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Response of Biofertilizers on Growth, Yield Attributes and Associated Protein Profiling Changes of Blackgram (*Vigna mungo* L. Hepper)

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Abstract: A field trial was conducted during 2009 growing season at the Botanical garden, Department of Botany, Annamalai University to study the effect of biofertilizers on the growth, yield as well as nitrogen components, total protein and protein profiles in black gram. The treatments consisted of biofertilizers alone or combination treatment viz., control (without any microbial inoculation), *Azotobacter, Azospirillum,* Phosphobacteria, *Rhizobium, Rhizobium* + *Azotobacter, Rhizobium* + *Azospirillum* and *Rhizobium* + Phosphobacteria replicated three times in randomized block design. The results revealed that biofertilization perform significant improvement in plant productivity and quality. The maximum germination percentage, fresh and dry weight, no. of pods per plant, seed yield per plant, hundred seed weight and root nodules per plant were increased with the inoculation of biofertilizers especially *Rhizobium* with Phosphobacteria treatment when compared to others. Generally, biofertilizers induced over or low gene expression of most mid and high molecular weight protein bands. Hither, they also induced new proteins of low molecular weight. These low molecular weights proteins may be used as an adaptive mechanism for biofertilizer application in plants to give the maximum yield. The molecular weight ranged between 14.3 and 97.4 kDa were observed in treated plants as well as control. But, *Rhizobium* with Phosphobacteria inoculated plants produced one new protein band with molecular weight of 77.5 kDa.

Key words: *Vigna mungo* · *Azotobacter* · *Rhizobium* · SDS-PAGE · Phosphobacteria

INTRODUCTION

Black gram is the third important pulse crop in India. It is annual pulse crop and native to central Asia. It is also extensively grown in West Indies, Japan and other tropics/subtropical countries. Black gram seeds are highly nutritious containing higher amount of protein (24-26 %) and are reported to be rich in potassium, phosphorus and calcium with good amount of sodium. It is also reported to be rich in vitamin A, B1, B3 besides nutritionally rich proteins, important mineral and vitamins. It has some medicinal properties, like curing diabetes, sexual dysfunction, nervous disorder, hair disorders, digestive system disorders and rheumatic afflictions [1]. Black gram seeds have shown antiantherogenic activity in guinea pigs.

Soil is a dynamic, living matrix that is an essential part of the terrestrial ecosystem. It is a critical resource not only for agricultural production and food security but also towards maintenance of most life processes. The functions of soil biota are central to decomposition processes and nutrient cycling. Soil is considered a storehouse of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space. Therefore, major microbial activity is confined to the 'hot-spot', i.e. aggregates with accumulated organic matter, rhizosphere [2]. Soil micro organisms play an important role in soil processes that determine plant productivity.

Biofertilizer or microbial inoculants can be generally defined as preparation containing live or latent cells of efficient strains of nitrogen fixing and phosphate solubilizing microorganism used for treatment of seed or soil. They are organic products containing living cells of different types of microorganisms, which have the ability to convert nutritionally important elements from unavailable to available form through biological processes [3]. They are composting the area with the objective of increasing the number of such microorganisms and accelerate microbial process to augment to extent of the availability of the nutrient in a form which can easily assimilated by plant [4]. The *Rhizobium* as fertilizer in

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pulses could fix 50-200 kg of N/ha/season and is able to meet 80-90% of the crop requirement for nitrogen. Inoculation in these crops was found to increase the crop yield by about 10-15% under on farm conditions [5]. It includes mainly the nitrogen fixing, phosphate solubilizing and plant growth-promoting microorganisms [6]. Among biofertilizers benefiting the crop production are *Azotobacter*, *Azospirillum*, blue green algae, *Azolla*, P-solubilizing microorganisms and mycorrhizae [7]. Therefore, an experiment was conducted to study the influence of biofertilizer on growth, yield and associated protein profiling changes in black gram.

MATERIALS AND METHODS

Seeds of black gram (*Vigna mungo* (L.) cv. ADT3) obtained from Rice Research Institute, Aduthurai, Tamilnadu, India. The different types of biofertilizers (*Azotobacter, Azospirillum,* Phosphobacteria and *Rhizobium* were obtained from Ramvijay Biofertilizers, Puducherry**.** Seeds were sown in the plotted field with spacing of 10×30 cm between plants and rows. Biofertilizers were used alone as well as in combination to inoculate the different combination of the field such as T0-control (without any microbial inoculation), T1-*Azotobacter,* T2-*Azospirillum,* T3-Phosphobacteria, T4-*Rhizobium*, T5-*Rhizobium* + *Azotobacter;* T6-*Rhizobium* + *Azospirillum* and T7-*Rhizobium* + Phosphobacteria. The seeds were grown in a randomized block design in three replications. From each entry, 10 plants were randomly selected for recording observations on important yield attributing characters, plant height, fresh and dry weight, total chlorophyll, carotenoid, no. of pods per plant, seed yield per plant, hundred seed weight, protein content and no. of nodules per plant during the plant growth period.

