Cadmium–Zinc interactions in a hydroponic system using *Ceratophyllum demersum* L.: adaptive ecophysiology, biochemistry and molecular toxicology

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The interaction between an essential micronutrient, Zn and a toxic non-essential element, Cd has been comprehensively reviewed based on our experiments conducted with *Ceratophyllum demersum* L. in a hydroponic system. Since Cd and Zn belong to the group IIB transition elements and show similarities in chemistry, geochemistry and environmental properties, it would be one of the elemental pairs of choice to investigate metal-metal interactions. Evidence in support of the protective role of Zn against Cd toxicity in *Ceratophyllum demersum* L. is presented in this review. Based on our experimental results, we conclude that the antioxidant properties of Zn play an important role in counteracting Cd toxicity.
Key words: antagonistic, antioxidant, enzyme conformation, metal interactions, redox pool.

Interações cádmio–zinco em um sistema hidropônico usando *Ceratophyllum demersum* L.: ecofisiologia adaptativa, bioquímica e toxicologia molecular: A interação entre um micronutriente essencial, Zn, e um elemento tóxico não essencial, Cd, é amplamente revisada com base em nossos experimentos com *Ceratophyllum demersum* L. em um sistema hidropônico. Desde que Cd e Zn pertencem ao mesmo grupo IIB de elementos de transição e mostram semelhanças nas propriedades química, geoquímica e ambiental, poderiam ser os elementos de escolha para investigar as interações metal-metal. Com base em nossos resultados experimentais, apresentamos, nesta revisão, evidências a favor de um papel de proteção antioxidativo de Zn contra a toxidade de Cd em *Ceratophyllum demersum* L.
Palavras-chave: antagonista, antioxidante, antagonístico, conformação enzimática, interação metálica, pool redox.

INTRODUCTION

Heavy metal pollution is of considerable importance and relevant to the present scenario due to the increasing levels of pollution and its obvious impact on human health through the food chain (Hadjiliadis, 1997). Aquatic ecosystems act as the receptacle for various contaminants from natural and anthropogenic sources such as effluents from mines, smelters, industries, excessive use of fertilizers and pesticides, agricultural runoff, and partially from aerial deposition (Ross, 1994; Kabata-Pendias 2001; Adriano 2001). It is known that unfavourable effects of heavy metals on plants are manifested, among others, by inhibiting the normal uptake and utilization of mineral nutrients (Trivedi and Erdei, 1992). Most of the experimental data on Cd toxicity leaves a dearth of information on the specifics of essential (Cu, Fe, Zn) and non-essential metals (Pb, Hg) (Rauser, 2000) and few studies have been designed specifically to address the effect of micronutrient status on toxicity from exposure to non-essential metals (Peraza et al., 1998). It is notable that metalliferous environments are often contaminated by more than one metal in potentially toxic concentrations (Wallace, 1982; Siedlecka, 1995) that may have synergistic, additive or antagonistic effects on plants (Siedlecka, 1995). Therefore, the effects of metal mixtures on model plant systems need to be investigated critically as the phytotoxicity and interactive aspects of metal mixtures are complex processes (Taylor, 1989; Rauser, 2000). Knowledge about the biochemical and molecular mechanisms by which plants tolerate multiple metal stress can bring a thorough understanding of the plasticity of metabolic pathways and their limits of functioning, which is essential for genetic engineering approaches aimed at improving the cellular defense mechanism (Xiang et al., 2001; Prasad and Strzałka, 2002; Prasad, 2004b).

Cadmium *vs* Zinc: Toxicology and occurrence

It is a common characteristic of all life forms that elements required for metabolism are accumulated and toxic metals excluded in certain plant species (Baker, 1981). Cadmium ($^{112.41}Cd_{48}$), a group IIB transition element is a non-essential and toxic element, without any metabolic significance. Cadmium pollution is increasing due to excessive mining, industrial usage and other anthropogenic activities (De, 1992; Prasad, 1995a). In addition, some phosphate fertilizers applied to crops have been found to contain high levels of Cd (He and Singh, 1994). Cadmium released into the environment tends to concentrate in soils and sediments, where it is potentially available to rooted plants. The available Cd thereby enters biogeochemical cycles, becomes bioconcentrated (Devi et al., 1996) and even affects human health (for example, the Itai-itai disease caused by Cd-contaminated rice in Japan) (Rivai et al., 1990). Cadmium has been classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC, 1993; Goering et al., 1994; Waalkes, 2000). On the other hand Zinc ($^{65.39}Zn_{30}$), another group IIB transition element, is one of the most essential micronutrients for the plant system. Zinc plays a fundamental role in several of the critical cellular functions such as protein metabolism, gene expression, chromatin structure, photosynthetic carbon metabolism and indole acetic acid metabolism (Vallee and Falchuk, 1993; Marschner, 1995; Prasad, 1995; Cakmak and Braun, 2001). Zinc is an important component of many vital enzymes having a catalytic, co-catalytic or structural role, as well as being a structural stabilizer for proteins, membrane and DNA-binding proteins (Zn-fingers) (Vallee and Falchuk, 1993), yet it is toxic at high concentrations. Cadmium and Zinc (IIB transition elements) have a similar electronic configuration and valence state, possessing equal affinities for sulphur, nitrogen and oxygen ligands (Nieboer and Richardson, 1980) and hence similar geochemical and environmental properties (Nan et al., 2002). Cadmium is often associated with Zn as a contaminant of up to 5 % in the processed Zn-ores of Zn mines and smelters (Adriano, 2001). Similarly, tyres containing ZnO and sewage sludge applied to agricultural soils also contain Cd (Sherlock, 1986) as a major contaminant. It has been hypothesized that elements whose physical and chemical properties are similar will act antagonistically to each other biologically (Das et al., 1997). In the recent years, a number of workers have documented responses of plants to combinations of Zn and Cd in soil as well as in solution culture (Thys et al., 1991; Smilde et al., 1992; Symeonidis and Karataglis, 1992; Mckenna et al., 1993, Zhou et al., 1994; Chaoui et al., 1997). This aspect has also been studied in soil-crop systems under field conditions (Nan et al., 2002), but here the study was limited to the bioavailability and bioaccumulation of Zn and Cd by the tested plant systems. Nevertheless, investigations focussing on the adaptive physiological and biochemical mechanisms of the interaction between Zn and Cd are rather scanty. Moreover, in view of the conflicting results in earlier studies, a clear understanding of potential interaction between Zn and Cd has yet to appear. We have initiated investigations on various aspects focussing on the biochemistry of Cd-Zn interactions (Aravind and Prasad, 2003; 2004a,b). Investigations on the same lines have begun with other systems also (Koleli et al., 2004). This review critically analyzes the adaptive ecophysiology, biochemistry, and the molecular toxicological aspects of Cd-Zn interactions.

***Ceratophyllum demersum* L. -an ideal aquatic macrophyte for toxicity bioassays**

Aquatic plants are known to accumulate heavy metals and are being considered for phytoremediation (Prasad et al., 2001). *Ceratophyllum demersum* L. belongs to the order Nymphaeales and family Ceratophyllaceae (the family of hornworts), grows in shallow, muddy, quiescent water bodies at low light intensities. *C. demersum* is a convenient plant for laboratory toxicity bioassays (Holder-Czytko, 1994; Kumar and Prasad, 2004a,b) and is also useful as an oxygenator in closed equilibrated biological aquatic systems (Voeste et al., 2003). Hence we have analyzed the interactions of Zn and Cd using *C. demersum* as the model plant system of study. *C. demersum* is known to be a filter of Cd (Ornes and Sajwan, 1993; Saygideger and Dogan, 2004; Keskinkan et al., 2004), Cu (Devi and Prasad, 1998), Cr (Garg and Chandra, 1990), Pb (Rai et al., 1995; Saygideger and Dogan, 2004) and Hg (Suckcharoen, 1979). As this plant has recently been recommended for use in remediation of toxic metals (Rai et al., 1995), metabolic studies on *C. demersum* are worthwhile using sensitive metabolic parameters (with significant stimulatory or inhibitory effects) that may be used as biomarkers for heavy metal stress, and in elucidating the plant response to multiple metal combinations. Moreover, *C. demersum* has unique features, which make it ideal for laboratory toxicity bioassays. *C. demersum* is a submerged floating rootless plant and this avoids the complication of soil-system and root-shoot metal partitioning (Best, 1986; Best and Meulemans, 1979). It is convenient for experimental handling, cost-effective in maintenance and multiplies rapidly, an important pre-requisite for choosing any macrophyte as a material for study. The

forked leaves of the plant and thin cuticle on the plant surface facilitate uptake of metals from water through its large surface. The response of an organism to deficient or excess levels of metal (i.e. bioassays) can be used to estimate metal impact. Such studies when carried out under defined experimental conditions can provide results that can be extrapolated to the natural environment.

