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INTERACTIVE EFFECT OF *RHIZOBIUM* STRAINS AND P ON SOYBEAN YIELD, NITROGEN FIXATION AND SOIL FERTILITY

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Abstract

Pot studies under natural conditions were undertaken to determine the effect of various exotic *Bradyrhizobium japonicum* strains viz., TAL 377, 379, 102 used alone or in mixture with and without phosphorus on soybean growth, yield and nitrogen fixation parameters. Surface sterilized soybean seeds (NARC-4 var.) coated with *Rhizobium* strains were sown in earthen pots. Phosphorus (P) was applied as single super phosphate (SSP) at the time of sowing in the soil. Nitrogenase activity, pink bacteriodal tissue volume and specific nitrogenase activity was determined at flowering stage. These parameters were relatively higher when mixed rhizobial strains were applied in combination with P. However, efficiency of different rhizobial strains for specific nitrogenase activity and other parameters was TAL102>TAL379>TAL377 either alone or in combination with P. Application of *Rhizobium* strain and P also increased the growth and yield of soybean and also improved soil fertility and NPK uptake by plant tissues. It is concluded that increase in soybean yield can be achieved by applying *Rhizobium* mixed culture with phosphorus, which also improved soil fertility for sustainable agriculture system.

Introduction

Soybean (*Glycine max* (L.) Merr.), a leguminous crop, is one of the most important and extensively grown crops that accounts for 30% of the world's processed vegetable oil and also has been employed as a source for bio-diesel fuels (Graham & Vance, 2003). In Pakistan, presently soybean covers about 3650 hectares mostly to meet the demand of edible oil. On account of the wide gap between production and consumption of edible oil in Pakistan, it is imperative to improve the yield of oil seed plants (Jalaluddin, 2005).

Use of *Rhizobium* inoculums in the establishment of legumes has been widely recognized, especially in areas where indigenous nodulation has been found to be inadequate. The benefits by the use of *Rhizobium* inoculants show that a quite good deal of money can be saved by marginal farmers by using quality tested inoculants on the farm. Further, it has been reported that the legumes crop enrich the fertility of the soil. Rhizobial inoculation to seeds is well studied and exploitation of this beneficial nitrogen fixing root nodule symbiosis represents a hallmark of successfully applied agricultural microbiology (Bruno, *et al*., 2003). Biological nitrogen fixation plays an essential role in crop establishment and yield, since no N fertilizer is applied and it fulfils most of plants need for nitrogen (Vargas & Hungria, 1997; Chen *et al*., 2002).

In Pakistan, phosphorus in soil is generally quite abundant but it reacts readily with iron, aluminium and calcium to form insoluble compounds. These reactions result in very low phosphorus availability and low efficiency of phosphorus fertilizer used by the plants (Jodie & Peter, 2000). Phosphorus is also an essential ingredient for *Rhizobium* bacteria to convert atmospheric N (N_2) into an ammonium (NH_4) form usable by plants. Inadequate P restricts root growth, the process of photosynthesis, translocation of sugars and other such functions which directly influenced N fixation by legume plants (Anon., 1999).

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This study has been designed to evaluate the effect of various exotic *Rhizobium* strains alone and combination with phosphorus on nitrogen fixation, weight of nodules, yield, its effects on soil nutrients level and mineral uptake by plants. This study was aimed to test the possibility of enhancing of soybean production among the small holder farmers in Pakistan through the use of mixture of *Rhizobium* inoculation and moderate applications of P.

Materials and Methods

Plant materials and growing conditions: Soybean seeds cv NARC-4 obtained from National Agricultural Research Centre (NARC) Islamabad, were surface sterilized by Mercuric chloride (0.1%) for 2 min., and thoroughly rinsed with distilled water. Thereafter, seeds were soaked in distilled water for 6 hours. For inoculation with peat-based inoculums (*Rhizobium* strain NifTAL 377, TAL 378, TAL 102), seeds were moistened in sugar solution (48%) before application of inoculums to get a thin uniform coating of inoculums on seeds immediate before sowing. Seeds were sown in earthen pots containing soil and sand 1:3 ratio. Phosphorus was applied in respective pots as single super phosphate in soil at the time of filling the pots. Four plants per pot were grown during mid July in natural environment. The experiment using laid out in Complete Randomized Design (CRD) with four replications. The treatments were as follows:

T1 = TAL377
T2 = TAL377+ Phosphorus
T3 = TAL379
T4 = TAL379+Phosphorus
T5 = TAL102
T6 = TAL102+ Phosphorus
T7 = TAL377+379+102
T8 = TAL377+379+102+ Phosphorus
T9 = Phosphorus
T10 = Control

At maximum flowering stage nodules were collected to determine nodule weight plant^{-1}, nitrogenase activity, volume of pink bacteroid tissue and specific nitrogenase activity. At maturity, plants were harvested and seeds were collected to determine yield per plant.

