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Effect of combined inoculation of *Rhizobium* and antagonistic bacteria on nodulation and growth of green gram *(Vigna radiata* (L.) Wilczek)

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Abstract : The experiment was conducted to study the efficiency of the peat based combined inoculant of *Rhizobium* (CoG 15, CRM 11 and CRM 8) and antagonistic bacteria (AB 3, AB 5 and AB 9) on the growth, nodulation and grain yield of green gram variety Co 3 under pot culture condition. A significant increase was noticed in *Rhizobium* and antagonistic bacterial combinations over the *Rhizobium* alone. Maximum number of nodules (30 No./pl) and nodule dry weight (30.27 mg/pl) was recorded by CoG 15 with antagonistic bacteria (AB 3) combinations. The total nitrogen and phosphorus content were also enhanced due to the combined effect of these organisms. More grain yield was recorded due to inoculation with *Rhizobium* alone (14.28 to 24.02%) or in combination with antagonistic bacteria (28.57 to 49.35%)

Key words: Nitrogen fixation, Rhizobium, Antagonistic bacteria, Green gram.

Introduction

Grain legumes are the major source of protein in our food. Being cheaper than other rich foods like meat, fish and milk, the legumes will continue to occupy a pivotal position in meeting the protein requirement of the masses. Besides nitrogen fixation, the legume - *Rhizobium* symbiotic partnership represents inexpensive alternate to the use of chemical nitrogen fertilizers in the production of food grains. Legume symbiosis contributes atleast 70 million tonnes of N per year for the 100-300 kg N/ ha/year (Subba Rao, 1977). Green gram *(Vigna radiata)* is one of the important legume containing 24% protein, 1.3% fat, 3.5% minerals, 5.7% carbohydrate, 0.435% phosphorus, 0.12% calcium, 0.07% iron, 0.04% fibre and 334 Kcal of energy from every 100 gram of grain legumes (Gopalan *et al.* 1977). Inoculation of grain legumes with suitable rhizobial strains is carried out in many countries to ensure effective nodulation (Thompson, 1980). Though it proves fruitful, yet the introduced rhizobial population is unable to fix sufficient nitrogen, because of the occurrence of many other antagonistic group of microorganisms. Many workers have reported the inhibition of *Rhizobium* growth by several antagonistic bacteria, fungi and actinomycetes of plant rhizosphere (Pugashetti *et al.* 1982). In view of the likely importance of intergeneric competition and native rhizobial competition in colonization of the legume rhizosphere and nodulation of *Rhizobium*, a new approach was attempted to reduce this competition (Murugesan, 1997). The approach involved here was joint inoculation of second microbe along with *Rhizobium* that might produce antagonistic compounds that hopefully suppress microbiota competing with root nodule bacteria but not *Rhizobium*.

Materials and Methods

Isolation of antagonistic bacteria

Antagonistic bacteria along with respective fungi and bacteria were isolated from different rhizosphere soils of green gram collected from New area and Millet Breeding Station of Tamil Nadu Agricultural University, Coimbatore during 2000. Plating was carried out with lower soil dilutions on nutrient agar medium and rose bengal medium respectively. The crowded plate technique facilitated to pick out colonies of bacteria that produced inhibition zones against soil fungi and bacteria. Antagonistic bacteria inhibiting fungal and bacterial colonies were selected. The antagonistic bacterium, was designated as AB, and serially numbered.

Duplication of antagonistic and susceptible microorganism were avoided during isolation based on their morphological characters, colony texture, colour etc. The isolated cultures were maintained in the respective media and stored for further studies.

Cross reaction of antagonistic bacteria with rhizobial isolates and susceptible bacteria and fungi

Fifteen ml of 1.8 per cent nutrient agar was spread over in sterile petridishes. Test tube containing 5 ml of 0.8 per cent soft nutrient agar in water bath maintained at 40°C was taken, cooled and mixed uniformly with 1 ml of 24 h old culture of the susceptible organisms, poured over the solid agar layer and spread uniformly throughout the plate. Using multiple inoculator, all the mid-log phase antagonistic bacteria were aseptically spotted on the seeded soft agar medium (Josey *et al.* 1979) and incubated at room temperature.

After incubation for 2-4 days at room temperature, the inoculated plates were observed for the inhibition zones. Exposure of all collected organisms against the antagonistic bacteria by this method, facilitated to screen more number of susceptible or resistant organisms. Efficiency of inhibitory action of antagonistic bacterium was tested by the following formula as described by Murugesan, (1997).

$$\text{Percentage of Antagonistic Efficiency (AE)} = \frac{\text{Total number of inhibited microbes}}{\text{Total number of organisms tested}} \times 100$$

An antagonistic bacterium with higher percentage of AE was considered to be the best and used for combined inoculation along with *Rhizobium*, in later studies.

