Recent Research in Science and Technology 2012, 4(1): 01-05
ISSN: 2076-5061
Available Online: http://recent-science.com/

Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an *in vitro* study

Ajay Kumar*±, Amit Kumar#, Shikha Devi±, Sandip Patil#, Chandani Payal# and Sushila Negi#

± Department of Microbiology, Shoolini Institute of Life Sciences and Business Management Solan, Himachal Pradesh-173212 India.
Faculty of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Solan, Himachal Pradesh,173212 India.

Abstract

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can be found in the rhizosphere, in association with roots which can enhance the growth of plant directly or indirectly. A large number of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium and Serratia* have reported to enhance plant growth. In the present study, six French bean rhizospheric soil samples were collected from different location of Shimla and Solan in H.P (India). A total of thirty bacteria were isolated and *in vitro* screening was done for different plant growth promotion activities i.e. phosphate solublization, IAA production, ammonia production, ACC deaminase activity, HCN production and catalase. In the present work twelve bacterial isolates were positive for phosphate solublization. IAA production was shown by almost all the bacterial isolates. Three isolates were positive for ammonia production. ACC deaminase activity was shown by nine isolates. Two isolates were positive for HCN production and all the isolates were found to be catalase positive. Seven isolates were showing maximum plant growth promotion activities and further identified on the basis of colony morphology, Gram staining and biochemical tests. These isolates were identified as *Acinetobacter* sp., *Bacillus* sp., *Enterobacter* sp., *Micrococcus* sp., and *Pseudomonas* sp. As PGPR are environmental friendly and offer sustainable approach to increase production of crops and health. Therefore, these isolates can be utilized for biofertilizer formulation under local agro-climatic conditions of Himachal Pradesh.

Keywords: Rhizobacteria, PGPR, Biofertilizers.

INTRODUCTION

The rhizosphere zone has been defined as the volume of soil directly influenced by the presence of living plant roots or soil compartment influenced by the root [1]. Rhizosphere supports large and active microbial population capable of exerting beneficial, neutral and detrimental effects on the plants. Rhizobacteria (root colonizing bacteria) that exert the beneficial effects on the growth of the host plant via direct or indirect mechanisms are termed as plant growth promoting rhizobacteria (PGPR) [2]. The plant-microbe interactions in the rhizosphere are responsible for increasing plant health and soil fertility [3].

PGPR strains use one or more direct or indirect mechanisms to enhance the growth and health of plants. These mechanisms can be active simultaneously or independently at different stages of plant growth[4].PGPR have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen, solublization of minerals such as phosphorus, production of siderophores, and synthesis of plant growth hormones i.e. Indole-3-acetic acid (IAA), gibberellic acid, cytokinins, and ethylene [5] Indirect mechanisms involves the biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzymes, hydrogen cyanide, catalase and siderophore or through competition for nutrients and space can improve significantly plant health and promote growth, as evidenced by increases in seedling emergence, vigor, and yield [6]. After N_2 fixation, Phosphate (P) solubilization is very important plant growth promoting activity. A large proportion of soluble inorganic phosphate added to the soil is fixed as insoluble forms soon after the application and become unavailable to the plants [7]. Several soil bacteria particularly belonging to genera *Bacillus* and *Pseudomonas,* posses the ability to change insoluble forms into soluble form by secreting organic acids as formic acid, acidic, propionic, lactic, glycolic, fumaric and succinic acid [8]. Biofertilizers such as microbial inoculants promote plant growth, productivity and increase the nutrient status of the host plant have internationally been accepted as an alternative source of chemical fertilizers [9]. Significant increases in crop yields have been reported by applying PGPR microbial inoculants [10]. So, keeping all this in view, the present study was carried out to isolate the various plant growth promoting strains from the rhizospheric soils of Himachal Pradesh.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected from the rhizosphere of French bean plants growing at different sites at Solan and Shimla of Himachal Pradesh in India. Intact root system was dug out and the rhizospheric soil samples were carefully taken in plastic bags and stored at 4^0C. Total of six soil samples were collected for the isolation of rhizosphere bacterial isolates.

Received: Nov 12, 2011; Revised: Dec 24, 2011; Accepted: Jan 7, 2012.

*Corresponding Author

Ajay Kumar
Department of Microbiology, Shoolini Institute of Life Sciences and Business Management Solan, Himachal Pradesh-173212 India.

