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Research Article

Effect of *Bradyrhizobium* Isolates for the maximization of growth and yield of black gram (*Vigna mungo* L.)

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Keywords

Black gram,
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Abstract

Black gram (*Vigna mungo* L.) is one of the important pulse crops gaining importance all over the world in recent years. It is rich in proteins and contains amino acids higher quantities than any other cereals and pulses. Black gram is an annual food legume. Black gram seeds are boiled and eaten whole or after splitting into dhal. The dried seeds contain approximately 9.7 % water, 23.4 % protein, 1 % fat, 57.3 % carbohydrate, 3.8 % fibre and 4.8 % ash. It is very nutritious and is recommended for diabetics. *Bradyrhizobium* fixes the nitrogen in pulse crops in this study *Bradyrhizobium* isolated from different locations in Cuddalore district of Tamil Nadu and isolates designated as BR-1 to BR-5 respectively. The isolate BR-3 was selected as an efficient strain based on Indole acetic acid (IAA) production and Exopolysaccharides (EPS) production. Among the five treatments, the treatment T_5 which contains BR-3 (*Bradyrhizobium*) + 75% recommended dosage fertilizer recorded maximum plant height, leaf area index, dry matter production, number of branches plant^{-1} and number of pods plant^{-1}.

Introduction

Black gram is one of the important pulse crops in India. It has been reported that Black gram has been cultivated in India since ancient times**.** It was disbelieved that the Black gram is a native of India and Central Asia and grown in these regions since prehistoric times. It is widely cultivated throughout the Asia, including India, Pakistan, Bangladesh, Sri Lanka, Thailand, Laos, Cambodia, Vietnam, Indonesia, Malaysia, South China and Taiwan. In Africa and U.S.A. it is probably recent. Black gram is a protein rich staple food. It contains about 25 per cent protein, which is almost three times that of cereals. It supplies protein requirement of vegetarian population of the country. It is consumed in the form of split pulse as well as whole pulse, which is an essential supplement of cereal based diet (Sureshkumar *et al*., 2011; Kanchana *et al*., 2013). The moong dal Khichdi is recommended to the ill or aged person as it is easily digestible and considered as complete diet. Roti with Moong dal and Moong dal chawal is an important ingredient in the average Indian diet. Tong and Sadowky (Tong *et al.*, 1994) developed a novel non-antibiotic containing medium which allows selective isolation of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains from soils. The medium, BSJM, in based on the resistance of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* to more than 40 μg of the metals ions Zn^2 and CO^2 per ml. BJSM does not allow the growth of *Rhizobium* sp strains (Anitha *et al*., 2010; Usharani *et al*., 2013; Sivasakthi *et al*., 2014).

The bacterial polysaccharides are necessary for a functional *Bradyrhizobium* legume symbiosis. Exopolysaccharide (EPS), Lipopolysaccharide (LPS), Capsular polysaccharides and cyclic β (1-2) glucon play essential role in the formation of the infection thread and in nodule development (Saranraj *et al.,* 2013). *Bradyrhizobium* fixes nitrogen in way of symbiotic nitrogen fixation occurs mainly through symbiotic association of legumes with N_2 fixing *Rhizobia* that convert elemental nitrogen fixation (BNF) in to ammonia. This type of biological nitrogen is therefore less costly and more sustainable as compared with nitrogen fertilizers for production of plant proteins. Scientific and technological progress has opened tremendous opportunities for the benefit of small farmers (Sivasakthi *et al.,* 2013; Sivasakthivelan and Saranraj, 2013).

Materials and Methods

Details of the locations

The survey was conducted at five locations in Cuddalore district of Tamil Nadu comprising Annamalai Nagar, Bhuvanagiri, Vayalore, Sivayam and Mangalam.

Microbiological analysis of soil samples

The population of bacteria, fungi and actinomycetes in the rhizosphere soil sample of Blackgram were estimated by Serial dilution and Pour plating method, using appropriate dilutions and the following media. The colonies were counted and expressed as cfu g^{-1}.

Isolation and enumeration of *Bradyrhizobium* sp. from rhizosphere of Black gram

The blackgram rhizosphere soil samples collected from five blackgram field of a particular locations were pooled and one ml of black gram rhizosphere soil sample was transferred to 100 ml of sterile distilled water in a 250 ml Erlenmeyer flask and incubated on a rotator shaker (100 rpm) for 30 minutes at ambient temperature. The well mixed suspension was then diluted appropriately upto 10^{-6} dilution. One ml of suspension from 10^{-4} and 10^{-5} dilution was aseptically transferred to sterile petriplates and 10 – 20 ml of selective Yeast Extract Mannitol agar (YEMA) medium was added and incubated at 37ºC for 24 hours. Three replications were maintained for each dilution. The colonies were counted by using Colony counter. The total number of colonies in the original samples was expressed as cfu g^{-1}.

