Indian J. Applied & Pure Bio. Vol. 36(2), 519-528 (2021). **ISSN: 0970-2091**
A web of Science Journal

Impact of graded concentration of NaCl on growth, Heterocyst differentiation and macromolecular content of Diazotrophic Cyanobacterium *Anabaena variabilis* [wild type and Multiple Herbicide Resistant strain(MHR)]

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Abstract

Salinity and alkalinity are the major problem of soil which greatly affects the fertility, and the ability of cyanobacteria to tolerate a wide range of adverse environmental conditions including salinity, makes them a good alternative of chemical fertilizers. So in this background and context an effort was made to evaluate the potential of two diazotrophic cyanobacterial strains *Anabaena variabilis* ,a rice field isolate and its multiple herbicide resistant mutant *A.variabilis* (MHR) ^{Ar,Al,B,D} exhibiting resistant to herbicide arozin, alachlor, butachlor and 2,4-D under graded concentration of NaCl to generate the knowledge about their mechanisms of actions and tolerance level towards salt(ionic and osmotic stress).

Excessive salinity in environment is the major factor, which depress the crop production world wide. Cyanobacteria acts as scavengers of Na^+, so they have been used for the reclamation of saline soil[32]. Although cyanobacteria need trace amount of Na^+ for normal growth and nitrogen fixation, this element is the predominant agricultural deterrent in saline habitats. Cyanobacteria do not accumulate Na^+ [2,3] but a transient net Na^+ uptake may occur in response to hyper saline upshock[28]. Salt stress induces many biological processes that assist organisms to survive in environment with high salt concentrations. The mechanisms responsible for tolerance to salt stress seem to be activated when cells are exposed to high salt concentrations. It has been reported that in *Synechocystis* sp. PCC 6803, the addition of NaCl to the medium enhanced expression of genes related to salt tolerance, such as those that express glucosyl glycerol-phosphate synthase[18,24].

The requirement of Na^+ for cyanobacterial growth is mostly because of its role as component of symport system for transport of NO_2, HCO_3 and PO_4 in cyanobacterial cells[19,26] had demonstrated a role of Na^+ in cyanobacterial pH homeostasis in addition to

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its role in cyanobacterial growth and photo-synthesis.

· Selection and use of stress tolerant cyanobacteria and understanding the mechanisms involved in such stress resistance are priority area of contempory cyanobacterial research[16].

In the present investigation two diazotrophic cyanobacteria *A. variabilis* wild type and its spontaneous mutant MHR (resistant for four types of herbicide-(Alachlor, Butachlor, 2,4D and Arozin) have been exposed under different concentrations of NaCl. Strain *Anabaena variabilis* is a local rice field isolate of Jabalpur region[33] and showed enhanced growth and other metabolic activities as compared to other local isolates.So an effort have been made to examine the tolerance level of these strains towards NaCl to understand the mechanism of and extent of salt tolerance, so that such strains can be further used in saline and alkaline soil as a potential biofertilizer and also will serve as gene bank for further biotechnological studies.

Impact of graded concentration of NaCl on growth and macromolecular contents of Anabaena variabilis (Wild type and MHR) (Determination of lethal and sublethal dosages): Impact of increasing concentration of salt NaCl on the growth and survival of diazotrophic cyanobacteria *Anabaena variabilis* wild type and MHR strarins were determined in N_2- medium by estimating the variation in the concentration of chlorophyll *a* pigment at the regular interval of 24 hours. Exponentially growing cyanobacterial cells (6-8 days old) were harvested, washed thrice with sterilized double distilled water and dispensed equally in 250ml capacity erlenmeyer flask containing 100ml sterile BG^{-}_{11} medium. Culture was added under photo autotrophic growth conditions followed by the addition of graded concentration of NaCl. Flasks were shaken vigorously to mix NaCl with test organism. Flasks containing no NaCl were served as a control. After addition of stress (NaCl) samples (3.0ml) were withdrawn in triplicate to extract and estimate the chlorophyll *a*[23] with the help of spectrophotometer. This was considered as 0 hour sample. The point at which complete lysis of culture occurred was consider as lethal dose, the dose just before the lethal dose at which some degree of survival found was consider as sub-lethal dose. These experiments were also re-confirmed by plating the cells with graded concentration of salts (NaCl). All other macromolecular contents except chlorophyll *a* and carotenoids were estimated at regular interval of 4 days.

