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Improving and effect of bio fertilizer on enhancement of the growth and bio chemical characteristic of photosynthesis on the Blackgram (*Vigna mungo* L.)

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Abstract Biofertilizer is an increasingly available in the market as one of the option in contrast to chemical fertilizer. The effectiveness of the growth of *Vigna mungo* L. utilizing biofertilizers, *Pseudomonas fluorescens*, and *Bacillus subtilis* was investigated. The rhizosphere soil sample collected from Dharmapuri District**.** The plants were raised in pots containing soil amended with various biofertilizers application (Control, *P. fluorescens, B. subtilis, P. fluorescens*+ *B. subtilis*). The seeds of *Vigna mungo* L. were transplanted in 4 pots of equal size, which were noted as treatment (T1-T4). The morphological parameters such as, shoot, root and leaf length, and biochemical constituents for example chlorophyll, carotenoids, carbohydrate and protein content were analyzed at 60 days respectively. Compare with all pots the combined inoculation of the biofertilizers (T4) *P. fluorescens*+ *B. subtilis* in 60 days showed better response in all the parameters was tested.

Keywords: Biofertilizer, Agriculture, *Vigna mungo* L., Yield, Growth

Introduction

India is increasing agricultural based country in agricultural land for various crop to improve yields. The nutrients for plant growing are nitrogen and phosphorus that plays the vital role to increase the productivity (Baset Mia and Shamsuddin, 2010). Rhizosphere soil the active microbial population of beneficial microorganism with neutralize and detrimental impacts on plant growth. There are various free-living soil bacteria that capable to apply beneficial impacts to the plants or protect environment through direct or indirect mechanisms (Antoun and Prevost, 2005). The microbial formulations are carrier–based preparations containing beneficial microorganisms that can be done by viable seed treatment and soil application. The inoculated seed treatment with plant growth promoting rhizobacteria (PGPR) is known to

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increase nodulation, nitrogen uptake, growth and yield responses of plants (Johri *et al.,* 2003). Biofertilizers are being used as one of the best modern tool for crop production ag. Biofertilizer is done as microbial inoculants containing living or dormant cells of effective strains of nitrogen fixing and phosphate solubilizing microorganism which utilized for seed and soil treatments. They are biological products containing living cells of various microorganisms which expressed the ability to convert nutritionally important nutrients from unavailable to available forms through biological process (Vessey, 2003). Biofertilizers are applied in the agricultural fields for substitution of chemical fertilizers which contains soil microorganisms or PGPR. It contains microorganisms to promote the adequate supplying nutrients to the plants for their appropriate growth and regulation in their physiology (Mishra *et al.,* 2013).

Blackgram (*Vigna mungo* L.) cultivar vembon belongs to the Fabaceae is one of the important pulses crops of India. Pulses crops are very important food crop in India and from all integral parts of the cropping system all over the country. They belong to Fabaceae or Leguminaceae and as an important nutrient which scientist reported the values of grain legumes as sources of protein, fiber and drawn attention to the complementary nature of their amniotic composition. It is primarily crown under rain fed condition and a low fertility neglected soil and India is the largest producer and consumer of the world (Saraswathi *et al.,* 2004).

The research finding was to test a combination of PGPR strains to enhance the growth of *Vigna mungo* L. under greenhouse conditions. Other objective was to combine two different PGPR testing for enhancing plant growth and yield of *Vigna mungo* L.

Material and methods

Soil sample collection

Soil sample were collected from Vellakkal in Dharmapuri District, TamilNadu, India. Sample was collected from the upper 5-15cm layer of the rhizosphere, deposited in sterilized plastic bags and transported to the laboratory in a cool box.

Soil analysis

The collected the soil sample was analyzed at Tamil Nadu Government Agriculture Department, Selanaickempatti, Salem District, TamilNadu. The physicochemical parameters such as pH, Nitrogen, Potassium and Phosphorous were investigated before and after treatments.

Seed collection

The experimental plant, seeds of Blackgram (*Vigna mungo* L.) cultivar Vembon were obtained from the pulses Division, TamilNadu Agricultural University, Coimbatore. Seeds with equal size, colour and weight were chosen for experimental purpose. Seeds were surface sterilized with 0.1% mercuric chloride solution and washed thoroughly with tap water and then with double distilled water.

