IJBPAS, December, 2013, 2(12): 2300-2311

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

SCREENING OF *AZOSPIRILLUM* FOR ENHANCED INDOLE ACETIC ACID PRODUCTION AND EXOPOLYSACCHARIDE FOR MAXIMIZING ITS SURVIVAL RATES IN DROUGHT PRONE PADDY FIELDS

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ABSTRACT

In the present study, the soil samples were collected from rhizospere region of 5 different paddy fields in Dharmapuri district and 13 isolates of *Azospirillum* were obtained. Morphological characteristics of isolates viz, shape, size, elevation, surface form, margins, surface texture and colour were observed for their characterization.

Among 13 isolates 5 were selected for further study based on the formation of subsurface pellicle and bacterial size in micrometry. These 5 isolates were screened for increased indole acetic acid and exopolysaccharide production. For IAA production, the isolates were inoculated on the respective medium with tryptophan or without tryptophan and incubated at 30-32°C temperature for 15 days. Development of pink colour indicated IAA production. Estimation of exopolysaccharide was done by Phenol Sulfuric method.

The soil parameters such as moisture and pH were evaluated. Gravimetric method of moisture estimation was done. Two isolates which had increased indole acetic acid and exopolysaccharide were selected for mass cultivation and seed dressing. Pot experiments were conducted with these 2 isolates and commercially available *Azospirillum* biofertilizer. Plant growth parameters were monitored under drought prone condition. This study reveals that increased indole acetic acid

and exopolysaccharide production by *Azospirillum* improves its survival rates under drought prone conditions.

Keywords: *Azospirillum* Indole Acetic Acid, Exopolysaccharide, Tryptophan

INTRODUCTION

Nitrogen is an essential plant nutrient; unfortunately, it is usually not present in soil at concentrations sufficient for agricultural production of commercial crops. It therefore must be provided to the crops in the form of fertilizer for the commercially important crops, such as cereals, corn, wheat, rice and barley. Biological nitrogen fixation is one of the most important processes in the natural environment. It is the major pathway for the reduction of dinitrogen molecules from air to give ammonia and subsequently glutamine and other nitrogen containing molecules.

Bacteria of the genus *Azospirillum* exert beneficial effects on plant growth and yield of many crops of a gronomic importance. *Azospirillum* is an efficient nitrogen fixing bacteria. *Azospirillum*species are described as gram negative, rod shaped, 1mm in diameter, highly motile. It utilizes glucose, lactate, succinate, fructose, malate, pyruvate, fumarate as carbon source, reduced nitrate and does notrequire biotin. *Azospirillum* species are highly adaptable, being able to grow under anaerobic conditions (nitrate used as electron acceptor) microaerobic (elemental or ammonia used as N source) fully aerobic conditions (ammonia, nitrate, aminoacid or combined N only).It also produces growth promoting substances like IndoleAcetic Acid (IAA), gibertellins and promotes root proliferation. It increases the rootlet density and root branching resulting in the increased uptake of mineral and water.

Azospirillum results in the following benefits **[1]**: a) Promotion of root hair development and branching b). increased uptake of N, P, K and microelements. c) improved water status of plant and d)increased dry matter accumulation and grain yield. Most species of the genus *Azospirillum* are known to act as Plant Growth- Promoting Rhizobacteria (PGPR) and stimulate plant growth directly either by synthesizing phytohormones or by promoting improved N nutrition through BNF **[2]** PGBR also produce several other growth promoting substances including IAA, moisture on wheat and maize seedlings grown under water stress conditions **[3]**. *Azosirillum* species isolated from moisture stressed conditions can GA3, zeatin and ABA **[4]** *Azospirillum* inoculation alleviates low soil improve tolerance of water stress, and thus they can be inoculated to promote plant

