BIOLOGICA NYSSANA
11 (1) September 2020: 35-44
DOI: 10.5281/zenodo.4060292

Isolation and impacts of rhizobacteria from *Saussurea obvallata* (DC.) Edgew. (Brahma Kamal)

Original Article

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Received: November 06, 2019
Revised: January 21, 2020
Accepted: January 26, 2020

Abstract:

The rhizospheric association of bacteria in terrestrial plants across the hilly region has tremendous potential for plant growth-promotion (PGP) and protection. This is the first study which aims to isolate some rhizo-bacteria from the different rhizospheric soil samples of *S. obvallata* in The Himalayan region. Genotypic, biochemical and PGP traits shows genetically similar bacteria species to have diverse PGP potential as shown by IAA production of 87.567 µg ml^{-1} by *Bacillus* sp. D5 and phosphate solubilization potential 30 mm halo zones are shown by *Bacillus* sp. D9. Growth of isolates was in the ranges of temperature (0-60 °C), salt (0-20%), which shows their tendencies towards surviving harsh environmental conditions. The isolates were further evaluated on *Amaranthus cruentus*, which shows a significant improvement in growth compared to control. The novel study finds the bacterial community at high altitudes to survive extreme climatic variability through association with plants by developing a commensalistic relationship with host.

Key words:

S. obvallata, indigenous rhizobacteria, plant growth promotion, amaranth

Apstract:

Izolacija i uticaj rizobakterija iz *Saussurea obvallata* (DC.) Edgew (Brahma Kamal)

Bakterijska rizosferna asocijacija na terestričnim biljkama širom brdskog regiona ima ogroman potencijal za stimulaciju biljnog rasta (PGP) i zaštitu. Ovo je prva studija koja ima za cilj izolovanje nekih rizobakterija iz različitih uzoraka zemljišta rizosfere vrste *Saussurea obvallata* u regionu Himalaja. Genotipska, biohemijska i PGP svojstva pokazuju da genetski slične vrste bakterija imaju raznovrstan PGP potencijal, kao što pokazuje IAA produkcija od 87.567 µg/ml od strane *Bacillus* sp. D5 i potencijali za solubilizaciju fosfata od 30 mm halo zone od strane *Bacillus* sp. D9. Rast izolata je bio u rasponu temperature (0-60°C) i soli (0-20%), što pokazuje njihove tendencije prema preživljavanju surovih uslova životne sredine. Izolati su dalje procenjeni na vrsti *Amaranthus cruentus*, što je pokazalo značajno poboljšanje rasta u odnosu na kontrolu. Nova studija otkriva da bakterijska zajednica na velikim nadmorskim visinama preživljava ekstremne klimatske varijacije povezivanjem sa biljkama i razvijanjem komensalističke veze sa domaćinom.

Ključne reči:

S. obvallata, autohtone rizobakterije, stimulacija biljnog rasta, *Amaranthus*

Introduction

Saussurea obvallata (DC.) Edgew. (Brahma Kamal), the state flower of Uttarakhand, India is an endemic herb of the Himalayan region (encompassing the Indian Himalayan Region, Northern Burma and Southwest China). The plant is distributed at an altitudinal range of 3000–4800 m (Semwal and Pant, 2013). Brahma Kamal is used for the preparation of traditional medicines by the local peoples in Tibet and other places including Garhwal Himalayas. It is well known that indigenous rhizobacteria exert beneficial effects on plants productivity and sustenance of soil health through their capacity for phosphate solubilization (Sarikhani et al., 2019), indole acetic acid (IAA) (Selvakumar et al., 2008), ammonia, HCN and cell wall degrading enzyme production (Tsegaye et al., 2019) etc. PGPR are heterogeneous groups of microbes associated with the plant's root in diverse ways (Nath et al., 2015). In the rhizobac-

terial population, 2-5% of total the beneficial rhizobacteria are involved in plant growth promotion (Bakker and Schippers, 1987; Antoun and Kloepper 2001; Ahemad and Kibret, 2014). Over 95% of the rhizobacteria existing in the roots and plants obtain many nutrients through the soil bacteria (Ji et al., 2014). Different PGPR, including associative and symbiotic bacteria such as *Pseudomonas* sp.*, Azospirillum* sp.*, Azotobacter* sp.*, Rhizobium* sp.*, Klebsiella* sp.*, Enterobacter* sp.*, Alcaligenes* sp.*, Arthrobacter* sp.*, Burkholderia* sp.*, Bacillus* sp.*, and Serratia* sp. groups have been used for their beneficial effects on plant growth and improvement (Kloepper and Beauchamp, 1992; Höflich et al., 1994). Rhizospheric microbial population mainly promote plant health, they stimulate plant growth directly by producing or changing the concentration of plant growth regulators (Kloepper et al., 1980; Ramette et al., 2003; Sparzak-Stefanowska et al., 2019) like gibberellic acid, IAA, cytokinins (Glick, 1995; Vessey, 2003), asymbiotic N_2 fixation (Ardakani et al., 2010; Bhattacharyya and Jha, 2012). These populations also achieve antagonism against phytopathogenic microorganisms by producing antibiotics, fluorescent pigment, enzyme, cyanide, phenolics, signal compounds (Niranjan et al., 2005), as well as solubilization of mineral phosphates and other nutrients (Johri et al., 1999; Sharma and Johri 2003). Inoculums have been used to increase plant yields in several countries, which is reported in research articles (Dursun et al., 2019) and commercial products are currently available (Backer et al., 2018).