Nitrogenase activity of nodules: Nitrogenase activity was measured by the acetylene reduction method at flowering stage as described by Bergerson (1980). Nodules with 1cm root segments were picked and incubated with acetylene for 1h at room temperature to determine the nitrogenase activity. Trace gas chromatograph GC – 2000 (Thermo Quest-C.E, instrument Italiana) with hydrogen flame ionization detector (FID) was used for acetylene reduction assay. The uncharged acetylene and ethylene produced were calculated as ratio on chrome card software. The nitrogenase activity was expressed as μ moles of C_2H_4 produced plant^{-1} h^{-1} g^{-1} nodules weight.

Measurements of volume of pink bacteroid tissue of nodules: The nodules were taken from roots at flowering stages. Thin sections of nodules (5μ) were made with the help of razor blade and the volume of pink bacteroid tissue containing leghaemoglobin present in the nodule cortex were measured under light microscope (Nikon Research Microscope, optiphat with HFX- II Camera) at 4X (Gretchen, 1967).

Specific nitrogenase activity of nodules:

Specific nitrogenase activity of nodules is expressed as μmoles C_2H_4 pink bacteroid tissue mm^3 $plant^{-1}$ h^{-1} =

$$\frac{C_2H_4\ \mu moles\ g^{-1}\ nodule\ plant^{-1}\ h^{-1}}{Pink\ bacteroid\ tissue\ mm^3\ plant^{-1}\ h^{-1}}$$

Available soil NPK and organic matter: Available P in soil samples was determined using method described by Watanable & Olsen (1965) and soil nitrogen was determined using O'Brain & Flore (1962) method. Potassium and organic matter were determined in soil samples using method described by Mc Keagece (1978).

NPK uptake by plant tissue (%): The plant tissue NPK uptake was determined in plant samples using method described by Winkleman *et al*., (1985).

Statistical analysis: The data was analyzed statistically by analysis of variance and differences among the significant treatments were determined by least significant difference (LSD) test.

Results

Physicochemical properties of the experimental soil: The experimental soil was loam in texture and available nitrogen was 2.73%, potassium 0.120 μgKg^{-1}, phosphorus 3.0 $mgKg^{-1}$, and organic matter 0.75%. CFU count showed presence of *Rhizobium* population $1.08x10^6$ g^{-1} soil and phosphorus solublizining bacterial population $1.4x10^6 g^{-1}$ soil.

Nodule weight, nitrogenase activity, volume of pink bacteriod tissue and specific nitrogenase activity: Results revealed that mixed rhizobial strains increased nodule weight 71.7%, (Table 1) while when inoculated with P, the weight increased upto 76% as compared with control. LSD values for nitrogenase activity and volume of pink bacteroidal tissue were with low difference either treated with *Rhizobium* culture alone or in combination with P as described in Table 2. Highest value of both (3.88umoles C_2H_2/g nodules/plant/h, 2.03mm^3, 1.89 C_2H_2 umoles/g nodules/plant/h, respectively) were observed when soybean was treated with all three *Rhizobium* strain cumulatively in combination with P. Different values with low differences were observed under the treatment of TAL 377 and 379, alone and in combination with P. TAL 102 proved best when inoculated alone or with P that produced 2.54 and 2.78g nodule wt/plant, respectively (Table 2). Maximum specific nitrogenase activity 1.8 and 1.89 mm^3 umoles C_2H_4/g nodule/plant/h were observed when all three rhizobial strains were applied cumulatively and in combination with P, respectively.

Seed weight $plant^{-1}$ (g): The LSD (Table 2) values shows that mixture of *Rhizobium* strains with and without phosphorus were found to promote seed yield by 63.3%, 60.1% respectively as compared to single strain over control. Among three strains TAL102 with and without phosphorus increases yield by 60.1% and 51%, respectively. Phosphorus alone increased 34.4% seed weight over control.

Table 1. Effect of various *Rhizobium* strains alone and in combination with phosphorus on yield, nodule weight and nitrogen fixation of soybean var. NARC-4.

