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Plant Growth Promoting Activities, Biofilm Formation and Root Colonization by *Bacillus* sp. Isolated from Rhizospheric Soils

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Efficient biofilm forming PGPR is expected to perform better under field conditions due to enhanced root colonization and survival under stress condition. Therefore, in this study 14 isolates of *Bacillus* sp. were selected after random screening based on multiple plant growth promoting traits like phosphate solubilization, production of indole acetic acid, siderophore, HCN, ammonia, EPS and ACC deaminase activity. Biofilm formation was studied *in vitro* by using 96 well polystyrene plate. Based on *in vitro* biofilm formation these isolates were grouped as moderate and strong biofilm former. Two isolates of *Bacillus subtilis* (BD6 and BCH4) identified by 16S rRNA gene sequence analysis exhibited multiple PGP traits but differed in their biofilm forming capacity were evaluated for root colonization and biofilm formation on the root surface. Biofilm was characterized by scanning electron and confocal laser scanning microscopy. Colonization studies revealed that isolate BCH4 colonized wheat roots more strongly compared to BD6 and form a biofilm on the root surface. Inoculation response to wheat showed an increase in plant vegetative growth parameters compared to control in a pot culture assay indicating that BCH4 a strong biofilm former with multiple PGP traits may be considered for further development as bioinoculant.

Key words: Confocal laser scanning microscopy; *Bacillus subtilis*; Biofilms; PGPR; *Triticum aestivum*; 16S rRNA gene analysis; root colonization.

Plant growth promoting rhizobacteria (PGPR) are the diverse class of microbes that inhabit the plant root as ectophytes or endophytes, enhancing the host plant growth by both direct and indirect mechanism. The application of beneficial rhizobacteria can help in reducing dependency on chemical fertilizers in enhancing agriculture production[1]. PGPR can stimulate plant growth by increasing the plant nutrition by several mechanisms like the production of plant growth hormones, nitrogen fixation and phosphate solubilization, siderophore production[2-4]. Another mechanism of plant growth promotion took place through the biocontrol activity and induced systemic resistance as well as by favoring rhizobia or mycorrhizal symbiosis[5].

Various species of genus *Bacillus* are well recognized for their plant growth promoting properties[6]. The members of this genus have unique characteristics due to the formation of endospore, production of peptide antibiotics and extracellular enzymes. These properties make this genus survive better in different environments for the long duration, under field condition[7].

In principle, plant growth promoting agent like the genus *Bacillus* as bioinoculant should build and sustain their critical population size to impart the benefits for efficient plant growth[8, 9]. Therefore, keeping in view of the problem of survival and long term sustenance after inoculation, it is proposed that PGPR with biofilm-forming capability can efficiently colonize, survive and sustain better under different field conditions compared to poor

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biofilm former. In the recent past, the significance of biofilm forming beneficial rhizobacteria in the survival of bioinoculant on plant root surfaces and plant-bacterial communication have gained increased attention[6, 10].

Biofilms are bacterial communities that flourish in different situations, surrounded by a protective extracellular matrix[6]. Plant root associated biofilms assist in the preservation of plant nutrients available in the form of rhizodeposits or root exudates and make them available to beneficial bacteria and, therefore, depriving the phytopathogens of these nutrients[11]. Several workers have suggested the application of biofilm forming PGPR in agriculture due to several advantages of biofilm[12].

Therefore, considering the importance of biofilm in plant growth and health and lack of concerted efforts from India, we have made an attempt in this study to isolate and assess *Bacillus* sp. exhibiting multiple plant growth promoting (PGP) traits and capable of forming biofilm under *in vitro* condition. Selected isolates differing in their biofilm forming capacity were evaluated for their colonization, biofilm formation on the root surface and their impact on plant growth.

MATERIALS AND METHODS

Isolation of *Bacillus* sp

Rhizospheric soil samples were collected from different crops grown in the vicinity of Aligarh city ($27^0 29^1$ latitudes and $72^0 29^1$longitudes), India. Rhizospheric soil samples (1 g) were suspended in 9 ml sterile distilled water and incubated in water bath at 80 ºC for 10 min and serially diluted for the isolation of *Bacillus* sp. using peptone yeast dextrose agar medium (PYD) (containing g l^{-1} peptone 2.0, yeast extract 2.0, dextrose 5.0, agar 15.0). Plates were incubated at 28 °C for 24-48h. Isolated pure colonies were subcultured on PYD agar medium for purification and maintained in glycerol as a stock culture at -80 ºC for further studies. The *Bacillus* sp. isolates were characterized biochemically using standard methods[13].

Assays for plant growth promoting activities

Indole acetic acid (IAA) production

IAA production was detected by the method of Brick *et al*.[14]. Quantitative analysis of IAA was performed using the spectrophotometric method of Loper and Scroth[15] in the presence of 100 µg ml^{-1} tryptophan. Bacterial cultures were grown in nutrient broth (NB) (containing g l^{-1} Yeast extract 1.5; Beef extract 1.5; Peptone 5.0; Sodium chloride 5.0) medium at 28°C for 48 h on the rotary shaker (120 rpm). Fully grown cultures were centrifuged at 825×g for 30 min, and two ml of supernatant was mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent. Development of pink color indicates IAA production. Optical density was measured at 530 nm with the help of spectrophotometer (UV5704SS, ECIL, India). The concentration of IAA produced by cultures was measured with the help of standard graph of IAA (Hi-Media, India) obtained in the range of 10–100 µg ml^{-1}.

