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Biochemical Responses of *Potamogeton pectinatus* L. Exposed to Higher Concentrations of Zinc

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Zinc is fairly abundant in nature and its ores are widely distributed (Kelly 1988). Although small amount of Zn is released through leaching of rocks and other natural processes, the level of Zn in inland waters are often greatly increased by anthropogenic activities viz., mining, smelting of metal ores, electricity generation through coal based thermal plants etc. (Greger and Kautsky 1991). Zn is one of the essential micronutrient which is required at low concentrations for normal growth and development of plants and is essential for several metabolic processes (Prasad et al 1999). However, the presence of Zn at higher concentrations induced several phytotoxic symptoms in plants (growth retardation, loss of photosynthetic pigments, protein contents, lipid peroxidation etc.) by interfering with many metabolic processes (Ebbs and Kochian 1997). These phytotoxic responses of a plant species could be used as bio-indicator of metal pollution (Baryla et al 2000).

The concentrations of zinc in natural water bodies have been found to vary between 0.001-3.059 ppm (Ali et al 1999; Singh 2001). *Potamogeton pectinatus* L. (Potamogetanaceae), a submerged aquatic plant grows luxuriantly in metal polluted (including Zn) water bodies (Adam et al 1980; Ali et al 1999). Therefore, physiological changes induced by zinc in *P. pectinatus* might be useful in indicating the level of the zinc in polluted water bodies. Hence, objective of the present investigation is to study the Zn accumulation potential of the *P. pectinatus* exposed to the higher level of the Zn and its effect on various biochemical and physiological parameters to understand the strategies adopted by *P. pectinatus* under Zn stress.

MATERIAL AND METHODS

Plants of *Potamogeton pectinatus* L. were collected from unpolluted water bodies and were acclimatized for more than six months in large hydroponic tubs in natural conditions (plants rooted in sediment receiving normal day light and dark period, temperature etc. which was prevailing in the study area i.e., Lucknow, U.P., India) at National Botanical Research Institute, Lucknow. *Potamogeton* spp. are reported to grow well in Hoagland solution (Ali et al 2000). Therefore, growing shoots of these plants were detached and cultured in 250 ml flasks containing 200 ml of 5% Hoagland solution for six weeks under laboratory conditions (light /dark cycle 14:10 h, temperature 25 ± 2° C, 115 μC mol m^{-2} s^{-1} illumination provided through day fluorescent tube light). Various concentrations of Zn (0.0, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0

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mM) were prepared by diluting the stock solution (100 mM Zn solution prepared from $ZnCl_2.7H_2O$) with 5% Hoagland solution. The acclimatized plant material (approximately 2 g fresh weight) were transferred to plastic beakers (250 ml) containing 200 ml Zn supplemented medium. Four sets (comprising of three beakers for each concentration), were placed separately in a growth chamber under above mentioned conditions. Hoagland solution (5%, v/v) contains 0.00035 μM zinc and plants placed in 5% Hoagland solution (without incrementing Zn concentration) served as control. Time 0 data of each parameter was recorded. Experimental cultures were aerated 6 h a day. One set of each concentration was harvested after 24, 48, 72 and 96 h of the treatment and washed three times with double distilled water. Fresh weight of the plants (control and treated) was recorded. Biomass of the plants was expressed on dry weight basis. The oven dried treated and control plants (1 g each) were digested in HNO_3 : $HClO_4$ (3:1, v/v) at 80° C and zinc concentrations were estimated by Flame Atomic Absorption Spectrophotometer (Perkin Elmer 2380). The experimental zinc concentrations were analytically confirmed by estimating the Zn test concentrations as described above. The standard reference materials of Zn (E-Merck, Germany) was used to provide calibration and quality assurance for each analytical batch. The efficacy of digestion of plant samples and test concentrations was determined by adding the reference material of Zn to the samples. After addition of the standard Zn solutions, samples were digested as above and Zn concentrations were measured. Mean recovery of Zn was 96 ± 4%. The detection limit of Zn was 0.005 μg ml^{-1}. Replicate (n=3) analyses were conducted to assess the precision of the analytical techniques. Triplicate analysis for each sample varied by no more than 5%.

