

Integrated Effect of *Rhizobium* and *Azotobacter* Cultures on the Leguminous Crop Black Gram (*Vigna mungo*)

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Abstract

A pot experiment was performed to evaluate the integrated effect of *Rhizobium* and *Azotobacter sp.* on the plant growth, nodule appearance, no of leaf, shoot length, root length, chlorophyll contents and carbohydrate content in black gram during 2016 growing period at the Department of Microbiology, Dr. Ram Manohar Lohia Avadh University Faizabad, UP, India. Different treatments viz., T₁: Control (Sterile soil+Seeds without culture treatment), T₂: Sterile Soil and Seeds both are treated with *Azotobacter sp.*, T₃: Sterile Soil and Seeds both are treated with *Rhizobium sp.*, T₄: Sterile Soil and Seeds both are treated with mixed culture of *Azotobacter sp.* and *Rhizobium sp.*, T₅: Sterile Soil+Seeds treated with *Azotobacter sp.*, T₆: Sterile Soil + Seeds treated with *Rhizobium sp.*, T₇: Sterile Soil+Seeds treated with mixed culture of *Azotobacter sp.* and *Rhizobium sp.* All experiments were carried out in triplicate set. The T₄ treatment showed maximum shoot length (51.6 cm), root length (17.3 cm), fresh and dry shoot biomass (12.99 and 3.21 g), fresh and dry root biomass (3.54 and 0.99 g), no. of leaves (20.4), root nodules per plant (18.2) and chlorophyll content (1.3 mg/g) and reducing (867.4 µg/g) and non-reducing sugar (1905.5 µg/g) content per plant biomass respectively. The *Azotobacter* and *Rhizobium sp.* have friendly associations and they have different physiology and habitat. Therefore, they help plant growth promotion by them own system. Therefore, such combination can be recommended for field application for sustainable agriculture. Excessive application of chemical fertilizers causes environmental and economic problems; hence the use of PGPR and *Rhizobium* bacteria can be acceptable due to cut contribution expenditure, increase in grain yield and environmental friendly.

Keywords: *Azotobacter*; Black gram; Co-culture; *Rhizobium*; Biofertilizers; *Vigna mungo*; Germination

Introduction

Black gram is one of the important pulse crops in India. It is also generally grown in other tropical/subtropical countries. Black gram is extremely nutritious due to having higher protein contents (24-26%) along with higher content of potassium, phosphorus, calcium, sodium and vitamins (retinoic acid, thiamine, riboflavin) [1]. It has several therapeutic properties, like curing diabetes, sexual dysfunction, nervous, hair, and digestive system disorders and rheumatic afflictions [2]. Black gram seeds have shown anti-anthrogenic activity in guinea pigs.

Chemical fertilizers are frequently used to achieve maximum crop production in agricultural field. These cost effective chemicals, however, when used roughly, have resulted in loss of soil fertility and consequently, the crop production [3]. Due to these reasons, focus in recent times has been shifted towards the use of cost-competitive biological resources such as Plant Growth Promoting *Rhizobacteria* that colonize the roots of plants following inoculation onto seeds and that enhance plant growth [4]. The PGPR used as biofertilizers have ability to mobilizing significant nutrients in the soil from unavailable to available form for most vegetation and important for crops [1,5,6]. Therefore, its use is an eco-friendly approach to reduce the utilization of chemical fertilizers, enhance soil fertility and increase crop production by their biological activity in the rhizosphere. Several bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Rhizobium*,

Flavobacterium, *Bacillus* and *Serratia*, *phosphobacteria* and VAM fungi have been used as biofertilizers supplement of nitrogen and phosphorus fertilizers for improved crop production [7-16].

Rhizobium bio-fertilizer approx fix 50-200 kg of N/ha/season and increase the crop yield about 10-15% agriculture field [17]. Bio-fertilizers comprised mostly the nitrogen fixing, phosphate solubilizing and plant growth-promoting microorganisms [18]. The main agents of biofertilizers are *Azotobacter*, *Azospirillum*, blue green algae, *Azolla*, P-solubilizing microorganisms and *mycorrhizae* [19]. However, apart from providing N to plants, *Azotobacter* promotes plant growth directly by secreting considerable amounts of biologically active substances like B vitamins, nicotinic acid, pantothenic acid, biotin, gibberellic acid, Indole-3 Acetic Acid (IAA) and cytokinin [20-22] and ammonia [23] or indirectly by protecting the plant from diseases [24]. Hence, a trial was performed to study the effect of bio-fertilizer on plant growth, yield and linked protein content changes in black gram. In this paper, we have observed and discussed the combined effect of *Rhizobium* and *Azotobacter* on growth promotion of black gram. In this aspect, a strain of *Rhizobium* and *Azotobacter* was isolated from methi plants and wheat rhizosphere respectively.

