JJBS

Volume 9, Number 2, June .2016
ISSN 1995-6673
Pages 79 - 88

Jordan Journal of Biological Sciences

Effects of Plant Growth Promoting Rhizobacteria on the Performance of Greengram under Field Conditions

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Received: January 28,2016 Revised: April 11, 2016 Accepted: April 28, 2016

Abstract

Despite reports on effects of single Plant Growth Promoting Rhizobacteria (PGPR) inoculation on legumes, response of greengram to combined inoculation with phosphate solubilizing asymbiotic *Azotobacter* and ACC deaminase positive symbiotic *Bradyrhizobium* sp. (vigna) under field conditions remains unexplored. The present study aims at identifying ACC deaminase producing and phosphate solubilizing bacterial strains and to assess their impact on greengram plants in order to find efficient and friendly co-cultures for developing effective bioinoculants for increasing sustainable production of legumes. Additionally, plant growth promoting activities of *Azotobacter* and *Bradyrhizobium* sp. (vigna) were determined using standard methods. The isolated bacterial cultures were characterized morphologically, culturally and biochemically and were identified as *Bradyrhizobium* sp. (vigna) and *Azotobacter chroococcum*. Dry matter accumulation in whole plants, symbiotic attributes, nutrient uptake and grain yields were significantly enhanced following co-inoculation of *A. chroococcum* and *Bradyrhizobium* sp. (vigna).The inoculation of *Azotobacter* with *Bradyrhizobium* increased seed yield by two fold and produced the highest grain protein. A- 75% and 52% increase in P concentration in root and shoots, respectively was observed for *A. chroococcum*, while P uptake was highest (0.52 mg/g) in shoots following combined inoculation of *A. chroococcum* with *Bradyrhizobium* at harvest. The highest N concentration in roots and shoot at harvest were observed with co-culture of *A. chroococcum* and *Bradyrhizobium* sp. (vigna). Gram negative *Azotobacter* and *Bradyrhizobium* solubilized insoluble phosphate, synthesized indole acetic acid, ammonia, cyanogenic compounds and exopolysaccharides while only *Bradyrhizobium* showed ACC deaminase activity. The results suggest that two unrelated bacteria belonging to symbiotic and asymbiotic group and capable of facilitating greengram production under field conditions and expressing multiple plant growth promoting activity can be used to produce composite bioinoculants for enhancing greengram production while saving the use of fertilizers.

Keywords: *Azotobacter*,ACC Deaminase,*Bradyrhizobium*,Greengram, Nutrient Uptake, Nodule,Seed Yield.

1. Introduction

In high input agricultural practices, chemical fertilizers are frequently used to optimize crop production. These expensive chemicals, however, when used injudiciously, have resulted in loss of soil fertility and consequently, the crop production (Lemanski and Scheu, 2014). Due to these reasons, focus in recent times has been shifted towards the use of inexpensive natural resources such as Plant Growth Promoting Rhizobacteria (PGPR): soil bacteria that colonize the roots of plants following inoculation onto seeds and that enhance plant growth (Kloepper and Schroth, 1978). The PGPR involving free living (asymbiotic) growth promoting rhizobacteria (Lugtenberg and Kamilova, 2009; Bhattacharya and Jha, 2012), symbiotic rhizobia (Ahmad *et al.,* 2013; Peix *et al.,* 2015) and phosphate solubilizers (Zaidi *et al.,* 2009; Nosrati *et al.,* 2014) have been used for enhancing the production of different crops (Mohite, 2013; Viruel *et al.,* 2104) including legumes (Noreen *et al.,* 2012; Singh *et al.,* 2013). Among non-nodule forming diazotrophs, *Azotobacter*, a free living nitrogen fixer, discovered and described in 1901 by the Dutch Microbiologist and botanist Martinus Beijerinck, play an important role in crop improvement by supplying mainly nitrogen (N) to plants. 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, pentothenic acid, biotin, gibberellic acid, Indole-3 Acetic Acid (IAA) and cytokinin (Ahmad *et al.,* 2005; Lenin and Jayanthi, 2012; Oskar *et al.,* 2014) and ammonia (Narula and Gupta, 1986) or indirectly by protecting the plant from diseases (Saini, 2012). Also, the secretion of 1-aminocyclopropane-1-carboxylate (ACC) deaminase by PGPR including nitrogen fixers have been found to reduce the level of plant stress hormone ethylene and consequently to enhance plant growth (Akhgar *et al.,* 2014; Hassan *et al.,* 2014; Magnucka and Pietr, 2015).The

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PGPR endowed with variable plant growth promoting activities have been used singly or in combination for enhancing legume production (Ahmad *et al.,* 2013). For example, Ara et al.(2009) conducted a field experiment to study the effects of *Bradyrhizobium* and *Azotobacter* inoculation on growth and yield of mungbean varieties viz. BARI mung-3 and BARI mung-4, using five bacterial and chemical fertilizers (20 kg N ha^{-1}). Application of *Bradyrhizobium* or *Azotobacter* or their combination significantly increased nodulation, and root and shoot weights at 35 days after sowing. Similarly, the number of seeds per pod and seed yield was significantly influenced by the bacterial biofertilizer. Also, seed inoculation with free living PGPR or symbiotic rhizobia have been shown to increase legume growth and productivity (Wani *et al.,* 2007) suggesting that use of mixed inoculants could be an effective method for sustainable production of greengram.

