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*Full Length Research Paper*

# *Bacillus cereus* and *Enterobacter cancerogenus* screened for their efficient plant growth promoting traits rhizobacteria (PGPR) and antagonistic traits among sixteen bacterial isolates from rhizospheric soils of Pigeon Pea

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**Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers and pesticides. Here, we have isolated and characterized PGPR traits from the rhizosphere soil of pigeon pea and tested for the growth enhancement of pigeon pea. Rhizosphere soils were collected from different areas of Samalkot, Pithapuram, Peddapuram and Kakinada in India. A total of 65 isolates have been identified and characterized for their morphological, cultural, staining and biochemical characteristics, of which 35 isolates have been selected for further screening of PGPR traits. Sixteen isolates were successfully characterized for the PGPR traits like indole acetic acid (IAA) production, phosphorus solubilization and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Among the sixteen isolates, seven were found to be high IAA producing. Six were found to be efficient phosphate solubilizers, two were shown to be promising in both IAA production, phosphate solubilization and mineralizing capacity. These isolates showed growth promotion in pigeon pea for seedling emergence, increase of shoot length, root length, dry matter production, nodule number and nodule mass. Furthermore, PGPR isolates remarkably increased seed germination of pigeon pea. The two promising isolates were identified for 16sRNA sequencing and found to be *Bacillus cereus* and *Enterobacter cancerogens*.**

**Key words:** Indole acetic acid (IAA), plant growth promoting rhizobacteria (PGPR), phosphorus solubilization, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), seed germination.

## INTRODUCTION

Bacteria that colonize the rhizosphere and plant root, and enhance plant growth are referred to as plant growth promoting rhizobacteria (PGPR) (Frankenberger and Arshad, 1995). In the context of increasing international concern for food and environmental quality (Cappuccino, 1992), the use of PGPR for reducing chemical inputs in agriculture is potentially important. PGPR have been applied to various crops to enhance growth, seed emergence and crop yield (Hameeda et al., 2005). A PGPR *Pseudomonas fluorescens* isolated from roots of graminaceous plants has been shown to colonize the roots of various plants and to increase the height, flower number, fruit number and total fruit weight of tomato plants (Gyaneshwar et al., 1998).

In addition to improvement of plant growth, PGPR are directly involved in increased uptake of nitrogen,

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**Table 1.** Identification of potential bacterial isolates based on biochemical tests.

| Name of the test | RB 1 | RB 3 | RB 6 | RB 7 | RB 8 | RB 9 | RB 10 | RB 11 | RB 12 | RB 13 | RB 16 | RB 22 | RB 23 | RB 27 | RB 29 | RB 30 | RB 31 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Motility | NM | M | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | M | NM | M | NM |
| Indole | - | + | + | - | - | + | + | - | + | + | + | + | + | - | + | - | - |
| Methyl red | - | + | - | - | - | - | + | + | + | + | - | + | + | + | - | - | + |
| Voges Proskeur | + | - | + | + | + | + | + | - | - | - | + | - | - | - | - | - | - |
| Citrate utilization | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Identification | *Z* | *Y* | *X* | *W* | *V* | *U* | *T* | *S* | *R* | *Q* | *O* | *N* | *M* | *K* | *I* | *H* | *G* |

*G = Bacillus cereus*, *H= Pseudomonas* sp., *I = Serrratia marcescens*, *J = Azospirillum brasiliense, K = Klebsiella pneumoniae*, *L = Pseudomonas* sp., *M = Psuedomonas* sp., *N = Pseudomonas* sp., *O = Serratia* sp., *P = Bacillus circulans, Q = Bacillus* sp., *R = Bacillus* sp., S=*Bacillus cereus T = Pseudomonas* sp., *U = Azotobacter* sp., *V = Rhizobium*, *W = Micrococcus* sp, X = *Klebsiella* sp., *Y = Clostridium* sp..

synthesis of phytohormones, solubilization of minerals such as phosphorus and iron by production of siderophores that chelate iron and make it available to the plant root (Renwick and Campbell, 1991; Pal et al., 2001). In order to make cultivation sustainable and less dependent on chemical fertilizers, it is important to study how to use PGPR that can biologically fix nitrogen, solubilize phosphorus and induce substances like indole acetic acid (IAA) that can contribute to the plant growth (Farah et al., 2006). The interactions in the rhizosphere by soil micro-organisms play a pivotal role in transformation, mobilization and solubilization from a limited nutrient pool in the soil and ensure uptake of essential nutrients by the crop plants (Gyaneshwar et al., 1998).