growth on stressed sites, such as semiarid and arid regions **[5]**. Salinity is a form of water related stress; crop loses, particularly in semiarid and irrigated agriculture. High salinity in soils results from naturally high salt levels or from local salt accumulation due to irrigation or the application of chemical fertilizers. Usually sulphates, chlorides and biocarbonates of Na +, K + Mg2 + and Ca2 + contributes to the salinity of the soil. Increase of elevated salinity osmolarity although water may be present in the surrounding of the cell it is not available to the cell and this hence creates osmotic stress. Living cells respond to osmotic salinity stress by modulating their cytoplasmic osmolality. The compounds which can be accumulated to high intracellular concentrations with minimal damage to cellular metabolism or enzyme function **[6]**. Osmoregulation mechanism adjust the compatible solute levels by modulating their uptake, biosynthesis, catabolism or efflux **[7]**. The identification of *Azospirillum* genes involved in plant interactions has relied on the homology between *Azospirillum* genes and plant interaction genes from other plant-associated bacteria. Hybridization probes have come from a variety of rhizosphere bacteria including: *Rhizobium* **[8]**.

Azospirillum halopraeferens which tolerates high salinity colonizes mangrove roots in sea water andenhances the growth of halophytes irrigated with sea water. Most strains of *Azospirillum* speciescould tolerate only limited levels of salt (NaCl). *A. brasilense* could tolerate 2% NaCl when co cultured with *Staphylococcus* species. The molecular understanding of azide mutagenesis has progressed significantly with the discovery ofthe metabolism of the azide and the identification of the product as azidoalanine. A discovery of the metabolism of the Azide is, a well known inhibitor of the terminal segment of the electron transport chain, but has also been repeated to have several effects on the growth of bacterial cells. In addition azide-resistant mutant cells are often defective in their control of cell division. Most genetics studies of*Azospirillum* have focused on *A. brasilense* Sp7. Initially, genetic research focused on nitrogen fixation, reflecting belief that this was the main mechanism for plant growth promotion by *Azospirillum* Later it became apparent that pathogens, symbionts, and the plant growth promoting rizobacteria share with *Azospirillum* spp. With regard to recognition of host plant and affinity phenomena, hormoneproduction, and root morphological modifications. Therefore, some of the bacterial genesinvolved in such

interaction may be similar **[9]**. Thus, one of the strategies for identification of *Azospirillum* genes involved in plant interaction has involved comparison of the *Azospirillum* genome with the genome of other bacteria associated with plants.

All strains of *A. brasilense* and *A. lipoferum* examined possess plasmids. The two species have 90-MDa plasmids (p90), that share conserved regions and carry several genes involved in the *A.brasilense* plant root interaction **[10]** in addition to *A. brasilense*Sp7 contain three other large plasmids with molecular masses greater than 300 MDa, *A. lipoferum* plasmid pi 15 is frequently lost form *A.brasilense* mutagenesis. Some strains contain a 150-MDaplasmid not present in *A.brasilense* strains. These mega plasmids are present in *Azospirillum*s trains at a rate of one copy per cell **[11]**.

In the present study the screening of *Azospirillum* for enhanced indole acetic acid production and exopolysaccharide production to enhance its survival rates in drought prone paddy fields.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples were collected from the rhizosphere region of five different paddy fields of Dharmapuri, in the regions of Karimangalam, Toppur, Makkanur, Pennagaram. Samples were collected in polythene bags from the selected sites at a depth of 10-15 cm. The samples were then immediately transported to lab for further processing.

Preparation of Medium

*Azospirillum*isolation agar was found to be the selective medium for the isolation of *Azospirillum*.

Composition of Medium

Part A (950 ml)

Malic acid: 5g; Dipotassium phosphate: 0.50g; Ferrous sulphate: 0.50g; Manganese sulphate: 0.01g: Magnesium sulphate: 0.20g: Sodium sulphate: 0.10g; Bromothymol blue: 0.002g; Sodium molybdate: 0.002g; Calcium chloride: 0.02g; Agar: 1.75g; Distilled water: 1000ml; pH: 6.8 + 0.2

PART B (50ml)

Potassium hydroxide: 4g

Basal Minimal Salt Agar

Composition of medium

Agar: 20.0g; L-Malic acid: 2.5 g; Sucrose: 2.5 g; Potassium hydroxide: 2.0 g; Potato Extract Solution: 950 ml; Bromothymol: 1.0 ml; Vitamin solution: 1.0 ml; Distilled water: 1000 ml; pH-7.0 ± 0.2

Isolation of *Azospirillum* [12]

Azospirillum was isolated from each sample by serial dilution and spread plate method. One gram (1g) of soil sample was mixed with 9 ml of autoclaved distilled water and was thoroughly mixed.From the major dilution 1 ml of the above solutions was transferred to 9 ml of sterile distilled water to obtain 10^{-2} dilution. Similarly 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} serial dilutions were made for each soil sample. After serial dilution0.1ml of each dilution was spread on *Azospirillum* isolation agar and incubated at room temperature for 72-80 hours. Colonies based on color, texture, elevation size and shape were picked for various biochemical characterization.

