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RESPONSE OF RICE TO INOCULATION WITH PLANT GROWTH PROMOTING RHIZOBACTERIA IN CONTROL LAB ENVIRONMENT AND FIELD EXPERIMENT

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Abstract

The present study was conducted to evaluate the effects of bacterial inoculation on different growth parameters of rice variety JP-5. Three bacterial strains (*Azospirillum brasilense* R1, *Azospirillum lipoferum* RSWT1 and *Pseudomonas* Ky1) were used to inoculate rice varietyJP-5 at control lab environment and field. Plant growth promotion was observed in all inoculated treatments over non-inoculated, which was evident from increase in root area, root length, number of tillers, straw and grain yields and total weight of plant. *Azospirillum brasilense* R1 was more effective in plant growth promotion than other strains and showed 19% increase in the straw weight and 39.5% increase in grain weight. Inoculation with *Azospirillum lipoferum* RSWT1 and *Pseudomonas* Ky1 increased grain weight by 18.5% and 13.8% respectively. The study revealed that beneficial strains of PGPR can be used as biofertilizer for rice.

Introduction

Rice (*Oryza sativa* L.) is an important Kharif crop of Pakistan, ranking second to wheat as a food staple. It is a high value cash crop and a major export item. Rice is a monocotyledonous plant belonging to the genus *Oryza* L., sub family Oryzoideae, in the family Poaceae (Graminae). Rice is cultivated between 36 ^{0} ES – 55 ^{0} EN and grows from sea level to an altitude of 2,500 M or even higher. Rice brings economic prosperity of the farmers as well as earns billions of rupees for country through its export. Rice has gradually moved to occupy a pre-dominant position in the agriculture economy of Pakistan. Rice account for 2.7% of the value added in agriculture and 0.6% GDP (Anon., 2013). In Khyber Pakhtunkhwa (KPK), rice cultivation stands next to wheat and maize and is characterized by being grown under two different agro-climate conditions i.e. the plain and the upper mountainous valleys. Most of the cultivated area (81%) is situated in the cooler, high altitude area of Malakand, Hazara Division and adjacent tribal areas of KPK. In Swat during 2008 – 2009 rice was grown in an area approximately 7349 hectares (Anon., 2012).

The average yield of rice in the country and particularly in KPK is far behind than other developing countries of the world. The export of rice from Pakistan decreased from US $ 2.18 billion in 2009-10 to US $ 1.92 billion in 2012-13, thus showing decline of 19% (Anon., 2013). The increase in yield of rice is largely depend on availability of essential nutrients like nitrogen and phosphorus in the soil which can be supplied as chemical fertilizers. This leads to environmental pollution and health hazards. The use of chemical fertilizers is also too costly, especially nitrogen, which is one of the common limiting factor in rice production (Ahemad & Khan, 2012). A substitute is the use of Plant Growth Promoting Rhizobacteria (PGPR) as bacterial inoculants for crops. These rhizobacteria are the dominant deriving force in the recycling of nutrients and help in improving soil fertility (Glick *et al.*, 2012). Ahmed *et al*., (2013) reported that inoculation of rice with *Azospirillum brasilense* increase grain yield by 22.7% over control. PGPR inoculation to rice may effectively increase the surface area of roots (Richardson, 2001), root weight (Cakmakci *et al*., 2007), production and quality (Mehnaz *et al*., 2010). The present study was conducted with objectives to evaluate the effect of PGPR inoculation on the growth and yield of rice in control lab environment and field.

Materials and Methods

Isolation of bacteria from rice roots and rhizosphere: Roots of rice (*Oryza sativa* L.) variety JP-5 along with the rhizosphere soil were collected and stored at 4^{o}C. One gram of roots along with adhering soil was ground well. Serial dilutions (10X) were made and 100 µL aliquots from 10^{-3}–10^{-5} dilutions were spread on LB plates (Maniatis *et al*., 1982). Semi solid NFM (Okon *et al*., 1977) was incubated with 100 µL of these serial dilutions. The inoculated plates and NFM vialswere incubated for 24-72 hrs at 30^{o}C. Morphologically different colonies appearing on the growth medium were selected for further purifications. Bacterial growth obtained in NFM medium was streaked on NFM agar plates and incubated at 30^{o}C for 24-72 hrs. Single colonies appearing on the agar plates were transferred to a drop of sterilized water on a microscopic glass slide and observed under the light microscope (Nikon Japan).The bacterial cultures obtained were grown at 30^{o}C for 24 hrs and preserved in glycerol (20%) at -20^{o}C.