Recently, there is growing interest in PGPR due to their efficacy as biological control and growth promoting agents in many crops (Kloepper et al., 1991; Sajjad et al., 2001). There is very little information regarding the use of PGPR as biofertlizer in pigeon pea. Therefore, the present study was undertaken to screen the PGPR strains (Rupela et al., 1991) which are compatible with *Cajanus cajan* and used as biofertilizer. PGPR are also known to produce antibacterial compounds that are effective in the control of certain plant pathogens and pests.

Thus, the need of the hour is to enhance the efficiency of small amount of external inputs by employing the best combinations of bacterial microbes for improved crop production.

## MATERIALS AND METHODS

### Isolation of rhizobacteria

Soil samples were collected from the rhizosphere of 2 to 3 month old pigeon pea plants in the fields of Samalkot, Pithapuram, Kakinada and Peddapuram regions of East Godavari, India. For collection of the rhizosphere soil in the field, soil samples were dug out at a depth of 10 to 20 inch deep and collected with intact root system in sterile cloth bags and polythene bags (Dubey et al., 2010). The rhizosphere soil was dug out and stored at 4°C. Samples of rice straw compost and vermicompost were also taken for screening the PGPR traits of indigenous organisms. The top of plant stems were cut, and the root system was used for collection of fine soil adhering to the roots carefully into Petri plate by tapping or with a smooth brush. The soil sample is used for dilution and plating.

Serial $10^{-}$ fold dilution technique was performed up to $10^{-7}$ dilution. An aliquot of this suspension was spread on the plates of Luria Bertani (LB) agar medium. Plates were incubated for 2 days at 28°C to observe the colonies of bacteria. Well isolated single colony was picked up and restreaked on fresh LB agar plate and incubated similarly. The technique was perpetrated thrice and cultures were made single colony type. Thus, a number of 65 isolates were selected out of which, 35 bacteria were sorted out in pure exhibiting different colony with morphological characteristics. The total number of bacteria was enumerated based on standard plate count method (SPC).

### Characterization of rhizobacteria

Bacteria were identified based on the Gram staining and cell morphology, placed in 4 groups, that is, Gram +ve rods, Gram +ve cocci, Gram -ve cocci and Gram -ve rods. Usually, the predominant bacteria in rhizosphere of crop plants are Gram -ve rods which may belong to *Pseudomonas*, Klebsiella based on colony characteristics and biochemical characteristics, and followed by Gram +ve rods of *Bacillus*, Clostridium. The isolates are identified up to genus level by the biochemical characteristics according to the Dubey and Maheshwari Practical Mannual level and results are given in Table 1.

### Phosphorus mobilization

Phosphorus is second only to nitrogen in mineral nutrients which is most commonly limiting in the growth of plants (Pikovaskaya, 1948). Many soil microorganisms are able to solubilize unavailable forms of bound P (Daniel et al., 1998). Visual detection and semi quantitative estimation of phosphate solubilizing ability of microorganisms is possible

by plate screening methods, that show clear zone around the microbial colonies in media containing insoluble mineral phosphates (tricalcium phosphate or hydroxyapatite) as sole P source (AMES, 1964; Rodriguez and Fraga, 1999). Quantitative estimation of phosphate solubilization was performed using Fiske Subba Row method of colorimetric analysis by inoculating individual bacteria in PVK broth and incubation for about 7 days. The drop in pH is also considered for the solubilization of inorganic phosphorus.

### IAA production

Plant hormones can be natural or synthetic. There are several phytohormone groups and the best known is the auxin group. Diverse soil microorganisms including bacteria, fungi and algae are also capable of producing physiologically active quantities of auxins (IAA). The isolates are incubated in the peptone broth enriched with tryoptophan broth to check for the production of indole acetic acid, a precursor of auxin which is an important plant hormone. The quantitative estimation of IAA is performed by using Salkowski method by using the reagent, 1 ml of $FeCl_3$, 0.5 mM in 35% $HClO_4$. Mixtures were incubated at room temperature for 25 min and observed for pink colour production and read colorimetrically.

### Antagonistic effect

The Antagonistic nature of the isolates towards pathogenic fungi *Macrophomina phaseolina* and *Fusarium udum* were tested. The initial antagonism of the bacterial cultures were tested using Dual plate method. The quantitative testing is performed by measuring the diameter of the growth inhibition zone of fungi using the bacterial cultures. The decrease in fungal biomass is also measured by inoculating the bacterial cultures along with the fungal mycelium in PDA broth. The suitable control is set up to know the growth inhibition. The decrease in fungal biomass is noted with the cultures RB 13 and RB16 on boh *Macrophomina phaseolina* and *Fusarium udum* the phytopathogenic fungi.

