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Isolation and screening of rhizobacteria for various plant growth promoting attributes

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Abstract
Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth through a variety of mechanisms that include improvement of plant nutrition, production and regulation of phytohormones, and suppression of disease causing organisms. A total of ten morphologically different bacterial strains were isolated from rhizospheric soil of leguminous and non-leguminous crops and were evaluated for their various PGPR activities. Most of the strains were *Pseudomonas* and *Bacillus* species followed by *Rhizobium*, *Mesorhizobium* and *Azotobacter* species. The PGP traits studied were ammonia production, indole acetic acid (IAA) production, HCN production, potassium solubilization, phosphate solubilization, siderophore production and zinc solubilization. Interestingly, strain PGP2, PGP5, PGP7, PGP8, PGP9 and PGP10 seems to be very promising and can be potentially used to promote plant growth and sustainable agriculture.

Keywords: Isolation, screening, plant growth promoting rhizobacteria, strains

1. Introduction
Agriculture is a major sector of national income in developing countries including India and it also ensures employment and food security. Interestingly, plant growth promoting rhizobacteria (PGPR) are considered as a potential tool to provide substantial benefits to agriculture. They are a heterogenous group of beneficial bacteria associated with the plant root that obtain nutrients from plants exudates and have beneficial effects on crop productivity and can help in sustainability of safe environment [1]. The PGPR strains can improve the plant growth and health by diverse mechanisms that include phosphate and potassium solubilization, siderophore production, biological nitrogen fixation, phytohormone production, 1-Aminocyclopropane-1-carboxylate (ACC) deaminase production, synthesis of antifungal metabolites, production of biocontrol agents, etc. [2]. PGPR confer beneficial effects on plant growth by direct and indirect means, but the specific mechanisms involved have not all been well characterized. Direct mechanisms of plant growth promotion by PGPR entails either providing the plant with a compound that is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment. The indirect promotion of plant growth occurs when the PGPR reduce or lessen the deleterious effect of plant pathogen on crop yield. This can happen by producing antagonistic substances or by inducing resistance to pathogens [3].
Moreover, PGPR can also produce antioxidant enzymes which can protect plants from environmental stresses like salinity, drought and heavy metal toxicity which leads to the generation of reactive oxygen species that cause cell damage. Direct enhancement of mineral uptake due to increase in specific ion flux at the root surface in the presence of PGPR has also been reported. PGPR strains may use one or more of these mechanisms in the rhizosphere. PGPR that synthesize auxins and cytokinins or that interfere with plant ethylene synthesis have been identified. Biological control using specific PGPR strains has been demonstrated against many plant pathogens e.g., *Pythium* sp., *Rhizoctonia solani*, *Fusarium* sp., *Pseudomonas* sp., etc. They protect plants against pathogens *via* the production of antibiotics, antifungal chemicals and insecticides [4]. PGPR strains can be genetically modified to improve the plant growth and also the disease resistance of agricultural crops.
The PGPR strains belong to various genera e.g. *Rhizobium*, *Azospirillum*, *Azotobacter*, *Alcaligenes*, *Bacillus*, *Arthrobacter*, *Pseudomonas*, *Mesorhizobium*, *Enterobacter*, *Erwinia*, *Acinetobacter*, *Flavobacterium*, etc. Several researchers have studied on the diversity, dynamics and significance of PGPR strains in enhancing crop productivity and its role in sustainability of safe environment [4, 5]. However, the results achieved under *in vitro* conditions may not be reproducible under field conditions. Also, the PGPR activity is affected due to variation in environmental conditions, soil characteristics, type of indigenous soil microbial

flora and their activity. Hence, it is necessary to explore the diversity in soil microbial flora for PGPR strains having multiple plant growth promoting activities and also, they could be promising in field conditions. So, keeping it in view, the present study was undertaken to isolate and screen bacteria from rhizospheric soils of Ayodhya (U.P., India) for various plant growth promoting activities.

2. Materials and Methods

2.1 Collection of soil samples

The soil samples were collected aseptically up to the depth of 15 to 20 cm from the rhizosphere zone of various leguminous and non-leguminous crops from different places grown in the vicinity of Ayodhya, Uttar Pradesh, India and packed in sterile plastic bags, labelled properly and stored at 4 ºC in the laboratory till use.

2.2 Isolation of rhizosphere bacteria

The rhizosphere soil samples were mixed thoroughly to make it a composite soil.Bacteria were isolated from rhizospheric soil employing serial dilution technique which wasprepared in normal saline up to 10-6 dilutions with the sterile blank.Total culturable bacteria were isolated and enumerated on nutrient agar by the standard pour plate technique[6]. Plates were incubated at 30±2°C for 24-36 h and the total number of bacteria was determined as colony forming units per milliliter (CFU per milliliter). Further purification of the isolated bacterialcolonies was done by repeated streaking on several nutrient agar plates and their purity was checked by Gram and negative staining.The purified colonies were further preserved at 4 °C on nutrient agar slants.

