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Burkholderia sp. from rhizosphere of *Rhododendron arboretum*: Isolation, identification and plant growth promotory (PGP) activities

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Abstract: Plant growth promoting rhizobacteria (PGPR) is beneficial bacteria that colonize plant roots and enhance plant growth by wide variety of mechanism like phosphate solubilisation, etc. Use of PGPR has steadily increased in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements. The present research work was designed to isolate and characterize the PGP activity of *Burkholderia* sp. For this purpose rhizospheric soil from *Rhododendron arboreum* of Kumaun Himalaya was collected and efficient bacterial strain was screened on the basis of phosphate solubilization. Further, assessment of various parameters of plant growth promotion activity was done and enhanced production of IAA (16.4 $\mu g ml^{-1}$) and (20.8 $\mu g ml^{-1}$) was observed in the presence of 250$\mu g ml^{-1}$ and 500 μg ml^{-1} of tryptophan, respectively. Correspondingly, in respect of 7.8 μg ml^{-1} IAA without tryptophan, and their confirmation was executed by TLC. A remarkable change in color from green to reddish-brown zone on CAS plates, suggests the positive result for siderophore production, and finally the seed germination and pot trial experiment depicted the growth index of wheat plant. Therefore, the present study suggests that *Burkholderia* sp. is beneficial for plant growth promotion.

Keyword: *Burkholderia* sp., IAA, PGPR, Phosphate solubilization, Siderophore

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are group of bacteria that actively colonize plant roots and increase plant growth and yield. The mechanism by which PGPR promote plant growth is not fully understood, but it is thought to include the ability to produce phytohormones, asymbiotic N fixation against phytopathogenic microorganisms by production of siderophores, synthesis of antibiotics, enzymes and fungicidal compounds (Ahmad *et al.*, 2006). In this connection the beneficial, root-colonizing, PGPR are defined by three intrinsic characteristics: (i) they must be able to colonize the root, (ii) they must survive and multiply in microhabitats associated with the root surface, in competition with other micro biota, at least for the time needed to express their plant promotion/protection activities, and (iii) they must promote plant growth. Several novel techniques to identify and characterize the PGPR and to study the colonization pattern and molecular determinants of root colonization have been extensively discussed (Gamalero *et al.*, 2004). PGPR can affect plant growth by different direct and indirect mechanisms (Naz *et al.*, 2012) like enhanced resistance to pathogenic diseases (Shobha and Kumudini, 2012). Direct methods of increasing plant growth are through production of phytohormones, such as auxin, cytokinin and gibberellin. Screening PGPR isolates for quantity of auxin production has been proposed as a mechanism for selecting PGPR species for wheat inoculation (Khalid *et al.*, 2004). These studies indicate that higher the plant growth promoting activities of rhizobacteria, higher is the chance of increased plant growth under different conditions. Hence, isolation and characterization of strains with higher plant growth promoting abilities, adapted under certain climates, can be very useful for the production of effective biofertilizers (Dastager *et al.*, 2010; Abbas *et al.*, 2010). A number of different bacteria promote plant growth, including *Azotobacter sp.*, *Azospirillum sp.*, *Pseudomonas* sp., *Bacillus sp.* and *Acetobacter sp.* (Turan *et al.*, 2006). Plant growth promoting bacteria are important in managing plant growth because of their effects on soil conditions, nutrient availability, growth and yields. Therefore, the aim of this study was to check various PGPR activities of isolated and screened, phosphate solubilizing bacteria. For this purpose the PGP rhizobacteria was isolated as well as screened out and in conclusion characterized on the base of their direct (IAA production) and indirect (Siderophore production) plant growth promoting activities along with the measurement of their growth

index.

