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Full Length Research Paper

Growth promotion by P-solubilizing, siderophore and bacteriocin producing rhizobacteria in *Zea mays* L.

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An isolation study of plant growth promoting rhizobacteria was carried out in rhizosphere soil of rice grown in raised bed fields at Kalashakaku, Pakistan. Eight rhizobacteria were isolated, purified and their colony morphology was recorded. All of them were positive for catalase test. These isolates variably showed P-soloubilization, bacteriocin and siderophore production abilities. On the basis of these traits, three isolates 4PKR, 12PKR and 13PKR from raised bed field of rice rhizosphere were selected for an investigation of their growth promoting potential on maize (*Zea mays* L.) in a pot experiment. Most of the isolates resulted in significant increase in growth of maize *(Z. mays* L.) seedling. Two isolates12PKR and 13PKR exhibited better performance.

Key words: Catalase, P-soloubilization, bacteriocin, siderophore.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are root colonizing bacteria that exert their beneficial effect on root growth. 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 (Minorsky, 2008). 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 (Bowen and Rovira, 1999). Significant increases in growth and yield of agronomical important crops in response to inoculation with PGPR have been reported (Bashan et al., 2004). Inoculation of plants with *Azospirillum* could result in significant changes in various growth parameters, such as increase in plant biomass, nutrient uptake, tissue N content, plant height, leaf size and root length of cereals. Biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzyme, hydrogen cyanide and siderophore or through competition for nutrient and space, can significantly improve plant health and promote growth by increasing seedling emergence, vigor and yield (Antoun and Kloepper, 2001).

Objectives of the study

There is lack of information on the potential and mechanisms of PGPR isolates from rhizosphere soil of rice grown in raised bed fields. The main objective of this research is to isolate and screen PGPR from rhizosphere soil of rice grown in raised bed fields, and also to see if PGPR isolates from rice rhizosphere could affect seed germination and growth parameters of maize seedling in greenhouse.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from rhizosphere soil of rice grown in raised bed fields at Kalashakaku, Pakistan. They were taken from 6 inches depth from the area surrounding the roots of three months

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old rice plants and carried to the laboratory in sterile plastic bags. This was followed by immediate processing, after which they were stored at 4°C in cold room.

Isolation

Isolation of PGPR isolates were made from rhizosphere soil of rice grown in raised bed fields and irrigated fields at Kalashakaku on Luria Bertani (LB) (g/L tryptone, 10; yeast extract, 5; NaCl, 10; agar, 18 and pH, 7.5). 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 at 120 rpm. One milliliter of suspension was added to 10 ml vial and shaken for 2 min. Serial dilution technique was performed up to 10^{-7} dilution. An aliquot (0.1 ml) of this suspension was spread on the plates of Luria Bartany (LB) agar medium. Plates were incubated for 3 days at 30°C to observe the colonies of the bacteria. Typical bacterial colonies were observed over the streak. Morphologically different colonies were selected, marked and re-streaked until pure cultures were obtained. Well isolated single colony was picked up and re-streaked on fresh LB agar plate and incubated similarly. The technique was perpetrated thrice and cultures made were of single colony type.

Morphology of isolates

Morphological characteristics of the colony of each isolate were examined on LB agar plates. All the isolates were streaked on LB agar plates. After 3 days of incubation, different characteristics of the colonies such as shape, size, elevation, surface, margin, color, odor etc. were recorded.

Catalase test

Fresh culture (24 h old) was used and bacterial colony was placed on a glass slide and one drop of H_2O_2 (30%) was dropped on the colony. The appearance of gas bubbles is the indicator of catalase enzyme (McFadden, 1980).

PGP characters

Phosphate solubilization

The plates were prepared with Pikovskya's medium. The culture of these isolates was streaked on the plates and incubated in an incubator at 28°C for 7 days.