Microbial strains

The selected microbial strains *Pseudomonas fluorescens* strain no. MK478897.1 and *Bacillus subtilis* strain no. MK483262.1 were obtained from in Soil Microbiology Laboratory, Department of Botany, Periyar University, Salem, TamilNadu, India (Figure 1). These strains were cultured individually by addition of bacterial strains in 50ml of nutrient broth and King's B medium. The flasks were placed in the incubator for 24-48 hours at 28℃ for their proliferation. Cultures were sub cultured to maintain for future work. The culture medium was added 1ml of grown pure culture of bacteria in 50ml broth at 28℃ for 24-48hours. The concentration of 10^9 cells/ml was adjusted for the experimental purpose.

Mass multiplication of biofertilizers

Bacillus subtilis strain no. MK4832621 was grown in nutrient broth which contains peptone 5g, beef extract 3g, sodium chloride 3g in 1 litre of distilled water, and adjusted pH7) then sterilized at 1.1 kg/ cm^2 pressure for 20 minutes. One full loop of *B. subtilis* culture was transferred and incubated for 24 hours served as mother culture. One litre of mother culture is transferred to 100 litres of sterilized nutrient broth in a fermenter. The bacterial growth is harvested after incubation for 72 hrs. Then, it is mixed with 250 kg of sterilized peat soil amended with 37 kg Calcium carbonate, dried in shade, packed in polythene bags and stored up to 6 months. It yielded *B. subtilis* biofertilizer (Figure 2).

Pseudomonas fluorescens strain no MK478897.1 is subcultured to sterilized Kings 'B' broth for 48 hours. The substrate talc powder is adjusted to pH 7 by calcium carbonate 150 g / kg. Those substrate is sterilized at 1.1 kg/cm 2 pressure for 30 minutes for two successive days. *P. fluorescens* suspension of 400 ml is added to 1 kg of substrate containing 5 g carboxy methyl cellulose

and mixed. The formulation is packed in Polythene bags and stored for one month. It yielded *P. fluorescens* biofertilizer (Figure 2).

Mixed biofertiizer

King's B and nutrient broths were used to transfer *P. fluorescens* and *B. subtilis* and incubated at 30±2 ℃ in shaking incubator for 48 h. The cultures were separately centrifuged to get pellets at 10,000 rpm at 4 ℃ for 15 min. The washed pellets were conducted by washing in saline solution 0.85% g (NaCl) to remove the residual culture broth. The washed pellets were then suspended in 1% carboxy methyl cellulose (CMC) suspension by mixing to get a uniform cell density of 10^7 CFU ml^{-1} (Mortensen *et al.,* 1992). It yielded mixed biofertilizer (*P. fluorescens +B. subtilis*).

Pot culture

Vigna mungo L. seeds were sown in 4 pots of equal size, 5kg of sterilized soil was used in each pot. The experimental pots were provided with water facilities, and weed control was also maintained.

The experiment was designed as a Randomized Complete Block Design (RCBD) with three replications. Treatments were non treated control (T1), *Pseudomonas fluorescens* Biofertilizer (T2), *Bacillus subtilis* Biofertilizer (T3) and mixed biofertilizers (*Pseudomonas + Bacillus subtilis)* as *(*T4). *B. subtilis* and *P. fluorescens* biofertilizers were tested. Each biofertilizers treatment was separately amended to sterilized soil surface (50 ml·pot−1) for three times a week after seedling emergence. Non-treated pots were made as control. There were 4 pots used in each treatment. All experimental pots were maintained in the open shade at under greenhouse condition with averaged relative humidity of 60% and temperature (20 ℃-30 ℃).

Morphological characterization of harvested plants

The plants were harvested after 60 days (Figure 3), and cleaned using running tap water to remove the adhered dust particles. The plant samples were dried using absorbent paper. The grown parameters eg. shoot, root and leaf length were measured by using centimeter scale and recorded.

Fresh weight and dry weight of plants

Plant samples were washed with tap water and sterile distilled water, then dried to remove the moisture content. The fresh weight was recorded by using electrical balance, separated and kept in a hot air oven at 80 ℃ for 24

hours, then kept in a desecrator, and dry weight was recorded using an electronic balance.

