

## Potential use of Iranian rhizobial strains as plant growth-promoting rhizobacteria (PGPR) and effects of selected strains on growth characteristics of wheat, corn and alfalfa

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### Abstract

Many agricultural researches have been performed to improve soil productivity. Nitrogen (N) and Phosphorus (P) are essential elements which are utilized by the plants in large amounts. Phosphorus can be provided by applying chemical fertilizers. Microorganisms convert insoluble phosphate to the soluble form and some bacteria such as rhizobacteria play an important role in this process. This research was designed to determine the plant growth promoting (PGP) abilities, especially phosphate solubilization, of different isolates of indigenous rhizobia and their effect on growth characteristics of wheat, corn and alfalfa. 446 isolates belonging to different species of rhizobia were examined regarding inorganic and organic P solubilization, siderophore, auxin (IAA and homologues), 1-aminocyclopropane-1-carboxylate (ACC) - deaminase and hydrogen cyanide (HCN) production. It was found that 7% of the strains could produce HCN, 86% siderophores and 74% IAA and 44% were able to solubilize phosphorus. 8 rhizobial isolates were found as superior plant growth promoting rhizobacterial (PGPR) strains. Green house experiments using these strains evaluated the promoting effects of different strains on legume (alfalfa) and non-legume (wheat and corn) plants. Total biomass to the above mentioned plants was determined and the amount of N, P and iron (Fe) in shoots were also measured. The results were analyzed by the RCBD contrast method using SAS software V6.12. In conclusion, the green house experiment showed that  $P_1B_2$  and a mixture of 4 plant growth stimulating rhizobacterial strains are the best suited as growth promoting inoculants.

**Keywords:** Phosphate solubilizing Rhizobacteria, Wheat, Corn, Alfalfa, PGPR

### 1. Introduction

Agricultural research has long been focused on improving productivity following the green revolution. However, the increasing population and decreasing soil fertility has overcome its positive effects. As far as soil fertility is concerned, nitrogen, phosphorus, etc. are required by the plant in large amounts, with phosphorus being of major significance. Microorganisms are known to solubilize insoluble phosphate through the production of organic acids and chelating oxo-acids from sugars (Antoun, & Kloepper,

2001; Peix *et al* 2001). Recently, some good phosphate solubilizers have been reported in agricultural systems. Plant growth promotion by microorganisms via phytohormone production (specially IAA), phosphorus solubilization or release of poorly characterized low molecular mass compounds such as siderophores are well documented in the literature (Abd-Alla 1994; Zimmer, & Bothe, 1988; Zaat, *et al* 1989).

Agricultural soils in Iran are predominately calcareous and have high pH and low amounts of the available phosphate ion ( $P_i$ ). Up to 75% of the P fertilizers added to crops may be converted to insoluble forms by reacting with free  $Ca^{+2}$  or  $Fe^{+3}$  and  $Al^{+3}$  ions in high and low pH soils, respectively (Goldstein, 1986). Organic P

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represents 50 to 80% of total soil P and most plants are unable to utilize this source of P (Richardson, & Hadobas, 1997). Rhizobia are able to solubilize both organic (Abd-Alla, 1994) and inorganic phosphates (Antoun, 1998). The main advantage of using rhizobia as phosphate solubilizing bacteria (PSB) is their dual beneficial nutritional effect resulting from mineral and organic P mobilization and N<sub>2</sub>-Fixation (Peix, et al 2001). The current study was designed to determine the ability of 5 selected plant growth promoting rhizobacterial (PGPR) strains to mobilize inorganic and organic phosphate and production of indole acetic acid (IAA), siderophore and hydrogen cyanide (HCN), in order to allow the identification of the best growth promoting rhizobacterial strains as appropriate inoculants for legume and non-legume plants in Iran.

## 2. Materials and methods

### 2.1. Characterization of rhizobial isolates

446 *Sinorhizobium meliloti* (*S. m*), *Rhizobium leguminosarum* bv. *Viciae* (*R. lv*), *R. l.* bv. *Trifol* (*R. lt*), *R. l.* bv. *phaseoli* (*R. lp*), *Mesorhizobium ciceri* (*M. c*) and *M. mediterraneum* (*M. m*), *Bradyrhizobium* sp. (*B. sp*) and *Bradyrhizobium Japonicum* (*B. j*) isolates obtained from all over Iran were examined for solubilization of inorganic and organic P qualitatively and quantitatively, according to the Sperber method (Sperber, 1958). Siderophore production was evaluated in culture medium containing chrome azerol S (CAS-Agar) using three different methods including direct plating (DP), half-half (HP) and

double layer culture (DL) medium based on the modified methods of Schwyn & Neilands (1987), Milagres et al. (1999) and the proposed method of this study, respectively. HCN production was determined according to Alstrom and Burns, (1989), Auxin production (IAA and homologues) was tested based on the method of Bric et al (1991) and production of ACC-deaminase was determined in certain superior rhizobacteria according to the method of Penrose and Glick (2003).

