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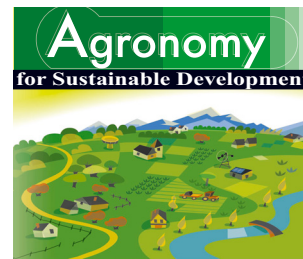
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Research article

Higher soybean yield by inoculation with N-fixing and P-solubilizing bacteria

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Abstract – Phosphorus availability for soybean growth is frequently low because P reacts with iron, aluminum and calcium in soil to form insoluble phosphates. The increasing price of phosphatic fertilizers is a major obstacle faced by farmers for application of recommended P doses. Low yield of soybean in Pakistan is also lowering adoption by farmers of this oilseed crop. These issues could be solved by inoculation of beneficial microorganisms that enhance the availability of N and P to the plant. Here, we tested the effect of inoculation of *Bradyrhizobium* or *Pseudomonas*, or both, in the presence and absence of P_2O_5 fertilizer, on soybean yield. Experiments were carried out in pots and the field during 2004 and 2005 under natural conditions. The bacterial strains were applied in broth culture in the pots at the seedling stage or as a seed treatment in the field. Our results showed that co-inoculation of *Bradyrhizobium* and *Pseudomonas* strains with the P_2O_5 treatment resulted in increased grain yield of 38% in pot experiments and 12% in the field experiment, compared with the P_2O_5 treatment alone. *Bradyrhizobium japonicum* strain TAL 377 produced 74.64 $\mu\text{g/mL}$ indole acetic acid and 261.2 $\mu\text{g/mL}$ gibberellic acid. Similarly, *Pseudomonas* strain 54RB produced 8.034 $\mu\text{g/mL}$ indole acetic acid and 1766 $\mu\text{g/mL}$ gibberellic acid. The survival efficiency of *Bradyrhizobium* was up to 46% higher due to co-inoculation and P_2O_5 as compared with its single inoculation. On the other hand, *Pseudomonas* survival efficiency was up to 33% higher with added P_2O_5 as compared with its single inoculation.

***Bradyrhizobium* / *Pseudomonas* / co-inoculation / soybean / survival efficiency**

1. INTRODUCTION

Soybean is one of the most important oilseed crops in the world. Soybean has the highest protein content (40–42%) of all other food crops and is second only to groundnut in terms of oil content (18 to 22%) among food legumes (Robert, 1986). In Pakistan, it is grown on a very small area at present, only in Sindh and the North-Western Frontier Province. Average yield of soybean in the country is low as compared with other soybean-growing countries. The problem is the low soil fertility, especially nitrogen and phosphorus deficiency in Pakistani soils due to intensive cropping, low inputs and P fixation in calcareous soils coupled with high fertilizer prices, especially phosphatic fertilizers. So, this is a comparative study of the effects of P_2O_5 application and inoculation with *Pseudomonas* and *Bradyrhizobium* on available P content of soil, and ultimately the soybean yield.

The P bioavailability is a major factor limiting nitrogen fixation (Israel, 1987) and P deficiency could inhibit the nodule development and thereby the total N_2 fixation (Miao et al., 2007). Although N and P fertilizers will continue to play a major role in intensive agriculture, depletion of natural resources,

high input costs, and long-term unsustainability necessitate alternative strategies be investigated and implemented to buffer against food insecurity and environmental degradation. Furthermore, because improved N and P use by plants has an immediate and direct benefit in extensive agriculture in developing countries where access to fertilizers is limited, efforts in research focused on improved nutrient efficiency should be a high priority (Vance, 2001).

Microorganisms involved in biofertilizer production can play a major role in increasing nutrient availability for high yield and reducing production cost by limited chemical fertilizer application. Phosphorus biofertilizers could help increase the availability of phosphate for plant growth by solubilization, promoting the plant growth through production of plant growth-promoting substances, and by increasing the efficiency of biological nitrogen fixation (Kucey et al., 1989). Although the role of phosphorus fertilizers in nodulation, nitrogen fixation, nutrient-use efficiency and growth of soybean has been reported (Israel, 1987; Tsvetkova and Georgiev, 2003; Israel, 2006; Miao et al., 2007), information is scanty regarding the role of phosphate-solubilizing bacteria in phosphorus bioavailability, growth promotion and also their interaction with N_2 -fixing bacteria.