### 16srRNA analysis of selected isolates:

RB 13, RB16 identified as promising in showing the growth promoting traits and antagonistic traits and subjected to partial 16srRNA gene sequencing for phylogenetic analysis. Genetic diversity was investigated using restricted patterns produced by amplified rDNA restriction analysis and were identified as *Bacillus cereus* and *Enterobacter cancerogenus.*

### Plant germination test

*Cajanus cajan* seed were collected from the local agricultural research station and surface sterilized using $HgCl_2$. The seed germination efficiency tested using the sterilized minimal media and along with cultures isolated. The seedlings transplanted in sterile soil media along with the isolated cultures individually to monitor leaf and shoot developments for a period of 7, 15 21, days successively. Suitable controls without the cultures are set up to compare the growth. Efficient shoot development leaf area increase, root development, biomass increase were found with cultures RB13 and RB16 and the results tabulated in Table 2. Figure 1 shows the seed germination percentage seedling growth and root growth in percentages when inoculated with the isolated Rhizobacterial cultures.

## RESULTS

Sixteen bacterial isolates were successfully characterized as shown in Table 1. The morphological and biochemical characteristics of PGPR isolates widely varied among isolates. The bacterial isolates showed varied biochemical characteristics, phosphate solubilization and IAA production. The varied efficiency of isolates in their phosphate solubilization, IAA production, ACC deaminase abilities, Antagonistic activities towards fungal pathogens are given in Table 3 and are ranked based on their performance levels. The isolates were subjected to further analysis like, auxin production and phosphate solubilization. The isolates were tested for growth promotion of pigeon pea the efficiency percentages shown in Table 2 and Figure 1. The two isolates are promising in their enhancement of growth, nodule formation and plant biomass.

The bacterial isolates were named as RB1, RB2 and RB3 for illustrations, of which 8 were showing positive to biochemical characterization, enzyme productions, and were further screened out for the overlapping production abilities like both IAA production, phosphate solubilization. The isolates are further ranked on their abilities of showing PGPR Traits and Antagonistic traits. RB13 and RB16 of the sixteen isolates were further characterized for phylogenetic identification.

These were promising in phosphate solubilization, IAA production, antagonism to phytopathogens. These two cultures were further identified based on 16sRNA sequencing and PCR-RFLP analysis and found to be *Bacillus cereus* and *Enterobacter cancerogens*

## DISCUSSIONS

PGPR colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms (Rupela et al., 2005). To be an effective PGPR, bacteria must be able to colonize roots because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects. The exact mechanism by which PGPR stimulate plant growth is not clearly estimated, although several hypothesis such as production of phytohormones, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved (Rupela and Kumar, 1999).

Of the twelve isolates, seven were found to be high IAA producing. Among them, it has been found that RB6, RB13 and RB16 were profound in their shoot and root enhancement of seed of pigeon pea in laboratory conditions.

In the experiment, six were found to be efficient phosphate solubilizers, and their phosphate solubilizing efficiency has been calculated and tested in plant growth. Seed germination was increased when seeds were pretreated with PGPR isolates. Thus, *B. cereus* can be

**Table 2.** Effect of seed treatment with bacteria from rhizosphere and PGPR on cultivar in pots having unsterilized soil.

| Isolate | Seed germination (%) | Seedling height (cm) | Root length (cm) | Dry weight (mg/plant) |
|---|---|---|---|---|
| control | 82.10 | 10.30 | 4.10 | 5.60 |
| RB1 | 84.10 | 12.30 | 4.50 | 6.40 |
| RB3 | 86.63 | 12.50 | 4.30 | 6.60 |
| RB6 | 84.31 | 11.80 | 4.40 | 6.40 |
| RB7 | 84.01 | 13.80 | 5.30 | 6.80 |
| RB10 | 86.11 | 10.90 | 4.60 | 6.50 |
| RB13 | 90.26 | 12.60 | 4.50 | 6.20 |
| RB16 | 92.59 | 13.10 | 4.80 | 6.60 |
| RB23 | 92.07 | 12.70 | 4.40 | 6.08 |
| RB24 | 90.15 | 13.20 | 4.50 | 6.50 |
| RB29 | 92.59 | 10.30 | 5.59 | 6.20 |

