Science Vision 14(4), 207-214

Original Research

2014
October-December
ISSN (print) 0975-6175
ISSN (online) 2229-6026

Changes in antioxidative enzymes of cyanobacterium *Nostoc muscorum* under copper (Cu^{2+}) stress

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Received 17 November 2014 | Revised 5 December 2014 | Accepted 15 December 2014

ABSTRACT

The effects of copper on the growth, photosynthetic pigments, and antioxidants of the cyanobacterium *Nostoc muscorum* were studied. Growth behavior under different concentrations of Cu showed strong inhibition with growth rate of 50% reduced at 2.5 μM after 96 h of treatment. There were total inhibitions at 7.5 μM and 10 μM concentrations. Photosynthetic pigments such as chlorophyll-a, phycocyanin and carotenoid contents decreased at the same extent after 96 h of Cu treatment and the inhibition was highest on phycocyanin content. Protein content was inhibited at the same pattern with photosynthetic pigments. Cu-induced lipid peroxidation was concentration and time dependent. Treatment with 10 μM Cu for 2 h resulted in 4.9 folds increase in malondialdehyde (MDA) level in comparison with the control. Similarly, 3.8 folds increased was observed after 5 h treatment with LC_{50} metal (2.5 μM Cu), compared to the control. The activities of antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were strongly increased following the increase of Cu concentrations-they reached 159%, 202%, 172% and 179% above the control, respectively.

Key words: Copper; superoxide dismutase; catalase; glutathione reductase; *Nostoc muscorum.*

INTRODUCTION

Heavy metal toxicity and uptake in cyanobacteria have been extensively discussed.[1-3] Microalgae, especially cyanobacteria can efficiently sequester toxic heavy metal ions by adsorption and by absorption from aquatic environments; therefore, they are widely used for phytoremediation and regulation of heavy metal polluted areas.[4] They are considered to be one of the most important factors improving contaminated soils as a result of their metabolic activity. They may bind up to 10% of their biomass as metals. Converti *et al.*[5] used *Spirulina platensis* biomass as adsorbent for copper removal from water solution while El-Sheekh *et al.*[6] found that copper was removed by 12.5-81.8% from wastewater by using cyanobacterial cultures of *Nostoc muscorum* and *Anabaena subcylindrica*. It was well docu-

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mented that some heavy metals could penetrate the cell walls and subsequently deposited into the cell ingredients, e.g. *Anabaena cylindrica* and *Plectonema boryanum* accumulated Al and Cd in the polyphosphate bodies.[7]

It was widely accepted that one possible course of action of heavy metals in plants as well as algae including cyanobacteria is generation of toxic reactive oxygen species (ROS). Copper, a redox-active metal induced formation of hydrogen peroxide (H_2O_2), hydroxyl radicals and other ROS by directly participating in the Haber-Weiss reaction and damage the membrane lipids and proteins. In order to survive under stress, every cell possesses a complex array of enzymatic and nonenzymatic antioxidant defense systems. Superoxide dismutase (SOD) is the first enzyme of the enzymatic antioxidative pathway to convert superoxide anion into peroxides, which are scavenged by catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). These antioxidant enzymes not only control the steady of the reactive oxygen species but also allow them to perform important functions at specific sites under a variety of environmental conditions and at different developmental stages of the organisms. It was found that catalase and ascorbate peroxidase activities were presented in *N. muscorum*, *Synechococcus* and *A. nidulans* cells.[8]

The problem concerning heavy metal detoxifying in algae and cyanobacteria remains still unsolved. The aim of the present work was to study changes in some antioxidative enzymes and their role in cell response against heavy metal injuries in the cyanobacterium *N. muscorum*.

