Salt tolerant mutant of *Anabaena doliolum* exhibiting efficient ammonium uptake and assimilation

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ABSTRACT

Effect of salinity (NaCl, 100 mM) on growth, nitrate reductase (NR) and glutamine synthetase (GS) activities, and uptake of NH_4^+ was studied in the wild type (WT) and the NaCl-tolerant mutant type (MT) of cyanobacterium *Anabaena doliolum*. Results obtained in the presence of salt showed significant reduction in the growth rate of both WT and MT cells of *A. doliolum* by about 77.8 and 40 %, respectively over without NaCl. Similarly rate of NR activity in both WT and MT strains was reduced by 45.5 and 44.5 %, respectively. On the contrary, rate of GS activity of both the WT and MT strains in the presence 100 mM of NaCl increased by 34 and 159 %, respectively. The results of this study indicate that tolerance to NaCl in *A. doliolum* is more dependent on NH_4^+ assimilation rather than on nitrate assimilation in relation to N-metabolism. The increased GS activity in MT cells of the cyanobacterium is possibly because of high rate of energy dependent NH_4^+ uptake. [Physiol. Mol. Biol. Plants 2009; 15(4) : 377-381] E-mail : dpsingh_lko@yahoo.com

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Abbreviations : ADP – Adenosine di-phosphate; GS - Glutamine synthetase; NR - Nitrate reductase; NTG -N-methyl-N-nitro-N-nitrosoguanidine; CCCP - Carbonyl. cyanide m-chlorophenyl hydrazone; DNP – 2, 4-Dinitrophenol; WT – Wild type; MT – Mutant type

INTRODUCTION

Increasing soil salinity in many parts of the world has become a major problem for plant productivity (Egamberdieva *et al.*, 2008). The widespread distribution of cyanobacteria under extreme conditions makes them a good tool to study the effect of stress conditions on cell metabolism. Inorganic forms of nitrogen nutrients are taken up by the plant cells through the membrane bound transport systems (Loque *et al.*, 2007; Li *et al.*, 2007), which are subsequently incorporated in amino acids and proteins (Masclaux-Daubresse *et al.*, 2006). In relation to uptake of nitrogen nutrients, it has been found that the highly specific membrane transporters are involved, which do not allow the transport of other nutrients through the same transport system as in the case of NH_4^+ transport (Gazzarrini *et al.*, 1999).

Though there are several experimental evidences about the impact of salinity on NH_4^+ uptake and assimilation in higher plants (Salehi *et al.*, 2008; Abdolzadeh *et al.*, 2008), such studies are rare in case of cyanobacteria. Apte and Bhagwat (1989) reported synthesis of salinity-stress-induced proteins in two nitrogen-fixing *Anabaena* strains. Recently, Soltani *et al.* (2007) have reported the effect of salinity on growth, photosynthesis, nitrogenase activity and antimicrobial effects in cyanobacterium *Fischerella* sp. FS18. (There is need to know about the response of nitrogen assimilation of cyanobacterium to NaCl stress due to reported involvement of nitrogen nutrition in amelioration of the NaCl toxicity to cyanobacteria) (Apte and Bhagwat, 1989; Waditee *et al.*, 2001; Soltani *et al.*, 2007). Further, it would be interesting to study the effect of salinity stress on the membrane transport of NH_4^+ , when the primary target of salinity is the cyanobacterial membrane system (Niu *et al.*, 1995; Binzel *et al.*, 1988).

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The present work is an effort to study the effect of NaCl-salinity stress on NH_4^+ uptake and key enzymes of nitrogen assimilation i.e., NR and GS in WT and NaCl-tolerant MT cells of cyanobacterium *A. doliolum*.

MATERIALS AND METHODS

Organism and growth condition

The seed culture of cyanobacterium *Anabaena doliolum* was obtained from the "Centre for Conservation and Utilization of blue green algae." Indian Agriculture Research Institute (New Delhi). Axenic culture of the *A. doliolum* was routinely grown in the Allen and Arnon's medium at $25 \pm 1^\circ\text{C}$ with 14/8 hours light/dark cycle (light intensity of 10 W m^{-2}).

