International Journal of Advanced Multidisciplinary Research (IJAMR)
ISSN: 2393-8870
www.ijarm.com

Research Articles

Effect of *Azotobacter* sp. For the improvement of growth and yield of Pearl Millet (Cumbu) (*Pennisetum glaucum* L.)

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Keywords

Azotobacter, Agriculture, Pearl millet, IAA, PHP and RDF.

Abstract

Agriculture is heavily dependent on the use of chemical fertilizers and pesticides to achieve higher yields. This dependence is associated with problems such as environmental pollution, health hazards, interruption of natural ecological nutrient cycling and destruction of biological communities that otherwise support crop production. To overcome this problem, biofertilizer is the best source to replace the chemical fertilizer. In this study, five locations were selected in Cuddalore district, Tamil Nadu, India to isolate the *Azotobacter* and designated as AZ-1 to AZ-5. This *Azotobacter* isolates has capacity to produce growth hormones facilitate to enhance the growth of Pearl millet is a major source of cereals in human diet. In Tamil Nadu, farmers plant pearl millet early and they do not have good yield. In this region, there is no clear optimum planting date for this crop. Based on production of IAA and PHP efficient strains selected and treated with pearl millet among the five treatments the plant growth recorded most excellent results in T_5 (100% RDF + *Azotobacter* sp.).

Introduction

Pearl millet (*Pennisetum glaucum* L.) is a drought - tolerant cereal grain typically grown as grain crop in India. All pearl millet production is used for a variety of food products. Pearl millet can potentially be planted as a double crop after winter wheat or barley in southern areas. It is planted in early summer when soils have warmed up. Many autochthonous pearl millet ecotypes have generated interest in millet as a substitute for sorghum because of its ability to reliably produce grain on sandy and low fertility soils of the south of India. Plant breeding efforts to develop pearl millet as summer annual forage crop to replace forage sorghum for low water-holding soils and short growing season areas of the Great Plains will take place in the future.

Azotobacter synthesizes and secretes considerable amounts of biologically active substance like B vitamin, nicotinic acid, pentothenic acid, biotin, heteroauxins, and gibberellins etc., which enhance root growth of plants. Other important characteristics of *Azotobacter* association with the presence of root exudates, which helps in modification of nutrient uptake by the plants (Narula and Guptha, 1986). *Azotobacter* has the ability to produce antifungal antibiotics and fungi static compounds against pathogens like *Fusarium*, *Alterneria* and *Trichoderma*. All these factors combined together produce positive effects on crop yield. The use of *Azotobacter* offers an attractive way to replace chemical fertilizer, pesticides, and supplements; most of the isolates result in a significant increase in plant height, root length, and dry matter production of shoot and root of plants (Sivasakthivelan and Saranraj, 2013; Kanchana *et al*., 2013; Usharani *et al*., 2014).

Materials and Methods

Details of Location

The survey was conducted at five locations in Cuddalore district of Tamil Nadu comprising Annamalai Nagar, Bhuvanagiri, Neyveli, Sivayam and Mangalam.

Collection of Pearl millet rhizosphere soil from different location

In each and every location of the survey area a field which has been under long behind mono culture practice was selected. The locations of rhizosphere soil sample were made at

different location of pearl millet field. The collected soil samples were brought to laboratory for further analysis.

Enumeration of *Azotobacter* population from rhizosphere of Pearl millet

The adhered soil of Pearl millet roots, collected from five Pearl millet plants of a particular location, were pooled and one gram of soil sample was transferred to 100 ml of sterile distilled water in a 250 ml Erlenmeyer flask and incubated on a rotator shaker (100 rpm) for 30 minutes at ambient temperature. The well mixed suspension was then diluted appropriately upto 10^{-6} dilution. One ml of suspension from 10^{-5} and 10^{-6} dilution was aseptically transferred to sterile petriplates and 10 – 20 ml of selective WB-77 medium was added and incubated at 28ºC for 48 hrs. Three replications were maintained for each dilution. The colonies were counted by using colony counter. The total number of colonies in the original samples was expressed as cfu g^{-1} of oven dry soil.

