Int.J.Curr.Microbiol.App.Sci (2017) *6*(10): 1-13

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 10 (2017) pp. 1-13**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.610.001

Effect of Mixed Bioinocula on Growth and Efficiency of *Azotobacter* Species

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Keywords

Plant Growth Promoting Rhizobia, *Azosprillum*, *Pseudomonas*, Efficiency parameter.

Article Info

Accepted:
04 September 2017
Available Online:
10 October 2017

A B S T R A C T

Azotobacter is an important PGPR (Plant Growth Promoting Rhizobacteria) that directly affect the growth of plant by increasing IAA production Phosphatase and other growth promoting activity. The *Azotobacter* population in the isolated from rhizosphere of maize significantly increased due to inoculation of (*Azosprillum* + *Pseudomonas*) over the un-inoculated control. Addition of Nitrogen in combination with these two bacteria *Azosprillum* and *Pseudomonas* increases the native population of *Azotobacter* remarkably during the active plant growth stage. The maximum population of *Azotobacter* and its various growth factor activities was recorded due to application of 50% N + *Azosprillum* + *Pseudomonas* combination. Increasing the dose of nitrogen has deteriorating effect on population of *Azotobacter*. Effect on nitrogenase enzyme activity shown a different trends but phophatase activity and IAA production increased in the same combination as above. Due to these factors growth of root and shoot length of lentil increased that was at par with 100% nitrogen.

Introduction

Area around the root zone *i.e.* rhizosphere is a rich environment for plant growth promoting bacteria (PGPR) which can be utilized in many way to improve the growth of crop and yield. In agriculture one of the limiting factors is providing plant nutrition particularly nitrogen and phosphorus to the crop. Different Plant-Growth Promoting Rhizobacteria (PGPR) such as *Azospirillum*, *Azotobacter*, *Bacillus*, *Clostridium* and *Pseudomonas* has been used for their beneficial effects (Ozturk *et al.*, 2003). Studies showed a positive effect of PGPR on growth of different crops in different climates and soils (Salantur *et al.*, 2006). Improvement of crop yields by inoculation of diazotrophs like *Azotobacter* and *Azosprillum* has been suggested as the eco -friendly technology (Choudhury and Kennedy 2004). Non-legumes after inoculation with *Azotobacter chroococcum* increase the yield of field crops by about 10% and of cereals by about 15 % - 20 % (Hussain *et al.*, 1987). *Azotobacter* has the ability to proliferate and colonize the rhizosphere of farm crops most effectively (Brown *et al.*, 1962; 1964). Being a sensitive bacterium its

establishment in soil is very poor but once established the bacterium has been found to synthesize plant growth promoting substances like auxins, gibberellins and cytokinins (Shende *et al.*, 1977) and some antibiotic metabolites (Dorosinskii, 1962 and Meshram, 1984). Several authors have shown the beneficial effects of *Azotobacter chroococcum* on vegetative growth and yields of maize (Pandey *et al.*, 1989; Mishra *et al.*, 1995), as well as the positive effect of inoculation with this bacterium on wheat (Elshanshoury, 1995). The beneficial effects of dual inoculation have been reported by many workers (Sreeramula *et al.*, 2000; Vassilev *et al.*, 2001) for certain plant species. Inoculation with *Azotobacter* sp. complements the symbiotic relationship between plant roots and AM (Arbusacular mycorriza) fungi due to its nitrogen fixation, phytohormones production and phosphate solubilization properties (Kumar *et al.*, 2001).

