

Dual Inoculation of Native *Rhizobium* spp. and Arbuscular Mycorrhizal Fungi: An Impact Study for Enhancement of Pulse Production

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(Received July 31, 2004)

Fifteen *Rhizobium* spp. from nodules of 6 common pulses collected from 6 districts of Assam were studied for their infectivity, intrinsic antibiotic resistance, nitrogenase activity and effect of dual inoculation with two native Arbuscular Mycorrhizal Fungi viz. *Glomus mosseae* (GM) and *Gigaspora gilmarie* (GG). Out of the 15 isolates 9 were found nodulation positive and 6 of them (AR1, BR8, BR12, AR10, UR10 & GR21) were subjected to intrinsic antibiotic sensitivity test of which AR1 showed resistance against all the 9 test antibiotics. Isolates AR1 and GR21 showed the highest (4.25 mole, gm⁻¹hour⁻¹) and the lowest (1.05 mole, gm⁻¹hour⁻¹) nitrogenase activity respectively. In Most Probable Number count, the maximum *Rhizobium* population 5.8×10⁵, was found in both Blackgram and Greengram variety of pulses. The maximum dry weight of nodules (3.14 g), dry weight of shoot (10.08 g), nitrogen content (7.68 mg, plant⁻¹), chlorophyll content (1.89 mg, g⁻¹), phosphorus content of shoot (6.17 mg, g⁻¹) and yield (535.67 kg, Ha⁻¹) were found when AR1 dually inoculated with GM in Blackgram.

KEYWORDS: Arbuscular mycorrhizal fungi, Dual inoculation, *Rhizobium*

Rhizobium has already been recognized as promoter of pulse production up to 30% (Subba Rao *et al.*, 1999). However, in convenient combination with *Arbuscular mycorrhizal* fungi (AM Fungi) the production increases up to 50% (Lukiwati *et al.*, 1995; El Ghandour *et al.*, 1996). Blending of *Rhizobium* with Mycorrhiza improve plant growth through increased availability of phosphorus together with higher nitrogen fixation in soil (Tilak *et al.*, 1992; Subba Rao *et al.*, 1999). Increase of pulse production in combination of *Rhizobium* and mycorrhizal fungi was also reported by workers from India and elsewhere (Singh *et al.*, 1993; Azcon *et al.*, 1993; Dixon *et al.*, 1993; Barthakur *et al.*, 1997; Kumar *et al.*, 2001, Ammani, 2002). This combination can be taken as cheapest way to enrich tropical soil with nitrogen and phosphorus, necessary for enhancement of pulse production in the region. Thus, the present investigation aims at to isolate and characterize the local species of *Rhizobium* and also to evaluate them in dual inoculation with native AM fungi with or without organic blending. So, it is expected to enhance pulse production in the North Eastern Region of India where this commodity is highly deficient to meet the current demand.

Materials and Methods

Species. Fifteen *Rhizobium* species and two AM fungal species used in the experiments were listed in Table 1.

Plant Materials. Greengram (*Phaseolus aureus*, Roxb.)

and Blackgram (*Phaseolus mungo*, Roxb.) were treated as the test pluses in all the experiments.

Antibiotics. Nine antibiotics viz. Streptomycin (ST), Kanamycin (K), Penicillin (P), Nalidixic acid (NA), Neomycine (N), Tetracyclin (T), Ampicilin (AP), Erythromycine (E), Chloramphenicol (C) from Nova Biotech Ltd. Calcutta, India were used as antibiotic markers in intrinsic antibiotics tests.

Species of rhizobia and AM fungi. *Rhizobium* spp. were obtained following the procedures of Vincent (1970) and Somasegaran and Hoben (1985), while AM Fungi were isolated, according to wet-sieving method (Gerde-mann and Nicolson, 1963; Mosse, 1973; Powell and Bag-yaraj, 1991) with graded sieves and identified according to *Mycorrhizal* manual (Schenck and Pervez, 1987, Bag-yaraj *et al.*, 1991).

Leonard jar test. Infectivity test of *Rhizobium* spp. was conducted with Leonard jar assembly according to Soma-segaran and Hoben (1985). This experiment was conducted using fifteen *Rhizobium* spp. with 3 replicates as mentioned earlier with control (both nitrogen-deficient and uninoculated).