Organisms and growth conditions :

Source of strains :

The axenic clonal culture of N_2-fixing cyanobacterium *Anabaena variabilis*, a rice field isolate[33] and its multiple herbicide resistant mutant *A. variabilis* $(MHR)^{Ar, Al, B, 2, 4D}$ exhibiting resistance to herbicides- Arozin, Alachlor, Butachlor, and 2,4-D[8,34] were routinely grown in BG^{-}_{11} medium [30,31] devoid of any combined nitrogen source (called as N_2-medium).

The axenic clonal culture of cyano-bacterial strains were maintained in a bacteria free state by routinely transferring (at intervals

of 7 days) the exponential phase cultures to 100ml fresh sterile N_2 medium in 250ml Erlenmeyer flask under a laminar flow hood (Klenzaids, Bombay, India). The cultures were grown photoautotrophically in an air conditioned culture room maintained at 25±°C and illuminated with cool day florescent lights (photon flux density 45μE m^{-2} s^{-1}) for 18 hours a day.

Measurement of growth :

Growth was measured at regular interval of one day.

Extraction and estimation of chlorophyll 'a' and carotenoids:

A known aliquot (3ml) of cyanobacterial sample was taken and centrifuged (3000Xg,5min) and supernatant was discarded. The pellet was resuspended in the same amount of methanol and shaken thoroughly. The tubes were kept for 15 minutes in a hot water bath maintained at 60°C and was centrifuged to discard pellet. The optical density of chlorophyll *a* and carotenoids solution was read at 665 nm[23] and 460nm[35] respectively against methanol blank using UV-VIS Spectrophotometer (model 118, Systronics, India).

Extinction coefficients :

13.42 x A665 = Chlorophyll in *'a'* μg ml^{-1}

12.0 x A460 = Carotenoids in μg ml^{-1}

Heterocyst frequency :

Heterocyst frequency was determined microscopically and in expressed in percentage as total number of heterocyst occurring per 100 vegetative cells of each cyanobacterial culture.

$$\text{Heterocyst Frequency} = \frac{\text{No. of heterocyst}}{\text{No. of vegetative cells}} \times 100$$

Determination of cellular constituents :

Estimation of total protein :

Total cellular protein content of cyanobacterial strain was estimated by the method of Lowry *et.al.*[21], and as modified by Herbert *et.al.*[15]

Reagents :

(i) 1.0 N Sodium hydroxide
(ii) 5% Sodium Carbonate
(iii) 0.5%Copper Sulphate ($CuSO4.5H_2O$) in 1% Sodium potassium tartarate. Working solution was prepared by adding 50ml of solution (ii) with 2ml of solution (iii)
(iv) Folin-Ciocalteau reagent (1N)- Commercially available reagent (LOBA Chemie, India) was diluted 1:1 before use.

Procedure :

To 0.5ml-algal sample, 0.5 ml of NaOH was added, heated in boiling water bath for 10 minutes and cooled in running tap water.

Subsequently, 2.5 ml of working reagent was added and incubated at room temperature for 10 minutes. This was followed by the addition of 0.5 ml Of Folin- 'Ciocalteau reagent and after 30 minutes, the intensity of the blue colour so developed as read at 750nm

in UV-VIS Spectrophotometer (Model 118, Systronics India) against appropriate blank (double distilled water was taken in the place of culture and rest of the reagents were same) the amount of total cellular protein was calculated by using standard curve prepared from Bovine Serum Albumin (BSA, Sigma, USA) as standard.

Estimation of carbohydrate :

Reagents :

1- 5% Phenol- 5g Phenol dissolved in 100 ml of distilled water.
2- Concentrated sulfuric acid (A. R. Grade Specific Gravity 1.89).