Purification of *Bradyrhizobium* sp. isolates

All the five *Bradyrhizobium* sp. isolates were purified by Streak plate method using Yeast Extract Mannitol agar (YEMA) medium frequently.

Designation of *Bradyrhizobium* sp. isolates

The *Bradyrhizobium* sp. isolates obtained from the rhizosphere of blackgram grown at five different location of Cuddalore district and were designated as BR and numbered randomly.

Characterization and authentication of the *Bradyrhizobium* isolates

Characterization and authentication of *Bradyrhizobium* isolates was carried out by testing the colony morphology, Gram staining, shape, size and biochemical characteristics.

Growth on yeast extract mannitol agar with Bromothymol blue as indicator

Each strain was streaked on the Bromothymol blue (5 ml/L) Yeast extract Mannitol agar medium and incubated for 3 days at 28°C for testing alkaline production. After incubation period, the colour of the medium changes from light green to dark blue indicating the *Bradyrhizobium* strains were able to change the medium of alkaline condition.

Growth on Hofer's alkaline medium

Hofer's alkaline medium was used to confirm whether the strains of *Bradyrhizobium* sp. were *Rhizobium* or *Agrobacteria*. The *Agrobacteria* can withstand higher pH levels, while *Rhizobium* cannot. A loopful of each strain was streaked separately on Hofer's alkaline medium.

Growth on Congo red agar medium

A loopful of each isolates was streaked on Congo red agar and the plates were incubated for a week. The *Bradyrhizobium* colonies appear as white, translucent, glistening, elevated, small ones with entire margin without absorbing red colour in contrast to red stained colonies of *Agrobacterium* sp.

Growth on Glucose peptone agar

The *Agrobacteria* readily utilize the glucose, grow and change its pH to yellow colour. On the other and *Bradyrhizobium* grow poorly in this medium.

Growth on Ketolactose agar

Ketolactose agar was prepared by replacing mannitol with lactose in YEMA medium. The isolates were streaked on this medium and incubated. After incubation, the plates were flooded with Benedict's solution and yellow colour formation of colonies after one hour incubation indicates the *Agrobacterium* contamination.

Quantitative estimation of Indole acetic acid (IAA) produced by *Bradyrhizobium* isolates

The Yeast extract mannitol broth in 100 ml quantities were prepared and supplemented with D, L-Tryptophan, at a concentration of 100 mg/litre after sterilization. This was followed by the addition of standard inoculum (1×10^7 cells/ml) of the isolates and incubated at 30°C under dark for a period of 7 - 12 days in order to prevent the photo inactivation of biologically active compounds. The solution was centrifuged at 7000 rpm for 30 minutes and the supernatant was reduced to 50 ml volume by flask evaporation under Vacum and IAA extracted into ethyl acetate and n-butanol by the procedure followed by (Tien *et al.* 1979). The IAA in the methanol fraction was determined by employing salper reagent. To 1.5 ml distilled water in a test tube 0.5 ml of methanol residue was mixed, 4 ml fresh salper reagent was rapidly added and kept in dark for one hour and read in calorimeter at 535 nm. From a standard graph prepared with known concentrations of IAA, the quantity of IAA in the filtrate was calculated. 1 division = 0.307 μg IAA.

Quantitative estimation of Exopolysaccharides

Two ml of the inoculum was added to 100 ml of YEM liquid medium and incubated on Psychrotherm incubator shaker at 28°C for 72 hours. The cells are harvested by centrifugation. Sixty ml of isopropyl alcohol was added to 20 ml of the supernatant fraction and let it stand at 4°C overnight to precipitate WSP. The precipitate was collected by filtering through Whattmann No. 42 filter paper and dried in an incubator at 60°C till a constant weight obtained Sutherland and Wilkinson (1971).

