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Cadmium-Inducible Proteins in *Ceratophyllum demersum* L. (a Fresh Water Macrophyte): Toxicity Bioassays and Relevance to Cadmium Detoxification

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Cadmium is used in a variety of industries viz., batteries, pigments, stabilizers, coatings, alloys and electronic compounds. Other sources of cadmium released into the environment are through non-ferrous metals (zinc, lead and copper), iron and steel, fossil fuels combustion, cement and phosphate fertilisers (Cook and Morrow 1995). Cadmium contamination of the aquatic environment is of a serious human health concern being cytotoxic, carcinogenic and mutagenic (Hadjiliadis 1997). Many aquatic plants synthesize metal chelating peptides and proteins known as phytochelatins (PCs) (analogous to metallothioneins of animals and some fungi), which play a key role in cellular metal-ion homeostasis and metal detoxification (Prasad 2004). Short chain PCs are attributed to low-level metal exposure, while long chain PCs are presumed to form as metal levels increase. PCs in macrophytes thus, serve as indicators to monitor elevated levels of heavy metals in natural and polluted water (Vachon and Campbell 1993) and also play an important role in phytoremediation of heavy metals (Prasad 2004).

Ceratophyllum demersum L. (Coontail or Hornwort), a submerged, free-floating rootless aquatic macrophyte of worldwide distribution grows in stagnated water. It is reported to scavenge Cd when present in low concentrations (Ornes and Sajwan 1993). It has been used not only for the removal of metals but also radionuclides (Bolsunovskii et al. 2002) phosphorus from agricultural runoff (Dierberg et al. 2002), aquatic modules for bioregenerative life support systems based on "Closed Equilibrated Biological Aquatic System" as an artificial aquatic ecosystem, novel laboratory approaches to multi-purpose aquatic bioregenerative closed-loop food production systems (Bluem and Paris 2001). In the present work Cd-induced proteins were investigated as a function of the Cd concentration (with environmentally realistic concentration) and duration of exposure. Cadmium bioaccumulation was correlated with Cd-induced proteins.

MATERIALS AND METHODS

Ceratophyllum demersum L. plants were collected from local fishponds and maintained in aquaria with 1/10 Hoagland's nutrient solution. Based on the dose response curve established by giving wide range of Cd-treatment concentrations

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(0.25–100 μM) for seven days (data not shown), mature plants (2.5 gm) were subjected to three types of treatments viz., short-term, medium-term and long-term by using AAS standard solution (1000 mg L^{-1}) in 250 ml glass containers. In short-term, plants were treated with 10, 15, 20, 25, 50, 75 and 100 μM Cd for 12 hr and samples were analyzed at 6 hr interval for protein and Cd accumulation. In medium-term, Cd treatment (2.5, 5, 7.5 and 10 μM) was given up to eight days and plants were harvested in two days interval for analyses. For long-term, 0.25, 0.50, 0.75 and 1.00 μM Cd treatment was extended up to three weeks. Plants were harvested at weekly intervals and analyzed for Cd induced proteins and Cd bioaccumulation. Only in long-term, treatment solutions were renewed at weekly intervals and plants washed with 10 μM EDTA before transferring to new solutions, whereas in short as well as medium-term treatments, solutions were not renewed, since analysis showed residual Cd. In all treatments, concentrations of the test solutions were verified by AAS using uniform analytical procedures. All the glassware was soaked in 15% HNO_3 for 3 days and rinsed in ultrapure water to minimize metal contamination. Certified reference materials (National Institute of Standard and Technology) were also digested along with experimental samples. The measured concentrations of Cd in aquaria showed 0.01 to 0.1 μM deviation. At the end of each treatment, protein was quantified in one gram of plant material by Lowry's method. A sub-sample of the protein was electrophoressed and silver stained. Cd bioaccumulation was determined by Atomic Absorption Spectrophotometer (GBC 932 plus, Australia) using Graphite furnace system (GBC GF 3000). Data were collected from five replications of each of the treatment and subjected to correlation and two-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Plants subjected to three different treatments showed differential response for the Cd-induced proteins and total protein content. In short-term treatment, total protein content, in general, decreased with increasing treatment concentration after 6 and 12 hr treatment. Interestingly, 10 and 15 μM Cd-treatments increased protein by 17.42% and 2.62% respectively over the control after 6 hr duration. Like wise, treatment for a duration of 12 hr increased protein content with 10 μM concentration (26.99%), which is significantly higher than the control (Table 1). After 6 hr treatment, 75 μM Cd significantly decreased the protein, whereas with 12 hr duration 25 μM significantly reduced the protein content, the corresponding internal Cd concentration being 128.29 and 154.65 μg g.d.wt.$^{-1}$. A 17 kDa protein was induced by Cd (10 μM) after 6 hr duration. However, this protein was not observed in other tested treatments. Total protein content showed significant negative correlation with treatment concentrations after 6 hr ($r=-0.9053$; $t=-5.2195$; $p=0.05$) and 12 hr ($r=-0.8839$; $t=-4.6285$; $p=0.05$) treatment durations. When the differential response of treatment concentrations and durations for the protein content were analyzed, the ANOVA revealed significant differences between treatment concentrations, whereas variation between the treatment periods ($p>0.05$) for total proteins was not significant (Table 2).

