academicJournals

Vol. 7(31), pp. 4010-4014, 2 August, 2013
DOI: 10.5897/AJMR2013.5375
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The applications of plant growth promoting rhizobacteria (PGPRs) as biofertilizers for cold area rice variety JP-5

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Accepted 29 May, 2013

The aim of present study was to evaluate the effect of bacterial inoculation on different growth parameters of rice variety JP 5. A total of 18 bacterial strains were isolated from roots and rhizosphere of rice. Three bacterial strains (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) were used to inoculate rice variety JP 5 at two experimental sites in Swat (Agriculture Research Institute (N) Mingora and Udigram). Plant growth promotion was observed in all inoculated treatments, which was evident from increase in number of tillers, straw and grain yields and total weight of the plants. *A. brasilense* strain R1 was more effective in plant growth promotion than other strains. At ARIN Mingora, Swat, inoculation of rice variety JP 5 with *A. brasilense* strain R1 showed 19% increase in the straw weight and 39.5% increase in the grain weight. At this experimental site, inoculation with *A. lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 increased grain weight by 18. and 13.8% respectively. At Udigram, Swat, any significant beneficial effect of inoculation with *A. lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 was not observed whereas inoculation with *A. brasilense* strain R1 showed positive results of 15.5 and 27.4% increase over control in straw weight and grain weight, respectively. The study reveals that beneficial strains of PGPR can be used as biofertilizer for rice.

Key words: *Azospirillum*, *Pseudomonas,* inoculation of rice, effects of inoculation.

INTRODUCTION

Rice (*Oryza sativa* L.) is an important Kharif crop of Pakistan, ranking second to wheat as staple food. Rice is a monocotyledonous plant belonging to the genus *Oryza* L, sub family Oryzoideae, in the family Poaceae (Graminae). Rice is cultivated between 36°ES - 55°EN and grows from sea level to an altitude of 2,500 M or even higher. Rice has gradually moved to occupy a predominant position in the agriculture economy of Pakistan. In Khyber Pakhtunkhwa (KPK), rice cultivation stands next to wheat and maize and is characterized by being grown under two different agro-climate conditions that is the plain and the upper mountainous valleys. Most of the

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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 to 2009 rice was grown in an area of approximately 7349 hectares. The average rice yield in the country and particularly in KPK is far behind what can be obtained from the potential of the crop (Anonymous, 2009).

The increase in yield of rice is largely dependent upon 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 a limiting factor for rice production (Cheng et al., 2012). A substitute is the use of Plant Growth Promoting Rhizobacteria (PGPR) as bacterial inoculants for cereal crops (Ryu et al*.,* 2005). PGPR inoculation to cereal crops may effectively increase the surface area of roots (Richardson, 2001), root weight (Cakmakci et al., 2007), productivity and quality (Mehnaz et al. 2010).

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 from the plants grown in the fields of Udigram and ARIN Mingora, Swat. The soil samples were stored at 4°C and used for further studies. One gram of roots along with adhering soil was ground well with the help of a pestle and mortar. Serial dilutions (10X) were made and 100 µL aliquots from 10^{-3}-10^{-5} dilutions were spread on Luria Bertani (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 vials were incubated for 24 to 72 h at 30°C.

Morphologically different colonies appearing on the growth medium were selected for further purifications. Isolated colonies were streaked on fresh plates with LB medium to get single-cell colonies. Bacterial growth obtained in NFM medium was streaked on NFM agar plates and incubated at 30°C for 24 to 72 h. 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°C for 24 h and preserved in glycerol (20%) at -20°C.

Identification and characterization of bacterial isolates

The bacterial strains were characterized by morphological and physiological tests, including pigment production on nutrient agar medium, cell morphology and motility and growth at 30°C on NFM. The cultural characteristics of the purified bacterial strains were studied using light microscope. All the strains were grown on Luria Bertani (LB) agar medium (Maniatis et al., 1982) and incubated at 30°C for 24-48 h. The purified cultures were maintained on LB agar slants. The colonies of bacterial culture were observed for colour, shape, size and motility. Single colony of each strain was suspended in 0.85% saline. A drop of this suspension was put on a glass slide, covered with cover slip and observed under light microscope. Each bacterial strain was tentatively identified on the basis of cell morphology that is size, shape and motility.

