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Isolation and screening of plant growth promoting rhizobacteria from rhizosphere of chilli

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Abstract
In the present study PGPRB were isolated from agricultural field of chilli (*Capsicum annum* L.) Venbavur, Perambalur and characterized for plant growth promoting characteristics. Total number of bacteria in rhizosphere and non rhizosphere soil were 54 ± 1.73 x 10^6 cfu/g and 27± 2.0 x 10^6 cfu/g and the R/S ratio was found to be 2.0± 0.17x 10^6. The PSI index of three isolates VPGPRB1, VPGPRB4 and VPGPRB6 were 1.83 ± 0.06, 3.0 ± 0.46 and 2.45 ± 0.19, identified and confirmed to be *Enterobacter* sp., *Bacillus* sp. and *Pseudomonas* sp. The results of the evaluation of plant growth promoting characterisitcs showed that all the three isolates produced IAA, ammonia and exopolysaccharide (0.76 ± 0.15 mg/ml, 1.37 ± 0.25 mg/ml and 0.43 ± 0.08 mg/ml). The amount of IAA production were (10.6 ± 1.3µg/ml, 28.3 ± 0.66µg/ml and 19.1 ± 1.65µg/ml) and phosphate solubilization were (123 ± 1.5 µg/ml, 214 ± 1.8 µg/ml and 158 ± 1.74 µg/ml) by *Enterobacter* sp., *Bacillus* sp. and *Pseudomonas* sp.

Keywords: rhizosphere, PGPRB, IAA, phosphate solubilization, exopolysaccharide and *Pseudomonas* sp

Introduction
Plant growth in agricultural soils is influenced by many abiotic and biotic factors. Rhizosphere is the immediate zone in the soil surrounding the roots of a plant where intense chemical and biological activities occur in a narrow sleeve around the axes of the root-hairs. A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere [1]. Bacteria are the most abundant among them. Plants select those bacteria that contribute most to their fitness by releasing organic compounds through exudates creating a very specific environment where diversity is low [2, 3]. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology to some extent, especially considering their competitiveness in root colonization [4].
Plant growth-promoting bacteria (PGPB) associated with many plant species and are commonly present in many environments. The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and closely adhering soil interface, the rhizosphere [5]. PGPR mediated plant growth promotion occurs by the alteration of the whole microbial community in rhizosphere niche through the production of various substances [6]. Generally, PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents [7, 8].
Hence, the use of beneficial microorganisms such as PGPR as biofertilizers and biocontrol agents has become more important in recent years in order to improve plant growth and manage plant diseases but also to avoid environmental pollution. In order to focus the said aspects the present work was aimed to isolate PGPR from rhizosphere of *Capsicum annum* L. agricultural field of Venbavur, Perambalur and evaluate their potential for the plant growth promoting characteristics.

Materials and Methods
Soil samples from rhizosphere and non rhizosphere were collected from the chilli field (*Capsicum annuum* L), Venbavur, Perambalur, Tamil Nadu. The rhizosphere and nonrhizosphere soil samples were serially diluted in normal saline up to 10^{-6} dilutions with the sterile blank. The number of bacteria was enumerated by spread plate technique on nutrient agar medium plates. The plates were incubated at 37^0C for 24 hrs. The quantitative rhizosphere effect of the plants was calculated using the formula [9, 10].

$$R/S = \frac{\text{Number of microorganism per gram of rhizosphere soil}}{\text{Number of microorganism per gram of non-rhizosphere soil}}$$

Screening of phosphate solubilization by plate assay
Randomly predominant morphologically different bacteria on the plates were selected and purified on nutrient agar slants. Seven bacterial isolate VPGPRB1 – VPGPRB7 were screened for phosphate solubilization on NBRIP medium (National Botanical Research Institute's phosphate growth medium) containing calcium phosphate as inorganic phosphate. Each isolate was spot inoculated onto the medium and incubated at 37°C for 72 hours. The phosphate solubilization index was calculated as the ratio between the total diameter (colony + halo) and the colony diameter [11, 12].

Identification of the PGPRB isolate
The PGPRB isolates were identified by morphological and biochemical test Gram stain, motility, spore stain, IMViC, lactose fermentation, oxidase and catalase test [13, 14] and identified according to Bergeys manual of determinative Bacteriology [15].