Table 1. Total protein content (mg g.f.wt.$^{-1}$) and internal Cd concentration (μg g.d.wt.$^{-1}$) in *Ceratophyllum demersum* exposed to short-term treatment.

Treatment concentration (μ*M*)	Treatment duration (hours)			
	6 hours		12 hours	
	Protein content	Internal Cd concentration	Protein content	Internal Cd concentration
Control	1.88 ± 0.45	0.00 ± 0.00	1.94 ± 0.11	0.00 ± 0.00
10	2.21 ± 0.28	32.52 ± 1.25	2.47 ± 0.02*	74.65 ± 0.76
15	1.94 ± 0.11	48.75 ± 2.40	1.89 ± 0.11	112.35 ± 5.11
20	1.83 ± 0.02	57.64 ± 3.75	1.86 ± 0.04	129.61 ± 4.26
25	1.76 ± 0.07	71.25 ± 3.41	1.79 ± 0.13*	154.65 ± 7.01
50	1.68 ± 0.03	106.53 ± 7.25	1.62 ± 0.02*	231.53 ± 6.52
75	1.39 ± 0.01*	128.29 ± 0.55	1.36 ± 0.05*	255.85 ± 5.38
100	1.33 ± 0.02*	151.98 ± 6.40	1.13 ± 0.02*	289.22 ± 8.35

* - significantly different (p=0.05) from the corresponding control (according to Dunnett's test).

In medium-term treatment, total protein content decreased with increased treatment concentration and duration. After two days treatment, protein content increased significantly in all concentrations over the control (Table 3). At 2.5 μ*M* concentration, protein content increased over their corresponding controls up to 6 days and decreased thereafter. At 5 and 7.5 μ*M* concentrations, increase was observed only up to 4 days. After 2 and 4 days treatment, the first concentration that significantly decreased the protein content was 10 μ*M*. On the other hand after 6 and 8 days, significant decrease was started at 7.5 and 2.5 μ*M* respectively. Protein content showed positive correlation with treatment concentrations only at 2 days treatment and with treatment durations in control plants (Table 3), which

Table 2. ANOVA for the effect of treatment concentration and duration on total protein content in different treatment methods of Cd.

Source of variation	DF	SS	MS	F – value
Short-term treatment				
Between treatment cons.	7	1.6690	0.2384	28.4569*
Between treatment periods	1	0.0001	0.0001	0.0144
Error	7	0.0586	0.0083	
Medium-term treatment				
Between treatment cons.	4	0.3695	0.0924	1.9777
Between treatment periods	3	0.2041	0.0681	1.4565
Error	12	0.5605	0.0467	
Long-term treatment				
Between treatment cons.	4	2.9063	0.7265	15.4919*
Between treatment periods	2	0.6999	0.3499	7.4618*
Error	8	0.3752	0.0469	

* F-values significant at p = 0.05

Table 3. Total protein content (mg g.f.wt.$^{-1}$) in *Ceratophyllum demersum* exposed to medium-term treatment and their correlation (r-value) with Cd treatment concentrations and duration.

Treatment Concentration (μM)	Treatment duration (days)				
	2 days	4 days	6 days	8 days	r-value
Control	1.14 ± 0.06	1.21 ± 0.04	1.35 ± 0.14	1.72 ± 0.05	0.9426
2.5	1.46 ± 0.02*	1.42 ± 0.06*	1.36 ± 0.05	1.24 ± 0.06*	-0.9142
5.0	1.42 ± 0.06*	1.38 ± 0.03*	1.29 ± 0.07	0.96 ± 0.10*	-0.9019
7.5	1.34 ± 0.04*	1.21 ± 0.05	1.14 ± 0.05*	0.77 ± 0.09*	-0.9362
10.0	1.29 ± 0.01*	1.04 ± 0.02*	1.03 ± 0.04*	0.67 ± 0.11*	-0.9453
r - value	0.2391	-0.5582	-0.9475**	-0.9434**	

* - significantly different (p=0.05) from the corresponding control (according to Dunnett's test).
** - significant at p=0.05

were not significant (p>0.05). Significant correlation between protein content and treatment concentration was observed only at 6 and 8 days duration. At none of the treatment concentrations, significant correlation was observed between protein content and treatment duration (Table 3). No significant difference for proteins was observed either between treatment concentrations or duration by two-way ANOVA (Table 2).

