SENRA Academic Publishers, Burnaby, British Columbia
Vol. 3, No. 1, pp. 669-673, 2009
ISSN: 1715-9997

Canadian Journal of pure&applied sciences

OSMOTIC STRESS IN THE CYANOBACTERIUM *NOSTOC MUSCORUM* OVERCOME BY THE ACCUMULATION OF PROLINE

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ABSTRACT

Growth and percent survival in the cyanobacterium *Nostoc muscorum* severely inhibited, when challenged with salinity (NaCl) and osmotic (sucrose) stresses. The spontaneously occurring NaCl-R and sucrose-R (Su-R) mutant clones were examined with regards to their cross-resistant relationships. The NaCl-R mutant when challenged with salinity and osmotic stresses simultaneously showing cross-resistance with the sucrose stress. In contrast, the Su-R mutant showing resistant only to osmotic stress and was salinity sensitive. The physiological response towards osmotic stress in both the mutant clones was the over-production of proline. The ionic component of the salinity stress in the NaCl-R mutant was overcome by the Na^+-efflux. The Su-R mutant clones missing Na^+ efflux mechanism, consequently, they became salt sensitive. These findings highlight that the organic osmolyte strategy in *N. muscorum* assured osmotic resistance.

Keywords: Cyanobacterium, intracellular proline, *Nostoc muscorum*, osmotic stress, salinity stress.

INTRODUCTION

Cyanobacteria are prokaryotic, photoautotroph growing and multiplying luxuriantly in natural and agricultural ecosystems, despite facing substantial fluctuation in the range of environmental conditions including salinity and osmotic stresses. They exhibit photo-assimilation of nitrogen, CO_2 and contribute to O_2 production. Some of the cyanobacterial forms are the progenitor of chloroplast evolution in eukaryotes through the process of endosymbiosis. Therefore, they are considered as evolutionarily the most important group of organisms (Douglas, 1994). Saline soil creates ionic stress as well as osmotic stress and inhibiting microbial and crop productivity by creating water stress and Na^+ toxicity. Therefore, the microorganisms that thrive in such stressful habitat alter their physiology to cope up with the changing environment. The ionic component of the stress factor is readily overcome by the Na^+/H^+ antiporter activity (Inaba *et al*., 2001; Elanskaya *et al*., 2002; Waditee *et al*., 2002). The cytoplasmic osmotic potential is balanced either by accumulation or by synthesis of compatible solutes (Csonka, 1989; Alia and Gahiza, 2007). The synthesis of compatible solutes is usually regulated by the cyanobacterial habitat. The fresh water cyanobacterial strains were found to be least tolerant and are known to produce sucrose, trehalose and proline as compatible solutes (Hagemann *et al*., 1996; Singh *et al*., 1996). The moderately halotolerant cyanobacteria produces glucosyl-glycerol as organic osmolyte for such adaptation (Borges *et al*., 2002; Hincha and Hagemann, 2004; Marin *et al*., 2006).

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In comparison, hyper saline cyanobacterial strains are known to produce glycine-betaine as compatible solutes (Warr *et al*., 1988).

The present study indicates that different biochemical mechanism operate to remove ionic (salinity) and non-ionic (osmotic) stresses in the *N. muscorum*. The ionic stress is overcome by Na^+ export and osmotic stress by the synthesis of low molecular weight organic compatible solute i.e. proline.

MATERIALS AND METHODS

The *Nostoc muscorum* used in the present study was a fresh water, filamentous, nitrogen fixing (diazotroph), heterocystous cyanobacterium. It was grown axenically in Chu No. 10 (Gerloff *et al*., 1950) at 28 ± 2°C and illuminated with fluorescent tubes having a photon fluence rate of 50 μmol m^{-2} s^{-1} with 16/8 h light/dark cycle. The culture medium was buffered to pH 7.5 with 10 mol m^{-3} HEPES-NaOH.

Salt and osmotic concentrations were adjusted by adding 100 mol m^{-3} NaCl and 250 mol m^{-3} sucrose to the diazotrophic growth medium. Such diazotrophically grown cultures were periodically examined (3, 6, 9, 12 hours) for their respective characteristics.

NaCl at the concentration of 100 mol m^{-3} and sucrose at the concentration of 250 mol m^{-3} were found lethal to the diazotrophic growth of the cyanobacterium *N. muscorum*. NaCl-Resistant (NaCl-R) and Sucrose-Resistant (Su-R) mutants of the cyanobacterium were isolated by plating $2.5\text{-}3.0\times10^7$ colony forming units (CFUs) on diazotrophic growth medium containing 100 mol m^{-3} NaCl and 250

mol m^{-3} sucrose. The spontaneously occurring mutant clones were checked for their stability by the method described previously (Bhargava and Singh, 2006).

