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# Cadmium Toxicity to *Ceratophyllum demersum* L.: Morphological Symptoms, Membrane Damage, and Ion Leakage

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Cadmium has no known metabolic significance to plants; however, it has several industrial applications. Owing to industrial uses, its emission poses human health risk being cytotoxic, carcinogenic and mutagenic (Cook and Morrow 1995; McLaughlin and Singh 1999). Aquatic macrophytes serve as convenient models for the assessment and monitoring of toxic metals (Prasad et al. 2001). *Ceratophyllum demersum* L. (Coontail or Hornwort) is a submerged, free-floating rootless aquatic macrophyte grows in stagnated fresh water throughout the world. It is reported to scavenge cadmium (Ornes and Sajwan 1993), mercury (Suckcharoen 1979), chromium (Garg and Chandra 1990) and radionuclides (Bolsunovskii et al. 2002). *C. demersum* is a suitable model system for laboratory toxicity bioassays. Ornes and Sajwan (1993) used lateral branches and stem internodes to examine the Cd imposed stress. Heavy metals are reported to accelerate senescence in aquatic plants (Jana and Choudhuri 1984, 1992). Therefore, the present study was undertaken to examine the morphological symptoms exhibited by leaves and stems exposed to varying concentrations of cadmium, and to establish a dose response curve.

## MATERIALS AND METHODS

*Ceratophyllum demersum* L. was collected from the local fishponds and maintained in aquaria with 1/10 Hoagland's nutrient solution. In order to study the morphological symptoms and dose response curve, mature plants (ten plants of uniform size, weight and age) were treated with different concentrations of Cd viz., 0.25, 0.5, 0.75, 1, 2.5, 5.0, 7.5, 10, 15, 20, 25, 50, 75 and 100 $\mu M$ for seven days. Five replicates were maintained for each treatment. Atomic absorption spectrophotometer (AAS) grade standard solution was used as the source for Cd. 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 and treatment solutions were verified by AAS (GBC 932 Plus, Australia) using uniform procedures. The measured concentrations of Cd in aquaria showed 0.01 to 0.1 $\mu M$ deviation. Membrane damage, ion leakage and lipid peroxidation were investigated by transferring mature plant material (2.5 gm) to 250 ml of

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nutrient solution supplemented with 2.5, 5.0, 7.5 and 10 μ*M* Cd. This is a single dose treatment, where the nutrient solutions have not been renewed as residual Cd was observed in the medium. At the end of six days treatment, plants were washed thoroughly with 10 m*M* EDTA solution for 5 minutes followed by a gentle wash with Millipore ultra pure distilled water in order to remove the adsorbed metal as well as nutrient ions from the surface of the plants. Thus, the cleaned plant material was transferred to de-ionised water and kept for 24 hours with gentle shakings intermittently. After 24 hours, the plants were removed and the water was checked for Electrical Conductivity (EC) (Digisun Electronics digital conductivity meter, model DI 909); leaked Na, K and Ca ions were measured by using Flame Photometer (Systronics Flame Photometer 128). Lipid peroxidation was measured at two-day intervals (Heath and Packer 1968). Data were collected from five replicates of each of the sample and subjected to correlations and analysis of variance (ANOVA) (Snedecor and Cochran 1968).

## RESULTS AND DISCUSSION

*Ceratophyllum demersum* plants exposed to various Cd concentrations (0.25–100 μ*M*) exhibited four types of morphological symptoms viz., curling of the tender leaves, mucilaginous plant parts, stem dissolution and leaf detachment (Table 1). These symptoms appeared from second day onwards at higher concentrations (75 and 100 μ*M*), whereas at lower concentrations (0.25-1.0 μ*M*), plants survived up to three to four weeks without showing the above symptoms. The symptoms viz., leaf curling and mucilaginous plant parts were observed at 0.5–20 μ*M*, while stem dissolution and leaf detachment in 25–100 μ*M* concentrations. The survival period was negatively correlated (r = -0.83) with the treatment concentration (Table 1).

The extent and magnitude of symptoms and damage of the plant parts increased with increasing treatment concentration of Cd. Based on the extent of damage and magnitude of symptoms, four survival categories viz., +, ++, +++ and ++++ have been recognized. These four categories were assigned arbitrary values viz., 0.25, 0.50, 0.75 and 1.00 units respectively, to facilitate the calculation of survival value of the plant. Plants with 75 per cent damage (= 25% survive) were identified as + category and assigned an arbitrary value 0.25. Likewise, plants with 50, 25 and 0 per cent damage were assigned with 0.50, 0.75 and 1.00 values respectively. At the end of seven days treatment period, survival values of the plants were calculated for these concentrations by using the following formula and the results were presented in Table 2.

