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CHARACTERIZATION AND CROP PRODUCTION EFFICIENCY OF DIAZOTROPHIC BACTERIAL ISOLATES FROM COASTAL SALINE SOILS

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Abstract: **The present study deals with the soil samples collected from various places at Cuddalore district, Tamil Nadu, India for isolation of *Azospirillum* isolates and investigated. The physico-chemical properties of rice soil samples were analyzed. Soil organic carbon content ranged from 0.272 to 0.55%, pH ranged from 7.3 to 8.3 and EC ranged from 0.59 to 2.70 dSm^{-1}. The N status was categorized as low ranged from 112 to 160 kg ha^{-1}. The available potassium ranged from 90 to 245.50 kg ha^{-1} and phosphorous ranged from 7.6 to 12.85 kg ha^{-1}. Five *Azospirillum* isolates were obtained, characterized and designated as SFA-1 to SFA-5. Out of five four belongs to *Azospirillum brasilense* and one belongs to *Azospirillum lipoferum*. One isolate observed to be nir^+ and the remaining four nir^-. The inoculation of stress tolerant SFA–1 to rice plants increased plant height, fresh and dry weight of shoot and root significantly, While other isolate treatment slightly increase the morphological parameters when compared to control (without adding N and *Azospirillum*). But, The *Azospirillum* SFA-1 inoculation at graded levels of nitrogen i.e., 75N + 25% *Azospirillum* increases the shoot and root growth, fresh and dry weight of shoot and root when compared to control considerably.**

Keywords: **Microorganism, isolation, *Azospirillum* isolates, appearance of *Azospirillum* culture, saline soil, rice plant.**

I. INTRODUCTION

On Sunday 26th December 2004, a large earthquake occurred at the west coast of Indonesia Island of Sumatra (IA) generating a Tsunami that caused over 2,00,000 fatalities in 12 countries across the Indian Ocean basin. The Tsunami was truly global event with significant activity and was recorded around the world [1]. Rice is the most important staple crops in the world. Substantial areas under rice cultivation on the tropics and subtropics are affected by the soil salinity. Plant productivity in saline soil is considerably reduced due to improper nutrition of plant plus the osmotic and drought stress [2]. Biological Nitrogen Fixation (BNF) based forming systems would enhance agriculture production in the long term in both economically viable and socially acceptable ways. Since it offers an eco-friendly and promising technology for nitrogen replenishment in cultivable soils.

Crop responses to inoculation with *Azospirillum* were demonstrated in many cereal and forage crops. The review of the data on the field inoculation study with *Azospirillum* over the past twenty years revealed that 60 to 70% success with statistically significant increases in yield was 5 to 30% of the trails [3]. In many cases, the reasons for failure to inoculation were attributed to biotic and abiotic factors that affects the performance of the inoculated *Azospirillum* in the field. The other main reason is the use of poor quality of inoculants.

Plant growth promoting rhizobacteria play a key role in nutrient cycling and maintenance of soil fertility and establish positive interaction with plant roots in agricultural environments. Improvement of the beneficial associations between microorganisms and plants particularly in the rhizosphere is an area of research of global interest.

The present study was undertaken with the following objectives, Survey for the occurrence of *Azospirillum* spp. in the saline soils of T.S. Pettai, Killai, Parangipettai, Devanampattinum and Periapattu places at Cuddalore district. Screening of *Azospirillum* isolates for its efficiency and to develop stress tolerant *Azospirillum* inoculants to study their shelf-life and to study the effectiveness of stress tolerant *Azospirillum* on rice under field conditions.

II. MATERIALS AND METHODS

The *in vitro* experiments were carried out in the microbiology laboratory, Department of Microbiology, Faculty of Agriculture, Annamalai University. The pot culture trails were conducted at the pot culture yard of the Department of Botany and the field trials were at botanical garden, Department of Botany, Annamalai University.