The photosynthetic pigments were extracted in 80% chilled acetone (v/v) and were estimated as per procedure of Arnon (1949). However, carotenoid concentrations in these extracts were calculated by the formula given by Duxbury and Yenstch (1956). Protein was estimated following the method of Lowry et al (1951) using bovine serum albumin as reference. The lipid peroxidation in the leaf tissue was measured in terms malondialdehyde (MDA) content following the method of Heath and Packer (1968). Cysteine and non-protein thiol contents were estimated following Gaitonde (1967) and Ellman (1959), respectively. A two way analysis of variance in complete randomized block design involving seven zinc concentrations, five treatment durations and three replications was performed to confirm the validity and variability of each set of the data. Duncan's Multiple Range Test (DMRT) was used to compare the means in each set of data. Simple linear regression and correlation analysis have been performed to describe zinc accumulation rates (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

P. pectinatus accumulated significant amount of Zn (ANOVA, $p<0.05$) in concentration and duration dependent manner. Maximum accumulation of zinc by test plant was achieved when plants were exposed to 10 mM zinc for 96 h (Table 1). Further, zinc accumulation rates were linear (Table 1) and significantly correlated with the level of zinc in the nutrient medium (correlation coefficient (r) significant at 5% level, df =5). Biomass of the *P. pectinatus* plants was not found affected by 0.1-1 mM zinc (Fig. 1A). Reduction in biomass was significant (DMRT $p>0.05$) only when plants were exposed to 5 and 10 mM zinc in nutrient medium. Zinc has adverse effect (ANOVA, $p<0.05$) on total chlorophyll contents of *P. pectinatus* (Fig. 1B). Its maximum reduction was observed when plants were exposed to 10 mM zinc for 96 h.

The zinc concentrations <5 mM increased carotenoid level at all the treatment durations (Fig. 1C) However, reduction in carotenoid contents was recorded when plants were exposed to zinc level > 1 mM (DMRT, $p<0.05$).

Similarly, zinc enhanced protein level in *P. pectinatus* when exposed to <0.5 mM zinc (DMRT $p<0.05$) at each treatment duration (Table 2). The adverse effect of zinc on protein content was first documented when plants were exposed to 5 mM zinc for 24 h (Table 2). The toxicity to protein content enhanced with increased concentration of zinc and treatment duration.

Table 1 Zinc accumulation by *P. pectinatus* exposed to different level of zinc.

Zinc	Treatment duration (h)				
(mM)	0	24	48	72	96
0.0	$1.940^{a}\pm0.087$	$1.962^{g}\pm0.08$	$2.621^{g}\pm0.13$	$2.968^{g}\pm0.15$	$3.123^{g}\pm0.16$
0.1	$1.942^{a}\pm0.078$	$16.62^{f}\pm0.73$	$17.72^{f}\pm0.88$	$19.84^{f}\pm0.90$	$26.49^{f}\pm1.10$
0.25	$1.948^{a}\pm0.088$	$33.45^{e}\pm1.50$	$38.62^{e}\pm1.80$	$45.12^{e}\pm2.25$	$50.88^{e}\pm2.30$
0.5	$1.950^{a}\pm0.076$	$67.50^{d}\pm3.10$	$78.55^{d}\pm3.82$	$87.12^{d}\pm3.15$	$99.80^{d}\pm3.78$
1.0	$1.945^{a}\pm0.072$	$120.12^{c}\pm5.80$	$144.2^{c}\pm6.20$	$163.3^{c}\pm7.13$	$191.19^{c}\pm9.55$
5.0	$1.942^{a}\pm0.086$	$184.65^{b}\pm8.16$	$172.49^{b}\pm7.52$	$329.71^{b}\pm15.3$	$409.96^{b}\pm20.45$
10.0	$1.943^{a}\pm0.090$	$239.71^{a}\pm10.8$	$412.02^{a}\pm18.5$	$486.86^{a}\pm22.5$	$617.27^{a}\pm28.5$