MATERIAL AND METHODS

N. muscorum which is a filamentous, heterocystous cyanobacterium was grown axenically in Chu-10 (without N_2) medium illuminated by fluorescent tubes under 8 h photoperiod at pH 7.5. The cultures were hand shaken at least 2-3 times daily. Growth was determined by measuring the optical density of cyanobacterial culture at 663 nm in a UV/VIS spectrophotometer (Systronics, India) on every third day up to 18th day by using reference blank of basal culture medium. The specific growth rate (μd^{-1}), based on absorbance was calculated for control and treatment after 96 h, using the equation:

$$\mu = [\ln (n_2/n_1)]/[t_2-t_1]$$

where μ stands for specific growth rate and n_1 and n_2 are absorbance of culture suspension at the beginning (t_1) and the end (t_2) of the selected time interval. Lethal concentration (LC_{50}) was determined using data of specific growth rate of the cyanobacterium under the stress as mentioned in Guillard.[9]

Protein content was determined by Lowry's method.[10] Chlorophyll-a and carotenoid contents were measured after extraction with 95% ethanol overnight at 4°C. The contents were determined and calculated according to Li.[11] To determine phycocyanin content, samples were resuspended in 0.05 M PBS 7.8 and then treated with freezing and thawing five times. The amount was calculated according to Myers and Kratz.[12]

Oxidative damage of lipid was measured in terms of the total content of 2-thiobarbituric acid -reactive substances (TBA) and expressed as equivalent of MDA using method of De Vos *et al.*[13] Lipid peroxidation in the test algae was determined after 2 h treatment with various concentrations of Cu and with lethal dose for different time periods.

Different concentrations of Cu-induced enzymatic antioxidants were studied. The cell pellet separated from exponentially growing cultures were suspended in cell lysis buffer [potassium phosphate buffer (pH 7.0), 1 mM EDTA and 1% (w/v) PVP] and subjected to sonication (350 mA for 2 min with six intervals of 20 sec each) in ice-cold condition (4°C). However, the above buffer additionally contained 1 mM ASA for APX assay. The sonicated sample was centrifuged at 15,000×g for 30 min at 4°C, and the resulting supernatant containing antioxidant enzymes was used for further assay. The total SOD activity was assayed by monitoring the inhibition of reduction of nitroblue tetrazolium according to the method of Robert *et al.*[14] Catalase activity was estimated by measuring the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 1 min.[15] APX activity was determined by measuring the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min in 1 ml reaction mixture.[15] GR activity was determined by measuring the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 5 min in 2 ml of assay mixture according to the method of Schaedle and Bassham.[16]

Statistical analysis

The observation was taken in triplicate. The mean data of triplicate value was put in statistical analysis by taking their standard error.

RESULTS

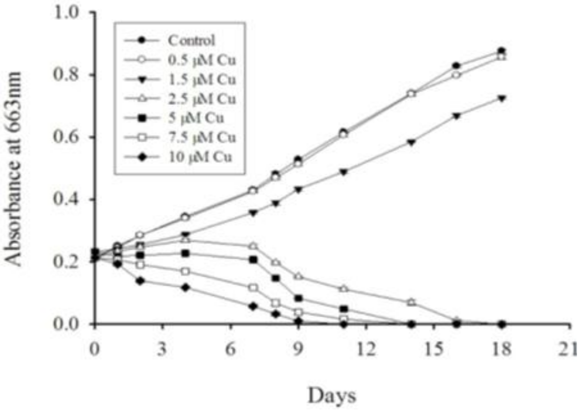

Figure 1. Growth pattern of *N. muscorum* in response to Cu treatment. Values are means of three replicates.

Figure 1 shows growth behavior of *N. muscorum* under different concentrations of Cu. A continuous decline in the growth of copper treated cells was observed. Table 1 shows specific growth rate of *N. muscorum* under various concentrations of Cu treatment after 96 h. The algal growth was significantly decreased in an increasing concentrations of Cu in the external medium and this reduction was more pronounced in the higher concentrations (7.5 µM, 10 µM), where there showed no growth. An approximate of 50% inhibition in specific growth rate of *N. muscorum* was observed at 2.5 µM of Cu after 96 h exposure. This dose was selected for further time-course study of different parameters. A general reduction in protein, chl-a, caroteniod and phycocyanin content (Fig. 2) was also observed in a metal concentration-dependent manner after 96 h of Cu treatment. Compared to the control, the chl-a content of *N. muscorum* showed 6.3%, 23.6%, 33.8%, 51.3%, 61.8% and 71.8% depletion with increasing Cu concentrations. Similarly, 7.692%, 26.025%, 37.694%, 55.258%, 65.773% and 79.873% and 1.159%, 16.225%, 28.146%, 41.887%, 50.331% and 58.704% respectively of phycocyanin and carotenoid content were also found to decrease. Among the photosynthetic pigments the inhibitory effect of metals on phycocyanin content was slightly higher than chl-a and carotenoid content at the highest concentration of tested

Table 1. Effect of different concentrations of Cu on percentage growth and inhibition of *N. muscorum* after 96h of treatment.

