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Dual inoculation with *Rhizobium* sp. and *Glomus fasciculatum* enhances nodulation, yield and nitrogen fixation in chickpea (*Cicer arietinum* Linn.)

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Summary Seed inoculation with Rhizobium and soil inoculation with *Glomus fasciculatum* increased nodulation, nitrogen and phosphorus concentration in plants and yield of chickpea (*Cicer arietinum*) var. BG 212 in pots containing unsterilized soil especially wih 50 kg P_2O_5 ha^{-1} in the form of superphosphate.

Inoculation with Rhizobium or *G. fasciculatum* separately or in combination significantly increased the N_2 fixed in straw and grain than uninoculated controls as determined by ^{15}N atom percent excess of plants grown in soil amended with labelled ammonium sulphate $(^{15}NH_4)_2SO_4$) at the rate of 20 kg N ha^{-1}. These increases were most pronounced when P was applied at 50 kg P_2O_5 ha^{-1}.

Introduction

The vesicular-arbuscular mycorrhizal (VAM) fungi are known to increase nutrient uptake, particularly phosphorus to the host plant[7,12,15,19], which in turn is known to stimulate nitrogen fixation[13]. Synergistic effects of inoculation of VAM with rhizobia in soybean[2], lucerne[17], clovers[1,8], french beans[3], groundnut[11], Pueraria and Stylosanthes[20] and cowpea[9] have also been reported. The present report deals with the results of pot experiments to assess the effects of inoculating seeds of chickpea with *Rhizobium* sp. and soil with a VAM fungus (*Glomus fasciculatum*) on nodulation, yield and N and P concentration in plants. The amount of nitrogen fixed in grain and straw of the legume was determined by estimating ^{15}N atom percent excess using isotopially labelled ammonium sulphate, to determine if *G. fasciculatum* with or without Rhizobium would enhance nitrogen fixation.

Materials and methods

Unsterilized farm soil of a sandy-loam nature from the fields of the Indian Agricultural Research Institute, New Delhi was used. The previous crops grown in this soil were wheat (*Triticum aestivum* Linn.) and maize (*Zea mays* Linn.) during winter and rainy seasons, respectively. It had the following composition: Total organic C – 0.52%, total N – 0.04%,

available P by Olsen method[10] was 5 ppm and pH 7.0. The soil was sieved through 5 mm mesh and distributed in 30 cm dia pots at the rate of 10 kg soil per pot. The plants were grown in a glasshouse receiving sunlight for 10 hr each day and were irrigated with tap water. The temperature in the glasshouse during the experiment ranged from 4°C (min) to 35°C (max).

Nitrogen was applied in the form of isotopically labelled $(^{15}NH_4)_2SO_4$ at the rate of 20 kg N ha^{-1} (5% atom excess), whereas phosphorus was applied at the rate of 50 kg P_2O_5 ha^{-1} (21.8 kg P ha^{-1}) in the form of superphosphate on soil weight basis. Both the fertilizers were applied at the rates of 0.47 g of $(NH_4)_2SO_4$ and 1.56 g of superphoshate per pot at the time of sowing and uniformly mixed with soil.

A soil:sand (1:1) mixture containing extramatrical chlamydospores and infected root segments of pearlmillet (*Pennisetum americanum* (Linn.)) Leeke infected with *Glomus fasciculatum* (Thaxter sensu Gerdeman) Gerdemann et Trappe obtained from Dr K R Krishna, International Crops Research Institute for Semi-Arid Tropics, Hyderabad, India, and grown for 90 days served as the inoculum. The purity of the inoculum was checked and it contained 350 chlamydospores $100\,g^{-1}$ soil. A thin layer of inoculum (200 ml) was placed 2 cm below the soil surface in pots before sowing to produce the mycorrhizal plants.