Growth of the *Anabaena* was measured in terms of increase or decrease in the turbidity of the cultures at 665 nm by using NH_4Cl (1.5 mM) as inorganic nitrogen source. Specific growth rate was calculated in the absence and presence of NaCl (100 mM). The nitrogen starvation of *Anabaena* cells was carried out for 48 hours before NH_4^+ uptake studies. The protein concentration was measured by method described by Lowry *et al.* (1951).

Isolation of Mutants

Exponentially growing cells of *A. doliolum* were mutagenized by using N-methyl-N-nitro-N-nitrosoguanidine (NTG) as described by Golden (1958). Rest of the conditions were the same as those described earlier (Singh and Kshatriya, 2002). Mutant was screened by using lethal doses of NaCl (400 mM).

Measurement of NH_4^+ uptake

The NH_4^+ uptake was measured in terms of loss of NH_4^+ from the external medium. Rate of NH_4^+ uptake was calculated in terms of $\text{nmol NH}_4^+/\text{mg protein/hr}$. The uptake was initiated with the addition of 100 mM of NH_4Cl . Samples were withdrawn at regular intervals and were centrifuged ($3000 \times g$, 5 min). The NH_4^+ concentration was determined according to Herbert *et al.* (1971). Cells were suspended in the HEPES buffer, cell culture grade (10 mM, pH 6.5) to see the effect of NaCl (100 mM) on the NH_4^+ uptake. NaCl (100 mM) and metabolic inhibitors were added to the uptake assay mixture prior to the addition of NH_4^+ . The experimental flask without NaCl and inhibitors served as control.

Measurement of NR and GS activity

Activity of NR enzyme (EC 1.6.6.3) was measured by the method as described by Manzano *et al.* (1976). NR activity was expressed in terms of $\text{n mol NO}_2^-/\text{formed/mg protein/hr}$. The Mn^{2+} dependent γ -glutamyl transferase (L-glutamate ammonia ligase ADP forming) was measured by determining the rate of γ -glutamyl hydroxamate formed/mg protein/hr by using the method as described by Shapiro and Stadtman (1970).

Statistical analyses

Statistical analyses were done on all samples ($N=6$). The values reported are means of two sets of three repeated observations $\pm 1\text{SE}$. The effect of NaCl and metabolic inhibitors on NH_4^+ uptake, NR and GS activity was determined through ANOVA.

RESULTS AND DISCUSSION

NH_4Cl dependent growth of *Anabaena doliolum* cells

Time dependent growth (up to 7 days) of both the MT and WT cells of *A. doliolum* were measured with or without NaCl (100 mM) and NH_4Cl (1.5 mM) as a nitrogen source (Table 1). The NaCl toxicity was more pronounced in the WT cells and its growth was reduced by approximately 77.8 % as compared to control (without NaCl). On the other hand growth of mutant cells responded to NaCl (100 mM) with growth reduction by approximately 40 %.

Photoautotrophs exposed to salinity stress condition mainly encounter two problems i.e. intracellular osmotic adjustment of the cell cytoplasm and ionic interference in the nutrient uptake (Reed *et al.*, 1985). However, plasma membrane is an important component (Binzel *et al.*, 1988) that can be the primary target of salinity stress irrespective of the mode of NaCl action. The earlier reports on the cyanobacteria *Nostoc muscorum* and *Fischerella* sp FS18 have shown NaCl induced decline in growth rate with increasing salinity (Bhargava and Singh, 2006; Soltani *et al.*, 2007). Results on the growth behaviour of WT and MT cells in the presence of combined nitrogen source (NH_4^+) exhibited differential response of both strains towards NaCl, due to more salinity tolerance by the MT cells.