Materials and Methods

Isolation, screening and identification of *Rhizobia* and *Azotobacter sp.*

For the isolation of *Rhizobia*, healthy plants root nodules from methi was used. All selected root nodules were washed with water and then immerse the nodules in HgCl₂ (0.1%) or H₂O₂ (3-5%) for five

minutes to surface sterilization. After that, nodules were washed in sterile water for 3-4 times. All nodules sticking to the root system were removed and surface sterilized by treating with 70% alcohol for 1 minute, after which they were treated by chloramine-T solution (1%) for 3 minutes and washed thoroughly by with sterile water. Now, nodules were crushed in 1000 µl of water with a sterile rod and make a suspension of *Rhizobia* with sterile water. Then suspension of *Rhizobium* (0.1 ml) was spread on the yeast extract agar medium plate which contain (g/l): Yeast extract: 1.0, K₂HPO₄: 0.5, K₂SO₄·7H₂O: 0.2, NaCl: 0.1, Mannitol: 10.0, Agar: 20 and 2.5 ml congo red solution (1%) with pH 6.9. The inoculated plates were incubated for 5-6 days at 26°C for proper growth. After that, culture was maintained on the same medium and stored at 4°C in the refrigerator. The culture was re-culturing after every 15 days.

For isolation of *Azotobacter*, one gram soil sample (Wheat rhizospheric soil) was added to 9 ml of sterile water, shake well by vortex allowing standing for 30 minutes. Then 1 ml of sample suspension was transferred to 9 ml sterile distilled water. Through this method, samples were serially diluted up to 10⁵ dilutions. After that, 1 ml of serial diluted samples (from 10³ to 10⁵ fraction) was used in a sterilized Petri Plates containing Ashby's medium and then incubated at 28 ± 2°C temperature for 2-3 days for proper growth. The isolates were purified through streak plate technique.

Preparation of inoculum of *Rhizobium* and *Azotobacter sp.*

One full loop of pure *Rhizobium* and *Azotobacter* culture was inoculated in 100 ml Nutrient broth for 24 h for preparation of inoculum, which was used for soil and seed treatment. The Soil sample (2 Kg) and seed sample (8 seeds) was treated with pure culture of *Rhizobium* and *Azotobacter* with 25 and 10 ml inoculum.

Soil collection and sterilization for enrichment by *Rhizobium* and *Azotobacter*

Soil samples were collected in sterile polybags (each bag contain 2 Kg Soil) from different garden of Avadh University Campus and then autoclaved at 15 lbs for 15 min. After that, sterile soil samples were supplemented with different combinations of *Rhizobium*, *Azotobacter* and mixed culture of *Rhizobium* and *Azotobacter sp.* Sterile soil sample without any culture treatment was work as control. Then treated and untreated soil samples were ready for soil sowing.

Seed preparation and sowing in treated and untreated soil

For seed preparation, the seeds of Black gram were collected and treated with 10 ml of *Rhizobium* and *Azotobacter* culture for 30 min, then sowing it into the treated and untreated soil samples and left it for its proper growth.

Optimization of different parameters for maximum plant growth promotion

Germination studies: Seeds were allowed to germinate in treated and non-treated soil sample in polybags under field conditions.

Site of experiment: The plants were maintained under natural condition in the garden, Dr. Ram Manohar Lohia Avadh University, Faizabad, UP, India in 2016. The weather was temperate and slightly wet at the time of starting the experiment. The temperatures ranged between 35°C to 38.5°C (maximum) and 25.6°C to 30.7°C (minimum), respectively during the experimental period.

Experimental plan and design (Treatments)

T₁: Control (Sterile soil+Seeds without culture treatment)

T₂: Sterile Soil and Seeds both are treated with *Azotobacter sp.*

T₃: Sterile Soil and Seeds both are treated with *Rhizobium sp.*

T₄: Sterile Soil and Seeds both are treated with mixed culture of *Azotobacter sp.* and *Rhizobium sp.*

T₅: Sterile Soil+Seeds treated with *Azotobacter sp.*

T₆: Sterile Soil+Seeds treated with *Rhizobium sp.*

T₇: Sterile Soil+Seeds treated with mixed culture of *Azotobacter sp.* and *Rhizobium sp.*

Seedlings: In one set of experiment, treated black gram seeds were sown in plastic bag containing sterile soil which treated with *Azotobacter sp.*, *Rhizobium sp.*, and mixed culture of *Azotobacter sp.* and *Rhizobium sp.*, and in another set of experiment treated black gram seeds were sown in plastic bag containing sterile soil without any culture treatment. In this experiment, sterile soil and seeds without any culture treatment were worked as control as mentioned earlier. Seven experimental replicates were set for all treatment. Black gram seeds were also treated with *Azotobacter sp.*, *Rhizobium sp.*, and mixed culture of *Azotobacter sp.* and *Rhizobium sp.*, after surface sterilized with sodium hypochloride (0.005%) for 45 min and wash twice with sterile distilled water and then grown into a 5 cm depth in polythene bag under natural condition for two months.

