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Evaluation of Plant Growth Promoting Activity of Rhizosphere Bacteria of Different Crop Plants

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are good sources of nourishment to soil and hence a potential alternative to chemical fertilizers. In the study, a total of 12 rhizobacterial isolates, designated as SI1 - SI12 were assayed for their plant growth promoting activity. Using available standardized protocols the phosphate solubilisation and nitrogen fixation capabilities of the bacterial isolates were determined. Indole acetic acid (IAA) production capability of all the isolates was observed, among which two of the isolates SI-2 and SI-9 were noted to produce IAA in considerable amounts. These PGPR are thus found to be a promising source of bio fertilizers, detailed experimental study of which is to be accomplished in future.

Keywords: Rhizosphere**,** Phosphorus solubilising bacteria, Nitrogen fixing bacteria, IAA production

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INTRODUCTION

Rhizosphere is the closely associated soil layer to the root system of plants. The root system provides support and aids in water and nutrient uptake for the plant. Apart from this, the root system of the plants also synthesizes and secretes certain exceptional range of compounds [1]. They act as chemical attractants for a large number of heterogeneous and diverse, metabolically active soil microflora. These chemicals are collectively called root exudates. These exudates alter the chemical and physical properties of the soil, thus modifying the soil microbial community [2]. In addition, the exudates also encourage the plant-beneficial symbiotic interactions and hinder the growth of the hostile plant species [3]. Moreover, microbial activity in the rhizosphere influences rooting patterns and the supply of available nutrients to plants. A portion of these plant-derived small organic molecules is further metabolized by microorganisms present in this area as carbon and nitrogen sources, and some microbes produces come important nutrient molecules that are subsequently re-taken up by plants for growth and development [4]. Hence, rhizosphere is any volume of directly influenced by plant roots and or in close association with the root hairs and plant-produced material [5].

Rhizosphere soil usually contains a hugely diversified bacterial population commonly addressed as rhizobacteria [6]. These bacteria can be beneficial, neutral or deleterious [7]. The bacterial colonies producing healthy activities are coined as PGPR or plant growth promoting rhizobacteria [8]. Rhizobacteria, the term indicates the bacteria that luxuriously colonize the rhizosphere [9]. The plant growth promoting rhizobacteria (PGPR), are distinguished by the following characteristics: (i) they must colonize the root surface abundantly, (ii) they must outlive, multiply and compete with other microbes, (iii) they must encourage plant growth [10]. They should possess the inherent nature of competing with the other microbial flora of the environment, suppress the growth of phytopathogens and exert a beneficial effect on the growth of plants [11]. To note, the exact mechanism behind plant growth promoting activity isn't clearly defined, though many traits of the PGPR are responsible for the plant growth [12]. These include production or alteration of plant growth hormones like Indole Acetic Acid, Gibberellic acid, cytokinins and ethylene; fix atmospheric nitrogen; suppress the growth of deleterious microorganisms by production of siderophore, β-1, 3-glucanase, chitinase, antibiotics and cyanide; dissolves phosphates and other nutrients [7] [13]. These properties show a positive change in the yield of a crop. In some cases quality of the yield is also altered to a better and enriched variety [14].

Fixation of atmospheric nitrogen is one of the most important and interesting biological phenomenon noted. Biological Nitrogen Fixation represents an economically beneficial and environmentally sound alternative to chemical fertilizers. The free living and symbiotic nitrogen fixers like *Azotobacter sp*, *Rhizobium sp*, etc. are some of the main organisms involved in this process of biological nitrogen fixation. Since nitrogen is one of the major constituent that contributes to the greenhouse gas effect, it is essential to maintain a balance of the free nitrogen available in the atmosphere. Moreover nitrogen is also one of the essential nutrients required for plant growth and metabolism [15] [16].

Phosphorus (P), the second important plant growth-limiting nutrient after nitrogen, is abundantly available in soils in both organic and inorganic forms [17]. In spite of this huge availability, the amount of P that can be taken up by plants is low, the reason being that the forms found in soil are mostly insoluble. Plants can utilize only the two soluble forms, monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions [18]. Immobilization of inorganic phosphorous in soil is a major concern. Soon after the addition of phosphate fertilizers the phosphorus becomes unavailable to the plant. Plants take up only small amounts of the applied fertilizers and the rest is quickly converted into insoluble complexes [19]. It has been reported that only 0.1% of the total phosphorus content of the soil is available to the plant for absorption [14]. It can be solved either by some enzymatic treatment or microorganism aided mobilization of the phosphorus to make it available for the plant uptake as one of its vital macro nutrient component. The availability of phosphorus is one of the indispensable stages of the phosphorus cycle in nature. Some of the commonly reported organisms involved in this process are *Rhizobium sp*, *Pseudomonas sp*, *Burkholderia sp* etc. [20].