MATERIALS AND METHODS

Isolation, screening and biochemical characterization of bacterial strain: Bacterial strain was isolated from rhizospheric soil of *Rhododendron arboreum* of Kumaun Himalayan region. The rhizospheric soil sample were taken from a depth of 0-10cm, kept in plastic bags and carried to the laboratory. The pure culture was isolated by using serial dilution and streak plate technique to obtain single colony. The culture was screened out on the basis of its ability to solubilize insoluble inorganic phosphate by spotting overnight grown cultures on Pikovskaya's agar plates and incubated at 30ºC. Isolated strain, showed clear zone in the plate around the colony after 48 to 72 hrs of incubation.

Further, the identification of bacterial strain BS_4 was carried out according to Bergey's manual on systematic bacteriology (Holt *et al.,* 1994). Catalase activity was determined by detective bubble formation with 3% H_2O_2 solution. Oxidase test was determined by using bacteriological differentiation oxidase disc (Hi-Media Laboratories, India). Other important biochemical properties were performed by using biochemical test kits (KB001, KB002 HiAssorted^{TM}).

Molecular characterization: Based on screening and biochemical experiment, potential strain BS_4 was characterized on the basis of 16S rRNA sequencing. Polymerase chain reaction (PCR) amplification of the partial 16S rRNA gene region was carried out with the bacterial primer set FD1: 5`-ccgaattcgtcgacaac AGAGTTTGATCCTGGCTCAG-3`and RP2: 5`-cccgggatccaagcttACGGCTACCTTGT TACGACTT-3`, using the Gene amp kit (Applied Biosystems). PCR System 9700, U.S.A Pikovaskaya, 1948. About 50 -100 mg of the purified DNA was sequenced using BigDye Terminator Cycle. Sequencing Kits (v3.1) (Applied Biosystems, USA) the templates were purified by Ethanol/EDTA precipitation method and sequenced on ABI 3730xls Genetic Analyzer (Applied Biosystems) at Bioserve Biotechnologies, Pvt. Ltd. Hyderabad, Andhra Pradesh, India. The sequence was then analyzed by basic local alignment search tool (BLAST) at National Center for Biotechnology Information, USA (NCBI) database. Further, the isolate was identified based on the similarity scores. Phylogenetic tree was constructed using the unweighted pair group mean average (UPGMA) tree method and the phylogenetic data were obtained by aligning the different sequences of the 16S rRNA of closely related strains.

Quantitative estimation of phosphate (Pi) and indole-3-acetic acid (IAA) production: The isolated strain BS_4 was further examined for its ability to release inorganic phosphate from tricalcium phosphate (TCP) in broth medium. One ml of overnight culture of the isolate was inoculated to 100 ml of Pikovskaya's broth (Pikovaskaya, 1948). The inoculated flask was incubated at 30°C. The amount of inorganic phosphate released in the broth was estimated by sampling broth culture at every 24 hours. The broth cultures were centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the cell growth and insoluble phosphate. The available Phosphate in the supernatant was estimated by phosphomolybdic blue colour method. One ml of the culture supernatant was taken in a 50 ml volumetric flask to which 10 ml of chloromolybdic acid was added and mixed thoroughly. The volume was made up to approximately three fourth with distilled water and 0.25 ml chlorostannous acid was added to it. Immediately, the volume was made to 50 ml with distilled water and mixed thoroughly. After 15 minutes, the blue colour developed was read in a spectrophotometer at 610 nm using a reagent blank. Concurrently, a standard curve was prepared using various concentrations of phosphate solution. The amount of phosphorus solubilized by the strain BS_4 was calculated using the standard curve.

Quantification of IAA, by strain BS_4 was done by inoculating three 250 ml conical flasks containing 100 ml of minimal salt medium each, supplemented with tryptophan at a concentration of 500 µg/ ml, 250 µg/ ml and control without tryptophan, and incubated at 30°C under shaking. The amounts of IAA produce in the broth of all flasks were estimated by sampling broth culture after every 24 hours. Broth culture was centrifuged at 7500 rpm for 10 minutes. Two ml of aliquot of the supernatant of the cultures was mixed with two drops of orthophosphoric acid and 4 mL of Salkowski reagent (50 ml, 35% perchloric acid; 1 ml 0.5 M $FeCl_3$) (Noori and Saud, 2012) and incubated at 30°C for 25 minutes. Absorption was read at 530 nm and the concentration of IAA in the bacterial strain was determined and quantified by comparison with a standard curve of IAA (Reddy *et al.,* 2011).