Isolation of *Azotobacter* from rhizosphere of Pearl millet

Ten g of air dried pearl millet rhizosphere soil sample was transferred to 100 ml of sterile distilled water in a 250 ml Erlenmeyer flask and incubated on a rotary shaker (100 rpm) for 30 minutes at ambient temperature. The well mixed suspension was diluted appropriately upto 10^{-6} dilution. One ml of the suspension from 10^{-5} and 10^{-6} dilution was aseptically transferred to sterile petriplates and 15 – 20 ml of selective WB-77 medium was added to each petriplates mixed well with agar by rotating at clockwise and anticlockwise and allowed to set. Then, the plates were incubated at 28±2ºC for 48 hrs. After the incubation period, the colonies were developed and transferred to WB-77 agar slants and maintained at 4ºC for further study.

Purification of *Azotobacter* isolates and designation of *Azotobacter* isolates

All the five *Azotobacter* isolates were purified by streak plate method using WB-77 medium frequently. The *Azotobacter* isolates, obtained from the rhizosphere of pearl millet grown at five different location of Cuddalore district, were designated as AZ and numbered randomly.

Characterization of A*zotobacter* isolates

All the five *Azotobacter* isolates, obtained from rhizosphere of Pearl millet grown at Cuddalore district were characterized based on LOPAT test.

Screening of *Azotobacter* isolates for plant growth promoting characteristics

The plant growth promoting characteristics *viz*., IAA production and ARA were examined with five *Azotobacter* isolates which were obtained from the rhizosphere of pearl millet with a view to screen them on the basis of their plant growth promoting efficiency. The preparation of inoculums for conducting these studies was a stated below under otherwise were maintained for each study.

Preparation of Inoculum

All the five *Azotobacter* isolates were grown in Waksman's base medium - 77 in a shaking bath at 28±2ºC for 24 hrs. Then, the medium was centrifuged at 5000 ×g for 10 minutes to harvest the log phase cells and the pellets washed three times with 0.1 M Phosphate buffer (pH 6.8). Finally, the cells were suspended in the same buffer to a cell concentration of 1 × 10^{10} cfu ml^{-1} by measuring the absorbance at 420 nm and used as inoculums.

Extraction and estimation of IAA from the medium

After determining the growth, the medium was centrifuged at 700 ×g for 30 minutes and the supernatant was reduced to 50 ml volume by flash evaporation under vacuum and IAA extracted into ethyl acetate and n-butanol by Tien *et al*. (1976). One ml of Methanol residue was taken in a test tube, 4 ml of Salper's reagent and 1.5 ml of distilled water were added. The reagent was added in a drop wise manner but rapidly with continuous agitation. The sample was incubated in dark for one hour and the absorbance of pink colour was measured at 535 nm against a solvent reagent blank. The quantity of IAA was estimated from a standard curve draw from known concentration of IAA (1Div-0.307 µg of IAA).

Determination of the di-nitrogen fixing ability of *Azotobacter* isolates (*in vitro*) under free living condition (Acetylene Reduction Activity - ARA)

Waksman's base medium-77 was prepared as detailed earlier, dispersed in 30 ml quantities in 100 ml vials and sterilized by autoclaving 0.5 ml log phase culture of each *Azotobacter* isolates were added to each vials individually and incubated for 48 hrs at 32 - 35ºC. Then, the cotton plugs were replaced with acetylene puncture rubber stopper, aseptically. Using a syringe, 10 per cent volume of the vial was replaced with acetylene gas and culture was also injected with acetylene gas. After the inoculation period, 0.5 ml of the gas sample was withdrawn from the vial and feel into a gas chromatography with following features

Gas chromatography : Chemicals, 3800 model,
Column : Porapah T (80-100)
3×3.1mm stainless steel.
Detector : Flame ionization detector
Carrier gas : Nitrogen, 40ml/min
Temperature
Injection part : 110ºC
Detector : 110ºC

Calculation
Peak height (in mm) of C_2H_4 (='a' mm) in injection volume,
Peak height (in mm) in one (='b' mm) in injection volume,
Nanomoles of C_2H_4 corresponding= 'C' n moles to 'b' mm peak height from standard curve,
Volume of the vials ='d' ml
Volume of medium 'b' ml = d – e = F ml
Volume of gas phase in vial of C_2H_4/vial = F × C for 12 hours incubation calculate the protein content of = mg of protein/'e' ml *Azotobacter* vial n mole of C_2H_4/mg protein / hr = F × C × 60/12 × x.