Estimation of Chlorophyll [8]: 0.5 mg of fresh leaf was ground in a mortar and pestle with 20 ml of 80 per cent acetone. The homogenate was centrifuged at 3000 rpm for 15 min. The supernatant was saved. The pellet was restricted with 5 ml of 80 per cent acetone each time, until it become colorless. All the supernatants were pooled and utilized for chlorophyll determination. Absorbance was measured at 645 and 663nm in spectrophotometer. The chlorophyll content was determined by using the following formulae.

Chlorophyll 'a' (mg/g fr. wt.) = (0.0127) × (OD663) - (0.00269) × (OD645)
Chlorophyll 'b' (mg/g fr. wt.) = (0.229) × (OD645) - (0.00488) × (OD663)
Total chlorophyll(mg/g fr. wt.)=(0.0202) × (OD645) - (0.00802) × (OD663)

Estimation of Carotenoid [9]: The same chlorophyll extract was measured at 480 nm, in spectronic-20 to estimate the carotenoid content.

$$\text{Carotenoid (mg/g fr. wt.)} = \frac{D \times F \times V \times 10}{wt. \times 2500} = (\text{OD } 480 + \text{OD } 114)\ ´\ (\text{OD } 663) - (\text{OD } 638\ ´\ \text{OD } 645)$$

Estimation of Protein [10]: 0.5 mg of plant materials was macerated with a pestle and mortar with 10 ml of 20 per cent trichloroacetic acid. The homogenate was centrifuged for 15 min at 600 rpm. The supernatant was discarded. To the pellet, 5 ml of 0.1 N NaOH was added and centrifuged for 5 min. The supernatant was saved and made up to 10 ml of 0.1 N NaOH. This extract was used for the estimation of protein. From this extract, 1 ml of sample was taken in a 10 ml test tube and 5 ml of reagent was added. The solution was mixed well and kept in dark for 10 min. Later 0.5 ml folinphenol was added and the mixture was kept in dark for 30 min. The sample was read at 660 nm in the spectronic-20. Blank prepared without protein sample was used for zero setting. The absorbance value was referred to the standard graph proteins prepared by using 5th fraction of Bovine's serum albumen.

Seed Nitrogen Content [11 and 12] and Seed Crude Protein [13 and 14]: Two hundred mg of dried seed sample was taken in a 100 ml kjeldahal flask. About 5 mg of salt mixture (potassium sulphate, cupric sulphate and selenium powder mixed in the ratio of 50:10:1) was added with 3 ml of concentrated sulphuric acid. After digestion, 10 ml of distilled water was added. The distillate was collected in a conical flask containing 10 ml of 4 per cent boric acid and 3 drops of mixed indicator (0.3 g bromocresol green and 0.2 g methyl red in 400 ml of 90% ethanol). This solution was titrated against 0.05 N HCl. Nitrogen content was estimated using the flowing formula.

$$\text{Nitrogen (\%)} = \frac{\text{Sample titre} - \text{blank titre} \times \text{Hot HCl}}{\text{Sample weight} \times 1000} \times 14 \times 100$$

Crude protein was calculated by multiplying the total nitrogen content by 6.25.

Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE) for Protein Analysis: At seed maturity harvest stage, seeds from different treatments were taken for protein analysis. Proteins were extracted from the seed samples in liquid nitrogen. The tris buffer system used was that originally devised by Laemmli [15], as described by Dunn [16], with 2% (w/v)

SDS and 5% (v/v) 2-mercaptoethanol to cleave the disulphide bonds. The slurry was centrifuged for 20 min at 12000 rpm. The samples were heated in a boiling water bath for 15 minutes before loading to ensure dissociation. Gel preparation was carried out according to Hames [17], where 15% resolving gel was used. Bromophenol blue (0.001%) tracking dye was used for marking the buffer front during electrophoresis. The gel was electophoresed at 25 - 30mA constant current, then at 200 V (for about 8 - 9 h). Staining was done using coomassie brilliant blue and a solution of 10% acetic acid and 45% methanol was used for destaining. In the quantitative analysis of the protein bands, laser densitometry was used to image the stained profiles. Data were analyzed and identified by gel documentation system (GDS) which comparing polypeptide maps, which include the use of band intensity, molecular weight and the rate of mobility of each polypeptide with standard markers.