Identification and characterization of bacterial isolates: The bacterial strains were grown on Luria-Bertani (LB) agar medium (Maniatis *et al*., 1982) and incubated at 30^{o}C for 24-48 hrs. The bacterial strains were characterized by morphological and physiological tests including pigment production on nutrient agar medium, cell morphology, colour, shape, size, motility and growth at 30^{o}C on NFM and tentatively identified.

Indole acetic acid (IAA) production: The bacterial strains were grown in conical flasks containing 50mL Nitrogen-free medium (Okon *et al*., 1977) supplemented with L-tryptophan (100 mg/L) and NH_4Cl 1g/L at 30^{o}C. The supernatant of the culture fluid was obtained by

centrifuging the stationary phase cultures at 10,000 rpm for 15 min. The pH was adjusted to 2.8 with 1N HCl. The auxins from the acidified culture medium were extracted with equal volumes of ethyl acetate (Tien *et al*., 1979), evaporated to dryness and re-suspended in one mL of ethanol. The samples were analyzed by HPLC (Varian Pro star) using UV detector and C-18 column. The mobile phase used in this reaction was Methanol: acetic acid: water (30:1:70 v/v/v) at the rate of 0.6 mL min^{-1} (Rasul *et al*., 1998). Pure indole-3-acetic acid was used as standard. The IAA of the samples was identified and quantified by comparing the retention time and peak area by using computer software (Varian).

Inoculation of rice grown in the lab in falcon tubes: The bacterial strains were grown in 100 mL of LB liquid medium in water bath at 25°C and 150 rpm. The cell suspensions were pelleted in sterile centrifuge tubes at 10,000 rpm for 10 min., washed once with distilled water and re-suspended in 100 mL of distilled water. The selected bacterial strains (*Azospirillum brasilense* R1, *Azospirillum lipoferum* RSWT1 and *Pseudomonas* Ky1) were used as inoculum for the rice variety JP 5. The seeds were surface sterilization with sodium hypochloride for 5 min and then washed with sterilized water. The seeds were sown in sterilized sand in 50 mL Falcon tubes and kept in the growth room (254µEm-^{2}S-^{1} and 25^{o}C). In each tube one seed was sown. One mL of the inoculum was used to inoculate each seedling two days after germination. One mL of Nitrogen-free Hoagland solution (1/2 strength) was added twice a week as nutrient source. Seedlings were harvested after 4 week of growth. For measuring the root area and root length, the plant roots were washed, separated and spread on a transparent polyethylene sheet. The sheet with roots was put on the desktop scanner and computer image of the roots was created. The root area and root length were measured on the P-IV IBM computer and scanner by using root image analysis programme developed by Washington State University, USA.

Raising of rice nursery and inoculation of rice in the field experiment: A fertile piece of land was selected for field experiment at Agriculture Research Institute North (ARIN) Mingora, Swat and seed were cultivated on 21 May 2009.In the early stages of growth the water was drained out daily at night. Afterward, the depth of water was kept 2-4 cm to suppress weeds. After 30 days i.e., on 20 June 2009, the nursery was transplanted to the field. During transplantation the water depth was kept 2 cm in the field. Bacterial inoculums were prepared and 50 mL of the inoculum of each bacterial strain (*Azospirillum brasilense* R1, *Azospirillum lipoferum* RSWT1 and *Pseudomonas* Ky1) was added to 2 L water and roots of the nursery seedling were inoculated for 1 hr. The right number of seedling was detached from the bundles and inserted in the soil not shallower than 1.5 cm and not deeper than 3 cm. Two seedlings per hill at 20 x 20 cm distance were planted. The missing hills were replaced about 10 days after transplantation.

