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Exploration of Different *Azospirillum* Strains from Various Crop Soils of Srivilliputtur Taluk

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Abstract

Exploration of different *Azospirillum* strains from various crop soils of Srivlliputtur Taluk was analysed. There are three different species of strains were found and analysed with different parameters like their morphology, motility, catalase and biotin content. However, the biology was also been studied which includes the vitamin and carbon utilization of the *Azospirillum*. In this results showed that there are two different species of *Azospirillum* were identified in all the soils namely *A.lipoferum*, and *A.brasilense*. The morphology, biochemical parameters of these two species of *Azospirillum* are varied according to the soil in which they are identified. It is concluded that the strains of *Azospirillum* will help to the plants for better growing by means of utilization of various parameters from the soil to the plants and these strains are used as very efficient biofertilizers in the crop plants from all over the world.

Keywords: Crop soils; Srivilliputtur Taluk; Exploration; *Azospirillum*

Introduction

Biofertilizer is a wide term which includes a diverse category of bioinoculants such as nitrogen fixers, phosphate solubilizers, phosphate mobilizers and plant growth promoting rhizobacteria. Biofertilizers are the organisms which are naturally present in all types of soils. Applications of these biofertilizers are environment friendly, means to supplement nutrient to the plants. Important biofertilizers are nitrogen fixers, phosphate solubilizers and phosphate mobilizers [1]. However, the concept of biofertilizers was developed with the discovery of nitrogen fixing *Azospirillum*. *Azospirillum* was first reported by *Beijerinck* [2] and it was named as *Spirillum lipoferum* by *Schroeder* [3]. It gained the reputation of being the most studied plant associative bacterium only after it was rediscovered by *Dobereiner J* [4] in the roots of *Digitaria decumbens*. The meaning of *Azospirillum* was from 'azite' a French word meaning nitrogen and 'spira' a Greek word meaning spiral. *Azospirillum* means a small nitrogen spiral. The name *lipoferum* means fat bearing; *brasilense* from brasiliensis pertaining to the country of Brazil, South America. They also explored the high potentiality of *Azospirillum* as microbial inoculant with tropical grasses and other crop plants. It has been enlarged to encompass other possible bacterial associations by adopting the terminology "Diazotropic biocoenosis".

Azospirillum is a gram negative, motile, curved rod of variable size, ranging from 0.5 – 1µm in length, exhibits spirillar movement and polymorphism, containing poly-β- hydroxy butyrate (PHB) granules and fat droplets [4,5] They contain peritrichous flagella and polar flagellum used for swarming [6]. It was associated with the root system of plants making use of the nutrients exuded by them. *Azospirillum* is colonized the root region of crop plants in large numbers and fixes substantial amount of nitrogen [7] and they exerted beneficial effects on plant growth and yield many crops of economic importance [8].It is used extensively in rice and other cereal crops as biofertilizers [8]. Tarrand et al. [9] and Magalhaes et al. [10] proposed as *Azospirillum*, the genus distinguished into two species based on physiological and morphological differences between various strains on DNA homology experiments like *Azospirillum brasilense* and *A. lipoferum* [9,10] Later, four additional *Azospirillum* species were described, *A. amazonense*. Isolated from many grasses in the Amazonian area of Brazil [11], the salt tolerant species *A. halopraeferans*, associated exclusively with roots of kallar grass [12], *A. irakense* [13] and *A. dodereinerae* [14].

Azospirillum is grown in N-free medium, it behaves as microaerophilic, fixes nitrogen and when supplemented with nitrogen it grows as an aerobe [4]. The population levels of *Azospirillum* were reported from 10^4 to 10^6 cells per gram of dry soil or root by Magalhaes et al. [10] and 7 x 10^4 per gram of fresh roots in *Stenocerus pruinosus* and 1.1 x 104 in *Opuntia ficus - indica* by Mascarua-Esparza et al [15]. Maximum *Azospirillum* numbers were detected in a laterite soil and the minimum in an extremely acid sulphate saline kari soil [16]. The present study aims to isolate, identify, screen and study the biology of *Azospirillum* spp from various soils of Srivilliputtur Taluk, Virudhunagar District, Tamil Nadu, India.

Methodology

Study area

Tamil Nadu is situated in Southern end of India, towards east of Kerala north of Andhra Pradesh and Karnataka. Several folds or parts of Western Ghats separate the states of Tamil Nadu and Kerala. The area of investigation, Srivilliputtur is located in the Southern side of Virudhunagar District; this area is a boundary of Theni and Madurai Districts in North, Tirunelveli District in South and Kerala in Southwest.

