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Biochemical and Nutritional Responses of Tripartite Soybean-*Rhizobium*-*Glomus* Association Under Low and High P Fertilization

K.S. Subramanian*, R.A. Jegan, M. Gomathy and S. Vijayakumar
Department of Nano Science & Technology
Tamil Nadu Agricultural University, Coimbatore - 641 003

A greenhouse experiment was conducted on soybean in black sandy loam soil and inoculated either with *Rhizobium japanicum* and *Glomus intraradices* singly or in combination with low (40 kg P_2O_5 ha^{-1}) and high (80 kg P_2O_5 ha^{-1}) levels of P. Soybean plant samples were analyzed for plant nutritional, biochemical and enzymatic changes. The results revealed that combined inoculation of *Rhizobium* and mycorrhizal fungus favorably increased all the nutritional, biochemical and enzymatic characteristics which contributed for the increased grain yield of soybean 10-25 per cent depending on the level of P added. This study suggests that tripartite association assists in improving the yield and nutritional qualities of soybean besides maintaining soil fertility.

Key words: *Glomus intraradices, Rhizobium,* soybean, phosphorus

The largest contribution of biological Nitrogen fixation to agriculture is derived from the symbiosis between legumes and species of *Rhizobium*. Among the leguminous crops, soybean produces a very few number of nodules which is closely associated with the P nutrition of crops. The infection mechanism of a legume root by its appropriate species of *Rhizobium*, particularly the process of N_2 fixation, has a high energy requirement (Shuman, 1988). The legume plants are to be nourished adequately with P nutrition as the key enzyme in N_2 fixation is dependent on ATP for the reduction of atmospheric dinitrogen to ammonia, approximately 21 moles of ATP are converted to ADP per molecule N_2 reduced. The deficiencies of phosphorus and micronutrients (Zn, Cu and Mo) in soil are known to limit rhizobial growth, nodulation or symbiotic N_2 fixation (Subramanian *et al.,* 2008).

Mycorrhizal fungi are ubiquitous, forming symbiotic association with nearly 90 per cent of the terrestrial plant species, contribute significantly for phosphorus nutrition (Subramanian *et al.*, 2009). Despite the fact that the mycorrhizal colonization is known to improve the nutritional status of P and other slowly diffusing mineral ions such as Zn and Cu, besides nitrate under drought conditions (Subramanian and Charest, 1997; Subramanian *et al.,* 2009), the nutritional responses of tripartite associate among soybean- *Rhizobium*-mycorrhizae is poorly understood especially under differential P fertilization. This study hypothesized that mycorrhizal colonization promotes P nutrition of soybean which facilitates proliferation of nodule and effective N fixation that collectively contribute for the overall productivity and nutritional quality of the crop.

*Corresponding author email: kssubra2001@rediffmail.com

Materials and Methods

Experimental conditions

A greenhouse experiment was conducted on a black sandy loam soil with slightly acidic pH (6.3), free from salinity (EC 0.12 dS m^{-1}) and extremely low in organic carbon status (0.3%). Soil had low available N (192 kg ha^{-1}), P (8.60 kg ha^{-1}) and high in available K (382 kg ha^{-1}). The DTPA (diethylene triamine penta acetic acid) extractable Zn and Cu were 1.2 and 1.4 mg/kg, respectively. Besides soil characteristics, the experimental soil was evaluated for its indigenous mycorrhizal status. Since the indigenous viable spore population was low (< 10 spores per 100 g soil) and therefore no attempt was made to fumigate the soil. The greenhouse had 24-28°C, light intensity (800 -1000 µmol /m^2/ s provided by natural light), relative humidity (60-65%) and 12-h photoperiod.

