inoculum turbidity become 0.5 O.D. (620 nm). Each well of the kit was inoculated with an aliquot of 50 µl of bacterial suspension and the plates were incubated at 35±2°C for 18-24 hrs. The experiment was done with three replicates. The result was observed in the form of color change of the medium in wells of the kit as per manufacturer's protocol. Based on carbon utilization profiles, a binary matrix comprising of 1 (positive) and 0 (negative) values was generated. The data were analyzed using a numerical taxonomy and multivariate analysis system (NTSYSpc 2.02i) software package (Rohlf, 2000). The dendrogram was based on the proximity matrix obtained from the Jaccard coefficient and sequential agglomerative hierarchical non-overlapping (SAHN) and clustering was done using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath and Sokal, 1973).

Other Plant Growth Promoting Attributes

Production of IAA

The isolates under investigation grown in culture tubes containing 10 ml of nutrient broth supplemented with 0.2 mM tryptophan (L-Trp) and incubated for 48 h at 26±2°C. The cultures were centrifuged at 10,000 rpm for 15 min and 2 ml of the supernatant was mixed with 2 ml of Salkowski's reagent (150 ml H_2SO_4, 250 ml distilled water, 7.5 ml $FeCl_3.6H_2O$ 0.5 M). The mixture was incubated at room temperature for 30 min and presence of IAA was determined by development of pink color, and IAA concentration was measured spectroscopically at 520 nm and quantified (Patten and Glick, 2002) using an IAA standard curve.

Aminocyclopropane-1-carboxylate (ACC) deaminase

The bacterial isolates were allowed to grow in LB medium (5 ml) for 24 h at 150 rpm at 28±2°C. Bacterial cell pellets, harvested by centrifugation at 9000 rpm for 5 min were washed with sterile distilled water and resuspended in 1ml of sterile water, were spot inoculated on petri plates containing DF salts minimal medium (Dworkin and Foster, 1958) supplemented with 3.0 mM ACC. The strains growing on the ACC supplemented plates after incubation for 48h at 28°C were considered positive for ACC deaminase activity.

Siderophore production

Siderophore production was tested qualitatively using the chrome azurol S medium (CAS-medium) (Husen, 2003). In this test, about 10 µl of the bacterial inoculum was dropped onto the center of a CAS plate. After incubation for three days at 25°C, the siderophore production was assessed which is indicated by the presence of orange halos around the colonies.

Protease

The protease activity of the isolates was tested on skim milk agar medium (composition/L: 5 g pancreatic digest of casein, 2.5 g yeast extract, 1 g glucose, 7% skim milk solution and 15 g of agar). Bacterial cells were spot inoculated in the petriplates containing above medium and after 2 days of incubation at 28°C, the proteolytic activity was observed by production of clear zone around the cells (Smibert and Krieg, 1994).

HCN production

The qualitative test for HCN production by bacteria was done following the method of Bakker and Schipper (1987). The bacterial culture was streaked on nutrient agar medium supplemented with

glycine (4.4 gm/l). Change in the color of the Whatman filter paper No. 1 (previously soaked in solution of 2% Na_2CO_3 in 0.05% picric acid) placed in-between base and lid of the culture-plate (sealed with parafilm) after an incubation at 27 ± 2°C for 96 h from yellow to orange-brown was considered positive for HCN production.

Chitinase

The chitinase activity of strains was tested by spot inoculation of the bacterial cells on the chitin agar medium having 1.62 g nutrient broth, 0.5 g NaCl, 6 g M9 salts, 8 g colloidal chitin and 15 g agar per liter of medium (Renwick *et al.*, 1991). After 5 days of incubation at 30°C, the chitinase activity was identified by appearance of clear zone around the bacterial cells.

Cellulase

Bacillus strains were screened for production of cellulase by plating them onto M9 medium agar amended with 10 g of cellulose and 1.2 g of yeast extract per liter. After 8 days of incubation at 28°C, colonies surrounded by clear halos were considered positive for cellulase production (Cattelan *et al.*, 1999).

Pectinase

The pectinase production ability of the isolates was determined by allowing them to grow on M9 medium amended with 4.8 g of pectin per liter. After 48 hrs of incubation (28°C) the plates were flooded with 2 mol l^{-1} HCl. The bacterial growth surrounded by clear halos was considered positive for pectinase production (Cattelan *et al.*, 1999).