Subsurface Pellicle Formation [13]

The loopful of culture was taken and inoculated on to semi solid nitrogen free basal medium.After inoculation it was incubated at 32°C for 48 hours and checked for sub surface pellicle formation.

Characterization of the Isolates

The isolates were characterized as per the biochemical characterization and the results were recorded.

Indole Acetic Acid Production (IAA) [14] and Extraction of Crude Exopolysaccharide (EPS) [15]

The isolates were inoculated on the respective medium with tryptophan (1, 2 & 5 mg/ml) or without tryptophan incubated at 30-32°C temperature for 15 days.After incubation cultures the cultures were divided into two parts. One part of the culture was centrifuged at 3000 rpm for 30 min. After centrifugation supernatant (2ml) was mixed with 4 ml of Solawaski's reagent (50ml, 35% Perchloric acid; 1ml 0.5% $FeCl_3$).Development of pink colour indicates IAA production. O.D was read at 530nm using UV spectrophotometer. The amount of IAA produced by the isolates was estimated by a standard IAA graph.

The other part of the culture was then centrifuged at 10,000 rpm for 20 min at 4°C.EPS were precipitated with three volumes of chilled 95% ethanol and standing overnight at 4°C. Then the precipitate,was collected by centrifugation at 9,000 rpm for 20 min at 4°C.The resulting precipitate was dissolved in 15 ml of Tricholoroacetic acid (TCA) and the solution was again centrifuged at 9,000 rpm for 15 min. at 4°C.The total sugar content of the EPS suspensions was estimated by the phenol/ sulfuric method.

Tests for Soil Parameters

a) **Soil Moisture test (Gravimetric Method)**

   Gravimetric method of moisture estimation is most widely used where the soil sample is placed in an oven at 105 °C and dried to a constant weight.

The difference in weight is considered to be water present in the soil sample.

Calculation:

$$\text{Moisture (\%)} = \frac{\text{Loss in weight}}{\text{Weight of tray - weight of soil}} \times 100$$

The corresponding moisture correction factor (mcf) for analytical results or the multiplication factor for the amount of sample to be weighed in for analysis is:

$$\text{mcf} = \frac{100 + \%\text{moisture}}{100}$$

b) Soil pH [16]

The soil pH is the negative logarithm of the active hydrogen ion (H+) concentration in the soil solution. It is the measure of soil, acidity or neutrality. It is very important estimation for soil, since soil pH influences to a great extent the availability of nutrients to crops.

Cultivation of Paddy Using Drought Soil

a) Mass cultivation of Azospirillum

Azospirillum was transferred to a flask containing 1000ml of sterile *Azospirillum* isolation broth. The flask was incubated on a rotary shaker at 32°C for 72-80 hours.

b) Seed Dressing

The seeds of paddy were bought from Tamilnadu Horticulture Department, Chennai. The seeds were surface sterillzed with 0.1% mercuric chloride solution for one minute. Then, it was washed with sterile distilled water several times and then the seeds were aseptically transferred to a flask containing *Azospirillum* and the flask was again incubated on a rotary shaker for 4 hours. After the incubation the seeds were dried in shade at room temperature.

c) Pot Cultivation

Three experimental set up for isolate 7 and isolate 9 were maintained individually. After dryingseeds were sowed in 3 sets of pots in drought prone soil conditions and maintained in duplicates.

Group 1- *Azospirillum* (isolate) seed dressed.

Group 2- only seed (no bacteria)

Group 3- *Azospirillum* (commercially available) biofertilizers seed dressed.

Study of Plant Growth Parameters

Assessment of Number of Leaves, Root and Shoot Length

For assessment of number of leaves, root and shoot length, saplings were randomly up rooted on 15th, 30th and 45th days of growth from inoculated and control pots and washed with running tap water and the results were tabulated.

