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Studies on Developing Salt Tolerant *Azospirillum* Strains from the Coastal Saline Soils of Tamil Nadu

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A B S T R A C T

Salinity decreases the water absorbing potential of plants, immediately decreases the growth rate, reduction of water stress and reduces the photosynthetic activity in plants. High salinity interfere to plant growth and can also leads to physiological drought conditions and ion toxicity. It can also injure cells in transpiring leaves, which leads to growth inhibition. Among the PGPR *Azospirillum* act as a major role in salinity soil *Azospirillum* is microaerophilic, gram negative and spiral shape bacterium. It is asymbiotic nitrogen fixer which able to fix the atmospheric nitrogen and make it available to plants. It is beneficial to plants through mechanisms related to enhancement of plant growth, increases the mineral uptake, increase the dry matter, improve the water absorption and improve the yield. The main aim of this study is to identify the salt tolerant *Azospirillum* strains from the coastal saline soils. From this study, we isolated *Azospirllum* strains from coastal saline soil which could grow in higher level of pH, NaCl, concentration and temperature range. The *Azospirillum* isolates AZST2, AZST5, and AZST7 were found to have ability to grow under varied conditions like high pH, NaCl concentration and temperature which showed the potential to use them as successful inoculants for coastal saline agriculture.

Introduction

In worldwide, Salinity is an important factor for affecting agricultural crop production. FAO reported that more than 800 million hectares of land and 20% of water irrigated agricultural fields are affected by the salinity in world 2008 (Zhang *et al*.,2018).Salinity decreases the water absorbing potential of plants, immediately decreases the growth rate

,reduction of water stress and reduces the photosynthetic activity in plants (Munns, 2002). High salinity interfere to plant growth and can also leads to physiological drought conditions and ion toxicity. It can also injure cells in transpiring leaves, which leads to growth inhibition (Zhu, 2002). Higher concentrations of sodium ions reduce the photosynthesis and production of reactive oxygen species.

The plant growth promoting rhizobacteria (PGPR) influence the crop productivity, by inhabiting in the rhizosphere region Several PGPR strains are presenting in the rhizosphere soil viz., *Achromobacter*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Microbacterium*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Serratia*, *Streptomyces* etc. PGPR usually improve plant growth promotion by triggering plant growth hormones, antioxidant system, produce siderophore and enhance nutritional capacity of the plants (Numan *et al*., 2018). Among the PGPR *Azospirillum* act as a major role in salinity soil *Azospirillum*is microaerophilic, gram negative and spiral shape bacterium. It is asymbiotic nitrogen fixer which able to fix the atmospheric nitrogen make it available to plants.

It is beneficial to plants through mechanisms related to enhancement of plant growth, increases the mineral uptake, increase the dry matter, improve the water absorption and improve the yield.

Azospirillum can help plants minimize the negative effects of abiotic stresses because, *Azospirillum* can survive the in absence of their host due to the presence of poly-β-hydroxybutyrate (PHB) and polysaccharide synthesis (Ibrahim *et al*., 2012). *Azospirillum* has nitrogenase activity shown to be more sensitive towards salt stress than cellular growth on combined nitrogen. The main aim of this study is to identify the salt tolerant *Azospirillum* strains from the coastal saline soils.

Materials and Methods

Sample collection

Soil samples were collected from coastal saline soil area in Ramanathapuram district of Tamil Nadu. The saline soil was collected from rhizosphere region of plant root in the depth of 15cm. The collected samples were kept in poly bags in laboratory the soil samples and preserved in refrigerator for further use.

Physiochemical parameters of saline soil

The collected soil samples were analysed for major physical and chemical soil quality parameters like pH, Electrical Conductivity (EC), Organic Carbon (OC), Nitrogen (N), Phosphorus (P) Potasium (K). The soil samples were air dried in shade, crush the soil clots lightly and grind with the help of pestle and mortar. Then pass the entire quantity through 2mm stainless steel sieve.

Isolation of *Azospirillum* from root samples

The root samples are washed free of soil particles and cut into small bits of 1-2 cm size. The root bits are first surface sterilized using mercuric chloride (0.1%) for one minute and followed by 80 per cent alcohol for one minute. Then root bits are washed in several times in sterile distilled water to remove the excess of chemicals.