Extraction of crude IAA and thin layer chromatography (TLC): Single bacterial colony of strain BS_4 was inoculated in 100 ml nutrient broth supplemented with 5 mg/ml tryptophan and incubated at 30°C under shaking. Bacterial culture were centrifuged at 7500 rpm for 10 minutes to separate the supernatant and subsequently acidified to pH 3 with 1N HCL. Extraction was done twice with ethyl acetate at double the volume of supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotary evaporator at 40^{o}C. The extract was dissolve in 0.5 ml methanol and kept it at -20^{o}C. TLC of extracted ethyl acetate fraction (10-30µl) was done and developed in chloroform: ethyl acetate: formic acid (5:3:2). Spot with RF value identical to standard IAA were identified under UV light (254 nm) by spraying the plates with salkowski reagent.

Seed germination test and pot experiment: Effect of IAA produced by strain BS_4 on the growth of plant

was also studied by seed germination test (Strom and Garhardson, 1988) and pot experiment (Kravchenko, 2004). Wheat (*Triticum aestivum*) seeds were surface sterilized by exposing to 95% ethanol and immersing in 0.2% $HgCl_2$ solution for 3 min. The seeds were then subjected to five times washing with sterile distilled water. One ml of overnight grown bacterial culture (10^7-10^6 cells/ml, 0.6 of O.D at 540 nm) was applied on each seed for 10 min and treated seeds were dried. For seed germination test the sterile non treated dried seeds as control, soaked with non inoculated media for 10 min and the treated dried seeds were sown on soft agar (0.8%) plates under axenic condition and incubated them at 30°C for 5 days. The percent seed germination and root length were measured. For Pot experiment, soil sample were collected, air dried, sieved and sterilized three times repeatedly by autoclaving before filling the pots. Finally, the sterile non treated dried seeds as control, soaked with non inoculated media and the treated dried seeds were transfer to pots containing sterile soil to a depth of 5 mm, the experiment was performed in triplicates. The pots were kept in green house for 15 days under daily observation. After 15 days the plant were uprooted carefully and the length of root and shoot were measured.

Detection of siderophore production: The analysis of siderophore production by the bacterial strain BS_4 was performed by Chrome Azurol S (CAS) method**.** The CAS plates were used to check for the presence of siderophore. The CAS assay is the universal chemical assay for the detection of siderophores. It is based on the high affinity of siderophores for ferric iron, where by ferric iron bound to dye, is released from the dye. The blue color of the medium is due to the dye iron complex. When siderophore is added, the siderophore binds to the ferric iron, releasing the free dye, which is orange in colour.

Fe3+ -dye (blue) + siderophore

Fe3+-siderophore + dye (orange)

Hence, the presence of siderophore is indicated by a color change from blue to orange.

The CAS plates were prepared in three separate steps.

(1) Preparation of CAS indicator solution.

(2) Preparation of basal agar medium.

(3) Preparation of CAS agar plates.

Preparation of CAS indicator solution: Initially, 60.5 mg of chrome azurol S was dissolved in 50 ml of ddH_2O. 10 ml of Ferric ion solution (27 mg $FeCl_3 \cdot 6H_2O$ and 83.3 µL concentrated HCl in 100 ml ddH_2O) was added, along with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml ddH_2O. The HDTMA solution was added slowly while stirring, resulting in a dark blue solution (100 ml total volume), which was then autoclaved.

Preparation of basal agar medium: In a 250 ml flask, 3 gm 3-(N-morpholino) propane sulfonic acid (MOPS) (0.1 M), 0.05 gm NaCl, 0.03 gm KH_2PO_4, 0.01 g ammonium chloride (NH_3Cl), and 0.05 gm L-asparagine was dissolved in 83 ml ddH_2O. The pH of the solution was adjusted to 6.8 using 6 M NaOH. The total volume was brought to 88 ml using RO water, and 1.5 g agar was added to the solution while stirring and heating until melted. The solution was then autoclaved.