The plates were then examined and data were recorded (Pikovaskya, 1948):

$$SI = \frac{\text{Colony diameter + halozone diameter}}{\text{Colony diameter}}$$

Assay for siderophore production

PSB isolates were assayed for siderophore production on the chrome azurole S agar (CAS) described by Clark and Bavoil (1994). Chrome azurole S agar plates were prepared and spot was inoculated with test organism and incubated at 30°C for 5 days. Development of yellow – orange halo around the colony was considered as positive for siderophore production.

Bacteriocin production

Bacteriocin activity was done by using a saturated culture of indicator strain VF39 grown in TY medium; diluted (10^{-2}) 1 ml of the diluent was mixed with 25 ml of molten and soft TY agar (0.6% wt/vol) containing 5 mM Ca^{+2}. The single colonies of strain to be verified for bacteriocin activity were inoculated into soft agar within two hours after agar solidification. Halozones are the indicators surrounding the stab inoculated cultures. The plates were incubated approximately for 48 h and the result was recorded (Oresnil et al., 1999).

Inoculation of maize (*Zea mays* L.) by PGPR isolates

Re-inoculation studies of selected isolates were performed on maize plants. Maize seeds of variety "Islamabad White (Sawan 3)" were collected from National Agriculture Research Centre, Islamabad. In preparation of inocula 24 h old fresh cultures were inoculated in 100 ml broth of LB media and kept on shaker for 48 h; and then centrifuged for 10 min at 10,000 rpm. Supernatant was discarded and pellet was diluted with distilled water up to 100ml, with the optical density measured as 1. Seeds were surface sterilized with 10% chlorox for 3 min and then washed with 95% ethanol for 3 min with constant shaking; and later washed with sterilized water. Sterilized seeds were soaked in broth for 2 to 4 h. After soaking, seeds were sown in pots containing autoclaved soil and sand in 3:1. Pots were placed in the green house of Quaid i Azam University and watered with autoclaved water. Sterilized water was put in pots. Seedlings were harvested after 15 days. Length, fresh and dry weight of root and shoot and leaf area were recorded.

RESULTS

Isolation of PGPR

In the present study, a total of eight bacterial isolates were isolated from rhizosphere soil of rice grown on raised bed at Kalashahkaku.

These eight bacterial isolates were selected on the basis of different morphological characters and were tested for their plant growth promoting qualities. They were designated as 3PKR, 4PKR, 6PKR, 7PKR, 8PKR, 12PKR and 13PKR.

Morphology of bacterial colonies

All bacterial isolates from rhizosphere soil of rice from raised bed fields were odourless, had round colonies with smooth and shiny surfaces and raised elevation, with size ranging from 1 to 4 mm. Most of them had entire margins except 3PKR and 4PKR which were having wavy margins. The color of 3 PKR, 6 PKR, 7 PKR, and 8 PKR was skin; 12 PKR and 13 PKR, green; 4PKR, yellowish. All the isolates were gram negative. The cell shape of 7PKR, 8PKR and 13PKR is rod, while other bacterial isolates were round in shape. Among them, some were paired and some were scattered in arrangement (Table 1).

Table 1. Morphological characteristics of 3 day old colonies of PGPR isolates.

Bacterial isolates	Shape	Size (mm)	Odor	Color	Elevation	Surface	Margins	Cell shape	Arrangement	Grams test
1PKR	Round	1.5	Odorless	Off white	Raised	Smooth shiny	Entire	Round	Paired	Gram negative
3PKR	Round	2	Odorless	Skin	Raised	Smooth shiny	Wavy	Round	Scattered	Gram negative
4PKR	Round	1.5	Odorless	Yellowish	Raised	Smooth shiny	Wavy	Round	Scattered	Gram negative
6PKR	Round	1	Odorless	Skin	Raised	Smooth shiny	Entire	Round	Paired	Gram negative
7PKR	Round	4	Odorless	Skin	Raised	Smooth	Entire	Rod	Scattered	Gram negative
8 PKR	Round	1	Odorless	Skin	Raised	Smooth shiny	Entire	Rod	Paired	Gram negative
12 PKR	Round	3.5	Odorless	Green	Raised	Smooth shiny	Entire	Round	Paired	Gram negative
13 PKR	Round	4	Odorless	Sea green	Raised	Smooth shiny	Entire	Rod	Scattered	Gram negative

1PKR, 3PKR, 4PKR, 6PKR: 5PKR, 7PKR, 8PKR, 12PKR, 13PKR: Bacterial isolates from rhizosphere soil of rice grown in raised bed fields at Kalashakaku.