For the measurement of Na^+ efflux cyanobacterial strains were Na^+ starved by growing them in diazotrophic growth medium lacking Na^+ for 72 hours. The cyanobacterial strains were centrifuged, washed and resuspended in DH_2O buffered by 10 mol m^{-3} HEPES-NaOH at pH 7.5. Cultures were incubated for 30 min at 28 ± 2°C under continuous light at a photon fluence rate of 50 μmol m^{-2} s^{-1}. NaCl at a concentration of 5 mol m^{-3} was added to the incubation mixture. The amount of Na^+ present in the buffer was measured with the help of Flame Photometer.

Estimation of growth, percent survival, chlorophyll *a* (Mackinney, 1941), protein (Lowry *et al*., 1951) nitrogenase activity, intracellular proline contents and proline oxidase activity was done as described previously (Bhargava and Singh, 2006).

RESULTS

NaCl-R mutant and Su-R mutant clones reported here arose with a mutational frequency of 0.6 ~ 0.8 × 10^{-7} suggesting that the resulting mutant phenotype is a product of point mutation in the chromosomal genes. The physiological response of *N. muscorum* and its spontaneously occurring NaCl-R and Su-R mutant clones were compared under NaCl stress and sucrose stress conditions. The percent survival decreases with the increasing time of stress exposure. A dose of 100 mol m^{-3} NaCl and 250 mol m^{-3} sucrose for 12 hours completely inhibit the growth of the wild type strain (Fig. 1).

A comparison of percent survival of the Su-R mutant under NaCl and sucrose stresses was done. The observation indicates that Su-R mutant clones were showing resistance only to sucrose stress. On further examination Su-R mutant clones were found to be sensitive to NaCl stress (Fig. 2). The NaCl-R mutant clones were compared for their percent survival in the diazotrophic growth medium containing 100 mol m^{-3} NaCl and 250 mol m^{-3} sucrose. The results (data not shown) suggested that NaCl-R mutant clones exhibited almost complete resistance to growth inhibitory action of 100 mol m^{-3} NaCl and 250 mol m^{-3} sucrose.

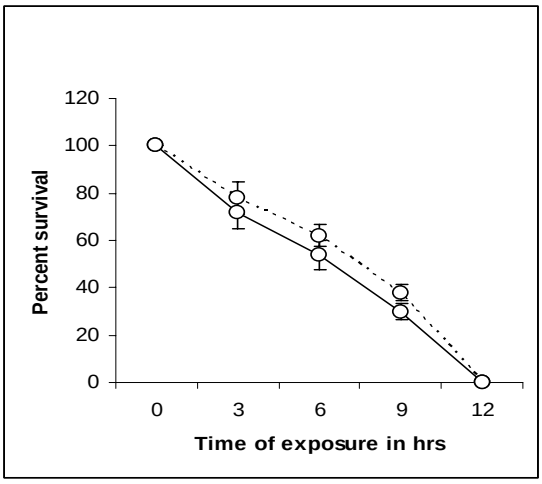

Fig. 1. Percent survival of the wild type *N. muscorum* challenged with NaCl stress (—) and under sucrose (---) stress conditions under diazotrophic growth condition. Each reading is an average (±SEM) of three independent experimental determinations.

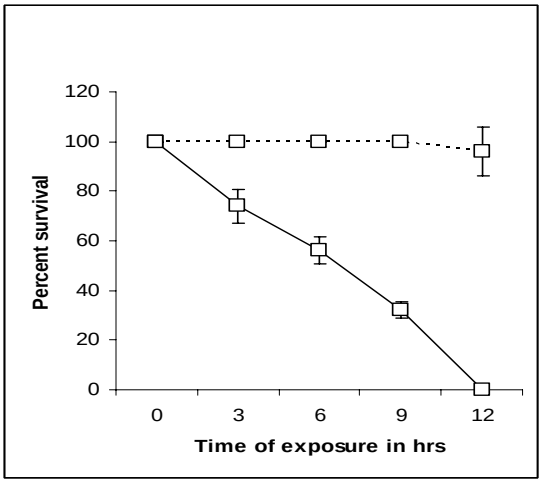

Fig. 2. Percent survival of the sucrose-R mutant clones challenged with NaCl stress (—) and sucrose stress (---) conditions under diazotrophic growth condition.

