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Co-inoculation Effects of Phosphate Solubilizing Microorganisms and *Glomus fasciculatum* on Green Gram-*Bradyrhizobium* Symbiosis

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Abstract: Experiments were conducted to evaluate the effects of nitrogen fixing (*Bradyrhizobium* sp. (Vigna)), phosphate solubilizing bacterium (Bacillus subtilis), phosphate solubilizing fungus (*Aspergillus awamori*) and AM fungus (*Glomus fasciculatum*) on the growth, chlorophyll content, seed yield, nodulation, grain protein, and N and P uptake of green gram plants grown in phosphorus-deficient soils. The triple inoculation of AM fungus, *Bradyrhizobium* sp. (Vigna) and *B. subtilis* significantly increased dry matter yield, chlorophyll content in foliage and N and P uptake of green gram plants. Seed yield was enhanced by 24% following triple inoculation of *Bradyrhizobium* + *G. fasciculatum* + *B. subtilis*, relative to the control. Nodule occupancy, determined by indirect enzyme linked immunosorbent assay (ELISA), ranged between 77% (*Bradyrhizobium* + *A. awamori*) and 96% (*Bradyrhizobium* + *G. fasciculatum* + *B. subtilis*) at flowering (45 DAS), decreasing at the pod-fill (60 DAS) stage with each treatment. Replica immunoblot assay (RIBA) revealed a greater variation in the rhizobial populations within nodules and the correlation between nodule occupancy and immunoblot counts was highly significant at 45 (r = 0.95) and at 60 DAS (r = 0.96). There was a negative effect on some of the measured parameters when *A. awamori* was used alone or added to the combination treatments. The present findings showed that rhizospheric microorganisms can interact positively in promoting plant growth, as well as N and P uptake of green gram plants, leading to improved yield.

Key Words: AM fungi, ELISA, green gram, N uptake

Introduction

Phosphorus is one of the major nutrients limiting plant growth. Most of the soils throughout the world are P deficient (Batjes, 1997) and therefore require P to replenish the P demand by crop plants. To circumvent the P deficiency in soils, P fertilizers are applied. However, after application, a considerable amount of P is rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils (Norrish and Rosser, 1983) or Ca in calcareous soils (Lindsay et al., 1989) before plant roots have had a chance to absorb it. Further, the use of rock phosphate as a phosphate fertilizer and its solubilization by microbes (Kang et al., 2002), through the production of organic acids (Maliha et al., 2004), have become a valid alternative to chemical fertilizers. Rock phosphate is widely distributed throughout the world, both geographically and geologically (Zapata and Roy, 2004). In conjugation with phosphate solubilizing microorganisms (PSM), rock phosphate provides a cheap source of P fertilizer for crop production. In this regard, several studies have conclusively shown that PSM solubilizes the fixed soil P and applied phosphates, resulting in higher crop yields (Zaidi 1999; Gull et al., 2004). The alternative approach is to use these PSM along with other beneficial rhizospheric microflora to enhance crop productivity. In this context, the simultaneous application of *Rhizobium* and PS microorganisms (Perveen et al., 2002) and PSM and arbuscular mycorrhizal (AM) fungi (Zaidi et al., 2003) has been shown to stimulate plant growth more than inoculation of each microorganism alone in certain situations when the soil is P deficient. AM fungi, on the other hand, encourage the plant roots to rapidly absorb solubilized P. Accordingly the increase in plant growth may be due to the release of certain plant growth promoting substances (Kucey et al., 1989) by the PS organisms or AM development and mycorrhizal

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formation (Azcon-Aguilar and Barea, 1985). However, the inoculation effects of the tripartite interaction between N_2 fixing, PSM and AM fungus on legume crops are relatively scarce (Zaidi et al., 2004).