### 2.2. Rhizobial inoculants preparation

Some (9 Isolates) of the rhizobial isolates identified as superior PGPR strains (Table2) were used in alfalfa, wheat and corn greenhouse experiments. The cultures were mixed with perlite to prepare the powder inoculants ( $5 \times 10^8$  cfu/gr perlite). Greenhouse experiments were designed to include three individual experiments which evaluated the promoting effects of different PGPR strains on various legume (alfalfa) and non-legume (wheat & corn) plants.

### 2.3. Soil Selection

Soil samples were prepared from Karaj's suburban areas. The samples were tested for their physical, chemical and biological characteristics (Table 1). The soil for experiment was selected according to the minimum number of *S. meliloti* and PSM, high total P, low P<sub>i</sub> and Fe. In addition percentage nitrogen content (N%), percentage calcium carbonate content (CaCO<sub>3</sub>%), pH and EC of the soil were appropriate to the experimental purposes.

Table 1. Physical, chemical and biological characteristics of the soil used in greenhouse experiments

Characteristics	Value
Soil texture class	Sandy loam
%Clay	16.0
%Silt	20.2
%Sand	63.8
pH	7.88
ECe (dS m <sup>-1</sup> )	0.83
SP	23.5
%F.C	17.13
%O.M	0.49
Ca <sup>+</sup> (me l <sup>-1</sup> )	4.5
Mg <sup>+</sup> (me l <sup>-1</sup> )	0.6
Na <sup>+</sup>	2.0
K <sup>+</sup>	0.13
%Lime	8.47
%N (total)	0.081
P- total (mg kg <sup>-1</sup> )	887.2
P ava. ( mg kg <sup>-1</sup> )	2.52
Fe-ava. ( mg kg <sup>-1</sup> )	3.6
PSM* (Cells gr <sup>-1</sup> soil)	150
<i>S. meliloti</i> (Cells gr <sup>-1</sup> soil)	31

\* Phosphate Solubilizing Microorganisms

Table 2. The PGPR characteristics and selection number of different rhizobial strains

ACC-deaminase <sup>5</sup>	PGPR Rhizobacteria indices					Selection number of applied bacteria	Bacteria
	P-Solubilization <sup>4</sup>	Sid <sup>3</sup>	IAA <sup>2</sup>	HCN <sup>1</sup>	%S.E		
-	-	-	-	-	-	Non-bacterial sample (Negative Control)	B <sub>0</sub>
+4	-	3	-	-	121	R <sub>125</sub> <i>Sm</i>	B <sub>1</sub>
+2.7	403	-	-	-	-	R <sub>419</sub> <i>Mc</i>	B <sub>2</sub>
-	-	-	2.7	-	-	R <sub>254</sub> <i>Rlp</i>	B <sub>3</sub>
-	-	-	-	5	-	R <sub>340</sub> <i>Rlv</i>	B <sub>4</sub>
+4	368	1.25	2.7	-	-	R320 <i>Rlv</i>	B <sub>5</sub>
+2	302	1.25	1.33	-	-	R307 <i>Rlp</i>	
-	315.6	1.33	1.08	5	-	R375 <i>Mc</i>	
-	-	3.8	1.25	-	-	R490 <i>Rlv</i>	
+3	-	-	-	-	121	R <sub>164</sub> <i>Sm</i>	

1) The level of HCN production

2) Colorful halo diameter, resulted from IAA production, to rhizobial colony diameter

3) Colorful halo diameter, resulted from Siderophore production, to Rhizobial colony diameter

4) Solubilized phosphorus concentration (mg l<sup>-1</sup>)