Inoculation of Rhizobium *and mycorrhizal fungus*

The treatments comprised of un-inoculated control, inoculated either with *Rhizobium japonicum* (Bradhyrhizobia) and *Glomus intraradices* (Schenk and Smith) singly or in combination with different levels of P (0, 40 and 80 kg/ha). Plastic pots measuring a dimension of 30 cm diameter and 30 cm depth were used for this study. Each pot was filled with 10 kg soil. There were 12 treatments each was replicated three times in a factorial randomized block design (FRBD) for sampling at 45 DAS. The pre-germinated soybean seeds (Co 3) were inoculated with *Rhizobium japonicum* (50g inoculum per kg seed) by using rice gruel as a sticking agent. Mycorrhizal inoculation was done by placing the seeds over a thin layer of vermiculite based mycorrhizal inoculum carrying *Glomus intraradices* at the rate of 5 g per pot. Vermiculite based mycorrhizal inoculum (*Glomus intraradices* TNAU-03-09) used in this study was obtained from the Department of Microbiology, TNAU, Coimbatore. This strain was cultured in maize plants and propagules comprised of infected root bits and spores blended in sterile vermiculite. The seeds were sown in pots containing eight kg of soil. Six seeds were sowed in each pot and irrigated. After emergence (7 days) seedlings were thinned to one plant in each pot. The full dose of N (20 kg ha^{-1}) and K (40 kg ha^{-1}) was applied in the form of urea and muriate of potash, respectively, as basal at the time of sowing. Basal dose of P as per treatment was applied in the form of single superphosphate. The plants were removed after six weeks of growth and the plant roots were systematically washed to eliminate soil particles and separated in to shoots and roots to analyze nutritional status (N and P) and biochemical changes. In addition, soil samples were analyzed for the available P and soil enzymes *viz.,* nitrate reductase (Nicholas and Naik, 1976), dehydrogenase (Stevenson, 1959) and acid phosphatase (Dodd *et al.,* 1987).

Results and Discussion

Mycorrhizal colonization measured by the external mycelium or number of arbuscules significantly ($P \leq 0.01$) increased in the treatment receiving combined inoculation of *Rhizobium* and arbuscular mycorrhizal fungus (AMF) than colonization by AMF alone (Table 1). The increase in the colonization was four times as that of control and 30 per cent higher than AMF alone. Incremental levels of P did not affect the mycorrhizal colonization process. The experimental soil is extremely low in P and thus added P had little importance in the lability of Phosphorus. Inhibiting effects of P fertilized plants was reported when the level exceeds 10 mg/kg (Bethlenfalvay *et al.,* 1987a). This higher colonization observed in combined inoculation may be attributed to the rhizobia which produced growth promoting substances that assisted in enhance ment of mycorrhizal colonization (Bethlenfalvay *et al.*, 1987b).

Table 1. Impact of Rhizobial and AMF inoculations under different P fertilized soybean on mycorrhizal colonization in soybean roots at 45 DAS

Treatments	Arbuscules (%)				External mycelium (%)			
	P0	P40	P80	Mean	P0	P40	P80	Mean
Control	14.00	30.60	27.30	23.97	17.00	15.50	19.00	17.17
Rhizobium	17.20	42.20	35.40	31.60	20.00	21.20	23.60	21.60
AMF inoculation	34.10	52.00	72.00	52.70	67.30	83.50	34.40	61.73
Rhizobium + AMF	64.70	79.40	98.70	80.93	76.20	92.20	94.80	87.73
Mean	32.50	51.05	58.35		45.12	53.1	42.95	45.12
CD (0.05)								
I		**				**		
P		NS				NS		
I × P		**				NS		

NS - Non significant; P0, P40 and P80 refers amount of P as P_2O_5 applied in kg ha^{-1}; ** refers significantly differ at $p \leq 0.01$

Dual inoculation of *Rhizobium* and AMF enhanced the plant nitrate reductase activities than the *Rhizobium* and AMF alone (Fig.1). Nitrate reductase activity was higher in shoots than roots of soybean. Nitrate reductase activity in AMF alone or combined inoculation of *Rhizobium* with *G. intraradices* treatments was significantly higher than control. This increased nitrate reductase activity might be due to nutritional improvement and greater levels of growth regulating compounds in the host plants. Nitrate reductase is a substrate inducible enzyme. Mycorrhizae colonized roots have greater access to transport utilizes nitrate nitrogen which is

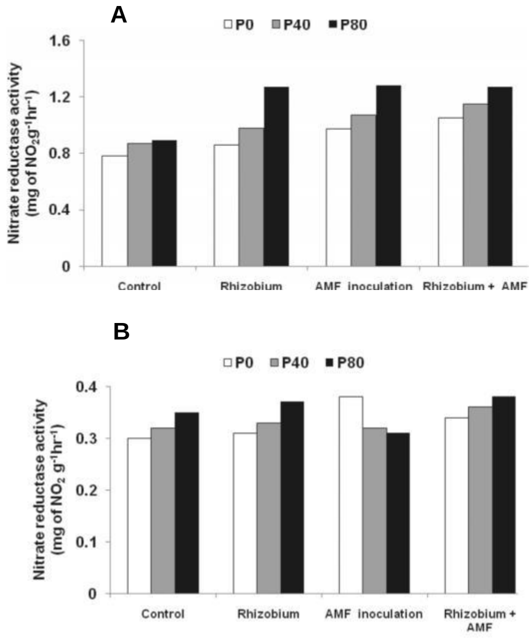

Fig. 1. Enzymatic changes of soybean under tripartite association at 45 DAS; A- shoot; B-root

unavailable to uninoculated plants. Mycorrhizal colonized maize roots have enhanced nitrate reductase activity as a consequence of nitrate nitrogen being transported by the external mycelium which serves as substrate for nitrate reductase besides mycorrhizal structures have a set of genes to regulate nitrate reductase activity (Subramanian and Charest, 1999). Higher enzyme activity in cluster bean was detected when treated with *Rhizobium* and AMF (Tarafdar and Rao, 2001).

