Tolerance of nitrogen-fixing cyanobacteria to NaCl

H. PADHI, B. RATH and S.P. ADHIKARY

P.G. Department of Botany, Utkal University, Bhubaneswar 751 004, India

Abstract

Eleven species of nitrogen-fixing cyanobacteria belonging to six genera (*Cylindrospermum, Anabaena, Nostoc, Calothrix, Scytonema,* and *Westiellopsis*) tolerate different concentrations of NaCl (from 0.05 to 0.35 M). Wide variation in the chlorophyll (Chl) *a* content of the species of the same genus and between genera in presence of NaCl was observed. The least tolerant (*Cylindrospermum* sp., Cy 6), the maximum tolerant (*Westiellopsis* sp., We 1), and the intermediate tolerant (*Westiellopsis* sp., We 6) species of cyanobacteria were selected, and their response to various concentrations of NaCl (growth, nitrogen-fixing capacity, and production of extracellular substances) was studied. Nitrogen fixing capacity of both the We 1 and We 6 was greatly impaired in comparison to the decrease in their Chl *a* content in the NaCl containing media. Cellular and extracellular saccharide and extracellular amino acid contents of the cyanobacteria species in the NaCl supplemented cultures were increased suggesting that presence of saccharides and amino acids enabled the cyanobacterial species to thrive under salt stress. Further, the We 1 did not adapt to the salt whereas Cy 6 showed adaptation to low concentrations of NaCl suggesting that the species which tolerate high concentrations of the salt may not possess the ability to adapt to NaCl.

Additional key words: amino acids, *Anabaena*, adaptation, *Calothrix*, chlorophyll, *Cylindrospermum*, extracellular saccharides, *Nostoc, Scytonema, Westiellopsis.*

Introduction

The reports on the response of cyanobacteria to NaCl (Hagemann *et al.* 1973, Guy *et al.* 1988, Saxena and Kaushik 1992, Kumar and Kaushik 1994) include Na^+ uptake by halotolerant species (Anantani and Vaidya 1983, Blumwald *et al.* 1983, Reed *et al.* 1985, Kaushik and Nagar 1993), osmotic adjustments (Blumwald and Tel-or 1982a, Reed *et al.* 1986, Anand and Parameswaran 1990, Reviere *et al.* 1990,

Received 28 August 1996, *accepted* 20 April 1997.
Acknowledgement: We are grateful to the Professor and Head of the Department of Botany, Utkal University for providing laboratory facilities. We thank authorities of Department of Biotechnology, Government of India for providing financial assistance (No.BT/R&D/02/06/93). One of us (B.R.) thanks C.S.I.R., New Delhi for a Senior Research fellowship.
Fax: (+91) 674 481850

Hagemann *et al.* 1991), ultrastructural changes during salt adaptation (Blumwald and Tel-Or 1982b), *etc.* In most of these studies a single species of cyanobacteria, mostly a halotolerant form was used for the experiments. No report is available on the response of diazotrophic cyanobacteria of the rice-fields of coastal regions to various concentrations of NaCl. These diazotrophic cyanobacterial species isolated from salt affected rice fields, due to sea water influx, may tolerate relatively high concentrations of NaCl. In the present report relative tolerance of several nitrogen-fixing cyanobacteria isolated from the rice fields of coastal regions of Orissa to NaCl was studied. Chlorophyll, saccharide and amino acid contents, nitrogen fixing capacity and adaptation to NaCl of selected species of cyanobacteria were investigated.