Growth parameters

The growth parameters were calculated on every 15th, 30th, 45th and 60th day of the plant growth in all the treatments.

Shoot length: The plants were uprooted carefully without damaging the root system. The shoot length of the plants was measured from the collar region to the tip of the plant, using a standard scale and values were recorded in centimeters.

Root length: The plants were uprooted carefully without damaging the root system. The root length of the plants was measured from the end region to the shoot of the plant, using a standard scale and values were recorded in centimeters.

Number of leaf: Number of completely opened leaf in all the treatments was calculated manually.

Number of root nodules: Number of completely developed nodules in all the treatments was calculated manually.

Fresh shoot biomass: The shoot part was segregated from the root and was blotted on the Whatmann No 1 filter paper to absorb the water content. The mass (in gram) of the shoot was measured by saltorious.

Fresh root biomass: The plants were carefully uprooted and roots were rinsed with tap water to remove the soil particles. The clean roots were blotted on the Whatmann No 1 paper and the roots were weighed using electrical balance in grams.

Dry shoot biomass: To obtain the fresh shoot biomass of the plants, the shoots were dried in a hot air oven at 60°C for 48 hours to get constant mass. After that, dry weights of the shoots were measured in grams by electrical balance.

Dry root biomass: After measuring the dry root biomass of the plants, it was dried in a hot air oven at 60°C, for 48 hours. After that, dry weights of the roots were calculated in grams by electrical balance.

Physiological parameters

Estimation of chlorophyll content: The chlorophyll content of the plant leaves were estimated in all the treatments on 15th, 30th, 45th and 60th days by Arnon method [25]. The leaves were expurgated from the plants and rinsed with water and then blotted dry. One gram of leaf sample was homogenized with 10 ml pre-chilled 80% acetone and a bit of CaCO₃ was supplemented to make easy grinding. After grinding, the extract sample was centrifuged at 10000 rpm for 10 minutes. The supernatant was filtered through Whatmann No 1 filter paper and the apparent supernatant was transferred to 1 cm quartz cuvette. The absorbance was measured using specific absorptions co-efficient for chlorophyll a and b at 645 nm and 663 nm using 80% acetone as blank in UV-Visible spectrophotometer. The following simultaneous equations were setup for measuring chlorophyll concentrations.

Chlorophyll“a”=(0.0127 × OD at 663 nm)-(0.00269 × OD at 645 nm).

Chlorophyll“b”=(0.0229 × OD at 645 nm)-(0.00468 × OD at 663 nm).

Estimation of carbohydrates: Reducing and non-reducing sugars were extracted from shoot and root from all the test treatments were estimated by Highkin and Frankel method [26]. The carbohydrate contents were estimated on 15th, 30th, 45th and 60th days of plant growth. Two hundred milligram of oven dry sample was extracted with 20 ml (80%) boiled ethanol and then centrifuged at 10000 rpm. The supernatant was reduced to a half volume (10 ml) on boiling water bath and cooled at room temperature. After that, 5 ml of saturated neutral lead acetate was added in the supernatant to precipitate proteins. Saturated aqueous disodium phosphate (10 ml) was also added in the supernatant to precipitate excess of lead. Then activated charcoal (300 mg) was added and the supernatants were shaken for 30 min and then filtered. The filtrate supernatant was diluted to 100 ml with distilled water and was used for reducing and non-reducing sugars estimation.

Results

Isolation, screening and identification of *Rhizobium sp.* and *Azotobacter sp.*

Azotobacter sp. and *Rhizobium sp.* were isolated on Ashby's and Yeast extract agar medium at 28°C from wheat sphere soil and methi plant. The appearance of *Azotobacter* is small and smooth white colony on Ashby's medium plate. Morphologically, it is rod shape and Gram-negative under microscopic study. The colonies of the isolate were smooth, convex, glistening and opaque on media (Figure 1). Biochemical tests revealed that are isolate P-12 showed positive results for Catalase, Oxidase and Starch hydrolysis, Indole, Motility tests and Citrate utilization. The isolate was positive for H₂S production, urea hydrolysis, and produced acid from glucose, maltose, fructose, Trehalose, and raffinose.



Figure 1: Sample of root nodules from Gram plant root.

Deep Glucose Agar test confirmed that the isolate was obligate aerobes. Halo-tolerance ability is a significant feature of *Azotobacter* for salinity affected areas. If it is possible to apply halo-tolerant *Azotobacter* as biofertilizer, the crops productivity will definitely increase. It is found that our isolate was salt resistant *Azotobacter* (10% NaCl) (Table 1a).

