Evidence for zinc, amino- and organic acids induced protection to cadmium exposed *Ceratophyllum demersum* L. A free floating aquatic macrophyte

Thesis submitted to the University of Hyderabad for the degree of
DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to Certify that the research work in the thesis titled **"Evidence for zinc,** amino- **and organic acids induced protection to cadmium exposed Ceratophyllum demersum L.: a free floating aquatic macrophyte"** has been carried out by Mr. P. Aravind under my supervision for the full period prescribed under the Ph D ordinance of this University and that this work has not been submitted for any other degree or diploma to any University or Institute.

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DECLARATION

I hereby declare that the work presented in the thesis titled **"Evidence for zinc,** amino- **and organic acids induced protection to cadmium exposed *Ceratophyllum demersum* L.: a free floating aquatic macrophyte"** has been carried out by me under the supervision of **Prof. M.N.V. Prasad, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad - 500 046,** and that this work has not been submitted for any other degree or diploma to any University or Institute.

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P. Aravind

Abbreviations

ANS	8-Anilino-1-naphthalenesulfonic acid
ANSA	l-Amino-2-naphthol-4-sulfonic acid
APX	Ascorbate peroxidase
AsA	Ascorbate
ATP	Adenosine triphosphate
CA	Carbonic anhydrase
CAT	Catalase
CDNB	l-Chloro-2,4-dinitrobenzene
CTAB	Hexadecyltrimethylammonium bromide
DCCD	N-N'-Dicyclohexylcarbodimide
DCPIP	2,6 Dichlorophenol indophenol
DCMU	3-(3,4-dichlorodiphenyl) 1, 1-dimethyl urea
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DMPO	5,5'-Dimethylpyrroline-N-oxide
DMSO	Dimethylsulfoxide
DNP	2,4-Dinitrophenol
DTNB	5, 5'-Dithio-bis-2-nitrobenzoic acid
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
γ-GCS	γ-Glutamylcysteine synthetase
GPX	Guaiacol peroxidase
GR	Glutathione reductase

GSH	Glutathione
GSH-PX	Glutathione peroxidase
GSSG	Glutathione disulfide
MDHAR	Monodehydroascorbate reductase
MSA	Methanesulfinic acid
MTT	3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium chloride
PMSF	Phenylmethylsulfonylfluoride
POD	Peroxidase
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBAP	Tetrabutylammonium perchlorate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'- Tetramethylethylenediamine

1. Introduction

Heavy metals As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Se and Zn have been considered as major environmental pollutants and their phytotoxicity is well established (Ross 1994, Prasad 2001, Prasad and Strzalka 2002). 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 one of the major receptacle other than terrestrial ecosystem for various contaminants generated through the unregulated release of effluents from mines, smelters, industries, excessive usage of agrochemicals, and from aerial deposition (Nriagu and Pacyna 1988, Kabata-Pendias 2001, Adriano 2002). Locations adjacent to agricultural areas pose a high risk to aquatic habitats because of the potential for significant pesticide runoff after rainfall events. The survival of a plant in a heavy metal contaminated environment is determined by its sensitivity to metal toxicity (Prasad 1997). Every, organism regardless of whether it lives in a metal-enriched environment or not has certain ability to cope with non-essential or excessively available essential elements although there are limits in metal tolerance (Ernst *et al* 1992). Therefore plants have to adapt themselves to the prevailing conditions for their survival, resulting in acquisition of a wide range of metal-tolerance mechanisms. It may be prudent to investigate the responses of plants to mixtures of metals in model systems, 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 gives us a thorough understanding of the plasticity of metabolic pathways and their limits of functioning, which is absolutely necessary for genetic engineering approaches (Czarenecka *et al*, 1984, Misra and Gedamu 1989, Clemens *et al*, 1998, Rugh *et al* 1998, Zhu *et al* 1999,

Leustek *et al* 2000, Clemens 2001, Xiang *et al* 2001, Prasad and Strzałka 2002, Haraguchi 2004, Prasad 2004b) aimed at improving the cellular defense mechanism.

1.1. Essential and non-essential elements

The existence of a complex metal homeostasis mechanism in the plant system has come into focus recently (Clemens 2001). 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). The rates of accumulation are necessarily governed by physiological requirements rather than toxicity (Frausto *et al* 2001). Out of the many naturally occuring elements of the earth's crust N, P, K, Ca, Mg and S are macronutrients absolutely required for plant growth, 53 are heavy metals, but only some of them (Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, V, Zn) have a direct bearing on the living system and are classified as essential elements. These elements function as micronutrients for the plant system, which may become toxic at higher concentrations. The other category comprises the non-essential elements (As, Ag, Cd, Hg, Pb, Sb), which have no known metabolic function and are toxic to the plants (Breckle 1991, Siedlecka 1995). Essential elements are designed such that their removal from the living system causes reproducible pathological changes and for the most part are unalterable at persistent deficiency. The deficiency induced alterations are reversed on the restoration of the element immediately. Essential elements have wide diversity of functions in plants ranging from their involvement in various enzymes and other physiologically active molecules to chlorophyll, protein and nucleic acid biosynthesis, metabolism of carbohydrates and lipids, regulation of gene expression and stress tolerance. Micronutrients are also involved in the structural and functional integrity of membranes and other cellular components (Rengel 2004).

1.2. Cadmium - a toxic and non-essential element

Cadmium (Cd_{48}) is a group IIB transition element without any known metabolic significance to the living system. Hence the presence of excess Cd in the environment would constitute a serious threat. Cadmium pollution is increasing due to excessive mining, industrial usage and other anthropogenic activities (De 1992). The principal use of Cd in industries constitutes Ni-Cd batteries (62%), rest comprising pigment formulations (16%), surface coatings and plating (9%), stabilizers for synthetics and plastics (9%), nonferrous alloys (2%) and other uses including electrooptics (2%) (Ross 1994). Cadmium has applications wherever high stability and resistance to heat, cold and light are required (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, gets bioconcentrated (Devi *et al* 1996) and even affects human health (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, Waalkes 2000). Cadmium has an extremely long biological half-life (30 years) that makes it a cumulative toxin and to date there is no proven treatment for chronic cadmium intoxication (Goering *et al* 1994). Cadmium ions directly affect growth, leaf cell expansion, stomatal opening and water content (Pearson and Kirkham 1981, Barcelo and Poschenreider 1990) in plants. Cadmium toxicity induces lipid peroxidation through reactive oxygen species, destroys lipid bilayer and thereby alters membrane permeability and cellular ion homeostasis (Prasad 1995a, Landberg and Greger 2002). The free-radical reactions initiated by Cd directly affect the structure and function of macromolecules by oxidative reactions (Stadtman and Oliver 1991, Szuster-

Ciesielska 2000). Cadmium interferes with the functional activities of enzymes involved in various synthetic and metabolic activities of the plant system (Van Assche and Clijsters 1990, Reddy and Prasad 1992a). Cadmium ions directly affect the biosynthesis of photosynthetic pigments and the associated photosynthetic processes, leading to impaired carbon-utilization and respiratory processes (Krupa 1988, Atal *et al* 1991, Greger and Ogren 1991, Tukendorf 1993, Prasad 1995b, Šeršeň and Kráľová 2001, Sanita di Toppi *et al* 2003, Prasad 2004a) in the plant.

1.3. Zinc - an essential plant micronutrient

Zinc ($^{65}_{39}Zn_{30}$) is one of the most essential micronutrient for the plant system. The chemical and metabolic significance of Zn has been reviewed thoroughly (Williams 1984, Cousins 1985). Its nutritional essentiality has focussed attention on the pathology and clinical consequences of both its deficiency and toxicity (Prasad 1995, Vallee and Auld 1990). Zinc is the only essential element of the group IIB comprising Zn, Cd and Hg, the latter two being non-essential elements. 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, Cakmak and Braun 2001). It is an important component of many vital enzymes having catalytic, co-catalytic or structural role, structural stabilizer for proteins, membrane and DNA-binding proteins (Zn-fingers) (Prasad 1995, Vallee and Auld 1990), but toxic in high concentrations. Carbonic anhydrase (CA; carbonate hydrolyase, E.C. 4.2.1.1) is one such ubiquitous enzyme existing among living organisms, which catalyzes the reversible interconversion of CO_2 and HCO_3^- (Lamb 1977, Pocker and Sarkanen 1978). Zinc has a catalytic role in plant and animal CA being coordinated to the imidazole rings of three histidines close to

the active site (Pocker and Sarkanen 1978, Graham *et al* 1984, Tripp *et al* 2001). Carbonic anhydrase has been known to directly reflect the intracellular levels of Zn as it represents nearly 1-2% of the total soluble leaf protein thus representing a significant pool of Zn (Rengel 1995). Carbonic anhydrase activity being affected by Zn deficiency (Bar-Akiva and Lavon 1969, Randal and Bouma 1973, Gibson and Leece 1981, Rengel 1995) and its regulation by Zn (Shiraiwa and Kikuyama 1989, Lane and Morel 2000) in different systems have been reported. Zinc also plays a critical structural role in several motifs, the transcriptional regulatory proteins, including the Zn finger, Zn cluster, and RING finger domains (Klug and Rhodes 1987, Reddy and Radhika, 2001, Maret 2004). It has been estimated that as many as 2% of all yeast gene products contain Zn-binding domains (Fox and Guerinot 1998). Zinc 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. Compared with other micronutrients Zn exists in biological systems in high concentrations particularly in biomembranes (Chvapil 1973, Bettger and O'Dell 1981, Cakmak 2000). Most of the critical functions of Zn in the cells are its ability to form tetrahedral co-ordination bonds in different vital cellular constituents (Bray and Bettger 1990). Cysteine, histidine, aspartate and glutamate are the major cellular ligands of Zn that form tetrahedral co-ordinations (Vallee and Falchuk 1993). The chemical properties of Zn are favourable to various metabolic reactions, since under physiological conditions Zn has a unique property of existing in a univalent state without any redox cycling (Vallee 1959, Vallee 1988). Elemental Zn has two outer shell electrons, which it readily loses 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° does not occur as there is no biological reductant strong enough (i.e 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 further to Zn^{2+} state (Schutzendubel and Polle 2002). Hence Zn is stable in biological medium whose oxidoreductive potential is subjected to continous 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. Zinc is amphoteric, existing as both aquo and hydroxo metal complex at pH value near neutrality. It has a variable co-ordination sphere and stereochemical adaptibility to assume multiple co-ordination geometries, contributing to its biochemical versatility (Vallee 1988, Vallee and Falchuk 1993). It has been reported that Zn deficiency, both in animals and plants induces oxidative stress to all the cellular components and alters the antioxidant enzyme activity, disturbs cellular ion homeostasis and induces severe oxidative damage to macromolecules suggesting that Zn does play an important role as an antioxidant (Chvapil 1973, Girotti *et al* 1985, Zago and Oteiza 2001). Antioxidant has been defined as any substance, which a) prevents the transfer of electrons to and from molecular oxygen and organic molecules b) stabilizes organic free radicals, and/or c) terminates organic free radical reactions (Bray and Bettger 1990). From a historical perspective it is very well known that Zn has been used for the past nearly 100 years to galvanize iron or steel, thereby preventing oxidation of the material (Berg and Shi 1996).

1.4. Metal-metal interactions - cadmium *vs* zinc

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 (Burzyński 1987, Trivedi and Erdei 1992). One of the crucial factors of heavy metals influence on plant metabolism and physiological processes are their relationships with other mineral nutrients (Marschner 1995, Siedlecka 1995). 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 there have not been much studies 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) and therefore the effect of metal mixtures on model plant systems bear exploring. Plant responses to combinations of metals in the growth medium can be divided into three categories (Taylor 1989, Symeonidis and Karataglis 1992):

1. *Additive:* Relative growth under conditions of multiple metal stress is equal to the product of the relative growth produced by the individual metals in isolation. (eg: Cu-Co)
2. *Antagonistic:* Relative growth under conditions of multiple metal stress is greater than that of the product of the relative growth produced by the individual metals in isolation (eg: Cu-Cd, Ca-Cd).
3. *Synergistic:* Relative growth under conditions of multiple metal stress is less than that of the product of the relative growth produced by individual metals in isolation (eg: Cu-Zn).

Earlier studies have demonstrated heavy metal-induced imbalances in the ratio and uptake of nutrients in various plant systems like *Beta vulgaris* (Greger and Lindberg

1987), *Cucumis saliva* (Burzynski and Buczek 1989), *Halimione portulacoides* (Reboredo 1994), *Holcus lanatus* (Symeonidis and Karataglis 1992), *Koeleria splendens* (Ouzounidou 1995), *Lactuca sativa* (Thys *et al* 1991), *Lycopersicon esculentum* (Khan and Khan 1983), *Oryza sativa* (Tanaka and Navasero 1966), *Phaseolus vulgar is* (Siedlecka and Krupa 1996), *Pisum sativum* (Hernandez *et al* 1998), *Solarium melongena* (Khan and Khan 1983), *Triticum aestivum* (Trivedi and Erdei 1992), and *Zea mays* (Walker *et al* 1987, Agriffoul *et al* 1998, Lagriffoul *et al* 1998). Cadmium and Zn belong to group IIB transition elements with similar electronic configuration and valence state, both having affinity to sulphur, nitrogen and oxygen ligands (Nieboer and Richardson 1980). Hence both these elements have similar geochemical and environmental properties (Nan *et al* 2002). Most of the ores are mixtures of metals where potentially toxic metals (As, Cd and Hg) other than the sought-after-elements may also be present. Following extraction, which varies in efficiency but never complete, the contaminant metals are also released into the environment freely. Ore extraction of Zn from mines and non-ferrous metal production processes in smelters with subsequent release of zinc effluents to the environment is normally accompanied by cadmium environmental pollution (Dudka *et al* 1996, Pichtel *et al* 2000, Sterckmann *et al* 2000) because of zinc ores (ZnS) generally containing 0.1-5% and sometimes even higher cadmium (Adriano 2001). Similarly tyres containing ZnO and sewage sludges applied to agricultural soils as fertilizers also contain Cd (Sherlock 1986) as a major contaminant. Thus, this association of Cd and Zn in the environment, their chemical similarity, and hence the interactive functions are of considerable importance of study (Das *et al* 1997). Moreover the regulation and control of uptake of essential and non-essential elements are vital at the organismal and cellular level (Clemens *et al* 2002). Furthermore because control of this accumulation is imperfect, plants have to cope up with the exposure to unwanted elements through

different mechanisms striking the exact balance between essential and non-essential elements. Cadmium has been described as an antimetabolite of Zn by scientists due to the observed Zn deficiency in most of the Cd treated systems (Peraza *et al* 1998). 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 (Lagerwerff and Biersdorf 1972, Haghiri 1974, Chaney *et al* 1976, Coughtrey *et al* 1979, Sharma *et al* 1985, Taylor and Stadt 1990, Thys *et al* 1991, Smilde *et al* 1992, Symeonidis and Karataglis 1992, Mckenna *et al* 1993, Dudka *et al* 1994, Zhou *et al* 1994, Chaoui *et al* 1997), in soil-crop system under field conditions (Nan *et al*, 2002), but the study was limited only to the bioavailability and bioaccumulation of Zn and Cd by the tested plant systems. However investigations focussing on the adaptive physiological and biochemical mechanisms of interaction between Zn and Cd are rather scanty. Moreover with the reported conflicting results, a clear understanding of potential interaction between Zn and Cd has yet to appear.

1.5. Metal uptake

Plants are able to take up metals from air and water as well as soil and sediment media, depending on the growth environment (Prasad 2004a) as well as the different retention times of the metals in different media (Forstner 1979, Marschner, 1995). Many environmental factors are known to modify the availability of metals in water to the aquatic macrophytes such as speciation, pH, temperature, salinity, light intensity, oxygen level, redox potential (Eh), organic chelators, humic substances and particles, complexing agents, and presence of other metal ions (Prasad *et al* 2001). In aqueous system most metal concentrations increase with decreasing pH upto 4 (Vesely and Majer 1994) and at pH >7 metals are in insoluble and immobilized forms (Franklin

et al 2000). Similarly the redox potential (Eh) also plays an important role in determining the availability of metal ions for the aquatic plants. At low redox potential metals become bound to sulfides in sediments and thus are immobilized, but at higher Eh, the metal availability increases (Forstner 1979). The uptake of metals is not linear in correlation to concentration increase because of saturation inside the plant after high uptake (Vymazal 1986). Metals are first taken up into the apoplast passively and then further distributed between apoplast and cell walls, attracted by negatively charged groups in the cell wall acting as cation exchangers. Part of the metal taken into the apoplast is further transported into the cell through active transport across a concentration gradient maintained at the plasma membrane (Greger 2004). 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 identified (Lasat *et al* 2000). Transport of non-essential elements like Cd is most likely to occur via transporters for essential cations (Simkiss and Taylor 1995). Three classes of membrane transporters involved in metal transport have been identified: i) members of the cation diffusion facilitator *(CDF)* family, ii) heavy metal ATPases possessing Cys-Pro motifs (*CPx* type), and iii) natural resistance-associated macrophage protein (*Nramp*) family (William *et al* 2000). Vast information is available on two related subfamilies of transporter proteins that are involved in Zn(II) and Fe(II) uptake in all organisms (Guerinot and Eide 1999). *ZIP* (zinc-induced permease) gene family, a novel cation transporter family is found in diverse array of eukaryotic organisms, all of them having eight transmembrane domains (Fox and Guerinot 1998). The *ZIP* family, represented by *ZIP1, ZIP2, ZIP3* genes complement yeast transport mutants that show Zn(II) deficiency. In addition *ZIP1* and *ZIP3* are expressed in roots upon induction by Zn deficiency, indicating that these genes undoubtedly play a direct role in Zn uptake in *Arabidopsis thaliana* (Grotz *et al* 1998).

Similarly in *Saccharomyces cerevisiae* the high and low affinity Zn transporters coded by *ZRT1* and *ZRT2* are induced under conditions of Zn deficiency (Eide 1997). The Zn(II) transporting activity of these proteins are inhibited by Cd(II), Co(II) and Cu(II), indicating that *ZIP* proteins may transport potentially toxic metals as well as nutrients (Kochian 1993). Apart from *ZIP* family of transporters, the iron transporters *(IRT1)* are required for normal iron utilization (Eide *et al* 1996). It is known that *IRT* transcripts accumulate in roots in conditions of Fe deficiency, similar to *ZIP* transcripts for Zn deficiency. Fe (II) uptake was not greatly inhibited by high concentrations of other physiologically relevant metal ions such as Cu(I), Cu(II), Mn(II), and Zn(II). Most interestingly, Cd has been shown to inhibit iron uptake by *IRT1*, thereby facilitating transport of other heavy metal divalent cations such as Cd^{2+}, Ni^{2+} 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 is dependent on the H^+ gradient generated by H^+ ATPase located in the plasma membrane (Michelet and Boutry 1995, Morsomme and Boutry 2000). This enzyme belongs to the family of P-type ATPases (Moller *et al* 1996), whose members share among other features, a characteristic phosphorylation of a conserved aspartic acid residue at the catalytic site, which mechanistically couples ATP splitting to ion pumping across the membrane (Morsomme and Boutry 2000, Portillo 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). In plants the activity of the proton pump is regulated by a large number of environmental factors at both transcriptional and post-translational levels (Portillo 2000). Ionic imbalance has been suggested as one of the first events of heavy metal toxicity to plants suggesting that ion transport system may be regulated to shift the transport mechanism to non-essential elements, instead of

the element of interest (Souza-Santos *et al* 2001). 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 *et al* 1995), *Lactuca saliva* (Costa and Morel 1994), Cu in *Saccharomyces cerevisiae* (Fernandes *et al* 1998), Fe in *Zea mays* (Souza-Santos *et al* 2001) and *Nicotiana tabacum* (Vansuyt *et al* 2003), and Mg in *Neurospora Crassa* (Brooker and Slayman, 1983). *AtNramp* genes isolated from *Arabidopsis* encode metal transporters and showed homology to the *Nramp* gene family in bacteria, yeast, other plants and animals. Expression of *AtNramp* cDNAs increased Cd^{2+} accumulation and Fe uptake in yeast revealing heterogeneity in the functional properties of *Nramp* transporters (Thomine *et al* 2000). Plants have the ability to accumulate essential and non-essential elements, which could be utilized for engineering plants to remove toxic metal ions from contaminated ecosystems (Prasad 2004b).

1.6. Metal toxicity induced oxidative stress

One of the primary responses evoked by heavy metals in a biological system is the production of toxic reactive oxygen species (ROS) through various mechanisms involving electron transfer (Dietz *et al* 1999). "Oxidative stress" is a condition where the balance of formation of oxidants exceeds the ability of various antioxidant systems to remove the destructive oxyradicals leading to significant physiological impairment (Shaw *et al* 2004). A variety of macromolecules including proteins, lipids, polysaccharides and nucleic acids can be oxidatively modified, and the manifestations of this damage are multifarious, running the gamut from altered membrane fluidity and permeability attributable to lipid peroxidation, through loss of conformation and enzyme activity to genomic damage arising from scission of DNA (Thompson *et al* 1987, Davies 2003). The species of reactive oxygen capable of causing oxidative

damage include the superoxide anion ($O_2^{\cdot-}$), perhydroxyl radical ($HO_2^{\cdot}$)- the protonated form of superoxide, hydrogen peroxide (H_2O_2), hydroxyl radical (OH), alkoxyl radical (RO), peroxyl radical (ROO), organic hydroperoxide (ROOH), singlet oxygen ($^1O_2$) and excited carbonyl (RO*) (Thompson *et al* 1987, Fleschin *et al* 2000). These are called reactive oxygen species (ROS), which participate in chemical reactions than molecular oxygen because of the unpaired electrons (Halliwell and Gutteridge 1990, Bergendi *et al* 1999). Superoxide can either act as an oxidant where it can oxidize sulphur, ascorbate (AsA) or NADPH or as a reductant reducing cytochrome C and metal ions or it can be dismutated to H_2O_2 non-enzymatically proceeding through $HO_2^{\cdot-}$ or in an enzyme catalyzed reaction (Gebicki and Bielski 1981). H_2O_2 is not a free radical in a real sense because all of its electrons are paired, but is capable of initiating reduction and $OH^{\cdot}$ formation, thereby being classified as an intermediate reduction product of oxygen (Shaw *et al* 2004). The well known reactivity of H_2O_2 is not due to its reactivity per se, but requires the presence of a metal reductant specifically Fe to form the highly reactive $OH^{\cdot}$ radical, the reaction called Haber-Weiss Fenton reaction involving coupling of reduction of Fe (III) by $O_2^{\cdot-}$ and reoxidation to Fe (II) by H_2O_2 (Girotti 1985).

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH''$$

Copper has also been reported to catalyze this reaction (Wardman and Cadeias, 1996). The damage caused by $OH^{\cdot}$ would therefore be site-specific whereby iron catalyst attached to membrane lipids on $OH^{\cdot}$ radical formation would lead to the destructive lipid peroxidation process or in the case of DNA strand breakage and base modifications are likely due to $OH^{\cdot}$ produced with metal catalyst associated with DNA (Thompson *et al* 1987).

1.6.1. Oxidative damage to membranes

One of the most damaging effects of ROS and their products in cells is the peroxidation of membrane lipids. This process is initiated by abstraction of hydrogen atom from the methylene group (-CH2-) of the polyunsaturated fatty acids of the membrane lipid by $OH^{\cdot}$. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond and so makes H^{+} removal easier (Kappus 1985). Abstraction of hydrogen atom leaves behind a carbon centered radical (CH), which stabilizes by molecular rearrangement to form conjugated dienes, which then further reacts with oxygen molecule to form peroxy radical (ROO) (Logani and Davies 1980). This inturn abstracts another hydrogen atom from adjacent lipid molecule propagating the chain reaction further finally forming lipid hydroperoxides (ROOH). An alternative fate of $ROO^{\cdot}$ is to form cyclic peroxides, which finally get fragmented to aldehydes (malondialdehyde-MDA) and various other polymerization products (Fridovich 1986). ROOH can decompose to form alkoxyl (RO) and peroxyl ($RO_2^{\cdot}$) radical which in turn can further propagate lipid peroxidation by chain branching (Tadolini *et al* 1989)

Initiation:	RH	$\longrightarrow$	$R^{\cdot}$
Propagation:	$R^{\cdot} + O_2$	$\longrightarrow$	$ROO^{\cdot}$
	$ROO^{\cdot} + RH$	$\longrightarrow$	$ROOH + R^{\cdot}$
Termination:	$2R^{\cdot}$	$\longrightarrow$	R-R
	$R^{\cdot}$ + ROO	$\longrightarrow$	ROOR
	$2ROO^{\cdot}$	$\longrightarrow$	ROOR + O_2

Lipid peroxidation can also be induced enzymatically by phospholipases and lipoxygenases (LOX), the former by lipolysis releases unsaturated fatty acids, which subsequently acts as the substrate for LOX, a non-heme Fe (III) dioxygenase yielding

cis-trans conjugated dienes as the main product (Lacan and Baccou 1998, Brash 1999, Oliw 2002).

1.6.2. Oxidative damage to chloroplasts

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), *Phaseolus vulgaris* (Padmaja *et al* 1990), *Spinacea oleracea* (Seršeň and Kral'ova 2001), *Triticum aestivum* (Atal *et al* 1991, Loggini *et al* 1999), *Vigna radiata* (Keshan and Mukherji 1992), 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 (Mohanty and Mohanty 1988) leading to changes and disorganization (Stoyanova and Tchakalova 1999) of the grana stacks with dilated thylakoid membranes observable as plastoglobules (Baszynski *et al* 1980). The levels of phosphatidylcholine and phosphatidylglycerol associated with the inner membrane of chloroplasts for the efficient PS II activity are known to be decreased (Baszynski 1984, Maksymiec and Baszynski 1988, Krupa *et al* 1994), with a simultaneous increase in galactolipase activity and hence degradation of acyl lipids monogalactosyl diacylglycerol specifically (Skorzyńska and Baszynski 1993). This ultimately leads to the inactivation of oxygen-evolving centers and impaired electron transport (Sanita di Toppi *et al* 2003). Metal ions specifically inhibit chlorophyll biosynthesis through δ-aminolevulinic acid dehydratase (ALA dehydratase) (Mysliwa-Kurdziel and Strzałka 2002) and protochlorophyllide reductase (Baszynski *et al* 1980, Gadallah 1995, Ouzounidou 1995, Mysliwa-Kurdziel *et al* 2003) because of the oxidation prone -SH group (Prasad and Strzalka 1999) leading to the lower production of 5-aminolevulinic acid (ALA), the first common precursor for all the tetrapyrroles, thereby impairing chlorophyll biosynthesis.

1.6.3. Protein oxidation

Oxidative attack of ROS on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis (Davies 1987). Since the rate constants for reaction of $O_2^{\cdot-}$ with amino acid side chains are higher than those with most other cellular targets, proteins would be the major targets for ROS (Ho Kim *et al* 2001, Davies 2003). ROS modify proteins directly or indirectly reach targets in protein through "secondary toxic messengers" such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) generated from fatty acid degradation (Esterbauer *et al* 1991), which unlike free radicals are long-lived and can therefore attack targets quite distant from their site of production (Cabiscol *et al* 2000). Conversion of -SH groups to disulfides and other species (eg: oxyacids- glycine to glyoxylic acid, alanine to acetaldehyde, acetic acid) is one of the earliest observable events during the radical-mediated oxidation of proteins (Davies 1987, Dean *et a*\ 1997). 4-Hydroxynonenal is specifically known to react with the sulphydryl groups to form stable covalent thiolether adducts. Similarly methionine can get modified to methionine sulfoxide carrying a carbonyl function (Stadtmann 1990). Elucidation of the chemistry of protein oxidation by ROS has indicated the oxidation of aliphatic amino acids to hydroxylated derivatives by $OH^{\cdot}$ radical (histidine to oxo-histidine; proline to hydroxyproline, glutamic semialdehyde etc.) and aromatic residues to phenoxyl derivatives [tyrosine to dityrosine, chlorotyrosine, dihydroxyphenylalanine (DOPA)] in the absence of any reductants (thiols, vitamin E) to repair amino-acid derived radicals (Wright *et al* 2002, Winterbourn and Kettle 2003), ultimately leading to peptide bond cleavage (Shacter 2000a), cross-linking (Davies *et al* 1987a, Stadtman and Levine 2000) and increased susceptibility to proteolysis (Wolff *et al* 1986, Davies *et al* 1987b). There are many indications that radical damaged proteins are rapidly removed *in vivo* in efficient

systems due to enhanced susceptibility to proteolysis, but in some cases inhibition of proteolysis due to inactivation of proteolytic enzymes, leads to the accumulation of oxidized proteins within cells completely impairing cellular function (Wolff *et al* 1986, Davies *et al* 1987b, Cabiscol *et al* 2000). The oxidative degradation of a protein is further enhanced by site-specific metal (Fe, Cu) catalyzed oxidations, where the bound transition metal reacts with H_2O_2 in a fenton reaction to form a amino acid side chain bound hydroxyl radical which is highly destructive to the protein (Stadtman and Oliver 1991, Requena and Stadtman 1999). Extensive oxidation leads to unfolding of the protein and loss of native fluorescence as well as specific tertiary interactions of the aromatic amino acid residues (Anfinsen 1973, Davies and Delsignore 1987, Ali *et al* 1999, Shacter 2000a, b). Further a strong correlation has been demonstrated between increased hydrophobicity on the surface of protein and oxidatively modified proteins (Pacifici and Davies 1990, Chao *et al* 1997). Oxidatively modified enzymes can have either mild or severe effects on cellular and systemic metabolism, depending on the percentage of molecules that are modified and the chronicity of modification (Shacter 2000a).

1.6.4. Oxidative damage to DNA

Nucleic acids are highly susceptible to metal-catalyzed oxidations with both nucleobases and sugar moieties being targets of ROS (Imlay and Linn 1986, Jabs *et al* 1996). The mediation of metal toxicity on DNA damage may be of direct nature (Hossain and Huq 2002a, b) or indirect in nature (Gichner *et al* 2004). Oxygen free radicals induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effect (Breen and Murphy 1995). The primary effect is the oxidation of sugar moiety by the $OH^{\cdot}$ radical, in a metal (bound to DNA by chelation to phosphodiester linkage) catalyzed reaction 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). Iron and Copper are potent inducers of DNA damage especially in the presence of H_2O_2 with other metals like Ni, Cd and Co also reported to induce base modifications. Various products of oxidized bases have been recorded such as cytosine glycol, 5,6-dihydroxycytosine, 8-oxoguanine, 7,8-dihydro-8-oxoguanidine, 7,8-dihydro-8-oxoadenine, 5-hydroxymethyl uracil, thymine glycol etc.(Dizgaroglu and Bergtold 1986). Cross-linking of protein to DNA is another consequence of $OH^{\cdot}$ attack on either the DNA or protein generating covalent linkages such as thymine-cysteine adducts making the DNA-protein inseparable thereby being lethal to the system if replication or transcription precedes repair mechanism (Olenick *et al* 1986, Hartwig 2001, Valverde *et al* 2001, Hengstler *et al* 2003). Current evidences suggest that DNA repair systems are also important targets for metals, leading to diminished removal of endogenous and exogenous DNA damage, which is extremely degenerative to the system (Hartwig and Schwerdtle 2002, Fatur *et al* 2003).

1.7. Detoxification mechanisms for reactive oxygen species

Although aerobic metabolism is efficient in living systems, the presence of oxygen in the cellular environment poses a constant oxidative threat to cellular structures and processes. Detoxification reactions must therefore involve right balance between the formation and detoxification of active oxygen species. The defense strategies for counteracting the potentially hazardous reactions initiated by ROS ranges from prevention, through interception, to repair. Since $OH^{\cdot}$ radicals are far too reactive to be controlled easily, defense mechanisms are based on the elimination of its precursors (Of and H_20_2). Removal of ROS and cellular homeostasis are regulated by

antioxidant systems, which includes the enzymatic as well as non-enzymatic components (Larson 1988). The enzymatic antioxidants include enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD) including ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) and a complex antioxidant system- the ascorbate-glutathione cycle (AGC) (Zhang and Kirkham 1996) and the associated glutathione metabolism enzymes (Rennenberg and Brunold 1994, Nagalakshmi and Prasad 2001) comprising γ-glutamylcysteine synthetase (γ-GCS), glutathione-S-transferase (GST), and glutathione peroxidase (GSH-PX). The endogenous non-enzymatic antioxidants include carotenoids, α-tocopherol, flavonoids, phenolic acids, amino acids, polyamines, ascorbate (AsA) (Horemans *et al* 2000, Pallanca and Smirnoff 2000), thiols (-SH) and glutathione (GSH) (Foyer *et al* 2001), which are effectively free radical scavengers. Singlet oxygen is mainly quenched by carotenoids (Polykov *et al* 2001) and superoxide radical is dismutated to molecular oxygen and H_2O_2 by SOD (Scandalios 1993). Since H_2O_2 is potentially capable of reacting with $O_2^{\cdot-}$ to form OH', H_2O_2 detoxifying mechanisms become pivotal in the defense against active oxygen species (Cakmak *et al* 1993). Subsequently H_2O_2 is scavenged by CAT and POD enzymes -APX as well as GPX in cytosol and peroxisomes and, in chloroplasts via coupling of reduction of H_2O_2 to the oxidation of GSH by GSH-PX (Asada 1994). The products of oxidative damage initiated by OH' radicals like 4-hydroxyalkenals, 4-hydroxynonenals (membrane lipid peroxides) and base propenals (products of oxidative DNA degradation) are highly cytotoxic. Glutathione-S-transferases (GSTs) detoxify such endogenously produced electrophiles by conjugation with GSH (Marrs 1996). Glutathione is a very important antioxidant in the cellular milieu responsible for maintenance of the antioxidative machinery of the cells intact under stress (Rennenberg 1982, Noctor and Foyer 1998). The chemical reactivity of this thiol group, its relative stability and high solubility in water makes

GSH a particular adequate electron donor/acceptor in physiological reactions (Potters *et al* 2002). Glutathione functions as a stress indicator occurring in two distinct redox forms, promptly responding to oxidative stress (May *et al* 1998, Devi and Prasad 1998). Glutathione is synthesized from glutamate, cysteine and glycine in two ATP-dependent reactions catalyzed by γ-GCS in the first step of glutamate-cysteine coupling and glutathione synthetase (GS) in the second step of glycine addition (Hell and Bergmann 1990). Glutathione also detoxifies metal ions by scavenging them through the formation of phytochelatins and thereby facilitate their transport to vacuole (Cobbett 1999, Rauser 2000). Ascorbate (AsA) is another vital antioxidant, which is an excellent electron donor participating in various reactions of the plant system even involved in photoprotective xanthophylls cycle (Smirnoff 1996, Horemans *et al* 2000). Ascorbate also aid in the generation of the lipophilic chloroplastic antioxidant α-tocopherol (vitamin E) from the α-chromanoxyl radical (Asada 1994, Arrigoni and De Tullio 2000). Ascorbate-glutathione cycle, a major H_2O_2 scavenging pathway operates both in chloroplast as well as the cytosol (Zhang and Kirkham 1996). In this cycle H_2O_2 is reduced to water by ascorbate peroxidase (APX) hence producing ascorbyl radical (monodehydroascorbate) and oxidized form of ascorbate (dehydroascorbate) (Hausladen and Kunert 1990). The regeneration of ascorbate from monodehydroascorbate and dehydroascorbate is catalyzed by NAD(P)H-dependent monodehydroascorbate reductase (MDHAR) (Hossain *et al* 1984) and GSH-dependent dehydroascorbate reductase (DHAR) (De tullio *et al* 1998), coupled with glutathione reductase (GR) (Smith *et al* 1989). Operation of the AGC not only maintains the reduced active forms of GSH and AsA in cells on a suitable level, thereby adjusting the cellular redox potential but also participates in ROS detoxification (Kingston-Smith and Foyer 2000, Ma and Cheng 2003).

A number of organic acids, amino acids and some members of mugineic acids occur in plant tissues and are possible ligands for metal complexation thereby conferring metal tolerance (Lobinski and Potin-Gautier 1998, Hall 2002). Amino acids are present in living systems upto concentrations of several millimoles per liter and therefore are major candidates for metal ion binding ligands stabilizing various macromolecules structurally and aid in vital cellular functions (Pohlmeier 2004). The sulphur atoms of cysteine are responsible for the major covalent cross-links in protein structures, where the disulfide bridge formed between two cysteine molecules is important in stabilizing protein conformation (Komarnisky *et al* 2003). Protective thiol group containing amino acids like methionine and cysteine are reported to prevent methylmercury toxicity by chelation (Peraza *et al* 1998). Similar reports exist for the Ni-histidine complex (Kramer *et al* 1996, Kerkeb and Kramer 2003) in the xylem sap of *Alyssum lesbiacum* and ***Brassica juncea***. Amino acids cysteine and glutamate are the basic components of GSH (Noctor and Foyer 1998), a crucial antioxidant of the plant cell as well as phytochelatins-PCs (Poly(γ-glutamyl-cysteinyl)-glycines), metal binding peptides which chelate free metal ions and transport them to vacuole (Rauser 2000). Therefore amino acids indirectly modulate detoxification of xenobiotic compounds and scavenging of free radicals and hence oxidative stress (Bray and Taylor 1993). Phytochelatins mainly consist of glutamic acid, cysteine and glycine in molar ratios of 2:2:1 to 11:11:1 and are also referred to as class III metallothioneins (Reddy and Prasad 1992b, Rauser 1995). Extensive studies on glutathione synthesis in plants have indicated that GSH synthesis is regulated by cysteine and glutamic acid availability, feedback inhibition by γ-GCS, transcriptional control of γ-GCS and translational regulation of γ-GCS by the ratio of reduced to oxidized glutathione (Xiang and Oliver 1998, Foyer *et al* 2001). Low molecular weight organic acids especially citric, oxalic and malic acids are capable of forming complexes with metals which can affect their fixation, mobility and availability

to plants. Metal-organic acids interactions in the soil-plant system are found to be important for solubilizing metals from highly insoluble phases (Cieslinski *et al* 1998, Jones 1998, Wu *et al* 2003). The toxicity of aluminium to plants is known to be handled efficiently by Al-citrate, Al-aconitate, Al-malate and Al-oxalate complexes (Ma 2000, Jonnarth *et al* 2000). Similar reports exist for Ni-citrate in *Sebertia acuminata* latex (Sagner *et al* 1998). Phytoremediation of Cd contaminated soils with organic acid amendments (Elkhatib *et al* 2001) also indicate the potential role of organic acids in detoxification of toxic metal ions.

1.8. Aquatic macrophytes for metal toxicity bioassays

Aquatic plants are represented by a variety of macrophytic species that occur in various types of habitats. Aquatic macrophytes are extremely important components of an aquatic ecosystem vital for primary productivity, nutrient cycling, sediment stabilization, habitat, food and refuge for a variety of organisms (Chilton 1990). Aquatic macrophytes are represented by various class of members ranging from submerged free floating plants (*Ceratophyllum, Hydrilla),* submerged but rooted to the sediment forms *(Potamogeton, Najas, Ruppia* etc..), surface living forms but attached to the sediments (*Nymphaea, Nelumbo),* emergent plants with submerged roots (*Ranunculus, Typha, Carex* etc.) to free floating forms on the surface of water (*Lemna, Eichhornia).* Aquatic plants are known to accumulate heavy metals producing an internal concentration several fold greater than their surroundings (Brix and Schierup 1989, Prasad *et al* 2001). The submerged plant thickets in polluted lakes are reported to accumulate trace metals to the tune of 10^3- 10^4 (Gersberg *et al* 1986) and reduce the water velocity thereby accelerating sedimentation of suspended fine paniculate trace metals, which otherwise are toxic to the biota when present in interstitial waters in available form (St-Cyr and Campbell 1994, St-Cyr *et al* 1994). The trend for greater dependence upon roots for

heavy metal uptake is in rooted floating leaved taxa with lesser dependence in submerged taxa (Denny 1980). The tendency to use shoots as sites of heavy metal uptake instead of roots increases with progression towards submergence and simplicity of root structure. Submerged rooted plants have some potential for the extraction of metals from water as well as sediments, while rootless plants extract metals rapidly only from water (Cowgill 1974). Extensive experimentation on various macrophytes like *Eichhornia* (Farago and Parsons 1983, Klump *et al* 2002, Sipauba-Tavares *et al* 2002, Soltan and Rasheed 2003), *Elodea* (Welsh and Danny 1980, Van der werff and Pruyt 1982, Greger and Kautsky 1991, Ribeyre and Boudou 1994, Mal *et al* 2002), *Lemna* (Staves and Knaus 1985, Wang 1986a, 1988, Jain *et al* 1989, Lytle *et al* 1995, Mallick *et al* 1996, Mohan and Hosetti 1997, 1999, Prasad *et al* 2001, Dirilgeren and Dogan 2002, Axtell *et al* 2003), *Myriophyllum* (Welsch and Danny 1980, Greger and Kautsky 1991, Cardwell *et al* 2002, Keskinkan *et al* 2003, Kamal *et al* 2004), *Potamogeton* (Welsh and Danny 1980, Jones *et al* 1986, Greger and Kautsky 1991, Lytle *et al* 1995, 1996, Cardwell *et al* 2002, Wolterbeck and Van der meer 2002, Wu and Guo 2002, Rai *et al* 2003, Tripathi *et al* 2003), and various other aquatic macrophytes exist indicating the potential utilization of macrophytes as biomonitor systems for toxic metals.

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

Ceratophyllum demersum L. belonging to the order Nymphaeales and family Ceratophyllaceae (the family of hornworts), grows in shallow, muddy, quiescent water bodies at low light intensities. Its forked, whorled and toothed leaves are usually crowded towards the branch tips giving the common name of 'coontail' as it is usually described. Vast literature exists on the metal uptake potential of various macrophytes as mentioned above. The earlier laboratory studies on *C. demersum* as a toxicity bioassay material (Raey 1972, Suckcharoen 1979, Garg and Chandra, 1990, Ornes and Sajwan

1993, Tripathi *et al* 1995, Rai *et al* 1995, Gupta and Chandra 1996, Szymanowska *et al* 1999, Kumar and Prasad 2004a, b, c) was limited only to the bioaccumulation capacity of various metals like Cd, Cr, Cu, Fe, Pb, and Hg by *C. demersum.* The physiological response of *C. demersum* to metal toxicity has not been discussed in detail. The only report existing on similar lines is in response of *C. demersum* to Cu stress (Devi and Prasad, 1998). The effects of non-metals like S (Wium Anderson *et al* 1983), N (Best 1980) and even abiotic factors such as light (Fair and Meek 1983), allelopathic effects of other macrophytes (Kulshreshtha and Gopal, 1983, Hofstra 1999, Nakai *et al* 1999) on *C. demersum* have been analyzed earlier. In addition growth rate and uptake kinetics has been analyzed in *C. demersum* under trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-l,3,5-triazine (RDX) treatments (Best *et al* 1997, 1999). Even the present studies are either concentrated on sediment resuspension (Horpilla and Nurminen 2003) and N loading effects (Tracy *et al* 2003) on *C. demersum* or metal adsorption capacity (Keskinkan *et al* 2004, Kumar and Prasad, 2004c), effect of UV-B radiation (Rozema *et al* 2002), capacity of C. *demersum* in the degradation of atrazine (Rupassara *et al* 2002), accumulation of P (Dierberg *et al* 2002), radionuclides (Bolsunovskii *et al* 2002) or the potential use of *C. demersum* as an oxygenator in aquarium and closed equilibrated biological aquatic system (Blum *et al* 2003, Kitaya *et al* 2003, Voeste *et al* 2003) in space flight experiments. As this plant has recently been recommended for use in remediation of toxic metals (Rai *et al* 1995), metabolic studies are worthwhile on C. *demersum* using sensitive metabolic parameters (with significant stimulatory or inhibitory effects) that may be used as biomarkers to heavy metal stress, and in elucidating the plant response to multiple metal combinations. The suitability of a test species is usually based on the specimen bioavailability, sensitivity to toxicant, reported data and the like (Mohan and Hosetti 1999). Moreover, *C. demersum* has unique features, thus ideal for laboratory toxicity bioassays. This macrophyte has universal

distribution and rapid reproductive rate, an important pre-requisite for choosing any macrophyte as the study material. *C. demersum* is a floating rootless form reducing the complication of the study system as well as root-shoot metal partitioning and is cost-effective in maintenance under laboratory conditions. Moreover the forked leaves of the plant provide large surface area for absorption and thin cuticle on the plant surface facilitates uptake of metals from water through the entire surface (Ornes and Sajwan 1993). Hence the integrated amounts of bioavailable metals in aqueous system are reflected directly in the plant, thereby directly reflecting the toxicity of metals in aqueous system. The response of an organism to deficient or excess levels of metal (i.e. bioassays) can be used to estimate metal impact. Such studies done under defined experimental conditions can provide results that can be extrapolated to natural environment.

1.10. Objectives of the study

1. To elucidate the mechanism involved in the competitive inhibition of Cd uptake by Zn supplementation.
2. To identify the potential role of Zn as an antioxidant of Cd-mediated reactive oxygen species, oxidative stress and responses of antioxidant enzymes.
3. To analyze the modulatory effect of supplementing Zn on the redox pool, cellular antioxidants of the plant system in conjunction with ascorbate-glutathione cycle and glutathione metabolic enzymes, powerful ROS detoxifying pathways.
4. To identify competitive displacement/substitution reactions between Cd and Zn in the active site of carbonic anhydrase (CA), an enzyme requiring Zn for its catalytic functioning, for the interconversion of CO_2 and HCO_3^-, vital for photosynthesis in submerged aquatic plants. Subsequently analyze the structural,

conformational stability and changes reflected under Cd toxicity and Zn supplemented Cd treatments.

5. To understand the influence of Zn supplements on Cd-induced structural damage to chloroplasts and functional impairment to the photosynthetic processes and electron transport chain.
6. To examine the effect of Zn supplements on Cd-induced oxidative damage to the structural integrity of DNA.
7. To investigate the responses of *C. demersum* to Cd treatments supplemented with amino acids (cysteine, glutamic acid and glycine) and organic acids (citric, oxalic and malic acids) individually and in different combinations and also with Zn supplements. Identify the specific detoxification mechanism adopted by amino acids and organic acids.

2. Materials and methods

Chemicals

All the chemicals used were of analytical grade. ATP, Sodium orthovanadate, Cytochrome C, Epinephrine, DMPO, TBAP, Riboflavin, *O*-Dianisidine, DTNB, Ascorbate oxidase, PVPP, Dehydroascorbate, MTT, ANS, L-amino butyrate, Glutathione reductase, DEAE Sephadex A 50, Sephadex G 25, Tricine, DCMU, CTAB, Phenol, RNase, Benzidine, CDNB, Cysteine, Glutamic acid and Glycine were purchased from Sigma chemical company, USA. Phenylmethylsulphonylfluoride was purchased from Boehringer Manheim, Germany. Fast Blue BB salt was purchased from Fluka, Switzerland. EB was purchased from Amresco, USA. Silver nitrate, H_2O_2 were purchased from E. Merck, India. Cadmium chloride, Zinc chloride, PVP, Linoleic acid, Titanyl sulphate, Liquor ammonia, NADPH, 2,4-Dinitrophenyl hydrazine, Guanidine-HC1, NBT, DCPIP, GSH, GSSG, Methionine, Triton-X-100, Ascorbic acid, *O*-Phthalaldehyde, *N*- Ethylmaleimide, ANSA, *P*-Nitrophenyl acetate, Diethylmalonate, Cd and Zn atomic absorption standards, Acrylamide, *N-N'* Methylene bis acrylamide, Ammonium persulphate, Glycine, Citric acid, Oxalic acid and Malic acids were purchased from Sisco Research laboratories, Bombay, India. Tris-Buffer, Guaiacol, DMSO, TEMED, Potassium ferricyanide from Qualigens, India. Rest of the chemicals of analytical grade were purchased from various other local establishments.

2.1. Plant material and growth conditions

Ceratophyllum demersum L. was collected from local ponds and maintained in multiple sets of aquaria in the following conditions. The plants were supplied with 0.1% Hoaglands nutrient solution (Hoagland and Arnon 1950) containing 1 mM $NH_4H_2PO_4$, 10 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, as macronutrients, 46 µM H_3BO_3, 9 uM $MnCl_2$. 4 H_2O, 0.76 uM $ZnSO_4$. 7 H_2O, 0.32 uM $CuSO_4$, 0.55 uM H_2MoO_4, as micronutrients and 78 uM Fe-EDTA as the iron source at room temperature and solution pH 6.0, under the natural day and night cycle in shade conditions (without the influence of direct sunlight) as found exactly in natural environment. The plants were not provided with any supplementary light source as it would be inhibitory for their growth. After the plants were acclimatized to laboratory conditions for more than one month they were used for further experiments.

2.2. Cadmium and zinc treatments

Plant material (2 g) was transferred to a 250 ml glass beaker with 200 ml of 0.1% Hoaglands solution (containing 0.764 nmoles Zn) and the chosen concentration of the metals. Cd treatment of 10 uM concentration was given to the plants using $CdCl_2$. Zn supplementation (10, 50, 100 and 200 uM) was done using $ZnCl_2$ to the Cd (10 uM) treated plants. Plants were also treated with only Zn (10, 50, 100 and 200 uM) for comparison. The same concentrations were also subjected to treatment with 10 uM 2,4-Dinitrophenol (DNP), an uncoupler of proton pumps and 100 µM N-N' Dicyclohexylcarbodimide (DCCD), an exclusive inhibitor of H^+ATPase (Tripathi *et al* 1995). The treatments were maintained for one week.