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Co-inoculation of *Bacillus* strains onto soybean plants with *Bradyrhizobium japonicum* provided the largest and most consistent increases in nodule number, nodule weight, shoot weight, root weight, total biomass, total nitrogen and grain yield (Bai-Yu Ming et al., 2003). The present investigation aimed to evaluate the efficiency of *Pseudomonas* and *Bradyrhizobium* in order to use them as a combination in biofertilizer for nutrient availability and subsequent yield improvement of soybean. An experiment under axenic conditions was also conducted to determine the survival efficiency of *Bradyrhizobium* and *Pseudomonas* alone and in co-inoculation in the rhizosphere during early vegetative growth of soybean, and also to estimate the magnitude of interaction of the two microorganisms applied simultaneously.

2. MATERIALS AND METHODS

The *Pseudomonas* sp. strain 54RB and *Bradyrhizobium* strain TAL 377 were obtained from the Soil Biology and Biochemistry Lab. of the National Agricultural Research Center (NARC), Islamabad. This *Pseudomonas* strain has not been evaluated before for its potential alone as a phosphorus solubilizer or in association with N-fixing bacteria for soybean. Soybean seeds (cv. NARC 7) were obtained from the Oil Seed Program, NARC, Islamabad.

2.1. Pot experiment

Clay pots (24 × 36 cm) were filled with 10 kg soil collected from uncultivated land situated in Shahdra near Quaid-I-Azam University, Islamabad, and well mixed with sand at 1:1 in order to make it porous for easy and accurate root nodule sampling. The final mixture had sandy loam soil texture, pH 7.9, EC 0.43 dsm⁻¹, P content 10 ppm and NO₃-N 15 ppm. Ten seeds were sown in each pot (six pots per treatment). After germination, five plants per pot were maintained for growth until maturity. The experiment was conducted under natural conditions in 2004 and 2005 with temperature varying from 20 °C–32 °C, total rainfall 481 mm, relative humidity ranging from 77–86% and the photoperiod ranging from 10–13 h.

A starter dose of nitrogen of 0.3 g/pot (equivalent to 30 kg/ha-recommended dose) was applied in the form of urea in each pot, whereas P₂O₅ was applied at 1.35 g per treatment pot (equivalent to 50 kg/ha-recommended dose) in the form of Single Superphosphate (SSP) only in treated pots. Plants were watered as required to maintain moisture at field capacity and harvested at maturity for grain and straw yield after a growth period of 11 weeks.

The treatments included a non-inoculated control, P₂O₅, *Pseudomonas*, *Bradyrhizobium*, *Bradyrhizobium* and P₂O₅, *Pseudomonas* and P₂O₅, *Bradyrhizobium* and *Pseudomonas*, and *Bradyrhizobium*, *Pseudomonas* and P₂O₅. Each treatment was replicated six times. The *Pseudomonas* inoculum was prepared in Pikovskaya medium (Pikovskaya, 1948) [containing insoluble tricalcium phosphate 2.5 g, glucose 13 g, (NH₄)SO₄

0.5 g, NaCl 0.2 g, MgSO₄·7H₂O 0.1 g, KCl 0.2 g, yeast extract 0.5 g, MnSO₄ trace, FeSO₄·7H₂O trace, pH adjusted to 7.2 and dissolved in 1000 mL distilled water]. *Bradyrhizobium* inoculum was prepared in yeast mannitol broth (Somasegaran and Hoben, 1985) containing K₂HPO₄ 0.5 g, mannitol 10.0 g, MgSO₄·7H₂O 0.2 g, NaCl 1.0 g, yeast extract 0.1 g, pH adjusted to 7.0 and dissolved in 1000 mL distilled water.

After the emergence of seedlings, broth of *Bradyrhizobium japonicum* strain TAL 377 and *Pseudomonas* strain 54RB were applied with the help of a micropipette at the base of each seedling using 1 mL containing 10⁸ cells/mL as measured by optical density using a Spectronic 21 at wavelength 520 nm.

Leaf samples were collected 70 days after sowing for sugar and protein analysis. Sugar and protein estimation in leaves was done following the method of Johnson et al. (1966) and Lowry et al. (1951), respectively. At maturity seeds were dried, ground, weighed and acid-digested for determination of N and P contents following the methods of Cottenie (1980) and Winkleman et al. (1984). Composite soil samples were taken from each treatment and extraction was done using ammonium bicarbonate diethylene triamine pentaacetic acid (AB-DTPA) for determination of soil NO₃-N and available P content.