Morphologic characters	Result	Biochemical characters	Result
Gram reaction	-	Catalase	+
Shape	Rod	Oxidase	+
Motility Test	+	Carbohydrate utilization	
Pigment	White	Glucose	A
Spore formation	-	Fructose	A
Colony Appearance	Smooth, convex, opaque	Maltose	A
Different NaCl concentration (%)		Raffinose	A
2%	+	Trehalose	A
4%	+	Starch	+
6%	+	Urease test	+
8%	+	Nitrate reductase	+

10%	+	H ₂ S production	+
Growth in different temperatures		Amylase	+
10	+	Voges-Proskauer	-
20	+	Indole	+
30	+	Lysine decarboxylase	-
40	+	Arginine dihydrolase	-
50	+	Culture	<i>Azotobacter</i> sp.

Table 1: (a) Morphological and Biochemical Characterization of *Azotobacter* sp.

The *Rhizobium* isolates were white pigmented, circular, elevated, semi translucent, mucilaginous with entire margin on yeast extract mannitol agar and Gram-negative rods, arranged singly corresponding to the general character of genus *Rhizobium*. The bacterial colony was circular, convex and semi- translucent on YEM agar. All bacteria were capsulated but did not form any spore (Table 1b). *Rhizobium* gives negative result with indole and TSI test and positive result with MR-VP

and catalase test. The cultural and biochemical properties exhibited by the bacteria isolated from legumes are in accordance to the cultural description of *Rhizobium* sp given in Bergey's manual [27]. When subjected to versatility in utilizing different carbon sources as energy the isolate utilized lactose, glucose, sucrose and starch, respectively (Table 1b). This shows more likeness of isolates towards lactose than other carbon sources.

Morphologic characters	Result	Biochemical characters	Result
Gram reaction	-	Catalase	+
Shape	Rod	Oxidase	+
Motility Test	+	Carbohydrate Fermentation	
Pigment	Redish	Glucose	+
Spore formation	-	Fructose	+
Colony Appearance	Smooth, convex, opaque	Maltose	+
Different NaCl concentration (%)		Raffinose	+
2%	+	Trehalose	+
4%	+	Sucrose	+
6%	+	Urease test	+
8%	-	Nitrate reductase	-
10%	-	H ₂ S production	-
Growth in different temperatures		Amylase	-
10	+	Voges-Proskauer	+
20	+	Indole	+
30	+	Gelatin hydrolysis	-
40	+	Arginine dihydrolase	-
50	-	Culture	<i>Rhizobium</i> sp.

Table 1: (b) Morphological and Biochemical Characterization of *Rhizobium* sp.

Effect of *Rhizobium* and *Azotobacter sp.* on black gram growth parameters

Both strains showed positive results in the experiments carried out on “Effect of *Azotobacter*, *Rhizobium* and mixed culture of both (*Azotobacter* and *Rhizobium sp.*) on the growth and biochemical aspects of black gram” were discussed in this section. To enhance a significant plant growth response, it is necessary to recognize the prominent strains of PGPRs for the sowing condition. It was in this context that an efforts were made to study the PGPRs of black gram with special reference to *Rhizobium* and *Azotobacter* and their mixed culture. The effects of enriched microbial inoculants in soil and on plant growth, biomass and biochemical characteristics were studied in polybag culture under natural condition. Soil which augmented with mix microbial inoculants was found to significantly increase shoot length, root length, number of leaf, number of nodules and fresh and dry weight of shoot and root, total fresh and dry weight of the plant. The microbial inoculants provide high-quality of plant nutrients has supported plant growth.

Effect on shoot length: In this experiment, the result showed that shoot length was greatly increased with the mixed culture of *Rhizobium* and *Azotobacter sp.* when compared with single culture of *Azotobacter sp.*, and *Rhizobium sp.*, at 30th, 60th, 90th and 120th days after planting. The maximum shoot length was achieved in the T₄ treatment with 20.1, 35.8, 44.3 and 51.6 cm, respectively (Figure 2). The mixed culture inoculations of prominent microorganisms have been reported to perform better than the single culture treatments.