Seeds of chickpea (*Cicer arietinum* Linn.) var. BG 212 were treated with carrier-based (soil and charcoal in 1/3 : 2/3 proportions) inoculant of *Rhizobium* sp. (strains C-1 and C-2) having a population of 6.5×10^9 cells/g air dry carrier at the rate of 20 g/100 g seed. The seeds were sown immediately. Four plants were grown in each pot. The control treatment received neither VAM inoculum nor the seed inoculation with *Rhizobium* sp.

The experiment was laid-out in a simple randomized block design consisting of 4 inoculation treatments and two levels of nitrogen (0 and 20 kg N ha^{-1}) and two levels of phosphorus (0 and 50 kg P_2O_5 ha^{-1}) each. Thus in all there were 16 treatments and each treatment was replicated 6 times. Three replicates were harvested at 45 days of plant growth, while the remaining three replicates were harvested at the time of plant harvest (130 days after sowing). Critical difference (C.D.) was calculated for treatments.

Percentage mycorrhizal infection was determined after 45 days of plant growth by the slide technique[4]. The root samples were cut into small segments approximately 1 cm. They were then floated in water in a dish and number of segments, varying from 100–150 depending on the size of the sample, were selected randomly. They were cleared with 10% KOH and stained with trypen-blue lactophenol[16]. All the infected and uninfected root segments were counted and the percent root colonization was calculated as follows:

$$\frac{\text{Number of VAM positive segments}}{\text{Total number of segments scored}} \times 100$$

The data on nodulation (number and dry weight of nodules) and dry weight of shoot were recorded at 45 days of plant growth. Total N and P contents in plants were estimated by kjeldahl and vanado-molybdate methods, respectively, at 45 days of plant age[8]. The yield (grain and straw) was recorded at the time of plant maturity (130 days of plant growth).

Nitrogen fixed in grain and straw was estimated by the determination of ^{15}N atom percent excess and total nitrogen yield using labelled ammonium sulphate $(^{15}NH_4)_2SO_4$. Wheat (*Triticum aestivum* Linn.) cv Sonalika was selected as a standard non-legume plant grown under conditions similar to chickpea experimental plants with 20 kg N ha^{-1} (5% atom excess $(^{15}NH_4)_2SO_4$). Wheat has physiological similarities to chickpea in terms of its growth period and climatic adaptation. The average ^{15}N atom percent excess in grain and straw of wheat at the time of plant maturity (130 days after sowing) were 0.25 and 0.46, respectively. The total nitrogen fixed by chickpea was calculated according to the following formula:

$$\text{Total } N_2 \text{ fixed} = 1 - \frac{\%\ ^{15}N \text{ atom excess (legume)}}{\%\ ^{15}N \text{ atom excess (non-legume wheat)}} \times \text{Total N yield}$$

Table 1. Intensity of root infection of chickpea cv. BG 212 in relation to inoculation with Rhizobium and *Glomus fasciculatum* at 45 days after sowing (mean of 4 replications)

Treatments	0 kg P_2O_5 ha^{-1}		50 kg P_2O_5 ha^{-1}		Mean (treatments)
	N_0	N_{20}	N_0	N_{20}	
Uninoculated control	15.5	20.5	25.0	28.5	22.4
Rhizobium sp.	20.5	25.5	28.5	28.0	25.6
Glomus fasciculatum	35.0	39.5	42.5	48.0	41.3*
Rhizobium × *G. fasciculatum*	55.0	50.5	60.5	65.0	57.8*
Mean – Nitrogen – 0 kg N ha^{-1}			35.3		
20 kg N ha^{-1}			38.2		
Phosphorus – 0 kg P_2O_5 ha^{-1}			32.8		
50 kg P_2O_5 ha^{-1}			40.8*		
Critical difference (C.D.) at 5%	5.85				

* Significant increase at 5% level over uninoculated control.

Results

Root infection by G. fasciculatum

Root infection by *G. fasciculatum* at 45 days of plant growth was more with combined inoculation of Rhizobium + *G. fasciculatum* than soil inoculation with *G. fasciculatum* alone. Not much variation was noticed between uninoculated control and seed treatment with *Rhizobium* sp. The uninoculated controls also showed the root infection by mycorrhiza which could be attributed to the presence of native VA-endophytes in the soil.

