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Research Article

CHARACTERIZATION OF PSEUDOMONAS SP FROM RHIZOSPHERE OF TOMATO PLANTS (*LYCOPERSICON ESCULENTUM*) AND ITS EFFICACY ON PLANT GROWTH PROMOTION

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are known to enhance growth of plants by involving various direct and indirect mechanisms. About sixteen rhizobacterial isolates were isolated from various rhizospheric soils of tomato crops in the fields of Dehradun. These bacterial isolates were phenotypically characterized and were subjected to *in vitro* screening for their plant growth promoting traits like ammonia and siderophore production, production of indole acetic acid (IAA), hydrogen cyanide (HCN) and phosphate solubilization. Out of sixteen bacterial isolate, three bacterial isolates proved to have potential PGPR activities. The production of indole acetic acid (IAA) by all rhizobacteria was investigated as possible mechanism for plant growth stimulation. All rhizobacterial isolates were shown to produce IAA *in vitro* in presence of L-tryptophan in the culture medium. The isolates were also tested for their efficacy in seed germination and seedling vigour by using tomato seeds in roll towel method. All rhizobacterial isolates also showed an increased in both the shoot and the root length of tomato seedlings as compared to the control. The seed germination ranged from 90 percent (uninoculated control) to 98.5 percent. All the three strains GKS-V, HPR-I, HPR-III significantly improved seed germination when compared to the uninoculated control (UIC). These isolate showed significantly increased in shoot and root length as well as enhanced vigour index of 124.54 and 741.45 after 6 and 16 days respectively. From biochemical tests it was concluded that all the three selected rhizobacteria belong to *Pseudomonas* species.

Key words: Plant growth promoting rhizobacteria, IAA production, phosphate solubilization, siderophore production, Seed germination, Roll towel method.

INTRODUCTION

Bacterial diversity has gained quite importance as most useful resource with considerable significance in the global form of biofertilizers, bioremediation and bioprospecting¹.

The vicinity of plant roots are surrounded by rhizosphere which is an extremely important and active area for root activity and metabolism. Hiltner first gave the concept of rhizosphere and described it as the narrow zone of soil surrounding the roots where microbe populations are stimulated by root activities². However, this concept has now been modified to include the soil surrounding a root in which physical, chemical and biological properties have been changed by root growth and activity³. The composition of bacterial community in the rhizosphere is important for the performances of the plant, as bacterial species can have beneficial, neutral or harmful relationships with the roots⁴. Rhizosphere consists of a large number of microorganisms such as bacteria, fungi, protozoa and algae in which bacteria are the most abundant of all. Plants secrete some organic compounds through their exudates which helps them in selecting such useful bacteria creating a very selective environment where diversity is low⁵⁻⁷. Bacteria being the most profuse microorganism in the rhizosphere, it is quite certain that they influence the plant physiology to a larger extent, especially considering their competitiveness in root colonization⁸.

Microorganisms colonizing the rhizosphere can be distinguished based on their effect on plants and the way they interact with

roots, some being pathogens whereas others trigger beneficial effects. Rhizobacteria that inhabit plant roots exert some positive effect ranging from direct influence mechanisms to an indirect effect. So, these rhizobacteria inhabiting the rhizosphere and exerting beneficial effect on plants are termed as PGPR⁹.

Plant growth-promoting rhizobacteria (PGPR) are a group of microorganisms in the rhizosphere that promote plant growth by increasing nutrient availability and may be used as inoculants for biofertilization, phytostimulation and biocontrol and can be classified according to their beneficial effects¹⁰. These rhizobacteria are benefited from the nutrients secreted by the plant root and in turn beneficially influence the plant in a direct or indirect way, resulting in enhanced plant growth. The interaction between rhizobacteria and plant roots can be beneficial, detrimental or neutral and this delicate balance is a consequence of both plant and soil type¹¹. These beneficial bacteria can also be symbiotic or free living, and are abundant near the roots¹². They benefit plants through, (a) Production of plant hormones, such as auxins¹³ (b) symbiotic N₂ fixation¹⁴ (c) antagonism against phytopathogenic microorganisms by production of antibiotics, siderophores, β -(1,3)-glucanase, chitinase¹⁵ and cyanide¹⁶ and (d) solubilization of mineral phosphates and other nutrients¹⁷. A number of PGPR such as *Bacillus*¹⁸, *Pseudomonas*¹⁹ and *Arthrobacter*²⁰ have been used for enhancement of plant growth performance. Moreover antibiotic production, growth promotion, biocontrol potential and induced responses of PGPR are used as an effective pathogens management tool. The potential to use PGPR in integrated