Raising of rice nursery and inoculation of rice in the field experiment

A fertile piece of land that has easy access to the water channel and convenient drainage system was selected to raise the rice nursery. The land was prepared by ploughed with tractor 3 times and then irrigated. Eradication of the weeds was carried out through ploughing and planking. During this process the water remained in the field. Seeds of the selected rice variety JP 5 were cultivated on 21 May 2009 at ARIN Mingora and Udigram Swat.

In the early stages of growth, water was drained out daily at night. Afterward, the depth of water was kept 2-4 cm to suppress weeds. After 30 days that is on 20 June 2009, the nursery was transplanted to the fields. During transplantation the water depth was kept 2 cm in the field. Bacterial inoculums were prepared by growing in 100 mL of LB liquid medium. Fifty milliliter (50 mL) of the inoculum of each bacterial strain (*A. brasilense* strain R1*, A. lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) was added to 2 L water and roots of the nursery seedling were inoculated for 1 h. After inoculation of the rice seedlings, the remaining bacterial suspension was distributed equally in their respective beds. The seedling was brought and distributed throughout the field in their respective beds in the form of small bundles. Seedling were detached from the bundles and inserted in the soil not shallower than 1.5 cm and not deeper than 3 cm. 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 m at ARIN Mingora and 3 x 5 m at Udigram, Swat. The number of rows at ARIN, Swat was 14 x15 with 210 plants per plot and at Udigram was 12x22 with 330 plants per plot. After one week of transplantation, recommended chemical fertilizers (120 kg ha^{-1} N, 60 kg ha^{-1} P and 40 kg ha^{-1} K) were applied. The plants were harvest from 30 - 35 days after flowering. Initially, 5 plants from each plot (that is 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°C for three days to estimate root dry weight. Similarly straw weight and grain weight of all 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°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 following the same methodology. Statistical calculations were carried out by using MSTAT C program and LSD tests.

RESULTS

Isolation of bacteria from rice roots and rhizosphere

A total o f 18 bacterial isolates were obtained from the roots and rhizosphere of rice collected from ARIN Mingora and Udigram Swat. Out of 18 bacterial strains, 10 were isolated from ARIN, Swat and 8 from Udigram, Swat. In addition to these, two bacterial strains (*A. lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) were obtained from the culture collection of Plant Microbiology Division, NIBGE, Faisalabad.

Identification and characterization of bacterial isolates

The morphological and physiological characterization of all the bacterial isolates was carried out on the basis of colony morphology (colour, shape and size), cell shape and motility. The cultural characteristics of the purified bacterial strains were studied using light microscope. Eleven isolates (MRSWT3, MRSWT4, MRSWT6, R1-1, R1-2, R3-1, R3-2, R4-1, R5-2, R6-1 and R6-2) were tentatively identified as *Pseudomonas* strains and four isolates (R1, R2, R3, R5) as *Azospirillum* strains. Most of the *Pseudomonas* strains formed white or off-white colonies on LB agar plates and the cells were thin, rod-shaped and motile. The *Azospirillum* strains like R1, R2, R3 and R5 formed pinkish colonies on LB agar plates, the cell were rod-shaped and showed spiral motility. Three strains, R2-1, R4-2 and MRSWT5 could not be identified. The white colonies of strain R2-1 were very hard and were submerged in the agar plates. The colony morphology of this strain was totally different from all others. The strain R4-2 formed fluffy colonies. The cells were rod shaped and slightly motile. The colonies of isolate MRSWT5 were also yellowish and fluffy. The cells showed morphological characteristics similar to those of strain R4-2.