Soil dehydrogenase and acid phosphatase activities were significantly higher in *Rhizobium* inoculated followed by dual inoculated treatments (Table 2). Available P was significantly higher in the P80 inoculated with AMF and *Rhizobium*. This could be correlated with higher nitrogenase activity of *Rhizobium* and AMF inoculated treatment which was due to improved P nutrition of inoculated plants. This may be attributed to the intense nodulation and enhanced nitrogen fixation in soybean which ultimately resulted in enhanced dry matter production. This increased availability of P in mycorrhizae inoculated plants might be due to the exploration of larger volume of soil beyond the rhizosphere region by AM fungi and increased the mobility of P in soil.

Table 2. Dehydrogenase and acid phosphatase activities and available (Olsen's) P in the single or dual inoculated soils at soybean differentially fertilized with P

Treatments	Dehydrogenase (change in OD min^{-1} g^{-1} of soil)				Acid phosphatase (µg of PNP released $g^{-1}min^{-1}$)				Olsen's P (Kg ha^{-1})			
	P0	P40	P80	Mean	P0	P40	P80	Mean	P0	P40	P80	Mean
Control	0.170	0.212	0.236	0.206	2.520	2.647	2.794	2.653	9.90	16.0	25.3	17.1
Rhizobium	0.762	0.922	1.344	1.009	2.987	3.140	3.354	3.160	9.36	14.5	27.8	17.2
AMF inoculation	0.120	0.155	0.190	0.155	2.072	2.172	2.273	2.172	8.73	19.5	33.4	20.5
Rhizobium + AMF	0.673	0.835	0.948	0.818	2.306	2.341	2.422	2.356	11.6	25.1	35.6	24.1
Mean	0.431	0.532	0.679		2.471	2.575	2.711		9.92	18.8	30.5	
CD (0.05)												
I		**				**				**		
P		**				**				**		
I × P		**				NS				**		

NS - Non significant; P0, P40 and P80 refers amount of P as P_2O_5 applied in kg ha^{-1}; ** refers significantly differ at p≤0.01

Table 3. Nutritional changes in soybean plants differentially P fertilized in the presence of single or dual inoculated treatments

Treatments	N content (%)								P content (%)							
	Shoots				Roots				Shoots				Roots			
	P0	P40	P80	Mean	P0	P40	P80	Mean	P0	P40	P80	Mean	P0	P40	P80	Mean
Control	2.56	3.06	3.37	3.00	1.33	2.00	2.43	1.92	0.16	0.22	0.29	0.22	0.22	0.30	0.39	0.30
Rhizobium	3.23	3.46	3.48	3.38	2.03	2.63	2.80	2.48	0.20	0.28	0.43	0.30	0.26	0.44	0.53	0.41
AMF inoculation	3.53	3.80	3.27	3.53	2.37	2.60	2.77	2.58	0.21	0.33	0.32	0.29	0.32	0.47	0.42	0.40
Rhizobium + AMF	3.17	4.07	4.50	3.91	2.67	2.53	3.20	2.80	0.28	0.35	0.50	0.37	0.40	0.48	0.53	0.47
Mean	3.12	3.60	3.65		2.10	2.44	2.80		0.21	0.29	0.38		0.30	0.42	0.47	
CD(0.05)																
I			**				**			**				**		
P			**				**			**				**		
I x P			**				NS			**				**		

NS - Non significant; P0, P40 and P80 refers amount of P as P_2O_5 applied in kg ha^{-1}; ** refers significantly differ at p≤0.01

The combined inoculation of *Rhizobium*, mycorrhizae and their interactions at varying levels of P significantly influenced the N and P contents of the host plants. The N and P contents were the highest in the shoots of dual inoculated plants regardless of P levels and the interaction was nonsignificant in N content of root (Table 3). The increased N content in the combined inoculated treatment could be due to the synergistic interaction between rhizobia and mycorrhizal fungi which stimulated the biological nitrogen fixation and P uptake by the soybean plants (Bethlenfalvay *et al.,* 1987b; Tarafdar and Rao, 2001). AMF colonization rates showed a significant positive correlation to the shoot P and N content in the bean plants (Aryal *et al.,* 2008).