strategies to reduce Nitrogen and Phosphorous fertilizers offers and appealing research area for those scientists engaged in growth promotion studies in dependence of biological control. There are several reports that PGPR's have plant promoted the growth of productive parameters of plant ranging from cereals, pulses, ornamentals, medicinal and aromatic plants, vegetable crops, and even tree species²¹. Treatment with PGPR has increased germination percentage, seedling vigor, emergence, plant stand, root growth, shoot growth, total biomass of the plant, seed weight, early flowering, increased grain, fodders, fruit yield etc^{19,22-23}. Inoculations of crop plants with certain strains of PGPR at an early stage of development improve biomass production through direct effects on root and shoot growth. Inoculation of agricultural crops with PGPR may result in multiple effects on early-season plant growth, as seen in the enhancement of seedling germination, plant vigor, plant height, shoot weight, and nutrient content of shoot tissues²⁴. PGPR are reported to influence the growth, yield and nutrient uptake by an array of mechanisms. There has been much research interest in PGPR and there is now an increasing number of PGPR being commercialized for various crops.

Furthermore the plant grows faster and greener with longer roots and shoots than the untreated plants. It has been established that *fluorescents Pseudomonas* enhance plant growth in several ways viz; producing plant growth promoter such as gibberellins, cytokinins, indole acetic acid, which can either directly or indirectly modulate the plant growth and development²⁵. In the present study soil samples were collected from tomato rhizosphere of Dehradun, Uttarakhand region for assessment of *Pseudomonads* showed *in vitro* PGPR property.

MATERIAL AND METHODS

Isolation of *Pseudomonas* species from the rhizosphere

Rhizosphere soil samples were collected from tomato fields growing in Dehradun. *Pseudomonas* species were isolated using dilution method with King's B medium (Protease peptone 20 g/L, K₂HPO₄ 1 g/L, MgSO₄·7H₂O 0.40 g/L, Agar 20 g/L). Rhizospheric soil samples (10 g) was suspended in 90 mL of 0.85% normal saline (pH 7.0) and shaken vigorously at 150 rpm at 37 °C for 1 h. The resulting slurry was serially diluted (100 µL) to 900 µL of 0.85% normal saline and appropriate dilution (10⁻⁵) of this suspension (0.1 mL or 100 µL) was spread plated in triplicate on K.B medium. Cultures were incubated at 37 °C±2 for 2 days. For experimental use, isolates were transferred when needed to KB medium that was stored at 4 °C. Each colony was assayed further for morphological and physiological characteristics. *Pseudomonas* species were identified by morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology²⁶.

Purification and maintenance of the recovered bacterial isolates

All bacterial isolates were purified by re-streaking on KB medium and maintained on glycerol-KB medium at deep frozen temperature 4°C as stock cultures.

Glycerol stock preparation

A 2.5 ml, autoclaved mixture of glycerol and KB broth (1:1 v/v) was added to 5 ml of overnight grown culture in the King's B broth. Glycerol stocks were maintained in cryovials and preserved at 4 °C.

Morphological characterization

Recovered bacterial isolates were phenotypically (morphotypic and functional) characterized. A total sixteen of bacterial isolates were thus randomly selected morphologically from tomato rhizosphere. Colony morphology of isolates was studied under a microscope. This included shape, edge, elevation, surface and pigmentation. Cellular morphology was based upon cell shape and Gram staining. Bacterial identification was carried out on the isolates by comparing the results obtained with the standard characterization definitions of Skerman²⁷ and that of Bergey's manual of determinative systematic bacteriology (1986).