Catalase test

All the isolates from rhizosphere soil of raised bed fields of rice were positive for catalase test (Table 2).

P solubilization

Solublization index based on colony diameter and halozone for each isolated rhizobacteria is presented in Table 2. Results showed that among eight isolates tested for solubilization index, only 4PKR, 6PKR, 12PKR and 13PKR were positive for P solubilizing potential.1PKI, 13PKI, and 13PKR showed significantly higher SI as compared to other PSB isolates. By observing pikovskaya's plates, it was concluded that halos were produced due to the solubilization of insoluble tricalcium phosphate (Table 2).

Siderophore production

Among the eight isolates from rhizosphere soil of rice from raised bed fields, 7PKR, 12PKR and 13PKR were positive for siderophore production; however, larger halo was formed around the colonies of 13PKR, medium halo was formed around the colonies of 12PKR and small halo was formed around the colonies of 7PKR (Table 2).

Bacteriocin production

Out of 8 PGPR isolates which were screened and tested for plant growth promoting characters, four isolates were positive for bacteriocin production against tested organism (VF39 strain of *Rhizobium leguminosarum*) by producing clear inhibition zones around the stab inoculated cultures on TY plates. The bacterial isolate, 12PKR showed maximum bacteriocin production by exhibiting inhibition zone of 12.3 mm (Table 2).

Re-inoculation studies

In this experiment, among all isolates, only 12PKR and 13PKR were positive for P solubilization, siderophore and bacteriocin production while 6PKR and 4PKR showed only P solubilizing potency. Bacterial isolates, 4PKR, 12PKR and 13PKR from isolates of rhizosphere soil of rice grown in raised bed fields of Kalashahkaku were selected for the evaluation of their effect on growth of maize (*Zea mays* L.). Most of the isolates significantly increased length of shoot and root fresh and dry weight of shoot and root as well as leaf area of maize seedlings.

Shoot and root length

All isolates showed significant increase in shoot and root length of maize as compared to uninoculated control. Among all bacterial isolates 13PKR showed maximum increase by 43 and 41.39% in increasing shoot and root length of maize respectively as compared to control. Bacterial isolates 4PKR and 12PKR showed insignificant difference in increasing shoot length between each other but significantly increased shoot length by 32.9% as compared to control (Figure 1).

Table 2. Solubilization index, catalase test, siderophore production and bacteriocin production of PGPR isolates from the rhizosphere soil of rice grown in raised bed fields at Kalashahkaku against VF39 strain of *Rhizobium leguminosarum*.

Bacterial isolates	Solubilization index	Catalase test	Siderophore production	Bacteriocin inhibition zone (mm)
1PKR	Not detected	+	Not detected	Not detected
3PKR	Not detected	+	Not detected	Not detected
4PKR	2.65^{bc}	+	Not detected	Not detected
6PKR	2.65^{bc}	+	Not detected	Not detected
7PKR	Not detected	+	+	Not detected
8 PKR	Not detected	+	Not detected	Not detected
12 PKR	2.32^{c}	+	++	12.3
13 PKR	2.98^{ab}	+	+++	10.6

1PKR, 3PKR, 4PKR, 6PKR, 5PKR, 7PKR, 8PKR, 12PKR, 13PKR: Bacterial isolates from rhizosphere soil of rice grown in raised bed fields at Kalashakaku. Where, **-** stands for negative in test, + stands for positive in test.