Procedure :

To 0.5 ml of cell suspension, 0.5 ml of 5% Phenol reagent was added thoroughly. This was followed by addition of 2.5 ml of concentrated $H2SO_4$. (AR grade, specific gravity 1.89) with a fast flowing pipette, directing the stream on surface of liquid. After 10 minutes the tubes were shaken and placed in water bath at 30°C for 20 minutes. Blank was prepared with 0. 5ml double distilled water and reagents. The intensity of straw colour so developed at the end of reaction was measured spectrophotometrically using UV-V1S Spectrophotometer (Model 118, Systronics, India) at 688 nm. The amount of total carbohydrate in the test sample was calculated from standard curve prepared from known concentration of D- galactose.[9]

Estimation of DNA and RNA :

Estimation of DNA and RNA was done by the method of Herbert *et al.*[15]

Extraction :

The cyanobacterial samples (5ml) were centrifuged (3000xg, 5 min) and pellets were treated with Ethanol: Ether (3:1V/V), This solvent was removed by the repeated centrifugation and decantation. Remaining pellet was resuspended in 3 ml cold 3.0 N Perchloric acid and the suspension was kept 4°C for 30 minutes to extract RNA in suspension. The residual material recovered after centrifugation was again suspended in 3 ml of 3.0 N Perchloric acid at 80°C for 30 minutes. The DNA extraction in supernatant was recovered by centrifugation.

DNA estimation :
Reagents :

1. Diphenylamine reagent (DPA)- 1-5g of steam distilled diphenylamine (DPA) dissolved in 100 ml glacial acetic acid, 1.5 ml of concentrated H_2S0_4 was added to this mixture and stored in dark.

Procedure :

To the known aliquot (3.0ml) of extracted DNA was added 1.0ml of diphenylamine reagent. The tubes were incubated in boiling water bath for 10 minutes, cooled to room temperature and intensity of blue colour so developed was read Spectrophotometrically at 610nm against double water- reagent blank. DNA content was calculated from standard curve prepared by using Herring Sperm DNA (HI Media Lab., India).

RNA estimation :
Reagents :

1. Ferric Chloride - 0.9 gram dissolved in 1.0 litter concentrated HC1 (AR grade, BDH

India)

2. Orcinol - 1.0 g Orcinol (Sigma, USA) dissolved in 100 ml distilled water and stored at 0°C.
3. Orcinol reagent - To 4.0 ml of reagent (2)1.0 ml of reagent (1) was added, this was prepared fresh immediately before use.

Procedure :

To RNA extract (3.0ml) was added 2.0ml orcinol reagent and heated in boiling water bath for 20 minutes. The tubes were subsequently cooled in running tap water. The intensity of the green colour so developed was read Spectrophotometrically at 610nm. Blank was prepared with double distilled water and reagent. The RNA content was calculated from standard curve prepared by using Torula Yeast RNA (Hi Media Lab., India).

Estimation of Phycocyanin (PC), Phycoerythrin (PE) and Allophycocyanin(APC) :

Estimation of PC, PE and APC the water soluble pigments was done by the method of Bennet and Bogorad[6]. A known volume (5ml) of cells was centrifuged (3000 x g, 5 min). The supernatant was discarded and to the pellet was added 5 ml of buffer saline (0.01 M Na_2HPO_4 and 0.15 M NaCl) in distilled water and supplemented with 8% toluene. (pH = 7.0). The content was cyclomixed vigorously and kept overnight in dark at room temperature for complete extraction. Again centrifugation (3000 X g, 5 min) was done and optical density of extracted pigments in the supernatant was read spectrophotometrically at 615 nm, 550 nm and 650 nm, for phycocyanin and phycoerythrin and allophycocyanin respectively against buffer saline as blank. Relative concentrations were found by using the formulae –

$$(PC) = \frac{OD_{615}\text{-}0.474(OD_{65o})}{5.34}$$

$$(APC) = \frac{OD_{650}\text{-}0.208(OD_{615})}{5.09}$$

$$(PE) = \frac{OD550\text{-}2.41\ (PC)\text{-}0.849(APC)}{9.62}$$

Effect of graded concentration of salt (NaCl) on growth,macromolecular contents, heterocyst differentiation of cyanobacterial isolates :