Tel:+919418130189; Fax: +911792226364
Email: micro_1978@rediffmail.com

Isolation of bacteria from rhizospheric soils

The P- solubilizers were isolated from the rhizospheric soil samples by serial dilution technique [11]. Appropriate dilution was spread on Pikovskaya agar plate containing insoluble ticalcium phosphate. Plates were incubated at 30±0.1 ^{0}C for 24-48 h. Colonies showed halo zone were considered as P-solubilizer. The P-solubilizers were purified by repeated streaking and stocked for further use.

Maintenance of isolates

All the isolates were maintained at 4°C in equal volumes of nutrient broth and 30% glycerol.

In vitro screening of isolates for different plant growth promoting activities.
Phosphate solubilization

The isolates were screened for phosphate solubilization as per methodology described by Gupta S. et al., [12]. On modified Pikovskaya agar with insoluble tricalcium phosphate (TCP), a loop full of each culture was placed on the centre of agar plates and incubated at 30±0.1 °C for 5 days. The solubilization zone was determined by subtracting the diameter of bacterial colony from the diameter of total zone.

Quantitative estimation of phosphat

Quantitative estimation of inorganic phosphate solubilization was done as per methodology described by Nautiyal [13] and Jackson [14]. Bacterial isolates were grown in National Botanical Research Institute's Phosphate (NBRIP) broth containing 0.5% tricalcium phosphate (TCP). The flasks containing 50 ml medium was inoculated with 500 µl bacterial culture in triplicates and incubated at 30±0.1 °C at 180 rpm for 5 days in Incubator Shaker. Simultaneously, the uninoculated control was also kept under similar conditions. The cultures were harvested by centrifugation at 10,000 rpm for 10 min. The phosphorus in supernatant was estimated by vanado-molybdate-yellow color method. To a 0.5 ml aliquot of the supernatant, 2.5 ml Barton's reagent was added and volume was made to 50 ml with de-ionized water. The absorbance of the resultant color was read after 10 min at 430 nm in UV/Visible Spectrophotometer. The total soluble phosphorus was calculated from the regression equation of standard curve. The values of soluble phosphate liberated were expressed as µg ml^{-1} over control. The pH of culture supernatants were also measured using a pH Meter.

Detection of IAA
Culture growth conditions

Fifty milliliter of Nutrient broth (NB) containing 0.1% DL-tryptophan was inoculated with 500 µl of 24 h old bacterial cultures and incubated in refrigerated incubator Shaker at 30±0.1 °C and 180 rpm for 48 h in dark. The bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. Estimation of indole-3-acetic acid (IAA) in the supernatants was done using colorimetric assay [15].

Colorimetric estimation

One millilitre of supernatant was mixed with 4 ml Salkowski reagent and absorbance of the resultant pink color was read after 30 min at 535 nm in UV/Visible Spectrophotometer. Appearance of pink color in test tubes indicated IAA production described by Gordon and Weber [16]. The IAA production was calculated from the regression equation of standard curve and the result was expressed as µg ml^{-1} over control.

1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity by Germinating seed bioassay

The effect of bacterial isolates on root elongation was studied by germinating seed bioassay as per methodology described by Dey et al., [17] and Belimov [18]. Seed surface of *Pisum sativum* and *Zea mays* were sterilized with 20% NaOCl for 3 min and washed three times with sterile de-ionized water. All the seeds were allowed to germinate at 25 °C in 1% water agar plates for 48 h. Germinated seeds were individually dipped for 1 h in 20 ml bacterial cultures grown for 48 h in NB and transferred to wet filter paper in Petri dishes taking three seeds per dish. The Petri dishes were incubated in dark at 30±0.1 °C with three replications for each treatment. The seedlings treated with uninoculated NB served as control. The root length of seedlings was measured in cm after 5 days of incubation.

Ammonia production

All the bacterial isolates were tested for the production of ammonia as described by Cappuccino and Sherman [19]. Overnight grown bacterial cultures were inoculated in 10 ml peptone broth and incubated at 30±0.1 °C for 48 h in Incubator Shaker. After incubation 0.5 ml of Nessler's reagent was added. The development of faint yellow to dark brown color indicated the production of ammonia.