Characterization of rhizobacteria for PGPR traits

Siderophore production

Siderophore production was tested qualitatively using Chromeazuroil's Agar Medium (CAS-medium)²⁸. Each *Pseudomonas* isolate was streaked on the surface of CAS agar medium and incubated at a temperature of 36 °C for 1 to 3 days. Siderophore production was indicated by orange halos around the colonies after the incubation, and this test was done in two replications.

Phosphate solubilization

Solubilization of tri-calcium phosphate was detected in Pikovskaya's Agar¹⁹. All bacterial isolates were streaked on the surface of Pikovskaya agar medium and phosphate solubilizing activity was estimated after 1 to 5 days of incubation at a temperature of 35 °C. Phosphate solubilization activity was determined by the development of the clear zone around bacterial colony.

Production of HCN

Production of hydrogen cyanide by the isolates was tested by adapting the method of Lorck²⁹. In this method the nutrient broth was amended with 4.4 g glycine/l and the isolates were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36±2 °C for 4 days. Development of orange to red colour indicated HCN production.

Qualitative assay of IAA production

The qualitative assay of IAA produced by Rhizobacterial strains was conducted on the basis of Brick³⁰ proposed method. In this method each petridish was inoculated with toothpicks. Then each inoculated Petri dish was overlaid with an 82-mm-diameter disk of nitrocellulose membrane. Petri dishes were inversely incubated into incubator (at 27 °C) for 2 to 4 days. When the diameter of appeared colonies on King's B media was about 2 mm, IAA production assay was conducted as following exposition:

The disks of nitrocellulose membrane including rhizobacterial grown colonies (about 2 mm) were treated with Salkowski reagent within other petridish including a reagent-saturated filter paper (Whatman no. 1). The reaction was allowed to proceed until adequate color developed. All reagent incubations were conducted at room temperature. Bacteria producing IAA were identified by the formation of a characteristic red halo within the membrane surrounding the colony (after time of 5 to 2 hours) and the color and size of the colonies differed in different isolates (depending on the amount of produced IAA by isolates).

Quantitative assay for Production of Indole acetic acid

Indole acetic acid (IAA) production was detected as described by Patten and Glick³¹. Bacterial cultures were grown for 48 h on their respective media at 37±2 °C. Bacteria were grown overnight in five ml of nutrient broth supplemented with L-tryptophan to achieve a final concentration of 0100 µg ml⁻¹. After incubation for 42 h, bacterial growth was measured using spectrophotometry at 600 nm; cells were removed from culture medium by centrifugation at 7,500 rpm for 10 min. 1 ml aliquot of supernatant was mixed with 4 ml of Salkowski's reagent. Samples were left at 28±2 °C for 25 min and absorbance was taken at 535 nm. The concentration of IAA was determined by referring to a standard curve.

Evaluation of effective PGPR strains on seed germination and seedling vigour Seed germination assay

The experiment was conducted to assess the influence of 3 selected efficient isolates on seed germination and tested for their plant growth promotion ability by the standard roll towel method in growth chamber²³.

Fluorescent bacteria were grown in King's B broth and were kept in a shaker (150 rpm) for 2 days. Then the cultures were centrifuged at 10,000 rpm for 5 minutes. The pellet was mixed with sterile carboxy methyl cellulose (CMC) suspension (1%). Tomato seeds were surface sterilized with 0.1% mercuric chloride for 5 min, rinsed with sterilized distilled water (SDW) and soaked in bacterial suspension (3×10⁸cfu/ml) mix with CMC for 24h and sterile blank nutrient broth served as control. Then the seeds were blot dried, placed in wet blotters and incubated in growth chamber which was maintained at 25±20 °C and 95±3% Relative Humidity. The percentage of germination was recorded at fourth day. About ten seedlings were taken at random from each replication and length of root and shoot measured separately at 6 and 16 days. Plant growth promotion of tomato seedling was assessed using Vigour Index (VI)¹⁸.