2.3 Screening of rhizospheric bacteria for growth on selective media

The growth of isolated rhizosphere bacteriawereevaluated on selective media plates using replica plate technique. The selective media used were yeast extract mannitol agar for Rhizobium and Mesorhizobium sp., King's B medium for Pseudomonas, Jensen's medium for Azotobacter and nutrient agar for Bacillus and other bacterial strains.The inoculated plates were incubated at 30±2°C for the desired duration and their growth was observed. All the bacteriological media as well as media ingredients were procured from HiMedia Pvt. Ltd. *In vitro s*creening of rhizobacteria for multiple plant growth promoting traits was carried out.

2.3.1 Phosphate solubilization test

The isolated bacterial strains were screened for their tri-calcium phosphate (TCP) solubilizing activity on Pikovskaya's (PKV) agar medium plates.Bacterial culture was aseptically spotinoculated on agar plates. The plates were incubated at 30±2°C for 5days.The presence of clear zone around the bacterial colonies indicated phosphate solubilization activity.

2.3.2 Potassium solubilizing activity

The selected bacterial isolates were spot inoculated aseptically on Alexsandrov agar medium plates and were incubated at 30±2°C for 4 days. The presence of clear zone around the rhizobacterial colonies indicated potassium solubilization activity.

2.3.3 Siderophore production test

Siderophore production was assayed on Chrome Azurol S (CAS) Agar medium plates. The tertiary complex Chrome Azurol S/ Fe+3/ hexadecyltrimethylammonium bromide served as an indicator. Spot inoculation of bacterial isolates was done on CAS agar plates and incubated at 30±2°C for 48-72 h. Development of yellow-orange clear halo zone around the bacterial growth was considered as positive test for siderophore production.

2.3.4 Indole Acetic Acid production

The bacterial strains were grown in sterilized nutrient broth amended with tryptophan (10 µg/ml) and incubated at 30±2˚C for 2-3 days in incubator shaker. The bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4˚C. To 2ml of the culturesupernatant, 4 ml of Salkowski reagent (50 ml of 35% perchloric acidin 1ml of 0.5 M $FeCl_3$) were added and incubated at 28°C for 20 minutes. Development of pink to red colour indicates IAA production. The absorbance was read at 530 nm using spectrophotometer. Concentration of IAA was measured with the help of standard graph of IAA obtained in the range of 0-5mg/ml. the values of IAA are expressed as µg/ml.

2.3.5 Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Overnight grown cultures were inoculated in 10 ml of sterile peptone waterand incubated at 30±2°C for 3-4 days. One ml of Nessler's reagent was added in each tube including control.Development of yellow to brown colour indicated the production of ammonia which is a positive test.

2.3.6 Hydrogen cyanide (HCN) production

The bacterial isolates were screened for the production of hydrogen cyanide. The King's B medium plateswere prepared aseptically and was inoculated by thestreakplate method. The Whatman filter paper No.1 disc (of ~petriplate diameter) was soaked in sodium picrate solution (0.5% picric acid in 2% sodium carbonate) and placed in the lid of each petriplate. The petriplates were sealed with parafilm and incubated at 30±2°C for 2-5 days.The change of the filter paper colour from deep yellow to orange and finally to dark brown indicated the production of hydrogen cyanide indicating positive test. If the colour of the filter paper remains deep yellow it then indicates negative HCN test.

2.3.7 Zinc solubilization test

The zinc solubilizing ability of the rhizobacterial isolates was determined by spot inoculation of the isolates on the Tris-minimal medium plates (per liter: Tris–HCl 6.06 g; NaCl 4.68 g; KCl 1.49 g; NH_4Cl 1.07 g; Na_2SO_4 0.43 g; $MgCl_2.2H_2O$ 0.2 g; $CaCl_2.2H_2O$, 30 mg, pH 7.0) supplemented with 1.5% agar and 0.1% (w/v) insoluble zinc in the form of zinc sulfate ($ZnSO_4$). The plates were incubated for 5-7 days at 30±2°C and examined for the formation of halo zones around the bacterial colonies for zinc solubilization.

2.4 Identification of the selected rhizobacterial isolates

The scheme of Cowan and Steel was followed for identification of the selected isolate [7,8]. Various morphological, physiological and biochemical tests were performed and the results were interpreted according to Bergey's Manual of Determinative Bacteriology [9].

3. Results and Discussion

3.1 Isolation and identification of plant growth promoting rhizobacteria

A total of ten morphologically distinct plant growth promoting rhizobacterial strains were isolated and purified from the rhizospheric soil. All isolates were showing significant PGPR activity. On the basis of morphological, cultural and biochemical characteristics they have been identified as *Rhizobium*, *Mesorhizobium*, *Pseudomonas*, *Azotobacter* and *Bacillus* species (Table 1). The present investigation gives information about the diverse type of plant growth promoting rhizobacteria present in the rhizospheric soil of leguminous and non-leguminous crops that possess one or more PGP traits. Plant rhizosphere is known to be preferred ecological niche for various types of soil microorganisms due to rich nutrient availability [4]. PGPR also modify root functioning, improve plant nutrition and influence the physiology of the whole plant [1]. For identification of efficient PGPR strains with multiple activities, microbial isolates were subjected to further studies to understand their plant growth promoting properties under *in vitro* conditions.

