Efficacy testing of in vitro diazotrophic activity of *Acinetobacter* on the field growth of soybean

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**ABSTRACT**

Nitrogenase enzyme activity as a measure of nitrogen fixation by *Acinetobacter* genospecies 3 A15 was found to be 49.56 µmoles ethylene/96h at 28°C. Efficacy testing of diazotrophic A15 inoculum for growth promotion at field I showed early germination, podding and flowering, increased post harvest parameters, freedom from weeds and diseases but complete absence of nitrogen fixing root nodules. The control plants were infected with mildew, stem rot and blights causing 36% mortality. The average yield of inoculated soybean was 12% over control. At field II, inoculated plants were poorly nodulated without indication of promotion of growth parameters or yield unlike control.

**Key words:** *Acinetobacter* seed inoculum, Biocontrol, Biometric parameters, Growth promotion, Nitrogenase, Root nodules, Yield.

**INTRODUCTION**

In nitrogen deficient farm soil, nitrogen requirement of legumes is either met by heavy input of nitrogenous chemical fertilizers or commercialized biofertilizers before and during cultivation (Subba Rao, 1986; Govindan and Thirumurugan, 2003). In recent times, farmers have preferred sustainable farming practices that involve use of renewable and ecofriendly biofertilizers. But the problem is not unavailability of growth promoting strains but their productively inconsistent field performance, a major hindrance to development of prospective legume bioinocula. Many investigations in the past have revealed an inability of plant growth promoting rhizobacteria (PGPR) to express plant growth promotion (PGP) characteristics such as fixing nitrogen, phosphate solubilization or production of plant growth regulators which were otherwise evidently detected in laboratory tests but failed to perform in open field conditions (Pandey et al., 1998; Hilali et al., 2001; Remans et al., 2008).

Though, *Acinetobacter* spp. is not included in the ‘Encyclopedia of soils in the environment’ (Bashan and Bashan, 2005) as PGPR; there are many reports from the past which designate its role in PGP. It was recounted as Indole acetic acid (IAA) and gibberellin producer (Kang et al., 2009), in phosphate solubilization (Gulati et al., 2010), hydrogen oxidation (Wong et al., 1986) and nitrogen fixation (Liba et al., 2006). Hemolysis, DNase and secondary metabolites like antibiotics produced by *Acinetobacter* can have significant application in biocontrol of phytopathogens (Hebbard et al., 1999; Indiragandhi et al., 2008). *Acinetobacter* spp. has also been reported in crop growth promotion (O’Connell, 1992; Huddedar et al., 2002; Prashant et al., 2009). In this study, it was decided to test efficiency of nitrogen fixing *Acinetobacter* at the field level for legume crop, soybean. The current investigation also aimed to justify two problems at 2 different field locations: whether soybean growth is benefitted from diazotroph other than its usual symbiotic partner *Bradyrhizobium* and whether nitrogen fixation is a widespread reality in rhizobacterial isolates irrespective of their legume or non-legume host plants.

**MATERIALS AND METHODS**

**Experimental culture:** *Acinetobacter* genospecies 3 (A15) was isolated by Huddedar et al. (2002) from rhizosphere of wheat. The isolate was identified and confirmed up to the genus level by chromosomal DNA transformation assay (Junji, 1972) and delineated into the genospecies by biochemical scheme recommended by Bouvet and Grimont (1987) by Huddedar et al. The strain A15 was borrowed from Huddedar et al for the present investigation.

