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Ameliorative Effect of Ca^{2+} on the Nitrogen Metabolism Changes Induced by Salinity in *Anabaena subcylindrica*

AMAL.H.EL-NAGGAR[1], MOHAMED.E.H.OSMAN, MOSTAFA.M.EL-SHEEKH AND MAHA, M.F. MAKLED†
Botany Department, Faculty of Science, Tanta University, Tanta, Egypt
†*The Technology Center for Education Development, Tanta, Egypt*
[1]Corresponding author's e-mail: elnagaramal@yahoo.com

ABSTRACT

The effect of salinity stress (0.3M NaCl) on the nitrogen metabolism of the cyanobacterium *Anabaena subcylindrica* (Borge) in absence or presence of $CaCl_2$ (0.03 or 0.05 M) was investigated. Salinity stress induced reduction in protein content, nitrogenase activity, some amino acids biosynthesis and nucleic acids content. On the other hand, salinity treatment stimulated the protease activity and the production of proline and cystine. Exogenous addition of $CaCl_2$ to the culture medium alleviated the toxic action induced by salinity. The protein electrophoretic pattern of the salinized *A. subcylindrica* showed disappearance of some protein bands (76,42 and 39 KDa) as compared with control, presence of $CaCl_2$ in the salinized culture caused the reappearance of these bands again. At the same time, 40 KDa protein appeared in salt and salt calcium treated cells.

Key Words: Amino acids; Calcium; Cyanobacteria; Protein patterns; Salinity

INTRODUCTION

Salinity is considered the most significant ecological factor affecting growth and metabolic activities of the different plants and microorganisms. Among the microorganisms which seriously affected by salinity are cyanobacteria, which play a fundamental role in supplying the crop plants with different nitrogen components, growth regulators, increase the yield and indirectly maintain the fertility status of soils. Salinity stress is well known to suppress algal growth (Lehtimaki *et al*., 1997; Masojidek *et al*., 2000 & Hagen *et al*., 2001). Moreover, it caused significant reduction in photosynthesis and respiration of some macroalgae (Karsten *et al*., 1991) and enhanced Golgi apparatus and endoplasmic reticulum (Berube *et al*., 1999). In previous communication (El-Naggar *et al*., 2004), salinity treatment (0.3M NaCl) induced pronounced reduction in growth, pigment fractions, carbohydrates, O_2-evolution, respiration, lipids content and increased some elements content (Na^+, K^+, Mg^{2+}, Fe^{3+}) in *Anabeana subcylindrica* and *Nostoc linckia*. In addition, presence of Ca^{2+} (0.03 or 0.05M) caused significant recovery in the above mentioned growth parameters and metabolic activities.

The cyanobacterial response to salinity is very rapid and varied with time and external concentration during stress (Apte & Bhagwat, 1998). Salt-responsive genes encode proteins and other products taking part in osmoregulation (Delauney & Verma, 1993), general defense (La Rosa *et al*., 1992), and cellular protection (Godoy *et al*., 1990). Many authors recorded inhibition in the synthesis of several proteins in response to salinity treatment (Apte & Bhagwat, 1998). On the other hand, an enhacement in the synthesis of certain proteins and *de novo* synthesis of a specific set of proteins in response to salinity was observed (Fulda & Hagemann, 1995). The information concerning the role of calcium in repairing damage in stressed algal cells is scarce. The aim of this study was to examine a possible role played by Ca^{2+} in ameliorating salt induced changes in the nitrogen metabolism in *A. subcylindrica*.

MATERIALS AND METHODS

Anabaena subcylindrica was the most dominant cyanobacterial species in the cultivated fertile soil in Tanta, Egypt. The organism was isolated, identified according to Prescott (1978). Axenic cultures of the organism were obtained by treating the cultures with antibiotics as described by Venkatarman (1969). The cultures were grown in the nutrient medium recommended by Allen and Stanier (1968), illuminated continuously with fluorescent light of 75 μ mol m^{-2} s^{-1} and maintained at 26$\pm$1°C. Cultures were aerated and shaken on rotatory shaker.