2.2.1. Quantification of cadmium and zinc content

Metal-treated plants (without DNP, DCCD and treatments with DNP, DCCD) were washed thoroughly with 10 mM EDTA to remove metals adsorbed to the surface. They were oven-dried at 80 °C for two days and acid-digested with 3:1 $HNO_3/HClO_4$. The digested material was dissolved in minimal volume of H_2O_2 and made up in distilled water. This solution was analyzed for Cd and Zn content using atomic absorption spectrometer (GBC 932 plus, Australia) (Greger and Lindberg 1987).

2.2.2. H^+ ATPas(E.C. 3.6.3.6) activity

The treated plant material (without DNP, DCCD and treatments with DNP, DCCD) was ground in 30 mM HEPES-Tris buffer (pH 6.0), 0.1 mM phenylmethylsulfonylfluoride (PMSF), 2% (w/v) polyvinylpolypyrrolidone (PVPP) and centrifuged at 13,000 x *g* for 20 min at 4 °C. The supernatant was used to measure the activity of the enzyme. The protein content in the supernatant was determined according to Lowry *et al* (1951).

H^+ ATPase was assayed according to Souza-Santos *et al* (2001). The assay mixture for H^+ ATPase consisted of 30 mM HEPES-Tris (pH 6.0), 50 mM KNO_3, 10 mM $MgCl_2$, 2 mM NaN_3, in the presence and in the absence of Na_3VO_4, an inhibitor of ATPase. The reaction was initiated by addition of 100 ug protein and 2 mM ATP. After 30 min incubation at 37° C, the reaction was quenched by the addition of TCA to a final concentration of 10% (w/v). The rate of ATP hydrolysis was determined by measuring the release of Pi colorimetrically according to the method of Fiske and Subbarow, (1925) utilizing 25 mg of a reaction mixture (1.28g sodium bisulfite, 1.28 g sodium sulfite, and 0.2g ANSA) and 2.5% ammonium molybdate. The phosphomolybdate so formed was estimated against phosphate standards at 660 run. The difference of vanadate-uninhibited and vanadate-sensitive ATP hydrolysis activity can be attributed

to H^+ATPase activity. The assay was performed in the same way for metal treatments with added uncoupler DNP and treatments with ATPase inhibitor DCCD.

2.3. Membrane damage

2.3.1. Lipid peroxidation

Plant material (300 mg) was homogenized with 3 ml of 0.5% *(w/v)* TBA in 20 % *(w/v)* TCA. The homogenate was incubated at 95 °C for 30 min and the reaction was stopped in ice. The samples were centrifuged at 10,000 x *g* for 10 min and the absorbance of the resulting supernatant was recorded at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance. The absorbance co-efficient of malondialdehyde (MDA)-155 mM^{-1} cm^{-1} was used in the calculation according to Heath and Packer (1968).

2.3.2. Lipoxygenase activity (LOX, E.C. 1.13.11.12)

The plant material (300 mg) was homogenized in 50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, 0.1 mM PMSF, 2% (w/v) polyvinylpyrrolidone (PVP), 1% (v/v) glycerol and 0.1% (v/v) tween 20. The extract was centrifuged at 15,000 x *g* for 20 min and the supernatant was immediately used for the assay of lipoxygenase activity according to Ederli *et al* (1997). Lipoxygenase activity was measured spectroscopically at room temperature by addition of 1 mM linoleic acid in 0.1 M sodium acetate buffer (pH 5.6) to the extract and measuring the increase of absorbance at 234 nm. The extinction coefficient of (25 $mM\ L^{-1})^{-1}$ cm^{-1} was used to convert absorbance values to μmoles of conjugated diene. One unit of activity was defined as the amount of enzyme catalyzing the synthesis of 1 μmol of hydroperoxide (HPOD) min^{-1}.

2.3.3. Ion leakage: electrical conductivity and flame photometry

The treated plant material (750 mg) was taken and washed with double distilled water. They were then transferred to 100 ml of deionized water and left for 24 h so as to facilitate the maximum ion leakage. The electrical conductivity of the water as a measure of ion leakage was measured after 24 h using a conductivity meter (Digisun, DI 909). The same samples were estimated for efflux of K^+, Na^+, and Ca^{2+} ions by flame photometer (128, Systronics, India) using respective standards (Cakmak and Marschner, 1988a).

2.4. Measurement of free radicals

2.4.1. NADPH dependent superoxide (Of) generation

The treated plant material (1g) was homogenized in 50 mM HEPES-Tris buffer (pH 7.5), 40 mM PMSF, 2% (w/v) PVPP and centrifuged at 13,000 x *g* for 20 min at 4° C to obtain the supernatant. Superoxide generation was assayed by measuring the rate of SOD-inhibitory cytochrome C reduction in the presence of NADPH (Cakmak and Marschner 1988b). The measurements were carried out immediately after the preparation of the extract. Reaction mixtures in the sample cuvette contained 50 mM HEPES-KOH (pH 7.8), 0.1 mM EDTA, 1 uM $K_4Fe(CN)_6$, 75 uM Cytochrome C, 100 ug protein and 50 uM NADPH in a total volume of 2 ml. The reference cuvette was identical to the sample cuvette except for the addition of 25 uM superoxide dismutase. After 1 min pre-incubation, the reaction was started by the addition of NADPH to both the cuvettes and the absorbance changes at 550 nm were followed for 1 min. Rates of $O_2^{\cdot-}$ generation were calculated using an extinction co-efficient of 21.0 mol^{-1} m^{-3} cm^{-1}. Under similar conditions NADPH oxidation was measured at 340 nm except that

cytochrome C was omitted from the reaction mixture. Rates of NADPH oxidation were calculated using extinction co-efficient of 6.2 mol^{-1} m^{-3} cm^{-1}.

2.4.2. Cellular levels of superoxide(O_2 ') radicals

The levels of superoxide radical were determined by the rate of epinephrine to adrenochrome with 1 mM NADPH as substrate. The absorbance difference (A_{485}-A575, e= 2.96 mM^{-1} cm^{-1}) was recorded at room temperature. The 3 ml reaction mixture contained 1 mM epinephrine and 100 µg of protein in 50 mM potassium phosphate buffer (pH 7.0) (Chen and Li 2001)

2.4.3. Measurement of hydrogen peroxide (H_2O_2)

Hydrogen peroxide was extracted and estimated according to the method of Mukherjee and Choudhari (1983). Isolation was made from 2g of the treated plant material in ice-cold 100% acetone. By addition of 5% (*w/v*) titanyl sulphate and conc. NH_4OH solution, the peroxide-titanium complex was precipitated. The precipitate was dissolved in 15 ml of 2M H_2SO_4, making the final volume to 20 ml in cold water. The absorbance of the resultant was read at 415 nm. The H_2O_2 content was calculated from a standard curve prepared in similar way.

2.4.4. Electron spin resonance spectra of hydroxyl(OH) radicals

ESR spectroscopy was carried out using a JOEL JES-FA series X-band spectrometer, where the sample was analyzed in a flat cell in the spectrometer cavity. Since commercial DMPO (spin trap) has varying amounts of paramagnetic impurities, it was purified through activated charcoal (1:3 diluted DMPO: 0.5 gms charcoal) atleast three times prior to use as suggested by Mason and Degray (1994) and pure form stored

under dark conditions in -20° C. The samples were homogenized in 50 mM potassium phosphate buffer (pH 7.5), containing 10 mM TBAP, which stabilizes superoxide anions for reaction (Van Doorsler *et al* 1999). The homogenate was centrifuged at 10, 000 x *g* for 10 min and the supernatant was immediately used for spectral recording. The standard mixture for the spectra consists of 100 mM DMPO, 1 mM NADPH, and phosphate buffer (pH 7.5) in a total volume of 300 µl (Li *et al* 2000). All the spectra were recorded at room temperature under the following conditions: microwave power- 1 mW, modulation frequency- 100 KHz, modulation amplitude 0.1 mT, scanning field: 335 ± 6 mT, sweep time- 3 min, time constant- 0.03 S. All spectra were measured at a frequency of 9.50 GHz (X-Band frequency).

2.4.5. Measurement of hydroxyl (OH) radical levels

Hydroxyl radical content was estimated according to Babbs and Gale (1987). The treated plant material (2 g) was homogenized in distilled water and 0.5 ml of 10 N H_2SO_4 was added to 2 ml of the sample with vortexing. The tubes were centrifuged to remove precipitated protein, the supernatant extracted twice with 2 ml of toluene-butanol (3:1) to remove interfering detergent-like substances and the organic layers were discarded. Butanol (4 ml), previously saturated with 1M sulfuric acid was added to the aqueous phase and vortexed thoroughly for 60 sec. Two ml of 0.5 M sodium acetate (pH 5) was added to the upper butanol phase, mixed vigorously, centrifuged at 1000 x *g* for 3 min and the colour reaction was run on methanesulfinic acid (MSA) extracted into the aqueous phase. The pH of the aqueous sample was adjusted to 2.5 by the addition of 0.3 ml of 1 N HC1 and 0.1 ml of 30 mM fast blue BB salt solution (prepared freshly and kept in dark) was added and incubated for 10 min in dark. Toluene-butanol (3:1) of volume 1.5 ml was added, vortexed for 2 min and the phases were separated by

centrifugation at 1000 x *g* for 3 min. The lower phase containing the unreacted diazonium salt was discarded and the toluene-butanol phase was washed with 2 ml of butanol-saturated water for 30 sec to remove remaining reagent. The tubes were centrifuged at 1000 x *g* for 3 min and the upper phase containing the diazosulfones was transferred to a cuvette. With 0.1 ml of pyridine-glacial acetic acid (95:5) added to each sample to stabilize the colour, the absorbance was recorded at 340-520 nm and the MSA content was calculated from the absorption maximum at 425 nm with reference to a standard curve.

2.4.6. Total protein content

One gram of the treated plant material was ground in 50 mM Tris-HCl (pH 7.5) under ice-cold conditions. Along with this 0.1 mM PMSF and 2% *(w/v)* PVPP were used as the protease and phenolics inhibitors respectively. The extract was centrifuged at 13,000 x *g* for 20 min and the supernatant was used for protein estimation according to Lowry *et al* (1951) with bovine serum albumin as standard. The total protein profile was analyzed in a 12.5% sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-Page) (Laemmli 1970)

2.4.7. Carbonyl content as a measure of protein oxidation

The protein extract from the previous step was directly used for this estimation. Protein of equal amounts was precipitated with 20% TCA. The tubes were centrifuged at 12,000 x *g* for 10 min and the pellet obtained was allowed to react with 500 µl of 10 mM 2,4-dinitrophenylhydrazine in 2 M HC1 and incubated at room temperature for 1 h with regular vortexing every 10-15 min. The samples were again precipitated with 500 µl of 20% TCA, centrifuged at 12, 000 x *g* for 5 min. The

resultant pellet was washed with ethanol: ethyl acetate (1:1) to remove excess reagent, allowing the samples to stand for 10 min before each centrifugation and supernatant was discarded. The precipitated protein was redissolved in 0.6 ml of 6 M guanidine-HCl. The resultant was read at 360-390 nm (e= 22, 000 mM^{-1} cm^{-1}) against a blank treated with 2 M HC1 instead of 2,4-dinitrophenylhydrazine (Reznick and Packer 1994).

2.5. **The antioxidant enzymes**

The treated plant material (1g) was ground in 50 mM sodium phosphate buffer (pH 7.8) for SOD and (pH 7.0) for CAT, POD, APX, and GPX respectively with 2% PVPP and centrifuged at 13,000 x *g* for 20 min at 4 °C. The supernatant was used to measure the activity of the enzymes. The protein content in the supernatant was determined according to Lowry *et al* (1951).

2.5.1. Superoxide dismutase (SOD, E.C. 1.15.1.1)

Superoxide dismutase activity was determined by the method of Beauchamp and Fridovich (1971). The required cocktail for SOD activity estimation was prepared by mixing 27 ml of sodium phosphate buffer (pH 7.8), 1.5 ml of methionine (300 mg ml^{-1}), 1 ml of NBT (14.4 mg 10 ml^{-1}), 0.75 ml of triton-X-100 and 1.5 ml of 2 mM EDTA. To 1 ml of this cocktail, 10 µl of riboflavin (4.4mg 100ml^{-1}) and 50 µg of protein were added. After mixing, the contents taken in a cuvette were illuminated for 8 min using three comptalux bulbs (100W, Philips India Ltd.). The temperature was maintained at 25 °C using a water bath. A tube with protein kept in dark served as blank, while the control tube was without enzyme and kept in light. The absorbance was measured at 560 nm. The reduction of NBT under illumination was measured without enzyme and also in the presence of the enzyme. Activity of SOD is the measure of NBT reduction in light without protein minus NBT reduction with protein. One unit of

activity is the amount of protein required to inhibit 50% initial reduction of NBT under light. The amount of each type of SOD was determined by its sensitivity to cyanide and H_2O_2. Cu/Zn-SOD is sensitive to cyanide, Fe SOD is sensitive to H_2O_2 and Mn-SOD insensitive to both.

Isozymes of SOD were visualized according to Beauchamp and Fridovich (1971) as modified by Rucinska *et al* (1999) in a 10% native gel under non-denaturing conditions at 4 °C, the principle involved being the inhibition of NBT reduction by superoxide ion radicals generated photochemically. After electrophoresis gels were soaked in 2.45 mM NBT for 20 min, followed by incubation in a solution containing 36 mM potassium phosphate buffer (pH 7.8), 28 mM TEMED, 24 µM riboflavin and subsequently illuminated until the SOD bands appear on a dark background.

2.5.2. Catalase (CAT, E.C. 1.11.1.6)

Catalase activity was measured according to the method of Chance and Meahly (1955). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 19 mM H_2O_2 and 100 µg of protein in a final volume of 3ml. The activity was determined by the oxidation of H_2O_2 at 240 run. The change of absorbance of 1 O.D. represents the oxidation of 25 µmoles of H_2O_2

Catalase isozymes were visualized in a 7.5% native gel according to Woodbury and Spencer (1971). After electrophoresis the gel was washed in three changes of water for 45 min to remove the buffer from the gel's outer surface. The gel was then incubated in 20 mM H_2O_2 and kept in dark for 10 min. The gel was then briefly washed and placed in a solution containing 1% ferric chloride and 1% potassium ferricyanide solution until catalase bands became visible.

2.5.3. Total peroxidase (POD, E.C. 1.11.1.7)

Peroxidase was analyzed by the method of Reddy and Prasad (1992a) spectrophotometrically in a 3 ml reaction mixture containing 75 ug of protein, 2.4 uM *O*-dianisidine and 20 mM H_2O_2 at 430 nm. One unit of peroxidase activity represents a change in absorbance of 0.001 at 430 nm/ 3 min.

Peroxidase isozymes were separated on 7.5 % native polyacrylamide gels run at non-denaturing conditions and stained for peroxidase activity according to Rao *et al* (1995). The gels were stained with 2 mM benzidine in DMSO, 50 mM sodium acetate (pH 4.5) with reaction initiated by 10 mM H_2O_2 and allowed to proceed until POD bands become completely visible.

2.5.4. Ascorbate peroxidase (APX, E.C. 1.11.1.11)

Ascorbate peroxidase was assayed by the method of Nakano and Asada (1981). The reaction mixture for measuring APX activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50 µg of protein. The activity was recorded as decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized was calculated from the extinction coefficient- 2.6 mM^{-1} cm^{-1}.

In-gel APX activity staining was performed according to Mittler and Zilinskas (1993) and Lee and Lee, (2000). Non-denaturing electrophoresis was performed in a buffer containing 2 mM ascorbate. Subsequent to electrophoresis the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM ascorbate for a total of 30 min with the equilibration buffer changed every 10 min. The gel was then incubated with 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 20 mM H_2O_2 for 20 min. H_2O_2 was added to the solution just prior to the incubation

of the gel. The gel was subsequently washed with sodium phosphate buffer (pH 7.8), 28 mM TEMED and 24 mM NBT with gentle agitation for approximately 10 min and stopped by brief wash with distilled water.

2.5.5. Guaiacol peroxidase (GPX, E.C. 1.11.1.7)

Guaiacol peroxidase was measured by the method of Mazhoudi *et al* (1997). The reaction mixture contained 50 mM phosphate buffer, 0.2 mM guaiacol, 10 mM H_2O_2 and distilled water in a total volume of 3 ml. The reaction was started by adding 50 µg of protein. The change in absorbance of one unit per min at 470 nm (extinction coefficient of 26.6 $mM^{-1}cm^{-1}$) gave the activity of guaiacol peroxidase.

Native gels for GPX activity staining were run under non-denaturing conditions according to Mazhoudi *et al* (1997). After pre-electrophoresis for 15 min the extracts were loaded onto the gel and separated at room temperature. Gels were stained for GPX activity by incubation in a freshly prepared solution of 18 mM guaiacol, 25 mM H_2O_2 in 25 mM potassium phosphate buffer (pH 7.0) until the peroxidase activity bands were completely visualized. Gels were preincubated in the phosphate buffer for 15 min to lower the pH.

2.6. Non-enzymatic cellular antioxidants

2.6.1. Measurement of total, protein bound and non-protein thiols

The treated plants were homogenized in 0.02 M EDTA under cold conditions. The thiol contents of the homogenates were measured using Ellman's reagent [5, 5'-Dithio-bis-(2-nitrobenzoic acid), (DTNB)] (Sedlak and Lindsay 1968).

2.6.1.1. Total thiols (T-SH)

Aliquots of 0.5 ml of the homogenates were mixed with 1.5 ml of 0.2 M Tris buffer (pH 8.2) and 0.1 ml of 0.01 M DTNB. The mixture was brought to 10 ml by the addition of 7.9 ml of absolute methanol. The colour was allowed to develop for 15 min. The absorbance of the clear supernatant was read at 412 nm (e= 13, 100).

2.6.1.2. Non-protein thiols (NP-SH) and protein bound thiols (PB-SH)

Aliquots of 5 ml of the homogenates were mixed with 4 ml of distilled water and 1 ml of 50% TCA. The contents were mixed and after 15 min the tubes were centrifuged at 10, 000 x *g* for 15 min. Two ml of the supernatant was mixed with 4 ml of 0.4 M Tris buffer (pH 8.9), 0.1 ml of DTNB and absorbance was read within 5 min at 412 nm against a reagent blank. The protein bound thiols (PB-SH) were calculated by subtracting the non-protein thiols from protein thiols (Sedlak and Lindsay 1968).

2.6.2. Cellular glutathione content

2.6.2.1. Estimation of reduced glutathione content (GSH)

The levels of reduced and oxidized glutathione were estimated fluorimetrically (Hissin & Hilf 1976). Plant material (1 g) was ground in 1 ml of 25% H_3PO_3 and 3 ml of 0.1 M sodium phosphate-EDTA buffer (pH 8.0). The homogenate was centrifuged at 10, 000 x *g* for 20 min. The supernatant was used for the estimation of GSH and GSSG in a Hitachi spectroflourimeter F-3010. The supernatant was further diluted 5 times with sodium phosphate-EDTA buffer (pH 8.0). The final assay mixture (2.0 ml) contained 100 µl of the diluted supernatant, 1.8 ml of phosphate-EDTA buffer and 100 µl of *O*- phthalaldehyde (1 mg ml^{-1}). After thorough mixing and incubation at

room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence at 420 run was measured after excitation at 350 nm.

2.6.2.2. Estimation of oxidized glutathione content (GSSG)

An aliquot of 0.5 ml of the supernatant was incubated at room temperature with 200 ul of 0.04 M *N*-ethylmaleimide for 30 min to interact with the GSH present in the supernatant. To this mixture, 4.3 ml of 0.1 N NaOH was added. A 100 ul portion of this mixture was taken for the measurement of GSSG, using the procedure outlined for GSH assay, except that 0.1 N NaOH was used as the diluent rather than phosphate-EDTA buffer (Hissin and Hilf 1976).

2.6.3. Cellular ascorbic acid (AsA) and dehydroascorbate (DHA) content

Ascorbate (AsA) and dehydroascorbate (DHA) were measured according to Logan *et al* (1998) with minor modifications as per Ma and Cheng (2003). Briefly 1 gm of treated plant material was ground in 1 ml of ice cold 6% (v/v) $HClO_4$. The extract was centrifuged at 10, 000 x *g* at 4° C for 10 min and supernatant was immediately assayed for AsA and DHA. 100 ul of the extract was neutralized with 30 ul of 1.5 mM Na_2CO_3. AsA was assayed spectrophotometrically at 265 nm (e = 14 mM^{-1} cm^{-1}) in 100 mM potassium phosphate buffer (pH 5.6), before and after 15 min incubation with 5 units of ascorbate oxidase. For total ascorbate, 100 ul of extract was neutralized with 30 ul of 2 mM Na_2CO_3 and incubated for 30 min at room temperature with equal volume of 20 mM GSH in 100 mM Tricine-KOH (pH 8.5). DHA was calculated as the difference between total ascorbate and AsA.

2.7. Ascorbate-glutathione cycle enzymes

The treated plant material was homogenized in 50 mM Tris-HCl (pH 7.5) with the addition of 40 mM PMSF and 2% PVPP. The extract was centrifuged at 13, 000 x *g* for 20 min and the resultant supernatant was used for all the assays. The amount of protein was calculated according to Lowry *et al* (1951).

2.7.1. Monodehydroascorbate reductase (MDHAR, E.C. 1.6.5.4)

MDHAR activity was assayed by monitoring the change in absorbance at 340 nm due to NADPH oxidation (E = 6.2 mM^{-1} cm^{-1}) for 1 min in a 3 ml reaction mixture containing 0.1 mM NADPH, 2.5 mM AsA, 50 mM sodium phosphate buffer (pH 7.6) and 100 µg of protein. The reaction was started by the addition of 4 units of ascorbate oxidase (Drążkiewicz *et al* 2003).

2.7.2. Dehydroascorbate reductase (DHAR, E.C. 1.8.5.1)

Spectrophotometric determination of DHAR activity was performed according to De Tullio *et al* (1998). The assay measured the formation of AsA at 265 nm (E= 14 mM^{-1} cm^{-1}) in a reaction mixture containing 0.1 M Na-phosphate buffer (pH 6.2) and 2 mM GSH. The reaction was started upon the addition of 1 mM DHA and 100 ug of protein. Changes in absorbance at 265 nm were followed for 1 min. The rate of non-enzymatic DHA reduction was corrected by subtracting the values obtained in the absence of enzyme extract.

In-gel enzyme activity staining was done following the method of De Tullio *et al* (1998) and Tommasi *et al* (2001). Native-gel electrophoresis (7.5% gel) was performed at 4° C. After the run, gels were incubated under agitation for 20 min in 0.1 M sodium phosphate buffer (pH 6.2) containing 4 mM GSH and 2 mM DHA. Gels were

then washed with distilled water and specifically stained for DHAR activity by incubation for 15 min in 0.1 N HC1 solution containing 0.1 % (w/v) ferric chloride and 0.1 % (w/v) ferricyanide, their reaction with AsA yielding a coloured product. Proteins representing DHAR activity were observed as dark blue bands on a blue background the latter due to non-enzymatic AsA formation occurring in the reaction between DHA and GSH.

2.7.3. Glutathione reductase (GR, EC. 1.6.4.2)

The assay was performed according to Jiang and Zhang (2001). The reaction mixture contained 500 ul of 0.2 M sodium phosphate buffer pH 7.0, 100 µl each of 10 mM GSSG, 1 mM NADPH and 180 ul of distilled water. The reaction was started by addition of 50 µg protein and NADPH oxidation was recorded as the decrease in absorbance at 340 nm for 1 min.

In-gel GR activity staining was performed in a 7.5 % native gel under cold conditions. GR activity was detected following the procedure of Lee and Lee (2000). GR was visualized in the native gel by incubation in 50 ml of 0.25 M Tris-HCl buffer (pH 7.5) containing 10 mg of MTT, 10 mg DCPIP, 3.4 mM GSSG and 0.5 mM NADPH. The bands were visualized as dark blue bands in a pale blue background.

2.8. Glutathione metabolism enzymes

2.8.1. γ-Glutamylcysteine synthetase (γ-GCS, EC 6.3.2.2)

The assay was performed following the methodology of Reugsegger *et al* (1992). The reaction mixture for assaying γ-GCS contained 50 µl each of 0.2 M sodium glutamate, 0.2 M L-amino butyrate, 40 mM Na_2-EDTA, 0.4% BSA, 100 ul each of 0.2 M $MgCl_2$, 50 mM Na_2-ATP and 500 ul of 0.2 M Tris-HCl (pH 8.2). It was pre-

incubated for 2 min at room temperature and the reaction was started by addition of 50 µg protein. The reaction mixture was incubated at 37° C for 30 min and the reaction was stopped by the addition of 100 ul of TCA. The mixture was centrifuged and the supernatant was used for the estimation of phosphate content by phosphomolybdate estimation at 660 nm (Fiske and Subbarow 1925) as previously described.

2.8.2. Glutathione-S-transferase (GST, E.C. 2.5.1.18)

The assay was according to Habig and Jacoby (1981). The reaction mixture for assaying GST consisted of 500 ul of 0.2 M potassium phosphate buffer (pH 7.0), 100 ul of 10 mM GSH, 10 ul of 0.1 M 1-chloro, 2,4-dinitrobenzene and 390 ul of distilled water. The reaction was started by the addition of 50 ug of protein and the increase in absorbance was measured at 340 nm for 1 minute.

2.8.3. Glutathione peroxidase (GSH-PX, E.C. 1.11.1.9)

The assay mixture contained 100 ul each of 0.2 M potassium phosphate buffer (pH 7.0), 10 mM Na_2-EDTA, 1.14 M NaCl, 10 mM GSH, 2 mM NADPH, and 2.5 mM H_2O_2. The reaction was started by adding 5 ul GSSG-reductase (500 units/2.8 ml), 344 ul of water and 50 ug protein. The disappearance of NADPH was recorded at 340 nm for 1 min (Nagalakshmi and Prasad 2001).

2.9. Structural, conformational and functional changes of carbonic anhydrase in response to Zn and Cd.

2.9.1. Carbonic anhydrase activity (CA, E.C. 4.2.1.1)

The treated plant material (1 g) was ground in ice-cold mortar and pestle with 50 mM Tris-HCl buffer pH 7.5, 40 mM PMSF and 2% (*m/v*) PVP. The

homogenate was filtered through two layers of muslin cloth to remove the cell debris and centrifuged at 15,000 x *g* for 20 min and the supernatant was used as the source of the enzyme. The amount of protein was estimated according to Lowry *et al* (1951). The assay was performed according to Armstrong *et al* (1966). This assay is based on the principle that carbonic anhydrase not only catalyses the hydration of CO_2 and dehydration of HCO_3^-, but also the hydrolysis of many esters and aldehydes. This assay utilizes p-nitrophenyl acetate as the ester for assaying the hydrolytic reaction. *p*-Nitrophenyl acetate (54.3 mg) was dissolved in 3 ml of acetone and millipore ultrapure water was added slowly with constant stirring to a final volume of 100 ml. This solution was prepared fresh everyday. In a quartz cuvette, 1 ml of this solution with 1.7 ml of water and 0.3 ml of 0.1 M diethylmalonate was added with rapid stirring and incubated for 2 ½ minutes. The increase in absorbance at 348 nm was recorded in a double beam spectrophotometer (Cintra 5, GBC Scientific, Australia) after the addition of 100 µg of protein. The activity was calculated using the molar absorptivity of 5.4 mM^{-1} cm^{-1}.

2.9.2. Purification of carbonic anhydrase

All the operations were carried out at 4° C. Only four treatments (Control, 10 µM Cd, Cd 10 uM +Zn 200 µM and Zn 200 uM) were chosen for the purification process. A modified procedure of Armstrong *et al* (1966), Atkins *et al* (1972), and Guliev *et al* (2003) was followed. Plant material (60 g) was washed thoroughly and homogenized in ice cold buffer- 50 mM Tris-HCl (pH 8.7), 40 mM PMSF and 2% (*m/v*) PVP. The homogenate was filtered through two layers of muslin cloth to remove the cell debris and centrifuged at 15, 000 x *g* for 20 min. The pellet was discarded and the supernatant was used as the enzyme source. Diethylaminoethyl (DEAE) sephadex A

50 (Sigma, USA) was washed thoroughly according to the manufacturer's instructions and packed to give a column of 2 X 35 cm. The column was equilibrated with three column volumes of 50 mM Tris-HCl (pH 8.7) buffer and the enzyme source was loaded onto the column with a flow rate of 20 ml h^{-1}. After two column washings the bound protein was eluted with 0.1 M Tris-HCl (pH 8.7) as collected in 2 ml fractions. The eluate was tested for protein content by checking the positive absorbance at 280 nm (ultraviolet absorbance specific for column eluates) and CA activity and the positive fractions were pooled up and concentrated by ammonium sulphate precipitation (80% saturation). The concentrate was dialysed against 3 volumes of 0.1 M Tris-HCl (pH 8.7) overnight under cold conditions. This concentrated enzyme was chromatographed on a sephadex G-25 (Sigma, USA) column (1.5 X 35 cm) equilibrated with 0.1 M Tris-HCl (pH 8.7) at a flow rate of 4.5 ml h^{-1}. One ml fractions were collected and tested for protein content by checking the positive absorbance at 280 nm (ultraviolet absorbance specific for column eluates) and CA activity. The activity rich fractions were pooled up and stored in the presence of sulphydryl protecting agents (15 mM dithiothreitol) and referred to as the purified carbonic anhydrase. Samples of the crude extract as well the DEAE sephadex elution fractions and purified carbonic anhydrase from sephadex G-25 fractions were subjected to SDS-PAGE as described by Laemmli (1970) to confirm the homogeneity of the purified enzyme and to estimate its molecular weight.

2.9.2.1. Zinc and cadmium content of purified carbonic anhydrases

The purified carbonic anhydrases were analysed for Zn in a GBC flame atomic absorption spectrometer in an air-acetylene flame system (GBC 932 plus, Australia). The purified sample was also analysed for Cd to verify whether there is any substitution of Zn by Cd.

2.10. Structural and conformational studies on carbonic anhydrases

2.10.1. Ultraviolet difference spectra of carbonic anhydrases

The purified CA of control, Cd-alone, Zn alone-treatments and Zn supplemented Cd treatments at a protein concentration of 100 μg in 0.1 M Tris-HCl (pH 8.7) were analysed for ultraviolet spectra at room temperature from 200-350 nm at 100 nm/min (Mailer *et al* 1984).

2.10.2. Circular dichroism (CD) spectra of carbonic anhydrases

Circular dichroism spectra of the purified CAs from different treatments (control, Cd-alone, Zn supplemented Cd treatments and Zn alone treatments) were recorded in near UV (240-300 nm) as well as far-UV (190-240 nm) using a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan). All spectra were recorded at room temperature (27 °C) with blank spectra of the solvent [0.1 M Tris-HCl (pH 8.7)] subtracted for precision readings (Beychok *et al* 1966). The spectrum was recorded with protein samples taken in a one-centimeter path length quartz cell. Measurements were performed at 0.5 nm spectral resolution with the protein concentration of 0.01 mg ml^{-1}. The molar ellipticity was calculated using the formula

$$[\theta]_{ME} = \theta/(10xCxL)$$

where 8 is the measured ellipticity in degrees,

C is molar concentration calculated from the formula $C = (1000xP_{conc})/M_r$

Where, P_{conc} is the protein concentration in g ml^{-1}, M_r is the molecular weight of the protein, and L is the cell path in cm (Yang *et al* 1986).

The percentage of each form of secondary conformation of the protein was calculated by the equation (Chen *et al* 1974)

$$[\theta]_{ME} = f_H\theta_H + f_\beta\theta_\beta + f_R\theta_R$$

where $[\theta]_{ME}$ is the molar ellipticity, θ_H, θ_β, and θ_R are the reference values of helix, beta and unordered from according to Chen *et al* (1974), *fafp,* and f_R are the fractions of helix, beta and unordered forms of the protein.

2.10.3. Fluorimetric studies of carbonic anhydrases

2.10.3.1. Intrinsic fluorescence -tryptophan, tyrosine and dityrosine

The fluorescence spectra of CAs purified from control, Cd-alone, Zn supplemented Cd treatments and Zn alone treatments were recorded in a Hitachi spectroflourimeter F-3010 in a 1 cm path-length quartz cell. The protein samples (0.02 mg ml^{-1}) were brought to room temperature (27 °C) and normalized for the solvent [0.1 M Tris-HCl (pH 8.7)] fluorescence by blank subtraction before recording the spectra for all measurements. For tryptophan fluorescence the samples were excited at 280 nm and the fluorescence emission was recorded from 300-400 nm (Eftink 1991, Bhattacharjee and Das 2000).

The intrinsic fluorescence of tyrosine and dityrosine (oxidised form of tyrosine) were estimated by excitation of the protein samples at 275 nm and 310 nm for tyrosine and dityrosine respectively. The fluorescence emission spectra was recorded from 250-350 nm and 350-450 nm for tyrosine and dityrosine respectively. (Eftink and Ghiron 1981, Eftink 1991).

2.10.3.2. Extrinsic fluorescence- surface hydrophobicity as a measure of protein unfolding

Hydrophobic interaction of 8-anilino-1-naphthalene sulfonic acid (ANS) with proteins is one of the widely used methods for characterizing and detecting folded/unfolded state of the proteins (Matulis and Lovrien 1998). The surface hydrophobicity of CAs purified from control, Cd-alone, Zn alone and Cd treatments with supplemented Zn were investigated by incubating 50 ug of the protein in 0.1 M

Tris-HCl (pH 8.7) with 75 uM ANS at 37 °C for 30 min. The change in the fluorescence associated with ANS-protein hydrophobic region binding was monitored as fluorescence emission from 400-600 nm after excitation at 380 nm. Samples were corrected for the inherent solvent fluorescence by blank subtraction before the spectral recording (Chao *et al* 1997).

2.11. Sulphydryl (SH) groups of carbonic anhydrases

Changes in the sulphydryl groups of the CAs purified from different treatments were measured using 100 ul of Ellman's reagent (DTNB). The absorbance of the clear supernatant was read at 412 nm (E= 13, 100) after an incubation period of 15 min for the colour to develop (Sedlak and Lindsay, 1968).

2.12. Carbonyl content as a measure of oxidative damage to carbonic anhydrases

The carbonyl content determining the oxidative modification of purified CAs from different treatments was measured with 10 mM 2,4-dinitrophenylhydrazine in 2 M HC1 and incubated at room temperature for 1 h with regular vortexing every 10-15 min. The samples were again precipitated with 500 ul of 20% TCA, centrifuged at 12, 000 x *g* for 5 min. The resultant pellet was redissolved in 0.6 ml of 6 M guanidine-HCl and carbonyl content was estimated at 360-390 nm (E= 22, 000 mM^{-1} cm^{-1}) (Reznick and Packer 1994).

2.13. Analysis of photochemical functions

2.13.1. Quantification of photosynthetic pigments

The treated plant material (1 g) ground in 80% acetone was centrifuged at 8,000 x *g* for 10 minutes and the supernatant was estimated for chlorophyll a, chlorophyll b (Arnon, 1949) and carotenoids (Kirk and Allen 1965).

2.13.2. Isolation of chloroplasts

Metal treated plants were homogenized in a pre-chilled mortar and pestle in ice-cold isolation buffer, which contained 400 mM sucrose, 10 mM NaCl and 20 mM Tricine (pH 7.8). The slurry was filtered through five layers of cheesecloth and the chloroplasts were sedimented at 3,000 x *g* for 5 minutes at 4° C. The supernatant was carefully discarded and the pellet retained. The pellet was washed and resuspended in a small volume of chilled suspension buffer that contained 100 mM sucrose, 10 mM NaCl, 2 mM $MgCl_2$ and 20 mM N-[2-hydroxyethyl]piperazine-N'-2-ethanesulphonic acid] (HEPES) (pH 7.5). Care was taken that the whole procedure was completely done in ice-cold conditions as quickly as possible to inactivate and prevent the degradation of chloroplast by proteolytic enzymes (Atal *et al* 1991, Dilnawaz *et al* 2001). Chlorophyll was extracted in chilled 80% acetone and estimated spectrophotometrically (Arnon 1949).

2.13.3. Intactness of isolated chloroplasts

The percentage intactness of the isolated chloroplasts was determined by the ferricyanide assay. This assay utilizes the property that chloroplasts with intact envelopes will not carry out reactions like reduction of exogenous oxidants such as NADP at a faster rate. In this assay isolated chloroplasts were given an osmotic shock and NH4 Cl as the uncoupling agent, which rendered them 100% envelope free. The rate of O_2 evolution from intact chloroplasts and envelope free uncoupled chloroplasts were measured using an oxygen electrode (YSI model 5300 biological oxygen monitor, Gilson med. Elec., Middleton WI). The percentage intactness was calculated from the difference in the rates of O_2 evolution from intact chloroplasts and envelope free uncoupled chloroplasts (Reeves and Hall 1980).

2.13.4. Rate of photosynthesis

The photosynthetic light mediated evolution of oxygen of the isolated chloroplasts was measured polarographically using oxygen electrode (YSI model 5300 biological oxygen monitor, Gilson med. Elec., Middleton WI). The assay medium contained 0.5 M sorbitol, 10 mM KC1, 0.5 mM $MgCl_2$, 0.05% (w/v) BSA, 10 mM $NaHCO_3$, and HEPES-KOH (pH 7.6) (Greger and Ogren 1991)

2.13.5. Activities of Photosystems II, I and whole chain electron transport:

2.13.5.1. PS II: The reaction mixture for assaying PSII contained 20 mM HEPES (pH 7.5), 100 mM sucrose, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM $K_4Fe(CN)_6$ and 0.1 mM phenylenediamine (Atal *et al* 1991).

2.13.5.2. PS I: The assay mixture contained 20mM HEPES (pH 7.5), 100 mM sucrose, 10 mM NaCl, 2 mM $MgCl_2$, 0.6 mM ascorbate, 1 mM sodium azide, 0.5 mM methyl viologen and 5 μM DCMU [3-(3,4 dichloro diphenyl, 1,1, dimethyl urea)] and 100μM DCPIP (2,6-dichlorophenolindophenol) as the electron donor (Atal *et al* 1991).

2.13.5.3. Whole chain electron transport: The reaction mixture for whole chain electron transport, contained 20 mM HEPES (pH 7.5), 100 mM sucrose, 10 mM NaCl, 2 mM $MgCl_2$, 0.5 mM methyl viologen and 1 mM sodium azide (Atal *et al* 1991).

All the photochemical assays were carried out at a saturating intensity of white light 800 $\mu E\ m^{-2}\ S^{-1}$) at 25 °C and chloroplasts equivalent to 30μg of Chl used.

2.14. Analysis of the structural integrity of DNA

2.14.1. Isolation of DNA

DNA extraction was carried out according to Doyle and Doyle (1990). Three grams each of control as well as treated plant tissue was ground in liquid nitrogen with 5% PVP. This was transferred to 15 ml of 2% CTAB buffer (previously incubated at 65° C), mixed thoroughly and incubated at 65° C for 90 min, with thorough mixing every 20-30 min. This was followed by equal volumes of chloroform extraction twice with centrifugation at 5,000 x *g* for 10 min and equal volume of chloroform: isoamyl alcohol (24:1) extraction once and centrifugation at 5,000 x *g* for 10 min. To the clear supernatant 15 ml of ice-cold isopropanol was carefully added from the sides of the tube and incubated at -20° C for 45 -60 min. The DNA visible as fine threads were obtained by centrifugation at 10, 000 *x g* for 10 min, washed with 70% ethanol, air dried thoroughly and dissolved in 3 ml of Tris-EDTA (TE) buffer with 5 ul of RNAse (1 mg ml^{-1}) in it. This was incubated for 12-15 hrs at 37° C. The sample was further extracted in phenol: chloroform (24:1) in equal volumes and centrifuged at 5,000 x *g* for 10 min. To this extracted sample 200 ul of 3 M sodium acetate was added and again DNA was precipitated with ice-cold isopropanol and incubated at -20° C for 45 -60 min. The DNA was obtained by centrifugation at 10, 000 x *g* for 10 min, washed with 100% ethanol, air dried thoroughly, dissolved in 100 ul of Tris-EDTA (TE) buffer and stored at 4° C.

2.14.2. Measurement of DNA damage by ethidium bromide (EB) binding assay

DNA damage was assessed by the fluorescence changes measured in a Hitachi spectroflourimeter F-3010 according to Cai and Cherian (2003). A 2 ml reaction mixture for estimating DNA damage consisted of TE buffer, 300 uM DNA and 10 ul of

1 mM EB. The Loss of fluorescence was recorded with excitation of the sample at 510 nm and emission recorded at 590 nm.

2.14.3. Gel mobility of DNA

To confirm the DNA damage assessed by EB binding assay, DNA gel electrophoresis was used to observe changes in the mobility (Cai and Cherian 2003). Non-denaturing agarose gels (1%) were casted with 2 µl of EB and 10 µl DNA (from the same samples as above) was loaded. Electrophoresis carried out in a TAE (Tris-glacial acetic acid-EDTA) buffer at 50 V for 2-3 h. The DNA bands were visualized in UV light and photographed.

2.15. Toxicity functions in Cd treatments with exogenous ami no **acids (cysteine, glutamic acid and glycine) and organic acids (citric, oxalic, and malic acids) supplementations.**

Amino acids (cysteine, glutamic acid and glycine) and organic acids (citric, oxalic and malic acid) of the concentrations 0.1, 0.2, 0.5 and 1 mM were supplemented along with Cd-10 µM treatments with and without Zn 200 µM individually and in the following combinations

Amino acids: 1 mM Cysteine + 0.5 mM Glutamic acid

1 mM Cysteine + 0.2 mM Glycine

0.5 mM Glutamic acid + 0.2 mM Glycine

1 mM Cysteine + 0.5 mM Glutamic acid + 0.2 mM Glycine

Organic acids: 1 mM Citric acid + 0.5 mM Oxalic acid

1 mM Citric acid + 0.5 mM Malic acid

0.5 mM Oxalic acid + 0.5 mM Malic acid

1 mM Citric acid + 0.5 mM Oxalic acid + 0.5 mM Malic acid.

In the above treatments the parameters of Cd and Zn uptake, levels of photosynthetic pigments, and lipid peroxidation levels were estimated as outlined in the earlier sections. In addition only in amino acids supplemented Cd treatments with and without Zn, the levels of cellular glutathione and γ-glutamylcysteine synthetase activity were also analyzed.

2.16. Statistics

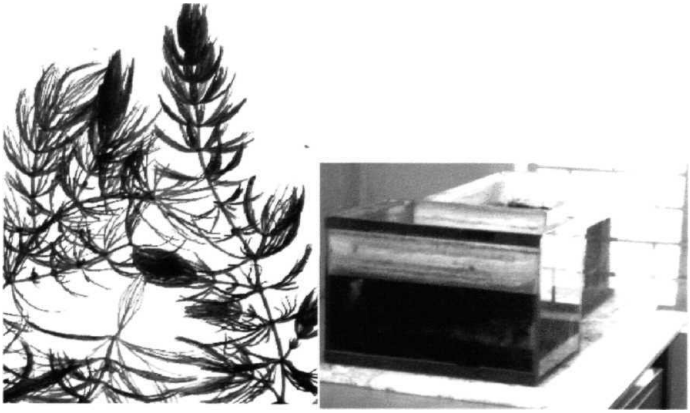
The values are means of three individual experiments with duplicates (value averaged to one) for each experiment and the results were subjected to statistical analysis by student's T-test (n=3). The level of significance was set at 0.01 ($p < 0.01$).

3. Results

3.1. Toxicity symptoms: *Ceratophyllum demersum* maintained under laboratory conditions (see figure 1a, b, page 55) treated with 10 uM Cd exhibited toxicity symptoms observed as chlorosis, necrosis, with stems and leaves of the plant showing complete disintegration and their survival was only up to a week. These symptoms were not observed in Cd-treated plants supplemented with Zn (10-200 uM) and Zn (10-200 μM)-alone treated plants, which showed healthy morphology indicating the non-toxic nature of the chosen Zn concentrations (see figure 2, page 55). Zn treatment was restricted upto 200 uM since Zn dose beyond 200 uM was found to be toxic to the plant.

3.2. Mechanism of metal uptake

Quantification of cadmium and zinc content: Cd-treated plants accumulated 143.39 ug g^{-1} DW. Whereas the Cd concentration decreased (26%) to 105.81 μg g^{-1} DW in treatments with supplemented Zn (especially at 200 uM) (see figure 3, page 56). Simultaneously the reduced (50%) amount of Zn (29 ug g^{-1} DW) in Cd-10 uM alone treatments was restored to 57.64 μg g^{-1} DW similar to control and accumulation increased (79%) upto 270.56 ug g^{-1} DW in Cd treated plants with supplemented Zn (200 uM). In the presence of 10 uM DNP and 100 uM DCCD there was reduction of Cd uptake in Cd-10 uM treatments up to 122.34 μg g^{-1} DW (15% reduction) and 110 ug g^{-1} DW (23% reduction) respectively (see figure 4, page 56). In addition Zn (10-200 uM) along with DNP and DCCD further reduced Cd uptake to 96 ug g^{-1} DW (33% reduction) and 88.22 ug g^{-1} DW (38% reduction). There was absolutely no effect on Zn uptake by DNP or DCCD. Zn-treated plants without any Cd treatment comparatively



a b

Figure 1 a. *Ceratophyllum demersum* L.- Habit
b. *C. demersum* maintained in aquaria under laboratory conditions

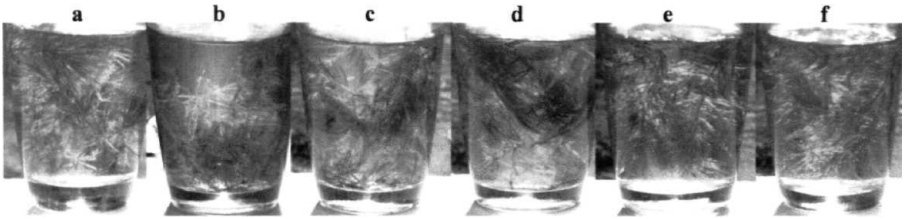


Figure 2. Toxicity bioassay under different metal treatments using *C. demersum*
a. Control plants without any treatments
b. Cd-10 µM
c. Cd-10 µM + Zn-10 µM
d. Cd-10 µM + Zn-50 µM
e. Cd-10 µM + Zn-100 µM
f. Cd-10 uM + Zn-200 uM treatments.
Note the expression of distinct symptoms by C. *demersum* under different treatments.

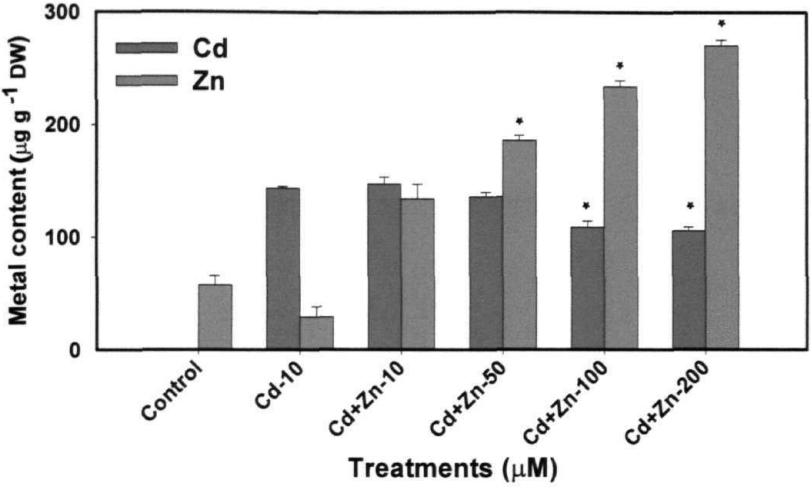


Figure 3. Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 μM and Zn (10, 50, 100, and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' for Cd content indicates significant difference from Cd treated plants; 'asterisk' for Zn content indicates significant difference from control plants.

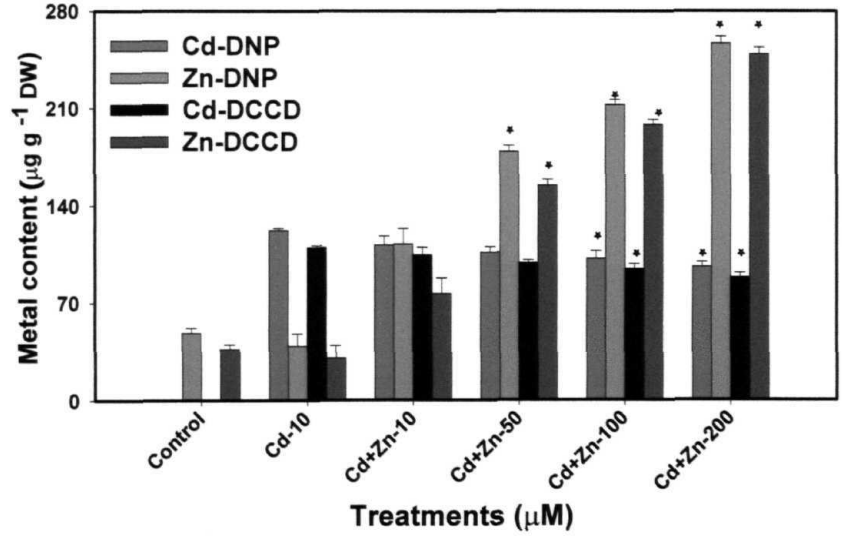


Figure 4. Metal accumulation in C. *demersum* treated with Cd-lO μM and Zn (10, 50, 100 and 200 μM)concentrations in the presence of metabolic inhibitors 2,4-Dinitrophenol (DNP) (10 μM) and N-N' Dicyclohexylcarbodimide (DCCD) (100 μM). Error bars represent standard errors and 'asterisk' for Cd content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

Table 1. Influence of Zn -alone treatments on metal content and H^+ ATPase activity with and without DNP, DCCD. Values in parenthesis indicate standard deviation (n=3) and "asterisk" indicates significant difference from the control ($p< 0.01$).