In long-term treatment, at all treatment concentrations and durations, protein content decreased than their corresponding controls. Protein content decreased with increasing treatment concentration as well as duration (Table 4). All the tested Cd concentrations resulted in significant decrease in the protein content. The first significantly decreased concentration was 0.25 μM with internal Cd concentration 21.06 µg g.d.wt^{-1} after one-week. At the end of one week, the proteins in the 29-42 kDa range reduced in the treatments above 0.5 μM Cd and a 17 kDa protein was induced in all treatment concentrations (Fig. 1), which was absent in control. At the end of 2 weeks 17 kDa and 68 kDa proteins were induced in all treatment concentrations. Total protein content showed negative correlation with treatment concentrations as well as treatment durations. Significant correlation values were observed at all treatment durations (Table 4). Contrastingly, no significant correlation (p>0.05) was observed for any of the treatment concentrations (Table 4). Two-way ANOVA revealed significant differences between treatment concentrations as well as treatment durations for total protein content (Table 2).

Intracellular Cd concentrations in short-term treatment after 6 and 12 hr at different treatment concentrations were given in Table 1. Bioconcentration of Cd increased with increased treatment concentration, which showed significant positive correlation (p=0.05) at 6 and 12 hr treatment (r=0.9837, t=12.23; r=0.9626, t=7.94 respectively). As no protein induction was observed in medium-

Table 4. Total protein content (mg g.f.wt.$^{-1}$) and internal Cd concentration (μg g.d.wt^{-1}) in *Ceratophyllum demersum* exposed to long-term treatment and their correlation (r-value) with treatment concentrations and durations.

Treatment concentration (μM)	Total protein content	Internal Cd concentration
	One week treatment	
Control	2.73 ± 0.11	0.00 ± 0.00
0.25	2.39 ± 0.06*	21.06 ± 2.45
0.50	2.31 ± 0.04*	36.22 ± 1.55
0.75	1.97 ± 0.01*	48.01 ± 2.59
1.00	1.58 ± 0.05*	57.15 ± 5.75
r-value	-0.9833**	0.9937**
	Two week treatment	
Control	2.86 ± 0.03	0.00 ± 0.00
0.25	2.85 ± 0.04	32.57 ± 1.07
0.50	2.79 ± 0.03*	43.04 ± 2.02
0.75	1.81 ± 0.03*	52.45 ± 3.55
1.00	1.53 ± 0.02*	59.92 ± 5.36
r-value	-0.9051**	0.9973**
	Three week treatment	
Control	2.37 ± 0.05	0.00 ± 0.00
0.25	2.32 ± 0.02*	34.25 ± 3.85
0.50	1.68 ± 0.02*	46.84 ± 3.28
0.75	1.45 ± 0.04*	56.35 ± 5.33
1.00	1.44 ± 0.01*	60.02 ± 7.50
r-value	-0.9380**	0.9741**
	r-value with treatment durations	
Control	-0.7184	---
0.25	-0.1382	0.9186
0.50	-0.5659	0.9867
0.75	-0.9754	0.9993**
1.00	-0.9805	0.8809

* - significantly different (p=0.05) from the corresponding control (according to Dunnett's test).
** - significant at p=0.05

term treatment, data on Cd accumulation has not been given. In long-term treatment, Cd bioconcentration showed positive correlation with treatment concentration as well as duration. At all treatment durations, bioconcentration showed significant correlation with treatment concentration. In contrary, no significant correlation (p>0.05) was observed between treatment duration and Cd bioconcentration at any of the treatment concentrations, except at 7.5 μM

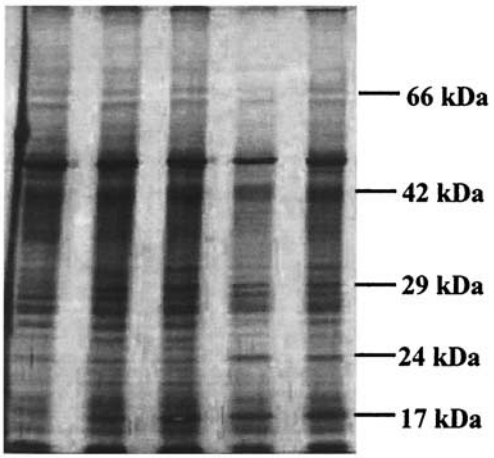

Figure 1. SDS-PAGE analysis of proteins from *Ceratophyllum demersum* treated with different concentrations of Cd for one week. The plants treated with Cd showed an induced protein of 17 kDa.

