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Efficiency of Rhizosphere Bacteria in Production of Indole Acetic Acid, Siderophore and Phosphate Solubilization

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Abstract: After the green revolution, agriculture has become more dependent on chemicals. Increase in agricultural productivity is mainly achieved through chemical fertilizers and fungicides. These agro chemicals pose serious threats like pollution of soil and water sources and can cause sterility of soil. Eco-friendly ways can be adopted to complement the action of the agro-chemicals and to decrease their use. One of the best ecofriendly practice is using Plant Growth Promoting Rhizobacteria (PGPR) which colonize plant roots and promote plant growth. Rhizobacteria act directly or indirectly as potential biocontrol agents, biofertilizers and biostimulating agents by producing various metabolites by various mechanisms. The potential environmental benefit of these PGPR is that they can help reduce use of agricultural chemicals and enhances sustainable agricultural practices. In the present study, the efficacy of rhizobacteria isolated from Siruvani (Western Ghats region, Tamil Nadu, India) was investigated for plant growth promoting (PGP) traits *viz*., IAA production, phosphate solubilization and siderophore production under *in vitro* conditions. A total of 59 PGP isolates were screened of which many isolates were positive for one or other traits. Among these PGP isolates, 5 isolates were found to possess all the three traits. The highly potential isolate A10 was characterized by molecular 16S rRNA gene sequencing and phylogenetic tree construction using neighbor joining method and identified as *Pseudomonas nitroreducens*.
Keywords: 16S rRNA gene sequencing, Indole Acetic Acid (IAA), Siderophore, Phosphate solubilization.

Introduction and Experimental

India is mainly an agricultural country and agriculture has been the backbone of the country's economy since time immemorial. Though green revolution had a tremendous effect in making India self-sufficient in food grain production and cash crops, it also has its own draw backs. Usage of chemicals in agriculture makes the soil lose its fertility and alters the natural composition of the soil thereby making it lose the natural beneficial organisms and the biodiversity. Reducing the use of chemicals and promoting eco-friendly practices is the need of the hour.

Plant growth-promoting rhizobacteria (PGPR) are bacteria that colonize the rhizosphere, plant roots, and enhance plant growth by various mechanisms. Internationally there is an increase in the concern for food and environmental quality, hence the potential use of PGPR for reducing chemical inputs in agriculture is an encouraging practice. PGPR have been applied to various crops to enhance growth, seed emergence and crop yield. Few PGPR have been commercialized[1,2,3]. PGPR also help in the increased uptake of nitrogen, solubilization of minerals such as phosphorous, and synthesis of phytohormones[4,5,6]. It is also reported that PGPR is capable of solubilizing both inorganic and organic phosphates in soil[7].

PGPR collectively denotes many types of bacteria[8]. Soil bacteria that flourish in the rhizosphere of plants, which may grow in and around plant tissues, and stimulate plant growth by a plethora of mechanisms are collectively known as PGPR. The use of PGPR as biofertilizers is one of the most promising tools to improve primary production with low inputs of chemical fertilizers, through any of the possible mechanisms such as biocontrol, nutrient mobilization, phytohormone production or nitrogen fixation[5].

Many rhizosphere bacteria have the capacity to synthesize Indole Acetic acid (IAA) that has pronounced effect on plant growth and development[9, 10]. The rhizosphere bacteria appear to have a greater potential to synthesize and release IAA as secondary metabolites than normal soil microbiota because of the relatively rich supply of nutrients from the root exudates in the rhizosphere[11, 9]. Production of IAA by microbial isolates varies greatly among different species or strains that depends on the availability of substrates.

The rhizosphere microbes also called as phosphate solubilizers have the capacity to solubilize the bound phosphate and rock phosphate into simple phosphate. Phosphorus solubilizing bacteria (PSB) play crucial role in phosphorus nutrition by enhancing its availability to plants through release from inorganic and organic soil phosphate pools by solubilization and mineralization[12]. Use of phosphorus solubilizing bacteria as inoculants in bioformulation can increase phosphorus uptake in plants.

Some PGPR can exert plant growth by producing siderophores which are low molecular weight iron chelating compounds. The iron sequestered by microbial siderophores cannot be scavenged by pathogens. Siderophore producing microorganisms can protect plants either by limiting the growth of pathogenic microorganisms or by triggering plant's defensive metabolism.

The aim of the present study is to identify and characterize the most efficient PGP isolates which could be further explored for biofertilizer development. The rhizobacterial population were isolated from selected crop fields of Siruvani regions of Coimbatore, India. The rhizosphere isolates were screened for phosphate solubilization and production of plant growth promoting metabolites like IAA and siderophores.