Bagyaraj and Menge (1978) studied the interaction between a mycorrhizal fungus and *Azotobacter* and their effects on rhizosphere micro flora and plant growth. They found larger population of bacteria and actinomycetes in the rhizosphere of tomato plants inoculated with mycorrhizal fungus *Glomus fasciculatum* and *Azotobacter chroococcum* than uninoculated treatment. Inoculation of G. *fasciculatum* increased the population of *A. chroococcum* in the rhizosphere and maintained the same for a longer period. Maman *et al.*, (2013) used four inoculants formula as a biofertilizer mixed with chemical fertilizer in different doses and reported that due to a group of certain microbial interaction in to the soil and their special ability to utilize plant nutrient the chemical dose of nitrogen and phosphorus can be reduced and sustainability of field can be remain longer for many years. Akthar *et al.*, (2012) tried inoculation of Azotobacter with IAA and studied effect on *Azotobacter* population. They reported satisfactory result on the yield of lentil crop. *Azosprillum* and *Pseudomonas* both are diazotrophic and plant growth promoting bacteria. They are known to secrete several hormone and organic acid which have positive effect on the growth and population of beneficial bacteria and have suppressive effect on plant pathogenic fungi and bacteria. The present experiments aimed to study the effect of *Azosprillum* and *Pseudomonas* enriched soil on population of *Azotobacter* isolated from the maize farm (Pusa, Samastipur) and their efficiency parameter as below:-

Production of IAA
Nitrogenase activity
Phosphate Solubilization
Effect on lentil crop in pot house experiment

Materials and Methods

Isolation and enumeration of *Azotobacter* (Norris and Chapman, 1968)

Preparation of field for sample collection

Total of seven combinations of microbes and fertilizer were studied. Plots were divided into seven subplots. The field was fertilized with different combination of (100ml each) *Azosprillum* and *Pseudomonas* (PGPR) and chemical fertilizer alone and/or indifferent combination. The combination was *Azosprillum* + *Pseudomonas* (T2), 50% N + 100% PK (T3), 50% N + *Azosprillum* + *Pseudomonas* (T4), 100% NPK (T5), 100 % N + *Azosprillum* + *Pseudomonas* (T6), 150% N (T7) and soil fertilized with Phosphorus (SSP) and Potash (MOP) as Control (T1). From each plots three samples were taken randomly and they were mixed into homogenous population to obtain true microbial population. The mixed homogenous sample was treated as representative soil sample. The entire sample was taken in

triplicates. These were then tested for various activities.

Microbial analysis of soil was done following methods (Subba Rao, 1982)

Taken 10 g of soil from representative sample into a sterilized 100 ml beaker. Prepared blank (Tuladhar, 1983) by adding 90 ml sterile water in to a 250 ml sterilized beaker. Add soil (10gm) in to blank and shake it for 15-20 minutes on a magnetic shaker (10^{-1}). From the first dilution, transferred 1 ml of the suspension while in motion, to the second dilution blank with a sterile and fresh 1 ml pipette diluting the original suspension/specimen to 100 times (10^{-2}). From the 10^{-2} suspension, transferred 1 ml of suspension to the third dilution blank with a fresh sterile pipette, thus diluting the original sample to 1000 times (10^{-3}). Repeated this procedure till the original sample had been diluted 10^{-7} times using every time a fresh sterile pipette (Schmidt and Caldwell, 1967).

Preparation of Jensen's medium (Jensen, 1951)

Weigh 20.0 gm sucrose, 0.5 gm $MgSO_4.7H_2O$, 0.1 g $FeSO_4.H_2O$, 1.0g K_2HPO_4, 0.5g NaCl, 0.005g Na_2MoO_4, 2.0 g $CaCO_3$, and mix it in a small volume of water. Add 16.0-18 g Agar and warm it till the entire constituent mixed properly. Dissolved all weighed amount of constituents in 1000 ml of distilled water in 2 litre flask. Distribute the media in to 250 ml flasks and autoclaved it at 15 psi at 121^0C for 15 minutes. Allowed to cool the medium (37^0C) and pour into sterilized Petri plates and the medium was allowed to solidify.

Inoculation and spread plating

0.1ml of inoculum was added from 10^{-1}, 10^{-2}, 10^{-3} serial dilution on Petriplates containing solidified Jensen's N free medium for *Azotobacter* and spread it evenly. Care should be taken during adding and spreading the inoculums that the media should be cooled. Incubate the petri plates at 28-30°C for 24-48 hr in BOD incubator. *Azotobacter* population was calculated on the basis of per gm of soil.

Number of bacteria per gm of oven dry soil:

$$= \frac{\text{No. of colony forming units X Dilution}}{\text{Dry weight of 1 gm moist soil X aliquot taken}}$$

After incubation select the colonies showing light brown to black pigment. Identify the pure isolates of *Azotobacter* on the basis of following characters

Large ovoid cells 1.5-2.0 μm or more in diameter.