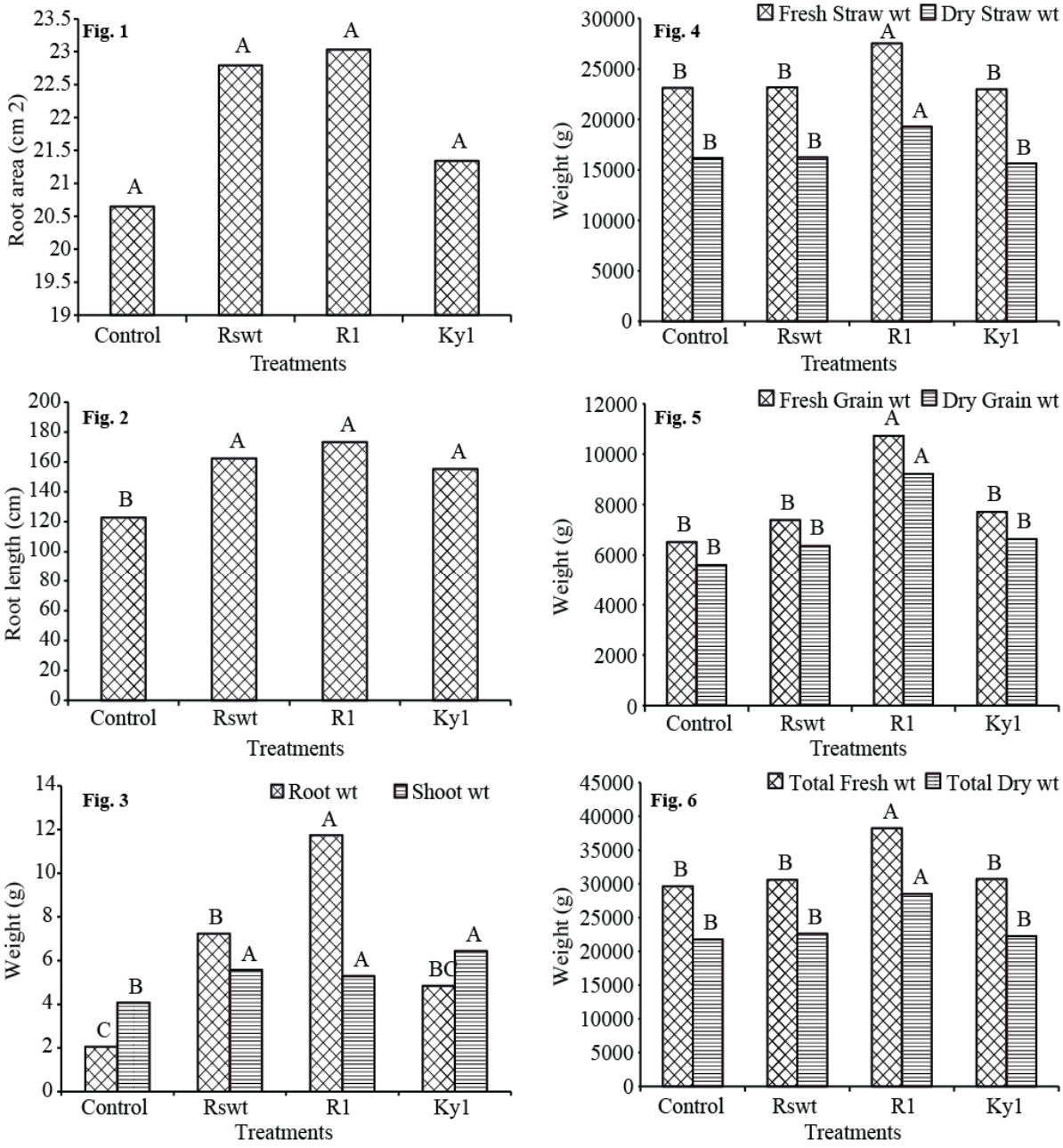
Randomized complete block design was used in the current investigation. The size of the plots was kept 3 x 3 meter. The number of rows was 14 x15 with 210 plants per plot. After one week of transplantation, recommended chemical fertilizers (120 kg ha^{-1} N, 60 kg ha^{-1} Pand 40 kg ha^{-1} K) were applied. The plants were harvest from 30–35 days after flowering. Initially, 5 plants from each plot (i.e. a total of 20 plants from each treatment) were up-rooted carefully to take out whole root system. Roots were washed carefully to remove adhering soil and kept at 55^{o}C for three days to estimate root dry weight. Similarly straw weight and grain weight of all the randomly selected plants was taken. To record the total grain weight and straw weight from each plot, the grains were separated from the straw and their fresh weight was recorded. In order to carry out dry weight study of grains and straw, 5 kg of grains were dried in oven at 55^{o}C for three days. After drying the plant material, dry weight was measured and the difference between fresh weight and dry weight was calculated. Keeping in view the loss per kg, the total dry grain weight of each treatment was calculated. The dry straw weight and total dry weight of the plants per plot and per treatment was also calculated. Statistical calculations were carried out by using MSTAT C program and LSD tests.