Detection and quantification of siderophore production

Production of siderophore was detected by using universal assay of Shwyn and Nielands (16). Bacterial isolates were further tested for the quantification of catechol-types phenolates using the method as described by Reeves *et al*. [17]. Briefly, the isolates were grown in Modi medium (containing g l^{-1} K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.4, NaCl 0.1, Mannitol 10, Glutamine 1.0, NH_4NO_3 1.0) at 28 °C for two days on rotary shaker at 120 rpm. The fully grown cultures were centrifuged at 9168×g for 20 minutes. The supernatant was adjusted at pH 2.0 with diluted HCl. An equal quantity of ethyl acetate was added twice in a separating funnel and separated. The resultant 60 ml ethyl acetate fraction was taken. Five ml ethyl acetate fraction was mixed with five ml of Hathway's reagent, and the absorbance was read at 700 nm for benzoate and 560 nm for salicylates. The concentrations of benzoates and salicylates were calculated with the help of standard 2-3 dihydroxy benzoic acid (1-10 µg ml^{-1}) and salicylic acid (1-30 µg ml^{-1}) respectively.

Detection of phosphate solubilization activity

The test bacterial isolates were screened for their phosphate solubilization activity on Pikovskaya's (PVK) medium[18]. Freshly grown bacterial cultures were spot inoculated on Pikovskaya's medium containing tricalcium phosphate (0.5%) and incubated at 28 °C for seven days. The development of a clear zone around the bacterial growth on culture plates was considered positive for phosphate solubilization.

Quantification of phosphate solubilization

The phosphate solubilization was quantified by measuring the soluble phosphate released in the culture medium, following the method of King[19] as described by Gaur[20]. 100 ml of sterile Pikovskaya's broth was separately inoculated with fully grown culture (10^7 cells ml^{-1}) of the bacterial isolates and incubated for seven days at 28°C on a rotary shaker (120 rpm). The uninoculated medium served as control. Ten ml of culture was removed from each flask and centrifuged at 9168×g for 30 minutes. Ten ml of supernatant is transferred to tubes. To each sample 10 ml, chloromolybdic acid and five drops of chlorostannous acid was added, and volume was adjusted to 50 ml with distilled water. The developed blue color was read at 600 nm. The amount of phosphate solubilized was calculated using the calibration curve of KH_2PO_4.

Ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated at 28 °C for 48-72 h. Subsequently 0.5 ml of Nessler's reagent was added in each tube. Development of brown to yellow color was a positive test for ammonia production[13].

Detection of hydrogen cyanide (HCN) production

The test bacteria were screened for the production of hydrogen cyanide by the method of Lork (21). Briefly, nutrient agar medium (Hi-Media, India) was amended with 4.4 g of glycine l^{-1} and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5 % picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28 °C for four days. Development of orange to the red color indicated HCN production.

Exopolysaccharides production

The exopolysaccharides (EPS) synthesized by the bacterial isolates was determined as suggested by Mody *et al*.[22]. For this, the bacterial isolates were grown in 100 ml capacity flasks containing the basal medium. Inoculated flasks were incubated at 28°C for 72 h on rotary shaker at 125 rpm. The culture broth was centrifuged at 5433×g for 30 min and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying at room temperature. Each experiment was repeated at least two times.

1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity

To determine the presence of ACC deaminase, the ability of the isolates to use 1-aminocyclopropane-1-carboxylate **(**ACC) as nitrogen source was checked by growing bacterial isolates onto Dworkin and Foster's salts minimal agar medium (23). The medium was supplemented with 3.0 mM ACC (Sigma-Aldrich, USA) per liter instead of $(NH_4)_2SO_4$ as a sole nitrogen source. The heat-labile ACC was filter sterilized through sterile 0.2 µm membrane filters (Merck Millipore, USA), and the filtrate was added to the salts medium after autoclaving. The isolates were inoculated in triplicate on DF agar medium plates amended with ACC. The plates were incubated at 28 °C in the dark for three days. The growth of the isolates on DF agar medium amended with ACC (DF-ACC agar) was considered as an indicator of the efficiency of isolates to utilize ACC and to produce ACC deaminase[24].

Seed germination assay

Triticum aestivum L. (var. PBW-343) seeds were surface sterilized with 5% sodium hypochlorite (Qualigens Fine Chemicals, India) solution for 10 min followed by subsequent washing with sterilized distilled water (SDW). Surface sterilized seeds were soaked in overnight grown bacterial cultures for two h. Seeds soaked in SDW alone served as control. Seeds were placed in Petri plates containing 0.8% agar and incubated at 25°C for seven days. Three replications were maintained for each treatment. Percent seed germination, shoot, and root lengths were recorded in treatments and control.