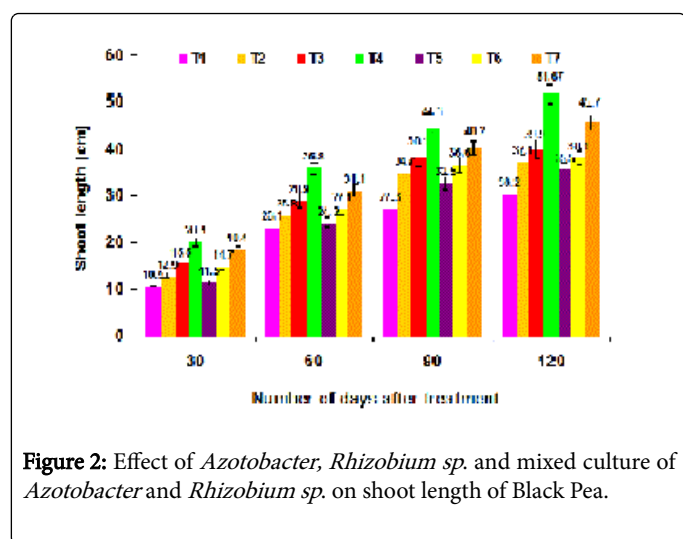


Figure 2: Effect of *Azotobacter*, *Rhizobium sp.* and mixed culture of *Azotobacter* and *Rhizobium sp.* on shoot length of Black Pea.

Effect on root length: The plant root length was increased considerably with the mixed culture of *Rhizobium* and *Azotobacter sp.* when compared with single culture of *Azotobacter sp.*, and *Rhizobium sp.*, at 30th, 60th, 90th and 120th days after planting. The maximum average root length was achieved in the T₄ treatment with 7.3, 12.1, 15.4 and 17.3 cm, respectively (Figure 3). The combined inoculations of beneficial organisms have been reported to perform better than the single inoculation treatments.

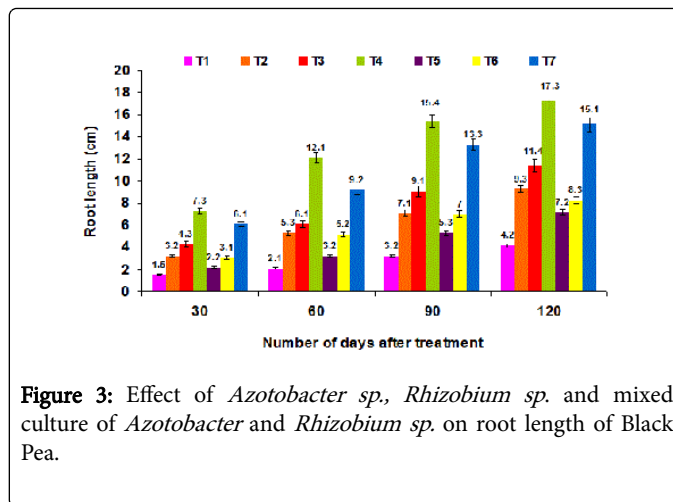


Figure 3: Effect of *Azotobacter sp.*, *Rhizobium sp.* and mixed culture of *Azotobacter* and *Rhizobium sp.* on root length of Black Pea.

Effect on leaves number: The plant leaves number were increased significantly with the mixed culture of *Rhizobium* and *Azotobacter sp.* when compared with single culture of *Azotobacter sp.*, and *Rhizobium sp.*, at 30th, 60th, 90th and 120th days after planting. The maximum average leaves number was recorded in the treatment, T₄ with 8.4, 12.3, 16.3 and 20.4, respectively (Figure 4). The combined inoculations of beneficial organisms have been reported to perform better than the single treatment.

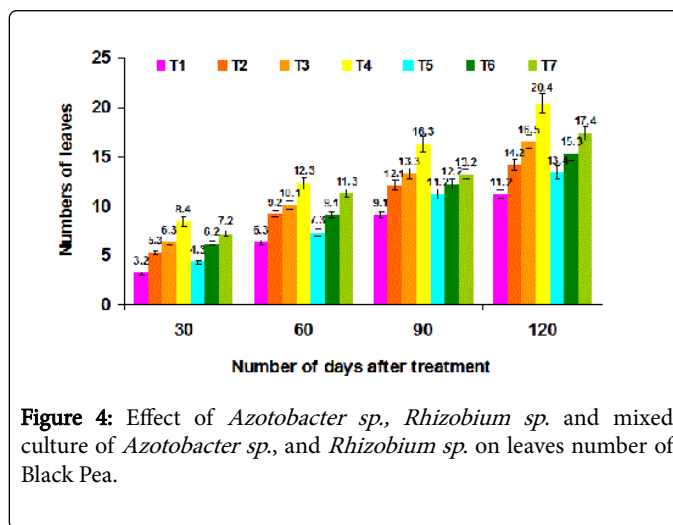


Figure 4: Effect of *Azotobacter sp.*, *Rhizobium sp.* and mixed culture of *Azotobacter sp.*, and *Rhizobium sp.* on leaves number of Black Pea.

Effect on nodules number: The plant root nodules number were increased significantly with the mixed culture of *Rhizobium* and *Azotobacter sp.* when compared with single culture of *Azotobacter sp.*, and *Rhizobium sp.*, at 30th, 60th, 90th and 120th days after planting. The maximum average root nodules number was recorded in the T₄ treatment with 6.1, 12.3, 16.3 and 18.2 respectively (Figure 5). The combined inoculations of beneficial organisms have been reported to perform better than the single inoculation treatments.