Preparation of CAS agar plate: The autoclaved basal agar medium was cooled to 50ºC in a water bath. The CAS indicator solution was also cooled to 50ºC, along with a 50% solution of glucose. Once cooled, 2 ml of the 50% glucose solution was added to the basal agar medium with constant stirring, followed by 10 ml of the CAS indicator solution, which was added carefully and slowly along the walls of the flask with constant stirring, but at a speed so as not to generate any bubbles. Once mixed thoroughly, the resulting solution (100 ml) was poured into sterile plastic plates, each plate receiving approximately 25 ml of blue agar. Under minimal iron conditions, siderophore is produced and released into the culture medium. To detect the siderophore production strain BS_4 was grown in iron-restricted (0.5 µM added iron) modified minimal medium. After 24 hours of growth, the culture was inoculated to CAS plates and the plate was incubated at 30°C temperature to develop. The production of siderophore was confirmed by the presence of an orange colour halo zone around the colony.

RESULTS AND DISCUSSION

PGPR colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms. The present study deals with the isolation and characterization of various plant growths promoting activity of strain BS_4, to execute this, the strain has been isolated, screened and assessed for various parameters indicative of both the direct and indirect mechanisms of plant growth promotion along with wheat growth index by pot trial experiment.

Bacterial isolation and screening: For bacterial isolation, soil sample were collected from rhizospheric soil of *Rhododendron arboreum* of Kumaun Himalayan region. Bacterial strain BS_4 was isolated from the soil sample on nutrient agar media and screen out on the basis of high phosphate solubilisation activity on Pikovaskya's agar plate (Fig. 1A). The positive result was determined by halo zone formation around the bacterial colony on the Pikovaskya's agar plate.

Biochemical characterization: Cellular morphology of screened bacterial strain was studied by performing gram staining and observing cells under light microscope. Biochemical characterization of the strain was done by different tests like catalase, nitrate reductase, dextrose, sucrose fermentation, voges pouskar test, oxidase test, H_2S production and methyl red test (Table 1). Attractively, the strain responded differently towards the entire biochemical test, this behavior suggests their

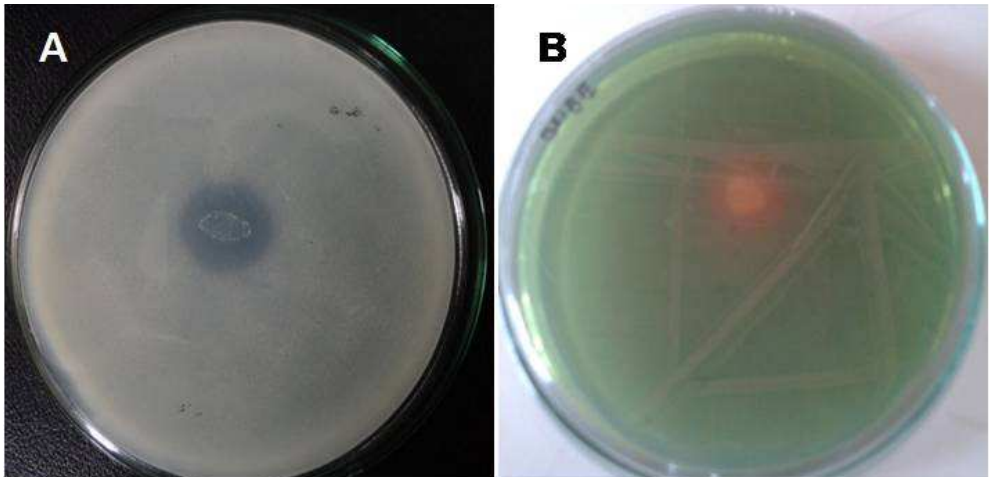

Fig.1. *Phosphate solubilization and Siderophore production: A- The zone of inorganic phosphate solubilised (halo zone) on Pikovskaya's agar plate. B- The siderophore produced (orange colour zone) from bacterial culture on CAS agar plate).*

