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Research paper

ISOLATION, SCREENING AND CHARACTERIZATION OF PGPR ISOLATES FROM RHIZOSPHERE OF RICE PLANTS IN KASHIPUR REGION (TARAI REGION)

Tarun Sharma*, Navin Kumar, Nishant Rai

Department of Biotechnology, Graphic Era University, Dehradun, India.
*Corresponding Author: tarunsharmamicro84@gmail.com

ABSTRACT: Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can be found in the rhizosphere, in association with roots which can enhance the growth of plant directly or indirectly. A large number of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium* and *Serratia* have reported to enhance plant growth. Here, we have isolated, screened and characterized the PGPR from the rhizosphere soil of rice field. Rhizosphere soils were collected from different areas of Kashipur region in Uttarakhand, India. Ten isolates of bacteria, designated as PGB1, PGB2, PGB3, PGB4, PGB5, PGB6, PGB7, PGB8, PGB9 and PGB10, were successfully isolated and characterized. Subsequently, to investigate the PGPR isolates for their antagonistic activity against phytopathogenic fungi such as *Fusarium oxysporum* and *Rhizoctonia solani*. In extend, the growth of PGPR isolates was optimized under different temperature conditions such as 10°C, 20°C, 28°C, 37°C and 45°C. Isolates PGB3, PGB4, PGB6, PGB7, PGB8, PGB9 and PGB10 induced the production of indole acetic acid (IAA), whereas only PGB8 isolate was able to solubilize phosphorus. In case of Siderophore production, the isolates PGB4, PGB6, PGB7, PGB8 and PGB10 were found to be positive. Most of the isolates grown best under the temperature of 20°C & 28°C when compared to 10°C & 37°C and very few can grown at 45°C. Furthermore, most of the PGPR isolates shown antifungal activity against *Fusarium oxysporum*, and *Rhizoctonia solani*. The present study, therefore, suggests that the use of PGPR isolates PGB6, PGB8 and PGB10 as inoculants/ biofertilizers might be beneficial for rice cultivation as they enhanced growth of rice due to the production of IAA, Phosphate solubilization, Siderophore production and also having antifungal activity against phytopathogenic fungi.

KEY WORDS: PGPR, **IAA, phosphorus solubilization, siderophore production, antagonism assay.**

INTRODUCTION

Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as plant growth-promoting rhizobacteria (PGPR). In the context of increasing international concern for food and environmental quality, the use of PGPR for reducing chemical inputs in agriculture is a potentially important issue. PGPR have been applied to various crops to enhance growth, seed emergence and crop yield, and some have been commercialized (Khalid *et al.,* 1997, Joseph *et al.,* 2007 and Leinhos *et al.,* 1994). Under salt stress, PGPR have shown positive effects in plants on such parameters as germination rate, tolerance to drought, weight of shoots & roots, yield, and plant growth (Bowen and Rovira., 1999, Mirza *et al*., 2001). Another major benefit of PGPR is to produce antibacterial compounds that are effective against certain plant pathogens and pests. Moreover, PGPR mediate biological control indirectly by eliciting ISR against a number of plant diseases (Yanni *et al*., 1997). Application of some PGPR strains to seeds or seedlings has also been found to lead to a state of induced systemic resistance in the treated plant (Biswas *et al*., 2000). PGPR have also been reported in cereal crops including rice (Haas and Defago, 2005). In addition to improvement of plant growth, PGPR are directly involved in increased uptake of nitrogen, synthesis of phytohormones, solubilization of minerals such as phosphorus, and production of siderophores that chelate iron and make it available to the plant root (Kloepper *et al*., 2004). It has also been reported that PGPR is able to solubilize inorganic and organic phosphates in soil (Herman *et al.,* 2008).