Pleomorphic, ranging from rods to Coccoid cells.

Aerobic

Production of water soluble and water insoluble pigments.

For characterization of *Azotobacter* Gram staining and Catalase test should be done

Morphological and biochemical characterization

Gram staining

For this 24 hr cultures of *Azotobacter chroococcum* was taken isolated from all the representative soil sample from seven plots.

Gram staining was done using standard staining method. Examined the slides microscopically and identified the gram reaction of culture and described the morphology and arrangement of the cells.

Efficiency assessment

IAA production

Azotobacter has ability to produce auxines which have favorable affect on plant growth (Tien *et al.,* 1979). The quantitative analysis of indole-3-acetic acid was performed by the method suggested by Gordan and Weber 1951 and later modified by Brick *et al.,* (1991). All Twelve strain of *Azotobacter* isolates were grown in Luria Bertani broth (g/l: tryptophan 10; yeast extract 5; NaCl 10 and pH 7.5).

A 100 ml of LB broth amended with tryptophan (10, 20, 60, 80 and 100 µg ml^{-1}) was inoculated with 1 ml of culture (No.x108 cells ml^{-1}) and incubated for 24 h at 28±1°C on a rotary shaker for 125 rpm.

After 24 h, 5 ml of each culture was centrifuged (10000 rpm) for 15 min and 2 ml of Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) was added to 2 ml of supernatant and incubated at 28°C in the dark for 1 h. the IAA concentration was determined using a spectrophotometer (λ 540 nm) against a standard curve. The experiments were conducted three times at different time intervals.

Nitrogenase activity

The N-fixing efficiency of these isolates was assessed by the acetylene reduction assay (ARA) as described by Hardy *et al.,* (1968-71). Briefly, 5ml of the *Azotobacter* broth in 12 ml vials was inoculated with ~10^4CFU/ml of each isolate and incubated at 28 °C for 48-96 h. Once visible growth was observed, the vials were plugged with rubber.

10% of the head space (7 ml) was injected with pure acetylene gas (C_2H_2) after removing an equal amount of air from vials by means of a disposable plastic syringe (Muthukumarasamy *et al.,* 1999; Ravi Kumar *et al.,* 2004). The gas samples (0.7 µl) were removed after 24 h incubation, and were assayed for ethylene production with a gas chromatograph. The chromatograph was fitted with Poropak N column and a H2-FID detector. The rate of nitrogen fixation was calculated and values were expressed as nanomoles of ethylene produced per hour per vial (nmoles C_2H_4 h^{-1} $vial^{-1}$) (Natheer *et al.,* 2012).

Qualitative estimation of P solubilization

Pikovskaya's medium was prepared by weighing following chemical Glucose10.0 gm. Tricalcium Phosphate 5.0 gm. Ammonium Sulphate 0.5 gm, Sodium Chloride 0.2 gm.

Magnesium sulphate 0.1 gm. Yeast extracts 0.5 gm. Ferrous Sulphate- Traces, Agar Traces Distilled water 1000 ml, pH.15.0. Sterilize at 10 lb pressure for 30 minutes Culture growth of *Azotobacter* was taken. Prepared 1liter Pikovskaya's medium and sterilized (by distributing the media in 5-6 small flasks). About 15-20 ml. medium is poured aseptically into sterilized petri plates. Inoculated *Azotobacter* (taken form 8-10days old slant). Inoculation is done in the middle of petri-dish in rounded manner and plate was kept in BOD incubator at 30◦C for 3-4days. The inoculated plates were incubated at 28°C.

The diameter of zone of clearance (halo) surrounding the bacterial colony as well as the diameter of colony were measured after 2, 4 and 7 days in triplicates. P_i solubilizing index (PSI) was calculated as the ratio of diameter of halo (mm)/ diameter of colony (mm) (Kumar and Narula, 1999). All the isolates were also assayed for phosphatase activity according to the method described by Malboobi *et al.,* (2009). The isolates were cultured on Pikovskaya medium in the

presence of soluble phosphate (KH_2PO_4) or insoluble phosphate (TCP) and incubated at 28°C. Phosphatase activities in isolates were compared based on scoring the intensities of blue stained colonies at 24, 48 and 72 hours.