Pot culture assay

A pot experiment was conducted to compare the plant growth promoting effects of different isolates on wheat. The experiment consists of 15 treatments and 3 replications. With pots distributed in completely randomized design (CRD): T1=control; T2=BD1(strong biofilm former); T3=BD2(moderate biofilm former); T4=BD3(strong biofilm former); T5=BD4(strong

biofilm former); T6=BD5(moderate biofilm former); T7=BD6(moderate biofilm former); T8=BD7(moderate biofilm former); T9=BCH1(strong biofilm former); T10=BCH2 (strong biofilm former); T11=BCH3 (strong biofilm former); T12=BCH4 (strong biofilm former); T13=BCH5 (strong biofilm former); T14=BCH6 (strong biofilm former); T15=BCH7 (strong biofilm former). Healthy surface sterilized seeds of wheat (var. PBW-343) were treated with bacterial cultures as described above. Ten seeds of wheat were sown in earthen pots of 3 kg capacity (25 x 22 cm) consisting of sandy loam soil. The pots were watered daily with tap water and maintained in an open field conditions. The plants were sampled after 45 days of growth for analysis of vegetative growth parameters.

16S rRNA gene sequencing and phylogenetic analysis

Sequencing of the 16S rRNA gene of isolates BD6 and BCH4 was done commercially by a DNA sequencing service (Macrogen Inc. Seoul, South Korea) using universal primers, 518F (5′CCAGCAGCCGCGGTAATACG3′) and 800R (5′TACCAGGGTATCTAATCC3′). The nucleotide sequences were found to be 799 (BD6), and 997 (BCH4) bp in size were submitted to GenBank sequence database (accession numbers: BD6 KT423119 and BCH4 KR996708). The partial sequences were analyzed with the Basic Local Alignment Search Tool (BLASTN) and compared to known bacterial sequences in NCBI GenBank using BLAST. Related sequences obtained from the database were used to construct a phylogenetic tree using MEGA5 software.

Microtitre plates assay for biofilm formation

The biofilm formation was performed using 96 well polystyrene microtitre plates as described by O'Toole and Kolter[25] with some modifications. Briefly, bacterial isolates were grown in PYD broth medium at 28 °C for 24h. Each well was filled with 200 µl (≈10^5 CFU ml^{-1}) of culture and incubated statically at 28 °C for 24 h. The wells were washed with phosphate buffer saline (PBS), followed by staining with 0.1% crystal violet for 20 min. After staining, the wells were washed with PBS. The bound crystal violet was solubilized with 95% ethanol, and absorbance was measured at 590 nm using plate reader (Thermo Scientific Multiskan EX, UK). A classification of the isolates into moderate biofilm formers, $(2 \times OD_{control}) < OD \leq (4 \times OD_{control})$, and strong biofilm formers, $(4 \times OD_{control}) < OD$, were made[26].

Confocal laser scanning microscopy of biofilms

The biofilm formation was performed on glass coverslips using the methods of O'Toole and Kolter[25] with little modifications. Briefly, bacterial isolates (BD6 and BCH4) were grown in PYD broth medium at 28 °C for 24 h. A 12 well microtiter plate containing coverslips were filled with two ml of cell culture (≈10^5 CFU ml^{-1}) per well and incubated statically at 28 °C for 24 h. Coverslips were rinsed with PBS and were stained with acridine orange for 10 min. Microscopic observations and image acquisitions were performed with a CLSM system (Olympus Fluoview FV1000). The excitation and emission wavelengths were 488 and 525 nm respectively. Images were obtained using 100x oil immersion lenses with UPlanSApo 100x/1.40 objective with an additional zoom of 3x. Each coverslip was scanned at five randomly selected positions for biofilm visualization.

Root colonization assays for *Bacillus* isolates

Triticum aestivum L. (var. PBW-343) surface sterilized seeds were treated with selected cultures (BD6 and BCH4) as described above. Treated and untreated seeds were sown directly in earthen pots containing sterile sand-soil mixture (autoclaved four times at a span of two days). The wheat plants were grown for 45 days under open pot conditions. Three replicates for inoculated and control treatment were maintained. The soil water content of each pot was adjusted daily with sterile distilled water until the seed germination and after the emergence of shoot the pot is watered daily with Hewitt plant nutrient solution (containing g 100 ml^{-1} $Ca(NO_3)_2$ 32.8, KNO_3 20.8, $MgSO_4.7H_2O$ 18.4, $NaH_2PO_4.2H_2O$ 20.8, $FeC_6H_5O_7.3H_2O$ 5.98, $MnSO_4.4H_2O$ 2.23, $CuSO_4.4H_2O$ 0.25, $ZnSO_4.7H_2O$ 0.29, H_3BO_3 1.86, $(NH)_6Mo_7.O_{24}.4H_2O$ 0.08) (27). Three to five plants were randomly uprooted at 15, 30, and 45 days after sowing. The wheat root samples (1gm) was macerated in 9ml sterile normal saline solutions (NSS) vortexed vigorously to remove root-adhering bacteria. Viable plate count of *Bacillus* isolates was determined by plating 0.1 ml of different dilutions on PYD agar medium containing 100 µg ml^{-1} rifampicin to which the isolates were resistant. Plates were incubated at 28

°C for 24 h, the number of colonies was counted and expressed as log CFU g^{-1} of roots.

