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Phosphate solubilizing potential of *Rhizobium* and *Bacillus* species for enhancing yield and available phosphorus in maize crop (*Zea mays*)

Naseem Akhtar*, Fakhar Mujeeb, Muhammad Amjad Qureshi, Munnaza Rafique, Aneela Riaz, Muhammad Asif Ali

Soil Bacteriology Section Agriculture Biotechnology Research Institute, AARI, Faisalabad, Pakistan

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Abstract

A field experiment was conducted to evaluate the separate and integrated effect of Rhizobium and Bacillus spp. on the growth of maize (Zea Mays L.). Inocula of Rhizobium and Bacillus were applied as seed coating. Recommended dose of fertilizer (120-60 kg NP ha-1) was applied at sowing. The treatments were implied according to Randomized Complete Block Design with three repeats. Inoculation had no significant effect on the leaf length (84cm) and internodal distance (18.3cm) compared to their respective control (80cm and16.5cm) but the photosynthetic rate (105.3µ mol-2s-1) , transpiration rate (13.2 mmolm-2s-1), plant height (259.3cm), leaf width (7.7cm), stem diameter (15.43mm), leaf area (644cm-2) and shoot fresh weight (79.6 tones ha-1) were significantly improved by co-inoculation. Effect of Bacillus was statistically at par with co-inoculation regarding transpiration rate (11.47 m mol m-2s-1), plant height (249.3 cm) and stem diameter (14.87 mm). Response of leaf width, stem diameter, leaf area and shoot fresh weight were significantly higher by Rhizobium application compared to the Bacillus inoculation, however, positive influence was observed by all the inoculation treatments over the control. These findings indicated that inoculation of Rhizobium and Bacillus has positive effect on the maize growth and their co-inoculation (Rhizobium+ Bacillus) showed more pronounced results.

* **Corresponding Author:** Naseem Akhtar ✉ nasimsajjad235@gmail.com

Introduction

Phosphorus is indispensable nutrient for the life cycle of plants. Its role starts from root development and culminates up to seed formation. But in our cropping systems almost 75-90% of added P-fertilizer is entered into immobile pools owing the presence of highly reactive Ca^{2+} of alkaline soils (Gyaneshwar *et al.,* 1999; Hao *et al.,* 2002; and Hinsinger *et al.,* 2001). On the other hand soil microbes, produce organic acids, to transform this immobile P into solution P which eventually becomes available to plants (Pradhan *et al.,* 2005; Chen *et al.,* 2006; Deubel *et al.,* 2005). *Rhizobium* and *Bacillus* are the microbes which enhance the crop yields through growth hormones and P solublization (Gull *et al.,* 2004). This microbial biomass assimilates the soluble P, and prevents it from adsorption or fixation (Khan et al., 2009). This bioavailability of soil inorganic P in the rhizosphere varies evidently with plant species and nutritional status of soil (Hoflich *et al*., 1995).

Many researchers have studied various species of the genus *Bacillus, Pseudomonas, Aspergillums* and *Penicillium* as P-solubilizer (Seshadri *et al.,* 2004). Among these the *Bacillus* was abundant in the rhizosphere. It has vital role in P solubilization and also acts as plant growth promoting rhizobacteria (PGPR) (Probanza *et al.,* 2002; Gutierrez *et al.,* 2003). It promotes plant growth by a number of mechanisms, including P solubilization and phyto hormone production such as Indole Acetic Acid (IAA) (Choudhary *et al.,* 2009; Lal *et al.,* 2009). Co-inoculation of *Bacillus* with *Rhizobium* stimulated the plant growth more than their separate inoculation depending upon the soil conditions (Askary *et al.,* 2009; Perveen *et al.,* 2002; Zaidi *et al.,* 2003).

The Phosphate solubilizer and PGPR can reduce the Phosphorus requirement of plant by 50% without reducing the crop yield [Yazdani *et al.,* 2009] and this may lessen the dependence on costly mineral fertilizers. Present study was planned to evaluate the effect of *Rhizobium* and *B*acillus alone and in combined form, on the yield parameters of maize and to explore the potential of *Rhizobium* as a P solubilizer for non-legumes.