Azospirillum spp (ATCC: 29145) was obtained from microbial type culture collection and gene bank, institute of Microbial Technology (IMTech) Chandigarh, India and was used as reference strain in the present study. It was maintained in nutrient agar slants at 32°C with monthly transfer.

A survey was carried out in different locations of salinity prone in Cuddalore district, viz., T.S. pettai, Killai, Parangipettai, Devanampattinam and Periyapattu, Tamilnadu state, India and the rice rhizosphere soil samples were carefully collected, transported to the laboratory. The *Azospirillum* isolated were enumerated, isolated and purified by following the standard procedure and maintained in nutrient agar and used in this entire study (NF medium – N_2 free bromothymol blue medium; N_2 free bromothymol blue medium semi solid. The soil samples collected from different locations were analyzed to determine organic carbon content, electrical conductivity, soil pH and available N, phosphorus and available potassium by atomic absorption spectrometer. A population of *Azospirillum* in the rhizosphere soil was enumerated by following the Most Probable Number (MPN) technique.

Isolation, Characterization and Identification of Azospirillum isolates

Isolation:

Ten gram of rhizosphere soil samples were transferred in 90 ml of sterile distilled water in 250 ml Erlenmeyer flasks and incubated on a rotary shaker (100 rpm) for 30 min at room temperature. The well mixed suspension was diluted appropriately and 0.1 ml of the suspension was aseptically transferred into test tubes containing 10 ml of semisolid nitrogen free malate medium (NFB) and incubated at 32°C for 24 to 72 h.

After the incubation period the inoculated tubes containing NFB semisolid medium were observed for the development of white subsurface pellicles, which is the presumptive indication of the growth of *Azospirillum.*

Characterization of Azospirillum Isolates:

Microscopic observation of the wet mounts of the 72 hold NFB medium cultures was carried out of the shape, motility and presence of poly-β-hydroxy butyrate granules. Gram staining was carried out as per Hucker's modifications [4]. Further, the *Azospirillum* isolates were subjected to various biochemical tests for their species level identification.

Requirement for Biotin:

The nitrogen free malate medium was used for assessing the requirement for biotin. Two sets of media were prepared with and without biotin (100 μg L^{-1}). The cultures were grown for 48 h in glucose peptone broth [5], centrifuged and washed twice with sterile distilled water and re-suspended in sterile distilled water to a uniform density. A quantity of 0.1 ml of this suspension was used to inoculate 5 ml volume of medium with and without biotin and incubated for 48 h at 30 ± 2°C and the growth of the isolates was observed. For each set an un-inoculated control was maintained.

Screening of Azospirillum Isolates for their Efficiency:

Five identified isolates of *Azospirillum* were screened for the efficiency of dinitrogen fixation and production of IAA in the culture media.

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Extraction and estimation of IAA produced:

After the incubation period, the broth cultures were centrifuged at 5000 rpm for 15 minutes in a lab centrifuge and the supernatant was filtered through Whatman No. 42 filter paper. The filtrate was adjusted to a pH 2.8 with 1.0 N HCl.

50 ml of filtrate were taken in a separating funnel and equal volume of peroxide free cold diethyl ether was added and mixed thoroughly. The contents of the funnel were allowed to stand for 4 h at 4°C with intermittent shaking. The aqueous phase was separated from organic phase. The filtrate was again subjected to two more extraction at 4 hrs interval and organic phase was pooled together. The other phase was evaporated to dryness and residue was dissolved in 2 ml of isopropyl alcohol. From this suspension, 0.5 ml was taken in test tubes and added with 1.5 ml of distilled water followed by 4 ml of Salper's reagent (1.0 ml of 0.5 M ferric chloride in 50 ml of 35% perchloric acid). The tubes were incubated in complete darkness for one hour at 28°C. The intensity of pink colour developed in test tubes was measured at 535 nm in spectronic-20 and the value was used to calculate the quantity of IAA produced by the test cultures.

Determination of growth parameters:

Plant height:

The plant height of the three plants was measured from the ground to tip of the main shoot on 30, 60 and 90 days and the values were expressed in centimeters and fresh, dry weight of shoot and root was measured by electronic balance and were expressed g plant^{-1}.