We are addressing for the first time isolation of high altitude rhizobacteria from *S. obvallata* (Brahma Kamal) rhizospheric soil in the Himalayan region and their beneficial effects, harsh environment survival strategy and plant growth promotion in *Amaranthus cruentus.* We proposed that these isolates may also have the ability to express activity towards promoting sustainability in harsh environments.

Material and methods

The four rhizospheric soil samples of *S. obvallata* were collected from different place of Kedarnath valley, Uttarakhand, India and samples collection sites were described by Semwal et al. (2018) and Mitra et al. (2019). The soil samples were serially diluted and 100 µL of dilution sample was plated onto nutrient agar medium (NAM) (HiMedia, MV002, India) for the isolation of potential PGP. All isolated strains were stored at -20 °C in nutrient broth containing 15% (v/v) glycerol.

Morphological and biochemical characterization tests *viz.* IMViC test, citrate utilization, starch hydrolysis, phenylalanine deaminase, urease test, casein hydrolysis, H_2S production, peroxidase test, nitrate utilization, and growth in different media - MacConkey's agar, esculine hydrolysis, mannitol salt agar tests were carried out followed by MacFaddin, 1985; Harley and Prescott, 2002; Anija, 2003; Dubey and Maheshwari, 2007; Cappuccino and Sherman, 2008; Brown, 2009 respectively. To identify the morphological characteristic of all isolates, Gram staining, negative staining, shape, margin, elevation, form, motility and pigment were recorded.

A loopful of bacteria was inoculated and incubated into pre-sterilized peptone broth containing 1% of tryptone for 48 h at 28 °C. After 48 h, 1 mL of Kovac's reagent (HiMedia, India) was added to all tubes including control, gently shaked for few second and left to react for 5-10 minutes. The appearance of red/ pink ring at the top is the clear indication of IAA production (Loper and Schroth, 1987; Dubey and Maheshwari, 2007). As described in Gordon and Weber (1951), the standard graph of IAA was prepared for the quantitative estimate of IAA production. Different IAA concentrations were prepared as aqueous solution of IAA ranging from 5-150 µg ml^{-1}. To each 1 ml of the standard, 2 mL of 2% $FeCl_3$ (0.5 M) in 35% perchloric acid *i.e.* Salkowaski reagent was added and readings were taken after 30 minutes at 535 nm using UV-Visible spectrophotometer (Thermo Scientific™ GENESYS-10S-UV). The standard graph was finally prepared by plotting concentration of IAA in ppm vs optical density at 535 nm. Bacterial cultures were grown for 48 h in nutrient broth. Fully grown cultures were centrifuged at 4000 rpm for 20 minutes at 4 °C. The supernatant (1 mL) was mixed with 4 mL of Salkowaski reagent. Samples were left at 28 °C for 30 minutes. Development of pink color indicates the production of IAA. Optical density was measured at 535 nm with a spectrophotometer. The concentration of IAA produced by bacterial cultures was measured with the help of standard graph of IAA (Loper and Schroth, 1987). Phosphate solubilization ability of the isolates was evaluated in Pikovskaya's (PKV) agar medium (HiMedia, India) incorporated with tri-calcium phosphate (TCP) [Ca_3 $(PO_4)_5$] as insoluble phosphate. PKV media plate was prepared and point inoculation was transferred in each plate at 28 °C for 5 days. After incubation, positive phosphate solubilizing bacteria which give clear zone around the colony were measured (Fiske and Subbarow, 1925). Isolated strains were grown overnight in 10% trypone soy agar supplemented with glycine (4.4 gly L^{-1}) (HiMedia, India) (Bakker et al., 2003). A Whatman no. 1 filter paper soaked in 2% sodium carbonate and 0.4% picric acid solution was placed to the underside of the petri dish lids (Macfaddin, 1980; results explained by Cook, 1993) and kept at 28 °C for 4 days. For the ammonia production, all isolated

bacterial strain was grown in 4% peptone broth for 48 h at 28 °C. Detection of ammonia production was done by adding dropwise of 1.5 mL Nessler's reagent (HiMedia, India) (Bakker et al., 2003). Cellulase enzyme production was determined from clear zone test in CMC (Carboxyl methyl cellulose) agar (CMC - 100.00 g, peptone - 5.0, agar: 15.00 g, distilled water - 1000 mL, pH: 7.2) and Czapek-mineral salt medium ($NaNO_3$ - 2.2 g, K_2HPO_4 - 1.0 g, $MgSO_4.7H_2O$ - 0.7 g, KCl - 0.5 g, CMC - 5.0 g, peptone - 2.5 g, agar - 18.0 g, distilled water - 1000 mL, pH - 6.0) (Bakthavatchalu et al., 2012). Two days after incubation, 2 mL of Congo red solution (0.1% Congo red in 1 M NaCl) was added in each plate. All the plates were then gently shaken. A positive result was indicated when a clear zone and light develops (Qadri et al., 1988). Protease activity (casein degradation) was determined from the clear zone in skim milk agar (skim milk powder - 100.0 g, peptone - 5.0 g, agar - 15.0 g, distilled water - 1000 ml and pH - 7.2) (Vijayaraghavan and Vincent, 2013).