Materials and methods

Isolation of Rhizobium and Bacillus

Rhizobium was isolated from nodules of chickpea, mung, vegetable pea and berseem (Russell *et al.,* 1982). For this purpose pink, healthy, undamaged nodules were selected and were immersed in 95% ethanol for 1-4 minutes. Then they were rinsed with sterile water and acidified mercuric chloride solution (0.1% W/V). Afterward, washed for 5-6 times in sterile distilled water and crushed under larger drop of sterilized water in a Petri dish. Their juice was transferred immediately to the Congo Red Yeast Mannitol Agar (CRYMA) media and this mixture was then placed in incubator at 28 ± 2oC (Vincent, 1970). The *Rhizobial growth* that could not attain the color of Congo red, were picked and re-streaked steadily to obtain pure cultures. The purified culture was stored at 4 ± 2^{o}C on slants and maintained for further experimentation. Wheat seed was inoculated by *Rhizobium* isolated from above mentioned legumes and grew in the Petri dishes for germination test under controlled conditions. Germination assay showed that *Rhizobium* isolated from chickpea, was better than other strains and was selected for experimentation.

Bacillus was isolated by dilution plate technique from the rhizosphere soil of maize growing at the Fodder Research Station, Ayub Agricultural Research Institute (AARI), Faisalabad. Dilutions up to $10^{-2,}$ 10^{-4} and 10^{-6} were prepared and placed in the oven for heat shock at 80^{o} C for 10 minutes and cooled down, then inoculated on the selective medium [Nautiyal, 1999]. Plates were incubated at 28 ± 2^{o} C for seven days. The growth of *Bacillus* was purified and screened out on the Pikovskaya medium (El-Komy, 2005). From each plate, the growth was selected and sub-cultured repeatedly to get a pure culture. Gram tests (Davies *et al.* 1983) and spore formation (Knaysi, 1935) was positive for this pure culture. Then respiration test was conducted through oil film (Claus *et al.,* 1986) which came negative indicating the

presence of *Bacillus megaterium*. The starch hydrolysis test (Vera *et al.,* 1980) and Voges-Proskauer tests were carried out which were positive and negative respectively, confirming the presence of *Bacillus megaterium* (Ljutov, 1963).

Auxin Biosynthesis and Phosphate solubilization of isolates

Screening of *Rhizobium* and *Bacillus* was carried out for their auxin biosynthesis potential. The isolates of *Rhizobium* were inoculated on the Yeast Mannitol Broth (YMB) and *Bacillus* on Pikovskaya's broth culture for 72 hours. The auxin biosynthesis potential was determined as Indole-3-acetic acid (IAA) equivalents using Salkowski's reagent (2 mL of 0.5M $FeCl_3$ + 98 mL of 35% $HClO_4$) [Sarwar et al., 1992]. *Rhizobium* and *Bacillus* isolates, exhibiting the highest auxin biosynthesis were selected for the study of P solubilization.

The solubilization indices of *Bacillus* and *Rhizobium* isolates were checked on the Pikovskaya's medium (Pikovskaya, 1948). Isolates solubilize insoluble phosphates in the Pikovskaya's medium by forming the halos. The growth and solubilization diameter were determined after incubation at 28 ± 2° C for seven days. On the basis of diameter of clearing halo zones, solubilization index (SI) [Vazquez *et al.,* 2000] was calculated using the following formula;

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

Auxin biosynthesis potential of *Rhizobium* ranged from 15.3-19.7 µg g^{-1} whereas that of *Bacillus* isolates was from 2.9 to 3.3 µg g^{-1}. Isolates of *Rhizobium* and *Bacillus* with highest Auxin biosynthesis potential and phosphate solubilization were selected for experiment (Table 1).

Broth cultures of the media were incubated at 28 ± 2° C under shaking at 100 rpm for three days. Leaf mold, as carrier, was processed and sterilized at 121° C and 15 psi pressure for one hour and inoculated with the cultures @10 mL 100^{-1} g of peat and incubated at 28 ± 2° C. It carries 10^8 CFU g^{-1} of leaf mold.