Effect of *Bradyrhizobium* sp. on the growth and yield of black gram (ADT 3)

The black gram variety cv. ADT 3 was chosen for the present study. The pot culture experiment was conducted to study the effect of *Bradyrhizobium* sp. on the growth and yield of black gram var ADT 3. The study was conducted at Department of Microbiology, Annamalai University, Annamalai Nagar, Tamil Nadu, India. The soil used in the pot culture experiment was clay loamy in nature. The experiment was arranged in Randomized Block Design (RBD) with three replications. The five treatments were: T_1 – Control; T_2 – 100% RDF; T_3 – 25% RDF + *Bradyrhizobium* sp.; T_4 – 50% RDF + *Bradyrhizobium* sp. and T_5 - 100% RDF + *Bradyrhizobium* sp. For sowing in inoculated pots, blackgram seeds were soaked with trehalose at 15 mM, polyvinyl pyrollidone (PVP) at 2% and glycerol for 30 min in different formulations (20 ml/kg of seeds), (Spacing, 15 cm × 10c m; 3 seedlings/hill and 12 seedlings/pot). Gap filling was done after 10 DAS. The crop was given hand weeding on 30th DAS and well protected against pests and diseases. A water level of 5 cm depth was maintained through the crop period. Five representative samples of plant hills in each pot were pegmarked for periodical observation.

Biometric observations of Black gram

For each treatment, three replications were chosen for measuring and recording the biometric observations of plants and were recorded at periodic intervals *viz.*, 20th, 40th and 60th days after sowing (DAS). The following biometric observations (growth components) were recorded.

Plant height

The plant height was recorded in the each treatment at 20th, 40th and 60th days after sowing.

Number of branches per plant

The mean numbers of branches of plants from all treatments were recorded at harvesting.

Leaf Area Index

In the selected plants, the leaf area index was recorded after harvesting by measuring the length and width of the leaves for each of the treatment, number of leaves per plant and the respective observations were also taken. From the observations leaf area index was calculated using the formula of Puttaswamy *et al.* (Puttaswamy *et al.*,1976).

$$LAI = L \times W \times N \times K$$

Where, L = length of the leaf in cm; W = maximum width of the leaf in cm; N = number of leaves per plant and K = constant (0.75 for cereals crop)

Dry matter production

The plants collected from each treatment were recorded after harvesting of intervals. The sample plants after removing the roots were initially air – dried and then oven dried at 60 ± 5˚C till a constant weight was reached and expressed as t/ha.

Number of pods per plant

The number of pods per plant from each treatment was counted and the mean number of pods per plant was recorded.

Test weight

Mean test weight of 100 grains per treatment was recorded at 14 per cent moisture content and expressed in grams

Grain yield

The grains were picked from each of the plant were weighed immediately after the harvest and weight of the harvest was recorded in kg/ha^{-1}.

Haulm yield

The dry weight of haulm yield from each plot was recorded and expressed in kg ha^{-1}.

Results and Discussion

Plant growth promoting rhizobacteria (PGPR) may promote growth directly by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus and potassium, production of siderophore

that solublize and sequester iron, or production of plant growth regulators (Sivasakthivelan *et al.,* 2013). The beneficial effects of *Bradyrhizobium* inoculation to various leguminous crop plants have been investigated by several workers (Kanchana *et al.,* 2013; Usharani *et al*., 2014). The beneficial effects of *Rhizobium* and *Bradyrhizobium* in legume in terms of biological N_2 fixation has been a main focus in the recent past (Deshwal *et al.,* 2003), as it is an important aspect of sustainable and environmental friendly food production and long term productivity. In the present study, the black gram rhizosphere soil was collected from five different locations in Cuddalore district and the details of sample collection was furnished in Table – 1.

Table – 1: Details of locations of sample collection

Name of the District	Name of the locations
Cuddalore	Annamalai Nagar
	Bhuvanagiri
	Vayalore
	Sivayam
	Mangalam

The collected soil samples were analyzed for the total microbial population and the results were presented in Table – 2. The results revealed that the total bacterial population ranged from 15.8 to 22.3 × 10^6 cfu g^{-1} of soil and the highest population of 22.3 × 10^6 cfu g^{-1} was observed in soil of Sivayam. The total fungal and actinomycetes population were ranged between 10.5 × 10^5 cfu g^{-1} to 18.0× 10^5 cfu g^{-1} and 8.9 × 10^3 cfu g^{-1} to 14.0 × 10^3 cfu g^{-1} of soil respectively.