Treatments (μM)	Metal uptake ug g^{-1}DW	Metal uptake with DNP ug g^{-1}DW	Metal uptake with DCCD μg g^{-1}DW	H^+ ATPase activity μmol Pi mg^{-1} protein	H^+ ATPase activity with DNP umol Pi mg'" protein	H^+ ATPase activity with DCCD umol Pi mg^{-1} protein
Control	57.85 (4.58)	57.85 (4.58)	57.80(4.58)	74.75 (4.80)	56.00 (5.59)	30.42 (5.6)
Zn-10	128.58 (32.32)*	125.00(12.62)*	122.58(32.32)*	74.80(2.51)	56.84 (6.80)	31.12(3.11)
Zn-50	162.16 (11.80)*	158.66(9.00)*	168.78 (11.80)*	75.00(2.87)	56.96(4.01)	30.98(4.01)
Zn-100	206.13(10.19)*	211.58 (10.19)*	206.90 (10.19)*	75.25(3.81)	56.98 (4.27)	30.66(1.72)
Zn-200	247.26 (27.33)*	240.06 (27.33)*	244.66 (27.33)*	75.25(1.97)	56.00 (2.46)	30.02(1.59)

showed lesser uptake of Zn in all treatments indicating increased Zn requirement in plants treated with both Cd and Zn (see table 1, page 57).

H^+ATPase activity: Cd-10 uM indicated an 18% increase in H^+ ATPase activity in treatments without any inhibitors, but there was only a negligible increase in Cd-10 uM treatments with DNP and DCCD inhibitors (see figure 5, page 59). In contrast there was a 27% decrease in H^+ ATPase activity in Zn supplemented Cd treatments (especially at 200 uM) without any inhibitors, 17% and 21% decrease respectively in DNP, DCCD supplemented treatments. Zn-alone treatments did not show distinct changes in ATPase activity (see table 1, page 57).

3.3. Membrane damage

Lipid peroxidation: The thiobarbituric assay, evaluating the degree of lipid peroxidation revealed a 37% increase at Cd-10 uM over the control. This was decreased up to 48% in Cd-treated plants with supplemented Zn (especially at Zn-200 uM) from the increased TBA-reactive substances value at Cd-10 uM (see figure 6, page 59). Zn treated plants however did not show any increase in TBA-RS levels (see table 2, page 62).

Lipoxygenase (LOX) *activity:* Cadmium showed a very high increase in activity (almost 3- fold) of 247% of this lipolytic enzyme. This was decreased in Cd-treated plants with supplemented Zn up to 264% (a 3 fold decrease) (especially at Zn-200 uM) from the increased activity indicating the inhibitory effect of Zn (see fig. 7, page 60). Plants with Zn treatments only, showed a very little increase in LOX activity (see table 2, page 62).

Ion leakage- electrical conductivity and flame photometry: Cd treatment led to a high increase in the electrical conductivity (29%) (see figure 8, page 61) and increased the

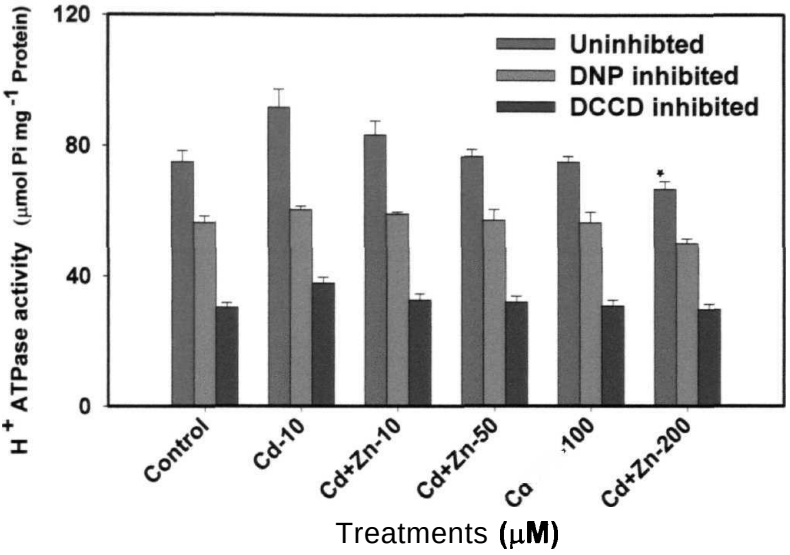


Figure 5. FT ATPase activity in *C. demersum* treated with Cd-10 µM and Zn (10, 50, 100 and 200 µM) in the absence of and in the presence of metabolic inhibitors 2,4-Dinitrophenol (DNP) (10 µM) and N-N' Dicyclohexylcarbodimide (DCCD) (100 µM). Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

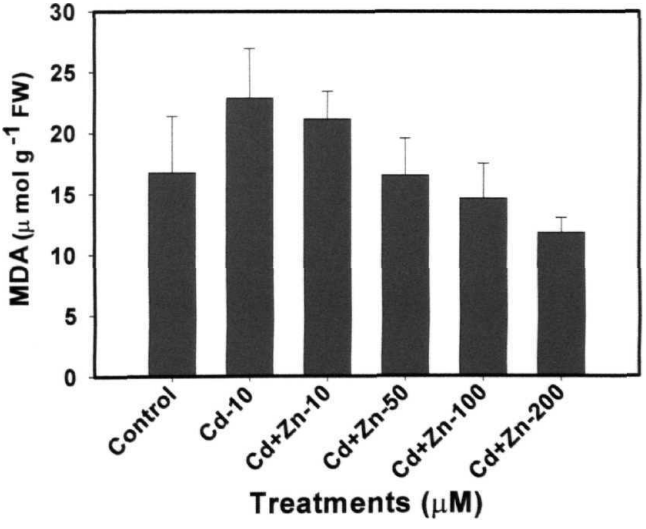


Figure 6. Level of lipid peroxidation as a measure of TBA -RS in *C. demersum* treated with Cd-10 µM and Zn (10, 50, 100 and 200 µM). Error bars represent standard errors.

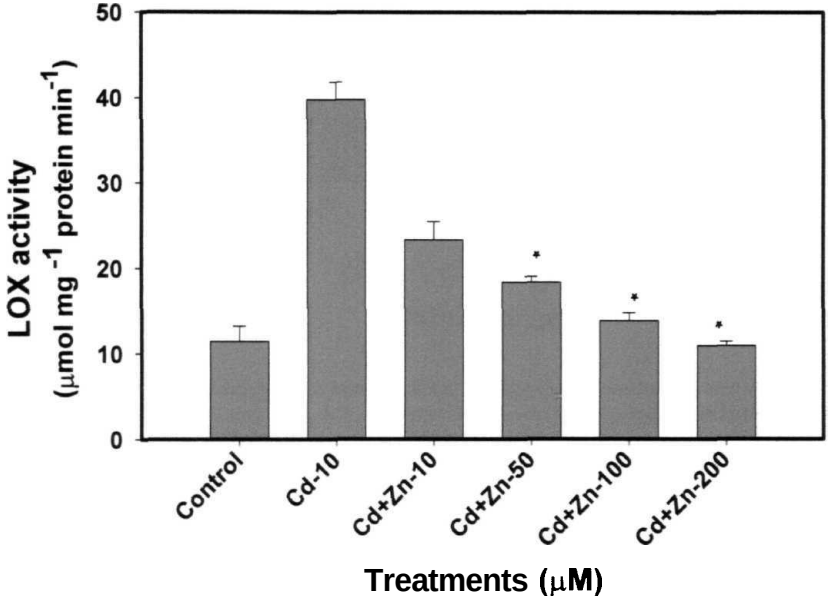


Figure 7. Activity of lipoxygenase in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM)concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

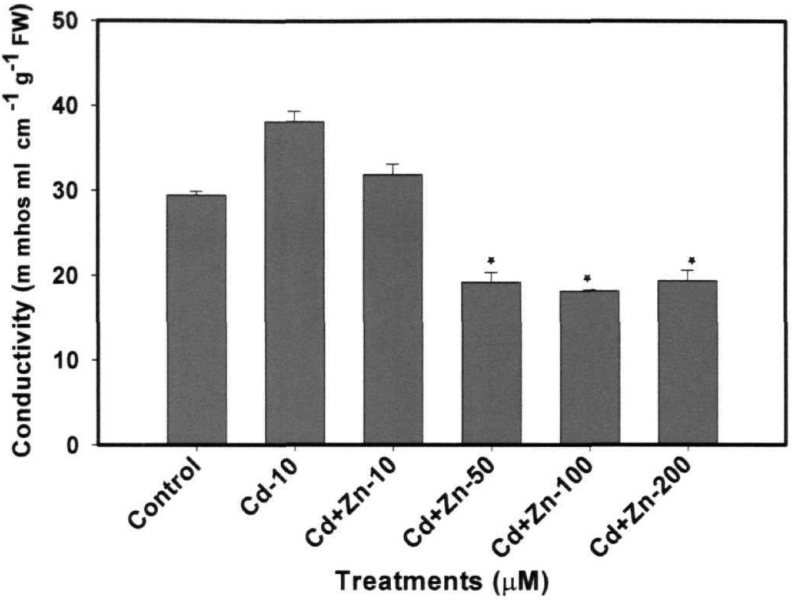


Figure 8. Electrical conductivity as a measure of ion leakage in *C. demersum* treated with Cd- 10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

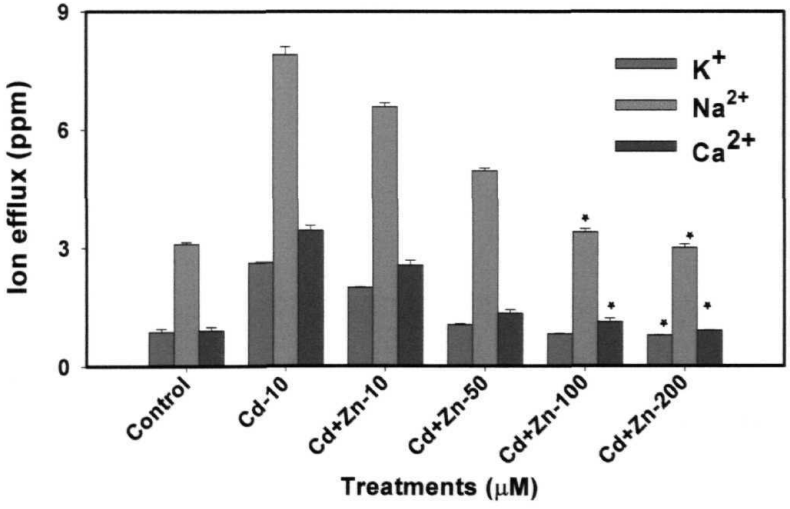


Figure 9. Ion efflux of K^+, Na^{2+} and Ca^{2+} in C. *demersum* plants treated with Cd-10 uM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

Table 2. Influence of Zn -alone treatments on lipid peroxidation, lipoxygenase activity, conductivity, and ion leakage Values in parenthesis indicate standard deviation (n=3).

Treatments (MM)	Lipid peroxidation ($\mu molgm^{-1}$ FW)	Lipoxygenase activity ($\mu mol\ mg$ 'protein min^{1})	Conductivity (m mhos ml cm^{1} g^{1} FW)	Ion efflux (ppm)		
				K^{+}	Na^{2+}	Ca^{2+}
Control	14.13(0.45)	**11.86 (2.50)**	30.23 (2.26)	0.87 (0.008)	3.10(0.05)	0.90 (0.006)
Zn-10	12.23(1.17)	12.53 (0.28)	30.83 (2.84)	0.88 (0.006)	3.25 (0.08)	0.88(0.01)
Zn-50	**11.86 (0.49)**	16.40(3.95)	23 (0.93)	0.86 (0.005)	**3.15 (0.09)**	0.88 (0.009)
Zn-100	**11.98 (0.18)**	18.80(2.82)	21.26(2.05)	0.86 (0.007)	3.12(0.04)	0.86(0.011)
Zn-200	13.52(0.28)	18.93(1.38)	19.83(1.47)	0.85 (0.009)	3.08(0.01)	0.86 (0.009)

efflux of electrolytes K^+, Na^+, and Ca^{2+} by 66%, 61% and 74% respectively from the plant cell (see figure 9, page 61). The conductivity in Cd-treated plants with supplemented Zn showed a 52% decrease (at Zn-200 uM), 70%, 62% and 74% decrease of ion efflux from the increased electrical conductivity and ion efflux at Cd-10 uM. Treatments with only Zn showed lesser differences in conductivity (see table 2, page 62).

3.4. Measurement of free radicals

NADPH dependent superoxide (Of) generation: Cd-10 uM treatments induced a 72% increase in NADPH oxidation and $O_2^{\cdot-}$ production which was completely inhibited by supplementing Zn (10-200 uM) to Cd treatments (see figures 10 and 11, page 64).

Cellular levels of superoxide radicals: With the enhanced oxidation of NADPH, Cd-10 uM concentration induced a 41% increase in the levels of $O_2^{\cdot-}$ radical (see figure 12, page 65).

Levels of hydrogen peroxide (H_2O_2): Subsequently there was a very high increase (60%) in H_2O_2 levels at Cd-10 uM treatments. Zn supplementation (10-200 uM) was very effective in decreasing $O_2^{\cdot-}$ radicals (47%) and hence subsequently H_2O_2 levels (69%) in the plant system (see figure 13, page 65).

Electron spin resonance spectra of hydroxyl (OH) radicals and hydroxyl radical levels: The electron spin resonance spectra analyzed for OH radical depicted a 1:2:2:1 quartet at g-value of 2.0060, hyperfine splitting values: $a^H = a^N =$ 14.84 G at a field center of 335 mT, characteristic of $OH^{\cdot}$ radical signal, referred with the spin trap database (http://hippo.niehs.nih.gov) and the calibrated standard values in the instrument (see figure 14, page 66). The 1:2:2:1 quartet represents the virtual identical coupling of the free electron to the nitroxide nitrogen (a^N =14.84 G) and beta hydrogen (a^H =14.84 G) of the pyrroline ring of the spin trap DMPO. There was a 74% increase in

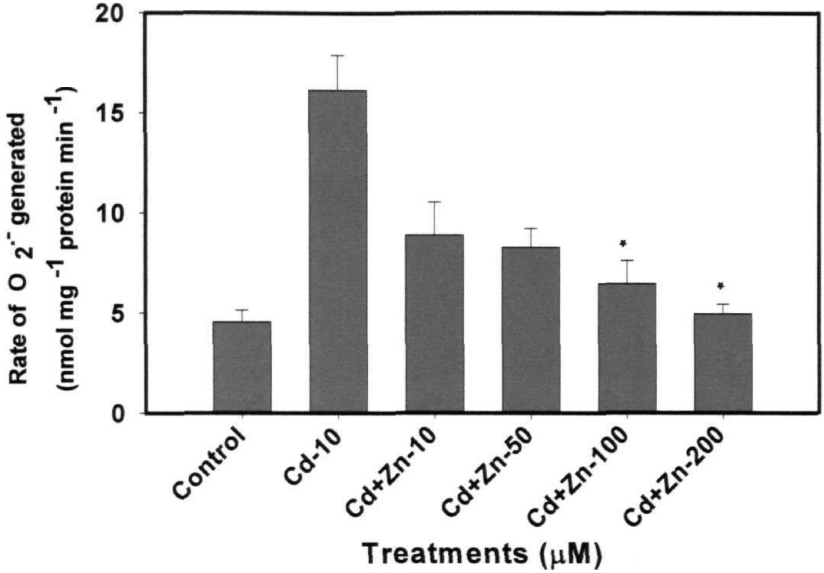


Figure 10. NADPH dependent superoxide generation in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

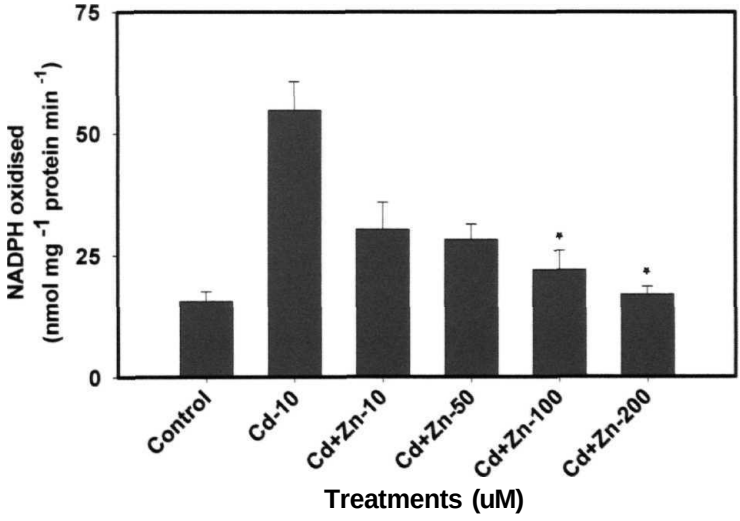


Figure 11. Rate of NADPH oxidation in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

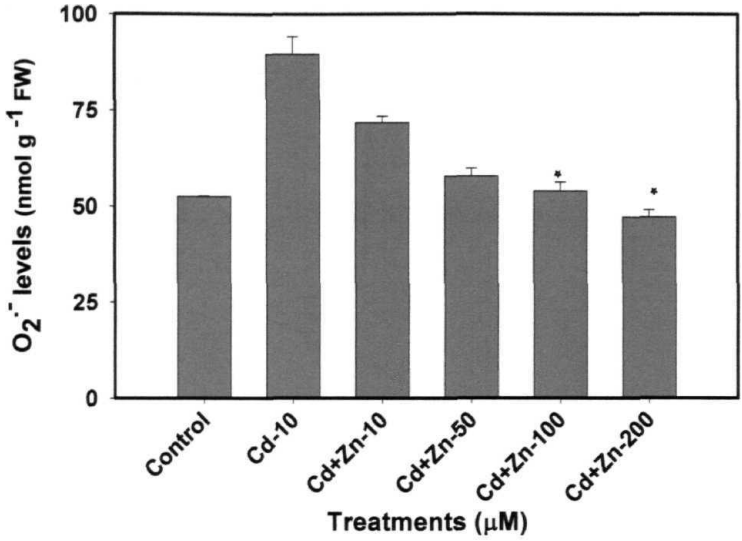


Figure 12. Superoxide radical levels in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

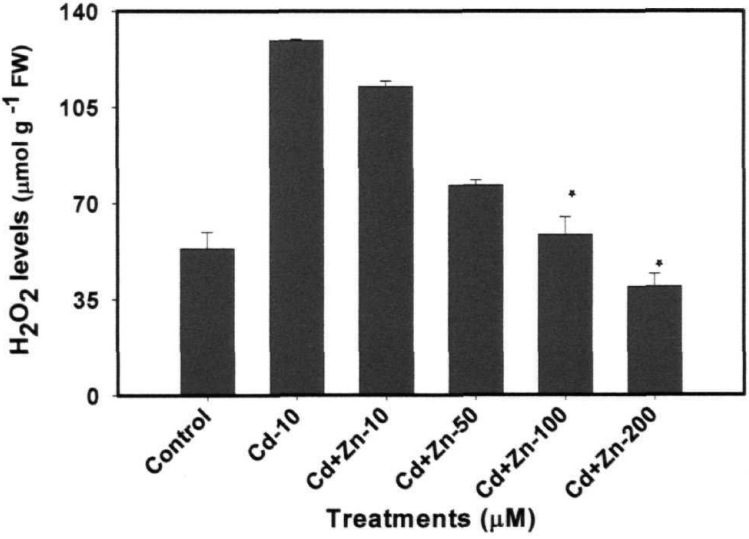


Figure 13. Levels of hydrogen peroxide in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

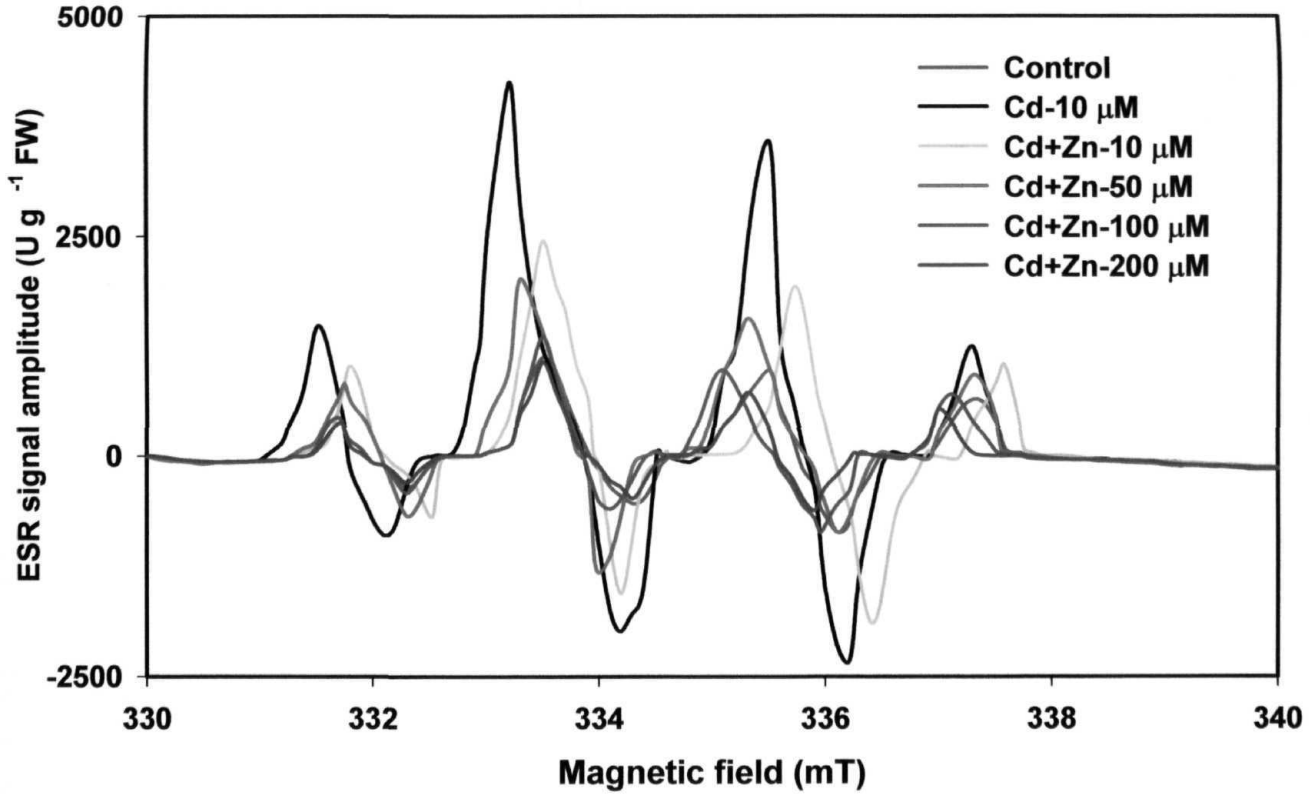


Figure 14. Electron spin resonance spectra showing the typical 1:2:2:1 quartet of the DMPO-OH adduct in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100, 200 μM) concentrations.

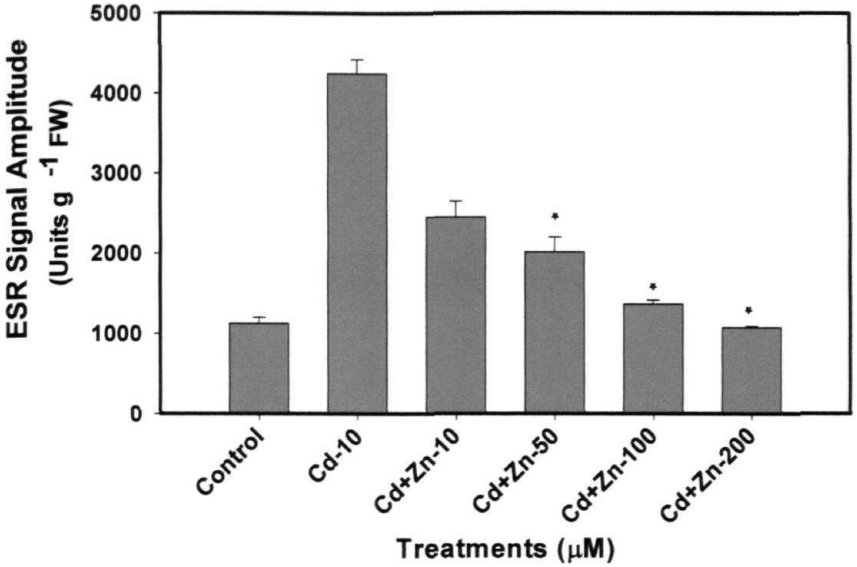


Figure 15. ESR signal amplitude changes recorded for DMPO-OH adducts in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

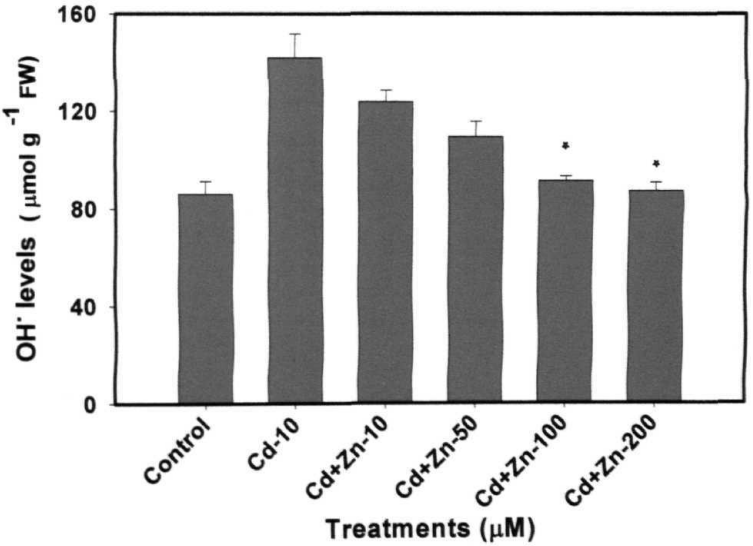


Figure 16. Hydroxyl radical levels in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

Table 3. Influence of Zn- alone treatments on free radical formation in *C. demersum*. The values are means of three individual experiments with duplicates. The values in parenthesis indicate standard deviation (n=3).

Treatments (HM)	Rate $O_2^{\cdot-}$ production (n mol mg^{-1} protein min^{-1})	NADPH oxidised (n mol mg^{-1} protein min^{-1})	$O_2^{\cdot-}$ levels (n mol g^{-1} FW)	H_2O_2 levels (μmol g^{-1} FW)	ESR-DMPO-OH Signal amplitude (Units g^{-1} FW)	OH levels (μmol g^{-1} FW)
Control	4.86(1.03)	16.66(3.48)	52.72 (3.82)	55.23 (9.66)	1132.97 (60.66)	86.66 (2.65)
Zn-10	5.51(0.42)	19.89(3.67)	50.92(1.40)	53.10(10.03)	1111.06(128.90)	86.55(5.41)
Zn-50	5.57 (0.62)	20.40 (2.46)	50.83 (2.40)	50.63(1.70)	1080.02(63.35)	86.76(3.29)
Zn-100	5.66 (0.88)	19.88(3.35)	49.05 (0.69)	45.13(2.06)	1029.41 (98.21)	85.99(2.55)
Zn-200	5.85 (0.52)	19.88(2.21)	43.53 (2.36)	42.43 (1.80)	976.19(58.53)	83.27(2.35)

the signal amplitude (see figure 15, page 67) of the DMPO-OH quartet peaks and hence a 40% increase in $OH^{\cdot}$ levels in Cd-10 uM treated plants (see figure 16, page 67). Zn supplements (10-200 uM) protected the plant cells from OH radicals as inferred from the complete reduction of the 1:2:2:1 quartet signal amplitude of DMPO-OH adduct to control levels (see figure 15, page 67) and hence a 38% decrease in $OH^{\cdot}$ radical levels. (see figure 16, page 67). Zn alone treatments did not influence free radical production (see table 3, page 68).

Total protein content and carbonyl content as a measure of protein oxidation:

The total protein content was decreased (35%) considerably by Cd treatment (see figures 17 and 18, page 70), thereby substantially increasing (48%) the oxidation of proteins measured as protein carbonyls (see figure 19, page 71). The decrease in protein content was not only actively restored (39%) by Zn supplementation but there was complete inhibition (54%) of protein carbonyl formation. Treatments with Zn alone also showed a slight increase in the total protein content, and decrease in carbonyl content indicating its non-toxic nature (see table 4, page 72).

3.5. The antioxidant enzymes

Superoxide dismutase (SOD, E.C. 1.15.1.1): Activity of SOD showed a 3.6-fold increase in plants supplemented with Zn-200 uM over the control as against just 1.5-fold increase in plants treated with Cd-10 uM only (see figure 20, page 73). Similarly Cu/Zn SOD and Mn SOD also showed 3.56 and 3.78-fold increase in plants supplemented with Zn-200 uM as against just 1.5 and 1.2-fold increase in activity at Cd-10 uM treatments indicating the efficiency of this antioxidant enzyme in the presence of Zn than without any Zn supplementation. The isozymes stained for SOD clearly depicted the result observed in the assay indicating enhanced enzyme activity

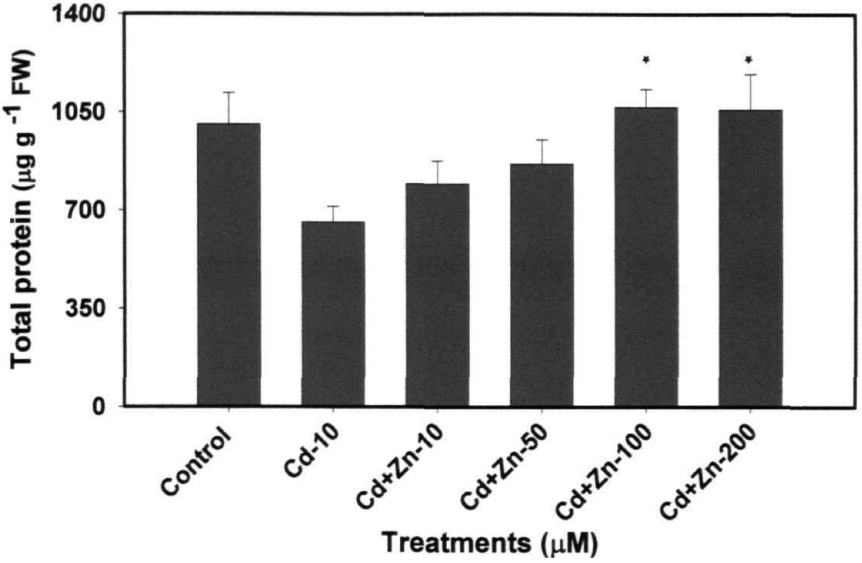


Figure 17. Total protein content in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

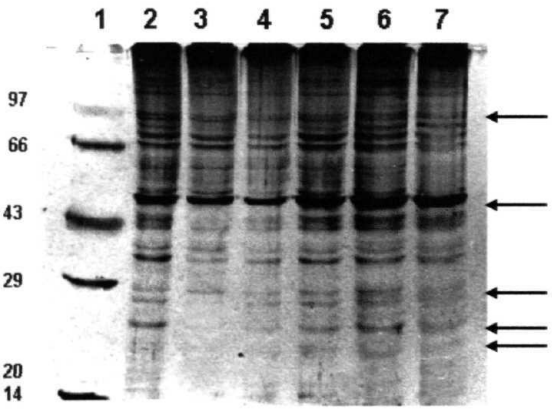


Figure 18. SDS-PAGE (12.5%) showing the total protein profile in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Lanes 1: molecular weight markers, 2: control, 3: Cd-10 μM, 4: Cd+Zn-10 μM, 5: Cd+Zn-50 μM; Cd+Zn-100 μM, 6: Cd+Zn-200 μM. The arrows indicate appearance of new bands.

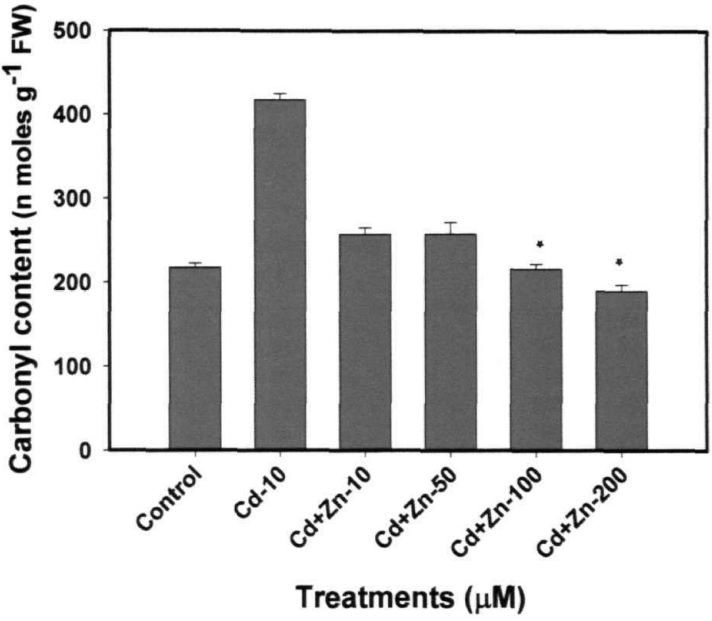


Figure 19. Levels of protein carbonyls in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

Table 4. Influence of Zn- alone treatments on the total protein and protein carbonyls content in *C. demersum*. The values are means of three individual experiments with duplicates. The values in parenthesis indicate standard deviation (n=3).

Treatments (μM)	Total protein content (ug g^{-1} FW)	Protein carbonyl content (n mol g^{-1} FW)
Control	1005.56(68.90)	213.50(3.34)
Zn-10	1010.45(32.46)	197.50(1.24)
Zn-50	1024.00(26.68)	193.00(2.45)
Zn-100	1052.36(46.86)	190.00(1.08)
Zn-200	1058.68(32.36)	188.06(2.24)

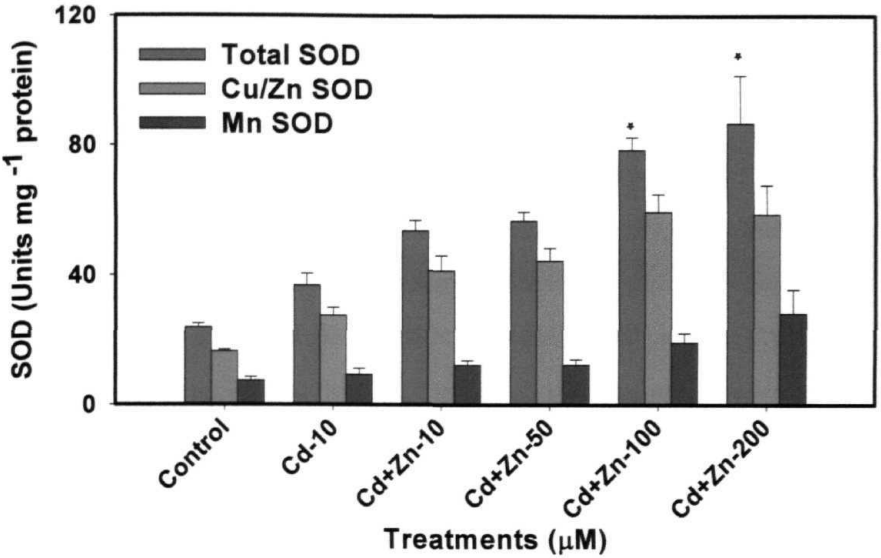


Figure 20. Activities of total superoxide dismutase (SOD), Cu-Zn SOD and Mn SOD in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100, and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

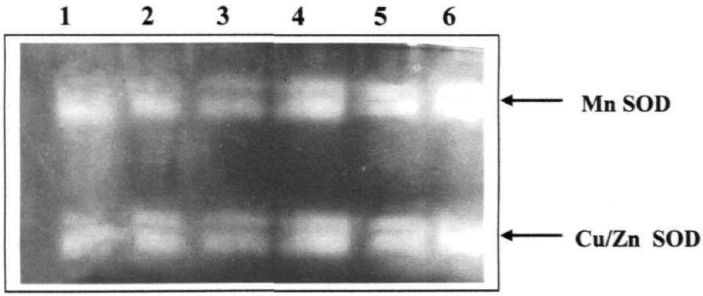


Figure 21. Native gel (10%) showing the isozymes of Cu/Zn and Mn SOD stained for in-gel enzyme activity in *C. demersum*. Lanes 1: control, 2: Cd-10 μM, 3: Cd+Zn-10 |xM, 4: Cd+Zn-50 μM, 5: Cd+Zn-100 μM, 6: Cd+Zn-200 μM. Note the increase in staining intensity at lanes indicating Zn supplemented Cd treatments.

observed as increased staining intensity at higher supplements of Zn to Cd treatments (see figure 21, page 73).

Catalase (CAT, EC. 1.11.1.6): CAT also showed the same trend as SOD, where Cd treatments with Zn addition showed a very high increase in activity than treatments with only Cd or Zn. In CAT 5.35 fold increase in activity was seen in Cd-treated plants supplemented with Zn-200 uM but, Cd-10 uM treatments did not affect CAT activity much over the control (see figure 22, page 75). Catalase isozymes stained for in-gel activity also indicated the above observation very well (see figure 23, page 75).

Total peroxidase (POD, E.C. 1.11.1.7): The activity of the hydrogen peroxide scavenging peroxidase showed a negligible increase in activity in Cd- 10 µM treatments (see figure 24, page 76), but a 3-fold increase in activity as well as induction of a new isozyme (see figure 25, page 76) was clearly observed in Zn (10-200 uM) supplemented Cd treatments indicating enhanced peroxidase activity aided by Zn supplementation.

Ascorbate peroxidase (APX, E.C. 1.11.1.11): Cd-treated plants with supplemented Zn showed a very high increase in APX activity than Cd or Zn-alone treated plants (see figure 26, page 77). This was visually depicted by the isozymes of APX in the native gel (see figure 27, page 77), whereby the Zn supplemented Cd treatments revealed induction of an additional isozyme, and increasing intensity of the three isozymes stained than control or Cd-alone treatments (with only two isozymes), due to the higher activity of APX. Zn-200 µM supplemented Cd-treatments showed 405% (5 fold) increase in activity as against 114% (2.14 fold) increase at Cd-10 uM, indicating the enhanced APX functioning due to Zn supplementation.

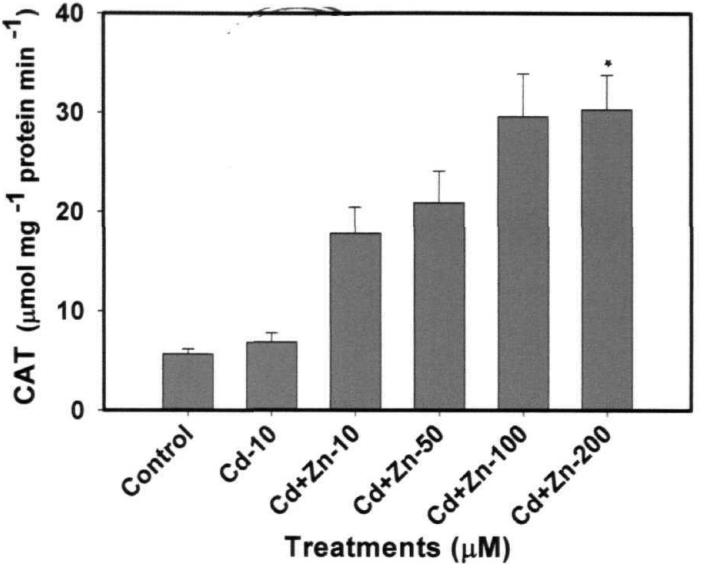


Figure 22. Catalase (CAT) activity in *C. demersum* treated with Cd-10 µM and Zn (10, 50, 100 and 200 µM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

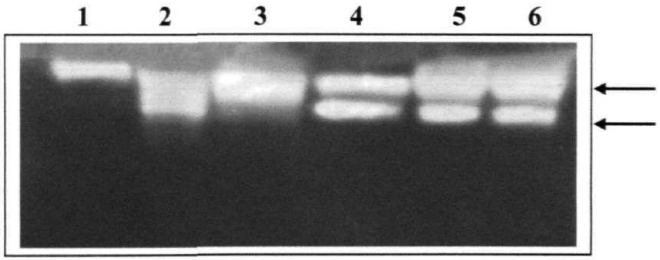


Figure 23. Native gel (7.5%) showing the isozymes of catalase (CAT) stained for in-gel enzyme activity in *C. demersum.* Lanes 1: control. 2: Cd-10 µM, 3: Cd+Zn-10 uM , 4: Cd+Zn-50 µM, 5: Cd+Zn-100 uM, 6: Cd+Zn-200 µM. The arrows indicate enhanced CAT activity at Zn supplemented Cd treatments.

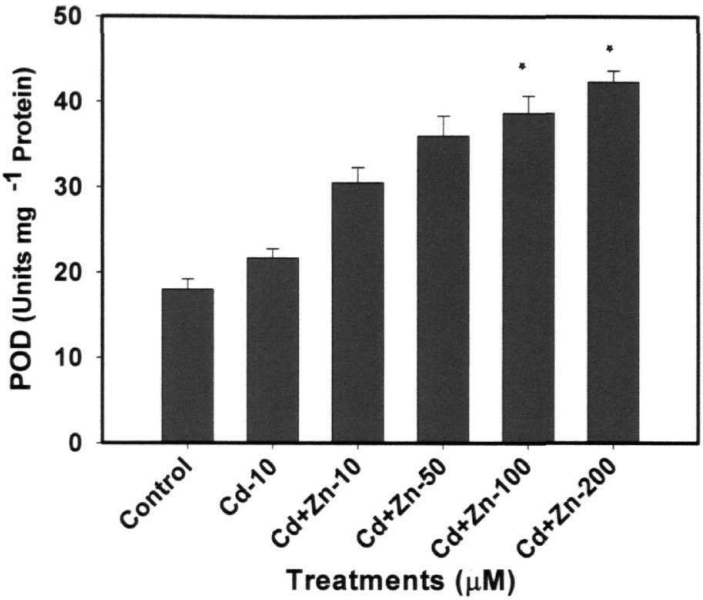


Figure 24. Peroxidase (POD) activity in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

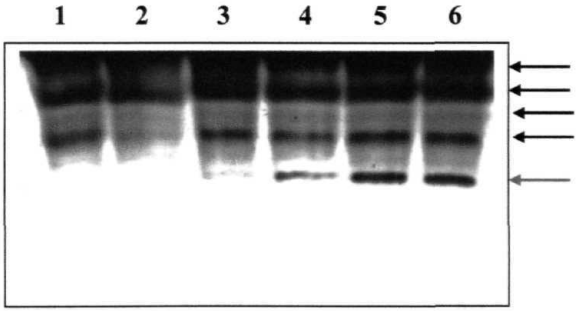


Figure 25. Native gel (10%) showing the isozymes of peroxidase (POD) stained for in-gel enzyme activity in *C. demersum*. Lanes 1: control, 2: Cd-10 μM, 3: Cd+Zn-10 μM, 4: Cd+Zn-50 μM, 5: Cd+Zn-100 uM, 6: Cd+Zn-200 μM. The arrows indicate enhanced POD activity and red arrow indicates induced isozymes at Zn supplemented Cd treatments.

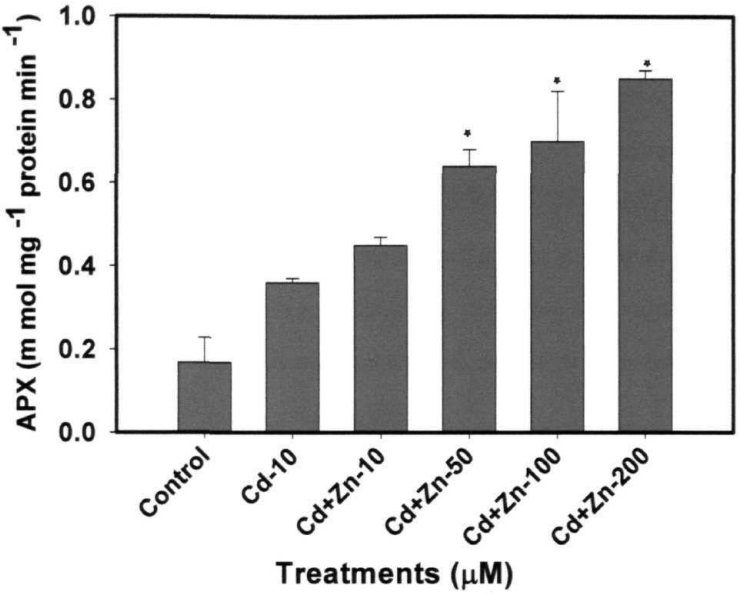


Figure 26. Ascorbate peroxidase (APX) activity in C. *demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

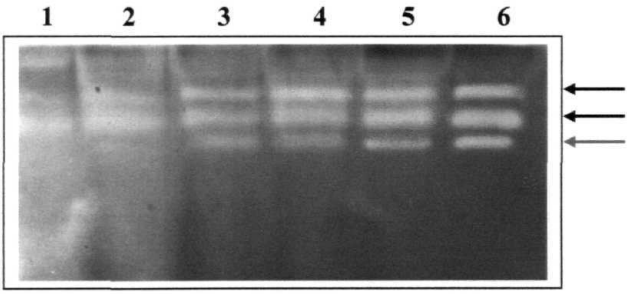


Figure 27. Native gel (12.5%) showing the isozymes of ascorbate peroxidase (APX) stained for in-gel enzyme activity in *C. demersum*. Lanes 1: Control, 2: Cd-10 μM, 3: Cd+Zn-10 μM , 4: Cd+Zn-50 uM, 5: Cd+Zn-100 uM, 6: Cd+Zn-200 uM. The arrows indicate enhanced APX activity and red arrow indicates induced isozymes at Zn supplemented Cd treatments.

Guaiacol peroxidase (GPX, E.C. 1.11.1.7): GPX showed a relatively less difference in activity and also in native gel stained specifically for GPX activity, at all the treatments when compared with other antioxidant enzymes. Cd treatments with Zn-200 uM supplement showed 41% and Cd-10 uM, 16% increase only (see figures 28 and 29, page 79).

In all the above assays Zn-alone treatments induced a slight increase in activity (see table 5, page 80) but not upto the levels of Cd treatments with supplemented Zn indicating that the responses of antioxidant enzymes are due to Cd induced oxidative stress and enhanced activity by Zn supplementation.

3.6. Non-enzymatic cellular antioxidants

Total, protein bound and non-protein thiol contents: The total thiol content (T-SH) indicating the levels of sulphydryl groups showed a drastic 49% reduction in Cd-10 uM treatments, while protein bound thiols (PB-SH) and non-protein thiols (NP-SH) showed 56% and 10% decrease respectively in Cd-10 uM treated plants in comparison with control (see figure 30, page 81). Zinc supplemented Cd treatments showed a 45% restoration of the T-SH levels and hence 51% in PB-SH and 19% in NP-SH levels. Zn alone treatments did not reflect much difference in the levels of thiols (see table 6, page 82).

Cellular glutathione content- Levels of reduced and oxidized glutathione (GSH)

The levels of reduced (GSH) and oxidized glutathione (GSSG) directly point towards the induction of oxidative stress by Cd. Reduced glutathione levels showed a 33% decrease in Cd-alone-treated plants and a 39% increase in the level of GSSG (see figure 31, page 81), thereby altering the GSH/GSSG ratio more towards the oxidized form. Zn supplementation to Cd-treated plants was effective in not only restoring GSH (57%

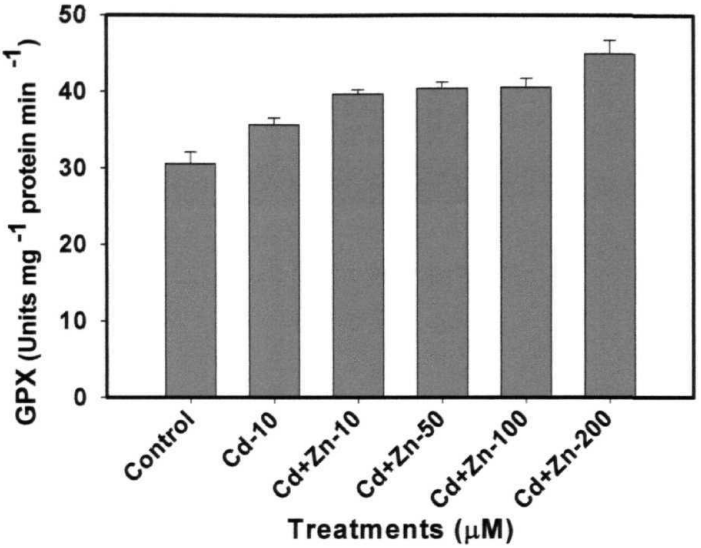


Figure 28. Guaiacol peroxidase (GPX) activity in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors.