MPN count. The most probable number (MPN) technique was conducted (Somasegaran and Hoben, 1985) to study the effect of host plants on *Rhizobium* population. Ten fold serial dilutions of *Rhizobium* spp. were prepared for all the soils. The seedlings were observed for nodula-

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Table 1. Species of *Rhizobium* and AM fungi used in this study

Rhizobium species	Source	Place (Districts)
AR1	Arhar (<i>Cajanus cajan</i> Mill.)	Kamrup
LR5	Lentil (<i>Lens culinaris</i> Medic.)	Nalbari
LR1	Lentil (<i>Lens culinaris</i> Medic.)	Kamrup
BR5	Black gram (<i>Phaseolus mungo</i> Roxb.)	Nalbari
BR8	Black gram (<i>Phaseolus mungo</i> Roxb.)	Nagaon
BR12	Black gram (<i>Phaseolus mungo</i> Roxb.)	Sonitpur
PR12	Pea (<i>Pisum sativum</i> L.)	Sonitpur
GR13	Green gram (<i>Phaseolus aureus</i> Roxb.)	Lakhimpur
AR10	Arhar (<i>Cajanus cajan</i> Mill.)	Sonitpur
UR10	Urdbean (<i>Dlichos labab</i> L.)	Sonitpur
BR13	Black gram (<i>Phaseolus mungo</i> Roxb.)	Lakhimpur
PR7	Pea (<i>Pisum sativum</i> L.)	Nagaon
PR16	Pea (<i>Pisum sativum</i> L.)	Jorhat
GM16	Green gram (<i>Phaseolus aureus</i> Roxb.)	Jorhat
GR21	Green gram (<i>Phaseolus aureus</i> Roxb.)	Kamrup
<i>AMF</i> species		
<i>Glomus mosseae</i>	Rice field soils	Kamrup
<i>Gigaspora gilmarie</i>	Rice field soils	Kamrup

tion up to six weeks.

Nitrogenase activity. The nitrogenase activity was determined by acetylene reduction method with Gas Liquid Chromatography technique (Somasegaran and Hoben, 1985).

Intrinsic antibiotic sensitivity test. Five discs, containing 10 µg each of the test antibiotics were placed in five positions of the 6 petri plates (6 cm) containing YEMA media (Somasegaran and Hoben, 1985) each plate inoculated with 6 highly nodulated *Rhizobium* spp. (AR1, BR8, BR12, AR10, UR10 & GR21) and incubated at 28±1°C. The experiment was replicated three times.

Dual inoculum. *Rhizobium* inocula were produced as per standard procedure (Somasegaran and Hoben, 1985). Inoculum production of AM Fungi was done by root organ culture technique in onion, grown on pots as per procedure of Subba Rao, 1993. Onion roots were harvested at maturity and preserved aseptically maintaining the moisture content (80%), temperature (25~35°C) and pH 7 with or without carrier material for 6 months.

For dual inoculation assay, plot size was taken 2 m×1 m where row-to-row spacing was 30 cm, plant-to-plant spacing was 20 cm and the seed rate was 25 kg, ha⁻¹. The experiment was done with fully randomized block design and replicated three times. The observations were made at different periods except the production, which was taken at harvest.

Results

Table 1 presented fifteen numbers of *Rhizobium* spp. viz.

AR1, LR5, LR1, BR5, BR8, BR12, PR12, GR13, AR10, UR10, BR13, PR7, GM16, PR16 and GR21 and two AM Fungi viz. *Glomus mosseae* (GM) and *Gigaspora gilmarie* (GG) with their sources and places of collection while in Table 2 it was observed that only nine test *Rhizobium* species viz. AR1, LR1, BR8, BR12, AR10, UR10, PR16, GM16 & GR21 could produce nodules in Blackgram and Greengram, while the other six could not. However, the highest nodule number in Blackgram (95) and Greengram (125) was produced by the species AR1,

Table 2. Nodulation test of *Rhizobium* species in Leonard jar with Blackgram and Greengram

Species	Nodule No. in Blackgram	Nodule No. in Greengram
AR1	95.00±2.89	125.00±2.89
LR5	0.00±0.00	0.00±0.00
LR1	87.00±0.58	118.67±1.86
BR5	0.00±0.00	0.00±0.00
BR8	68.67±0.88	102.67±1.45
BR12	74.67±2.91	114.67±2.19
PR12	0.00±0.00	0.00±0.00
GR13	0.00±0.00	0.00±0.00
AR10	65.33±2.91	72.00±4.73
UR10	39.33±2.96	42.00±2.89
BR13	0.00±0.00	0.00±0.00
PR7	0.00±0.00	0.00±0.00
PR16	37.33±1.45	47.00±1.15
GM16	29.67±2.40	37.67±1.76
GR21	55.00±1.15	79.00±2.08
Control	0.00±0.00	0.00±0.00

*=mean of 3 replications mean±SE.