Mean ± SD (n=3); ANOVA ($p<0.05$). Identical superscripts denote no significant ($p>0.05$) difference between means in a column according to DMRT. Simple linear regression and correlation coefficient (r):Y_0 =1.945-0.0001X, r= -0.156*;Y_{24} =2.41+21.99X, r = 0.920; Y_{48} =2.41+36.05X, r = 0.960; Y_{72} = 49.71+46.70X, r = 0.971; Y_{96} = 56.33 +59.61X, r = 0.977; Tabular value of r at 5% level =0.754; * = not significant

Zinc induced lipid peroxidation (enhanced MDA levels) in *P. pectinatus*. Maxium MDA level was observed when plants were exposed to 10 mM zinc for 96 h (Table 2). The increase in MDA content was concentration and duration dependent (ANOVA $p<0.05$). Zinc (upto 1 mM) exposed test plants accumulated significant amount (ANOVA $p<0.05$) of NP-SH and cysteine contents. The concentrations >1 mM significantly (DMRT $p< 0.05$) reduced the NP-SH and cysteine contents (Table 3).

During the present study, *P. pectinatus* accumulated high amount of Zn in a concentration-duration dependent manner. Several other aquatic plants are also reported to accumulate zinc in their different plant parts (Marquenie-Vander and Ernst 1979; Dunbabin and Bowmer 1992). In addition, it was shown to accumulate other toxic metals including cadmium (Greger and Kautsky 1991). Cadmium is a toxic metal, which is released with Zn into the environment. Biomass of the *P. pectinatus* was not found affected by the zinc upto 1 mM. Our this finding reveals tolerance of the *P. pectinatus* to the elevated level of zinc in nutrient medium. The occurrence of *P. pectinatus* in polluted lakes has also been reported by Adam et al (1980) and Ali et al (1999).

Heavy metal accumulation in plants may lead to some physiological and biochemical changes. These phytotoxic symptoms may be used as indicator for metal pollution (Baryla et al 2000). Production of free radicals in plants under heavy metal stress has