Table - 2: Microbiological analysis of soil samples

Rhizosphere soil sample	Bacteria cfu x 10^6 g^{-1}	Fungi cfu x 10^5 g^{-1}	Actinomycetes cfu x 10^3 g^{-1}
Annamalai Nagar	19.4	18.0	8.9
Bhuvanagiri	17.6	12.6	12.3
Vayalore	18.0	16.3	9.6
Sivayam	22.3	13.0	14.0
Mangalam	15.8	10.5	11.9

The total bacterial population, *Bradyrhizobium* population and percentage of *Bradyrhizobium* population were estimated and the results were presented in the Table - 3. The location, namely Sivayam recorded maximum of 7.71 cfu x 10^6 g^{-1} community population of *Bradyrhizobium* from Mangalam recorded least population of 7.21 cfu x 10^6 g^{-1} in the rhizosphere. All other locations recorded the community population of *Bradyrhizobium* species. Five strains of *Bradyrhizobium* were isolated from various areas from Cuddalore district. They were designated as "BR" series and numbered randomly. The details of designation of the isolates their rise of collection are presents in Table – 4.

The occurrence of *Rhizobium* populations in desert soil and the effective nodulation of legumes growing therein emphasize the fact that the *Rhizobium* can exist in soils with limiting moisture levels, however population densities tend to be lowest under the most desolated conditions and to increase as the moisture stress is relieved Tate (1995). It was well known that some free living *Rhizobium* was capable of survival under salt stress or low water potential (Fuhrmann *et al.,* 1986).

Table - 3: Occurrence of community *Bradyrhizobium* population from rhizosphere of rice at Cuddalore District

Rhizosphere soil sample	*Bradyrhizobium* (cfu x 10^6 g^{-1})
Annamalai Nagar	7.65
Bhuvanagiri	7.55
Vayalore	7.66
Sivayam	7.71
Mangalam	7.21

Table - 4: Designation of *Bradyrhizobium* isolates from five locations of Cuddalore District

Rhizosphere soil sample	*Bradyrhizobium* Designation
Annamalai Nagar	BR – 1
Bhuvanagiri	BR – 2
Vayalore	BR – 3
Sivayam	BR – 4
Mangalam	BR – 5

The blackgram root nodule isolates obtained from five different locations were designated as BR - 1 to BR - 5 and was presented in Table - 5. The isolates were authenticated as *Bradyrhizobium* sp. by conducting several confirmative tests *viz*., Infectivity test, Gram staining, growth on Congo red agar, Hofer's alkaline medium, Glucose peptone agar medium and growth on Ketolactose agar medium.

The isolates formed white, translucent, mucoid colonies on Congo red agar and YEMA. The isolates are Gram negative, rod shaped, did not absorb colour in Congo red agar, and no growth on Glucose peptone agar and Hofer's alkaline medium. No yellow colorations in Ketolactose agar medium. These results confirmed that all the isolates were *Bradyrhizobium* sp.

Table – 5: Authentication of *Bradyrhizobium* isolates obtained from black gram of Cuddalore Districts of Tamil Nadu

S. No	Name of the isolates	Infectivity test	Gram staining	Growth on Congo red	Growth on Glucose peptone agar	Growth on Ketolactose agar	Growth on Hofer's alkaline medium
1	Annamalai Nagar	+	-ve	NA	NG	NC	NG
2	Bhuvanagiri	+	-ve	NA	NG	NC	NG
3	Vayalore	+	-ve	NA	NG	NC	NG
4	Sivayam	+	-ve	NA	NG	NC	NG
5	Mangalam	+	-ve	NA	NG	NC	NG

+ - Positive; -ve – Negative; NA – No Absorbance; NG – No growth and NC – No colour change.

Table - 6: Screening of *Bradyrhizobium* species for IAA and EPS production

S. No	Isolates	IAA («g ml$^{-1)}$	EPS (µg ml$^{-1)}$
1	BR – 1	1.80	22.25
2	BR – 2	0.86	21.30
3	BR – 3	4.95	275.50
4	BR – 4	3.75	249.00
5	BR – 5	1.75	68.75

The effect of *Bradyrhizobium* sp. for Indole acetic acid (IAA) production was investigated and the results were given in Table – 6. Maximum IAA production was observed in *Bradyrhizobium* BR – 3 (4.95 µg ml^{-1}). Least IAA production was noticed in *Bradyrhizobium* BR – 2 (0.86 µg ml^{-1}). It has been postulated that indole acetic acid (IAA) as the most probable initiator substance to cause the curling effect Subba Rao (1997). The ability to produce curling of root hair is necessary for a *Rhizobium* strain to be able to cause nodulation (Sahlaman *et al.*, 1962). In the present study, all five isolates had the ability to produce IAA under *in vitro* condition. The production of IAA ranged from 4.95 to 0.86 µg ml^{-1}.