Results and Discussion

A variety of beneficial bacteria colonize the roots and aerial parts of rice. Interest in beneficial rhizobacteria associated with rice has increased due to their potential use as biofertilizer (Mehnaz *et al*., 2010).A total of 18 bacterial isolates were obtained from the roots and rhizosphere of rice and 17 produced IAA. Production of IAA was higher in R4-1 strain with 45.6 µg/mL, followed by R2 with 45.5 µg/mL and MRSWT6 with 35.8 µg/mL of IAA. *Pseudomonas* Ky1 also produced higher amount (32.5 µg/mL) of IAA. The value of IAA production by the isolates R4-2 and R6-2 were comparatively low (4.5 µg/ mL and 5.6 µg/mL respectively) while the isolate R5-2 did not show any IAA production in pure culture. Production of IAA by the *Azospirillum* and *Pseudomonas* has been reported by many researchers (Adewusi *et al.,* 2008; Bhattacharyya & Jha *et al.,* 2012).

The inoculated strains showed positive effects on both the root area and root length of rice plant though the root area was not significantly different from control. Maximum increase in the root weight and shoot weight was recorded for the plants inoculated with *Azospirillum brasilense* R1 (Figs. 1-3). A similar response was observed by Ahemad & Khan (2012) who reported significant increase in the root length, shoot weight and grain weight of the inoculated plants. Maximum beneficial effects on most growth parameters (root fresh weight, shoot fresh weigh, shoots dry weight, grain fresh and dry weight) were observed in plants inoculated with *Azospirillum lipoferum* RSWT1.Plant growth promotion was observed in all inoculated treatments over non-inoculated, which was evident from increase in root area, root length, number of tillers, straw and grain yields and total weight of plant (Table 1). Complete yield data collected from whole plots indicated that the inoculated bacterial strains resulted in more yields than non-inoculated ones. The effect of the inoculated strains was

positive on both fresh and dry weight. *Azospirillum brasilense* R1 showed the maximum growth promotion of rice variety JP 5 as compared to other inoculants used in this study. Inoculation with this strain resulted in 19% increase in fresh straw weight, 39.3% increase in fresh grain weight and 29% increase in the total fresh weight over non-inoculated control. The increase in the dry straw weight, dry grain weigh, dry total weight were 19%, 39.5%, 30.8%, respectively. *Azospirillum lipoferum* RSWT1 and *Pseudomonas* Ky1 also showed significant increase of 18.5% and 13.5% respectively in the total grain yield (Figs. 4-6).The results are parallel to the finding of Ahmed *et al*., (2013) who reported that inoculation of rice with *A. brasilense* R1 result an increase of 16.6% in dry straw weight, 22.7% in dry grain weight and 19.8% in total plant dry weight. This result was also in consistence to the work of Tariq *et al*., (2007) who stated that the application of PGPR maintain the concentration of Zn in the soil and increase the grain yield and total biomass of rice crop.



Figs. 1-6. Effect of inoculated bacterial strains on growth and yield of rice variety JP 5; F1: root area; F2: root length; F3: root and shoot weight; F4: fresh and dry straw weight at ARIN; F5: fresh and dry grain weight at ARIN; F6: total weight (straw+grain) at ARIN; control: non-inoculated; RSWT: *A. lipoferum*; R1:*A. brasilense*;Ky1:*Pseudomonas*; the values are an average of 4 replicates;the statistical calculations were carried out by using MSTAT C program and LSD tests;different letters given above the bars in the graphs show that value are different at 5 % level of significance.

Table 1. Effect of bacterial inoculation on different growth parameters of rice.

S. No.	T	PL (cm)	RD (g)	SD (g)	GD (g)	NG	NSG	NT	NP	DP (g)
1	T1	133.7 (±3.5)	8.5 (±2.1)	38.4 (±1.8)	27.9 (±7.1)	1366.6 (±440)	119.8 (±114)	10.4 (±1.7)	9.6 (±2.1)	0.8 (±0.2)
2	T2	130.3 (±2.7)	10.1 (±1.7)	56.0 (±11.1)	39.3 (±11.7)	1563.2 (±24.1)	19.6 (±20.6)	13.6 (±3.3)	13.2 (±2.7)	1.2 (±0.5)
3	T3	130.1 (±2.2)	15.7 (±2.8)	63.5 (±13.6)	39.8 (±10.1)	1993.4 (±350)	21 (±21.9)	14 (±1.8)	13.2 (±2.7)	1.6 (±0.7)
4	T4	128.2 (±7.7)	13.9 (±5.1)	59.2 (±52.1)	37.1 (±15.4)	1517.6 (±685)	22.4 (±18.6)	14.6 (±5.0)	13.8 (±5.3)	1.3 (±0.6)

T1: control; T2: *A. lipoferum*; T3*: A. brasilense*; T4: *Pseudomonas*; PL: plant length; RD: root dry wt; SD: shoot dry wt; NG: number of grains; NSG: number of sterile grains; NT; number of tillers; NP: Number of panicles; DP: Dry wt of panicles; the values are average of 20 plants and the values in brackets represents Standard Deviation.

