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Effect of *Azotobacter* and *Pseudomonas* inoculation on microbiology of rhizosphere soils of Okra

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Abstract
An experiment was carried out involving characterization of *Azotobacter* and PSB inoculations applied for enhancing growth performance of okra (*Abelmoschus esculentus*). Variety- Suraksha. The experiment was laid out in green house with six treatments and four replications. Treatments consisting of T_1 (100% NPK), T_2 (100% NPK + Vermicompost), T_3 (75% NPK + Vermicompost + PSB), T_4 (75% NPK + Vermicompost + *Azotobacter*), T_5 (75% NPK + Vermicompost + *Azotobacter* + PSB), T_6 (50% NPK + Vermicompost + *Azotobacte*r + PSB). Both the isolates showed positive reaction for starch hydrolysis, catalase test. TSI test showed glucose, lactose and sucrose fermentation occurred with *Azotobacter* whereas in PSB no fermentation was seen. The results showed that the treatments T5 and T6 produced significantly highest growth of okra and better microbial property of rhizosphere soils of okra. Significantly highest PSB and *Azotobacter* population density in rhizosphere soil of pot grown okra was observed (21.14 X 10^4 and 48.54X 10^4 per g of soil) due to (T5) followed by T6. Dual inoculation with *Azotobacter* and PSB treatments also showed maximum dehydrogenase activity in rhizosphere soils of pot grown okra in green house condition. Both the treatments showed significant effect on okra over rest of the treatments.

Keywords: *Azotobacter,* PSB, microbial population, dehydrogenase and Okra

Introduction

Among the biofertilizers *Azotobacter* as nitrogen fixer and *Pseudomonas* as phosphate solublizer have gained much importance and there has been encouraging response to inoculation with *Azotobacter* and PSB. *Azotobactor* is free living bacteria and has been reported to fix 20 kg N ha^{-1} in field of non-legume crop and also secretes some growth promoting substances. The most feasible and economically viable fertilizer package is one which improves the crop yield without deterioration soil health. *Azotobacter* not, only provides nitrogen, but also produces a variety of phyto hormones i.e. indol acetic acid, and gibberellins. It stimulates vegetative growth by cell division and incenses the development of large stems and leaves and tends to produce succulence in Okra fruits. Phosphate solubilizing Bacteria (PSB) significantly helps in the release of this insoluble inorganic phosphate and makes it available to the plants. PSB are a group of beneficial bacteria capable of hydrolyzing organic and inorganic phosphorus from insoluble compounds. P-solubilization ability of the microorganisms is considered to be one of the most important traits associated with plant phosphate nutrition.

Fertilizers are generally applied to improve the crop yield, nutritional quality and aesthetic value of crops. Bio-fertilizers are the most advanced bio technology can increase the output, improve the quality. Bio-fertilizers are organisms that help to provide and keep in the soil all the nutrients and micro-organisms required for the benefits of the plants. Application of biofertilizers results in mineral and water uptake, root development, vegetative growth and nitrogen fixation. (Siddiqui *et al.,* 2014) [16]. Okra (*Abelmoschus esculentus*) is one of the most important vegetable crops grown for its tender green fruits in almost all parts of the world. Therefore proper attention must be given to these nutrients on plant nutrition (Khalil, 2006) [7]. Whereas, chemical fertilizers are quite expensive input and their use over a long period deplete the soil fertility and soil health. Besides chemical fertilization of the crops which involves high cost, whereas bio-fertilizers are cheaper and renewable sources and contribute to the development strategies. Bio-fertilizers are necessary to support developing organic agriculture, sustainable agriculture, with no pollution.

Nitrogen fixing and phosphate mobilizing microorganisms in bio-fertilizer enhance the availability of plant nutrients in the soil and thus, sustain the agricultural production. As per particular agroclimatic condition, effective bioinoculants should be developed which indicates that there is an urgent need for their characterization to ability to survive and nodulate in soils of Chhattisgarh (Gupta *et al*., 2005) [5]. Therefore the present investigation was carried out in laboratory to characterize of *Azotobacter* and *Pseudomonas* species on the basis of bio chemical and morphological characters and applying as bioinoculants for increasing growth of okra.