The green gram (*Vigna radiata* L. Wilczek) is a grain legume grown widely in the tropics and fixes about 40-50 kg N ha^{-1}. In India, the green gram is grown in an area of 3 x 10^6 ha with an annual production of 1 x 10^6 t of grain (Sharma, 2000). Therefore, it is of great practical importance to evaluate the effect of tripartite symbioses where each partner plays a specific role in plant growth and yield. The objective of the present work was to evaluate the efficiency of the interaction between N_2 fixing (*Bradyrhizobium* sp. (Vigna)), PS bacterium (*Bacillus subtilis*), PS fungus (*Aspergillus awamori*) and AM fungus (*Glomus fasciculatum*) on the growth, nodule occupancy, yield and N and P uptake by green gram plants under non-sterilized soils, in clay pots.

Materials and Methods

The cultures of *Bradyrhizobium* sp. (Vigna) and *G. fasciculatum* were obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India. The PS bacterium, *Bacillus subtilis* (MTCC 121) and PS fungus (*A. awamori*) were procured from the Institute of Microbial Technology, Chandigarh, India. *Bradyrhizobium* sp. (Vigna) was grown in yeast extract mannitol broth in flasks shaken at 125 rpm at 28 ± 2 °C for 7 days to a cell density of 4 x 10^8 cells ml^{-1}. *Bacillus subtilis* and *A. awamori* were grown in National Botanical Research Institute Phosphate (NBRIP) growth medium (Nautiyal, 1999) for 6 and 3 days, respectively, at 28 ± 2 °C to a cell density of 2.3 x 10^8 and 4.5 x 10^6 cells ml^{-1}, respectively. *Glomus fasciculatum* was multiplied on rhodes grass (*Chloris gayana* Kunth) following the open pot culture method (Gilmore, 1968).

Seeds of green gram var. Pusa Baisakhi were surface sterilized (Vincent, 1970), rinsed 6 times with sterile water and dried. The surface disinfected seeds were coated by soaking seeds in liquid culture medium of each organism for 2 h using 10% gum arabic as adhesive to deliver 10^8 cells $seed^{-1}$ *Bradyrhizobium*, and 10^7 cells $seed^{-1}$ *B. subtilis*. For combined inoculations, the liquid cultures of each organism were mixed in equal proportion and then seeds were dipped in it. In combined treatments with *G. fasciculatum,* the bacterized seeds were sown in soils having 100 g of the mycorrhizal inoculum (infected roots and spores). Spore suspensions (4 ml) of 2 x 10^6 ml^{-1} of *A. awamori* was added to soils 48 h before sowing. The uninoculated seeds served as a control treatment for comparison. Rock phosphate (P_2O_5 23%) was added as phosphatic P (20 mg kg^{-1}) to the soil before seeding, and was common in all treatments, except the control, which had 20 mg kg^{-1} N (urea) and 40 mg kg^{-1} P (single super-phosphate).

The inoculated seeds were sown in earthen pots (10 seeds pot^{-1}) having 12.5 kg of unsterilized sandy clay loam soil (alluvial, organic C 0.4%, pH 7.4, WHC 0.44 ml g^{-1}, Olsen P 16 mg kg^{-1} and Kjeldahl N 0.75 g kg^{-1}). The seeds were sown during the summer of 2003 and were repeated with the same treatments during the summer of 2004. The pots with different treatments were arranged in a randomized complete block design with 9 replications of each treatment. The pots were kept at 22 ± 2 °C and 60% relative humidity. Seedlings were thinned to 4 plants per pot 5 days after emergence. The plants were watered using tap water as and when required. The treatments were as follows: T_1 *Bradyrhizobium* sp. (Vigna); T_2 *Aspergillus awamori*; T_3 *Glomus fasciculatum*; T_4 *Bacillus subtilis*; T_5 *Bradyrhizobium* sp. (Vigna) + *A. awamori*; T_6 *Bradyrhizobium* sp. (Vigna) + *G. fasciculatum*; T_7 *Bradyrhizobium* sp. (Vigna) + *B. subtilis*; T_8 *A. awamori* + *G. fasciculatum*; T_9 *A. awamori* + *B. subtilis*; T_{10} *G. fasciculatum* + *B. subtilis*; T_{11} *Bradyrhizobium* sp. (Vigna) + *A. awamori* + *G. fasciculatum*; T_{12} *Bradyrhizobium* sp. + *G. fasciculatum* + *B. subtilis*; T_{13} *A. awamori* + *B. subtilis* + *G. fasciculatum*; and T_{14} Un- inoculated control ($N_{20}P_{40}$). The experiments were repeated consecutively for 2 years during the summer to ensure the reproducibility of the results.