Antagonism Assay Against Phytopathogenic Fungi

These *Bacillus* isolates were assayed for antifungal activities against *Alternaria burnsii* using half concentration of Nutrient Agar and Potato Dextrose Agar (PDA) medium. *Bacillus* isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi (inoculated at the center of the petriplates). Antagonist activity was investigated for 4 to 7 days after incubation at room temperature. The barrier between *Bacillus* isolate and fungi indicate antagonist interaction between them. Percent inhibition of fungus was estimated using the formula: $1-(a/b) \times 100\%$ (Kumar *et al.*, 2002); where, *a*: distance between fungi in the center of petridish to *Bacillus* isolate, *b*: distance between fungi in the center of petridish to blank are without *Bacillus* isolate.

16S *r*RNA Gene Sequence Analysis

The 16S *r*RNA gene sequence analysis was conducted in order to investigate the species taxa of these potential isolates. DNA from the strains were isolated employing GeneiPure Bacterial DNA Purification kit (Merck Specialities Private Limited, Mumbai, India). The 16S *r*RNA gene fragment amplification was carried out by PCR using universal primers corresponding to positions 8-27 for the forward primer, 27F (5′-AGAGTTTGAT CMTGGCTCAG-3′) and 1492-1510 for the reverse primer, 492R (5′-TACGGYTACCTTGTTAGA CTT-3′). The Polymerase Chain Reaction comprised of predenaturation at 94°C for 5 min; followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, polymerization for 2 min at 72°C, and post PCR extension for 10 min at 72°C. The PCR product was amplified agarose gel (1%) along with 500 bp DNA ladder (GeNei, India). The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen) and sequencing of the 16S rRNA gene was done using Big Dye terminator cycle sequencing kit (Applied BioSystems, Waltham, USA).

The products were resolved on an automated DNA sequencing system (Applied BioSystems, USA) and nucleotide sequences were determined. These sequences thus obtained were subjected to similarity search with the reference species already present in the genomic database using NCBI BLAST (Basic Local Alignment Search Tool). The sequences were aligned (ClustalW alignment algorithm), phylogenetic position was inferred using UPGMA method, and evolutionary distances were computed using Maximum Composite Likelihood method using MEGA5 (Tamura *et al.*, 2011).

RESULTS

Isolation of Bacillus from the Rhizosphere

The *Bacillus* isolates obtained from the rhizosphere of cumin plants showed characteristics of *Bacillus* based on physiological and morphological criteria, including Gram-positive reaction, rod-shaped cells, and production of endospore.

Phosphate Solubilization

From among the fifty *Bacillus* isolates, only ten of them could grow efficiently on the Pikovskaya's agar medium, solubilized tri-calcium phosphate and were able to survive on the nutrient agar medium supplemented with 1% glycerol (w/v). These ten isolates were further studied for their functional and genetic characteristics for use as PGPR agents. These isolates showed increased phosphate solubilization with incubation (Table 1). The isolate BCRh10 proved most efficient phosphate solubilizer, which could solubilize 37.84 μg ml^{-1} of P after two days of incubation. For all the isolates and for all the incubation, a decline in the pH was regitered.

Biochemical Characterization

The biochemical characterization of these bacteria was done based on carbon utilization pattern. The selected isolates were found positive for fructose, sucrose, dextrose, dextrose, glycerol and esculin utilization. They showed negative test for utilization of melibiose, adonitol, melizitose, ONPG, citrate, malonate and sorbose.

However, the isolates behaved differently for utilization of other carbon sources such as lactose, xylose, maltose, galactose, raffinose, trehalose, L-arabinose, mannose, inulin, sodium gluconate, salicin, dulcitol, inocitol, sorbitol, mannitol, arabitol, erythritol, a-methyl-D-mannoside, rhamnose, cellobiose, a-methyl-D-mannoside and D-arabinose. Among them, the isolate BCRh10 was tested most versatile in utilizing various carbon sources and was positive for utilization of maltose, fructose, dextrose, galactose, raffinose, trehalose, sucrose, L-arabinose, mannose, glycerol, salicin, inocitol, sorbitol, mannitol, arabitol, a-methyl-D-mannoside, rhamnose, cellobiose, a-methyl-D-mannoside, xylitol and esculin (Table 2).