Indole acetic acid (IAA) is a phytohormone. The common name to this group of hormone is auxin. Auxins are secondary metabolite of the microbial metabolism and are reported for their indigenous property of enhancing the growth rate of the plants. IAA production enhances the plant growth by increasing the number of root hairs and lateral roots [21]. Microbial biosynthesis of IAA is influenced by tryptophan secreted in the root exudates or decaying cells [22] [23]. IAA promotes plant cell division, elongation and differentiation, it stimulates seed and tuber germination, increases the rate of xylem and phloem development, controls the

process of vegetative growth, and mediates responses to light [24]. They also form a part of the defence mechanism by inhibiting the growth of phytopathogens [25]. Therefore IAA by PGPR is recognized as an effective compound in plant-microbe interactions, both in pathogenesis and phytostimulation [26].

The soil texture and the availability of nutrients play an important role in the colonization of the microorganisms. The greater the nutrient content more is the microbial metabolism which in turn is involved in the growth promoting activities. Temperature, pH, moisture content are some of the physio-chemical parameters to be maintained accurately to obtain the expected outcome. All these factors collectively help in maintaining and improving the fertility of the soil to obtain products of high value [27].

Our present study was focused on the isolation and screening of plant growth promoting microorganisms from rhizosphere of different crop plants. The colony characters and morphology of the isolates were examined. A detailed soil analysis study was performed for each soil sample used in the study. These isolates were examined for their ability to fix free nitrogen from atmosphere and solubilise phosphorus to its organic form. Their potential to produce Indole acetic acid was also determined using the standards.

MATERIALS AND METHODS

Chemicals

Chemicals and Media were purchased from Sisco Research Laboratories Pvt. Ltd., Merck Specialities Pvt. Ltd., Mumbai, India and Hi Media Private Limited, Mumbai, India respectively.

Sample collection

The rhizosphere soil samples from six different farms under cultivation were collected from the Vellore district (12.92°N, 79.13°E), Tamil Nadu, India during the month of August 2013. Sampling was done using randomised block deign method and the soil was collected from 15-20cm depth. Samples were collected aseptically in sterile plastic bags using sterile spatula and immediately transported to the laboratory for further treatment of the soil.

Soil analysis

100g of each of the soil sample was oven dried and sieved through a 2mm sieve. The sieved soils were analysed for their physicochemical properties, using standard Ohio University protocols for - soil texture, lime status, pH, electrical conductivity, and available Nitrogen, available Phosphorus, available Potassium, Iron, Manganese, Copper and Zinc [27].

Isolation of Rhizosphere bacteria

Wet soil sample was used for the isolation of rhizobacteria. A tenfold serial dilution of each soil sample was prepared in normal saline from 10^{-1} to 10^{-7}. 0.1 ml aliquots from10^{-4} to 10^{-6} dilutions of the soil samples were plated in triplicates on Nutrient agar medium. The plates were incubated at 30±1°C for 24-48 hours.

Screening for free living nitrogen fixing bacteria

Pure isolates obtained from serial dilution and plate technique in nutrient agar media were used. The isolates were streaked in Ashby's agar medium for selective growth of free living nitrogen fixing Azotobacter species. Plates were incubated at 30±1°C for 24-48 hours [25].

Screening for phosphate solubilising micro organisms

Pure isolates were streaked on Pikovskaya's agar medium for selective growth of phosphate solubilising microorganisms. Plates were incubated at 30±1°C for 24-48 hours [28].

Screening for dual nature of the isolates

The isolates obtained after screening for nitrogen fixation and phosphate solubilisation were further tested to determine their dual ability. The phosphate solubilizing isolates were inoculated on Ashby's agar plates to check their ability to fix the nitrogen. Likewise, the nitrogen fixing isolates were and plated on Pikovskaya's agar plates to check their ability to solubilize the inorganic phosphate.

Production of IAA by isolates

The morphologically characterized potential isolates were tested qualitatively for their ability to produce IAA. Each isolate was inoculated in tryptone broth supplemented with 1µg/ml L-Tryptophan and incubated at room temperature for 48 hours. The cultures were then centrifuged at 4000rpm for 20 minutes. 1ml of the supernatant was treated with 4ml of Salkowski Reagent [1ml 0.5M $FeCl_3$ in 50ml of 5%$HClO_4$]. The tubes were kept undisturbed for 20minutes. Pink colouration indicates positive test results. The quantification of the IAA production was done spectrophotometrically at 530nm.