Hydrogen cyanide production and catalase

Screening of bacterial isolates for hydrogen cyanide (HCN) production was done as per methodology described by Castric [20]. Bacterial cultures were streaked on nutrient agar medium containing 4.4 g per liter of glycine. A Whatman filter paper No. 1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate. Plates were sealed with parafilm and incubated at 30±0.1 °C for 4 days. Development of light brown to dark brown color indicated HCN production.

Catalase activity

Catalase test was performed by taking a drop of 3% hydrogen peroxide was added to 48 hr old bacterial colony on a clean glass slide and mixed using a sterile tooth-pick. The effervescence indicated catalase activity.

Biochemical identification of selected beneficial bacterial isolates

Seven isolates which showed maximum PGPR activity were further characterized by Gram staining and biochemical tests as per methodology described by Krieg and Holf [21]. The various tests performed were Oxidase, MR-VP, Indole, Citrate, Urease, Nitrate reduction and fermentation of various sugars.

RESULTS

Thirty bacterial isolates were isolated from the total of six rhizospheric soil samples from French bean. All the isolates were designated as shown in Table no. 1. All isolates has shown significant PGPR activity. A total of thirty bacterial isolates were screened for phosphate solublization on modified PVK agar, of which twelve isolates showed the development of sharp phosphate solublization zones, ranging from 4 mm to 20 mm. Other isolates showed the development of hazy zones. FBJ6 and FBS4 showed highest phosphate solublization i.e 20.0 mm and 13.5 mm, respectively. Five isolates i.e. FBJ1 (12.0 mm), FBJ6 (20.0 mm), FBK1(10.5 mm), FBK3 (11.0 mm) and FBS4 (13.5 mm) produced zone greater than 10 mm, were further selected for quantification of phosphate solublization and further characterized for various PGPRs activities. In quantitative estimation, range of tri calcium phosphate solublization was between 15 to 60 µg/ml observed (Table 2). Most of the bacterial isolates produced plant growth promoting hormone i.e. IAA (Table 3). The range of IAA production was 0.2 to 213 µg/ml. Among all isolates, FBK3 produced high IAA (213.15 µg/ml) whereas, FBJ6, most efficient P-solubilizer was found to produced 198.08 µg/ml of IAA. Ammonia production is another important trait of PGPR that indirectly influence the plant growth. All the five efficient isolates were able to produced ammonia. Production of HCN was detected in two isolates i.e. FBJ6 and FBS4. Catalase activity was detected in most of the bacterial isolates that may be potentially very

Table 1. Description of the Bacterial isolates.

Sample number	Location of French bean rhizosphere soil	No. of isolates	Isolate Codes
Sample 1	Tank road ,Solan (H.P.)	Five	FBT1,FBT2,FBT3,FBT4, FBT5
Sample 2	Arki kunihar , Solan (H.P.)	Four	FBA1,FBA2,FBA3,FBA4
Sample 3	Chail paindly, Shimla (H.P.)	Five	FBC1,FBC2,FBC3,FBC4,FBC5
Sample 4	Junga chyora,Shimla(H.P.)	Six	FBJ1, FBJ2, FBJ3, FBJ4, FBJ5, FBJ6
Sample 5	Kandaghat, Solan (H.P.)	Five	FBK1,FBK2,FBK3,FBK4,FBK5
Sample 6	Sadhupul,Solan (H.P.)	Five	FBS1,FBS2,FBS3,FBS4, FBS5

Table 2. Bacterial isolates showing Quantitative P-solubilization

Isolate Codes	P- solublization (µg/ml)
FBJ1	443.26
FBJ6	562.34
FBK1	362.72
FBK3	396.57
FBS4	482.64

Table 3. Bacterial isolates showing different plant growth promotion activities.

Isolate Codes	P Solublization	IAA production	Ammonia production	ACC Deaminase activity	HCN production	Catalase Activity
FBJ1	+	139.84 µg/ml	+	+	-	+
FBJ6	+	198.08 µg/ml	+	+	+	+
FBK1	+	107.58 µg/ml	+	+	-	+
FBK3	+	213.15 µg/ml	+	+	-	+
FBS4	+	163.72 µg/ml	+	+	+	+

Table 4. Biochemical characterization of bacterial isolates.