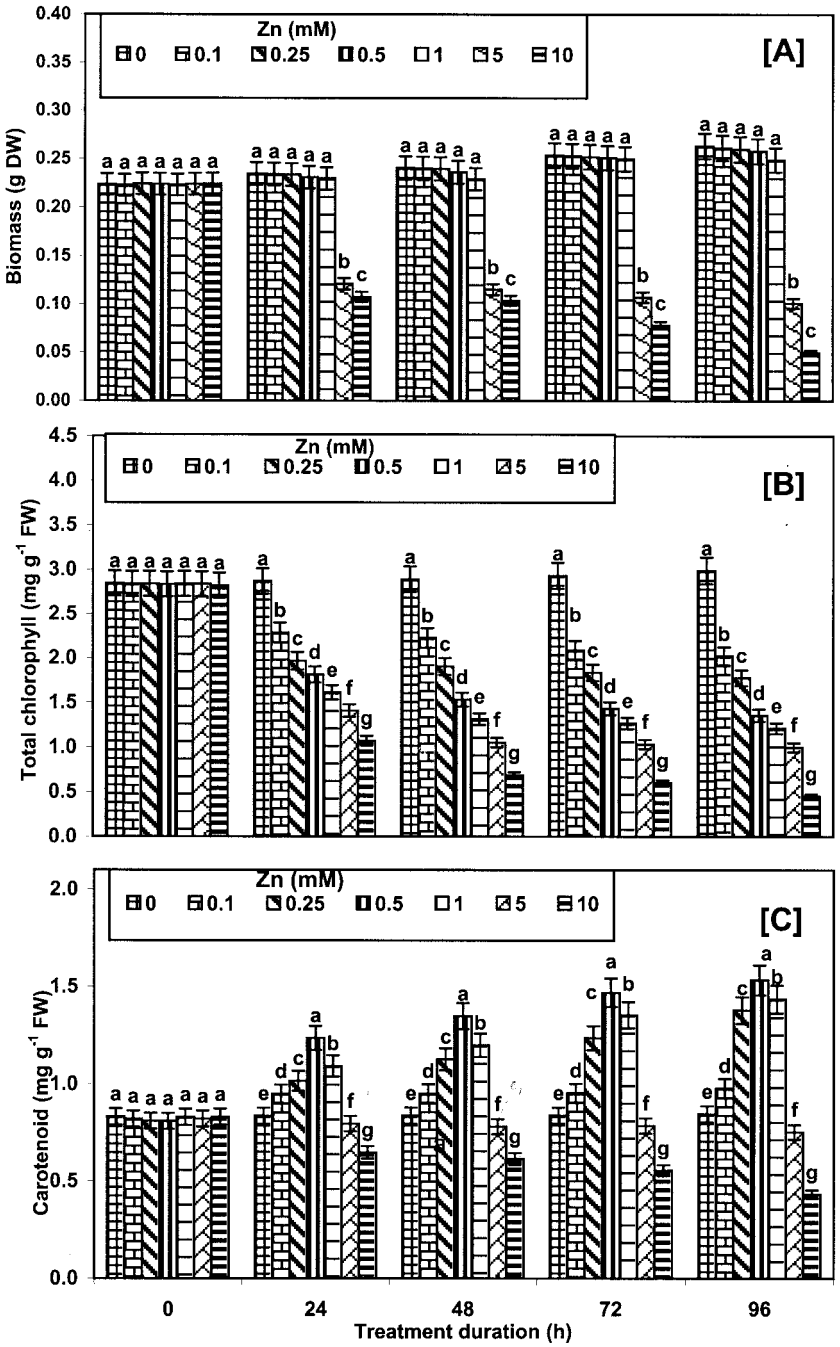

Figure 1. Biomass (A), total chlorophyll (B) and carotenoid (C) contents of. *P. pectinatus* as affected by zinc accumulation. ANOVA ($p<0.05$). Identical superscripts denote no significant ($p>0.05$) difference between bars (for each treatment duration) according to DMRT.

Table 2 Effect of zinc on protein, MDA, non-protein thiol (NP-SH) and cysteine contents of *P. pectinatus*.