Table 1
Solubilization of tricalcium phosphate by *Bacillus*

	Incubation period (days)									
	2		*4*		*6*		*8*		*10*	
Strain	*P solubilized* [#]	*pH*	*P solubilized*	*pH*	*P solubilized*	*pH*	*P solubilized*	*pH*	*P solubilized*	*pH*
BCRh1	21.98 ± 0.91	6.5	40.56 ± 1.44	6.0	51.06 ± 1.33	5.7	63.18 ± 1.26	5.2	77.97 ± 1.42	4.9
BCRh2	10.71 ± 0.41	6.5	19.72 ± 1.45	6.1	35.37 ± 1.20	5.6	52.45 ± 1.66	5.4	72.25 ± 2.12	4.8
BCRh3	11.49 ± 0.53	6.6	23.77 ±1.46	6.2	31.51 ± 0.98	6.0	47.27 ±1.44	5.9	63.36 ± 1.78	5.8
BCRh4	20.80 ± 0.88	6.4	42.45 ± 1.75	5.9	64.23 ± 1.21	5.7	76.55 ± 1.84	5.2	82.41 ±1.69	4.8
BCRh5	17.75 ± 0.48	6.4	34.41 ± 1.70	6.2	49.71 ± 1.65	5.8	52.57 ± 1.66	5.5	63.84 ± 1.57	5.3
BCRh6	18.32 ± 0.54	6.4	38.72 ± 1.94	6.2	53.83 ± 1.26	5.8	54.15 ± 1.42	5.5	62.74 ± 2.05	5.2
BCRh7	13.26 ± 0.55	6.4	34.23 ± 1.24	6.0	53.38 ± 1.44	5.7	68.57 ± 1.85	5.2	78.04 ± 1.50	4.9
BCRh8	11.27 ± 0.60	6.5	25.18 ± 0.87	6.1	35.56 ± 1.16	5.9	46.50 ± 1.51	5.7	53.31 ± 1.79	5.5
BCRh9	23.21 ± 1.00	6.3	39.33 ± 1.73	5.6	48.37 ± 1.63	5.2	63.89 ± 0.96	4.8	86.44 ± 0.71	4.4
BCRh10	37.84 ± 0.87	6.2	52.02 ± 1.91	5.8	72.70 ± 2.02	5.6	86.03 ± 1.90	5.2	96.76 ± 1.53	4.8
SEm±	0.400±		0.958		0.661		0.893		1.010	
CD	1.189±		2.846		1.965		2.653		3.002	
CV	6.366±		8.200		4.003		4.383		4.112	

[#] Tricalcium phosphate solubilization ha been expressed in unit of μg ml^{-1} ± standard error (SE)

Table 2
Carbon utilization profile of the *Bacillus* isolates

Carbon source	*Bacillus endophyticus (BCRh1)*	*Bacillus megaterium (BCRh2)*	*Bacillus megaterium (BCRh3)*	*Bacillus cereus (BCRh4)*	*Bacillus cereus (BCRh5)*	*Bacillus cereus (BCRh6)*	*Bacillus megaterium (BCRh7)*	*Bacillus megaterium (BCRh8)*	*Bacillus endophyticus (BCRh9)*	*Bacillus amyloliquefaciens (BCRh10)*
Lactose	+	–	–	–	–	–	–	–	+	–
Xylose	+	–	–	–	–	–	–	–	+	–
Maltose	+	–	–	+	+	+	–	–	+	+
Fructose	±	±	+	±	±	±	±	+	–	±
Dextrose	+	+	+	+	+	+	+	+	+	+
Galactose	–	–	–	–	–	–	–	–	–	+
Raffinose	–	–	–	–	–	–	–	–	–	+
Trehalose	+	±	+	+	+	+	±	–	+	+
Melibiose	–	–	–	–	–	–	–	–	–	–
Sucrose	±	±	+	+	+	+	±	±	+	+
L–Arabinose	–	±	+	–	–	–	+	+	–	+
Mannose	±	±	±	–	–	–	±	±	±	+
Inulin	+	–	–	–	–	–	–	–	+	–
Sodium gluconate	–	–	–	–	+	+	–	–	–	–
Glycerol	+	+	+	±	+	+	+	+	+	+
Salicin	+	+	+	–	–	–	±	+	+	±
Dulcitol	–	–	–	–	+	+	–	–	–	–
Inositol	–	–	–	–	+	+	–	–	–	+
Sorbitol	–	–	–	–	–	–	–	–	–	+
Mannitol	–	–	–	–	–	–	–	–	–	±
Adonitol	–	–	–	–	–	–	–	–	–	–
Arabitol	–	–	–	–	–	–	–	–	–	+
Erythritol	–	–	–	–	±	–	–	–	–	–
a–Methyl–D–glucoside	–	–	–	–	±	–	–	–	–	±
Rhamnose	–	–	–	–	+	+	–	–	–	±
Cellibiose	–	–	–	–	+	–	–	–	–	+
Melezitose	–	–	–	–	–	–	–	–	–	–
a–Methyl–D–mannoside	–	–	–	–	–	–	–	–	–	±
Xylitol	–	–	–	–	–	–	–	–	–	+