5) The level of ACC-deaminase production

#### 2.4. Alfalfa greenhouse experiment

This experiment was carried out in a factorial randomized complete block design (RCBD) using 14 treatments with 5 replications. Experimental treatments were: Two levels of phosphorus (P<sub>0</sub>, P<sub>1</sub>) and seven rhizobial strain inoculants (B<sub>0</sub> and B<sub>1</sub> to B<sub>6</sub>). One positive control (P.C) containing nitrogen (70 mg kg<sup>-1</sup>) and phosphate fertilizer [(20mg kg<sup>-1</sup>) as KH<sub>2</sub>PO<sub>4</sub>] without rhizobial inoculants (P<sub>20</sub>N<sub>70</sub>B<sub>0</sub>) and one negative control (N.C) without rhizobial inoculants, nitrogen and phosphorus (P<sub>0</sub>N<sub>0</sub>B<sub>0</sub>) were also included in the experiments. The P<sub>0</sub> and P<sub>1</sub> treatments consisted of pots without any P and with 1 gr of Iranian rock phosphate (Asfordi-Yazd) containing 39% P<sub>2</sub>O<sub>5</sub> respectively. The amount of rock phosphate in 4.8 kg of soil in each pot was approximately equal to 600 kg of phosphate per hectare (34 mg kg<sup>-1</sup> in each pot). For alfalfa cultivation, appropriate Iranian alfalfa (cv. Hamedani) seeds were disinfected according to the method of Vincent (14 Vincent JM (1970)). Each pot received 6 alfalfa seeds and 0.1 gr of inoculants which was added to the seeds' surface and mixed with the soil around it. The pot moisture was adjusted to 65±15% F.C and kept covered until early growing stage. Finally 5 alfalfa plants remained in each pot. 200 ml of Hoagland solution (Fe free) was also added to provide micronutrients for each pot. The period of light and darkness were equal to approximately 12 hours. The pots were kept at a temperature of between 20-30°C for 140 days and watered daily. Plant dry matter (Biological yield) was harvested, weighed and recorded as total biomass. In addition, the amount of N, P and Fe of shoots and also the available soil P and Fe were measured. Plant Fe content was

measured according to the DTPA procedure. All the results were analyzed by the RCBD contrasts method using SAS V6.12 software.

#### 2.5. Wheat greenhouse experiment

The treatments were: two phosphorus levels (P<sub>0</sub>, P<sub>1</sub>, as the alfalfa experiment) and six rhizobial strain inoculants (B<sub>0</sub>, B<sub>1</sub> to B<sub>5</sub>). All rhizobial inoculants were prepared as explained above. 5 pots representing positive controls (P<sub>20</sub>B<sub>0</sub>), received 20 mg/kg of available P (KH<sub>2</sub>PO<sub>4</sub>) without rhizobial inoculants. Appropriate Iranian spring wheat seeds (CV. Tajan) were selected and disinfected as for alfalfa seeds. Disinfected seeds were placed on sterile water-agar (%1) medium and incubated at 25°C to germinate. 1 gr of Iranian rock phosphate (Asfordi-Yazd) was added to the P<sub>1</sub> treatment pots and mixed completely with the soil. Then 5 germinated wheat seeds were planted and 0.1 gr of rhizobial inoculant was added to the soil surrounding the seeds. Pots' water content were adjusted to 65±15% F.C and the pots were covered until early growing stage. After seven days of plant growth, plant numbers were thinned to 4. All pots were irrigated daily with distilled water. Nitrogen and potassium supplement were provided as required. 200ml of Hoagland Solution (Fe free) was added to prevent micronutrient deficiency. This experiment was performed in a Randomized Complete Block factorial Design (RCBD) with 12 treatments (two levels of phosphorus and six bacterial strains) and 5 positive controls with 5 replications. Minimum and maximum temperatures were 20 and 30°C and the periods of light and darkness were adjusted to 12 h. Plant growth characteristics such as shoots dry matter (Biological Yield) were evaluated. The

results were analysed by the RCBD contrast method using SAS V 6.12 software for statistical analysis.