Materials and Methods

An experiment was carried out involving characterization of *Azotobacter* and PSB (*Pseudomonas*) inoculations applied for enhancing growth performance of okra (*Abelmoschus esculentus*) Variety- Suraksha. The experiment was laid out in green house with six treatments and four replications. Treatments consisting of T_1 (100% NPK), T_2 (100% NPK + Vermicompost), T_3 (75% NPK + Vermicompost + PSB), T_4 (75% NPK + Vermicompost + *Azotobacter*), T_5 (75% NPK + Vermicompost + *Azotobacter* + PSB), T_6 (50% NPK + Vermicompost + *Azotobacte*r + PSB). The promising *Azotobacter* isolates and PSB isolate were obtained from Microbiology repository of Department of Agricultural Microbiology, College of Agriculture, Raipur. Laboratory experiment was conducted to characterize *Azotobacter* and PSB (*Pseudomonas*) isolates by Gram staining, colony morphological characters, Starch Hydrolysis Test, Catalase test, Triple Sugar Iron Agar test (TSI test). 7 days old culture was used for morphology, colony characters and Gram's reaction. One ml of appropriate dilution of *Azotobacter* and PSB isolates were transferred into the petriplates containing Jensen Media and Pikovskaya media. The phenotype and growth pattern were observed comprised colony morphology, including parameters like diameter, margin, elevation, form, transparency and colour (Aneja, 2003) [1]. The Gram's staining technique was followed as suggested by (Graham and Parker, 1964) [4]. The bacteria that appeared purple were referred to as Gram positive and those which appeared pink were described as Gram- negative (Aneja, 2003) [1].

The isolates were subjected to different biochemical tests viz, Starch hydrolysis Test, Catalase Test and TSI (Triple sugar Iron Agar test). Hydrolysis of starch was examined by streaking the organism on starch with nutrient agar and iodine solution (Graham and Parkar, 1964) [4]. Starch agar medium (5g/L peptone, 2g/L Potato starch, 3g/l beef extract, 15g/L agar, pH 7.0) were inoculated with culture and incubated at 29ºC temperature for 48 hours. Drop of iodine solution 0.1N were spread on 48 hours old culture grown on petriplates. Formation of blue colour indicated non - utilization of starch and vice-versa. Catalase activity was tested by adding 2-3 drops 3% H2O2 to a drop of matured broth of *Azotobacter* and PSB isolates to see whether any bubbles are evolved or not (Graham and Parkar, 1964) [4]. If catalase is present resulted in immediate formation of gas bubbles. Triple Sugar Iron agar media is used for the differentiation of microbes by ability to determine carbohydrate fermentation and hydrogen sulfide production. Organisms that ferment glucose produce a variety of acids, turning the colour of the medium from red to yellow. Triple sugar iron agar medium was prepared by dissolving 16.13 g TSI medium in 1000 ml of distilled water and slants were prepared to set in sloped form with a butt about 1 inch long. Slants were streaked, incubated at 28±2ºC for48 hrs and results were observed.

Application of Biofertilizers, Seed sowing and plant growth

Once the pure culture of *Azotobacter* and PSB has been established and confirmed, *Azotobacter* cells were immobilized on carriers, which is an inert material used for mixing broth. The carrier (charcoal) was powdered and dried in sun to get 5% moisture level. Then it is screened through 100-200 mesh sieves and neutralized by mixing with calcium Carbonate powder. The broth containing *Azotobacter* and PSB cells were mixed with carrier and kept in tray. The moisture content was maintained to about 35-40%. After proper mixing it is left for 2-10 days by covering the tray with polythene at 22-24ºC. During this period *Azotobacter* and PSB cells multiplied, a process called curing. Thereafter, inoculants can be used directly or packed and stored.