The effect of graded concentration of NaCl (0-500mM) on growth and survival of cyanobacterial isolates *Anabaena variabilis (*wild type) and *Anabaena variabilis* (MHR) was monitored by estimating chlorophyll *a* content upto 16 days at an interval of 24h. by treating N_{2}- grown exponential (6 days old) culture. The growth pattern of salt (NaCl) treated and untreated cultures are shown in fig (1.1-1.2).

The growth kinetics of wild type and multiple herbicide resistant mutant of *A. variabilis* was compared under diazotrophic growth conditions. Cyanobacterial isolates showed gradual inhibition in the growth with increasing dosage of salt (NaCl). Complete lysis of the cultures occurred at 400mM (for wild type and MHR) of NaCl on 8^{th} day of incubation.

These dosages were considered as the lethal dosages and the dosages lower than these were considered as the sub-lethal dosages (Table 1).

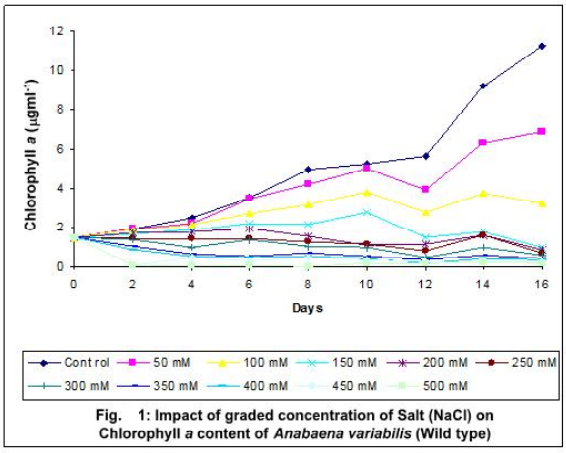

Fig. 1: Impact of graded concentration of Salt (NaCl) on Chlorophyll *a* content of *Anabaena variabilis* (Wild type)

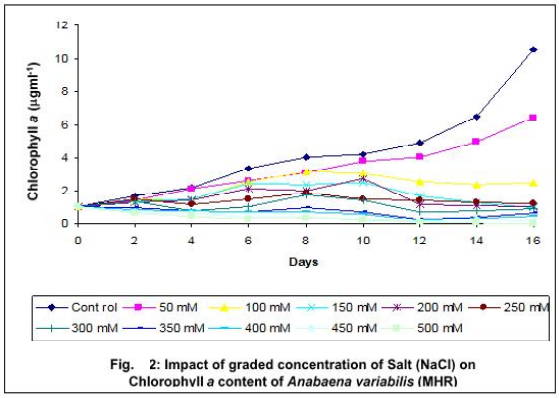

Fig. 2: Impact of graded concentration of Salt (NaCl) on Chlorophyll *a* content of *Anabaena variabilis* (MHR)

Table-1 Lethal, and Sub-lethal values of NaCl for wild type and MHR strain of *A variabilis*

Cyanobacterium	NaCl			
	Lethal		Sub-Lethal	
A. variabilis	Wild type	MHR	Wild type	MHR
	400	400	350	350

Values given in Mm

Similarly the lethal and sub-lethal dosages of salt (NaCl) for *Anabaena Variabilis* (wild type and MHR) were re-confirmed on solid agar plates by performing survival test of the cyanobacterial isolates. Slight variation in lethal and sub-lethal dosages were observed in survival assay on agar than liquid culture assay.

Substantial reduction in chlorophyll *a*, carotenoid, phycobilin pigments, total protein content, heterocyst frequency,DNA and RNA content was observed under graded concentration of salt (NaCl) in both wild type and MHR strain. The results of such experiments are shown in Table (2-5).