Cont. table 1

Table 2
Carbon utilization profile of the *Bacillus* isolates

Carbon source	*Bacillus endophyticus (BCRh1)*	*Bacillus megaterium (BCRh2)*	*Bacillus megaterium (BCRh3)*	*Bacillus cereus (BCRh4)*	*Bacillus cereus (BCRh5)*	*Bacillus cereus (BCRh6)*	*Bacillus megaterium (BCRh7)*	*Bacillus megaterium (BCRh8)*	*Bacillus endophyticus (BCRh9)*	*Bacillus amyloliquefaciens (BCRh10)*
ONPG	–	–	–	–	–	–	–	–	–	–
Esculin	+	+	+	+	+	+	+	+	+	+
D–Arabinose	–	±	±	–	–	–	±	±	–	–
Citrate	–	–	–	–	–	–	–	–	–	–
Malonate	–	–	–	–	–	–	–	–	–	–
Sorbose	–	–	–	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–	–	–	–

Carbon utilization pattern: + Positive utilization; – Negative utilization; ± Negligible utilization

Other Plant Growth Promoting Attributes

The *Bacillus* isolates were able to produce IAA in various concentrations in the medium supplemented with 0.2 mM tryptophan after incubation for two days. *Bacillus* sp. designated as BCRh10 synthesized highest quantity of IAA (94.82 µg/mL). Expression of PGPR characters listed in Table 3 by the *Bacillus* isolates shows the indispensible plant-microbe and microbe-microbe interaction in the rhizospheric region.

These isolates were studied for their abilities to produce certain enzymes (*e.g.* ACC deaminase, protease, cellulase, and pectinase) necessary or various biochemical reactions and for production of certain metabolite necessary for inhibition of fungal plant pathogen (chitinase activity, siderophore and HCN production). The isolates varied widely for these attributes and showed presence of more than one type of biochemical activities. The isolates BCRh2 and BCRh10 were found positive for the production of the ACC deaminase, protease, cellulase, and pectinase enzymes; but BCRh2 was unable to produce chitinase and HCN whereas BCRh10 produced all the three metabolite (chitinase, siderophore and HCN) for inhibition of fungal plant pathogen.

Antagonism Against Phytopathogenic Fungi

Co-inoculation of PGPR *Bacillus* isolates and *Alternaria burnsii* showed differential level of inhibition (Table 3), which was reflected as barrier zone (brown or relatively dark) due to the antagonistic interaction between the two organisms. Careful microscopic examination revealed discoloration of fungal mycelium due to inhibition/ lysis effects by the bacteria. BCRh10 (*Bacillus amyloliquefaciens*) proved most efficient (45%) inhibition of *Alternaria burnsii* whereas BCRh3 showed least inhibition of fungal pathogen.