All plants in 3 pots were uprooted each at 45 (flowering stage) and 60 days (pod-fill stage) after seeding (DAS) and were used for nodulation analysis. The plants were uprooted carefully and the adhering soil particles were removed by washing under water, and intact nodules were detached from the roots, counted, oven dried (80 °C) and weighed. Nodule occupancy was determined by indirect ELISA (Kishinevsky and Bar-Joseph, 1978) while the rhizobial populations within nodules were quantified by replica immunoblot assay (RIBA) (Khan et al., 2002). Nodule occupancy was calculated as % nodule occupancy = (Number of nodules positive to ELISA) / total number of nodules tested x 100.

The rhizobial populations in each nodule was quantified as number of purple spots on the nitrocellulose membrane/ dilution factor x volume of inoculum. Plants removed at flowering (45 DAS) and at harvest (80 DAS) were oven dried before the weights of roots and shoots and total plant biomass were determined. The remaining pots were maintained until harvest. Plants were finally harvested after complete maturity (80 DAS) and seed mass and protein contents in grain (N x 6.25) were recorded. Total chlorophyll contents in foliage were determined at 45 DAS (Mechenny, 1941). Total N contents in roots, shoots and straw was measured at 80 DAS as suggested by Iswaran and Marwah (1980). Total P contents in whole plants was estimated at 45 and 60 DAS by the method of Jackson (1958). The data on 2-year trials were pooled and subjected to analysis (ANOVA).

Results

The inoculation effects of N_2 fixing, PSM and AM fungus on the green gram, used either singly or in combinations, were variable (Table 1). The single inoculation of *Bradyrhizobium* sp. (Vigna) significantly ($P \leq 0.05$) increased the dry matter accumulation in shoots at 45 and 80 DAS and total biomass of green gram plants only at 45 DAS compared to the control. In contrast, the single inoculation of *A. awamori* and *B. subtilis* significantly decreased the dry matter accumulation in roots at 45 and 80 DAS, while the *G. fasciculatum* inoculation reduced the root weight only at 80 DAS. Among dual inoculation treatments, the combination of *Bradyrhizobium* sp. + *B. subtilis* significantly enhanced the dry matter accumulation in the roots, shoots and dry weight of whole plant at flowering (45 DAS) and at harvest (80 DAS) relative to the control. In comparison,

Table 1. Co-inoculation effects of N_2 fixing and phosphate solubilizing microorganisms and AM fungus on growth, seed yield and chlorophyll content in foliage of green gram plants.