CD (at 5% level) for Black Gram 4.794 CD (at 5% level) for Green Gram 5.489.

Table 3. MPN count of 6 *Rhizobium* species

Rhizobium species	No. of <i>Rhizobium</i> per gm of soil		
	Before growing	After growing	
	Black gram & Green gram	Black gram	Green gram
AR1	3.1×10 ⁵	5.8×10 ⁵	5.8×10 ⁵
BR8	5.8×10 ³	3.1×10 ⁴	5.8×10 ⁴
BR12	1.7×10 ⁴	3.1×10 ⁵	3.1×10 ⁵
AR10	3.1×10 ³	5.8×10 ⁴	5.8×10 ⁴
UR10	3.1×10 ⁴	1.7×10 ⁵	3.1×10 ⁵
GR21	1.7×10 ⁴	3.1×10 ⁵	5.8×10 ⁵

which differs significantly with the other species. Nodule numbers were recorded after 45~55 days of sowing. Table 3 presented the population of *Rhizobium* in the field both before and after growing of the test pulses. The maximum (5.8×10⁵) *Rhizobium* population were achieved by spp. AR1 while the minimum (3.1×10⁴) was by species BR8 and BR12 in Blackgram and Greengram respectively. To study the effect of dual inoculation of *Rhizobium* and AM Fungi, a field experiment was conducted with Blackgram and Greengram to determine dry weight of nodules, dry weight of shoot, nitrogenase activity, nitro-

gen, chlorophyll and phosphorus content along with the yield. Table 4 presented the effect of dual inoculation of six *Rhizobium* species. with GM on the growth and production of Blackgram. The maximum dry weight of nodules (3.14 g), shoot dry weight (10.08 g), nitrogen & chlorophyll content (7.68 mg, plant⁻¹; 1.89 mg, plant⁻¹), phosphorus content of shoot (6.17 mg, plant⁻¹) and yield (535.67 kg, Ha⁻¹) were observed with the species AR1 with GM. Table 5 presented the effect of dual inoculation of GG and the *Rhizobium* species on the above parameters including production of Blackgram. All the parameters were found superior over the control and the single application of GG. Dual inoculation of AR1 with GG showed the best results for nodule dry weight (3.84 g), shoot dry weight (6.30 g), nitrogen & chlorophyll content (8.36 mg, plant⁻¹; 1.78 mg, g⁻¹), phosphorus content on shoot (7.41 mg, g⁻¹) and also for the yield (527.33 kg, Ha⁻¹). Table 6 presented the effect of dual inoculation of GM and the *Rhizobium* species on growth and production of Greengram. All the parameters showed best results over the control and over single inoculation with GM. The maximum nodule dry weight (2.99 g), shoot dry weight (9.59 g), nitrogen & chlorophyll content (7.78 mg, plant⁻¹; 1.93 mg, plant⁻¹) phosphorus content of shoot (6.33 mg,

Table 4. Effect of dual inoculation of six *Rhizobium* species and *Glomus mosseae* on growth and yield of Blackgram

Species	Nodule dry weight (in gm)	Shoot dry weight (in gm)	N-content mg plant ⁻¹	Total chlorophyll content (mgg ⁻¹ fresh weight)	Phosphorus content in shoot (mgg ⁻¹)	Yield (kgHa ⁻¹)
Control	1.44±0.13	7.16±0.04	4.27±0.22	1.38±0.04	3.60±0.19	307.33±1.86
GM	1.55±0.07	7.00±0.29	4.33±0.34	1.47±0.01	4.64±0.15	366.33±17.17
AR1+GM	3.14±0.09	10.08±0.09	7.68±0.04	1.89±0.05	6.17±0.16	535.67±8.99
BR8+GM	2.76±0.14	8.84±0.60	7.40±0.15	1.76±0.03	5.92±0.22	494.67±5.36
BR12+GM	2.30±0.15	8.22±0.33	6.70±0.12	1.63±0.03	5.57±0.29	449.67±10.73
AR10+GM	2.11±0.06	7.59±0.36	5.77±0.15	1.54±0.04	5.10±0.20	417.00±17.56
UR10+GM	3.03±0.04	9.26±0.38	7.14±0.30	1.72±0.04	6.01±0.41	517.33±17.34
GR21+GM	2.19±0.13	7.04±0.26	5.77±0.15	1.70±0.05	5.00±0.17	367.33±32.20
CD (at 5% level)	0.330	1.087	0.609	0.115	0.086	49.395