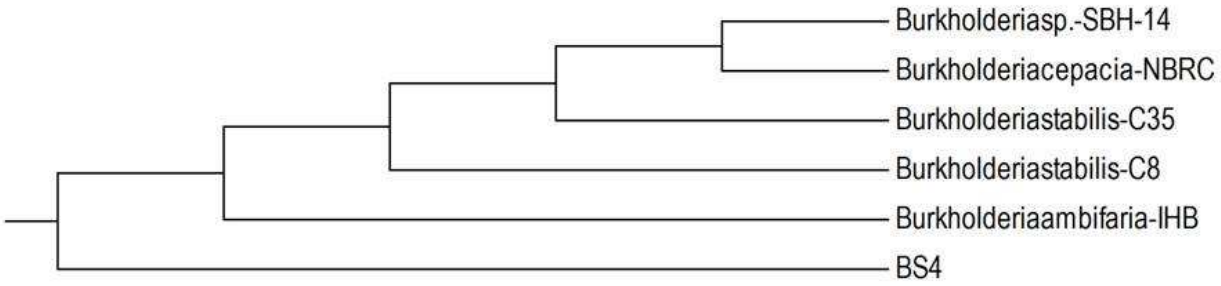

Fig. 2. *Phylogenetic tree for the 16S rRNA sequences of the bacterial strain BS4 constructed by using MEGA4 software by UPGMA method.*

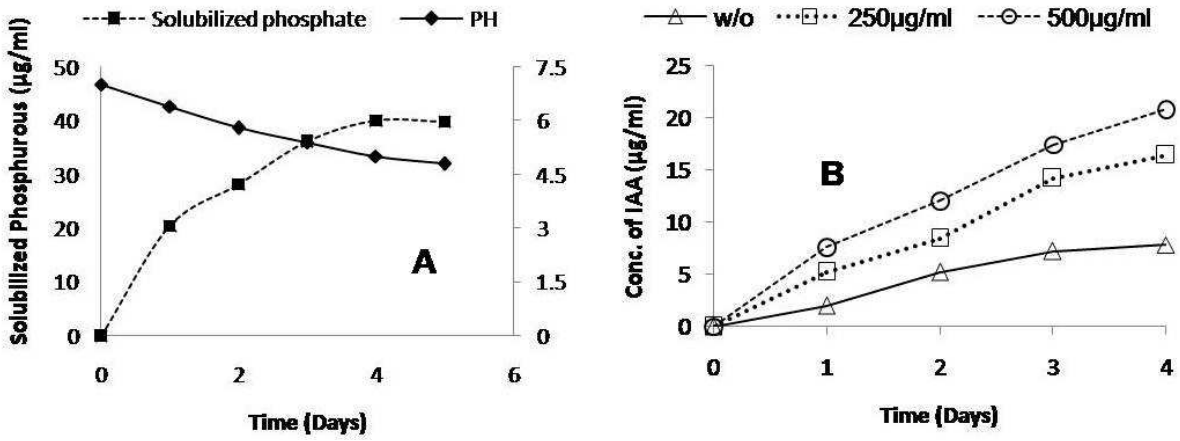

Fig. 3. *Assessment of phosphate solubilizing and IAA producing activity of the strain BS4 as a function of time. A- The concentration IAA on divergent level of tryptophan. B- The amount of soluble phosphate determined from the absorbance data using the calibration curve with KH_2PO_4 at 600 nm. The right-axis indicates the change in pH of the Pikovskaya's medium during growth.*

unique metabolic potential and categorized them putatively into the group of *Burkholderia*.