NR and GS activity

The results on NR activity showed NaCl induced decrease in the NR activity of both the WT and MT cell by approximately 45.5 % and 44.5 %, respectively

Table 1. Effect of NaCl on specific growth rate (μ /h), nitrate reductase (NR) activity (n mol NO_2^- /mg protein/h) and Transferase assay of glutamine synthetase (GS) activity (n mol- γ -glutamyl monohydroxamate /mg protein/h) measured in both the wild and mutant cells of *Anabaena doliolum*. Values are means of two sets of three repeated observations $\pm$ 1 SE (N=6).

Parameters	WT cells	MT cells	Significance level
Growth rate (without NaCl)	0.18 $\pm$ 0.08	0.15 $\pm$ 0.05	$P < 0.01$
Growth rate (100 mM NaCl)	0.04 $\pm$ 0.01	0.09 $\pm$ 0.03	$P < 0.001$
NR activity (without NaCl)	0.22 $\pm$ 0.12	0.18 $\pm$ 0.09	$P < 0.001$
NR activity (100 mM NaCl)	0.12 $\pm$ 0.02	0.10 $\pm$ 0.03	$P < 0.01$
GS activity (without NaCl)	32 $\pm$ 0.29	52 $\pm$ 0.54	$P < 0.001$
GS activity (100 mM NaCl)	83 $\pm$ 0.87	70 $\pm$ 0.67	$P < 0.001$

WT = Wild type; MT=Mutant type

(Table 1). On the contrary, the GS activity was found to be stimulated by NaCl (100 mM) in both strains. The extent of NaCl induced stimulation of GS activity in the WT cells (159 %) was higher than that of the MT cell (34.6 %) when compared with their corresponding control values obtained in the absence of NaCl. Many findings have revealed that results obtained concerning the effect of NaCl on NR activity are not always consistent (Aslam *et al.*, 1984; Martinez and Cerda, 1989). In the light of present findings, it could be suggested that NR enzyme in both the WT and MT cells respond to NaCl stress in a similar manner,

irrespective of the level of salinity tolerance. However, results on GS activity in both the WT and MT cells are found to be reverse to NR activity in the presence of NaCl. The result may be interpreted in terms of NaCl induced nitrogen starvation like condition within the cells as evident from the reduced rate of NR activity. Similar reports have appeared regarding the stress induced decrease in the rate of NR activity leading to nitrogen deficiency within the cells (Louchli *et al.*, 1990). However, increased GS activity in presence of NaCl could be interpreted as more intracellular demand for nitrogen as suggested by Berteli *et al.* (1995).

Table 2. Effect of metabolic inhibitors on NH_4^+ uptake (nmol NH_4^+ / mg protein/hr) in wild and mutant type strains of *Anabaena doliolum* in the presence of NaCl (100 mM). The rate of NH_4^+ uptake obtained in the absence of NaCl and inhibitors was taken as control (100 %). The values in parentheses indicate the % uptake in relation to the control. Values are means of two sets of three repeated observations $\pm$ 1 SE (N=6).

Metabolic Inhibitors	NH_4^+ uptake (n mol/ mg protein/hr)		Significance level
	WT cells	MT cells	
Control (without NaCl)	25.3 $\pm$ 0.97 (100)	20.3 $\pm$ 0.76 (100)	$P < 0.001$
NaCl (100 mM)	8.2 $\pm$ 0.24 (56.2)	12.3 $\pm$ 0.34 (80.0)	$P < 0.001$
NaCl + CCCP (10 μ M)	6.0 $\pm$ 0.21 (43.8)	7.5 $\pm$ 0.34 (53.2)	$P < 0.001$
NaCl + DNP (100 μ M)	14.5 $\pm$ 0.43 (87.2)	15.0 $\pm$ 0.46 (86.8)	$P < 0.01$
NaCl + Monensin (50 μ M)	16.3 $\pm$ 0.76 (70.9)	18.0 $\pm$ 0.87 (87.4)	$P < 0.001$

WT = Wild type; MT=Mutant type

Effect of metabolic inhibitors

Since, the cyanobacterial membrane is the primary target of the NaCl stress, it was interesting to study NH_4^+ uptake property of *A. doliolum* in the presence of NH_4^+ (100 μM) alone or in combination with the NaCl (100 mM). Results (Table 2) showed that the NH_4^+ uptake rate in the WT cells was drastically reduced (by approximately 43.8 %), while the rate of uptake in the MT cells registered lesser decline (by 20 %) in the presence of NaCl, when the values were compared with respective control (without NaCl, 100 %).

