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BRIEF COMMUNICATION

Impact of copper on reactive oxygen species, lipid peroxidation and antioxidants in *Lemna minor*

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Abstract

Lemna minor L. treated with 20, 50, or 100 μM $CuSO_4$ accumulated Cu and reactive oxygen species (hydrogen peroxide and superoxide radical) in frond and root cells. The time-course analysis of lipid peroxidation showed high increment in malondialdehyde production only after 12 and 48 h of Cu treatment. Guaiacol peroxidase and superoxide dismutase activities decreased after 48 h while glutathione reductase activity enhanced 48 h after Cu-treatment. Ascorbate and glutathione contents increased with the increasing Cu stress.

Additional key words: ascorbate, glutathione, duckweed, malondialdehyde, oxidative stress, peroxidase.

Aquatic environments are often subjected to heavy metal contamination and the ability of many aquatic plants to accumulate heavy metals from water has been well documented. Aquatic macrophytes are known to accumulate heavy metals and their cell concentrations have been found several times greater than those in surrounding substrate (Greger 1999). Cu is essential for plant in trace amounts but at higher concentrations [20 - 30 mg kg^{-1}(d.m.)] it is severely toxic to cells (Marschner 1995). Reduction in biomass, symptoms of chlorosis, decline in pigment content and alterations in the chloroplast and thylakoid ultrastructure have been reported (Baszynski *et al.* 1988, Patsikka *et al.* 1998) in plants under Cu stress. Toxic concentration of Cu results in generation of reactive oxygen species (ROS) like hydrogen peroxide, superoxide radical, and hydroxyl radical that exerts oxidative stress in cells (Panda *et al.* 2003). In response to oxidative stress the antioxidant system involving enzymes like catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), superoxide dismutase (SOD) and low molecular mass antioxidants are induced (De Vos *et al.* 1992, Luna *et al.* 1994, Panda 2003, Panda *et al.* 2003, Wang *et al.* 2004). The antioxidative response, however, varies from plant to plant and in many cases it has been found that ascorbate-glutathione cycle has been important in reducing the deleterious effects of Cu (Drazkiewicz *et al.* 2003).

In this study, *Lemna minor* was used as model plant system. *L. minor* is widely distributed in aquatic ecosystem and often subjected to heavy metal stress (Bassi and Sharma 1993, Grager 1999) and thus can be reliably used to access Cu toxicity levels in aquatic environment. Investigation will be focused on analysis of oxidative stress in frond and root tissue.

Fresh samples of duckweed (*Lemna minor* L.) were collected from uncontaminated ponds in Silchar, India during the August, 2004 - May, 2005. Plants were brought carefully to the laboratory in clean plastic jars. Healthy plants were selected and then cultured in 1:40 (v/v) Hogland's nutrient solution as suggested by Bassi and Sharma (1993). Plants were cultured in transparent plastic tumblers inside the growth chamber for 7d prior to Cu treatment and white light with photon flux density of 52 μmol m^{-2} s^{-1} (PAR) was provided with 16-h photoperiod. Cu treatment in the form of $CuSO_4$ was given at concentrations 20, 50 and 100 μM and both fronds and roots were harvested after 24 or 48 h of treatment for various biochemical analysis whereas sampling for lipid peroxidation was done after 2, 6, 12, 18, 24 and 48 h.

The hydrogen peroxide (H_2O_2) content was estimated

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Abbreviations: APX - ascorbate peroxidase; GPX - guaiacol peroxidase; GR - glutathione reductase; MDA - malondialdehyde; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloroacetic acid.

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as per the method of Sagisaka (1979). Superoxide radical ($O_2^{\bullet-}$) was measured as per the method of Elstner and Heupel (1976) Lipid peroxidation was measured in terms of malondialdehyde (MDA) content determined by thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968).

The copper content was measured following the method suggested by Sidhu and Brown (1996). Fronds and roots were washed with distilled water to remove the extracellular unbound cations, followed by 20 mM nickel chloride ($NiCl_2$) to displace the extracellular exchangeable cations. The shoots were dried overnight at 80 °C and dry mass was measured. In order to displace the intracellular cations, both fronds and roots were rinsed in 1 M HNO_3 for 2 h. The samples were soaked dry and digested with 5 cm^3 concentrated solution of HNO_3 till the solution turns clear. The sample volume was adjusted to 20 cm^3 with distilled water. The total Cu content was measured using atomic absorption spectrometer (*Perkin-Elmer 3110*, Germany).