The biofilm experiment was performed in 10 mL glass bottles. Three milliliters of nutrient broth (without NaCl) was dispensed and inoculated with an overnight experiment culture. The bottles were incubated under the static condition for 5 days, the wells were washed thrice with distilled water and the biofilm was stained with 1% crystal violet dye solution for 15 min, and again the wells were rinsed thrice with distilled water to wash off the unbound dye. Quantification of the biofilm biomass was done by absolute alcohol and recording the absorbance at 600 nm according to Srinandan et al. (2010). Antibiotic resistance testing was determined by disc diffusion method (penicillin: SD028-1VL, erythromycin: SD083-1VL, streptomycin: SD091-1VL, chloramphenicol: SD153-1VL and tetracycline: SD133-1VL, HiMedia, India) using nutrient agar plates. The bacterial suspension was inoculated on the nutrient agar plate by swabbing to give a smooth lawn and antibiotic discs were properly placed on the plate for overnight. The plates were observed and recorded the zone of inhibition around the antibiotic disc. All isolates antibiotic profile diversity was analyzed by NTSYS 2.02e software, where 0 denotes susceptible and 1 denotes resistance to a particular antibiotic source. The data were analyzed using the Similarity for Qualitative data subroutine of NTSYS-PC2 package. The isolates were grouped as per Jaccard's similarity index by the unweighted paired group method using arithmetic means (UPGMA) and depicted as dendrogram (Ansari et al., 2014). Antibiotics test result were graphically determined by the R-Heatmap plot analysis where 0 denotes susceptible and 1 denotes resistance to a particular antibiotic source.

To find out the optimal conditions for the growth of bacterial isolate, the effect of different temperature levels on the development of isolates was examined. 0 °C, 4 °C, 28 °C, 37 °C and 60 °C were mainly used and bacterial suspension (100 µL) was placed in a tube containing 10 mL nutrient broth. The tube was incubated at the predetermined temperatures for 48 hours. The effect of different salt concentration on bacterial growth was studied with nutrient broth containing salt concentrations ranging from 0-20%. The effect on different salt concentration (0% and 8% NaCl) for indole-3-acetic acid production in tryptone broth. This was investigated by using tryptone broth containing 0% and 8% NaCl and following 48 h of incubation, the effects on growth rate were measured by spectrophotometric measurements at 660 nm.

The pot experiment was conducted (two times) in the net-house, Graphic Era (Deemed to be University), Dehradun (30°19'N, 78°04'E, 650 m) with two times sterile soil. *Amaranthus cruentus* seeds were surface sterilized, the seeds were dried for 2 hours in sterile conditions and subsequently planted into the pots prepared for treatments. There were five replicates per treatment with 15 seeds planted per pot. Two bacterial isolates *viz. Bacillus* sp. D5 and *Bacillus* sp. D9 were selected based on PGP activity. Soil treatments consisted of 0.5 kg Lignite (carrier material) and 100 mL of inoculum (D5 and D9; separately) solution and sterile distilled water was the control. After 30 days, the length and mass of shoots and roots were taken to determine the effect of treatments on the growth of *A. cruentus* plants. Diversity and statistical analysis were done by using NTSYS 2.02e software. Experimental data were analyzed using standard analysis of variance (ANOVA) and HAU, Hisar online statistical analysis software

Results

Ten PGPR isolates (*Pseudomonas* sp. D1, *Bacillus* sp. D2, *Bacillus* sp. D3, *Bacillus* sp. D4, *Bacillus* sp. D5, *Bacillus* sp. D6, *Bacillus* sp. D7, *Bacillus* sp. D8, *Bacillus* sp. D9 and *Bacillus* sp. D10) were chosen from a total of eighty-five isolates using the serial dilution technique of rhizospheric soil samples of *S. obvallata.* All isolates were screened and selected on the basis of growth rate, morphological (**Fig. 1**) and biochemical characteristics. This is the first research report that the D1, D2 and D3 isolated from Hathi Parwat (HP), D4 and D5 from Maha Panth (MP), D6 and D7 from Madhu Ganga (MG) and D8, D9 and D10 from Cheer Ganga (CG) of Kedarnath valley. Similarly, Mitra et al. (2019) reported that some dominant fungi *viz. Phanerochaete chrysosporium, Aspergillus fumigatus* and *Trichoderma longibrachiatum* was found and identified through 16S rDNA sequencing (ITS4 and ITS5) from the same

