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Isolation and Characterization of Salinity Tolerant Free Living Diazotrophs

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Abstract

Twenty seven free living diazotrophs (*Azotobacter* spp.) were isolated from soil samples with different electrical conductivity. These isolates were tested *in vitro* to investigate the ability to tolerate different NaCl concentration, characterized for their different morphological, biochemical and beneficial properties like IAA, ammonia excretion and nitrogen fixed. All the isolate showed 40.7—100% of tolerance to 2—8% of NaCl. The total nitrogen fixed differed and varied from 6—14 mg/g sugar consumed. Out of 27 strains ST3, 6, 9, 17 and 24 showed all highest amount of IAA (5.56—13.62µg/ ml) and ammonia (2.23—4.72 µg/ml) as compared to other strains.

Key words : *Azotobacter,* Salinity tolerance, Nitrogen fixation, Indole acetic acid (IAA).

Saline soil is a major agricultural problem and significantly affects plants and the microbial activity. Agricultural productivity is severely affected by soil salinity and the damaging effect of salt accumulation in soils and has become an important environmental concern. Salinity is known to affect almost all the phases of plant growth from germination to maturity (1). Soil salinity has been a major problem for agricultural production. This problem is increasing at alarming rates due to mismanagement of agricultural practices. It is estimated that 7% area of the world's soil is under salinity (2). The beneficial effects of *Azotobacter* application are largely associated with the protection against root pathogens (3), formation of physiologically active substances of various types (4), stimulation of rhizospheric micro-organisms, modification of nutrient uptake, siderophore production, and enhancement of nitrogen fixation (5). Seed inoculation with *Azotobacter* also inhibited the occurrence of bacterial, fungal and viral diseases of plants. Application of *Azotobacter chroococcum* and *Streptomyces niveus* as reported by Magda et al. (6) to maize plants grown under saline condition, influenced the content of total soluble sugars, total free amino acids, proline, tital soluble proteins, DNA and RNA in shoots and roots resulting in higher salt tolerance of the plants and positive results under different salinity levels were observed (7).

The effects of three salt tolerant *Azotobacter* strains (*A. chroococcum, A. vinelandii* and *A. beijerinckii*) increased the root and shoot dry biomass by 75.8%, 56.12% respectively in *Ceriops decandra* and *Avicennia marina* (8). The inoculation of *Azotobacter* in *Brassica olerancea* var italica and wheat also resulted in greater plant parameters (9, 10). Till now no biofertilizer for various crops are available to function under saline conditions. Therefore the present investigation was undertaken to isolate such type of isolates which can be used as biofertilizer for different crops under saline conditions.

Table 1. Colony characteristics of the various soil isolates. All isolates were found to be Gram negative. Four samples collected.

Colony characteristics	No. of soil isolates		Gram reaction
Large, watery, round, raised	8	(ST1, ST3, ST6, ST7, ST8, ST14, ST15, ST20)	–
Medium, round, watery	7	(ST13, ST16, ST19, ST21, ST23, ST25, ST26)	–
Large, round, opaque	6	(ST12, ST17,ST18, ST22, ST24, ST27)	–
Small, round, raised, opaque	6	(ST2, ST4, ST5, ST9, ST10, ST11)	–

Table 2. Carbon utilization test of various salinity tolerant (ST) soil isolates. 5 + = Excellent, 4 + = Very good, 3+ = Good, 2 + = Fair, + = Average, ± = Poor, – = Negative, Sucrose =100%, Malonate = 92.5%, Rhamnose = 51.85%, Caprylate = 25.9%, Mannitol = 77.7%, Benzoate = 40.7%.

	Soil isolates	Carbon utilization						Starch hydrolysis
		Sucrose	Malonate	Rhamnose	Caprylate	Mannitol	Benzoate	
1.	ST1	5+	+	–	–	2+	–	+
2.	ST2	5+	5+	3+	±	3+	±	–
3.	ST3	5+	5+	–	–	3+	+	+
4.	ST4	5+	5+	3+	–	±	±	–
5.	ST5	5+	5+	2+	2+	2+	+	–
6.	ST6	5+	5+	2+	3+	3+	+	–
7.	ST7	5+	5+	2+	3+	3+	2+	–
8.	ST8	+	5+	2+	±	±	–	–
9.	ST9	5+	5+	+	+	3+	+	–
10.	ST10	5+	5+	–	–	±	–	+
11.	ST11	5+	5+	–	–	±	±	+
12.	ST12	3+	3+	–	–	2+	±	+
13.	ST13	5+	3+	+	±	+	±	–
14.	ST14	5+	2+	+	–	+	+	–
15.	ST15	5+	3+	–	–	+	+	+
16.	ST16	5+	±	–	–	±	+	+
17.	ST17	5+	5+	–	–	2+	+	+
18.	ST18	5+	5+	2+	+	+	+	–
19.	ST19	5+	5+	2+	+	+	–	–
20.	ST20	3+	±	–	–	+	–	+
21.	ST21	4+	3+	–	–	+	±	+
22.	ST22	4+	3+	–	–	2+	±	+
23.	ST23	5+	5+	2+	–	+	–	–
24.	ST24	3+	5+	–	–	+	+	+
25.	ST25	5+	3+	2+	–	2+	–	–
26.	ST26	5+	5+	2+	+	2+	±	–
27.	ST27	5+	5+	–	–	±	±	+

