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Copper-Induced Oxidative Stress in *Scenedesmus bijugatus:* Protective Role of Free Radical Scavengers

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Metal sequestering potential of algae is being considered for the removal of heavy metals from contaminated aquatic ecosystems (Cai et al. 1995). Copper (Cu) is a bioelement and essential for metabolism (Kaim and Rall 1996). However, it is toxic at high concentrations and Cu salts are popular algicides. Hence, the mechanisms of toxicity and tolerance acquired by algae are of considerable significance (Prasad et al. 1998). Cu induces oxidative stress by generating reactive oxygen species like superoxide and hydroxyl radicals via Haber-Weiss and Fenton reactions. Oxidative stress directly damages proteins, amino acids, nucleic acids, porphyrins and phenolic substances etc. Free radicals also cause peroxidative degradation of polyunsaturated fatty acid chains of membrane lipids as evident by the increased formation of malondialdehyde (MDA), a product of lipid peroxidation (Shen et al. 1997a,b). The free radicals are also scavenged by non-enzymic substances like ascorbate, glutathione, α-tocopherol and glucose (De Vos and Schat 1991).

Scenedesmus bijugatus, a green alga, was chosen as the experimental material to conduct laboratory toxicity bioassays. In this study, degradation of chlorophyll a (Chl a) and lipid peroxidation were investigated as parameters of Cu toxicity. Mannitol, sodium benzoate, butylated hydroxy toluene and glutathione have been used as free radical scavengers. The activities of oxidative enzymes viz. superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) were also investigated as a function of Cu toxicity.

MATERIALS AND METHODS

Scenedesmus bijugatus cells were cultured in Kessler's medium under continuous light of about 8000 lux at 24-26 °C. Growth was measured as the increase in Chl a content. Chlorophyll was extracted in hot methanol at 60 °C and absorbance was read at 663 nm (Mackinney 1941). Subcultures were treated with Cu (100 and 200 µM), Cu with mannitol (10 mM), sodium benzoate (10 mM), butylated hydroxy toluene (1 mM) and glutathione (2 mM). The amount of MDA-TBA (malondialdehyde-thiobarbituric acid) formed due to Cu toxicity was taken as a measure of lipid peroxidation (Heath and Packer 1968). Cells were homogenized using a

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sonicator in 50 mM Tris-HCl for 3 min and centrifuged at 25000 g for 20 min. The supernatant was used for assaying oxidative enzymes such as APX (1.11.1.11), CAT (1.11.1.6), GPX (1.11.1.7) and SOD (1.15.1.1) following the methods of Nakano and Asada 1981; Chance and Maehly 1955; Shinshi and Noguchi 1975; Beauchamp and Fridovich 1971, respectively. Glutathione and ascorbic acid were measured according to Guri (1983).

RESULTS AND DISCUSSION

The synthesis of Chl a was stimulated at 25 µM concentration of Cu which is an essential trace element for metabolism (Kaim and Rall 1996). Chl a gradually decreased with increased Cu concentration from 50 µM onwards and the lag phase was lengthened compared to control. This may be due to the slowing down of chlorophyll synthesis in the presence of Cu or due to the degradation of chlorophyll by free radicals generated by Cu (Butler et al. 1980; Halliwell 1978) (Fig 1). Cu has also been reported to inhibit cell division and cell volume in several of the microalgae (Nalewajko and Olaveson 1995; Prasad et al. 1998; Twiss et al. 1993).

Cu induced lipid peroxidation and increased antioxidant enzymes viz. APX, SOD, CAT and GPX (Luna et al. 1994). The activities of APX, SOD, CAT and GPX increased by 56%) 63 % , 35 % and 84 % , respectively in the presence of 100 µM concentration of Cu (Table 1). APX is the main H_2O_2 scavenging enzyme in algae whose primary function is dismutation of H_2O_2 (Asada 1992). Enhanced activity of APX indicates increased availability of reactive oxygen species. Guaiacol peroxidase is one of the common plant peroxidases for which the oxidation of substrates by H_2O_2 serves a physiological function. Their primary function is not disproportionation of H_2O_2. Hence, an increase in activity of GPX shows not only an oxidative defence but also a physiological reaction. CAT is the main H_2O_2 scavenging enzyme in animals, but in plants it is present exclusively in peroxisomes. Superoxide radicals and SOD inactivate CAT (Salin 1988) and hence the disproportionation of H_2O_2 is carried out mainly by APX in algae

