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RESEARCH ARTICLE

EFFECT OF SALT CONCENTRATIONS ON THE GROWTH AND EFFICIENCY OF THERMOTOLERANT *Azospirillum* STRAINS UNDER SALT STRESS CONDITIONS

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Abstract

Bacteria of the genus *Azospirillum* are widely distributed in soil and associated with the roots of forage grasses, cereals and non gramineous plants. *Azospirillum* sp. are widely distributed soil nitrogen fixing bacteria that play an important role in the promotion of plant growth. The genus *Azospirillum* comprises free - living nitrogen fixing rhizosphere bacteria to a group that exerts beneficial effects on plant growth, namely the plant growth promoting rhizobacteria. In the present study, five different thermotolerant *Azospirillum* strains and Reference strain Sp – 7 were tested for their NaCl tolerance level in broth culture condition by adding different concentrations of NaCl *viz*., 0.0%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. Maximum growth, Acetylene reductase activity and Phytohormones production was observed in the *Azospirillum* isolate MZA-36 at 0.0% NaCl concentration followed by MZA – 13, MZA – 4, MZA – 3 and MZA – 2. Best results were noticed at 0.0% NaCl concentration and the least results were observed at 3.0%.

Keywords: Salt stress, Thermotolerant *Azospirillum*, Growth, Acetylene reductase activity and Phytohormones production.

Introduction

Salinity became a serious problem for agriculture, all over the world. Salinity, water shortage and low water quality are the main problems for agriculture production. Under such circumstances, salt stress reduces the free energy of water in soils available to plants and results in negative water potential in soils. This drop in water potential is accompanied by specific ion toxicities, deficiencies, retardation of water uptake and nutritional imbalances in plants which affect enzymatic and physiological functions reducing growth and yield of crops (Shanon, 2013). Salinity is one of the most serious critical constraints and environmental problems influencing crop growth throughout the world (Ravikumar, 2008). In India, out of an estimated area of 187.7 million ha of total degraded lands, 8.1 million ha are salt affected in which 3.1 million ha are in the coastal regions (Tripathi *et al*., 2007).

Salinity is a major factor reducing crop productivity and a major cause of the abandonment of lands and aquifers for agricultural purposes. Developing salt -tolerant crops has been a much desired scientific goal but with little success to date, as few major-determinant genetic traits of salt tolerance have been identified (Munns and Tester, 2008; Schubert *et al*., 2009). An alternative strategy to improve crop salt tolerance may be to introduce salt-tolerant microbes that enhance crop growth. As soil microbes have enhanced the growth of many different crops grown in a wide range of root-zone salinities as discussed below, this approach may succeed where it has proved difficult to develop salt-tolerant germplasm. Indeed, several recent studies have demonstrated that local adaptation of plants to their environment is driven by genetic

differentiation in closely associated microbes (Rodriguez and Redman, 2008).

Plant growth promoting rhizobacteria play a key role in nutrient cycling and maintenance of soil fertility, and establish positive interactions 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 (Bilal *et al*., 2014). Colonization of the plant roots by bacteria is affected by biotic and abiotic factors such as dynamics of microbial populations, plant characteristics and soil types. Alterations in the biological or chemical environment of the rhizosphere are expected to influence plant health as many rhizosphere microorganisms mobilize nutrients, produce phytohormones and suppress pathogens.

Bacteria of the genus *Azospirillum* are widely distributed in soil and associated with the roots of forage grasses, cereals and non gramineous plants (Bashan and Holguin, 1997). *Azospirillum* sp. are widely distributed soil nitrogen fixing bacteria that play an important role in the promotion of plant growth (Steenhoudt and Vandeleyden, 2000). The genus *Azospirillum* comprises free - living nitrogen fixing rhizosphere bacteria to a group that exerts beneficial effects on plant growth, namely the plant growth promoting rhizobacteria (PGPR). Because of those properties, numerous studies on *Azospirillum* ecology, physiology and biochemistry have been carried out during the past 15 years (Vande Broek and Vandeleyden, 1995). *Azospirillum* are widespread in soils and comprise diverse diazotrophic rhizobacteria stimulating plant growth and development of polysaccharide components of the surface *Azospirillum* play an important role in the formation of associations with other rhizobacteria (Yogorenenkova *et al*., 2001).