Seed protein was calculated according to the formula:

$$\% \text{protein} = \% \text{N} \times 5.61 \text{ (factor) (Mosse, 1990).}$$

2.2. Field experiment

The experimental plot located at Quaid-I-Azam University, Islamabad (longitude 70°10'E and latitude 33°40'N) was divided into twenty-four sub-plots, consisting of three rows, each measuring 3 m with row-to-row distance of 30 cm in each plot. Three replicates and eight treatments, as described above for the pot experiments, were arranged in a completely randomized block design. Weeds were removed manually as and when required. The soil was clay loam with pH 7.8, ECe 0.42 dsm⁻¹; P and NO₃-N were 8 ppm and 11 ppm, respectively. The same field was used for two consecutive years of experiments (2004 & 2005).

Growing conditions were the same as described above for the pot experiments. Nitrogen at 30 kg/ha (recommended dose) in the form of urea was applied in each plot, whereas P₂O₅ at 50 kg/ha (recommended dose) in the form of single super phosphate (SSP) was applied per treatment plot at the time of sowing. Inoculation was done using sterilized carrier material (NARC carrier, Khalil et al., 1991). The carrier material was inoculated with the broth cultures of *Bradyrhizobium* and *Pseudomonas* (single and mixed). For this purpose, seeds were soaked in sugar solution and coated with the carrier material containing inocula at approx. 10⁸ cells mL⁻¹.

Leaf sugar and protein analyses were carried out (70 days after sowing) following the methods of Dubo et al. (1956) as modified by Johnson et al. (1966) and Lowry et al. (1951), respectively. Number of nodules per plant was counted at flowering. For this purpose, five plants per plot were uprooted gently and washed with tap water carefully before counting nodules. At maturity (106 days after sowing), harvesting was done and

shoot weight and root weight were determined on a per plant basis (five plants per plot). For grain yield, plants were harvested (1 m² per plot) and threshed manually, and seeds were weighed in g/m² and the value was converted into kg/ha. The seed N, seed P, soil N and soil P were determined as described for the pot study.

2.3. Solubilization index

A loopful of culture of *Pseudomonas* (54RB) was used to inoculate Pikovskaya's agar (Pikovskaya, 1948) plates [containing insoluble tricalcium phosphate 2.5 g/L] and incubated at 28 °C for 7 d. The Solubilization index was determined using the formula (Premono et al., 1996):

$$\text{Solubilization Index} = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}}$$

2.4. Indole acetic acid and gibberellic acid production

For detection and quantification of indole acetic acid and gibberellic acid production by bacterial strains, broth culture (100 mL) of *Bradyrhizobium* and *Pseudomonas* was prepared in yeast mannitol agar and Pikovskaya's media, respectively. In the broth, L-tryptophan (100 mg/L) was added as a precursor for biosynthesis of indole acetic acid. After 7 days of growth at 28 °C on a rotary shaker, the bacterial cells were harvested by centrifugation at 10 000 rpm for 10 min. The pH of the supernatant (i.e., cell-free growth medium) was adjusted to 2.8 with HCl. Extraction of phytohormones was carried out by the method of Tien et al. (1979). An equal volume of ethyl acetate (Merck, Germany) was added to the cell-free liquid culture media adjusted to pH 2.5–3 and was mixed vigorously in a separating funnel. Ethyl acetate extraction was repeated three times and extracts were evaporated to dryness under a rotary film evaporator. The residues were dissolved in 1 mL methanol (Merck).

The samples were analyzed on HPLC (Shimadzu, C-R4A Chromatopac; SCL-6B System Controller) using a UV detector and C18 column (39 × 300 mm). Pure indole acetic acid and gibberellic acid (Sigma, USA) were used as standards for identification and quantification of indole acetic acid and gibberellic acid produced by bacterial cells. Methanol: acetic acid:water (30:1:70; v/v) was used as a mobile phase at the rate of 1.5 mL/min with a run time of 8 min. For identification of hormones 100 µL samples filtered through a 0.45 Millipore filter were injected into the column. The growth hormones were identified on the basis of the retention time of the standard indole acetic acid and gibberellic acid using a UV detector at 280 nm wavelength. The concentration of indole acetic acid and gibberellic acid was calculated on the basis of peak area.

2.5. Experiment conducted in axenic conditions

Soil collected from a farmer's field, air-dried and well mixed with sand (1:1), was autoclaved (three times) at 120 °C

at 15 lb pressure for 1 h. The soil was checked for sterility before starting the experiments, by plating aliquots from serial dilutions of sterilized soil on agar plates and monitoring any growth. In sterilized plastic pouches (0.5 kg soil capacity) five seeds were sown and after germination thinned to 3 plants per pouch. The seedlings were grown for four weeks (July–August 2006) at a temperature of 25 °C–30 °C, photoperiod of 12–13 h and relative humidity varying from 75–80%. A starter dose of nitrogen of 0.01 g per pouch was applied in the form of urea, whereas P₂O₅ was applied at 0.135 g per treatment. Plants were watered daily to maintain moisture at field capacity. The colony-forming units were determined at weekly intervals for 4 weeks.