All the antagonistic bacteria isolated previously were tested for their reaction with rhizobial isolates along with standard green gram strains available in the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore.

Double layer agar technique described earlier was followed. One ml of *Rhizobium* (10^8 cells ml^{-1}) was seeded in 0.8 per cent tryptone yeast extract soft agar (Beringer, 1974) medium and plated. The antagonistic bacteria were spotted over the seeded medium and inhibitory effect was observed. If the seeded *Rhizobium* was found inhibited to a particular antagonistic bacterium (AB), it was considered as a susceptible (S) and the resistant (R) rhizobia were the ones, which were not inhibited by antagonistic bacteria. One of the antagonistic bacteria which was found to be non-inhibitory to maximum number of rhizobial cultures was considered as compatible and was used in further combined inoculation studies, as suggested by Murugesan (1997).

Combined inoculation of Rhizobium and antagonistic bacteria

A pot culture experiment was conducted to study the efficiency of peat based combined inoculant of *Rhizobium* and antagonistic bacteria in green gram *(V. radiata)* var. CO3. Three isolates COG. 15, CRM 11 and CRM 8 exhibiting varying degree of resistance to antagonistic bacteria AB 3, AB 5 and AB 9 respectively were seed inoculated together. The influence of combined inoculation on the growth, nodulation and yield of green gram was studied. The pot contained 10 Kg of soil (red soil and sand mix 1:1) amended with 2% organic matter. Seed bacterization of *Rhizobium and* antagonistic bacteria was done and seeds were sown to the pots. The treatments were:

1. *Rhizobium* (COG 15)
2. *Rhizobium (CRM* 11)
3. *Rhizobium* (CRM 8)
4. COG 15 + Antagonistic Bacteria (AB 3)
5. CRM 11 + Antagonistic Bacteria (AB 5)
6. CRM 8 + Antagonistic Bacteria (AB 9)
7. Control (No inoculation)

Initially the pots contained five plants and after 15 days they were thinned to three per pot. The pots were watered regularly. The treatments were randomized with three replications. The shoot length, root length, nodule number and dry weight, plant dry weight, total nitrogen and phosphorus were recorded on 45th day after sowing. At harvest, pod yield was recorded. Total N and P of plant samples were recorded by the method of Humphries (1956) and Jackson (1973) respectively.

Results and Discussion

Isolation of antagonistic bacteria

Eleven antagonistic bacteria *viz.* AB1, AB2, AB3, AB4, AB5, AB6, AB7, AB8, AB9, AB10 and AB11 were isolated from rhizosphere soils of green gram. The inhibitory activity

varied from one organism to another. Some organisms exhibited larger inhibition zones while some exhibited smaller inhibition zones. Organisms which developed away from antagonistic bacteria in the crowded plate, too, were taken and tested against all antagonistic bacteria.

Cross-reaction of antagonistic bacteria with soil microorganisms including rhizobia

All the antagonistic bacteria were cross checked with susceptible and other organisms in order to find out their wide spectrum of action. The organisms that were inhibited by antagonistic bacteria were considered as susceptible (S) while uninhibited organisms were considered as resistant (R). Bacteria other than rhizobia or bradyrhizobia multiplied more rapidly and attained greater densities in the rhizosphere of legumes (Routt and Katzelson, 957; Lennox and Alexander, 1981; Hossain and Alexander, 1984). The green gram isolates (CRM 8, 11 and COG15) exhibited resistant to antagonistic bacteria were identified. An introduced organism such as rhizobia grows slower than many indigenous species of the rhizosphere because of competitive disadvantage (Amarger, 1981). Some beneficial strains compete aggressively for sites on roots or in the rhizosphere, where nutrients are available. Occupation of these places allow introduced bacteria to pre-empt establishment of fungi on the roots. Certain strains of fluorescent pseudomonads introduced through seed or planting material control plant diseases caused by soil borne pathogens or promote growth by suppressing deleterious rhizosphere microorganisms (Suslow, 1982).

Plant growth parameters

The shoot length, root length and plant dry weight of green gram as influenced by inoculation with *Rhizobium* and antagonistic bacteria under pot culture conditions is presented in Table-1. The data revealed that combined inoculation of *Rhizobium* and antagonistic bacteria significantly increased the shoot length, root length and plant dry weight than the individual inoculation of *Rhizobium*. All the combined inoculations were statistically on par. A significant increase was noticed in *Rhizobium* and antagonistic bacterial combinations over uninoculated control. Maximum growth was observed in COG 15 plus AB 3 combinations.

Nodulation

Inoculation of *Rhizobium* either alone or in combination with antagonistic bacteria enhanced the nodule number and nodule dry weight significantly compared to uninoculated control. Maximum nodule number (30.0 no pl^{-1}) and nodule dry weight (30.27 mg pl^{-1}) were recorded in COG 15 with antagonistic bacteria (AB 3). The differences were statistically significant.