Rhizobium and mycorrhizal inoculated treatments recorded significantly higher values of dry matter when compared to control (Fig.2). This increased dry matter production might be due to increased leaf canopy because of biological

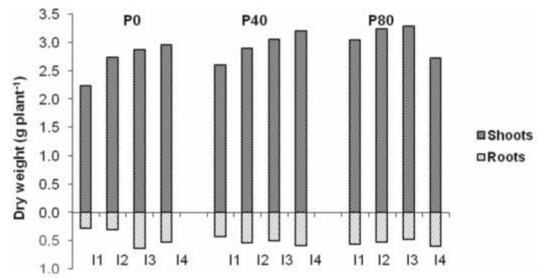

Fig. 2. Influence of tripartite association in dry weight of soybean roots and shoots
I1 - Control ; I2 - Rhizobium ; I3 - AMF inoculation; I4 - Rhizobium +AMF

nitrogen fixation and mycorrhizal colonization which resulted in higher CO_2 fixation through photosynthesis. The carbon might have translocated to roots and enhanced the mycorrhizal fungal population. The dual inoculation of mycorrhizae and *Rhizobium* significantly enhanced the number and dry weight of nodules over control (Fig.3). Significant difference was observed between AMF alone and

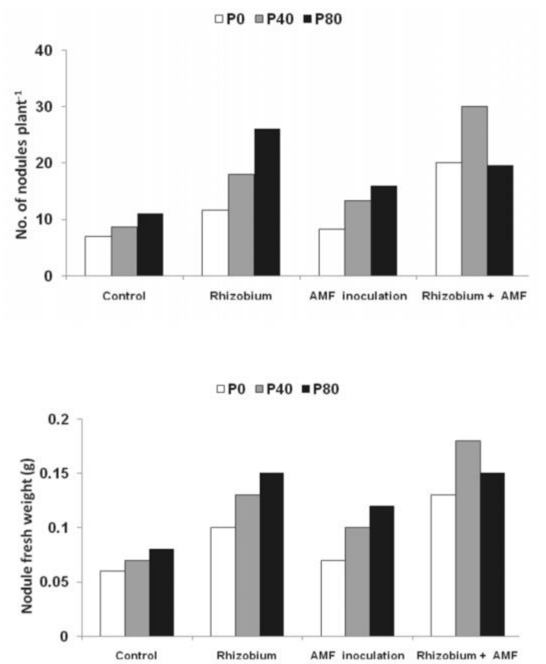

Fig. 3. Nodulation of soybean under differentially P fertilized single or dual inoculated treatments. A - Nodule number per plant; B - Nodule weight per plant

AMF with *Rhizobium*. The interaction of mycorrhizae and *Rhizobium* revealed that higher number of nodules and the nodule fresh weight were higher in the P_{40}. Similarly, significant nodulation in different pulse crops by *Rhizobium* and mycorrhizal fungal combination. Higher number of nodules due to inoculation with various strains of Bradhyrhizobia. The presence of a mycorrhizal association had a positive impact on nutrient uptake and biomass production.

Soybean yield was significantly higher in *Rhizobium* and VAM inoculated treatments than control (Fig.4). The results clearly indicated that tripartite association resulted in enhanced plant

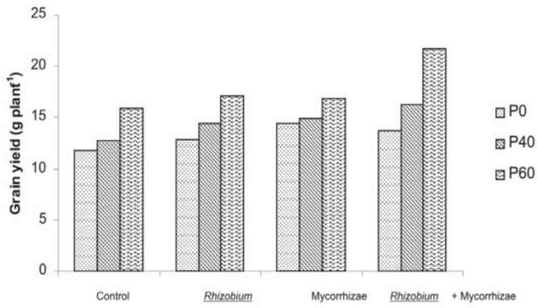

Fig. 4. Grain yield of soybean under tripartite association

growth which resulted in increased yield. Similar results were obtained where the groundnut yield increased due to the inoculation of *Rhizobium* and VAM. This increased yield could be correlated with the higher number of nodules, availability of P and enhanced enzymatic activity in the plant.

Conclusion

Overall the data suggest that *Rhizobium*-mycorrhiza-soybean tripartite association assists in the acidification of the rhizosphere by enhanced acid phosphatase activity that facilitate availability of P besides stimulated the nitrate reductase activity. These biochemical changes collectively contribute for the higher biomass productivity and grain yield of soybean.The response to mycorrhizal colonization increased with the addition of P especially when native soil is extremely deficient in phosphorous status.

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