Several mechanisms, other than Biological Nitrogen Fixation mentioned above, have been postulated to explain how *Azospirillum* enhances growth and development of plants, such as phytohormone production and nitrate reduction (Steenhoudt and Van der Leyden, 2000). Nevertheless, to date no unique mechanism had been established to explain the growth promotion capability of these bacteria. Instead, the most accepted hypothesis postulates that a sum of events accounts for the general plant growth promotion effect (Bashan and Holguin, 1997).

Materials and Methods

The growth efficiency of thermotolerant strains were studied at the salinity levels of 1.0, 2.0, 2.5 and 3.0 per cent NaCl concentrations respectively.

Effect of salt concentrations on the growth of thermotolerant *Azospirillum* strains

The Nfb broth without bromothymol blue was prepared and distributed in 100 ml quantities into 250 ml Erlenmeyer flasks and supplemented with different levels of NaCl *viz.,* 1.0, 2.0, 2.5 and 3.0 per cent and sterilized. The flasks were inoculated with 1 ml of standard inoculum of thermotolerant *Azospirillum* isolates and reference strain and incubation at room temperature with intermittent shaking to maintain aerobic condition. After 72 hrs incubation period, the growth was assessed by serial dilution and plating method.

Effect of salt concentrations on Acetylene reduction activity of thermotolerant *Azospirillum* strains

Nitrogen free malate medium supplemented with yeast extract at 100 mg^{-1} was prepared and dispensed in 30 ml quantities in 300 ml penicillin vials and different levels of NaCl *viz*., 1.0, 2.0, 2.5 and 3.0 per cent were also added and sterilized. After cooling, the vials were inoculated with 1.0 ml of standard inoculum of each of thermotolerant strains of *Azospirillum* and incubated at room temperature for 3 days under static conditions and the ARA and cell protein were determined .

Effect of salt on the growth hormone production by thermotolerant *Azospirillum* strains

Indole acetic acid production

A quantity of 100 ml of nitrogen free malate broth (without bromothymol blue indicator) was prepared and sterilized. Freshly prepared, filter sterilized solution of L-tryptophan was added to each flask to a final concentration of 100 mg L^{-1}. One ml of culture broth of *Azospirillum* isolates were inoculated to each flask and incubated at 37°C in dark for seven days.

After incubation, the cultures were centrifuged at 6000 rpm for 5 min to remove the bacterial cells. The supernatant was brought to pH 2.8 with 1 N HCl. Fifteen ml of the acidified supernatant was taken in 100 ml conical flask and to it equal volume

of diethyl ether was added and incubated in dark for 4 h. IAA extraction was done at 4°C in a separating funnel using diethyl ether. The organic phase was discarded and the solvent phase was pooled and evaporated to dryness. To the dried material, two ml of methanol was added, pooled and the IAA present in the methanol extract was determined using the method of Gorden and Paleg (1957). To 0.5 ml of the methanol extract, 1.5 ml of distilled water and four ml of Salper's reagent (1.0 ml of 0.5 M $FeCl_3$ in 50 ml of 35 per cent perchloric acid) were added and incubated in dark for one hour. The intensity of pink colour developed was read at 535 nm in a spectrophotometer. From a standard curve prepared with known concentrations of IAA, the quantity of IAA in the culture filtrate was determined and expressed as µg per ml of culture medium.