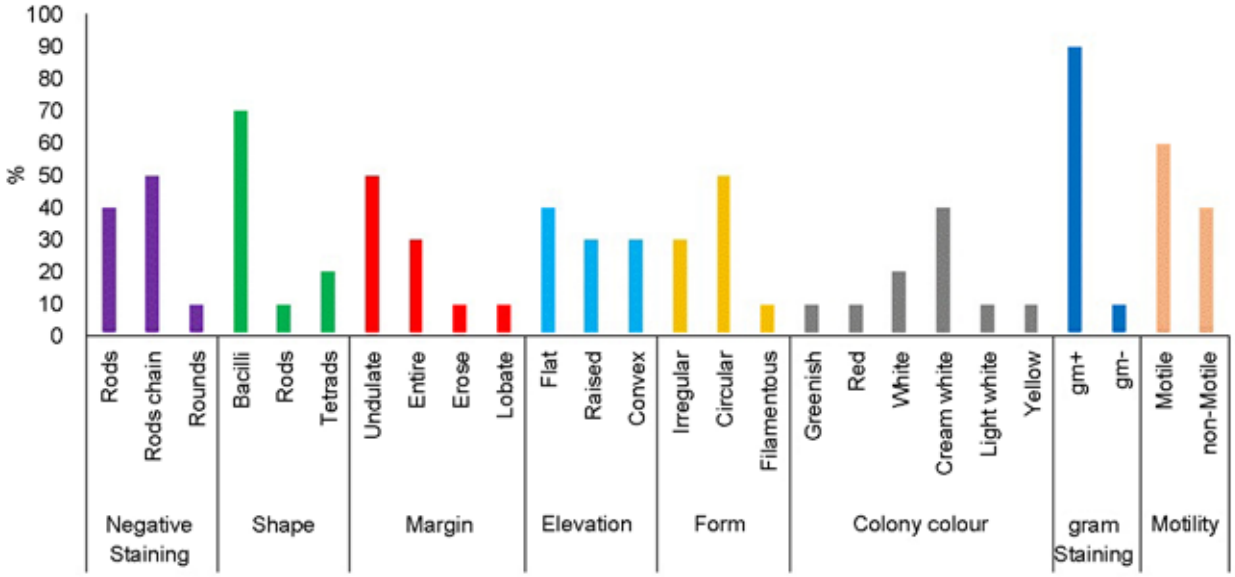

Fig 1. The figure shows the percentage abundances of bacteria as determined under microscope

rhizospheric soil samples of *S. obvallata.*

Biological communities in the Indian Himalayas include one of the biggest altitudinal encompass in the world and a portion of the more extravagant gatherings of wild and medicinal plants are found in this area (Chauhan et al., 2015). Presentation and exploitation of PGPR in agro-biological systems improve plant-microbes interactions that may influence biological communities supportability, farming efficiency, and ecological quality (Zahid et al., 2015). The rhizosphere is a special specialty for different kinds of microorganisms in the soil (Agrawal and Johri, 2014). Semwal et al. (2018) reported that *S. obvallata* rhizospheric and non-rhizospheric soils macro - micro nutrients analysis and range of different nutrients availability *viz.* nitrogen, phosphorus, potassium, zinc, copper, iron, manganese in normal and suggested that they are helpful for development and growth for this endangered plant. So, in our present study, ten PGPR bacterial isolates were isolated and screened for PGP abilities (Kloepper et al., 1988).

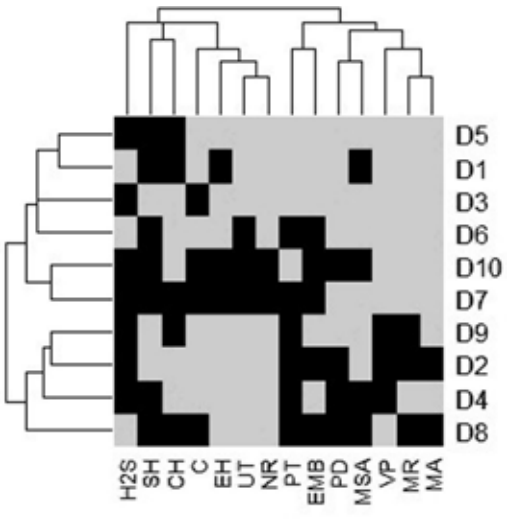

Fig 2. Biochemical test results represented by Heatmap plot (MR and VP: MR/VP test, C: Citrate utilization, SH: Starch hydrolysis, PD: Phenylalanine deaminase, UT: Urease test, CH: Casein hydrolysis, H2S: H2S production, PT: Peroxidase test, NR: Nitrate utilization, MA: MacConkey's agar, EH: Esculine hydrolysis and MSA: Mannitol salt agar)

This study was conducted to isolate the native low temperature growing potential PGPR from the Himalayan region rhizospheric soil of *S. obvallata* plant and showed that some common PGPRs like *Bacillus* sp. and *Pseudomonas* sp. were present in this rhizospheric area. Similarly, Majeed et al. (2015) reported that they are isolated some PGPR for the root endosphere and rhizosphere of wheat from the Himalayan region and isolates showed PGP ability like IAA production, N_2 Fixation and P - solubilization. Agrawal and Johri (2014) reported that fifty-five rhizobacteria were isolated, characterized and screened PGP attribute from the central Himalayan region, forest sites; altitude 3500, 12000 and 3100 ft. Biochemical test results of all bacterial isolates were analyzed by Heatmap plot and represented the results where black colour denotes negative and gray colour denotes positive activity (**Fig. 2**). Heatmap plot colour analysis showed that most (approx. <50%) isolates has positive biochemical activity.