Methods

Soil samples of different electrical conductivity were collected from different villages (such as Dobhi (EC 1.7, 1.1), Shakhpura (EC 3.6) and Kirdhana (2.7) of Haryana state (India). The top layer of soil was gently removed from 0—6 unit depth. Three random samples were collected from one location and mixed together, visible large soil particles were removed mechanically. Soil was used for isolation of free living diazotrophs by enrichment culture technique using Jensen's nitrogen free medium (JM) (11); 10g of soil sample was inoculated in 90 ml JM broth and incubated at 30C for 4—6 days; 10 ml from above were transferred to fresh 90 ml JM broth and incubated at 30C for six days. Samples were serially diluted, plated on JM plates and incubated at 30C for 6 days. Isolated colonies were purified,maintained on JM slants and stored at 4C. Isolates were transferred to fresh JM slants at regular intervals. Composition of Jensen's medium : (g/l, pH 7); sucrose 20.0, $K_2HPO_4$1.00, $MgSO_4$. $7H_2O$.50, NaCl.50, $CaCl_2$.10, $Na_2MoO_4.2H_2O$.005, $FeSO_4.7H_2O$.050 and Agar-agar 20.0g/l in 1000 ml distilled water.

Soil isolates were identified on the basis of their morphological, chosen biochemical properties and plant growth promoting properties according to Bergey's Manual of Systematic Bacteriology (12). Carbon utilization test included various carbon source viz. caprylate, malonate, mannitol, sucrose, rhamnose and benzoate. Starch hydrolysis test was performed for all isolates using basal agar media (13) supplemented with starch. The various *Azotobacter* isolates were tested for their growth on JM media containing different sodium chloride concentrations viz. 2, 4, 6 and 8%. Chosen isolates were grown in JM broth and streaked on plates containing different concentrations of salt (NaCl) and incubated at ± 30C for 3—4 days.

Table 3. Salt tolerance (ST) among various soil isolates. 3+ = Excellent, 2+ = Very good, + = Good, ± = Poor, – = Negative. Growth % : 2% = 100%, 4% = 92.59%, 6% = 74.07%, 8% = 40.7%.

	Soil isolates	NaCl % 2	4	6	8
1.	ST1	+	–	–	–
2.	ST2	3+	2+	2+	2+
3.	ST3	2+	2+	2+	2+
4.	ST4	2+	+	+	–
5.	ST5	2+	±	–	–
6.	ST6	3+	3+	3+	3+
7.	ST7	3+	3+	2+	2+
8.	ST8	2+	2+	2+	+
9.	ST9	2+	2+	2+	±
10.	ST10	2+	2+	2+	+
11.	ST11	2+	+	±	–
12.	ST12	+	±	–	–
13.	ST13	3+	3+	2+	+
14.	ST14	2+	+	–	–
15.	ST15	3+	+	+	–
16.	ST16	+	–	–	–
17.	ST17	2+	2+	2+	±
18.	ST18	+	±	–	–
19.	ST19	3+	2+	+	–
20.	ST20	2+	2+	±	–
21.	ST21	2+	+	±	–
22.	ST22	+	±	–	–
23.	ST23	+	±	±	–
24.	ST24	3+	3+	3+	3+
25.	ST25	3+	3+	2+	+
26.	ST26	3+	2+	+	–
27.	ST27	3+	2+	+	–

Table 4. Production of beneficial properties by various salinity tolerant (ST) soil isolates. * Salkowski method (15). **Method Chaney and Marbach (14). ***Kjeldhal's method (16). ND = Not detected.