Quantitative determination of phosphate solubilization
Quantitative estimation of phosphate solubiliztion by the bacterial isolate was performed by vanadomolybdophosphoric method [16, 17]. 1 ml of the culture 0.5 OD at 600nm was inoculated into the 100ml of NBRIP broth and incubated at 37ºC for 7 days. An uninoculted NBRIP broth was used as control. Each experiment was performed in triplicate. After incubation the cell suspension was centrifuged at 10000rpm for 20mins. To the one ml of the supernatant 2.5ml of Barton's reagent was added in a 50ml standard flask and the volume was made upto 50ml with distilled water and kept for 10 mins. The intensity of yellow colour developed was read at 430nm in a spectrophotometer. The amount of phosphate solubilized was extrapolated from standard curve drawn using potassium di-hydrogen phosphate.

Indole acetic acid production
Indole acetic acid produced by bacteria was determined as described by Brick *et al*. [18]. Bacterial cultures were grown in NB amended with tryptophan (100µg/ml) at 30°C for 48 hrs on shaker (120 rpm). The cultures were centrifuged at 3000 rpm for 30 minutes. The supernatant (2 ml) was mixed with two drops of *o*-phosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl3 solution) and incubated for 25 mins at room temperature in dark. Development of pink color indicate IAA production. The intensity of pink color was read at 530 nm spectrophotometrically and the amount of IAA produced was extrapolated from the standard curve [19].

Ammonia production
Bacterial isolates were grown in peptone water. 1% inoculum was added to 5 ml of peptone water in each tube and incubated for 72 hrs at 30°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production [20].

Exopolysaccharide (EPS) production
The ability of exopolysaccharide production of the isolated rhizobacteria was screened by growing it in the medium (g/l) 0.2 g KH2PO4; 0.8 g K2HPO4; 0.2 g MgSO4.7H2O; 0.1 g CaSO4.2H2O; 2.0 mg FeCl3; Na2MoO4.2H2O (trace); 0.5 g extract yeast 20 g sucrose with pH7.2 using sucrose as sole carbon source [21]. The isolate was inoculated onto 50ml of the medium and incubated on a rotary shaker at 200rpm for 3 days.

Extraction of Exopolysaccharide
After the end of the incubation period the medium was centrifuged at 10000rpm for 10 mins. The supernatant was separated from the bacterial cell. Then to the supernatant three times the volume of cold acetone was added to precipitate exopolysaccharide and it was centrifuged at 15000 rpm for 30 mins and repeated. Precipitates were collected on a preweighed petriplate. The precipitates were allowed to dry overnight or until air dry and reweighed the petriplate. The increase in the weight of petriplate is noted which is amount of the EPS produced by the isolate.

Results and Discussion
Rhizosphere and non rhizosphere soil samples were collected from the chilli field (*Capsicum annum* L.) of Venbavur, Perambalur, Tamil Nadu, India. The total number of bacteria present in the rhizosphere soil and non rhizosphere soil of *Capsicum annuum* L. was found to be 54 ± 1.73 x 10^6 cfu/g and 27± 2.0 x 10^6 cfu/g (fig 1). The number of bacteria was more in the rhizosphere soil than in the non-rhizosphere soil. The results can be related with the reports of the [22] who found maximum rhizosphere population of 8 x 10^8 CFU/g and non- rhizosphere bacteria was 2.35 x 10^8 CFU/g. The rhizosphere effect of *Capsicum annuum* L. was 2.0± 0.17x 10^6**.** The greater the rhizospheric effect the higher will be microorganism's number. Greater rhizosphere effect is seen with bacteria than the actinomycetes and fungi and only negligible changes are noted with regard to protozoa and algae [9]. The rhizosphere effect greatly decreases as we move away from the root [23]. The varying types and quantities of rhizodeposits have been postulated to act as key factors influencing the density and diversity of the rhizospheric microorganisms [24]. Several organisms were present in the rhizosphere of *Capsicum annuum* L.

Screening of Bacteria for Plant growth promoting characteristics
A total of seven predominant morphologically different bacteria (VPGPRB1 – VPGPRB7) were selected randomly and screened for phosphate solubilizing activity by plate assay showed that three bacterial isolate exhibited phosphate solubilization activity. Phosphate solubilization index (PSI) were 1.83 ± 0.06, 3.0 ± 0.46 and 2.45 ± 0.19 (Table 1). These three isolates were selected for further study.