Cd-Zn interaction in *C. demersum*: Ecophysiology

a) Competitive inhibition of Cd uptake by Zn: Our studies on Cd-Zn interaction clearly identified the potential toxicity of Cd and the role of Zn in antagonizing Cd toxicity. The distinct toxicity symptoms of chlorosis and necrosis observed under the toxic treatments of Cd were clearly alleviated by Zn (figure 1). The inhibitory effect of Zn on Cd was reflected directly by analysis of metal accumulation by *C. demersum.* Zn supplements (10, 50, 100 and 200 µM) to Cd-treated plants reduced (26 %) the accumulation of cadmium especially at the 200 µM concentration (figure 2). Simultaneously the reduced (50 %) amount of Zn found in Cd-10 µM alone treatments was restored to control levels and accumulation increased (79 %) when Cd-treated plants were supplemented with Zn (200 µM). This suppression in Cd uptake due to the increase in Zn accumulation indicates a strong competition between Zn and Cd. Since Cd and Zn, both taken up as divalent cations, have a similar chemistry (Nieboer and Richardson, 1980), Cd can readily inhibit most of the Zn-dependent processes. Consequently, increased Zn concentration is able to replace a non-physiological metal like Cd, which may bind to the crucial and functional membrane and enzyme active sites and inactivate their functions (Van Assche and Clijsters, 1990; Shaw et al., 2004). Cadmium has been described as an antimetabolite of Zn due to the observed Zn deficiency in most of the Cd-treated systems (Peraza et al., 1998), an observation true to our system also. Other reported evidence such as in *Glycine max* (Cataldo et al., 1983), *Holcus lanatus* (Symeonidis and Karataglis, 1992) and *Lactuca sativa* (Thys et al., 1991) also point to depressed Cd uptake in the presence of Zn.

b) Zn regulates membrane transporter-mediated Cd uptake: There are specific metal ion uptake systems in cells especially for essential nutrients that are tightly controlled at both transcriptional and post-transcriptional levels with specific regulatory mechanisms (Lasat et al., 2000). Transport of non-essential elements like Cd is most likely to occur via transporters of essential cations (Fox and Guerinot, 1998; Souza-Santos et al., 2001). The Zn(II) transporting activity of *ZIP* proteins is inhibited by Cd(II), Co(II) and Cu(II), indicating that *ZIP* proteins may transport potentially toxic metals as well as nutrients (Kochian, 1993; Grotz et al., 1998). Apart from *ZIP* family of transporters, the iron transporters (*IRT1*) are required for normal iron utilization (Eide, 1997). Cd has also been shown to inhibit iron uptake by *IRT* transporters, indicating the control of entry through nutrient transporters (Bereczky et al., 2003). In *Thlaspi caerulescens, ZNT1* encodes a high affinity transporter, also mediating low affinity Cd transport (Lasat et al., 2000). Many essential physiological processes in plants including the uptake of minerals are dependent on the H^+ gradient generated by H^+ ATPase located in the plasma membrane (Michelet and Boutry, 1995, Morsomme and Boutry, 2000). These ATPases belong to the CPx type transporters, which have conserved intramembranous Cys-Pro-Cys or Cys-Pro-His motifs (Solioz and Vulpe, 1996; Portillo, 2000). There are reports on regulation (up/down) of H^+ ATPase by various metals in different systems such as Cd in *Glycine max* (Cataldo et al., 1983), *Helianthus annus* and *Triticum aestivum* (Fodor

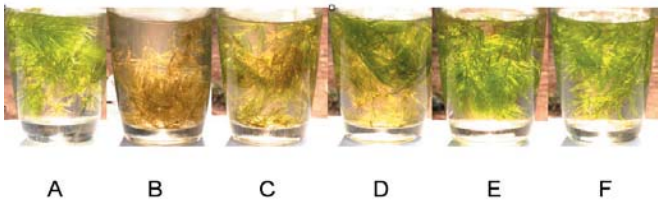

Figure 1. Toxicity bioassay under different metal treatments using *C. demersum*. A) Control plants without any treatments; B) Cd-10 µM; C) Cd-10 µM + Zn-10 µM; D) Cd-10 µM + Zn-50 µM; E) Cd-10 µM + Zn-100 µM; F) Cd-10 µM + Zn-200 µM treatments.

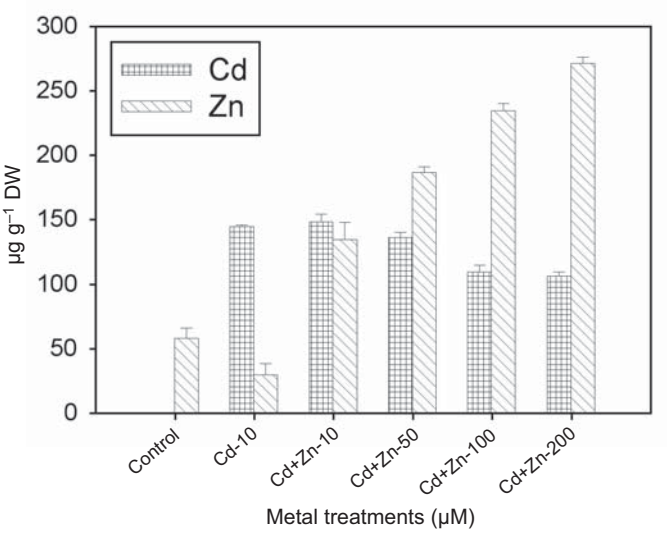

Figure 2. Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 µM and Zn (10, 50, 100, and 200 µM). Error bars represent standard errors and an 'asterisk' for Cd content indicates significant difference from Cd-treated plants; an 'asterisk' for Zn content indicates significant difference from control plants.

et al., 1995), *Lactuca sativa* (Costa and Morel, 1994), and Fe in *Zea mays* (Souza-Santos et al., 2001). The uptake of Cd by the plant system decreased (15 %) in the presence of the metabolic inhibitor 2,4-dinitrophenol (DNP) indicating the proton gradient-mediated uptake as one of the routes of active transport into the plant cell (figure 3). According to the chemiosmotic hypothesis, DNP increases proton permeability of biomembranes and thereby breakdown of proton gradients that could explain the pronounced inhibition of Cd uptake (Poole, 1978). Furthermore, the association with H^+ATPase is reinforced by the observation of a decrease (23 %) in Cd uptake in the presence of N-N'-dicyclohexylcarbodimide (DCCD), a proteolipid-binding H^+ATPase specific inhibitor. This was further justified from the assay of H^+ATPase which indicated an increase (27 %) in activity in Cd treatments in the absence of metabolic inhibitors corresponding to the higher uptake of Cd (figure 4). These results are in agreement with the earlier work on Cd uptake in *C. demersum* (Tripathi et al., 1995) and *Lactuca sativa* (Costa and Morel, 1994). It should be noted that in Cd treatments with added DNP and DCCD the accumulation of Cd decreased with a simultaneous decrease in H^+ATPase activity. Zn supplementation (10-200 μM) along with DNP and DCCD further reduced Cd uptake (33 % and 38 % reduction respectively). There was absolutely no effect on Zn uptake by DNP or DCCD. The H^+ATPase activity as well as Cd uptake was further reduced by Zn supplements along with DNP and DCCD indicating direct evidence for the regulation of the H^+ATPase-mediated proton pumping mechanism by Zn, through which Zn can indirectly antagonize Cd entry into the plant cell.