Zinc	Treatment duration (h)				
(mM)	0	24	48	72	96
	Protein content (mg g^{-1} FW)				
0.0	11.29^{a}±0.50	11.69^{d}±0.40	11.89^{d}±0.57	11.97^{d}±0.55	12.11^{c} ±0.77
0.1	11.26^{a}±0.54	13.76^{c}±0..59	14.84^{c}±0.62	15.12^{c}±0.76	16.59^{b} ±0.68
0.25	11.32^{a}±0.50	16.60^{b}±0.75	16.98^{b}±0.78	18.24^{b}±0.81	19.48^{a} ±0.80
0.5	11.24^{a}±0.48	19.60^{a}±1.30	19.68^{a}±0.88	21.98^{a} ±1.05	15.98^{b} ±0.72
1.0	11.30^{a}±0.52	13.89^{c}±0.58	15.01^{c}±0.65	14.662^{c}±0.63	12.80^{c} ±0.65
5.0	11.25^{a}±0.48	9.29^{e}±0.45	8.67^{e}±0.43	7.89^{e}±0.32	7.12^{d} ±0.32
10.0	11.28^{a}±0.50	7.75^{f}±0.39	6.28^{f}±0.25	5.08^{f}±0.22	4.50^{e} ±0.18
	MDA content (μ mol g^{-1} FW)				
0.0	4.80^{a}±0.20	4.95^{g}±0.18	5.62^{g}±0.28	6.32^{g}±0.25	7.15^{g}±0.30
0.1	4.83^{a}±0.17	9.91^{f}±0.45	10.40^{f}±0.49	11.84^{f}±0.52	14.68^{f}±0.70
0.25	4.85^{a}±0.18	16.47^{e}±0.56	17.56^{e}±0.69	19.31^{e}±084	22.46^{e}±1.10
0.5	4.75^{a}±0.24	21.20^{d}±1.02	22.43^{d}±0.98	24.25^{d}±1.10	25.86^{d}±1.21
1.0	4.80^{a}±0.20	26.23^{c}±1.11	28.25^{c}±1.41	31.72^{c}±1.48	38.53^{c}±1.80
5.0	4.88^{a}±0.16	32.10^{b}±1.50	33.30^{b}±1.32	36.18^{b}±1.50	40.12^{b}±1.86
10.0	4.79^{a}±0.19	39.13^{a}±1.85	40.52^{a}±2.03	42.82^{a}±1.72	48.52^{a}±2.10
	Non-protein thiol (μ mol g^{-1} FW)				
0.0	17.49^{a}±0.80	17.69^{e}±0.78	17.90^{e}±0.57	18.26^{e}±0.55	19.41^{e}±0.77
0.1	17.42^{a}±0.78	24.78^{d}±1.12	25.60^{d}±0.62	26.47^{d}±0.76	30.18^{d}±0.68
0.25	17.48^{a}±0.83	33.93^{b}±1.59	38.38^{b}±0.78	40.27^{b}±0.81	43.58^{b}±0.80
0.5	17.32^{a}±0.72	43.16^{a}±2.16	48.64^{a}±0.88	51.32^{a} ±1.05	56.52^{a} ±0.72
1.0	17.46^{a}±0.85	30.12^{c}±1.40	33.56^{c}±0.65	35.38^{c}± 0.63	38.13^{c}±0.65
5.0	17.40^{a}±0.70	12.23^{f}±0.51	11.60^{f}±0.43	10.50^{f}±0.32	9.88^{f}±0.32
10.0	17.36^{a}±0.83	9.12^{g}±0.30	8.82^{g}±0.25	7.95^{g}±0.22	7.50^{g}±0.18
	Cysteine (μ mol g^{-1} FW)				
0.0	32.88^{a}±1.40	33.01^{e}±1.60	33.22^{e}±1.46	33.57^{e}±1.32	34.38^{e}±1.55
0.1	32.80^{a}±1.50	40.08^{d}±1.98	41.76^{d}±1.96	47.40^{d}±2.30	52.39^{d}±2.50
0.25	32.70^{a}±1.30	50.04^{c}±2.50	54.72^{c}±2.53	68.32^{c}±2.98	67.38^{c}±3.10
0.5	32.98^{a}±1.30	66.68^{a}±2.99	68.11^{a}±3.12	75.40^{a}±3.60	86.77^{a}±3.80
1.0	32.88^{a}±1.20	57.12^{b}±2.50	60.32^{b}±2.66	62.8^{b}±2.97	80.22^{b}±4.20
5.0	32.91^{a}±1.10	27.39^{f}±1.30	26.61^{f}±1.20	26.18^{f}±1.30	24.06^{f}±1.20
10.0	32.75^{a}±0.99	19.32^{g}±0.86	18.12^{g}±0.90	15.68^{g}±0.65	13.62^{g}±0.62

Values are mean ±SD (n=3); ANOVA $p<0.05$; FW = fresh weight; DW = dry weight; Identical superscripts denote no significant ($p>0.05$) difference between means in a column (Protein/MDA/NP-SH/Cysteine) according to DMRT.

been documented (Toppi and Gabbrieli 1999). Like other metals, zinc also induces free radical generation in plants (Fang and Kao 2000). The excess of zinc promoted MDA production through excessive generation of free radicals. During present study, zinc stimulated MDA production in *P. pectinatus*. A similar response was observed in some terrestrial plants treated with high concentrations of Zinc (Prasad et al 1999). Recently, it has been reported that evaluation of lipid peroxidation as a toxicity

bioassay for plants in heavy metal contaminated habitats could be regarded as reliable indicator for metal pollution (Baryla et al 2000).