Inoculant development has been most successful to deliver biological control agents of plant disease i.e. organisms capable of killing other organisms pathogenic or disease causing to crops. Different bacteria which are predominantly

studied and increasingly marketed as the biological control agents includes the genera *Bacillus*, *Streptomyces*, *Pseudomonas* and *Agrobacterium*. They suppress plant disease through at least one mechanism, induction of systemic resistance and production of siderophore or antibiotics. Exposure to the PGPR triggers a defence response by the crop as if attacked by pathogenic organisms (Minorsky., 2008).

IAA producing PGPR strains can able to enhance the growth and development of plants by interfering in the concentration of known phytohormones (Dey *et al.*, 2004). One of the most important ways that those bacteria affect growth and development is by producing Indole-3-acetic acid (IAA) that this hormone is led to plant root system development and subsequently nutritional uptake increase by plant. Many of rhizobial species enable to produce IAA (Husen., 2003). In order to produce Indole-3-acetic acid (IAA), the bacteria use Tryptophan (L-Trp) as precursor (Verma *et al*., 2001). This substance can be converted to IAA by soil beneficial bacterial activities. In addition to root growth, the synthesized IAA by bacteria in some case by promoting enzyme 1-aminocyclopropane- 1-carboxylate (ACC) synthase and increasing the synthesis of ACC, precursor of ethylene, can be caused a reverse result. The excess of produced ethylene, so-called named stress ethylene, causes to decrease vegetative period and finally, yield (Kloepper *et al*., 1999).

2. MATERIAL AND METHODS

2.1. Isolation of PGPR from rice rhizosphere

Soil samples were collected from the rhizosphere of 2 month old rice plants in different areas of Kashipur region (Tarai Region) in India. The rhizosphere was dugout with intact root system. The samples were placed in plastic bags and stored at 4°C in refrigerator. Ten grams of rhizosphere soil were taken into a 250 mL

of conical flask, and 90 mL of sterile distilled water was added to it. The flask was shaken for 10 min on a rotary shaker. One milliliter of suspension was added to 10 ml vial and shaken for 2 min. Serial dilution technique was performed upto 10^7 dilution. An aliquot (0.1 ml) of this suspension was spread on the plates of Nutrient agar medium. Plates were incubated for 3 days at 28°C to observe the colonies of bacteria. Bacterial colonies were streaked to other Nutrient agar plates and the plates were incubated at 28°C for 3 days. Typical bacterial colonies were observed over the streak. Well isolated single colony was picked up and re-streaked to fresh Nutrient agar plate and incubated similarly. The technique was repeated thrice and cultures were made single colony type.

2.2. Characterization of isolates

Morphological characteristics of the colony of each isolate were examined on NA plates. All the isolates were streaked on Nutrient agar plates. After 3 days of incubation, different characteristics of colonies such as shape, size, elevation, surface, margin, color, odor, pigmentation, etc were recorded. A loopful of bacterial culture from each isolates was diluted into a test tube containing 1 mL sterile distilled water and was vortexed. A loopful was then taken on a glass slide and smeared. The slide was air dried and fixed by heating on a Bunsen flame. The slide was flooded with crystal violet solution for 3 min. The slide was washed gently in flow of tap water and air dried. The slide was observed under microscope and the shape was recorded. A drop of sterile distilled water was placed in the center of glass slide. A loopful of growth from young culture was taken, mixed with water, and placed in the center of slide. The suspension was spread out on slide using the tip of inoculation needle to make a thin suspension. The smear was dried in air and fixed through mild heating by passing the lower side of the slide 3 to 4 times over the flame. The smear was then flooded with

crystal violet solution for 1 min and washed gently in flow of tap water. Then the slide was flooded with iodine solution, immediately drained off, and flooded again with iodine solution. After incubation at room temperature for 1 min, iodine solution was drained out followed by washing with 95% ethanol. After that, it was washed with water within 15 to 30 seconds and blot dried carefully. The smear was incubated with safranin solution for 1 min. The slide was washed gently in flow of tap water and dried in air. The slide was observed under microscope and data were recorded. Motility of bacteria was observed by hanging drop method. A loopful of 2 day old bacterial culture was suspended in 1 mL of nigrosin solution. A drop of suspension was taken on a cover slip. The cover slip was hanged on a hollow slide with vaseline. The slide was then observed under microscope to test the motility of bacteria.