Yield parameters

Plants were selected at random from each pot and the number of pod per plant, pod length, number of seeds per pod were recorded. Randomly selected plants were uprooted or removed at 60 DAI along with soil mass.

Determination of Photosynthetic pigment assay in harvested plant samples

Estimation of chlorophyll content was done as 0.5mg of fresh leaves was crushed in a mortar and pestle with 20ml of 80% acetone. The homogenate leave sample was centrifuged at 3000rpm for 15 minutes, and supernatant was corrected. The pellets were restricted with 5ml of 80% percent each time, until it become colorless. All the supernatants were pooled and utilized for chlorophyll determination by using absorbance to measure at 645, and 663nm in the UV-visible spectrophotometer (Arnon, 1949).

Estimation of carotenoid content was done as same chlorophyll extract to measure at 480 nm, in spectrophotometer to determinate the carotenoid content (Krik and Allen, 1965).

Total carbohydrates

Total carbohydrates were estimated following the work of Hedge *et al*., (1962), 100mg sample heated in 5ml HCl (2.5M), and boiled for 3.5h. The mixture was allowed to cool at room temperature, then Na_2CO_3 was added to neutralize the reaction mixture. A final volume of 25 ml was prepared utilizing double distilled water. A mixture of 0.5ml extract and 2ml of Anthrone reagent was heated for 10 min and allowed to cool, the absorbance was estimated at 630nm.Total carbohydrate were estimated using D-glucose as a standard in mg^{-1} dry weight.

Protein assay

Protein assay was done using 100mg of leaves samples, then ground in phosphate buffer with a mortar and pestle as described method of Lowry and Rosebrough (1951). The stock standard was prepared at 1mg/ml (BSA), then the different concentrations of BSA were designed as 0.2, 0.4, 0.6, 0.8, 1.0ml and put into test tubes. The final volume was prepared to 1ml using sterile

distilled water, pipetted to 0.2ml of tested samples in test tube, and the final volume is done to 1ml by sterile distilled water, and 5ml of analytic reagent was added to each test tube, and then mixed well. It was incubated at room temperature for 10min before adding with 0.5ml of folin –ciocateaue to each test tube, and incubated for 30min. The absorbance was seen at 660nm using UV spectrophotometer.

Determination of some nutrients status of soil

To determine the nutrients status of the soil, soil sample of the experiment were analysed for pH, organic carbon, available N, P, K content.

Total microbial amounts

The amounts of cultivable microbe including bacteria were determined by plate counting method (Figure 4). One gram of each sample was weighed and added to 9ml of filter-sterilized water. Soil suspensions were diluted and plated on King's B and Nutrient Agar medium. The quality of microbial colony was counted after 3 to7 day incubation at 28 ℃.

Bacterial population was counted in 1 gram of soil sample in an Erlenmeyer filled with 90 ml of 0.8% sterile NaCl. The Erlenmeyer was operated in a 120 rpm shaker for 30 minutes and diluted into 10^1-10^7 dilutions. Each dilution was 0.2 ml dropped in a sterile petri dish, then poured with King's B and NA media. The media utilized for *Pseudomonas fluorescens* and *Bacillus subtilis* consecutively were Kings B and Nutrient Agar media. The quantity of viable cells was estimated by CFU/g (colony forming unit) after 7 days incubation.

Results

Soil analysis

The soil physicochemical parameters were tested increased after inoculation of biofertilizer such as pH (6.8), Nitrogen (37.82mg/Kg), Phosphorus (25.26 mg/Kg) and Potassium (13.05 mg/Kg).

Pot experiment

Plant growth parameters

The data pertaining the plant growth parameters (shoot and root length, leaf length) is presented in (Table 1, Figure 3). Shoot length was showed that treatments inoculated with of *Pseudomonas fluorescens* (27.2cm) followed by

Bacillus subtilis (25.9cm), *Pseudomonas fluorescens*+ *Bacillus subtilis* (28.92cm) respectively, root length was showed that treatments inoculated with of *Pseudomonas fluorescens* (12.76cm) followed by *Bacillus subtilis* (8.76cm), *P. fluorescens*+ *Bacillus subtilis* (10.86) and Leaf length was showed that treatments inoculated with of *P. fluorescens*+ *Bacillus subtilis* (7cm) followed by *Bacillus subtilis* (6.43cm), *fluorescens*+ *Bacillus subtilis* (7.06cm) when compared to uninoculated plants. Which indicated that, the highest plant growth parameters was found in T4. The lowest number of Number of pods per plant was recorded at T1.