(Table 4). ANOVA revealed significant differences for bioconcentration of Cd between treatment concentrations as well as treatment periods.

Solutions of non-polluted soils contain Cd concentrations ranging up to 0.3 μ*M* (Wagner 1993). Most of the experimental studies use Cd concentrations above 1 μ*M*. Chronic stress resulted by exposure of plants for a long time at very low concentrations is managed by general cellular homeostasis whereas; acute stress by high concentrations for very short duration involves a variety of responses (Sanità di Toppi and Gabbrielli 1999). In present study, both methods have been adopted to study the effects of Cd–inducible proteins. Differential response of treatment concentration and duration for total protein content and Cd-induced proteins suggest that the induction of proteins is greatly influenced by the concentration and duration of the metal (Vachon and Campbell 1993).

Evidence for Cd-induced proteins has been derived from the thiol content of the Cd-induced complex by DTNB (5,5'-dithiobis-2-nitrobenzoic acid) test and their low molecular weight (Devi and Prasad 1998; Leblova et al. 1986; Prasad 2004). Cd-induced proteins up to certain treatment concentration and duration and their non-induction or disappearance in further concentrations and durations might be due to denaturation of complexes formed between Cd and proteins by Cd itself (Sanità di Toppi and Gabbrielli 1999). Bioconcentration of Cd seems to play an important role in the induction of metal binding proteins. Protein content in short-term as well as long-term treatments showed significant negative correlation with the internal concentration of Cd at all treatment durations except at two weeks. In short-term treatments, Cd inducible protein was observed at 10 μ*M* after 6 hr where, the Cd bioconcentration was 32.52 μg gr.dr.wt.$^{-1}$. Increase in Cd

bioconcentration at 12 hr (74.65 μg gr.dr.wt^{-1}) suppressed the induced protein. Like wise, in long-term treatment, inducible proteins were observed after 1 and 2 weeks, where the bioconcentration of Cd ranged from 21.06 to 46.84 μg gr.dr.wt^{-1} and further increase in Cd bioconcentration might have suppressed the proteins (Sanità di Toppi and Gabbrielli 1999). Protein content decreased in all three types of treatments. Though, in general, the total protein content decreased with increased treatment concentration and duration, a notable observation in this study was hormetic response, a dose-response phenomenon characterized as a low dose stimulation, high dose inhibition (Calabrese and Baldwin 2003), at certain low treatment concentrations and durations in short-term and medium-term treatments, where high (10–100 μ*M*) and moderate concentrations (2.5–10 μ*M*) of Cd were used respectively, that might be due to Cd stimulated mRNA synthesis, which in turn leads to increase in total proteins (Hirt et al. 1989). No such hormetic response of Cd was observed in long-term treatment, where very low (0.25–1 μ*M*) Cd concentrations were tested, which indicates that the hormetic response of Cd is also dependent on treatment duration and also attributed to Cd inhibited mRNA transcription only at low concentrations but not at moderate concentrations (Hirt et al. 1989). Nascarella et al. (2003) reported hormetic response of Cd in one developmental stage (pupation) while the subsequent adult emergence displays a stage specific toxicity. In present study, all three types of treatments in total showed a J-shaped dose response, which is characteristic of hormetic effect. This is mainly dose dependent. Such a dose-dependent increase of inducible-protein synthesis was reported in *Acetabularia calyculus* during short-term exposure to mercury (Garcia and Reyes 2001). In several species, Cd exposure induced the synthesis of a considerable number of stress proteins with a molecular mass ranging from 10-70 kDa. Rapid induction of proteins after 6 hr treatment indicate that the mechanism for short-term synthesis is reported to be controlled and expressed by a cytoplasmic pathway (Garcia and Reyes 2001); whereas synthesis of Cd-induced-proteins in long-term exposure is a gene-regulated (De Miranda et al. 1990).

The results obtained in this study suggest that the internal Cd concentration would greatly influence the induction of the metal binding proteins/peptides. Chronic treatment (long-term treatment at lower Cd concentrations) would be more effective for inducing the proteins than the acute treatment.

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