Table 1. Activity of oxidative enzymes in different treatment

Cu µM	APX µkat/mg protein	SOD U/mg protein/hr	GPX U/mg protein/s	CAT nkat/mg protein
0	4.87	42.46	5.53	22.95
25	9.02	71.01	2.77	25.89
50	10.64	95.67	22.17	30.52
100	11.28	116.74	36.07	35.68

(Asada 1992). SOD disproportionates $O_2^{-\bullet}$ into H_2O_2 and O_2. The Cu-mediated increase in the activity of SOD may be the result of either a direct effect of these

ions on the gene for SOD or an indirect effect mediated via an increase in levels of superoxide radicals (Chongpraditnum et al. 1992).

All the four free radical scavengers were supplied exogenously and their relative capacities to protect Chl a from degradation and reducing lipid peroxidation were studied. After 6 days of treatment there was no difference in the actions of the free radical scavengers. However, after 10 days distinct differences were noticed. The level of Chl a was greater in the presence of mannitol. Mannitol is known as a hydroxyl radical scavenger *in vitro* (Elstner 1987; Smirnoff and Cumbes 1989) and *in vivo* (Shen et al. 1997). It has been shown that the hydroxyl radical may be the active agent in chloroplasts involved in the toxicity of H_2O_2 (Shen et al. 1997a,b). Hence, the protection of Chl a by mannitol may be a direct consequence of scavenging of hydroxyl radicals (Fig. 2).

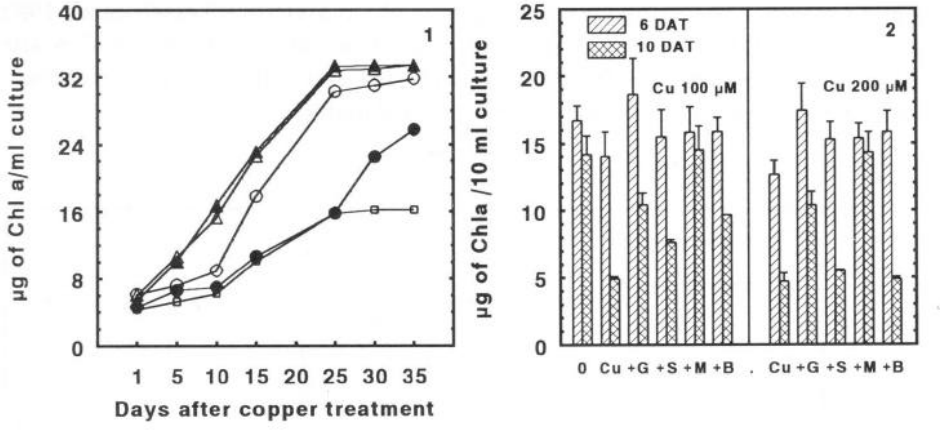

Figure 1. Growth curves of Cu exposed and unexposed cells of *S. bijugatus* as a function of increase in chl a content/l0 ml of culture. Symbols: Hollow triangle = control; Solid triangle = 25 µM $CuSO_4$; Hollow circle = 50 µM $CuSO_4$; Solid circles = 75 µM $CuSO_4$ and Hollow square = 100 µM $CuSO_4$

Figure 2. Protection of free radical scavengers against Cu^{2+} mediated degradation of Chl a after 6 and 10 DAT (days after treatment). Values are the means of results from three experiments with two replicated measurements. Vertical bars represent standard deviation. +G = Cu with glutathione (2 mM); +S = Cu with sodium benzoate (10 mM); +M = Cu with mannitol (10 mM); +B = Cu with butylated hydroxy toluene (1 mM).

Removal of H_2O_2 by APX requires ascorbate and glutathione. Ascorbate is the principal electron donor for APX. It is oxidized to dehydroascorbate in the

reaction of APX with H_2O_2 and is reduced back to ascorbate in the presence of glutathione by dehydroascorbate reductase (Zhang and Kirkham 1996). Hence, the levels of these antioxidants play an important role in oxidative defence. The decrease in amount of these two antioxidants (glutathione 46% and ascorbate 20%) in Cu (200 µM) treated cells was dose dependent (Fig. 3).