Broth culture of *Bradyrhizobium* and *Pseudomonas* containing 10⁸ cells/mL was applied 10 days after sowing at the base of each seedling at 1 mL per plant. Plants were uprooted and soil was gently removed from the root surface and rhizosphere to make dilutions. Colony-forming units (CFU) of *Bradyrhizobium* and *Pseudomonas* per gram soil were determined by plating 100 µL of tenfold serial dilution on respective culture media. The CFU was calculated according to James (1978).

$$\text{Colony-forming unit} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of inoculum}}$$

2.6. Statistical analysis

The data obtained in the study were averaged for two years for pot as well as field experiments and then subjected to analysis of variance using MSTAT-C software (Bricker, 1991) and means were compared by Duncan's Multiple Range Test (Duncan, 1955). Correlations between growth parameters were also calculated (Bricker, 1991).

3. RESULTS AND DISCUSSION

3.1. Phytohormone production and phosphate solubilization

Bradyrhizobium japonicum strain TAL 377 produced 74.64 µg/mL indole acetic acid (IAA) and 261.2 µg/mL gibberellic acid, and *Pseudomonas* strain 54RB produced 8.034 µg/mL IAA and 1766 µg/mL gibberellic acid, and exhibited a phosphorus solubilization index of 4.1. It was observed that this strain, which produced the highest amount of auxins and gibberellins, also caused maximum increase in growth and yield.

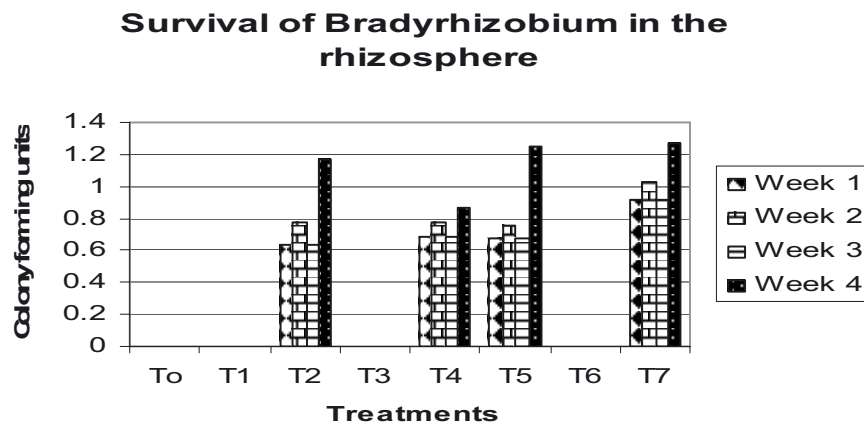
3.2. Pot experiment

Shoot/root weight, plant height, number of pods, grain yield, and leaf sugar and leaf protein content showed maximum increase over control in the *Bradyrhizobium Pseudomonas* P₂O₅ treatment (Tab. I). Increase in root/shoot

Table 1. Effect of *Bradyrhizobium*, *Pseudomonas* and P₂O₅ on agronomic traits, plant biochemical analysis, soil and plant nutrient analysis and grain yield of soybean (average data, pot experiments 2004 & 2005).