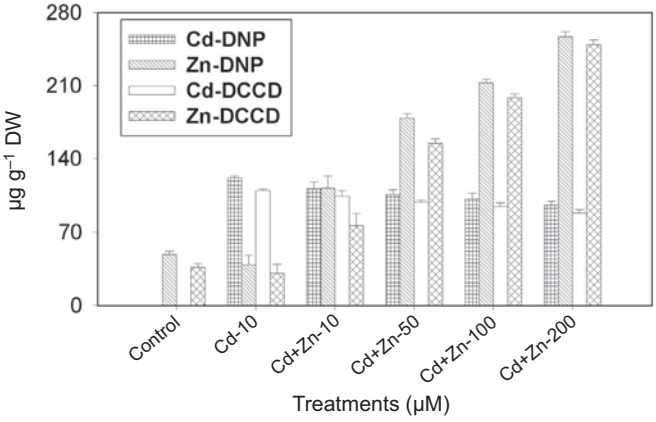

Figure 3. Metal accumulation in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations in the presence of metabolic inhibitors 2,4-dinitrophenol (DNP) (10 μM) and N-N' dicyclohexylcarbodimide (DCCD) (100 μM). Error bars represent standard errors and an 'asterisk' for Cd content indicates significant difference from Cd treated plants; an 'asterisk' for Zn content indicates significant difference from control plants.

Zn-Cd interaction in *C. demersum*: biochemistry

a) Zn alleviates Cd-induced oxidative stress: Accumulation of Cd is known to cause oxidative stress through free radicals [superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2)], (Shaw et al., 2004). A variety of macromolecules including proteins, lipids, polysaccharides and nucleic acids are oxidatively modified, and the manifestations of this damage are multifarious (Davies, 2003). In *C. demersum* Cd induced strong oxidative stress as evidenced by high levels of lipid peroxidation, electrical conductivity, increased Na^{2+}, Ca^{2+}, and K^+ leakage and lipoxygenase activity (table 1) indicating higher lipolytic activity and oxidation of membrane-bound fatty acids causing propagation of lipid peroxidation and hence ion leakage. Cadmium is known to bind to sulphydryl groups of membrane-bound proteins and destabilize the membrane system, by inducing the formation of disulphide links leading to distortion in structure and function of membrane ion channels and hence regulation of uptake/efflux of essential nutrients (Prasad, 1995a). Hence it leads to distortion in membrane structure increasing the electrolyte leakage and loss of ions. Reports in various animal and plant systems related to studies on Zn-deficiency have highlighted the increase in peroxidative damage and oxidative stress, which was completely inhibited under Zn-sufficient conditions (Cakmak and Marschner, 1998a,b; Cakmak, 2000). This indicates that Zn might play an important role in modulating production of ROS and in maintaining cellular homeostasis (Powell, 2000). Zn supplementation reduced Cd-induced oxidative stress in *C. demersum* as observed by the decrease in these parameters

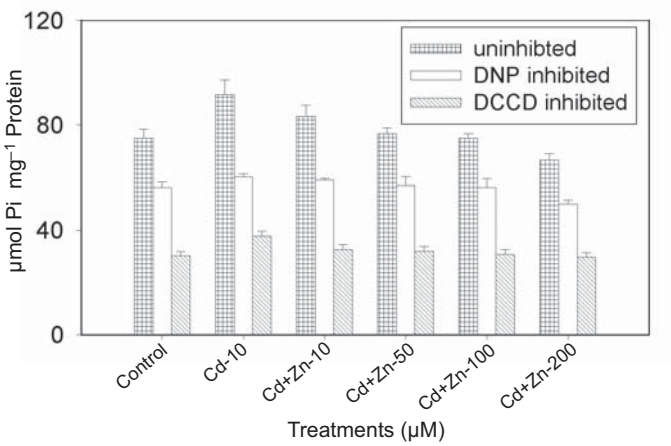

Figure 4. H^+ ATPase activity in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) in the absence of and in the presence of metabolic inhibitors 2,4-dinitrophenol (DNP) (10 μM) and N-N' dicyclohexylcarbodimide (DCCD) (100 μM). Error bars represent standard errors and an 'asterisk' indicates significant difference from Cd treated plants.

Table 1. Various parameters of oxidative stress investigated in *Ceratophyllum demersum* L. treated with Cd-10 µM alone and Cd-10 µM supplemented with Zn (10, 50, 100 and 200 µM) and Zn- alone (10, 50, 100 and 200 µM).

Parameters investigated	Results of the investigations			References	Inferences
	Cd-10 µM Treatments *	**Zn supplemented Cd treatments****	**Zn-alone treatments***		
A. Membrane damage					
1. Lipid peroxidation	37 % ↑	48 % ↓	↕	Heath and Packer, 1968 Ederli et al., 1997	Zn supplements inhibited Cd-induced damage to membrane structure and prevented loss of ions.
2. Lipoxygenase (E.C. 1.13.11.12) activity	3-fold ↑	3-fold ↓	↕	Cakmak and Marschner, 1998a	
3. Ion leakage: electrical conductivity	29 % ↑	52 % ↓	↕		
B. Estimation of free radicals					
1. NADPH dependent superoxide generation	72 % ↑	70 % ↓	↕	Cakmak and Marschner, 1998b	Zn inhibited NADPH oxidation and thereby excess production Cd-induced free radicals.
2. Cellular levels of superoxide radicals	41 % ↑	47 % ↓	↕	Chen and Li, 2001	
3. Measurement of hydrogen peroxide	60 % ↑	69 % ↓	↕	Mukherjee and Choudhari, 1983	
4. Electron spin resonance signals of hydroxyl radical	74 % ↑	72 % ↓	↕	Li et al., 2000	
5. Measurement of hydroxyl radical levels	40 % ↑	38 % ↓	↕	Babbs and Gale, 1987	
C. Protein oxidation					
1. Total protein content	35 % ↓	39 % ↑	↕	Lowry et al., 1951	Zn supplements effectively protected the total protein of the system from ROS-mediated oxidation.
2. Carbonyl content as a measure of Protein oxidation	48 % ↑	54 % ↓	↕	Reznick and Packer, 1994	

↑ indicates increase in the measured parameter; ↓ indicates decrease in the measured parameter; ↕ indicates negligible or no change in the measured parameter; * indicates changes in comparison with control; ** indicates changes in comparison to Cd-10 µM treatments.

indicating the intact membrane structural integrity. The chemical properties of Zn are favourable to various metabolic reactions, since under physiological conditions Zn has the unique property of existing in a univalent state without undergoing redox cycling (Vallee and Auld, 1990). Elemental Zn has two outer shell electrons, which are readily lost in water at pH 7.4 to form Zn^{2+}. Zinc carries out its biochemical functions as a divalent cation primarily when bound to enzymes and other proteins (Vallee and Falchuk, 1993). Further reduction to Zn^{+1} or Zn^{0} does not occur as there is no biological reductant strong enough (i.e. having a high enough redox potential) to reduce Zn. Similarly Zn cannot be oxidized further to Zn^{3+} since it possesses a full complement of '3d' electrons and removing one of these would require more energy than any known biological oxidant could mobilize (Vallee and Falchuk, 1993). Furthermore, due to filled *d*-shell orbitals, Zn^{2+} has a ligand-field stabilization energy of zero (McCall et al., 2000). Zinc has a very low electrochemical potential, higher charge density, and hence very high ionization energy would be required to remove or add electrons beyond the Zn^{2+} state (Schűtzendűbel and Polle, 2002). Hence Zn is stable in biological medium where the oxidoreductive potential is subjected to continuous flux (Vallee and Falchuk, 1993; Cakmak, 2000; Powell, 2000; Zago and Oteiza, 2001). This property forms the basis for the efficient functioning of Zn in biological systems. Zn-alone-treated plants however did not show any variation in the above parameters (table 1). Zinc prefers binding to the –SH groups of the membrane protein moiety and protects the phospholipids and proteins from thiol oxidation and disulphide formation (Chvapil, 1973) either by direct binding or to a site close to the sulphydryl group or through a conformational change. This results in an apparent stability of the enzymes, membrane proteins as well as the lipid structure (Bray and Bettger, 1990; Powell, 2000) and hence affords protection from Cd-induced sulphydryl oxidation and structural damage.