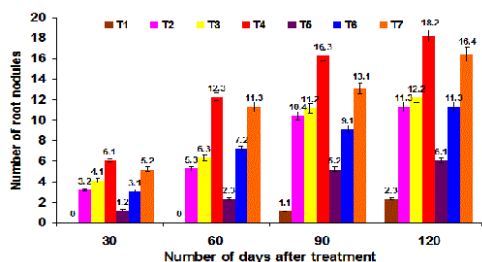


Figure 5: Effect of *Azotobacter sp.*, *Rhizobium sp.* and mixed culture of *Azotobacter sp.*, and *Rhizobium sp.* on root nodules number of Black Pea.

Fresh and dry shoot and root biomass: Accordingly, the root and shoot growth, the fresh and dry content in root and shoot as well as total dry contents of black gram were also increased due to the combined action of both strain. The maximum root and shoot fresh and dry weight was achieved in the T₄ treatment. The T₄ Treatment enhanced the root fresh and dry content of 3.54 and 0.99 g per plant and shoot fresh and dry content of 12.99 and 3.21 g per plant over the control (Tables 2 and 3). Mix-culture could increase the total root and shoot fresh and dry biomass of 16.5 and 4.2 g per plant, respectively (Tables 2 and 3).

Treatments	Shoot fresh biomass				Root fresh biomass				Total fresh biomass			
	Days after treatment											
	30	60	90	120	30	60	90	120	30	60	90	120
T ₁	0.85	1.46	2.11	4.81	0.32	0.77	1.01	1.6	1.17	2.23	3.12	6.1
T ₂	1.33	2.73	4.27	7.55	0.45	1.18	1.31	2.1	1.78	4.53	5.58	9.65
T ₃	1.68	3.98	5.41	9.01	0.51	1.23	1.51	2.40	2.19	6.91	6.92	11.41
T ₄	2.87	6.95	9.53	12.9	0.67	1.48	1.73	3.54	2.58	8.45	10.26	16.53
T ₅	1.21	2.38	3.87	5.90	0.40	1.0	1.28	2.0	1.61	3.30	5.69	7.9
T ₆	1.42	2.77	4.91	7.01	0.5	1.08	1.38	2.2	1.92	3.85	6.29	9.21
T ₇	1.58	3.75	6.72	10.1	0.62	1.28	1.41	2.38	2.2	5.03	8.13	12.43

Table 2: Effect of *Azotobacter sp.*, *Rhizobium sp.* and mixed culture of *Azotobacter*, and *Rhizobium sp.* on fresh bio mass weight (g) of Black Pea.

Treatments	Shoot dry biomass				Root dry biomass				Total dry biomass			
	Days after treatment											
	30	60	90	120	30	60	90	120	30	60	90	120
T ₁	0.29	0.67	0.91	1.48	0.11	0.26	0.44	0.65	0.40	0.93	1.35	2.13
T ₂	0.43	0.84	1.08	1.95	0.21	0.4	0.55	0.85	0.64	1.24	1.63	2.8
T ₃	0.68	1.0	1.35	2.90	0.29	0.47	0.58	0.89	0.97	1.47	1.93	3.79
T ₄	0.81	1.13	2.56	3.21	0.33	0.5	0.69	0.99	1.14	1.63	3.25	4.2
T ₅	0.39	0.76	0.9	1.5	0.18	0.37	0.44	0.65	0.57	1.13	1.34	2.15
T ₆	0.58	0.92	1.12	2.38	0.26	0.41	0.49	0.82	0.84	1.33	1.61	3.2
T ₇	0.78	1.0	2.29	3.0	0.3	0.42	0.5	0.78	1.08	1.42	2.79	3.78

Table 3: Effect of *Azotobacter sp.*, *Rhizobium sp.* and mixed culture of *Azotobacter sp.*, and *Rhizobium sp.* on dry biomass (g) of Black Pea.

Effect on chlorophyll content: Chlorophyll content was increased in T₄ treatment (1.3 mg/g) followed by T₇ treatment (0.95 mg/g) at 30, 60, 90 and 120 days after sowing which was significantly better over the control (0.53 mg/g). Enhanced chlorophyll content can be achieved due to the existence of rhizomicrobes in the rhizosphere, promoting

the crop roots to secrete growth promoting substances, which in turn might have improved the growth of N₂-fixers, P-solubilizing microorganisms in situ, while a synergistic effect might have achieved in the T₄ and T₇ treatment (Figure 6). Enhancement of rhizospheric microorganism in case of treatment T₄ might be due to inoculation of

Azotobacter sp. and *Rhizobium sp.* consortium, where more root hairs become liable for rhizo-microbial infection and also might be due to better condition for P-availability by P-solubilizers.