Table 1. Some important features of isolates tested during the investigation.

Isolates	*IAA equivalents (µg mL^{-1})*	*Gram reaction*	*Solubilization index (SI)*
Rhizobium (Chickpea)	19.7	Negative	2.3
Rhizobium (Mung)	15.3	Negative	2.1
Rhizobium (Vegetable pea)	17.0	Negative	2.2
Rhizobium (Berseem)	18.0	Negative	1.9
Bacillus	3.3	Positive	3.5
Bacillus	2.9	Positive	3.0

Treatments and experimental design

Field study was conducted in two consecutive years with medium textured soil having pH 8.0, ECe 1.7 dSm^{-1}, nitrogen 0.028% and available P 8.2 mg kg^{-1} at Soil Bacteriology Section, Agriculture Biotechnology Research Institute, AARI, Faisalabad. Recommended dose of fertilizers (120, 60 NP kg ha^{-1}) was applied to all the treatments. The experiment was laid out in Randomized Complete Block Design (RCBD) with three replications.

Treatments

T_1: Control

T_2: *Rhizobium* inoculation

T_3: *Bacillus* inoculation

T_4: Co-inoculation (*Bacillus* + *Rhizobium* 1:1)

Growth parameters

Crop growth was monitored over the entire vegetative period. At tasseling (58 days after sowing) harvesting was done. Plant height was measured up to the top of

the terminal leaf of the plant. Vernier callipers were used to measure the stem girth at the fifth internode and leaf breadth of nearby leaf was also recorded. Inter nodal distance between the fourth and a fifth node was measured from each plant in each set. Data regarding photosynthetic rate and transpiration rate were observed by IRGA CI.340. After harvesting, shoot fresh weight was recorded. Dry weight was measured following air oven drying at 65°C for 48 hour. Phosphorus and N contents of soil and plant were also recorded. Nitrogen was determined according to Kjeldhals method (Bremmner and Mulvany, (Bremner *et al.,* 1982) while P by modified Olsen method [Olsen and sommers, 1982].

Statistical analysis

Data were subjected to statistical analysis following RCBD using standard procedures (Steel and Torrie, 1997). The difference among the treatment means were compared by applying the Duncan's Multiple Range tests (Duncan, 1955).

Results

Leaf length, leaf width and leaf area

Positive effect of co-inoculation was observed on growth of the crop. Leaf width significantly while leaf length was non-significantly increased by co inoculation. Leaf area varies considerably by inoculation (Table 2). Maximum leaf length was observed by co inoculation (84 cm) followed by *Rhizobium* (83.7 cm) compared to control (80.0 cm). Leaf width was significantly increased by co inoculation (7.7 cm) followed by *Rhizobium* (7.0 cm) and *Bacillus* (6.5 cm) inoculation compared with control (6.3cm). Co-inoculation significantly improved the leaf area (644 cm^2) compared to all other treatments followed by 585 cm^2 by *Rhizobium* and 527 cm^2 by *Bacillus* which were significantly higher than control (491cm^2). Increase in leaf area by co inoculation was 31.2 % compared to control.

Table 2. Effect of Rhizobium and Bacillus on leaf parameters (mean of 3 repeats).

Treatments	*Leaf length (cm)*	*leaf width (cm)*	*leaf area (cm^2)*
Control	80.0	6.3	491
Rhizobium inoculation	83.7	7.0	585
Bacillus Inoculation	81.0	6.5	527
Co-inoculation (*Rhizobium* + *Bacillus)*	84.0	7.7	644
LSD	NS	0.5544	68.75

Transpiration rate/ Photosynthetic rate

Data regarding transpiration rate and photosynthetic rate is given in (Table 3). *Bacillus* (11.47 mmol $m^{-2}s^{-1}$) and co inoculation (13.2 mmol $m^{-2}s^{-1}$) showed similar but significantly enhanced rate of transpiration than control (8.17 mmol $m^{-2}s^{-1}$). Regarding transpiration, *Rhizobium* showed non significant increase as compared to control. Photosynthetic rate was significant higher by co-inoculation (105.3 µmole $m^{-2}s^{-1}$) than separate inoculation of *Rhizobium* (95.3µmol $m^{-2}s^{-1}$) and *Bacillus* (90µmol$em^{-2}s^{-1}$) and it was 25.8 % higher than control. *Rhizobium* and *Bacillus* were statistically at par with respect to each other. However, they showed significantly higher photosynthesis rate than control (83.790µmol$em^{-2}s^{-1}$).