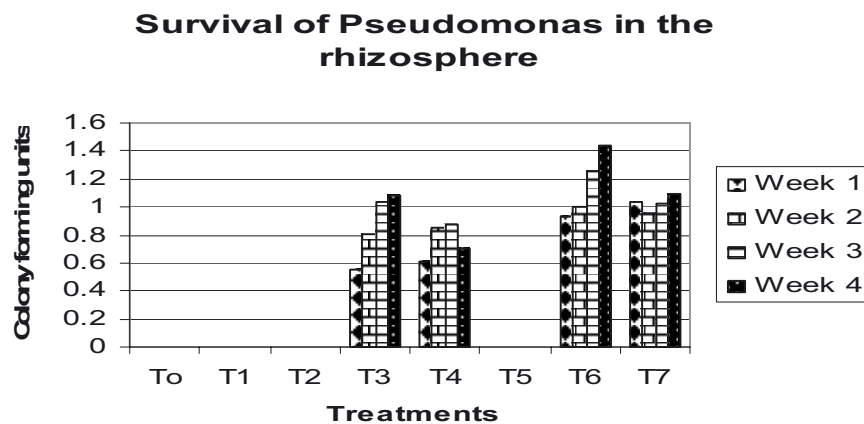
Treatments	Shoot wt. (g)/plant	Root wt. (g)/plant	No. of nodules	Plant height (cm)	No. of pods	Grain wt. (g)/pot	Leaf sugar (µg)	Leaf protein (µg)	Seed N (%)	Seed protein (%)	Seed P content (%)	Soil P content (ppm)	Soil N (ppm)
Control	1.29 d	0.23 e	0.00 d	23.6 e	4.67 e	2.46 e	48.2 d	538 b	4.6 d	28.1 e	0.67 c	5.2 d	6.2 e
P ₂ O ₅	2.54 b	0.39 b	0.00 d	32.3 ab	10.7 b	5.33 bc	54.6 bc	729 a	5.1 c	29.8 d	0.76 b	5.7 cd	7.2 d
<i>Bradyrhizobium</i>	1.28 d	0.24 e	4.83 b	25.7 de	6.67 d	3.61 e	51.2 cd	797 a	5.7 b	32.3 bc	0.71 bc	5.3 d	8.7 c
<i>Pseudomonas</i>	1.67 c	0.28 de	2.50 c	28.4 cd	8.33 cd	4.32 de	56.1 ab	774 a	5.4 b	31.0 cd	0.75 b	7.4 b	6.3 e
<i>Bradyrhizobium</i> + <i>Pseudomonas</i>	2.54 b	0.31 cd	9.30 a	30.7 bc	10.2 bc	4.71 cd	54.9 bc	747 a	5.5 b	31.8 bc	0.81 a	6.0 c	10.6 b
<i>Bradyrhizobium</i> + P ₂ O ₅	2.66 ab	0.39 b	4.83 b	32.0 ab	11.8 b	5.95 b	56.1 ab	747 a	5.6 b	32.5 bc	0.72 bc	5.8 cd	10.0 b
<i>Pseudomonas</i> + P ₂ O ₅	2.70 ab	0.35 bc	8.17 a	29.8 bc	12.2 b	6.94 a	57.4 ab	743 a	5.7 b	33.0 b	0.84 a	8.6 a	8.13 c
<i>Bradyrhizobium</i> + <i>Pseudomonas</i>	2.88 a	0.45 a	9.17 a	35.0 a	14.5 a	7.35 a	59.8 a	849 a	6.1 a	35.3 a	0.87 a	8.6 a	12.4 a
LSD value	0.2283	0.0554	1.418	2.930	1.963	0.787	4.069	110.8	0.2215	1.518	0.0554	0.667	0.798

Values followed by different letters in a column are significantly different ($P < 0.05$), using Duncan's multiple range test. P₂O₅ @ 1.35 g/pot = 50 kg/ha.



To. control T1. P_2O_5 T2. *Bradyrhizobium* T3. *Pseudomonas* T4. *Bradyrhizobium* + *Pseudomonas* T5. *Bradyrhizobium* + P_2O_5 T6. *Pseudomonas* + P_2O_5 T7. *Bradyrhizobium* + *Pseudomonas* + P_2O_5 .

Figure 1. Colony-forming units of *Bradyrhizobium* per gram rhizosphere soil as affected by different treatments. These counts were taken after the 1st, 2nd, 3rd and 4th weeks of seedling growth of soybean. The values represent the average of three replicates multiplied by 10^6 .



To. control T1. P_2O_5 T2. *Bradyrhizobium* T3. *Pseudomonas* T4. *Bradyrhizobium* + *Pseudomonas* T5. *Bradyrhizobium* + P_2O_5 T6. *Pseudomonas* + P_2O_5 T7. *Bradyrhizobium* + *Pseudomonas* + P_2O_5 .

Figure 2. Colony-forming units of *Pseudomonas* per gram rhizosphere soil as affected by different treatments. These counts were taken after the 1st, 2nd, 3rd and 4th weeks of seedling growth of soybean. The values represent the average of three replicates multiplied by 10^6 .

weight of soybean due to co-inoculation of *Bradyrhizobium japonicum* and phosphate-solubilizing bacteria could be attributed to phytohormone production by these microbes, resulting in root proliferation, as has been reported by Bai-Yu Ming et al. (2003), Gautam and Pant (2002) and Tomar et al. (2004).