Identification of the PGPRB Isolate
Three bacterial isolate VPGPRB1, VPGPRB4 and VPGPRB6 which showed positive for phosphate solubilization by plate assay were selected and identified to be *Enterobacter* sp., *Bacillus* sp. and *Pseudomonas* sp. by morphological and biochemical characteristics (Table 2).

Evaluation of PGPRB characteristics of bacteria
Quantitative determination of Phosphate Solubilization
The amount of phosphate solubilized by the three isolates were determined by vanadomolybdophosphoric method showed that *Bacillus* sp. solubilized high amount of phosphate 214 ± 1.8 µg/ml followed by *Pseudomonas* sp. 158 ± 1.74 µg/ml and *Enterobacter* sp.
produced solubilized low amount of phosphate 123 ± 1.5 µg/ml (fig 2). Similar result was reported that *Bacillus megaterium*01-A3 and *Pseudomonas* sp. solubilized 85.57 µg/ml and 71.51 µg/ml of phosphate after 72 hrs of incubation [25].

Indole acetic acid production

Auxin is the most investigated hormone among plant growth regulators. The most common, best characterized and physiologically most active auxin in plant is indole-3-acetic acid (IAA). IAA is known to stimulate both a rapid response (e.g. increased cell elongation) and a long term response (e.g. cell division and differentiation) in plants [26]. In the present study the results showed that the bacterial *Enterobacter* sp., *Bacillus* sp. and *Pseudomonas* sp. produced pink colour after the addition of Salkowski reagent which indicates that it was able to produce indole-3-acetic acid (table.1) and *Bacillus* sp. produced high amount of IAA 28.3 ± 0.66µg/ml followed by *Pseudomonas* sp. 19.1 ± 1.65µg/ml. and *Enterobacter* sp. produced low amount of IAA 10.6 ± 1.3µg/ml (fig 2). IAA production is an important trait of PGPR because it is most important phytohormones and function as signal molecule in the regulation of plant development. The production of IAA by PGPR can vary among different species and strains, as observed during the present study, also influenced by culture condition, growth stage and substrate availability [27]. Other research workers also recorded IAA production in Azotobacter isolates are in agreement with other authors [27, 28] and high level of IAA production by Pseudomonas. Production of IAA and soluble phosphate are the most common mechanisms of actions implicated in PGPR and indeed microbes demonstrating the same are wide spread in rhizosphere [29].

Production of ammonia

A brown colour developed after the addition of Nessler's reagent which indicate that the test is positive and the PGPR isolates *Enterobacter* sp., *Bacillus* sp. and *Pseudomonas* sp. produced ammonia (Table 1). The ammonia is useful for plant as directly or indirectly. Ammonia production by the plant growth promoting bacteria helps influence plant growth indirectly. Ammonia released by diazotrophs is one of the most important traits of PGPR's which benefits the crop [30] This accumulation of ammonia in soil may increase in pH creating alkaline condition of soil at pH 9-9.5. It suppresses the growth of certain fungi and nitrobacteria due to it potent inhibition effect. It also upset the microbial community and inhibits germination of spores of many fungi [30]**.** Joseph *et al.* [31] reported ammonia production in 95% of isolates of *Bacillus* followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45%) isolates. Kumar *et al.* [32] isolated thirty two bacterial isolates from rhizosphere soil and reported that three bacterial isolates were positive for ammonia production.

Exopolysaccharide (EPS) production

Exopolysaccharides are polysaccharides synthesized by soil bacteria and secreted into the external environment. They are natural polymers and have distinctive chemical structures and physiochemical properties. Both plant growth promoting rhizobacteria and phytopathogenic bacteria are known to produce EPS for the establishment in soil and root environment, protection from predation and sustenance during nutrient starvation and desiccation. EPS forms soil aggregates and maintain soil