**Growth on nitrogen free medium:** A15 was screened for its ability to grow on nitrogen free Burk’s medium with composition gram per liter of distilled water: (Sucrose, 20;
Detection of nitrogenase enzyme and measurement of diazotrophic activity: Standard acetylene reduction test (ART) was performed (Hardy \textit{et al.}, 1968; Capone and Carpenter, 1982) to detect the presence of nitrogenase enzyme in \textit{Acinetobacter}. Culture growth of A15 from Luria agar was inoculated in Burk’s liquid medium and incubated for 24h under shaking at 150rpm and 28°C. Loopful from this culture broth was inoculated onto Burk’s agar slants prepared in glass vials (20ml); uninoculated slants were kept as control. The experiment was performed in 2 sets. The test and control slants were incubated for 168h, 144h, 120h, 96h, 72h, 48h and 24h. This was set (I) and kept at 10°C for 30 days after completion of the incubations. After 30days, Set (II) was prepared similarly but was not kept at 10°C but incubated at 28°C. Both the sets were assayed for acetylene reduction at the same time. At the time of assay, cotton plugs of glass vials were replaced with serum stopper seals under aseptic conditions. Immediately acetylene gas (99.5% purity, UHP grade, pC$_2$H$_2$=1KPa; pO$_2$=0.1) was filled at 20% head space volume of vial. All the vials were incubated for 1h at 28°C. Gas Chromatograph (GC, Chemito 1000) was set up with Poropak-N column (Chromatopack Corp. 2 meter length, 1’8” diameter). The original protocol of Capone and Carpenter (1982) was modified with respect to temperature setting up (60°C) of GC to the column temperature at 100°C while the detector and injector temperature set at 140°C. The mobile gas phase consisted of nitrogen (4Bar), hydrogen (2Bar) and air (2Bar). The parameters were set such that the standard ethylene peak was registered between 1.2 and 2.0 min. The standard reading of ethylene (C$_2$H$_2$) and acetylene (C$_2$H$_4$) were made by injecting about 500µl of standard ethylene (100% purity) and acetylene separately using air tight syringe (UTek Chromatography). At the end of incubation period, 500µl of gas from headspace of vials was quickly injected into GC. The peak area and retention time for each sample was recorded. The amount of acetylene reduced per ml of sample and ethylene produced in nmoles/h/sample was calculated (Sadasivam and Manickam, 2004).

Detection of plant growth promoting (PGP) characteristics other than nitrogen fixation: As a prospective PGPR, A15 was also characterized in \textit{vitro} for PGP properties like IAA production by Salkowskii assay (Gordon and Weber, 1951), Chitinase activity (Renwick \textit{et al.}, 1991), protease activity (Boemare and Akhurst, 1988); tricalcium phosphate solubilization on Pikovskaya’s agar (Bhawasr \textit{et al.}, 2011) and antimicrobial activity against selected phytopathogens was determined by agar disc diffusion method. Antifungal and antibacterial activity was tested against phytopathogens \textit{Sclerotia minor}, \textit{Fusarium oxysporum} and \textit{Proteus} spp., \textit{Enterobacter aerogenes} respectively. Besides, A15 has already been reported for its bioemulsifier mediated antimicrobial activity (Bhawasr \textit{et al.}, 2011).

Preparation of \textit{Acinetobacter} inoculum and soybean seed bacterization: Bioinoculum was prepared in carrier lignite powder (mesh size 75m) as a semisolid preparation. Before inoculum preparation, lignite powder was autoclaved at 15lbs pressure and 121°C for 1h and cooled. Its pH was adjusted by 10% CaCO$_3$ to 7.3 prior to addition of inoculum suspension of A15. Bacterial suspension was prepared by inoculating a colony growth from Luria agar to 500ml Luria broth and incubated on rotary shaker at 28°C for 48h. Inoculum was aseptically added to trays containing sterile lignite powder at the rate of 40ml of broth culture/60 grams of lignite. It was kept static for half an hour at room temperature so that bacterial cells had a chance to adequately coat the carrier particles and stabilize. Bioinoculum so prepared was filled in sterile plastic bags and incubated for 24h at room temperature; bioinoculum was thus ready for seed application. \textit{Acinetobacter} population from each inoculum was monitored at the interval of 30, 60, 90, 120, 150, 180 and 210 days on shelf by spread plate method. The total viable count (TVC) was determined by serial dilution of inoculum prepared in triplicate on LB agar plates. Total viability was determined as colony forming unit (CFU) per gram at the monthly interval for 6 months. Viability of inoculum bacteria in the field was also determined as a test for root colonization at VE (Early emergence), R2 (Full flower) and R4 (Full pod) stages by CFU counts of antibiotic rifampicin resistant (Rif$^R$) spontaneous mutants of A15 (Suslow \textit{et al.}, 1979; Huddedar \textit{et al.}, 2002).