Pot house experiment

Preparation of seed

Ten seeds/Pot of Lentil (ver Aruna) was taken for experiment. Surface sterilized the seeds with 95% Ethanol and 0.1% $Hgcl_2$ for 3 min followed by successive washings (at least ten times) with sterile distilled water to remove traces of $Hgcl_2$. Soaked the seeds in culture broth of seventy two hours old culture of all twelve strain of *Azotobacter* for about one hour. The number of cell was taken approx 10^7 cells /ml. drained off the excess culture broth from the seeds. Kept at least 3 replicates for each treatment.

Pot experiment

Materials required: Earthen pots, Sandy soil, Filter paper.

Procedure: Pot experiment was carried out in the year in the month of Oct-Nov (2008-09). Approximately 14 kg sandy soil having pH 8.0 was mixed with proper dose of N, P, K according to treatment. Seeds were surface sterilized with 95% ethanol and 0.1 % mercuric chloride. Seed was treated with 10 ml culture of the *Azotobacter* prepared from the all the treated plot and shown in the soil (Pots were taken in triplicate for each treatment).

The treatments (T1-T7) were placed in controlled environment and watering was done regularly. The length of root and stem as well as weight of root and stem was taken for further observation.

Results and Discussion

From (Table 1) in T2 combination (Soil enriched with both *Azosprillum* + *Pseudomonas*) population of *Azotobacter* ($5.48x10^4$) increased with respect to control ($1.3x10^4$). This number further increased ($29.0x10^4$) with the addition of nitrogen up to 50% as compared to Control (T4) and Soil enriched with chemical fertilizer ($15.5x10^4$) alone (T3) without any other inoculation with microorganism, the population of *Azotobacter* increased upto 100% nitrogen in presence of the other two microorganism (T6) but less than the treatment T4. As the nitrogen percent increased from 100-150%, population of *Azotobacter* decreased ($5.025x10^4$ to $3.0x10^4$) in T5 and T7 respectively. Highest population was observed in T4 which was approximately two times higher than the T3.

The population of *Azotobacter* again decreased in T5 due to higher availability of nitrogen. In T6 effect of bio nutrient consortium is clear from the data as increased population was obtained as compared to T5. Increasing the level of nitrogen has deteriorating effect on population of *Azotobacter*. Therefore conclusion can be drawn that the optimum level of nitrogen for maximum population of *Azotobacter* is 50% in combination with *Azosprillum* and *Pseudomonas*. When the nitrogen percent was increased (100%) *p*opulation of *Azotobacter* was decreased (in T6) as compare to T_4 treatment. There was a negative correlation between microbial numbers of *Azotobacter* and the content of N-fertilizer during the cultivation period. These results were in agreement with the findings of Ali *et al.*, (2002) who explained population of *Azotobacter* was associated with the presence of chemicals exuded by the plant roots and the extra presence of combined nitrogen. These probably explained the suppression of *Azotobacter* in the rhizosphere soil of Maize

treated with high levels of nitrogenous fertilizer. Inoculation with *Azosprillum* and *Pseudomonas* resulted in a considerable increase in the density of *Azotobacter* colonized in the rhizosphere region. Similar results were obtained by Mujiyati and Supriyadi (2001), Saad and Mostaffa (2009), Jha *et al.,* (2013) and Dutta *et al.,* (2014). Variation in morphology and biochemical characteristic has been observed in the strain of *Azotobacter* isolated from seven treated plot. A total of twelve strains of *Azotobacter* were identified from the representative seven samples. Based on morphological and biochemical studies (Table 2) some bacteria were Gram-negative bacilli, coccus, or oval shape bacteria.

All are aerobic in nature as growth was observed on the upper surface of the culture media. Colonies was white, transparent, viscous and moist which turn dark brown black or non-pigmented after 5-7 days of incubation on a mannitol N-free agar medium. Similar morphological and biochemical analysis was reported by Daddok *et al.,* (2014), Tejera *et al.,* (2005) and Lu and Huang (2010). Same type of strain was clubbed together for the convenience of experiment. They were listed in the table given below with their characteristic color for further experimentation viz. IAA production, Nitrogenase, Phosphatase, and Pot house experiment.