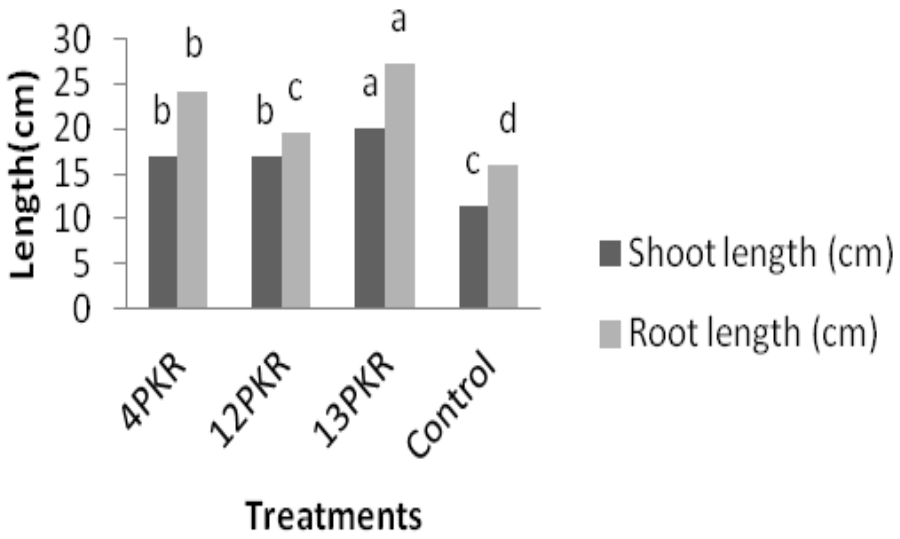

Figure 1. Effect of PGPR (isolated from rhizosphere soil of rice grown in raised bed field at Kalashahkaku) on shoot and root length of maize (*Zea mays L*.).

Shoot and root fresh weight

All bacterial isolates showed stimulatory effect on fresh weight of shoot of maize as compared to uninoculated control. 13PKR and 12PKR showed insignificant difference between each other in increasing fresh weight of shoot, while they showed maximum increase by 58.9 and 56.3%, respectively, as compared to control. 13PKR was most efficient in enhancing root fresh weight that is, by 50% as compared to control (Figure 2).

Shoot and root dry weight

All the inoculants tested in this experiment showed significant increase in shoot dry weight as compared to uninoculated control but exhibited insignificant difference among each other. Significant increase in root dry weight was shown by all inoculants as compared to uninoculated control. 13PKR exhibited maximum increase by 45.9% in root dry weight as compared to control (Figure 3).

Leaf area

Among all six bacterial isolates used as inoculants only12PKR and 13PKR showed significant difference in increasing leaf area of maize as compared to control. Maximum increase was shown by 12PKR that is, by 75.81% (Figure 4).

Statistical analysis

The data were analyzed statistically by Analysis of Variance technique (Steel and Torrie, 1980) and