Maximum numbers of nodules were produced with *Bradyrhizobium* and *Pseudomonas*. This result may be due to the greater survival efficiency of these microbes as observed by higher colony-forming unit values when *Bradyrhizobium* and *Pseudomonas* were co-inoculated (Figs. 1 and 2), indicating the synergistic effect of the two microbes on root nodulation. The minimum number of nodules was noted in plants where only *Pseudomonas* was applied. It explains the role of phosphorus solubilizers in the process of nodule formation and N fixation through phosphorus bioavailability in the root zone. The importance of P solubilizers is also obvious from the

observation that no nodule was produced in P_2O_5 treatment alone. The possibility of contamination could not be ignored, but similar results in the field open a gateway to further research on nodule formation with species other than *Bradyrhizobium* on one hand, and the effectiveness of *Bradyrhizobium* in the presence of any P solubilizer, on the other hand. The co-inoculation treatment of *Bradyrhizobium* and *Pseudomonas* in the presence of P_2O_5 increased number of pods (36% over P_2O_5), grain yield (38% higher than P_2O_5), seed N and P, and available P and N content (51 and 72% over P_2O_5 , respectively) of soil, indicating the positive role of these microbes in nutrient mobilization and ultimately increasing yield of soybean.

In most studies interpretation of the effects of phosphorus on host plant growth, nodule formation and N fixation has been made. In the present study, the host plant was also inoculated with phosphorus-solubilizing bacteria along with phosphorus

Table II. Effect of *Bradyrhizobium*, *Pseudomonas* and P₂O₅ on agronomic traits, plant biochemical analysis, soil and plant nutrient analysis and grain yield, of soybean (average data, field experiments 2004 & 2005).

Treatments	Shoot wt. (g)/plant	Root wt. (g)/plant	No. of nodules /plant	Plant height (cm)	No. of pods	100 grain wt.(g)	Grain wt. (kg/ha)	Leaf sugar (µg)	Leaf protein (µg)	Seed N (%)	Seed protein (%)	Seed P (%)	Soil P (ppm)	Soil N (ppm)
Control	2.54 c	0.58 c	0.00 d	33.3 c	16.7 e	10.2 b	1532 e	55.4 bc	572 c	4.2 e	24.0 e	0.52 b	4.5 e	3.90 c
P ₂ O ₅	5.82 a	1.61 a	0.00 d	55.0 ab	32.5 c	10.9ab	2199 c	56.3 abc	716 b	4.3 e	24.4 e	0.59 a	8.1 b	8.70 b
<i>Bradyrhizobium</i>	5.50 a	1.57 ab	5.67b	53.3 ab	29.7 cd	10.4ab	2251 bc	52.0 c	843 a	5.3 b	30.2 b	0.60 a	4.5 e	10.4 ab
<i>Pseudomonas</i>	4.14 b	1.21 b	2.67 c	47.5 b	27.7 d	10.2 b	1953 d	59.6 ab	755 ab	5.2 bc	29.3 bc	0.58 a	6.4 d	5.30 c
<i>Bradyrhizobium</i> + <i>Pseudomonas</i>	5.36 a	1.70 a	9.17 a	54.7 ab	42.8 a	11.6 a	2220 c	55.4 bc	759 ab	6.0 a	34.0 a	0.57 a	7.0 c	8.30 b
<i>Bradyrhizobium</i> + P ₂ O ₅	5.74 a	1.45 ab	5.33 b	54.7 ab	38.3 b	10.9ab	2319 b	59.4 ab	728 b	4.8 d	27.2 d	0.62 a	7.8 b	10.4 ab
<i>Pseudomonas</i> + P ₂ O ₅	5.56 a	1.33 ab	8.17 a	51.7 ab	45.7 a	11.3ab	2407 a	58.2 ab	739 ab	5.1 c	29.0 c	0.63 a	9.2 a	10.8 ab
<i>Bradyrhizobium</i> + <i>Pseudomonas</i>	5.68 a	1.48 ab	8.17 a	57.5 a	45.7 a	11.4 a	2453 a	60.1 a	843 a	6.1 a	34.7 a	0.63 a	9.4 a	11.8 a
LSD value	0.819	0.355	1.269	6.858	3.76	0.365	71.86	4.019	97.14	0.156	0.917	0.055	0.583	2.368

Values followed by different letters in a column are significantly different ($P < 0.05$), using Duncan's multiple range test. P₂O₅ @ 50 kg/ha.

fertilizer (recommended dose only) for enhanced supply of phosphorus for nodule formation, N fixation and ultimately, growth of the host legume plant, and the results indicated that this treatment has a profound effect on nodule number, nutrient uptake, growth and final grain yield of soybean. This bacterial strain has not been tested before for its potential as a co-inoculant with *Bradyrhizobium* in soybean at the pot and field level.

In pot experiments broth culture inoculum application in the rhizosphere is feasible and is generally practiced by the scientific community for testing the potential of new bacterial strains to be used as biofertilizer. A comparison of the pot and field studies with respect to broth inoculation and seed coating methods indicated that in general both these methods are effective and produce similar results in the pot and field, although some exceptions were also observed.