Table 1: Identification of plant growth promoting rhizobacterial strains

Bacterial strains	Organism identified
PGP 1	Rhizobium sp.
PGP 2	Pseudomonas sp.
PGP 3	Azotobacter sp.
PGP 4	Bacillus sp.
PGP 5	Rhizobium sp.
PGP 6	Bacillus sp.
PGP 7	Pseudomonas sp.
PGP 8	Bacillus sp.
PGP 9	Pseudomonas sp.
PGP 10	Mesorhizobium sp.

3.2 Screening of plant growth promoting traits in rhizobacterial isolates

The screening results of rhizobacterial strains for various plant growth promoting traits are depicted in Table 2. Out of ten isolates tested, phosphate solubilization was observed in nine isolates which was confirmed by halo zone formation in Pikovskaya's (PKV) medium while only the PGP-3 isolate was unable to solubilize inorganic phosphate. It has been reported that phosphate-solubilizing bacteria native to acid soil had ability to promote Phaseolus vulgaris growth [10]. The study was conducted to characterize three bacterial strains in solubilising rock phosphates as well as their impact in promoting soybean growth under pot grown conditions. Among 10 rhizobacterial isolates, 7 isolates were able to solubilize potassium on Alexsandrov agar medium which was confirmed by the presence of clear zone around the rhizobacterial colonies. The occurrence of potassium solubilizing bacteria in the rhizosphere soil as compared to non- rhizosphere soil has also been reported [3].

Table 2: Various plant growth promoting traits of rhizobacterial strains

Bacteria	NH_3 production	HCN production	IAA Production	Potassium solubilization	Phosphate solubilization	Siderophore production	Zinc solubilization
PGP 1	+	-	+	+	+	+	-
PGP 2	+	+	+	+	+	+	+
PGP 3	-	+	-	-	-	-	+
PGP 4	+	-	-	-	+	-	+
PGP 5	+	-	+	+	+	+	+
PGP 6	-	-	+	-	+	-	+
PGP 7	+	+	+	+	+	+	+
PGP 8	+	+	+	+	+	-	+
PGP 9	+	+	+	+	+	+	+
PGP 10	**+**	**-**	**+**	**+**	**+**	**+**	**+**

(+) Indicates: Positive / (-) Indicates: Negative

Siderophore production was detected in only six isolates as they produced visible orange halo zones around the bacterial colonies. Further, out of 6 isolates PGP-9 exhibited strong (+++) siderophore production followed by PGP-2 and PGP-10. In the remaining three isolates moderate (++) siderophore production was observed. Siderophore production is a very important PGP trait that influences plant growth by binding to the available Fe^{3+} in the rhizosphere. Through this process iron is made available to the plants but unavailable to the phytopathogens [11]. The siderophore production by *Rhizobial* strains has been considered as a potential way to improve nodulation and N_2 fixation in iron deficient conditions [12].

The results of Indole Acetic Acid production showed that out of 10 isolates tested, only eight isolates were able to produce IAA. Further, out of 8 isolates PGP-2 showed maximum IAA production, followed by PGP-9, PGP-5 and PGP-10. It has been reported that IAA production by PGPR can vary among different bacterial species and strains and that it is also influenced by the culture conditions, growth stage and substrate availability [5]. Further out of the 10 rhizobacterial isolates tested in the present study, eight isolates were able to produce ammonia significantly, whereas, the remaining 2 isolates *viz.*, PGP-3 and PGP-6 were scored as weak (+) for ammonia production. It is one of the very important trait of PGPR as it directly influences the plant growth [13].

In the present investigation, five rhizobacterial strains were potential HCN producer. HCN is a potent inhibitor of cytochrome- C oxidase and of several other metalloenzymes. HCN producing bacteria can help plants in their defense against fungal pathogens [5]. This property was predominantly described among *Pseudomonas* strains [14]. Interestingly, the zinc solubilizing ability was observed in nine rhizobacterial isolates which was confirmed by halo zone formation in

selective medium while only one isolate PGP-1 was unable to solubilize the zinc from insoluble zinc sulfate.

Multiple PGP activities among plant growth promoting rhizobacteria have been reported by some researchers while such findings on indigenous rhizobacterial isolates of India are less commonly explored. The present work clearly indicates that some PGPR, such as PGP2, PGP5, PGP7, PGP8, PGP9 and PGP10 seems to be very promising and can be potentially used to promote plant growth and sustainable agriculture.

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