Table 3 Summary of first effect levels of zinc to various parameters of *P pectinatus* exposed to different concentrations of Zn.

Parameters	**Concentrations of the Zn exhibiting first effects (mM)**	
	Stimulatory	**Inhibitory**
Total chlorophyll	-	0.1
Carotenoid	0.1	5
Protein	0.1	5
MDA	0.1	-
Non protein thiol	0.1	5
Cysteine	0.1	5

The oxidative stress promotes synthesis of cellular antioxidants viz., glutathione, ascorbate and carotenoids in plants (Pichorner et al 1993; Kumar et al 2002). These cellular antioxidants play a significant role in providing resistance to plants from metals by protecting labile macromolecules against attack by free radicals which are produced under heavy metal stress (Devi and Prasad 1998). The enhanced level of non-protein thiols, cysteine and carotenoids in zinc treated plants (upto 1 mM) in comparison to control plants suggest their active participation in detoxification of free radicals directly. Besides, increased non protein thiol and cysteine contents are considered to be an indicator of phytochelatin synthesis (Mehra and Tripathi 1999). Phytochelatins (PCs) are metal binding peptides, derived from tripeptide glutathione and have the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (where n=2-11). Therefore, an increase in non protein thiol and cysteine content in Zn treated plants of *P. pectinatus* may indicate strategic phytochelatin synthesis to protect the vital processes of plant from zinc toxicity. The increased activities of sulphate reduction enzyme (ATP sulphurylase and adenosine 5' phosphosulphate sulphotransferase) leading to more cysteine content have also been reported under heavy metal stress (Nussabaum 1988). The decrease in cysteine, non- protein thiol and carotenoid levels at zinc concentrations >1 mM was probably due to toxic effects of this metal. Reduction in non- protein thiol and cysteine contents has been reported in *Potamogeton crispus* when exposed to higher level of Hg^{2+} (Ali et al 2000).

Zinc reduced total chlorophyll content in *P. pectinatus*. The reduction in total chlorophyll contents might be attributed to the phytotoxic effect of Zn at some stages in biosynthesis of chlorophyll as zinc is supposed to compete with Fe, leading to chlorosis and reduced rate of photosynthesis (Van Assche and Clijsters 1990). Lipid peroxidation mediated decrease of photosynthetic pigments has also been reported (Somashekaraiah et al 1992; Kumar et al 2002). Therefore, reduced photosynthetic pigments in zinc exposed plants of *P. pectinatus* might be attributed to the phytotoxic consequences of the Zn induced lipid peroxidation and interference of this metal to the vital processes of chloropyhyll biosynthesis.

Heavy metals are reported to induce stress proteins in plants (Reddy 1992; Toppi and Gabbrielli 1999) Hence, increased protein content in *P. pectinatus* treated by Zn (0.1-1 mM) might be attributed to the synthesis of stress proteins. The decrease in protein level at concentrations >1 mM may be due to lethal effects of lipid peroxidation

induced under zinc stress. Further, oxidative stress may denature or fragment the proteins (Davies 1987). A decrease in protein content in the presence of zinc ions may also be due to the increased activity of protease or other catabolic enzymes which are activated and finally destroyed the protein.

It could be concluded from the present study, that *P. pectinatus* could survive in waters containing high level of Zn. The species accumulates higher amounts of zinc in its leaves. *P. pectinatus* prevented its vital metabolic processes from lipid peroxidation by accumulation of cysteine, NP-SH and carotenoids. The zinc induced MDA level could be considered as an important tool for diagnosing zinc pollution in aquatic ecosystems.

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