### 2.6. Corn greenhouse experiment

Experimental treatments were exactly the same as the wheat experiment. Phosphorous fertilizers were applied in form of  $\text{KH}_2\text{PO}_4$  at rate of 25 mg/kg at planting time. Corn seeds (single cross 647) were disinfected as described by Vincent (1970) and transferred to a Petri - dish (15 cm) containing water agar and incubated at 25 °C. Then 4 germinated seeds were planted in each pot after addition of rock phosphate (Asfordi-Yazd) and 0.1 gr of rhizobial inoculant was added to the soil around the seeds. The moisture level of pots was adjusted approximately to 80% F.C. Pots were kept covered until germination. The plants were subsequently thinned to 3. All pots were irrigated daily to supply  $65 \pm 15\%$  F.C. Nitrogen (equal to 350 kg urea /he) was added as ammonium nitrate solution. Mineral elements and potassium amounts were exactly the same as the alfalfa and wheat experiments. This experiment was also carried out in a RCBD which involved 12 treatments (2 levels of P and 6 rhizobial inoculants,  $B_0$ ,  $B_1$  to  $B_5$ ) with 5 replications and 5 pots as positive controls. Maximum and minimum temperatures were 30 and 22°C, respectively. After 145 days of growth, plant shoot length, ear numbers and shoot dry matter (Biological Yield) were measured. All of the shoots (including the ear) were dried completely and their N content, P and Fe concentrations were measured by chemical analysis. The data were analyzed with the RCBD contrast method using the SAS V 6.12 software.

## 3. Results and discussion

### A) *In vitro* Tests

1) Approximately 7% of the rhizobial bacteria capable of producing HCN were classified as cyanogenic bacteria. The capacity of producing HCN and the abundance of cyanogenic bacteria are different among the *rhizobium* group. The greatest capability as well as the abundance of cyanogenic species belongs to that of *R. lv.* None of the slow growing *rhizobia* were capable of producing HCN.

2) Despite the superior growth potential of the rhizobial bacteria on Tri-calcium phosphate Yeast Extract Mannitol Agar (TYMA) medium (compared to Sperber), no halos resulting from

the solubilization of mineral phosphate were observed on the TYMA culture. Therefore, mannitol medium seems to be unsuitable for evaluating the capability of solubilization of mineral phosphate. 44% of *rhizobia* were recognized as being capable of solubilizing tri-calcium phosphate. The largest number of phosphate solubilizing bacterial strains belonged to the *M. c* species (approximately 70%) and strains with the greatest potential solubilize phosphate belonged to the *R. lv.* and *M. c* groups. None of the slow growing *Rhizobia* showed any capability for solubilizing mineral phosphates. Based on the analysis of variance (not shown) there were significant differences ( $P < 0.001$ ) among the rhizobial groups as well as among the strains within each group in their potential for solubilizing phosphate minerals.

3) 76% of the rhizobial isolates were capable of solubilizing organic phosphates. The rhizobial strains from the *S. m* and the *R. lv.* groups contained the largest number of such bacteria (95% and 93%, respectively) and were recognized as the best organic phosphate solubilizers. The greatest potential of solubilizing organic phosphate also belonged to the *M. c* and *S. m* groups. A small percentage (about 6%) of the slow growing rhizobial strains (genus *Bradyrhizobium*) was found to be capable of solubilizing organic phosphate. Based on the analysis of variance (not shown), there were significant differences ( $P < 0.01$ ) among the rhizobial groups as well as among the strains within each group in their potential for solubilizing organic P.

4) The capability of solubilizing mineral phosphate in liquid Sperber medium was compared to semi quantitative analysis method on solid Sperber medium. The results showed that strains in the *R. lv.* group were highly capable of (197.1 mgP/ml) solubilizing mineral phosphates. According to the analysis of variance there were significant ( $P < 0.001$ ) differences at various time intervals in the pH values and phosphorus concentrations of cultures belonging to different rhizobial groups and strains within each group.

5) There were significant differences ( $P < 0.01$ ) among different methods of preparing CAS-agar culture for evaluation of siderophore production. The greatest bacterial growth potential (statistically) was determined on HP and DL culture media, while DP resulted in the greatest degree of growth suppression of the rhizobia (Table 3). As a whole, the best method for evaluation of siderophore production was the HP method for pure samples and the DL

method especially for soil samples. Some (86%) of the rhizobial bacteria (72% fast growing and approximately 14% slow growing) were shown to be capable of producing siderophores. The ratio of the halo's diameter to the colony diameter varied from 1 to 7 for various siderophore producing strains with the most capable group being the strains of *S. m*. The rate of halo formation varied from 0 to 2.93 mm/day.