Details of Location and Source of Azospirillum Isolates:

In Cuddalore district (T.S. Pettai, Killai, Parangipettai, Devanampattinum and Periyapattu) were selected for the survey of *Azospirillum* isolates in saline soil (Tsunami affected soils), which are usually prone to saline soil condition. The samples were randomly collected and five isolates of *Azospirillum*, were isolated from the different variety of rice grown at each location. The isolated were numbered from SFA-1 to SFA-5.

III. RESULTS AND DISCUSSION

The physico-chemical properties of the soil sample collected from five location at Cuddalore districts and the data was given in Table - 1. The soil sample belongs to different textural type *viz.*, clay loam soil and sandy loam soil. The soil pH ranging from 7.3 to 8.3 and soil EC ranging from 0.59 to 2.70 dS m^{-1}. The organic carbon ranging from 0.272 to 0.55, the available nitrogen ranging 112 to 160 (kg ha^{-1}), phosphorus 7.62 to 12.85 (kg ha^{-1}) and potassium ranging from 90 to 245.50 (kg ha^{-1}).

TABLE I: PHYSICO-CHEMICAL PROPERTIES OF THE RICE RHIZOSPHERE SOILS

Sl. No.	Name of the location	Soil type	pH	EC (dSm^{-1})	Soil organic carbon (%)	Available nitrogen (kg ha^{-1})	Available phosphorus (kg ha^{-1})	Available potassium (kg ha^{-1})
1.	T.S. Pettai	Clay loam	7.9	1.75	0.49	115.00	12.85	245.50
2.	Killai	Clay loam	7.5	2.70	0.45	112.00	7.95	220.00
3.	Parangipettai	Sandy loam	7.3	0.59	0.47	152.00	7.6	90.00
4.	Devanampattinum	Sandy loam	8.3	1.80	0.55	150.00	8.6	90.00
5.	Periyapattu	Sandy loam	7.8	1.29	0.27	160.00	9.5	103.00

The analysis of physico-chemical properties of the rice soils revealed that there was a positive correlation between the population of total bacteria and organic matter content between *Azospirillum* population and organic carbon content. The role of organic matter content in augmenting the microbial population was well documented [6]. But there existed no correlation between the available nutrients *viz.*, nitrogen, phosphorus and potassium and the *Azospirillum* population. The *Azospirillum* population was relatively less in soils with pH 7.8 and above. Interestingly, the highest population of *Azospirillum* 54.5 x 105 cfu g^{-1} was recorded in Periyapattu soil and the maximum relative occurrence of 1.37% of *Azospirillum to* total bacteria was recorded in T.S. Pettai. The higher population of *Azospirillum* in T.S. Pettai may be due to the availability of higher organic carbon content, whereas the higher relative occurrence of *Azospirillum* to total bacteria in Devanampattinum soil may be due to the increased rhizo-competence of the *Azospirillum* strains.

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Quantitative Occurrence of *Azospirillum* in rice Rhizosphere Soil*:*

The total bacterial population and *Azospirillum* population in the rice rhizosphere soil sample were enumerate and relative occurrence of *Azospirillum* were determined and presented in Table 2. The total bacterial population in the rhizosphere soil of rice ranged from 12.6 cfu g^{-1} to 46.2 x 107 cfu g^{-1} and the *Azospirillum* population ranged from 8.6 cfu g^{-1} to 54.5 x 105 cfu g^{-1} of soil the relative occurrence of *Azospirillum* to the total number of bacteria ranged from 0.49 to 1.37% in the soil samples tested. The highest bacterial population of 46.2 x 107 cfu g^{-1} and the highest total *Azospirillum* population 54.5 x 10^{-5} cfu g^{-1} was recorded at Devanampattinum area soils. However the relative population of *Azospirillum* total bacterial population was the highest of 1.37 % in T.S. Pettai and lowest percentage recorded at Periyapattu 0.49%.