Soybean variety ‘JS335’ (MPKV, Rahuri) was used for both the field trials. The seeds were treated with bioinoculum at the rate of 10Kg seeds/250 grams of inoculum and mixed properly such that every seed was coated by uniform layer of inoculum. Such treated seeds were dried in shade and sown on prepared plots. The inoculum applied seeds were assayed along with untreated seeds (control) after a week to calculate \textit{in vitro} % seed germination by wet filter paper method developed by Quartberg (Seefeldt, 2012).
**Field trials:** The field experiments were performed at two different locations namely, Field I and Field II. Experimental plots were different with respect to size, location, soil physio-chemical and microbiological characteristics but the treatments (inoculum treated seeds as test and uninoculated control) were similar, arranged in completely randomized block design (Table 1). The field observations were noted as growth promotion effects (seed germination, plant height, nodulation, shoot length, number of nodes, leaf width, flowering and pod maturity), biocontrol (presence of visible symptoms of bacterial, fungal or insect diseases) and postharvest parameters (thousand grain weight, dry weight, yield and percent optimum stand) at all or selective growth stages (VE to R8) of soybean crop for the period of 6 months. Before the field experiment, life cycle of soybean crop was studied in detail from the literature available (www. clemson.edu/soybeans/growth; www.soybeanstation.org).

**Statistical analysis and comparison:** All the measurements were taken in triplicates by considering minimum 25 plantlets selected at randomly in experimental plots. The significant difference between observations of control and experimental plants in relation to the parameters reflecting nitrogen fixation within the same field and between two fields were analysed statistically. Parameters ‘plant height,’ ‘nodulation’ and ‘soil N’ content before and after harvest were selected to be analysed statistically by mean, S.D. ANOVA, coefficient of variation and comparisons of means (Panse and Sukhatme, 1954).

**RESULTS AND DISCUSSION**

**Growth on nitrogen free medium:** Bacterial growth on Burk’s nitrogen free medium and LB medium showed changes in colony morphology characters. The colonies on LB medium were mucoid, non-glistening and peach orange pigmented. The growth of very mucoid, glistening colonies with the loss of usual pigmentation on Burk’s medium was observed. Mucoidy was found to enrich after long term storage at 28°C and 10°C. These changes may be ascribed to stress condition created by lack of nitrogen in the Burk’s medium. Exopolysaccharide like compounds produced in nitrogen deficiency impart mucoidy and glistening appearance to colonies (Buckmire, 1984). Apparently pigmentation under nutrient stress is presumed insignificant as bacterium tries to save metabolic energy by shutting down pigment synthesis; this could be the reason for depigmentation.

**Nitrogenase (Diazotrophic) activity:** ART is usually done to show diazotrophic ability of a strain and in this study, acetylene reduction to the production of ethylene confirmed the presence of active nitrogenase in A15. The standard ethylene peak was registered in between 1.2-1.8. The highest peak height and retention time (RT) shown by A15 was proportional to its nitrogenase activity (Figure 1). The maximum nitrogenase activity was found to be 49.56 µmoles ethylene per vial per unit time at 28°C at 96h of incubation. Nitrogenase activity was noted to be time dependent. It was lesser at 0-24h to 0-96th h incubation and increased at 6th and 7th day of incubation. Presence of nitrogenase activity added an important trait to plant growth promoting potential of A15.