6) Approximately 74% of the rhizobial strains were found to be capable of producing IAA. The greatest number of IAA<sup>+</sup> types (>90%) belonged to the fast growing groups and the smallest number (34.34%) belonged to the *M. c* group. According to the analysis of variance, there were significant differences ( $P < 0.001$ ) among the various *rhizobium* groups and among the strains within the groups in their potential for producing IAA. The most capable strains belonged to the *R. lp.*, *R. lv* and *S. m* groups. The *R. lt* and *B. j* groups on the other hand did not show the potential of IAA production.

*In vitro* tests of the strains showed that most of the rhizobial strains isolated from around the country had PGP properties such as organic and inorganic phosphate solubilization, Acc deaminase, siderophors, auxin (IAA) and HCN production which is in agreement with previous reports on PGPRs (Abd-Alla, 1994; Antoun, et al 1998; Zaat et al 1989). Based on the PGP

properties of the above strains, 9 strains were selected as superior strains and considered for greenhouse experiments.

#### B) Greenhouse Experiments

The effects of the superior rhizobial strains (PGPR) on alfalfa (legume), wheat and corn (non-legumes) were studied in a green house test with three separate completely randomized block factorial experiments. The results of each experiment were as follows:

1) The alfalfa greenhouse experiment showed that the treatments P<sub>1</sub>B<sub>5</sub> and P<sub>1</sub>B<sub>2</sub> (Table 2) increased biological yield significantly (Figure 1). The superiority of P<sub>1</sub>B<sub>5</sub> as compared to P<sub>1</sub>B<sub>2</sub> indicated that the rhizobial strains used in P<sub>1</sub>B<sub>5</sub> caused an increased in yield by solubilizing phosphate in addition to producing the plant growth phytohormones IAA, siderophore and ACC- deaminase. The presence or absence of rock phosphate had no significant effect on improving alfalfa growth, except in the presence of the phosphate solubilizing *rhizobium* (B<sub>2</sub> and B<sub>5</sub> treatments). The total uptake of N, P and Fe were significantly greater in the P<sub>1</sub>B<sub>2</sub> and P<sub>1</sub>B<sub>5</sub> treatments than those the other *rhizobium* treatments (Figure 2). The levels of plant available phosphorus were greater in the soils treated with P<sub>1</sub>B<sub>5</sub> and P<sub>1</sub>B<sub>2</sub> than those treated with other *rhizobia*.

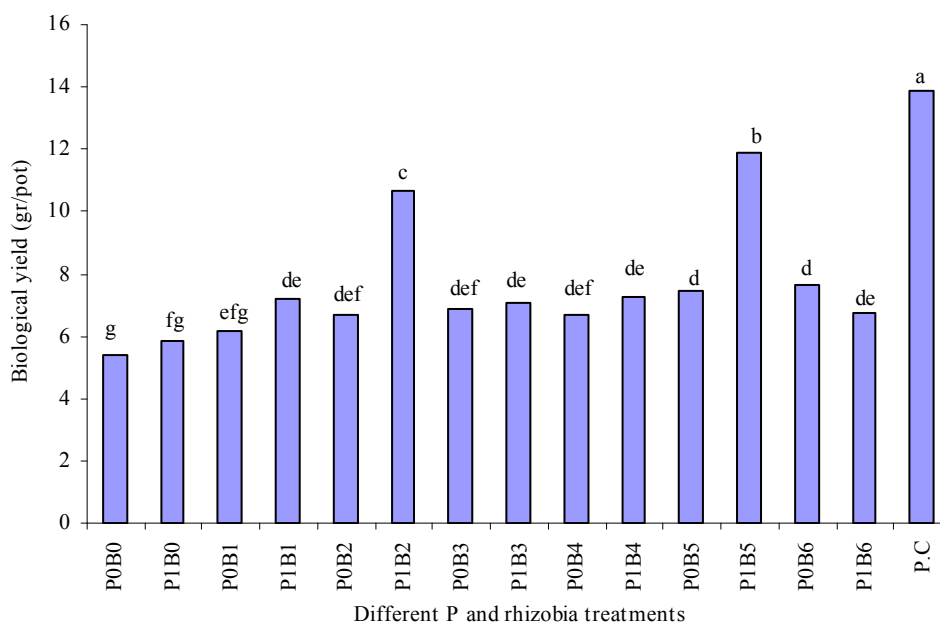


Fig. 1. The biological yield of Alfalfa in different treatments of phosphorus and plant growth promoting rhizobia by Duncan's multiple range test

- P0, P1 and B0-B6 are different phosphate and bacterial treatments respectively (see text)
- Means with the same letters had no significant ( $p < 1\%$  or  $5\%$ ) differences