The bits are then aseptically transferred to test tubes (1-2 root bits/test tube) containing 5 ml of Nitrogen Free semi solid Malic acid medium and incubated for 3-5 days at 30º C. The tubes are observed for growth of

Azospirillum after incubation. For comparison uninoculated tubes are maintained as controls. The positive tubes are observed for the development of small white globular subsurface pellicles in the medium. More over the change of colour of the medium from yellowish green to blue is an indication of the growth of *Azospirillum***.**

Characterization of *Azospirillum*

Morphological characterization

The morphological traits *viz.,* colony morphology, cell morphology, gram staining of *Azospirillum* was identified by following the methods of Gerhardt *et al.,* (1981).

Gram staining

Based on the method of Gerhardt *et al.,* (1981) the gram staining was performed for all *Azospirillum* strains. Take a clean glass slide, then thin smear of *Azospirillum* isolates were made on the glass slide, air dried and heat fixed. After heat fixing the smear primary strain (crystal violet) was added and kept for 1 min and washed using tap water/distilled water.

Next to primary stain, Lugol's iodine (mordant) was added and kept for 30 sec, then washed and air dried followed by decolorizer (ethanol/alcohol) was added and kept it for 30 sec and dried.

Atlast counter stain (saffranin) was added and kept it for 30 sec and washed in tap water. Then the slides are air dried and viewed under microscope.

Biochemical characterization

Isolated *Azospirillum* strains were subjected to different biochemical test *viz.,* starch hydrolysis test, catalase test, were carried out.

Starch hydrolysis test

The *Azospirillum* strains were streaked on the plates containing starch agar medium then incubated it for 2-5 days at room temperature. After 2-5 days of incubation the plates were tested by using iodine solution as an indicator (Aneja, 1993). Development of yellow colour around the cultures shows the hydrolysis of starch indicates positive result shows the presence of amylase enzyme and blue colour indicates the negative result.

Catalase test

The *Azospirillum* isolates were streaked on the plates containing nutrient agar and kept for incubation at 30±2ºC for 2-4 days. After incubation few drops of 3% hydrogen peroxide was flooded over the grown cultures, prompt effervescence indicates positive result for catalase test (Gerhardt *et al.,* 1981b).

Growth of *Azospirillum* at different levels of NaCl concentration

Laboratory studies to the tolerance of *Azospirillum* to different NaCl concentration were conducted. For that NFB media plates were prepared with different concentrations of NaCl at 100,200, 300, 400, 500, 600, 700and 800 mM. Thent he isolates were streaked over the plates and incubated for seven days at room temperature.The growth was reported as no growth (-), poor growth (+), medium growth (++), good growth (+++) and very good growth (++++) (Tensingh Baliah and Rajalakshmi, 2015).

Growth of *Azospirillum* at different pH levels

NFB broth was prepared and adjusted to different pH levels by incorporating 1N NaOH or Hcl. Further the broth was transferred to the sterilized test tubes aseptically and inoculated

with the *Azospirillum* isolates separately. After incubation for four days, growth was observed visually in terms of turbidity developed (Usha and Kanimozhi, 2011).

Growth of *Azospirillum* at different temperature levels

The ability of different isolates to grow at different temperature levels was examined. The plates were streaked with different isolates and incubated at temperature ranging from 10 to 50^0C. After four days of incubation, the results were recorded (Tensingh Baliah and Rajalakshmi, 2015).

Results and Discussion

The coastal saline soil samples were collected from 15 different locations of Ramathapuram district of Tamil Nadu. The physico chemical properties of the soil samples were summarized in (Table 1). The soil was sand loamy clay with the maximum pH of 8.86 and minimum pH 7.45.The total nitrogen content (327.60 Kg/ha^{-1}) phosphrous content (140 Kg/ha^{-1}) potassium (531.60 Kg/ha^{-1}) and organic carbon (0.48 g/kg) were recorded.

Isolation of *Azospirillum*

Totally 15 strains were isolated from saline soils of Ramanathapuram district of Tamil Nadu. Observations of morphological characters like motility, vegetative cell shape and staining reaction by *Azospirillum* isolates were presented in Table 2.