RESULTS AND DISCUSSION

Growth and Yield Characters: The growth and yield parameters of black gram such as germination percentage, plant height, fresh and dry weight, total chlorophyll, carotenoid, no. of pods per plant, seed yield per plant, hundred seed weight, protein content and no. of root nodules per plant were significantly increased by plant growth promoting rhizobacteria (PGPR) application in all concentrations when compared to control. Utilization of biological fertilizer increased fresh and dry weight, no. of pods per plant, seed yield and hundred seed weight that it could be due to increasing other nutrient absorption, also biological phosphate fertilizer can be used as a solution for increasing phosphate and micronutrient sorption in the alkaline soil. Zahir *et al*., [18] in maize both qualitative and quantitative characteristics were significantly increased by phosphate-solubilizing microorganisms and also increased the growth and resistance of plants in water deficit conditions (Ehteshami *et al*., 2007). Hoshang Naserirad *et al.* [19] and Asad Rokhzadi [20] indicated that inoculation with biofertilizers containing *Azotobacter* and *Azospirillum* increased plant height, leaf number per plant, fruit mean weight and yield in compare to control (without biofertilizer).

Azotobacter and *Azospirillum* fixed N_2 from the atmosphere and released plant available N forms to soil, resulting in increased uptake and plant height. These data agree with the previously reported results on the effects *Azotobacter* and *Azospirillum* on the plant height of bhendi [21].

P- solubilizing activity of phosphobacteria associated with the release of organic acids and a drop in the pH of the medium. Different kinds of organic acids, namely citric acid, gluconic acid, lactic acid, succinic acid, propionic acid and three other unknown organic acids were produced from the cultures of these isolates. The utilization of phosphate-solubilising microorganisms, account for about 45% of the total biofertiliser production and use. This bacterium helps in increasing crop productivity by way of helping in solubilization of insoluble phosphorus, stimulating growth by providing hormones, vitamins and other growth factors [22]. The availability of phosphorus to legume crop is a key constraint to its production. The soil micro organisms are responsible for transfer of the immobilized soil phosphorus into available form through which phosphorus becomes easily available to these legume crops [23].

The stimulatory effects of biofertilizers used are in accordance with the results obtained by Chauhan *et al*., [24] who found that inoculation of *Azospirillum*-as a biofertilizer-markedly increased pods number and seed yield of *Brassica napus* L. plants over the non inoculated plants. In addition, Buragohain [25] found that sugarcane yield was significantly higher in the cultivated crops with *Azotobacter* than uninoculated crops. In addition, Goel *et al*., [26] reported that, the inoculation with certain plant growth-promoting rhizobacteria (PGPR) may enhance crop productivity either by making the other nutrients available or protecting plants from pathogenic microorganism (allelopathic effects). Zodape [27] also concluded that, the increase in yield productivity with biofertilizer application is due to micro-element and plant growth regulator contained in the fertilizer.

Co-inoculation can benefit plant growth by different mechanisms [28]. However one of the most commonly reported plant growth promotion mechanism by bacteria is the changing of morphological and physiological changes in root system [29]. An increase in the number of lateral roots and root hairs cause addition of root surface available for nutrients and water uptake. Higher water and nutrient uptake by inoculated roots caused an improved water status of plant, which in turn could be the main factor enhancing plant growth [30, 31]. Increasing nutrient uptake such as NO^{-3}, NH_4, $Po^{-2}{}_4$, K^+, Fe^{2+} in the various inoculated plants have reported [32]. For example, single and double inoculation with *Azotobacter*, *Azospirillum* and *Streptomyces* increased P, Mg and N content in wheat grains [33]. Co-inoculation of *Sorghum* with *Azospirillum* and *Glomus* significantly increased P, N, Zn, Cu and Fe content. Thus co-inoculation may substitute partially as P and N fertilizer [34].

Protein Content: Results obtained from variance analysis showed that used bacteria treatment and interaction of these agents had significant difference. It is observed in mean comparison, that double-inoculation of *Rhizobium* with Phosphobacteria yielded more protein content than single and non-inoculation (control). Shehata and EL-Khawas [35] studied bio-fertilizers including growth prompting bacteria improved the sunflower yield and qualitative parameters in compared with control (non-inoculation) treatment and as a result caused to increasing protein content. Studying interaction of bacteria treatment showed that double-inoculation caused to nitrogen content and yielded highest protein content using double-inoculation. Since *Rhizobium* with Phosphobacteria is nitrogen fixing bacteria and nitrogen is basic matter to forming protein treatment.