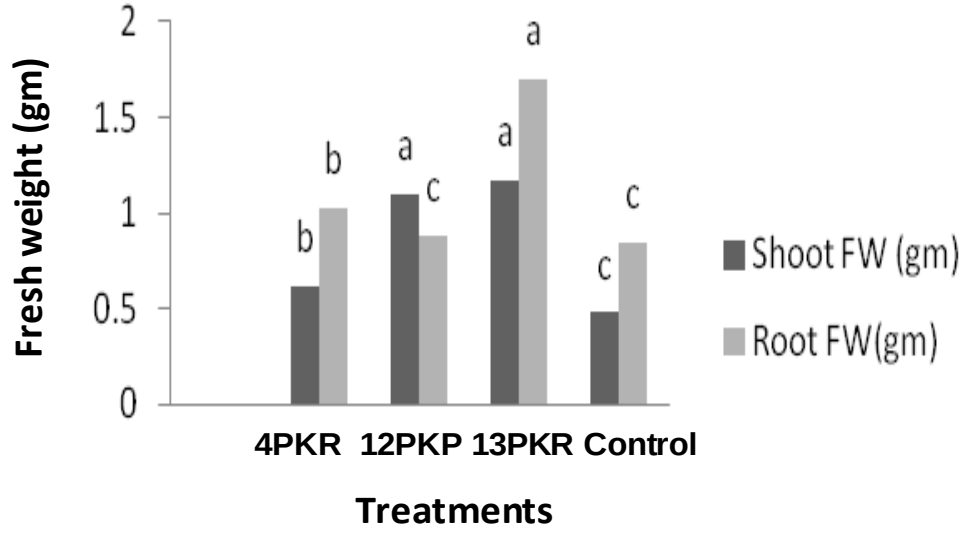

Figure 2. Effect of PGPR inoculation (isolated from rhizosphere soil of rice grown in raised bed field Kalashahkaku) on shoot and Root fresh weight of maize (*Zea mays* L.).

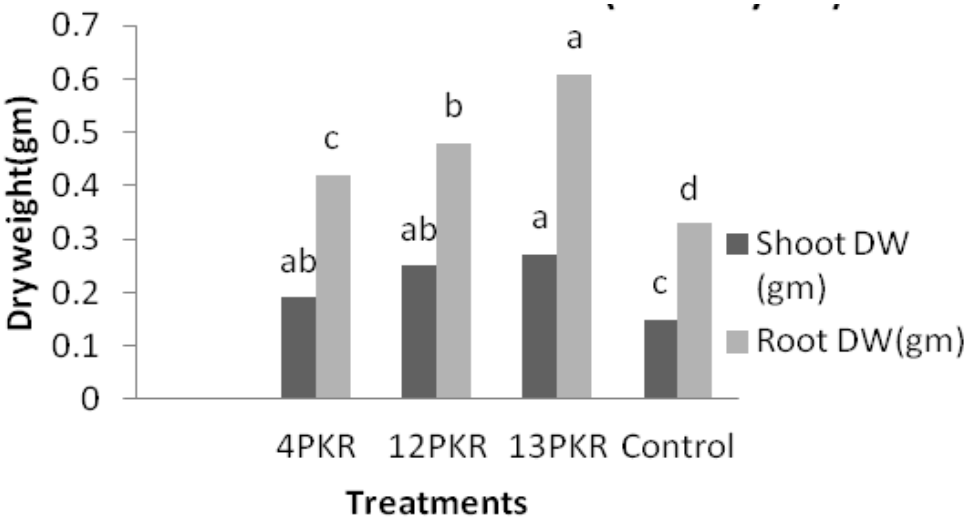

Figure 3. Effect of PGPR (isolated from rhizosphere soil of rice grown in raised bed field at Kalashahkaku) on dry weight of shoot and root of maize (*Zea mays* L.).

comparison among treatment means was made by Duncan's Multiple Range Test (DMRT) (Duncan's, 1955).

DISCUSSION

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. Most phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by plants (Pradhan and Sukla, 2005). PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to rice that represents a possible mechanism of plant growth promotion under field conditions (Verma et al., 2001). Acidification, due to low molecular weight organic acids released by soil microorganisms/degradation of complex organic molecules, is a major mechanism of mineral P solubilization (Zaidi et al., 2009; Khan et al., 2007). Accordingly, inoculation of PSB in soils has been shown to improve the availability of P and subsequently crop yields (Linu et al., 2009; Hameeda et al., 2008). In present study, results showed that among eight isolates

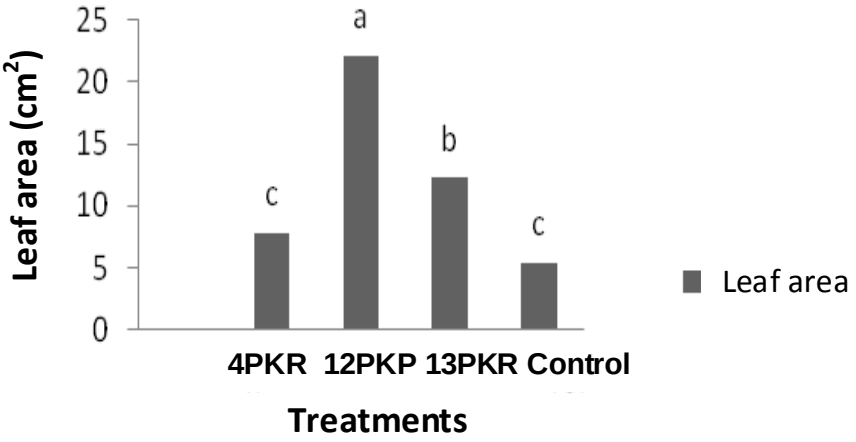

Figure 4. Effect of PGPR (isolated from rhizosphere soil of rice grown in raised bed field at Kalashahkaku) on leaf area of maize (*Zea mays* L.).