**Other PGP properties of Acinetobacter A15:** *In vitro* analysis showed that A15 produced IAA which is important growth regulating auxin, solubilized insoluble phosphatic compounds (Prikry et al., 1985) and phosphorus in solution is important macronutrient, only second to the nitrogen for plant growth (Singh et al., 2008). A15 showed oil emulsification, emulsifier production helps to break down complex organic molecules in the soil and rhizosphere into assimiable simple substrates (Mishra and Sundari, 2013). Chitinase, protease activity and antimicrobial activity shown against test plant pathogenic fungi and bacteria means inoculum could shield host plant from pathogen attack and

### TABLE1: Comparative analysis and layout of field I and II.

<table>
<thead>
<tr>
<th>Soil characteristics</th>
<th>Field I</th>
<th>Field II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humidity Status</td>
<td>87% Moist</td>
<td>66% Dry</td>
</tr>
<tr>
<td>Color</td>
<td>Brown</td>
<td>Black</td>
</tr>
<tr>
<td>Type</td>
<td>Clay loam</td>
<td>Clay loam</td>
</tr>
<tr>
<td>PH</td>
<td>7.5</td>
<td>7.7</td>
</tr>
<tr>
<td>N:P:K (ppm)</td>
<td>0.26:10:38</td>
<td>0.11:8:81</td>
</tr>
<tr>
<td>Zn:Cu:Fe:Mn (ppm)</td>
<td>8.12:7.7:3.43:37.87</td>
<td>1.26:4.5:3.7:36.50</td>
</tr>
<tr>
<td>Soil Rhizobium (TVC) cells/gram soil</td>
<td>110³</td>
<td>1.10¹³</td>
</tr>
</tbody>
</table>

**Field layout and treatments**

<table>
<thead>
<tr>
<th>Size of the field (ft)</th>
<th>48×32</th>
<th>12×5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rows</td>
<td>15/treated and 1/control</td>
<td>8/treated and 1/control</td>
</tr>
<tr>
<td>Number of soybean plants / row</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>Total soybean plantlets</td>
<td>1125</td>
<td>200</td>
</tr>
</tbody>
</table>

No fertilizer treatment was applied before sowing at both the fields.
diseases (Renwick et al., 1991). One and all of these properties are known to contribute to the plant growth promotion or biocontrol by various bacterial crop inoculants (Malik et al. 1995; Hegde et al., 1999).

Viable count, root colonization and in vitro % seed germination: The highest TVC was found to be 100.2 × 10⁸ CFU/gm in carrier lignite at 120 days, after that it was decreased at the rate of 10 to 42%. The average shelf life for all strains in lignite was found to be 4 months (120 days). TVC of 47, 58, 127 and 68 (×10⁶ CFU/gm) of RifRA15 mutants at VE (Early emergence), R2, R4 and R6 (Full seed) stages respectively on soybean roots suggested efficient root colonization and hence establishment by A15 inoculum culture. It was also an indicator of positive cooperation between soybean and newly introduced A15 on the field. Based on these results and reports by Suslow et al. (1979), it was ascertained that effective root colonization was the basis of growth promotion or biocontrol as observed in field trial. On the contrary at field II, TVC of mutants was less than 25 ×10⁸ CFU/gm and growth promotion response was found to be less than control. *In vitro* germination frequency of inoculum treated (test) soybean seeds was found to be 100% at 48th hour of sowing while as untreated control showed germination frequency of 83.3% under similar experimental conditions. This was later evident under field conditions at VE stage.

**Soil analysis:** Considerable difference was found in pre and postharvest soil analysis from both the fields. Soil analysis of both the fields after harvest showed increase in nitrogen, phosphorous and potassium (NPK) content. These results were supported by previous field trials where luxurious growth of inoculum treated plants than control was observed due to augmented concentration of macronutrients (Orhan et al., 2006; Das and Singh, 2014) or contribution of nitrogen by legume growth in deficient soils (Mapfumo et al. 2001; Porpavai et al. 2011).

**Nodulation:** Nodulation of soybean plants (test and control) at 2 fields was different. It was expected to obtain increased nodulation count in test plants as promoted by diazotrophic A15 but results were not likely.