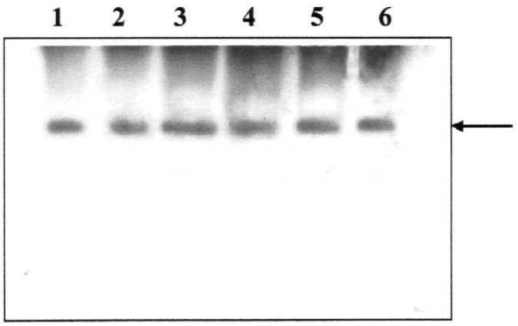


Figure 29. Native gel (7.5%) showing the isozymes of guaiacol peroxidase (GPX) stained for in-gel enzyme activity in *C. demersum*. Lanes 1: control, 2: Cd-10 μM, 3: Cd+Zn-10 μM , 4: Cd+Zn-50 μM, 5: Cd+Zn-100 μM, 6: Cd+Zn-200 μM.

Table 5. Influence of Zn- alone treatments on the antioxidant enzyme activities in *C. demersum*. The values are means of three individual experiments with duplicates. The values in parenthesis indicate standard deviation (n=3) and 'asterisk' indicates significant difference from control.

Treatments (μM)	Superoxide dismutase (Units mg^{-1} protein)	Catalase (μMol mg^{-1} protein min^{-1})	Peroxidase (Units mg^{-1} protein min^{-1})	Ascorbate peroxidase (mM mg^{-1} protein min^{-1})	Guaiacol Peroxidase (Units mg^{-1} prot min^{-1})
Control	22.42(0.71)	5.16(0.43)	18.00(1.09)	0.16(0.027)	30.54(1.19)
Zn-10	25.49 (0.56)*	7.31 (0.43)*	19.23 (0.73)	0.30 (0.023)*	35.45 (4.54)
Zn-50	31.75(0.82)*	8.65 (0.39)*	22.20 (1.11)	0.41 (0.1003)*	35.08(1.88)
Zn-100	46.07(12.16)*	11.25 (0.93)*	23.96(0.55)	1.43 (0.046)*	38.84 (3.27)
Zn-200	48.62 (7.92)*	17.91 (2.74)*	25.78(1.27)	0.48 (0.016)*	40.58 (2.65)*

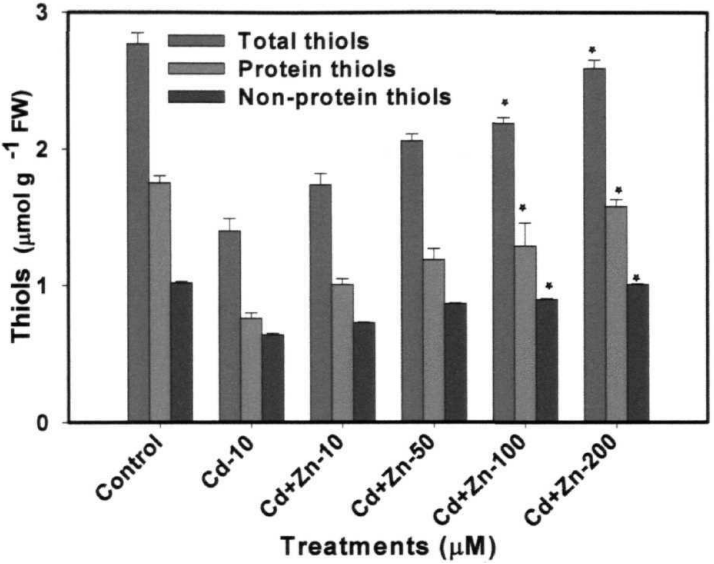


Figure 30. Levels of total thiols, protein- and non-protein thiols in *C. demersum* plants treated with Cd-10 µM and Zn (10, 50, 100 and 200 µM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

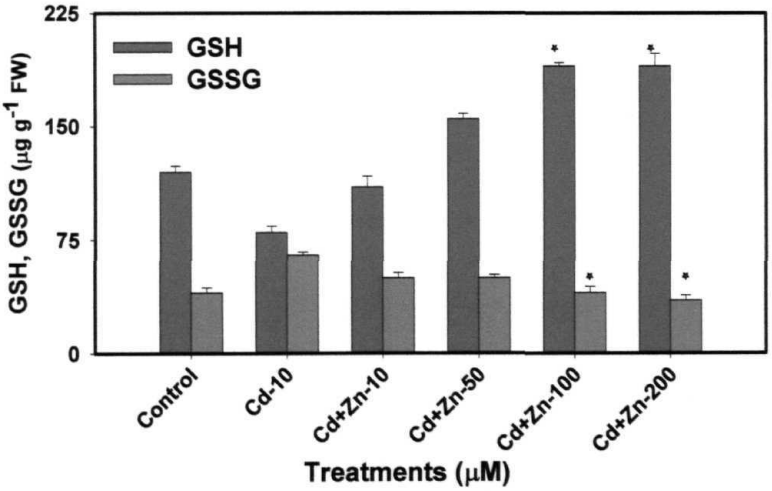


Figure 31. Cellular glutathione content: GSH and GSSG levels in *C. demersum* plants treated with Cd-10 pM and Zn (10, 50, 100 and 200 µM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

Table 6. Influence of Zn on the levels of total thiols, protein bound and non-protein thiols, reduced and oxidized glutathione and total ascorbate, ascorbate and dehydroascorbate contents in *C. demersum*. The values are means of three individual experiments with duplicates. The values in parenthesis indicate standard deviation (n=3).

Treatments (μM)	Total thiols (μmoles g^{-1} FW)	Protein thiols (μmoles g'FW)	Non-protein thiols (umoles g'" FW)	Glutathione reduced (ugg'FW)	Glutathione oxidized (μg g^{-1} FW)	Total ascorbate (μmoles g'FW)	Ascorbate (μmoles g'FW)	Dehydroascorbate (μmoles g'FW)
Control	2.76(0.14)	1.75(0.01)	1.01 (0.01)	120(2.04)	40.0(1.52)	85.93 (3.26)	50.63(2.81)	35.25 (2.64)
10	2.90(0.15)	1.86(0.08)	1.04(0.01)	125(4.22)	42.4(1.38)	91.50(2.30)	56.24 (0.80)	35.26 (0.70)
50	2.93 (0.15)	1.88(0.07)	1.05(0.03)	130(3.42)	42.4(1.04)	96.90 (0.70)	36.92 (2.30)	36.92 (2.30)
100	2.95 (0.09)	1.90(0.14)	1.05(0.04)	150(2.04)	44.0 (0.52)	100.63(3.33)	35.80(1.73)	35.80(3.73)
200	2.98 (0.08)	1.92(0.08)	1.06(0.03)	155(2.04)	45.0 (0.38)	101.63(5.20)	34.82 (2.92)	34.81 (4.92)

restoration), but also indicated an increase in GSH levels (36% increase) with simultaneous decrease of GSSG (47% decrease) (see figure 31, page 81) and hence restored the shift of glutathione from an oxidized state to the active reduced state. Zn alone treatments maintained the GSH/GSSG ratio without indicating any oxidative shift (see table 6, page 82).

Cellular ascorbic acid (AsA) and dehydroascorbate (DHA) content: Total AsA, indicated a very high decrease (64%) whereby the ratio of DHA to AsA was altered more to the oxidized AsA form (DHA) (DHA/AsA ratio of 1.87) in Cd-alone treated plants when compared to control (DHA/AsA ratio of 0.68) (see figure 32, page 84). Total ascorbate was restored completely (72%) with a slight increase (21%) in AsA form and a simultaneous decrease in DHA (DHA/AsA ratio of 0.45 only) as well in Cd-treated plants with supplemented Zn. Zn alone treatments maintained the redox pool of ascorbate (see table 6, page 82).

3.7. Enzymes of ascorbate-glutathione cycle (AGC)

Monodehydroascorbate reductase (MDHAR): In contrast to APX, MDHAR displayed differential response to Cd toxicity. In Cd-10 uM treated plants MDHAR considerably decreased by 34% in comparison to control (see figure 33, page 84). In Cd-treated plants with supplemented Zn there was a restoration (52%) as well as an increase (26%) in MDHAR activity.

Dehydroascorbate reductase (DHAR): The assay of DHAR activity revealed a trend similar to MDHAR, whereby Cd-10 uM treatments reduced DHAR activity (38% reduction). Zn was effective in restoring (51% restoration) as well as increasing DHAR activity (see figure 34, page 85). In-gel activity staining of DHAR proteins (see figure 35, page 85) also indicated increase in DHAR activity at Zn supplemented Cd

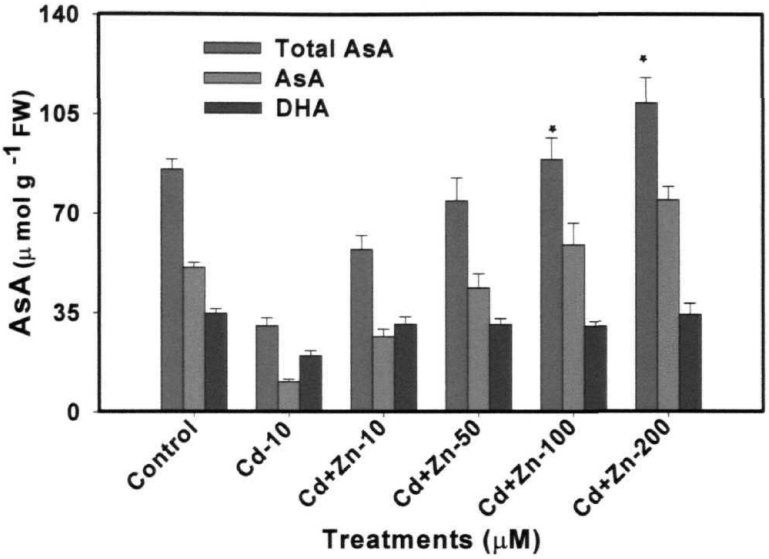


Figure 32. Levels of total ascorbate, ascorbate and dehydroascorbate in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

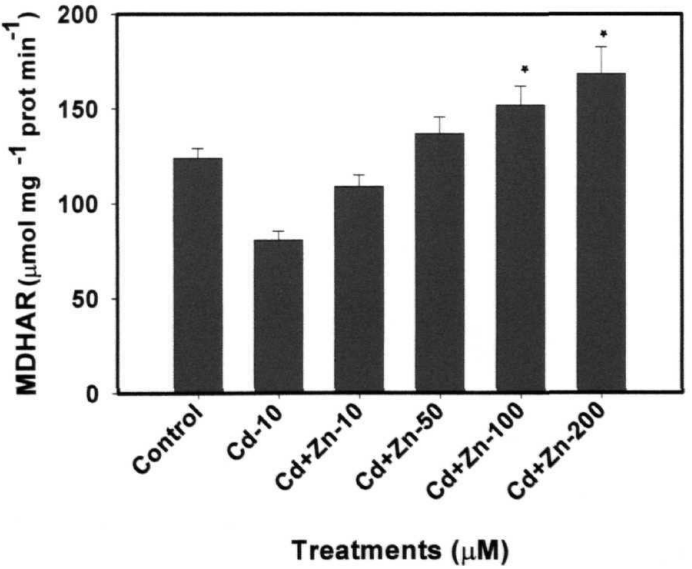


Figure 33. Monodehydroascorbate reductase activity in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

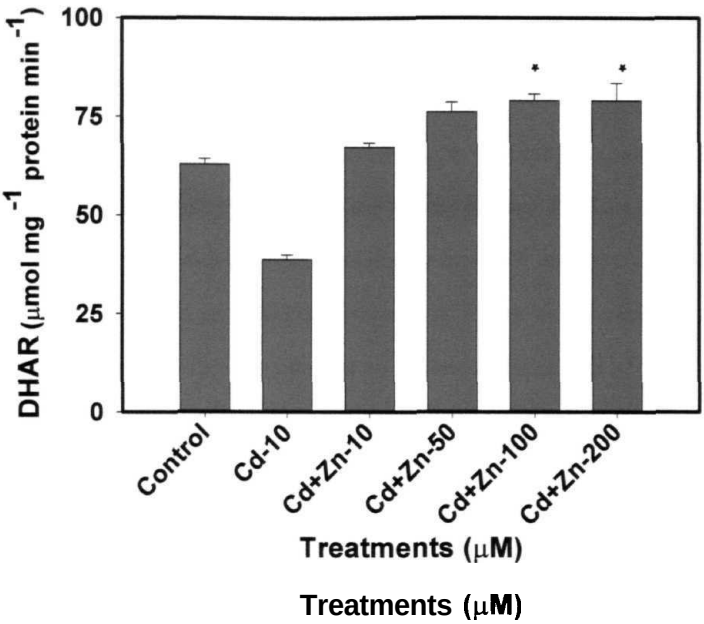


Figure 34. Dehydroascorbate reductase (DHAR) activity in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 (iM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

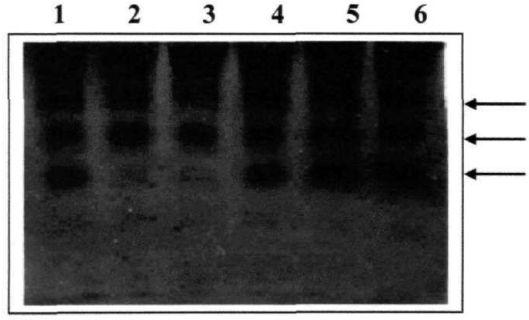


Figure 35. Native gel (7.5%) showing the isozymes of dehydroascorbate reductase (DHAR) stained for in-gel enzyme activity in *C. demersum.* Lanes 1: control, 2: Cd-10 uM, 3: Cd+Zn-10 μM, 4: Cd+Zn-50 μM, 5: Cd+Zn-100 uM, 6: Cd+Zn-200 μM. The arrows indicate enhanced DHAR activity at Zn supplemented Cd treatments.

treatments. The gel showed three isozymes of DHAR stained with increasing intensity at Zn supplemented Cd treatments.

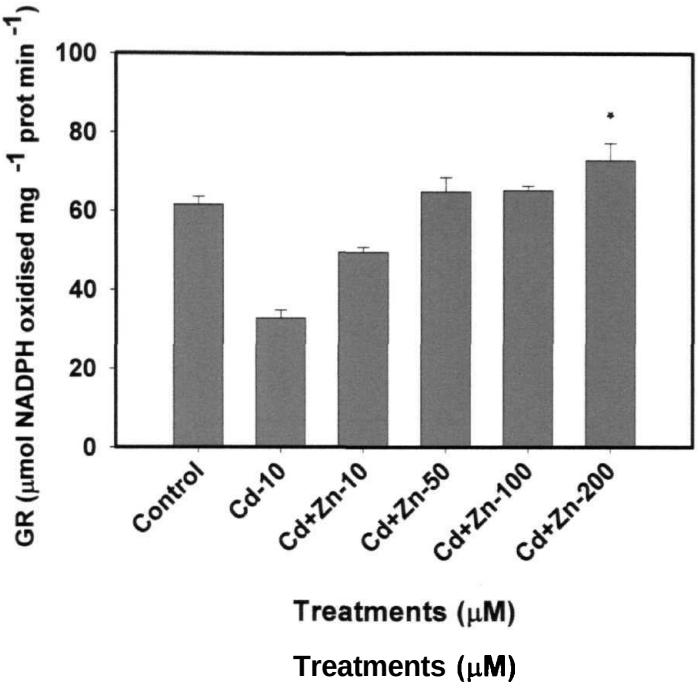
Glutathione reductase (GR): GR activity was drastically reduced (46% decrease) in plants treated with 10 uM Cd. Supplementing Zn to Cd treatments restored (55%) this impairment in GR activity efficiently (see figure 36, page 87). The enzyme activity staining of GR also depicted the same result (see figure 37, page 87). Zn supplemented Cd treatments induced an additional isozyme at higher concentrations due to Zn-enhanced GR activity. This isozyme was not noticed in the control or Cd-alone treated plants, which displayed only four isozymes.

Zn-alone treatments did not affect the functioning of AGC in any way reflecting the non-toxic nature of the Zn concentrations chosen (see table 7, page 88). Thus, the overall cycle of ascorbate and glutathione utilizing antioxidant pathway was impaired due to Cd toxicity and the results presented also suggest that Zn plays an active role in alleviating Cd-induced toxicity through the efficient functioning of AGC. Similar but unique effects were noticed on the glutathione metabolism enzymes in *C. demersum* in response to Cd alone and Cd together with Zn treatments.

3.8. Glutathione metabolism enzymes

γ-Glutamylcysteine synthetase (γ-GCS): The toxicity imposed by Cd on *C. demersum* did not affect y-GCS, an important enzyme involved in GSH biosynthesis. There was only a slight decrease in Cd treatments, which was restored by Zn (see figure 38, page 89).

Glutathione S-transferase (GST, E.C. 2.5.1.18): GST, an important cellular detoxifier of metabolites involved in oxidative stress indicated a 14% increase in Cd-10 uM alone



Treatments (μM)

Figure 36. Glutathione reductase (GR) activity in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

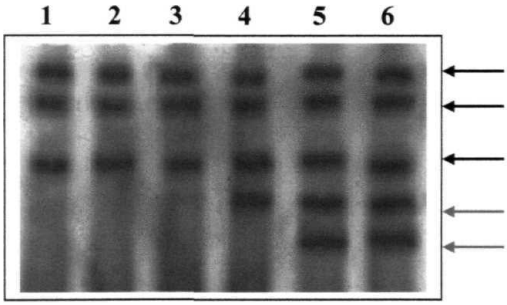


Figure 37. Native gel (7.5%) showing the isozymes of glutathione reductase (GR) stained for in-gel enzyme activity in *C. demersum.* Lanes 1: control, 2: Cd-10 μM, 3: Cd+Zn-10 μM, 4: Cd+Zn-50 μM, 5: Cd+Zn-100 μM, 6: Cd+Zn-200 μM. The arrows indicate enhanced GR activity and red arrow indicates induced isozymes at Zn supplemented Cd treatments.

Table 7. Influence of Zn on the ascorbate-glutathione cycle enzymes (APX, MDHAR, DHAR and GR) in *C. demersum.* The values are means of three individual experiments with duplicates. The values in parenthesis indicate standard deviation (n=3).

Treatments (μM)	Monodehydroascorbate reductase (μ moles mg^{-1} protein min^{-1})	Dehydroascorbate reductase (μ moles mg^{-1} protein min^{-1})	Glutathione reductase (μ moles NADPH oxidized mg^{-1} protein min^{-1})
Control	123.11(4.05)	62.37(3.18)	61.82(6.31)
10	127.68 (6.28)	64.04(2.13)	61.80(8.87)
50	133.92(4.40)	65.32 (0.98)	65.15(7.29)
100	133.79(4.87)	65.37 (4.53)	65.58 (3.28)
200	140.21 (10.79)	65.40 (5.96)	66.66 (4.05)

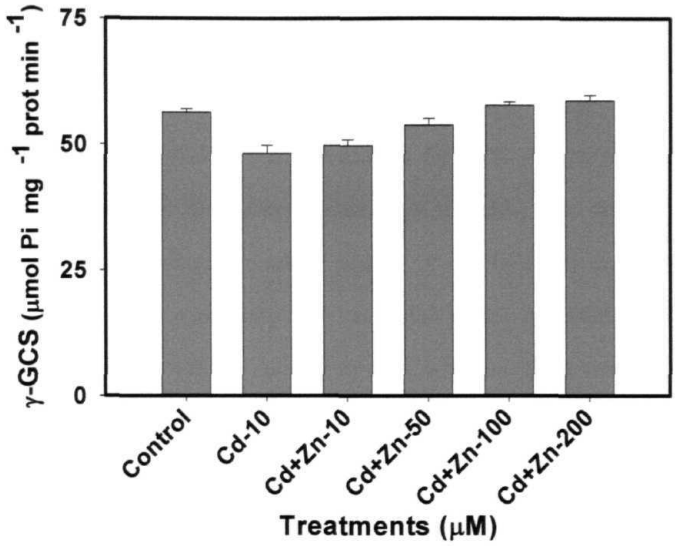


Figure 38. γ-Glutamylcysteine synthetase activity in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

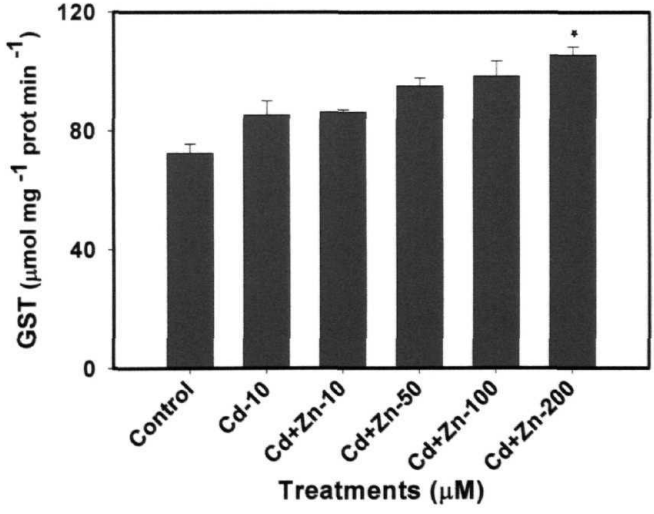


Figure 39. Glutathione-S-transferase activity in C. *demersum* treated with Cd-10 μM and Zn (10, 50, 100, and 200 (iM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

treated plants, whereas Cd-treated plants with supplemented Zn stimulated (31 %) GST activity accounting for the increased detoxification of stress metabolites (see figure 39, page 89).

Glutathione peroxidase (GSH-PX, E.C. 1.11.1.9): GSH-PX is an important GSH utilizing H_2O_2 scavenger. Since care was taken not to expose GSH to air and GSSG-reductase was included in the reaction mixture, it can be safely assumed that the observations made are specifically due to GSH-PX activity. GSH-PX showed a significant decrease of 40% in its activity in Cd-10 uM alone treated plants. Zn supplementation to Cd treatments efficiently restored as well as increased (57%) the activity of GSH-PX accounting for increased ROS scavenging induced by Zn (see figure 40, page 91). Zn alone treatments did not show much variation in the above parameters indicating the non-toxic nature of the Zn concentrations chosen (see table 8, page 92)

3.9. Structural, conformational and functional changes of carbonic anhydrase in response to Zn and Cd

Carbonic anhydrase activity (CA, E.C. 4.2.1.1): The results of the assay of CA revealed a drastic 50% reduction in the activity of this enzyme in Cd 10 uM alone treated plants when compared to the control plants (see figure 41, page 91). The activity was not only restored (67%) completely by Zn in Cd-treated plants with supplemented Zn, but it was also increased (34%) beyond the control level. However plants treated with only Zn showed a slight increase in CA activity (see table 8, page 92), but not upto the levels of Cd + Zn-200 uM treatments.

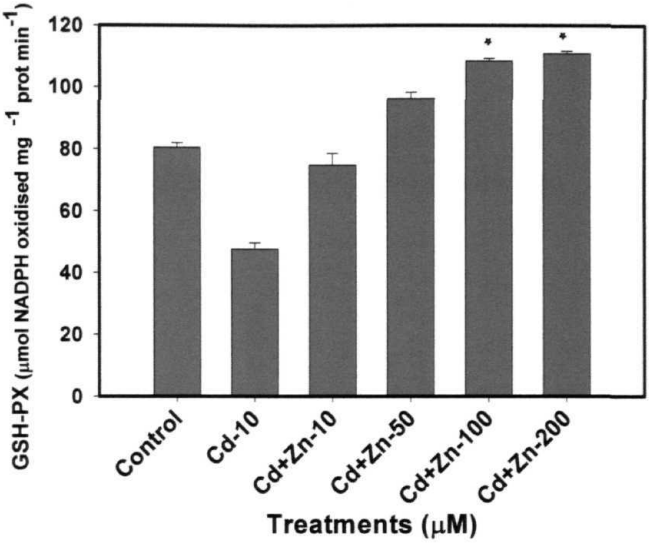


Figure 40. Glutathione peroxidase activity in *C. demersum* treated with Cd-10 µM and Zn (10, 50, 100 and 200 µM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

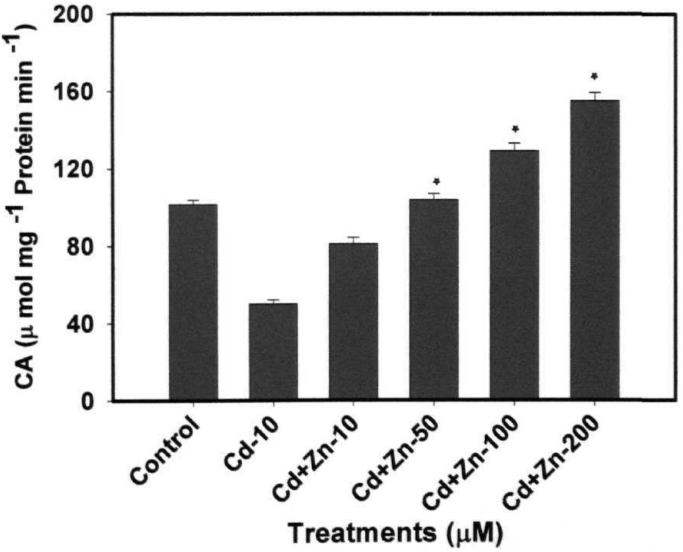


Figure 41. Carbonic anhydrase activity in *C. demersum* treated with Cd-10 (iM and Zn (10, 50, 100 and 200 µM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

Table 8. Influence of Zn on glutathione metabolism enzymes **(γ-GCS,** GST, GSH-PX) and carbonic anhydrase activity in *C. **demersum.*** The values are means of three individual experiments with duplicates. The values in parenthesis indicate standard deviation **(n=3)**

Treatments (uM)	γ-Glutamylcysteine synthetase (umoles Pi liberated mg^{-1} protein min^{-1})	Glutathione-s-transferase (umoles mg^{-1} protein min^{-1})	Glutathione peroxidase (umoles NADPH oxidized mg^{-1} protein min^{-1})	Carbonic anhydrase (umoles mg^{-1} protein min^{-1})
Control	56.30(1.23)	72.70 (3.27)	80.32 (4.56)	102.77(3.91)
10	54.43 (0.68)	78.71 (6.73)	81.93(4.71)	102.77(4.65)
50	53.32(1.02)	79.05 (4.87)	86.12(2.01)	**115.43 (4.97)**
100	54.43 (0.90)	83.67(1.14)	86.12(1.21)	119.13(1.52)
200	55.57(1.56)	86.52(1.83)	93.54(1.66)	127.64(2.67)

***Purification of carbonic anhydrase*:** The purification profile of CAs from different treatments are shown in tables 9-12 (see pages 98, 99) and the elution profiles are shown in figures 42-49 (see pages 94-97). There was a 8.5 fold purification of the enzyme from the soluble plant protein in all the treatments. There were large differences only in the recovery of the active enzyme from each treatment. Control plants (without any metal treatments) showed a 32% recovery of the active purified protein (see table 9, page 98). Cd-treated plants showed toxicity to the enzyme as seen in the form of a very low recovery (22%) of the purified active form (see table 10, page 98). Zn supplementation to Cd-treated plants proved to be beneficial as a highly active CA with a higher recovery of 43% was purified (see table 11, page 99). Treatments with only Zn also did not affect the enzyme activity and its purification (see table 12, page 99) and a 33% recovery was observed for the purified enzyme. Electrophoresis on a 13% gel revealed the homogeneity of the purified enzyme and the molecular weight of purified CA was 43 kDa (see figure 50, page 100) with an additional closely associated isozyme stained as a second band very near to the purified enzyme.

Zn and Cd content of the purified carbonic anhydrase: Estimation of the metal content in the purified form of CAs revealed interesting results. Zn content was drastically reduced in Cd-alone-treated plants showing a 73% reduction in the Zn content accounting for the impaired CA activity in Cd-treated plants. A very important observation found in our results was the detection of trace amounts of Cd ($0.028 \mu g\ mg^{-1}$ protein) in CA purified from Cd-treated plants (see figure 51, page 100). In contrast in Cd-treated plants with supplemented Zn there was a total restoration of the Zn content (77%) as well as a slight increase in Zn content indicating the nature of Zn in overcoming Cd toxicity. Zn-alone treated plants however did not show differences in the Zn content from the CA purified from control. Except in CA purified from Cd-treated plants there was no trace detection of Cd in other treatments.

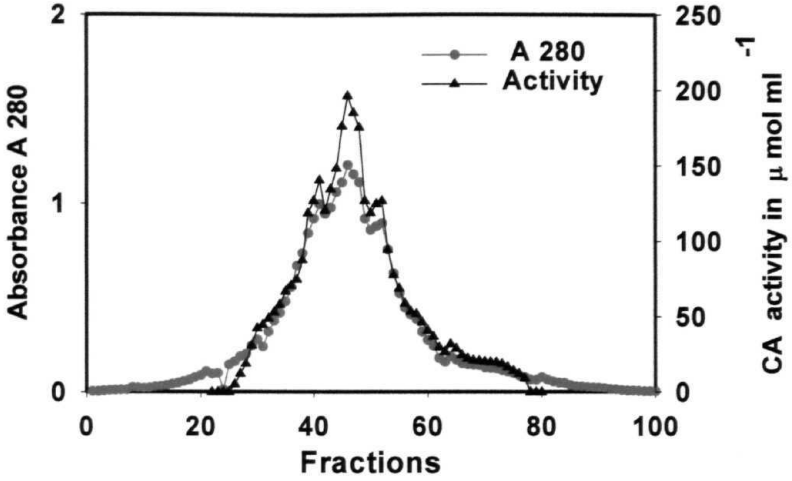


Figure 42. Elution profile of the purification of carbonic anhydrase from control plants of *C. demersum* through DEAE Sephadex column.

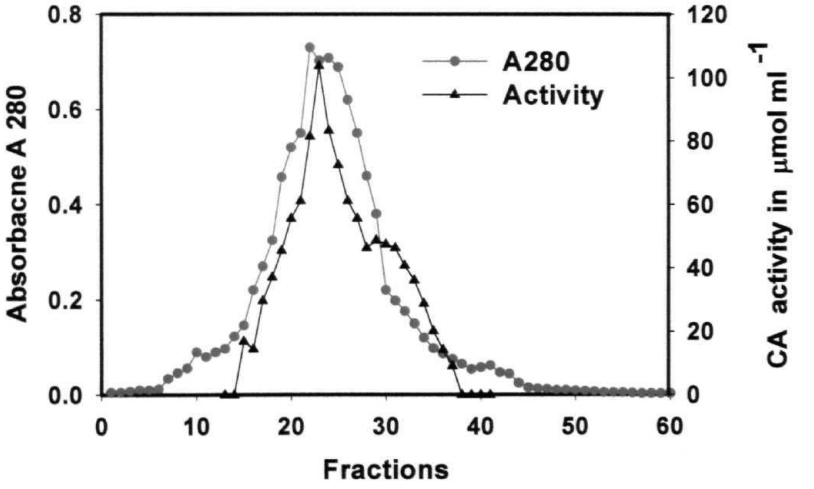


Figure 43. Elution profile of the purification of carbonic anhydrase from control plants of *C. demersum* through Sephadex G25 column.

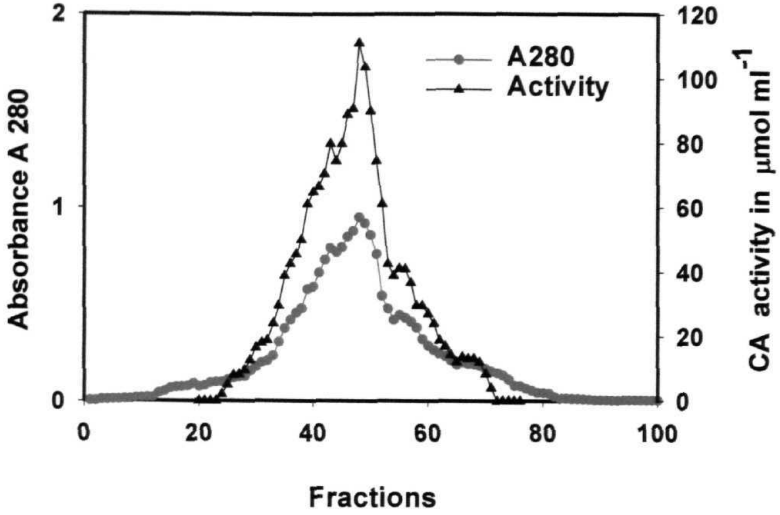


Figure 44. Elution profile of the purification of carbonic anhydrase from Cd 10 µM treatments of C. *demersum* through DEAE Sephadex column.

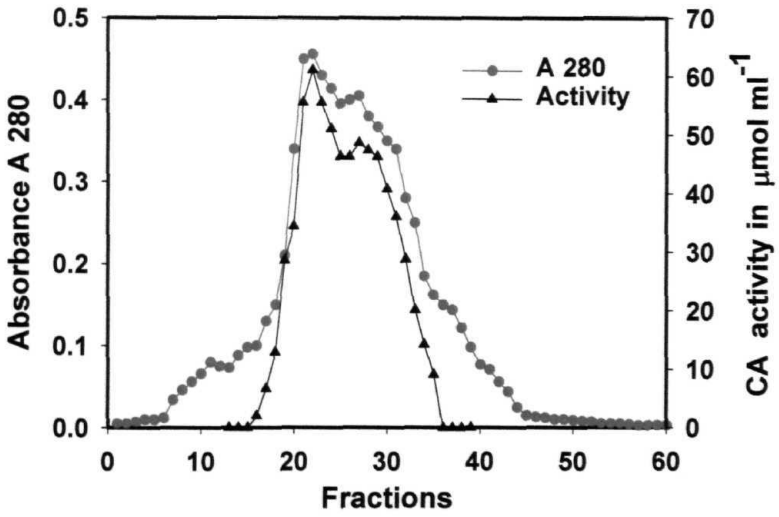


Figure 45. Elution profile of the purification of carbonic anhydrase from Cd 10 µM treatments of *C. demersum* through Sephadex G25 column.

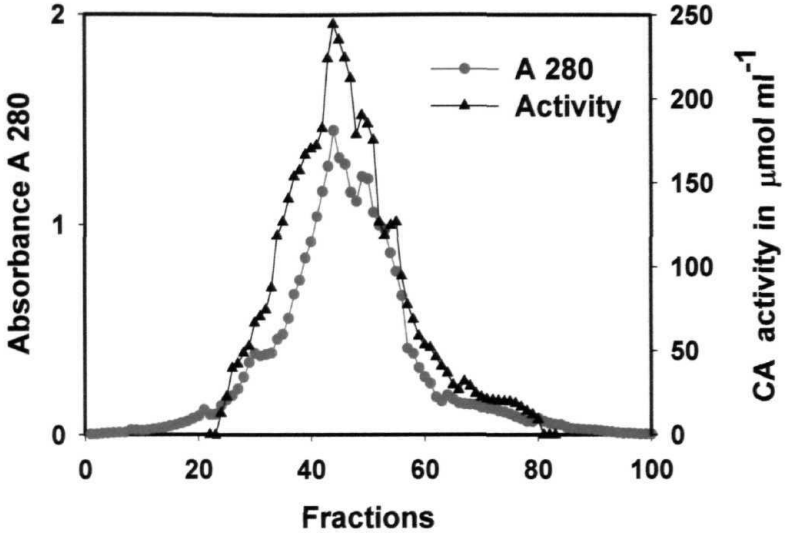


Figure 46. Elution profile of the purification of carbonic anhydrase from Zn -200 µM supplemented Cd 10 uM treatments of *C. demersum* through DEAE Sephadex column.

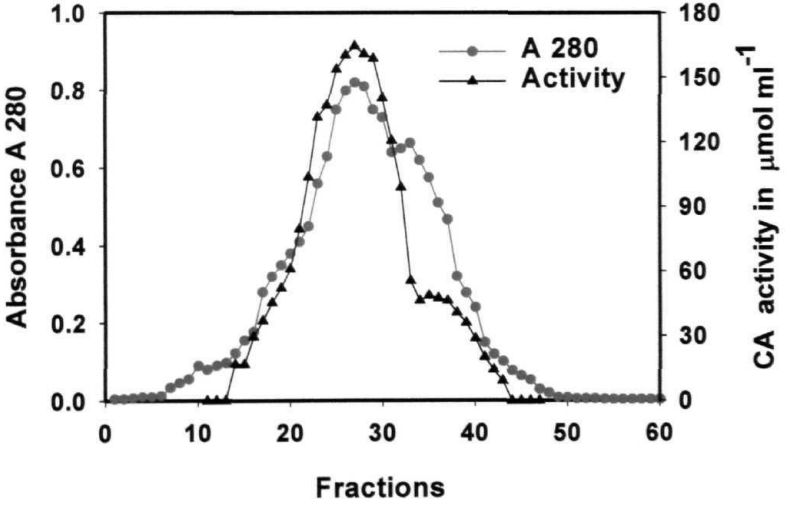


Figure 47. Elution profile of the purification of carbonic anhydrase from Zn -200 supplemented Cd 10 µM treatments of *C. demersum* through Sephadex G25 column.

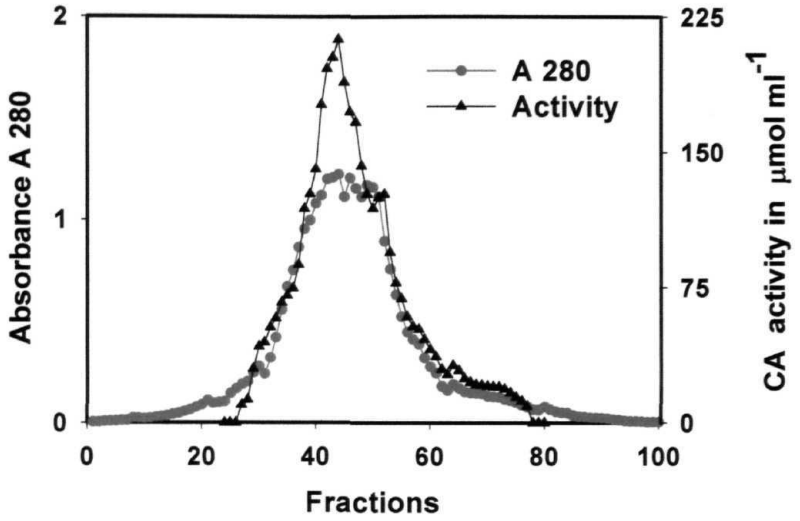


Figure 48. Elution profile of the purification of carbonic anhydrase from Zn -200 µM treatments of *C. demersum* through DEAE Sephadex column.

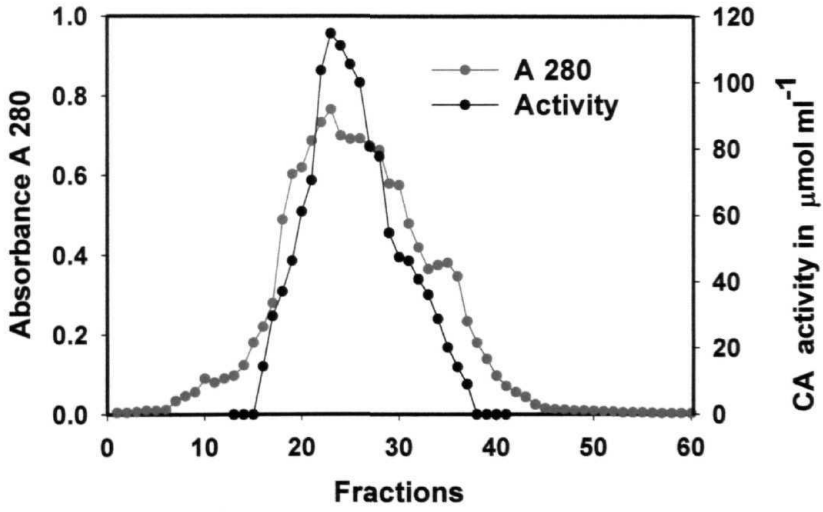


Figure 49. Elution profile of the purification of carbonic anhydrase from Zn -200 µM treatments of *C. demersum* through Sephadex G25 column.

Table 9. Purification profile of carbonic anhydrase from control plants of *Ceratophyllum demersum.*

Protein source	Protein content (mg ml^{-1})	Activity (umol ml^{-1})	Volume of the protein (ml)	Total protein	Total activity	Specific activity (umol mg^{-1} protein)	Yield (%)	Fold
Homogenate supernatant	5	59	60	300	3540	11.8	100	1
Pooled "active" fractions from DEAE- sephadex	1.8	65	30	54	1890	36.11	53.4	3.06
Pooled "active" fractions from Sephadex G-25	0.77	77	15	11.55	1155	100	32.5	8.47

Table 10. Purification profile of carbonic anhydrase from Cd-10 uM treated plants *of Ceratophyllum demersum.*

Protein source	Protein content (mg ml^{-1})	Activity (µmol ml^{-1})	Volume of the protein (ml)	Total protein	Total activity	Specific activity (µmol mg^{-1} protein)	Yield (%)	Fold
Homogenate supernatant	3.2	25	60	192	1500	7.81	100	1
Pooled "active" fractions from DEAE-sephadex	1.27	30	24	30.48	720	23.62	48	3.02
Pooled "active" fractions from Sephadex G-25	0.58	38	9	5.22	342	65.51	22.8	8.38

Table 11. Purification profile of carbonic anhydrase from Cd +Zn-200 uM treated plants *of Ceratophyllum demersum.*

Protein source	Protein content (mg ml^{-1})	Activity (µmol ml^{-1})	Volume of the protein (ml)	Total protein	Total activity	Specific activity (µmol mg^{-1} protein)	Yield (%)	Fold
Homogenate supernatant	6.7	85.88	60	402	5152.8	12.81	100	1
Pooled "active" fractions from DEAE-sephadex	3.0	116.81	35	105	4088.35	38.93	79.35	3.03
Pooled "active" fractions from Sephadex G-25	1.15	125.92	18	20.7	2266.56	109.49	43.5	8.54

Table 12. Purification profile of carbonic anhydrase from Zn-200 µM treated plants *of Ceratophyllum demersum.*

Protein source	Protein content (mg ml^{-1})	Activity	Volume of the protein (ml)	Total protein	Total activity	Specific activity (µmol mg^{-1} protein)	Yield (%)	Fold
Homogenate supernatant	5.8	70.37	60	348	4222.2	12.13	100	1
Pooled "active" fractions from DEAE-sephadex	2.1	77.77	32	67.2	2488.64	37.03	59	3.05
Pooled "active" fractions from Sephadex G-25	0.87	88.88	16	13.92	1422.08	102.16	33.7	8.42

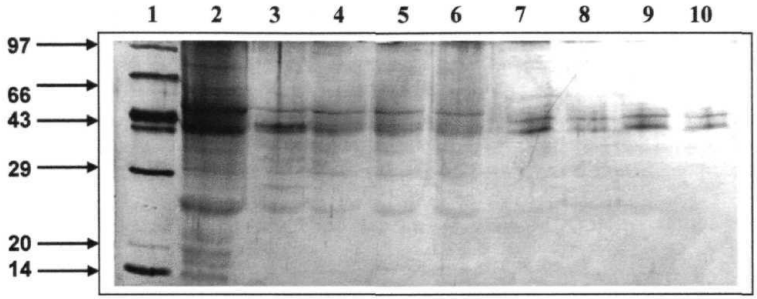


Figure 50. 13% SDS PAGE showing the protein profile at different stages of purifcation. Lane 1: molecular Wt. Markers, 2: crude extract, Lanes 3-6: DEAE Sephadex elution fractions of Control, Cd-10 |xM, Cd+Zn-200 μM and Zn-200 μM, Lanes 7-10: Sephadex G-25 elution fractions showing the purified carbonic anhydrase in the same order as above.

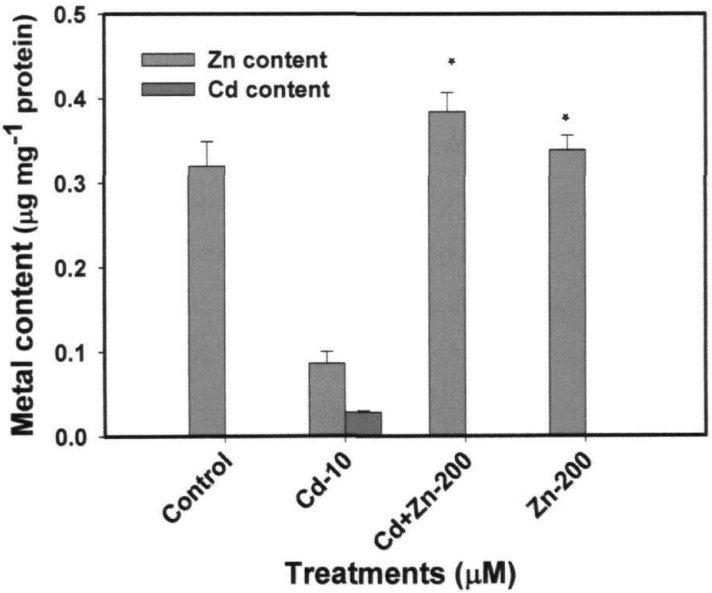


Figure 51. Zn and Cd content of purified carbonic anhydrase from *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

3.10. Structural and conformational studies on carbonic anhydrases

Ultraviolet difference spectra of carbonic anhydrases: The difference spectra of CAs from control, Cd alone treated, Cd treatments with supplemented Zn and Zn alone treatments gave a good indication of the nature of Zn in protecting and restoring the native state of functional CA. The UV spectra of CAs from different treatments are shown in figure 52, page 102. The spectra of native CA from control showed absorbance maxima at the range of 270-285 nm indicating contributions predominantly from tyrosine and tryptophan residues. It can be clearly observed that Cd treatments decreased (37%) the UV absorbance of the purified CA compared to control. This was restored (43%) by Zn supplements to Cd treatments indicating the nature of Zn in maintaining the structure of CA. Zn alone treatments did not reflect any change in the UV spectra.

Circular dichroism (CD) spectra of carbonic anhydrases: CD spectra was used as the tool to evaluate the extent of Zn-Cd interactions on the secondary structure conformation of CA. Figure 53 on page 102 Shows the near UV-CD spectra of native CA from control plants, CA purified from Cd-alone treated, Cd-treatments with supplemented Zn and Zn alone treated plants. There is a sizable negative signal (0= -2,466) centered at 274 nm for the native enzyme, which increased to a more negative extremum in Zn supplemented Cd treatments (0= -2990) and Zn alone treatments (θ= -2780). However in Cd-alone-treated plants there was a striking qualitative difference in the peak exhibiting a drastic decrease (θ= -323) in ellipticity. At shorter wavelengths of near UV, the CD spectrum indicated a strong positive peak (θ= 612) at 248 nm in the native CA from control plants. The same pattern was observed in CA isolated from Cd treatments with supplemented Zn (0= 548) and Zn alone treated

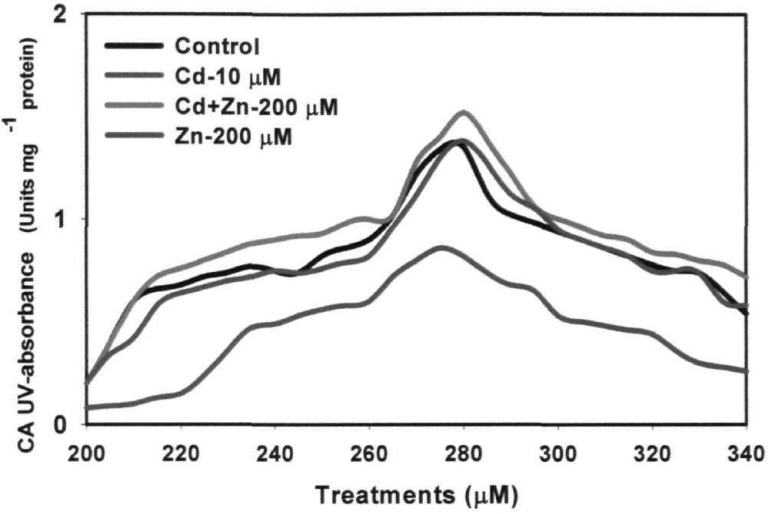


Figure 52. Ultraviolet (UV) difference spectra of CAs purified from control, Cd-10 μM, Cd-10 uM supplemented with Zn 200 uM and Zn 200 uM treatments of *C. demersum.*

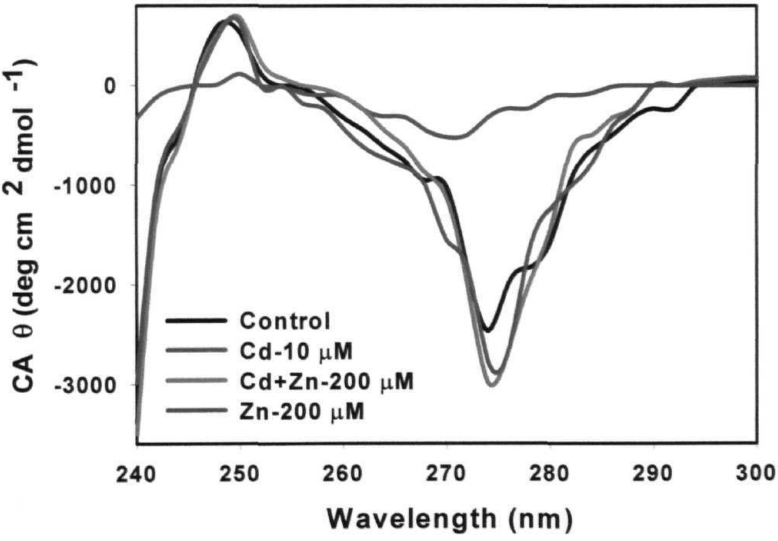


Figure 53. Near UV-Circular dichroism spectra of CAs purified from control, Cd-10 μM, Cd-10 uM supplemented with Zn 200 μM and Zn 200 uM treatments of *C. demersum.*

plants (θ= 512) but not in CA from Cd-alone treatments which indicated a reduced almost diminished signal (θ= 14) at this wavelength.