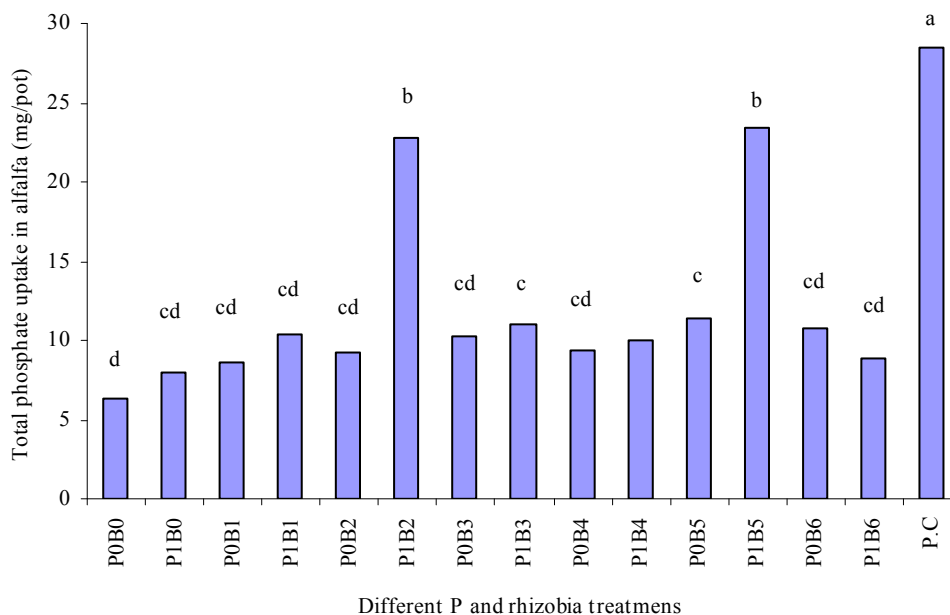


Fig. 2. Comparison of total P-uptake of alfalfa in different treatments of phosphorus and plant growth promoting rhizobia by Duncan's multiple range test

- P0, P1 and B0-B6 are different phosphate and bacterial treatments respectively (see text)
- Means with the same letters had no significant ( $p < 0.01$  or  $0.05$ ) differences

2) The biological yield of wheat plant in the  $P_1B_5$  and  $P_1B_2$  treatments were significantly greater than those of other treatments (Figure 3). The biological yield for other *rhizobium* strains that were only capable of producing siderophore, IAA or HCN were the same as that of the negative control ( $p_0B_0$ ). Similar results were obtained with respect to the other growth indices, especially the average length of cluster, the number of seeds and the crop yield. The total phosphorus uptake by the wheat and grain were significantly higher in the  $P_1B_5$  and  $P_1B_2$  treatment than other rhizobial treatments (Figure 4). The seed yield was also higher in  $P_1B_2$  than that of the other rhizobial treatments. The total Fe absorption by the wheat plant and grain was the same for  $P_1B_1$  and  $P_0B_1$  but considerably greater than that of  $P_1B_2$ . The level of available Fe was greater for  $P_1B_5$  than the other rhizobial treatments (data not shown).

3)  $P_1B_2$  treatment produced the maximum corn biological yield (figure 5) as well as corn ears (on the dry weight basis) which were significantly different from other treatments. With respect to the yield and ear dry weights,  $P_1B_5$  treatment and positive control P.C followed by  $P_1B_2$  were the same but

significantly higher than the other treatments. The  $P_1B_2$  treatment was significantly more effective than the other treatments regarding total P and Fe absorption (Figure 6) and different from treatment  $P_1B_5$  with respect to total Fe absorption. Plant available P was significantly greater in the  $P_1B_1$  treated soil than in other samples.

In conclusion, the green house experiment showed that a mixture of plant growth stimulating rhizobial strains as used in the  $P_1B_5$  treatment is the best suited growth stimulating (PGPR) inoculum.