Gibberellic acid production

The thermotolerant strains of *Azospirillum* were aseptically cultured in 500 ml N free malate medium (NFb) with 1 mM NH_4Cl under shaking condition for 7 days at 30ºC, 40ºC, 50ºC and 55ºC. The seven days old culture was centrifuged for 15 min at 800 xg and the supernatant was taken. The cell pellet was reextracted with phosphate buffer (pH 8.0) and again centrifuged. Both supernatants were pooled, acidified to pH 2.5 using 5N HCl and partitioned with equal volumes of ethyl acetate for five time. The ethyl acetate phase was dried at 32ºC and the residue redissolved in 2 ml of distilled water containing 0.05 per cent Tween 80. Fifteen ml ethyl acetate fraction was taken and 2 ml of zinc acetate solution was added. After 2 min, 2 ml of potassium ferrocyanide solution was added and the mixture was centrifuged at 8000 x g for 10 min. Five ml of supernatant was added to 5 ml of 30 per cent hydrochloric acid and the mixture was incubated at 20ºC for 75 min. The blank was prepared with 5 per cent hydrochloric acid. The absorbance was measured at 254 nm in spectronic 1001 spectrophotometer.

Results and Discussion

Soil salinity is an important limiting factor in agriculture economy. In addition to traditional breeding and genetic modification of plants (Blumwald, 2000), recent focus of research involves implication of plant growth promoting rhizobacteria to combat salt stress. The plant growth promoting rhizobacteria increase water use efficiency, fresh and dry weight of plants (Mayak *et al*., 2004) and render the plants more tolerant to salt stress by improving antioxidant status and physiological response (e.g proline used as osmoregulant) of plants (Han and Lee, 2005). PGPR also produce several other growth promoting substances including IAA, GA_3, zeatin and ABA (Perrig *et al*., 2007).

The selected five different thermotolerant *Azospirillum* strains and Reference strain Sp – 7 were tested for their NaCl tolerance level in broth culture condition by adding different concentrations of NaCl *viz*., 0.0%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% for a period of 96 hours and the final populations were estimated and the findings were tabulated in Table - 1. The growth of thermotolerant strains were in the order of MZA -36 > MZA – 13 > MZA – 4 > MZA – 2 > MZA – 3. The increase of salt concentration decreased the growth of thermotolerant *Azospirillum* isolates. Maximum growth was noticed at 0.0% NaCl concentration followed by 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. Highest growth was recorded by the *Azospirillum* isolate MZA – 36 (8.15×10^8 cfu ml^{-1}) followed by the Reference strain (8.03×10^8 cfu ml^{-1}) and the lowest growth was recorded by the isolate MZA – 3 (7.00×10^8 cfu ml^{-1}).

Zahran (1999) found that the nitrogen fixing bacteria was able to grow in media containing 300 mM NaCl. Ravikumar *et al*. (2002) found that the *Azospirillum brasilense* could tolerate the concentration 3% in the medium on media containing 3% NaCl. Siddiqui *et al*. (2003) also reported that high level of salinity may cause a dramatic fall in bacterial populations in the rhizosphere and roots and consequently may reduce the growth-promoting potential on plants. Similarly, Ravikumar *et al.* (2004) reported that some types of *Azotobacter* isolated from mangrove sediments (*A. chroococcum, A. berijerinkii, A. vivelandii*) and *Azospirillum* (*A. lipoperum, A. brasilense, A. haloprefens* and *A. irakense*) tolerant to high salinity concentrations upto 35 g/L.

The effect of thermotolerant *Azospirillum* strains on Acetylene reduction assay was estimated in comparison with Reference strain Sp – 7 at different concentrations of NaCl *viz*., 0.0%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% and the results were furnished in Table – 2. The ARA activity of thermotolerant strains was found to be gradually decreasing with increase in NaCl concentration. Maximum ARA activity was observed at 0.0% NaCl concentration followed by 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. At 0.0% NaCl concentration, highest

Table - 1: Effect of NaCl on the growth of thermotolerant *Azospirillum* isolates