IAA production is a natural property of rhizobacteria that enhance and facilitate plant growth and development. Quantitative assay of IAA production of rhizobacteria was done by the spectrophotomet-

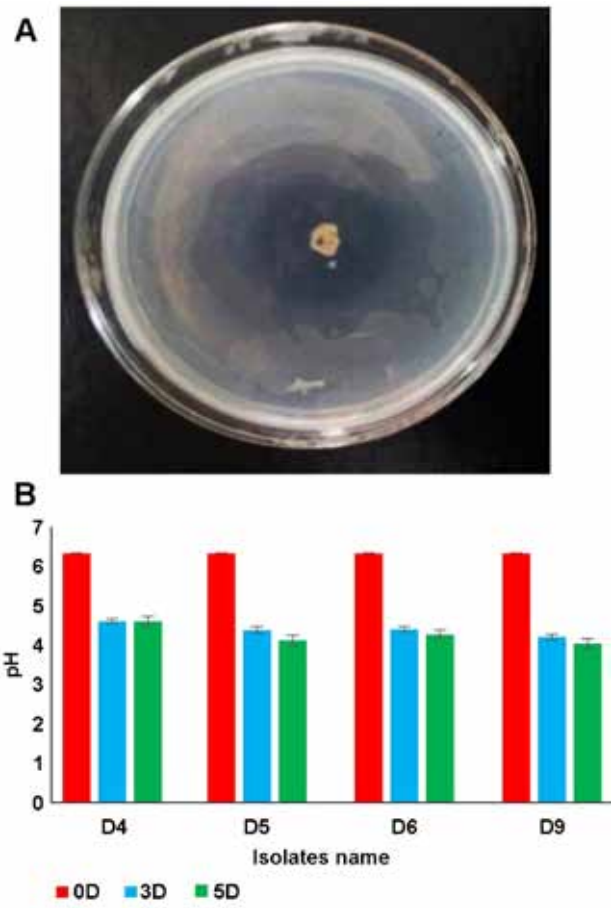

Fig 3. A - *Bacillus sp*. D9 isolate phosphate solubilization zone on PKV media after 5 days; B - pH level in PKV broth

ric analysis. The concentration of IAA produced by bacterial cultures was measured with the help of the standard graph of IAA. In this study, six qualitative screened positive bacterial isolates were taken for the quantitative assay and D5 isolate IAA production is 87.567 µg ml^{-1}. Phosphate solubilizing bacteria used as inoculants simultaneously increases phosphate uptake by the plant and crop yield (Rodríguez and Fraga, 1999). Phosphate solubilization activity was determined by the development of the clear zone around the bacterial colony. Isolate D9 is a high phosphate solubilizer bacterial strain which is used for the treatments. D9 showed the 30 mm zone (diameter) on PKV agar media (**Fig. 3A**) after 5 days of incubation. Four phosphate solubilizer bacteria strains were grown in PKV medium (pH 6.35) for 3 and 5 days at 28 °C. After the given time, pH of the culture medium was checked by pH meter and decreased pH rate can be observed in **Fig. 3B**. In the similar study, Panday et al. (2002) reported and isolated *P. corrugata* from the low-temperature location of Sikkim and reported that all isolates could grow and solubilize phosphate in the range of 4 to 35 °C.

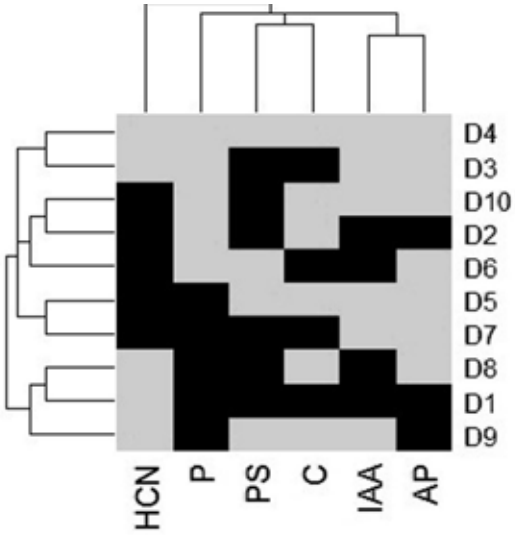

Fig 4. Isolates plant growth-promoting test results are evaluated graphically by Heatmap plot where gray color: (+) PGPR and black colour: (-) PGPR. (IAA: IAA production, PS: Phosphate solubilization, HCN: HCN Production, AP: Ammonia Production, C: Cellulase Test and P: Protease test)

All isolates were grown in 10% tryptone soy agar with glycine (4.4 gly L^{-1}) on the plates sealed with parafilm®. The production of HCN was determined by the change in colour of filter paper from yellow to red-brown. After the incubation of all bacterial culture in medium, the Nessler's reagent was added. Ammonia produced by bacterial isolates formed the yellowish-brown colour in the medium. Cell wall degrading enzyme production was determined by congo red method and protease activity was determined by clear zone formation on the test plate. By this, the production of the most important plant growth hormones was recorded (**Fig. 4)**.