Materials and methods

Eleven different species of diazotrophic cyanobacteria belonging to the genus *Cylindrospermum, Anabaena, Nostoc, Calothrix, Scytonema* and *Westiellopsis* were used as the experimental material. Two species of each genus were selected for experiments in order to find out the inter- and intra-generic response of cyanobacteria to NaCl. The organisms were isolated from the rice fields of coastal region of Orissa state and maintained in pure culture in nitrogen free BG 11 medium (Rippka *et al.* 1979). Growth experiments were done in 150 × 15 mm cotton stoppered hard glass test tubes containing 10 cm^3 of medium and various concentrations of NaCl (0.05, 0.10, 0.15, 0.2, 0.25, 0.3 and 0.35 M). Stock solutions of NaCl were prepared in distilled water, sterilized separately, and then added aseptically to the BG 11 medium to obtain the desired concentration. Equal amount of homogenised culture suspension from exponential growth phase of each organism was inoculated into the experimental tubes and incubated for 10 d at 25 ± 1 °C under a 7.5 W m^{-2} irradiance provided from fluorescent tubes. Growth was expressed on chlorophyll basis. For estimation of chlorophyll (Chl) *a* content, the cultures were centrifuged at 3 000 *g* for 20 min followed by extraction of the pigment with 80 % (v/v) acetone. Absorbance of the acetone extract was measured at 660 nm in a *Systronics 105* spectrophotometer (*Systronics*, Ahmedabad, India) and the Chl amount was determined using the specific absorbance given by Mackinney (1941). The extracellular amino acid content of the cultures was estimated by Moore and Stein's ninhydrin reaction method modified by Spies (1957). Amount of amino acids was determined from the standard curve using alanine (*Qualigens*, Thane, India). Cellular and extracellular saccharide contents of the experimental cultures were estimated with anthrone reagent (Herbert *et al.* 1971) and the amount was calculated from the standard curve prepared using glucose (*Qualigens*). Total nitrogen of the cyanobacterial cultures was determined by Micro-Kjeldahl digestion and distillation method as described by Herbert *et al.* (1971). The amount of nitrogen fixed by the organisms was calculated after substracting the amount of N present in the respective initial inoculum.

Results

Cylindrospermum sp. (Cy 6) and both the species of *Calothrix* tested (Ca 1 and Ca 5) were relatively more susceptible to high concentrations of the NaCl; Chl *a* content of these organisms decreased to zero in the presence of 0.10 - 0.15 M NaCl in the culture medium (Fig. 1). From the eleven tested species of cyanobacteria, *Westiellopsis* (We 1) was the most tolerant species followed by *Nostoc muscorum* (No 4).

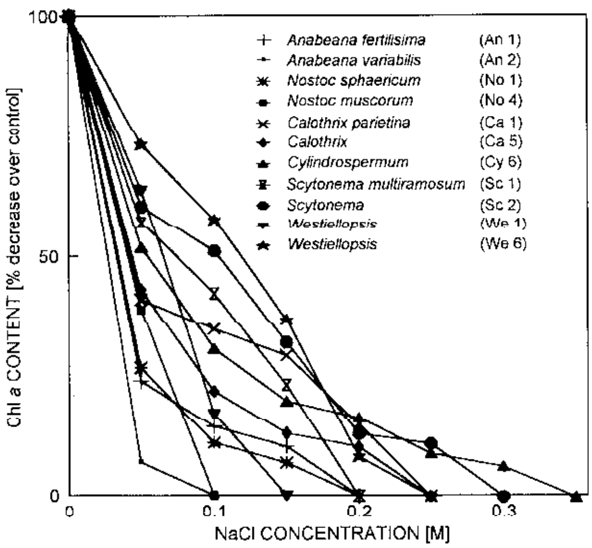

Fig. 1. Chlorophyll (Chl) *a* content of 11 different species of rice field cyanobacteria in presence of different concentrations of NaCl in the culture medium. Cultures were grown up for 10 d at 25 ± 1 °C and 7.5 W m^{-2} irradiance.