Molecular characterization and analysis phylogenetic tree: Selected strain BS_4 was subjected to 16S rRNA sequencing and was analyzed using different bioinformatics tools. The partial sequence data of the isolates was analyzed by BLAST search that showed unambiguous similarity (98-99%) with *Burkholderia* sp. Further, the phylogenetic tree was constructed using MEGA4 software by neighbour-joining tree method and the phylogenetic data were obtained by aligning the different sequences of the 16S rRNA of closely related strains (Fig. 2).

Siderophore production and quantification of solubilized phosphate: Siderophore production of by *Burkholderia* sp. was confirmed by CAS assay.

Table 1. Morphological and biochemical characteristics of bacterial strain BS4.

S. N.	Biochemical Test	Response
1.	Gram Staining	Gram negative
2.	Cellular morphology	Rod shape
3.	Citrate Utilization	Positive
4.	Lysine Utilization	Positive
5.	Ornithine Utilization	Positive
6.	Urease	Negative
7.	Phenylalanine Deaminase	Negative
8.	Nitrate Reduction	Positive
9.	H_2S Production	Negative
10.	Catalase Test	Positive
11.	Lactose	Positive
12.	Xylose	Positive
13.	Maltose	Positive
14.	Fructose	Positive
15.	Dextrose	Positive
16.	Galactose	Positive
17.	Raffinose	Negative
18.	Trehalose	Negative
19.	Melibiose	Negative
20.	Sucrose	Negative
21.	L-Arabinose	Negative
22.	Mannose	Positive
23.	Adonitol	Negative
24.	Sorbitol	Negative
25.	Glucose	Positive
26.	Oxidase	Positive

Appearance of a reddish-brown zone on CAS plates suggests the positive result for siderophore production (Fig.1B). This low molecular weight iron binding protein is well known to exhibit antagonistic activity against phytopathogenic fungi. Involvement of the siderophore in the suppression of *Pythium*-induced damping-off of tomato by *P. aeruginosa* TNS K2 has been demonstrated using pyoverdine-deficient mutants (Buysens *et al.,* 1996). Similar increase in the biocontrol potential of the siderophore (Bakthavatchalu *et al.,* 2012) over producing mutant MPS 16 M-1 of *Pseudomonas* sp. against *Rhizoctonia solani* in chickpea has also been reported (Goel *et al.,* 2002). Comparable finding related to siderophore production by different bacterial strains were documented by Sakthivel and Karthikeyan (2012).

Although, The efficiency of *Burkholderia* sp. was carried out in Pikovskaya's broth and amount of soluble phosphate released in liquid medium due to solubilization of tri-calcium phosphate has been determined using the calibration curve of KH_2PO_4 at 600nm to be (40.0 $mgml^{-1}$) upon 5 days of growth. The data showed the time-dependent increase in the amount of solubilised phosphate, and inverse relationship with the pH of the medium. Periodic monitoring of pH of the culture filtrate revealed a significant reduction from pH 7.0 to 4.6 (Fig. 3B). The relationship of decrease in pH of the culture medium with the increase amount of phosphate solubilization is well recognized (Kumar *et al.,* 2012; Anwar *et al.,* 2014). Thus, the results suggest the enhanced phosphate solubilization activity with the screened bacterial strain could be due to result of higher acid production. The production of organic acid and its role in phosphate solubilization is well known, reported in earlier (Kaur and Sharma, 2013; Sujatha and Ammani, 2014).

IAA Production, quantification and confirmation with TLC: Estimation of IAA produced by *Burkholderia* sp. was carried out using the calibration curve of IAA. The extended incubation of culture up to 4 days showed some reduction in cell viability. Fig. 4 A, clearly demonstrates the production of significant amount of IAA in LB broth medium. However, sufficient bacterial biomass persisted in the stationary phase culture