Estimation of plant biomass

The data pertaining the plant biomass (fresh and dry weight) plant is presented in (Table 1). Fresh weight was showed that treatments inoculated with of *P. fluorescens* (49.56) followed by, *Bacillus subtilis* (59.03g), *Pseudomonas fluorescens*+ *Bacillus subtilis* (63.63), followed by dry weight was showed that treatments inoculated with of *Pseudomonas fluorescens* (27.46g/plant) followed by, *Bacillus subtilis* (26.20g), *Pseudomonas fluorescens*+ *Bacillus subtilis* (28.06) Which indicated that, the highest result for combined inoculation compared to uninoculated control plants was recorded at T1.

Yield parameters of Vigna mungo L.

Number of pods per plant

The data pertaining the number of the number of pods per plant is presented in (Table 2). Number of pods was showed that treatments inoculated with of *Pseudomonas fluorescens* (26) followed by *Bacillus subtilis* (25.3), *Pseudomonas fluorescens*+ *Bacillus subtilis* (27.6) which indicated that the highest Number of pods per plant was found in T4.The lowest number of Number of pods per plant was recorded at T1.

Length of the pod (cm)

The data of pod length was collected at harvesting stage. It was further analyzed and tabulated in (Table 2). Length of pod was showed that treatments inoculated with of *Pseudomonas fluorescens* (5.36cm/plant) followed by *Bacillus subtilis* (5.06 cm/plant), *Pseudomonas fluorescens*+ *Bacillus subtilis* (5.90 cm/plant). The highest length of the pod was recorded at T4 treatment which was significantly different from the test of the treatment and the lowest pod length was recorded at T1 treatment.

Number of seed per pod

Number of seeds per pod was showed that treatments inoculated with of *Pseudomonas fluorescens* (7.66) followed by *Bacillus subtilis* (8), *Pseudomonas fluorescens+ Bacillus subtilis* (8.66). The data pertaining the number of seeds per pod is presented in (Table 2). Which indicated that, the highest number of seeds per pod was found in T3.The lowest number of seeds per pod was recorded at T1.

Photosynthetic pigment (mg/g FW)

Total chlorophyll content significantly increased in *Vigna mungo* L. leaf which was inoculated with of followed by T2 (1.068 mg/g FW) T3 (0.075 mg/g FW) T4 (1.505 mg/g FW), respectively, when compared to uninoculated T1 (0.069 mg/g FW), plants. Carotenoids content also increased in inoculation of T2 (0.135 mg/g FW) followed by T3 (0.148 mg/g FW), T4 (0.163 mg/g FW) when compared with uninoculated control T1 (0.071 mg/g FW) plants (Table 3).

Estimation of carbohydrates

Total carbohydrates content significantly increased in *Vigna mungo* L. leaf which was inoculated with of T2 (13.58mg) followed by T3 (15.21 mg), T4 (24.03 mg) respectively, when compared to uninoculated T1 (11.71) plants (Table 3).

Estimation of protein content

Total protein content significantly showed in leaf which was inoculated with of T2 (0.173mg) followed by T3 (0.175mg), T4 (0.355mg), respectively, when compared to uninoculated (0.143mg) plants (Table 3).

Initial and post-harvest analysis of soil samples

Soil analysis was done after the field experiments with bioinoculant. In the treated soil, a highly amount of nitrogen content followed by phosphorus and potassium content was noticed, whereas the control soil recorded low amount of nitrogen, phosphorus and potassium content. In case of the treated plants recorded in comparison with uninoculated control plants (Table 4).The application of biofertilizer and organic fertilizer fundamentally increase the soil bacterial population which was counted on harvesting. The highest number of population was seen on the soil inoculated with the combination of *Pseudomonas fluorescens + Bacillus subtilis* Biofertilizers.