RESULTS AND DISCUSSION

Azospirillum spp are commonly used free living plant growth promoting rhizobacteria capable of affecting the yield of numerous plant spp of agronomic importance. This study aims at promoting the survival capacity of the bacterium in drought prone conditions, with increased indole aceticacid and exopolysaccharide. Dharmapuri district was selected as sample site and divided into geographical planes. Fields from karrimangalam, toppur, makkanur, pennagaram, and dharmapuri was selected for sample collection.

All samples were collected from fields which had a 45 days growth of paddy and most were drought prone which lacked water logging conditions due to lack of water for irrigation central part of dharmapuri maximum number of 4 isolates were collected as farmers took their own measures in providing water to the fields where as in Toppur, the southern part, only 1 isolate was obtained could be because of extreme increased temperature and complete lack of water. The isolated strains were rod and vibriod in shape. This result was confirmed by Doberenier and Baldani **[17]** and Krieg *et al*., **[18]** and also reported that microscopic examination revealed polymorphism, but the dominant forms on a solid malate medium are characteristic curved rods of various sizes with predominant refractive fat droplets. The isolates showed 4 distinct morphological appearances, they were all gram negative and motile.

Figure 1 shows they fix the formation of subsurface pellicle formation in *Azospirillum* isolation broth as it has the characteristic feature of micro-aerophilic nature of *Azospirillum* of the 13 isolates isolate 5, 6, 7, 9 and 13 formed distinct subsurface pellicles. Dobereiner and Day **[19]** reported micro aerophilic growth in semisolid agar stagnant conditions were helpful for the inoculation of the organism. Since *Spirillum lipoferum* grows in a typical pellicle 1 to 4mm below the surface, this method was particularly useful for studying the substrates and growth conditions for nitrogen fixation.

Bigger size of *Azospirillum* as isolates 3,5,6,7,8,9,and 13 were having a size > 0.8 µm which is a characteristic feature of *Azospirillum* spp. Based on the above isolates of 3,5,6,7,8,9,and 13 were chosen for further study. Eckert reported that all these features were very similar to other *Azospirillum* sp. The trait that differentiates, the species from other based on its ability to use several sugars and some minute genetic detail optimum growth occurs at 30°C and at pH values between 6.0 and 7.0 but not 37°C.

Biochemical characteristics of the 5 isolates were all the isolates were found to reduce nitrate, utilize biotin and use all the 3 sugars as a carbon source.

Figure 2 shows the utilization of tryptophan at concentration of 1, 2 and 5 mg for production of indole acetic acid , isolate 9 showed maximum production followed by isolate 7,13,and 6 were as isolate 5 had least indole acetic acid production. However at a higher concentration of tryptophan, the production of IAA is higher which might exert an adverse effect on plant growth.

Figure 3 depicts the percentage of exopolysaccharide produce by the 5 isolates, isolate 7 showed a distinct increase in exoploysaccharide with 52 % followed by isolate 7,13,6 & isolate 5 had very minimal % of exoplysaccharide, from the above 2 tables it is clear that isolate 7 & 9 could be used under drought prone conditions by mass cultivation and checked for plant growth promot in properties. The total sugar content of the EPS was estimated by phenol/ sulfuric method **[15]**.

The moisture and pH of the soil before and after plant growth did not have any significant increased or decreased plant growth.

The isolates screened for exopolysaccharide and indole acetic acid were sowed in pots and maintained in duplicates. The growth of paddy in pots inoculated with, commercially available *Azospirillum*. Cultures showed a good increase in the length of the paddy shoot and the paddy crop grown with isolates 7 & 9 showed and an equally good shoot length even under drought prone conditions where as uninoculated paddy showed shunted growth and unhealthy small paddy leaves.

Cristyakova and Kalininskaya, **[20]** reported that bacteria of the genus *Azospirillum* are widespread in the soilsof various regions and usually occur in the rhizosphere of vascular plants. In pot culture experiment, the results indicated that the growth of *Azospirillum* treated paddy seedlings excelled over the untreated ones. The seed germination studies revealed that the percentage germination of seeds were higher in *Azospirillum* treated seeds than in control.