Cu2$^+$ (µM) in medium	**Specific growth rate (µd^{-1})**	**% growth**	**% inhibition in growth**
0	0.121 ± 0.004	100	0
0.5	0.119 ± 0.004	98.071	1.929
1.5	0.075 ± 0.007	61.709	38.291
2.5	0.058 ± 0.004	48.760	51.24
5	0.016 ± 0.008	13.223	86.777
7.5	No growth	0	100
10	No growth	0	100

All values are presented as the mean ± SE of three replicates.

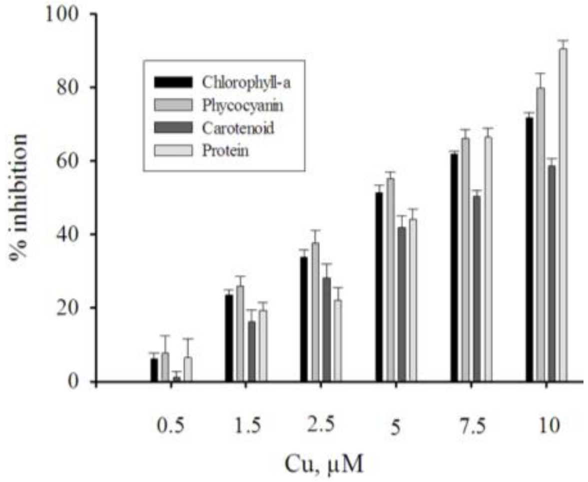

Figure 2. Effect of Cu on chrorophyl-a, phycocyanin, carotenoid and protein contents after 96 h of treatment. Values are means of three replicates.

metal.

Lipid peroxidation was used to measure oxidative damage in the test algae. Cu-induced lipid peroxidation was concentration and time dependent. MDA level significantly increases with increasing metal concentrations. The responses of the MDA contents to applied concentrations of Cu are depicted in Figure 3. MDA level significantly increases with increasing metal concentrations and also increases with time period. Treatment with 10 µM Cu for 2 h resulted in 4.9 folds increase in MDA level in comparison with the control (Fig. 3B). Similarly, 3.8 folds increased was observed after 5 h treatment with LC_{50} metal (2.5 µM Cu), as compared with the control (Fig. 3A).

Different concentrations of Cu-enhanced antioxidative enzyme (SOD, CAT, GR, and APX) activities are shown in Table 2. All the tested enzymes showed highest activity in stressed cells with increase in the concentrations of treatment. Effect of Cu on several antioxidative enzymes such as SOD, the enzyme for catalyzing the dismutation of O_2^- to O_2 and H_2O_2, CAT, the enzyme mainly responsible for eliminating H_2O_2 in the peroxisomes, and APX and GR, the two key enzymes of the Halliwell-Asada pathway for removal of H_2O_2 in the chloroplast. SOD content showed highest activity at 10 µM of Cu, this being 1.6 fold as compared to the control. Same trend were found in other antioxidative enzymes studied. CAT (2.0 fold) showed maximum activity after 10 µM Cu treatment followed by GR (1.7 fold) and APX (1.6 fold) as compared with their respective controls.

DISCUSSION

The study demonstrates effects of copper on growth, photosynthetic pigments, oxidative