The dry weight was determined according to the method used by Ahmed and Osman (1973). The total soluble proteins were determined by the method described by lowry *et al.* (1951). Total free amino acids were estimated according to Moore and Stein (1958) using a Beckmann amino acid analyzer (model 119CI). The quantity of each amino acid was calculated as gm amino acid / 100 gm protein. Proline content was estimated using acid ninhydrin method as described by Bates *et al*. (1973).

The protein electrophoretic pattern was determined by the method cited by Laemmli (1970) using the extraction buffer recommended by Hawkesford and Belcher (1991). The extract was also used for protein estimation.

The protease activity was determined, in the enzyme

extract prepared according to the method described by Yomo and Varner (1973). The enzyme activity was assayed and the amino acid produced were determined by ninhydrin reagent according the method cited by Lee and Takahashi (1966). The nitrogenase activity was estimated using the method described by Hardy *et al*. (1973). Nucleic acids were determined according the method developed by Marmur (1961) and modified by Mohamed and Capesius (1980).

Statistical analysis. To investigate the relationships between concentrations of $CaCl_2$ with NaCl and contents of different variables at different ages, the Pearson correlation coefficient (r) was calculated at 0.05, 0.01 and 0.001 probability levels and the test of significance was performed. One way analysis of variance was performed at the above P levels of significance

RESULTS AND DISCUSSION

Treatment of *Anabaena subcylindrica* with 0.3 M NaCl caused reduction in the protein content throughout the experimental period which attained 42% after 15 days (Fig. 1). The reduction in protein content in response to high levels of salinity was reported in some green microalgae by Ahmed *et al*. (1984); in *Synechocystis* sp. by Mikkat *et al.* (1997); in *Dunaliella salina* by Dondini *et al.* (2001). Inhibition of protein accumulation by NaCl alone may be attributed to the inhibitory effect on the enzymatic reactions responsible for protein biosynthesis.

However addition of $CaCl_2$ (0.03 or 0.05 M) to salinized culture of *A. subcylindrica* caused significant increase in the protein content. The percentage of increase amounted to 118% compared with salinized culture after 15 days of incubation. In accordance with the present results, cytosolic Ca^{2+} is involved in signaling pathways in plant responses to salinity/ drought (Sanders *et al*., 1999).

During adaptation to stress, cyanobacteria gene expression is altered resulting in the induction of special stress proteins (Borrbely *et al*., 1985). It could be suggested that accumulation of protein by addition of $CaCl_2$ in salinized culture may be one of the ways through which the algae can abolish the inhibitory effect of salinity, and or may be due to increased respiration leading to the utilization of carbohydrate in increased nitrogen metabolism.

To assess the inhibitory impact of salinity stress and its alleviation by calcium on protein synthesis, an electrophoretic profile of proteins for *A. subcylindrica* treated with NaCl and $CaCl_2$ was detected (Fig.2). Scanning of the gel showed the disappearance of proteins with an apparent mol. mass of 76, 42 and 39 kDa from the salinized cells compared to the control. At the same time 40 kDa protein has developed in both salinized and Ca^{+2} treated cultures which was not detected in the control. The induction of 40 kDa protein in response to salinity has been shown earlier in *Anabaena sp*. by Sinha and Hader (1996) and in *Synechocystis sp.* by Fulda *et al.* (1999). This accumulated protein in response to salinity may be involved in the rapid accumulation of proline or glycine betaine during stress (Weigel *et al*., 1986). These alterations in the proteins pattern in response to salinity coincides with Apte & Bhagwat (1998) who reported that salinity altered the protein patterns of two *Anabaena* strains by inducing the synthesis of a specific set of proteins called the salt-stress proteins. These proteins were distributed in a molecular mass range of 12 to 155 kDa and they are strain dependent. However, the 76 and 42 kDa proteins disappeared under NaCl stress was initiated again by the addition of 0.03 M $CaCl_2$ to the salinized culture of *A. subcylindrica*. Whereas, the 39 kDa band was observed in response to treatment with 0.05 M $CaCl_2$ after 15 days of incubation (Fig. 2). These results are in agreement with those obtained by Hoyos and Zhang (2000), who found that reversible protein phosphorylation / dephosphorylation play important roles in signaling the plant adaptive responses to salinity /drought stresses. A protein kinase with molecular mass of 40 kDa is activated in tobaco cells exposed to NaCl, and named HOSAK (high osmotic stress-activated kinase). This HOSAK is a component in a Ca^{2+} pathway that may lead to plant adaptation to hyperosmotic stress.