Greengram [*Vigna radiata* (L.) wilczek] is a popular grain legume cultivated in the tropics and forms a highly specific symbiosis with *Bradyrhizobium* sp. (vigna). Greengram contains 24% protein with excellent digestibility, 1-3% fats, 50.4% carbohydrates, 3.5-4.5% fibers and 5.5% ash, while Ca and P are 132 and 367 mg per 100 grams of seed, respectively (Frauque *et al.*,2000). Greengram is a short duration legume (maturing in 55 to 70 days), and hence, it fits well into many cropping systems, including rice and sugarcane under both rain fed and irrigated conditions. Moreover, greengram fixes about 40-50 kg N ha^{-1}. In India, it is grown in an area of 3 x 106 ha with an annual production of 1 x 106 t of grains (Sharma, 2000). Since greengram is one of the most important grain legumes, improving its production using low cost technology such as biofertilizer is urgently required to fulfill the increasing demands of pulses for ever growing population over a limited land. Even though there are no direct symbiotic connections between *Azotobacter* and greengram plants as observed between rhizobia and greengram plants, Perveen *et al.* (2002) observed a substantial increase in the yield of *Azotobacter* inoculated greengram plants which were attributed to some plant growth regulators secreted by *Azotobacter*. However, little is known about the response of greengram to combined inoculation with phosphate solubilizing asymbiotic nitrogen fixing bacteria and nodule forming *Bradyrhizobium* under field conditions. Considering the importance of symbiotic rhizobia and phosphate solubilizing *Azotobacter* and lack of information on the use of mixed cultures in greengram production, the present study was aimed at identifying ACC deaminase producing and phosphate solubilizing bacterial strains and to evaluate the effect of single and combined inoculations of notable plant growth promoting rhizobacteria belonging to asymbiotic N_2 fixer (*A.chroococcum*) and symbiotic N_2 fixer (*Bradyrhizobium* sp. (vigna) group on growth, symbiotic attributes, nutrient uptake and yield attributes of greengram, grown under field soils fertilized with/without nitrogenous (urea) and phosphatic (diammonium phosphate, DAP) fertilizers in order to find a suitable composite bacterial culture for producing effective bioinoculants for enhancing the production of greengram.

2. Materials and Methods

2.1. Isolation and Identification of Bacterial Cultures

Bradyrhizobial strains were isolated from nodules of greengram plants grown in the fields of Agricultural Sciences, Aligarh Muslim University, Aligarh, ($27^0 29^1$ latitude and 72^0 29^1 longitude) using Yeast Extract Mannitol (YEM) medium (Somasegaran and Hoben, 1985). For this, healthy and undamaged nodules were surface sterilized with sodium hypochlorite (2.5 % for 2 min.). Then, sodium hypochlorite treated nodules were rinsed in 95 % ethanol (v/v) and washed several times with distilled water. Surface sterilized nodules were then crushed gently in Normal Saline Solutions (NSS). Nodule suspensions were serially diluted in NSS and 100 µl of each diluent was spread plated on YEM agar medium (HiMedia Laboratories Pvt. Ltd. Mumbai, India) containing 2.5% Congo red indicator dye. The inoculated plates were incubated at 28±2 °C for 5 days. A- single colony was picked and streaked 4 times on the same medium to ascertain the purity of the cultures and each chosen strain was maintained on YEM (g/l: mannitol 10; K_2HPO_4 0.5; $MgSO_4$. $7H_2O$ 0.2; NaCl 0.1; yeast extract 1; $CaCO_3$ 1 and pH was adjusted to 7) agar medium at 4 °C until use. Bradyrhizobial strain was subjected to plant infection test using greengram as a host plant in order to validate its host specificity. For *Azotobacter* isolation, soils were collected from chilli (*Capsicum annuum*) rhizosphere in sterilized polythene bags (15×12 cm2). Soil samples were serially diluted in sterile NSS and 100 µl of diluted suspension was spread plated (Buck and Cleverdon, 1960) on Ashby's mannitol agar medium (g/l: mannitol 20.0; Dipotassium hydrogen orthophosphate 0.2; Potassium sulphate 0.1; Mg SO_4 0.2; $CaCO_3$ 5.0; NaCl 0.2) (HiMedia Laboratories Pvt. Ltd. Mumbai, India). Plates were then incubated at 28±2 °C for 5 days and colonies producing brown to black pigment (melanin) were picked and used for further experiment. Both *Bradyrhizobium* and *Azotobacter* were subjected first to Gram reaction which is an important step in the identification of bacteria to species level. The bacterial isolates were then subjected to further identification using various morphological, cultural and biochemical tests (Holt *et al.,* 1994) that included indole reaction, citrate utilization test, methyl red test, Voges-Proskauer, catalase, oxidase test, starch and gelatin hydrolysis etc. which led to their identification.

2.2. Bacterial Preparation, Seed Treatments and Planting

Azotobacter chroococcum isolated from chilli rhizosphere and *Bradyrhizobium* sp. (vigna) recovered from greengram nodules were grown in Ashby and YEM broth, respectively, to a cell density of 3x105 cells/ml (*A. chroococcum*) and 6x108 (*Bradyrhizobium*). Healthy seeds of greengram var. K-851 were purchased from Prakash Agrochemicals and seeds, Aligarh, Uttar Pradesh, India. Seeds were surface sterilized with 70% ethanol for 3 min. and 3% sodium hypochlorite, 3 min. (Vincent, 1970). Surface sterilized seeds were rinsed 6 times with sterile water and dried. Surface sterilized seeds were bacterized by soaking seeds in liquid culture medium for 2h using 10% Arabic gum as sticker to deliver

approximately 10^8 and10^5 cells ml-1 each of *Bradyrhizobium* sp. (vigna) and *A. chroococcum*, respectively. For co-inoculations, the liquid culture of each organism was mixed in equal proportion to soak the sterile seeds (100 g seeds in 200 ml broth solution for 2h). The non-coated (without inoculant) sterilized seeds soaked in sterile water served as control. The non-inoculated and inoculated seeds were sown in soil (sandy clay loam; 0.4% organic carbon, 0.75 g/kg Kjeldahl N, 16 mg/kg Olsen P, pH 7.2 and 0.44 ml/g water holding capacity (WHC), 11.7 cmol kg-1 cation exchange capacity, 5.1 cmol/kg Anion exchange capacity, 59.1 µSm-1 salt conductivity) using seed-drill method at a soil depth of 10–12cm in 5m×5m plots at 45 cm row distance (RxR) and 5-10 cm plant distance (PxP) within the rows. The treatments were:

T1= control (neither fertilized nor inoculated:NFNI);

T2= recommended dose of urea (25 kg/ha);

T3= recommended dose of diammonium phosphate (DAP) (85 kg/ha);

T4= *A. chroococcum*;

T5=*Bradyrhizobium* sp. (vigna);

T6=*A. chroococcum* with 25 kg/ha urea;

T7=*Bradyrhizobium* sp. (vigna) with 85 kg/ha DAP;

T8=*A. chroococcum* with *Bradyrhizobium* sp. (vigna);

T9=urea (25 kg/ha) with DAP (85 kg/ha).

The plots receiving inoculation treatments remained unfertilized. All nine treatments were replicated six times and experiment was laid out in a completely randomized design. The field experiments were repeated for two successive years under identical environmental conditions using the same treatments to ensure the reproducibility of the results.

2.3. Measurement of Biological and Chemical Characteristics of Plants

All plants in three replicates for each treatment were removed 50 Days After Sowing (DAS) and remaining plants in three replicates for each treatment were maintained until harvest (80 DAS). Randomly selected plants removed at 50 DAS were used to observe the extent of nodulation. Nodules collected randomly from each treatment were counted, oven dried at 80 °C and weighed. The leghaemoglobin (Lb) content in fresh nodules recovered from the root systems of greengram plants were quantified by the method of Sadasivam and Manickam (1992).For this, fresh nodules were crushed using mortar and pestle in 5 ml sodium phosphate buffer (pH 7.4) and filtered through two layers of cheese cloth. The nodule debris was discarded. The turbid reddish brown filtrate was clarified by centrifugation at 10000 g for 30 min. The supernatant was diluted to 10 ml with sodium phosphate buffer (pH 7.4). The extract was divided equally into two glass tubes (5 ml /tube) and equal amount of alkaline pyridine reagent was added to each tube. The haemochrome formed was read at 556 and 539 nm after adding a few crystals of potassium hexacyanoferrate and sodium dithionite, respectively. The leghaemoglobin content was calculated using the formula–

$$\text{Lb content (mM)} = \frac{[A_{556} - A_{539}] \times 2D}{23.4}$$

where, D= initial dilution.

The dry matter accumulation in whole plants (with intact roots, shoots and leaves) was measured both at 50 and 80 DAS. Total N content in roots and shoots was measured at harvest by micro-Kjeldahl method of Iswaran and Marwah (1980), while P content in roots and shoots was measured by the method of Jackson (1967). Seed yield and grain protein (Lowery *et al.*, 1951) were measured at harvest.

2.4. Statistical Analysis

The resulting data of the measured variables were pooled together and subjected to analysis of variance by applying two-way ANOVA [for two-factors (inoculation and fertilizer)] at 5% probability level using software, Mini-Tab 10.

2.5. In Vitro Bioassay of Plant Growth Promoting Activities

2.5.1. ACC Deaminase Activity, P Solubilization and Indole Acetic Acid Content

Using the spot inoculation method, 5 µl of each bacterial inoculum was placed on a section of plate (marked in 16 equal parts) containing Dworkin and Foster (DF) salt minimal medium (g.l-1: KH2PO4 4; Na_2HPO_4 6; $MgSO_4.7H_2O$ 0.2, glucose 2.0; gluconic acid 2.0; citric acid 2.0; $FeSO_4.7H_2O$ 0.001; H_3BO_3 0.01, $MnSO_4.H_2O$ 0.011; $ZnSO_4.7H_2O$ 0.124; $CuSO_4.5H_2O$ .078; MoO_3 0.01; $(NH_4)_2SO_4$ 2.0; pH 7.2) (Dworkin and Foster,1958) supplemented with 3 mM ACC (instead of $(NH_4)_2SO_4$) and incubated at 28±2 °C for 72 h and bacterial growth was checked daily. The ACC deaminase activity (EC 4.1.99.4) of *A. chroococcum* and *Bradyrhizobium* sp. (vigna) was measured by the method of Penrose and Glick (2003). The ACC deaminase activity was expressed as the amount of α-ketobutyrate produced per milligram of protein per hour. A positive control [*Mesorhizobium* LMS-1 containing pRKACC plasmid was also run along the experiment. All samples were tested in duplicate and each individual experiment was repeated three times.For phosphate solubilizing activity, culture suspensions of both *Bradyrhizobium* and *Azotobacter* weres pread plated on Pikovskaya agar (Pikovskaya, 1948) medium (HiMedia Laboratories Pvt. Ltd. Mumbai, India). Plates were incubated at 28±2 °C for 7 days. The bacterial cultures exhibiting clear halo around growth following incubation were considered as P-solubilizers. The halo forming bacterial strains were further used to determine the extent of P-solubilization in Pikovskaya broth ($g.l^{-1}$): glucose 10; $Ca_3(PO_4)_2$ 5; $(NH_4)_2SO_4$ 0.5; NaCl 0.2; $MgSO_4.7H_2O$ 0.1; KCl 0.1; yeast extract 0.5; $MnSO_4$ and $FeSO_4$ trace; pH 7) by the chlorostannous-reduced molybdophosphoric acid blue method (King, 1932). Briefly, 100 ml of Pikovskaya broth was inoculated with 1 ml of 10^8 cells ml^1-of *A. chroococcum* and *Bradyrhizobium* sp. (vigna). The inoculated flasks were incubated for 7 days with shaking (200 r/min.) at 28±2 °C. A 20 ml culture broth from each flask was removed and centrifuged (9000g) for 30 min. To 10 ml of supernatant, 10 ml chloromolybdic acid and 5 drops of chlorostannous acid was added and volume was adjusted to 50 ml with distilled water. The absorbance of developing blue color was read at 600 nm using spectrophotometer Spectronic 20 D+. The amount of