Isolation of Pgpr Bacterial Strains from Rhizosphere Soil Sample

Rhizosphere soil samples were collected in Siruvani Region (Sample A – Marakkadu tomato rhizosphere, Sample B - Nallurvayal brinjal rhizosphere, Sample C – Sadivayal brinjal rhizosphere) of Coimbatore, Tamilnadu, India, and brought to the laboratory in polythene bags and shade dried under sterile conditions[13].

The rhizosphere soil samples were separated and sieved and samples were serially diluted upto 10^{-6} dilution. Nutrient agar plates were aseptically prepared and 0.1ml aliquots from 10^{-3} to 10^{-6} dilutions were transferred to each plate and spread evenly with 'L' rod. The plates were incubated at 30°C for 24 hrs. Morphologically different colonies were picked and streaked on fresh agar plates to obtain pure cultures. Pure cultures of the isolates were subcultured and stored at 4°C for further investigation. The culture characters of the isolates were noted. Actinomycetes and bacterial colonies were distinguished by morphological characters and Gram's staining.

***In Vitro* Screening for IAA Production by the Rhizosphere Isolates**

The IAA production by the isolates was screened under *in vitro* conditions by the standard method described by Loper and Scroth[14]. Isolates were grown in nutrient broth supplemented with tryptophan (1mg/ml) at 30°C for 72 hrs in shaker incubator to get confluent growth. After incubation, cultures were centrifuged and the supernatant was taken. To 2 ml of the supernatant, 2 drops of 10 mM Orthophosphoric acid and 4 ml of Salkowski's reagent (50 ml 35% Perchloric acid; 1 ml 0.5 $FeCl_3$) were added and incubated at room temperature for 25 mins. Pink color formation confirmed the presence of IAA in the supernatant.

Quantification of IAA produced by the PGPR was done spectrophotometrically by reading the absorbance of the treated supernatant at 535 nm was read Hitachi U – 2910 spectrophotometer. The concentration of IAA produced by the isolate in the broth was quantified by comparing the standard graph made using standard IAA procured from Himedia.

***In Vitro* Screening of Phosphate Solubilizing Activity of the Rhizosphere Isolates**

The phosphate solubilization by the isolates from inorganic sources was screened by using Pikovskaya agar[15] amended with insoluble tricalcium phosphate. Solubilization of the complex insoluble tribasic calcium phosphate was indicated by the zone of clearance around the bacterial colony.

Solubilization of organic complex phosphates by the isolates was screened by using lecithin (inositol phosphate) as phosphate source in the media. Solubilization of lecithin by the isolates was indicated by the formation of clear zone around the bacterial colony.

In Vitro Screening of Siderophore Production by the Isolates

Siderophore production by the rhizosphere isolates was determined by using the protocol devised by Schwyn and Neilands[16]. The Siderophore production was demonstrated by formation of orange color around the bacterial colony in CAS – blue agar medium containing chrome azurol S dye and incubated at 28°C for 2 weeks[17].

Molecular Identification of Efficient Rhizosphere Isolate Using 16S rRNA Gene Sequencing

Molecular characterization of the efficient PGPR isolate was done by 16Sr RNA gene sequencing. DNA of the isolate was extracted by modified method of Sambrook and Russel[18]. The gene was amplified using universal bacterial 16S rRNA gene primers[13] under following conditions; Initial denaturation at 95°C for 5mins, 30 cycles of denaturation at 95°C for 30 secs, annealing at 52°C for 30 secs, extension at 72°C for 2 mins and final extension for 10 mins in Eppendorf thermocycler. The amplified products were purified by electrophoresis in 1.2% agarose and extracted by QIA quick gel elution kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The purified PCR products were sequenced. The sequence was compared with the available sequences in the NCBI database using BLAST program. Phylogenetic tree was constructed by neighbor joining method using tree view software[19].

Result

Isolation and Characterization of Rhizosphere Isolates from Siruvani Region

The colonies formed in the nutrient agar were enumerated and 59 morphologically distinct colonies were identified. The isolates were pure cultured onto fresh medium separately and designated according to the area and crop of isolation. From the soil samples A, B and C, 13, 18 and 24 morphologically distinct isolates were subcultured respectively. They were stored at 4ºC until further studies.

Gram's staining was performed for all the isolates. Most of our isolates were Gram positive and almost 48 isolates were either rods or small rods. The isolates A5, A6, A12, B2, and B3 had filamentous structures. The isolates A1, A2, A4, B8, C11 and C21 were cocci shaped.

