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Studies on the Effect of Co-Inoculation of PPFM and Rhizobium on the Enhancement of Nodulation, N_2 Fixation and Yield of Rice Fallow Black Gram

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ABSTRACT

The co-inoculation effect of Methylobacterium and Rhizobium revealed the positive influence of the co-inoculated microbes in augmenting the growth, dry weight, total nitrogen content, number of pods and yield of black gram. Interestingly the individual application of either Methylobacterium or Rhizobium could augment the plant growth stimulation and yield parameter of black gram but effect was more pronounced when the isolates were co-inoculated to black gram plants. Instead of trying single strain with a single trait to get more benefit in plant-bacteria association, try to use microbial consortia, with multiple benefits, for better plant-bacteria interaction.

Keywords: Nodulation, Co-inoculation, Black gram, Nitrogen fixation, Methylobacterium

INTRODUCTION

Pulses form an important part of Indian dietary. They are an important source of protein which are essential adjustments to a predominantly central based diet and enhance the biological value of protein consumed.

Black gram (*Phaseolus mungo*) or mung bean is grown about 2.5 million hectares, spread all over the country, with a production of 0.8 MT/ha. They are grown as rice fallow, catch crop during the summer period (January-March) in Pondicherry region.

Now-a-days, research in the area of Biological Nitrogen Fixation, a purely microbiological process is gaining momentum. The legume- Rhizobium symbiosis, a most promising plant-bacteria association, is a suitable illustration for Biological Nitrogen Fixation. The legume- Rhizobium interaction fixes nitrogen in root nodules of host plants which can very well be utilized as substitute for costly commercial nitrogenous fertilizers. To get maximum benefit, the legume-Rhizobium interaction needs thorough investigation.

Multiplication of inoculated Rhizobium in spermosphere and rhizosphere regions is greatly influenced by ecology of that locale. Currently, the genus Methylobacterium has attracted the attention of microbiologists, agronomists, ecologists due to its ability to form nodulation and fix nitrogen in association with certain rhizobial strain during co-ninoculation.

Jourand et al. [1] reported the symbiotic association *Methylobacterium nodulans*. Jaftha et al. [2] characterized the nitrogen fixing isolates from the nodules *Latanansis baimsii* as pigmented methylotrophic bacteria. Madhaiyan et al. [3] investigating the survival of Methylobacterium, as symbionts of tropical legume and reported the positive occurrence of the same.

Methylotrophs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins [4,5] and to alter agronomic traits like branching, seedling vigor, rooting and heat/cold tolerance [6].

Co-inoculation of several legumes with different plant growth promoting rhizobacteria (PGPR) was shown to benefit plant growth in the greenhouse and in the field production and nitrogen content of dually inoculated plants may be attributed to earlier and enhanced nodulation, higher nitrogen fixation rates and a general improvement of root development.

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MATERIALS AND METHODS

Dinitrogen fixing efficiency of Methylobacterium isolates

100 ml volume of the AMS broth was taken into 250 ml Erlenmeyer flasks and sterilized by autoclaving. The flasks were separately inoculated with (1×10^7 CFU/ml) 48 h old cultures of Methylobacterium aseptically. Then, the flask was incubated at 30+2°C for one week under stationary condition.

Dinitrogen fixing efficiency of Rhizobium isolates

100 ml volume of the YEM broth was taken into 250 ml Erlenmeyer flasks and sterilized by autoclaving. The flasks were separately inoculated with (1×10^7 CFU/ml) 48 h old cultures of Rhizobium aseptically. Then, the flask was incubated at 30+2°C for one week under stationary condition.

Microkjeldahl assay [7]

Reagent used:

Digestion mixture:

Potassium Sulphate 10 g

Cupric Sulphate 1 g

Selenium metal powder 0.1

Salicylic-sulphuric acid mixture:

Salicylic acid 0.1 g

Sodium Thiosulphate 1 g

Conc. Sulphuric acid 30 ml

100 ml of the AMS and YEMA medium was taken into 250 ml Erlenmeyer flasks and sterilized by autoclaving. The flasks were separately inoculated with 1 ml of (1×10^7 CFU/ml) of 48 h culture of Methylobacterium and Rhizobium isolates (PPFM-1 to PPFM-5 and RZ-1 to RZ-5), aspetically. Then, the flasks were incubated at 30+2°C for one week under stationary condition. After the incubation period, 1 ml of the broth was transferred to 50 ml Pyrex Microkjeldahl flask, separately. A quarter teaspoonful of the digestion mixture (10 g of reagent grade potassium sulphate, 1 g of cupric sulphate and 0.1 g of selenium metal powder) and 4 ml of Salicylic-Sulphuric acid mixture (0.1 g salicylic acid, 1 g of sodium thiosulphate and 30 ml of concentrated sulphuric acid) were introduced into it. The contents were slowly heated till frothing ceased and then heated strongly. Completion of the digestion was indicated by the solution turning bluish to green. After cooling, about 15 ml of distilled water was added to the flask, swirled and cooled. The contents were transferred into the distillation units and 25ml of 40% sodium hydroxide was added. The ammonia was steam distilled for 15 min into an excess of 0.1 N Sulphuric acid (10 ml) containing 2 drops of methyl red. The contents were back titrated against 0.1 N Potassium hydroxide till the appearance of golden yellow color.

The 'N' content of the sample was calculated using the factor:

1 ml of 0.1 N H_2SO_4=0.0014 g of N

SCREENING THE RHIZOBIUM ISOLATES ON THE BASIS OF THEIR NODULATION EFFECIENCY IN BLACKGRAM

Leonard Jar technique [8]

The neck of the bottle is plugged with cotton and the bottle filled with equal mixture of coarse and fine washed sand (grain size 1-3 mm). The jar contained 500 ml of the plant nutrient solution. The jar must be wide mouthed to take the inverted sand bottle which is made up of standard Indian beer bottle. The bottle is 7.5 cm in diameter and 25 cm in height. The bottom of these bottles were cut and opened. The bottle holds approximately 900 g of sand. The bottle was filled with dry sand without any air left inside the sand column. The bottle was placed upside down inside the jar. About 100 ml of plant nutrient solution was poured through the sand and the remaining 400 ml added to jar. The bottle and the jar were covered with craft paper bag, 27.5 cm long and 12.5 cm wide. The closed end of the bag was cut. The bag was inserted on to the jar and bottle assembly so that it forms a jacket around the assembly. A square piece of paper was placed on the tops folded down and fixed by rubber bands in place. The whole assembly was then sterilized in an autoclave for one hour at 15 lb pressure. After autoclaving the sand jar assemblies were transferred to the black house. The bench top was swabbed with Lysol before placing the sand jar assemblies. The black gram seeds were germinated and planted into the sand at the rate of 4 to a jar with radical down. The surface of the sand was moistened with sterile water. The paper cap was replaced when the seedlings were established. After the seedlings were established excess over 2-3 seedlings per jar was removed.

When seedlings were 7-10 days old, 1 ml of the Rhizobium broth culture was added by means of sterile pipette to each seedling in the jar. Uninoculated control was also maintained. After suitable period of growth, the plants were removed with roots and nodules intact and washed with water. The tops, roots and nodules were separated and wet as well as dry weight of the plants and the numbers of nodules were determined.

COINOCULATION EFFECT OF METHYLOBACTERIUM AND RHIZOBIUM ON THE AUGMENTATION OF GROWTH AND YIELD PARAMETERS IN BLACK GRAM

Pot culture experiment

A pot culture experiment was conducted to study the co-inoculation effect of Methylobacterium and Rhizobium on various growth and yield parameters of black gram. The experiment was conducted during the month of Jan-2018 at PASIC experimental farm, Arasur, Pondicherry. The

treatments and the details of experiments were given as stated below:

Treatments: The black gram was applied with organisms (Methylobacterium and Rhizobium). The details of the treatments are as follows:

T1-Control

T2-Methylobacterium alone

T3-Rhizobium alone

T4-Methylobacterium and Rhizobium co-inoculation

EFFECT OF DIFFERENT FORMULATION OF METHYLOBACTERIUM AND RHIZOBIUM ON VARIOUS GROWTH AND YIELD PARAMETES IN BLACK GRAM

Pot culture experiment

Seed inoculation: Black gram seeds were surface sterilized with 0.1% mercuric chloride for 2 min and washed several times with sterile distilled water. They were then treated for 12 h with bacterial suspension (PPFM-1 Methylobacterium, RZ-4 Rhizobium) at 1 × 10 CFU/seed for 5-10 h, shade dried and used as inoculum.

An inoculums level of 1×10^{-7} CFU/kg of soil was applied after 4 DAS.

Circular mud pots with 30 cm diameter were filled with paddy field soil and sterilized at 20 lb pressure for 1 h. The inoculated seeds were sown in each pot and three replications were maintained for each experiment with control pots. Black gram plants in sterilized pots were regularly watered with sterile distilled water.