The plant tissue was homogenized with phosphate buffer, pH 6.8 (0.1 M) in pre chilled motor and pestle. The extract was centrifuged at 4 °C for 15 min at 12 000 *g* in a cooled centrifuge. The supernatant was used for the assay of guaiacol peroxidase (GPX, EC 1.11.1.7) as per the method of Chance and Maehly (1955), superoxide dismutase (SOD, EC 1.15.1.1) as per the method of Giannopolitis and Ries (1977), and glutathione reductase (GR, EC 1.6.4.2) by the method of Smith *et al.* (1988). The extraction and estimation of glutathione was done according to Griffith (1980) method. The ascorbate extraction and estimation was done by the method of Oser (1979).

All the experiments were carried out thrice. The data was analyzed statistically with *SPSSS 7.5* statistical package (*SPSS Inc*., Chicago, USA) by *ANOVA* followed by Tukey test to evaluate the significance of the treatment effects.

Treatment of fronds and roots of *Lemna minor* with different concentrations of Cu increased H_2O_2 content after 48 h (Table 1) and the maximum increase was observed at the highest concentration (100 μM). H_2O_2 content increased by 60 % in fronds while 146 % in roots after 48 h at 100 μM of Cu, indicating roots to be primary site of Cu injury and ROS production. The $O_2^{\bullet-}$ content also increased in frond and root cells with increase in concentration of Cu in the culture medium and with increase in treatment period. The root cells showed higher $O_2^{\bullet-}$ content than fronds (Table 1). It is well established that oxidative stress can lead to deleterious effects on cell components like proteins, lipids and nucleic acids. However, many important signaling roles of ROS have been hypothesized (Scandalios 2002, Schutzendubel and Polle 2002). H_2O_2 production has been reported in plants under heavy metal stress (Dietz *et al.* 1999, Panda *et al.* 2003, Choudhury and Panda 2004, Panda and Choudhury 2005a,b, Österås and Greger 2006.). It induced DNA damage and inactivated thiol containing enzymes of the chloroplast. $O_2^{\bullet-}$ is incapable of crossing the biological membrane and together with H_2O_2 in the presence of Cu complexes can form highly reactive hydroxyl radicals *via* Haber-Weiss reaction (Panda 2002) that might lead to severe oxidative load on cells.

The elevated concentration of Cu was recorded in fronds and roots after 48-h treatment. The Cu concentration was quite proportional with concentration of Cu used to induce stress. The roots accumulated significantly more Cu than shoots (Table 1).

Table 1. Effect of different concentrations of Cu on contents of hydrogen peroxide, superoxide radical, Cu, ascorbate and glutathione, and activities of SOD, GPX and GR in fronds and roots of *Lemna minor* after 48-h treatment. The data presented are mean of three separate experiments ± SE. * - indicates differences from control at $P < 0.05$ according to Tukey test.

Parameters		0 μM Cu	20 μM Cu	50 μM Cu	100 μM Cu
H_2O_2	fronds	1.50 ± 0.00	1.73 ± 0.03	1.87 ± 0.03	2.10 ± 0.06*
[μmol g^{-1}(f.m.)]	roots	1.49 ± 0.01	2.23 ± 0.03	3.23 ± 0.03	3.67 ± 0.03
$O_2^{\bullet-}$	fronds	3.07 ± 0.03	3.07 ± 0.03	3.47 ± 0.03	4.03 ± 0.03*
[μmol g^{-1}(f.m.)]	roots	3.17 ± 0.03	4.07 ± 0.03*	5.07 ± 0.03*	5.60 ± 0.06*
Cu	fronds	1.33 ± 0.18	7.30 ± 0.15*	9.33 ± 0.18*	13.27 ± 0.13*
[μg g^{-1}(f.m.)]	roots	2.67 ± 0.09	13.47 ± 0.24*	19.37 ± 0.19*	24.60 ± 0.31*
Ascorbate	fronds	8.60 ± 0.09	8.67 ± 0.12	9.20 ± 0.09	10.67 ± 0.09*
[μmol g^{-1}(f.m.)]	roots	11.63 ± 0.06	12.20 ± 0.09	12.63 ± 0.12*	12.63 ± 0.09*
Glutathione	fronds	6.10 ± 0.06	7.13 ± 0.07	8.17 ± 0.09*	8.70 ± 0.10*
[μmol g^{-1}(f.m.)]	roots	11.20 ± 0.12	12.27 ± 0.13*	13.30 ± 0.15*	13.80 ± 0.15*
SOD	fronds	2.07 ± 0.03	2.23 ± 0.03	2.20 ± 0.06	1.80 ± 0.06*
[U g^{-1}(f.m.) min^{-1}]	roots	2.33 ± 0.03	2.33 ± 0.03	2.03 ± 0.03	1.57 ± 0.03*
GPX	fronds	10.17 ± 0.17	11.17 ± 0.17	10.17 ± 0.17	9.17 ± 0.17*
[U g^{-1}(f.m.) min^{-1}]	roots	22.33 ± 0.33	29.33 ± 0.33*	20.67 ± 0.33	20.67 ± 0.33*
GR	fronds	3.40 ± 0.12	4.50 ± 0.06	5.13 ± 0.07	6.20 ± 0.12*
[U g^{-1}(f.m.) min^{-1}]	roots	5.10 ± 0.06	6.23 ± 0.03	6.37 ± 0.09*	8.60 ± 0.06*