The most abundant amino acids of the total free amino acids in control *A. subcylindrica* were aspartic acid (14%), glutamic acid (13%) and arginine (10%) (Table I). Treatment with NaCl reduced the total free amino acids contents by 19% compared to the control. However, addition of 0.05 M $CaCl_2$ reversed the alterations in free amino acids composition induced by salinity and raised its content by 27% above that of the control. Application of 0.3 M NaCl to the culture medium caused marked reduction in the dicarboxylic amino acids (aspartic & glutamic), aliphatic amino acids (threonine, glycine, alanine, valine, isoleucine and leucine) and basic amino acids (histidine, lycine and arginine). However, addition of 0.05 M $CaCl_2$ to the salinized culture induced marked increase in these amino acids content reaching 64 and 96% in threonine and alanine, respectively (Table I).

Concerning mono amio-monocarboxylic amino acids containing sulphur, inclusion of 0.3 M NaCl induced stimulation in the biosynthesis of cystine (3.3 fold). Whereas, no detectable change in methionine content was observed. From aromatic amino acids, the biosynthesis of phenylalanine was stimulated by 12% above the control level in response to salinity, whereas, the tyrosine content was reduced by 19% below the control level. However, addition of 0.05 M $CaCl_2$ caused marked increase in both aromatic and sulphur containing amino acids as compared with control. The reduction in the amino acids content in response to NaCl treatment, was observed earlier in many plants (Agarwal & Gupta, 1995 and Mattioni *et al*., 1997).

Arginine and methionine are suggested to be the precursors of polyamine biosynthesis, therefore, reduction in their contents under salinity stress is expected to affect

Table I. Effect of 0.05 M $CaCl_2$ on the amino acids content (gm amino acids/100 g dry weight) of salinized culture of *Anabaena subcylindrica* after 15 days of incubation

Amino acids	Control	0.3 M NaCl	0.3 M NaCl + 0.05 M $CaCl_2$
Aspartic acid	6.4	3.6	6.6
Threonine	2.8	1.4	3.2
Serine	2.4	1.3	2.7
Glutamic acid	5.8	5	7.5
Glycine	2.4	1.9	3
Alanine	3.2	2	5.1
Cystine	0.03	0.1	0.2
Valine	2.6	2.3	3.4
Methionine	0.7	0.7	0.8
Isoleucine	2.5	2.5	3.7
Leucine	3.5	2.4	4.4
Tyrosine	1.6	1.3	1.9
Phenylalanine	1.7	1.9	2.7
Histidine	1	0.6	1.2
Lysine	2	1.9	2.8
Arginine	4.7	2	4.9
Total	44.83	36.45	56.9

Table II. Effect of two different concentrations of $CaCl_2$ (0.03 or 0.05 M) on the proline content (mg/100ml algal suspension) of cells and culture filtrate of salinized cultures of *Anabaena subcylindrica*

	Control		0.3 M NaCl		0.3 M NaCl + 0.03 M $CaCl_2$		0.3 M NaCl + 0.05 M $CaCl_2$	
Age (days)	Cells	Filtrate	Cells	Filtrate	Cells	Filtrate	Cells	Filtrate
3	0.6	0.8	1.3	1.7	1.3	1.6	1.4	1.9
6	0.7	0.85	2	2.3	1.5	1.8	1.5	1.95
9	0.7	0.85	2.5	2.7	1.7	2	1.7	2.2
12	0.75	0.9	2.9	3.1	1.7	2.2	1.9	2.4
15	0.8	1.0	3.1	3.6	1.2	1.6	1.6	1.8

F-value	Algal cells	Culture filtrate
Day	$1.5E^{+03**}$	$2.8E^{+03**}$
Conc.	$2.4E^{+04**}$	$2.4E^{+04**}$
Day x Conc.	$2.4E^{+04**}$	$1.6E^{+03**}$