Effect of bacterial inoculation on growth of rice in the field experiment

The present investigation showed significant effects of plant growth promoting rhizobacteria on different growth parameters of rice variety JP 5 at both experimental sites (that is ARIN, Swat and Udigram, Swat) when compared to non-inoculated control. At the experiment conducted at ARIN Swat, the *A. brasilense* strain R1 showed 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 plant fresh weight over non-inoculated control. The increase in the dry straw weight, dry grain weigh, dry total plant weight were 19, 39.5, and 30.8% respectively. Inoculation with *A. lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 showed significant increase (18.5 and 13.57 % respectively) in the dry grain weight of rice variety JP 5 (Figure 1; F1-F3).

At the experimental site Udigram, Swat, growth promotion effect of inoculation with *A. brasilense* strain R1 was observed on rice variety JP 5. Inoculation with this strain resulted in 15.9 % increase in fresh straw weight, 27.5 % increase in fresh grain weight and 19.5% increase in total fresh plant weight over the non-inoculated control. The values for dry weight were 15.5% increase in straw weight, 27.4% increase in grain weight and 16% increase total plant weight over non-inoculated control. In the same variety any significant beneficial effect of inoculation with *A. lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 was not observed on straw weight, grain weight and total plant weight (Figure 1; F4-F6).

DISCUSSION

The root, rhizosphere and aerial parts of cereal crops are colonized by a number of beneficial bacterial strains (Mehnaz et al., 2010). Complete yield data collected from whole plots indicated that the inoculation of bacterial strains in both experimental fields resulted in significant increase in the yields than the control treatment. The effect of the inoculated strains was positive on both fresh and dry weight. Inoculation with *A. brasilense* strain R1 at Agriculture Research Institute (N) Migora Swat, resulted to increase in fresh straw weight (19%), fresh grain weight (39.3%) and total fresh weight (29%). The increase in the dry straw weight, dry grain weight and total dry weight was 19%, 39.5%, 30.8% respectively. *A. lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 also showed significant increase (18.5% and 13.5% respectively) in the total grain yield**.** The results are parallel to the finding of Okon and Hadar (1987) who reported 10 - 30% increase in grain and forage field due to inoculation of rice with *Azospirillum* strains. The results are also in consistence with the finding of Ahmed et al. (2013) who reported that inoculation of rice with *A. brasilense* strain 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.

In the field experiment at Udigram Swat, *A. brasilense* strain R1 was found to be the best inoculant as maximum increase in straw, grain and total plant weight was obtained in plants inoculated with this strain. This result is also in consistence to the work of Omar et al. (1989). They reported 15-20% increase in grain yield due to inoculation of rice with *A. brasilense*. Negative effect of the inoculated strains on certain growth parameters was also observed. In the whole plant study, the height of inoculated rice variety JP 5 plants at ARIN was less than non-inoculated control. Similarly the shoot weight of the inoculated rice variety JP5 at Udigram, Swat was comparatively less than non-inoculated treatment. 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 and Alexander, 1988; Ahmed et al. 2013).