3.3. Field experiment

Maximum shoot weight was observed with the P₂O₅ treatment (129% over control), followed by the *Bradyrhizobium* and P₂O₅ treatment (126% over control) (Tab. II). This result showed that inoculation did not play its role in improving plant shoot weight in the field. The difference in response to inoculation between the field and pot may be attributed to the difference in root exudation from host plants, production of microbial metabolites and soil nutrients exposed to the larger area of the field, which are under the influence of climatic conditions accompanied by losses due to leaching. Maximum root weight and the highest number of nodules was recorded in the *Bradyrhizobium* and P₂O₅ treatment. It was interesting to note that plants inoculated with *Pseudomonas* alone also produced nodules. The possibility of contamination cannot be overruled. However, increased nodulation due to phosphate solubilizer (*Sordaria fimicola*) and phosphorus sources has previously been reported by Rasal et al. (2004) and Paratey and Wani (2005). Increasing available phosphorus frequently increases N₂ fixation in *Rhizobium* (Andrade et al., 1998). Tilak et al. (2006) reported that the PGPR isolates used did not antagonize the introduced *Rhizobium* strain and dual inoculation with either *Pseudomonas putida*, *P. fluorescens* or *Bacillus cereus* resulted in a significant increase in plant growth, nodulation and enzyme activity over *Rhizobium*-inoculated and uninoculated control plants. The nodule occupancy of the introduced *Rhizobium* strain increased from 50% (with *Rhizobium* alone) to 85% in the presence of *Pseudomonas putida*. Increase in number of nodules in *Bradyrhizobium* and *Pseudomonas* co-inoculation may be due to *Pseudomonas*-induced phosphorus solubilization as well as increased production of gibberellic acid, which increases root proliferation and stimulates plant growth. Number of nodules was correlated with leaf protein (r = 0.64**), seed N content (r = 0.85**) and soil N content (r = 0.67**).

Similarly to the pot experiments, co-inoculation of *Bradyrhizobium* + *Pseudomonas* in the presence of P₂O₅ resulted in maximum increase in plant height, number of pods, grain yield, leaf sugar and protein content. Increased plant

height by co-inoculation of *Bradyrhizobium* and *Pseudomonas* along with P₂O₅ may possibly be attributed to the inoculation-induced increase in indole acetic acid and gibberellic acid production, which subsequently increased the cell division and cell elongation. The grain yield with co-inoculation of *Bradyrhizobium* and *Pseudomonas* in the presence of P₂O₅ was 37.89% in pot experiments and 11.55% in the field compared with P₂O₅ alone. In a comparison of the last two treatments (Tabs I and II) it was noted that in the field study the grain yield was 09% higher than P₂O₅ when *Pseudomonas* was used along with P₂O₅ and 12% higher than P₂O₅ when *Bradyrhizobium* and *Pseudomonas* were used along with P₂O₅ as compared with the pot study, where this increase was 38 and 30%, respectively. Increased plant growth of soybean inoculated with *Bradyrhizobium* and *Pseudomonas* has been reported by Zaidi and Singh (2001). However, in our study supplemented P₂O₅ further enhanced the yield. The size of grain as indicated by 100-grain weight was significantly higher in the co-inoculation treatment even in the absence of P₂O₅. Usman et al. (2004) have reported increased number of pods, grain weight and yield with a mixture of rhizobial strains including TAL 377 in soybean. Increase in grain yield due to co-inoculation of *Bradyrhizobium* and phosphate-solubilizing bacteria has been reported by Zaidi et al. (2004) in green gram, and Tapas and Gupta (2005) and Jain and Trivedi (2005) in soybean. In our study it was observed that co-inoculation of synergistic microbes is benefited by phosphatic fertilizer application. It is evident from the colony-forming unit (CFU) values of the applied microbes in the rhizosphere as well. Number of pods was highly correlated with grain yield, seed N and seed P. These results may be due to the production of growth-promoting substances and high colonization ability of rhizobacteria such as *Pseudomonas* because they enhance the nitrogen fixation of soybean when co-inoculated with *Bradyrhizobium japonicum* (Chebotar et al., 2001).