In general, the IAA production increased with increasing physiological precursor of L-Tryptophan by all strain.

It is clearly indicated that production of IAA by *Azotobacter* is maximum in plot T4 (21.8) as compared to performance of *Azotobacter* (6.9) with double inoculation (17.1) in plot T6 having 100% nitrogen. Further his may be due to production of any physiological factor that enhances the growth and population of *Azotobacter* with simultaneous increased efficiency at 50% nitrogen only.

Without nitrogen and excess of nitrogen may have deteriorating effect on the ability to produce IAA (Plot1 and T7). Similar study was done by Kanapiran and RamKumar (2011) they reported that joint inoculation of different PGPR bacteria have positive effect on IAA production.

Table.1 Enumeration of *Azotobacter* population in Maize rhizospheric soil having different Nutrient status

Representative soil Sample (Homogenous mixture of three replicates)	**Average Population(Three replicates) in cfu/g dried soil**
T_1	$1.3x10^4$
T_2	$5.48x10^4$
T_3	$15.5x10^4$
T_4	$29.0\ x10^4$
T_5	$5.025x10^4$
T_6	$19.5x10^4$
T7	$3.0x10^4$

Table.2 Morphological and biochemical characteristics of *Azotobacter species*

Strain	Gram reaction	Shape	Aerobic anaerobic	Pigment	Catalase test
A_1	-ve	Oval	Aerobic	Black	+ve
A_2	-ve	Coccus	Aerobic	White	+ve
A_3	-ve	Oval	Aerobic	Brown	+ve
A_4	-ve	Rod/Cocci	Aerobic	Black	+ve
A_5	-ve	Oval	Aerobic	Brown	+ve
A_6	-ve	Oval	Aerobic	Black	+ve
A_7	-ve	Coccus	Aerobic	White	+ve
A_8	-ve	Oval	Aerobic	White	+ve
A_9	-ve	Rod	Aerobic	White	+ve
A_{10}	-ve	Oval	Aerobic	Brown	+ve
A_{11}	-ve	Oval	Aerobic	Black	+ve
A_{12}	-ve	Oval	Aerobic	Black	+ve

Table.3 Description of strain derived from different plot

Plot	**Belonging strain**	**Color of strain**
T1	A_2	White
	A_9	White
T2	A_3	Brown
	A_7	White
T3	A_1	Black
	A_5	Black
T4	A_4	Black
	A_8	White
T5	A_6	Black
	A_{12}	Black
T6	A_{10}	Brown
T7	A11	Black

Table.4 IAA production by different strain

Plot	Strain	Production of IAA µgml^{-1} at different concentration of L-Tryptophan µgml^{-1}					
		10	20	40	60	80	100
T1	A_2	1.8	2.9	3.5	4.2	4.8	6.2
	A_9	1.5	2.1	2.3	3.4	4.2	5.5
T2	A_3	2.5	2.9	3.8	5.8	6.2	6.9
	A_7	2.3	2.7	2.8	5.5	6.1	6.7
T3	A_1	2.9	3.5	4.5	6.3	6.9	7.9
	A_5	2.7	3.2	3.9	6.1	6.3	6.7
T4	A_4	6.7	15.9	17.5	18.2	21.1	21.8
	A_8	5.9	6.5	11.1	15.5	18.1	19.8
T5	A_6	2.7	3.2	3.3	5.9	6.2	6.6
	A_{12}	3.2	4.4	4.6	5.9	6.9	8.5
T6	A_{10}	6.6	12.8	14.2	15.6	16.5	17.1
T7	A_{11}	2.6	3.1	3.8	4.8	5.1	5.8

Table.5 Nitrogenase activity in different selected strain with mean of triplicates followed by standard deviation