To elicit the mode of NaCl induced inhibition of NH_4^+ uptake, different metabolic inhibitors like CCCP (10 μM), DNP (100 μM) and Monensin (50 μM) were used. However, the effect of CCCP was more pronounced in the WT cells than MT cells. On the contrary, addition of DNP (100 μM) - an inhibitor of phosphorylation, could revert the effect of NaCl on NH_4^+ uptake in both the WT and MT cells to the level of approx. 87 % as compared to the control (without NaCl, 100 %). Monensin (50 μM) - an inhibitor of Na^+ transporting channel, also caused reversal of the inhibitory effect of NaCl as compared to the control. Taken together, effects of these inhibitors were more pronounced in the WT than the MT cells. Results on the NaCl induced inhibition of NH_4^+ uptake showed a reduced susceptibility of uptake process of the MT cells towards NaCl as compared to that of the WT, due to an elevated level of NaCl tolerance of MT. Use of metabolic inhibitors suggested that NaCl induced inhibition of NH_4^+ uptake in the *Anabaena* is mediated by Na^+/H^+ anti-port system as evident from the inhibitory effect of Monensin - an inhibitor of Na^+/H^+ anti-port system (Karpel *et al.*, 1991). The DNP induced reversal of NaCl effect on the NH_4^+ uptake might be due to its effect on the excess accumulation of proton, which regulates the intake of both the Na^+ as well as NH_4^+ ions.

From the above discussion, it was deduced that NaCl has relatively more pronounced effect on NH_4^+ uptake and NR and GS activities of WT cells. While the lesser susceptibility of the MT to NaCl as compared to WT might be explained based on increased NaCl tolerance of the MT cells.

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REFERENCES

- Abdolzadeh A, Shima K, Lambers H and Chiba K (2008). Change in uptake, transport and accumulation of ions in *Nerium oleander* (rosebay) as affected by different nitrogen sources and salinity. *Ann. Bot.*, 102: 735-46.
- Apte SK and Bhagwat AA (1989). Salinity stress induced protein in two N_2 -fixing *Anabaena* strains differentially tolerant to salt. *J. Bacteriol.*, 171: 909-915.
- Aslam M, Huffaker RC and Rains DW (1984). Early effects of salinity on nitrate assimilation in barley seedlings. *Plant Physiol.*, 76 : 321-325.
- Berteli F, Corrales E, Guerrero C, Ariza MJ, Pliego F and Valpuesta V (1995). Salt stress increases ferredoxin dependent glutamate synthase activity and protein level in the leaves of tomato. *Plant Physiol.*, 93: 259-264.
- Bhargava S and Singh K (2006). Differential response of NaCl-resistant mutants of the cyanobacterium *Nostoc moscurum* to salinity and osmotic stresses. *World J. Microb. Biotech.*, 22: 783-789.
- Binzel ML, Hess FD, Bressan RA and Hasegawa PM (1988). Intracellular compartmentation of ions in salt adapted tobacco cells. *Plant Physiol.*, 86: 607-614.
- Egamberdieva D, Kamilova F, Validov S, Gafurova L, Kucharova Z and Lugtenberg B (2008). High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan. *Env. Microbiol.*, 10: 1-9.
- Gazzarrini S, Lejay L, Gojon A, Ninnemann O, Frommer WB and Von-Woren N (1999). Three functional transporters for constitutive, diurnally regulated and starvation-induced uptake of ammonium into *Arabidopsis* roots. *Plant Cell* 11: 937-948.
- Golden SS (1958). Mutagenesis of cyanobacteria by classical and gene transfer-based method. *Methods Enzymol.*, 167: 714-727.
- Herbert D, Phipps PJ and Strange RE (1971). Chemical analysis of microbial cells. In: *Methods in Microbiology* (Eds. Norris JR and Ribbons DW), Academic Press, London, pp. 209-234.
- Karpel R, Alon T, Glaser G, Schuldiner S and Padan E (1991). Expression of a sodium proton antiporter (Nha A) in *Escherichia coli* is inducing by Na^+ and Li^+ ions. *J. Biol. Chem.*, 266: 21753-21759.
- Lauchli A (1990). Calcium, salinity and the plasma membrane. In: *Calcium in Plant Growth and Development* (Eds Leonard RT and Hepler PK), *Am. Soc. Plant Physiol.*, Rockville, MD., pp. 26-35.
- Li W, Wang Y, Okamoto M, Olive F, Crawford NM, Siddiqi MY and Glass ADM (2007). Dissection of the *AtNTR2.1*: *AtNTR2.2* inducible high affinity nitrate transporter gene cluster. *Plant Physiol.*, 143: 425-433.