2.3.Growth under different temperature conditions

The culture of 10 isolates were streaked on Nutrient agar plates and incubated at 10, 20, 28, 37 and 45°C. The change in growth and color was observed and recorded after 3 days of incubation.

2.4. 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 culture of 10 isolates was 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 calorimetrically.

2.5. Phosphate solubilization

Phosphorus is only second to nitrogen in mineral nutrients which is most commonly limiting in the growth of plants. Many soil microorganisms are able to solubilize unavailable forms of bound P (Kokalis-Burelle *et al.*, 2006). The plates were prepared with Pikovaskya's medium. The cultures of ten isolates were spot inoculated on the plates and incubated in an incubator at 28°C for 3-5 days. Formation of clear zone around the microbial colonies indicated phosphate solubilization.

2.6. Siderophore production

Siderophore production was tested qualitatively using chrome azurol S medium (CAS-medium) (Pradhan and Sukla., 2006). The culture of 10 isolates were streaked on the surface of CAS agar medium and incubated at room temperature for 2 to 4 days. Siderophore production was indicated by orange halos around the colonies after the incubation, and this test was done in two replications.

2.7. Antagonism assay against phytopathogenic fungi

All the 10 isolates were assayed for antifungal activities against *Fusarium oxysporum*, *Rhizoctonia solani* by using Potato Dextrose Agar (PDA) medium. The isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at the center of the medium. The barrier between isolates and fungi indicated antagonist interaction between them. Antagonist activity was investigated for 4 to 7 days after incubation at room temperature. The value of inhibition was measured using the formula described by Kumar *et al*., 2002. (Minorsky, 2008) which is 1-(a/b) x 100% (a: distance between fungi in the center of Petri dish to test isolate, b: distance between fungi in the center of Petri dish to blank are without *Bacillus* isolate).

3. RESULTS

3.1. Isolation of PGPR

Ten bacterial isolates were successfully isolated from the rhizosphere soils of rice field from different areas in Kashipur (Table 1). They were designated as PGB1, PGB2, PGB3, PGB4, PGB5, PGB6, PGB7, PGB8, PGB9 and PGB10.

Table 1: Description of the PGPR isolates

S.No.	Isolates	Location of rhizosphere soil
1.	PGB1	Jaspur
2.	PGB2	Jaspur
3.	PGB3	Kashipur
4.	PGB4	Nadehi sugar mill
5.	PGB5	Ramnagar Van
6.	PGB6	Ramnagar Van
7.	PGB7	Narayanpur
8.	PGB8	Dhyan nagar
9.	PGB9	Kunda Village
10.	PGB10	Hariyawala

3.2. Morphological characteristics of PGPR isolates

As shown in Table 2, the morphological characteristics of PGPR isolates widely varied. The isolates were found to be first growers. All the isolates produced round shaped and raised colonies having smooth shiny surface with smooth margin. They differed in color but all were odorless. No pigmentation was observed in the colonies on Nutrient agar plates. Diameter of the colonies of isolates varied from 0.2 to 2 mm.