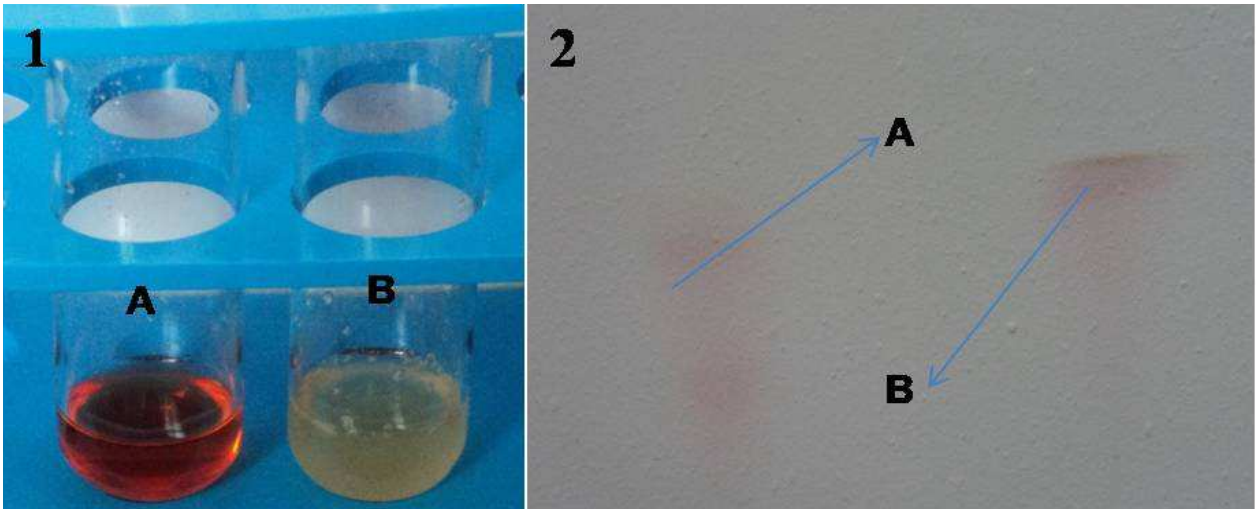

Fig. 4. *Estimation and confirmation of IAA production: 1- Positive result of IAA production (B) with compare with negative control (A): 2- TLC of indole-3-acetic acid detected by Salkowiski reagent; A) Sample, B) Standard.*