EFFECT OF PLANT HEIGHT

The height of the black gram from each treatment was measured on 30th day after sowing (DAS). Then mean value of the plants from 3 replications was recorded.

EFFECT OF DRY WEIGHT OF PLANT

The dry weight of the black gram plant was taken on 30th day after sowing (DAS). Three plant samples were drawn, washed, air dried and later dried to constant weight in an oven at 60°C. The oven dried weight of the black gram samples was recorded.

'N' CONTENT OF PLANT

The nitrogen content of the soil was estimated using the microkjeldahl method suggested by Humphries (1956) and computed to $Kg^{-1}ha^{-1}$.

Number of pods per plant

The number of pods per plant was counted and their means was expressed as number of pods per plant at 25 and 45 DAS.

RESULTS

Results are shown in **Tables 1-3**.

Table 1. Di-nitrogen fixing efficiency of rhizobium isolates under free living condition (Microkjedahl assay).

Isolate Number**	Amount of N fixed* (mg/g of Mannitol)[a]
BR-1	15.56 ± 0.45
BR-2	13.45 ± 0.18
BR-3	13.14 ± 0.20
BR-4	14.90 ± 0.22
BR-5	16.20 ± 0.12
BR-6	13.85 ± 0.17
BR-7	12.10 ± 0.31
BR-8	12.55 ± 0.25
BR-9	14.20 ± 0.16
BR-10	12.80 ± 0.27

*** Inoculum level at 1 × 10^{7} CFU mL^{-1}*

** Amount of 'N' fixed by Bradyrhizobium mg g^{-1} of Mannitol*

[a] *values are average of three replication ± SD*

Table 2. Screening the Rhizobium isolates collected from the Rhizosphere of black gram for their nodulation efficiency (Leonard Jar technique).

Treatments	Number of nodules on 25th DAS		
	Total	Pink	White
BR-1	1.3	0.9	0.4
BR-5	2.7	1.1	1.6

Table 3. Studies on the co-inoculation effect of Methylobacterium and Rhizobium on the augmentation of growth and yield parameters in black gram (Pot culture) after 25th DAS.

Treatments	Plant height (cm)	Root dry weight (g)	Shoot dry weight (g)	'N' content of plant (%)	Number of Pods	Yield (Kg/ha)
Control	12.3	0.10	0.080	0.89	25.02	480
Methylobacterium	16.04	0.023	0.121	1.30	32.4	700
Rhizobium	18.6	0.026	0.136	1.43	38.6	750
Methylobacterium and Rhizobium CoI	20.2	0.035	0.154	1.67	62.4	800
Methylobacterium and Rhizobium CoI	20.2	0.035	0.154	1.67	62.4	800

CoI=Co-inoculation; DAS= Days after Sowing

DISCUSSION

Two isolates viz., BR-1 and BR-5 constituted the first category. Five isolates viz., BR-2. BR-3, BR-4, BR-6 and BR-9 constituted second category and the remaining three isolates were ranked in the third category. Rubiya [9] reported that the efficiency of Rhizobium to fix 'N' per g of mannitol was 0.9 to 12.5 mg. The results of the present study revealed a maximum amount of atmospheric dinitrogen (16.20 mg 'N' fixed g^{-1} of mannitol) fixed by Rhizobium isolate viz., BR-5. The other isolates showed 'N' fixation in a range of 12.10-15.56 mg 'N' fixed g^{-1} of mannitol.

BR-1 and BR-5 on the enhancement of nodulation was studied under *in vitro* condition (Leonard jar assembly). It was observed that the existence of inter strain differences between the two rhizobial isolates in augmenting the above parameters in groundnut plant. The Rhizobium viz., BR-5 recorded a high level performance in augmenting the above parameters followed by the isolate BR-1. Asseva and Kirillova [10], Rangaswami and Oblisami [11] and Obaton [12] reported the interstrain difference of Rhizobium on the nodulation and nitrogen fixation in legume plants,

"Intergeneric PGPB co-inoculation" consisting of Rhizobium and Methylobacterium genera augmented the growth parameters viz., height, dry weight, N content of plant yield parameters of haulm yield to a higher level when compared to co-inoculation and single strain application of PGPB cells.

CONCLUSION

It was concluded that "Intergeneric PGPB co-inoculation of Rhizobium and Methylobacterium on the enhancement of nodulation, N_2 fixation and yield of rice fallow black gram.

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