**The nodulation picture at Field I:** Test plants showed very scanty nodulation only at R1 (Beginning of flowering) and R2 stages. At R1 and R2 stages nodulation count was in the range 1-2 and 20-160 respectively; while 0-2 (R1) and 1-130 (R2) nodules were counted in control plants. Though all test plants exhibited normal healthy growth over control, nodulation count was suddenly decreased after R2 and till the harvest they remained without any nodules. There was no such decrease in nodules of control plants which remained profusely nodulated in later stages also.

**The nodulation picture at Field II:** Inoculum treated test as well as control plants were nodulated; however % nodulation in test plants was considerable. Examination of nodule morphology indicated that nodules present on test and control plants belonged to *Rhizobium* spp. Table (2) indicates comparative nodulation count and percentage of control and test plants.

### Table 2: Soybean nodulation count and % nodulation taken at R2 (Full flower) stage

<table>
<thead>
<tr>
<th>Test/ Control</th>
<th>Number of nodules (mean of 5 plants)</th>
<th>Dry weight of nodules in grams</th>
<th>% nodulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test I</td>
<td>23.8</td>
<td>0.68</td>
<td>14.85 (54.05)</td>
</tr>
<tr>
<td>Control I</td>
<td>40.8</td>
<td>0.83</td>
<td>25.46 (21.23)</td>
</tr>
<tr>
<td>Test II</td>
<td>43.8</td>
<td>0.9</td>
<td>27.34 (15.44)</td>
</tr>
<tr>
<td>Control II</td>
<td>51.8</td>
<td>1.25</td>
<td>32.33 (9.68)</td>
</tr>
</tbody>
</table>

Values in parentheses indicate % decrease in nodulation count of test as compared to control

Test I and Test II: Inoculated soybean plants from Field I and II respectively
Control I and Control II: Uninoculated soybean plants from Field I and II respectively

**FIG 1:** A. GC-FID analysis of standard ethylene and acetylene B. Ethylene production by A15 at 96h, 28°C on Burk’s N₂ free medium
Out of the many reasons described in the literature (Subba Rao, 1986; Bohner, 2009), following reasons could be the possibilities for less or no-nodulation of inoculated plants at field I and II. Field I was first time planted by soybean, the opportunity of nodulation was missed as roots might have grown past where the inoculant was established or by the time native bradyrhizobia had had the chance to nodulate roots, moreover A15 cells might had already taken the place on roots as indicated by root colonization test. Quite the opposite was the situation in field II, which had a history of soybean plantation for consecutive 3 years; A15 failed to replace established rhizobia in soil and rhizosphere region and hence could not colonize successively. Nodule morphology also indicated presence of rhizobia. Therefore inoculum had no chance to express its PGP properties and hence showed no growth promotion as compared to control. From the results of 2 fields, it was concluded that: 1). Proper native strains that can nodulate soybean were absent 2). A15 was not stimulator of nodulation by native rhizobia and/or 3). There was enough nitrogen present in the soil (Table 1) so growth of soybean was benefitted from inoculant’s PGP properties rather than diazotrophy.

**Growth promotion indicators on field:** Although, it was expected to get similar growth promotion effects from 2 field trials but in reality the efficacy of inoculum in terms of total yield was found to be greatly affected by field conditions. The performance of bioinocula under field condition depends on several factors like soil physicochemical environment, native bacterial population (Munishamanna and Hegde, 1994) and cropping practice in the history (Subba Rao, 1986). Alike differences in soil analysis and environmental conditions of two fields, there was the variance of effects obtained from 2 field trials. Neither of the growth parameters under consideration from both the fields showed any similarity nor distinct differences that could be correlated. It was therefore concluded for not the one but many of the concealed reasons were responsible for variation in field results (Zaidi et al., 2009). As observed by Pandey et al. (1999), the reason for multi-PGP effects at field I could be A15 inoculum which might have acted as stimulus for activation of other native microbial communities.