Scanning electron and confocal microscopy of biofilms formed on the roots of wheat

Surface sterilized seeds of *Triticum aestivum* L. (var. PBW-343) were sown in sterile sand-soil mixture (autoclaved four times at a span of two days) for raising seedlings. The soil water content of pot was adjusted daily with sterile distilled water until the seed germination and after the emergence of shoot the pot is watered once in every three days with Hewitt plant nutrient solution (27). After 15 days of growth, the plant was removed from the pot, and roots were washed with sterile distilled water. Roots of the wheat were inoculated by soaking for 24 hrs in 10 ml diluted overnight cultures of Bacillus sp. BD6 and BCH4 ($\approx 10^5$ CFU ml^{-1}) prepared in PYD broth medium. In the control plants, SDW was used. After 24 h root samples had been fixed in 2.5% glutaraldehyde, they were washed in the same buffer and then fixed in osmium tetraoxide. The samples were dehydrated through a graded ethanol series and dried in CO_2 with a critical point-dryer, mounted on stubs, and shadowed with gold, (Sputter Coater - Polaron SC7640, UK) for viewing under an SEM (Carl Zeiss EVO 40, Germany) at 20 kV at various magnifications. All images were computer-processed. For CLSM, the 24 h treated root samples were stained with acridine orange for 10 min and images was acquired as mentioned in CLSM of coverslip biofilms.

Statistical analysis

All experiments were repeated at least two times in triplicate. The data obtained on seed germination, and pot experiments were analyzed using the analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at $P \leq 0.05$ with the software SPSS ver.21.00.

RESULTS

Bacillus isolates from rhizospheric soil samples were isolated and characterized as Gram-positive rod and found positive for the biochemical tests such as citrate, nitrate reduction, gelatin liquefaction, and utilized various carbohydrates (Table 1). Isolates exhibiting morphological, cultural and biochemical traits similar to *Bacillus subtilis* were considered for further investigation. All the isolates were screened for plant growth promoting activities. The selected, 14 isolates showed multiple plant growth promoting activities, like the production of IAA, siderophore, hydrogen cyanide, ammonia, ACC deaminase, exopolysaccharides and phosphate solubilization (Table 2). The quantitative analysis showed that Indole acetic acid production in the presence of 100 µg ml^{-1} of tryptophan range from 17.80 to 48.53µg ml^{-1}. Phosphate was solubilized in the range of 92.86 to 300.86 µg ml^{-1}. Siderophore production was found in the range of 0.24 to 32.40 µg ml^{-1} for salicylates and 0.63 to 7.75 µg ml^{-1} for benzoates. All the test isolates were found to produce the significant amount of exopolysaccharides ranging from 0.77 to 3.72 g l^{-1}. Ammonia production was exhibited by all the isolates. ACC deaminase activity was exhibited by all the isolates except BD2 and BCH6. Whereas hydrogen cyanide production was detected in fifty percent isolates.

Table 1. Biochemical characteristics of *Bacillus* isolates

Biochemical characters	*Bacillus* sp.
Pigmentation	-
Colony morphology	White, fragile, smooth colony, fried egg type, serrated margins
Gram reaction	Gram +ve
Cell shape	Rods
Growth at 4°C	-
Growth at 41°C	+
Growth at 44.5°C	+
Oxidase	-
Growth on N_2 free medium	-
Nitrate reduction	+
Indole	-
Methyl red	-
Voges-Proskuer	+
Citrate utilization	92
Catalase	100
Hydrolysis of Starch	84
Gelatin liquefaction	100
Carbohydrate utilization	
Glucose	96
fructose	100
Sucrose	100
Mannitol	94

* Values are in percent of isolates tested

Table 2. *In vitro* plant growth promoting activities of *Bacillus* sp

Isolate no.	IAA* (µg ml^{-1})	Phosphate solubilization (µg ml^{-1})	Siderophores (µg ml^{-1})		EPS g l^{-1}	ACC deaminase	HCN	NH_3
			SA	2,3-DHBA				
Control**	19.84±1.05	38.28 ± 2.90	1.20 ± 0.01	0.57 ± 0.6	0.70 ±0.30	-	-	+
BD1	35.95±1.98	223.86±10.0	4.45±0.36	1.67±0.21	0.86±0.25	+	-	+
BD2	15.90±1.09	ND	ND	ND	0.78±0.10	-	-	+
BD3	34.91±1.96	202.86±7.91	4.63±0.21	2.75±0.25	0.95±0.09	+	+	+
BD4	33.01±2.10	212.35±11.1	4.51±0.25	1.85±0.26	1.09±0.05	+	-	+
BD5	26.62±1.23	153.99±9.23	2.67±0.35	ND	0.77±0.04	+	+	+
BD6	23.81±1.56	94.45±4.23	1.79±0.25	ND	1.45±0.09	+	+	+
BD7	17.80±2.11	101.86± 9.33	0.24±0.10	ND	1.07±0.18	+	-	+
BCH1	40.01±1.96	92.86±8.11	6.20±0.40	3.19±0.30	1.86±0.20	+	+	+
BCH2	31.73±0.98	248.86±12.1	1.96±0.21	ND	1.66±0.21	+	-	+
BCH3	32.59±0.67	203.99±11.5	4.51±0.20	2.92±0.30	0.79±0.10	+	-	+
BCH4	48.53±1.39	300.86±15.2	32.40±1.29	6.63±0.26	3.72±0.32	+	+	+
BCH5	29.32±1.02	222.35±14.3	3.62±0.23	2.57±0.15	2.05±0.15	+	+	+
BCH6	43.95±0.91	198.86±10.9	2.67±0.30	0.63±0.09	2.56±0.20	-	+	+
BCH7	45.11±0.86	294.86±12.1	30.85±0.19	7.75±0.10	2.09±0.20	+	-	+