Number of Nodules per Plant: The increased nodulation, N_2 fixation and yield of legume crops following inoculation with biofertilizers have been reported by many workers [36]. The number of nodules increased significantly in treatments with pure cultures of *Rhizobium* and carrier based inocula treated plants. It has already been reported by Hunter *et al.* [37]. The significant increase in dry matter and nodule weight with *Rhizobium* inoculation could be higher number of bacteria present under inoculated conditions. Vijle and Jebaraj [38] also reported similar results. The combined inoculation of *Rhizobium* + Phosphobacteria resulting in the significant increase in nodule weight than *Rhizobium* or PSB alone**.** It might have resulted due to more competitive ability of microbes in carrier than in pure culture against native rhizobial population. Since roots are the sites for microbial infection, well-developed root system provides more evidence for infection resulting in greater number of nodules.

NP Contents of Soil: Microbial inoculums not only increased the nutritional assimilation (total N, P and K) of plants, but also improved soil properties. In this study, soil was tested before sowing and after harvest of plants for residual NPK contents. Available N contents increased significantly in inoculated soil than uninoculated control. It may be due to reason that as the microbes fixes nitrogen and this nitrogen fixation suppresses nitrate uptake as demonstrated by Zarin *et al.* [39] who reported that soil nitrate was higher when hormones were applied to nitrogen fixing chickpea plants. It correlates with maximum nodulation influenced by microbes [40]. Moreover, significant increase in N content may be due to the fact that legumes contribute to the total pool of nitrogen in the soil as observed by Ahmad *et al*. [41]. Some nodules get sloughed off from senescing plants at harvest and results in increased NO^3-N and other macronutrients. Higher P content may be due to inoculation and availability of P nutrients in soil by microbes. Soil pH, organic C, total N, P and K, available N, P and K content were significantly increased by the application of biofertilizer application. Concerning micro-nutrients i.e., iron, zinc and manganese, the data in Table 1 show that the biofertilized treatments induced lower values in comparison with the mineral ones for iron and zinc while little differences between treatments were found regarding manganese element.

Table 1: Effect of biofertilizers on seed germination and morphological parameters of *Vigna mungo* (L.) Hepper

Treatments	Germination (%)	Plant height (cm)	Fresh weight (mg/g)	Dry weight (mg/g)	Total chlorophyll	Carotenoid	No. of pods per plant	Seed yield per plant (g)	Hundred seed wt.(g)	Protein content	No. of root nodules per plant
To	83.8±2.51	45.1±1.35	36.7±1.10	21.0±0.63	2.05±0.06	0.532±0.015	28.9±0.86	6.2±0.18	4.67±0.14	4.17±0.13	20.1±0.60
T1	87.2±2.62	50.1±1.50	39.5±1.19	22.7±0.68	2.11±0.06	0.542±0.016	31.5±0.95	7.3±0.21	4.43±0.13	4.20±0.13	76.6±2.30
T2	85.5±2.57	53.9±1.61	38.2±1.15	21.9±0.66	2.03±0.06	0.558±0.017	29.1±0.46	6.8±0.20	4.96±0.15	4.22±0.12	73.8±2.21
T3	87.2±2.62	54.2±1.63	39.0±1.17	22.4±0.67	2.63±0.08	0.520±0.016	30.0±0.91	7.0±0.21	5.10±0.15	4.34±0.13	76.6±2.29
T4	87.6±2.63	53.5±1.61	51.2±1.53	27.7±0.83	2.61±0.07	0.572±0.017	30.9±0.93	7.2±0.22	5.01±0.15	4.61±0.14	79.2±2.37
T5	87.8±2.63	53.5±1.61	48.2±1.45	23.9±0.92	2.47±0.07	0.533±0.016	26.6±0.80	7.8±0.23	4.91±0.15	4.29±0.13	70.2±2.11
T6	86.4±2.59	55.1±1.65	52.0±1.56	28.7±0.86	2.23±0.06	0.561±0.017	29.6±0.89	8.1±0.24	4.92±0.14	4.49±0.13	74.2±2.23
T7	88.5±2.65	56.7±1.70	54.0±1.62	29.6±0.88	2.85±0.08	0.572±0.017	31.8±0.95	8.5±0.26	5.14±0.15	4.81±0.14	80.2±2.41