Plant height, Shoot fresh and dry weight

Plant height and shoot fresh weights (table 4) were significantly increased by inoculation compare to control. Maximum shoot weight was observed by co-inoculation (79.6 ton ha^{-1}) followed by 77.3 by *Rhizobium* and 74.0 t ha^{-1} by *Bacillus* inoculation. Increase in shoot weight by co inoculation was 10.5% over control. Shoot dry weight was significantly higher by co inoculation (10.7 t ha^{-1}) than all other treatments. *Rhizobium* and *Bacillus* inoculation produced shoot dry weight (10.0 and 9.8 t ha^{-1} respectively) statistically at par with each other but

higher than control. Plant matter proportion on drying was 13.44% by co inoculation, 13.2 % by *Bacillus* and 12.9% by *Rhizobium* while it was 10.9% in control.

Table 3. Effect of Rhizobium and Bacillus on Transpiration &Photosynthetic rate (mean of 3 repeats).

Treatments	*Transpiration rate (m mol $m^{-2}s^{-1}$)*	*Photosynthetic rate (µmol $m^{-2}s^{-1}$)*
Control	*8.17b*	*87.7c*
Rhizobium inoculation	*8.63b*	*90.0b*
Bacillus Inoculation	*11.47a*	*95.3b*
Co-inoculation (Rhizobium + Bacillus)	*13.2a*	*105.3a*
LSD	*1.889*	*5.844*

Table 4. Effect of Rhizobium and Bacillus on Plant height, Shoot fresh and dry weight (mean of 3 repeats).

Treatments	*Plant height (cm)*	*Stem diameter (mm)*	*Shoot fresh ($T\ ha^{-1}$)*	*dry weight ($T\ ha^{-1}$)*
Control	233.7b	12.30c	72.0d	7.87c
Rhizobium inoculation	254.0a	13.43b	77.3b	10.00b
Bacillus Inoculation	249.3a	14.87a	74.0c	9.80b
Co-inoculation (*Rhizobium* + *Bacillus)*	259.3a	15.43a	79.6a	10.70a
LSD	14.58	0.7475	2.209	0.6967

Plant and soil analysis

Data regarding N and P contents in plant and soil are presented in (Table 5). All the inoculation treatments significantly affect the P contents of the plant. Co inoculation showed significantly higher P (0.025%) followed by *Bacillus* (0.23 %) and 0.21% by *Rhizobium* compared to control (0.18%).

Table 5. Effect of Rhizobium and Bacillus on soil and plant nutrient (mean of 3 repeats).

Treatments	*Plant P (%)*	*Plant N (%)*	*Soil P (ppm)*	*Soil N (%)*
Control	0.18c	1.18d	9.1c	0.031b
Rhizobium inoculation	0.21b	1.43b	10.9b	0.035a
Bacillus Inoculation	0.23ab	1.27c	11.0b	0.032b
Co-inoculation (*Rhizobium* + *Bacillus)*	0.25a	1.51a	11.9a	0.035a
LSD	0.0266	0.0583	0.5287	1.489

Nitrogen % in plant matter varies significantly by inoculation. Co-inoculation showed 1.15 % N which is significantly higher than all other treatments. It was 1.43 % by *Rhizobium* and 1.27% by *Bacillus,* which were significantly higher than control (1.18 %). Inoculation with *Rhizobium* and *Bacillus* produced

higher % of soil N and available P as compared to control. Soil N was 0.035% by *Rhizobium* and co-inoculation as well. Nitrogen % was not significantly affected by *Bacillus*. Co-inoculation exhibited maximum available P (11.9 mg kg^{-1}) that differed significantly from *Bacillus* and *Rhizobium* inoculation (11.0 and 10.9 mg kg^{-1} respectively). Co-inoculation showed 30.7% increase in available P compared to control.