In vitro Screening for Iaa Production

Among the 59 isolates screened for *in vitro* IAA production, 11 isolates were found to produce IAA in varying levels in the presence of tryptophan (1mg/ml) (Fig. 1). The IAA produced by the isolates ranged from 13.83 µg/ml to 90.17 µg/ml (Table 1).

Table 1. Consolidated result showing the concentration of IAA (µg/ml) produced by the Rhizosphere Isolates under *in vitro* conditions

S. No.	Isolate	Mean Conc. ± SD (µg/ml)
1	A10	13.83 ± 3.2
2	B10	42.167 ± 36.3
3	B11	29 ± 26.7
4	C5	50.83 ± 12.4
5	C10	67 ± 19.6
6	C11	30.17 ± 9.3
7	C13	26.67 ± 12.5
8	C14	54.17 ± 15.8
9	C15	90.17 ± 5.3
10	C16	82.5 ± 22.6
11	C23	76.5 ± 1.3

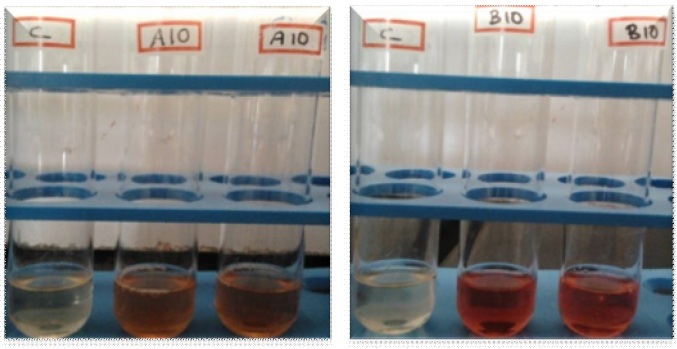


Fig. 1. IAA Production by rhizosphere isolates showing pink color formation after addition of Salkowski's reagent

In vitro Phosphate Solubilization

Among the 59 isolates screened for phosphate solubilizing ability in Pikovskaya agar, 10 isolates showed formation of clear zone around the colony (Fig. 2). The zone of clearance which is directly proportional to the phosphate solubilizing efficacy was measured in Pikovskaya agar and it ranged from 0.1 to 0.9 cm. The highest phosphate solubilizing activity was shown by the isolate A10 with a zone of clearance 0.9 cm (Table 2).

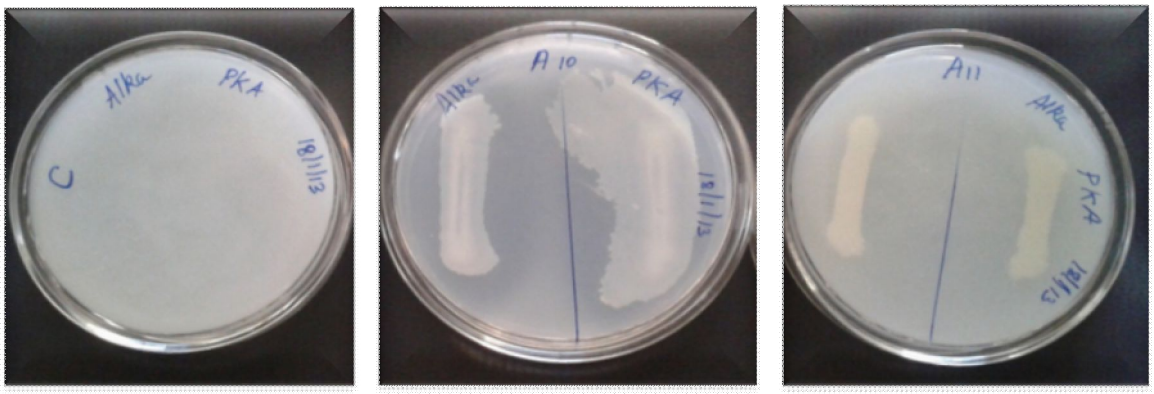


Fig. 2. Inorganic Phosphate solubilization seen as clear zone around the colony in Pikovskaya's medium (with Tricalcium Phosphate)

Table 2. Solubilization of Inorganic Phosphate in Pikovskaya medium

S. No	Isolate No.	Result	Zone of Clearance (cm)
1	A10	+	0.9
2	A11	+	0.3
3	B10	+	0.5
4	B11	+	0.3
5	B12	+	0.12
6	B17	+	0.1
7	B18	+	0.3
8	C5	+	0.2
9	C10	+	0.5
10	C28	+	0.2

The isolate A10 alone was able to solubilize organic phosphate and showed 0.5 cm zone of clearance with 0.02% lecithin amended medium after 7 days of incubation (Fig. 3).