Treatment	Mean dry mass (g plant^{-1})				Total dry mass (g plant^{-1})		Seed mass (g 1000 seed^{-1})	Chlorophyll (mg plant^{-1})
	Root		Shoot					
	45 d	80 d	45 d	80 d	45d	80 d		
T_1 *Bradyrhizobium* sp. (Vigna)	0.5	0.7	1.5*	1.8*	2.0*	2.5	34.5*	2.7*
T_2 *A. awamori*	0.3*	0.4*	0.8	1.1	1.1	1.5	27.6*	1.8
T_3 *G. fasciculatum*	0.6	0.6*	0.8	0.9	1.4	1.5	33.6*	2.1
T_4 *B. subtilis*	0.4*	0.6*	0.7	0.9	1.1	1.5	19.2*	2.1
T_5 *Bradyrhizobium* + *A. awamori*	0.5	0.6*	0.8	0.9	1.3	1.5	25.4*	1.8
T_6 *Bradyrhizobium* + *G. fasciculatum*	0.9*	1.1*	1.1*	1.4	2.0*	2.5	34.8*	1.6
T_7 *Bradyrhizobium* + *B. subtilis*	0.3*	1.3*	1.5*	1.8*	1.8*	3.1*	32.2*	2.9*
T_8 *A. awamori* + *G. fasciculatum*	0.4	0.8	1.4*	1.8*	1.8*	2.6	24.4*	2.0
T_9 *A. awamori* + *B. subtilis*	0.4	0.7	0.8	1.4	1.2	2.1	26.6*	1.9
T_{10} *G. fasciculatum* + *B. subtilis*	0.8*	1.3*	1.7*	2.3*	1.5	3.6*	35.2*	3.2*
T_{11} *Bradyrhizobium* + *A. awamori* + *G. fasciculatum*	0.7	1.2*	1.5*	2.5*	2.2*	3.8*	30.2	3.1*
T_{12} *Bradyrhizobium* + *G. fasciculatum* + *B. subtilis*	1.5*	1.8*	2.4*	3.5*	3.9*	5.3*	37.4*	3.9*
T_{13} *A. awamori* + *B. subtilis* + *G. fasciculatum*	0.9*	1.4*	0.9	1.5*	1.8*	2.9	36.6*	3.2*
T_{14} Control ($N_{20}P_{40}$)	0.6	0.8	0.8	1.2	1.4	2.0	30.2	2.1
LSD ($P \leq 0.05$)	0.12	0.18	0.21	0.25	0.36	1.02	1.3	0.52

Values presented are means of 3 replicates, where each replicate constituted 4 plants per pot. * In this and the subsequent table indicates significant difference over the control at $P \leq 0.05$

the co-inoculation of *G. fasciculatum* + *B. subtilis* significantly enhanced the dry matter accumulation in roots and shoots at 45 and 80 DAS and total plant biomass at 80 DAS only, compared to the control. Addition of *G. fasciculatum* to *Bradyrhizobium* sp. (Vigna) + *B. subtilis* resulted in a significant ($P \leq 0.05$) increase in the dry mass of roots and shoots and total plant biomass of green gram plants both at 45 DAS and at 80 DAS compared to T_{14}. In contrast, *A. awamori* + *B. subtilis* + *G. fasciculatum* significantly enhanced the dry matter accumulation in roots at 45 and 80 DAS, the dry matter accumulation in shoots at 80 DAS, and total biomass at 45 DAS, relative to the control. The triple inoculation of *Bradyrhizobium* sp. (Vigna) + *G. fasciculatum* + *B. subtilis* augmented the total dry weight of green gram plants at 80 DAS by 165%, compared to the control, and was superior to all the other treatments. Seed mass in general increased significantly with all the treatments except T_2 and T_4 or combined inoculation where *A. awamori* was included, relative to the control. Seed yield increased by 14% and 11% due to inoculation with *Bradyrhizobium* sp. (Vigna) and *G. fasciculatum,* when used alone, respectively. Seed yield increased even further, by 15% and 17%, respectively, when *Bradyrhizobium* + *G. fasciculatum* and *G. fasciculatum* + *B. subtilis* were used together, over the control. However, the seed mass declined by 36% and 9% with T_4 and T_2 inoculation, respectively, compared to the control. The efficiency of *Bradyrhizobium* sp. (Vigna) was more pronounced when it was applied in combination with *G. fasciculatum* + *B. subtilis* and increased seed yield by 24%, which was followed by a 21% increase with the triple inoculation of *A. awamori* + *B. subtilis* + *G. fasciculatum* (T_{13}). The total mass of green gram plants and seed yield was positively correlated ($r = 0.4$). Chlorophyll contents increased significantly at the flowering (45 DAS) stage with *Bradyrhizobium* sp. (Vigna) alone, and composite cultures of *Bradyrhizobium* sp. (Vigna) + *B. subtilis* and *G. fasciculatum* + *B. subtilis*. The increase in chlorophyll content following the application of 3 organisms together, i.e. T_{13} and T_{12}, ranged between 52% and 86%, respectively, in comparison with the control (Table 1).