Combined inoculation of rhizobial strains COG 15, CRM 8 and CRM 11 with antagonistic bacteria AB 3, AB 5 and AB 9 respectively were on par in nodule number and nodule dry weight (Table 2). Hossain and Alexander (1984) conducted a study to increase colonization of *R. japonicum* resistant to benomyl, streptomycin and erythromycin in the soybean rhizosphere. The addition of streptomycin and erythromycin to soil stimulated the growth of *R. japonicum* but inhibited other bacteria in the presence or absence of soybean. When a bacteriocin producing strain was introduced along with, proportion of nodulation was enhanced many fold, due to the inhibitory effect of bacteriocin producer on the population of indigenous rhizobia (Hodgson *et al.*1985).

Plant analysis

The strain COG 15, CRM8 and CRM 11 significantly increased the total nitrogen and phosphorus content of the plant, either alone or in combination with respective antagonistic bacteria. A significant increase in plant nitrogen content due *to Rhizobium* plus antagonistic bacteria inoculation was observed over individual and uninoculated control (Table 2). Total phosphorus content of the plant was maximum with combined inoculation of *Rhizobium* with antagonistic bacteria (Table.2). Plant phosphorus content in combined inoculation were statistically on par with each others. Maximum P content was recorded in COG15 and AB3 inoculation (0.32%) than the individual inoculation (0.26%) and uninoculated control (0.21%). The same trend as observed in total N content was also noticed in total P content.

Table 1. Effect of co-inoculation of *Rhizobium* with antagonistic bacteria on shoot length, root length and plant dry weight of green gram

Treatments	Shoot length (cm)	Root length (cm)	Plant dry weight (g)
COG 15 + AB 3	52.63	16.50	3.43
CRM 11 + AB 5	49.33	17.26	2.96
CRM 8 + AB 9	49.63	17.90	2.93
COG 15	48.59	14.53	2.43
CRM 11	45.35	13.96	2.60
CRM 8	45.71	14.39	2.61
Control	41.45	11.36	2.11
SEd	1.86	0.59	0.03
CD	3.90	1.24	0.06

Table 2. Effect of co-inoculation of *Rhizobium* plus antagonistic bacteria on nodule number, nodule dry weight, total N and P content of green gram

Treatments	Nodule number (No. pl^{-1})	Nodule dry weight (mg)	Total Nitrogen (%)	Total Phosphorus (%)
COG 15 + AB 3	30.00	30.27	1.32	0.32
CRM 11 + AB 5	28.00	27.51	1.31	0.29
CRM 8 + AB 9	27.99	27.43	1.32	0.28
COG 15	26.72	24.17	1.25	0.26
CRM 11	24.70	24.22	1.21	0.24
CRM 8	24.74	24.19	1.26	0.24
Control	19.33	20.47	0.80	0.21
SEd	1.55	1.45	1.50	0.01
CD	3.25	3.04	3.15	0.03

Table 3. Effect of co-inoculation of *Rhizobium* plus antagonistic bacteria on grain yield of green gram

Treatments	Grain yield (g pl^{-1})	% increase over control
COG 15 + AB 3	1.03	68.85
CRM 11 + AB 5	0.98	60.65
CRM 8 + AB 9	0.96	57.32
COG 15	0.87	42.62
CRM 11	0.79	29.50
CRM 8	0.72	18.03
Control	0.61	–
SEd	0.09	
CD	0.19	

Grain yield

More grain yield was recorded due to inoculation with *Rhizobium* alone or in combination with *Rhizobium* with antagonistic bacteria (Table 3). Combined inoculation of *Rhizobium* (COG 15) and antagonistic bacteria (AB 3) recorded the maximum grain yield (49.35%) followed by CRM11 plus AB5 (29.32%) and, CRM 8 plus AB 5 (28.57%). Significant increases over the individual inoculation as well as uninoculated control were observed.

All combined inoculation treatments were on par with each other. A significant increase was observed in all the treatment over the control. Li and Alexander (1988) reported that the colonization of alfalfa rhizosphere and nodulation by *R. meliloli* were enhanced by inoculation with *Pseudomonas* sp in soil containing $2.7x10^5$ *R. meliloti* g^{-1}. Natarajan and Gunasekaran (1991) reported that seed and soil inoculations of *R. japonicum, Bacillus megaterium* increased the plant growth and grain yield. Dual inoculation of *Rhizobium and Bacillus* gave higher seed yield than individual inoculation. Co-inoculation of field beans with *R.legumonosaram and Pseudomonas putida* R 105 increased the number of nodules and acetylene activity (ARA) significantly (De Freitas *et al.* 1993)

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