GM=*Glomus mosseae*.

*=mean of 3 replications mean±SE.

Table 5. Effect of dual inoculation of six species of *Rhizobium* and *Gigaspora gilmarie* on growth and yield of Black Gram

Species	Nodule dry weight (in gm)	Shoot dry weight (in gm)	N-content mg plant ⁻¹	Total chlorophyll content (mgg ⁻¹ fresh weight)	Phosphorus content in shoot (mgg ⁻¹)	Yield (kgHa ⁻¹)
Control	1.94±0.04	5.19±0.27	5.18±0.30	1.57±0.18	2.18±0.09	273.00±13.32
GG	2.62±0.11	5.34±0.10	6.40±0.10	1.49±0.09	3.68±0.34	319.33±9.94
AR1+GG	3.84±0.07	6.30±0.31	8.36±0.14	1.78±0.17	7.41±0.13	527.33±8.97
BR8+GG	3.43±0.25	5.77±0.62	8.17±0.31	1.59±0.11	6.03±0.34	512.33±14.38
BR12+GG	2.47±0.04	5.98±0.37	6.68±0.34	1.70±0.17	5.71±0.28	426.00±8.08
AR10+GG	2.39±0.06	5.27±0.03	7.45±0.29	1.54±0.04	4.68±0.29	374.67±27.09
UR10+GG	2.39±0.04	6.29±0.30	8.28±0.08	1.53±0.06	6.70±0.33	491.00±30.41
GR21+GG	2.42±0.06	5.30±0.04	7.22±0.48	1.47±0.03	5.75±0.25	381.67±36.35
CD (at 5% level)	0.319	0.944	0.857	1.57	0.291	63.721

GG=*Gigaspora gilmarie*.

*=mean of 3 replications mean±SE.

Table 6. Effect of six *Rhizobium* species and dual inoculation of *Glomus mosseae* on growth and yield of Greengram

Species	Nodule dry weight (in gm)	Shoot dry weight (in gm)	N-content mg plant ⁻¹	Total chlorophyll content (mgg ⁻¹ fresh weight)	Phosphorus content in shoot (mgg ⁻¹)	Yield (kgHa ⁻¹)
Control	1.38±0.19	6.52±0.28	4.15±0.30	1.30±0.00	3.60±0.19	302.33±1.86
GM	1.42±0.01	7.28±0.40	4.10±0.12	1.46±0.03	4.38±0.24	364.00±17.62
AR1+GM	2.99±0.01	9.59±0.20	7.78±0.12	1.93±0.04	6.33±0.27	532.67±9.62
BR8+GM	2.37±0.19	8.61±0.31	7.70±0.15	1.33±0.09	5.72±0.39	480.00±13.43
BR12+GM	2.18±0.14	8.49±0.41	6.47±0.26	1.64±0.04	5.72±0.38	448.67±11.70
AR10+GM	2.33±0.13	7.59±0.36	5.53±0.23	1.40±0.10	5.03±0.18	446.00±37.64
UR10+GM	2.89±0.22	9.31±0.42	7.23±0.32	1.79±0.07	5.50±0.41	517.33±17.34
GR21+GM	2.04±0.23	6.83±0.44	5.70±0.21	1.67±0.07	5.03±0.18	381.33±41.45
CD (at 5% level)	0.48	1.08	0.67	0.19	0.88	68.41

GM=*Glomus mosseae*.

* = mean of 3 replications mean±SE.