S. No.	Strain	Nitrogenase activity ($nmolC_2H_4\ h^{-1}$)
T1	A_2	132.7±3.62
	A_9	128.2±3.56
T2	A_3	151.7±4.24
	A_7	142.7±4.68
T3	A_1	87.2± 2.61
	A_5	78.6±3.12
T4	A_4	91.1±3.65
	A_8	98.1 ±2.84
T5	A_6	51.3±4.6
	A_{12}	48.6±4.61
T6	**A_{10}**	68.2.±3.71
T7	**A_{11}**	42.1±4.71

Table.6 Phosphatase activity in different isolates (variation ±1)

Sl no	Strain	PSI (Pi Solubilizing index) (Days) diameter in (mm)			Phosphatase Activity (hr)					
					With soluble Pi (hr)			With insoluble Pi(hr)		
		2	4	8	24	48	72	24	48	72
T1	A_2	1.4	1.5	1.8	+	+	+	+	+	+
	A_9	0	1.1	1.3	+	+	+	+	+	+
T2	A_3	1.6	1.7	2.8	+	+	+	+	+	+
	A_7	1.7	2.3	2.5	++	++	++	++	++	++
T3	A_1	1.9	2.5	3.0	+	+	+	+	+	+
	A_5	1.9	2.7	2.9	++	++	++	++	++	++
T4	A_4	3.1	3.9	4.5	+++	+++	+++	+++	+++	+++
	A_8	2.9	3.5	4.1	+++	+++	+++	+++	+++	+++
T5	A_6	0	1.2	2.3	-	-	-	-	_	-
	A_{12}	1.2	1.4	2.6	+	+	+	+	+	+
T6	**A_{10}**	2.6	2.8	3.2	+	+	+	+	+	+
T7	**A_{11}**	1.6	2.5	2.8	-	-	-	-	-	-

Table.7 Result of pot house experiment by application of different treatment on Lentil (Var Aruna)

Plot	Sample/Strain	Root length(cm)	Shoot length(cm)	Root wt(g)	Shoot wt(g)
		10.1	10.5	2.5	2.65
T1	A_2				
	A_9	10.5	10.2	2.1	2.22
T2	A_3	13.1	16.1	3.3	4.1
	A_7	12.9	15.9	2.9	4.2
T3	A_1	13.5	16.4	3.6	4.02
	A_5	13.3	16.3	3.7	4.01.
T4	A_4	14.5	17.2	3.9	4.25
	A_8	13.9	16.9	3.8	4.23
T5	A_6	15.0	18.5	3.5	4.5
	A_{12}	15.5	18.3	4.5	4.4
T6	A_{10}	17.1	20.5	3.3	5.5
T7	A_{11}	12.8	15.5	7.0	3.7

A very different trend of nitrogenase activity was observed with the increased use of nitrogen (Table 5). In (T2) maximum nitrogenase activity (151.7±4.24) was noticed due to stimulatory and additive action of two bacteria viz., *Azosprillum* + *Pseudomonas* on *Azotobacter* activity. Lowest value of (42.1±4.71) was observed in the case of (T7). In T1 intermediate value was noticed because no nitrogen is applied. So it can be seen that as soon as these two bacteria were mixed with nitrogen (50 and 100%) decrease in nitrogenase activity was notice (T4 and T6) with the increasing dose irrespective of the presence of these two bacteria. This can be justified with the fact because these all bacteria are diazotrophic so presence of nitrogen has negative effect on the synthesis of nitrogenase enzyme. Yin *et al.,* (2015) also investigated that different levels of N supplied to *Herbaspirillum seropedicae* (Z78) have significant effects on nitrogenase activity and auxin production. The highest nitrogenase activity and the lowest auxin production of *H. seropedicae* (Z78) were both recorded at 0 gL^{-1} of NH_4Cl. Higher levels of external N caused a significant decrease in the nitrogenase activity and an increased production of auxins. Decreased nitrogenase enzyme activity of *A. diazotrophicus* due to high concentrations of NH_4Cl and ammonium nitrate (NH_4NO_3) has been also recorded by (Muthukumarasamy *et al.,* 2002). Excess of NH_4^+ causes a reduction in the number of diazotrophs and a decrease in BNF activity also notice by (Rivera *et al.,* 1991). The justification may be that the BNF involves symbiotic or associative relationships between the diazotroph and the host plant, as the diazotrophic nitrogenase enzyme (encoded by the diazotroph *nifHDK* genes) catalyses the reduction of gaseous nitrogen (N_2) to NH_3 (de Campos *et al.,* 2006). However, the process is suppressed in many bacterial species when an ample or excess supply of fixed N is available. In the presence of excess fixed N, the diazotrophic nitrogenase enzyme is no longer active, either due to down-regulation of protein synthesis and/or inactivation of the protein (Rudnick *et al.,* 1997).