b) Influence of Cd-Zn interaction on the free radical species and antioxidants: Divalent cations such as Cd are known to trigger the oxidation of NADPH leading to superoxide radical production ($O_2^{\cdot -}$) (Kawano et al., 2001) furthering the reaction to hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) formation (Fleschin et al., 2000). Cadmium enhanced free radical production in *C. demersum* by mediating enhanced NADPH oxidation (table 1). The protective effect of Zn has been shown to be due to its ability to inhibit NADPH oxidation and oxygen-centered free radical generation as analyzed under Zn deficient conditions where the NADPH-

dependent $O_2^{\cdot -}$ radical production increased (Cakmak and Marschner, 1988b,c). This is in complete agreement with our results where Zn supplementation effectively inhibited NADPH oxidation and hence $O_2^{\cdot -}$ radical formation, thereby preventing ROS formation and maintenance of the NADPH redox pool. Cd increased OH radical levels as revealed by the very high increase in signal amplitude in electron spin resonance spectra of OH· signals (table 1) using 5,5-dimethyl pyrroline-N-oxide as a spin trap. Our observations also indicated a decrease in the total protein content and enhanced oxidation of proteins measured as carbonyls under Cd toxicity (table 1). Our results clearly exhibit the true antioxidative and ROS scavenging capacity enhanced by Zn supplementation as there was complete inhibition of formation of ROS in the plant system, an observation that would definitely justify Zn as an element with potential antioxidant properties. Zn supplements were effective in not only inhibiting free radical formation but also restored and slightly enhanced the total protein content by inhibiting the oxidation of proteins measured as carbonyls (table 1). This is in agreement with several studies in a number of plant species (Cakmak et al., 1989; Hossain et al., 1997), indicating that Zn is indeed one of the key elements regulating protein synthesis. Moreover the reduction of protein carbonyl formation indicated protection of cellular protein from ROS-mediated oxidative damage.

Variations in the responses of antioxidant enzymes to Cd toxicity are well known (Van Ascche and Clijsters, 1990). The enhanced SOD activity of both Cu/Zn SOD and Mn-SOD observed in Zn-supplemented Cd-treated plants (table 2) indicate enhanced $O_2^{\cdot -}$ scavenging and dismutation to H_2O_2. This metalloenzyme possesses Zn as one of its active site metals for its co-catalytic functioning (Vallee and Falchuk, 1993) along with Cu in Cu/Zn SOD. The enzyme has been shown to decrease in activity under Zn-deficient conditions (Cakmak and Marschner, 1993; Cakmak et al., 1997) but not under Cu deficiency, indicating a direct role of Zn in regulating SOD activity. Zn is known to stabilize superoxide dismutase and hence a higher Zn supplement is able to enhance dismutation of the Cd-increased $O_2^{\cdot -}$ radical, facilitating its detoxification in the subsequent steps utilizing CAT and APX. These observations indicate the probable role of Zn in stimulating the biosynthesis of antioxidant enzymes (Cakmak, 2000) as evidenced by a variety of biological systems that have shown a relationship between the relative tolerance to oxidative stress and the capacity of an organism to enhance intracellular antioxidant enzyme activity by induction of *de novo* protein synthesis (Miszalski et al., 1998). It is likely

Table 2. Responses of the antioxidant enzymes and non-enzymatic cellular antioxidants (redox pool) to Cd-Zn interaction in *Ceratophyllum demersum* L. Plants were treated with Cd-10 µM alone and Cd-10 µM supplemented with Zn (10, 50, 100 and 200 µM) and Zn- alone (10, 50, 100 and 200 µM).

Parameters investigated	Results of the investigations			References	Inferences
	Cd-10 µM Treatments *	**Zn supplemented Cd treatments ****	**Zn-alone treatments ***		
A. Antioxidant enzymes					
1. Superoxide dismutase (E.C. 1.15.1.1)	1.5-fold ↑	3.6-fold ↑	2.16 fold ↑	Beauchamp and Fridovich, 1971	Zn supplements enhanced the activity of antioxidant enzymes to a greater extent thereby alleviating Cd toxicity through increased quenching of free radicals enzymatically
2. Catalase (E.C. 1.11.1.6)	↕	5.35 fold ↑	3.47 fold ↑	Chance and Meahly, 1955	
3. Ascorbate peroxidase (E.C. 1.11.1.11)	2.14 fold ↑	5 fold ↑	3 fold ↑	Nakano and Asada, 1981	
4. Guaiacol peroxidase (E.C. 1.11.1.7)	16 % ↑	41 % ↑	24 % ↑	Mazhoudi et al., 1997	
B. Non-enzymatic cellular antioxidants					
1. Total, protein- and non-protein thiols	49 % ↓	45 % ↑	↕	Sedlak and Lindsay, 1968	Zn regulated Cd-impaired redox status of the plant cell by inhibiting redox shift of the cellular antioxidants, which are otherwise highly susceptible to oxidative stress
2. Cellular glutathione content	33 % ↓ GSH, 39 % ↑ GSSG	57 % ↑ GSH, 47 % ↑ GSSG	22 % ↑ GSH, ↕ GSSG	Hissin and Hilf, 1976	
3. Ascorbic acid and dehydroascorbate	64 % ↓ DHA/AsA ratio: 1.87	72 % ↑ DHA/AsA ratio: 0.45	15 % ↑ DHA/AsA ratio: 0.68	Logan et al., 1998	

↑ indicates increase in the measured parameter; ↓ indicates decrease in the measured parameter; ↕ indicates negligible or no change in the measured parameter; * indicates changes in comparison with control; ** indicates changes in comparison to Cd-10 µM treatments.

that excess production of ROS by Cd would have inactivated SOD and CAT as very high levels of H_2O_2 inhibit Cu/Zn SOD (Casano et al., 1997; Cakmak, 2000) through Cu^{2+} to Cu^{+} reduction and formation of excess hydroxyl radicals or inactivation of the enzyme-bound heme group (Luna et al., 1994) in CAT by $O_2^{\cdot -}$ leading to impaired CAT activity. Zn stimulated CAT, POD and APX activity (table 2) effectively scavenging Cd-increased H_2O_2 levels in the plant system (Anabel et al., 2002). The Zn-enhanced ROS scavenging antioxidant enzyme activity directly indicates the efficiency of Zn as an antioxidant thereby acting against Cd-induced oxidative stress.