- Loque DS, Lalonde S, Looger LL, Von-Wiren N and Frommer WB (2007). A cytosolic trans-activation domain essential for ammonium uptake. *Nature* 446: 195-198.
- Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ (1951). Protein measurements by Folin-phenol reagent. *J. Biol. Chem.*, 193: 266-275.
- Manazano C, Candau P, Gomez-Moreno C, Relimpio AM and Losada M (1976). Ferredoxin-dependent photosynthetic reduction of nitrate and nitrite by particles of *Anacystis nidulans*. *Mol. Cell Biochem.*, 10: 161-169.
- Martinez V and Cerda A (1989). Nitrate reductase activity in tomato and cucumber leaves as influenced by NaCl and N₂ source. *J. Plant Nutri.*, 12: 1335-1350.
- Masclaux-Daubress C, Resisdorf-cren M, Pageau K, Lelandais M, Grandjean O, Kronberger J Valadier MH, Feraud M, Loughlent T and Suzuki A (2006). Glutamine synthetase-glutamate synthetase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. *Plant Physiol.*, 140: 444-456.
- Niu X, Bressan RA, Hasegawa PM and Pardo JM (1995). Ion homeostasis in NaCl stress environments. *Plant Physiol.*, 109: 735-742.
- Reed RH, Richardson DL and Stewart WDP (1985). Na⁺ uptake and extrusion in cyanobacterium *Synechocystis* PCC 6714 in response to hypersaline treatment : Evidence for transient changes in plasmalemma Na⁺ permeability. *Biochem. Biophys. Acta.*, 814: 347-355.
- Salehi M, Salehi F, Poustini K and Sharifabad HH (2008). The effect of salinity on the nitrogen fixation in 4 cultivars of *Medicago sativa* L. in the seedling emergence stage. *Res. J. Ag. Biological Sci.*, 4: 413-415.
- Shapiro BM and Stadtman ER (1970). Glutamine Synthetase. In: *Methods in Enzymology* (Eds. Taber H and Tabor CW), Academic Press, New York, London, pp. 910-922.
- Singh Devendra P and Kshatriya Kavita (2002). Characterization of Salinity-Tolerant Mutant of *Anabaena doliolum* Exhibiting Multiple Stress Tolerance. *Curr. Microbiol.*, 45:165-170.
- Soltani N, Zarrini G, Ghasemi Y, Shokravish S and Bafttechchi L (2007). Characterization of a soil cyanobacterium *Fischerella* sp. FS18 under NaCl stress. *J. Biol. Sci.*, 7: 931-936.
- Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A and Takabe T (2001). Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na⁺/H⁺ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. *J. Biol. Chem.*, 276: 36931-36938.