$$\text{Survival Unit} = \sum \left[ \begin{array}{l}\text{Percentage of plants} \\ \text{present in a category}\end{array} \times \begin{array}{l}\text{Value assigned} \\ \text{to the category}\end{array} \right]$$

Dose response curve has been illustrated by plotting the survival values of the plants against the treatment concentrations (Fig. 1). Cadmium concentration present in the medium was found to have differential effect on the magnitude of damage. The data on magnitude and extent of damage of plant due to cadmium-

**Table 1.** Morphological symptoms and their appearance in *Ceratophyllum demersum* plants treated with cadmium.

| Cd Conc.(μ*M*) | Toxic symptoms observed | Appearance of Symptoms (DAT) | Lethal to plant (DAT) |
|---|---|---|---|
| 0.25 – 5.0 | • No symptoms were observed | > 6 days | > 2 weeks |
| 7.5 | • Curling of tender leaves<br>• Mucilaginous plant parts | 6 days | 13 ± 1 days |
| 10 | • Curling of tender leaves<br>• Mucilaginous plant parts | 4 days | 10 ± 1 days |
| 15 | • Curling of tender leaves<br>• Mucilaginous plant parts | 3 days | 8 ± 1 days |
| 20 | • Curling of tender leaves<br>• Mucilaginous plant parts | 3 days | 6 ± 1 days |
| 25 | • Curling of tender leaves<br>• Mucilaginous plant parts<br>• Detachment of some leaves | 3 days | 5 ± 0 days |
| 50 | • Mucilaginous stem<br>• Detachment of some leaves | 2 days | 4 ± 0 days |
| 75 | • Detachment of leaves<br>• Dissolution of stem | 1 day | 3 ± 0 days |
| 100 | • Detachment of leaves<br>• Dissolution of stem | 1 day | 2 ± 0 days |

DAT = days after treatment

**Table 2.** Percentage plants present in each survival category and the survival units of *Ceratophyllum demersum* plants treated with different concentrations of cadmium.

| Conc. of Cd (μ*M*) | Survival category | | | | | Survival Unit |
|---|---|---|---|---|---|---|
| | 0.00 | 0.25 (+) | 0.50 (++) | 0.75 (+++) | 1.00 (++++) | |
| 0.0 | - | - | - | - | 100 | 100 |
| 0.5 | - | - | - | - | 100 | 100 |
| 1.0 | - | - | - | - | 100 | 100 |
| 2.5 | - | - | - | 20 | 80 | 9 |
| 5.0 | - | 10 | 10 | 50 | 30 | 75 |
| 7.5 | - | 20 | 30 | 40 | 10 | 35 |
| 10 | 10 | 40 | 20 | 20 | - | 5 |
| 15 | 100 | - | - | - | - | 0 |
| 20 | 100 | - | - | - | - | 0 |
| 25 | 100 | - | - | - | - | 0 |
| 50 | 100 | - | - | - | - | 0 |
| 75 | 100 | - | - | - | - | 0 |
| 100 | 100 | - | - | - | - | 0 |

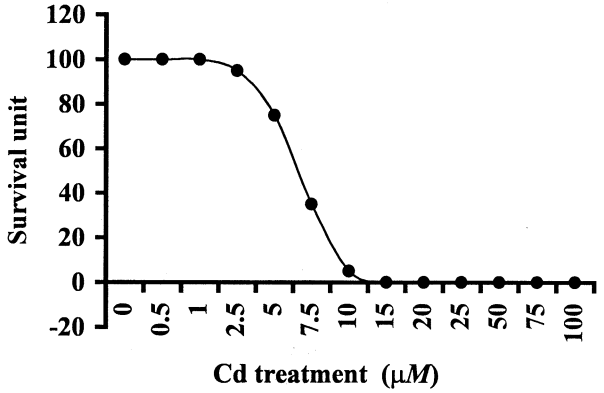


**Figure 1.** Dose response curve of *Ceratophyllum demersum* against the different treatment concentra tions of cadmium in solution cultures after seven days.

imposed stress was subjected to one-way ANOVA; significant difference (p=0.05) was observed between the treatment concentrations.

Since most of the symptoms were related to membrane damage, additional experimentation has been carried out to examine the membrane damage as a function of electrical conductivity (EC), ion leakage and lipid peroxidation. The treatment concentrations viz., 2.5, 5.0, 7.5 and 10.0 μ*M* of Cd, in which the plants possessed 5–95 survival units and showed varying degree of symptoms after one week treatment, were selected.