solubilized P was calculated using the calibration curve of KH2PO4. IndoleAcetic Acid (IAA) was determined by the method described by Brick et al. (1991). Bacterial cultures were inoculated at 108 cells ml-1 and were incubated for 24 h at 28±2 °C with shaking at 125 r/min. for 72 h for *Bradyrhizobium* and 48 h for *Azotobacter* on Luria Bertani (LB) broth (g. l^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5) supplemented with different concentrations of tryptophan (0, 100, 200, 400 and 500 µg/ml) at 28 °C. Fully grown cultures were centrifuged at 9000g for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink color indicated IAA production. Optical density was taken at 530 nm using spectrophotometer Spectronic 20 D+. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard.

2.5.2. Siderophores, Ammonia, Cyanide and Exo-Polysaccharides

The release of siderophores by *Bradyrhizobium* and *Azotobacter* was assayed using Chrome Azurol S (CAS) agar medium following the method of Alexander and Zuberer (1991). Chrome Azurol S agar plates were prepared separately and divided into equal sectors and spot inoculated with 10 µl of 10^8 cells/ml of *Bradyrhizobium* and 10^5 cells/ml of *Azotobacter* and incubated at 28±2 °C for five days. Development of yellow orange halo around the bacterial growth was considered as positive for siderophore synthesis. Each individual experiment was repeated three times. Siderophore was also detected quantitatively using Modi medium (K_2HPO_4 0.05%; $MgSO_4$ 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1%; NH_4NO_3 0.1%). Modi medium was inoculated with 108 cells/ml of *Bradyrhizobium* and 10^5 cells/ml of *Azotobacter* and incubated at 28±2 °C for 5 days. Catechol type phenolates were measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride-ferricyanide reagent of Hathway. Ethyl acetate extracts was prepared by extracting 20 ml of supernatant twice with an equal volume of solvent at pH 2. Hathway's reagent was prepared by adding one milliliter of 0.1 M ferric chloride in 0.1 N HCl to 100 ml of distilled water, and to this, was added one milliliter of 0.1 M potassium ferricyanide (Reeves *et al.,* 1983). For the assay, one volume of the reagent was added to one volume of sample and absorbance was determined at 560 nm for salicylates with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2, 3- Dihydroxy Benzoic Acid (DHBA) as standard.

The HCN synthesized by bacterial strains was determined following the method of Bakker and Schipper (1987). The synthesis of ammonia by the bacterial strains was detected using peptone water. Freshly grown bacterial strains (200 µl of 10^8 cells ml^{-1}) were inoculated in 20 ml peptone water in tubes and incubated at 28±2 0C for 4 days. One millilitre of Nessler reagent was added to each tube. Development of yellow colour indicated a positive test for ammonia production (Dye, 1962). Exo-Polysaccharides (EPS) secreted by *Bradyrhizobium* sp. (vigna) and *A. chroococcum* was quantitatively detected by allowing each organism to grow in 100 ml basal medium containing 5% sucrose and incubated for five days at 28±2 °C on rotary shaker (100 r/min.). After incubation, culture broth was centrifuged (5433g) for 30 min. and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying at 80 °C.

3. Results

The present work was undertaken to identify symbiotic and asymbiotic nitrogen fixing bacteria from greengram nodules and chilli rhizospheres, respectively. The selected bacterial cultures were used to assess their impact on the performance of greengram under field soils. Also, the plant growth promoting activities of the symbiotic and asymbiotic nitrogen fixing bacteria were determined.

3.1. Characterization and Identification

In the present study, a total of 20 *Azotobacter* strains were isolated from chilli rhizosphere and primarily identified by morphological, cultural and biochemical tests. Of these, strain AZ19 was selected due to its ability to exhibit greater P- solubilizing efficiency and IAA synthesis and was characterized and identified (Table 1). Moreover, isolate AZ19 produced brown to black pigment called melanin on Ashby medium after 5 days incubation. Strain AZ19 was found Gram negative and showed positive reaction to methyl red, nitrate reduction, Vogues Proskauer, and catalase. Strain AZ19 could further hydrolyze starch and gelatin and displayed variable carbohydrate utilization efficiency. Comparing these properties with those given in Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994), strain AZ19 was presumptively identified as Azotobacter. Of the 20 bradyrhizobial isolates, strain RB6 was especially chosen due to its better P solubilizing activity and ACC deaminase producing ability. The RB6 was Gram negative, short rods and produced circular mucoid colony on YEMA plates. The isolate RB6 showed a variable biochemical reaction (Table 1). In order to ensure the identity and specificity strain RB6 was used to inoculate greengram plants (infection test) for nodulation in sterile soil and subsequently following nodulation, it was identified as *Bradyrhizobium* specific to greengram.