The most salt susceptible *Cylindrospermum* sp. (Cy 6), the most tolerant, *Westiellopsis* sp. (We 1) and an intermediate tolerant (We 6) species of cyanobacteria were further selected and their response to selected concentrations of NaCl at different growth phases was studied. In We 6 and We 1 the lowest used NaCl concentration had a tendency to stimulate Chl synthesis in the lag phase (Fig. 2), all other NaCl concentrations had only an inhibitory effect. Nitrogen-fixing capacity of all the three test species was greatly reduced even in presence of 0.02 M NaCl (Table 1). In contrast to the NaCl effects on the Chl content, the effects on nitrogen fixation were not strikingly different in the three tested species. Cellular and extracellular saccharide contents of the tested organisms generally decreased with increase of NaCl concentration in the medium (Fig. 3), an exception was a small stimulation by 0.02 M NaCl in We 1 observed after 8 d of cultivation. Most differences in extracellular saccharides were statistically not significant, but usually there was a tendency to decline with increasing NaCl concentration. Similar changes were found in secretion of extracellular amino acids (Fig. 4). Highest contents of saccharides and highest secretion of amino acids were usually found in We 1.

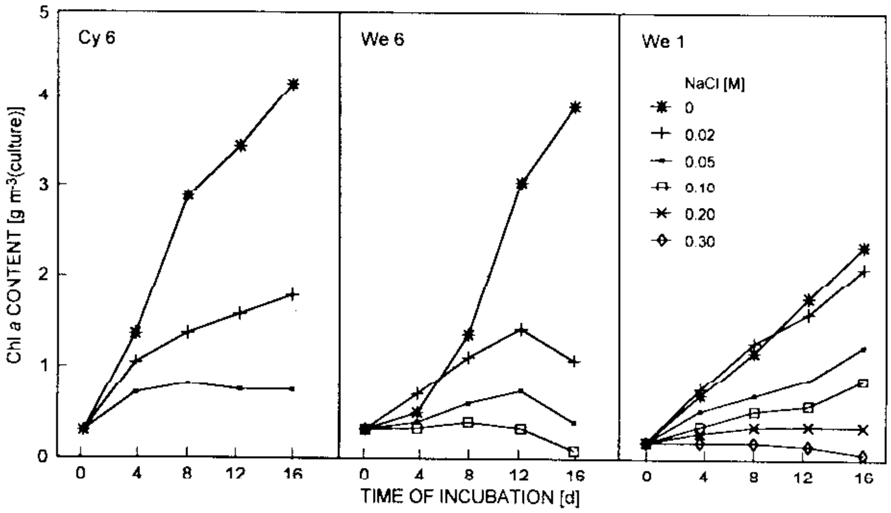

Fig. 2. Chlorophyll (Chl) *a* content of three different species of cyanobacteria (*Cylindrospermum* - Cy 6, *Westiellopsis* - We 1 and We 6) in the presence of different concentrations of NaCl in the BG 11-N medium.

Table 1. Effect of various concentrations of NaCl on the nitrogen fixation of three different species of rice-field cyanobacteria. Means of 3 independent determinations ± S.D.; values in parenthesis represent % decrease over control. The amount of nitrogen-fixed by the organisms in different experimental cultures was determined after subtracting the amount of N present in the initial inoculum.

Species	Strain	NaCl concentration [M]	Nitrogen fixed [g m^{-3}(culture)]
Cylindrospermum sp.	Cy 6	0	46 ± 8
		0.02	31 ± 5 (-32.61)
		0.05	16 ± 3 (-65.22)
		0.1	0
Westiellopsis sp.	We 1	0	61 ± 7
		0.02	46 ± 4 (-26.59)
		0.05	15 ± 5 (-75.41)
		0.1	12 ± 3 (-80.32)
		0.2	0
Westiellopsis sp.	We 6	0	31 ± 5
		0.02	15 ± 2 (-51.61)
		0.05	10 ± 2 (-67.74)
		0.1	0

These three selected cyanobacteria species were further grown under different concentrations of NaCl up to 20 d followed by washing in sterile distilled water and reincubating in NaCl containing medium for further 10 d. It was thought that an organism which grew better in the same concentration of NaCl in which it was