Treatments	Seed wt. $plant^{-1}$ (g)		Nodule wt. $plant^{-1}$ (g)		N_2 ase activity (µmoles C_2H_4 g^{-1} nodule $plant^{-1}$ h^{-1})		Vol. of pink bacteroid Tissue (mm^3)		Sp N_2ase activity of (mm^3 µmoles C_2H_4 g^{-1} nodule $plant^{-1}$ h^{-1})	
(T1)377	4.49^{EF}	(16.9%)	1.33^{G}	(36.8%)	2.58^{CD}	(37.5%)	1.58^{E}	(12.6%)	1.62^{ABCD}	(27%)
(T2)377+P	5.72^{CD}	(34.2%)	1.76^{F}	(52.2%)	2.82^{BCD}	(46.8%)	1.59^{E}	(13.3%)	1.76^{AB}	(32.9%)
(T3)379	5.17^{DE}	(27.8%)	1.84^{EF}	(54.3%)	2.36^{D}	(31.7%)	1.72^{D}	(19.7%)	1.37^{CDE}	(13.8%)
(T4)379+P	6.38^{C}	(41.5%)	1.97^{E}	(57.3%)	2.39^{D}	(51%)	1.76^{D}	(21.6%)	1.35^{DE}	(12.5%)
(T5)102	6.69^{BC}	(44.2%)	2.54^{D}	(66.9%)	2.39^{BCD}	(51%)	1.85^{C}	(25%)	1.58^{BCD}	(25%)
(T6)102+P	7.70^{B}	(51.5%)	2.78^{C}	(69.7%)	3.1^{BC}	(62.5%)	1.88^{BC}	(26.6%)	1.64^{ABC}	(28%)
(T7)377+379+102	9.36^{A}	(60.1%)	2.97^{B}	(71.7%)	3.41^{AB}	(65.9%)	1.92^{B}	(28%)	1.80^{AB}	(34%)
(T8)377+379+102+P	10.16^{A}	(63.3%)	3.5^{A}	(76%)	3.88^{A}	(70%)	2.03^{A}	(65%)	1.89^{A}	(37%)
(T9)P	5.69^{CD}	(34.4%)	1.36^{G}	(38%)	2.68^{CD}	(39.9%)	1.74^{D}	(20.6%)	1.52^{BCD}	(22%)
(T10) CONTROL	3.73^{F}		0.84^{H}		1.61^{E}		1.38^{F}		1.18^{E}	
LSD	1.073		0.1435		0.6041		0.05425		0.2819	

α level 0.05, * percentage differences over the control are given in parenthesis.

Table 2. Effect of various *Rhizobium* strains alone and in combination with phosphorus on NPK both in soil and plant tissue and organic matter of soil of soybean var. NARC-4.

Treatments	Plant			Soil			
	N (%)	P (%)	K (%)	N (%)	P (mg Kg $^{-1}$)	K (mg Kg $^{-1}$)	Org Mater (%)
(T1)377	1.53^{G} (8.49%)	0.10^{D} (0%)	1.46^{HI} (2.7%)	0.02^{AB} (50%)	3.03^{E} (7.5%)	99.0^{B} (30%)	0.47^{F} (48%)
(T2)377+P	1.54^{G} (9%)	0.14^{CD} (28%)	1.48^{H} (4%)	0.04^{AB} (75%)	4.13^{B} (32.2%)	99.6^{B} (30.7%)	0.55^{EF} (56%)
(T3)379	1.60^{F} (12.5%)	0.10^{D} (0%)	1.64^{G} (13%)	0.02^{AB} (50%)	3.13^{DE} (10.8%)	80.6^{BCD} (14%)	0.51^{EF} (52%)
(T4)379+P	1.70^{E} (17.6%)	0.13^{CD} (23%)	1.74^{F} (18%)	0.03^{AB} (66%)	3.83^{C} (26.8%)	90.3^{BC} (23%)	0.65^{DE} (63%)
(T5)102	2.11^{c} (33.6)	0.18^{ABC} (44%)	1.92^{D} (26%)	0.02^{AB} (50%)	3.03^{E} (7.5%)	70.3^{CD} (1.8%)	0.65^{DE} (63%)
(T6)102+P	2.72^{B} (48%)	0.20^{AB} (49%)	1.98^{C} (28%)	0.03^{AB} (66%)	3.23^{D} (13.3%)	70.3^{CD} (1.8%)	0.83^{C} (71%)
(T7)377+379+102	2.85^{A} (50.8%)	0.21^{AB} (52%)	2.18^{B} (34%)	0.06^{AB} (83%)	2.83^{F} (1.1%)	60.0^{D} (-15%)	1.16^{B} (79%)
(T8)377+379+102+P	2.86^{A} (51%)	0.23^{A} (56%)	2.50^{A} (52%)	0.07^{A} (85%)	3.03^{E} (7.5%)	60.0^{D} (-15%)	1.53^{A} (84%)
(T9)P	2.00^{D} (30%)	0.17^{BC} (41%)	1.86^{E} (23%)	0.03^{AB} (66%)	5.83^{A} (51.9%)	120.7^{A} (42%)	0.74^{CD} (67%)
(T10) CONTROL	1.40^{H}	0.10^{D}	1.42^{I}	0.01^{B}	2.80^{F}	69.0^{CD}	0.24^{G}
LSD	0.0542	0.05425	0.0542	0.1956	0.05425	24.65	0.1534