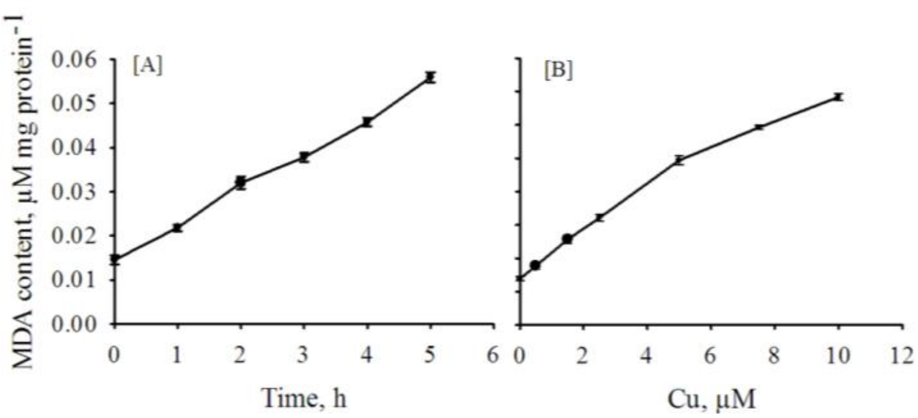

Figure 2. Effect of Cu on MDA content of *N. muscorum*. Exponentially growing algal cultures were treated with 2.5. µM Cu for 1,2,3,4, and 5 h (A).; and with different concentrations of Cu (B). Vertical bars represent standard error of the mean (n=#).

Table 2. Changes of SOD, CAT, APX and GR activities in *N. muscorum* cells after 3h treatment with different concentrations of Cu.

Cu concentration in µM	**Enzyme Activities**			
	U SOD mg^{-1} protein	**CAT (µM min^{-1} mg^{-1} protein)**	**GR (µM min^{-1} mg^{-1} protein)**	**APX (µM min^{-1} mg^{-1} protein)**
Control	0.0203 ± 0.001	0.0154 ± 0.002	0.0831 ± 0.003	0.328 ± 0.022
0.5	0.0213 ± 0.0009	0.0161 ± 0.0008	0.0870 ± 0.002	0.334 ± 0.014
1.5	0.0238 ± 0.004	0.0200 ± 0.001	0.0960 ± 0.002	0.393 ± 0.036
2.5	0.0277 ± 0.001	0.0231 ± 0.0009	0.119 ± 0.004	0.475 ± 0.014
5	0.0289 ± 0.001	0.0258 ± 0.001	0.132 ± 0.003	0.516 ± 0.022
7.5	0.0304 ± 0.001	0.0279 ± 0.0006	0.134 ± 0.004	0.551 ± 0.022
10	0.0323 ± 0.0009	0.0312 ± 0.002	0.143 ± 0.005	0.586 ± 0.036

All values are presented as the mean ± SE of three replicates.

stress and defense systems in *N. muscorum*. Cyanobacterium *N. muscorum* showed a series of physiological and biochemical alterations when exposed to various concentrations of Cu. Reduction in percent survival and growth of *N. muscorum* at increasing concentrations of copper confirmed the toxic potential of Cu (Fig. 1 & Table 1). The toxicity of metal may be due to either the disruption of the permeability of the cell membrane or inhibition of photosynthetic pigment and enzyme activities. Similar to present study several reports are available showing the inhibition of growth and metabolism of algae and cyanobacteria by Cu.[17] Another reason for reduction of growth might be the inhibition of cell division due to binding of Cu to sulphydryl groups which is responsible for regulation of cell division in plants. Further, it showed a significant reduction in Chl-a, phycocyanin, carotenoid and protein content in increasing Cu concentrations (Fig. 2). Metal treatment decreased in chl-a, phycocyanin and carotenoid content might be due to the active oxygen-species-induced damaging effects or intereaction with biosynthetic processes of these pigments. Active oxygen species are formed due to leakage of electrons at various sites of photosynthetic and respiratory electron transport chain under stress condition. The strong damaging effect of copper on phycocyanin may be due to the direct intereaction of metal with phycocyanin as it is located on the outer surface of thylakoid membrane. Finding on inhibition of chlorophyll content by Cu is in agreement with those of Tripathi *et al.*[18] Decrease in carotenoid content in the *N. muscorum* seems surprising as earlier reports suggest that carotenoid has protective role in restoration of photosynthesis. Many organisms tend to increase their carotenoid content under diverse kinds stress[19] and carotenoid accumulation is often regarded as one of the mechanisms to counteract stress in organisms.[20] However, the present study showed a concentration dependent decline in the level of carotenoids following exposure to Cu which is supported by the reports of Tripathi *et al.*[18] Similar finding was also observed in cyanobacteria under metal stress by Rahman *et al.*[21] Cu treatment also resulted in reduction of protein content extensively. This decilne may be due to production of ROS, which is known to damage protein, therefore disturbs the cellular homeostasis. Similar finding was also observed in *A. doliolum* exposed to Cadmium and UV-B stressed.[22] The present showed that metal affects negatively the total protein content at higher doses. It could be suggested that accumulation of protein at low heavy metal concentrations may be one of the ways through which the algae can abolish their toxic effects, or increase respiration leading to the utilization of carbohydrate in favor of protein accumulation.[23] Whereas the suppression of protein accumulation may be attributed to shortage of carbon skeleton results from low photosynthetic rate. Such results are in accordance with those of Fathi *et al.*[24] However, some authors[23,25] reported that the toxic action of heavy metals on the enzymatic reactions is responsible for protein biosynthesis.