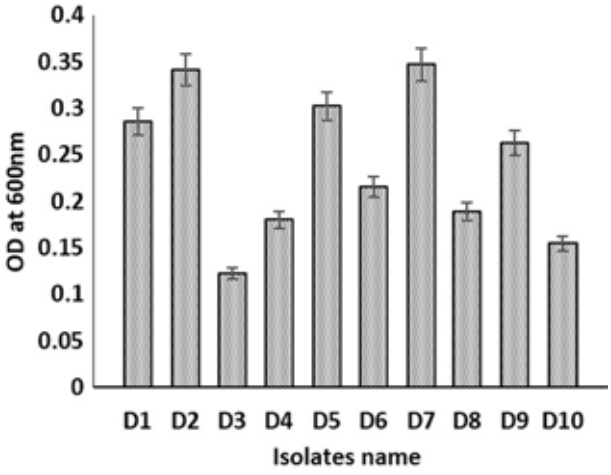

Fig 5. Biofilm formation by isolates

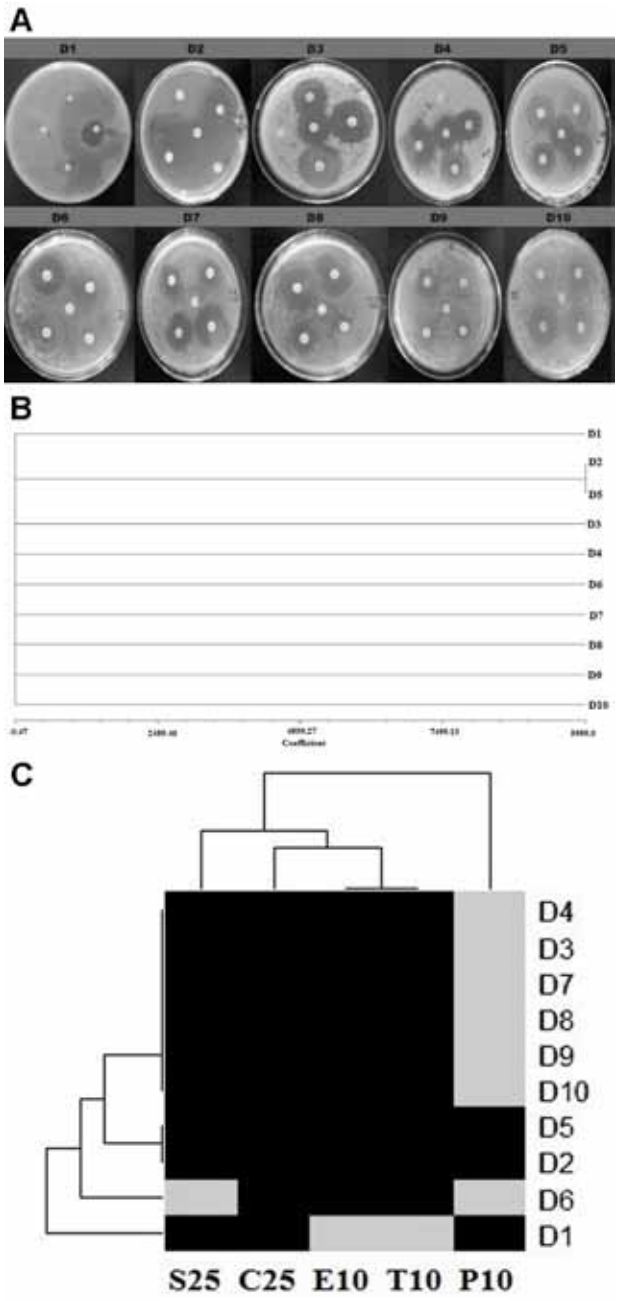

Fig 6. A - Effect of antibiotics to different isolates; B - Antibiotics diversity of isolates analyzed by NTSYS; C - Antibiotics test results analyzed by Heatmap plot where gray color: Resistance and black color: Susceptibility (P10: Penicillin, E10: Erythromycin, S25: Streptomycin, C25: Chloramphenicol, T10: Tetracycline

Quantification of the biofilm biomass results by the bacterial isolates are presented in **Fig. 5**. Isolates D2, D5, D7 and D9 showed high biofilm formation after 5 days of incubation.

The antibiotic sensitivity was determined by disc diffusion method, where five antibiotics were used and the results of resistance and susceptibility of the isolates are presented in **Fig. 6A.**

Antibiotic diversity of the isolates was analyzed by NTSYS and dendrogram, where D2 and D5 showed sensitivity to all tested antibiotics (**Fig. 6B**). Heatmap plot showed that D6, D7, D8, D9 and D10 are resistant to penicillin and D1 showed resistance to erythromycin and tetracycline (**Fig. 6C**).

Bacterial growth under different temperature and NaCl concentration were determined by measuring the optical density (OD) at 660 nm by spectrophotometer (**Fig. 7A, B)**. In this study D2, D5 and D9 bacterial isolates demonstrated the highest growth rate at 0 C, while 28 and 37 were optimal growth temperatures for most other isolated strains. All isolates showed low growth rate at 60 °C. Our study suggested that the most suitable salt concentrations differed among the strains. The result showed that optimal growth and survival were at 0% for D8, 0.5% for most of the strains (D1, D2, D3, D7, D9 and D10), while three strains (D4 D5 D6) had the best growth at 5% NaCl concentration (**Fig. 7B**).

IAA is a common product of L-tryptophan metabolism by numerous microorganisms but in the medium salt concentrations affects the growth rate of microorganism. All isolates were grown in tryptone broth with different salt concentration. D6 and D7 growth rate were higher at 8% NaCl and D9 growth rate was constant at 0% and 8% NaCl (**Fig. 8**).