A well mixed 8 kg mixture of soil, sand and compost in 3:1:1 ratio was filled in each pot. Inorganic fertilizer Nitrogen, Phosphorous and Potassium were given @ 100:50:40 kg/ha (100%) through Urea, SSP and MOP as per the treatment. Vermicompost were incorporated @ 2.5 t ha^{-1} (10 g/ pot) as per treatment to respective pots prior to sowing. Application of biofertilizers *Azotobacter* and PSB @ 10 g/pot were applied to soil in the pot before sowing as per treatment description.

About 24 hours before sowing, all the pots were irrigated. The seedlings were regularly watered and hand weeded in the pots as and when considered necessary to keep the seedlings free from weeds. The observations of okra plants were recorded at regular interval. Five plants were randomly selected from each replication and treatment and tagged. Height of each tagged plant was measured at 30 days interval and at harvest from base of the plant to tip of the main shoot by meter scale and average height of five plants was recorded as mean plant height in centimeter.

Soil microbial analysis after experiment

About 25g of soil sample was kept as such from polypot in sealed polythene bag to prevent the moisture loses and properly stored in refrigerator for quantitative analysis of microbes.

Enumeration of microbial population in rhizosphere soil

Rhizosphere soil samples from different treatments were collected after completion of experiment for enumeration of *Azotobacter* and PSB. Enumeration was done by serial dilution and plating technique.

Number of *Azotobacter /PSB* per gram of oven dry soil:

$$\frac{\text{No of colony forming units (cfu) x dilution}}{\text{Dry weight of one g moist soil sample x aliquot taken}}$$

The operation of making serial dilutions, setting of plates and inoculation with appropriate media was done in sterilized atmosphere of Laminar flow.

Dehydrogenase activity

Dehydrogenase activity in rhizosphere soil was determined with adding 3% Triphenyl Tetrazolium Chloride solution followed by incubated for 24 hrs at 37^0C. Clear pink colored supernatant was withdrawn and readings were taken with a colorimeter. The amount of TPF formed was calculated from

the standard curve drawn in the range of 10 mg to 90 µg TPF g^{-1}soil h^{-1}.

Results and Discussion

Retrieval of *Azotobacter* and PSB isolates

Azotobacter and PSB isolates (Table 1) present in culture deposits of department were revived on Jensen's medium and Pikovskaya's medium of pH (7.0) agar plates and incubated at 29^0C (Aneja, 2003) [1]. After two days of incubation, colonies were obtained. The isolates were maintained on slopes of respective medium. Pure isolates were used to study the morphological, cultural and biochemical characteristics and all tests were performed in triplicates.

Table 1: Cultural and Morphological characteristics of *Azotobacter* and PSB isolates

Isolates	Colony morphology	Gram staining
Azotobacter isolates in Jensen's medium		
Azotobacter	Gummy, round and convex, entire margin, whitsh in colour	Gram -ve
PSB isolates in Pikovskaya's medium		
PSB	Smooth round, entire margin, showing clearing zone, yellowish in colour	Gram -ve

Colony morphology and Gram staining

Azotobacter isolates produced translucent, nearly round and gummy colonies which varied in size between 1.5 to 2.00 mm and whitish in colour (Table 1). *Pseudomonas* bacterial strains were found capable of forming a clear zone on solid Pikovskaya's media. Colonies of all the isolates were found to be round and yellowish in color. Similarly using Gram staining technique as described by Aneja, 2003 [1] pink colored Gram negative rods were observed under light microscope for *Azotobacter* and PSB isolate (Table-1). The authentication of the isolates was performed using sub culturing method. The isolated bacteria were identified in consultation with Bergey's manual of Systematic Bacteriology. Based on the morphological tests, PSB isolates was found to be belonged to *Pseudomonas* sp.

The identification studies are also in coordination with the works done by Gadagi and Sa (2002) [3] and Dsouky *et al.* (2003) [2]. *Pseudomonas sp*. act as efficient solubilizers of Phosphorous is in line with the findings of Komy (2005) [8] also observed similar colony characteristics in PSB.