** NRRL-B-14596, *IAA at 100 ?g ml?1 of tryptophan, IAA: Indole acetic acid, SA: Salicylic acid, 2, 3-DHBA: 2, 3-dihydroxybenzoic acid, EPS: Exopolysaccharides, ACC: 1-Aminocyclopropane-1-carboxylate, HCN: Hydrogen cyanide, NH3: Ammonia., ND: Not detected.

Table 3. Inoculation effect of *Bacillus* isolates with varying capacity of biofilm formation on growth of wheat plant under pot conditions after 45 days of sowing

Treatment	Root length (cm)	Shoot length (cm)	Fresh weight (g plant^{-1})	Dry weight (g plant^{-1})
Control	5.08±0.12^{n}	23.36±0.15^{m}	1.53±0.10^{l}	0.69±0.09^{g}
BD1	8.38±0.12^{c}	30.1±0.15^{i}	2.91±0.07^{e}	1.18±0.02^{cde}
BD2	7.41±0.07^{i}	32.10±0.10^{c}	2.48±0.17^{h}	1.03±0.06^{def}
BD3	8.61±0.07^{b}	31.76±0.15^{d}	3.76±0.08^{b}	1.53±0.07^{ab}
BD4	8.13±0.06^{d}	31.15±0.05^{e}	3.02±0.06^{de}	0.93±0.06^{efg}
BD5	7.58±0.12^{gh}	30.56±0.15^{g}	3.50±0.13^{c}	0.89±0.09^{efg}
BD6	7.76±0.15^{fg}	28.20±0.10^{l}	2.31±0.11^{i}	0.93±0.06^{efg}
BD7	7.0±0.10^{j}	29.61±0.12^{k}	3.10±0.10^{d}	0.83±0.05^{fg}
BCH1	8.01±0.07^{de}	32.61±0.07^{b}	3.81±0.07^{b}	1.25±0.05^{bcd}
BCH2	8.70±0.17^{b}	30.90±0.10^{f}	4.0±0.09^{a}	1.38±0.15^{abc}
BCH3	7.83±0.15^{ef}	29.91±0.07^{j}	2.71±0.07^{g}	0.71±0.53^{g}
BCH4	9.85±0.05^{a}	32.91±0.03^{a}	4.15±0.05^{a}	1.58±0.07^{a}
BCH5	6.79±0.01^{l}	29.45±0.08^{k}	2.85±0.06^{ef}	0.90±0.07^{efg}
BCH6	6.60±0.20^{m}	30.30±0.10^{hi}	1.93±0.07^{k}	0.95±0.05^{efg}
BCH7	6.81±0.07^{k}	30.40±0.22^{gh}	2.1±0.10^{j}	1.38±0.15^{abc}

*Column values followed by a different letters are significantly different ($p \leq 0.05$) in Duncan's multiple range test

The level of biofilm formation among *Bacillus* was assessed under *in vitro* conditions using the crystal violet staining method. It was observed that the isolates express their biofilm forming ability as rings formed at the side of wells in 96 well polystyrene microtitre plate. Our study demonstrated that ten isolates (BD1, BD3, BD4, BCH1, BCH2, BCH3, BCH4, BCH5, BCH6, and BCH7) form strong biofilm where as remaining four isolates (BD2, BD5, BD6 and BD7) exhibited moderate biofilm forming ability (Figure 1). Above, fourteen isolates were further tested for their plant growth promoting effect on wheat in plate assay and open pot cultures methods (Figure 2 and Table 3). These isolates enhance varying level of seed germination, seedling growth and vegetative parameters of a wheat plant compared to control. Interestingly, isolate BCH4 was found most effective as compared to other isolates. Further, two isolates BD6 and BCH4 with different *in vitro* biofilm forming ability were subjected to 16S rRNA gene sequence analysis, showed close relatedness with *Bacillus subtilis* (Figure 3) with accession numbers KT423119, KR996708 for BD6 and BCH4 respectively.

Root colonization potential of selected two isolates (BD6 and BCH4) having different *in vitro* biofilm formation capability was evaluated. Surface sterilized seeds of wheat treated with BD6 and BCH4 were sown in different pots and investigated for their root colonization potential by determining viable plate count at different time intervals after sowing (Figure 4). The viable count of treated seeds was ≈ log 8.30 CFU per seed. The study revealed that the population count of BCH4 was comparatively higher than the population of BD6.