tested for solubilization index, only 12PKR and 13PKR were positive for P solubilizing potential. 13PKR showed significantly higher SI as compared to 12PKR isolates.

Siderophores directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria and may function in local and systematic host resistance in plants (Joseph et al., 2007; Wani et al., 2008). In fact, the vast majority of research on microbial siderophores in the rhizosphere is associated with their biocontrol activities due to their competitive effects with plant pathogens (Hiifte et al., 1994). Belimov et al. (2005) reported that AY197010 isolate of *Pseudomonas* and AY197006 and AY197009 isolates of *Flavobacterium* could manufacture siderophore. In this study, 7PKR, 12PKR and 13PKR were positive for siderophore production. By considering this, these bacterial isolates could also be used as bio-control agents.

The bacterial isolate 12PKR showed maximum bacteriocin production by exhibiting inhibition zone of 12.3mm. Bacteriocins in agriculture are used as a biological control for soil borne or phyllosphere-inhabiting bacterial plant pathogens (Herlache and Triplett, 2002) and play key role in determining the competitiveness for nodulation (Oresnil et al., 1999).

According to a large body of evidence, PGPR enhance growth, seed emergence and crop yield, and contribute to the protection of plants against certain pathogens and pests (Dey et al., 2004; Kloepper et al., 2004; Kokalis-Burelle et al., 2006; Herman et al., 2008; Minorsky, 2008). Rhizobacteria are known to enhance plant productivity by solubilizing mineral P (Khan et al., 2007).

Iron is an essential nutrient of plants, but it is relatively insoluble in soil solutions. Plant roots prefer to absorb iron as the more reduced ferrous (Fe^{2+}) ion (Salisbury and Ross, 1992). While, siderophores (like SA and DHBA) synthesized by soil microbial communities chelate iron and supply them to plants under iron-limiting conditions (Indiragandhi et al., 2008). However, there is a controversy in the significance of bacterial Fe^{3+} - siderophore uptake to the iron nutrition of plants. Some believe that the contribution of these siderophores to the overall iron requirements of plants is small (Glick, 1995). *Pseudomonas aeruginosa* strain PS1 enhances growth parameters of green gram (*Vigna radiata* (L.) in insecticide-stressed soils (Munees and Mohammad, 2011). The incorporation of bio-fertilizers (N fixers) plays major role in improving soil fertility, yield attributing characters, with final yield being reported by many workers (Subashini et al., 2007; Kachroo and Razdan, 2006; Son et al., 2007). Significant increase in plant height was observed as a result of Azospirillum inoculation compared to unincoclated control (Ashrafi and Seiedi, 2011). In this study bacterial isolate13PKR showed significant maximum increase by 41.3% in root length of maize as compared to control. Erturk et al. (2010) also observed significant increase in root length of *Bacillus simplex* RC19 (8.63 cm); and *Comamonas acidovorans* RC41 (7.70 cm), respectively compared to uninoculated control of kiwifruit (*Actinidia deliciosa*). 13PKR exhibited maximum increase by 45.9% in root fresh weight as compared to control. Similar results were exhibited by Shaharoona et al. (2007). 13PKR exhibited maximum increase by 45.9% in root dry weight as compared to control. Bhromsiri and Bhromsiri (2010) observed that the PGPR10 inoculation increased shoot dry weight by about 23%, whilst PGPR9 and PGPR5 inoculations improved root dry-weight by about 32 and 28% respectively ($P<0.05$).

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