Figure 1. Mass production of Biofertilizers

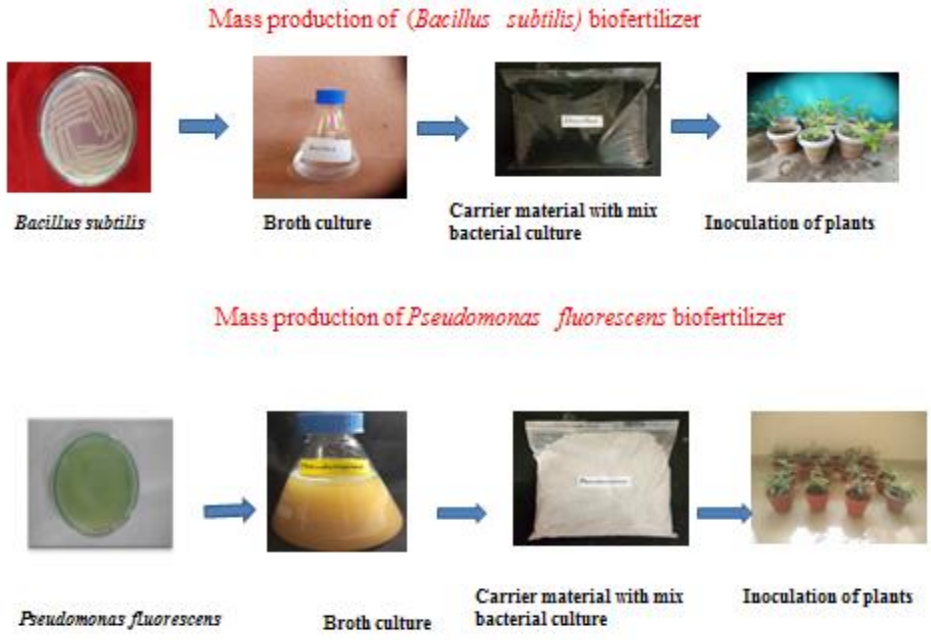

Figure 2. Preparation of biofertilizer using pot experiment

Figure 3. Effects on plant growth parameters of black gram plants treated with biofertilizer at 60 DAI

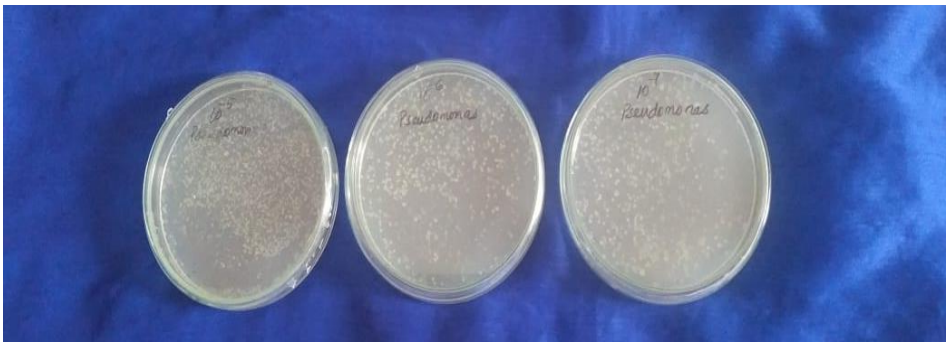

Figure 4. Soil microbial population

Table 1. Physical characteristic of post-harvest studies *Vigna mungo* L. (60 DAI)

Treatment	Shoot length (cm/plant)	Root length (cm/plant)	Leaf length (cm)	Fresh Weight (g/plant)	Dry Weight (g/plant)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
T1	32.3 ±1.01	10.10 ±1.67	5.46 ±0.25	29.96 ±1.91	16.83 ±1.40
T2	36.1 ±0.45	12.63 ±0.73	9.03 ±0.61	38.43 ±0.80	23.43 ±1.10
T3	33.8 ±0.70	12.06 ±0.77	10.66 ±0.56	37.80 ±0.43	21.36 ±1.56
T4	38.7 ±0.36	13.30 ±0.52	11.73 ±0.58	46.16 ±0.45	29.56 ±1.55
Fvalues	**50.382*** **	**5.410*** **	**81.687*** **	**84.546*** **	**40.149*** **

Data are represented by the mean of three replicates ± standard deviation. Significant difference *, **, *** = Extent of Significance LSD (P < 0.05).

Table 2. Yield parameters of *Vigna mungo* L.