Biofilm formed by BD6 and BCH4 were further studied on the glass surface using confocal laser scanning microscopy as depicted in Fig. 5. The results revealed that BCH4 formed dense biofilm compared to BD6. Confocal laser scanning microscopy (CLSM) analysis of biofilm formed on glass coverslips showed bacterial accumulation and biofilm formation whereas some aggregation and weak biofilm formation was observed in BD6. The average thickness of the BCH4 biofilm was found to be 26.54µm.

The interaction of these test isolates with wheat seedlings were evaluated using scanning electron and confocal laser scanning microscopy. Root sections of wheat bacterized with BD6 and BCH4 were examined. The scanning electron microscopy images revealed that cells of BCH4 were evenly scattered on the surface of roots (Figures 6e to j) compared to BD6, which showed

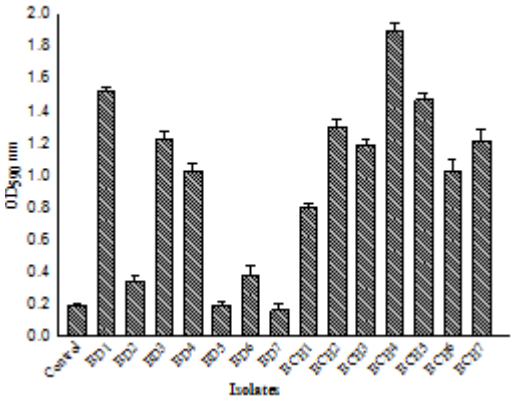

Fig. 1. Biofilm formation on 96 well polystyrene microtiter plate under stationary conditions. Biofilm was examined after 24 h of incubation. Error bars represent SD

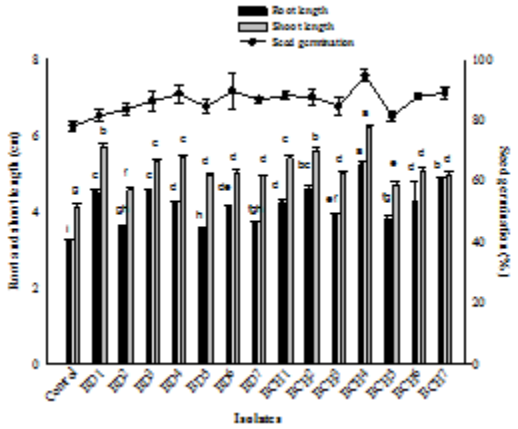

Fig. 2. Inoculation effect of *Bacillus* isolates on seed germination and growth of wheat. Bar values followed by a different letters are significantly different (P≤ 0.05) in Duncan's multiple range test

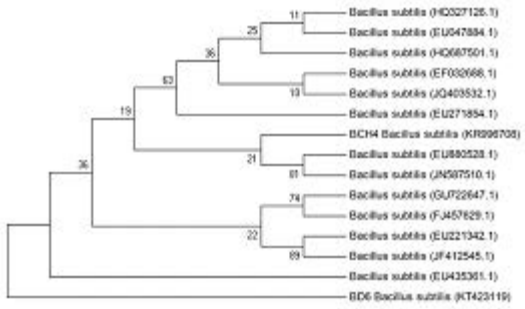

Fig. 3. Phylogentic tree of *Bacillus subtilis* strains (BD6, BCH4) based on 16S rRNA gene sequence. Numbers in brackets are GenBank accession numbers

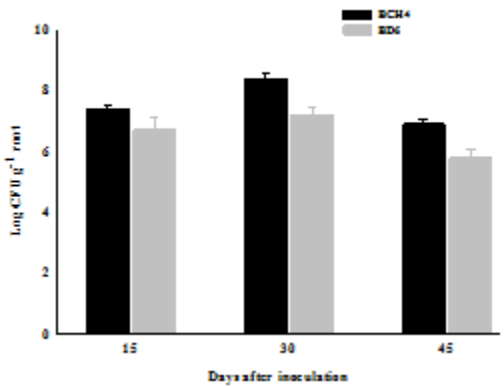

Fig. 4. Survival and colonization of BD6 and BCH4 with wheat rhizoplane sterile soil conditions after 45 days of sowing. Error bars represent SD