*= significant difference at $p \leq 0.05$, **= $p \leq 0.01$, ***=$p \leq 0.001$

polyamine content, which in turn inhibits algal growth and development (Bouchereau *et al*., 1999). Glutamic acid and glycine are known as precursors of glutathione, one of the antioxidant enzymes detected in higher rates in stressed plant cells (El-Shintinawy & El-Shourbagy, 2001). Thus, the decline in the contents of these amino acids may affect the rate of glutathione biosynthesis that has a role in protecting the plant cell from oxidative damage induced under salinity stress (Hernandez *et al*., 1993).

Addition of 0.3 M NaCl caused a significant rise in the free proline content in both cyanobacterial cells and culture filtrates amounted to 2.8 and 2.6 folds, respectively (Table II) after 15 day of incubation. However, the presence of 0.05 M $CaCl_2$ in the salinized culture counteracted the salinity effect by reducing the proline content by 49% and 50% in cells and culture filtrate, respectively after 15 days of incubation. The enhancement of proline content by salt stress is a common metabolic response observed in higher plants (Agarwal & Gupta, 1995) and algae as in *Cyclotella cryptica* (Liu & Hellebust, 1976) and in *Chlorella* (Laliberte & Hellebust, 1989). As a result of proline accumulation, a reduction in arginine and glutamic acid contents was observed, which regarded as proline precursors (Guerrier, 1998).

The recovery of the biosynthesis of the different amino acids by Ca^{2+}, except proline, are in agreement with the data obtained by Ahmed *et al.* (1989) and Hamada (1994). Thus, Ca^{2+} addition can alleviate the above mentioned alterations in amino acids composition by increasing the total free amino acids to exceed that of control.

Treatment of *A. subcylindrica* with 0.3 M NaCl caused significant stimulation in the protease activity throughout the experimental period (Fig. 3). The increase in protease activity in response to salinity was reported in *Vicia faba* by Sallam (1999). On the other hand, Hegazi *et al*. (1995) observed that protease activity of wheat plants was reduced under saline conditions. However, addition of $CaCl_2$ reversed the effect of salinity by inducing significant reduction in the protease activity. In this respect, Liao *et al*. (1993) reported that the divalent cations affected extracellular enzymes production. Moreover, Liao *et al*. (1992) suspected that calcium may serve as an environmental signal which is directly involved in the activation of rep genes required for the synthesis and/or export of extracellular enzymes. The applied high calcium concentration may be directly or indirectly involved in the inhibition of the rep genes. Palmieri *et al*. (2001) found that new extracellular protease activity is positively affected by calcium. A ten-fold decrease in the K_m value in the presence of calcium ions can reflect an induced structural change in the substrate recognition site region. The reduction in protease activity after addition of $CaCl_2$ to the salinized culture may support our results which indicated that addition of $CaCl_2$ to the salinized culture caused significant increase in the protein content. Also, the increase in protease activity in response to NaCl may support the previous

Table III. Effect of two different concentrations of $CaCl_2$ (0.03 or 0.05 M) on the nucleic acids (mg/g fresh weight) of salinized cultures of *Anabaena subcylindrica*

	Control		0.3 M NaCl		0.3 M NaCl + 0.03 M $CaCl_2$		0.3 M NaCl + 0.05 M $CaCl_2$	
Age (days)	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
3	1.1	3.1	0.8	2.2	2.1	5.1	2.3	7.4
6	2.1	4.9	1.1	3.5	2.5	7.9	2.8	9.4
9	2.4	5.8	1.5	5.9	2.8	9.3	3	9.9
12	3.1	7.4	2	6.8	3.5	10.6	3.7	11.9
15	4	9.9	2.4	7.4	4.2	12.7	4.4	13.8

F-value	Algal cells	Culture filtrate
Day	$9.9E^{+04**}$	$3.8E^{+05**}$
Conc.	$9.6E^{+04**}$	$1.0E^{+06**}$
Day x Conc.	$1.4E^{+03**}$	$9.5E^{+03**}$