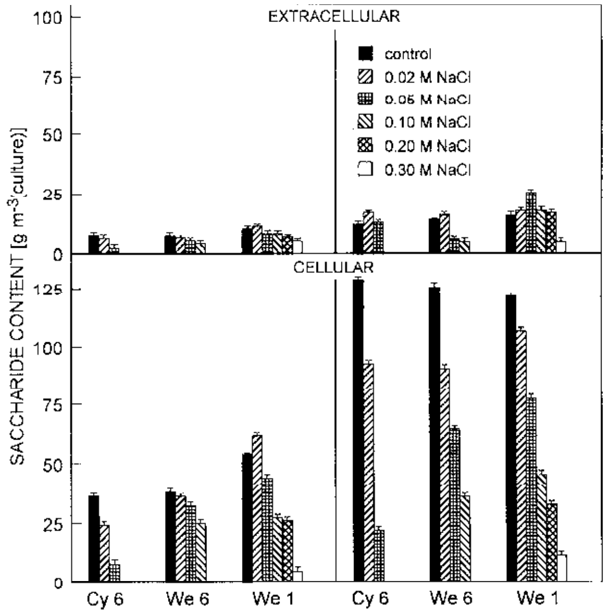

Fig. 3. Cellular and extracelluar saccharide contents [g(glucose equivalent) m^{-3}(culture)] of three species of cyanobacteria in the presence of various concentrations of NaCl in the culture medium.

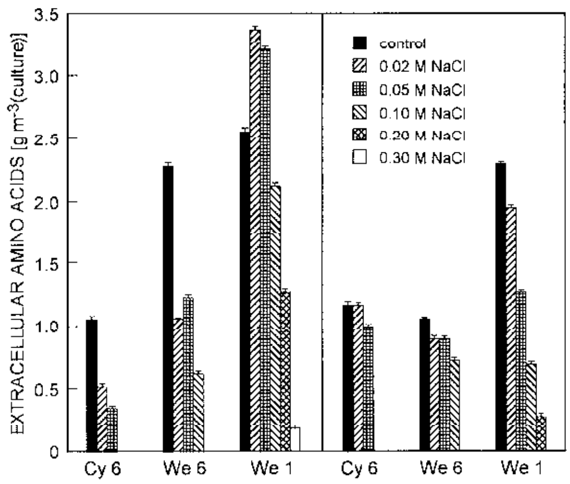

Fig. 4. Extracelluar amino acid concentration [g(alanine equivalent) m^{-3}(culture)] of three species of cyanobacteria in the presence of various concentrations of NaCl in the culture medium.

previously grown, were adapted to NaCl. The results showed that though the We 1and We 6 tolerated comparatively higher concentration of NaCl than the Cy 6, only the latter species previously grown in the presence of NaCl grew better under the same concentration of the salt (Table 2). Thus the species of cyanobacteria which tolerate high concentrations of NaCl necessarily would not adapt to the salt at repeated culturing in the salt containing medium.

Table 2. Chlorophyll (Chl) *a* content of three species of cyanobacteria cultivated in the presence or absence of various concentrations of NaCl. Means of 3 independent determinations ± S.D. Values in parenthesis show % decrease over control.

Species	Strain	NaCl concentration [M]	NaCl pretreatment	Chl *a* content [g m^{-3}(culture)]
Cylindrospermum sp.	Cy 6	0		2.28 ± 0.24
		0.05	–	0.18 ± 0.01 (-92.15)
		0.05	+	0.58 ± 0.12 (-74.51)
Westiellopsis sp.	We 1	0		2.10 ± 0.20
		0.05		1.09 ± 0.16 (-47.85)
		0.05	+	0.42 ± 0.12 (-79.81)
		0.2	–	0.33 ± 0.13 (-84.04)
		0.2	+	0.18 ± 0.04 (-91.48)
Westiellopsis sp.	We 6	0		1.36 ± 0.20
		0.05	–	0.33 ± 0.06(-75.42)
		0.05	+	0.15 ± 0.06 (-88.55)
		0.1	–	0.20 ± 0.06 (-85.25)
		0.1	+	0