Table 1. Growth (OD change at 663nm), heterocyst frequency (HF %), nitrogenase activity (m mol C_2H_4 formed g^{-1} Chl *a* h^{-1}) and proline oxidase activity (m mol proline oxidized g^{-1} Chl *a* h^{-1}) of the *N. muscorum* and its various mutant clones.

Parameters	Wild type	NaCl-R	Sucrose-R
Growth	0.82 ± 0.08	0.78 ± 0.07	0.80 ± 0.08
HF%	7-8	7-8	7-8
Nitrogenase activity	12.28 ± 0.78	11.15 ± 0.81	11.54 ± 0.79
Proline oxidase activity	2.56 ± 0.14	0-0	0-0

Non-heterocystous NH_4^+ grown cultures were source of inoculum for the experiments. Such cultures were grown for six days in diazotrophic growth medium and then used for estimation of their characteristics.
Each reading is an average (±SEM) of three independent experimental determinations.

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Further experiments were done to compare various mutant clones with its wild type in respect to photoautotrophic growth, heterocyst frequency, nitrogenase activity, proline oxidase activity, intracellular proline contents and Na^+ uptake. The results as shown in the table 1 suggested that mutant strains showed almost similar level of photoautotrophic growth, heterocyst frequency and nitrogenase activity in the diazotrophic medium. The wild type strains did not produce heterocyst and nitrogenase activity in the growth medium containing 1 mol m^{-3} proline suggested that *N. muscorum* is capable of assimilating proline as a nitrogen source and its mutation to NaCl-R and Su-R phenotypes have resulted in loss of this ability. Thus it can be concluded that mutant clones have lost genetically proline repression control of heterocyst and nitrogenase activity. Further analysis in respect of proline catabolizing enzyme i.e., proline oxidase revealed the presence of this enzyme in the wild type and its absence in the mutant clones. Consequently, mutation to resistant phenotype resulted in over-production of proline and acquisition of osmotic resistance in the cyanobacterium.

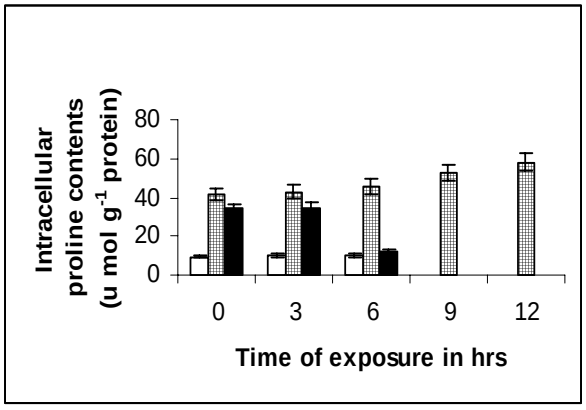

Fig. 3. Effects of salinity stress on intracellular contents of proline in the wild type *N. muscorum* (blank bars) and its NaCl-R (check bars) and sucrose-R (dark bars) mutant clones.
Each reading is an average (±SEM) of three independent experimental determinations.

The additional noteworthy characteristic of mutant clones (NaCl-R and Su-R) were its higher contents of the proline than that found in the wild type. In addition the proline contents of the NaCl-R mutant showed slight rise when stressed with NaCl (Fig. 3) or sucrose (Fig. 4). In comparison wild type with or without stress has not exhibit any variation in its proline contents. Similarly Su-R mutant was found similar to the wild type in intracellular proline contents under NaCl stress.

The pattern of Na^+-uptake was also examined in the wild type and in the NaCl-R and Su-R mutant strains. The results as shown in the figure 3 indicates that Na^+ uptake pattern in the wild type and its Su-R mutant was more pronounced as compared to the NaCl-R mutant. This observation suggested that Na^+ uptake pattern in the Su-R mutant remained unaltered, while the acquision of NaCl-R phenotype mitigates the Na^+ entry.

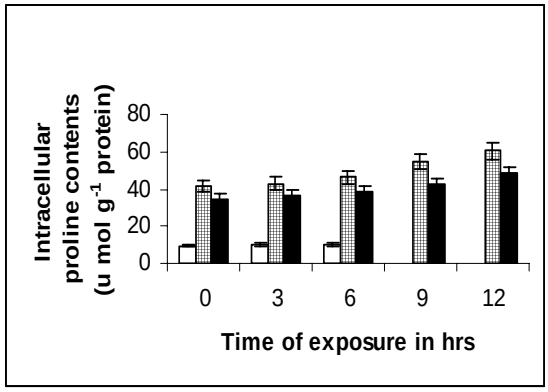

Fig. 4. Effects of osmotic stress on intracellular contents of proline in the wild type *N. muscorum* (blank bars) and its NaCl-R (check bars) and sucrose-R (dark bars) mutant clones.
Each reading is an average (±SEM) of three independent experimental determinations.