Further differences were clearly observed in the far UV-CD signal pattern of CA (see figure 54, page 104). Native enzyme purified from control plants exhibited strong negative peaks at 222 nm (θ= -15,500) and 208 nm (0= -10,986) with strong positive peak at 192 nm (0= 5555) characteristic of a typical a-helix secondary conformation. The ellipticity of these peaks clearly showed that CA purified from C. *demersum* predominantly exists in a-helix conformation in its secondary state of folding. Far UV-CD spectra of CA purified from Cd-alone-treated plants neither showed the 222 nm peak distinctly nor an observable 208 nm peak or 192 nm peak. This indicates disruption and unfolding of the a-helix conformation to a random and open coiling state induced by Cd-toxicity. Zinc supplementation to Cd treatments clearly shows the protection of CA native structure as observed by distinct α-helical peaks with an increased ellipticity (222: 0= -16,138, 208: 0= -11,012, and 192: 0= 5900). Carbonic anhydrase purified from Zn alone treatments did not reflect much variation in the helical ellipticities from control CA, indicating the non-toxic nature of the Zn concentrations chosen. Table 13 on page 104 shows the percentage of each form of secondary structure in CA purified from control, Cd alone and Zn alone treatments and Cd treatments with supplemented Zn. The results clearly indicate the exact pattern depicted by the spectra. CA purified from control, Cd treatments with supplemented Zn, and Zn alone treatments showed high percentage of a-helix conformation (43%, 45% and 44% respectively), whereas CA isolated from Cd-alone treated plants showed a clear deprival of the ordered a-helix conformation (only 29%) and increase in random coil state (41%).

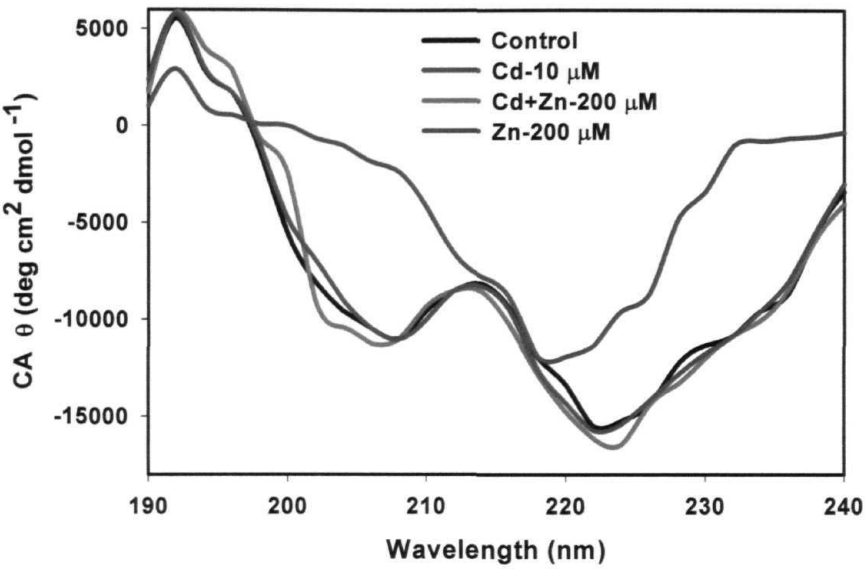


Figure 54. Far UV-circular dichroism spectra of CAs purified from control, Cd-10 uM, Cd-10 uM supplemented with Zn 200 uM and Zn 200 uM treatments of *C. demersum.*

Table 13. Percentage of different forms of secondary structure of CA identified from CD spectral analysis of CA purified from control, Cd 10 uM and Cd 10 uM supplemented with Zn 200 uM and Zn 200 uM concentrations of C. *demersum.* The value of each contributing form was calculated as given in methods according to Chen *et al* 1974.

Treatments	Forms of secondary' structure (%)		
	α-Helix	P-structure	Random coil
Control	43	25	32
Cd 10 μM	29	30	41
Cd 10 μM + Zn 200 μM	45	26	29
Zn 200 μM	44	26	30

Fluorimetric studies of carbonic anhydrases

Intrinsic fluorescence- tryptophan: Conformational changes accompanying Cd stress to CA and Zn modulated structural changes in Zn supplemented Cd treatments and Zn alone treated plants were monitored in comparison with control by the tryptophan fluorescence spectrum. The emission spectrum of native CA from control plants showed a maximum at 335 nm (see figure 55, page 106). In contrast CA purified from Cd 10 μM-alone treated plants showed decreased fluorescence intensity (53%) at 335 nm. An important observation noted from this spectrum was the shift in the emission maxima from 335 to 348 nm in CA purified from Cd 10 μM-alone treatments. On the other hand CA from Zn supplemented Cd treatments showed restoration and clear protection of protein folding as observed by the restored fluorescence (57%) of tryptophan residues with maxima at characteristic 335 nm. Zn alone treatments did not induce any change in tryptophan fluorescence.

Tyrosine and dityrosine fluorescence: The emission spectra of the aromatic side chain amino acid tyrosine of CAs from different treatments are shown in figure 56, page 106. Intact native enzyme purified from control plants displayed high fluorescence intensity with emission maxima at 310 nm. CA from Cd 10 μM-alone treated plants displayed the same trend of decrease (36%) in emission intensity followed by shift in emission maxima to 317 nm in tyrosine fluorescence. In continuation of tyrosine modification dityrosine, a product of its oxidation was also monitored. Dityrosine clearly showed increased accumulation in CA from Cd 10 μM-alone treated plants as observed by an increase (24%) in fluorescence intensity compared to native CA from control (see figure 57, page 107). Zn supplementation showed completely functional and unoxidized form of tyrosine as depicted by the fluorescence spectra, whereby tyrosine fluorescence was

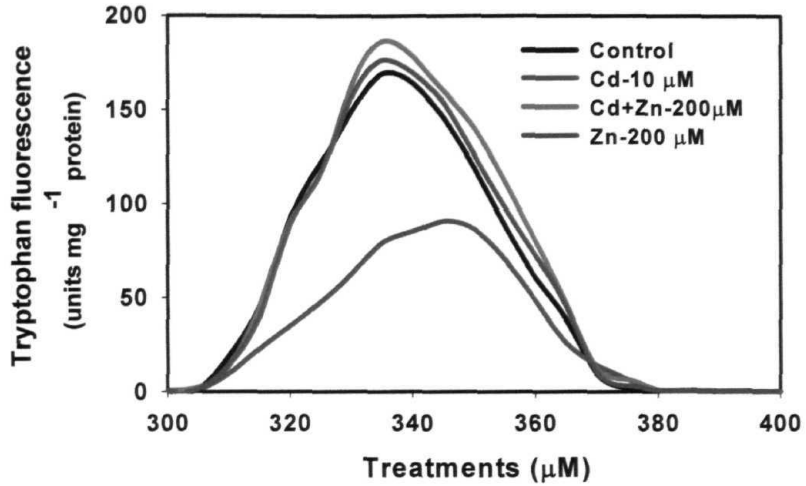


Figure 55. Spectra showing intrinsic fluorescence of tryptophan in CAs purified from control, Cd-10 uM, Cd-10 uM supplemented with Zn 200 μM and Zn 200 μM treatments of *C. demersum.*

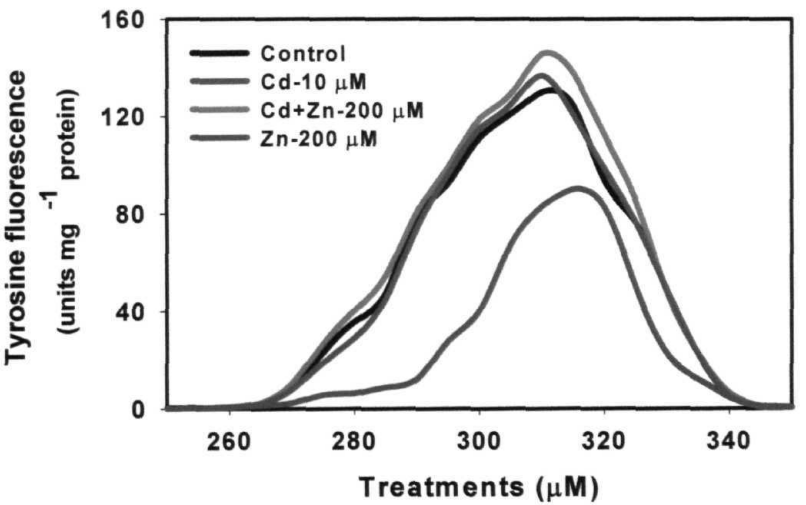


Figure 56. Spectra showing intrinsic fluorescence of tyrosine in CAs purified from control, Cd-10 uM, Cd-10 μM supplemented with Zn 200 μM and Zn 200 uM treatments of *C. demersum.*

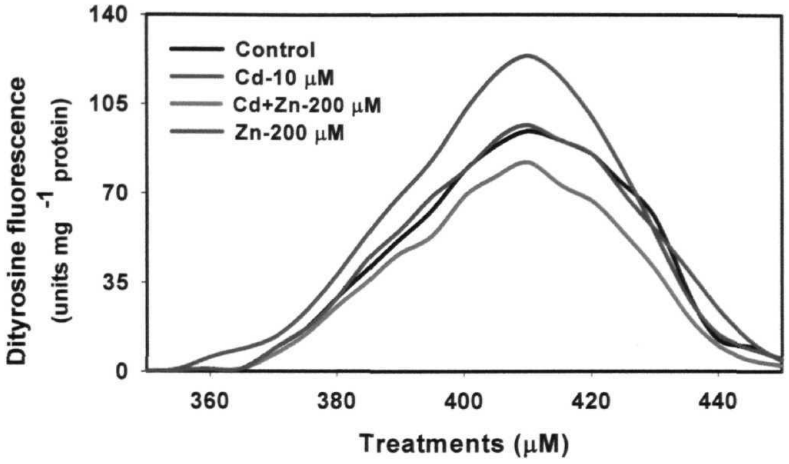


Figure 57. Fluorescence spectra indicating dityrosine levels in CAs purified from control, Cd-10 uM, Cd-10 uM supplemented with Zn 200 uM and Zn 200 uM treatments of C. *demersum.*

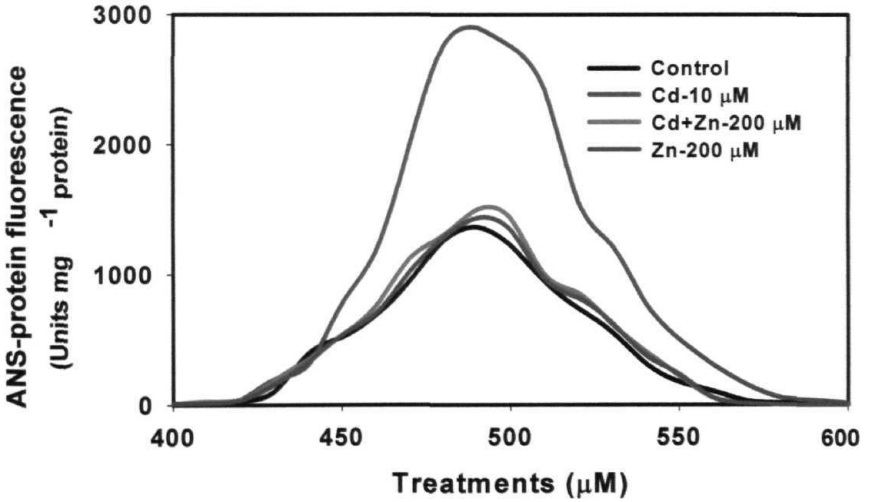


Figure 58. ANS-protein fluorescence as a measure of surface hydrophobicity in CAs purified from control, Cd-10 uM. Cd-10 uM supplemented with Zn 200 uM and Zn 200 uM treatments of *C. demersum.*

restored (43%) and increased at its peak (310 nm maxima) with a simultaneous reduction (34%) in dityrosine fluorescence. Zn alone treatments did not indicate variation from the spectrum of CA from control plants.

Extrinsic fluorescence- surface hydrophobicity as a measure of protein unfolding: The measurement of surface hydrophobicity through 8-anilino-1-naphthalene sulfonic acid (ANS)-protein binding fluorescence indicated Cd-induced surface modification to CA in Cd-alone treatments, clearly observed as high increase (53%) in ANS-protein fluorescence with a concomitant blue shift of the spectrum (see figure 58, page 107) in comparison with native CA from control plants. Zn supplementation to Cd treatments reduced the hydrophobicity of CA observed as a significant decrease (48%) in ANS-protein fluorescence. Zn alone treatments did not indicate many changes in hydrophobicity.

3.11. Sulphydryl (SH) groups of carbonic anhydrases: The oxidative stress induced by Cd was clearly reflected in the sulphydryl (SH) groups estimated as thiol content using 5, 5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB). The thiol content reduced (27%) in CA purified from Cd stressed (10 uM-alone) treatments when compared to control (see figure 59, page 109) . In Cd treatments with supplemented Zn, there was restoration (20%) of thiol content almost equalising the control.

3.12. Carbonyl content as a measure of oxidative damage to carbonic anhydrases: Estimation of the carbonyl content of CA showed direct evidence of oxidative stress conferred upon CA by Cd. CA purified from Cd 10 uM-alone treated plants showed a drastic increase (55%) in the carbonyl formation (see figure 59, page 109) indicating that Cd promoted high oxidation of the protein. In contrast Zn reduced (69%) the amount of carbonyls formed as estimated in Zn supplemented Cd treatments. Zn alone treatments did not induce much variation in the thiol or carbonyl content.

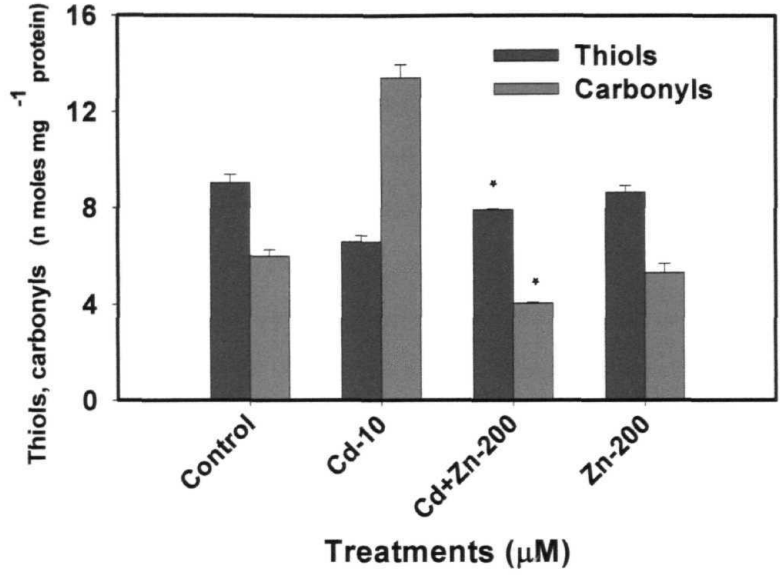


Figure 59. Thiols and carbonyls content of CAs purified from control, Cd-10 uM, Cd-10 uM supplemented with Zn 200 uM and Zn 200 uM treatments of *C. demersum*.

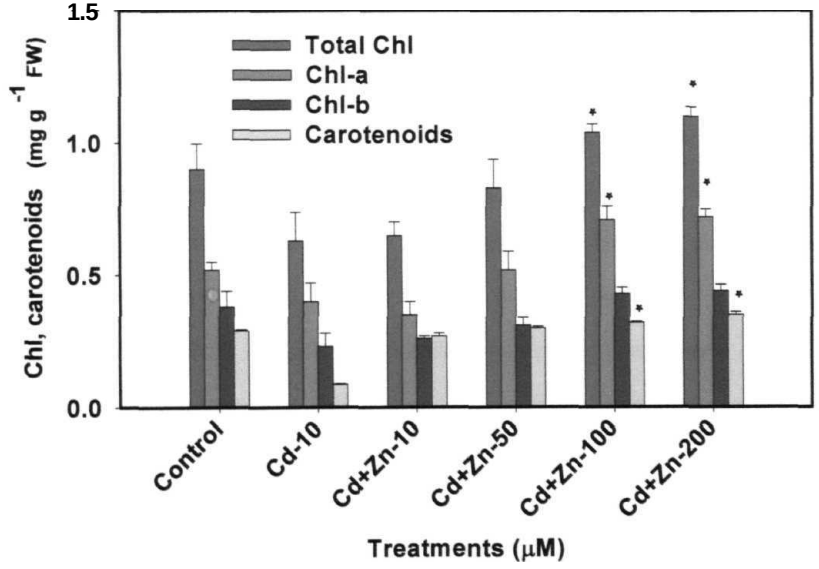


Figure 60. Levels of photosynthetic pigments in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

3.13. Analysis of photochemical functions

Quantification of photosynthetic pigments: The levels of chlorophyll also indicated the toxic nature of Cd to the plant system. Chlorophyll a and b showed 23% and 40% reduction in Cd treated plants respectively. Cd-treated plants with supplemented Zn however showed 44% and 47% restoration of the chlorophyll levels (see figure 60, page 109). Carotenoids were also equally affected as chlorophyll with reduction in carotenoids level in Cd treated plants being a drastic 70% from the control. Cd treated plants with supplemented Zn showed active restoration (75%) of the decreased carotenoid levels indicating its efficiency in protecting pigment levels in the system (see figure 60, page 109). However Zn alone treatments retained the levels of chlorophyll and carotenoids in the plant system (see table 14, page 112).

Intactness of chloroplasts: The intactness of isolated chloroplasts revealed the extensive membrane damage caused by Cd treatments. Cd-treated plants showed a 40% reduction in chloroplast intactness when compared to the chloroplasts isolated from control plants without any metal treatment. Zn restored (41.18 %) the intactness of the isolated chloroplasts in Cd-treated plants with supplemented Zn indicating its active role in protecting chloroplast thylakoid membranes from peroxidative damage (see figure 61, page 111). Treatments with only Zn did not reflect much change in the chloroplast intactness indicating the non-toxic nature of Zn (see table 14, page 112).

Rate of Photosynthesis and electron transport processes: Cd treatments showed a drastic reduction in the rate of photosynthesis and electron transport processes in the system. Rate of photosynthesis showed a 39% reduction in Cd-treated plants against control. This impairment was effectively controlled and restored completely by Zn (39.19%) in Cd-treated plants with supplemented Zn (see figure 62, page 111).

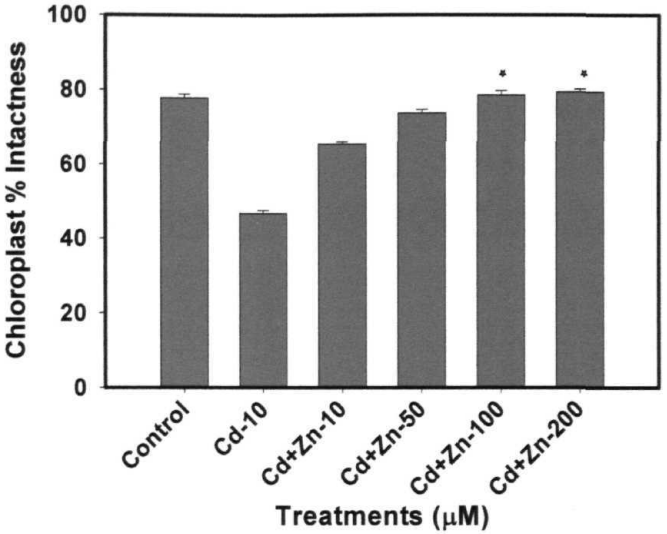


Figure 61. Intactness of isolated chloroplasts from *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

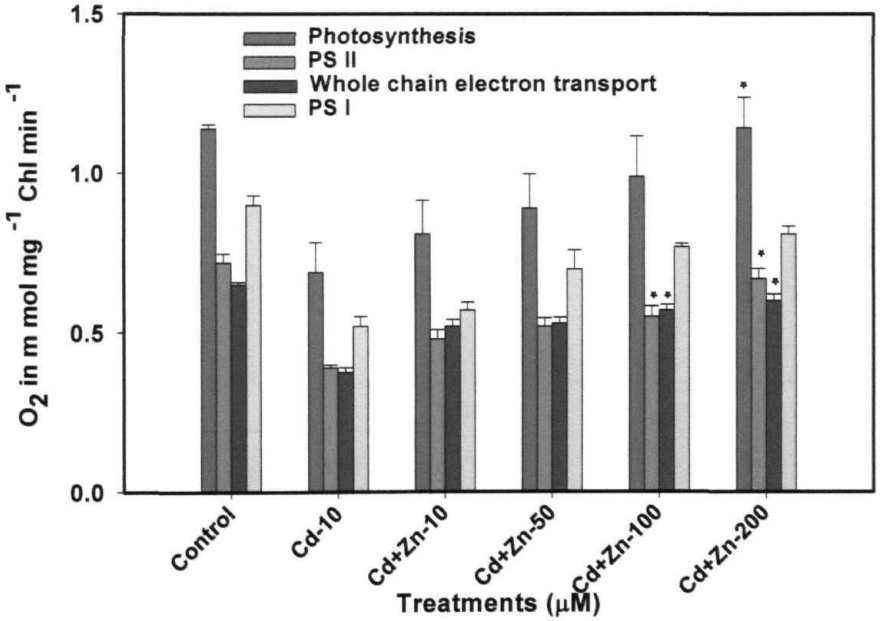


Figure 62. Rate of photosynthesis and electron transport processes in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100, and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

Table 14. Influence of Zn alone treatments on EB-DNA fluorescence as a measure of DNA damage and the levels of chlorophyll, carotenoids, and intactness of chloroplast in *C. demersum*. Values are means of three individual experiments with duplicates. Values in parenthesis indicate standard deviation (n=3).

Treatments (MM)	Ethidium bromide-DNA fluorescence units μg^{-1} DNA	Chlorophyll a mg g^{-1} FW	Chlorophyll b mg g^{-1} FW	Carotenoids mg g^{-1} FW	Intactness of isolated chloroplasts (%)
Con	62.00(1.20)	0.51 (0.022)	0.38(0.061)	0.3 (0.004)	77.73(1.22)
Zn-10	63.12(2.24)	0.51 (0.048)	0.39 (0.060)	0.29 (0.011)	77.56(1.05)
Zn-50	64.40(1.06)	0.52 (0.030)	0.40 (0.083)	0.31 (0.020)	78.10(1.56)
Zn-100	64.88 (0.98)	0.55 (0.038)	0.41 (0.041)	0.32 (0.006)	78.60(1.20)
Zn-200	65.56 (0.80)	0.55 (0.04)	0.41 (0.067)	0.32(0.015)	79.23 (0.90)

Table 15. Influence of Zn alone treatments on the rate of photosynthesis and electron transport activities of whole chain, PS II and PS I in *C. demersum*. Values are means of three individual experiments with duplicates. Values in parenthesis indicate standard deviation (n=3).

Treatments (μM)	Rate of Photosynthesis Amount of O_2 in m mol mg^{-1} Chl min^{-1}	Whole chain electron Transport ativity Amount of O_2 in m mol mg^{-1} Chl min^{-1}	PS II activity Amount of O_2 in m mol mg^{-1} Chl min^{-1}	PS I activity Amount of O_2 in m mol mg^{-1} Chl min^{-1}
Con	1.11 (0.035)	0.72 (0.023)	0.71 (0.018)	0.92 (0.072)
Zn-10	1.14(0.032)	0.70 (0.046)	0.65 (0.025)	0.86(0.141)
Zn-50	1.09(0.025)	0.68 (0.002)	0.65 (0.025)	0.86(0.141)
Zn-100	1.02(0.007)	0.68 (0.002)	0.62 (0.089)	0.85 (0.032)
Zn-200	1.01 (0.016)	0.67 (0.007)	0.62 (0.072)	0.85 (0.056)

Whole chain electron transport ($H_2O \longrightarrow MV$), PSII and PSI showed 42.15%, 45.83% and 42.22% reduction respectively in Cd treated plants. The addition of Zn to Cd treatments was effective in restoring the electron transport processes almost completely (see figure 62, page 111) [37.33% for ($H_2O \longrightarrow MV$) and 41.79% for PSII, and 35.80% for PS I] indicating complete protection of electron transport processes from Cd toxicity. Treatments with only Zn showed very little differences in all the above parameters (see table 15, page 112).

3.14. Analysis of the structural integrity of DNA

DNA damage and gel mobility: Cd-10 uM treatments induced strong oxidative damage to DNA (40%) assessed by the loss of fluorescence (40%) associated with ethidium bromide binding to DNA (see figures 63 and 64, page 114). In a 1% agarose gel visible increase in DNA migration in the form of a streak was observed in the DNA isolated from Cd- 10 uM treated plants (see figure 65, page 115). Zn supplements to Cd treatments effectively inhibited DNA damage assessed by restored ethidium bromide-DNA binding fluorescence in DNA isolated from Zn supplemented Cd treatments and absolutely inhibited mobility of the DNA on 1% agarose gel, visible as a single DNA band similar to that of the intact DNA from control. Zn-alone treatments did not induce DNA damage (see table 14, page 112).

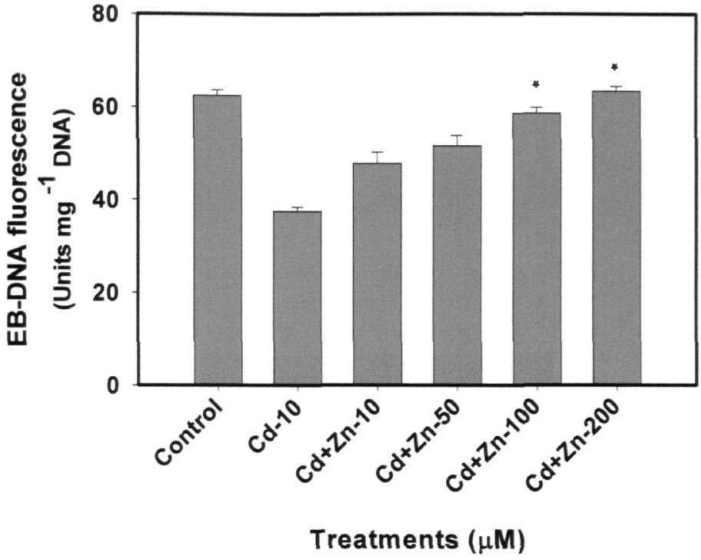


Figure 63. Ethidium bromide-DNA fluorescence as a measure of DNA damage in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

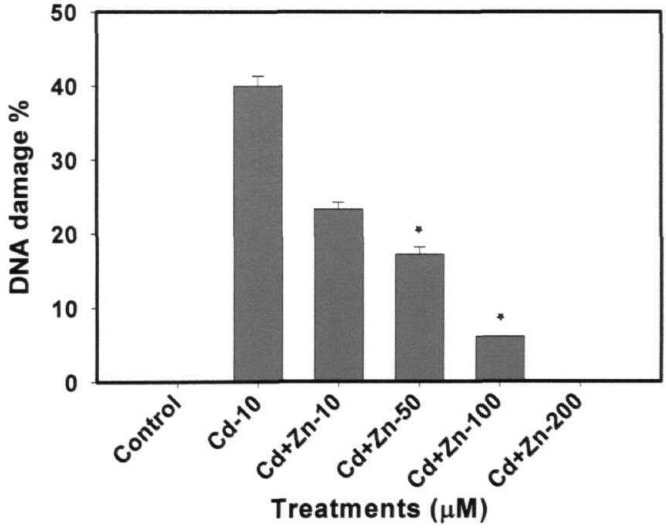


Figure 64. Levels of DNA damage in *C. demersum* plants treated with Cd-10 μM and Zn (10, 50, 100, and 200 μM) concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

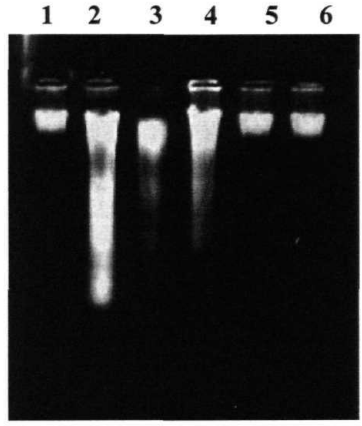


Figure 65. Agarose gel (1%) showing the DNA mobility of DNAs isolated from *C. demersum* plants. Lanes 1: control, 2: Cd-10 μM, 3: Cd+Zn-10 μM, 4: Cd+Zn-50 μM, 5: Cd+Zn-100 μM, 6: Cd+Zn-200 μM.

3.15. Toxicity functions in Cd treatments with exogenous amino acids (cysteine, glutamic acid and glycine) and organic acids (citric, oxalic, and malic acids) supplementations.

Amino acids: In cysteine (Cys), glutamic acid (Glu) and glycine (Gly) (all 0.1- 1 mM concentrations) individually as well as in different combinations Cys, Glu and Gly were found to be very effective in influencing the experimented parameters at 1.0 mM, 0.5 mM and 0.2 mM respectively. Hence only the effective concentrations were used for combinations of amino acids. The entire results are presented in tables 16 and 17, pages 117 and 118.

Organic acids: In citric acid (CA), oxalic (OA) and malic acid (MA) (all 0.1- 1 mM concentrations) individually as well as in different combinations CA, OA as well as MA were very effective in influencing the experimented parameters at concentrations of 1 mM, 0.5 mM and 0.5 mM respectively. Hence the effective concentrations used for combination treatments. The results are presented in tables 18 and 19, pages 132 and 133.

Table 16. Effect of Cys, Glu and Gly supplements (0.1-1.0 mM) on different parameters tested in *C. demersum*.

S.No	Treatments	Metal uptake	Total Chlorophyll	Carotenoids	Lipid peroxidation	Glutathione content		Y-GCS activity
	Amino acid supplements + Cd (10 μM)	Cd	% restored	% restored	% decrease	GSH % restored	GSSG % decrease	% restored
1.	Cys (1.0 mM)	I	30%	73%	51%	54%	51%	28%
2.	Glu (0.5 mM)	↕	28%	73%	50%	57%	51%	40%
3.	Gly (0.2 mM)	I	28%	73%	41%	41%	46%	26%
4.	Cys (1.0 mM) + Glu (0.5 mM)	↕	30%	27%	64%	62%	46%	31%
5.	Cys (1.0 mM) + Gly (0.2 mM)	t	28%	72%	48%	58%	38%	33%
6.	Glu (0.5 mM) + Gly (0.2 mM)	↕	25%	69%	56%	54%	54%	29%
7.	Cys (1.0 mM) + Glu (0.5 mM) + Gly (0.2 mM)	↕	34%	71%	65%	67%	62%	40%

↕ denotes no change in the tested parameter- Refer Figures 66-85, pages 119-131.

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Table 17. Effect of Zn (200 μM) and Cys, Glu and Gly supplements (0.1-1.0 mM) on different parameters tested in *C. demersum*.

	Treatments	Metal uptake		Total Chlorophyll	Carotenoid	Lipid peroxidation	Glutathione content		y-GCS activity
	Zn + Amino acid supplements + Cd (10μM)	Cd	Zn	% restored	% restored	% restored	GSH % restored	GSSG % decrease	% restored
1.	Zn (200 μM) + Cys (1.0 mM)	31%↓	79%t	33%	74%	52%	64%	60%	28%
2.	Zn (200 uM) + Glu (0.5 mM)	30%|	79%T	30%	72%	52%	62%	38%	40%
3.	Zn (200 uM) + Gly (0.2 mM)	28%1	79%↑	28%	72%	48%	62%	54%	33%
4.	Zn (200 uM)+Cys(1.0 mM) + Glu (0.5 mM)	28%↓	79%t	37%	72%	65%	63%	42%	39%
5.	Zn (200 uM)+Cys(1.0 mM) + Gly (0.2 mM)	26%↓	79%↑	36%	72%	48%	60%	46%	36%
6.	Zn (200 μM)+Glu (0.5 mM) +Gly (0.2 mM)	30%↓	79%T	30%	75%	56%	58%	38%	28%
7.	Zn (200 uM) + Cys (1.0 mM) + Glu (0.5mM) + Gly (0.2 mM)	33%↓	80%↑	33%	77%	64%	67%	62%	41%

↑ denotes increase and ↓ denotes decrease in the tested parameter- Refer figures 66-85, pages 119-131.

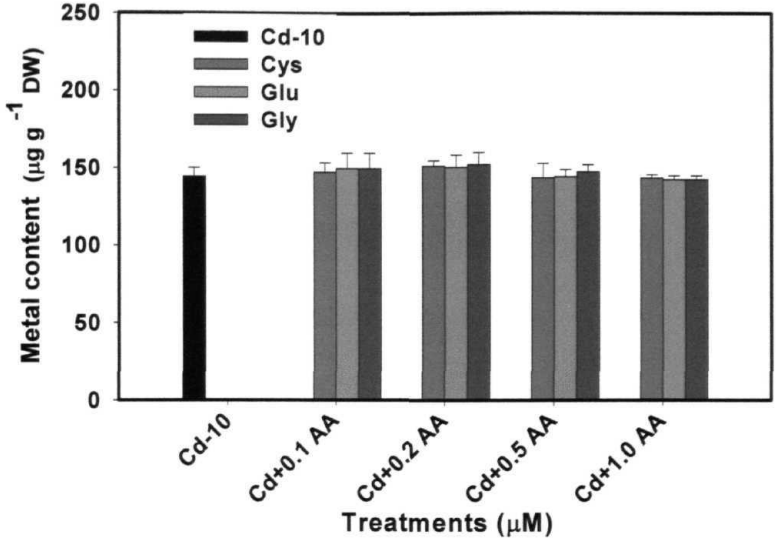


Figure 66. Cd accumulation in *C. demersum* treated with Cd-10 µM supplemented with amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors. AA-Amino acids.

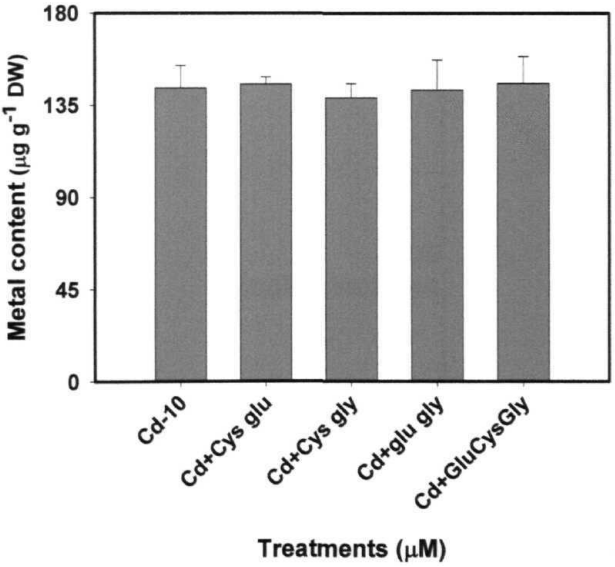


Figure 67. Cd accumulation in C. *demersum* treated with Cd-10 µM supplemented with amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors.

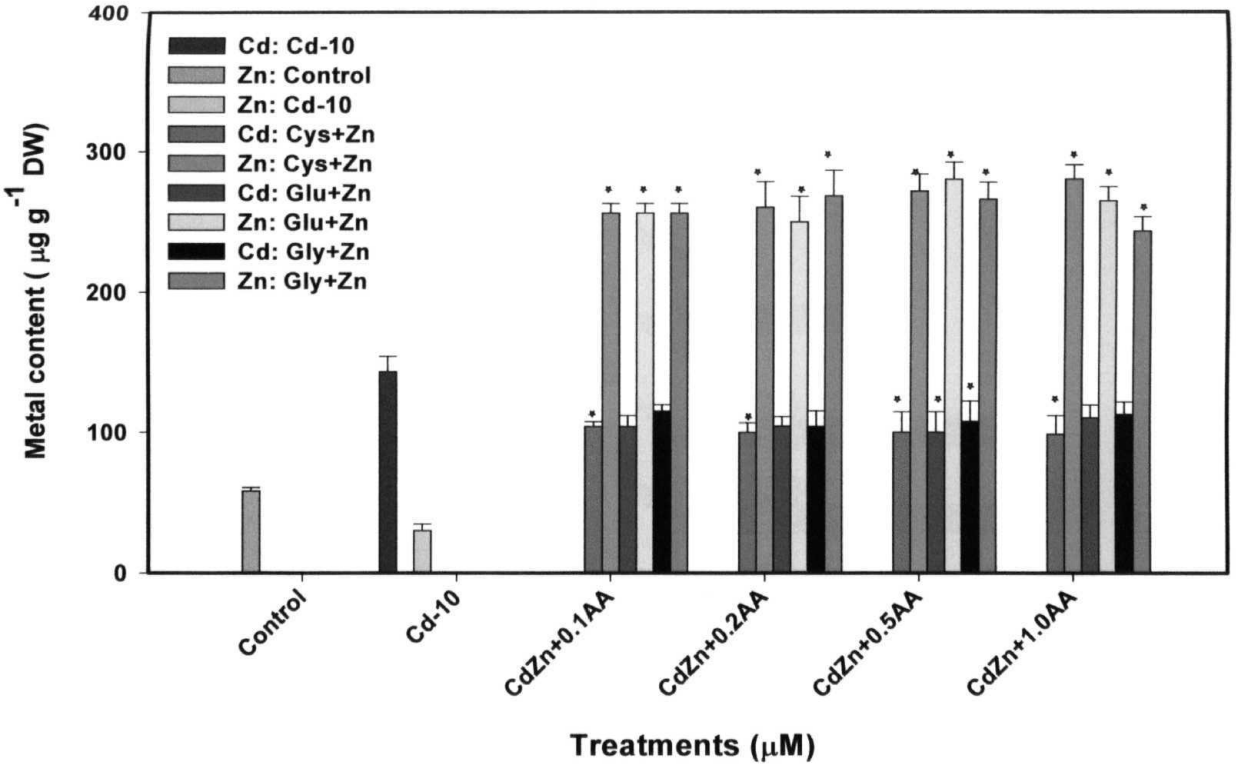


Figure 68. Cd and Zn accumulation in *C. demersum* treated with **Cd-10** μM supplemented with Zn-200 **μM** and amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 **mM** concentrations. Error bars represent standard errors and 'asterisk' for Cd content indicates significant difference from Cd treated plants and for Zn content indicates significant difference from control. AA-Amino acids.

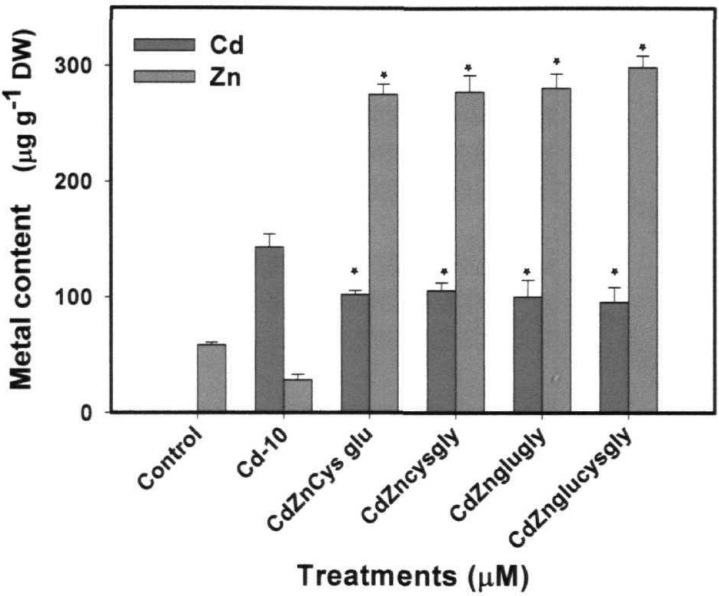


Figure 69. Cd and Zn accumulation in *C. demersum* treated with Cd-10 µM supplemented with Zn-200 µM and amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' for Cd content indicates significant difference from Cd treated plants and for Zn content indicates significant difference from control.

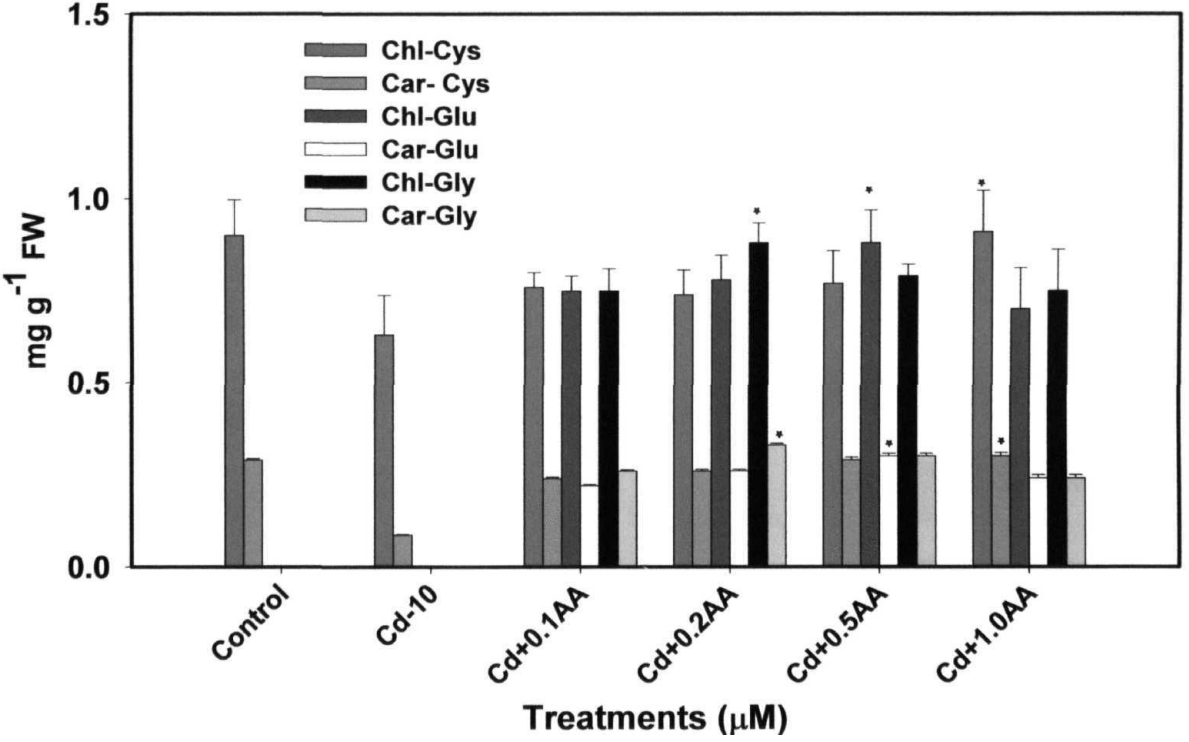


Figure 70. Chlorophyll and carotenoid levels in *C. demersum* treated with Cd-10 μM supplemented with amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA-Amino acids.

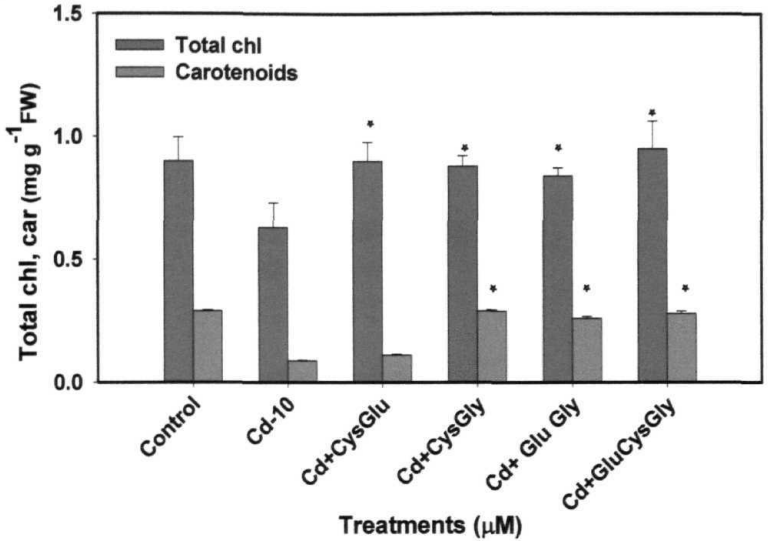


Figure 71. Chlorophyll and carotenoids levels in *C. demersum* treated with Cd-10 μM supplemented with amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

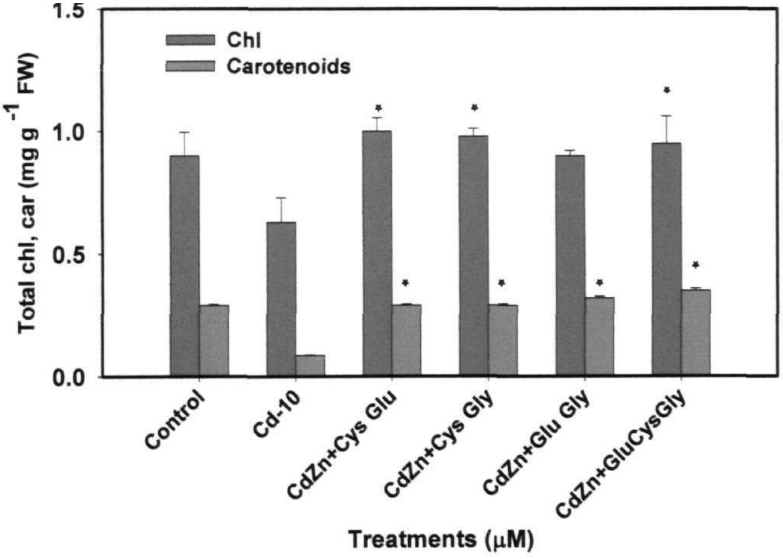


Figure 72. Chlorophyll and carotenoids levels in *C. demersum* treated with Cd-10 |aM supplemented with Zn 200 μM and amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

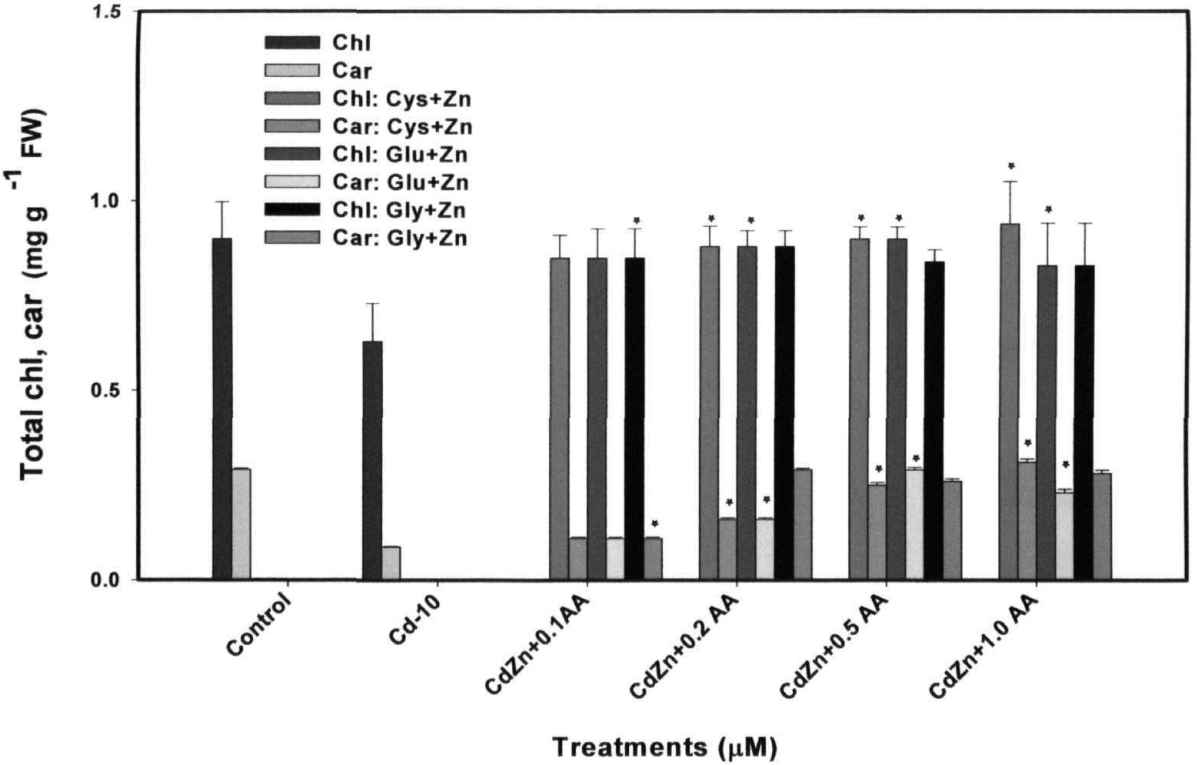


Figure 73. Chlorophyll and carotenoid levels in *C. demersum* treated with Cd-10 μM supplemented with Zn-200 μM and amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA-Amino acids.