*= significant difference at $p \leq 0.05$, **= $p \leq 0.01$, ***=$p \leq 0.001$

Fig. 1. Effect of two different concentrations of $CaCl_2$ on the total soluble protein content (g/100 g dry weight) of salinized culture of *Anabaena subcylindrica*

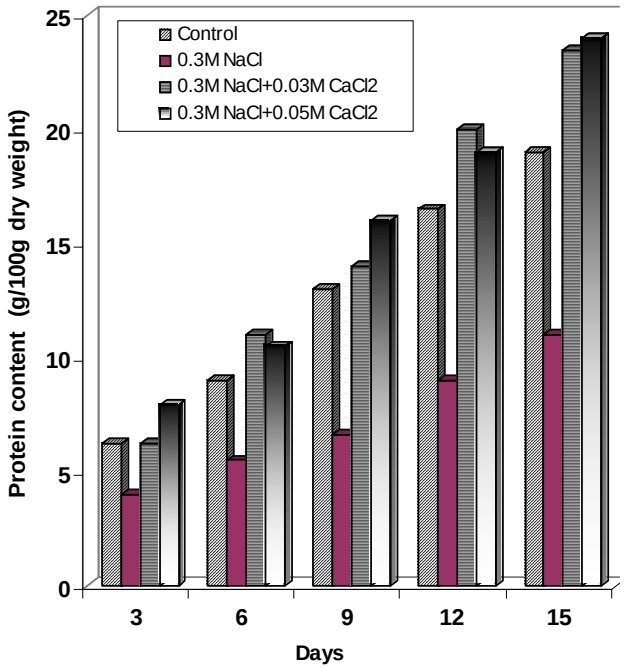


Fig. 2. The SDS-PAGE profile of total proteins of *Anabaena subcylindrica.* Lane 1, Molecular weight markers (kDa); lane 2, control; lane 3, 0.3 M NaCl - treated cells; lane 4, 0.3 M NaCl + 0.03 M $CaCl_2$ - treated cells ; lane 5, 0.3 M NaCl + 0.05M $CaCl_2$ - treated cells

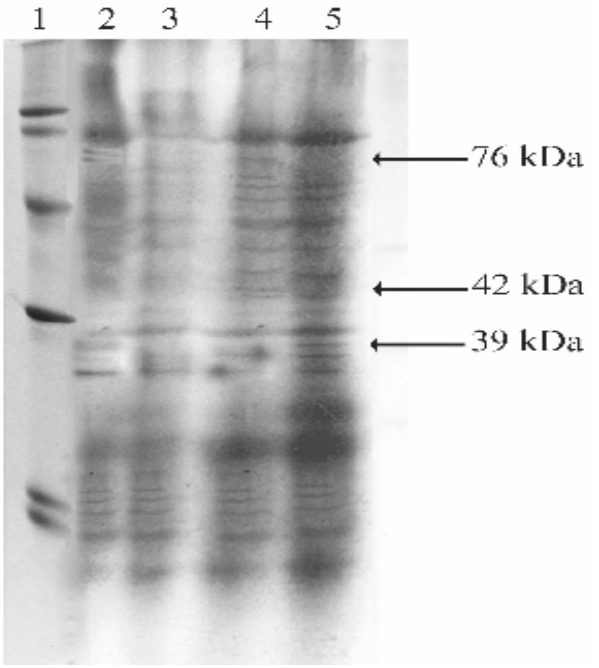


Fig. 3. Effect of two different concentrations of $CaCl_2$ on the protease activity (µmol amino acid released / h /100 mL algal suspension) of salinized culture of *Anabaena subcylindrica*