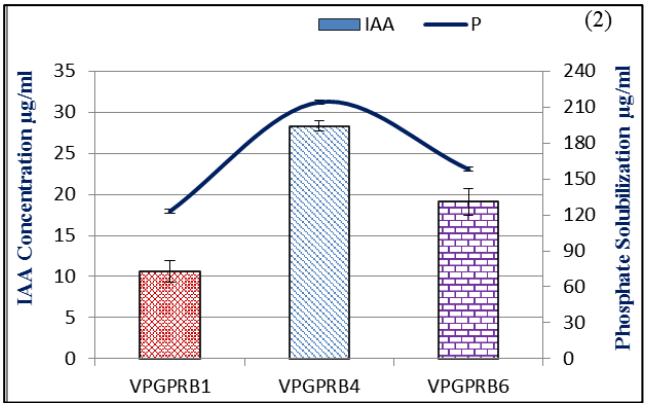

Fig 1: Total heterotrophic bacteria in the rhizosphere and non-rhizosphere soil of *Capsicum annuum* L.

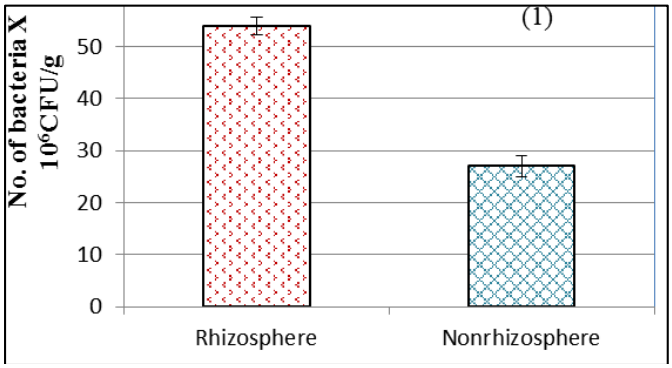

Fig 2: Quantitatve determination of IAA and Phosphate solubilization of PGPRB.

Table 1: Plant growth promoting characteristics of the Isolate

Isolate	Phosphate solubilization- plate assay			IAA Production	Ammonia production	Exo Poly Saccharide mg/ml
	Colony diameter (cm)	Halozone diameter (cm)	(PSI)			
VPGPRB1	1.2 ± 0.17	1.0 ± 0.1	1.83 ± 0.06	+	+	0.76 ± 0.15
VPGPRB4	1.3 ± 0.26	2.6 ± 0.2	3.0 ± 0.46	+	+	1.37 ± 0.25
VPGPRB6	0.9 ± 0.18	1.3 ± 0.22	2.45 ± 0.19	+	+	0.43 ± 0.08

Table 2: Morphological and biochemical characteristics of the PGPRB isolate

S No	Characteristics	VPGPRB1	VPGPRB4	VPGPRB6
1	Gram stain	Negative	Positive	Negative
2	Shape	Rods	Rods	Rods
3	Motility	Motile	Motile	Motile
4	Spore staining	-	+	-
5	Lactose fermentation	+	-	-
6	Indole	-	-	-
7	MR	-	+	-
8	VP	+	-	-
9	Citrate	+	+	+

10	Oxidase	-	-	+
11	Catalase	+	+	-

Water potential during dry season which is one of the vital soil characteristics. The PGPR isolates *Enterobacter* sp., *Bacillus* sp. and *Pseudomonas* sp. produced 0.76 ± 0.15mg/ml, 1.37 ± 0.25mg/ml and 0.43 ± 0.15mg/ml of exopolysaccharide (Table 1). Borgio *et al*. [33] reported three bacterial strains, *Bacillus subtilis* NCIM 2063, *Pseudomonas aeruginosa* NCIM 2862 and *Streptococcus mutans* MTCC 1943 were examined for their exopolysaccharide (EPS) producing ability at the laboratory level. The highest EPS production was recorded in *P. aeruginosa* (226 µg/ml) grown in nitrogen free medium followed by *S. mutans* and *B. subtilis* (220 and 206 µg/ml respectively) in nitrogen free medium after 7 days of incubation at 37°C.

Conclusion

In the present study three bacterial isolates *Enterobacter* sp., *Pseudomonas* sp. and *Bacillus* sp. exhibited plant growth promoting acitvities IAA production, phosphate solubilization, ammonia production and exopolysaccharide production. Among the three bacterial isolates the *Bacillus* sp. found to produce high amount of IAA and solubilized high amount of phosphate. PGPR ability can be exploited further by using it as bio-fertilizers in the field of medicinal plant, agriculture and crop plantation after further field studies to support these findings.

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