The results of the soil treatments with potential PGPR isolates *viz*. D5 and D9 on the growth of *A. cruentus* showed significant effect in enhancing the plant's shoot - root height and weight of inoculated plants as compared to uninoculated control (**Fig. 9**). In experiment 1, D5 and D9 showed a positive result in the development of shoot but in experiment 2, D5 showed high effect in enhancing shoots and roots as compared to control.

Discussion

PGPR are originally free-living soil bacteria that colonize the rhizosphere or the tissues of living plants. Once the bacteria are associated with the plant by colonizing the rhizosphere around the roots, the rhizoplane (root surface) or the root itself (Rodriguez-Navarro et al., 2007), the bacteria can increase the development of the plant through indirect or direct mechanisms. In our study, PGPR isolates *viz.* D4, D5, D6 and D9 showed positive for phosphate solubilization and pH ranged from 4.00 – 5.00. D9 bacterial isolates showed the highest zone in PKV plate. Highest biofilm formation showed by rhizobacterial isolates D1, D2, D5, D7 and D9 was very evident at 600 nm readings. From the compatibility results, D1, D5 and D9 isolates were selected for the pot experiment to assess its performance in terms of growth, nutrient uptake and stress conditions survival ability. Pot experiment results showed

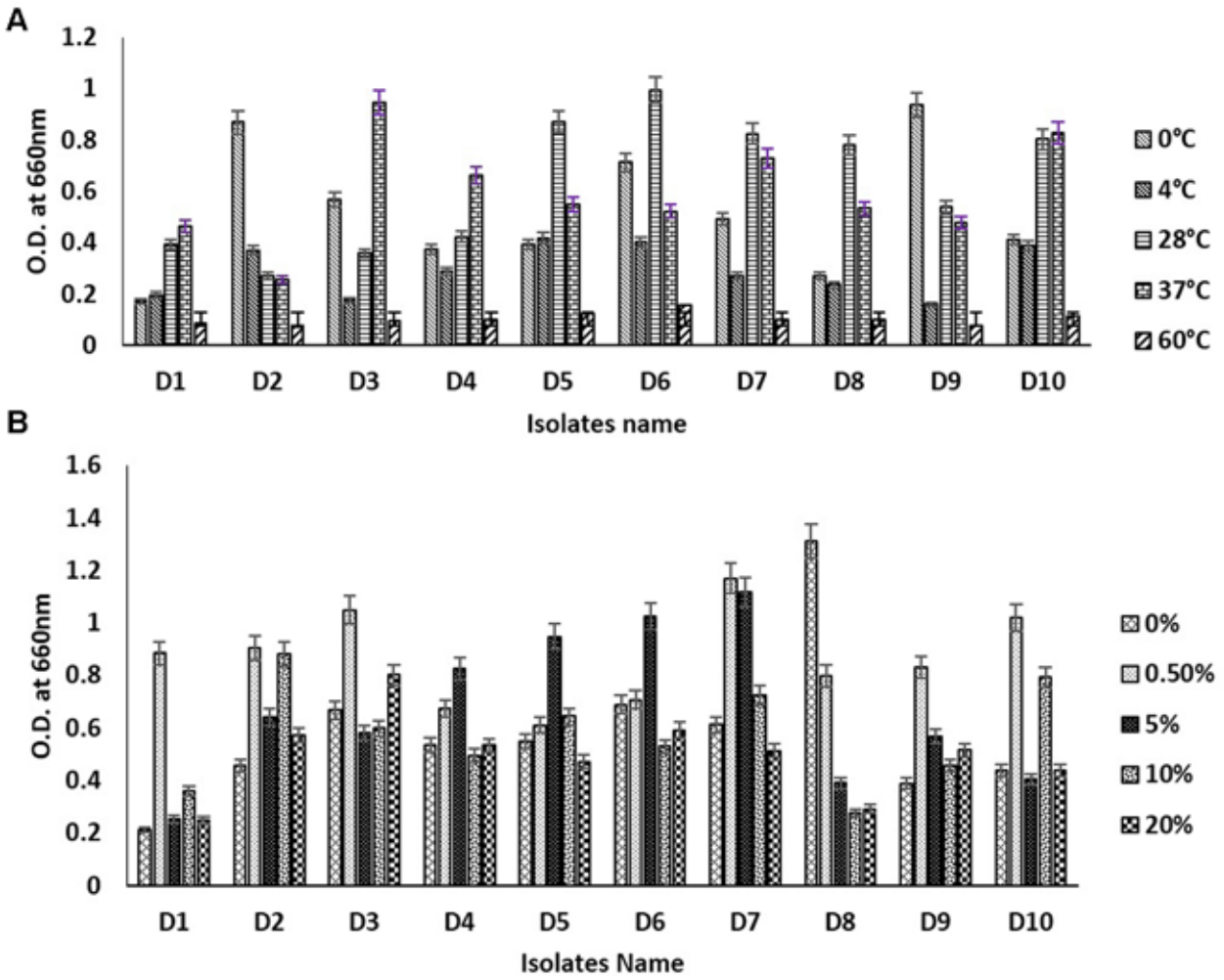

Fig 7. A - Isolates growth under the different temperature; B - Isolates growth under the different salt concentration