Biochemicl tests	Cat	Oxi	I	MR	VP	C	Lac	Glu	Man	Su	U	Nit	Mo	Organism
FBJ1	+	-	-	-	-	+	A	A	-	-	-	-	-	*Acinetobacter sp.*
FBJ6	+	+	-	-	-	+	-	A	-	-	+	+	+	*Pseudomonas sp.*
FBK1	+	-	-	-	+	+	AG	AG	AG	AG	-	+	+	*Enterobacter sp.*
FBK3	+	-	-	-	-	+	-	A	-	A	-	+	-	*Micrococcus sp.*
FBS4	+	+	-	+	+	+	-	A	A	A	-	+	+	*Bacillus sp.*

advantageous. Five isolates positively affected the germination of *Pisum sativum* and *Zea mays* seeds. Highest root elongation was recorded when seeds were pre-treated with FBJ6 isolate. Bacterial isolates FBJ1, FBK1 and FBS4 also showed the better ability to increase the length of root. These isolates were further identified by biochemical characterization. On the basis of biochemical characterization (Table 4) the most efficient P-solubilizer (FBJ6) was identified as *Pseudomonas* sp.

DISCUSSION

PGPR colonize roots of plant and promote plant growth and development through a variety of mechanisms. The exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion [22, 23]. There are many papers related to the advantages and screening of PGPR from crop plants particularly rice, maize and sugar cane but few on French bean. Little information about screening and using PGPR with French bean is available. In present study, beneficial bacteria were isolated from French bean rhizosphere. Isolated bacteria were screened for different plant growth promotion activities and characterized by biochemical tests. Five bacterial isolates were showed more than 10 mm zone of phosphate solubilization. The isolate FBJ6 (*Pseudomonas* sp.) showed highest phosphate solubilization zone (20 mm) in PVK agar. It has been reported that higher concentrations of phosphate-solubilizing bacteria are commonly found in the rhizosphere soil as compared to non-rhizospheric soil [24]. IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development. It has been reported that IAA production by PGPR can vary among different species and strains, and also influenced by culture conditions, growth stage and substrate availability [25]. Higher level of IAA production by *Pseudomonas* was recorded by other research workers [26]. In our study most of the bacterial isolates were positive for IAA production. Another important trait of PGPR is the production of ammonia that indirectly influences the plant growth. All the isolates were able to produce ammonia. All the bacterial isolates in the present study were able to produce catalase. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. A number of studies suggest that PGPR enhances the growth, seed emergence, crop yield, and contribute to the protection of plants against certain pathogens and pests. [18, 27, 28, 29, 30]. In current study, out of five isolates, isolate FBJ6 (*Pseudomonas* sp.) significantly increased the root length of *Pisum sativum* and *Zea mays* seeds showed high ACC deaminase activity. HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens [31]. In the present work, two bacterial isolates were positive for HCN production, which acts as an inducer of plant resistance. Multiple PGP activities among PGPR have been reported by some other workers while such findings on indigenous isolates of India are less commonly explored [32]. In the present study isolate FBJ6 (*Pseudomonas* sp.) was found to be most efficient PGPR which solubilized insoluble phosphorus, produced IAA, produced ammonia, showed ACC deaminase activity and produced HCN and catalase. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers.

ACKNOWLEDGEMENT

We are thankful to Prof. M.P Vohra (Dean, life Sciences) SILB Solan (H.P) for giving valuable guidance from time to time. We also have deep gratitude to Dr. Pratibha Mishra (Asst. Professor) SILB Solan (H.P). for their help and encouragement.

REFERENCES

[1] Hiltner, L. 1904. UÈ ber neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbackteriologie und unter besonderer BeruÈcksichtigung der GruÈnduÈngung und Brache. ArbeitenDeutscher Landwirtschafts Gesellschaft 98:59-78.

[2] Juanda, J. I. H. 2005. Screening of soil bacteria for Plant Growth Promoting Activities in Vitro. *J. Agri. Sci.* 4:27-31.

[3] Khan, M. S. 2006. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. 163:173-181.

[4] Ahmad, F., I. Ahmad and M. S. Khan. 2008. Screening of free-living rhizospheric bacteria for their multiple growth promoting activities. *Microbiol.* 163:173-181.

[5] Nelson, L. M. 2004. Plant growth promoting rhizobacteria (PGPR): Prospect for new inoculants. *Online. Crop Management* doi: 10. 1094/CM-2004-0301-05-RV.

[6] Khan, M. S. 2006. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. 163:173-181.

[7] Rodriquez, H. and R. Fraga. 1999. Phosphate solublizing bacteria and their role in plant growth promotion. *Biotechno*l.17:319-339.