Table 2: The effect of biofertilizers oh physico-chemical analysis of soil before sowing and after harvesting

Treatments	pH	EC (dS m^{-1})	Available N (kg/ha)	Available P (kg/ha)	Available K (kg/ha)	Available Ca (ppm)	Available Mg (ppm)	Available Zn (ppm)	Available Cu (ppm)	Available Fe (ppm)	Available Mn (ppm)
Before sowing	7.67	0.41	112.6	4.5	100.7	2.09	9.15	4.70	0.20	7.72	4.32
					After harvesting						
T0	7.60	0.44	119.2	5.9	114.2	2.18	9.37	5.72	0.26	8.16	5.01
T1	7.26	0.74	147.6	6.09	12.05	2.26	9.57	5.82	0.29	8.99	5.24
T2	7.52	0.98	162.7	8.12	120.1	2.32	9.61	5.44	0.23	8.26	5.33
T3	7.46	0.95	149.1	9.03	122.1	2.06	9.74	5.24	0.24	8.34	5.16
T4	7.38	0.94	160.1	8.2	120.6	2.87	9.62	6.07	0.32	9.26	5.21
T5	7.42	0.93	162.0	8.01	124.1	2.36	9.59	5.68	0.27	9.01	5.26
T6	7.46	0.93	152.0	7.6	121.0	2.44	9.62	5.71	0.31	8.39	5.06
T7	7.33	0.98	159.6	9.1	125.1	2.90	9.70	6.04	0.38	9.10	5.47

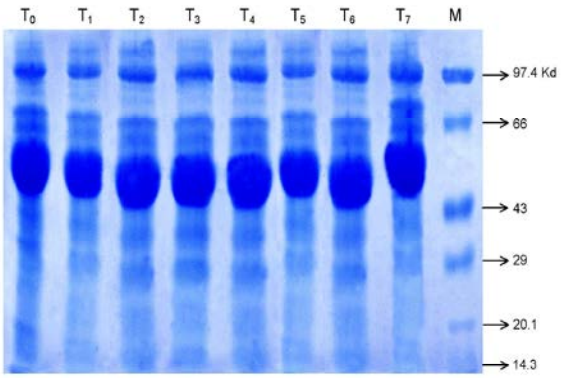

Fig. 1: Protein patterns of biofertilizer treated seeds in *Vigna mungo* L. The control (T0) represents without any microbial inoculation. The other groups of seeds are T1-*Azotobacter*, T2-*Azospirillum*, T3-Phosphobacteria, T4-*Rhizobium*, T5-*Rhizobium* + *Azotobacter,* T6-*Rhizobium* + *Azospirillum* and T7-*Rhizobium* + Phosphobacteria. M=marker

SDS-Protein Electrophoresis: Little investigations were carried out to study the effect of biofertilizer application on the protein content and protein profile. In this investigation, the total soluble protein content was significantly increased, the increase was more pronounced with biofertilizers (Table 2). Seed protein content was increased in response to biofertilizer application to soybean [40] and these results are in accordance with those obtained by Sharma and Namdeo [41] in mungbean and blackgram. The SDS-electrophoretic pattern showed that one new band having molecular weight 77.5 kDa that appeared by the effect of biofertilizer (*Rhizobium* with Phosphobacteria) treated seeds among the treatments under investigation in Figure 1. This combination treatment is characteristic by the appearance of one new inducible protein and this may be an adaptive mechanism for the biofertilizer absorption in plants to give higher yield productivity.

CONCLUSION

Bio-fertilizers are ecofriendly and environmentally safe. They form not only part of integrated nutrients but are low cost which is of immense help to the farming community. Utilization of biofertilizer increased all growth and yield promoting traits. As in this study maximum yield in compared to control was obtained by the application of biofertilizer. It seems that combination of *Rhizobium* with Phosphobacteria fertilizer was necessary for obtained maximum performance. Co-inoculation caused to increasing yield through synergistic effects by improving growth prompting hormones, controlling pathogenesis and growth reducing agents due to producing fungicide antibiotics and compounds (antagonistic effect) and also air molloculal nitrogen fixing and also producing growth prompting hormones such as auxin, cytokinin and gibberellin and solving mineral compound. The highest stimulatory effect of root associative beneficial bacteria, especially strains of *Rhizobium* + Phosphobacteria have potential to be used as biofertilizer increase the productivity of blackgram. Overall utilization of biofertilizers with single and combined treatments in addition to increased yield could be a strategy to achieve sustainable agriculture.

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