Biochemical character

Both *Azotobacter* and PSB isolates showed positive for catalase, amylase activity (Table 2). Positive results were obtained from the starch hydrolysis assay. On subjecting inoculated plates to Iodine test, clear zones around the colonies were seen and colonies turned yellow in appearance, whereas blue colour appears on no growth area. This indicates that the isolates have potential to hydrolyze starch present in the medium. The test was performed to determine capabilities of strains to use starch as carbon source (Oliveria *et al*., 2007) [12]. In presence of starch, the production of extracellular enzymes occurs indicating the potential of organisms to use starch as carbon source. De Oliveira, 2007 also observed that *Rhizobium* strains can utilize starch obtained from different sources. Results on catalase activity indicated that they were positive to catalase activity. TSI test showed Glucose, Lactose and Sucrose fermentation occurred with *Azotobacter*, but there was no gas evolution and H_2S production whereas in PSB no Glucose, Lactose and Sucrose fermentation was there and also negative result for production Gas or H_2S (Plate 1).

Table 2: Biochemical characterization of *Azotobacter* and PSB isolates

Isolates	Biochemical Test						
	Starch hydrolysis test	Catalase test	TSI test				
			Glucose fermen.	Lactose fermen.	Sucrose fermen.	Gas prod.	H2S prod.
Azotobacter	+	+	+	+	+	-	-
PSB	+	+	-	-	-	-	-

Plate 1: Triple sugar iron agar test (TSI test) of *Azotobacter* & PSB isolates used as inoculation in Okra

Effect of inoculation on Okra

Once the pure culture of *Azotobacter* and PSB has been established and confirmed for its various activities, the next step was conversion of *Azotobacter* and PSB broth into a form for application as biofertilizer they were applied as biofertilizers; in Okra in a green house experiment. Effect of these *Azotobacter* and PSB inoculants were evaluated on the growth performances of okra in pot experiment under Green house condition. Dual inoculation involving *Azotobacter* and PSB along with 75% NP K dose + Vermicompost significantly influenced the growth over control and remained at par with 50 % NPK + Vermicompost + *Azoto* + PSB. Both the treatments showed superior effect on okra over rest of the treatments. Significantly highest shoot length (112.91cm) was found in T5 followed by 110 cm in T6 and 104.81cm in T4 at harvest, however T5 and T6 were at par. In Inorganic fertilization only, shoot length was found (85.43cm) (Fig.1). Data of plant height recorded at three different stages of (30, 60, and 90 DAS) crop growth presented in Fig. 1.

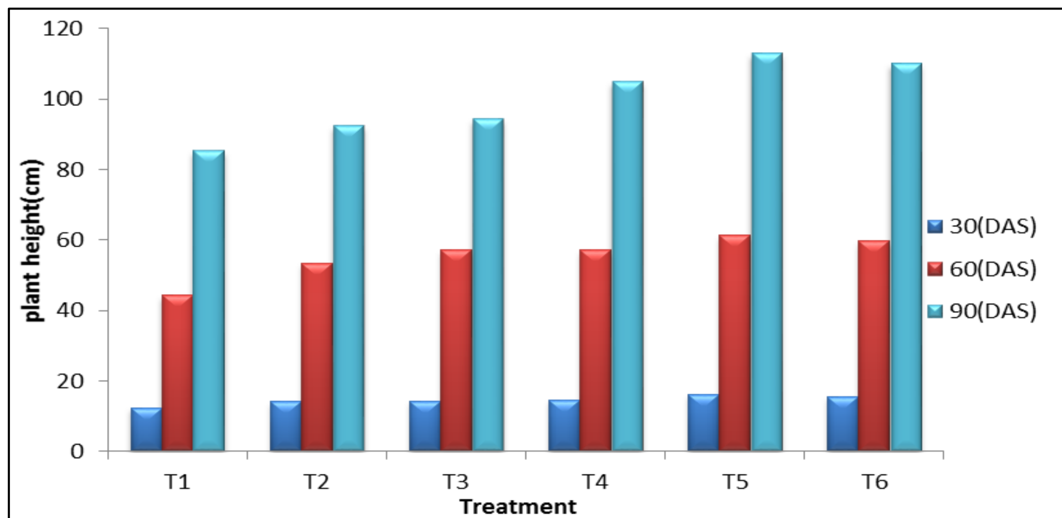

Fig 1: Effect of bioinoculants and vermicompost on plant height of Okra plant

Microbiology of rhizosphere soil of Okra

Data on population per g of soil in rhizosphere of okra as affected by different treatments effects at 90 DAS was given in (Table 3).