Table 7. Effect of dual inoculation of six species of *Rhizobium* and *Gigaspora gilmarie* on growth and yield of greengram

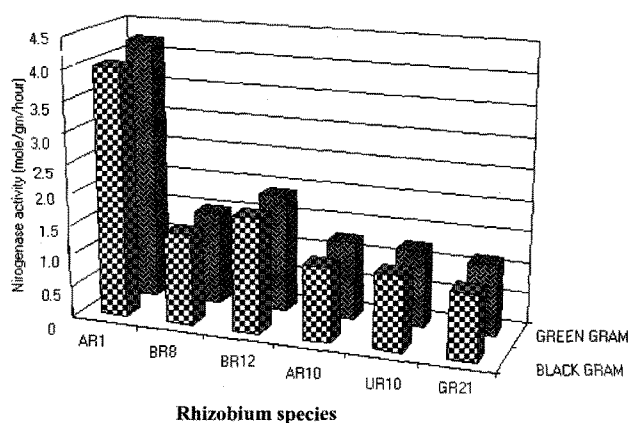
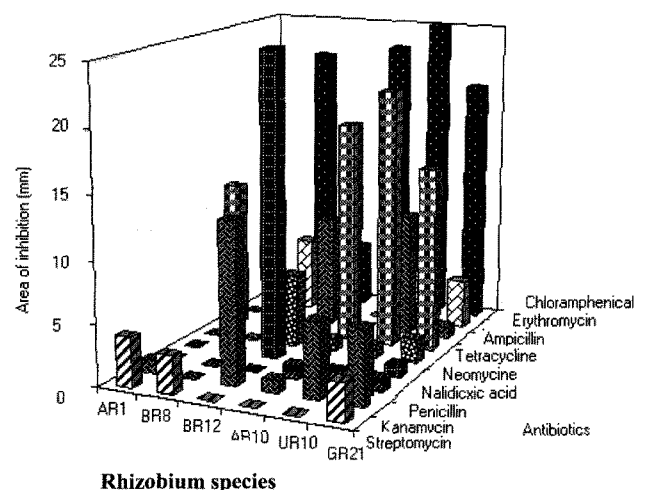
Species	Nodule dry weight (in gm)	Shoot dry weight (in gm)	N-content mg plant ⁻¹	Total chlorophyll content (mgg ⁻¹ fresh weight)	Phosphorus content in shoot (mgg ⁻¹)	Yield (kgHa ⁻¹)
Control	1.80*±0.08	4.88±0.47	4.92±0.14	1.58±0.18	2.17±0.10	269.33±9.77
GG	2.47±0.12	5.39±0.08	6.30±0.04	1.56±0.04	3.57±0.40	321.67±6.36
AR1+GG	3.48±0.25	6.18±0.26	8.16±0.10	1.71±0.16	7.28±0.19	524.67±8.19
BR8+GG	3.38±0.22	5.44±0.30	8.15±0.28	1.60±0.10	5.96±0.48	493.00±24.70
BR12+GG	2.40±0.02	6.00±0.39	6.56±0.41	1.47±0.03	5.48±0.37	417.67±9.39
AR10+GG	2.41±0.05	5.27±0.03	7.40±0.30	1.51±0.05	4.68±0.41	358.67±27.74
UR10+GG	2.40±0.05	6.11±0.28	8.07±0.04	1.41±0.21	6.55±0.23	472.67±25.98
GR21+GG	2.42±0.06	5.11±0.11	7.18±0.52	1.67±0.14	5.70±0.29	365.33±39.92
CD (at 5% level)	0.39	0.84	0.84	0.39	0.99	66.57

GG=*Gigaspora gilmarie*.

* = mean of 3 replications mean±SE.

plant⁻¹) & the yield (532.67 kg, Ha⁻¹) were found in AR1 with GM. Table 7 represented the effect of dual inoculation of GG and the *Rhizobium* species. All the parameters showed best results over the control and over single inoculation with GG. However, the maximum nodule dry weight (3.48 g), shoot dry weight (6.18 g), nitrogen & chlorophyll content (8.16 mg, plant⁻¹, 1.71 mg, plant⁻¹)

phosphorus content of shoot (7.28 mg, plant⁻¹), & the yield (524.67 kg, Ha⁻¹) were found in AR1 with GG. Figure 1 showed the nitrogenase activity of the *Rhizobium* species, AR1 showed the highest nitrogenase activity both in Blackgram (4.02) and Greengram (4.25). Figure 2