Application of phosphatic fertilizer at the rate of 50 kg P_2O_5/ha brought about increased mycorrhizal infection in root over control. Nitrogenous fertilizer application at 20 kg N/ha did not bring about any additive effect on mycorrhizal infection. However, the interaction of N and P fertilizers when 20 kg N ha^{-1} and 50 kg P_2O_5 ha^{-1} was given with *G. fasciculatum* resulted in maximum mycorrhizal root infection (Table 1).

Nodulation, dry matter, nitrogen and phosphorus content of plants

Soil inoculation with *G. fasciculatum* alone improved nodulation (number and dry weight of nodules), dry matter content and N concentration of shoots and was almost equivalent to the effects of seed inoculation with Rhizobium. Application of 20 kg N ha^{-1} did not influence the number and weight of nodules, dry matter and N content of shoots at 45 days of plant growth, but the addition of 50 kg P_2O_5 ha^{-1} resulted in appreciable improvement in all these parameters over

Table 2. Dual inoculation effect on chickpea (*Cicer arietinum*) at 45 days of plant growth (mean of 4 replicate pots – each pot having 4 plants)

Treatment	No. of nodules per pot	Dry wt. of nodules per pot (g)	Dry wt. of shoot per pot (g)	Nitrogen content of shoot (%)	Phosphorus content of shoot (mg/g)
0 kg P_2O_5 ha^{-1}					
Without N					
Uninoculated control	55	0.12	3.3	2.9	1.95
Glomus fasciculatum	61*	0.20*	3.3	3.0	2.72*
Rhizobium sp.	58*	0.21*	3.5	3.0	2.70*
Rhizobium + *G. fasciculatum*	70*	0.26*	3.6*	3.3*	2.73*
With N (20 kg N ha^{-1} ($^{15}NH_4)_2SO_4$)					
Uninoculated control	53	0.13	3.5	2.9	2.00
G. fasciculatum	66*	0.21*	4.8*	3.4*	2.25
Rhizobium sp.	60*	0.22*	4.2*	3.4*	2.25
Rhizobium + *G. fasciculatum*	68*	0.29*	5.2*	3.4*	2.70*
50 kg P_2O_5 ha^{-1}					
Without N					
Uninoculated control	71	0.24	3.2	3.0	2.00
Glomus fasciculatum	82*	0.41*	3.5*	3.0	3.47*
Rhizobium sp.	83*	0.38*	3.4	3.3	3.20*
Rhizobium + *G. fasciculatum*	89*	0.47*	4.9*	3.3	4.35*
With N (20 kg N ha^{-1} ($^{15}NH_4)_2SO_4$)					
Uninoculated control	58	0.33	4.5	3.2	2.70
G. fasciculatum	78*	0.42*	4.7	3.3	3.97*
Rhizobium sp.	58	0.34	4.6	3.1	3.47*
Rhizobium + *G. fasciculatum*	83*	0.50	5.0*	3.5	4.67*
Critical difference at 5%	2.5	0.65	0.25	0.40	0.65

* Significant increase at 5% level over corresponding uninoculated control.

0 kg P_2O_5 ha^{-1} level, especially with dual inoculation of Rhizobium and *G. fasciculatum*. Soil application of *G. fasciculatum* alone increased the phosphorus concentration in shoots although dual inoculation with Rhizobium and *G. fasciculatum* registered the highest P status, especially at $N_{20}P_{50}$ levels of fertilizer application (Table 2). Although there was nodulation in uninoculated plants, these nodules were not effective as evident from data on dry weight (Table 2).