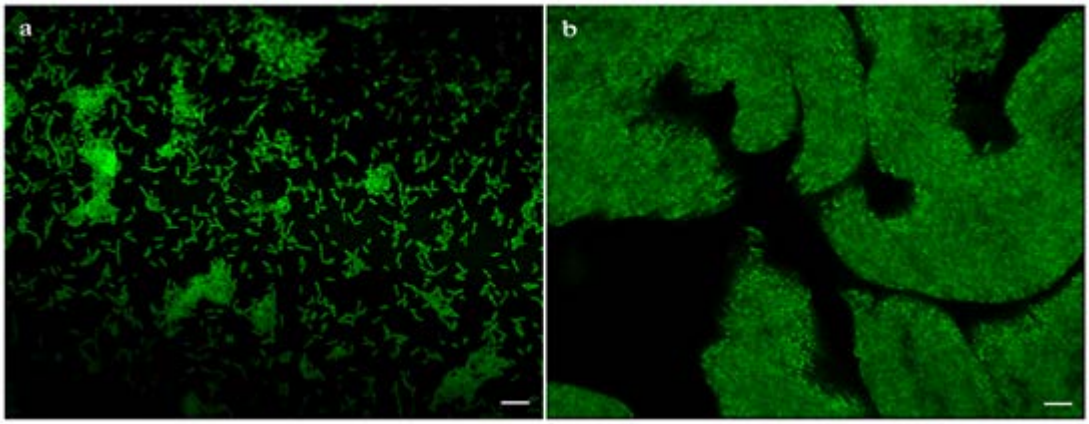

Fig. 5. Confocal laser scanning micrographs of biofilm formed by *Bacillus subtilis* on glass coverslip. (a) BD6, (b) BCH4. Biofilms were incubated for 24 h and stained with acridine orange. Magnification is 100x. Scale bars = 5 µm.

sparsely scattered bacterial cells and thin biofilm (Figures 6a to d). Figures (6g to h) clearly showed that *Bacillus subtilis* BCH4 heavily colonizes the root surface forming dense carpet-like configuration of test bacterial cells attached to the root surface. Whereas few attached bacterial cells were observed in control (Figures 6k to l). In the same way, when BCH4 biofilm was assessed using confocal laser scanning microscopy identical setup of colonization and biofilm formation on the root surface and hairs of wheat seedlings was revealed (Figures 7c to e). Whereas the moderate level of biofilm formation was depicted by isolate BD6 (Figures 7a to b).

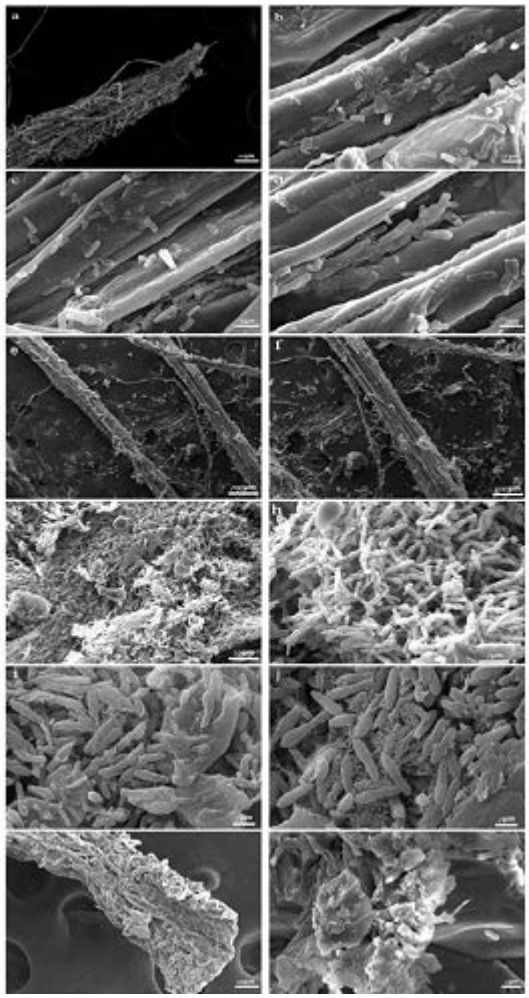

Fig. 6. Scanning electron microscopy images of *Triticum aestivum* seedling roots colonized by *Bacillus subtilis.* (a-d) Colonization and diminished biofilm formation on the root surface by BD6. (b) Magnified image of figure 6a. (c-d) Magnified images of figure 6b. (e-j) Colonization and biofilm formation on *Triticum aestivum* roots by strain BCH4. (f) Magnified image of figure 6e. (g-h) Large scale colonization and biofilm formation with extracellular matrix. (i-j) Magnified images of figure 6j. (k-l) Section of root in control seedlings.

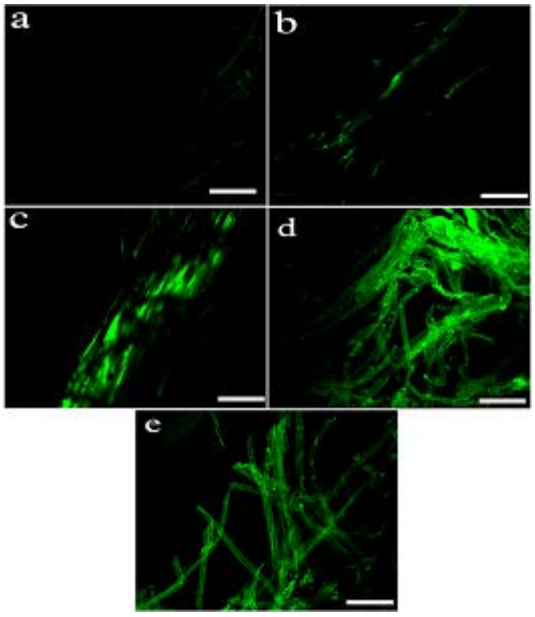

Fig. 7. Confocal laser scanning microscopy images of *Bacillus subtilis* biofilm grown on *Triticum aestivum* roots. (a-b) Colonization and diminished biofilm formation by strain BD6. (c-e**)** Colonization and biofilm formation on the root hairs and root surface by BCH4. Biofilm were examined 15 days after sowing and stained with acridine orange. Magnification is 20x. Scale bars = 50 µm.