Screening the *Azotobacter* isolates for Polyhydroxy butyrate (PHB) production

PHB is the alternative source of plastic which has similar physical properties like polypropylene and it can be easily bioderdable aerobically and anaerobically. Bacterial cell mass was collected by centrifugation and the dry weight was measured. Lipids were removed by adding methanol (40 times the volume of the collected cells) and incubated at 95ºC for an hour. Cells were recovered by vacuum filtration. Chloroform (50 times the dry cells weight) was added and incubated at 95ºC for 10 minutes. It was cooled to room temperature and the solution was mixed over night by stirring. The solution was filtered to remove cell debris. PHB was precipitated by adding a mixture of methanol and water (7:3v/v) at the rate of 5 volume of chloroform to the filtrate. The precipitated PHB was washed with acetone and dried.

Effect of *Azotobacter* sp. on the growth and yield of Pearl millet (Cumbu)

The pot culture experiment was conducted to study the effect of *Azotobacter* sp. on the growth and yield of Pearl millet (Cumbu). The study was conducted at Department of Microbiology, Annamalai University, Annamalai Nagar. The soil used in the pot culture experiment was clay loamy in nature. The experiment was arranged in Randomized Block Design (RBD) with three replications. For sowing in inoculated pots, Pearl millet (Cumbu) seeds were soaked with trehalose at 15 mM, polyvinyl pyrollidone (PVP) at 2% and glycerol for 30 min in different formulations (20 ml/kg of seeds), (Spacing, 15 cm × 10 cm: 3 seedlings/hill and 12 seedlings/pot). Gap filling was done after 10 DAS. The crop was given hand weeding on 30th DAS and well protected against pests and diseases. A water level of 5 cm depth was maintained through the crop period. Five representative samples of plant in each pot were peg marked for periodical observation.

Treatment schedule
Design-RBD
Replications-3
T_1 – Control
T_2 – 100% RDF
T_3 – 25% RDF + *Azotobacter* sp.
T_4 – 50% RDF + *Azotobacter* sp
T_5 - 100% RDF + *Azotobacter* sp.
(The growth and yield parameters will be analyzed after harvesting)

For each treatment, three replications were chosen for measuring and recording the biometric observations of plants and were recorded at periodic intervals *viz.*, 20th, 40th and 60th days after sowing (DAS).

Results and Discussion

The occurrence of *Azotobacter* population in the rhizosphere of Pearl millet variety Prabanikaranthi grown at five selected locations were designated as AZ-1 to AZ-5 respectively and numbered randomly. The total bacterial population, *Azotobacter* population and percentage of *Azotobacter* population were estimated and above selected pearl millet variety Prabanikaranti and results are presented in the Table – 1 and Table - 2. The location, namely Sivayam recorded maximum of 1.18 per cent as community population of *Azotobacter*, from Mangalam recorded least population of 0.40 per cent as community population in the rhizosphere. All other locations recorded the community population of *Azotobacter*. The occurrence and activities of *Azotobacter* in the rhizosphere have already reported by many researchers (Saranraj *et al*., 2013; Sivasakthi *et al*., 2013; Kanchana *et al*., 2013; Usharani *et al*., 2013; Sivasakthi *et al*., 2014; Usharani *et al*., 2014).

Five strains of *Azotobacter* were isolates from various area from Cuddalore District as described in "Materials and Methods". They were designated as "AZ" series and numbered randomly. The details of designation of the isolates their rise of collection are presents in Table – 2.

All the five isolates of *Azotobacter* were screened for their nitrogen fixing capacity, PHB production and plant growth promoting characteristics namely production of IAA and results are presented in Table – 3. The results of the present study revealed that the ability of all five *Azotobacter* isolates to produce PHB and IAA. The maximum production of PHB (0.92 g/l) and IAA (3.4 µg/ml) were recorded in AZ-4 isolate when compare to other isolates.