Table 1. Morphological and biochemical properties of bacterial cultures

Characteristics	Bacterial Strains	
	BR6	AZ19
Morphological		
Colony	Transparent, circular and mucoid	Serrated, muciliginous, Dark pigmented
Cell Shape	Short rods	Rod
Gram reaction	-ve	-ve
Biochemical Properties		
Citrate utilization	-	-
Indole	+	-
Methyl red	+	+
Nitrate reduction	+	+
VogesProskauer	+	+
Catalase	+	+
Oxidase	-	-
Hydrolysis		
Starch	+	+
Gelatin	+	+
Carbohydrate utilization		
Glucose	+	+
Lactose	-	+
Mannitol	+	-
Sucrose	+	+
Arabinose	+	+
Xylose	-	+
Inositol	-	-
Mannose	+	+
Melanin production	-	+
Identification	*Azotobacter*	
Nodulation test	*Bradyrhizobium* sp. (vigna)	

3.2. Field Experiments

3.2.1. Total Dry Biomass and Symbiotic Characteristics

Dry matter accumulation in greengram plants measured at 50 and 80 DAS varied among treatments (Figure 1). The recommended rates of urea and DAP and mixture of both fertilizers enhanced dry biomass accumulation from 1.77 g/plant (control) to 2.16 g/plant (31%), 2.27 g/plant (38%) and 2.78 g/plant (67%), respectively at 50 DAS. Similarly, at 80 DAS, the total dry matter accumulation in greengram plants varied significantly among treatments. Furthermore, the sole application of *A. chroococcum* and *Bradyrhizobium* improved dry matter accumulation in plants by 32 and 37%, respectively at 50 DAS compared to control plants. In comparison, the composite application of fertilizers and microbial inocula for example, urea with *A. chroococcum* enhanced the total dry biomass by 42% at 80 DAS compared to un-inoculated and untreated control plants. Total dry biomass was increased further by 72%, at 50 DAS following *Bradyrhizobium* inoculation with *A. chroococcum*. In general, the bacterial cultures used either alone or in combination with fertilizers consistently increased the dry biomass of whole greengram plants (with intact roots, shoots and foliage) grown in fields.

The nodule formation and leghaemoglobin accumulation in fresh nodules following bacterial inoculations and fertilizer application varied among treatments (Table 2).Among single-inoculations, *Bradyrhizobium* maximally enhanced nodule numbers, nodule mass and leghaemoglobin significantly ($P \leq 0.05$) by 162% (55 nodules/plant), 155% (191 mg/plant) and 143% [0.17mM $(gfm)^{-1}$], respectively above control at 50 DAS. The composite application of A. chroococcum and *Bradyrhizobium* further enhanced the nodule numbers, nodule mass and leghaemoglobin significantly by 210% (65 nodules/plant), 199% (224 mg/plant) and 200% compared to control plants. Among fertilizers, mixture of urea (25 kg/ha) and DAP (85 kg/ha) showed maximum increase in nodule numbers (143%) and Lb content (143%) compared to control.

3.2.2. Nutrient Uptake, Seed Yield and Grain Protein

Among single inoculations, *A. chroococcum* showed maximum increase in P contents in roots (0.35 mg/g) while *Bradyrhizobium* resulted in maximum increase in shoots (0.45 mg/g). Generally, bacterial cultures in the presence of urea and DAP further enhanced N and P concentrations in plant organs. For example, *Bradyrhizobium* inoculated plants grown in soil fertilized with DAP had the maximum N contents in shoots (68 mg/g) and P contents in shoots (0.53 mg/g) whereas *A. chroococcum* inoculated plants had maximum shoot N (65 mg/g) and shoot P (0.50 mg/g) when grown in fields fertilized with urea. Similarly, the mixture of *A.chroococcum* and *Bradyrhizobium* sp. (vigna) showed maximum increase in the measured parameters compared to other single bacterial treatments, fertilizer application, or uninoculated and unfertilized plants (Table 2). For example, dual inoculation of *A.chroococcum* and *Bradyrhizobium* sp. (vigna) augmented the root and shoot P by 120% and 79%, respectively over control. Among bacterial treatments, the sole inoculation of *A.chroococcum* and *Bradyrhizobium* significantly ($P \leq 0.05$) increased the seed yield by 65% (5.6g/plant) and 74% (5.9 g/plant), respectively relative to control (3.4 g/plant).While comparing the effect of fertilizers and mono bacterial cultures, *Bradyrhizobium* sp. (vigna)showed maximum increase in seed yield over urea and DAP. Moreover, in the presence of 85 kg DAP/ha, *Bradyrhizobium* sp. (vigna)gave maximum seed yield (103% increase) while *A.chroococcum* in the presence of 25 kg urea/ha increased the seed yield by 94% compared to control. The dual inoculation of *A.chroococcum* and *Bradyrhizobium* increased the grain yield maximally by 109% (7.1 g/plant). The increase in grain yield following mixture of urea and DAP (7.1 g/plant) was however, comparable to those observed for combined inoculation of *A.chroococcum* and *Bradyrhizobium*(7.0 g/plant). Protein content in seeds even-though were similar among treatments yet it was greater in inoculated plants compared to control ones. The maximum increase in grain protein was observed for dual inoculations of *A. chroococcum* and *Bradyrhizobium* sp. (vigna). Two-way ANOVA in general revealed that the individual effect was significant ($P \leq 0.05$) for all the measured parameters.

Table 2. Single and co-inoculation effects of asymbiotic *Azotobacter chroococcum* and ACC deaminase positive *Bradyrhizobium* sp. (vigna) on symbiotic characteristics, nutrient uptake and seed attributes of field grown greengram.