Table-2. Effect of graded concentrations of NaCl on percent inhibition/increase on growth, macromolecular contents and heterocyst frequency of wild type strain of A *variabilis* on 8th day of diazotrophic growth

Parameters	NaCl (in mM)									
	50	100	150	200	250	300	350	400	450	500
Chlorophyll *a*	14.6↓	43.9↓	55.6↓	68.4↓	74.1↓	79↓	86.6↓	89.5↓	91.2↓	96.7↓
Carotenoid	13.67↓	39↓	23↓	52.1↓	56.4↓	68.4↓	68.3↓	70.3↓	76↓	77.5↓
Phycocyanin	7.1↓	12.2↓	23.1↓	37.4↓	69.8↓	69.1↓	71.1↓	72.2↓	80.2↓	86↓
Phycoerythrin	26.7↓	32.1↓	50.4↓	53.7↓	73.3↓	74.1↓	74.1↓	76.8↓	78.3↓	80↓
Protein	10↑	13.5↑	17.1↑	12.4↑	17.6↑	44↓	73. 3↓	81.3↓	85. 9↓	87.5↓
Carbohydrate	24↑	36↑	53↑	66↑	72↑	50.8↑	5.2↑	2.29↓	10.1↓	13.9↓
DNA	12.2↓	17.5↓	28.7↓	44.8↓	55.4↓	57.1↓	60.8↓	68.7↓	77.6↓	78.5↓
RNA	19.3↓	37.1↓	45.3↓	60.2↓	63.7↓	70↓	76.2↓	71.8↓	85.3↓	89.2↓
Heterocyst frequency	15.2↓	17.3↓	ND	ND	ND	ND	ND	ND	ND	ND

The data are the mean of triplicate readings which did not vary by more than 5%

↓ ND = Not detectable

↑ = percent inhibition

= percent increase

Table-3 Effect of graded concentrations of NaCl on percent inhibition/increase on growth, macromolecular contents, heterocyst frequency of MHR of A *variabilis* on 8th day of diazotrophic growth

Parameters	NaCl (in mM)									
	50	100	150	200	250	300	350	400	450	500
Chlorophyll *a*	22.4↓	22.1↓	41.3↓	50.2↓	53.9↓	57.3↓	75.5↓	81.2↓	89.7↓	92.0↓
Carotenoid	10.9↓	12.7↓	28.6↓	45.5↓	55.5↓	62.5↓	66.0↓	83.7↓	88.8↓	92.8↓
Phycocyanin	12.9↓	20.5↓	37.0↓	52.3↓	58.2↓	72.8↓	79.1↓	82.1↓	83.2↓	83.1↓
Phycoerythrin	11.3↓	17.3↓	22.5↓	44.3↓	54.7↓	59.2↓	62.6↓	70.4↓	71.6↓	71.2↓
Protein	9.7↑	12.5↑	18.2↑	22.4↑	27.6↑	34↓	80.2↓	87.3↓	92.8↓	94.6↓
Carbohydrate	1.71↑	26.1↑	22.2↑	23.6↑	28.1↑	32.7↑	6.46↑	11.7↓	27.9↓	42.9↓
DNA	19.5↓	23..0↓	42.6↓	44.8↓	52.1↓	56.4↓	60.9↓	67.67↓	70.1↓	74.3↓
RNA	16.8↓	18.5↓	32.1↓	38.9↓	43.0↓	57↓	61.9↓	65.4↓	68↓	68.8↓
Heterocyst frequency	11.6↓	16.2↓	ND	ND	ND	ND	ND	ND	ND	ND

The data are the mean of triplicate readings which did not vary by more than 5%
ND = Not detectable

↑ = percent increase

↓ = percent inhibition

Table-4. Effect of graded concentration of salt (NaCl) on Heterocyst frequency (%) of *Anabaena variabilis* wild type

Days	Con-trol	50mM	100 mM	150 mM	200 mM	250 mM	300 mM	350 mM	400 mM	450 mM	500 mM
0	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
4	4.5	4.1	4.0	Fragmented							
8	4.4	3.9	3.8	Fragmented							
12	4.4	3.8	3.2	Fragmented							
16	4.0	3.4	3.1	Fragmented							