The Indole acetic acid (IAA) production by the isolates was studied in comparison to a standard IAA graph. Stock solution of IAA was prepared in acetone (1mg/ml). The standards used in quantification were 5µg/ml, 10µg/ml, 20µg/ml, 50µg/ml and 100µg/ml. A control was maintained which did not contain IAA. The tubes were incubated at room temperature for 25 minutes followed by corresponding spectrophotometric readings at 530nm against acetone as blank [25]. A standard graph was plotted and the corresponding values were noted.

Biochemical characteristics

The basic biochemical characteristics of the two potential isolates were determined by following standard protocols of Bergey's Manual of Determinative Bacteriology.

RESULT AND DISCUSSIONS

Soil Analysis

The results of the soil analysis (the texture of soil, lime content, pH determination and electrical conductivity of the soil) were given in Table 1. These physical characteristics determine the type of soil and thus provide insights to the suitable cultivation methods that might be carried. The nutrient chart helps in determining the fertility of the soil along with the prediction of the possible micro flora residing in that particular type of soil.

Table 1: Physico-chemical properties of the soil samples

Sample code	ST	LS	Ph	EC	N	P	K	Fe	Mn	Cu	Zn
A	SCL	Nil	7.7	0.2	157	7.9	142	6.96	4.56	2.87	1.03
B	SCL	Nil	7.7	0.2	165	9.2	117	7.40	5.92	1.90	1.09
C	SCL	Nil	7.7	0.1	143	7.9	136	6.07	6.38	2.00	0.92
D	SCL	Nil	7.7	0.1	146	9.2	130	4.75	3.92	3.46	1.06
E	SCL	Nil	7.7	0.2	217	7.9	134	8.40	5.63	3.07	1.35
F	SCL	Nil	7.7	0.1	195	7.9	138	8.62	5.91	1.95	1.58

ST=soil texture (sandy clay loam), LS=lime status, EC=electrical conductivity (ds/m), NPK (kg/acre), Fe/Mn/Cu/Zn (ppm) Source of isolation-A: *Cajanus cajan*, B: *Arachis hypogaea*, C: *Oryza sativa*, D: *Cicer arietinum, E: Sorghum vulgare, F: Pisum sativum*

Isolation and Screening of Bacteria from different samples

A total of 65 isolated colonies were obtained in preliminary isolation on Nutrient agar medium. Among them, 22 isolates showed selective growth on Ashby's agar medium and 25 isolates showed selective growth on Pikovskaya's medium.

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Screening for dual nature of the isolates

From the isolates obtained on Pikovskaya's agar and Ashby's agar medium, 12 bacterial isolates exhibited nitrogen fixing and phosphate solubilising capabilities. These colonies were designated as SI-1 to SI-12.

Morphological Identification of isolates

The colony morphology of the potential isolates showing dual nature of mobilizing inorganic phosphorus and ability to fix free nitrogen was recorded (Table 2).

Table 2: Colony Morphology

S.No	Shape	Color	Margin	Size	Elevation	Consistency
SI-1	Circular	Transparent	Entire	2mm	Raised	Sticky
SI-2	Circular	White	Entire	3mm	Raised	Powdery
SI-3	Circular	White	Entire	4mm	Raised	Powdery
SI-4	Circular	Transparent	Entire	2mm	Raised	Sticky
SI-5	Circular	White	Entire	3mm	Raised	Powdery
SI-6	Circular	Transparent	Entire	2mm	Raised	Sticky
SI-7	Circular	Pale yellow	Entire	4mm	Raised	Powdery
SI-8	Circular	Transparent	Entire	3mm	Raised	Sticky
SI-9	Circular	Transparent	Entire	2mm	Raised	Sticky
SI-10	Circular	Pale yellow	Entire	4mm	Raised	Powdery
SI-11	Circular	Off-white	Entire	3mm	Flat	Powdery
SI-12	Circular	White	Entire	3mm	Raised	Powdery

The gram nature of the isolates was studied under the high power oil immersion lens (100X) of a light microscope. Slides of each isolates were prepared following the standard gram staining technique. Motility was checked using hanging drop method (Table 3).