16S *r*RNA Gene Sequence Analysis

The 16S-rRNA gene amplification of these isolates yielded a specific band of approx. 1500bp. The resulting 16S-*r*RNA gene sequences were submitted in the NCBI GenBank with accession numbers sequentially from KT153597 to KT153606. BLAST search analysis of 16S-rRNA genes sequences revealed similarity with *Bacillus* sp as per GenBank

Table 3
Other plant growth promoting attributes of the phosphate-solubilizing *Bacillus* isolates

Strain	*Bacillus spp.*[#]	*IAA*[##] *(µg/mL)*	*ACC deaminase*	*Proteinase*	*Cellulase*	*Pectinase*	*Chitinase*	*HCN production*	*Siderophore*	*Percent inhibition of A. burnsii*
BCRh1	*Bacillus endophyticus* (KT153597)	87.76	+	+	–	+	+	+	–	35
BCRh2	*B. megaterium* (KT153598)	79.24	+	+	+	+	–	–	+	25
BCRh3	*B. megaterium* (KT153599)	68.46	+	–	–	–	–	–	+	9
BCRh4	*Bacillus cereus* (KT153600)	70.66	–	+	+	+	–	+	–	20
BCRh5	*B. cereus* (KT153601)	82.46	–	+	+	+	–	–	–	15
BCRh6	*Bacillus cereus* (KT153602)	72.82	–	–	+	–	–	+	–	27.5
BCRh7	*Bacillus megaterium* (KT153603)	79.60	–	–	+	+	–	–	+	30
BCRh8	Bacillus megaterium (KT153604)	82.64	–	–	–	–	–	–	–	25
BCRh9	*Bacillus endophyticus* (KT153605)	86.32	–	+	–	+	+	+	–	38
BCRh10	*B. amyloliquefaciens* (KT153606)	94.82	+	+	+	+	+	+	+	45

[#] Information in the paranthesis indicate Accession number of the *Bacillus* isolates
[##] IAA production by isolates indicate mean of three replicates

databases. According to the phylogenetic tree (Figure 1), all the plant growth promoter of *Bacillus* was divided into two groups; the first group comprised of the nine isolates whereas BCRh10 (*Bacillus amyloliquefaciens*) alone represented the second group (group D). The first group was further divided in to two at a dissimilarity co-efficient of 0.34; the first subgroup (group A) of this cluster comprised of four strains (BCRh-02, BCRh-03, BCRh-07, and BCRh-08) and represented *Bacillus megaterium* while group B contained the isolates BCRh-01 and BCRh-09 (*Bacillus endophyticus*). However, the second cluster (group C) was represented by the strains BCRh-04, BCRh-05, BCRh-06 (*Bacillus cereus*).

DISCUSSION

PGPR refers to such beneficial soil bacteria, which possess the ability to colonize plant roots and promote growth and development of plants (Kloepper and Schroth 1978), and the colonization of rhizosphere must be at population densities sufficient to produce a beneficial effect. In the present study, we have selected cumin rhizosphere soil for isolation of *Bacillus* species, as this crop is mainly grown in the semi arid tract, which normally receive scanty rainfall and is largely comprised of nutrient deficient and marginal land.

Moreover, *Bacillus* sp. is considered ubiquitous in agroecosystems and is one of the most important and efficient biofertilizer due to sporulation and free-living nature, which encourage their use for increased adaptation to commercial formulations and field application (Liu and Sinclair, 1993). Moreover, these isolates are able to utilize glycerol. Glycerol serves as an excellent carbon source as it activates the cells of *Bacillus megaterium* for higher production of poly b-hydroxy butyrate (Sathianachiyar and Devaraj, 2013) and promotes biofilm formation in *B. licheniformis* and *B. cereus* (Shemesh and Chai, 2013). Shemesh and Chai (2013) further indicated the presence of highly conserved signaling pathway for biofilm formation in *Bacillus*. Glycerol possibly acts as inducer of the glucose dehydrogenase-pyrroloquinoline quinone (GDH-PQQ) holoenzyme activity, which allows the induction of mineral phosphate solubilization via gluconic acid production (Sathianachiyar and Devaraj, 2013).