**Seed germination:** Early VE and cotyledon development (VC) as compared to control plants was the first growth response observed in test plants. Sprouts of 1-2cm length above ground and 1-1.5cm long adventitious roots observed 2 days after sowing. Extensive and long root were observed throughout the life cycle of test plants but not in control plants. Observations suggest role of A15 in root and shoot development and hence possibly increased mineral and water uptake and transport (Cattelan et al., 1999). At field II, control and test plots at VE and VC stages gave the impression of similar treatments. Unless stated in further parameters, the results of field II results of test and control are accounted to be similar and results of field I only are discussed for the justification.

**Plant height:** At Field I, all test plants showed considerable increment in shoot length with potential height difference of about 25-50% to control plants. The height difference was significant observation suggestive of role of IAA and phosphate solubilization for which A15 was found to be positive. Extensive and long roots along with increased plant height due to IAA have previously been reported in many field trials with other crop plants (Fuentes et al., 1993; Bhattacharya and Jain, 2000; Huddedar et al., 2002).

**Shoot length, leaf width and nodes on the stem:** The maximum shoot length measured above ground level was found to be 101cm and 51.4cm in test and control plants respectively at R3 (Beginning pod) stage. Maximum leaf width was found to be 9.9cm for test soybean whilst 6.2cm for control. Starting from V1 (First trifoliolate) to the end of R7 (Beginning maturity) stage there was average difference of about 2.9cm in span of control and test and leaves of test plants were more in width by 36.78% over control plants. Broader leaf span and width, was found more in inoculum treated plants and can be directly be correlated with high yield with respect to control, such results have been obtained in *Azotobacter* and *Azospirillum* crop inoculations (Rokhzadi et al., 2008; Naserirad et al., 2011). Total number of nodes and leaves were also more (15 leaves / treated plant over control) at every stage of life cycle. At V1 stage test plants showed more than one nodes on main stem which resulted in increasing number of leaves in the range 3-21. Node formation continued till the end of full pod stage (R4). These results are in correlation with previous field trial of *Acinetobacter* sp. on wheat (Huddedar et al., 2002).

**Flowering:** Test plants showed early flowering as well as early pod maturity. First bloom that is the beginning of reproductive stage (R1) of test was observed before control which represented 1-6 bloomed purple tricarpellary flowers. Control plants showed late blooming as compared to test plants by a gap of 2 weeks. Blooming of flowers lasted at the end of R4 stage. This was the largest period for blooming ever noted in past soybean cultivations as reported by the farmer of Field I. Apparently, this stage assured high yield of test crop as compared to control where blooming stared late and ended very soon. Very few workers have mentioned stimulated flowering followed by bioinoculation by PGPR (Ownley et al., 1999; Farina et al., 2012) as obtained in this field trial.
Pod maturity: Pod development followed by seed formation was very rapid with respect to time and quantity. Test plants had maximum of 15-45 pods at R3 stage in proportion to bloomed flowers. Seed development in all test plants began at R4 to R5 (Beginning seed) stage and pod cavity was filled with large green seeds by 8 days; however control plants were only at R3 stage. Within a week's time test plants passed R6 and R7 stages towards maturity. The greenish color of pods changed to their mature yellow color and seeds also transformed into yellow hard seeds. R8, the stage of full maturity (95%) was attained about 15 days earlier than actually expected and for all test crops at the same time. Nevertheless control plants' plot neither appeared in uniform growth stage nor could particular growth stage identified. The results were similar to those obtained by Chauhan et al. (1995) on Brassica napus L. after Azospirillum inoculation. There was no comparable variance in the different growth stages at Field II; test and control plants were alike in growth pattern.