c) Zn-enhanced functioning of the ascorbate-glutathione cycle and glutathione metabolism enzymes - Antioxidant pathways involved in Cd detoxification: The endogenous non-enzymatic antioxidants include carotenoids, α-tocopherol, flavonoids, phenolic acids, amino acids, polyamines, ascorbate (AsA) (Pallanca and Smirnoff, 2000), thiols (-SH) and glutathione (GSH) (Foyer et al., 2001), which are effectively free-radical scavengers (Alscher et al., 1997). Cadmium-induced oxidation of –SH groups is one of the earliest events observable in a living system. Cadmium induced a severe decrease in the redox pool of the plant cell – thiols, glutathione (GSH) as well as total ascorbate levels, and simultaneously oxidized ascorbate (AsA) to dehydroascorbate (DHA) and glutathione (GSH) to its oxidized form (GSSG) (table 2), a true indication of oxidative stress. GSH functions as a stress indicator shifting the redox state in response to oxidative stress (Noctor et al., 1998; Devi and Prasad, 1998). The formation of oxidized GSSG in Cd-10 µM treatments could be due to the reaction of GSH with oxyradicals generated by the toxic concentration of Cd as in any other metal stress resulting in elevated GSSG (Nagalakshmi and Prasad, 2001). The Cd-decreased GSH pool and altered GSH/GSSG ratio would apparently render the cell more sensitive to other forms of stress such as photooxidation, ozone etc (Xiang et al., 2001). Zinc clearly restored the lost thiol pools, a natural phenomenon associated with Zn (Cakmak 2000). It also restored and enhanced GSH to a great extent, with a simultaneous decrease in the oxidized GSSG. Zn probably modulates GSH levels by regulating its biosynthesis or by protecting the reactive cysteine residue of GSH (Cakmak, 2000) or through the efficient functioning of GR (Foyer and Halliwell, 1976). Zn-protected AsA would react efficiently with $O_2^{\cdot -}$, singlet oxygen (directly), ozone (chemically), and H_2O_2 (enzymatically through APX), and thereby assist in neutralizing these potential toxicants (Smirnoff et al., 2001). Cd-induced changes in the levels of cellular antioxidants drastically affected and impaired the functioning of the ascorbate-glutathione cycle (Zhang and Kirkham, 1996). The ascorbate-glutathione cycle (AGC) is a major H_2O_2 scavenging antioxidant pathway that operates both in chloroplasts as well as the cytosol (Zhang and Kirkham, 1996), where cycling of the redox molecules (AsA, GSH) takes place with effective utilization of the AGC enzymes [ascorbate peroxidase-APX, monodehydroascorbate reductase-MDHAR, dehydroascorbate reductase- DHAR and glutathione reductase- GR]. Zn supplementation effectively recovered the AGC pathway (table 3) simultaneously enhancing the activity of all the involved enzymes and thereby maintaining the GSH and AsA levels (table 2). The toxicity induced by Cd inhibited the activity of MDHAR, as well as DHAR there by increasing dehydroascorbate (DHA) formation. Extremely high DHA accumulation is generally considered as a negative event for cell metabolism (De Gara et al., 2000). Therefore a higher MDHAR activity and hence DHAR, was adopted as the strategy for inhibiting MDHA disproportionation and DHA reduction (Drażkiewicz et al., 2003) by Zn to counteract Cd-induced disturbances of the AsA redox status in *C. demersum*. Zn induced a very high increase in the activity of Glutathione reductase (GR) in *C. demersum* (table 3), which could have resulted from de novo GR protein synthesis connected to the trigger mechanism initiated by Cd-induced oxidative stress (Drażkiewicz et al., 2003). GR is extremely sensitive to inhibition by heavy metal ions like Cd^{2+}, Cu^{2+}, Fe^{3+}, and by compounds that react with the –SH groups due to the presence of thiol groups at the active site of the enzyme (Smith et al., 1989). Active participation in thiol protection (Cakmak, 2000; Powell, 2000) is a function particularly associated with Zn, and leads to enhanced activity of GR ensuring efficient cycling and utilization of the pyridine nucleotide reducing power .

Glutathione metabolism involves many reactions where glutathione is synthesized, degraded, conjugated or oxidized (Noctor et al., 1998). Our own experimental evidence showed that Cd as well as Zn did not have a great affect on γ-glutamylcysteine synthetase (γ-GCS), an enzyme of GSH synthesis (table 3). Glutathione-S-transferases (GST) catalyze both GSH-dependent conjugation and reduction thereby playing an active role in detoxification since GST mRNA responds very quickly to oxidative stress (Conklin and Lasat, 1995). The observed limited role of GST in Cd-treated plants may be due to its inhibition by peroxidation of GST protein or inactivation of GST genes. On the other hand Zn supplementation to Cd-10 µM treatments activated GST

Table 3. Effect of Cd-Zn interaction on the ascorbate glutathione cycle (AGC) enzymes and glutathione metabolism enzymes in *Ceratophyllum demersum* L. Plants were treated with Cd-10 µM alone and Cd-10 µM supplemented with Zn (10, 50, 100 and 200 µM) and Zn-alone (10, 50, 100 and 200 µM).

Parameters investigated	Results of the investigations			References	Inferences
	Cd-10 µM Treatments *	**Zn supplemented Cd treatments ****	**Zn-alone treatments ***		
A. Ascorbate-glutathione cycle enzymes					
1. Monodehydroascorbate reductase (E.C. 1.6.5.4)	34 % ↓	52 % ↑	↕	Drażkiewicz et al., 2003	Zn supplements effectively modulated the inhibition of AGC enzymes by Cd and thereby enhanced the cycling of the redox molecules for efficient antioxidant functions.
2. Dehydroascorbate reductase (E.C. 1.8.5.1)	38 % ↓	51 % ↑	↕	De Tullio et al., 1998	
3. Glutathione reductase (E.C. 1.6.4.2)	46 % ↓	55 % ↑	↕	Jiang and Zhang, 2001	
B. **Glutathione metabolism enzymes**					
1. γ-Glutamylcysteine synthetase (EC 6.3.2.2)	↕	↕	↕	Reugsegger et al., 1992	Zn antagonized the toxic effects of Cd on the functional activities of GSH metabolism enzymes in conjunction with GSH levels and AGC.
2. Glutathione-S-transferase (E.C. 2.5.1.18)	14 % ↑	31 % ↑	↕	Habig and Jacoby, 1981	
3. Glutathione peroxidase (E.C. 1.11.1.9)	40 % ↓	57 % ↑	↕	Nagalakshmi and Prasad, 2001	

↑ indicates increase in the measured parameter; ↓ indicates decrease in the measured parameter; ↕ indicates negligible or no change in the measured parameter; * indicates changes in comparison with control; ** indicates changes in comparison to Cd-10 µM treatments.

to a great extent (table 3) indicating increased detoxification. Zn, by triggering GSTs, detoxifies endogenously-produced electrophiles like 4-hydroxy alkenals and base propenals (Marrs, 1996). The enzyme GST conjugates itself to these products formed by oxidative degradation of lipids and nucleic acids and prevents them from propagating further damage (Nagalakshmi and Prasad, 2001; Foyer et al., 2001). Glutathione peroxidase (GSH-PX) is a part of the arsenal of protective enzymes, and is involved in coupling reactions with glutathione and cellular detoxification of H_2O_2, as a response to oxidative stress (Drotar et al., 1985). Zn played a key role in enhancing Cd-inhibited GSH-PX activity and hence its H_2O_2 scavenging activity.

Plants treated with Zn only did not show significant changes in the above parameters indicating that the chosen range of Zn concentrations did not affect the physiology of the plants in any way. In conclusion, the higher reduction state of the ascorbate and glutathione pool, consistent with higher activities of APX, MDHAR, DHAR and GR, as well as GST and GSH-PX enzymes in Zn-supplemented Cd-treated plants, indicate that the specific role of Zn in promoting ROS detoxification through AGC enzymes also involves GSH-metabolism.