Azotobacter produces substances which have been like 3-indole acetic acid (IAA) and Gibberrelic acid in bioassay. Bacteria that colonize roots effectively are termed as rhizobacteria (Scroth Hncock, 1982). *Azotobacter* produces substances which have been like 3-indole acetic acid (IAA) and Gibberrelic acid (GA_3) in bioassay. The highest concentrations of IAA like and GA_3 like substances detected in culture media have been 11 µg/ml. Glick *et al*. (1995) has

Table – 1: Occurrence and community population of *Azotobacter* from the rhizosphere of Bhendi grown at Cuddalore district

S. No	Location for sample collection	Rhizosphere population (log cfu g^{-1})		
		Total Bacterial population	*Azotobacter* Population	% of *Azotobacter* population
1	Annamalai nagar	7.65	6.63	0.53
2	Bhuvanagiri	7.55	5.59	0.64
3	Neyveli	7.36	6.74	0.61
4	Sivayam	7.71	6.83	0.41
5	Mangalam	7.21	6.42	0.40

Table – 2: Designation of *Azotobacter* isolates

S. No	Location	*Azotobacter* Designation
1.	Annamalai Nagar	AZ-1
2.	Bhuvanagiri	AZ-2
3.	Neyveli	AZ-3
4.	Sivayam	AZ-4
5.	Mangalam	AZ-5

Table – 3: Screening the *Azotobacter* isolates for plants growth promoting characteristics and PHB production

Isolates	IAA production (µg ml^{-1})	Acetylene Reduction Assay (n moles of C_2H_4/mg of protein/hr)	PHB (g/l)
Az-1	3.2	250.6	0.58
Az-2	2.0	220.7	0.78
Az-3	2.2	200.9	0.41
Az-4	3.5	202.3	0.36
Az-5	1.8	210.4	0.54

Table - 4: Effect of *Azotobacter* sp. on the growth parameters of Pearl millet

Treatments	Plant height (cm)	Leaf area index	Dry matter production (t ha^{-1})	Number of branches $plant^{-1}$	Number of pods $plant^{-1}$
T_1 – Control	26.34	2.85	1.5	7.60	20.16
T_2 – 100% RDF	34.12	3.30	2.5	7.95	27.34
T_3 – 25% RDF + *Azotobacter* sp.	30.77	2.98	2.1	7.65	22.88
T_4 - 50% RDF + *Azotobacter* sp.	32.17	3.15	2.2	7.70	25.50
T_5 - 75% RDF + *Azotobacter* sp.	34.85	3.53	2.7	8.05	27.80

been reported more secretion of IAA takes place at shaking condition.

Pearl millet (*Pennisetum glaucum*) is the most widely grown type of millet and one of the major field crops in Yemen. In 1999, the total cultivated area of millet is 649551 hectare and the total production was 464240 tons. The crop is principally produced for both human and animals. Nitrogen is one of the most important nutrients for the growth and yield of several crop. Nitrogen is the most limiting nutrient for cereal crops production. Also, it is an essential component of structural amino acid, amides, nucleotides, nucleoproteins and is essential to cell division, expansion and nonstructural components of plant cells. Ammonium and urea in soils are the two main sources of available N for plant growth. Although, other nitrogen sources such as fertilizer applications have noticeable effects on growth of *Pennisetum glaucum* plants. The effect of *Azotobacter* sp. on the growth and yield of pearl millet was investigated and the results were furnished in Table – 4. Maximum growth and yield parameters were observed in the treatment T_5 - 75% RDF + *Azotobacter* sp. followed by the treatment T_2 - 100% RDF. The treatment T_5 was on par with the treatment T_2. Minimum growth and yield parameters were recorded in the control treatment T_1.

Conclusion

In this present study, *Azotobacter* enhanced the growth of Pearl millet by production of IAA and PHP. This study recorded 75% of recommended dose of fertilizer and *Azotobacter* combination (T_5) recorded maximum plant height (34.85 cm), Leaf area index (3.53), Dry matter production (2.7 t ha^{-1}), Number of branches (8.05 $plant^{1}$), Number of pods (27.80 $plant^{-1}$).

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