EC of the medium, in which plants were grown, varied with treatment duration as well as concentration (Table 3). EC of the medium increased with increasing treatment concentration at all treatment durations. In control, the conductivity decreased with increasing treatment duration. After two days, significant increase

**Table 3.** Electrical conductivity (mMhos [cm(gm f.wt.)] $ml^{-1}$) of the hydroponic medium in which plants were grown for medium-term treatment.

| Treat. Con. (μ*M*) \ Treat. Per. | 0 Days (Initial) | 2 Days | 4 Days | 6 Days | 8 Days |
|---|---|---|---|---|---|
| 0 (Control) | 229 ± 2 | 223 ± 3 | 216 ± 3 | 212 ± 2 | 205 ± 3 |
| 2.5 | 243 ± 3* | 226 ± 2 | 226 ± 2* | 250 ± 3* | 278 ± 3* |
| 5.0 | 257 ± 2* | 248 ± 3* | 234 ± 2* | 260 ± 2* | 296 ± 4* |
| 7.5 | 274 ± 3* | 267 ± 3* | 270 ± 3* | 289 ± 4* | 315 ± 3* |
| 10.0 | 297 ± 4* | 269 ± 5* | 284 ± 4* | 299 ± 3 * | 328 ± 5* |

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

**Table 4.** ANOVA for the effect of Cd concentrations on membrane damage as a function of increase in electrical conductivity in the hydroponic medium in which plant were treated and lipid peroxidation.

| Source of variation | DF | SS | MS | F – value |
|---|---|---|---|---|
| Electrical conductivity | | | | |
| Between treatment concentrations | 4 | 19345.6 | 4836.4 | 26.6212* |
| Between treatment periods | 4 | 4873.6 | 1218.4 | 6.7065* |
| Error | 16 | 2906.8 | 181.7 | |
| Lipid peroxidation | | | | |
| Between treatment concentrations | 4 | 78.348 | 19.5870 | 23.2809 * |
| Between treatment periods | 3 | 40.124 | 13.3747 | 15.8969 * |
| Error | 12 | 10.096 | 0.8413 | |

* - significant at p=0.05

was observed at 5.0 μ*M*, while in the further duration, it was observed at 2.5 μ*M*. Electrical conductivity of the medium at different treatment concentrations and durations were analyzed; the two-way ANOVA revealed significant differences between treatment concentrations as well as durations (Table 4).

EC as well as leakage of Na, K and Ca ions increased with increasing treatment concentration. The increase of EC was highest (330% over the control) at 10 μ*M* treatment concentration. Interestingly, all treated values were greater than the control values, which indicated that the increase in EC was due to ion leakage caused by membrane damage resulting from Cd exposure. Potassium leakage was more (247.37% over the control) followed by sodium (149% over its control) and calcium at 10 μ*M* treatment. Interestingly, no Ca leakage was observed in the control plants (Table 5). The lowest concentration at which significant increase in EC and Na, K and Ca leakage observed was 2.5 μ*M*. Electrical conductivity, Na,

**Table 5.** Electrical conductivity and ion (Na, K, Ca) leakage from the plants after six days treatment with different concentrations of Cd and their correlation (r-value) with Cd treatment concentrations in *Ceratophyllum demersum* transferred to de-ionised water.

| Cd treatment Concentration | Electrical Conductivity | Sodium (Na) (in ppm) | Potassium (K) (in ppm) | Calcium (Ca) (in ppm) |
|---|---|---|---|---|
| Control | 13 ± 3 | 2.70 ± 0.45 | 0.38 ± 0.05 | 0.0 ± 0.0 |
| 2.5 μ*M* | 25 ± 2* | 4.48 ± 0.58* | 0.72 ± 0.03* | 0.5 ± 0.04* |
| 5.0 μ*M* | 38 ± 2* | 5.14 ± 1.01* | 0.97 ± 0.04* | 0.6 ± 0.02* |
| 7.5 μ*M* | 49 ± 3* | 5.79 ± 0.93* | 1.12 ± 0.05* | 0.8 ± 0.05* |
| 10.0 μ*M* | 56 ± 5* | 6.73 ± 1.35* | 1.32 ± 0.08* | 0.8 ± 0.07* |
| r - value | 0.9948** | 0.9773** | 0.9881** | 0.9141 |

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

** - r-value significant at p=0.05

K and Ca leakage into de-ionised water showed positive correlation with the Cd treatment concentration. Only the former three were found to be significant, whereas Ca leakage showed lack of significant correlation with treatment concentration (Table 5).

In order to study the membrane damage biochemically, lipid peroxidation was studied and the results were presented in Table 6. Compared to the control, the level of MDA (malondialdehyde) was increased with increasing treatment concentration as well as duration. Plants treated with Cd (7.5 $\mu M$) for 2 days, showed significant increase whereas at further treatment durations, all the concentrations showed significant increase in MDA reactive substances. The MDA reactive substances were positively correlated with treatment duration at all tested concentrations and vice versa (Table 7). In former case, only at 7.5 and 10.0 $\mu M$ concentrations MDA reactive substances were significantly correlated with treatment durations. On the other hand, in latter case, at all treatment durations, the level of reactive substance was found to be significantly correlated with treatment concentration (Table 7). When this differential response of treatment concentrations and duration for MDA levels were analyzed; the ANOVA revealed significant differences between treatment concentrations as well as treatment durations (Table 4).