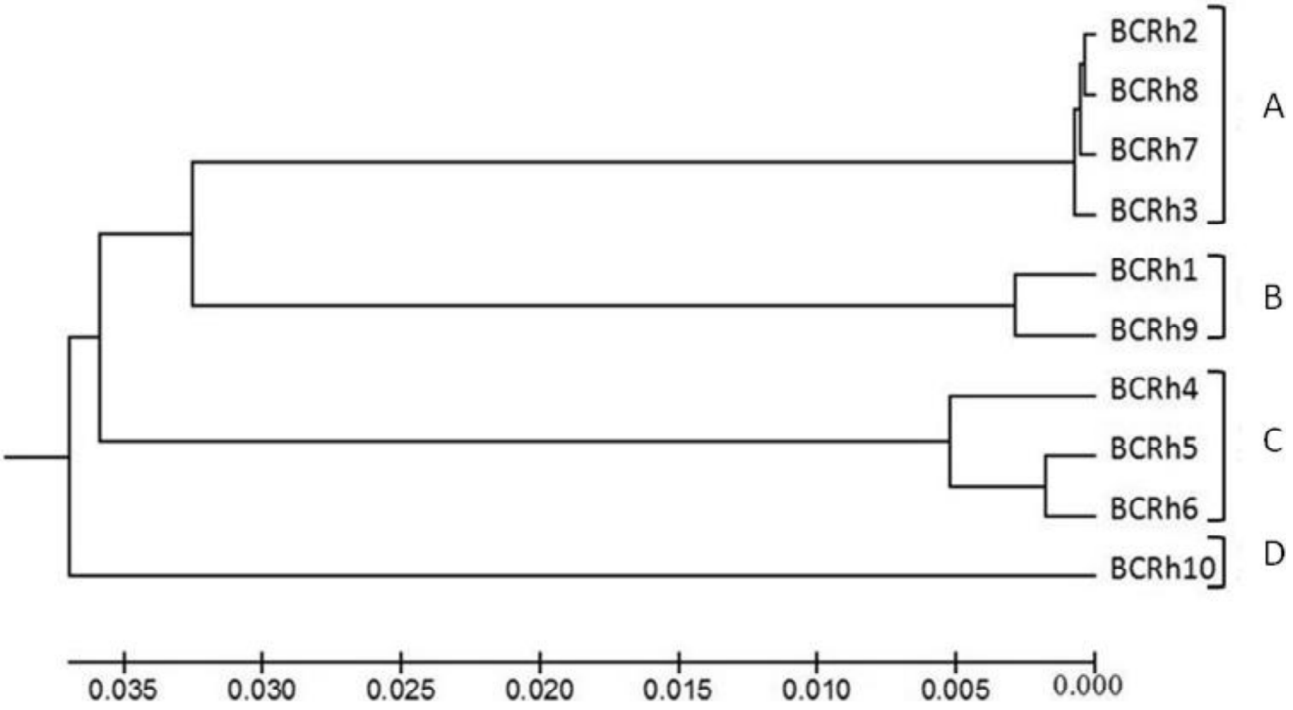

Figure 1: Dendrogram showing diversity of *Bacillus* isolates based on sequence of 16SrRNA gene

Phosphorus is required for growth and development of crop plants and is absorbed by plants mainly in two soluble forms, the monobasic ($H_2PO_4^-$) and the diabasic (HPO_4^{-2}) ions (Bhattacharyya and Jha, 2012). However, these anions are extremely reactive and form insoluble metal complexes with calcium or iron/aluminum ions respectively in calcareous or acidic soils. These metal ion complexes fix about 80% of the added phosphatic fertilizer and only a marginal part of it (1%) gets utilized by the plants (Qureshi *et al.*, 2012). This necessitates frequent application of P-fertilizers, which is costly and environmentally undesirable. In such a situation, use of PGPR having phosphate-solubilizing activity, is highly recommended as an economic, eco-friendly, and sustainable solution for improved crop production and sustaining the soil health.

Solubilization of inorganic phosphorus in soil occurs due to the low molecular weight organic acids synthesized by soil microorganisms (Zaidi *et al.*, 2009). However, phosphatases (catalyze hydrolysis of phosphoric esters) produced by microbes play an instrumental role in mineralization of organic phosphorus (Glick, 2012) and results in acidic pH of the medium. The present study also shows a decline in pH of the growth medium due to solubilization of tri-calcium phosphate, which may have been probably facilitated by the production of various organic acids by the bacteria. Zaidi *et al.* (2009) have also shown that the phosphate-solubilizing bacteria, besides providing P to the plants, also augment plant growth by stimulating the efficiency of biological nitrogen fixation, increased availability of trace elements, and by synthesizing important plant growth promoting substances.