Treatments	Symbiotic attributes			Nutrient uptake				Seed yield (g/plant)	Seed protein (mg/g)
	[a]Nodules no./plant	[a]Nodule biomass (mg/plant)	[a]Leghaemoglobin mM (g .f m)$^{-1}$	N content (mg/g)		P content (mg/g)			
				Root	Shoot	Root	Shoot		
Control	21^{e}±4	75^{f}±8	0.07^{d}±0.02	28^{e}±3	44^{d}±3	0.20^{d}±0.03	0.29^{d}±0.02	3.4^{d}±0.3	254^{b}±11
Urea (25 kg/ha)	33^{de}±4	124^{ef}±11	0.12^{c}±0.03	40^{c}±4	58^{c}±5	0.27^{c}±0.04	0.37^{c}±0.03	4.7^{c}±0.3	266^{a}±12
DAP (85 kg/ha)	35^{d}±5	138^{e}±13	0.12^{c}±0.03	39^{d}±3	56^{cd}±4	0.33^{bc}±0.03	0.46^{b}±0.03	4.9^{c}±0.4	265^{ab}±12
[1]*Azotobacter*	44^{c}±5	161^{d}±12	0.14^{bc}±0.02	43^{c}±3	60^{c}±5	0.35^{b}±0.04	0.44^{b}±0.04	5.6^{b}±0.4	265^{a}±13
[2]*Bradyrhizobium*	55^{b}±4	191^{c}±15	0.17^{b}±0.03	47^{b}±5	67^{a}±4	0.34^{b}±0.03	0.45^{b}±0.03	5.9^{b}±0.4	267^{a}±11
Azotobacter +Urea	44^{c}±6	181^{cd}±17	0.14^{bc}±0.03	50^{b}±4	65^{b}±5	0.40^{ab}±0.03	0.50^{a}±0.03	6.6^{ab}±0.5	270^{a}±10
Bradyrhizobium +DAP	56^{b}±4	211^{b}±19	0.18^{ab}±0.03	53^{a}±4	68^{a}±6	0.42^{a}±0.04	0.53^{a}±0.04	6.9^{a}±0.6	267^{a}±11
Azotobacter + *Bradyrhizobium*	65^{a}±6	224^{a}±21	0.21^{a}±0.03	54^{a}±5	70^{a}±5	0.44^{a}±0.04	0.52^{a}±0.04	7.0^{a}±0.5	270^{a}±12
Urea+ DAP	51^{c}±5	201^{bc}±19	0.17^{b}±0.03	54^{a}±4	69^{a}±4	0.46^{a}±0.04	0.54^{a}±0.04	7.1^{a}±0.5	272^{a}±11
LSD	4.2	11.2	0.03	2.7	3.4	0.04	0.06	0.5	3.1

[a]Nodule number, [a]Nodule dry biomass and [a]Leghaemoglobin content in fresh nodule was determined at 50 DAS of greengram growth; [1]*Azotobacter* indicates *A. chroococcum*;[2]*Bradyrhizobium*represents *Bradyrhizobium* sp. (vigna); DAP-diammonium phosphate; ± indicates mean value± SD; In this table each value is a mean of six replicates where each replicate constituted three plants/plot. Mean values are significant at P ≤0.05. Means followed by similar alphabets are not significantly different from each other according to post hoc Tukey HSD.

3.2.3. Bioassay of Plant Growth Promoting Substances

The *Bradyrhizobium* sp. (vigna) isolated from root nodules and *A. chroococcum* recovered from chilli rhizosphere were assessed for their ability to synthesize plant growth promoting substances (Table 3). Of these, only *Bradyrhizobium* sp. (vigna) produced 211 µmol α ketobutyrate/mg protein/h while *A. chroococcum* did not produce ACC deaminase. Even though, both *Bradyrhizobium* sp. (vigna) and *A. chroococcum* could solubilize insoluble P but the P-solubilization by *A.chroococcum* was 45.2% greater than those observed for *Bradyrhizobium* sp. (vigna). Also, A.chroococcumshowed 30% increase in IAA over *Bradyrhizobium* sp. (vigna) grown in Luria Bertani broth treated with 100 µg/ml tryptophan. Interestingly, both *Bradyrhizobium* sp. (vigna) and *A.chroococcum* secreted siderophores, EPS, HCN and ammonia, but no significant difference was observed among growth regulators.

Table 3. Quantitative assay of active biomolecules secreted by *Bradyhizobium* sp. (vigna) and *Azotobacterchroococcum.*

Bacterial strain	ACC deaminase activity (µmol α-ketobutyrate/mg protein/h)	P-solubilized (µg/ml)	IAA (µg/ml)	Siderophore			EPS (µg/ml)	HCN	Ammonia
				Zone on CAS agar (mm)	DHBA (µg/ml)	SA (µg/ml)			
Bradyhizobium sp. (vigna)	211±15	148±24	74±8	12±1.5	14±1.6	25±2.1	15±3	+	+
A. chroococcum	ND	215±11	96±9	12±1.1	15±1.6	28±2.4	19±2	+	+

Abbreviationsof plant growth promoting substances: CAS: Chrome Azurol S agar, DHBA: 2,3 Dihydroxy benzoic acid, SA: Salicylic acid, EPS: Exo-polysaccharides, ND: not detected, +: positive qualitative reaction, Values indicate mean ± S.D. of three independent replicates.