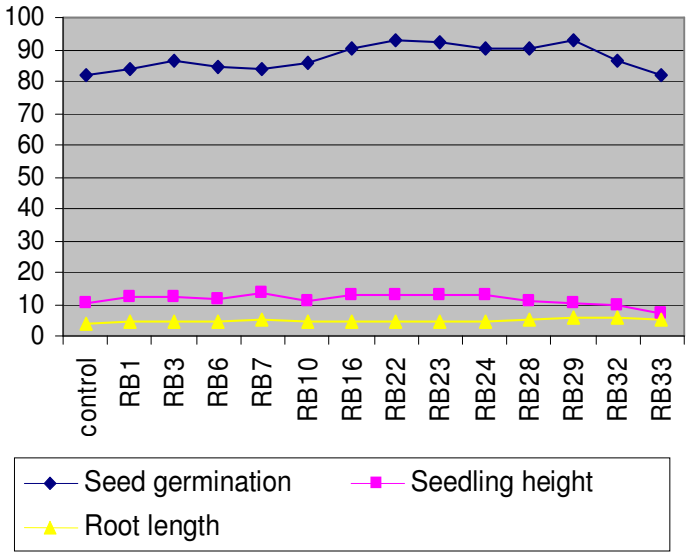


**Figure 1.** Effect of seed treatment with bacteria from rhizosphere and PGPR on cultivar in pots having unsterilized soil.

**Table 3.** Ranking of plant growth promoting bacteria for different traits used in the study.

| Isolates | PSB | Antagonism | Indole |
|---|---|---|---|
| RB1 | 2 | 2 | 1 |
| RB3 | 3 | 3 | 1 |
| RB6 | 3 | 3 | 0 |
| RB7 | 2 | 0 | 1 |
| RB9 | 2 | 0 | 2 |
| RB10 | 3 | 3 | 2 |
| RB11 | 2 | 3 | 1 |
| RB12 | 3 | 2 | 3 |
| RB13 | 1 | 0 | 0 |
| RB16 | 3 | 1 | 3 |
| RB22 | 2 | 0 | 0 |
| RB24 | 3 | 0 | 0 |

used not only as efficient phosphate solubilizer but also as a biopesticide against the fungal pathogens that attack the plants during the nodulation stage. *E. cancerogens* is profound in showing the antagonism to phytopathogens as it secretes certain colicins. Further investigations, including efficiency test under greenhouse and field conditions, are needed to clarify the role of PGPR as biofertilizers that exerts beneficial effects on plant growth and development. The *B. cereus* strain isolated showed antagonism equivalent to *Bacillus thuringenesis* according to the phylogenetic tree construction.

## REFERENCES

Ames BN (1964). Assay of inorganic phosphate, total phosphate and phosphatases.

Cappuccino JC, Sherman N (1992). In: Microbiology: A laboratory Manual 3rd ed New York: Benjamin/Cumming Pub. Co.

Daniel PS, Robert JR, Ayling SM (1998). Phosphorus uptake by plants, from soil to cell, pp. 447-453.

Dubey RC, Maheshwari DK, Harish K, Kamlesh C (2010). Assessment of diversity and plant growth promoting attributes of rhizobia from Cajanus cajan, Afr. J. Biotechnol., 9(50): 8619-8629.

Farah A, Iqbal A, Khan MS (2006). Screening of Free-living Rhizospheric Bacteria for their Multiple Plant Growth Promoting Activity, Microbiol. Res., 163: 173-181

Gyaneshwar P, Naresh KG, Paresk LJ, Poole PS (1998). Effect of buffering on the P- solubilizing ability of microorganisms World J. Microbial. Biotechnol., pp. 669-673.

Gyaneshwar P, Naresh KG, Paresk LJ, Poole PS (1998). Role of soil microorganism ms in improving P nutrition of plants. Plant Soil, 245: 83-93.

Hameeda Bee, Harini G, Rupela OP, Gopal R (2005). Growth Promotion of maize by phosphate solubilizing bacteria isolated from composts and macrofauna. Microbiol. Res., pp. 234-242.

Kloepper JW, Antoun H (1991). Plant Growth Promoting Rhizobacteria. Encyclopedia of Genetics, London, Academic Press.

Pikovaskaya RI (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Micobiologiya, 17: 362-370.

Renwick AR, Campbell A (1991). Assessment of *in vivo* screening systems for potential biocontrol Agents of Gaeumannomyces graminis. Plant Physiol., 40: 524-532.

Rodriguez H, Fraga R (1999). Phosphate solubilizing bacteria and their role in plant growth promotion, Biotechnol. Adv., 17: 319-339.

Rupela OP, Gowda CLL, Wani SP, Hameeda B (2005). Evaluation of

crop production systems based on locally available biological inputs.
Rupela OP, Kumar RA (1999). Novel methods for the identification and enumeration of microorganisms with potential for suppressing fungal plant pathogens, pp. 131-134.
Rupela OP, Kumar Rao JVDK, Sudarshana MR, Usha K, Anjaih A (1991). Rhizobium germplasm resources at ICRISAT center, Research Bulletin no. 15, Patancheru, A.P, India, pp. 21-40.