Table 2. Time course changes in MDA content [μmol g^{-1}(f.m.)] in fronds and roots of *Lemna minor* subjected to different Cu concentrations. Others the same as in Table 1.

	Cu [μM]	2 h	6 h	12 h	18 h	24 h	48 h
Fronds	0	2.93 ± 0.03	2.93 ± 0.03	3.07 ± 0.03	2.93 ± 0.03	3.07 ± 0.03	3.10 ± 0.06
	20	2.93 ± 0.03	2.93 ± 0.03	3.47 ± 0.03	2.93 ± 0.03	3.27 ± 0.03	5.10 ± 0.06
	50	2.93 ± 0.03	2.93 ± 0.03	4.43 ± 0.03	3.20 ± 0.06	3.53 ± 0.03	5.87 ± 0.03
	100	3.03 ± 0.03*	2.93 ± 0.03*	4.67 ± 0.03*	3.43 ± 0.03*	3.67 ± 0.03*	6.07 ± 0.03*
Roots	0	4.87 ± 0.03	4.87 ± 0.03	4.87 ± 0.03	5.08 ± 0.06	4.93 ± 0.03	5.03 ± 0.06
	20	5.07 ± 0.03	4.87 ± 0.03	5.13 ± 0.03	5.17 ± 0.03	4.93 ± 0.03	6.40 ± 0.06
	50	5.07 ± 0.03	4.87 ± 0.03	5.60 ± 0.06	5.37 ± 0.03	5.50 ± 0.06	7.10 ± 0.03
	100	5.20 ± 0.06*	4.87 ± 0.03	6.33 ± 0.03*	5.63 ± 0.03*	5.67 ± 0.03*	7.37 ± 0.03*

In fronds, the content of AsA increased significantly at 100 μM Cu. After 48 h of Cu treatment, the content of AsA was increased both in frond and root cells. The GSH content was also enhanced in both fronds and roots after 48 h of 20, 50 or 100μM of Cu treatment with respect to control (Table 1). The increase in both ascorbate and glutathione in *Lemna* suggested a potential non-enzymatic antioxidant protection from Cu-induced oxidative stress. An increase in glutathione content was in accordance with the fact that plants exposed to Cu produce phytochelatins, thiol-rich peptides synthesized from glutathione (GSH), which may chelate Cu and reduce toxicity (Cobbett and Goldsborough 2002).

The Cu induced increase in SOD activity only in fronds after 24-h treatment. The SOD activity decreased at 100 μM Cu after 48 h in both fronds (13.04 %) and roots (32.62 %). A decline in the GPX activity was observed after 48 h in roots. GPX activity in fronds, however, did not show significant variation. Maximum decrease in GPX activity was recorded at 100 μM of Cu after 24 h (20.7 % with respect to controls). GR, on the other hand, showed enhancement in activity after 24- or 48-h Cu treatment. In fronds, the GR activity was enhanced by 50.88 and 82.35 % after 48 h at 50 and 100 μM Cu, respectively. In roots, the GR activity was increased by 24.90 and 68.63 % after 48 h at 50 and 100 μM of Cu, respectively (Table 1).

Both duration of treatment and concentration of Cu showed notable variations in MDA content (Table 2). Analysis of the MDA content after 2, 6, 12, 18, 24 and 48 h revealed the period of onset of oxidative stress in the plant under Cu stress. The MDA content remained substantially unchanged after 2 or 6 h of Cu treatment. This response was similar in fronds and roots. The MDA content increased from 12 till 48 h of treatment. This indicates that oxidative stress was initiated after 12 h onwards of Cu. Thus lipid peroxidation increased at higher concentration of Cu and also with the increasing duration of treatment and it was higher in roots than in fronds. Cu induced lipid peroxidation has been previously reported (Halliwell and Gutteridge 1989, Panda *et al.* 2003, Panda 2003, Wang *et al.* 2004, Maksymiec and Krupa 2007).

The present findings suggest that Cu affects the physiological and biochemical processes of *Lemna minor* by inducing ROS formation, enhancing lipid peroxidation and regulating the antioxidant system.

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