Among the isolates, AZST 2, AZST3 , AZST7 , AZST11, AZST12 and AZST14 showed curved shape of cells, while AZST 5 AZST 9 and AZST 13 showed rod shaped. Eight isolates were found to be gram negative and others were gram positive. The motility test, showed that the cells of the selected isolates are motile, which is one of the characteristics of *Azospirillum* (Tilak *et al.,* 2010).

The *Azospirillum* isolates responded differentially to biotin requirement. Eight isolates out of 15 require biotin for their growth. Rest did not require biotin for their growth. The same was also reported by Tensingh Baliah and Rajalakshmi (2015).

Out of 15 strains, 10 strains were able to hydrolyse starch due to presence of enzyme amylase, while rest could not hydrolyse starch. The result was in conformity with the findings of Usha and Kanimozhi, (2011) and Hossain *et al.* (2015).

The growth of *Azospirillum* at different NaCl concentration level was given in the Table- 3. All the isolates grow well in the minimum level of 100 mM NaCl concentration and slowly decreased their growth as the concentration increased. The isolates, AZST2, AZST5, AZST7, AZST10, AZST12, and AZST14 were found to be tolerant to 800mM of NaCl concentration. Other isolates did not even grow at 700mM Nacl concentration.

Though the cultures were isolated from coastal saline soil, they could able to grow up to 600 mM concentrations. The above six isolates were found to have some mechanisms to grow in high concentrations of NaCl. The similar works were carried out by Usha and Kanimozhi (2011).

To study the growth of *Azospirillum* at varying pH levels, experiments were conducted. All the fifteen isolates showed good growth up to the level, 7.5.Almost half of the isolates only could growth in the pH level of 8.0. Tolerance to pH varied within the selected strains of *Azospirillum* (Table 4 and 5).

Table.1 Physico-chemical parameters of coastal saline soil

S,no	Soil	pH	EC	N in Kg/ha^{-1}	P in Kg/ha^{-1}	K in Kg/ha^{1}	OC g/kg
1	S1	8.72	0.15	165.20	40.00	293.4	0.42
2	S2	8.19	0.17	246.40	10.00	399.7	0.44
3	S3	8.46	0.23	170.80	50.00	675.6	0.42
4	S4	8.21	0.9	103.60	10.00	247.7	0.41
5	S5	8.70	0.50	42.00	20.00	248.8	0.42
6	S6	8.22	0.41	81.20	40.00	329.8	0.42
7	S7	8.55	1.45	98.00	10.00	285.3	0.42
8	S8	8.43	0.16	137.20	10.00	332.8	0.44
9	S9	8.45	0.06	117.60	10.00	278.9	0.46
10	S10	8.41	1.03	98.00	140.00	350.5	0.43
11	S11	8.65	0.37	75.60	80.00	369.2	0.41
12	S12	8.69	0.39	137.20	50.00	402.9	0.43
13	S13	8.86	0.17	327.60	30.00	265.3	0.46
14	S14	8.65	0.53	168.00	20.00	294.1	0.45
15	S15	8.89	1.33	95.20	50.00	339.6	0.43

Table.2 Morphologicl and biochemical characteristics of *Azospirillum* isolates

S.NO	Gram staining	Vegetative cell	Motility	Oxidase test	Catalase test	Starch hydrolysis	Biotin require
AZST1	+	curved	+	+	+	+	NR
AZST2	-	curved	+	+	+	_	R
AZST3	+	curved	+	+	+	+	R
AZST4	+	curved	+	+	+	+	NR
AZST5	-	rod	+	+	+	_	R
AZST6	+	curved	+	+	+	+	NR
AZST7	-	curved	+	+	+	_	R
AZST8	+	curved	+	+	+	+	NR
AZST9	-	rod	+	+	+	_	R
AZST10	+	curved	+	+	+	+	NR
AZST11	-	curved	+	+	+	_	NR
AZST12	-	curved	+	+	+	_	R
AZST13	-	rod	+	+	+	+	NR
AZST4	+	curved	+	+	+	_	R
AZST15	+	curved	+	+	+	+	NR