VI = per cent germination x mean total length of seedling (root length + shoot length)

Biochemical characterization

Rhizobacteria were further characterized by their biochemical characterization viz., catalase test, oxidase test, carbon source utilization, citrate utilization, and gelatin hydrolysis.

RESULTS AND DISCUSSIONS

Morphological characterization

A total of sixteen bacterial isolates were recovered from rhizospheric soil from different sites of tomato field of Dehradun. Pure colonies were obtained by subsequent subculturing by using serial dilution plate technique in King's B agar media. Isolated rhizobacteria were morphologically characterized on the basis of colour, morphological characteristics viz., colony morphology (shape, margin, elevation and surface) and cell morphology (Gram's reaction, cell shape and arrangement) and were studied in detail (Table 1). Bacteria exhibited wide morphological variation. A wide range of variation in colony shape was seen viz., irregular, circular, fusiform and round. Maximum of the obtained bacterial isolates were Gram negative, rod shaped with dry texture, entirely edged, flat and smooth, and creamy in appearance as tabulated in Table 1. They were further recognized as *Pseudomonas*

species. Bergey's manual of determinative of bacteriology was used as a reference to identify the isolates³².

In- vitro screening of Plant growth promoting (PGP) activities

All bacterial isolates were evaluated for their plant growth-promoting traits like HCN production, IAA production, ammonia production, Siderophore and Phosphate solubilizing activity (Table 2). In search of efficient PGPR strains with multiple activities, soil bacteria were isolated from soil of central and upper Himalayan region with a view to screen/evaluate their plant growth promoting potential¹⁹.

The isolates were tested *in vitro* for phosphate solubilizing bacteria in Pikovaskaya's agar plates. A total of seven isolates showed positive test for phosphate indicating conversion of insoluble phosphates into soluble forms through the production of organic acids.

Another important trait of PGPR is the production of ammonia that indirectly influences the plant growth. Total eight isolates were found to be positive for ammonia production indicating conversion of ammonia into nitrates and similar chemical compounds that plants can absorb and use.

HCN is known to be both beneficial and harmful for plants³³. The production of HCN in excess may play a critical role in the control of fungal diseases¹⁶. The bacterial isolates were tested for HCN production and all the isolates did not showed this activity. Isolates which gave negative result had PGPR property because HCN is a lethal bi-product which is harmful for the plant growth promotion.

The qualitative test for IAA production was done to check the production of the most important phytohormone indole acetic acid (IAA) which stimulates growth of plant directly. A total of ten isolates showed positive test for IAA production. The amount of indole acetic acid produced was detected in the presence of different concentration of tryptophan, which led to the elevation in indole acetic acid production as compared in the absence of tryptophan. Rhizobacterial isolates were recovered from rhizospheric soil associated with *Withania somnifera* and characterized as efficient Producers of IAA. The IAA production was further confirmed by thin layer chromatography (TLC). IAA producing bacteria may be efficient biofertilizer inoculants to promote plant growth and protecting the medicinal plants for the future generation³⁴.

Another important PGPR trait is the siderophore activity. A total of ten isolates were found positive for siderophore activities which were indicated by orange halo zone. The siderophore activity was also tested in the tube method which gave the clear indication of presence of siderophores. Siderophores are low molecular weight, extracellular compounds with a high affinity for ferric iron, that are secreted by microorganisms to take up iron from the environment³⁵ and their mode of action in suppression of disease were thought to be solely based on competition for iron with the pathogen³⁶.

Results of this study showed that in the rhizobacteria recovered from rhizospheric soil of the tomato plant exhibit various traits like phosphate solubilization, HCN production, ammonia production and siderophore production (Table 2).