On enumerating the *Azospirillum* load in the soil samples inoculated with isolates 7 & 9. They showed a good survival rate, compared to commercially available *Azospirilllum*.

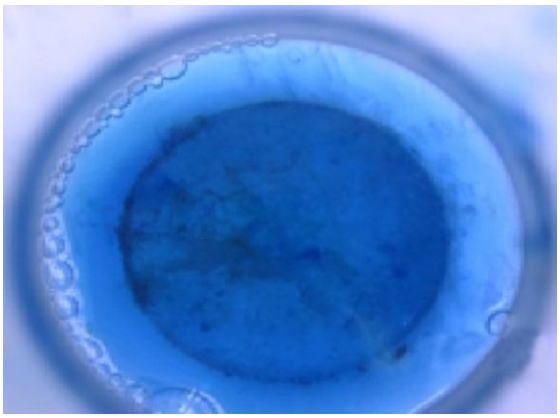

Figure 1: Sub Surface Pellicle Formation By Azospirillum

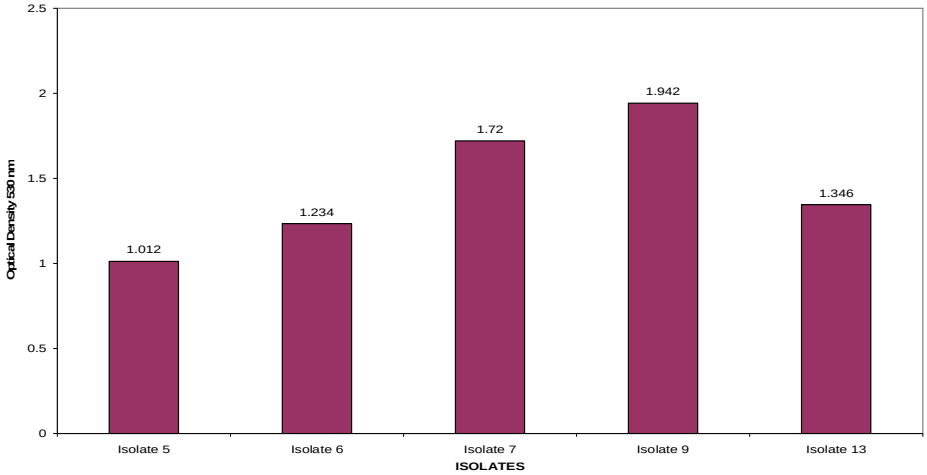

Figure 2: Utilization of Tryptophan by by

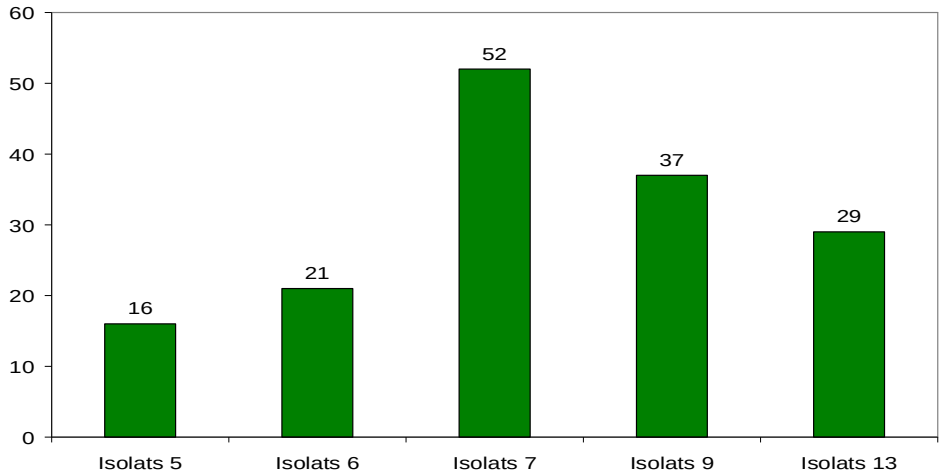

Figure 3: % of Exopolysachharide Produced

CONCLUSION

This study reveals that increased indole acetic acid and exopolysaccharide production by *Azospirillum* improves its survival rates under drought prone conditions. But the mechanism

by which the bacteria is capable of doing this needs further in site.

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