4. Discussion

In high input agricultural practices the use of biofertilizers especially those prepared from PGPR involving both phosphate solubilizers and N_2 fixers for optimum crop production has received greater attention due to low cost, easy production and lack of hazardous impact. Considering these properties, the hunt for soil microbiota capable of expressing variable plant growth activity has increased alarmingly. Realizing the importance of free living non symbiotic and phosphate solubilizing PGPR and symbiotic rhizobia in sustainable production of legumes, the present study was aimed at identifying and testing the influence of selected PGPR on overall performance of greengram under field soils fertilized with or without synthetic fertilizers. In the present study, strain AZ19was identified as Azotobacter by morphological, cultural and biochemical analysis and through melanin production while strain RB6 was identified as *Bradyrhizobium* by standard microbiological and plant infection test. There are four important species of *Azotobacter* viz., *A. chroococcum*, A. agilis, A. paspali and *A. vinelandii* of which *A. chroococcum* is most commonly found in Indian soils. A unique differentiating character of *Azotobacter* is its ability to form pigments. The pigment forming ability and types of pigment produced by each Azotobacter species is therefore, used as a marker for species differentiation. In the present study, aged cultures of *Azotobacter* formed an insoluble brown-black pigment commonly attributed to the presence of melanin. This melanin is formed as a result of oxidation of tyrosinase, a copper containing enzyme. Since Azotobacter produced melanin on Ashby medium, it was identified as *A. chroococcum* and hence, *Azotobacter* is considered *A. chroococcum* throughout the manuscript. In other studies, melanin excretion by nitrogen-fixing non-virulent bacterium *Azotobacter* was used as a marker for isolation and identification of *A. chroococcum* from soil samples (Aquilianti *et al.,* 2004; Tejera *et al.,* 2005; Banerjee *et al.,* 2014). However, other *Azotobacter* species forms fluorescent pigment (Jensen, 1954; Johnstone, 1955). Numerous authors have also isolated Bradyrhizobium from greengram nodules (Wani *et al.,* 2007a;Ahemad and Khan, 2011a).

The symbiotic nitrogen fixer *Bradyrhizobium* sp. (vigna) and asymbiotic nitrogen fixer *A. chroococcum* applied singly or as co-culture in the presence or absence of fertilizers favourably stimulated growth, nodulation, plant nutrition (N and P) and yield of greengram plants under field conditions. Generally, the combined application of *Bradyrhizobium* sp. (vigna) and *Azotobacter* showed a significant increase in overall performance of greengram plants compared to other single (fertilizers) treatments or simultaneous application of bacterial cultures. The composite culture of Bradyrhizobium sp. (vigna) and *A. chroococcum* among all treatments demonstrated highest increase in dry biomass of greengram plants at harvest (Figure 1). Similarly, the dual culture of *Bradyrhizobium* sp. (vigna) and *Azotobacter* resulted in maximum increase in nodule numbers (65/plant), nodule dry biomass (224 mg/plant) and leghaemoglobin [0.21 mM (g f m)-1], 50 days after sowing greengram plants compared to other treatments including the mixed application of urea and DAP. Additionally, since N and P are the two major elements affecting critically the growth and development of plants, the N and P contents in roots and shoots of inoculated and uninoculated plants were measured at harvest. In general, the N and P contents were at the maximum in shoots than in roots of field grown greengram plants inoculated with or without bioinoculants. Interestingly, the co-culture of *Azotobacter* and *Bradyrhizobium* sp. (vigna) producedlargest contents of N in roots(54 mg/g) and shoots (70 mg/g) while P contents in roots (0.46 mg/g) and shoots (0.54 mg/g) were found maximum following application of mixture of urea (25 kg/ha) and DAP (85 kg/ha) compared to other bacterial or fertilizer treatments. Combining an improved plant nutrient supply with N (*Bradyrhizobium*) and P (both by *Azotobacter* and *Bradyrhizobium*) together with other growth promoting substances with plant-growth promotion appears to have additive and possibly even multiplicative effects on greengram plants. Unlike other measured parameters, seed yield and seed protein were marginally better with combined application of urea and DAP over all treatments including co-culture of *Bradyrhizobium* sp. (vigna) and *Azotobacter*. However, the composite culture of *Azotobacter* and *Bradyrhizobium* sp. (vigna) significantly enhanced the seed yield and seed protein by 106% and 6%, respectively relative to control. The quantity and quality of greengram seeds were however, marginally better in *Bradyrhizobium* inoculated plants compared to *Azotobacter* inoculated plants. Similar increase due to inoculation with *Rhizobium* and *Azotobacter* in growth, symbiotic properties, nutrient uptake and seed attributes of legumes for example chickpea (Siddiqui *et al.,* 2014) and greengram following dual application of *Bradyrhizobium* and *Azotobacter* inoculants have been reported (Perveen *et al.,* 2002; Ara *et al.,* 2009). Additionally, the siderophore-producing rhizobial strains have been found more effective in nodulating legumes for instance chickpeas than other rhizobia (Duhan *et al.,* 1998). Similarly, the increased availability of P nutrition by phosphate solubilizing rhizobia (Kumar *et al.,* 2014) is reported to promote the rhizobial activity and concomitantly the biological N2 fixation (Giller, 2001) leading eventually to a substantial increase in the overall performance of legumes.