Discussion

Supplementation of 0.4 M NaCl decreased the biomass accumulation, Chl *a* content and cell protein content in *Calothrix brevissima* isolated from saline sodic soils whereas the sensitive *Anabaena* sp. tolerated NaCl only up to 0.1 M (Kumar and Kaushik 1994). The growth of nitrogen-fixing cyanobacterium *Nostoc muscorum* was completely inhibited by 0.4 M NaCl (Blumwald and Tel-or 1982a). The unicellular cyanobacterium *Microcystis firma* tolerated salinity up to 1.0 M NaCl but another unicellular species *Synechocystis aquatilis* showed salt tolerance only up to 0.3 M NaCl (Hagemann *et al.* 1973). None of our tested species tolerate NaCl > 0.35 M even though they were isolated from rice fields of coastal regions which received sea water due to a periodic influx. Thus the tolerance to elevated concentration of NaCl by an individual species was mostly due to its genetically determined capacity. There are reports that sheath layers around the cells/trichome of cyanobacteria enable the organisms to tolerate high concentrations of pesticides and other agrochemicals (Sahu *et al.* 1992, Rath and Adhikary 1994). Our work showed that species of *Calothrix* and *Scytonema* which possessed well defined sheath layers around their trichome did not tolerate higher concentrations of NaCl in comparison to sheathless forms suggesting that external envelope played a least significant role in protection against the salt stress.

Several mechanisms have been put forth for salinity tolerance in cyanobacteria. These include curtailment of Na^+ influx (Apte *et al.* 1987), accumulation of K^- (Miller *et al.* 1984) or accumulation of organic solutes (Borowitzka *et al.* 1980). Salinity stress in cyanobacteria was also relieved by a variety of exogenous saccharides like pentoses, hexoses and alcohols (Reed *et al.* 1986). A large number of amino acids may protect against salt stress in *Calothrix brevissima* (Kumar and Kaushik 1994). In our study increase of cellular saccharide content was found in the We1 when grown in the presence of NaCl. Further, considerable amounts of extracellular saccharides and amino acids were excreted into the medium during exponential growth, all of which might provide protection to the We 1 against salt stress. Our results also showed that nitrogen-fixing activities of all the three cyanobacteria (Cy 6, We 1 and We 6) were more affected by increased contents level of NaCl in the medium than the contents of Chl *a*. Further, the species of the cyanobacteria which could tolerate high NaCl levels did not possess the ability to adapt to NaCl. Even if any cyanobacterium of the rice fields showed adaptation to the salt, it tolerated only a limited concentration of NaCl and the adaptation process was slow. Thus due to increase of salinity of rice fields due to depletion of ground water or by sudden influx of salts to the rice fields located in the coastal regions, the nitrogen-fixing activity of these microorganisms would greatly be impaired thereby decreasing the overall nitrogen economy of the rice field soils.

References

Anand, N., Parameswaran, P.: Salinity responses of a unicellular blue-green alga (cyanobacterium) *Chroococcus minor*. - Seaweed Res. Util. **13**: 23-27, 1990.

Anantani, Y.S., Vaidya, B.S.: Growth characteristics and pigment levels in a halotolerant cyanophyte. - Phykos **22**: 101-104, 1983.

Apte, S.K., Reddy, B.R. Thomas, J.: Relationship between sodium influx and salt tolerance of nitrogen-fixing cyanobacteria. - Appl. environ. Microbiol. **53**: 1934-1939, 1987.

Blumwald, E., Tel-or, E.: Structural aspects of the adaptation of *Nostoc muscorum* to salt. - Arch. Microbiol. **132**: 163-167, 1982a.

Blumwald, E., Tel-or, E.: Osmoregulation and cell composition in salt-adaption of *Nostoc muscorum*. Arch. Microbiol. **132**: 168-172, 1982b.