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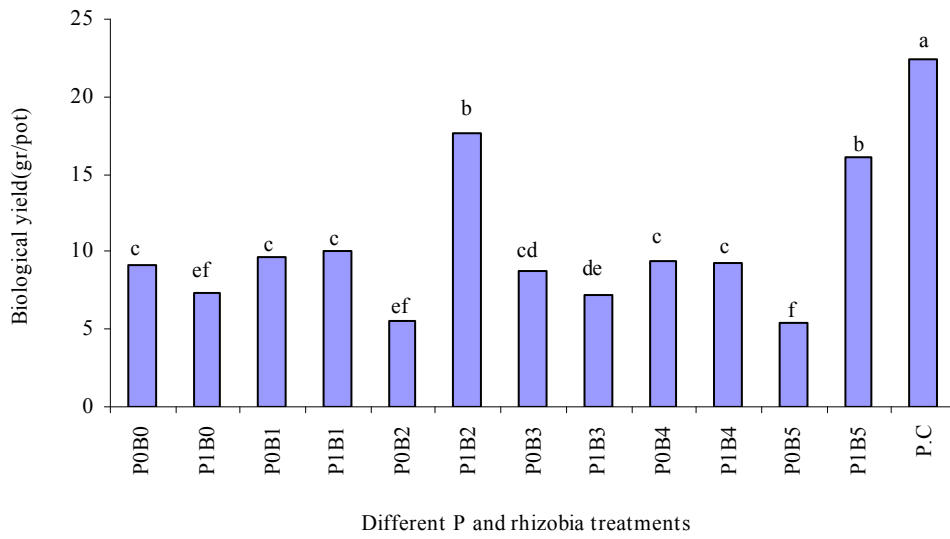


Fig. 3. Comparison of biological yield of wheat in different treatments of phosphorus and plant growth promoting rhizobia by Duncan's multiple range test

- P0, P1 and B0-B5 are different phosphate and bacterial treatments respectively (see text)
- Means with the same letters had no significant ( $p < 1\%$  or  $5\%$ ) differences

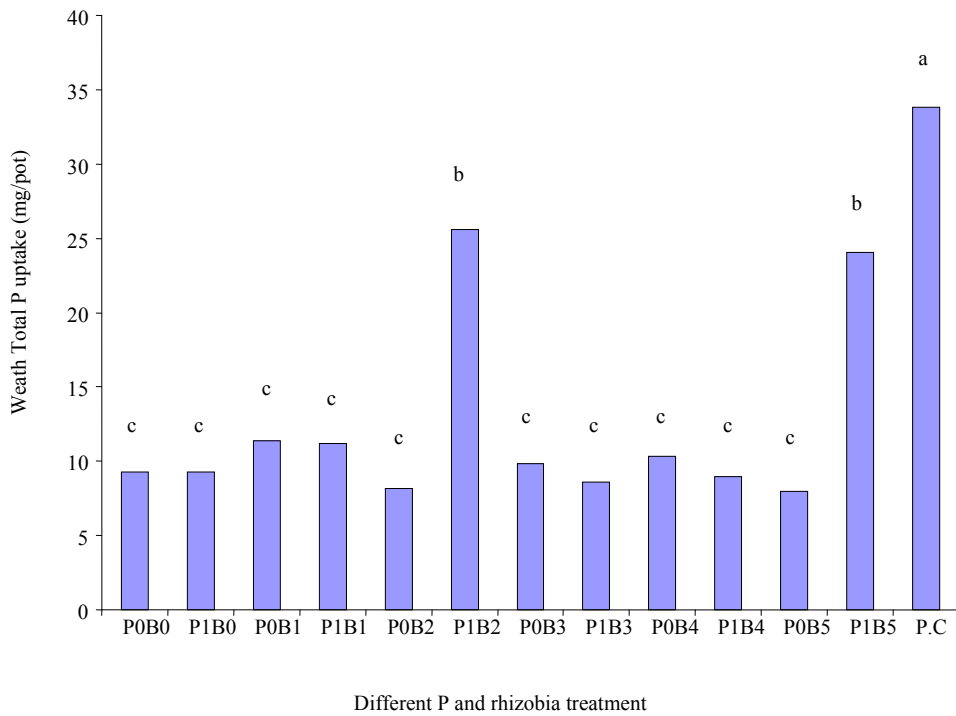


Fig. 4. Comparison of total P uptake by wheat seed in different treatments of phosphorus and plant growth promoting rhizobia by Duncan's multiple range test

- P0, P1 and B0-B5 are different phosphate and bacterial treatments respectively (see text)
- Means with the same letters had no significant ( $p < 1\%$  or  $5\%$ ) differences