Maximum seed P, N and protein were recorded by *Bradyrhizobium*-*Pseudomonas*-P₂O₅ treatment. Similarly, maximum soil P as well as soil NO₃-N was recorded with *Bradyrhizobium*-*Pseudomonas*-P₂O₅. *Pseudomonas* alone also improved soil P. Zaidi et al. (2004), Govindan and Thirumurugan (2005), and Zaidi and Singh (2001) reported significant increases in seed P of different legumes due to co-inoculation of *Rhizobium* and PSB over control. Jain and Trivedi (2005) and Lanje et al. (2005) have reported increases in seed protein of soybean due to *Bradyrhizobium japonicum* and P solubilizers alone or in combination. Govindan and Thirumurugan (2005) reported significantly increased post-harvest soil N and P levels in soybean due to co-inoculation of *Bradyrhizobium* and *Pseudomonas*. Addition of P fertilizer was again comparable with these studies.

The results indicate that although *Bradyrhizobium*-*Pseudomonas*-P₂O₅ was the best treatment for most of the growth and yield parameters, this is followed by *Pseudomonas*-P₂O₅ in the case of yield, sugar content and seed as well as soil P content. *Bradyrhizobium*-P₂O₅ was also effective for improving root/shoot weight, plant height, number of pods and grain yield of soybean. *Bradyrhizobium* has a favorable

effect on number of nodules, and soil and seed N content, as well as leaf and seed protein content.

3.4. Experiment conducted under axenic conditions

The colony-forming unit (CFU) of *Bradyrhizobium* was non-significantly affected in the presence of *Pseudomonas* or P_2O_5 applied separately, but in the presence of both *Pseudomonas* and P_2O_5 the colony-forming unit of *Bradyrhizobium* was significantly higher at all measurements for 4 weeks. In measurements made 4 weeks after inoculation, *Bradyrhizobium* showed a decrease in colony-forming unit when co-inoculated with *Pseudomonas*, but a significant increase in colony-forming unit was recorded in the presence of P_2O_5 or *Pseudomonas*- P_2O_5 . There was no significant effect of *Bradyrhizobium* inoculation on *Pseudomonas* until the 2nd week of measurement; thereafter, co-inoculation of *Bradyrhizobium* and *Pseudomonas* resulted in a significant decrease in colony-forming unit count. However, co-inoculation with *Bradyrhizobium* in the presence of P_2O_5 significantly stimulated colony-forming unit counts of *Pseudomonas* at all measurements for 4 weeks. The magnitude of the increase was significantly higher for *Pseudomonas* in the presence of P_2O_5 only. Phosphorus plays a key role in improving the density of *Rhizobium* in the rhizosphere (Anonymous, 1999). Tapas and Gupta (2005) reported that dual inoculation of bacterial cultures enhances the rhizobial and phosphate-solubilizing bacteria population in the rhizosphere. It is inferred that survival of *Bradyrhizobium* and *Pseudomonas* was higher when they were co-inoculated in the presence of P_2O_5 as compared with when they were single-inoculated. This may be due to availability of phosphorus in the form of P_2O_5 . The benefit of P_2O_5 to *Pseudomonas* was evident from the 1st week, while *Bradyrhizobium* benefited from P_2O_5 at a later stage. The survival efficiency of *Bradyrhizobium* was up to 46% higher due to co-inoculation and P_2O_5 as compared with its single inoculation while, on the other hand, *Pseudomonas*' survival efficiency was up to 33% higher with added P_2O_5 as compared with its single inoculation. So it is inferred that *Bradyrhizobium* requires P sources along with P solubilizer for increased survival in the rhizosphere.

4. CONCLUSION

It is inferred from this investigation that co-inoculation of these N-fixing and P-solubilizing microorganisms in the presence of P_2O_5 is very effective in increasing grain yield of soybean on a sustainable basis by up to 12–38% as compared with only P_2O_5 fertilizer. This study strengthens the efforts of combating the phosphorus fixation issue for wise use of this non-renewable natural resource for leguminous crops. Available soil P content was improved following inoculation with P-solubilizing bacteria and *Pseudomonas* also assisted *Bradyrhizobium* in increasing soil N level. The response to inoculated microorganisms showed a similar trend in pot and field experiments, with some exceptions. The co-inoculation of two

microbes has been reported to have both synergistic and antagonistic responses; however, during the present investigation the co-inoculation of *Bradyrhizobium* and *Pseudomonas* increased the number of these microbes in the presence of P_2O_5 , suggesting that P_2O_5 may assist *Bradyrhizobium* colonization and growth. Production of plant growth regulators, P solubilization activity and increased colonization in the rhizosphere suggest the mechanism of action of co-inoculated N-fixing and P-solubilizing microorganisms. It is suggested that in developing countries such as Pakistan, beneficial microbial consortia should be formulated for important crops and disseminated to farmers by extension workers for improvement in yield on a sustainable basis with less use of costly non-renewable depleted natural resources.

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