DISCUSSION

The associations of beneficial bacteria with the plant roots play a significant role in shaping the course of plant growth and development. The plant growth promoting rhizobacteria are well known for their contribution in several ecosystem processes. The PGPR are supposed to survive and increase their population in rhizosphere microhabitat and compete with indigenous microbial population, at least for the duration required to impart their beneficial effects on plants[28]. Therefore, many new approaches are needed for the efficient use of PGPR, to maintain the sustainable agro-technological practice. It is now common knowledge that PGPR form biofilm[6]. Thus, biofilm forming PGPR are supposed to colonize and survive more efficiently

for the longer period and can increase the plant growth consistently. In view of this, we isolated *Bacillus* from the rhizosphere of different crops mainly wheat and chickpea cultivated in the vicinity of Aligarh, India and assessed for their plant growth promoting (PGP) activities. Fourteen isolates exhibiting cultural traits similar to *Bacillus subtilis* and multiple PGP traits were selected in this study. Varying level of indole acetic acid (IAA) at 100 µg ml^{-1} was recorded. Isolate BCH4 produced the highest amount of IAA Similar results for IAA production in the presence of tryptophan have been described by other workers[29-31]. The presence of tryptophan in plant root secretions increases the IAA concentration, consequently increasing the plant growth[32]. It is reported that IAA produced by plant-associated bacteria help in increasing root growth[33]. The soil is a reservoir for a variety of phosphate, in addition to inorganic and organic phosphate. Many PGPR including *Bacillus* can solubilize insoluble phosphate through various mechanisms[34]. In our study all the isolates except BD1 were found positive for phosphate solubilization. *Bacillus* sp. has been demonstrated earlier for phosphate solubilization including other PGPR[3]. It has been reported that many PGPR increase the plant growth through siderophore production. Siderophore plays a significant role in plant growth and development. Siderophores secreted by microbes get attached with iron with high precision and blocked the iron supply to plant pathogens[35,36]. In our study, varying the level of siderophore production was recorded in 93 % isolates. The test bacterial isolates were found positive for hydrogen cyanide and ammonia production. A similar result has also been reported by Ahmad *et al,*[30]. The possession of 1-aminocyclopropane-1-carboxylate (ACC) deaminase is a key trait that facilitates the plant growth. This enzyme is capable of splitting the ethylene precursor, ACC, into ammonia and α-ketobutyrate[37]. Therefore, by reducing the levels of ACC in plants, ACC-deaminase producing microorganisms decrease the ethylene levels, which when present in high concentration can lead to stunted growth and ultimately, plant death[35]. In our study, all the isolates except BD3 and BCH6 were found positive for ACC deaminase activity. The test isolates were also found to produce exopolysaccharides. Bacterial exopolysaccharides work as a binding agent for soil, provide protection to microbes from different environmental stress conditions and assist in root colonization and biofilm formation[38].

Inoculation of wheat seeds with *Bacillus* enhances the growth of wheat seedlings, where BCH4 was found to significantly ($p= 0.05$) increase the growth as compared control. Moreover, a key component that should be considered during evaluation of PGPR is their activity in different soil and climatic conditions in which they will be supposed to be applied. In our investigation, the bacterial isolates significantly enhanced the vegetative plant growth parameters compared to uninoculated control after 45 days of sowing. Our study is in agreement with the recent report by Rana *et al.,*[39].

Further, the *in vitro* biofilm forming capability of all the fourteen isolates demonstrated that BCH4 forms the strong biofilm that may provide bacterium an additional advantage for its ecological fitness. Among all the investigated isolates, BD6 and BCH4 were selected based on their PGP traits, biofilm forming ability and level of plant growth promotion and identified as the new strain of *Bacillus subtilis* by 16S rRNA gene sequence analysis.

Colonization and the persistence of beneficial bacteria on plant roots are significant in imparting benefits for plant growth and development. For this, we selected two isolates (BD6 and BCH4) with different biofilm forming potential *in vitro* and examined for their ability to form the biofilm on plant root surfaces. The biofilms formed were characterized using confocal laser scanning, and scanning electron microscopy. The observation revealed that the isolate BCH4 showed strong biofilm forming capability on plant roots as compared to BD6. The root colonization data of these two isolates revealed that BCH4 colonized the wheat root more efficiently and survived up to 45 days after inoculation as compared to BD6, which might be due to its biofilm forming ability. Our findings are in support with some workers who have demonstrated biofilm by *Bacillus* sp. from other parts of the world[6].

CONCLUSION

This is probably the first report from India, highlighting the role of *Bacillus* biofilm in selecting the efficient strain for bioinoculant development. BCH4 could be considered as new promising PGPR for further investigation. On the other hand, the role of biofilm in rhizosphere under the natural condition is to be explored to understand the complex mechanism of plant-microbe interaction.

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