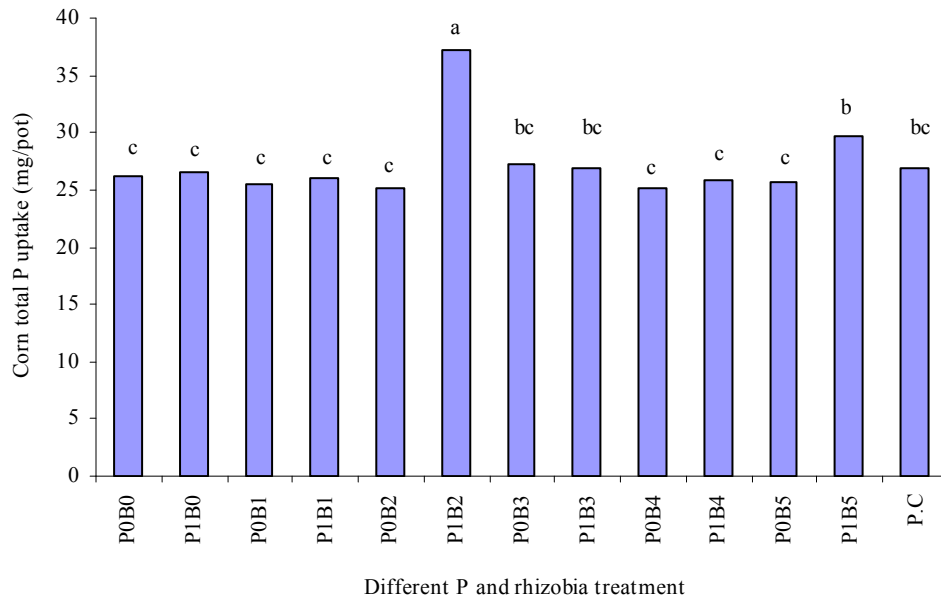


Fig. 5. Comparison of corn biological yield average in different P and PGPR treatments by Duncan's multiple range test

- P0, P1 and B0-B5 are different phosphate and bacterial treatments respectively (see text)
- Means with the same letters had no significant ( $p < 1\%$  or  $5\%$ ) differences

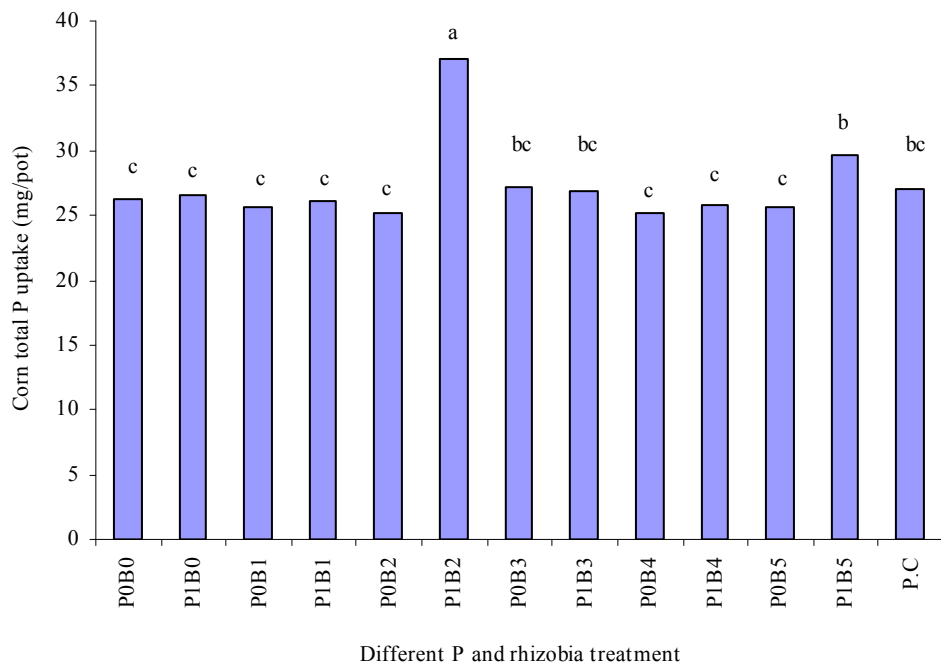


Fig. 6. Comparison of corn total P uptake in different P and PGPR treatments by Duncan's multiple range test

- P0, P1 and B0-B5 are different phosphate and bacterial treatments respectively (see text)
- Means with the same letters had no significant ( $p < 1\%$  or  $5\%$ ) differences



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