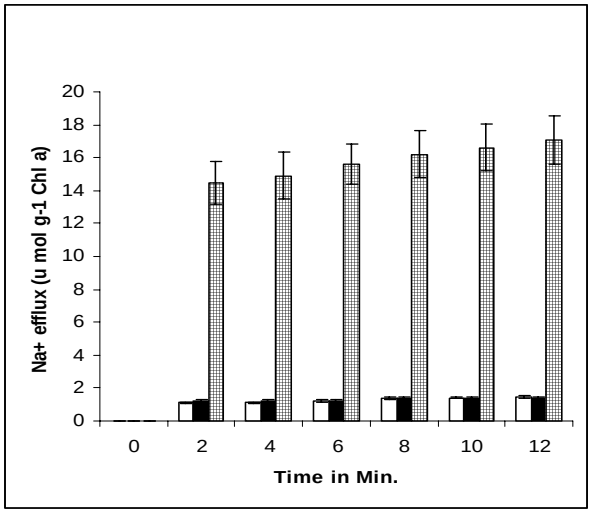

Fig. 5. Na^+ efflux pattern in the wild type *N. muscorum* (blank bars) and its NaCl-R (check bars) and sucrose-R mutant strains (dark bars).
Each reading is an average (±SEM) of three independent experimental determinations.

DISCUSSION

The physiological impact of salt and osmotic stresses has been analyzed in the *N. muscorum* and its spontaneously occurring mutant clones resistant to growth inhibitory action of salt and sucrose. NaCl-R mutant and Su-R

mutant were also analyzed for cross-resistant relationship with salt and osmotic stress. The NaCl-R mutant clones show cross relationship with osmotic stress, therefore, we suggest that mutation to NaCl-R phenotype may involve two different kinds of mutation one leading to ionic stress and another leading to osmotic stress components. On the contrary Su-R mutant clones showing resistant to sucrose stress only and are NaCl sensitive. Therefore, it can be concluded that Su-R phenotype involves mutation only in osmotic stress component.

Wild type *N. muscorum* and its mutant clones differ in a most noticeable way with regards to their intracellular proline contents. Wild type with or without stress has not exhibit any variation in its proline contents. In comparison NaCl-R and Su-R mutant exhibit manifold higher intracellular proline contents under normal growth condition. Since sucrose-R phenotype is not found cross-resistance to NaCl stress while having high intracellular proline contents the obvious cause for lack of cross-resistance of NaCl in Su-R mutant seem to be the efflux mechanisms which might have remain unaltered in the sucrose-R mutant clones.

The common molecular component in NaCl-R and Su-R mutants is high intracellular levels of proline. This common molecular phenotype correlates well with the physiological phenotype of osmo resistance (Mikkat *et al*., 1996; Marin *et al*., 1998; Marin *et al*., 2006). We may conclude that intracellular accumulation of proline is the common cause of osmotic resistance in the mutant clones. Our observation regarding the compatible solute synthesis during osmotic stress is in agreement with results already published on *Synechocystis* (Marin *et al*., 2006). The two genes *viz*. *put*P and *put*A are known to involve in accumulation or synthesis of proline in bacterial systems (Csonka, 1989). Mutational inactivation of the *put*A gene inhibits the catabolism of proline therefore such strains are proline-overproducer. Our findings supported by the above fact because the mutant clones reported here are also proline overproducer.

Singh *et al*. (1996) reported that enzyme proline oxidase is required to assimilate exogenous proline in the cyanobacterium *N. muscorum*. The mutants clones reported here missing proline oxidase activity, this could be the reason of proline over-production in the mutant clones. Similar findings were reported in bacterial systems by Dendinger and Brill (1970).

The NaCl-R mutant reported here involved two mutations leading to resistant phenotype. The osmotic stress component is overcome by the synthesis of compatible solute i.e. proline and the ionic component by the active efflux of Na^+. Our findings are quite in agreement with the previous reports regarding Na^+ efflux mechanism in bacterial and cyanobacterial systems (Padan and Schuldiner, 1994; Blumwald *et al*., 1984; Apte *et al*., 1987; Inaba *et al*., 2001; Elanskaya *et al*., 2002).

ACKNOWLEDGEMENTS

SB and SD are thankful to the MAPCOST, Bhopal (M.P) for the financial support. RM and VKS are thankful to the Director, RGTU, Bhopal (M.P) for providing laboratory facility.

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