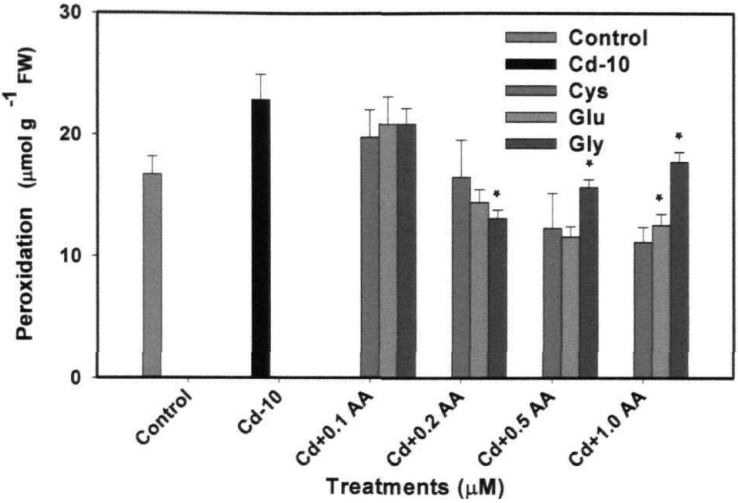


Figure 74. Lipid peroxidation in *C. demersum* treated with Cd-10 µM supplemented with amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA- Amino acids.

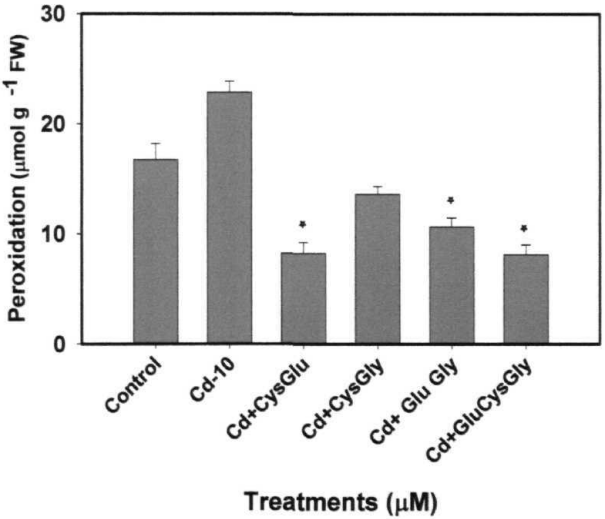


Figure 75. Lipid peroxidation in *C. demersum* treated with Cd-10 µM supplemented with amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

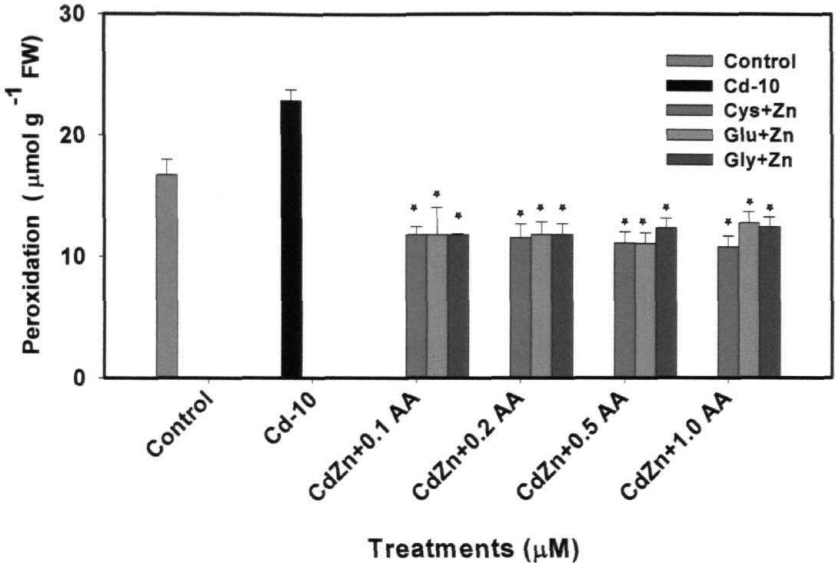


Figure 76. Lipid peroxidation in *C. demersum* treated with Cd-10 µM supplemented with Zn-200 µM and amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA-Amino acids.

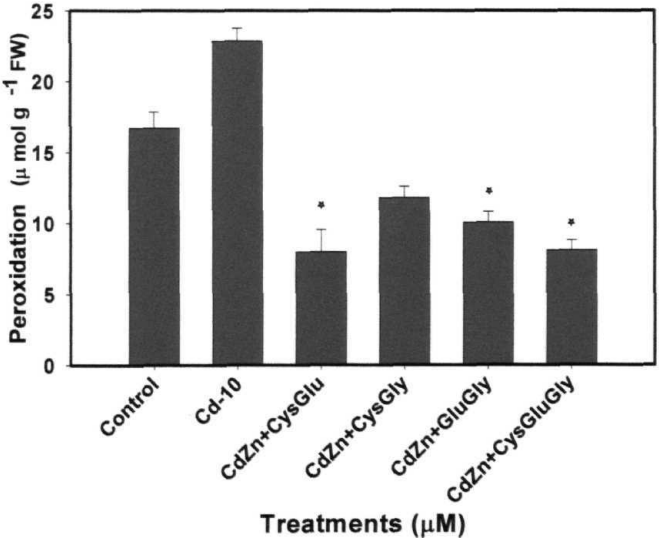


Figure 77. Lipid peroxidation in *C. demersum* treated with Cd-10 µM supplemented with Zn-200 µM and amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

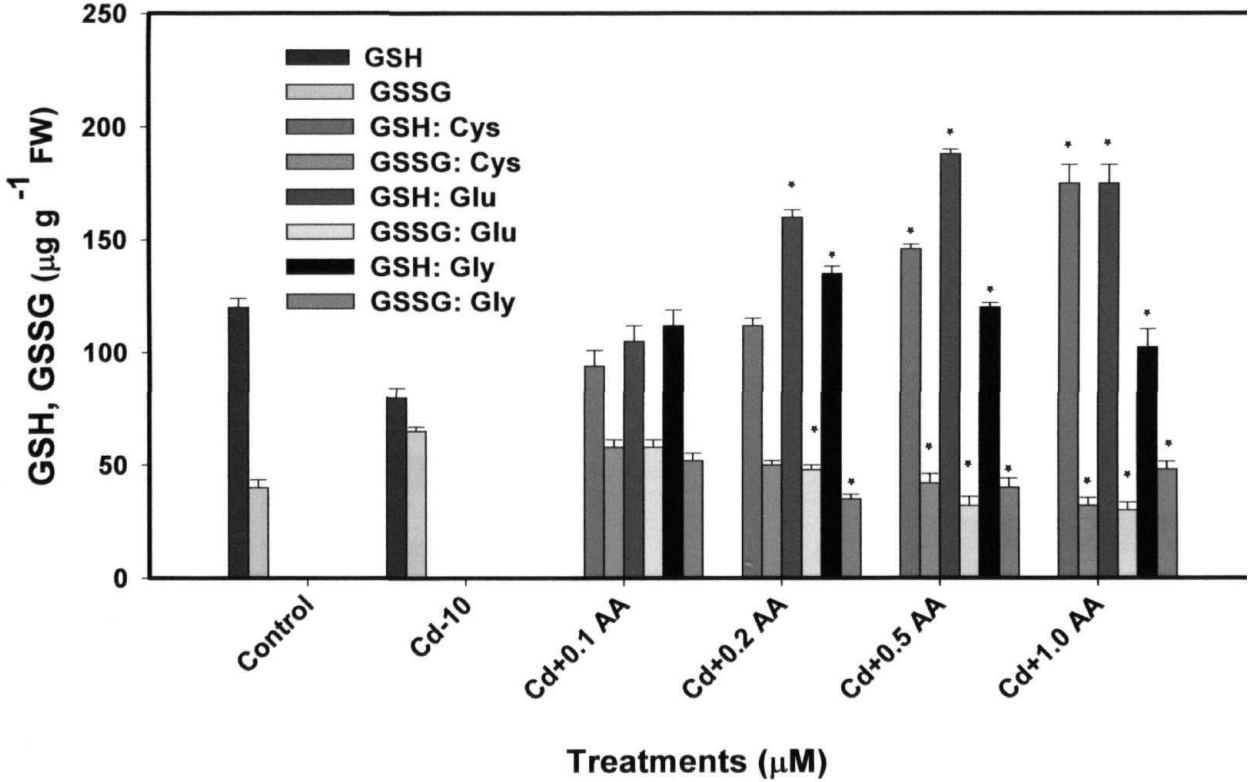


Figure 78. Cellular glutathione content in *C. demersum* treated with Cd-10 μM supplemented with Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA-Amino acids.

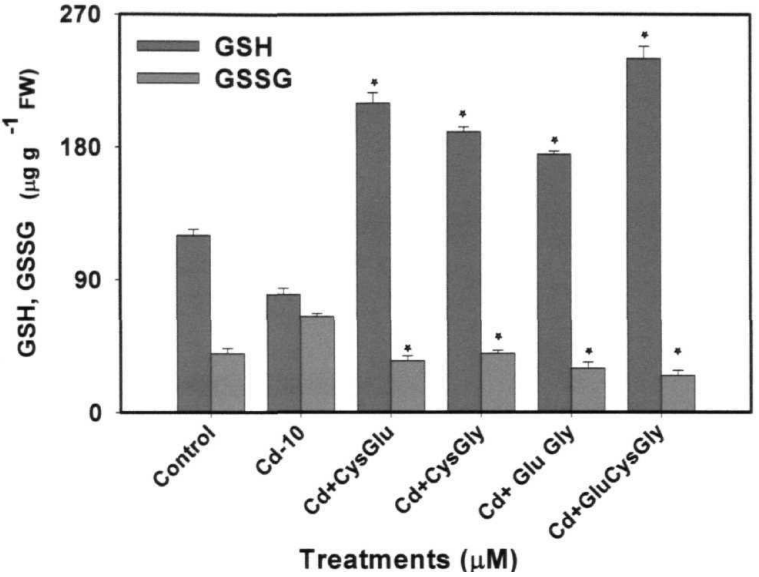


Figure 79. Cellular glutathione content in *C. demersum* treated with Cd-10 μM supplemented amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

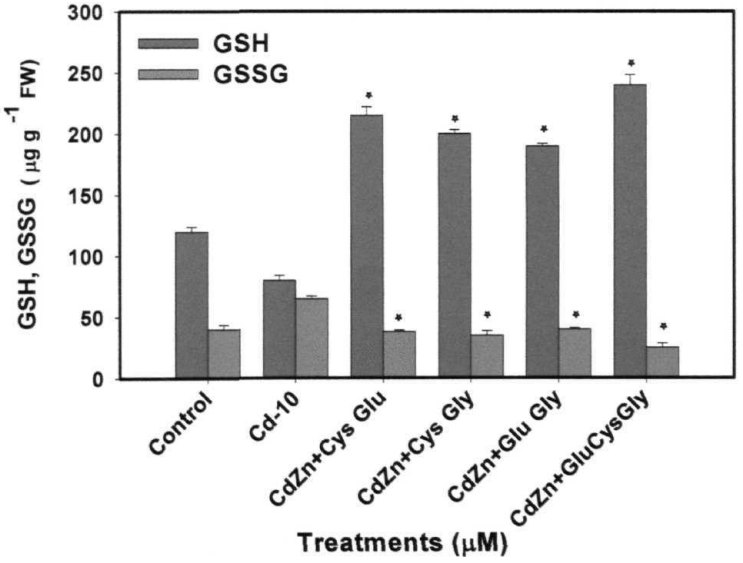


Figure 80. Cellular glutathione content in *C. demersum* treated with Cd-10 μM supplemented with Zn-200 μM and amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

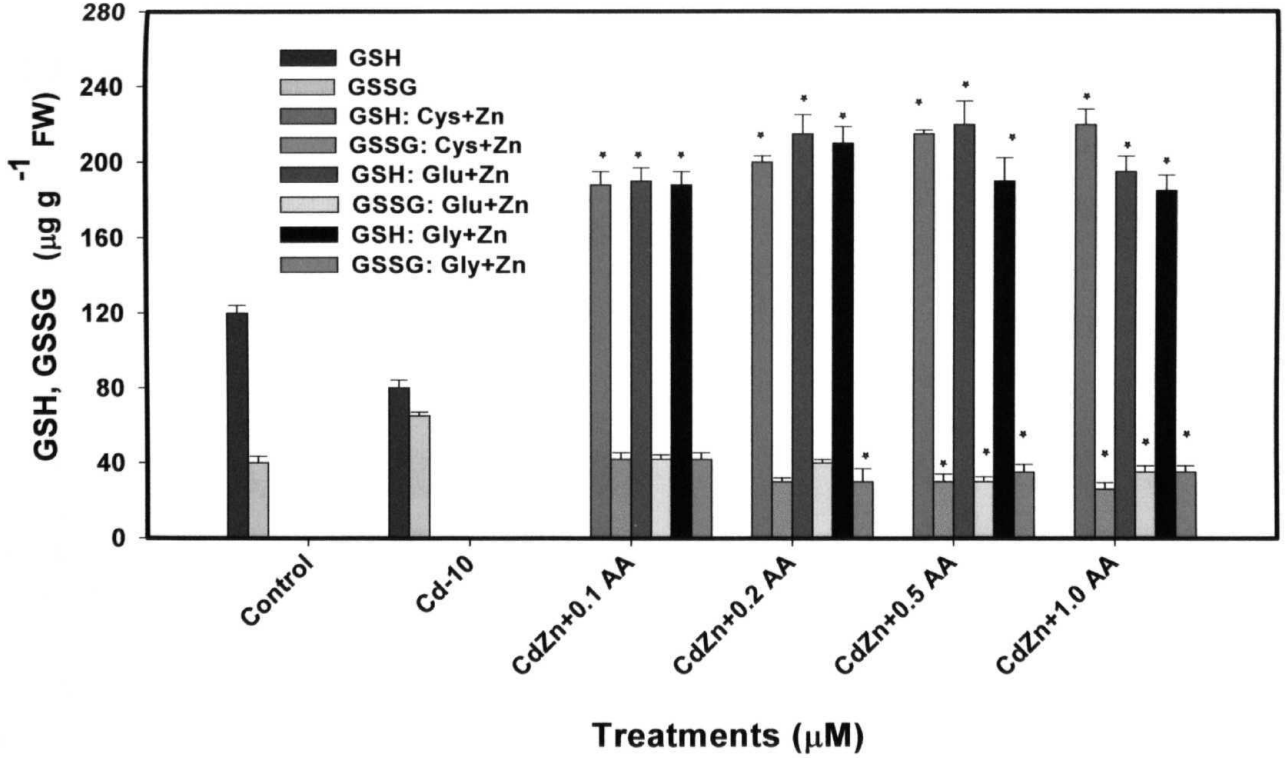


Figure 81. Cellular glutathione content in *C. demersum* treated with Cd-IO μM supplemented with Zn-200 μM and amino acids amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA-Amino acids.

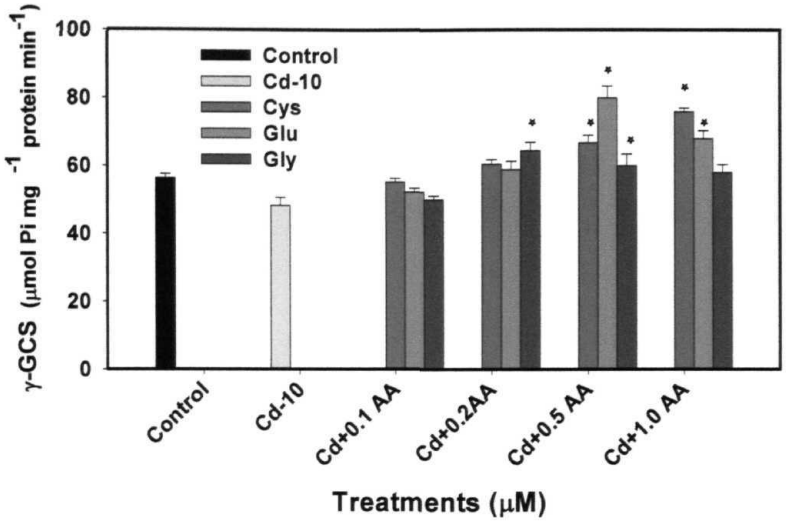


Figure 82. γ-Glutamylcysteine synthetase activity in *C. demersum* treated with Cd-10 μM supplemented with amino acids amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA-Amino acids.

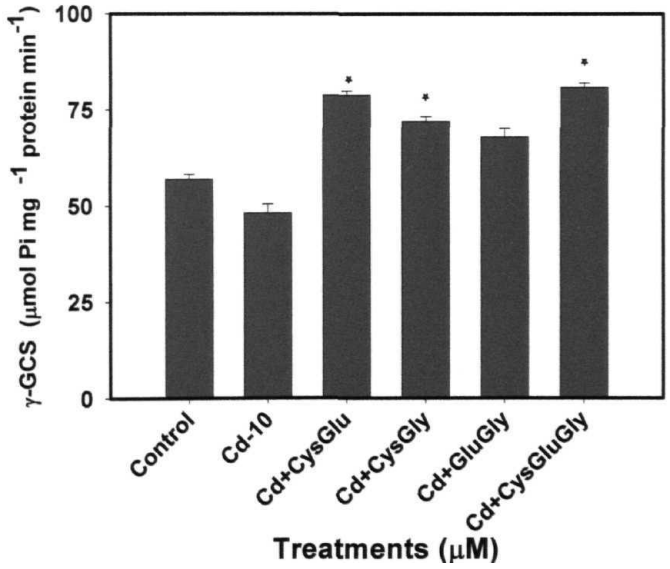


Figure 83. γ-Glutamylcysteine synthetase activity in *C. demersum* treated with Cd-10 μM supplemented with amino acids Cys, Glu, Gly in different combinations: 1.0 Cys + 0.5 Glu, 1.0 Cys + 0.2 Gly, 0.5 Glu + 0.2 Gly, 1.0 Cys + 0.5 Glu + 0.2 Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

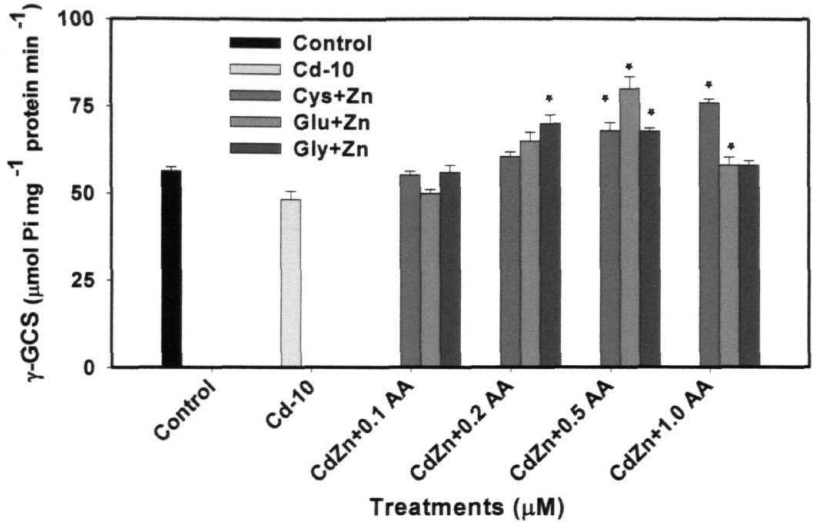


Figure 84. γ-Glutamylcysteine synthetase activity in *C. demersum* treated with Cd-10 μM supplemented with Zn-200 uM and amino acids Cys, Glu, Gly at 0.1, 0.2, 0.5, and 1 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. AA-Amino acids.

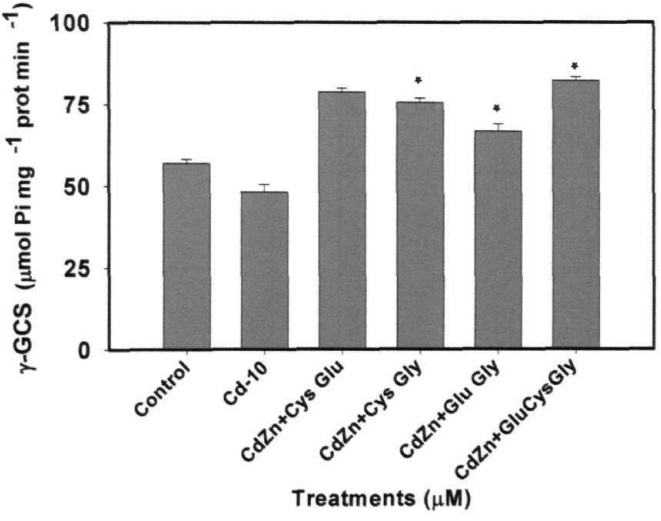


Figure 85. γ-Glutamylcysteine synthetase activity in *C. demersum* treated with Cd-10 μM supplemented with Zn-200 μM and amino acids Cys, Glu, Gly in different combinations: 1.0Cys+0.5Glu, 1.0Cys+0.2Gly, 0.5Glu+0.2Gly, 1.0 Cys+0.5Glu+0.2Gly. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

Table 18. Effect of citric, oxalic and malic acid supplements (0.1-1.0 mM) on different parameters tested in *C. demersum*.

S.No	Treatments	Metal uptake	Total Chlorophyll	Carotenoids	Lipid peroxidation
	Organic acid supplements + Cd (10 μM)	Cd	% restored	% restored	% decrease
1.	Citric acid (1.0 mM)	8%t	33%	73%	48%
2.	Oxalic acid (0.5 mM)	10%T	19%	72%	36%
3.	Malic acid (0.2 mM)	8%t	29%	72%	36%
4.	Citric acid (1.0 mM) + Oxalic acid (0.5 mM)	7% ↑	25%	67%	54%
5.	Citric acid (1.0 mM) + Malic acid (0.5 mM)	5%t	38%	72%	51%
6.	Oxalic acid (0.5 mM) + Malic acid (0.5 mM)	4%T	37%	72%	55%
7.	Citric acid (1.0 mM) + Oxalic acid (0.5 mM) + Malic acid (0.5 mM)	9% ↑	33%	73%	41%

denotes increase in the tested parameter- Refer figures 86-97, pages 134-141.

Table 19. Effect of Zn (200 uM) citric, oxalic and malic acid supplements (0.1-1.0 mM) on different parameters tested in *C. demersum*.

S.No	Treatments	Metal uptake		Total Chlorophyll	Carotenoids	Lipid peroxidation
	Organic acid supplements + Cd (10 uM)	Cd	Zn	% restored	% restored	% decrease
1.	Zn (200 uM) + Citric acid (1.0 mM)	9T	77%t	33%	72%	48%
2.	Zn (200 uM) + Oxalic acid (0.5 mM)	9%	73%T	19%	73%	36%
3.	Zn (200 uM) + Malic acid (0.2 mM)	8%t	77%t	29%	73%	36%
4.	Zn (200 uM) + Citric acid (1.0 mM) + Oxalic acid (0.5 mM)	2%↑	78%T	37%	66%	22%
5.	Zn (200 uM) +Citric acid (1.0 mM) + Malic acid (0.5 mM)	6%t	78%T	38%	69%	30%
6.	Zn (200 uM) +Oxalic acid (0.5 mM) + Malic acid (0.5 mM)	9%t	78%↑	37%	72%	37%
7.	Zn (200 uM) + Citric acid (1.0 mM) + Oxalic acid (0.5 mM) + Malic acid (0.5 mM)	10%t	78%↑	33%	73%	34%

t **denotes increase and ↓ denotes decrease in the tested parameter- Refer figures 86-97, pages 134-141.**

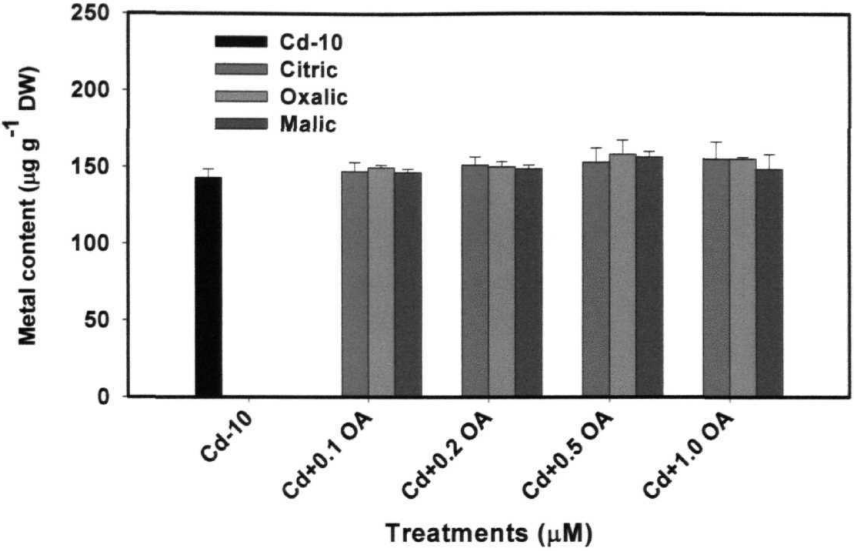


Figure 86. Cd uptake in *C. demersum* treated with Cd-10 µM supplemented with organic acids Citric (CA), Oxalic (OA) and Malic acids (MA) at 0.1, 0.2, 0.5, and 1.0 mM concentrations. Error bars represent standard errors. OA-Organic acids.

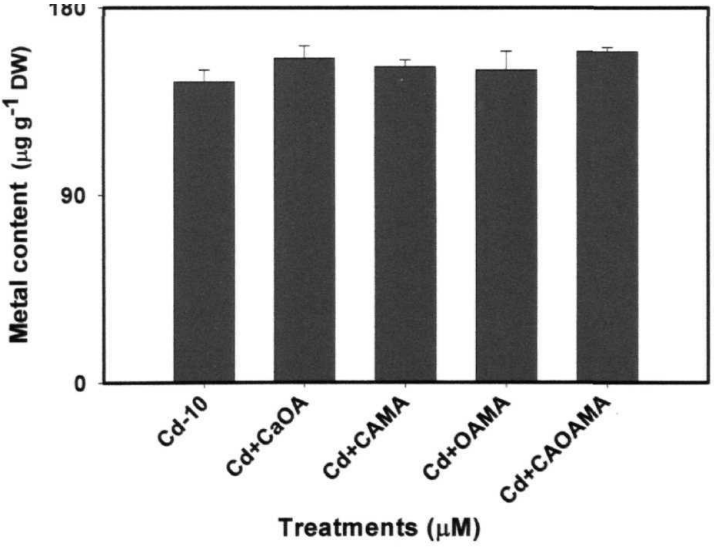


Figure 87. Cd uptake in *C. demersum* treated with Cd-10 uM supplemented with organic acids Citric (CA), Oxalic (OA) and Malic (MA) acids at different combinations (in mM)- 1.0 Citric + 0.5 Oxalic, 1.0 Citric + 0.5 Malic, 0.5 Oxalic + 0.5 Malic, 1.0 Citric + 0.5 Oxalic + 0.5 Malic acids. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

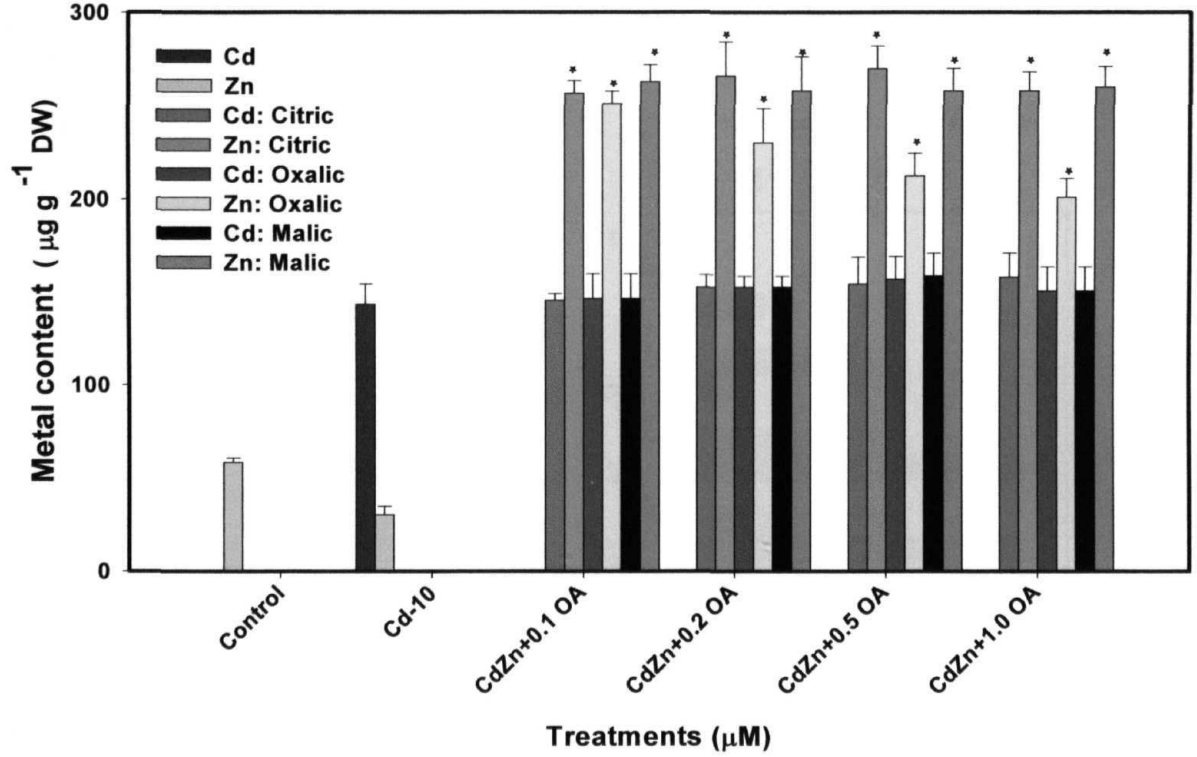


Figure 88. Cd and Zn uptake in *C. demersum* treated with Cd-10 (xM supplemented with Zn-200 μM and organic acids Citric, Oxalic and Malic acids at 0.1, 0.2, 0.5, and 1.0 mM concentrations. Error bars - standard errors, 'asterisk' indicates significant difference from Cd treated plants. OA-Organic acids

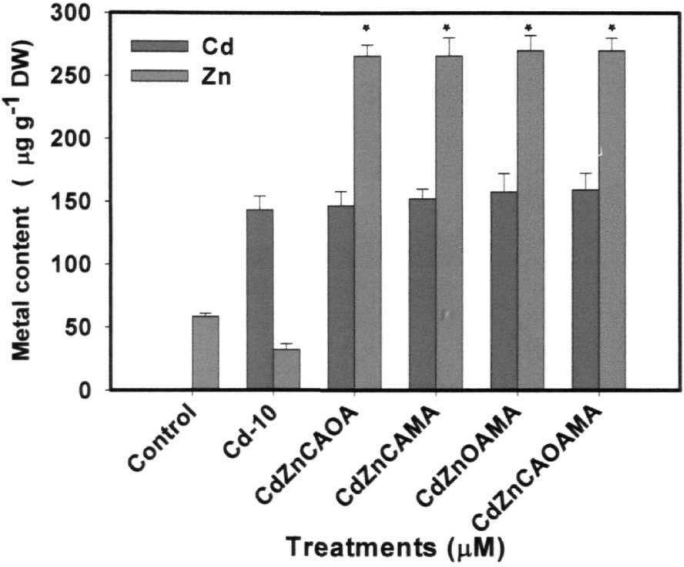


Figure 89. Cd uptake in *C. demersum* treated with Cd-10 µM supplemented with Zn-200 µM and organic acids Citric (CA), Oxalic (OA) and Malic (MA) acids at different combinations (in mM)- 1.0 Citric + 0.5 Oxalic, 1.0 Citric + 0.5 Malic, 0.5 Oxalic + 0.5 Malic, 1.0 Citric + 0.5 Oxalic + 0.5 Malic acids. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

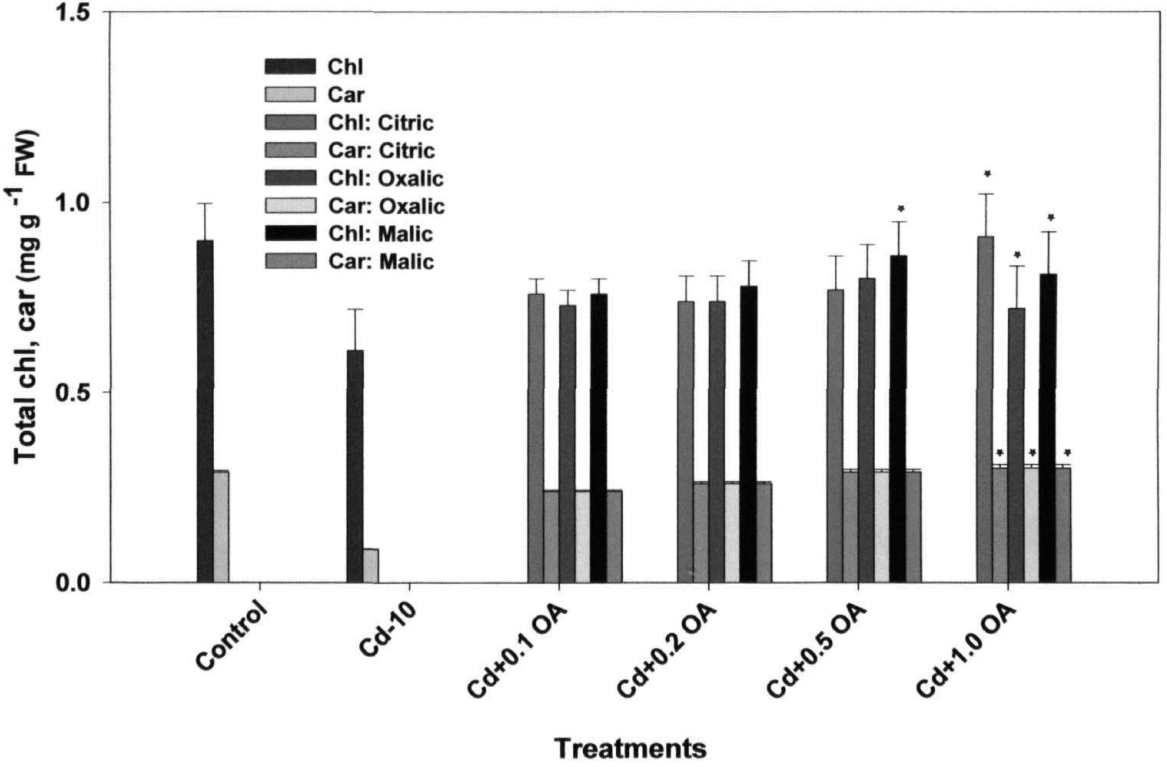


Figure 90. Photosynthetic pigments level in *C. demersum* treated with Cd-10 μM supplemented with organic acids citric, oxalic and malic acids at 0.1, 0.2, 0.5, and 1.0 mM concentrations. Error bars - standard errors and 'asterisk' indicates significant difference from Cd treated plants. OA-Organic acids.

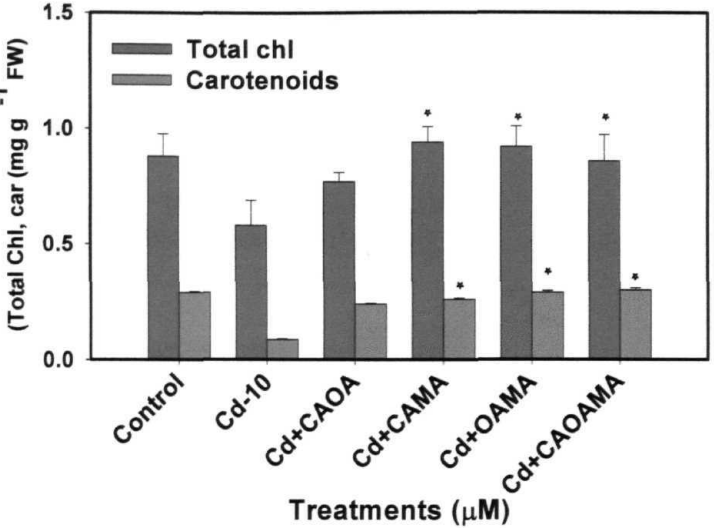


Figure 91. Photosynthetic pigments levels in *C. demersum* treated with Cd-10 μM supplemented with organic acids: Citric, Oxalic and Malic acids at different combinations (in mM)- 1.0 Citric + 0.5 Oxalic, 1.0 Citric + 0.5 Malic, 0.5 Oxalic + 0.5 Malic, 1.0 Citric + 0.5 Oxalic + 0.5 Malic acids. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

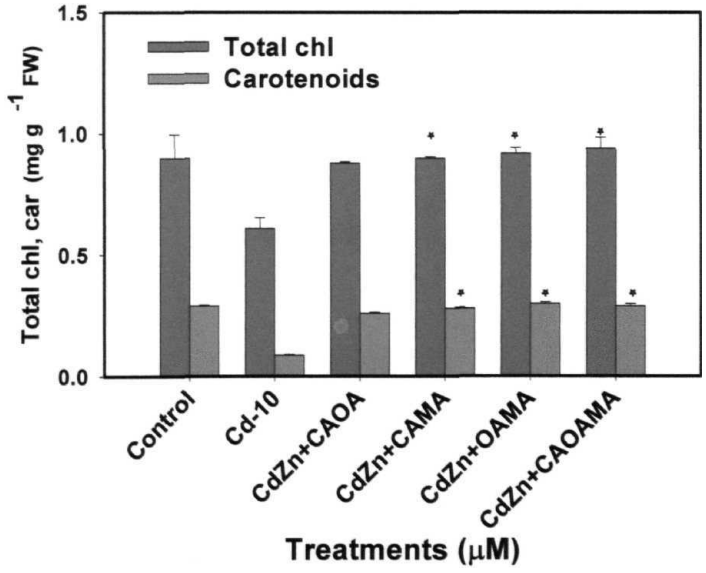


Figure 92. Photosynthetic pigments levels in *C. demersum* treated with Cd-10 μM supplemented with Zn-200 μM and organic acids: Citric, Oxalic and Malic acids at different combinations (in mM)- 1.0 Citric + 0.5 Oxalic, 1.0 Citric + 0.5 Malic, 0.5 Oxalic + 0.5 Malic, 1.0 Citric + 0.5 Oxalic + 0.5 Malic acids. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

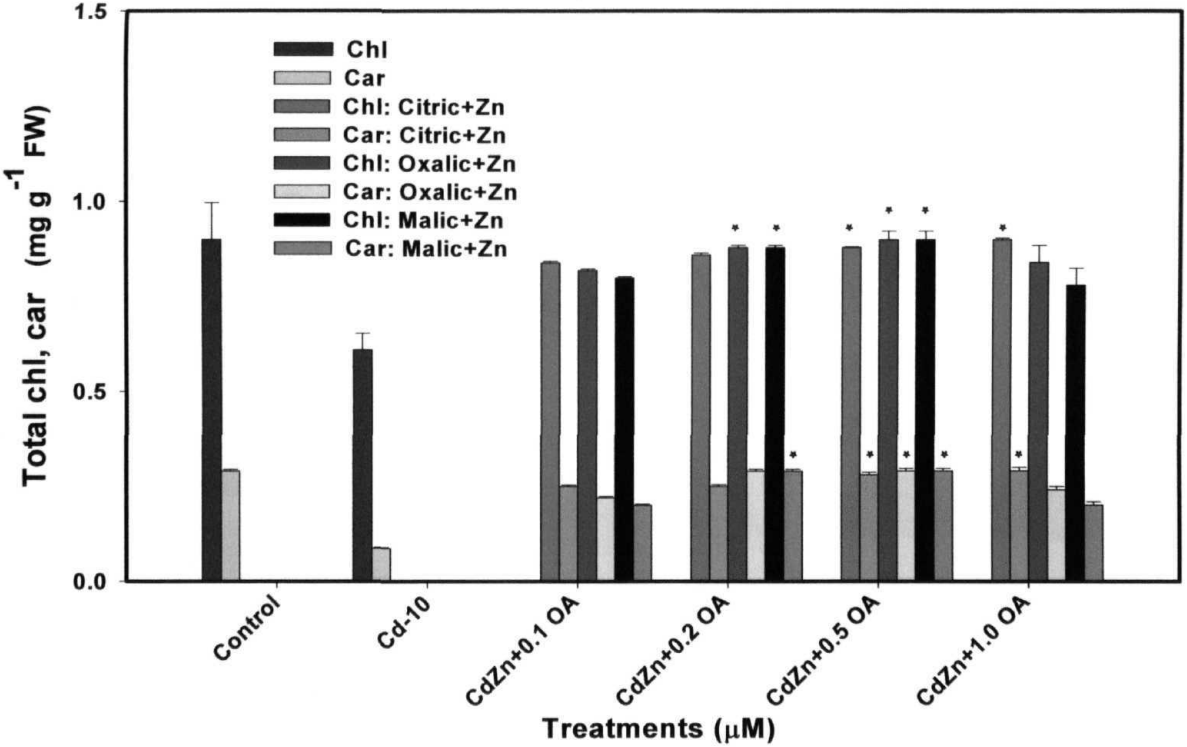


Figure 93. Photosynthetic pigments level in *C. demersum* treated with Cd-IO μM supplemented with Zn-200 μM and organic acids Citric, Oxalic and Malic acids at 0.1, 0.2, 0.5, 1.0 mM concentrations. Error bars- standard errors and 'asterisk' indicates significant difference from Cd treated plants. OA-Organic acids

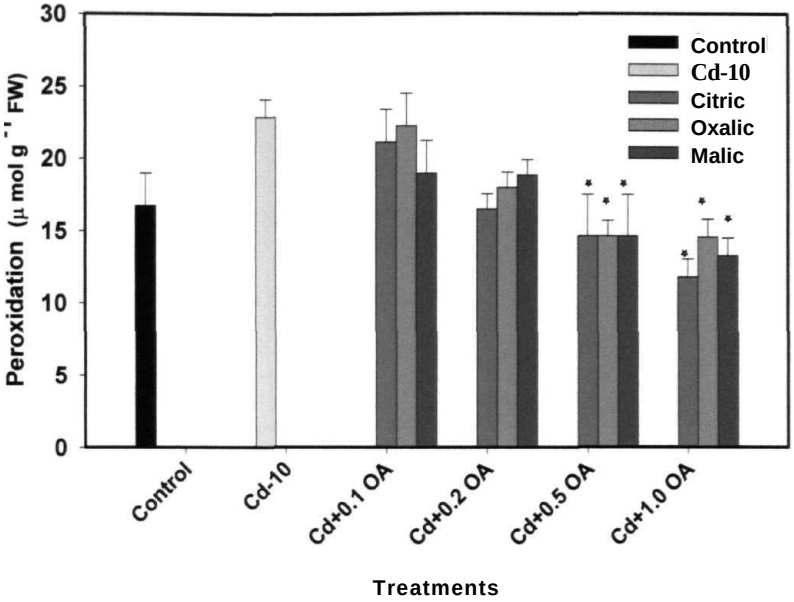


Figure 94. Lipid peroxidation in *C. demersum* treated with Cd-10 μM supplemented with organic acids Citric, Oxalic and Malic acids at 0.1, 0.2, 0.5, and 1.0 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants. OA-Organic acids

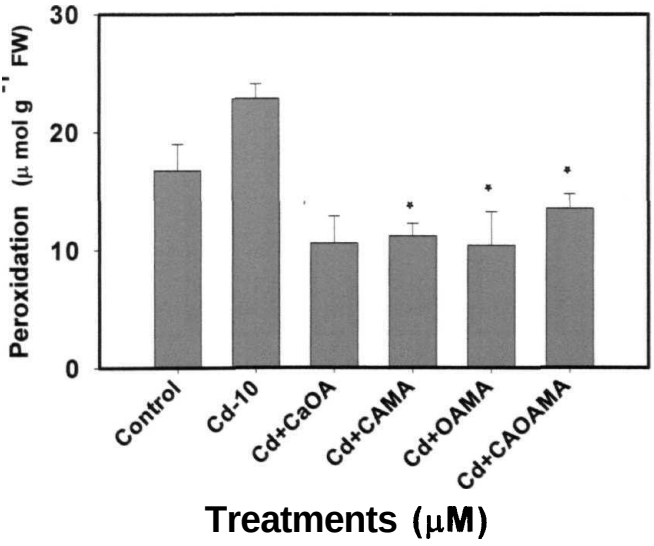


Figure 95. Lipid peroxidation in *C. demersum* treated with Cd-10 μM supplemented with organic acids: Citric, Oxalic and Malic acids at different combinations- 1.0 Citric + 0.5 Oxalic, 1.0 Citric + **0.5 Malic, 0.5** Oxalic + 0.5 Malic, 1.0 Citric + 0.5 Oxalic + 0.5 Malic acids. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

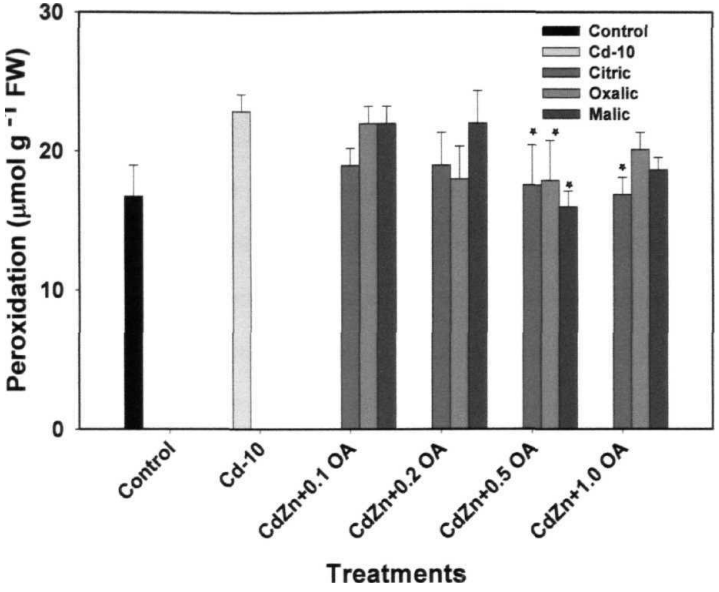
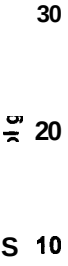


Figure 96. Lipid peroxidation in *C. demersum* treated with Cd-10 µM supplemented with Zn-200 |iM and organic acids Citric, Oxalic and Malic acids at 0.1, 0.2, 0.5, and 1.0 mM concentrations. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.



Treatments (µM)

Figure 97. Lipid peroxidation in C. *demersum* treated with Cd-10 µM supplemented with Zn-200 µM and organic acids: Citric, Oxalic and Malic acids at different combinations- 1.0 Citric + 0.5 Oxalic, 1.0 Citric + 0.5 Malic, 0.5 Oxalic + 0.5 Malic, 1.0 Citric + 0.5 Oxalic + 0.5 Malic acids. Error bars represent standard errors and 'asterisk' indicates significant difference from Cd treated plants.

4. Discussion

This study was undertaken to identify the mechanism of interaction between Zn, an essential nutrient and Cd, a non-essential and toxic element. An important part of current research in plant metal nutrition is directed towards elucidating the role and interactions between metals at cellular and molecular level. Thorough understanding of the metal interaction mechanism could be useful for genetic engineering aimed at improving the defense strategies. The convenience and potential of using *Ceratophyllum demersum* L. as a laboratory toxicity bioassay material could also be understood by the experimentation performed.

4.1. Regulation of Cd uptake by Zn

The present investigation showed reduced Cd uptake at increasing Zn concentrations and simultaneously increased Zn accumulation in the plant tissue (see figure 3, page 56). This suppression in Cd uptake due to increase in Zn accumulation indicates a strong competition between Zn and Cd. Since Cd and Zn, both taken as divalent cations belong to the group IIB transition metals with similar configuration (Nieboer and Richardson 1980), Cd can readily inhibit most of the Zn-dependent processes and hence 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, Agriffoul *et al* 1998, Shaw *et al* 2004). Cadmium has been described as an antimetabolite of Zn by scientists due to the observed Zn deficiency in most of the Cd treated systems (Peraza *et al* 1998), an observation true to our system also. Zinc has already been shown to antagonize other transition metals like Cu and Fe (Powell 2000, Zago and Oteiza 2001). The reduction of intracellular levels of Cd ions would prevent redox-cycling and

concomitant production of oxidant species. It should be noted that Zn uptake is lesser in plants treated with only Zn (see table 1, page 57), as the Zn concentration is already maintained in the plant system. Other reported evidences 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 the depressed Cd uptake in the presence of Zn. The uptake of Cd by the plant system decreased in the presence of metabolic inhibitor DNP indicating the proton gradient mediated uptake as one of the routes of active transport into the plant cell (see figure 4, page 56). As per chemiosmotic hypothesis DNP increase proton permeability of biomembranes and thereby breakdown of proton gradients which must have resulted in the pronounced inhibition of Cd uptake (Poole 1978). This mechanism further seems to be directly associated with H^+ATPase with the observed decrease in Cd uptake to a greater extent with DCCD, a proteolipid-binding H^+ATPase specific inhibitor. This was further justified from the assay of H^+ATPase indicating an increase in activity in Cd treatments without any metabolic inhibitors corresponding to the higher uptake of Cd (see figure 5, page 59). These results are in agreement with the earlier work on Cd uptake in *C. demersum* (Tripathi *et al* 1995) and also in *Hordeum vulgare* (Cutler and Rains 1974) 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 also. The H^+ATPase activity as well as Cd uptake was further reduced by Zn supplements along with DNP and DCCD indicating a direct evidence for the regulation of H^+ATPase involved proton pumping mechanism by Zn, thereby indirectly antagonizing Cd entry into the plant cell.