Post-harvest parameters
Yield, thousand grain weight and optimum stand: Potential increase in grain yield is assumed to evaluate plant growth promotion by inoculant and so was obtained in field I trial. At field I, the average yield increase was found to be 12% over control. Crop yield increase by Azospirillum inoculation has been reported in soybean (Bashan et al., 1990). Dry weight and thousand grain weight of test plants was about 42.21% and 22.02% more than control. Increased dry and thousand grain weights suggested enhanced plant biomass, productivity and hence yield increase in comparison to control (Dileep Kumar et al., 2001). Percent optimum population stand for control was 63.56 % and for test plants it was in average of 95.6 %; this decreased yield of control plot was due to less optimum stand. Similar effect of higher optimum stand on yield has been reported (Mishra et al., 2010). At field II, growth promotion effect of A15 inoculum could not be justified. There was no apparent difference between parameters indicating growth promotion of test and control plants. Yield difference was also unremarkable; it was not decreased or increased as in field I.

On field observation of biocontrol: Observations and identification of insect pests, fungal and bacterial infections on soybean plants was done with the help of experts from Plant Pathology and Microbiology section, College of Agriculture, Pune. During R1 stage, control plot of Field I received 2 applications of insecticide Monocrotophos at the rate of 500ml/acre within 15 days of first application. Monocrotophos is systemic and contact action insecticide but still some of the control plants were found heavily infested with white flies and leaf rollers. Control plants (2 in 10) were found infected by downy mildew as white colonies below the leaf. Its incidence was increased to 15 in 25 plants. Besides, yellowing of leaves, blight and stem rot were also identified. Unlike control, test plants were free from pathogens or diseases. Various lytic activities of A15 like chitinase, protease and anti-microbiosis might have kept fungal and bacterial diseases from test soybean at boundary. However, effect of lytic enzymes was not ascertained under field condition. The results of % optimum stand and increased yield reflected efficacy of these biocontrol mechanisms of inoculant on test plants. Goel et al (1999) have stated that inoculants with PGP enhance crop productivity either by making nutrients available to plants or by allelopathy but during this investigation it was difficult to trace effect of particular trait on plant growth as inoculum bacteria possessed more than one criterion that could promote plant growth. The biocontrol characteristics responsible for healthy growth of test plants needs further investigation. Control plot also received single application of weedicide Imazethapyr at the rate of 300ml/acre to destroy extensively growing common grass weeds but it was not sufficient as after a gap of 15 days there was emergence of new weeds in the control field. Otherwise growth of weeds in soybean control field was one of the reasons behind retarded growth and yield reduction of control plants. Growth of weeds and other herbs was totally absent in inocula treated field. Since there were no weeds in test plants' plot, it can be concluded that inoculation of A15 might have vital role in the suppression of growth of nonhost weeds.

Statistical analysis: Most reliable parameter to indicate effectiveness of diazotrophy is nodulation and which was the purpose of this experiment but at field I nodulation was literally absent while at field II nodulation picture of test and control plants was almost similar and could not be compared. PGP parameter ‘plant height’ was therefore selected for statistical analysis so as to evaluate effectiveness of growth promotion of soybean by inoculation of A15. Means between and within the treatments were compared using ANOVA test and t-distribution. Statistically, the tests were insignificant. Factually, insignificant outcome of this experiment was significant enough to prove positive effect of PG on soybean growth but no apparent effect of diazotrophy on test plants. In other words, A15 as a PGPR promoted growth and yield in this legume on account of various PGP it possess but in vitro diazotrophic property failed to assist or enhance nodulation.
CONCLUSIONS

It was revealed from this investigation that *Acinetobacter* inoculum could not express its diazotrophic property under field conditions. At field I, the growth promotion and yield increase was due to PGP properties other than nitrogen fixation. At field II, neither PGP properties nor diazotrophy was effective to promote growth of soybean. Results showed that *in vitro* diazotrophic activity of *Acinetobacter* was ineffective on field soybean growth promotion. Consistent and consecutive field trials are necessary to answer one or more queries which arose from field trials and to ascertain conditions that could make diazotrophy of A15 efficient in plant growth promotion.

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www.clemson.edu/soybeans/growth; Soybean vegetative and generative growth stages.