The beneficial impact of free living *Azotobacter* and nodule forming *Bradyrhizobium* sp. (vigna) on the biological and chemical characteristics of greengram plants has prompted us to identify factors involved in plant growth promotion. In order to find reasons forhow single or joint inoculation of *Azotobacter* and *Bradyrhizobium* sp. (vigna) facilitated the growth of greengram plants, the plant growth promoting activities of both bacterial cultures were assessed. Interestingly, both *A. chroococcum* and *Bradyrhizobium* sp. (vigna) used here produced considerable amounts of plant growth–promoting substances for example, both bacterial cultures solubilized insoluble P, secreted IAA, produced siderophores and EPS and showed a positive reaction to HCN and ammonia. However, ACC deaminase was

secreted only by *Bradyrhizobium* sp. (vigna) under in vitro conditions. Among plant growth regulators, ACC deaminase is an important plant growth regulator that induces metabolic changes and hence, increases the growth of plants indirectly by inhibiting/reducing ethylene synthesis (Glick *et al.,* 2007; Bal *et al.,* 2013; Magnucka and Pietr, 2015). Similar ACC deaminase production by rhizobia is reported (Ma et al. 2003; Bhattacharjee *et al.,* 2012). Solubilization of inorganic P is yet another important trait by which PGPR supplies soluble P and consequently enhance the growth of plants. In a P deficient soil, P is applied from external sources to fulfill P demands of crops. Interestingly, both *Azotobacter* and *Bradyrhizobium* used in this study solubilized insoluble P and following application, enhanced the yields of greengram. Therefore, both bacterial partners exhibited additional property of P solubilization in addition to their intrinsic nitrogen fixation ability. This finding could be of special interest for legume growers for soils deficient in both N and P sinceapplication of both *Bradyrhizobium* and *Azotobacter* together are likely to overcome the N and P deficiency. Furthermore, A. chroococcum showed 30% increase in IAA production over *Bradyrhizobium* sp. (vigna). Secretion of IAA by both *Azotobacter* and *Bradyrhizobium* sp. (vigna) is yet another microbiological trait that greatly influences the development of plants. IAA secreted as a secondary metabolite due to rich supply of substrates by PGPR is reported to control cell elongation and division, phototropism and apical dominance in plants (Remnas *et al.,* 2008; Ali *et al.,* 2009). Also, IAA aid in the production of longer roots with increased number of root hairs and lateral roots which are involved in nutrient uptake (Datta and Basu, 2000). Indole acetic acid also inhibits or delay abscission of leaves, induces flowering and fruiting (Zhao, 2010) and secretion of IAA by PGPR is reported (Shahab and Nasreen, 2009) Synthesis of siderophores may indirectly affect the growth of plants including legumes (Datta and Chakrabartty, 2014). Siderophores released by PGPR forms a complex with iron (Fe3+) in the rhizosphere and limits its availability to the phytopathogens and concomitantly prevent phytopathogens from causing damage to plants. In the present investigation, both *Bradyrhizobium* and *Azotobacter* showed siderophore activity as indicated by the development of orange color zone on CAS agar plates. The size of colored zone produced both by *Azotobacter* and *Bradyrhizobium* on CAS agar plates were nearly identical. However, *A. chroococcum* demonstrated 12% increase in salicylate compared to *Bradyrhizobium*. This result showed that both *Bradyrhizobium* and *Azotobacter* sp. can be beneficial for their antagonistic activity towards fungal pathogens. Similar siderophore production by *Azotobacter* (Muthuselvan and Balagurunathan, 2013) and rhizobia (Datta and Chakrabartty, 2014) is reported. The secretion of exopolysaccharides by *Bradyrhizobium* sp. (vigna) and *A. chroococcum* is another indirect plant growth promoting activity which is reported to favorably affect root colonization and consequently root ramification (Hirsch 1999) besides providing protection to bacterial cells against desiccation. Even though, both *Azotobacter* and *Bradyrhizobium* secreted considerable amounts of EPS, *Azotobacter* produced 27% more EPS than *Bradyrhizobium*. *Bradyrhizobium* and *Azotobacter* showed positive reaction to both HCN and ammonia also. Similar synthesis of plant growth promoting substances as observed in this study has also been reported to increase the growth and development of plants including legumes (Wani *et al.,* 2007; Ghosh *et al.,* 2015).The production of multiple plant growth regulators by *Bradyrhizobium* and *Azotobacter* suggest that the PGPR tested in this study could be exploited to develop co-culture and consequently to enhance the growth of legumes including greengram. However, the observed benefits following dual inoculations clearly indicate that the cumulative effect of these organisms which provided N (nitrogen fixers) and available P (*Bradyrhizobium* and *Azotobacter*) and improved nutrient absorption in addition to growth promoting substances resulted in overall improvement in greengram plants under field conditions.

5. Conclusion

The symbiotic nitrogen fixer *Bradyrhizobium* sp. (vigna) and asymbiotic nitrogen fixer *A. chroococcum* were isolated and identified from the greengram nodules and chilli respectively. The combined inoculation of *A. chroococcum* and *Bradyrhizobium* sp. (vigna) in the presence or absence of fertilizers was found superior than other treatments and enhanced growth, symbiosis, nutrient uptake and grain yields considerably. Moreover, the effects of mixed cultures of *A. chroococcum* and *Bradyrhizobium* were relatively greater than the sum of the individual inoculation effects, suggesting synergisms beyond simple additive effects. In addition, the maximum accumulation of N and P in inoculated plants suggested that both asymbiotic and symbiotic bacteria can live favourably in a microhabitat. The present finding therefore, strongly suggests that mixture of compatible pairing of asymbiotic bacterium (*A. chroococcum*) and symbiotic *Rhizobium* (*Bradyrhizobium*) endowed with multiple plant growth promoting activities can be used safely and sustainably to promote growth, symbiosis, nutrient uptake and yield of field-grown greengram while saving the chemical fertilizers.

Acknowledgments

One of the authors (Ees Ahmad) is grateful to the University Grants Commission, Government of India, for the financial support offered for the present study.

Author's Contributions

Designing and planning of the experiments were done by Prof. Mohd. Saghir Khan, and experiments were conducted and executed by Dr. Ees Ahmad. The overall editing, statistical analysis and preparation of the manuscript, according to the style of journal, were done by Dr. Almas Zaidi.

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