- Negative; + Positive; NR =Not Required and R = Required

Table.3 Growth of *Azospirillum* in different NaCl concentration (mM)

s.no	*Azospirillum* isolates	Growth of *Azospirillum* in different NaCl concentration (mM)							
		0.58 % 100	1.16% 200	1.75% 300	2.3% 400	2.9% 500	3.5% 600	4.0 % 700	4.6% 800
1	AZST 1	+++	++	++	++	++	+	+	_
2	AZST 2	+++	++	++	++	++	++	+	+
3	AZST 3	++	++	++	++	++	++	+	-
4	AZST 4	+++	+++	++	++	+	+	+	+
5	AZST 5	++++	++++	+++	+++	++	++	++	+
6	AZST 6	++++	++++	+++	+++	+++	++	+	+
7	AZST 7	++++	+++	++	++	++	+	_	_
8	AZST 8	++++	+++	+++	++	++	+	+	_
9	AZST 9	+++	++	++	++	++	++	+	
10	AZST 10	++++	+++	+++	+++	++	++	+	+
11	AZST 11	++	++	++	++	+	+	_	_
12	AZST 12	+++	+++	+++	+++	+++	++	+	+
13	AZST 13	++++	+++	+++	++	++	+	_	_
14	AZST 14	+++	+++	+++	+++	++	++	+	+
15	AZST 15	++++	++++	+++	++	++	+	+	_

- Negative; + Positive

Table.4 Growth of *Azospirillum* isolates in different pH levels

S.No	*Azospirillum* Isolates	pH range				
		5.0	6.0	7.0	7.5	8.0
1.	AZST1	+	++	+++	+++	+
2.	AZST2	+	++	+++	+++	+
3.	AZST3	+	++	+++	+++	+
4.	AZST4	+	++	+++	+++	-
5.	AZST5	+	++	+++	+++	+
6.	AZST6	+	++	+++	+++	_
7.	AZST7	+	++	+++	+++	+
8.	AZST8	+	++	+++	+++	+
9.	AZST9	+	++	+++	+++	-
10.	AZST10	+	++	+++	+++	-
11.	AZST11	+	++	+++	+++	-
12.	AZST12	+	++	+++	+++	+
13.	AZST13	+	++	+++	+++	+
14.	AZST14	+	++	+++	+++	+
15.	AZST15	+	++	+++	+++	-

- Negative; + Positive

Table.5 Growth of *Azospirllum* isolates in different temperature level

S.No	*Azospirillum* strains	15°C	20°C	30°C	40°C	50°C
1.	AZST1	+	++	+++	+++	-
2.	AZST2	+	++	+++	+++	+
3.	AZST3	+	++	+++	+++	_
4.	AZST4	+	++	+++	+++	-
5.	AZST5	+	++	+++	+++	+
6.	AZST6	+	++	+++	+++	-
7.	AZST7	+	++	+++	+++	+
8.	AZST8	+	++	+++	+++	-
9.	AZST9	+	++	+++	+++	_
10.	AZST10	+	++	+++	+++	-
11.	AZST11	+	++	+++	+++	-
12.	AZST12	+	++	+++	+++	-
13.	AZST13	+	++	+++	+++	-
14.	AZST14	+	++	+++	+++	-
15.	AZST15	+	++	+++	+++	-

- Negative; + Positive

These variations are mainly due to the nature of strains and the nature soil conditions. The pH ranges for the optimum growth of *A. amazonense*, *A. lipoferum* and *A. brasilense* strains isolated from variety of habitats were found to be 5.7 - 6.5, 5.7 - 6.8 and 6.0 -7.3 respectively (Baldani *et al.*, 1986). The *Azospirillum* isolates were exposed to different temperature levels starting from 15°C to 50°C. All the isolates showed good growth up to 35°C. Only three isolates viz., AZST2, AZST5 and AZST7 were found to grow at 50°C.The results are in agreement with findings of the El-Akhdar *et al.* (2019). The ability to withstand high temperature is one of the important characteristics for an inoculant. Further, these isolates, which were isolated from coastal salinity had twin ablity of tolerance to high temperature level, and high NaCl concentration. Among the plant growth promoting rhizobacteria, *Azospirillum* is very active and popular nitrogen fixer in laboratory as well as in field conditions and improves the growth and yield of the agricultural crops. From this study, we isolated *Azospirllum* strains from coastal saline soils which could grow in higher level of pH, NaCl concentration and temperature range. The *Azospirillum* isolates AZST2, AZST5, and AZST7 were found to have ability to grow under varied conditions like high pH, NaCl concentration and temperature which showed the potential to use them as successful inoculants for coastal saline agriculture

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