Blumwald, E., Mehlhorn, R.J. Packer, L.: Ionic osmoregulation during salt adaptation of the cyanobacterium *Synechococcus* 6311. - J. Plant Physiol. **73**: 377-380, 1983.

Borowitzka, L.J., Demmerle, S., Mackay, M.A., Norton, R.S.: Carbon-13 NMR study of osmoregulation in blue-green algae. - Science **210**: 650-651, 1980.

Guy, R., Vonshak, A., Guy, M.: The response of the filamentous cyanobacterium *Spirulina platensis* to salt stress. - Arch. Microbiol. **150**: 417-420, 1988.

Hagemann, M., Erdmann, N., Schiewer, U.: Salt adaptation of the cyanobacteria *Microcystis firma* and *Synechocystis aquatilis* in turbidostat cultures. I. Steady state values. - Arch. Hydrobiol. **9**: 450-481, 1973.

Hagemann, M., Teechel, D., Rensing, L.: Comparison of salt and heat-induced alterations of protein synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. - Arch. Microbiol. **155**: 587-592, 1991.

Herbert, D., Phipps, P.J., Strange, R.E.: Chemical analysis of microbial cells. - Methods Enzymol. **5B**: 209-234, 1971.

Kaushik, B.D., Nagar, A.P.: Sodium uptake by halotolerant *Westiellopsis prolifica* in presence of K^+, Ca^{2+}, Mg^{2+}, Fe^{2+} and Li^+. - Indian J. Microbiol. **33**: 93-96, 1993.

Kumar, H., Kaushik, B.D.: Response of *Calothrix brevissima* to salt. - Indian J. Microbiol. **34**: 37-41, 1994.

Mackinney, G.: Absorption of light by chlorophyll solutions. - J. biol. Chem. **140**: 315-322, 1941.

Miller, A.G., Turpin, D.H., Canvin, D.T.: Na^+ requirement for growth, photosynthesis and pH regulation in the alkalotolerant cyanobacteria *Synechococcus leopoliensis*. - J. Bacteriol. **159**: 100-106, 1984.

Rath, B., Adhikary, S.P.: Relative tolerance of several nitrogen-fixing cyanobacteia to commercial grade furadan (Carbofuran, 3 %). - Indian J. exp. Biol. **32**: 213-215, 1994.

Reed, R.H., Richardson, D.L., Stewart, W.D.P.: Na^+ uptake and extrusion in the cyanobacterium *Synechocystis* PCC 6714 in response to hypersaline treatment. Evidence for transient changes in plasmalemma Na^+ permeability. - Biochim. biophys. Acta **814**: 347-355, 1985.

Reed, R.H., Borowitzka, L.J., Mackey, M.A., Chudak, J.A., Foster, R., Warr, S.R.C., Moore, D.J., Stewart, W.D.P.: Organic solute accumulation in osmotically stressed cyanobacteria. - FEMS Microbiol. Lett. **39**: 51-56, 1986.

Reviere, M.E., Arrio, B., Steffen, I., Moliter, V., Kuntner, O., Peschek, G.A.: Changes of some physical properties of isolated and purified plasma and thylakoid membrane vesicles from the freshwater cyanobacterium *Synechococcus* 6301 (*Anacystis nidulans*) during adaptation to salinity. - Arch. Biochem. Biophys. **280**: 159-166, 1990.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y.: Generic assignments, strain histories and properties of pure cultures of cyanobacteria. - J. gen. Microbiol. **111**: 1-61, 1979.

Sahu, J., Das, M.K., Adhikary, S.P.: Reaction of blue-green algae of rice-field soils to pesticides application. - Trop. Agr. (Trinidad) **69**: 362-364, 1992.

Saxena, S., Kaushik, B.D.: Polysaccharide (biopolymers) from halotolerant cyanobacteria. - Indian J. exp. Biol. **30**: 433-434, 1992.

Spies, J.R.: Colorimetric procedures for amino acids. - Methods Enzymol. **3**: 467, 1957.