Data presented in Table 3 revealed that PSB population density at 90 DAS increased significantly over control. However, significantly highest population density of PSB in soil was observed due to inoculation with PSB and *Azoto*. At 90 DAS, PSB population density was observed in between 6.14×10^4 to 21.14×10^4 gram^{-1} of soil among different treatments. The PSB population was significantly maximum recorded 21.14×10^4 was found in T5, (75%+ NPK+ Vermicompost + *Azotobacter* + PSB). The minimum PSB population 6.14×10^4 was found in T1, (100% NPK). Results of present investigation are in confirmation with the findings of Saxena (2010) [15], Rudresh *et al*. (2005) [13], Walpola and Yoon (2013) [18] who reported that remarkable increase in the PSB population was observed in PSB-inoculated rhizosphere soil when compared with uninoculated soil. This work is strongly supported by Krishnaveni M.S. *et al.* (2010) [9], Kelel M. *et al.* (2014) [6].

Data presented in Table 3 revealed that *Azotobacter* population density at 90 DAS increased significantly over control under different treatments. However, highest population density of *Azotobacter* in soil was observed due to inoculation treatments. At 90 DAS *Azotobacter* population density was observed in between $18.41\times10^4$to 48.54×10^4/g of soil among treatments and in T1 was found 18.41×10^4/g of soil. The *Azotobacter* population was significantly maximum recorded 48.54×10^4 was found in T5 (75%NPK+Vermicompost+*Azotobacter*+PSB). The minimum *Azotobacter* population $18.41\times 10^4$was found in T1, (100%NPK). Results of present investigation are in confirmation with the findings by Torres *et al*. (2000) [17] (Table 3).

The data on the effect of different treatments on dehydrogenase activity (DHA) in rhizosphere soils of pot grown okra were presented in (Table 4). At 90 DAS the dehydrogenase activity was increased due to application of *Azotobacter* and PSB isolates alone and in combination. T6 and T5treatments had shown significant increment in dehydrogenase activity over control being maximum in T5.Above observations were in close agreement with Nowark (1996) [11] and Wyszkowska and Kucharski (2004) [19] who claimed that dehydrogenase activity reflects of the biological status of soil.

Table 3: Effect of bioinoculants and vermicompost on *Azotobacter* and PSB population after experiment

Treatments	Treatments detail	Microbial population	
		Azotobacter Population ($\times10^4$) CFU/g of soil	PSB Population($\times10^4$) CFU/g of soil
T1	100% NPK	18.41	6.14
T2	100%NPK+Vermi	21.62	8.28
T3	75%NPK+Vermi+PSB	24.14	18.34
T4	75%NPK+Vermi+*Azoto*	42.36	10.71
T5	75%NPK+Vermi+*Azoto*+PSB	48.54	21.14
T6	50%NPK+Vermi+*Azoto*+PSB	46.12	20.36
	SEm±	1.01	0.39
	CD (0.05 %)	3.04	1.18

Table 4: Effect of bioinoculants and vermicomposting dehydrogenase activity in rhizosphere soils in pot grown okra after harvest.

Treatments	Treatments detail	Dehydrogenase activity of soil (µTPF g^{-1} soil h^{-1})
T1	100% NPK	11.55
T2	100%NPK+Vermi	14.29
T3	75%NPK+Vermi+PSB	18.45
T4	75%NPK+Vermi+*Azoto*	23.44
T5	75%NPK+Vermi+*Azoto*+PSB	30.96
T6	50%NPK+Vermi+*Azoto*+PSB	30.51
	SEm±	0.84
	CD (0.05 %)	2.54

Dual inoculation with *Azotobacter* and PSB treatments were highly significant as compared to single inoculation with respect to microbiology of soil rhizosphere of pot grown okra in green house condition and also had significant effect on performance of Okra crop.

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