Phosphate can be solublized by *Azotobacter* species also. That can be estimated by phosphorous clearing zone appeared on Pikovskaya plate. Phosphate solubilization was measured in all isolates; as shown in Table 6, all twelve isolates were able to produce clear zone or blue color in minimal medium containing insoluble P_i or synthetic substrate. The largest clear zone was observed for A_4 (4.5 mm) followed by A_8 (4.1 mm) followed by A_{10} (3.2) after 8 days. Whereas A_2, A_9 isolate shows small zone. T2 plot shows increased diameter of hallo production due to microbial inoculation and plot T4 shows maximum efficiency due to *Azotobacter* along with nitrogen application and the two micro flora (*Azosprillum* + *Pseudomonas).* Phosphatase activity was not detected in T5 and T7 due to heavy dose of nitrogen which have deteriorating effect on the growth of microorganism. Vazquez *et al.,* 2000 studied the Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants and found positive impact on the each other. Enzyme activity was found to increase as compared to uninoculated one. Maman *et al.,* (2013) studied the use of bioinoculant formula as certain microbial group used as a stimulator of plant growth viz. *Azosprillum*, *Azotobacter*, *Rhizobium*, *Lactobacillus* etc. and found the decreased doses of nitrogen and phosphorus due to increased mobilization of phosphate and nitrogen to the plants.

The increased uses of nitrogen have positive correlation with root, shoot length Root

weight and Shoot weight (Table 7). But the Combined effect of microbes (T4) with 50% nitrogen gave almost same result as 100% and 150% nitrogen (T5 and T7). So the use of excess nitrogen is not necessary for the proper wt and length of root and shoot. This effect can curtail the use of chemical fertilizer. Similar experiment was done by Akthar *et al.*, (2012). They used Microbial combination of *Azotobacter* and *Rhizobia* with different doses of nitrogen level and noted that the co inoculation of *Azotobacter* with *Rhizobia* with 50% of nitrogen gave the same result of Shoot and Root length and weight as that of 100% nitrogen. These results were in agreement with those reported in some previous studies (e.g. Akhter *et al.,* 2004; Rothballer *et al.*, 2005). They reported that the stimulatory effect of bacterial inoculation is probably due to the bacterial production of growth-promoting substrates such as nitrogen, phosphorus, nitrite and indole-3-acetic acid in the plant rhizosphere.

The same effect was notice by Kuan *et al.*, (2016) and reported decreased use of nitrogen for growth of plants when microbial inoculation is done. Rajaramamohan *et al.*, (1987) reported bacterial inoculation improves plant growth and rice yield but not uniformly. The yield response to inoculation is more pronounced in the presence of moderate levels of fertilizer N.

So improvement on yield and yield attributes were noted by *Azotobacter* species in combination with fifty percent nitrogen along with other two PGPR namely *Azosprillum* + *Pseudomonas* due to their positive interaction, nutritional, stimulatory and therapeutic abilities that includes availability of nitrogen and phosphorus to plants and promoting other growth attributing factors like IAA production etc. Initiation of seed emergence by producing plant growth promoting substances like IAA and its antagonistic approach to plant pathogens respectively also benefited seed in one or in another way.

Acknowledgement

Support from Dr. M.N. Jha PI (AINP), Department of Microbiology, Dr. CRAU, Pusa, Samastipur is gratefully acknowledged.

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How to cite this article:

Sonia Kumari, Leelawati, Rajni Kant and Upendra Singh. 2017. Effect of Mixed Bioinocula on Growth and Efficiency of *Azotobacter* Species. *Int.J.Curr.Microbiol.App.Sci.* 6(10): 1-13.
doi: https://doi.org/10.20546/ijcmas.2017.610.001