Nodulation response to experimental treatments under non-sterilized pot soil was variable (Table 2). A significantly ($P \leq 0.05$) greater number of nodules formed per plant on the root systems of green gram plants inoculated with *Bradyrhizobium* sp. (Vigna) alone, and the dual and triple inoculation treatments (except T_9 and T_{10}) both at 45 and 60 DAS, compared to the control. Among the dual inoculation treatments, *Bradyrhizobium* sp. (Vigna) + *G. fasciculatum* had the greatest positive effect on the number of nodules at the flowering (45 DAS) and pod-fill (60 DAS) stages relative to the control and showed an increase of 7% and 6% at the flowering and pod-fill stages, respectively, over T_1. A significantly higher number of nodules was recorded in triple inoculations of *Bradyrhizobium* sp. (Vigna) + *G. fasciculatum* + *B. subtilis* compared to T_1 at 45 DAS (48 nodules plant^{-1}). Inoculation of *A. awamori* with *Bradyrhizobium* sp. (Vigna) or *Bradyrhizobium* sp. (Vigna) + *B. subtilis* significantly ($P \leq 0.05$) increased the number of nodules at both stages of plant growth compared to the control but the number of nodules was considerably lower compared to that observed for T_1. Generally, the number of nodules was more at 45 DAS compared to 60 DAS. Nodule occupancy as determined by indirect ELISA ranged between 77% (*Bradyrhizobium* sp. (Vigna)+ *A. awamori*) and 96% (*Bradyrhizobium* sp. (Vigna) + *G. fasciculatum* + *B. subtilis*) at 45 DAS, while it varied between 63% (T_{11}) and 96% (T_{12}) at 60 DAS. Nodules collected from treatments not inoculated with *Bradyrhizobium* sp. (Vigna), however, showed no positive immunoreaction in the ELISA test. Moreover, the host plant differed significantly with regard to their nodule dry weight, and the correlation between number of nodules and its dry mass was highly significant at 45 DAS ($r = 0.99$) and at 60 DAS ($r = 0.95$).

The establishment and survival of *Bradyrhizobium* sp. (Vigna) in nodules as determined by RIBA test varied considerably (Table 2). The populations of *Bradyrhizobium* sp. (Vigna) within nodules were significantly higher at 45 DAS (156×10^5 cells ml^{-1}) and at 60 DAS (145×10^5 cells ml^{-1}) in the nodular suspension prepared from nodules of the triple inoculation treatment (*Bradyrhizobium* sp. (Vigna) + *G. fasciculatum* + *B. subtilis*). The nodular suspension of *Bradyrhizobium* sp. (Vigna) + *A. awamori* + *G. fasciculatum* treatment, however, revealed the lowest rhizobial counts at 45 DAS (66×10^5 cells ml^{-1}) and at 60 DAS (57×10^5 cells ml^{-1}). The nodule occupancy and immunoblot counts were highly correlated at both 45 DAS ($r = 0.95$) and at 60 DAS ($r = 0.96$). A maximum increase of 42% in grain protein (GP) was found with *Bradyrhizobium* sp. (Vigna)

Table 2. Co-inoculation effects of nitrogen fixing and phosphate solubilizing microorganisms and AM fungus on nodulation, immunoblot counts and grain protein, and N and P content in green gram plants.