S. No.	*Azospirillum* Isolate Number	*Azospirillum* population x 10^8cfu ml^{-1}					
		Levels of NaCl (%)					
		0.0	1.0	1.5	2.0	2.5	3.0
1	MZA-2	7.53	5.32	4.80	1.12	0	0
2	MZA- 3	7.00	4.95	3.90	1.05	0	0
3	MZA-4	7.62	6.50	5.05	1.70	1.38	0
4	MZA-13	7.90	7.75	7.36	2.80	1.96	0
5	MZA-36	8.15	8.10	7.95	4.50	3.29	2.05
6	Sp -7	8.03	7.80	7.50	3.35	2.28	1.12
SE_D		0.17	0.55	0.69	0.56	0.53	0.35
CD (P = 0.05)		0.35	1.10	1.38	1.12	1.07	0.72

Table - 2: Effect of NaCl on the ARA of thermotolerant *Azospirillum* isolates

S. No.	*Azospirillum* Isolate Number	ARA (n moles of ethylene formed $h^{-1}mg^{-1}$ protein)					
		Levels of NaCl (%)					
		0.0	1.0	1.5	2.0	2.5	3.0
1	MZA-2	227.3	175.3	158.7	15.8	1.6	0
2	MZA- 3	213.5	152.3	121.5	50.4	0	0
3	MZA-4	242.6	200.3	183.4	10.6	2.5	1.4
4	MZA-13	290.7	243.3	220.5	116.5	5.2	3.3
5	MZA-36	337.4	326.4	306.4	142.1	30.7	15.6
6	Sp -7	306.2	282.5	272.8	120.30	10.6	5.3
SE_D		20.03	27.13	28.58	23.47	4.70	2.41
CD (P = 0.05)		40.06	54.26	57.16	46.94	9.40	4.82

ARA activity was recorded by the *Azospirillum* isolate MZA – 36 (337.4 n moles of ethylene formed h^{-1} mg $^{-1}$of protein) followed by the Reference strain Sp - 7 (306.2 n moles of ethylene formed h^{-1} mg $^{-1}$of protein) and the lowest ARA activity was recorded by the isolate *Azospirillum* MZA – 3 (213.5 n moles of ethylene formed h^{-1} mg $^{-1}$of protein).

The ARA in *Setaria italica* inoculated plants increased from 27°C to 32°C (Kapulnik *et al.,* 1981). Albrecht *et al.* (1989) reported that variation in temperature from 24 to 40°C had only a small influence on nitrogen fixation by *Azospirillum.* Wani, (1990) reported that the sorghum plants inoculated with *Azospirillum* recorded higher acetylene reduction activities at 34°C and 4°C than the plants incubated at 29°C.

The IAA (indole-3-acetic acid) is the member of the group of phytohormones and is generally considered the most important native auxin (Ashrafuzzaman *et al*., 2009). It functions as an important signal molecule in the regulation of plant development including organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, and gene regulation (Ryu and Patten, 2008). Diverse bacterial species possess the ability to produce the auxin phytohormone IAA. Different biosynthesis pathways have been identified and redundancy for IAA biosynthesis is widespread among plant - associated bacteria. Interactions between IAA - producing bacteria and plants lead to diverse outcomes on the plant side, varying from pathogenesis to phytostimulation.

Azospirillum uses tryptophan as precursor for IAA production (Reynders and Vlassak, 1982). *A. brasilense* produced extremely high amounts of IAA compared to *A. lipoferum* (Horemans *et al*., 1986). On the other hand, it was contradicted stating that the concentration of IAA was low during logarithmic growth phase and increased rapidly with the beginning of stationary phase (Baca *et al*., 1994). Confirmatory studies on IAA production by several strains of *Azospirillum* showed that production depended on the type of culture media and availability of tryptophan as a precursor. *A. brasilense* produced the highest level of 380 µ mol of IAA L^{-1} among the strains

Table - 3: Effect of NaCl on the IAA production of thermo tolerant *Azospirillum* isolates