The decrease in ascorbate level may be correlated with the increased activity of APX or it may be due to its participation in the ascorbate-glutathione antioxidative pathway. GSH is a scavenger of many reactive oxygen radicals. Hence, during oxidative stress, GSH is utilized by the cells, thereby leading to its depletion. Further, GSH is the precursor of metal-chelating peptides i.e. phytochelatins $(\gamma Glu\text{-}Cys)_n Gly$ which are synthesized enzymatically (Grill et al. 1989). Several of the microalgae sequester heavy metals by synthesizing phytochelatins (Gekeler et al. 1988). Thus, presumably phytochelatin synthesis (detection is in progress) might be another reason for GSH depletion in this study.

Lipid peroxidation was another consequence of Cu toxicity ((Kessels et al. 1985, Sandmann and Boger 1980). Lipid peroxidation increased by 66% and 67% after 10 days of Cu treatment (100 and 200 µM). However, after 6 days of treatment, there was only a 25% increase in lipid peroxidation (Fig 4).

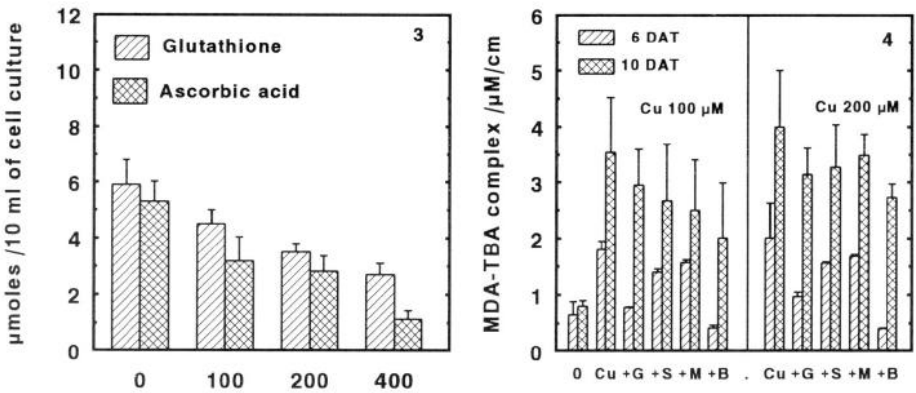

Figure 3. Glutathione and ascorbic acid levels in the cells 7 days after Cu treatment. Values are the means of results from three experiments with two replicated measurements. Vertical bars represent standard deviation.

Figure 4. Effects of free radical scavengers on the Cu^{2+} mediated increase in levels of MDA-TBA complex after 6 and 10 DAT (days after treatment). Values are the means of results from three experiments with two replicated measurements. Vertical bars represent standard deviation. +G = Cu with glutathione (2 mM); +S = Cu with sodium benzoate (10 mM); +M = Cu with mannitol (10 mM); +B = Cu with butylated hydroxy toluene (1 mM).

The free radicals generated by Cu initiate a chain of reactions in which alkoxyl and peroxyl radicals are formed. These radicals are involved in lipid peroxidation and eventually damage membrane permeability. The concentration of MDA-TBA complex was lower in the presence of BHT, indicating that BHT is a better scavenger with respect to lipid peroxidation. The peroxidation of membrane lipids, though initiated by hydroxyl radicals, is continued in a chain reaction by other free radical intermediates. BHT is known to react with peroxyl radicals formed in the reaction (Larson 1988), thus interfering with the peroxidative degradation of membrane lipids. Glutathione was capable of reducing the degradation of both Chl a and lipids by 25% and 22%, respectively.

Therefore, it is concluded that mannitol and BHT are potent scavengers of free radicals compared to sodium benzoate and glutathione. From this study it is evident that presence of exogenous antioxidants confers tolerance to copper toxicity in *Scenedesmus bijugatus*. Isolation/engineering of mutants/strains overexpressing genes for synthesis of endogenous antioxidants and tolerance to copper-induced oxidative stress could eventually lead to the development of microalgal metal scavengers, a feasible technology for removal of copper from contaminated aquatic environment.

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