Table 3: gram characteristics and motility

Sl.No	Gram nature	Motility
SI-1	Gram-ve rod	Non-Motile
SI-2	Gram-ve rod	Motile
SI-3	Gram+ve rod	Non-Motile
SI-4	Gram+ve cocci	Non-Motile
SI-5	Gram+ve rod	Non-Motile
SI-6	Gram+ve cocci	Motile
SI-7	Gram+ve rod	Motile
SI-8	Gram+ve cocci	Non-Motile
SI-9	Gram-ve rod	Motile
SI-10	Gram+ve rod	Non-Motile
SI-11	Gram+ve rod	Non-Motile
SI-12	Gram+ve rod	Motile

Production of IAA

Among the 12 isolates studied for IAA production, 2 isolates SI-2 and SI-9 showed significant results of 20.55µg/ml and 23.16µg/ml respectively. The results are as shown in Figure 1.

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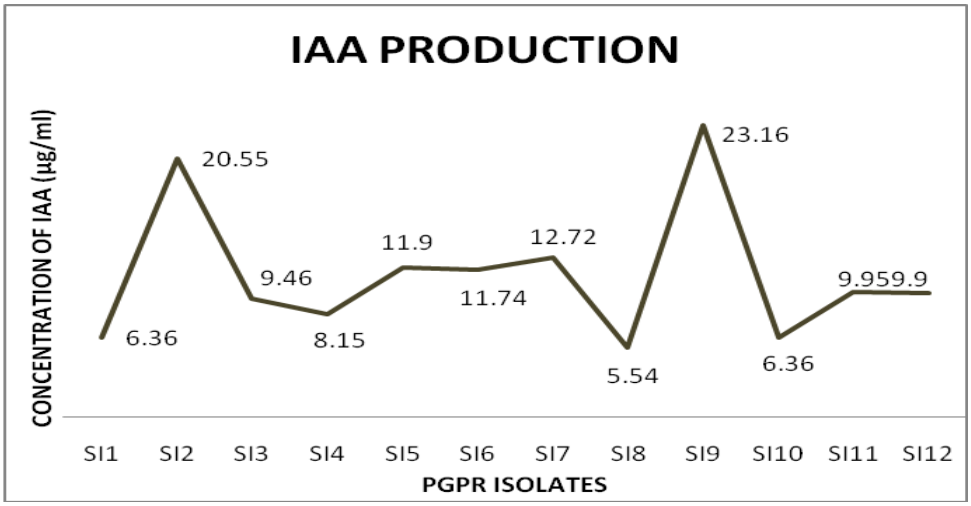

Figure 1: IAA PRODUCTION

Biochemical characteristics

Based on the biochemical test results the two potential isolates were tentatively identified as the *Pseudomonas sp*. (Table 4)

Table 4: Biochemical characteristics

Biochemical Test	SI-2	SI-9
Nitrate Reduction	+	+
Phosphate Solubilisation	+	+
IAA Production	+	+
Indole Test	-	-
MR-VP Test	-	-
TSI Test	-	-
Oxidase Test	+	+
Catalase Test	+	+
Citrate Utilization	+	+
Urease Test	-	-

(+) - Positive, (-) - negative

Isolates SI-2 and SI-9 showed most promising results (Fig 1) and (Table 5). They are capable of free nitrogen fixation and phosphate solubilisation in rhizosphere soil environment. Isolate SI-2 is a gram -ve rod showing powdery white colonies and isolate SI-9 is again gram -ve rod showing transparent sticky growth. IAA production as studied with reference to the standard IAA graph was quantified to be 20.55µg/ml and 23.16µg/ml produced by SI-2 and SI-9 respectively. Hence, these isolates can be said to possess considerable PGPR potential. These morphologically characterized isolates can be further tested for other growth promoting factors like siderophores production, acetylene reduction and HCN production along with its molecular characterization. In the present study bacterial isolates SI-2 and SI-9 were obtained from soil sample collected from rhizospere of *Arachis hypogea* suggesting that root exudates of this plant encourages growth of plant growth promoting rhizobacteria.

Table 5: Nitrate reduction, phosphate solubilization and IAA production

Nitrate Utilization	SI-2	SI-9
	+	+
Phosphate Reduction	+	+
IAA Production (µg/ml)	20.55	23.16

Acinetobacter sp., *Bacillus* sp., *Enterobacter* sp., *Micrococcus* sp., and *Pseudomonas* sp were isolated from six French bean rhizospheric soil samples by Ajay Kumar et al [29]. These isolates were found actively secreting IAA, solubilize phosphate and positive for catalase production. Research on isolation and characterization of PGPR from cauliflower roots was done by [30] Kushwaha et al in 2013. IAA is one of the most important phytohormones and is 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 [31]. However, in our study out the 12 isolates screened for IAA production all the isolates showed positive results. The highest amount was found to be 23.16 µg/ml. Elevated levels of IAA production by Pseudomonas was recorded by other research workers [32].

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