Treatment	Number of pods /plant	Pod length (cm)	Number of seeds /pod
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
T1	13 ±2.0	7.6 ±0.30	10 ±1.00
T2	29.33 ±3.05	9.33 ±0.45	13 ±1.00
T3	26.33 ±1.52	8.26 ±0.35	11.33 ±1.52
T4	30.66 ±3.78	9.9 ±0.36	14 ±1.00
F values	**26.207*** **	**23.535*** **	**7.063***

Data are represented by the mean of three replicates ± standard deviation. Significant difference *, **, *** = Extent of Significance LSD ($P < 0.05$).

Table 3. Effect of biofertilizer on chlorophyll, carotenoids, carbohydrates, protein content in *Vigna mungo* L. at 60 DAI

Treatments	Chlorophyll (mg/g FW)	Carotenoids (mg/g FW)	Carbohydrates (mg/g)	Protein (mg/g)
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
T1	0.069 ±0.005	0.071 ±0.009	11.71 ±0.365	0.143 ±0.005
T2	1.068 ±0.135	0.135 ±0.065	13.58 ±0.634	0.173 ±0.004
T3	0.075 ±0.008	0.148 ±0.007	15.21 ±0.371	0.175 ±0.011
T4	1.505 ±0.544	0.163 ±0.019	24.03 ±0.821	0.355 ±0.012
F.values	**19.954*** **	**36.326*** **	**264.940*** **	**332.160*** **

Data are represented by the mean of three replicates ± standard deviation. Significant difference *, **, *** = Extent of Significance LSD ($P < 0.05$).

Table 4. Physico-chemical analysis of before and after treatments in soil (*Vigna mungo* L.)

Nutrition	Before sowing soil analysis	Post harvesting soil analysis			
		T1	**T2**	**T3**	**T4**
pH	6.80	6.68	6.59	6.41	6.46
EC (dS/m)	0.654	0.668	0.563	0.609	0.586
Nitrogen (mg/Kg)	37.82	35.5	35.6	34.9	36.3
Phosphorus (mg/Kg)	25.26	24.62	27.8	26.9	28.3
Potassium (mg/Kg)	13.05	11.5	13.1	17.6	19.6

Discussion

The soil sample was analyzed to Tamil Nadu Agriculture Department, Salem. The physicochemical parameters such as pH, EC, Nitrogen, Potassium, Phosphorus content were tested before and after inoculation of biofertilizers. The impact of biofertilizers treatment on growth development and yield of blackgram was essentially higher in single or combined inoculation than control plants. Pot culture experiments was conducted to find out the effect of biofertilizers (*Pseudomonas fluorescens*, *Bacillus subtilis*) on growth , photosynthetic pigments, carbohydrates and protein of blackgram (*Vigna mungo* L.). In the current study, the highest fresh and dry weight of blackgram where recorded in combined application of biofertilizers *(Pseudomonas fluorescens + Bacillus subtilis*) when compared to control as well as biofertilizers alone. Similar results were observed with Maheshwari *et al.* (2014) carried their research work on effectiveness of the growth development of *Vigna mungo L.* utilizing biofertilizers.

The results showed that *Pseudomonas fluorescens+Bacillus subtilis* treated plants had the highest chlorophyll and protein contents. Similarly results were reported by the biofertilizers essentially improved chlorophyll concentration in chilli and blackgram (Tamilzhiniyan and Selvakumar, 2011). In this experiment, the highest yield parameters number of pods per plants, number of seeds were recorded in the crop grown under the combined applications of biofertilizers (*Pseudomonas fluorescens+Bacillus subtilis)* when compared with control as well as other treatments.this findings are in with other reports on maize (Wu *et al.,* 2005), rice and maize (Rajeswar and Ariff Khan, 2010) and soybean (Zarei *et al.,* 2012).

Hence my present investigation was clearly highlighted that combined inoculation of biofertilizers such as *P. fluorescens+B. subtilis* could enhance the morphological parameters such as shoot, root and leaf length, fresh and dry weight, number of pods, pod length, number of seeds and biochemical constituents such as chlorophyll, carotenoids, carbohydrates, protein. Compared to individual inoculation and control. Biofertilizer produced higher bacterial population than control plant combination. Biofertilizer of course have ability to replace the traditional chemical fertilizers and carrier based biofertilizers and play an important role in restoring the soil health. Hence it is justifiably concluded that the microbial inoculants could be detailed and delivered as biofertilizers bioinoculants to farmers.

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