α level 0.05, * percentage differences over the control are given in parenthesis.

NPK uptake by plant tissue (%) and its level in soil (kgmg^{-1}) after harvesting: The results pertaining to NPK uptake by plant tissue and its level in soil under the effect of different treatments are presented in (Table 2). Statistical analysis revealed that mixed *Rhizobium* inoculation with as well as without phosphorus significantly increased NPK uptake (2.86%, 39% and 20%, respectively) in shoot of soybean as compared to control. Soil N level was significantly increased upto 84% with phosphorus and 79% without phosphorus in the pots having mixed *Rhizobium* treatment as compared to control.

Organic matter in soil (%): The data regarding organic matter of soil is presented in (Table 2). Maximum organic matter (69%) was analyzed in the pots where seeds were inoculated with mixed *Rhizobium* strain and phosphorus than that for control. Phosphorus also increases organic matter (36%) in the soil when applied alone.

Discussion

Mineral nutrient deficiencies are the major constraints limiting legume N fixation and yield (O'Hara *et al.*, 1988). In leguminous crop this deficiency can be over come by applying *Rhizobium* strain and providing essential nutrients in the soil. In this experiment the beneficial effect of *Rhizobium* strains NifTAL 377, TAL379 and TAL102 single/mixture with and without P application was determined on soybean growth, yield and nitrogen fixation capability of soybean.

Nodule weight, nitrogenase activity, volume of pink bacteriod tissue and specific nitrogenase activity: The nitrogen fixation capacity is one of the most important selection criteria for superior legume varieties because nitrogen fixation is basic demand for high yield production.

Nodulation phenotypic parameters as nodule weight per plant, N2 fixation capacity are dependent upon Rhizobium strain and affectivity of gene that effect on Nod-factor structure and also on availability of nutrients to the plant. In the present study, efficiency of different rhizobial strains for specific nitrogenase activity was TAL102> TAL379> TAL377 and same rating was observed when inoculated with phosphorus individually. Nodule formation by Rhizobium strains describes genetic relationship between Rhizobium strains to soybean but difference is due to host specificity of nodulation loci, specificity of Rhizobium for lipo-polysacchrides of soybean roots and competition between microsymbionts for host carbohydrates. But combined effect of all three Rhizobium strain and phosphorus was maximum that describes relationship between the tribes. Gull *et al.*, (2003) reported that nitrogenase activity, nodules weight, shoot P was significantly enhanced by co-inoculation. The results are also in close conformity with the findings of Lukiwatid & Simanungkalit (2002). Presence of phosphorus in the soil with Rhizobium strains, individually or cumulative effect on nodule weight per plant and nitrogen fixation parameters while Kauas *et al*., (2005) stated that the intrinsic characteristics of the nodules (individual biomass and size, P concentration and efficiency of N fixation) did not depend on P availability. Phosphorus is required for the normal functioning of nitrogen fixing bacteria and has favorable effect on the number and weight of the effective nodules formation on the root system (Anon., 1999).