d) Carbonic anhydrase - A model enzyme with relevance to Cd-Zn interaction: Carbonic anhydrase (CA) has been extensively studied in many organisms from cyanobacteria to higher plants, animals and human systems (Armstrong et al., 1966; Atkins et al., 1972; Smith and Ferry, 2000; Tripp et al., 2001). CA has been presumed to have an active function in photosynthetic organisms (Sultmeyer et al., 1993) and has been the topic of interest in many aquatic systems where the availability of CO_2 is low (the predominant form of dissolved inorganic carbon being HCO_3^-) and further complicated by its slower diffusion rates in water, being 1 x 10^4 times slower than in air (Badger, 1987). In order to maintain photosynthetic efficiency under low CO_2 availability, a carbon-concentrating mechanism (CCM) functions (Lane and Morel, 2000). This CCM has two components, namely a mechanism for taking up HCO_3^- and a Zn-requiring CA that catalyses the interconversion of HCO_3^- to CO_2 (Badger and Price, 1994). Our investigations with *C. demersum* revealed the existence of an active Zn-dependent CA in *C. demersum* as in other aquatic angiosperms (*viz. Myriophyllum, Hydrilla, Elodea)* where HCO_3^- diffusion and then conversion to CO_2 by CA plays a vital role in maintaining the inorganic carbon levels (Elzenga and Prins, 1988). The toxicity of Cd-10 µM drastically affected the functioning and activity of CA. However, when Cd-treated plants were

supplemented with Zn, not only was the CA activity (table 4) restored but activity increased with higher concentrations of Zn. This demonstrates the complete dependence of the active form of this enzyme to the presence of sufficient levels of Zn. It has been suggested that CA activity is a suitable indicator of the levels of physiologically active Zn in plant tissues (Gibson and Leece, 1981). Cadmium possesses properties identical to Zn (Nieboer and Richarson, 1980). Hence Cd can readily inhibit most of the Zn-dependent processes either by competing for similar sites or displacement/ substitution reactions in occupying the active sites of the Zn-metalloproteins (Nieboer and Richardson, 1980; Siedlecka, 1995). CA purified from *C. demersum* subjected to different treatments can directly corroborate the above statement. There was a distinct loss of recovery as well as catalytic activity in Cd-treated plants, where the novel and important observation that Cd substituted Zn in the active site of CA was also observed (table 4). This was subsequent to a reduction in the Zn content of the purified enzyme. Thus the theory of Cd occupying the active sites of important Zn-metalloproteins has been proved by our findings. Carbonic anhydrase molecules devoid of their usual Zn content would be non-functional and Cd-induced Zn deficiency *per se* would lead to impaired protein synthesis (Marschner and Romheld, 1991). Hence the associated photosynthetic processes would also be affected extensively. In many cases it has been reported that removal of Zn from CA leads to irreversible loss of catalytic activity (Guliev et al., 1992), which corroborates our findings. Cd is known to affect enzymes and proteins through its interaction with –SH groups as well as inducing redox cycling (Powell, 2000). Moreover the S-P orbital energy separation for Cd is less than that of Zn, suggesting that excitation of the valence state may be easier for Cd than for Zn due to its low charge density (Schűtzendűbel and Polle, 2002). Therefore, a higher state of redox cycling by Cd and destruction of the Zn-thiolate bonds of the enzyme due to oxidation of disulphide groups by an inactive and toxic metal like Cd would completely impair CA functioning. The CA purified from Cd-treated plants with supplemented Zn showed a higher recovery as well as a highly active CA with the Zn content estimated to be slightly higher than that of the control (table 4). The increased uptake of Zn would explain the enhanced CA biosynthesis and catalytic activity. Zinc-alone treatments also produced an active purified enzyme with a Zn content closely comparable to that of the control. The presence of sufficient levels of Zn in Cd-treated plants with supplemented Zn strengthens the Zn-metalloprotein interaction through protection of the –SH groups from thiol oxidation and intramolecular disulphide formation, a function primarily associated with Zn (Cakmak, 2000; Cakmak and Braun, 2001).

The cadmium-substituted CA enzyme revealed a distorted α-helical conformation with unfolding of the α-helix to an open coiled structure, as indicated by near UV circular dichroism analysis (table 4). Since CA purified from Cd-10 μM treatments showed metal substitution reactions with Zn, the natural active site co-factor of CA, the electrostatic interactions within the protein molecule would be drastically affected when Zn is replaced by Cd (Creighton, 1993). This would also induce destabilizing intramolecular repulsive interactions between like charges which would act as the driving force for the unfolding of the macromolecule (Anfinsen, 1973) ultimately distorting the ordered secondary structure. Cadmium substitution had profound effects on the specific tertiary interactions of the aromatic amino acid residues inferred from the spectra of far UV-circular dichroism and UV spectra. Cadmium substitution reduced and shifted the emission maxima of the native fluorescence of tyrosine and tryptophan and increased 8-anilino-1-naphthalene sulfonic acid (ANS)-protein binding. It may be concluded from this observation that the exposure of aromatic amino acid residues from the interior of the hydrophobic pockets of the unfolded enzyme to the external solvent lead to the changes in fluorescence intensity with a concomitant shift in the emission maxima. Thus the exposed hydrophobic pockets of the enzyme react with external 8-ANS dye thereby increasing the fluorescence indicative of protein unfolding. A potent mode of direct attack on protein derives from site-specific metal-catalyzed oxidation (MCO), in which the reduced form of a protein-bound transition metal reacts with H_2O_2 to form a reactive intermediate in the immediate proximity of amino acid side chains (Stadtman and Levine, 2000). The reduction of –SH groups and consequently high levels of protein carbonyls in CA from Cd-alone treated plants (table 4) give good support for the above statement. In contrast, CA purified from Zn-supplemented Cd treatments clearly showed restoration and perfect α-helical conformation (table 4) similar to that of the native CA, indicating that Zn protects CA from Cd-induced damage, restoring its original conformation and hence physiological function. Zn being catalytically associated as a co-factor of CA, necessary for its active functioning, competitively replaces Cd from the Cd-substituted enzyme active site thereby maintaining the spatial relationships with the different amino acid residues in the polypeptide backbone and hence the charge interactions favouring intact protein conformation (Pocker and Sarkanen,

Table 4. Structural, conformational and functional changes of carbonic anhydrase (CA) in response to Zn and Cd in *Ceratophyllum demersum* L. Plants were treated with Cd-10 µM alone and Cd-10 µM supplemented with Zn (10, 50, 100 and 200 µM) and Zn- alone (10, 50, 100 and 200 µM).

Parameters investigated	Results of the investigations			References	Inferences
	Cd-10 µM Treatments *	**Zn supplemented Cd treatments ****	**Zn-alone treatments ***		
A. Functional studies					
1. Carbonic anhydrase activity (E.C. 4.2.1.1)	50 % ↓	67 % ↑	20 % ↑	Armstrong et al., 1966 Armstrong et al., 1966, Atkins et al., 1972, and Guliev et al., 2003	Zn and Cd show competition for the active site of CA. Zn supplements at higher concentrations completely eliminated trace levels of Cd substituted in the active site and restored the Zn content of CA, thereby restoring the functional activity of CA.
2. Purification of carbonic anhydrase					
a) percentage recovery	31 % ↓	49 % ↑	↕		
b) Specific activity	35 % ↓	40 % ↑	↕		
3. Zinc and cadmium content of purified carbonic anhydrases	Cd- trace levels Zn- 73 % ↓	Cd- not detected Zn- 77 % ↑	Cd-not detected Zn-↕		
B. Structural and conformational studies on carbonic anhydrases					
1.Ultraviolet difference spectra of carbonic anhydrases	37 % ↓	43 % ↑	↕	Mailer et al.,1984	The supplements of Zn effectively protected the structure and conformation of CA. Cd-impaired enzyme folding as well as its fluorescence properties were completely restored by Zn. Further the oxidation of functional groups were also inhibited by Zn . All the above results indicate Zn as a structural stabilizer of macromolecules, a function in contrast to Cd.
2. Circular dichroism spectra of carbonic anhydrases	33 % ↓ α-helix, 22 % ↑ random coil	35 % ↑ α-helix , 29 % ↓ random coil	↕ in α-helix or random coil	Yang et al.,1986, Chen et al.,1974	
3. Fluorimetric studies of carbonic anhydrases					
a) Intrinsic fluorescence –tryptophan	53 % ↓	57 % ↑	↕	Eftink 1991, Bhattacharjee and Das, 2000	
b) Tyrosine fluorescence	36 % ↓	43 % ↑	↕		
c) Dityrosine fluorescence	24 % ↑	34 % ↓	↕		
d) Extrinsic fluorescence- surface hydrophobicity as a measure of protein unfolding	53 % ↑	48 % ↓	↕	Matulis and Lovrien, 1998	
C. Sulphydryl groups of carbonic anhydrases	27 % ↓	20 % ↑	↕	Sedlak and Lindsay, 1968	
D. Carbonyls in carbonic anhydrases.	55 % ↑	69 % ↓	↕	Reznick and Packer, 1994	

↑ indicates increase in the measured parameter; ↓ indicates decrease in the measured parameter; ↕ indicates negligible or no change in the measured parameter; * indicates changes in comparison with control; ** indicates changes in comparison to Cd-10 µM treatments.