Treatment	Nodule						Immunoblot counts ($x10^5$ cells ml^{-1})		Grain protein (%)	N content (mg $plant^{-1}$)			P content (mg $plant^{-1}$)	
	No. $Plant^{-1}$		Occupancy (%)		Dry mass (mg $plant^{-1}$)									
	45 d	60 d	45 d	60 d	45 d	60 d	45 d	60 d		Root	Shoot	Straw	45 d	60 d
T_1 *Bradyrhizobium* sp. (Vigna)	41*	34*	86	75	18.6*	12.8*	112	90	25.2*	16.4	30.5*	20.4	1.2	2.4
T_2 *A. awamori*	-	-	-	-	-	-	-	-	15.5*	11.1*	19.6	21.2	1.3	2.4
T_3 *G. fasciculatum*	4	3	-	-	3.6	3.2	-	-	20.4	10.7*	14.4*	12.8*	1.2	2.2
T_4 *B. subtilis*	5	4	-	-	2.8	2.5	-	-	17.4	11.2*	15.0*	17.5	1.4	2.6
T_5 *Bradyrhizobium* + *A. awamori*	34*	23*	77	68	13.4*	12.4*	127	120	21.5	16.8	32.8*	26.6*	1.6*	3.1*
T_6 *Bradyrhizobium* + *G. fasciculatum*	46*	36*	87	86	20.3*	20.5*	131	110	26.7*	19.4	33.4*	28.4*	1.8*	3.1*
T_7 *Bradyrhizobium* + *B. subtilis*	44*	23*	93	89	21.2*	18.2*	126	120	25.2*	19.8	40.2*	29.6*	1.7*	3.4*
T_8 *A. awamori* + *G. fasciculatum*	-	-	-	-	-	-	-	-	11.4*	15.4	31.6*	28.5*	1.8*	2.9*
T_9 *A. awamori* + *B. subtilis*	5	3	-	-	3.6	3.3	-	-	19.8	17.6	19.4	12.4*	2.6*	4.2*
$T1_0$ *G. fasciculatum* + *B. subtilis*	5	4	-	-	3.2	3.0	-	-	22.0	19.7	20.5	22.6	2.2*	3.8*
T_{11} *Bradyrhizobium* + *A. awamori* + *G. fasciculatum*	31*	22*	83	63	12.5*	10.4*	66	57	25.4*	20.7	26.0	20.6	2.5*	3.9*
T_{12} *Bradyrhizobium* + *G. fasciculatum* + *B. subtilis*	48*	31*	96	96	19.8*	18.4*	156	145	28.6*	26.8*	30.3*	27.2*	2.8*	4.2*
T_{13} *A. awamori* + *B. subtilis* + *G. fasciculatum*	-	-	-	-		-	-	-	26.5*	19.0	25.0	22.2	2.8*	4.3*
T_{14} Control (N20P40)	6	5	-	-	3.6	3.4-	-	-	20.1	17.2	20.8	19.6	1.2	2.3
LSD ($P \leq 0.05$)	4.1	3.7	11.5	11.4	2.7	2.6	Nd	Nd	4.4	3.8	5.8	4.9	0.3	0.6