The percentage of PGP traits shown by isolates was also evaluated. The isolates exhibited 62.5% of siderophore and indole acetic acid activity, 43.75% of phosphate solubilization

and 50% ammonia production. These characterizations are beneficial as these tests indicate direct plant growth promoting activity by bacterial isolates which depicts efficient PGPR strains. Rhizosphere bacteria were isolated from the soil of Central Himalayan region and to evaluate their plant growth promoting potential as an alternative of chemical fertilizer for sustainable, environment friendly agriculture and assessment of their phylogenetic characterization¹⁹.

Quantitative assay for production of indole acetic acid

Production of IAA was detected by appearance of pink colour. Briefly three bacterial isolates such as were selected for quantitative analysis of IAA production based on their qualitative analysis. This was done by growing the isolates in tryptophan as substrate. Isolates GKS-V showed maximum IAA production and least by isolates HPR-III. Indole acetic acid (IAA) production was detected as described by Brick³⁰. The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant³⁴. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities^{23,37}. All three bacterial isolates were then subjected to biochemical characterization for the determination of probable microorganism. Biochemical and morphological characterization suggests that the above three isolates belong to *Pseudomonas* Species (Table 3). Production of IAA by *Pseudomonas* is a general characteristic of our test isolates. Higher level of IAA production by *Pseudomonas* was recorded by other workers³⁸. IAA producing rhizobacterial isolates exhibiting improved seed germination also supported improved root and shoot length^{23,34}. Seeds coated with rhizobacterial isolates derived from soil representing modern farming practices (MFB-1R-3) showed maximum germination (38.36%), root length (9.41 cm) and shoot length (2.83 cm) (Figure 1).

Evaluation of efficacy PGPR strains on seed germination and seedling vigour

After inoculating the seeds of tomato with the three bacterial isolates such as GKS-V, HPR-I and HPR-III, root and shoot length were measured and percentage of seed germination and vigour index was calculated. The data on seed germination and seedling vigour of tomato as influenced by seed bacterization

with different rhizobacterial isolates by roll towel method are given in (Figures 2 and 4).

In general, the seed germination ranged from 90 per cent (uninoculated control) to 98.5 per cent (GKS-V). Other strains such as HPR-I and HPR-III also showed significant improvement in seed germination when compared to the uninoculated control (UIC). The isolate GKS-V showed significantly increased seed germination (98.5%) and also shoot and root length as well as enhanced vigour index of 124.54 and 741.45 after 6 and 16 days respectively (Figure 3). Similarly, Agrawal et al.,²³ reported that Bacterized lentil seeds showed improved plant growth compared to untreated control. A significant difference in percentage of germination was observed compared to control; values ranged between 14.18 to 38.36%. The growth promoting activity of rhizobacterial isolates of PGPR was also tested for seed germination and Seedling vigour by using Ashwagandha seeds in roll towel methods. In roll towel method, the PGPR strains WSNb3, WSNb5 and WSNb6 produced higher shoot and root length of Ashwagandha seedlings and showed more than 90 percent seed germination and with enhanced vigour index after 21 days. The maximum vigour index of 1035.5 was recorded in WSNb3 treated seedling. The data on seed germination and seedling vigour of Ashwagandha as influenced by seed bacterization with different rhizobacterial isolates³⁴.

CONCLUSION

The isolated organisms were identified as *Pseudomonas* spp. from Tomato plants (*Lycopersicon esculentum*). Rhizobacterial isolates showed potential PGPR activities. The productions of indole acetic acid (IAA) by all rhizobacteria were investigated as important mechanism for plant growth stimulation. Rhizobacterial isolates produced IAA *in vitro* by the addition of L-tryptophan, in the culture medium. The PGPR isolates tend to evaluate the root and shoot length, seed germination and vigour index as compared to the uninoculated seeds. These results led to the selection of effective PGPR inoculants that are beneficial for the growth of various crops. These inoculants if applied directly to the plant or its parts can be effective in growth stimulation.