TABLE II: RELATIVE OCCURRENCE OF *AZOSPIRILLUM* POPULATION IN RICE RHIZOSPHERE SOILS

Sl. No.	Name of the location	Total bacterial population (x 10^7)	Total *Azospirillum* population (x 10^5)	% of *Azospirillum* to total bacteria
1.	T.S. Pettai	25.8	35.3	1.37
2.	Killai	18.6	15.8	0.85
3.	Parangipettai	'12.6	12.3	0.98
4.	Devanampattinum	46.2	54.5	1.18
5.	Periyapattu	17.6	8.6	0.49

In the present study five cultures of *Azospirillum* SFA-1 were isolated from rice rhizosphere soil samples collected from five different locations, which included Cuddalore districts of Tamil Nadu, India. These cultures were identified based on the general characterization and biochemical tests. All the five isolates found to be number of genus *Azospirillum* based on the formation of sub surface pellicle in NFB semi solid medium formation of pink colour colonies on BMS agar, cell shape, motility and gram negative character and presence of PHB [7].

Interestingly results were obtained when the five isolates of *Azospirillum* were subjected to different biochemical tests for their species level identification. Out of five isolates, four isolates were identified as *Azospirillum brasilense* (80%) and one isolates were identified as *A. lipoferum* (20%). In C_4 plants, *A. lipoferum* was the predominant species and *A. brasilense* was the predominant species associated with C_3 plants in tropical conditions [8].

Characterization of Azospirillum

Five isolated *Azospirillum* were characterized for their speciation.

TABLE III: GENERAL CHARACTERISTICS OF *AZOSPIRILLUM* ISOLATES

Isolates No.	Sub surface pellicle formation of NFB semisolid medium	Pink colour colonies on BMS agar	Cell shape	Motility	Gram reaction	Presence of PHB
SFA-1	+	+	Curved rods	+	-	+
SFA-2	+	+	Curved rods	4	-	
SFA-3	+	+	Curved rods	+	-	+
SFA-4	+	+	Curved rods	+	-	+
SFA-5	+	+	Curved rods	+	-	+

The isolation of *Azospirillum* was carried out by enrichment culture technique. The characteristics dense white sub-surface pellicles were observed in all the five isolates and also the change of nitrogen free malic acid medium (NFB) from yellowish green to blue was noticed the appearance of typical pink colour wrinkled colonies on potato infusion agar (BMS) agar confirmed *Azospirillum.* The characteristics curved rod shaped cells and spiral movement of the wet microscopic mount added additional confirmation of *Azospirillum.* All the five *Azospirillum* isolates showed gram negative reaction and the presence of PHB (Table 3).

Bio-chemical test for species level characterization:

The five isolates of *Azospirillum* were further subjected to various biochemical test *viz.*, the production of acid from glucose utilization of different carbon sources biotin requirement and denitrification property for the species of level identification and results are presented in Table 4.

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Production of acid and glucose was noticed in one isolates and remaining four isolates did not produce acid. The acid producing *Azospirillum* are grouped under *Azospirillum lipoferum* and the isolates which did not produce acid from glucose were grouped under *Azospirillum brasilense*.

TABLE IV: BIOCHEMICAL TESTS FOR THE SPECIES CHARACTERIZATION OF *AZOSPIRILLUM* ISOLATES

Isolate No.	Acid production from glucose	Utilization of different carbon sources						Biotin requirement	Nitrate reductase activity	Nitrite reductase activity	Speciation
		Malate	Succinate	Lactate	Glucose	Mannitol	a-Keto glutarate				
SFA-1	-	+++	+++	++	+	+	+	-	+	-	*A. brasilense*
SFA- 2	-	+++	+++	++	+	+	+	-	+	+	*A. brasilense*
SFA- 3	-	+++	+++	++	+	+	+	-	+	+	*A. brasilense*
SFA–4	+	+++	+++	++	+++	++	++	+	+	+	*A. lipoferum*
SFA–5	-	+++	+++		+		+	-	+	+.	*A. brasilense*

No correlation could be observed in many cases between the preference of host physiotype and the species of *Azospirillum* [9]. The present study clearly revealed the predominance of *A. brasilense* in rice soils in Tamil Nadu. The genus *Azospirillum* was classified in to two species based on the physiological and biochemical characters [10].