Surprisingly, all the isolates in the current study were able to produce IAA at varying concentration when the nutrient broth medium was supplemented with L-tryptophan (0.2 mM). IAA production by the bacterial inoculant is an influential trait because this phytohormone enables plant to develop highly organized root system, which improve uptake of nutrients from the soil (Tsavkelova *et al.*, 2007). *Azotobacter* strain (Azo-8) isolated from the dryland agricultural field could produce 20.24 ppm of IAA and save about 20 KgN/ha at the same time promote better crop yield (Singh *et al.* 2013).

The various *Bacillus* isolates showed differential expression of the various hydrolytic enzymes like, protease, chitinase, cellulase, and pectinase and the metabolite siderophores and HCN

production, which are also instrumental in suppressing plant pathogens. In the present study, the isolate BCRh10 (*Bacillus amyloliquefaciens*) produced most of the hydrolytic enzymes and metabolites necessary for better nutrition and inhibition of the plant pathogens (Table 3).

Several species of *Bacillus* produce multiple antibiotics and hydrolytic enzymes like protease, cellulase, pectinase and chitinase that can suppress one or more plant pathogens (Saha *et al.*, 2012). The cell wall degrading hydrolytic enzymes, especially chitinases, glucanases and proteases, are involved in hyperparasitism of phytopathogenic fungi and may be important factors in biological control (Kim and Chung, 2004). The cellulase enzyme, produced by *Bacillus* species, has the capacity to degrade the fungal cell wall and this is an important mechanism of fungal inhibition. Pectinase enzyme produced by bacteria catalyses the breakdown of pectic compounds (Reetha *et al.*, 2014). These enzymes help the bacteria in penetration of the plant host and promote induced systemic resistance. The hydrolytic enzymes may also complement the other plant growth promoting activities of bacteria like, nitrogen or phosphorus availability or production of phytohormones, hydrogen cyanide, ammonia, and/or siderophores.

Four of the ten *Bacillus* isolates, representing *Bacillus cereus, Bacillus megaterium,* and *Bacillus amyloliquefaciens,* were positive for the ACC deaminase activity. The bacterial ACC (1-aminocyclopropane-1-carboxylate) deaminase enzyme hydrolyzes plant ACC (immediate precursor of the phytohormone ethylene), prevents the production of plant growth inhibiting levels of ethylene, and reduces drought stress (Patten and Glick, 2002). This aspect is more important for an inoculant, which is proposed to be used for crops in the semi-arid region. Three isolates of *Bacillus megaterium* and one of *Bacillus amyloliquefaciens* produced siderophores. Siderophores are low molecular weight organic compounds, which contain high-affinity set of ligands that coordinate Fe_3^+ and help the bacteria in acquiring iron from their environment (Crosa and Walsh, 2002) and at the same time deprive other plant pathogenic organisms of this valuable nutrient. Siderophore production ability of PGPR confers them additional advantage to compete with other organisms and efficiently colonize the rhizospheric region.

Bacilluis endophyticus, Bacillus cereus, and *Bacillus amyloliquefaciens* in present study were able to produce hydrogen cyanide (HCN) which play an important role as antagonist against fungal pathogens. Siderophores, HCN and chitinase production are regarded some of the influential PGPR traits that indirectly promote plant growth and protect plants against phyto-pathogens (Zhou *et al.*, 2012). Of the ten isolates, four PGPR strains showed siderophore production, three chitinase, and five isolates showed HCN production. Hence, along with direct plant growth promoting traits, indirect PGP traits are equally important for overall plant growth and development. In the present study, we have tested important plant growth promoting traits of the *Bacillus* isolates *in-vitro* and their anti-fungal activity against the test fungus *Alternaria burnsii.*

CONCLUSIONS

The present study reflects the role of *Bacillus* species as an excellent PGPR for an ecologically fragile and nutritionally poor soil and climatic condition. The phosphate solubilizing *Bacillus* isolate varied widely for various plant growth promoting traits like, production of IAA, ACC deaminase, protease, pectinase, cellulase, chitinase, and hydrogen cyanide and carbon utilization.

The *Bacillus* isolates showed variation in their functional role within a species as well as between species. Among them, *Bacillus amyloliquefaciens* strain BCRh10 adjudged most efficient plant growth promoting agent and showed both direct as well as indirect PGP traits. Several of the PGP agents also showed proteinase, cellulase, pectinase, and chitinase activity as well as siderophores and HCN production, and hence, regarded potent PGPR agents.

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