1978). Zn reduced carbonyl formation and maintained the thiol level in CA of Zn-supplemented Cd treatments indicating inhibition of side chain oxidations and sulphur group protection (Vallee and Falchuk, 1993; Zago and Oteiza, 2001). It can be suggested that Zn probably induces molecular "chaperones" which also prevent aggregation of the enzyme molecule and aid in proper protein folding (Rozema and Gellman, 1996). Loss of tryptophan and tyrosine fluorescence, plus the shift in emission maxima, were completely restored in Cd treatments with supplemented Zn, indicating intact folded protein conformation. The formation of dityrosine (oxidized tyrosine) was also completely inhibited by supplementing Zn to Cd treatments. The protection of -SH groups from thiol oxidation by Zn in Cd treatments with supplemented Zn would have strengthened the Zn-metalloprotein interaction (Cakmak, 2000; Zago and Oteiza, 2001) and hence prevented formation of destructive dityrosine. 8-Anilino-1-naphthalene sulfonate-protein fluorescence was totally reduced to control levels in CA from Zn-supplemented Cd treatments indicating protected hydrophobic regions in the intact and folded state of native CA, similar to that of the control. Zinc-alone treatments did not affect conformation, nor the fluorescence properties of native CA. Wolff et al. (1986) have previously indicated that Zn might play a crucial role in the decreased formation of oxidized products of protein. Since Zn possesses antioxidant properties (Girotti et al., 1985; Bray and Bettger, 1990; Powell, 2000; Zago and Oteiza, 2001), an inverse correlation may exist between Zn status and protein oxidation, with radical damage to proteins being accentuated by Zn deficiency (Cakmak, 2000). Our experiments with CA using mixed treatments of Zn and Cd conclusively proved the above statement that Zn counteracts the ROS-mediated oxidative attack on, proteins induced by Cd stress, further substantiating the role of Zn as an antioxidant.

e) Zn protects chloroplast photochemical functions from Cd toxicity: Metal ions are well known to affect the structure and function of chloroplasts in many plant systems such as *Beta vulgaris* (Greger and Ogren, 1991), *Spinacea oleracea* (Šeršeň and Král'ová, 2001), *Triticum aestivum* (Atal et al., 1991), and *Zea mays* (Prasad, 1995b). Reactive oxygen species directly affect the structure of the thylakoid membrane through peroxidation and oxidative stress. This alters the lipid composition of the thylakoid membranes leading to changes and disorganization (Stoyanova and Tchakalova, 1999) of the grana stacks with dilated thylakoid membranes observable as plastoglobules (Baszyński et al., 1980) This ultimately leads to the inactivation of oxygen-evolving centers and impaired electron transport (Krupa et al., 1994). Metal ions specifically inhibit chlorophyll biosynthesis through δ-aminolevulinic acid dehydratase (ALA dehydratase) (Myśliwa-Kurdziel and Strzał ka, 2002) and protochlorophyllide reductase (Baszyński et al., 1980; Myśliwa-Kurdziel et al., 2004) through the oxidation-prone –SH group leading to diminished production of 5-aminolevulinic acid (ALA), the first common precursor for all the tetrapyrroles. In our study system of *C. demersum* Cd also drastically affected chlorophyll and carotenoid levels (table 5). On the other hand, when Cd-treated plants were supplemented with Zn, there was full protection and restoration of the chlorophyll levels. Zinc probably maintains chlorophyll synthesis through –SH group protection of the oxidation-prone δ-aminolevulinic acid dehydratase (ALA dehydratase) and protochlorophyllide reductase (Baszyński et al., 1980; Myśliwa-Kurdziel and Strzałka, 2002; Myśliwa-Kurdziel et al., 2004). Since ALA dehydratase catalyzing the conversion of ALA to porphobilinogen requires Mg^{2+} or Zn^{2+} for its efficient functioning (Beale, 1999), Zn possibly plays a role in activating this enzyme, and hence protochlorophyllide to chlorophyllide conversion facilitating the formation of the complete chlorophyll moiety. Our results clearly support not only the protection of chlorophyll but also an increase in chlorophyll indicating that Zn may be involved in furthering chlorophyll biosynthesis above the control level (table 5). Probably Zn also maintains the rate of synthesis of carotenoids in *C. demersum.* It has been reported that Cd affects the lipid structure around PSII, especially the light-harvesting chl *a*/*b* protein complex II (Prasad, 1995b), leading to a loss of major fatty acids (Peters and Chin, 2003) and the production of lipid hydroperoxides thereby crippling the photosynthetic process. Cd-induced loss of intactness is clearly observed in our case (table 5). Zn on the other hand shows a clear restoration of the lost photosynthetic activity indicating its action against the toxic nature of Cd. Zn is known to have a stabilizing and protective effect on biomembranes (Chvapil, 1973; Cakmak 2000). This, in addition to increased carotenoid synthesis triggered by Zn, protects the thylakoid membrane from ROS-mediated peroxidative damage and hence the loss of thylakoid proteins also, conclusively proved by our results of chloroplast intactness. Cd^{2+} ions have been reported to replace Mn^{2+} ions at the oxygen-evolving centers, the primary source of electrons from water to PSII, thereby inhibiting the reactions of PSII (Baszyński et al., 1980; Prasad, 1995b) and associated proteins of the PSII reaction centers, especially the D1 polypeptide. Cd complexes with aromatic amino acid residues like tryptophan

Table 5. Investigations on the effect of Cd-Zn interaction on the photosynthetic functions in *Ceratophyllum demersum* L. Plants were treated with Cd-10 µM alone and Cd-10 µM supplemented with Zn (10, 50, 100 and 200 µM) and Zn- alone (10, 50, 100 and 200 µM).

Parameters investigated	Results of the investigations			References	Inferences
	Cd-10 µM Treatments *	**Zn supplemented Cd treatments ****	**Zn-alone treatments ***		
Analysis of photochemical functions					
1. Quantification of photosynthetic pigments.	Chl-a 23 % ↓ Chl-b 40 % ↓ Carotenoids 70 % ↓	Chl-a 44 % ↑ Chl-b 47 % ↑ Carotenoids 75 % ↑	↔ ↔ ↔	Arnon, 1949; Kirk and Allen, 1965	Zn protected the structure and functional activities of chloroplasts impaired by Cd toxicity.
2. Intactness of isolated chloroplasts		41 % ↑	↔	Reeves and Hall 1980	
3. Rate of photosynthesis	40 % ↓	39 % ↑	↔	Greger and Ogren 1991	
4. Electron transport activities	39 % ↓		↔	Atal et al., 1991	
a) PS II		37 %	↔	Atal et al., 1991	
b) PS I	42 % ↓	42 %	↔	Atal et al., 1991	
c) Whole chain electron transport	45 % ↓ 42 % ↓	38 %	↔		

↑ indicates increase in the measured parameter; ↓ indicates decrease in the measured parameter; ↕ indicates negligible or no change in the measured parameter; * indicates changes in comparison with control; ** indicates changes in comparison to Cd-10 µM treatments.

(Šeršeň and Král'ová, 2001) and PSII-D1 polypeptide. This Cd-D1 complex interferes with the degradation of D1 protein by a protease, a normal operation of the PS II reaction center, leading to impaired PS II activity (figure 6) (Hideg et al., 1994). This dysfunction propagates throughout the electron transport chain in the system. Zn, by controlling the levels of Cd entering into the system, controls its intracellular levels. Furthermore, the replacement of a toxic metal, Cd, by a physiologically active metal like Zn, maintains the integrity at the oxygen-evolving centers and prevents oxidative burst at antenna chl molecules and binding of Cd to the major thylakoid proteins like D1 of PSII. An active PSII initiates a proper electron transport and hence an active functioning of PSI and the complete photophosphorylation process.