It was also of interest to note that both nir^+ and nir^- strains existed in the 5 *Azospirillum* isolates obtained. About 80% of *A. brasilense* and 20% of A *lipoferum were* found to be nir^+. Predominance of nir^+ strains in both species of *A. lipoferum* and *A. brasilense* was reported [11].

All the five isolates of *Azospirillum* were evaluated for their efficiency in dinitrogen fixation and IAA production under *in vitro* conditions. Based on the amount of N fixed g^{-1} of malate utilized, the five isolates were grouped into two categories one that fixed above 10.0 µg of N g^{-1} of malate and the other that fixed below 10.0 µg of N g^{-1} of malate, four isolates of *Azospirillum*, 60% of the total fell under the category I. The remaining 1 isolates 5% of total fell under the II category. *A. brasilense* fixed 80 to 12.5 mg N g^{-1} of malate and *A. lipoferum* fixed 11.0 to 16.5 N g^{-1} malate [12], But in the present study, no such correlation existed between the amount of N fixed and the species of *Azospirillum.*

The variation in the production of IAA by salt *Azospirillum* strains has been reported [13]. Beneficial effect of growth promoting substances produced by this diazotroph on root development was also reported [14]. The five *Azospirillum* isolates were categorized into two groups. Category I comprised or isolates (40%) that produced IAA above 1.0 µ*g* ml^{-1} of the culture filtrate and category II comprised three isolates (60%) produced IAA below 1.0 g ml culture the *Azospirillum* isolates which in under the category I were selected for the further study to screen for salt tolerance.

Shoot length and root length (cm plant^{-1})

Inoculation of rice with stress tolerant SFA-1 is applied for saline soil and the plant growth of shoot and root length is given in Table 5. The maximum was observed at 75% N + 25% *Azospirillum* and this was 70.4 in shoot and 15.5 in root cm $plant^{-1}$ higher when compared to control. The lowest growth morphology of shoot and root length observed with control (saline soil); no *Azospirillum* and no NPK on 90th days respectively.

TABLE V. EFFECT OF *AZOSPIRILLUM* SFA-1 INOCULATION AT GRADED LEVELS OF NITROGEN ON THE SHOOT LENGTH AND ROOT LENGTH OF RICE ADT 43
(mean value n = 3 replicates)

SI. No.	Treatment	Shoot length (cm plant^{-1})			Root length (cm plant^{-1})		
		30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS

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1	Control (saline soil)	31.0 ±1.53	42.5 ±2.12	51.4 ± 2.57	3.5 ±0.17	8.4 ±0.42	12.6 ±0.62
2.	100% *Azospirillum* alone	48.5 ±2.4	54.4 ±2.72	56.6 ±2.83	2.2 ±0.11	6.5 ± 0 .32	10.3 ± 0.51
3.	100% Nitrogen alone	49.4 ±2.55	60.32 ± 3.01	63.2 ±3.16	4.5 ±0.22	8.1 ±0.40	10.5 ±0.52
4.	75% Nitrogen alone	49.4 ±2.42	55.5 ±2.77	60.2 ±3.01	6.4 ±0.32	10.5 ±0.52	13.4 ±0.67
5.	75% N + 25% *Azospirillum*	60.2 ±1.55	66.4 ±3.32	70.4 ±3.42	8.4 ±0.42	14.4 ± 0 .72	15.5 ±0.77

The maximum fresh weight of shoot 30.65 and root 3.40 (g plant^{-1}) was recorded in the treatment of 75% N + 25% *Azospirillum* (Table 6). The inoculation effect in terms of fresh weight between 100% *Azospirillum*, 100% nitrogen and 75% nitrogen is less when compared to 75% N + 25% *Azospirillum.*