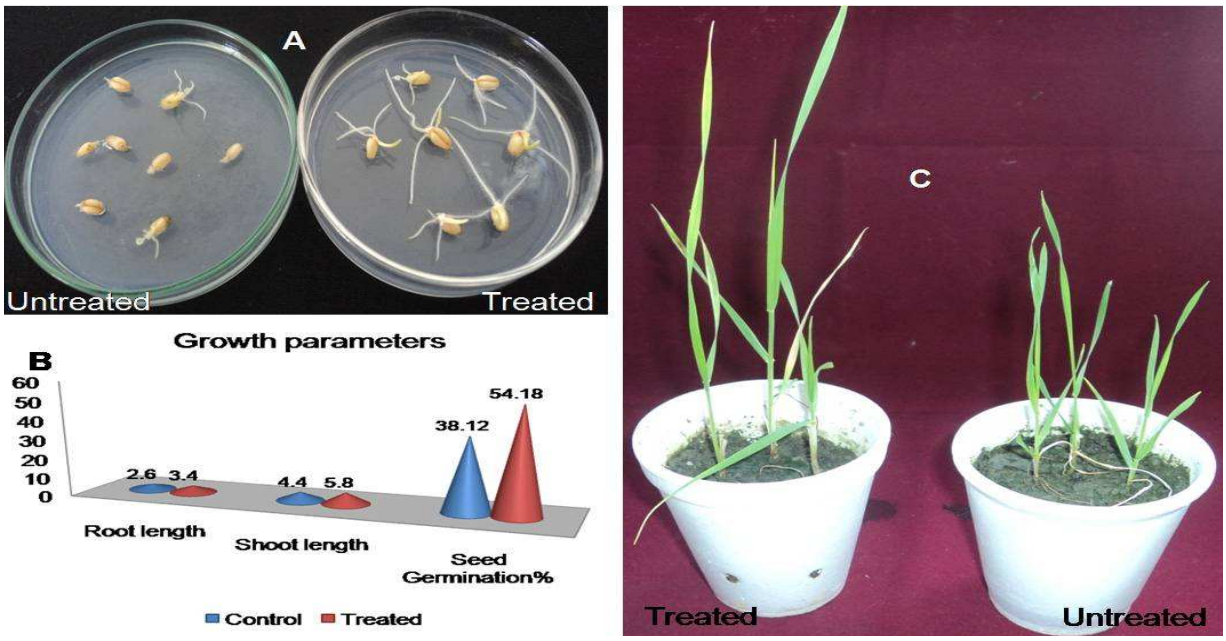

Fig. 5. *Evaluation of plant growth promoting activity (A) Seed germination (B) Growth index (C) Shoot/root length*

even on 4th day of incubation. The culture filtrate at different time intervals showed a linear and time dependent increase in IAA production. Enhanced production of IAA (16.4 µgml^{-1}) and (20.8 gml^{-1}) was observed in the presence of 250 µgml^{-1} and 500 µg ml^{-1} tryptophan, respectively, and 7.8 µg ml^{-1} IAA without tryptophan (Fig. 3A) and similar findings related to IAA quantification were documented by Sakthivel and Karthikeyan (2012). Furthermore, linear increase in IAA production up to 4th day exhibited stability of the metabolic cells during stationary phase. These results correspond with the earlier observations indicating IAA production in stationary phase of culture (Rani and Reddy, 2012). Confirmation of IAA production was done by TLC method (Fig. 4B), in which the culture filtrate of *Burkholderia* sp. was used to extract IAA for characterization. The spots of ethyl acetate extract of culture and standard IAA were tested in the solvent mixture of chloroform: ethyl acetate: formic acid (5:3:2). Chromatograms of spots were sprayed with salkowski reagent that shows almost the same RF value (0.85). The findings of TLC on the basis of RF value are in agreement with reports by other scientist (Ahmad *et al.*, 2005; Prasanna *et al.*, 2010; Sahasrabudhe, 2011).

Seed germination test and pot experiment: The evaluation of effect on plant growth parameter of these IAA producing *Burkholderia* sp. were further studied in seed germination and pot experiment (Fig.5)**.** In seeds germination test, the percentage seed germination of treated seed were found high (54.18%) as compared to control (38.12%) (Fig.5B). Maximum effect on root length was observed in germinated seed compared to control seed (Fig. 5A), the strain also shows greater length of root and shoot in the harvested plant as compared to control (Fig. 5C). The data obtained from seed germination and pot experiment demonstrated positive effect on root and shoot elongation compared to control. This indicates that the *Burkholderia* sp. can improve the plant growth development and thus considered as an effective PGPR. Similar type of results was reported by Bharucha *et al.* (2013), Sivakumar *et al.* (2012) and Mia *et al.* (2012).

Conclusion

Many bacteria strains present in rhizospheric region have been shown to possess plant growth promoting property and plant productivity enhancing activity due to which they are collectively designated as plant growth promoting rhizobacteria (PGPR). PGPR have several characteristics which help them to promote the growth and yield of plant. As elucidated by Ahmad *et al.* (2006) that to establish a bacterial culture as PGPR, they should have some characteristics like the ability to produce phytohormones, asymbiotic N fixation against phytopathogenic microorganisms by production of siderophores, synthesis of antibiotics, enzymes and fungicidal compounds. So, in the present study, a bacterial isolate *Burkholderia sp.* was isolated from *Rhododendron arboreum* rhizosphere of Kumaun region and characterized for the PGP activity. On the parameters of phosphate solubilisation, IAA production, siderophore production, PGP activity of the present strain was established which was further elaborated by evaluating the effect of bacterial culture on seed germination and growth index of wheat plant in a pot experiment. Hence, the present isolate can act as potential candidates for the development of bio-inoculants for crop plants.

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