**Fig. 1.** Nitrogenase activity test of 6 species of *Rhizobium* in respect of Blackgram and Greengram.**Fig. 2.** Area of growth inhibition of 6 *Rhizobium* species against 9 antibiotics.

revealed that the six *Rhizobium* species were resistant against the 9 test antibiotics. Species AR1 showing the lowest area of inhibition in 8 antibiotics and became the highly resistant one, while the species GR21 showing the highest area of inhibition became the highly susceptible one. Among the rest of the species BR8 and BR12 showed resistance to 7 and 6 antibiotics respectively while AR10 and UR1 showed resistance against 5 antibiotics. After 18 hrs of incubation the area of inhibition was calculated with the following formula and accordingly the resistance and susceptibility of the species towards the test antibiotics were presented in Fig. 2.

$$\text{Area of inhibition} = \frac{\text{Diameter of inhibition area}^2}{4} - \frac{\text{Diameter of disc area}^2}{4}$$

Discussion

Agro climatic condition of the North Eastern Region of India differs significantly from the rest of the country. The soils of this region is highly acidic having soil pH upto 3.5. Hence local species of *Rhizobium* and AM Fungi have been preferred in this piece of research work. Six species of *Rhizobium* viz. AR1, BR1, BR12, AR10, UR10, GR21 and two AM Fungi viz. *G. mosseae* and *G. gilmarie* showed good nodulation, nodule dry weight, shoot dry weight, nitrogen & chlorophyll content, phosphorus content of shoot and also the yield of two local pulses viz. Blackgram and Greengram. Similar results were also observed by Singh and Singh, 1992; Borbora Phookan and Shadeque, 1994; Rana *et al.*, 1998 and Sharma *et al.*, 2000.

Root colonization in legume rhizosphere by *Rhizobium* has been reported by Varma *et al.* 1998 and Subba Rao *et al.*, 1999. They also reported that due to this enhanced number of population of this above Plant Growth Promoting Regulators, the size and number of nodules increased that fixes more amount of nitrogen from the atmosphere. Our present results showed an overall increase of *Rhizobium* population after growing Greengram and Blackgram in the field. This could, perhaps, be due to either application of *Rhizobium* spp. or due to root colonization of resident *Rhizobium* spp. Mostly due to root exudates of the host roots. Similar results of increase of *Rhizobium* population and its as residual effect were observed by Kumar and Rao, 1998.

However, increase of nodule dry weight as observed in dual inoculation may be interpreted that it might be due to enhanced amount of soluble phosphorus made available by the presence of AM Fungi in blending with *Rhizobium* spp. A synergistic effect due to dual inoculation of *Rhizobium* spp. and AM Fungi on growth and production of pulses both in greenhouse and in extensive field trial was observed by various workers of India and elsewhere

viz. Ross and Haper, 1970; Schenck and Hinson, 1973; Crush, 1974; Sanni, 1976; Islam *et al.*, 1980; Barea *et al.*, 1980; Asimi *et al.*, 1980; Kehri and Chandra, 1989; Kumar *et al.*, 2001; Lukiwati and Simanungkalit, 2002. Their reports showed an enhanced rate of growth and production of pulses.

Our present results also showed that *Rhizobium* spp. in combination with AM Fungi produced higher nodule dry weight, shoot dry weight, nitrogen and phosphorus content along with chlorophyll and certainly because of this reason the production of pulses was increased in dual inoculation over the control and lone inoculation of AM Fungi.

We observed that all the six *Rhizobium* species showed better results when inoculated dually with AM Fungi viz. *G. mosseae* and *G. gilmarie* both in Blackgram and in Greengram. *Rhizobium* spp. AR1 produced the best results in respect of dry weight of nodules (3.14 g), dry weight of shoot (10.08 g), nitrogen content (7.68 mg, plant⁻¹), chlorophyll content (1.89 mg, g⁻¹), phosphorus content of shoot (6.17 mg, g⁻¹) and yield (535.67 kg, Ha⁻¹) when dually inoculated with GM in Blackgram.

Seasonal variations in growth and production of pulses have been reported by Subba Rao *et al.*, 1999. Our present experiment also showed that Kharif season (September to February) produced better growth in respect of nodule dry weight, shoot dry weight, nitrogen, chlorophyll and phosphorus content of shoot and yield than Rabi season (March to August).

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