Oxidative stress causes lipid peroxidation and thereby causing destruction of cell membranes. An elevated level of copper induced oxidative stress in *N. muscorum* was evident from enchanced lipid peroxidation and disrupted the cell membrane, thus causing a concomitant efflux of K^+ ions when algal cells are exposed to copper solutions. The present study showed increased MDA content with increasing concentrations of Cu after 2 h exposure to various concentrations of the test metal and with the lethal dose (2.5 μM) treatment for different time periods. Cu-induced generation of hydrogen peroxide, hydroxyl radicals and other ROS has been directly co-related with damage to membrane lipid and proteins.[26,27]. The present work shows stimulation of lipid peroxidation by Cu similar to the earlier reports by Mehta and Gaur.[28] Excess of Cu increased lipoxygenase activity and hence lipid peroxidation. Cu-induced lipid peroxidation in *N. muscorum* (Fig. 3A, B) finds support from Tang *et al*.[29] and Srivastava[30] who reported lipid peroxidation in *Scytonema javanicum* and *N. muscorum*, respectively exposed to salt stress. The prime consequence of lipid peroxidation is a disturbance in membrane fluidity, and thus ion balance, resulting in altered metabolism and ROS production.

A number of studies indicated that the degree of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of antioxidant system. The detoxicification of these reactive species is undertaken by non-enzymatic and enzymatic scavengers and quenchers. Present study showed activation of the protective enzymes SOD, CAT, GR and APX in Cu treated cells. Increased activity of antioxidant enzymes can be expected to reduce oxidative stress to algal cells. In fact, transgenic plants with enhanced activities of antioxidant enzymes have been shown to be tolerant to oxidative stress.[31] In *Nostoc* sp., the activity of the enzyme SOD that converts superoxide into H_2O_2, increased during the first 3 h of treatment and decreased with further increase in time (data not shown). However, SOD activity increases with increasing Cu concentrations. Induction of SOD activity is therefore, a requirement of the cell to encounter oxidative damage. Further, a decrease in the SOD activity can be explained in light of the fact that Fe-SOD is sensitive to prolonged and severe oxidative stress.[32] Activity of CAT, GR and APX under Cu stress has also been studied. It was observed that their activity increased significantly with increasing concentrations of copper. CAT play an important role in the fine regulation of ROS concentration through the deactivation of H_2O_2.[33] The increased activity of CAT is contrary to the finding of CAT sensitivity to salt stress in *A.doliolum.*[34] The other peroxide scavenging enzyme, APX, was also found to induced under Cu stress (Table 2). This increase may be attributed to a H_2O_2-mediated induction of the *apx* operon.[35] Therefore, it seems that H_2O_2 can be detoxified by a combine effort of both CAT and APX in *N. muscorum*. Increased GR activity is corroborated with induced APX activity owing to their direct relation in the Halliwell-Asada pathway. This result is supported by the findings of Srivastava[30] in salt stress of *N. muscorum.* Cu-induced GR activity may be due to its product GSH being required to maintain cellular homeostasis, and thus inducing NADPH level *vis-a-vis* dependent enzyme like GR under Cu stress.

ACKNOWLEDGEMENT

The authors acknowledge the host institution for providing necessary infrastructure facilities for the present work. Y. Mrinamala Devi is grateful to UGC, New Delhi for providing financial support in the form of Rajiv Gandhi National Fellowship.

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