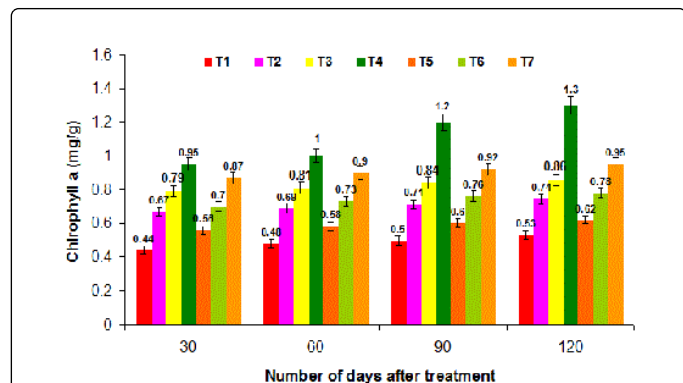


Figure 6: Effect of *Azotobacter sp.*, *Rhizobium sp.* and mixed culture of *Azotobacter sp.*, and *Rhizobium sp.* on Chlorophyll a content of Black Pea.

Effect on non-reducing and reducing sugar content: In the present study, mixed culture of *Azotobacter* and *Rhizobium sp.* treatment (T₄) increased reducing (867.8 mg/g) and non-reducing (1509.5 mg/g) sugars quantity (Figures 7 and 8). Non-reducing sugar contents were increased due to the possible reasons to enhance in carbon fixation, activation of enzymes and improved photosynthetic rate.

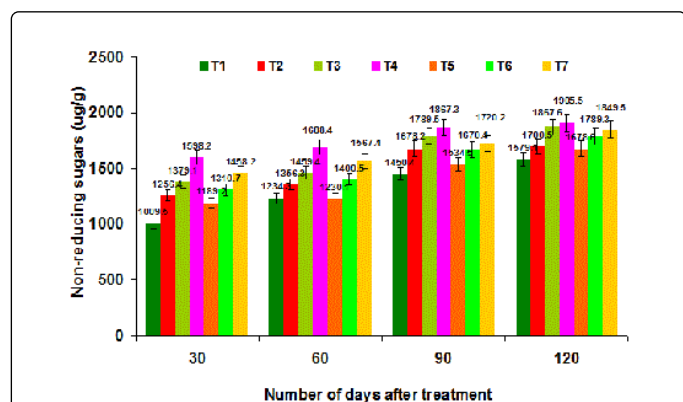


Figure 7: Effect of *Azotobacter sp.*, *Rhizobium sp.* and mixed culture of *Azotobacter sp.*, and *Rhizobium sp.* on non-reducing sugar contents of Black Pea.

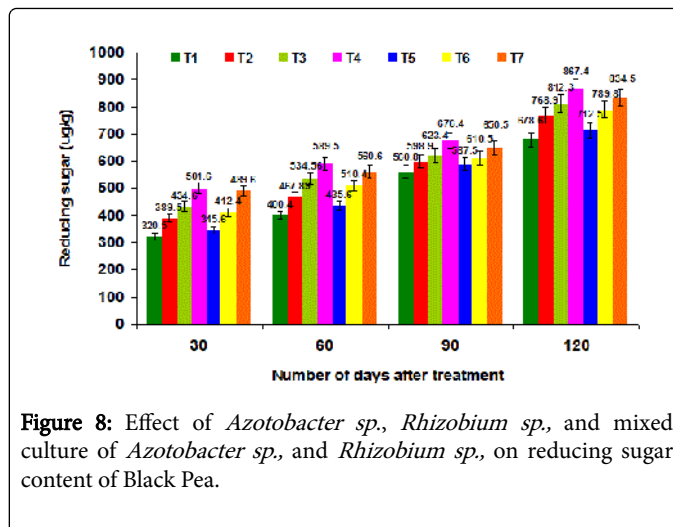


Figure 8: Effect of *Azotobacter sp.*, *Rhizobium sp.*, and mixed culture of *Azotobacter sp.*, and *Rhizobium sp.* on reducing sugar content of Black Pea.

Discussion

Application of biofertilizers is a satisfactory approach for higher yield and better quality of crops, which is healthy for human consumption. Our results showed that mixed culture of *Azotobacter* and *Rhizobium* gave positive result to the different studied parameters. The plant shoot length, root length, leaves numbers and nodules numbers were increased significantly with the mixed culture of *Rhizobium* and *Azotobacter sp.* when compared with single culture of *Azotobacter sp.*, and *Rhizobium sp.* Kamil et al. [28] have also reported that the mixed culture inoculations of beneficial strains were always performed better than the single inoculation treatments. Mixed culture of *Azotobacter* and *Rhizobium* significantly enhanced the fresh and dry shoot and root biomass along with the other parameters (chlorophyll content and reducing and non-reducing sugar content).