Yield and nitrogen fixed in grain

Seed inoculation with *Rhizobium* sp. or soil inoculation with *G. fasciculatum* increased the grain yield of chickpea over uninoculated control, especially in the presence of 20 kg N ha^{-1} and combined

Table 3. Grain yield and nitrogen fixation in grain of chickpea (*Cicer arietinum*) as influenced by dual inoculation at 130 days of plant growth (mean of 4 replicate pots – each having 4 plants)

Treatment	Grain yield g/pot	Increase In yield over uninoculated control (%)	^{15}N atomic excess (%)	Nitrogen yield (mg/pot)	N_2-fixed in grain (mg/pot)
0 kg P_2O_5 ha^{-1}					
Without N					
Uninoculated control	3.2	–	–	–	–
Glomus fasciculatum	5.4*	68.8	–	–	–
Rhizobium sp.	6.5*	103.1	–	–	–
Rhizobium + G. fasciculatum	8.5*	165.6	–	–	–
With N (20 kg N ha^{-1} ($^{15}NH_4$)$_2SO_4$)					
Uninoculated control	4.4	–	0.130	134.6	58.7
G. fasciculatum	7.1*	61.4	0.141	220.0	95.1*
Rhizobium sp.	7.4*	68.2	0.127	224.8	108.8*
Rhizobium + G. fasciculatum	8.7*	97.7	0.133	240.7	111.7*
Critical difference at 5%	–	grain yield: 1.2;			
50 kg P_2O_5 ha^{-1}					
Without N					
Uninoculated control	6.3	–	–	–	–
Glomus fasciculatum	6.2	–	–	–	–
Rhizobium sp.	8.0*	27.0	–	–	–
Rhizobium + G. fasciculatum	9.3*	47.6	–	–	–
With N (20 kg N ha^{-1} ($^{15}NH_4$)$_2SO_4$)					
Uninoculated control	6.4	–	0.112	205.8	119.1
G. fasciculatum	8.8*	37.5	0.116	278.1	147.1*
Rhizobium sp.	8.8*	37.5	0.105	293.9	170.4*
Rhizobium + G. fasciculatum	11.7*	82.8	0.108	326.3	184.3*
Critical difference at 5%	–	N_2 fixed in grain: 12.5			

* Significant increase at 5% level over uninoculated control.

inoculation with both the organisms significantly enhanced grain yield. Application of phosphorus was found to be beneficial to yield in conjunction with 20 kg N ha^{-1}, especially with dual inoculation with both the organisms at $N_{20}P_{50}$ level, amounting to 82.8 percent. The total nitrogen fixed in the grain was higher with dual inoculation at $N_{20}P_{50}$ level of fertilizer application when compared with either Rhizobium or *G. fasciculatum* inoculation (Table 3).

Table 4. Straw yield and nitrogen fixation in straw of chickpea (*Cicer arietinum*) as influenced by dual inoculation at 130 days of plant growth (mean of 4 replicate pots – each pot having 4 plants)

Treatment	Straw yield (g/pot)	Increase in yield over uninoculated control	^{15}N atomic excess (%)	Nitrogen yield (mg/pot)	Total N_2 fixed in straw (mg/pot)
0 kg P_2O_5 ha^{-1}					
Without N					
Uninoculated control	6.9	–	–	–	–
Glomus fasciculatum	6.9	–	–	–	–
Rhizobium sp.	7.8	13.0	–	–	–
G. fasciculatum + *Rhizobium*	7.8	13.0	–	–	–
With N (20 kg N ha^{-1} ($^{15}NH_4)_2SO_4$)					
Uninoculated control	7.6	–	0.152	89.6	59.5
G. fasciculatum	8.5	11.8	0.173	73.6	46.2
Rhizobium sp.	9.5	25.0	0.179	104.0	62.1
Rhizobium + *G. fasciculatum*	10.6*	39.5	0.199	99.6	55.6
Critical difference at 5%	–	Straw yield: 2.8;			
50 kg P_2O_5 ha^{-1}					
Without N					
Uninoculated control	8.1	–	–	–	–
Glomus fasciculatum	10.1	23.5	–	–	–
Rhizobium sp.	10.9*	34.6	–	–	–
G. fasciculatum + *Rhizobium*	11.5*	42.0	–	–	–
With N (20 kg N ha^{-1} ($^{15}NH_4)_2SO_4$)					
Uninoculated control	8.8	–	0.101	69.5	54.4
G. fasciculatum	13.5*	53.4	0.174	96.3	70.1
Rhizobium sp.	11.4	29.5	0.107	112.6	74.5*
Rhizobium + *G. fasciculatum*	15.9*	80.7	0.208	153.6	86.6*
Critical difference at 5%	–	N_2 fixed in straw: 16.5			