Collection of soil and root samples

Soil and Root samples were collected from different crop soils in various places of Srivilliputtur Taluk, Virudhunagar District. The soil

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samples (Rhizosphere soil) were air dried under shade and used for isolation and enumeration of *Azospirillum*.

Isolation and enumeration *Azospirillum*

For the isolation of *Azospirillum*, the rhizosphere soil samples were serially diluted from 104 to 106 using sterile water. One mille liter of the soil diluents from each dilution was transferred to the tubes containing 10 mille liter of nitrogen free malic acid semi solid medium and kept it in incubation for three days at 37±2°C.

Enumeration of *Azospirillum* in soil samples were carried out by Most Probable number method (MPN). One mille liter successive dilutions of 10^4, 10^5 and 10^6 soil samples were transferred to test tubes containing nitrogen free malic acid semi solid medium. Then the tubes were incubated at room temperature for 3 days. The positive tubes were counted and the population was calculated and expressed as number of *Azospirillum* per gram dry weight of soil samples.

$$\textit{Azospirillum}\ \text{population} = \frac{\text{MPN value x middle dilution x middle dilution used}}{\text{Dry weight of the soil sample}}$$

Identification and screening of *Azospirillum*

The selected bacterial strains were identified using standard biochemical tests as listed in the Bergey's Manual of Determinative Bacteriology. The following tests/methods were used for isolation and identification of *Azospirillum* from the soils.

Cell morphology: To study the cell morphology, log phase cultures of *Azospirillum* were properly diluted with sterile distilled water. Smear was prepared on clean glass slides by using a loopful of culture dilution. The smears were air-dried, heat fixed and stained with crystal violet. The cells were observed under light microscope with oil immersion objective to see the size and shape.

Motility: Motility was tested by hanging drop method. Slides were prepared with cultures and motility was observed under oil immersion.

Biotin requirement: The biotin requirements of the bacterial isolates are tested using semisolid nitrogen free malic acid medium prepared in two sets of tubes, one set of medium prepared with the addition of biotin (100 µg l^{-1}) and other without biotin. The growth was observed by the change in colour from yellowish green to blue.

Catalase test: Selected strains were inoculated on LB agar plates and incubated at 28°C for 24 h. About 3 to 4 drops of 3 percent H_2O_2 solution was allowed to flow over the culture. Formation of bubbles or effervescence indicated a positive result.

Biology of *Azospirillum*

Utilization of carbon, nitrogen, amino acid and vitamin sources

The utilization of different carbon, nitrogen, amino acid and vitamin sources of different *Azospirillum* isolates were estimated in LB broth. Filter sterilized carbon, nitrogen, amino acid and vitamin sources were inoculated aseptically into the sterile medium at 1 percent level. The *Azospirillum* cultures were inoculated at the rate of 1.0 mille liter and incubated at room temperature. The growth was observed by the turbidity of the broth read at 560 nm.

Results and Discussion

Totally fifteen *Azospirillum* strains were isolated from the soil samples collected from different crop plants at various places of Srivilliputtur Taluk, Virudhunagar district. The isolated strains were brought to pure culture by several subcultures. The purified *Azospirillum* strains were maintained in nutrient agar slants and stored at 4°C for future use (Table 1). The result showed that the population level of *Azospirillum* was higher in cotton followed by tomato. Among different crop plants, *Azospirillum* population was least in soil samples collected from bhendi (Table 1). According to Haahtela et al. [17] and Eckert et al. [14] Azospirilla were isolated from a wide variety of plants including many grasses and cereals from all over the world, in tropical, temperate and cold climates. In addition to isolation of *Azospirillum*, enumerates the population level in soil samples were also collected from different crop plants. *Azospirillum* population was higher in cotton and least in soil samples collected from bhendi. The association of these organisms with the roots of non-Graminae family plants was also reported by [18]. Seasonal variation in MPN counts, which showed a similar pattern of decrease or increase with the variable climatic conditions, confirm that all types of microorganisms were influenced by the temperature fluctuations in a similar fashion in tea soils Govindan and Purushothaman [19]. Plantation crops like areca

S.No	Crop Plants	Code Number	Number of Strains Isolated	Population level (x 10^5/g soil dry wt.)
1	Tomato	T1 T2 T3	3	5.7
2	Bendi	BD1 BD2 BD3	3	0.6
3	Bringal	B1 B2 B3	3	2.0
4	Chillies	C1 C2 C3	3	3.6
5	Cotton	CT1 CT2 CT3	3	16.6

Table 1: Isolation of Total Number of *Azospirillum* Strains and their Population level in different Crop Plants.