The effect of *Bradyrhizobium* sp. on Exopolysaccharides (EPS) production was investigated and the results were given in Table – 6. Maximum EPS production was observed in *Bradyrhizobium* BR – 3 (275.50 µg ml^{-1}). Least IAA production was noticed in *Bradyrhizobium* BR – 2 (21.30 µg ml^{-1}). Kannerberg and Brewin (Kannenberg and Brewin (1994) pointed out that bacterial polysaccharides are necessary for *Rhizobium* - legume symbiosis. Exopolysacchairdes (EPS), Lipopolysaccharide (LPS), Capsular polysaccharides and cyclic (1-2) glucon play an essential role in the formation of the infection thread and in nodule development. All the 30 isolates from green gram produced EPS and the production ranged from 21.30 to275.50 µg ml^{-1} in culture broth. The effect of *Bradyrhizobium* sp. on the growth and yield of blackgram was investigated and the results were furnished in Table – 7. Maximum growth and yield parameters were observed in the treatment T_5 - 75% RDF + *Bradyrhizobium* sp. followed by the treatment T_2 - 100% RDF. The treatment T_5 was on par with the treatment T_2. Minimum growth and yield parameters were recorded in the control treatment T_1. The yield components *viz.*, number of pods per plant, pod length, number of seeds per pod, seed yield and hundred seed weight were significantly influenced by *Bradyrhizobium* sp. foliar spray. Application of phosphobacteria, DAP and *Bradyrhizobium* sp. 3% foliar spray at different stages of the crop led to better photosynthetic activity of the plant and more extensive root system in the soil there by resulting in better development Ramesh Thatikunta and Yakadri (2002). The above findings favour our results which was obtained in our present investigation.

Tomar (2002) reported that the application of *Bradyrhizobium* sp. significantly increased the plant height, dry matter production, grains and yield of blackgram. Parthasarathi and Ranganathan (2002) reported that the supplementation of vermicompost and *Bradyrhizobium* sp. with NPK enhanced the growth and yield in the leguminous crop *Vigna mungo*, which support our results very well. Application of phosphobacteria and organic manures helped in supplying phosphorus to the crop. In addition to this, foliar spray increased the plant height due to increase in leaves and cell growth. The results of the present study are in line with the findings of Durai Singh *et al.* (Durai Singh *et al.*, 2002). Application of *Bradyrhizobium* sp. and organic manures might have increased the plant height and leaf area index, resulting in better light interception and photosynthetic rate thereby contributing to higher growth components. The results are in agreement with the findings of Sathish Kumar *et al.* (Sathish Kumar *et al.*, 2003).

Chaudhary *et al.* (Chaudhary *et al.,* 2003) also reported that application of *Bradyrhizobium* sp. caused significant increase in LAI, DMP and number of branches per plant which was essential for the growth and development of crops (Kanchana *et al.,* 2014, Usharani *et al.,* 2013, Sivasakthi *et al.,* 2014, Usharani *et al.,* 2014).

Table - 7: Effect of *Bradyrhizobium* sp. on the growth parameters of blackgram

Treatments	Plant height (cm)	Leaf area index	Dry matter production (t ha^{-1})	Number of branches $plant^{-1}$	Number of pods $plant^{-1}$
T_1 - Control	27.45	2.90	1.3	7.70	21.18
T_2 – 100% RDF	35.23	3.35	2.3	8.05	28.36
T_3 – 25% RDF + *Bradyrhizobium* sp.	30.88	3.03	1.9	7.85	23.90
T_4 - 50% RDF + *Bradyrhizobium* sp.	33.28	3.20	2.0	7.91	26.52
T_5 - 75% RDF + *Bradyrhizobium* sp.	35.96	3.58	2.5	8.21	28.82

Conclusion

In this present study, it was concluded that the *Bradyrhizobium* promote the growth of black gram by symbiotic nitrogen fixation. Nitrogen is one of the major important nutrients essential for plant growth. The economic and environment importance of legume crops is largely due to their ability to fix atmospheric dinitrogen in a black gram performs N_2 fixation by establishing a symbiotic relationship with the *Rhizobium*. Symbiotic nitrogen fixation resulting from mutual beneficial interaction between black gram and soil nodule bacteria provides a significant boost to N fertilization and additionally, does not cause any hazard to environment. This study state that from the five isolate of *Bradyrhizobium* the BR-3 was (*Bradyrhizobium* isolated from Vayalore) recorded as maximum IAA production and EPS production IAA production was observed in *Bradyrhizobium* BR – 3 (4.95μg ml^{-1}) and EPS production was observed in *Bradyrhizobium* BR – 3 (275.50 μg ml^{-1}).

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