Molecular aspects of Zn reversal of Cd-induced damage to DNA integrity

Nucleic acids are highly susceptible to metal-catalyzed oxidations with both nucleoside bases and sugar moieties being targets of ROS. The mediation of metal toxicity on DNA damage may be direct (Hossain and Huq, 2002a) or indirect in nature (Gichner et al., 2004). The toxicity of Cd on the structural integrity of DNA was identified utilizing the principle of the formation of a fluorescent complex between double-stranded DNA and ethidium bromide (EB) (Cai and Cherain, 2003). DNA, after exposure to the toxic levels of Cd (10 µM), resulted in a distinct loss of fluorescence due to the decreased binding of EB with DNA (figures 5 and 6). Oxygen free radicals induce numerous lesions in DNA that cause

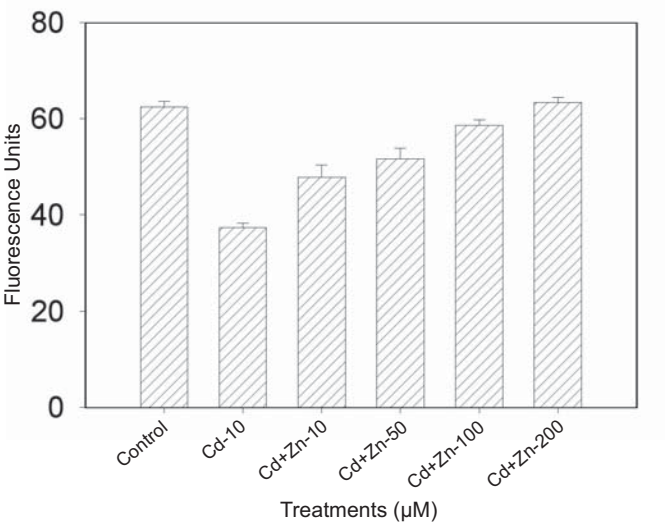

Figure 5. Ethidium bromide-DNA fluorescence as a measure of DNA damage in *C. demersum* plants treated with Cd-10 µM and Zn (10, 50, 100 and 200 µM). Error bars represent standard errors and an 'asterisk' indicates significant difference from Cd treated plants.

deletions, mutations and other lethal genetic effect (Breen and Murphy, 1995). The primary effect is the oxidation of the sugar moiety by the OH· radical, in a metal-catalyzed reaction (the metal bound to DNA by chelation to the phosphodiester linkage), thereby leading to the oxidation of the adjacent sugar or nitrogenous base which in turn provokes a broad spectrum of DNA lesions (Halliwell and Gutteridge, 1990). The DNA lesions include DNA single- and double-strand breaks, apurinic/apyrimidinic sites, DNA-protein cross-links and base modifications (Hartwig and Schwerdtle, 2002). Fe and Cu are potent inducers of DNA damage especially in the presence of H_2O_2 while other metals like Ni, Cd and Co are also reported to induce base modifications. Cross-linking of protein to DNA is another consequence of OH· attack on either the DNA or protein. This generates covalent linkages such as thymine-cysteine adducts making the DNA–protein inseparable and is lethal to the system if replication or transcription precedes the repair mechanism (Hartwig and Schwerdtle, 2002). DNA gel mobility assays also clearly determined the enhanced mobility of the DNA on the agarose gel due to loss of the double-stranded nature and fragmentation (Szuster-Ciesielska et al., 2000; Cai and Cherian, 2003) in DNA isolated from Cd-10μM treatments (figure 7) when compared with the intact DNA isolated from the control. First, direct binding of Cd to DNA bases (specifically G, A and T), the interaction of malondialdehyde with DNA and the interaction of Cd-induced ROS with DNA (Bhanoori and Venkateswerlu, 1998) all lead to extensive DNA damage. Second, metal ions are also known to inhibit the DNA repair processes (Hartwig and Schwerdtle, 2002). The DNA isolated from Zn-supplemented Cd treatments showed complete restoration of the lost fluorescence of EB-DNA interaction (figures 5 and 6) indicating intact structural integrity brought about by Zn ions favouring enhanced binding of EB with the DNA. Similarly, the DNA also showed complete inhibition of its mobility indicating the non-fragmentation of the DNA strands (figure 7). Zn ions act as the framework with which the folding of the domain is stabilized for a high affinity and site-specific binding of the double-stranded DNA (Klug and Rhodes, 1987; Wu and Wu, 1987). Zn ions were shown to inhibit DNA fragmentation and apoptosis induced by various stimuli in different animal and human systems (Szuster-Ciesielska et al., 2000; Cai and Cherian, 2003). Zn probably modulates the protection of DNA from Cd-induced damage either by inhibition of Ca^{2+}/ Mg^{2+}-dependent endonuclease or by inhibiting metal catalyzed oxidative damage through OH· attack on either the DNA or protein both by –SH group protection and inhibition of redox cycling of the associated metal ions thereby preventing the generation of covalent linkages such as thymine-cysteine adducts (Bray and Bettger, 1990; Ebadi et al., 1996). The latter seems to be the most probable mechanism in the present system of study.

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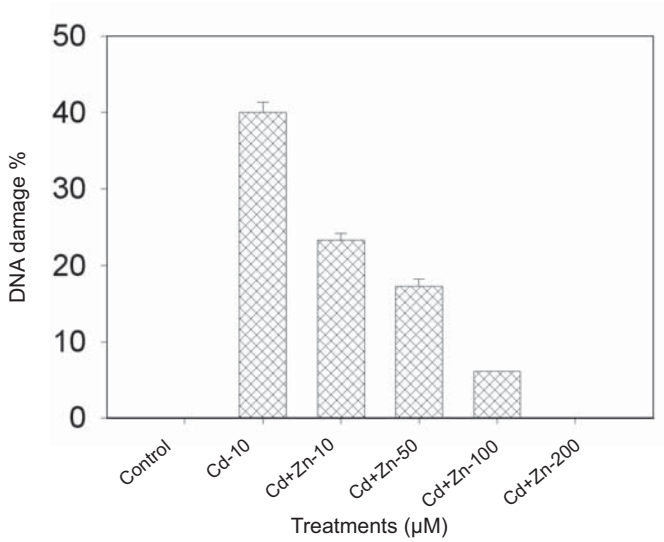

Figure 6. Levels of DNA damage in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100, and 200 μM). Error bars represent standard errors and an 'asterisk' indicates significant difference from Cd treated plants.

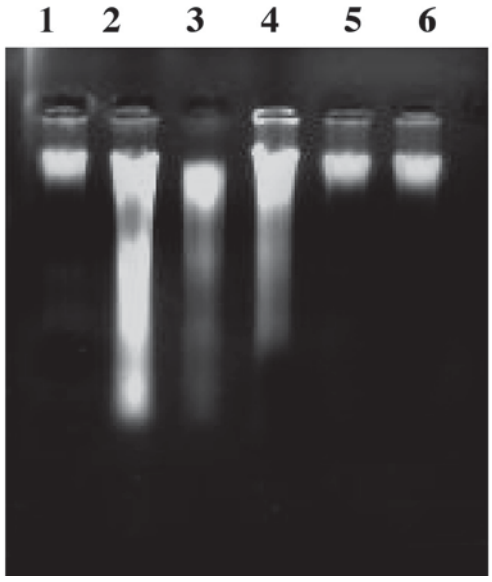

Figure 7. Agarose gel (1 %) showing the DNA mobility of DNAs isolated from *C. demersum* plants. Lanes 1: control, 2: Cd-10 μM, 3: Cd+Zn-10 μM, 4: Cd+Zn-50 μM, 5: Cd+Zn-100 μM, 6: Cd+Zn-200 μM.

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