S. No	COLOUR CHANGE				pH Change	Gram staining
	Strains	1st day	2nd day	3rd day	pH	Gram stain
1.	T1	Light blue	Blue	Dark blue	9.6	-ve
2.	T2	Green	Light blue	Dark blue	9.3	-ve
3.	T3	Light blue	Blue	Dark blue	9.8	-ve
4.	BD1	Light blue	Light blue	Dark blue	9.6	-ve
5.	BD2	Light blue	Light blue	Dark blue	9.5	-ve
6.	BD3	Light blue	Light blue	Dark blue	9.4	-ve
7.	B1	Light blue	Blue	Dark blue	9.4	-ve
8.	B2	Green	Light blue	Dark blue	9.6	-ve
9.	B3	Light blue	Blue	Dark blue	9.7	-ve
10.	C1	Light blue	Blue	Dark blue	9.8	-ve
11.	C2	Light blue	Light blue	Dark blue	9.7	-ve
12.	C3	Light blue	Light blue	Dark blue	9.7	-ve
13.	CT1	Light blue	Blue	Dark blue	9.4	-ve
14.	CT2	Green	Light blue	Dark blue	9.6	-ve
15.	CT3	Light blue	Blue	Dark blue	9.8	-ve
16.	Control	Green	Green	Green	7.0	--

Table 2: Results of colour changes, pH and Gram staining in the medium used for the growth of various *Azospirillum* strains.

nut, cashew, cocoa, rubber, cardamom and sapota grown in acid soils colonized by *Azospirillum* in their root system [20].

Selection of Efficient Strains

The results indicated that the *Azospirillum* strains were differed in their ability to change colour intensity of the medium. The difference between the regions in nitrogen fixing ability was related to geographic variables, including soil type [21]. In this study, the isolated strains were screened under *in vitro* condition. The isolated *Azospirillum* strains were screened by noting the changes of colour and pH in the *Azospirillum* growing medium. After selection the *Azospirillum* strains were screened by noting the colour and pH change of the medium. These two observations were used for the screening of *Azospirillum* in the laboratory conditions. In the nitrogen free malate broth, *Azospirillum* strains were able to change colour. The initial green colour changed into blue colour. The ability of colour change was differed between the strains. The result revealed that all the strains were able to change the colour and pH of the medium on 3rd day. But some strains started to change the colour even in the first day after inoculation. Among fifteen strains, the T1, T3, B1, B3, C1, CT1 and CT3 strains were superior in colour change than others (Table 2), however, among fifteen strains, the ability to pH changes is differed between the strains. In strains T3, C1 and CT3 were increased in pH than other strains (Table 2).

S.No	Strains	Identification	Catalase	Biotin
1	T1	*A.brasilense*	+	-
2	T2	*A.brasilense*	+	-
3	T3	*A.lipoferum*	-	+
4	BD1	*A.lipoferum*	-	+
5	BD2	*A.lipoferum*	-	+
6	BD3	*A.lipoferum*	-	+
7	C1	*A.lipoferum*	-	+
8	C2	*A.lipoferum*	-	+
9	C3	*A.lipoferum*	-	+
10	CT1	*A.lipoferum*	-	+
11	CT2	*A.lipoferum*	-	+
12	CT3	*A.lipoferum*	-	+

Table 3: Identification, catalase and biotin contents of various Azospirillum strains in different crop plants.

S. No	Strains	Carbon Source				
		Glucose	Lactose	Sucrose	Fructose	Maltose
1.	T1	0.357 +	0.357 +	0.349 +	1.191 +++	0.831 ++
2.	T2	0.562 ++	0.765 ++	0.760 ++	1.463 +++	0.760 ++
3.	T3	0.636 ++	0.670 ++	0.914 ++	1.437 +++	0.815 ++
4.	BD1	1.094 +++	1.003 +++	0.931 ++	0.932 ++	1.063 +++
5.	BD2	1.169 +++	0.799 ++	1.066 +++	1.413 +++	0.972 ++
6.	BD3	1.146 +++	0.683 ++	0.947 ++	1.451 +++	0.914 ++
7.	B1	0.845 ++	0.604 ++	1.025 +++	1.467 +++	0.713 ++
8.	B2	0.930 ++	0.683 ++	0.811 ++	1.543 +++	0.741 ++
9.	B3	1.050 +++	0.512 ++	1.106 +++	1.512 +++	0.834 ++
10.	C1	0.891 ++	0.865 ++	1.141 +++	1.455 +++	1.136 +++
11.	C2	0.830 ++	0.637 ++	1.473 +++	1.551 +++	1.212 +++
12.	C3	1.126 +++	0.820 ++	0.712 ++	1.543 +++	0.971 ++
13.	CT1	0.956 ++	0.998 ++	1.029 +++	1.521 +++	0.973 ++
14.	CT2	0.760 ++	0.851 ++	1.360 +++	1.462 +++	1.070 +++
15.	CT3	0.890 ++	0.712 ++	0.814 ++	1.568 +++	1.114 +++

+ Fair ; ++ Good; +++ Excellent

Table 4: Carbon utilization by *Azospirillum* strains.