+ *G. fasciculatum* + *B. subtilis*, which was followed by a 33% increase due to dual inoculation treatment of *Bradyrhizobium* sp. (Vigna) + *G. fasciculatum* over the control. *Aspergillus awamori*, either alone or in dual inoculation treatments, however, depressed the seed mass and GP. Single inoculation of *A. awamori* and combined inoculation of *A. awamori* + *G. fasciculatum* decreased the GP significantly by 23% and 43%, respectively, relative to the control. Single inoculation treatments in general (except T_1) depressed the N content in all the measured parts of plants, relative to the control. In contrast, the dual inoculations (except T_9 and T_{10}) significantly stimulated the N contents in the shoots and straw of green gram plants compared with the control. Among the dual inoculations, the composite application of *Bradyrhizobium* sp. (Vigna) + *B. subtilis* augmented the N contents in roots, shoots and straw by 15%, 93% and 51% over the control, and 33% and 9% in shoots and straw over T_{12} (Table 2). The triple inoculation of *Bradyrhizobium* sp. (Vigna) + *G. fasciculatum* + *B. subtilis* (T_{12}) enhanced the N contents in roots, shoots, and straw of green gram plants by 29%, 17% and 34%, respectively, compared to T_{11}, and 41%, 21% and 23% over T_{13}. The increase in plant biomass was positively correlated with the N contents of roots ($r = 0.8$), shoots ($r = 0.5$) and straw ($r = 0.2$). The single inoculation treatments, in general, did not have a significant effect on P contents of green gram plants at 45 and 60 DAS, compared to the control. In comparison, the dual and triple inoculation treatments significantly ($P \leq 0.05$) improved the P contents at both 45 and 60 DAS, relative to the control. Among the dual inoculation treatments, the co-inoculation of *A. awamori* + *B. subtilis* (T_9) showed the greatest positive effect on P contents and increased the P contents by 117% and 83% at 45 and 60 DAS, respectively, over the control. The addition of *G. fasciculatum* to the combination of *A. awamori* + *B. subtilis* further improved the P contents by 133% and 83% at 45 and 60 DAS, respectively, over T_{14}, and a marginal increase over the best performing pairing of T_9. The effect of microbial inoculation on total biomass and P uptake was positively correlated.

Discussion

The increased nodulation, N_2 fixation and yield of legume crops following inoculation with N_2 fixing and PSM have been reported by many workers (Algawadi and Gaur, 1988; Gupta, 2004). Presently, rock phosphate is being chiefly employed to sustain soil P levels in an available form for plants. In this context, PSM have been reported to solubilize the rock phosphate through the production of organic acids, ion chelation and exchange reaction in the growth environment (Yadav and Dadarwal, 1997). As a result of this activity, PSM play an important role in supplementing P to the plants, allowing a sustainable use of phosphatic fertilizers. In the present study, the addition of rock phosphate along with microbial cultures greatly enhanced the plant growth, symbiosis and nutrient uptake of green gram plants. It is generally thought that PSM in addition to solubilizing inorganic P also release growth-promoting substances (Kucey et al., 1989), which improve the germination and growth of plants and stimulate microbial activity in the rhizosphere. The present study thus clearly indicated that rock phosphate when used along with microbial treatments was transformed into available forms of P, as indicated by the increased P in plants, by the PSM (*A. awamori* and *B. subtilis*), and then was used up by growing plants and consequently enhanced the overall growth of green gram plants (Table 1). In some co-inoculation treatments, the growth of roots and shoots was poorly stimulated, suggesting the inadequate solubilization of rock phosphate by PSM and consequently the poor availability of P to the plants. However, the tripartite cultures in general were significantly effective compared to the other treatments. The explanation of this fact is that mycorrhizal endophyte could be stimulated in quantity, efficiency and longevity by metabolic product released from the inoculated bacteria. Moreover, root exudation and plasticity might have been changed by PSM inoculation, which could also affect AM development (Poi et al., 1989). Further, the N and P contents in plants were increased in the present study, which in turn positively and synergistically affected the development of green gram (Table 2). Generally, the addition of *A. awamori* to the N_2 fixer, AM fungus or both, was either inferior or negatively affected the measured parameters. The result of this relationship could be due to a negative interaction that may have occurred between PS fungus and nodule bacteria or AM fungus (Zaidi et al., 2004). The resulting inhibitory effect of *A. awamori* on the associative partner could be due to the release of inhibitory metabolites in the growing environment, which in turn adversely affected the plant growth. Furthermore, the P-releasing fungi produce

more organic acids (Venkateswarlu et al., 1984) than do bacteria, which enhance the solubilization of phosphate. However, most rhizobia prefer neutral or alkaline conditions for the establishment of a functional symbiosis and therefore the increased acidity might have changed the microenvironment, which possibly decreased the survival of nodule bacterium or colonization of AM fungus in the green gram rhizosphere.