* Significant increase at 5% level over uninoculated control.

Straw yield and nitrogen fixed in straw

Inoculation with Rhizobium or VA-mycorrhizal fungus alone increased the straw yield of chickpea over uninoculated control at 0 and 20 kg N ha^{-1} and at 0 and 50 kg P_2O_5 ha^{-1} levels. At these levels of fertilizer no significant difference existed between either Rhizobium or *G. fasciculatum* treatments. However, dual inoculation with both the organisms enhanced straw yield and the maximum increase was noticed with Rhizobium + *G. fasciculatum* treatment at $N_{20}P_{50}$ level. Maximum nitrogen fixation in straw was seen at $N_{20}P_{50}$ level with Rhizobium +

G. fasciculatum treatment followed by Rhizobium and *G. fasciculatum* treatments. At this level of fertilizer application, there was little difference between *G. fasciculatum* and Rhizobium treatments. In the absence of phosphatic fertilizer there was no significant difference between treatments as far as the N_2 fixation in straw of chickpea was concerned (Table 4).

Discussion

The results obtained by this study on dual inoculation with *Rhizobium* sp. and *Glomus fasciculatum* show that nodulation, growth, yield and nitrogen and phosphorus contents of chickpea are enhanced by the VAM fungus, especially with the application of 50 kg P_2O_5 ha^{-1} 21.8 kg P ha^{-1}) in the form of superphosphate. Soil inoculation with *G. fasciculatum* resulted in a significant increase in root infection by VAM fungus over uninoculated control. The effect was marked particularly in association with *Rhizobium* sp. Rhizobial inoculation is known to increase the yields of several legumes by way of increasing the nodulation and the biomass of root and shoot[6,18], and the increased root system might have facilitated VA-endophyte to colonize more effectively.

The use of isotopic nitrogen to quantitatively measure symbiotically fixed nitrogen appears to be the most promising technique[5]. Under field conditions where environmental factors cannot be regulated, it is practical to apply labelled N source in the form of isotopically labelled ammonium sulphate ($(^{15}NH_4)_2SO_4$) to the soil and determining nitrogen fixation by dilution of the labelled nitrogen taken up by the plant from the soil and labelled source by the unlabelled atmospheric nitrogen that becomes fixed. This requires a non-fixing plant as a control and wheat was selected for this purpose in the present study to determine the isotopic composition of the nitrogen taken up from the labelled source and from the soil without dilution by atmospheric nitrogen. The major assumptions are that the applied source of nitrogen in the soil are absorbed in the same ratio by the fixing and non-fixing plants, the isotopic composition remains the same and the fixation by the legume does not alter the availability of soil nitrogen[5]. However, the root exudates of both fixing and non-fixing plants differ which may alter the density of diazotrophs in the rhizosphere of both the plants and this in turn may affect the calculation of the symbiotically fixed nitrogen. Notwithstanding the aforesaid limitations, by using this technique, it was noted in the present study that N_2 fixation in grain and straw (as revealed by the determination of ^{15}N atom

percent excess), also increased by inoculation with *G. fasciculatum* with or without Rhizobium, thereby providing definitive evidence for the enhancement of N_2 fixation by dual inoculation. It is noteworthy to point out that *G. fasciculatum* alone enhanced nodulation and N_2 fixation in grain and straw to a marginal extent, which could be attributed to an undetermined secondary effect leading to better N_2 fixation[14].

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