S. No	*Azospirillum* Isolate Number	IAA produced (µg ml^{-1}) Levels of NaCl (%)					
		0.0	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**
1	MZA-2	8.4	6.0	4.4	1.0	0.6	0
2	MZA- 3	8.0	5.2	3.3	0.4	0	0
3	MZA-4	9.8	7.2	4.7	2.5	1.2	0
4	MZA-13	13.2	9.2	8.5	4.9	2.8	0.8
5	MZA-36	13.9	11.3	10.6	7.2	6.1	2.2
6	Sp -7	13.6	10.0	9.4	5.8	4.7	1.5
SE_D		1.11	0.97	1.24	1.12	0.99	0.38
CD (P = 0.05)		2.23	1.94	2.48	2.25	2.00	0.75

Table - 4: Effect of NaCl on the GA production of thermo tolerant *Azospirillum* isolates

S. No	*Azospirillum* Isolate Number	GA produced (µg L^{-1}) Levels of NaCl (%)					
		0.0	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**
1	MZA-2	7.5	5.2	3.8	2.7	1.8	0.4
2	MZA- 3	6.3	4.8	2.6	1.5	0.9	0
3	MZA-4	8.2	5.6	4.4	3.6	2.2	0.6
4	MZA-13	9.4	8.6	7.8	4.3	2.8	1.3
5	MZA-36	11.5	10.9	10.2	6.0	5.2	2.7
6	Sp -7	10.4	9.6	9.2	5.2	4.3	2.0
SE_D		0.78	1.05	1.28	0.67	0.65	0.42
CD (P = 0.05)		1.57	2.10	2.56	1.34	1.30	0.85

tested (El-Khawas and Adachi, 1999). The pH has a significant effect on the amount of IAA produced (Ona *et al*., 2003).

The effect of thermotolerant *Azospirillum* strains on phytohormones production (Indole acetic acid & Gibberrelic acid) was studied in comparison with Reference strain Sp – 7 at various NaCl levels *viz*., 0.0%, 1.0%, 1.5%, 2.0%, 2.5% & 3.0% and the results were showed in Table – 3 and Table - 4. The phytohormones production of thermotolerant strains were in the order of MZA -36 > MZA – 13 > MZA – 4 > MZA – 2 > MZA – 3. Maximum phytohormones production was observed at 0.0% NaCl concentration followed by 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. At 0.0% NaCl concentration, highest phytohormones production was recorded by the *Azospirillum* isolate MZA – 36 (Indole acetic acid – 13.9 µg ml^{-1} & Gibberrelic acid – 11.5 µg L^{-1}) followed by the Reference strain Sp - 7 (Indole acetic acid – 13.6 µg ml^{-1} & Gibberrelic acid – 10.4 µg L^{-1}) and the lowest phytohormones production was recorded by the isolate *Azospirillum* MZA – 3 (Indole acetic acid – 8.0 µg ml^{-1} & Gibberrelic acid – 6.3 µg L^{-1}).

The results obtain about strains growth on medium with different concentrations of NaCl are similar to result demonstrated by Usha *et al*. (2011) and indicating in the presence of 700 mM NaCl that the increase in growth rate was purely osmotic and not the result of specific ions present in high concentration. It has been proven that the mode of action of many PGPR is to increase the availability of nutrients for the plant, it is mainly the trais PGPR, as the production of IAA, GA_3, siderophores and solubilization of phosphates than some strains that have promote the growth of wheat.

Cassan *et al*. (2009) identified and quantified GA_3 on Gas chromatography – Mass spectrometry with selective ion monitoring (GC-MS) produced by *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109. Alonso-Ramirez *et al*. (2009) have reported that GA-responsive gene and exogenous addition of Gas are able to counteract the inhibitory effects of different adverse environmental conditions in seed germination and

seedling growth of Arabidopsis through modulation of SA biosynthesis. The same trend was also observed by Hamayun *et al*. (2010) and Iqbal and Ashraf (2007).

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