Table 2: Morphological characteristics of 3-day-old colony of PGPR isolates

Isolates	Shape	Size (mm)	Elevation	Surface	Margin	Color	Odour	Pigmentation
PGB1	Round	0.8-1.0	Raised	Smooth shiny	Smooth	Off white	Odourless	None
PGB2	Round	0.9-1.1	Raised	Smooth shiny	Smooth	Off white	Odourless	None
PGB3	Round	0.9-1.1	Raised	Smooth shiny	Smooth	Brownish	Odourless	None
PGB4	Round	1.0-1.3	Raised	Smooth shiny	Smooth	Yellowish	Odourless	None
PGB5	Round	1.0-1.5	Raised	Smooth shiny	Smooth	Whitish	Odourless	None
PGB6	Round	1.8-2.0	Raised	Smooth shiny	Smooth	Yellowish	Odourless	None
PGB7	Round	0.3-0.7	Raised	Smooth shiny	Smooth	Off white	Odourless	None
PGB8	Round	0.9-1.1	Raised	Smooth shiny	Smooth	Milky	Odourless	None
PGB9	Round	0.5-1.0	Raised	Smooth shiny	Smooth	Milky	Odourless	None
PGB10	Round	0.8-1.2	Raised	Smooth shiny	Smooth	Brownish	Odourless	

3.3. Microscopic observation of PGPR isolates

Microscopic observations were performed to investigate some characteristics of PGPR isolates such as shape, Gram reaction and motility (Table 3). Seven isolates were rod shaped while PGB2 and PGB5 showed ellipsoidal shape and PGB8 was coccus shaped. All the isolates were motile and Gram negative in reaction. It was also noted that the growth of isolates on nutrient agar plates varied in temperature (Table 4). The growth of all isolates was good in the temperature ranges of 20 to 28°C. In addition, PGB3 and PGB4 isolates were found to grow at 45°C.

Table 3: Cell shape, Motility and Gram reaction of PGPR isolates

Isolates	Cell Shape	Motility	Gram reaction
PGB1	Rod	Motile	Gram negative
PGB2	Ellipsoidal	Motile	Gram negative
PGB3	Rod	Motile	Gram negative
PGB4	Rod	Motile	Gram negative
PGB5	Ellipsoidal	Motile	Gram negative
PGB6	Rod	Motile	Gram negative
PGB7	Rod	Motile	Gram negative
PGB8	Coccus	Motile	Gram negative
PGB9	Rod	Motile	Gram negative
PGB10	Rod	Motile	Gram negative

Table 4: Growth of PGPR isolates at different temperature condition

Isolates	Temperature				
	10º C	**20º C**	**28º C**	**37º C**	**45º C**
PGB1	+	++	++	+	-
PGB2	+	++	++	+	-
PGB3	+	++	++	++	+
PGB4	++	++	++	++	++
PGB5	+	++	++	++	-
PGB6	+	++	++	+	-
PGB7	+	++	++	-	-
PGB8	-	++	++	+	-
PGB9	+	++	++	+	-
PGB10	-	++	++	++	-

- = No Growth, + = Weak Growth, and ++ = Good Growth.

3.4. Production of IAA and solubilization of phosphorus

We have investigated the IAA production and phosphorus solubilization of PGPR isolates. As shown in Table 5, isolates PGB3, PGB4, PGB6, PGB7, PGB8, PGB9 and PGB10 induced the production of IAA. Isolates PGB4 and PGB10 were found to be good producers of IAA. On the contrary, PGB8 was found to be a medium producer of IAA in comparison to the weak producer isolates PGB3, PGB6, PGB7 and PGB9. On the other hand, only PGB8 isolate had ability to solubilize the phosphorus (Table 5).

Table 5: Production of IAA and Phosphorous solubilization by PGPR isolates

Isolates	IAA production	Phosphorus solubilization	Siderophore production
PGB1	-	Not solubilize	Negative
PGB2	-	Not solubilize	Negative
PGB3	+	Not solubilize	Negative
PGB4	+++	Not solubilize	positive
PGB5	-	Not solubilize	Negative
PGB6	+	Not solubilize	positive
PGB7	+	Not solubilize	positive
PGB8	++	solubilize	Positive
PGB9	+	Not solubilize	Negative
PGB10	+++	Not solubilize	positive

(-) = *No production;*(+) = *Weak producer;* (++) = *Medium producer;* (+++) = *Good producer*

3.5. Siderophore production

Out of 10 PGPR isolates, 5 isolates (PGB4, PGB6, PGB7, PGB8 and PGB10) were able to produce siderophore and it is confirmed by the development of orange halos surrounding those colonies (Table 5).