In the present study, treatment concentration and duration seem to play an important role to express the toxicity symptoms. As *C. demersum* is a rootless aquatic plant, it absorbs minerals and ions through entire plant surface mainly through the stem. At lower concentrations, symptoms appeared after longer duration (chronic), whereas at higher concentrations symptoms appeared immediately (acute) in shorter durations. Curling and mucilaginous leaves can be considered as chronic symptoms, which were exhibited slowly after 4-6 days at lower concentrations (5-20 $\mu M$), while the symptoms viz., stem dissolution and detachment of leaves (at higher concentrations i.e., 25–100 $\mu M$) as acute which were exhibited immediately after 24 hr.

Heavy metal tolerance, which is a relative measure, is expressed as a comparison

**Table 6.** Level of peroxidation products measured as thiobarbituric acid reactive substances (μmoles/gm.f.wt) in *Ceratophyllum demersum* exposed to different concentrations of Cd for different durations of time.

| Cd treatment Concentration | 2 days | 4 days | 6 days | 8 days |
|---|---|---|---|---|
| Control | 7.3 ± 0.75 | 7.1 ± 0.68 | 7.5 ± 0.77 | 7.9 ± 0.82 |
| 2.5 $\mu M$ | 7.5 ± 0.83 | 9.8 ± 1.32* | 10.4 ± 0.85* | 11.1 ± 1.15* |
| 5.0 $\mu M$ | 8.2 ± 1.15 | 10.1 ± 1.55* | 11.8 ± 1.45* | 11.9 ± 1.65* |
| 7.5 $\mu M$ | 9.1 ± 1.33* | 11.6 ± 1.62* | 13.1 ± 1.33* | 13.6 ± 1.52* |
| 10.0 $\mu M$ | 9.8 ± 1.12* | 12.8 ± 1.24* | 14.7 ± 1.55* | 15.9 ± 1.65* |

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

**Table 7.** Correlation of lipid peroxidation with Cd treatment concentrations in *Ceratophyllum demersum*.

| Cd concentration/ treatment duration | r - value | t - value |
|---|---|---|
| Control | 0.8315 | 2.1168 |
| 2.5 $\mu M$ | 0.9435 | 4.0266 |
| 5.0 $\mu M$ | 0.9488 | 4.2479 |
| 7.5 $\mu M$ | 0.9583 | 4.7425* |
| 10.0 $\mu M$ | 0.9805 | 7.0559* |
| 2 Days | 0.9829 | 7.5488* |
| 4 Days | 0.9713 | 5.7749* |
| 6 Days | 0.9856 | 8.2431* |
| 8 Days | 0.9837 | 7.7365* |

* - significant at p=0.05

either between treatments or species (Köhl and Lösch 1999). In present study, it was expressed between the treatments and given for seven days (short-term). In the short-term studies, a single value of the tolerance is given for easy comparison. Tolerance Index (TI) and different effective concentration (EC) values viz., NOEC, EC50 and EC100 (Köhl and Lösch 1999) are most commonly used. In case of heavy metal tolerance, root elongation method is most commonly used (Köhl and Lösch 1999). But *C. demersum* is a rootless macrophyte, so the magnitude of damage has been taken as a parameter to calculate the tolerance capacity. Multi-concentration tests give a dose-response curve to reveal an EC-value. But dose-response curves are strongly dependent on the individual plants in a concentration series. In our experiments Cd concentration increased stepwise until lethal to the plant.

In control plants, the conductivity of the nutrient medium decreased with increasing treatment duration due to depletion of nutrients in the medium, whereas in media supplemented with selected concentrations of Cd, EC decreased up to $4^{th}$ day and then showed an increase thereafter. Instead of decrease in EC, media supplemented with Cd showed increase in their EC, which indicated the ion leakage due to membrane damage caused by Cd, and it also revealed that though the morphological symptoms in these concentrations were observed apparently after 4-6 days, damage in the membrane was started from 2-4 days. Metals interact with components in the membranes resulting in changed conformation and rigidity, causing elevated leakage of elements such as $K^+$ (Devi and Prasad 1998; DeVos et al. 1989; Ouzounodou et al. 1992). The present study revealed that Cd imposed stress increased the membrane damage, as is evident from increased values of EC in nutrient media, de-ionised water, ion leakage and lipid peroxidation.

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