[8] Vazquez, P., G. Holquin, M. E. Puente, A. Lopez-Cortez and Y. Bashan. 2000. Phosphate solubilizing microorganism associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biol. Fert. Soil* 30: 460-468.

[9] Vessey, J. K. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* 255: 571-586.

[10] Salamone, I. E. G. 2000. Direct beneficial effects of cytokinin producing rhizobacteria on plant growth. *Ph.D. Thesis*, University of Saskatchewan, Saskatoon, SK, Canada; 128.

[11] Johnson, L. F. and E. A. Curl. 1972. Method for research on ecology of soil borne pathogens. *Burgress Soc.,* 4:97–102.

[12] Gupta, R. S., S. Rekha, Aparna. and R. C. Kuhad.1994. A modified plate assay for screening phosphate solubilizing microorganisms. *J. Gen. Appl. Microbiol.* 40:255–260.

[13] Nautiyal C.S and S. Mehta. 2001. An Efficient Method for Qualitative Screening of Phosphate-Solubilizing Bacteria. *Microbiol.* 43:51-56.

[14] Jackson, M. L. 1973. Estimation of phosphorus content. Soil chemical analysis, Printer Hall, New Delhi (India).

[15] Loper, J. E. and M. N. Schroth. 1986. Influence of bacterial sources of indole-2-acetic acid on root elongation of sugar beet. Phytopath 76:386-389.

[16] Gordon, S. A. and R. P. Weber. 1951. Colorimetric estimation of indole acetic acid. Plant Physiol. 26:192–195.

[17] Belimov, A.A., V. I. Safronova and T. Mimura. 2002. Response of spring rape to inoculation with plant growth-promoting rhizobacteria containing 1aminocyclopropane-1 carboxylate deaminase depends on nutrient status of the plant. *Can. J. Microbiol.* 48:189–199.

[18] Dey, R., K. K. Pal, D. M. Bhatt and S. M. Chauhan. 2004. Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. *Microbio. Res*. 159:371-394.

[19] Cappuccino, J. G. and N. Sherman.1992. Biochemical activities of microorganisms. In: Microbiology, A Laboratory Manual. The Benjamin / Cummings Publishing Co. California, USA.

[20] Castric, P. A. 1975. Hydrogen cyanide, a secondary metabolite of *Psuedomonas aeruginosa*. *Can. J. Microbiol.* 21:613-618.

[21] Kreig, N. R. and J. G. Holf. 1984. Bergeys Manual of Systematic Bacteriology. William and Wilkins, Baltimore, USA.

[22] Glick, B. 1995. The enhancement of plant growth by free living bacteria. *Microbiol.* 41:109-117.

[23] Lalande, R., N. Bissonnette, D. Coutlée, H. Antoun. 1989. Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. *Plant Soil.* 115: 7-11.

[24] Reyes, V. A. and Z. Valduz. 2006. Phosphate solubilizing microorganisms isolated from the rhizospheric and bulk soils of colonizer plants at an abandoned rock phosphate mine. *Plant Soil* 287: 69-75.

[25] Mirza, M. S., W. Ahmad, F. Latif, J. Haurat, R. Bally, P. Normand and K. A. Malik. 2001. Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. *Plant Soil* 237:47-54.

[26] Xie, H., J. J. Pasternak and B. R. Glick. 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 that overproduce indoleacetic acid. *Curr. Microbiol.* 32:67-71.

[27] Herman, M. A. B., B. A. Nault and C. D. Smart. 2008. Effects of plant growth promoting rhizobacteria on bell pepper production and green peach aphid infestations in New York. *Crop Protect.* 27:996-1002.

[28] Kloepper, J. W., C. M. Ryu and S. Zhang. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopath.* 94:1259-1266.

[29] Kokalis-Burelle, N., J. W. Kloepper and M. S. Reddy. 2006. Plant growth-promoting rhizobacteria as transplant amendments and their effects on indigenous rhizosphere microorganisms. *Appl. Soil Ecol.* 31:91-100.

[30] Minorsky, P.V. 2008. On the inside. *Plant Physiol.* 146: 323-324.

[31] Voisard, C., C. Keel, D. Haas and G. Defago. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *EMBOJ.* 8:351-358.

[32] Abbas, Z. and Y, Okon. 1993. Plant growth promotion by *Azotobacter paspali* in the rhizosphere. *Soil Biol. Biochem.* 25:1075-1083.