Combined inoculations further enhanced the total root and shoot fresh and dry biomass of 16.5 and 4.2 g per plant (Tables 2 and 3). It is well documented that the non-symbiotic microorganisms have two beneficial aspect for plants, first is the nitrogen fixing capability and second is to produced growth promoting substances (vitamins, hormones and amino acids) [29,30] which attributed the combined effects of the consortium reside with friendly associations by receiving more colonization in the rhizosphere of the crops. The friendly association of *Rhizobium* and *Azotobacter* may be suggested for better results instead of single culture.

Chlorophyll content was increased in the mixed culture inoculation treatment (T₄) over the uninoculated control. Similar results in respect to increase in chlorophyll content in several plants have also been reported by several workers [31]. Enhanced chlorophyll content can be certified that the occurrence of microorganisms in the rhizosphere promoting the plant roots to produce growth promoting substances, which improved the growth of N₂-fixers, P-solubilizing microorganisms in situ and a synergistic effect might have attained. Improvement of rhizomicrobial growth in case of treated plant might be due to inoculation of *Azotobacter sp.* and *Rhizobium sp.* consortium, where more root hairs become susceptible for rhizomicrobial infection and also might be due to better provision for P-availability by P-solubilizers [32]. Furthermore, N-fixers like *Azotobacter* and *Rhizobium sp.* is recognized to improve the plant growth [33]. Enhanced quantity of reducing and non-reducing sugars

is due to improved carbon fixation, activation of enzymes and improved photosynthetic rate [34,35]. Growth parameters improved due to the mixed culture treatments.

Several methods have been recommended to elucidate the fact of plant growth enhancement by *Azotobacter* is due to increase in the nitrogen fixation, production of different hormones (auxins, gibberellins, cytokinin, and ethylene), phosphorus solubilization, sulfur oxidation, accessibility of nitrate, production of antibiotics, lytic enzyme, hydrocyanic acid, increase in root permeability, firm antagonism for the existing and root spot, inhibition of harmful rhizobacteria and improvement in the uptake of fundamental plant nutrients etc. [36-39].

It clearly indicate that *Rhizobium* nodulation (number and size) might have due to the presence was *Azotobacter* which could fix atmospheric N₂ and supported plant growth from initial growth of seedlings. In the early stage, plant roots might have supported the *Azotobacter* population. Such finding indicate that *Rhizobium* does not complete far soil organic compound and other minerals with the *rhizobium* because *Rhizobium* get its nutrients from the crops plant and that is why did not grow effect the *Azotobacter* and perhaps due to such condition, *Azotobacter* increase 5-6% time. The significant finding must be study along with other nutrients like Cr, Mg, Zn ability in soil Mg gives other finding regarding some combination further it's also important that how *Azotobacter* could permitted the plant growth higher it is through and plant growth hormones in the experiment the *Azotobacter* are isolate and tested for N₂ fixing.

The *Azotobacter sp.* is an aerobic organism that can fix atmospheric nitrogen as demonstrated by the grown in the flask at the liquid/air interface. The presence of oxygen can inactivate the nitrogenase enzyme and inhibit the nitrogen fixation. The *Azotobacter sp.* compensates for this problem by maintaining a very high respiration rate which effectively uses the oxygen as soon as it enters into the cell. *Rhizobium sp.* can only fix atmospheric nitrogen under microaerophilic conditions, such as inside the root nodule. It would be necessary to duplicate conditions found in nature in a strict artificial environment to prove that the *Rhizobium sp.* was able to fix atmospheric nitrogen.

Conclusion

The novel concept of this study is that combined application of *Azotobacter* and *Rhizobium* is more effective than single one. It is always found that *Rhizobium* is recommended for leguminous crops along due to node forming status by symbiotic relations. *Rhizobium* require more time in nodule formation and then N₂ fixation starts, while *Azotobacter* starts fixing N₂ asymbiotically in the soil and help better plant growth at the initial stage of seedling growth. Such effects make plants healthy and diseases free. Therefore, application of such combinations should be recommended to the farmers for better yield response. The consortium of *Azotobacter sp.*, and *Rhizobium sp.* could improve the growth and yield response of black gram by improving physiology of the crop by supplying nitrogen through symbiotically and asymbiotically. Therefore, the shoot length, root length, number of leaves, number of root nodules, fresh and dry biomass of root and shoot were improved as compared to individual culture of *Azotobacter sp.*, and *Rhizobium sp.* The chlorophyll and carbohydrates contents were also increased in the treatment plants. The application of consortium of *Azotobacter sp.*, and *Rhizobium sp.* as bioinoculants in agriculture, horticulture and other land plants should be

recommended for better yield of crops and improvement of soil health through increasing the biotic component in order to reduce the risk of chemical toxicity.

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