The data are mean of triplicate readings which did not very by more than 5%

Table-5. Effect of graded concentration of Salt (NaCl) on Heterocyst frequency (%) of *Anabaena variabilis* MHR

Days	Con-trol	50mM	100 mM	150 mM	200 mM	250 mM	300 mM	350 mM	400 mM	450 mM	500 mM
0	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3
4	4.3	4.2	4	Fragmented							
8	4.3	3.8	3.6	Fragmented							
12	4.1	3.6	3.2	Fragmented							
16	4.0	3.3	3.0	Fragmented							

The data are mean of triplicate readings which did not very by more than 5%

As compared to untreated control cultures at sub lethal dose of NaCl chlorophyll *a* was reduced to 86.6% and 75.5% carotenoid to 68.3% and 66%, PC to 71.1% and 79.1% PE to 74.1% and 62.6%, Protein content to 73.3% and 80.2%, DNA to 60.8% and 60.9%, RNA to 76.2% and 61.9%, in wild type and MHR strain respectively at the 8th day of growth. Heterocyst frequency could not be calculated due to the fragmentation of filaments. The reason behind the fragmentation of filaments might be the toxic action of NaCl. Carbohydrate content was increased to 5.23% and 6.46% in wild type and MHR strain respectively at 8th day of growth.

Results shows that lower concentration of NaCl increase the protein content of both, wild type and MHR strain. Carbohydrate content was also increased in the experimental set –up treated with lower concentration of NaCl.

The results shows gradual inhibition in growth with increasing concentration of salt NaCl. Both wild type and MHR strain tolerated NaCl up to 100mM, complete lysis occurred in 400mM of NaCl . Na^+ is an essential ion for most cyanobacteria[1,10] and Na^+ is needed for the uptake of several inorganic for nutrients viz. inorganic carbon,

nitrate, and phosphate[5,20] as well as for photosynthetic electron transport at the O_2 evolving complex[36] and these might be the reasons for high tolerance of both wild type and MHR strain of *A. variabilis*, towards NaCl .

Cyanobacteria have several kinds of mechanisms that allow them to acclimate to salt stress for example, the inducible synthesis of compatible solutes such as sucrose is synthesized in salt-sensitive strains of cyanobacteria such as *Synechococcus*[13,17, 22,29]; glucosylglycerol is synthesized in strains with intermediary tolerance such as *Synechocystis* sp. PCC 6803 [13,14,17,25]; glycinebetaine is synthesized in salt tolerant *Synechococcus* sp. PCC 7418 [13,17,22,29]. The protein content of wild type and MHR strains increased up to 250mM of NaCl (ionic+osmotic stress) both induce expression of several common stress protein and their corresponding genes in nitrogen-fixing *Anabaena* cells[7,11]. Although these osmo- responsive genes which have been shown to make important contribution to osmotolerance but play no significant role in tolerance to the ionic component of salinity stress. The tolerance to ionic component of NaCl has earlier been shown to be mediated by the curtailment of Na^+ influx and eventual Na^+ exclusion[4,27].

· To understand the mechanisms of salt tolerance in cyanobacteria, halo tolerant and halosensitive strains of *Nostoc muscorum* were investigated for the synthesis of some primary metabolic[12] and it was concluded that protein synthesis was stimulated upto 0.05M NaCl only halo tolerant strain qualitative changes in protein showed the presence of salt sensitive (37KD) proteins and emergence of 42.5, 27,72KD proteins providing tolerance to halo tolerant stains.

The results suggest the present of different degree of inherent natural tolerance among the diazotrophic cyanobacteria *Av* wild type and MHR isolated from the specific agroclimatic zone of Madhya Pradesh. These region specific cyanobacteral strains would prove potential biofertilizer to enhance soil fertility.

I would like to express my deep sense of gratitude to Algal Bioteechnology Laboratory, Department of Post Graduate Studies and Research in Biological Sciences, Rani Durgavati University, Jabalpur (M.P.) for providing the facilities and encouragement.

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