Identification of *azospirillum*

Based on the present study, the *in vitro* experiment shows that the totals of 15 *Azospirillum* strains were screened for their efficiency using various biochemical tests. In Gram stain all the fifteen strains were Gram negative because all the strains were stained with safranin rather than crystal violet. Based on the staining, fifteen strains were revealed Gram negative (Table 2), but the size of the selected strains were ranged from 1-2.0µm in diameter and 2.0-3.5µm in length. Microscopic examination of the isolates revealed that they were vibroid in shape and in the motility levels of all the fifteen strains were showed spiral movement. In catalase and biotin content of *Azospirillum*, all the strains except T1, T2 were negative to catalase test. (Table 3). The selected fifteen strains were cultured in nitrogen free semisolid medium with or without biotin. Biotin test is one of the important test to differentiate the *Azospirillum* species mainly *A. lipoferum* and *A. brasilense* (Table 3). According to Okon and Itzigsohn R [22], *Azospirilla* was gram negative,

S. No	Strains	Vitamin source			
		(Vitamin B) Nicotinic acid	(Vitamin B1) Thiamine	(Vitamin B6) Pyridoxine	(VitaminB12) (Myoinositol
1.	T1	0.713 ++	1.298 +++	0.678 ++	1.234 +++
2.	T2	0.443 +	0.978 ++	0.896 ++	1.340 +++
3.	T3	0.772 ++	1.236 +++	0.834 ++	0.978 ++
4.	BD1	0.725 ++	0.631 ++	0.428 +	1.456 +++
5.	BD2	1.273 +++	0.748 ++	0.853 ++	1.389 +++
6.	BD3	0.813 ++	0.734 ++	0.896 ++	1.467 +++
7.	B1	0.461 +	0.967 ++	0.438 +	1.389 +++
8.	B2	0.678 ++	0.834 ++	0.942 ++	1.271 +++
9.	B3	0.616 ++	0.616 ++	0.836 ++	0.945 ++
10.	C1	0.483 +	0.976 ++	1.134 +++	1.432 +++
11.	C2	0.893 ++	1.343 +++	1.224 +++	1.446 +++
12.	C3	0.842 ++	0.941 ++	1.036 +++	1.258 +++
13.	CT1	1.282 +++	1.134 +++	1.454 +++	1.468 +++
14.	CT2	0.841 ++	0.964 +++	0.916 ++	1.396 +++
15.	CT3	1.125 +++	0.978 ++	0.822 ++	1.464 +++

+ Fair ; ++ Good; +++ Excellent

Table 5: Utilization of Vitamins by *Azospirillum* strains.

the cured rods of variable size exhibits spirillar movement and contain PHB as reserve food material.

Utilization of carbon sources

The *Azospirillum* strains utilized different types of chemicals as carbon sources. The utilization of these different types of carbon sources varied from strain to strain. There was a marked difference between the *Azospirillum* species in the pattern of carbohydrate utilization. The preferential carbon sources varied from strain to strain. Most of the strains preferred fructose and maltose as carbon source but sucrose was moderately utilized while lactose was found to be a poor source (Table 4). The disaccharides except lactose, starch and mannitol supported little growth of isolates belonging to both species, while trisaccharides, polysaccharides did not support their growth. This suggests that sugars are supposed to be very poor substrates of carbon and energy for A. brasilense but better source for *A. lipoferum* [23].

Utilization of vitamin source

All *Azospirillum* strains were utilized for various vitamin sources. The preferential vitamin sources varied from strain to strain. Most of the strains preferred myoinosital, as vitamin source but nicotinic acid was also moderately utilized while, thiamine, pyridoxine was found to be a poor source (Table 5).

Conclusion

Our results demonstrate remarkably different strains of *Azospirillum* from various crop soils in Srivilliputtur Taluk.

The isolated strains were contains different features in their morphology, carbon utilization and their vitamin contents. This type of results which would be very useful in the field of agriculture to develop organic/bio fertilizer.

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