3.6. Antagonism assay against phytopathogenic fungi

Opposition assay was used to determine the isolates that inhibit the growth of *R. solani*, *F. oxysporum*. Numerous isolates were able to inhibit *F. oxysporum* and *R. solani* in various level of inhibition percentage. In this study, among 10 PGPR isolates that significantly promoted plant growth of rice seedling, there were only 3 isolates (PGB5, PGB6 and PGB10) that were able to inhibit *F. oxysporum*, 6 isolates (PGB1, PGB5, PGB6, PGB8, PGB9 and PGB10) inhibited *R. solani* (Table 6).

Table 6: Antagonism assay of PGPR isolates against phytopathogenic fungi

Isolates	**Antagonism assay against phytopathogenic fungi**	
	R.solani	***F.oxysporum***
PGB1	+	-
PGB2	-	-
PGB3	-	-
PGB4	-	-
PGB5	+++	++
PGB6	++	++
PGB7	-	-
PGB8	++	-
PGB9	+++	-
PGB10	++	++

+ = Low inhibition percentage (< 30 %); ++ = Moderate inhibition percentage (30% to 40%); +++ = Strong inhibition percentage (>40%)

4. DISCUSSION

PGPR colonize plant roots and exert beneficial effects on plant growth

and development by a wide variety of mechanisms. 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 established, although several hypotheses such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved(Kloepper *et al.,* 2004, Herman *et al*., 2008).

Although several naturally occuring auxins have been described in the literature, indole-3- acetic acid (indole acetic acid, IAA) is by far the most common as well as the most studied auxin, and much of the scientific literature considers auxin and IAA to be interchangeable terms (Liu *et al.,* 1992, Neeru *et al*., 2000). IAA may function as important signal molecule in the regulation of plant development. Out of ten isolates seven isolates are positive for IAA production (Table 5). Among them two Isolates PGB4 and PGB10 are found to be good producers of IAA (Table 5). It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Spaepen *et al.,* 2007). Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil (Patten and Glick., 1996).

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Sarwar and Frankenberger., 1994). The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can enhance the

availability of phosphorus and iron for plant growth. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to rice that represent a possible mechanism of plant growth promotion under field conditions (Jetiyanon and Kloepper., 2002). In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere (Whipps., 2001). In our experiments, only PGB8 isolate was able to solubilize phosphate in the rhizosphere soil (Table 5). Furthermore, this isolate was found to be medium producer of IAA. It is important to note that several phosphate solubilizing bacilli occur in soil but their numbers are not usually high enough to compete with other bacteria commonly established in the rhizosphere.

Siderophore is one of the biocontrol mechanisms belonging to PGPR groups under iron limiting condition. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi. In addition to siderophore, there are other mechanisms of biocontrol including antibiotics compounds, elicitation of induced systemic resistance (ISR) of plant, and lytic enzyme secretion.

This study has demonstrated that, the 10 PGPR isolates classified as plant growth promoter and produced siderophores such as PGB4, PGB6, PGB7, PGB8 and PGB10, and these isolates already found to be IAA producer. Based on the siderophore produced by those isolates, it has been determined that, all the isolates which produced the bioactive compound siderophore were able to inhibit the phytopathogenic fungi (Table 6). The results of this study suggest that, siderophore produced by those bacteria functions as suppressor to the growth of

phytopathogenic fungi such as *F. oxysporum, R. solani*.

CONCLUSION

It can be concluded from the above discussion that PGPR enhance the plant growth. Collectively, our results indicated that the use of PGPR isolates PGB6, PGB8 and PGB10 as inoculants/ biofertilizers might be beneficial for rice cultivation as they enhanced growth of rice due to the production of IAA, Phosphate solubilization, Siderophore production and also having antifungal activity against phytopathogenic fungi. Simultaneous screening of PGPR from field is a good tool to select effective PGPR for biofertilizer development technology.

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