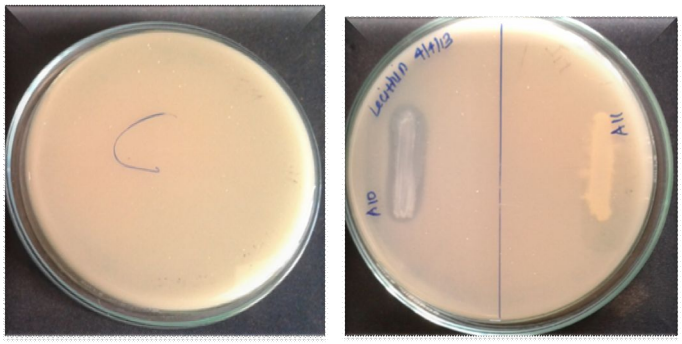


Fig. 3. Organic phosphate solubilization in Lecithin medium seen as clear zone around the bacterial colony

In vitro Screening for Siderophore Production by the Isolates

The *in vitro* screening for siderophore production showed that 10 out of 59 isolates were able to produce orange coloration in chrome azurol S blue agar, which indicated the production of siderophores (Fig. 4). The most efficient siderophore producers were the isolates A10, C10, C23 and C28 (Table 3).

Fig. 4. Siderophore production by rhizosphere isolates in CAS – Blue agar

Table 3. Siderophore Production in CAS - Blue Agar

S. No.	Isolate No.	Observation	Result
1	A10	orange (halo)	+
2	B2	orange (halo)	+
3	B10	orange	+
4	B11	orange	+
5	C5	orange	+
6	C10	orange	+
7	C13	orange	+
8	C15	orange	+
9	C23	orange (Halo)	+
10	C28	orange (Halo)	+

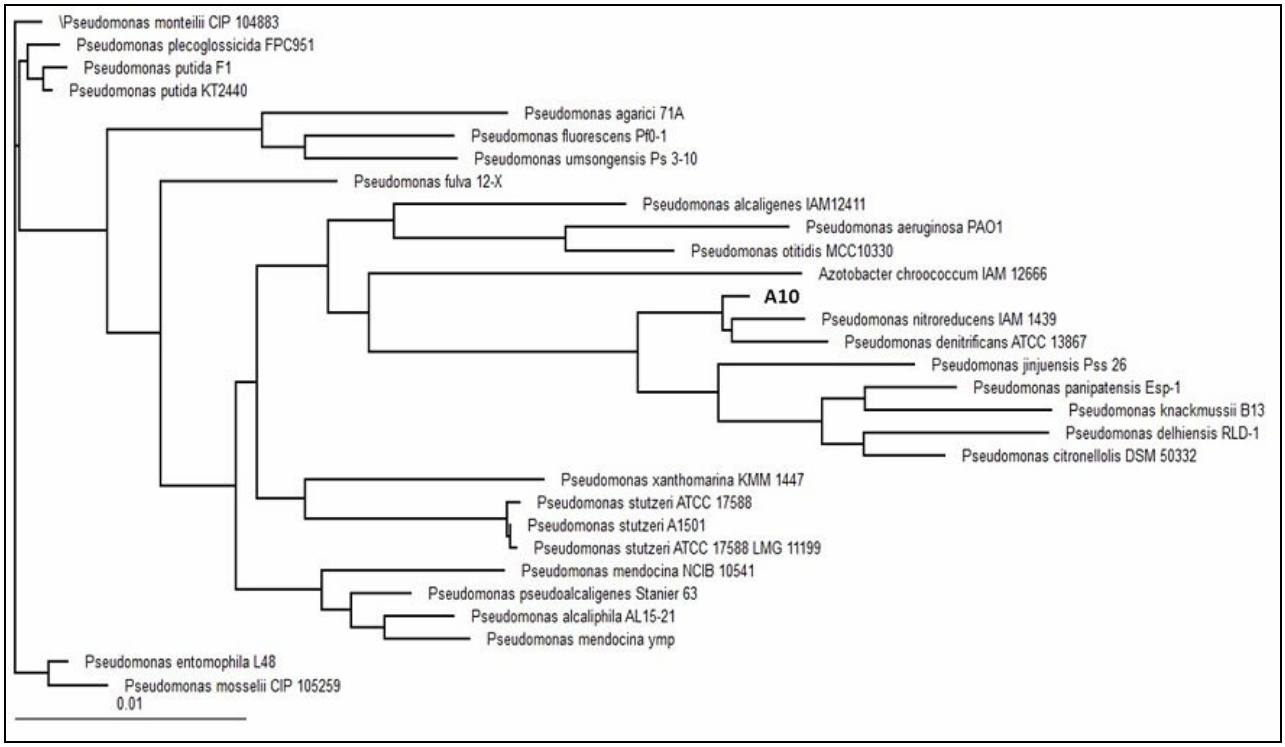
Molecular Characterization of the Potential Pgpr Isolate by 16S rRNA Gene Sequencing

The efficient isolate, A10 (shown in Table 4) was characterized by molecular 16S rRNA gene sequencing and phylogenetic tree construction using neighbor joining method and identified as *Pseudomonas nitroreducens* (Fig. 5).