4.2. Zinc supplementation protected Cd-induced membrane damage

The main site of attack by any redox active metal in a plant cell is usually the cell membrane where, oxidative damage through cascade of electron transfer reactions ultimately leads to peroxidation and production of secondary lipid-derived radicals (Gallego *et al* 1996a,b, Bhattacharjee 1998, Dietz *et al* 1999, Shaw *et al* 2004), which in turn induce dimerization and polymerization, highly destructive to the membranes (Logani and Davies 1980, Wolff *et al* 1986). Detection of malondialdehyde commonly known as thiobarbituric acid (TBA) assay, involving reaction of oxidized lipids with TBA has been used widely both *in vivo* and *in vitro* for detection of lipid peroxidation (Logani and Davies 1980). It is evident from the results of estimation of lipid peroxidation that Cd at 10 uM concentration increased TBA-RS level to a great extent (see figure 6, page 59). Increase in LOX activity (see figure 7, page 60) by Cd suggests higher lipolytic activity on the membrane and oxidation of membrane-bound fatty acids causing propagation of lipid peroxidation (Laccan and Baccou 1998, Oliw 2002). The results in this investigation also indicate a very high increase in ion leakage of K^+, Na^{2+}, Ca^{2+} measured as electrical conductivity as well as by flame photometry in Cd 10 uM treatments (see figures 8 and 9, page 61). Cadmium like any other transition metal is known to bind to sulphydryl groups of membrane bound proteins and destabilize the membrane system, by inducing the formation of disulfide links leading to the distortion in the structure and function of membrane ion channels and hence regulation of uptake/efflux of essential nutrients (Prasad 1995a, Choui *et al* 1997b, Dietz *et al* 1999). Hence it leads to distortion in membrane structure increasing the electrolyte leakage and loss of ions. It is evident from the results that Zn supplementation reduced lipid peroxidation, conductivity and LOX activity thereby maintaining the ionic homeostasis in the plant system. Calcium is absolutely required for the maintenance of membrane integrity along with a balanced ratio of K and Na (Marschner 1995) with the

requirement of Zn as a stabilizer (Cakmak and Marschner 1988a). Since Zn does not undergo oxidation-reduction reactions (Vallee and Falchuk 1993) at physiological pH, the membrane lipid packing is protected from ROS and peroxidation thereby inhibiting ion leakage due to regulated ion channel gating by Zn (Bray and Bettger 1990, Cakmak 2000). Similar observation has been reported in erythrocyte membranes of animal systems (Girotti *et al* 1985). In plant treatments with only Zn (no Cd treatment) all the above parameters did not show any significant change (see tables 1 and 2, pages 57 and 62 respectively) indicating that the plant system is affected only because of the presence of toxic Cd concentration. 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 by conformational change resulting in apparent stability of the enzymes, membrane proteins as well as the lipid structure (Bray and Bettger 1990, Powell 2000). Increase in membrane permeability altering the cellular ion status reported in *Gossypium hirsutum, Triticum aestivum* and *Lycopersicon esculentum* (Cakmak and Marschner 1988a) as a result of Zn deficiency point to the absolute requirement of sufficient Zn levels in the plant system.

4.3. Influence of Zn supplementation on Cd-induced free radicals.

A growing body of evidence indicates that transition metals act as catalysts in inducing oxidative burst through free radicals (Stohs and Bagchi 1995, Dalzuro *et al* 1997, Foyer *et al* 1997). 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). In Cd-10 uM treatments the superoxide generation through NADPH oxidation (see figures 10 and 11, page 64) was enhanced to a great extent thereby leading to high levels of $O_2^{\cdot -}$ radical (see figure. 12, page 65). Such a shift from reduced to oxidative state is very

characteristic of metal toxicity. The protective effect of Zn has been shown 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. Subsequent to enhanced $O_2^{\cdot-}$ radical higher levels of H_2O_2 could be detected in Cd treated plants (see figure 13, page 65) indicating an upsurge of free radical production. Electron spin resonance spectrometry utilizing spin-traps is one of the most sensitive techniques for detecting free radicals (Mason and Degray 1994, Rucinska *et al* 1999). Since hydroxyl radicals are comparatively more stable than other free radicals it can be exploited well for analysis using nitrone spin trap DMPO (Madden and Taniguchi 2001). The dramatic increase in OH radical levels in Cd-10 µM-alone treated plants assessed by ESR DMPO-OH signal intensity (see figures 14 and 15, pages 66 and 67) and hydroxyl radical estimation (see figure 16, page 67) would induce extensive peroxidation and oxidation of macromolecules as OH radicals are one of the most reactive and stable free radical species known in aerobic cells (Shaw *et al* 2004). The $O_2^{\cdot-}$ radical produced itself is deleterious which indirectly produces a potent oxidant, hydroxyl radical (OH) by reacting with H_2O_2 in a site-specific Haber-Weiss Fenton reaction, involving cycling of Fe (II) and Fe (III) (Gutteridge *et al* 1990). Since Fe (III) is strongly associated with polyanionic targets such as proteins, DNA or cell membranes these oxidants are produced in their immediate vicinity causing the repetition of this reaction (Fridovich 1986, Halliwell and Gutteridge 1990). 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. Zinc by eliminating the precursors of $OH^{\cdot}$ radical viz.. $O_2^{\cdot -}$ radical and H_2O_2 could effectively control $OH^{\cdot}$ radical formation further preventing oxidative damage to cellular components. The estimation of total protein content and protein carbonyls indicated a drastic reduction in total protein content (see figure 17, page 70) and enhanced formation of protein carbonyls (see figure 19, page 71) as well indicating potential toxicity of Cd at 10 μM treatments. This was completely reversed by Zn supplementation with a slight increase in total protein content as well (see figure 17, page 70). The 12.5 % SDS-PAGE also revealed induction of new bands at higher supplementations of Zn (see figure 18, page 70). This is in agreement with several studies in a number of plant species (Kitagashi and Obata 1986, 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. Zn-alone treatments did not enhance ROS production indicating that the chosen Zn concentrations did not affect the cellular redox status.

4.4. The responses of the antioxidant system to Zn-Cd interaction

4.4.1. ***Antioxidant*** *enzymes*

The ROS scavenging antioxidant enzymes SOD, CAT, and PODs- APX and GPX play a vital role in scavenging the destructive oxidant species. By catalyzing detoxification of $O_2^{\cdot -}$ to O_2 and H_2O_2, SOD blocks $O_2^{\cdot -}$ driven cell damage (Scandalios 1993, Cakmak 2000). Catalase (CAT) and peroxidases- APX and GPX breaks down H_2O_2 to H_2O and O_2 (Scandalios *et al* 1997). Variations in the responses of antioxidant enzymes to Cd toxicity are well known (Van Ascche and Clijsters 1990, Bhattacharjee 1998, Stroinski *et al* 1999, Vitoria *et al* 2001). The enhanced SOD activity of both Cu/Zn SOD and Mn-SOD observed in Zn-supplemented Cd treated plants (see figure

20, page 73) indicate the enhanced $O_2^{\cdot -}$ scavenging and dismutation to H_2O_2. This metalloenzyme, which possess Zn as one of its active site metal for its co-catalytic functioning (Vallee and Falchuk 1993) along with Cu in Cu/Zn SOD has been shown to decrease in activity under Zn-deficient conditions (Del Rio *et al* 1978, Vaughan *et al* 1982, Cakmak and Marschner 1993, Cakmak *et al* 1997) but not in Cu deficiency indicating a direct role of Zn in regulating SOD activity, an observation which is also clearly depicted by the isozymes stained for SOD activity (see figure 21, page 73). Superoxide dismutase has been reported to be stabilized by Zn and hence a higher Zn supplement is able to enhance the $O_2^{\cdot -}$ dismutation activity facilitating its detoxification in the subsequent steps. In the next step CAT activity was also enhanced (see figure 22, page 75) by Zn supplements which could be visualized again in the 7.5% native gel stained for CAT isozymes indicating enhanced staining for higher activity of CAT at Zn supplemented Cd treatments (see figure 23, page 75). The combined action of SOD and CAT would efficiently eliminate H_2O_2 and $O_2^{\cdot -}$ indirectly protecting the plant from highly toxic $OH^{\cdot}$ radical. The above results indicate the probability of the role of Zn in stimulating the biosynthesis of antioxidant enzymes (Cakmak 2000) as evidences from a variety of biological systems 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). In plants treated with only Cd 10 μM, SOD showed a slight increase in activity (see figure 20, page 73) but not CAT (see figure 22, page 75). The excess production of ROS by Cd would have inactivated the antioxidant enzymes. It has been suggested that very high levels of H_2O_2 inhibits Cu/Zn SOD (Casona *et al* 1997) through Cu^{2+} to Cu^{+} reduction and formation of excess hydroxyl radical. Similarly the observed indifference in CAT activity in Cd treatments indicates inactivation of the enzyme bound heme group (Luna *et al* 1994) leading to impaired CAT activity. Since CAT is very sensitive to $O_2^{\cdot -}$ it

could be inactivated by the higher levels observed in Cd-10 μM treatments (Scandalios *et al* 1997, Cakmak 2000). On the other hand PODs especially APX is reported to be stimulated by high levels of H_2O_2 (Oidaira *et al* 2000) which was found be true in our system as well, especially in Cd treatments supplemented with different concentrations of Zn. The stimulated POD (see figure 24, page 76) and APX activity (see figure 26, page 77) due to higher H_2O_2 levels coupled with enhanced activity aided by Zn supplementation would effectively scavenge H_2O_2 in the plant system (Asada 1992, Anabel *et al* 2002). The isozymes of POD as well as APX in the native gel also revealed induction of a new isozyme clearly depicting enhanced activity (see figures 25 and 27, page 76 and 77). Guaiacol peroxidase did not show a significant increase in H_2O_2 scavenging activity or in-gel activity staining indicating its secondary nature of action in comparison with APX (see figures 28 and 29, page 79). The enhanced ROS scavenging antioxidant enzyme activity by Zn directly indicates the efficiency of Zn as an antioxidant thereby combating Cd-induced oxidative stress. Plants treated with only Zn did not show a very high increase in activity (see table 5, page 80) in comparison with Zn supplemented Cd treatments indicative of the fact that in Zn supplemented Cd treatments the observed changes are due to the extreme oxidative stress induced by Cd and protection against this by induction of high levels of antioxidant enzymes by Zn.

4.4.2. Zn modulates cellular antioxidants and ascorbate-glutathione cycle to function against Cd toxicity

The reactive oxygen species are also regulated by various endogenous non-enzymatic antioxidants to maintain the equilibrium between free radical production and defense reactions in favour of the latter (Alscher *et al* 1997). The results of thiols and GSH-GSSG estimation in Cd-10μM treatments indicate a drastic reduction of protective thiol groups (including protein thiols) (see figure 30, page 81) with decrease in reduced

glutathione (GSH), and simultaneous increase in oxidized glutathione (GSSG) (see figure 31, page 81), a true indication of oxidative stress. The toxicity of Cd is usually checked by various endogenous antioxidants whereby the thiol pool of the plants plays an important role (Potters *et al* 2002). GSH, an important component of the thiol pool (Noctor and Foyer 1998), functions as a stress indicator shifting the redox state in response to oxidative stress (May *et al* 1998, Devi and Prasad 1998). The formation of oxidized GSSG in Cd-10 uM 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 stresses also like photooxidation, ozone etc (Xiang *et al* 2001). Zinc clearly restored the lost thiol pools (see figure 30, page 81), a natural phenomenon associated with Zn (Cakmak 2000), restored and enhanced GSH to a great extent, with simultaneous decrease in the oxidized GSSG form (see figure 31, page 81) as seen from the results of Cd-treated plants with supplemented Zn. Zn probably modulates GSH levels by regulating its biosynthesis or through the reactive cysteine residue of GSH by disulphide protection (Cakmak 2000) or by the efficient functioning of GR (Foyer & Halliwell 1976). Moreover a reduced GSSG level in Cd-treated plants with supplemented Zn would induce proper protein synthesis as higher levels of GSSG has been proved to interact with the initiation factors and inhibit stress resistant metabolites formation (Alscher, 1989).

Cadmium at 10 uM concentration affected the total AsA (AsA + DHA) pool to a great extent (see figure 32, page 84). In plants, AsA plays a pivotal role in protecting cells against oxidative stress (Smirnoff 1996, Smirnoff *et al* 2001). Its key role is illustrated by an *Arabidopsis thaliana* mutant *(vtcl)* containing 30% of the wild type AsA, is hypersensitive to oxidative stress (Conklin *et al* 1995). Ascorbate is one of

the best-known antioxidants involved in the scavenging of ozone and ozone-derived breakdown products (Luwe *et al* 1993). Ascorbate reacts with $O_2^{\cdot-}$, singlet oxygen (directly), ozone (chemically), and H_2O_2 (enzymatically through APX), and will thus assist in neutralizing these potential toxicants (Potters *et al* 2002). Ascorbate is one of the important redox molecules participating in the AGC (Noctor and Foyer 1998, Horemans *et al* 2000) and hence its redox status directly affects the functioning of AGC (Foyer 1993, Di Cagno *et al* 2001). The first enzyme of AGC- APX, which utilizes AsA in reduced form as a reductant is one of the most potent H_2O_2 scavengers in the plant cell (Shigeoka *et al* 2002). Since APX utilizes AsA as its reductant, AsA forms ascorbyl radical (monodehydroascorbate- MDHA), which may undergo disproportionation to oxidized AsA form (DHA) depending on the cellular redox status (Potters *et al* 2002).

Cadmium toxicity directly affected the total AsA content enhancing the oxidation of AsA to DHA (see figure 32, page 84). The changes in the ratio of AsA to DHA are crucial for the cell to sense oxidative stress and respond accordingly (Di Cagno *et al* 2001, Potters *et al* 2002). Zn supplementations to Cd-treated plants maintained the ratio of AsA to DHA in the correct proportion required for efficient cellular functions. Zn mediated decrease in DHA must be through an active AsA regeneration by MDHAR and DHAR, which is directly proven by our results of MDHAR and DHAR enzyme assays (see figures 33 and 34, pages 84 and 85) and the activity staining of DHAR (see figure 35, page 85) which revealed increased activity at Zn supplemented Cd treatments. The data from Cd-10 uM alone treated plants indicated a drastic reduction in the activities of MDHAR and DHAR, which corroborate with our findings on the altered ratio of AsA to DHA. The toxicity induced by Cd inhibited the activity of MDHAR. This in conjunction with an impaired DHAR activity and enhanced monovalent oxidation (Cuypers *et al* 2000) lead to increased non-enzymatic disproportionation of MDHA and hence increased formation of the oxidized form of

AsA. Extremely high DHA accumulation is generally considered as a negative event for the cell metabolism (Morell *et al* 1997, 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 (Drazkiewicz *et al* 2003) by Zn to counteract Cd-induced disturbances to AsA redox status in *C. demersum*.

It has been reported that the conversion of DHA to AsA requires extensive utilization of GSH (De Pinto *et al* 2000), but Zn supplementation to Cd treatments revealed unaffected redox status of GSH. This could have been possible either due to GSH biosynthesis or GR involved conversion of GSSG to GSH. It could be observed from our results that Zn induces a very high increase in the activity of GR (see figure 36, page 87), which could have resulted from *de novo* GR protein synthesis connected with the trigger mechanism initiated by Cd-induced oxidative stress (Drążkiewicz *et al* 2003). Glutathione reductase 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, Hernandez *et al* 1999). Glutathione reductase was inhibited by Cd, shifting the redox equilibrium of GSH to a more oxidative side (GSSG). Zn by its active participation in thiol protection (Cakmak 2000, Powell 2000), a function particularly associated with Zn, enhanced the activity of this enzyme ensuring efficient cycling and utilization of the pyridine nucleotide reducing power (Kocsy *et al* 2001). This inference can also be drawn from the native gel staining of GR where, Zn supplements induce additional isozymes clearly depicting Zn enhanced GR activity (see figure 37, page 87). It has been reported that redox perturbations especially in cellular GSH levels act as signal in stimulating defense genes (Wingate *et al* 1988, May *et al*, 1998). Recent experiments also indicate that GSH may function as a cellular sensor to maintain the NADPH pool also (May *et al* 1998).

Zn being non-redox cycling in nature (Vallee & Falchuk 1993, Zago & Oteiza, 2001) would maintain the chemical potential of the redox molecules.

4.4.3. Alleviation of Cdtoxicity by Zn utilizing glutathione metabolism enzymes

Glutathione metabolism involves many reactions where glutathione is synthesized, degraded, conjugated or oxidized (Meister 1989, Noctor *et al* 1998). The maintenance of GSH pool is not only because of GR but also suggestive of the involvement of γ-GCS, which is the rate-limiting enzyme in GSH synthesis. Overexpression of γ-GCS rather than GSH-synthetase has been suggested to enhance GSH levels (Noctor *et al* 1998). Our experimental evidences showed that Cd as well as Zn did not affect this enzyme to a great extent (see figure 38, page 89). This suggests that Zn does not activate GSH biosynthesis in any way but only involves itself in maintaining the redox status of GSH. The above observations also suggest that Zn does not mediate phytochelatin synthesis in our experimental situation in *C. demersum*, because of the observed non-involvement of Zn in increasing thiols level or GSH synthesis, which is a pre-requisite for the formation of any metal-binding protein (Rauser 2000). Glutathione-S-transferases (GST) catalyze both GSH-dependent conjugation and reduction thereby playing an active role in detoxification as GST mRNA responds very quickly to oxidative stress (Conklin and Lasat 1995). Cadmium triggered the activity of GST only to a little extent (see figure 39, page 89). This 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 to a great extent 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 eliminate them from propagating further damage (Nagalakshmi and Prasad 2001, Foyer *et al* 2001). Another important H_2O_2 scavenger associated with GSH is GSH-PX. This enzyme is a part of the arsenal of the protective enzymes, involving coupling reactions with glutathione and cellular detoxification of H_2O_2, as a response to oxidative stress (Drotar *et al*, 1985). Cadmium did not stimulate the peroxide scavenging activity of GSH-PX. Instead this enzyme was inhibited in its function to a great extent (see figure 40, page 91). In contrast Zn supplementation promoted the enzyme activity and hence its H_2O_2 scavenging activity.

Plants treated with only Zn did not show significant changes in the above parameters (see tables 6-8, pages 82, 88 and 92 respectively) indicating that the chosen 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 the higher activities of APX, MDHAR, DHAR and GR as well as GST and GSH-PX enzymes in Zn supplemented Cd-treated plants indicate the specific role of Zn in promoting ROS detoxification through AGC enzymes also involving GSH-metabolism.

4.5. Carbonic anhydrase: changes in structure, conformation and function in response to Cd toxicity and Zn supplementation

Carbonic anhydrase (CA) has been extensively studied in many organisms from cyanobacteria to higher plants, animals and human systems (Smith and Ferry 2000, Atkins *et al* 1972, Graham *et al* 1984, Rengel 1995, Armstrong *et al* 1966, 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 less (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 air (Walker 1983, Badger 1987). To maintain the photosynthetic efficiency in spite of low CO_2 availability the carbon-concentrating mechanism (CCM) functions (Lane and Morel 2000, Rascio 2002). This CCM has two components namely a mechanism for taking up HCO_3^- and a Zn-requiring CA that catalyses interconversion of HCO_3^- to CO_2 (Badger and Price 1994).

The present investigation revealed the existence of active Zn-dependent CA in *C. demersum* like other aquatic angiosperms *{eg: Myriophyllum, Hydrilla, Elodea)* where HCO_3^- diffusion and then conversion to CO_2 by CA plays a very vital role in maintaining the inorganic carbon levels (Prins *et al* 1982, Elzenga and Prins 1988, Shiraiwa and Kikuyama 1989). The toxicity of Cd-10 μM drastically affected the functioning and activity of CA. However Cd-treated plants when supplemented with Zn, showed not only active restoration of the CA activity, but also increase in activity at the highest concentration of Zn proving the complete dependence of the active form of this enzyme on the presence of sufficient levels of Zn (see figure 41, page 91). Zinc being a stable non-redox cycling element maintains the chemical potential of the Zn-enzymes and associated proteins protecting them from peroxidative damage and oxidative stress. Activity of CA has been suggested to be a suitable indicator of the levels of physiologically active Zn in the plant tissue (Bar-Akiva and Lavon 1969, Gibson and Leece, 1981).

Cadmium possess properties identical to Zn (Nieboer and Richarson, 1980). Hence Cd can readily inhibit most of the Zn-dependent processes either by competition for similar sites or displacement/substitution reactions in occupying the active sites of the Zn-metalloproteins (Nieboer and Richardson 1980, Siedlecka 1995). The purification of CA from *C. demersum* revealed this toxic function of Cd towards CA. There was a reduction in the recovery as well the activity of the enzyme purified from

Cd 10 µM-alone-treated plants (see table 10, page 98) (see figures 44 and 45, page 95). The estimation of Zn content in the purified enzyme not only showed a drastic reduction in the amount of Zn but also traces of Cd (see figure 51, page 100) as recorded by flame atomic absorption spectrometry. Our experiments showed a reduction of 73% in Zn content and its substitution by Cd would impair the structure as well as the activity of CA. 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 disturbed protein synthesis (Marschner and Romheld 1991). Hence the associated photosynthetic processes also would 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 corroborate with our results. Redox active metals like Cu, Fe and other transition metals such as Cd, Hg are known to primarily affect the enzymes and proteins through its interaction with the -SH groups and induce redox cycling (Powell 2000). Moreover the S-P orbital energy seperation 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 low charge density (Schützendübel 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 to 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 highly active CA (see figures 46 and 47, page 96) (see table 11, page 99). The Zn content estimated was also slightly higher (see figure 51, page 100) than that of the control accounting for the increased uptake of Zn for enhanced CA biosynthesis and catalytic activity (see figure 41, page 91). Zinc alone treatments also showed an active enzyme purified with Zn content closely comparable with that of the

control (see figures 48, 49 and 51, page 97, 100) (see table 12, page 99). The presence of sufficient levels of Zn in Cd-treated plants with supplemented Zn strengthens the Zn-metalloprotein interaction by protection of the -SH groups from thiol oxidation and intramolecular disulphide formation, a function primarily associated with Zn (Cakmak 2000, Cakmak and Braun 2001). Zn by associating with the -SH groups renders them inactive by steric hindrance and hence their susceptibility towards disulphide formation (Bray and Bettger 1990). In conclusion Zn restored the Cd impaired CA activity in C. *demersum*.

Cadmium (like several other heavy metals) is known to affect the structure, and conformation of enzymes through ROS-mediated oxidation (Van Assche and Clijsters 1990, Siedlecka and Krupa 2002). This could be well depicted by analyzing the conformation of CA using circular dichroism (CD) spectra. Circular dichroism is one of the most sensitive physical techniques for determining structures, also affording a direct interpretation of the changes in protein secondary structural conformation (Johnson 1990, Kelly and Price 1997) especially in the far UV region where both the shape and intensity of dichroic band depend on protein secondary structure (Venyaminov *et al* 1996). The far UV-CD spectrum showed a perfect α-helical conformation for the native CA purified from control plants without any metal treatment (see figure 54, page 104). In contrast Cd 10 µM-alone-treated plants showed loss of ellipticity, distorted a-helical conformation and transformation into open and random coiling, a clear indication of unfolding of the native CA. The main forces of interaction responsible for the protein structure have been identified as hydrophobic, electrostatic, valence, dispersion and repulsive forces (Anfinsen 1973, Creighton 1993). Since CA purified from Cd-10 µM treatments showed metal substitution reactions with Zn, the enzyme co-factor in the active site of CA, the electrostatic interactions within the protein molecule would be

drastically affected (Creighton 1993). This would also induce destabilizing intramolecular repulsive interactions between like charges acting as the driving force for the unfolding of the macromolecule as they fail to overcome the interactions of hydrophobic forces, salt bridges and metal ion-protein interactions that favour folding (Anfinsen 1973, Ali *et al* 1999). This creates distortion in the ordered secondary structure as clearly depicted in the CD spectra of CA from Cd 10 µM-alone-treated plants, thereby decreasing the native helical conformation (see table 13, page 104). Enzyme unfolding drastically affects other physicochemical characteristics such as solubility, stability and pK_a of the enzyme to a great extent leading to impaired functioning (Fleschin *et al* 2000).

In contrast CA purified from Zn supplemented Cd treatments clearly showed restoration and perfect α-helical conformation (see figure 54, page 104) similar to that of the native CA with restored P-form and decreased unordered conformation (see table 13, page 104), indicating that Zn protects CA from Cd-induced damage, restoring its original conformation and hence physiological function. Zinc being catalytically associated as a co-factor with CA, 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 1978). In a metalloenzyme like CA even a slight change in the protein conformation by substitution of catalytically inactive Cd would cause distorted unfoldings to form a totally impaired structure (Vallee and Auld 1990, Vallee and Falchuk 1993, Prasad 1995).

The decrease in the ellipticity observed in Cd-10 µM alone treated plants (see figure 54, page 104) could be accounted for insoluble aggregation of the fragmented protein. Free radicals, especially OH generated by Cd stress irreversibly oxidize amino acids by reaction with a-hydrogen atom of amino acid residues to form

carbon centered radical, further producing alkoxyl radical leading to peptide bond cleavage (Cabiscol *et al* 2000, Shacter 2000a) and increased susceptibility to proteolysis (Wolff *et al* 1986, Davies *et al* 1987b). Such carbon-centered radicals of each fragment react with another one to form protein-protein cross linked derivatives, leading to reduction of effective peptide concentration (Stadtman and Levine 2000) seen as decreased ellipticity in the CD of CA from Cd-10 uM alone treated plants. Carbonic anhydrase from Zn supplemented Cd treatments show a much higher ellipticity values indicating an intact conformation and non-aggregation of the enzyme molecule proving the nature of Zn in protecting CA from Cd toxicity.

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 (eg: Fe, Cu) reacts with H_2O_2 to form a reactive intermediate in the immediate proximity of amino acid side chains (Stadtman and Oliver 1991, Stadtman and Levine 2000). Two amino acids highly prone to such oxidation are cysteine and methionine because of their susceptible sulphur atom. Similarly other amino acyl moieties lysine, arginine, proline and threonine incur formation of carbonyl groups (aldehydes and ketones) on the side chains (Amici *et al* 1989, Shacter 2000b). In the course of this process CA finally becomes catalytically inactive or less active (Cabiscol and Levine 1995) and more thermolabile. The reduction of –SH groups and consecutively high levels of protein carbonyls estimated in CA from Cd-alone treated plants (see figure 59, page 109) give good indication of the above statement. In contrast Zn reduced the carbonyl formation and maintained the thiol level in CA from 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 prevent aggregation of the enzyme molecule and aid in proper protein folding (Rozema and Gellman 1996).

The near UV-CD spectra (240-300 nm) gave a conclusive picture of the role of Zn in restoring the intact conformation of CA and protection from Cd toxicity. The near UV-CD spectrum is used to monitor the tertiary structure of the proteins (Strickland 1974) where, the observable cotton effects are derived mainly from tyrosine and tryptophan residues due to their interaction with the asymmetric environment (Kahn 1979). The spectra of native CA from control clearly shows characteristic peak at 274 nm (see figure 53, page 102) indicating the specific tertiary interactions of the aromatic residues. Similarly the observed positive peak at 248 nm is suggestive of disulphide and partially phenylalanine residues in the enzyme molecule (Kahn 1979). Cadmium induced denaturation of the native structure of CA lead to disappearance of these cotton effects at 248 and 274 nm as the protein unfolded to form a distorted structure in Cd-10uM alone treated plants. Whereas in Zn supplemented Cd treatments the cotton effects were clearly visible with increase in ellipticity due to intact protein conformation and asymmetric interactions maintained by Zn in the enzyme molecule. Previous investigations have also shown the importance of tryptophan association with CA in the near UV-CD region contributing to the strong signals observed (Freskgard *et al* 1994, Boren *et al* 1996). This important relation could be well depicted by the analysis of UV difference spectra and fluorescence characteristics of CA, where the aromatic amino acid residues residing in the protein hydrophobic clusters reveal absorbance properties as reflected by changes in the environment and configuration of the protein. UV difference spectra of CA from Cd alone treatments (see figure 52, page 102) indicated a drastic change in the environment of the aromatic amino acid residues as observed by the decrease of intensity in spectral maxima from 270-285 nm. Similar observations could be made from the intrinsic tryptophan and tyrosine fluorescence of CA from Cd-10uM alone treated plants, which, revealed a substantial decrease in fluorescence as well as shift in the maxima in comparison to the native CA purified from control plants

(see figures 55 and 56, page 106). It can be stated from this observation that the exposure of aromatic amino acid residues from the interiors of the hydrophobic pockets of the unfolded enzyme to the external solvent lead to the changes in the intensity of fluorescence with concomitant shift in the emission maxima. It has been reported that a fully exposed tryptophan in an aqueous buffer is expected to have a maxima well over 350 nm (Eftink and Ghiron 1981, Eftink 1991, Ptitsyn 1993). Replacement of tryptophan by point mutation with alanine in β-lactoglobulin showed substantial reduction in fluorescence indicating its interactive contribution to the protein structure (Bhattacharjee and Das 2000). Thus the quenching effects of the tryptophan and tyrosine fluorescence observed in Cd-10uM alone treated plants are due to interaction with charged groups in the proximal aqueous environment rather than protein moiety, contributing to the loss of emission intensity observed in the unfolded state. Nevertheless, tyrosine fluorescence and tyrosine oxidation products are biologically meaningful indicators and hence studies on these would give a better picture of the antagonizing effect of Zn against Cd stress. Dityrosine production is a sensitive, simple and useful marker for protein modification by OH radical (Giulivi and Davies 1994, Wright *et al* 2002) as, intermolecular covalent (non-disulfide) dityrosine formation is certainly one mechanism for protein aggregation in the absence of reductants to repair oxidised tyrosine (Thiols, Vitamin E) (Dean *et al* 1997), although many other cross-links have also been reported (Davies *et al* 1987a,b). Cadmium treatments induced extensive tyrosine oxidation and formation of dityrosine as observed by the intensive fluorescence in CA from Cd treated plants (see figure 57, page 107). The reduction of protective thiol groups in CA from Cd treated plants would have lead to this extensive protein cross-linking through dityrosine formation. Under such conditions hydroperoxide formation can be expected on N-terminal tyrosines and those with the adjacent lysine residues also (Gebicki and Gebicki 1993, Morgan *et al* 2002, Wright *et*

al 2002) further enhancing hydroperoxide formation through lysine rich regions (Winterbourn and Kettle 2003). There are many indications that radical damaged proteins are rapidly removed *in vivo* in efficient systems, but polypeptides, which undergo radical modification with subsequent inhibition of proteolysis due to inactivation of proteolytic enzymes by metal stress, accumulate within cells completely impairing cellular function (Wolff *et al* 1986).

Loss of tryptophan and tyrosine fluorescence, and shift in emission maxima was completely regained in Cd treatments with supplemented Zn indicating intact folded protein conformation. There was complete inhibition in the formation of dityrosine (oxidized tyrosine) also 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.

Changes in the protein surface structure were monitored by studying the binding of 8-anilino-1-naphthalene sulfonic acid (ANS), as a conformation sensitive probe (Cardamone and Puri 1992, Bhattacharjee and Das 2000). It has been established very well that interaction of such hydrophobic fluorescent probes with the exposed hydrophobic sites on the surface of unfolded and denatured protein molecules would result in considerable increase of the dye fluorescence intensity and a blue shift in its spectrum (Uversky *et al* 1996, Matulis and Lovrien 1998). This was clearly depicted by the ANS fluorescence spectrum of CA from Cd-10uM alone treated plants (see figure 58, page 107) showing a very high increase in ANS binding fluorescence and a blue shift of 10 nm indicating exposed hydrophobic pockets due to considerable structural damage to CA. 8-Anilino-1-naphthalene sulfonate-protein fluorescence was totally reduced to control level in CA from Zn supplemented Cd treatments indicating protected hydrophobic regions in intact and folded state of native C A, similar to that of

control. Zinc alone treatments did not affect the α-helical conformation, the fluorescence properties of aromatic amino acid residues and thiol content of the native CA (see figures 52-59, pages 102, 104, 106, 107 and 109 respectively). Further, there was absolutely no increase in oxidative products (dityrosine and carbonyls) or ANS-hydrophobicity in Zn alone treatments indicating that the experimental concentration of Zn chosen for our study did not affect the native structure and conformation or the properties of CA in any way. 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), there can be an inverse correlation existing between Zn status and protein oxidation, with radical damage to proteins being accentuated due to Zn deficiency (Cakmak 2000). We have conclusively proved the above statement of Zn combating oxidative attack on proteins, induced by Cd stress with the results obtained by our investigations on C A, further substantiating the role of Zn as an antioxidant.

4.6. Zinc protects chloroplasts and associated photochemical functions front Cd toxicity

The present findings indicated a protective role of Zn to plant chloroplast functions in Cd-treated *C. demersum*. Our studies showed that chlorophylls a, b and carotenoids were severely reduced in Cd-treated plants in the absence of Zn supplementation (see figure 60, page 109). In contrast in Cd-treated plants with supplemented Zn, it was observed that there was full protection and restoration of the chlorophyll levels. Zinc probably maintains chlorophyll synthesis through sulphydryl group protection of the oxidation prone δ-aminolevulinic acid dehydratase (ALA dehydratase) and protochlorophyllide reductase (Baszynski *et al* 1980, Gadallah 1995, Ouzounidou 1995, Mysliwa-Kurdziel *et al* 2002, Mysliwa-Kurdziel *et al* 2003). Since

ALA dehydratase catalyzing the conversion of ALA to porphobilinogen requires Mg^{2+} or Zn^{+} 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 complete chlorophyll moiety (Lebedev and Timko 1998). 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.

The decreased carotenoid content observed in Cd-treated plants would increase the membrane damage due to loss of ROS scavenging function. Carotenoids are known to quench the oxidizing species and triplet state of the chlorophyll and other excited molecules in the pigment bed, which are seriously involved in disrupting metabolism through oxidative damage to cellular components (Larson 1988, Polykov *et al* 2001, Candan and Tarhan 2003). In Cd-treated plants with supplemented Zn, the carotenoid levels were well maintained (see figure 60, page 109). Perhaps Zn is able to increase or restore the biosynthesis of carotenoids. Thus Zn influences the proper development of the chloroplast pigments, which would subsequently maintain the photosynthetic activity of the system.

In plants treated with toxic Cd- alone, isolated chloroplasts showed a significant loss of photosynthesis, water oxidation capacity (whole chain transport), PS II and PS I catalyzed electron transport system (see figure 62, page 111). Metal stress impairs electron transport activity by inducing peroxidation and loss of thylakoid membrane integrity. It is very clear from our results of chloroplast intactness that chloroplasts isolated from Cd-treated *C. demersum* showed a very high loss of intactness accounting for the ROS mediated membrane damage and integrity (see figure 61, page 111). It has been reported that Cd affects the lipid structure around PSII especially the light harvesting chl a/b protein complex II (Krupa *et al* 1987, Prasad

1995b) leading to loss of major fatty acids (Peters and Chin 2003) and production of lipid hydroperoxides. This induces release of several Chl a-protein complexes and thylakoid membrane proteins including manganese stabilizing protein (MSP) and plastocyanin (PC) (Gartska and Kanuiga 1988). An impaired PC remains as a block in further electron transport process. This severe extent of peroxidative protein loss is ultimately responsible for the crippled photosynthetic process. Zn on the other hand shows clear restoration of the lost photosynthetic activity indicating its action against the toxic nature of Cd (see figure 62, page 111). Zn is known to have a stabilizing and protective effect on the biomembranes (Chvapil 1973, Cakmak 2000). This in addition with increased carotenoid synthesis triggered by Zn protects the thylakoid membrane from ROS mediated peroxidative damage and hence the loss of thylakoid proteins also. This is conclusively proved by our results of chloroplast intactness where Cd-treated plants with supplemented Zn showed higher intactness of isolated chloroplasts indicating intact thylakoid membrane (see figure 61, page 111).

In aquatic plants growing in a heavy metal contaminated environment the metabolically active metal ions of chloroplasts have been reported to be substituted by heavy metals (Prasad and Strzałka 1999). 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 (Baszynski *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 (Šeršeň and Kráľová 2001) and PSII-D1 polypeptide. This Cd-D1 complex interferes with the degradation of Dl protein by a protease, a normal operation of the PS II reaction center, leading to an impaired PS II activity (see figure 62, page 111) (Hideg and Spetea 1994). This dysfunction propagates throughout the electron transport chain in the system. Zn by controlling the levels of Cd entering into the system not only controls its intracellular

levels, but also the replacement of a toxic metal, Cd by a physiology 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 the PSII. An active PSII initiates a proper electron transport and hence an active functioning of PSI and complete photophosphorylation process. In conclusion our experimental results indicate that in Cd treated plants with supplemented Zn there is active protection of photosynthetic apparatus and hence restoration of photosynthetic as well as electron transport activities. Zn also enhanced the biosynthesis of photosynthetic pigments viz. chlorophyll and carotenoids ultimately proving beneficial for the photosynthetic machinery of the plant system.

4.7. Zinc reversal of Cd-induced damage to DNA.

The toxicity of Cd on the structural integrity of DNA was identifed utilizing the principle of formation of fluorescent complex between double-stranded DNA and ethidium bromide (EB) (Cai *et al* 1995, 1998, Cai and Cherain 2003). DNA damage after exposure to the toxic levels of Cd (10 μM) resulted in distinct loss of fluorescence due to the decreased binding of EB with DNA (see figures 63 and 64, page 114). This can be a contribution of several types of DNA damage including strand scission- single- and double-strand breaks, base oxidation and base liberation (Breen and Murphy 1995, Gichner *et al* 2004) leading to DNA lesions and hence decreased interaction with EB. DNA gel mobility assay also clearly determined the enhanced mobility of the DNA on the agarose gel due to loss of double stranded nature and fragmentation (Szuster-Ciesielska *et al* 2000, Cai and Cherian 2003) in DNA isolated from Cd-10μM treatments when compared with the intact DNA isolated from the control (see figure 65, page 115). Various possible mechanisms have been explained to be involved in the process of DNA damage as described in the introduction section. Firstly direct binding

of Cd to DNA bases specifically G, A and T, interaction of malonialdehyde with DNA (Mukai and Goldstein 1976, Summer field and Tappel 1984) and interaction of Cd induced ROS with DNA (Bhanoori and Venkateswerlu 1998) lead to extensive DNA damage. Secondly metal ions are also known to inhibit the DNA repair processes (Hartwig and Schwerdtle 2002, Fatur *et al* 2003). The DNA isolated from Zn supplemented Cd treatments showed complete restoration of the lost fluorescence of EB-DNA interaction (see figures 63 and 64, page 114) indicating intact structural integrity maintained by Zn ions favouring enhanced binding of EB with the DNA. Similarly the DNA also showed complete inhibiton of the mobility indicating the non-fragmentation of the DNA strands (see figure 65, page 115). 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 $OH^{\cdot}$ attack on either the DNA or protein through -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, Mishima *et al* 1995, Ebadi *et al* 1996). The latter seems to be the most probable mechanism in the present system of study.

4.8. Exogenously supplied amino acids (cysteine, glutamic acid and glycine) and organic acids (citric, oxalic and malic acids) alleviate Cd toxicity.

4.8.1. Amino acids (cysteine- Cys, glutamic acid- Glu and glycine- Gly)

Cysteine, glutamic acid and glycine supplements to 10 uM Cd treatments were ineffective in reducing Cd uptake by *C. demersum* (see figures 66 and 67, page 119), but morphologically the plants were healthy without showing any toxicity symptoms. It can be inferred from this that the detoxification mechanism triggerred by amino acid supplements involves a pathway operational within the plant system and there is absolutely no competition for metal uptake with Cd by the amino acids supplemented. In contrast Zn 200 uM combined with amino acid supplements (Cys at 1.0 mM, Glu at 0.5 mM and Gly at 0.2 mM concentrations respectively) effectively reduced Cd uptake (see figures 68 and 69, pages 120 and 121) by the plant system to a greater extent thereby decreasing the intracellular Cd concentration and hence toxicity due to Cd ions at the first step itself. In only amino acid supplemented Cd treatments the Cd-induced decrease in chlorophyll and carotenoid content was completely restored, but without any enhancement in the levels of pigments (see figures 70 and 71, pages 122 and 123). Cd-increased lipid peroxidation was completely reduced by the amino acids indicating enhanced protection against peroxidation and membrane damage (see figures 74 and 75, page 125). Similarly the Cd-impaired balance between GSH/GSSG ratio was restored/ maintained in amino acid supplemented Cd treatments (see figures 78 and 79, pages 127 and 128). An important observation is the increase in the levels of GSH which could possibly be because of the enhanced synthesis of GSH, an inference which could be directly drawn from the assay of γ-GCS (see figures 82 and 83, page 130). Zn 200 uM in addition to amino acid supplemented Cd-10 uM treatments were effective in just restoring photosynthetic pigments (see figures 72 and 73, pages 123 and 124), reduced peroxidation rate to a greater extent (see figures 76 and 77, page 126),

enhanced GSH levels (see figures 80 and 81, pages 128 and 129), but did not further enhance y-GCS activity. (see figures 84 and 85, page 131). These results indicate the direct involvement of Zn in metal elimination and detoxification process, but no role in GSH synthesis. The enzymatic assembly of amino acids Cys, Glu and Gly into the functional tripeptide GSH is well characterized (Grill *et al* 1985, Rauser 1995) and GSH dependence on Cys and Glu levels has been directly demonstrated (Foyer *et al* 2001). The results of the experiments give conclusive proof of the involvement of GSH-mediated detoxification of Cd ions. Amino acids Cys and Glu being the basic components of GSH (Noctor and Foyer 1998), a crucial antioxidant of the plant cell are also important for the synthesis of phytochelatins-PCs (Poly (γ-glutamyl-cysteinyl)-glycines)- metal binding peptides which chelate free metal ions and transport them to vacuole (Cobett 2000, Goldsbrough 1998, Rauser 2000). Biosynthesis of GSH, an important step in the progress of cell towards detoxification through GSH and PCs is known to proceed via the formation of γ-glutamylphosphate which reacts with Cys. It has been proposed that γ-glutamylcysteine synthetase initially reacts with ATP to form a phosphorylated enzyme, which subsequently reacts with L-glutamate to yield a y-glutamyl enzyme intermediate (Sekura and Meister 1977). However Following Cd exposure there are many reports suggesting depletion of GSH pools during PC synthesis (Berger *et al* 1989). It is also well known that GSH synthesis is regulated in part, by nonallosteric inhibiton of γ-glutamyl cysteine synthetase by GSH itself (Richman and Meister 1975). Reports also indicate the increase of γ-GCS activity several fold in many systems (Rennenberg and Brunold 1994, Ruesegger *et al* 1992, Herschbach et al, 1998), a response not noticeable in Cd treatments with Zn supplements but distinct in amino acid supplemented Cd treatments. Therefore supplements of amino acids favoured GSH synthesis and hence GSH-mediated metal detoxification which could probably involve PCs. Cadmium-induced toxicity reversal by Cys, Glu and Gly in *Scenedesmus*

quadricauda (Reddy and Prasad 1992b), Cys and GSH inhibiting Cu toxicity to *Chlorella pyrenoidosa* and *Nitzchia closterium* (Stauber and Florence 1986) have been reported earlier.

4.8.2. Organic acids (citric, oxalic and malic acids)

Citric, oxalic and malic acid supplements to Cd treatments slightly enhanced the uptake of Cd by *C. demersum* (see figures 86 and 87, page 134). Inspite of enhanced Cd accumulation the plant was morphologically healthy without displaying any toxicity symptoms, indicating a distinct detoxification mechanism operational within the plant. An increase in Cd uptake from Cd treatments with increasing supplementation of organic acids may be ascribed to the tendency of Cd to interact with organic ligands resulting in labile, organically-bound Cd and thus enhancing its plant accumulation (Stevenson and Fitch 1994). Consequently the levels of Cd-reduced photosynthetic pigments- chlorophyll and carotenoids showed complete restoration (see figures 90 and 91, pages 137 and 138). Similarly Cd-increased lipid peroxidation was also effectively controlled by organic acid supplements to Cd-10 uM treatments (see figures 94 and 95, page 140). The non-toxic behavoiur of Cd, inspite of Cd accumulation in plants can be ascribed to the fact that organic ligands not only enhance the solubility of trace metals, but also reduce their toxicity to plants. The free metal ions have been reported to be more toxic compared with organically complexed molecules (Dongser *et al* 1995, Krishnamurti *et al* 1997). Organic acids and their role as detoxification agents and in mobilization of essential micronutrients have been widely discussed (Jones 1988). Organic acids-bound Cd with enhanced accumulation and less toxicity have been reported in *Triticum aestivum* (Nigam *et al* 2000). Higher concentrations of organic acids have been reported in tolerant plants rather than sensitive plants (Yang *et al* 1997). Secretion of organic acids is one of the principal

responses of many plants exposed to toxic concentrations of Al (Ma 2000). Therefore our results clearly substantiate the chelation ability of organic acids in detoxifying Cd. Zn 200 uM addition did not show any distinct variation in the above tested parameters, not even reduction in Cd uptake a phenomenon observed in Cd+Zn 200 uM and Cd+ amino acids+ Zn-200 uM treatments (see figures 88, 89, 92, 93, 96 and 97, pages 135, 136, 138, 139 and 141 respectively). This indicates the ineffectiveness of Zn supplementation to organic acid supplemented Cd treatments, due to the enhanced binding affinity of organic acids to Cd, a toxic metal rather than Zn, a nutrient and hence enhanced Cd uptake rather than Zn.

5. Conclusions

1) Cadmium at 10 uM concentration clearly exhibited toxicity to the plants assessed as oxidative stress to all the cellular components.

Zn showed an antagonistic interaction with Cd and alleviated Cd toxicity as:

2) Zinc inhibited Cd uptake directly by competition and indirectly by controlling H^+ATPase leading to a reduced intracellular concentration of Cd.

3) Zinc reduced Cd-increased peroxidation, membrane leakage and lipolysis, formation of reactive oxygen species triggered by Cd-influenced NADPH dependent oxidation.

4) Zn substantially increased the antioxidant enzymes activity SOD, CAT, POD, APX to a great extent effectively combating ROS.

5) Zinc exerted its influence considerably on the maintenance and cycling of the redox pool (AsA, GSH and -SH) of the cells through modulation of the ascorbate-glutathione cycle involving APX, MDHAR, DHAR and GR, enhanced GSH-PX and GST activity indicating enhanced detoxification of ROS.

6) The restoration and enhanced functioning of CA in Zn supplemented Cd treatments by competitive substitution of Cd by Zn in the impaired enzyme and the protection of enzyme conformation and properties by Zn conclusively prove the antagonistic nature of Zn towards Cd toxicity and the active role of Zn as a stabilizer of protein structure.

7) The studies on photochemical functions provide a clear picture of the protective role of Zn against structural and functional damage to plant chloroplasts

8) Zinc supplements effectively prevented the oxidative damage to the DNA.

9) Amino acids as well as organic acids efficiently protected the plant from Cd toxicity adopting different routes of toxicity alleviation, amino acids being GSH-mediated and organic acids by chelation mechanism. Zn supplements in addition to amino and organic acids were influential in enhancing amino acid detoxification system but not organic acids.

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7. Publications

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Original article

Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L.: a free floating freshwater macrophyte

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Abstract

The potential mechanism by which Zn antagonizes Cd toxicity was investigated in *Ceratophyllum demersum* L. (Coontail), a free floating freshwater macrophyte, to understand the interaction between two metals, one a micronutrient and another a toxic metal. *C. demersum* was treated with 10 uM Cd along with Zn (10, 50, 100 and 200 uM). Treatments with Zn only (10, 50, 100 and 200 uM) were also given to the plant material. Cd uptake was suppressed by Zn and simultaneously, Zn concentration also increased in the plant Cd-induced strong oxidative stress as indicated by high levels of lipid peroxidation, electrical conductivity and lipoxygenase (LOX, E.C. 1.13.11.12) activity. Zn supplementation efficiently reduced this as observed by the decrease in these parameters. Zn-alone-treated plants, however, did not show any variation in the above parameters. The activity of antioxidant enzymes superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6), ascorbate peroxidase (APX, E.C. 1.11.1.11) and guaiacol peroxidase (GPX, E.C. 1.11.1.7) showed a very high increase in activity in Cd + Zn-treated plants as compared to Cd- or Zn-alone-treated plants. This indicates the efficient antioxidative and reactive oxygen species scavenging activity by Zn against Cd-induced free radicals and oxidative stress. Plants treated with only Zn concentrations did not show very high Zn uptake or high antioxidant enzyme activity. These results suggest that Zn supplementation proved to be inhibitory for Cd-induced oxidative stress and beneficial for the plant's survival.