Seed bacterization temporarily changes the balance of the rhizosphere populations and such changes may sometimes enhance the plant growth, yield and uptake of nutrients depending upon the establishment of the introduced cultures. Accordingly, the *B. subtilis*, *Bradyrhizobium* and AM fungus used in the present study were good competitors since growth, nutrient uptake and yield of green gram in the present study increased to a greater extent. The fact that plant growth and nutrient uptake increased in the presence of AM fungi suggested a strong synergistic relationship between root colonization, P uptake and growth promotion. In agreement with these findings, Zaidi et al. (2003) observed that in low P soils plant growth and nutrient uptake in chickpea were greater after inoculation with tripartite culture of *Mesorhizobium*, PSB and *G. fasciculatum* than after inoculation with each organism alone.

The number of nodules produced on legume plants is generally used an index for assessing the N_2 fixing efficiency of nodule bacteria. However, this does not reflect the true efficiency of particular rhizobial strains since manually counted nodules may also include nodules produced by the indigenous populations. Therefore, special attention was paid to establish the fact that the nodules produced on the root system of the green gram were produced only by the introduced rhizobia. Surprisingly, nodules formed on the root systems of the green gram that were even not inoculated with *Bradyrhizobium* sp. (e.g., control and some other treatments). Such nodules were therefore also subjected to indirect ELISA analysis in order to ensure the specificity of nodules (Table 2). During this study, none of the nodules collected from any of the treatments, except those inoculated with *Bradyrhizobium* sp. (Vigna), were positive to homologous antisera in the ELISA test. This finding suggested that nodules produced on the roots of treatments other than *Bradyrhizobium* sp. were produced by indigenous rhizobia. All the nodules collected from *Bradyrhizobium* inoculated plants, however, showed 100% immuno-reactivity with their homologous antisera in the ELISA test. The better nodulation in the case of composite inoculation at the flowering stage appeared to be a result of the favorable effects of PSM in making more P soluble and available to the plants, which consequently promoted root development. In the present study, a positive correlation between the plant biomass and nodule numbers and N and P contents of green gram plants further suggested the involvement of N and P in the establishment of an effective *Bradyrhizobium*–green gram symbiosis, which consequently increased the biological N_2 fixation, and yield of green gram plants. Similar evidence on the effect of P in N_2 fixation in the French bean (Saber et al., 2005) and wheat (Zaidi and Khan, 2005) is reported.

In the present study, the establishment and survival of *Bradyrhizobium* sp. (Vigna) in the nodules were determined using a most sensitive and rapid serological method, RIBA. The nodular suspension of each treatment when tested individually with the homologous antisera produced 84%-98% purple spots on the nitrocellulose membrane in the RIBA test, indicating a greater degree of rhizobia specificity in the nodules of inoculated plants (Table 2). Interestingly, nodules produced on the root system of green gram plants inoculated either with *Bradyrhizobium* alone or treatments receiving *Bradyrhizobium* showed 2%-16% serologically unrelated (white) spots on the nitrocellulose membrane, suggesting the appearance of an indigenous rhizobial population within a single nodule. Others have also reported such rhizobial diversity in the nodules (Galiana et al., 1994; Khan et al., 1999). The nodule occupancy and immunoblot counts of rhizobial populations within nodules thus provided strong evidence of an effective symbiosis, evident from the correlation between nodule occupancy and immunoblot counts at 45 ($r = 0.95$) and at 60 DAS ($r = 0.96$).

In conclusion, this study revealed that the mixed inoculation of N_2 fixing bacterium, PSM and AM fungus improved plant vigor, and nutrient uptake and dramatically increased the yield of green gram in unsterilized soil. Further, the combination of *Bradyrhizobium* sp. (Vigna) + *B. subtilis* + *G. fasciculatum* was more effective than other single, dual or triple inoculation treatments. This combination along with rock phosphate can be used for increasing the yield of green gram, concomitantly saving considerable amounts of N and P fertilizers, and it can also be used under field conditions.

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