Table 4. Isolates Showing more than one plant growth promoting (PGP) traits

S. No.	Isolates	Siderophore Production	Phosphate solubilization	IAA Production
1	A10	+	+	+
2	B10	+	+	+
3	B11	+	+	+
4	C5	+	+	+

5	C10	+	+	+
6	C13	+	-	+
7	C15	+	-	+
8	C23	+	-	+
9	C28	+	+	-



(The bar represents 0.01 nucleotide substitution per site)

Fig. 5. Phylogenetic tree based on the partial 16S rRNA gene sequencing of the isolate A10, constructed with Treeview software using neighbor joining method with bootstrap analysis of 1000 resampling.

Discussion

Plant Growth Promoting Rhizobacteria (PGPR) play a major role in crop protection, growth promotion and in improvement of soil health. As rhizosphere isolates live in close association with the plant roots, they directly benefit the plants by secreting the plant growth hormones and other growth stimulating factors in the immediate vicinity of the plant roots. Therefore this study was carried out as an attempt to characterize the rhizosphere bacteria from the unexplored Siruvani regions in Coimbatore, for their PGP activity. *In vitro* studies on IAA production, siderophore production and phosphate solubilization was done to identify the most efficient PGPR isolate.

Under *in vitro* conditions, IAA was synthesized as secondary metabolite by bacteria upon induction with tryptophan during stationary phase. Earlier studies reported the production of IAA by various rhizosphere isolates such as *Enterobacter* sp, *Klebsiella* sp, *Azotobacter* sp and *Pseudomonas* sp[20, 21, 22]. There are evidences that the IAA concentration increased as the tryptophan concentration in the medium was increased[22].

Solubilization of complex phosphate is another important trait in plant growth promotion. Rhizosphere microflora can assimilate the soluble phosphate and prevents from adsorption or fixation in the soil thereby making the phosphates available to the plants. Some rhizobacteria can mineralize organic phosphate and solubilize precipitated phosphate in the soil. Thus Phosphate solubilizers could reduce phosphate fertilizer application by 50% without any significant reduction of crop yield[12]. In our present study, among the 59 isolate screened for phosphate solubilization, 10 isolates were able to solubilize inorganic phosphate. The isolate A10 showed solubilization of both inorganic phosphate in the form of tricalcium phosphate and organic phosphate in

the form of lecithin. These phosphate solubilizing isolates can be used with phosphate fertilizers to prevent tribasic phosphate accumulation in the soil.

Siderophores is associated with plant growth improvement either directly by carrying iron or indirectly limiting noxious organisms in the soil by sequestering iron available to them. The isolates A10, B2, B10, B11, C5, C10, C13, C23 and C28 were able to produce siderophores.

The isolates A10, B10, B11, C5 and C10 obtaining in this study proved to be efficient PGPR as they possessed all three plant growth promoting traits (IAA production, Siderophore production and Phosphate solubilization) and the isolate A10 was able to solubilize both inorganic and organic forms of phosphate. A10 was identified by 16S rRNA gene sequencing as *P. nitroreducens*.

It is widely reported that bacteria belonging to the genus *Pseudomonas* are known for their PGP activity. Various studies have reported that *Pseudomonas* are excellent candidates of PGPR and can produce HCN, siderophores, protease, antimicrobial compounds and possess phosphate solubilizing activity[23]. Reports also have shown that the organisms belonging to the genus Pseudomonas have plant growth promoting properties as well as biocontrol properties because they produce siderophores. Various siderophores produced by the Pseudomonads are proved to be involved in suppression of various phytopathogenic fungi[24].

Our present study shows that *P. nitroreducens* strain is efficient PGPR isolate. This PGPR strain holds good prospects in future for sustainable agricultural practice with minimal chemical inputs and enhances organic farming. Production and utilization of biofertilizer formulation using these rhizobacterial strains in agricultural fields can increase soil fertility and can increase the yield by the multifaceted PGP action of these PGPR isolates. These beneficial effects can considerably reduce the use of chemical fertilizers.

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