	Soil isolates	*IAA (µg/ml) Days of Incubation 4	6	**Ammonia (µg/ml) 4	6	***Total nitrogen (mg/g of sugar consumed)
1.	ST1	3.13	2.23	ND	ND	7.70
2.	ST2	3.73	1.93	ND	ND	8.75
3.	ST3	7.40	3.93	2.23	3.89	12.95
4.	ST4	4.23	1.26	ND	ND	7.35
5.	ST5	4.80	2.06	ND	ND	8.75
6.	ST6	5.56	1.86	2.34	3.12	12.25
7.	ST7	4.13	0.73	ND	ND	10.5
8.	ST8	6.56	2.60	ND	ND	9.10
9.	ST9	10.70	6.60	2.27	3.90	10.15
10.	ST10	7.96	7.26	ND	ND	9.45
11.	ST11	5.20	2.06	2.70	ND	9.45
12.	ST12	7.56	3.93	1.32	ND	8.40
13.	ST13	6.86	0.73	ND	ND	9.45
14.	ST14	5.23	4.80	ND	ND	9.80
15.	ST15	7.23	6.56	ND	ND	10.15
16.	ST16	8.56	6.60	1.80	1.27	8.40
17.	ST17	11.86	8.00	4.29	3.70	12.25
18.	ST18	9.83	8.23	2.30	1.60	8.05
19.	ST19	9.06	7.23	2.06	1.71	8.75
20.	ST20	15.56	10.23	1.90	1.29	11.20
21.	ST21	2.50	2.16	1.78	1.49	7.00
22.	ST22	7.40	2.66	1.58	1.64	8.05
23.	ST23	9.93	7.53	2.33	3.01	7.35
24.	ST24	13.62	9.93	3.99	4.72	14.0
25.	ST25	4.26	3.30	3.85	3.80	8.40
26.	ST26	6.93	1.76	1.26	1.72	6.65
27.	ST27	6.93	6.30	3.57	2.95	9.45

The various beneficial properties like ammonia and indole acetic acid were estimated by Chaney and Marbach method (14) and Salkowski mehod (15) respectively, in all the soil isolates.Total nitrogen isolates were estimated by Kjeldhal's method (16) and was expressed on the basis of mg per g of sugar consumed.

Results and Discussion

Total 27 isolates were isolated from four soils with different electrical conductivity. Ability to grow on Jensen's nitrogen-free medium is accepted as preliminary criterion for the isolation of potential free living nitrogen fixers from soil (17). These soil isolates exhibited different morphological characters and gram—ve reaction (Table 1). Biochemical tests were performed for all soil isolates according to Bergey's manual and (11).

Isolates showed 100% growth on sucrose, 92.5% on malonate, and 77.7% on mannitol, 51.85% on rhamnose, 25.9% on caprylate and 40.7% on benzoate respectively (Table 2). Positive starch hydrolysis was observed by 13 isolates. Specific carbohydrate utilization test hinted that these isolates belong to *Azotobacter* spp. As described in Bergey's manual soil isolates which can not grow on rhamnose, caprylate and can also hydrolyze starch belong to *A. chroococcum* and those growing on rhamnose, caprylate and not hydrolyzing starch belong to *A. vinelandii*. Our results revealed that most of the isolates ST1, ST3, ST10, ST11, ST12, ST15, ST16, ST17, ST20, ST21, ST22, ST24 and ST27 belong to *A. chroococcum*. Rest of the isolates seems to be close

to *A. vinelandii.*

Salt tolerance of all the soil isolates was tested by incorporating different concentrations of NaCl viz. 2—8% and tolerance pattern was observed in the following manner; 100 at 2%, 92.59 at 4%,74.07 at 6% and 40.7% at 8% NaCl (Table 3). Tolerance of the isolates to NaCl was not found to be related to the place of origin from where they were isolated as no specific pattern emerged. Our work is supported by Sayeb and Hasnain (18).

Fixed nitrogen was obtained in the range of 5—15 mg/g sugar consumed. Maximum nitrogen was fixed by ST24 (14 mg/g sugar consumed) followed by ST3. Maximum IAA production was observed by ST20 (15.56 µg/ml), followed by ST24 (13.16), ST17 (11.83) and ST9 (10.70). ST24 was found to be the highest ammonia excretor (4.72µg/ml) among all the salinity tolerant soil isolates tested (Table 4). Possession of these traits favors their use as a biofertilizer for non-legumes and cereals (19—21). The use of *Azotobacter* as a bioinoculant has economic relevance which will increase further with the intensification of research and development. To see the full effect as biofertilizer of best isolates obtained during our studies, it is suggested that isolate should be tested under field in saline soil condition.

Also appropriate management practices under different stress conditions like salinity, sodicity and high temperature need to be provided to ensure maximum contribution from bioinoculants.

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