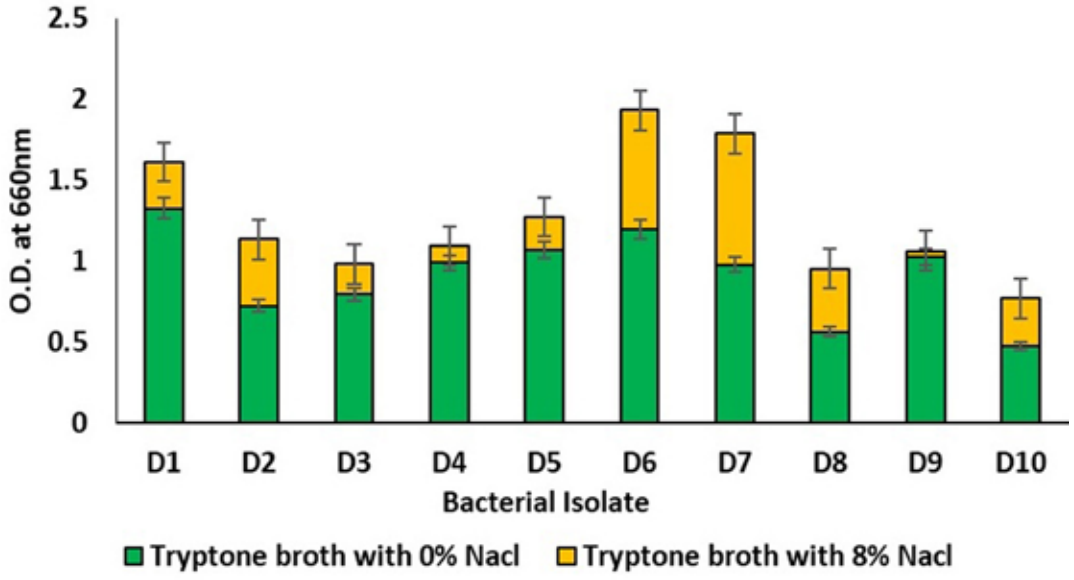

Fig 8. Growth rate in tryptone broth with different salt concentration

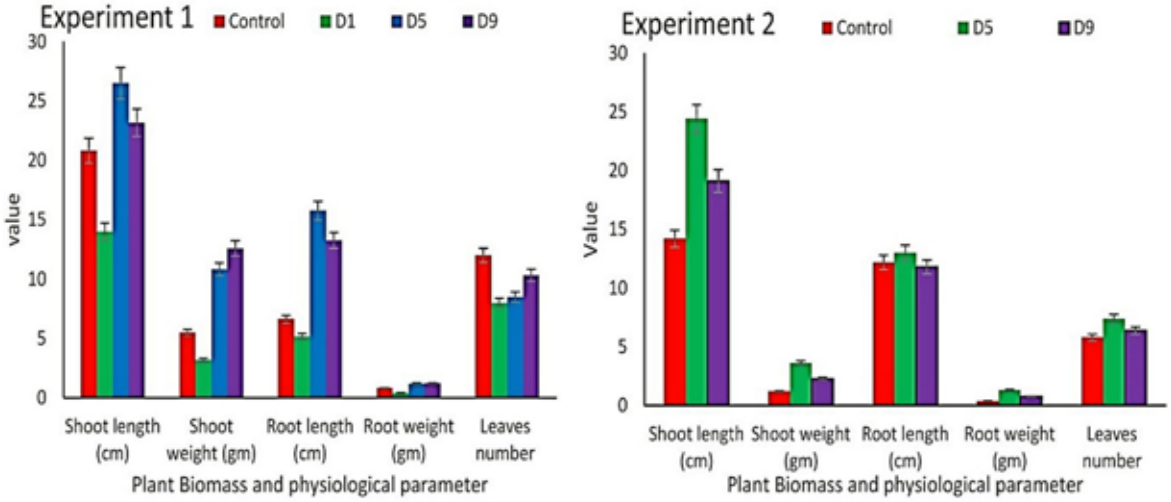

Fig 9. Biomass and physiological parameter of the treated plant after 30 days

that soil treatments with these isolates significantly enhanced the plant growth of inoculated amaranth plants as compared to uninoculated control.

Conclusion

In our study, we used *Bacillus* sp. D5 and *Bacillus* sp. D9 as a bio-inoculum and phosphate solubilizer from the rhizospheric soil samples of *S. obvallata* (Brahma Kamal) in The Himalayan region. The present study has clearly demonstrated the PGP trails and application on amaranths with *Bacillus* sp. D5 and *Bacillus* sp. D9 having a positive influence in growth parameter in comparison with the control. The study suggested that low-temperature bacterial community growth has multifunctional characteristics in growth promotion and develops a green biofertilizer for hill farming.

Acknowledgements. We are grateful to the Founder and President, Prof. (Dr.) Kamal Ghanshala, Graphic Era (Deemed to be University), Dehradun and giving student research grants for this research work. We are also grateful to Prof. Ashish Thapliyal, Professor and Head, Department of Biotechnology, Graphic Era (Deemed to be University), Dehradun. First author thanks to Devvrat, Ph.D. Scholar, Graphic Era (Deemed to be University), Dehradun, India and Ansuman Senapati, SRF, ICAR-NRRI, Cuttack, India for his help in data analysis

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