TABLE VI: EFFECT OF *AZOSPIRILLUM* SFA-1 INOCULATION AT GRADED LEVEL OF NITROGEN AND THE FRESH WEIGHT OF RICE ADT 43 (mean value n = 3 replicates)

SI. No.	Treatment	Fresh weight of shoot (g plant^{-1})			Fresh weight of root (g plant^{-1})		
		30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS
1	Control (saline soil)	4.39 ±0.21	7.60 ±0.38	8.15 ±0.40	0.51 + 0.02	0.86 ±0.04	0.95 ± 0.04
2.	100% *Azospirillum* alone	6.68 ,±0.33	11.50 ± 0.57	12.65 ±0.63	1.68 ±0.08	1.99 ±0.09	2.05 ±0.10
3.	100% Nitrogen alone	6.08 ±0.30	13.60 ± 0.68	14.15 ±0.73	1.59 ± 0.07	1.72 ±0.08	1.915 ± 0.09
4.	75% Nitrogen alone	5.21 ±0.26	17.05 ± 0.85	20.05 ±1.00	1.67 ±0.08	2.14 ±0.10	2.25 ±0.11
5.	75% N + 25% *Azospirillum*	8.96 ±0.44	25.6 ±1.28	30.65 ±1.53	2.67 ±0.13	3.05 + 0.1	3.40 + 0.17

Dry weight (g plant^{-1})

Inoculation of rice with stress tolerant SFA-1 increases the plant dry matter production over. Un-inoculated control in proportion to the level of nitrogen applied (Table 7). The maximum dry matter production of shoot 3.15 and in root 1.20 (g plant^{-1}) was recorded in the treatment 75% N + 25% *Azospirillum.* The other treatment *viz.*, 100% *Azospirillum*, 100% nitrogen and 75%. Nitrogen slightly increased the dry matter production when compared to control plants.

Table VII: Effect of *Azospirillum* SFA-1 inoculation at graded level of nitrogen on the dry weight of rice ADT 43 (mean value n = 3 replicates)

SI. No.	Treatment	Dry weight of shoot (g plant^{-1})			Dry weight of root (g plant^{-1})		
		30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS
1	Control (saline soil)	0.92 ±0.04	1.16 ±0.05	1.24 ±0.06	0.31 ±0.01	0.46 ±0.02	0.66 ±0.03
2.	100% *Azospirillum* alone	1.07 ±0.05	1.30 ±0.06	1.44 ±0.07	0.52 ±0.02	0.71 ±0.03	0.80 ±0.04
3.	100% Nitrogen alone	1.25 ±0.06	1.46 ±0.07	1.52 ±0.07	1.30 ±0.01	0.55 ± 0.02	0.64 ±0.03
4.	75% Nitrogen alone	1.95 ±0.09	2.15 ±0.10	2.31 ±0.11	0.59 ±0.02	0.74 + 0.03	0.80 ±0.04
5.	75% N + 25% *Azospirillum*	1.59 ±0.07	2.99 ±0.14	3.15 ±0.15	0.74 ±0.03	0.90 ±0.04	0.79 ±0.03

Azospirillum under stress condition enhance the plant growth by fixing atmosphere nitrogen and by the production of growth promoting substance and influencing root development causing increased uptake of minerals from the land and inhibiting pathogenic fungi and bacteria in the rhizosphere [15]. *Azospirillum* inoculation contact significantly increased the growth in terms of height number of leaf plant^{-1}, length and breadth of leaf and fresh and dry weight plant^{-1} of rice plant [16, 17]. *Azospirillum* is known to fix a substantial amount of atmospheric nitrogen and supplies to the crop enhances the fertilizers use efficiency, soil fertility grade and ensures partial saving of nitrogenous fertilizers. The ability of the *Azospirillum* to multiply in the rhizosphere of crop suggests its ability to get better the nutrient availability to the plants and can supplement the expensive inorganic and organic fertilizer.

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