Discussion

Co-inoculation positively affects all the growth parameters. Results of present study depicted that *Rhizobium* and *Bacillus* were efficient P-solubilizer and Auxin producers (Table 1). Previous studies also comply with the role of microbes for auxin production, P-solubilization and plant growth promotion (Martins et al., 2004). Significant increase was observed in leaf width and leaf area by co-inoculation (Table 2). *Rhizobium* fixed atmospheric N in legumes while in non legumes it acted as PGPR and enhanced the growth by colonizing the root of pepper and tomato (Garcıa *et al.,* 2012). Increased yield parameters of the barley by P-solubilizing microbe inoculation were also reported (Mehrvarz *et al.,* 2008).

Photosynthesis and transpiration rates were significantly increased by inoculation (Table3) which resulted into more plant growth. This might be due to the increased leaf width and larger leaf surface area by the Inocula application. More the transpiration more will be the water and nutrient uptake ensuing higher fodder yield. Similar results were also observed by co-inoculation of P-solubilizing microbes and PGPR inoculation in maize (Afzal, and Bano, 2008; Egamberdiyeva, 2007).

Stem diameter is positively affected by co-inoculation and *Bacillus* (Table 4). It is due to the veracity that phosphate solubilizing microbe (PSM) provided sufficient P to boost crop stand. It is evident that PSM enhanced the plant growth by increasing P availability (Ponumurugan and Gopi, 2006). Increase in inter nodal distance was also observed due to inoculation. Sufficient N and other nutrients were taken up by inoculation of *Rhizobium* and *Bacillus* which resulted in increased plant height and inter nodal distance.

Increased leaf area, plant height, transpiration and photosynthesis obviously enhanced the fresh and dry weight of fodder (Table 4). Yield augmentation by PGPR and *Bacillus* inoculation was previously observed in chickpea (Sharma *et al.,* 2007) and wheat (Galal, 2003).

Nutrient uptake by crop depends on availability of nutrients. *Rhizobium* and *Bacillus* are the most important P solubilizers and their co-inoculation solubilized 38% more P compared to control (Table 5). It happened due to the solubilization of P in the rhizosphere by microorganism which became readily available to plant.

Nitrogen % in plant matter is positively affected by inoculation. Significantly more N contents (1.51%) were observed by co-inoculation followed by *Rhizobium* (1.43%). Results are held up by previous findings that biofertilizers with half dose of NP fertilizers give the crop yield up to full dose of NP fertilizer [Jilani *et al.,* 2007. Many researchers reported increased seed P content by P-solubilizing microbes (Son *et al.,* 2006; Kumar *et al.,* 2008).

In our study significantly more soil N and P contents were observed by inoculation. Microbes like *Bacillus* and *Rhizobium,* produce organic acids that lower the soil pH, solubilize the fixed P and make it available to plant. Similar results were given by Khan *et al,* 2006 (Khan *et al.,* 2006) who reported that integrated inoculation of *Rhizobium* and *Bacillus* result in more soil N and available P.

Conclusion

It was concluded that bioavailability of precipitated Phosphorus was increased by *Bacillus* and *Rhizobium*. Their combined application increased soil Phosphorus content up to 31 % over control (without any inoculation). Co-inoculation of *Rhizobium* and *Bacillus* improved the shoot fresh weight of maize up to 10.0 %compared to control. This owed to provision

of growth hormones and increasing the nitrogen and phosphorus contents of plant by the application of microbial consortia. Soil nutrient status was also improved. Nitrogen contents were increased by 13% while increase in phosphorus was 30.7% due to co-inoculation of both the microbes. Thus application of *Rhizobium* and *Bacillus* provide wholesome environment for the subsequent crops.

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