Seed weight plant^{-1} (g): *Rhizobium* strains treatments to soybean significantly increased seed weight per plant either alone or in combination with P. Each *Rhizobium* strain has own synergetic effect on production of seed weight as seed weight also increased when *Rhizobium* strains were applied individually in combination with P. That shows P application is important nutritional element to get better soybean yield. The similar results were observed by Jalaluddin (2005). Khan (2000) reported that P application and *Rhizobium* inoculation significantly increased pod formation, grain yield and dry matter production as compared with un-inoculated treatments. This study revealed that *Rhizobium* inoculation with P fertilizer increased sufficient yield of grain, these findings are also supported by Amos, (2004). Shabayev *et al*., (1996) findings also supported the results that the yield increase was higher in P-treated plants inoculated with mix bacterial culture than the plants inoculated with nodulated bacteria alone. Seed yield is directly correlated with nodule weight per plant because seed contain nitrogenous compounds (proteins) that are affected by formation of nodules on plant root to fulfill nitrogen requirement. Therefore same pattern for nodule weight per plant was observed as for seed weight per plant. The highest nodule weight 3.5g was observed under the effect of *Rhizobium* strains with P leading from 2.97g per plant due to all *Rhizobium* strains without P.

NPK uptake by plant tissue (%) and its level in soil (kgmg^{-1}) after harvesting: Mixed *Rhizobium* inoculation with as well as without phosphorus significantly increased NPK uptake in shoot of soybean as compared to control. This increase may be due to supply of P that seems important for *Rhizobium* to fix relatively more nitrogen from soil, which resulted in increased plant growth and N uptake by root and then to shoots. Phosphorus plays a vital role in physiological and developmental process in plant life and favorable effect of this important nutrient might have accelerated the growth process that increases N uptake in plants. Similar results were shown by Basir *et al.,* (2005) and Sarawgi *et al*., (1999). Sustainable crop production will obviously require enhance flows of nutrients to crops.

Plant yield, shoot NPK contents, nodulation efficiency and nitrogenase activity were significantly enhanced by phosphorus application with mix *Rhizobium* strain. These results were strongly supported by Biswas (1998), who observed that *Rhizobium* inoculation significantly increased uptake of NPK by rice plants compared with uninoculated plants. Wu (2000) also revealed the effect of nitrogen fixing organism on N P and K uptake, some enzyme activities and linked to yield in Island cotton. Rodelas (1999) also observed increased root development and water and mineral uptake by root with the application of mix *Rhizobium* strain on *Faba beans.* Biswas *et al*., (2000) reported that rhizobial inoculants may also induce an increased number of roots hairs and lateral roots thereby favoring nutrient uptake by exploration of a greater soil volume. While Olivara *et al.,* (2004) reported that phosphorus application to leguminous crops increase plant biomass including nodule biomass and shoot phosphorus content increased by the rate of nitrogen fixation. Similarly, *Rhizobium* inoculation significantly increased nitrogenase activity, nodule mass that's ultimately increased plant NPK uptake. These results shows cumulative effect of *Rhizobium* strains and phosphorus on nodule weight, nitrogenase activity, volume of pink bacteriodal tissue, specific nitrogenase activity, yield and nutrient uptake by plant tissue. The low phosphorus and potassium level in soil after harvesting shows maximum uptake by plant due to the efficiency of *Rhizobium* strain because certain strains of rhizobia are able to solubilize precipitated P components (Chabot *et al.,* 1996). The higher P concentration in plant benefits the bacterial symbiont and the functioning of its nitrogenase activity, leading to increased nitrogen fixation.

Organic matter in soil (%): Maintenance of soil organic matter at a certain level is essential for sustained crop yield. The soil of Pakistan is low in organic matter; moreover, the prevailing climatic conditions are conducive to its rapid decomposition. Presence of organic matter in soil and biological activity ameliorates direct soil–based constraints to plant growth, such as nutrient and water availability, soil crusting, erosion and extreme pH or element toxicities (Woomer *et al*., 1994). The data regarding organic matter of soil reveals that maximum organic matter (69%) was analyzed in the pots inoculated with mixed *Rhizobium* culture and phosphorus than that for control. Phosphorus also increases organic matter (36%) in the soil when applied alone. Soil organic matter effects on plant growth and referred to as the "glue" that hold soil particles together that also promotes development of soil tilth and texture.

Conclusion

Nitrogen fixation is a dynamic process regulated by a suite of stage- dependent factors including P availability and nitrogen fixing bacteria that adds substantial amounts of nitrogen and organic matter into this system. Present study shown that mixed *Rhizobium* strains with phosphorus significantly increase soybean yield and few correlations can be drawn between Nod-factor substituents and the ability to nodulate specific legumes. That is due to increase in nodule weight production and efficient nitrogen fixing mechanism. Presence of organic matter and increased available nitrogen in soil after harvesting the crop is surplus point for sustainable agriculture system.

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