Table 1: Morphotypic characterization of rhizobacteria associated from tomato rhizosphere

Name of Isolate	Grams Reaction	Colour of Colony	Margin	Elevation	Surface	Form
Morphotypic characterization of rhizobacteria recovered from Garden Soil						
GKSI	Gram-Ve, Coccobacillus	Creamy	Undulate	Flat	Dry	Irregular
GKSII	Gram-Ve, Short Rods	Creamy Brown	Undulate	Slightly Raised	Mucoid	Irregular
GKSV	Gram -Ve, Rod shaped	Creamy Yellow	Entire	Umbonate	Mucoid	Irregular
GKSVI	Gram -Ve, Rods	Creamy	Undulate	Raised	Slightly Mucoid	Irregular
GKSVII	Gram +Ve, Long Rods	Creamy White	Auriculate	Flat	Mucoid	Fusiform
GKSVIII	Gram +Ve, Long Rods	Opaque	Filamentous	Flat	Dry	Irregular
Morphotypic characterization of rhizobacteria recovered from Home Soil						
HPSI	Gram-Ve, Coccobacillus	Creamy	Entire	Slightly Raised	Mucoid	Round
HPSII	Gram-Ve, Coccus	Off-White	Entire	Convex	Mucoid	Round
HPSIV	Gram -Ve, Coccus	Pale Yellow	Undulate	Raised	Smooth	Irregular
HPSV	Gram +Ve, Short Rods	Off-White	Curled	Flat	Dry	Irregular
HPR I	Gram -Ve, Short Rods	Off-White	Entire	Convex	Mucoid	Round
HPRII	Gram-Ve, Coccobacillus	Pale Yellow	Repand	Umbonate	Mucoid	Round
HPRIII	Gram-Ve, Coccobacillus	Off-White	Auriculate	Flat	Mucoid	Irregular
HPRIV	Gram-Ve, Thick Rods	Creamy	Entire	Convex	Mucoid	Round
HPRV	Gram -Ve, Coccus	Creamy	Undulate	Flat	Mucoid	Irregular
Morphotypic characterization of rhizobacteria recovered from Field Soil						
FPSII	Gram +Ve, Bacillus	Creamy	Auriculate	Umbonate	Mucoid	Irregular

Table 2: *In vitro* plant growth promoting attribute of rhizobacteria of tomato plant

Isolate Name	HCN production	Siderophore Production	Phosphate Solubilization	Ammonia Production	IAA Production
GKS-I	-	-	+	+	-
GKS-II	+++	+	-	+	+
GKS-V	-	+	+	+	++
GKS-VI	+++	-	-	-	-
GKSVII	-	-	-	-	-
GKS-VIII	-	+	-	+	+
HPS-I	++	-	-	-	+
HPS-II	+	+	+	-	-
HPS-IV	+++	-	+	-	-
HPS-V	++	+	-	+	+
HPR-I	-	+	+	+	++
HPR-II	+	+	-	-	+
HPR-III	+	+	+	+	++
HPR-IV	+	+	-	-	+
HPR-V	-	-	-	-	-
FPS-II	+++	+	+	+	+

Table 2: *In vitro* plant growth promoting attribute of rhizobacteria of tomato plant

Isolate Name	HCN production	Siderophore Production	Phosphate Solubilization	Ammonia Production	IAA Production
GKS-I	-	-	+	+	-
GKS-II	+++	+	-	+	+
GKS-V	-	+	+	+	++
GKS-VI	+++	-	-	-	-
GKSVII	-	-	-	-	-
GKS-VIII	-	+	-	+	+
HPS-I	++	-	-	-	+
HPS-II	+	+	+	-	-
HPS-IV	+++	-	+	-	-
HPS-V	++	+	-	+	+
HPR-I	-	+	+	+	++
HPR-II	+	+	-	-	+
HPR-III	+	+	+	+	++
HPR-IV	+	+	-	-	+
HPR-V	-	-	-	-	-
FPS-II	+++	+	+	+	+

Figure 1: IAA production by isolated rhizobacteria

Figure 2: Influence of PGPR strains on seed germination

Figure 3: Influence of rhizobacterial isolates on root and shoot length

Figure 4: Influence of rhizobacterial isolates on seedling vigour

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