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

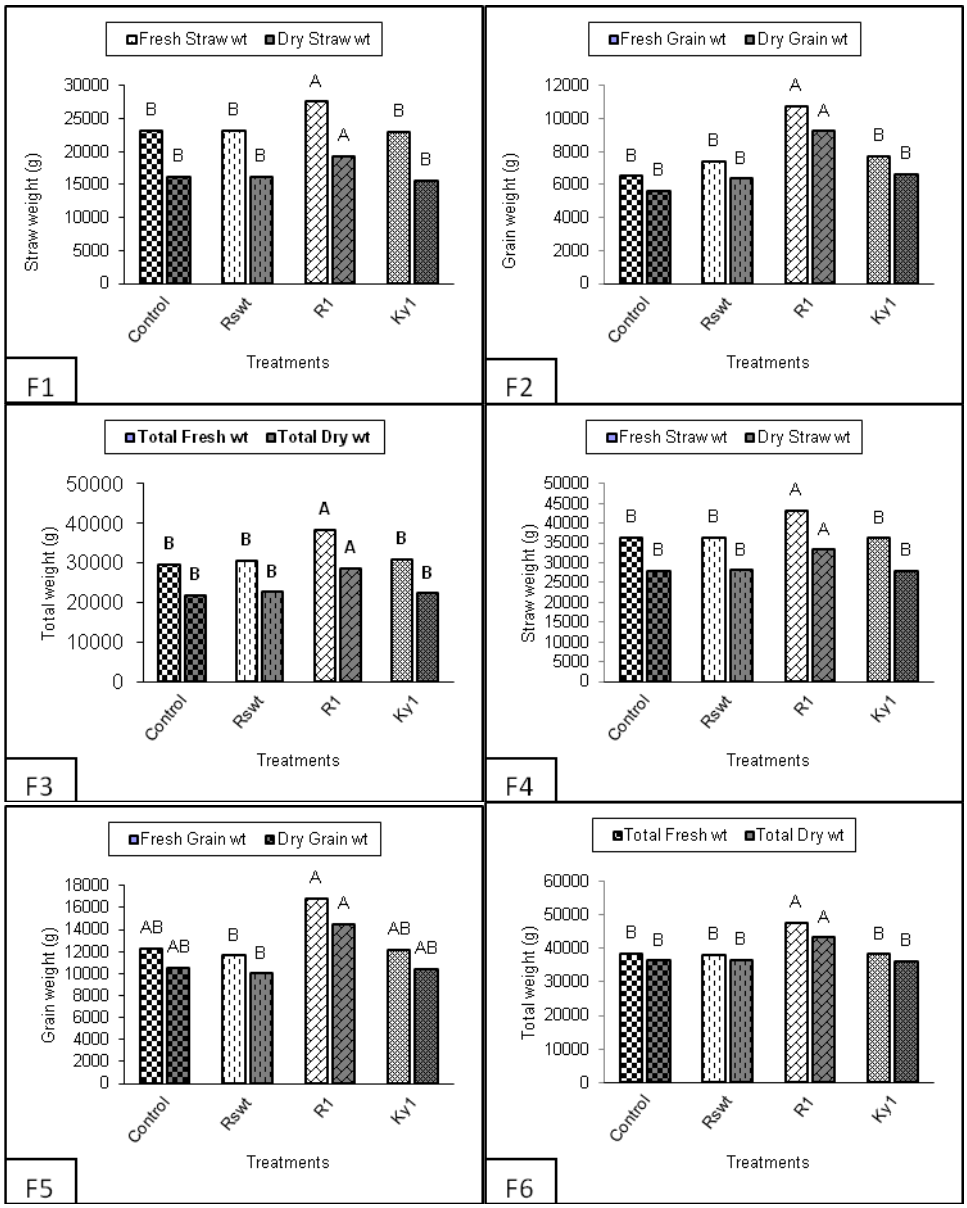

Figure 1. Effect of inoculated bacterial strains on growth and yield of rice variety JP 5; F1: Fresh and dry straw weight at ARIN; F2: fresh and dry grain weight at ARIN; F3: total weight (straw+grain) at ARIN; F4: fresh and dry straw weight at udigram; F5: fresh and dry grain weight at udigram; F6: total weight (straw+grain) at udigram; 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.

than when locally isolated PGPR were used as inoculants, more crop yield than inoculation with type strain and control without inoculation was obtained (Fulchieri and Frioni, 1994; Ahmed et al., 2013). Inoculation of indigenous strains of *Azospirillum* to rice led to increased plant height at some growth stages (40 and 75 days after transplantation) and the increase was significant over the control (Gunarto et al., 1999). Baldani et al. (2000) also observed 42-64% increases in growth of rice plants when locally isolated *Burkholderia brasilensis* and *B. vietnamiensis* were inoculated under gnotobiotic conditions.

Additional field studies in Swat area are required to confirm the beneficial role of bacterial inoculum 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.

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