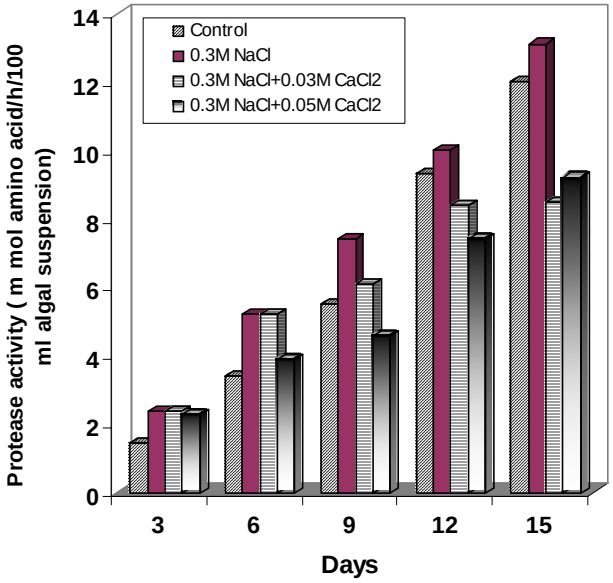
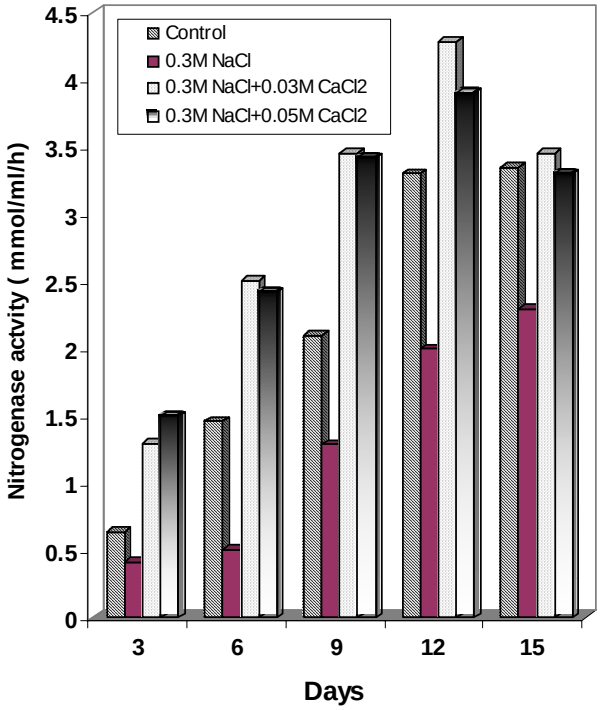


Fig. 4. Effect of two different concentrations of $CaCl_2$ on the nitrogenase activity (µmol C_2H_4/mL/h) of salinized culture of *Anabaena subcylindrica*



results which indicated the significant reduction in the protein content in response to salinity treatment.

Salinization of *Anabaena subcylindrica* with 0.3 M NaCl was accompanied by gradual reduction in nitrogenase activity (Fig. 4). The reduction in nitrogenase activity in response to salinity was observed earlier in *Anabaena* sp. by Rai and Tiwari (1999). The reduction in nitrogenase activity may be due to the reduction in the number of heterocysts caused by NaCl as reported in *Anabaena doliolum* by Rai and Abraham (1993).

However, addition of $CaCl_2$ to NaCl treated culture caused considerable recovery in nitrogenase activity. Rodriguez *et al*. (1990) reported the requirement of Ca^{2+} for nitrogenase activity in two strains of *Anabaena*. Calcium may function as a component of a signal transducing system in some cyanobacteria (Onek & Smith, 1992). Moreover, the role of Ca^{2+} seem to be related the protection of nitrogenase complex from oxygen.

The nucleic acid contents (DNA & RNA) showed significant reductions in response to salinity treatment throughout the experimental period (Table III). In accordance with our results, inhibition of nucleic acids content in response to salinity was reported in *Nostoc muscorum* by Pandey and Chatterjee (1999) and in wheat by Hegazi *et al*. (1995).

Again, addition of $CaCl_2$ in the culture medium to the salinized culture caused a significant increase in the nucleic acids content. In accordance with the results present in this study, Pardo *et al*. (1998) indicated that in yeast a Ca^{2+} and calmodiolin-dependent calcineurin (CaN) signal transduction pathway regulates determinants of salt tolerance required for stress adaptation. Modulation of this pathway by expression of an activity regulating intermediate substantially enhanced salt tolerance.

As a consequence of the intracellular regulatory effect of calcium, it is reasonable to believe that extra concentrations of Ca^{2+} in the soil could have a regulatory effect in the ecosystem.

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