Negative effect of the inoculated strains on certain growth parameters was also observed. In the whole plant study, height of inoculated plant was less than non-inoculated control. Negative effects of bacterial inoculations on legumes and other plant species due to over production of growth hormones, production of antibiotics and competition with *Rhizobium* for attachment sites on root surfaces have also been reported (Li & Alexander, 1988; Ahmed *et al.,* 2013).Comparatively analysis of the lab experiment, single plant data and whole plot data showed variation. In the single plant study, *Pseudomonas* Ky1 showed more positive response on shoot weight and grain weight than other inoculated strains. Such variation between single plants and whole plot analysis proved that on the basis of single plant analysis or small scale cultivation, we cannot draw exact picture about the effectiveness of a particular strain or comparing different inoculations.

This is the first study from Pakistan to demonstrate that PGPR can increase yield, growth and development of rice variety JP 5. In the present study both the *Azospirillum* strains used as inoculants were isolated from rice grown in the same area. It has been reported than when locally isolated PGPR were used as inoculants, more crop yield than inoculation with type strain and control (Ahmed *et al.,* 2013). Additional field studies in Swat area are required to confirm the beneficial role of bacterial inocula on growth and yield of other cereal crops and also locally isolated PGPR may be tested on other rice varieties grown in the area. This would help in developing a biofertilizer (inoculant) for use in agriculture in the future.

References

Adewusi, H.G., S.O. Bada, D.O. Ladipo and T. Husain. 2008. Nodulation in *Millettiathonningii* (Schum & Thonn.) Baker; native Rhizobia and seed interaction from southwest Nigeria. *Pak. J. Bot*., 5: 2237-2242.

Ahemad, M. and M.S. Khan. 2012. Evaluation of plant growth promoting activities of rhizobacterium *Pseudomonas putida* underherbicide-stress. *Ann. Microbiol*., 62: 1531-1540.

Ahmed, B., Midrarullah and M.S. Mirza. 2013. Effects of inoculation with plant growth promoting Rhizobacteria (PGPRs) on different growth parameters of cold area rice variety, Fakhre Malakand. *Afr. J. Microbiol. Res*., 7: 1651-1656.

Anonymous. 2012. Agricultural statistics of Pakistan. Govt. of Pakistan.Ministry of food, agriculture and livestock (Economic wing Islamabad).

Anonymous. 2012. Agriculture Research Institute Mingora Swat, Statistical Division, Crop Reporting Services Swat, Pakistan.

Bhattacharyya, P.N. and D.K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol*., 28: 1327-1350.

Cakmakci, R., M. Erat, U.G. Erdogan and M.F. Donmez. 2007. The influence of PGPR on growth parameters, antioxidant and pentose phosphate oxidative cycle enzymes in wheat and spinach plants. *J. Plant Nutr. Soil Sci*., 170: 288-295.

Glick, B.R. 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Hindawi Publishing Corporation, *Scientifica*.

Li, D.M. and A. Alexander. 1988. Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. *Plant Soil*, 108: 211-219.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory, U.S.A. p. 545.

Mehnaz, S., D.N. Baig and G. Lazarovits. 2010. Genetic and phenotypic diversity of plant growth promoting rhizobacteria isolated from sugarcane plants growing in Pakistan. *J. Microbiol. Biotechnol*., 20: 1614-1623.

Okon, Y., S.L. Albercht and R.H. Burris. 1977. Method for growing *Spirillumlipferum* and for counting the pour cultures and in association with plant. *Appl. Environ. Microbiol*., 33: 85-88.

Rasul, G., M.S. Mirza, F. Latif and K. A. Malik. 1998. Identification of plant growth hormones produced by bacterial isolates from rice, wheat and kallar grass. In: *Nitrogen Fixation with Non-legumes*. (Eds.): K.A. Malik, M.S. Mirza and J.K. Ladha. pp. 25-37. Kluwer Academic Publishers, Dordrecht, Netherlands.

Richardson, A.E. 2001. Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *J. Plant Physiol*., 28: 897-906.

Tariq, M., S. Hameed, K.A. Malik and F.Y. Hafeez. 2007. Plant root associated bacteria for Zinc mobilization in rice. *Pak. J. Bot*., 93: 245-253.

Tien, T.M., M.H. Gaskins and D.H. Hubbell. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl. Environ. Microbiol*., 37: 1016-1024.

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