Keywords: Antioxidant enzymes; Zinc protection; Cadmium; Free radicals; Oxidative stress; Peroxidation; Reactive oxygen species

1. Introduction

Heavy metals at extremely micro-concentrations affect different cellular components, thereby interfering with the normal metabolic functions [28]. The co-existence of essential and non-essential elements in the ecosystem leads to interactions that may be additive, antagonistic or synergistic [29]. It is well known that Zn is an important component of many vital enzymes, a structural stabilizer for proteins, membrane and DNA-binding proteins (Zn-fingers) [33]. On the other hand, Cd is extremelytoxic without any metabolic significance and its presence in aquatic environment is of concern in terms of health for man and biosphere. Oxygen free radicals, which includes superoxide ($O_2^{\bullet-}$), hydroxyl radicals (OH*) and hydrogen peroxide (H_2O_2), the ubiquitous products of single electron reductions of dioxygen are amongst the most reactive compounds known to be produced during a heavy metal stress [13]. They can lead to multifold effects such as membrane peroxidation, loss of ions, protein cleavage, inactivation and damage, and even DNA strand breakage [35]. To minimize this, cells are equipped with enzymatic and non-enzymatic mechanisms to eliminate or reduce their damaging effects. These protective mechanisms include antioxidant enzymes such as superoxide dismutase (SOD, E.C. 1.15.1.1) catalase (CAT, E.C. 1.11.1.6), peroxidases like ascorbate (APX, E.C. 1.11.1.11) and guaiacol peroxidase (GPX, E.C. 1.11.1.7) and other antioxidant compounds such as glutathione, carotenoids, etc. [20]. Zinc is known to have a stabilizing and protective effect on the biomembranes against oxidative and peroxidative damage, loss of plasma membrane integrity and also alteration of the permeability of the membrane [2]. Zinc ions bind to ligands containing sulfur, nitrogen and, to a lesser extent, oxygen and

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; HPOD, hydroperoxides; GPX, guaiacol peroxidase; NBT. nitro blue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA-RS, thiobarbituric acid reactive substances; TCA, trichloro acetic acid.

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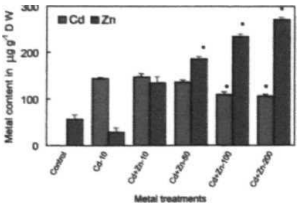


Fig. 1. Metal accumulation in C *demersum* treated with Cd-10 uM and Zn (10, 50, 100 and 200 uM) concentrations. The values are means of three individual experiments with duplicates. $n = 3$ for ± S.D., $P < 0.01$. The error bars represent standard errors and 'asterisk' for Cd content indicates significant difference from Cd-treated plants: asterisk' for Zn content indicates significant difference from control plants.

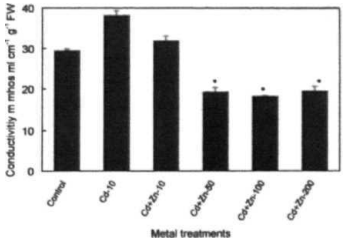


Fig. 2. Electrical conductivity as a measure of ion leakage in *C. demersum* treated with Cd-10 uM and Zn (10, 50, 100 and 200 μM) concentrations. The values are means of three individual experiments with duplicates. n - 3 for ±S.D., $P < 0.01$. The error bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

preferentially bind to the membrane proteins [2]. The balance between free radical generation and free radical defense determines the survival of the system. Therefore, Zn may have a role in modulating free radicals and its related processes through antioxidant properties [36]. Thus, studies on interactions of metals would elucidate the stress and resistance mechanisms exhibited by plants due to heavy metal exposure. In polluted ecosystems, bioconcentration of heavy metals by aquatic macrophytes is of environmental concern as several aquatics functions as biofilters of toxic elements [24]. *Ceratophyllum demersum* is known for its ability to accumulate industrial radionuclides and heavy metals as well [3,16,30,31]. C. *demersum* L. is a powerful scavenger of Cd at low concentrations (0.1–0.5 ppm) but does not survive higher concentrations (1 ppm-10 μM) [26]. However, no information is available on the role of nutrients against toxic metal in this system. Hence, the purpose of this study was to investigate Zn-Cd interactions with respect to oxidative stress in *C. demersum.*

2. Results

C. demersum plants treated with 10 uM Cd showed leaf and tissue dissolution, chlorosis and their survival was only up to a week. These symptoms were not seen in Cd-treated plants supplemented with Zn (10–200 uM) and the survival period was up to 3 weeks. Cd-treated plants accumulated 143.39 μg Cd g^{-1} DW, whereas, the Cd concentration decreased up to 105.81 ug Cd g^{-1} DW in treatments with supplemented Zn (especially at 200 uM). Simultaneously, Zn accumulation increased from 57.64 ug g ' DW in control to 270.56 μg g ' DW in Cd-treated plants with supplemented Zn (200 μM) (Fig. 1). Zn-treated plants without any Cd treatment comparatively showed lesser uptake of Zn, indicating increased Zn requirement in plants treated with both Cd and Zn (Table 1).

2.1. Electrical conductivity as a measure of ion leakage

Cd treatment led to a high increase in the electrical conductivity (29%). The conductivity in Cd-treated plants with supplemented Zn showed a 52% decrease (especially at Zn. 200 uM) from the increased electrical conductivity at Cd-10 uM indicating membrane protection (Fig. 2). Treatments with only Zn showed lesser differences in conductivity (Table 1).

2.2. Lipid peroxidation level

The thiobarbituric assay, evaluating the degree of lipid peroxidation revealed a 37% increase at Cd-10 μM over the control. This was decreased up to 48% from the increased TBA-reactive substances value at Cd-10 μM in Cd-treated plants with supplemented Zn (especially at Zn, 200 uM). This indicates the action of Zn against Cd at the membrane level (Fig. 3). Zn-treated plants, however, did not show any increase in TBA-RS levels (Table 1).

Table 1
Influence of Zn on lipid peroxidation, conductivity, lipoxygenase activity and metal content in C *demersum.* The values are means of three individual experiments with duplicates. The values in parentheses indicate standard deviation *in* • 3) and "asterisk" indicates significant difference from the control (a = 0.01). FW, fresh weight; DW, dry weight

Treatments (uM)	Lipid peroxidation (u mol g m^{-1} FW)	Conductivity (m mhos ml cm^{-1} g^{-1} FW)	Lipoxygenase activity (μkat mg ' protein min ')	Metal uptake (ug gm^{-1} d.wt)
Control	14.13(0.45)	30.23(2.26)	11.86 (2.50)	57.85 (4.58)
10	12.23 (1.17)	30.83 (2.84)	12.53 (0.28)	128.58 (32.32)*
50	11.86 (0.49)*	23 (0.93)	16.40 (3.95)	162.16 (11.80)*
100	11.98 (0.18)*	21.26 (2.05)	18.80 (2.82)	206.13 (10.19)*
200	13.52(0.28)	19.83 (1.47)	18.93(1.38)	247.26 (27.33)*

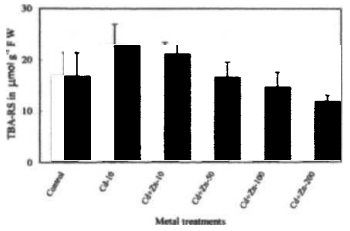


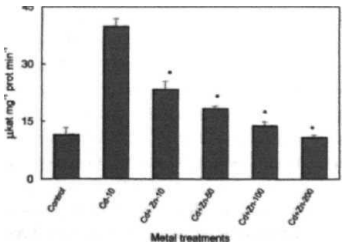
Fig. 3. Level of lipid peroxidation as a measure of TBA-RS in *C. demersum* treated with (d-10 μM and Zn (10, SO, 100 and 200 μM). The values are means of three individual experiments with duplicates. n • 3 for ±S.D., $P <$ 01 Error bars represent standard errors.

1.3. Lipoxygenase (LOX) activity

The results of the activity of LOX also confirm the role of Zn in membrane stabilization. Cd showed a very high increase in activity (almost 3-fold) of 247% of this lipolytic enzyme. This was decreased in Cd-treated plants with supplemented Zn up to 264% (a 3-fold decrease) (especially at Zn, 200 μM) from the increased activity, indicating the inhibitory effect of Zn on this fatty acid oxidizing enzyme and restoring the membrane stability (Fig. 4). Plants with Zn treatments only showed a very little increase in LOX activity Table 1).

'.4. Antioxidant enzyme activity

Analysis of antioxidant enzymes—SOD, CAT, APX and GPX revealed a high increase in their activities in Cd-treated slants with supplemented Zn than Cd-alone-treated plants. The activityof SOD increased up to 263% (3.6-fold increase) D plants supplemented with Zn (200 uM) over the control as igainst just 54".. (1.5-fold increase) in plants treated with d-10 uM only. Similarly, Cu/Zn SOD and Mn SOD also showed 256% (3.56-fold) and 279% (3.78-fold) increase in plants supplemented with Zn-200 μM as against just 67% 1 5-fold) and 25% (1.2-fold) increase at Cd-10 uM treat-



g. 4. Activity of lipoxygenase in C *demersum* plants treated with Cd-) μM and Zn (10, 50, 100 and 200 μM) concentrations. Values are means of ree individual experiments with duplicates. n 3 for ±S.D., *P* S 0.01. The far bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

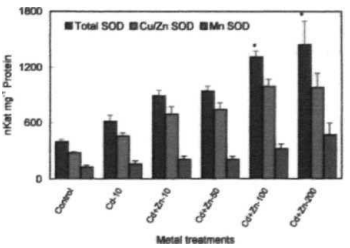


Fig. 5. Activities of total SOD, Cu-Zn SOD and Mn SOD in *C. demersum* treated with Cd-10 μM and Zn (10, 50, 100 and 200 μM) concentrations. The values are means of three individual experiments with duplicates. *n* 3 lor ±S.D., $P < 0.01$. The error bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

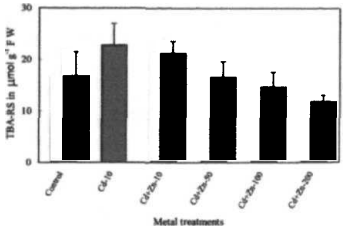
ments, indicating the efficiency of this antioxidant enzyme in the presence of Zn than without any Zn supplementation (Fig. 5). However, in treatments with only Zn, the activity increase of total SOD, Cu/Zn SOD and Mn SOD was not as high as Cd + Zn-supplemented plants (Table 2).

CAT and APX also showed the same trend as SOD, where Cd treatments with Zn addition showed a very high increase in activity than treatments with only Cd or Zn (Table 2). In CAT, 435% (5.35-fold) increase in activity was seen in Cd-treated plants supplemented with Zn-200 uM and only 21% increase at Cd-10 uM treatments over the control (Fig. 6). For APX, the increase was 405% (5-fold) in plants with Zn-200 uM supplement as against 114% (2.14-fold) increase at Cd-10 uM (Fig. 7).

GPX showed a relatively less difference in activity in all the treatments when compared to other antioxidant enzymes. Cd treatments with Zn-200 uM supplement showed 41% and Cd-10 μM, 16.78% increase only (Fig. 8). Thus, these results suggest that the Cd-induced membrane damage and oxidative stress is combated efficiently by Zn with an active antioxidant enzyme system controlling membrane peroxidation and damage.

3. Discussion

This study was performed to analyze the mechanism by which Zn antagonizes Cd toxicity in *C. demersum*. This interaction between a nutrient and a non-essential element may be important for understanding, analyzing and improving the defense strategies through various parameters. The present investigation showed reduced Cd uptake at increasing Zn concentrations and simultaneously increased Zn accumulation in the plant tissue. This suppression in Cd uptake and increase in Zn accumulation indicates a strong competition between Zn and Cd in the plant system. Since Cd and Zn. both taken as divalent cations belong to the group II transition metals with eight electrons in their outer orbital, Cd can readily inhibit most of the Zn-dependent processes [29] and hence increased Zn concentration is able to replace a non-physiological metal like Cd, which may bind to the crucial



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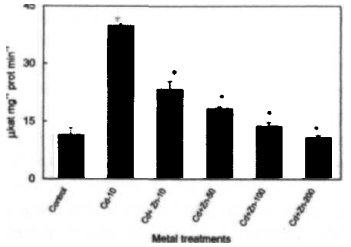

Fig. 4. Activity of lipoxygenase in C *demersum* plants treated with Cd-10 uM and Zn (10, 50, 100 and 200 μM) concentrations. Values are means of three individual experiments with duplicates. $n = 3$ for ±S.D., $P \leq 0.01$. The error bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

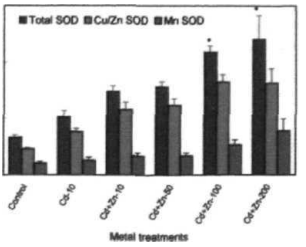

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Table 2
Influence of Zn on the activities of antioxidant enzymes in *C. demersum*. The values are means of three individual experiments with duplicates. The values in parentheses indicate standard deviation (n = 3) and "asterisk" indicates significant difference from the control (a • 0.01)

Treatments (uM)	SOD (nkat mg ' protein)	CAT (µkat mg ' protein min ')	APX (µkat mg ' protein min ')	GPX (nkat mg • protein min ')
Control	374.46 (11.98)	5.16(0.43)	166.39 (11.86)	510.01 (11.35)
10	422.44(9.54)*	7.31 (0.43) *	299.79 (25.82) *	592.12(43.75)
50	530.45 (13.81) *	8.65 (0.39) *	426.39 (7.29) *	585.83(18.18)
100	769.23 (203.22) *	11.25(0.93)*	435.19(26.99)*	648.62(31.52)
200	812.00 (132.34) *	17.91 (2.74) *	483.00(10.04)*	677.68(25.55)*

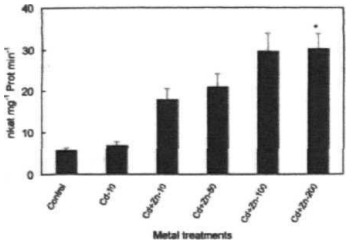

Fig. 6. Activity of CAT in *C. demersum* treated with Cd-10 uM and Zn(10, 50, 100 and 200 uM) concentrations. The values are means of three individual experiments with duplicates. $n = 3$ for ±S.D., $P < 0.01$. The error bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

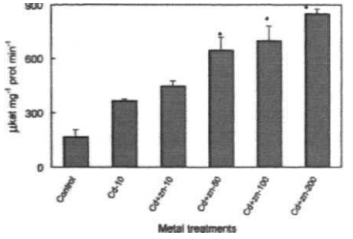

Fig. 7. Activity of APX in *C. demersum* treated with Cd-10 µM and Zn (10, 50, 100 and 200 uM) concentrations. The values are means of three individual experiments with duplicates. $n = 3$ for ±S.D., $P < 0.01$. The error bars represent standard errors and 'asterisk' indicates significant difference from Cd-treated plants.

and functional membrane and enzyme active sites and inactivate their functions. Zn has already been shown to antagonize other transition metals like Cu and Fe in the same manner [27,36]. This prevents the local redox-cycling and concomitant production of oxidant species. It should be noted that Zn uptake is lesser in plants treated with only Zn, as the Zn concentration is already maintained in the plant system. Several other evidences such as in lettuce [32] and soya bean [9] also point to the depressed Cd uptake in the presence of Zn.

The main site of attack by any redox active metal in a plant cell is usually the cell membrane. Heavy metals induce severe lipid peroxidation due to the removal of hydrogen by-ROS from unsaturated fatty acids leading to lipid radical

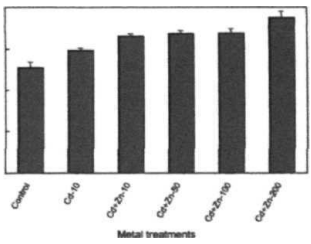

Fig. 8. Activity of GPX from *C. demersum* treated with Cd-10 µM and Zn (10, 50, 100 and 200 µM) concentrations. The values are means of three individual experiments with duplicates. $n = 3$ for ±S.D., $P \leq 0.01$. The error bars represent standard errors.

formation. This leads to a cascade of cyclical reactions leading to repetitive formation of short chain alkanes and lipid acid aldehydes totally destroying the lipid structure. This further leads to dimerization and polymerization of proteins, which is considered to be most damaging to the membranes [21]. This is evident from the results of lipid peroxidation, where Cd increases TBA-RS level. LOX activity increase by Cd suggests higher lipolytic activity on the membrane and oxidation of membrane-bound fatty acids causing propagation of lipid peroxidation [19]. The results in this investigation also indicate a very high increase in ion leakage measured as electrical conductivity in Cd treatments. Cd, like any other transition metal, is known to bind to sulfydryl groups and destabilize the membrane system, by inducing the formation of disulfide links leading to the distortion in the structure and function of membrane ion channels [11]. Hence, it leads to a leaky and damaged membrane, increasing the electrolyte leakage and loss of ions. It is evident from the results that Zn supplementation reduced lipid peroxidation, conductivity and LOX activity. Zn has a unique property of existing in a univalent state, without any redox-cycling, thereby being stable in a biological medium, whose oxidoreductive potential is subjected to continuous flux [34]. Due to this property of Zn, the membrane lipid packing is protected from ROS, peroxidation and this prevents ion leakage from ion channels [4]. In plant treatments with only Zn (no Cd treatment), all the above parameters did not show any significant change, indicating that the plant system is affected only because of the presence of toxic Cd concentration. Zn prefers binding to the -SH groups of the membrane protein moiety and protects the phospholipids and proteins

from thiol oxidation and disulfide formation [11] either by direct binding or to a site close to the sulfydryl group or by conformational change resulting in apparent stability of the enzymes, membrane proteins as well as the lipid structure [27].

Divalent cations such as Cd are known to trigger the oxidation of NADPH leading to superoxide radical production ($O_2^{\bullet-}$) [18]. The protective effect of Zn has been shown due to its ability to inhibit NADPH oxidation and oxygen-centered free radical generation [6,7]. The superoxide radical ($O_2^{\bullet-}$) produced itself is deleterious which indirectly produces a potent oxidant, hydroxyl radical (OH*) by reacting with H_2O_2. This reaction called site-specific Haber-Weiss reaction is catalyzed by Fe (III) reduction to Fe (II), followed by the reduction of H_2O_2 by Fe (II). Since Fe (III) is strongly associated with polyanionic targets such as DNA or cell membranes, these oxidants are produced in their immediate icinity causing the repetition of this reaction [15].

The ROS scavenging antioxidant enzymes SOD, CAT, VPX and GPX play a vital role in scavenging these destructive oxidant species. By catalyzing detoxification of $O_2^{\bullet-}$ to O_2 and H_2O_2, SOD blocks O_2*" driven cell damage [5]. CAT and peroxidases like APX and GPX break down H_2O_2 to H_2O and O_2. The results of this work indicate that in Zn-supplemented plants, there is a very high activity of SOD, CAT and APX, indicating the efficient ROS scavenging activity in the system. GPX did not show a significant increase in hydrogen peroxide scavenging activity indicating that it acts secondary to APX in this. The combined action of SOD and CAT efficiently eliminates hydrogen peroxide and superoxide and indirectly protects the plant against more toxic hydroxyl radical. In plants treated with only Cd, without any Zn supplementation, the activity of all these enzymes increased only to a little extent. The same trend was also seen in plants treated with only Zn, indicating that the increase in activity in Cd + Zn treatments was because of extreme oxidative stress induced by Cd and protection against this by induction of high levels of antioxidant enzymes by Zn. Probably, Zn is able to increase the biosynthesis of antioxidant enzymes [5]. In plants treated with only Cd, the excess production of ROS by Cd would have inactivated the antioxidant enzymes. It has been suggested that very high levels of H_2O_2 inhibits Cu/Zn SOD [8] through Cu^{2+} to Cu^{+} reduction and the formation of excess hydroxyl radical also in turn inhibits Cu/Zn SOD. Similarly, the CAT enzyme is also sensitive to O_2* ~ and can be inactivated by its increasing levels [5]. In conclusion, Zn strongly protects *C. demersum* from Cd-induced oxidative stress by inhibiting the peroxidation of membrane lipids, lipolytic activity of the LOX and facilitates proper functioning of ion channels and membrane proteins. The increase in the ROS scavenging antioxidant enzyme activity proves the role of Zn as an antioxidant and its action against oxidative stress. Hence, it can be concluded that Zn supplementation proved to be beneficial for the system in combating Cd toxicity. Since in Cd-treated plants, the Zn concentration was reduced, the plant system was unable to cope with the increasing ROS production and decreased antioxidant enzyme activity.

4. Methods

4.1. Plant material

C. demersum L. plants were collected from local ponds and maintained under laboratory conditions in aquaria in 1/10 Hoaglands solution.

4.2. Cadmium and zinc treatment

Plant material (2 g) was transferred to a 250 ml glass beaker with 200 ml of 1/10 Hoaglands solution (containing 0.764 nmol Zn) and the required concentration of the metals. Cd treatment of 10 uM concentration was imparted to the plant material using $CdCl_2$. Zn supplements (10, 50, 100 and 200 uM) were given to the plant as $ZnCl_2$ along with the Cd concentration. Treatments with Zn only(10, 50, 100 and 200 uM) were also given to the plant. The plants were kept under the natural day and night cycle for 1 week. Cd at a concentration of 10 uM was found to be toxic to the plant as seen in the form of chlorosis and tissue dissolution. The concentrations of Zn above 200 uM were also found to be toxic to the plant.

4.3. Cadmium and zinc analysis

Metal-treated plants were washed thoroughly with 10 mM EDTA to remove metals adsorbed to the surface. They were oven-dried at 80 °C for *2* d and acid-digested with 3:1 $HNO_3/HClO_4$. The digested material was dissolved in minimal volume of H_2O_2 and made up in distilled water. This solution was analyzed for Cd and Zn content using atomic absorption spectrometer (GBC 932 plus, Australia).

4.4. Electrical conductivity as a measure of ion leakage

The treated plant material (750 mg) was taken and washed with double distilled water. They were then transferred to 100 ml of deionized water and left for 24 h to facilitate maximum ion leakage. The electrical conductivity of the water as a measure of ion leakage was measured after 24 h according to [12].

4.5. *Lipid peroxidation*

Plant material (300 mg) was homogenized with 3 ml of 0.5(%) TBA in 20(%) TCA (w/v). The homogenate was incubated at 95 °C for 30 min and the reaction was stopped in ice. The samples were centrifuged at 10,000 × g for 10 min and the absorbance of the resulting supernatant was recorded at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance. The absorbance coefficient of malondialdehyde (MDA)-155 mM^{-1} cm^{-1} was used in the calculation according to [17].

4.6. Lipoxygenase activity (LOX, E.C. 1.13.11.12)

The plant material (300 mg) was homogenized in 50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF), 2% (w/v) polyvinylpyrrolidone (PVP), 1% (v/v) glycerol and 0.1% (v/v) tween 20. The extract was centrifuged at 15,000 × *g* for 20 min and the supernatant was immediately used for the assay of lipoxygenase activity according to [14]. LOX activity was measured spectroscopically at room temperature by the addition of 1 mM linoleic acid in 0.1 M sodium acetate buffer (pH 5.6) to the extract and by measuring the increase of absorbance at 234 nm. The extinction coefficient of (25 mM $l^{-1})^{-1}$ cm^{-1} was used to convert absorbance values to micromoles of conjugated diene. One unit of activity was defined as the amount of enzyme catalyzing the synthesis of 1 umol of hydroperoxide (HPOD) min^{-1}. The enzyme activities were expressed in katals.

4.7. Activities of antioxidant enzymes

The treated plant material (1 g) was ground in 50 mM sodium phosphate buffer (pH 7.8) for SOD and (pH 7.0) for CAT, APX, and GPX, respectively, and centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was used to measure the activity of the enzymes. The protein content in the supernatant was determined according to [22].

4.7.1. Superoxide dismutase (SOD, E.C. 1.15.1.1)

SOD activity was determined by the method of Beauchamp and Fridovich [1]. The required cocktail for SOD activity estimation was prepared by mixing 27 ml of sodium phosphate buffer (pH 7.8), 1.5 ml of methionine (300 mg ml^{-1}), 1 ml of NBT (14.4 mg, 10 ml^{-1}), 0.75 ml of triton-X-100 and 1.5 ml of 2 mM EDTA. To 1 ml of this cocktail, 10 µl of riboflavin (4.4 mg, 100 ml^{-1}) and 50 ug of protein were added. After mixing, the contents taken in a cuvette were illuminated for 8 min using three comptalux bulbs (100 W, Philips India Ltd.). The temperature was maintained at 25° C using a water bath. A tube with protein kept in the dark served as a blank, while the control tube was without the enzyme and kept in the light. The absorbance was measured at 560 nm. NBT reduction under illumination was measured without the enzyme and also in the presence of the enzyme. The activity of SOD is the measure of NBT reduction in light without protein minus NBT reduction with protein. One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light. The amount of each type of SOD was determined by its sensitivity to cyanide and H_2O_2. Cu/Zn SOD is sensitive to cyanide; Fe SOD is sensitive to H_2O_2 and Mn SOD is insensitive to both. The enzyme activity was expressed in katals.

4.7.2. Catalase (CAT, E.C. 1.11.1.6)

CAT activity was measured according to the method of Chance and Maehly [10]. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 19 mM H_2O_2 and 100 ug of protein in a final volume of 3 ml. The activity was determined by the oxidation of H_2O_2 at 240 nm. The change of absorbance of 1 OD represents the oxidation of 25 µmol of H_2O_2. The enzyme activity was expressed in katals.

4.7.3. Ascorbate peroxidase (APX, E.C. 1.11.1.11)

APX was assayed by the method of Nakano and Asada [25]. The reaction mixture for measuring APX activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50 ug of protein. The activity was recorded as the decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized was calculated from the extinction coefficient of 2.6 mM^{-1} cm^{-1}. The enzyme activity was expressed in katals.

4.7.4. Guaiacol peroxidase (GPX, E.C. 1.11.1.7)

GPX was measured by the method of Mazhoudi et al. [23]. The reaction mixture contained 50 mM phosphate buffer, 0.2 mM guaiacol, 10 mM H_2O_2 and distilled water in a total volume of 3 ml. The reaction was started by adding 50 ug of protein. The change in absorbance of one unit per min at 470 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) gave the activity of GPX. The enzyme activity was expressed in katals.

4.8. Statistics

The values are means of three individual experiments with duplicates (value averaged to one) for each experiment and the results were subjected to statistical analysis by student's r-test (n = 3). The level of significance (a) was set at 0.01

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ARTICLE

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Carbonic anhydrase impairment in cadmium-treated *Ceratophyllum demersum* L. (free floating freshwater macrophyte): toxicity reversal by zinc

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The influence of Cd (non-essential) and Zn (essential) on the functioning of carbonic anhydrase was investigated. Zinc restored the cadmium impaired carbonic anhydrase in *Ceratophyllum demersum* L. (coontail), a free floating freshwater macrophyte. Evidence from purified carbonic anhydrase in a two-step process using diethylaminoethyl (DEAE) Sephadex A50 and Sephadex G-25 (m = 43 kDa by SDS-PAGE), and Zn content by flame atomic absorption spectrometry, are presented.

Toxic heavy metal contamination of aquatic ecosystems is a cause of serious environmental concern for the health of biota. Cadmium (Cd) is a non-essential and toxic element, without any metabolic significance, whereas zinc (Zn) is an important component of many vital enzymes having a catalytic, co-catalytic or structural role,[1] structural stabilizers for proteins, membrane and DNA-binding proteins (Zn-fingers),[2-4] but toxic in high concentrations. Both these elements have similar geochemical and environmental properties. Non-ferrous metal production processes and subsequent release of zinc to the environment is normally accompanied by cadmium environmental pollution because of zinc ores (ZnS) generally containing 0.1-5%, and sometimes even more, cadmium.[5] The trace elements concerned are available to plants mainly from sludge-borne heavy metal applications and industrial wastewater irrigation, and partially from aerial deposition and the use of fertilizers and pesticides. This association of Cd and Zn in the aquatic environment and their chemical similarity can lead to interaction between cadmium and zinc.

Aquatic plants are known to accumulate heavy metals.[6] *Ceratophyllum demersum* is known to accumulate heavy metals and radionuclides,[7-9] especially Cd at low concentrations (0.1–0.5 ppm[10-12]). Interactions between Cd and Zn and their transfer in a soil–crop system under actual field conditions, in solution culture experiments, have been reported recently.[1314] However, there is no information on Zn restored carbonic anhydrase in Cd exposed aquatic plants and the importance of carbonic anhydrase in *C. demersum*.

Carbonic anhydrase (CA; carbonate hydrolyase, E.C. 4.2.1.1) is a ubiquitous enzyme among living organisms which catalyses the reversible interconversion of CO_2 and HCO_3^-. CA have been extensively studied in many organisms from cyanobacteria[15,16] to higher plants,[17,18] animals and human systems."[20] CA has been presumed to have an active function in photosynthetic organisms[21] and has been the topic of interest in many aquatic systems where the availability of CO? is less (the predominant form of dissolved inorganic carbon being H((), ı and further complicated by its slower diffusion rates in water, being 1 x 10^4 times slower than air." To maintain the photosynthetic efficiency in spite of low CO_2 availability the carbon-concentrating mechanism (CCM) functions.[23,24] This CCM has two components, namely a mechanism for taking up HCO_3^- and a Zn-requiring CA that catalyses interconversion of HCO_3^- to CO_2. In aquatic angiosperms it has been suggested that HCO_3^- diffusion and then conversion to CO_2 by CA plays a very vital role in maintaining the inorganic carbon levels.[26] Zn has a catalytic role in plant and animal CA, being coordinated to the imidazole rings of three histidines close to the active site.[27,28] In an earlier investigation we demonstrated that zinc alleviates cadmium-induced toxicity by using C *demersum* as an experimental system. Since CA activity being affected by Zn deficiency[29,30] and its regulation by Zn^{23} in different systems have been reported, we used CA to examine the mode of interaction between zinc and cadmium and analyze the role of Zn in CA in C *demersum*.

Experimental

Plant material

Ceratophyllum demersum L. plants were collected from local ponds and maintained under laboratory conditions in aquaria in 1/10 Hoaglands solution, a nutrient solution containing a combination of macro- and micronutrients essential for plant growth.[31]

(admium and zinc treatment

Plant material (2 g) was transferred to a 250 ml glass beaker with 200 ml of 1/10 Hoaglands solution (containing 0.764 nmol of Zn) and the chosen concentration of the metals. Cd treatment of 10 uM (using $CdCl_2$) concentration was given to the plants. Zn supplementation (10, 50, 100, and 200 uM) was carried out using $ZnCl_2$ on the Cd (10 uM) treated plants. Plants were also treated with only Zn (10, 50, 100, and 200 uM) for comparison. The control plant has 57 ug g~' DW Zn content in it, as reported earlier by us.[13] All the above experimental plant material was kept under the natural day and night cycle for one week. Only four treatments (Control, 10 uM Cd, Cd 10 uM + Zn 200 uM and Zn 200 uM) were chosen for purification process.

Extraction and assay of carbonic anhydrase

The treated plant material (1 g) was ground in an ice-cold mortar and pestle with 50 mM Tris-HCl buffer pH 7.5, 40 mM PMSF (phenylmethyl sulfonyl fluoride) and 2% (m/v) PVP (polyvinyl pyrrolidone). The homogenate was filtered through two layers of muslin cloth to remove the cell debris and centrifuged at 15,000 x g for 20 min and the supernatant was used as the source of the enzyme. The amount of protein was estimated according to Lowry *et al.*.[32] The assay was performed according to Armstrong *et al.*.[33] This assay is based on the

principle that carbonic anhydrase not only catalyses the hydration of CO_2 and dehydration of HCO_3^-, but also the hydrolysis of many esters and aldehydes. This assay utilizes *p*-nitrophenyl acetate as the ester for assaying the hydrolytic reaction. 54.3 mg of *p*-nitrophenyl acetate was dissolved in 3 ml of acetone and Millipore ultrapure water was added slowly with constant stirring to a final volume of 100 ml. This solution was prepared fresh everyday. In a quartz cuvette, 1 ml of this solution was mixed with 1.7 ml of water, 0.3 ml of 0.1 M diethyl malonate was added with rapid stirring and the solution incubated for 2½ min. The increase in absorbance at 348 nm was recorded in a double beam spectrophotometer (Cintra S, GBC Scientific, Australia) after the addition of 100 µg of protein. The activity was calculated using a molar absorptivity of 5.4 mM^{-1} cm^{-1}.

Purification of carbonic anhydrase

All the operations were carried out at 4 °C. We have followed a modified version of the procedure of Atkins *et al.*,[1] Armstrong *et al.*,[33] and Guliev *et al.*[34] Plant material (60 g) was washed thoroughly and homogenized in ice-cold buffer—50 mM Tris-HC1 pH 8.7, 40 mM PMSF and 2% (m/v) PVP. The homogenate was filtered through two layers of muslin cloth to remove the cell debris and centrifuged at 15000 x *g* for 20 min. The pellet was discarded and the supernatant was used as the enzyme source. Diethylaminoethyl (DEAE) Sephadex A 50 (Sigma, USA) was washed thoroughly according to the manufacturer's instructions and packed to give a column of 2 x 35 cm. The column was equilibrated with three column volumes of 50 mM Tris-HCl pH 8.7 buffer and the enzyme source was loaded onto the column with a flow rate of 20 ml h^{-1}. After two column washings the bound protein was eluted with 0.1 M Tris-HCl, pH 8.7, and collected in 2 ml fractions. The eluate was tested for protein content by checking the positive absorbance at 280 nm (ultraviolet absorbance specific for column eluates) and CA activity and the positive fractions were pooled and concentrated by ammonium sulfate precipitation (80% saturation). The concentrate was dialysed against 3 volumes of 0.1 M Tris-HCl, pH 8.7, overnight under cold conditions. This concentrated enzyme was chromatographed on a Sephadex G-25 (Sigma, USA) column (1.5 x 35 cm) equilibrated with 0.1 M Tris-HCl pH 8.7 at a flow rate of 4.5 ml h^{-1}. One ml fractions were collected and tested for protein content by checking the positive absorbance at 280 nm (ultraviolet absorbance specific for column eluates) and CA activity. The activity rich fractions were pooled and stored in the presence of sulfhydryl agents (15 mM dithiothreitol) and referred to as the purified carbonic anhydrase.

SDS–polyacrylamide gel electrophoresis

Samples of the crude extract as well the DEAE Sephadex elution fractions and purified carbonic anhydrase from Sephadex G-25 fractions were subjected to sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as described by Laemmli, to confirm the homogeneity of the purified enzyme and to estimate its molecular weight.

Zn and C d content in purified carbonic anhydrases by FAAS

The purified carbonic anhydrase was analysed for Zn in a GBC flame atomic absorption spectrometer in an air-acetylene flame system (GBC 932 Plus, Australia). The purified sample was also analysed for Cd to verify whether there is any substitution of Zn by Cd.

Statistic*

The values are means of three individual experiments with duplicates (value averaged to one) for each experiment and the results were subjected to statistical analysis by Student's /-test (n = 3). The level of significance (et) was set at 0.01 ($p \leq 0.01$).

Results

The results of our investigation demonstrated that Zn supplementation to a Cd poisoned plant restored CA functions, as reflected by the changes in its activity, recovery of active purified enzyme and Zn content in *C. demersum*. Further, we have also observed chlorosis in Cd 10 uM alone treatments, with the stem and leaf tissue becoming mucilagenous due to tissue degeneration. These symptoms were not observed in Zn supplemented Cd treatments. However, Zn treatments above 200 uM were found to be toxic to the plant. Zn alone treatments did not show any visible symptoms of toxicity indicating the non-toxic nature of the Zn concentrations chosen.

Carbonic anhydrase (CA) activity

The results of the assay of CA revealed a drastic 50% reduction in the activity of this enzyme in Cd 10 uM alone treated plants when compared to the control plants (Fig. 1). The activity was not only restored (67%) completely by Zn in Cd-treated plants with supplemented Zn, but it was also increased (34%) beyond the control level. However, plants treated with only Zn showed a slight increase in CA activity (Fig. 1), but not up to the levels of Cd + Zn 200 uM treatments.

Purification of carbonic anhydrase

The purification profile of CA of each treatment is shown in Tables 1-4 and the elution profiles are shown in Figs. 2-5. There was a 8.5-fold purification of the enzyme from the soluble plant protein in all the treatments. There were large differences only in the recovery of the active enzyme from each treatment. Control plants (without any metal treatment) showed a 32% recovery of the active purified protein (Table 1). Cd-treated plants showed toxicity to the enzyme as seen in the form of a very low recovery (22%) of the purified active form (Table 2). Zn supplementation to Cd-treated plants proved to be beneficial as a highly active CA with a higher recovery of 43% was purified (Table 3). Treatments with only Zn also did not affect the enzyme activity and its purification (Table 4) and a 33% recovery was observed for the purified enzyme.

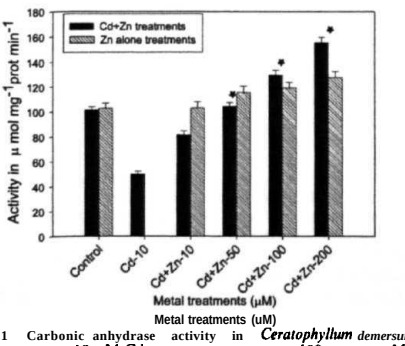

Fig. 1 Carbonic anhydrase activity in *Ceratophyllum demersum* L. treated with 10 µM Cd and Cd + Zn (10, 50, 100 and 200 µM) Error bars represent standard errors and an asterisk specifically indicates significant differences of Cd + Zn treatments from 10 µM Cd-alone treatments. Note the Cd-induced decrease in CA activity, being restored by supplementing Zn to Cd treatments.

Table 1 Purification profile of carbonic anhydrase from control plants of *Ceraiophyllum demersum*

Protein source	rngmP[1]	Protein content/	Activity/ umol ml"'	Volume of the protein/ml"	Total protein*	Total activity[c]	Specific activity[d]/ umol mg^{-1} protein	Yield (%)[e]	Fold[f]
Homogenate supernatant		5	59	60	300	3540	11.8	100	1
Pooled "active" fractions from DEAE Sephadex		1.8	65	30	54	1890	36.11	53.4	3.06
Pooled "active" fractions from Sephadex G-25		0.77	77	15	11.55	1155	100	32.5	8.47

[a] Denotes the amount of crude *C demersum* protein extract initially used as the starting material to purify CA. [b] Total protein refers to the total protein content of the entire volume of crude protein extract used. [c] Total activity refers to the CA activity of the entire volume *of* crude protein extract used. [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. [e] Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. [f] Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

Table 2 Purification profile of carbonic anhydrase from Cd 10 uM treated plants of *Ceraiophyllum demersum*

Protein source	Protein content/ mg ml^{-1}	Activity/ umol ml^{-1}	Volume of the protein/ml"	Total protein*	Total activity'	Specific activity[d]/ umol mg^{-1} protein	Yield (%)[e]	Fold[f]
Homogenate supernatant	3.2	25	60	192	1500	7.81	100	1
Pooled "active" fractions from DEAE Sephadex	1.27	30	24	30.48	720	23.62	48	3.02
Pooled "active" fractions from Sephadex G-25	0.58	38	9	5.22	342	65.51	22.8	8.38

" Denotes the amount of crude *C demersum* protein extract initially used as the starting material to purify CA. * Total protein refers to the total protein content of the entire volume of crude protein extract used. ' Total activity refers to the CA activity of the entire volume of crude protein extract used. [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. [e] Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. [f] Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

Table 3 Purification profile of carbonic anhydrase from Cd + Zn 200 uM treated plants of *Ceraiophyllum demersum*

Protein source	Protein content/ mg ml^{-1}	Activity/ umol ml"'	Volume of the protein/ml[a]	Total protein*	Total activity[c]	Specific activity[d]/ umol mg~' protein	Yield (%)[e]	Fold[f]
Homogenate supernatant	6.7	85.88	60	402	5152.8	12.81	100	1
Pooled "active" fractions from DEAE Sephadex	3.0	116.81	35	105	4088.35	38.93	79.35	3.03
Pooled "active" fractions from Sephadex G-25	1.15	125.92	18	20.7	2266.56	109.49	43.5	8.54

" Denotes the amount of crude *C. demersum* protein extract initially used as the starting material to purify CA. * Total protein refers to the total protein content of the entire volume of crude protein extract used. [c] Total activity refers to the CA activity of the entire volume of crude protein extract used [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. ' Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. [f] Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

Table 4 Purification profile of carbonic anhydrase from Zn 200 uM treated plants of *Ceraiophyllum demersum*

Protein source	Protein content/ mg ml^{-1}	Activity/ umol ml^{-1}	Volume of the protein/ml[a]	Total protein*	Total activity[c]	Specific activity[d]/ umol mg~' protein	Yield (%)[e]	Fold[f]
Homogenate supernatant	5.8	70.37	60	348	4222.2	12.13	100	1
Pooled "active" fractions from DEAE Sephadex	2.1	77.77	32	67.2	2488.64	37.03	59	3.05
Pooled "active" fractions from Sephadex G-25	0.87	88.88	16	13.92	1422.08	102.16	33.7	8.42

" Denotes the amount of crude C *demersum* protein extract initially used as the starting material to purify CA. * Total protein refers to the total protein content of the entire volume of crude protein extract used. ' Total activity refers to the CA activity of the entire volume of crude protein extract used. [d] Specific activity is activity specific to the protein of interest. It is calculated as activity of protein/protein content. The specific activity will increase as the required protein is being purified from the crude sample. ' Yield refers to the percentage recovery of the specific protein of interest from the crude protein extract. This indicates the amount of final pure form of the protein of interest. Fold purification compares the specific activity of the purified protein to the specific activity of the crude protein extract, indicating the level of purification of the specific protein of interest attained through various purification stages.

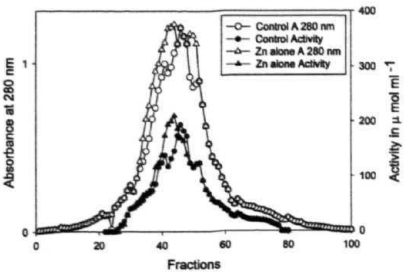

Fig. 2 **Elution** profile of control and Zn-alone (Zn 200 **μM**) treated C *demersum* fractions from DEAE Sephadex column. The active fractions were pooled for the next purification step. Note the slight increase in activity of CA in Zn-alone treatments compared with control indicating the non-toxic and catalytic role of Zn in CA activity.

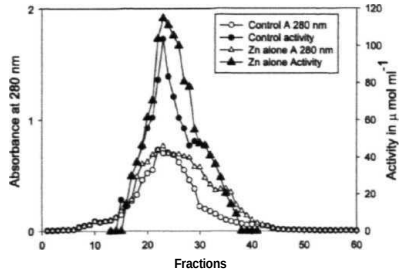

Fig. 3 Elution profile of control and Zn-alone (Zn 200 **μM**) treated *C. demersum* fractions from Sephadex G 25 column. The active fractions were pooled. Note the high increase in activity **of** purified CA in Zn alone treatments compared with control indicating the catalytic role of Zn in enhancing CA activity.

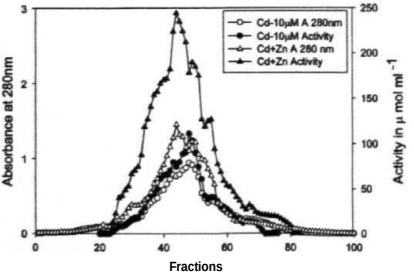

Fig. 4 Elution profile of **10** uM Cd treated and Cd + Zn 200 **μM** treated *C. demersum* fractions from DEAE Sephadex column. The active fractions were pooled for the next **purification** step. Note the drastic decrease in activity of CA in Cd-alone treatments and the restoration of CA activity in Cd treatments with supplemented Zn indicating the catalytic role of Zn in CA.

SDS-PAGE of the purified protein

Electrophoresis on a **13%** gel revealed the homogeneity of the **purified** enzyme and the molecular weight of purified CA was 43 kDa (Fig. 6), with an additional closely associated **isozyme** stained as a second band very near to the purified enzyme.

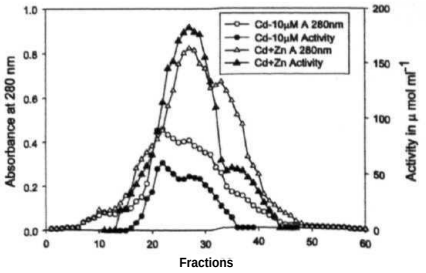

Fig. 5 Elution profile of 10 **μM** Cd treated and Cd + Zn 200 **μM** treated C *demersum* fractions from Sephadex G-25 column. The active fractions were pooled. Note the drastic decrease in activity **of CA** in **Cd-alone** treatments and the restoration of CA activity **in** Cd treatments with supplemented Zn, indicating the catalytic role or Zn in enhancing CA activity.

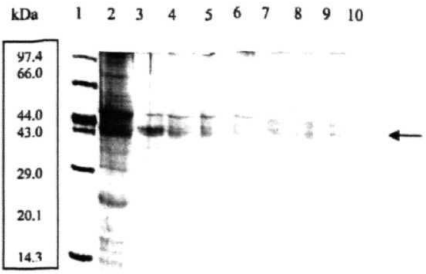

Fig. 6 **13%** SDS PAGE showing the protein **profile** at different stages of purification. Lane **1**: molecular weight markers; lane 2: crude extract; lanes **3–6**: DEAE Sephadex elution fractions **of** control, Cd **10** uM, Cd + Zn 200 **μM** and Zn 200 uM; lanes **7–10**: Sephadex G-25 elution fractions showing the purified 43 kDa carbonic anhydrase in the same order as above.

Zn and Cd content of the purified CA

Estimation of the metal content in the purified form of CA revealed interesting results. Zn content was drastically reduced in Cd-alone treated plants showing a 73% reduction in the Zn content accounting for the impaired CA activity in Cd-treated plants. A very important observation found in our results was the detection of trace amounts of Cd (0.028 **μg mg**$^{-1}$ protein) in CA **purified** from Cd-treated plants (Fig. 7). In **contrast,** in Cd-treated plants with supplemented Zn there was a total restoration of the Zn content (77%) as well as a slight increase in Zn content indicating the nature of Zn in overcoming Cd toxicity. Zn-alone treated plants, however, did not show differences in the Zn content from the CA purified from control. Except in CA purified from Cd-treated plants there was no trace detection of Cd in other treatments.

Discussion

This study was undertaken to identify the mechanism of interaction between Cd and Zn with CA as the enzyme, an important enzyme in aquatics for bicarbonate assimilation. Our earlier investigations have shown that Zn alleviates Cd-induced oxidative stress by its antioxidative capacity through the **influence** of antioxidant enzymes.[3] The present investigation revealed the existence of active Zn-dependent CA in

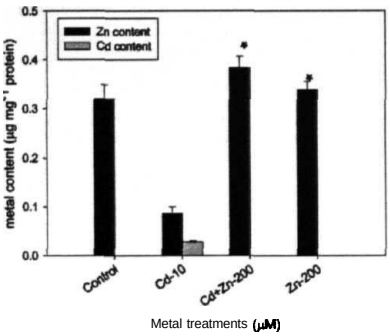

Fig. 7 Zn and Cd content in purified carbonic anhydrase from control, 10 uM Cd, Cd + Zn 200 uM and Zn 200 uM treatments Error bars represent standard errors and an asterisk specifically indicates significant differences from 10 uM Cd treatments. It can be inferred that traces of Cd detected, substituting Zn in the active site of CA, were efficiently removed and restored by Zn supplements to Cd treatment.

C. demersum like other aquatic macrophytes, although its location, whether periplasmic or intracellular, is yet to be investigated.[26] The results of our experiments indicated that CA is impaired by Cd (Fig. 1). Cadmium (like several other heavy metals) is known to affect the structure and functioning of enzymes[36] through peroxidation and production of toxic reactive oxygen species (ROS),[37] which would affect the functioning of the enzymes and proteins. ROS induce fragmentation of protein and impose oxidative modification, rendering cells susceptible to enzymatic proteolysis and hydrolysis." However, Cd-treated plants, when supplemented with Zn, showed not only active restoration of the CA activity, but also an increase in activity at the highest concentration of Zn, demonstrating the complete dependence of the active form of this enzyme on the presence of sufficient levels of Zn (Fig. 1). Zn does not undergo oxidation or reduction reactions and hence is stable in the biological medium, whose oxidoreductive potential is subject to continous flux.[39] This property of Zn helps in maintaining the chemical potential of the Zn enzymes and associated proteins, protecting them from peroxidative damage and oxidative stress.[13]

Cd, Hg and Zn form the group II transition metals with eight electrons filled in their outer orbital. Since Cd and Zn, both taken as divalent cations, are similar in configuration, Cd can readily inhibit most of the Zn-dependent processes, either by displacement or by occupying the active sites of the Zn metalloproteins.[40,41] The purification of CA (Fig. 6) from *C. demersum* revealed this toxic function of Cd towards CA. There was a reduction in the recovery as well the activity of the enzyme purified from Cd 10 uM-alone treated plants (Table 2, Figs. 4 and 5). Thus, the theory of Cd occupying the active sites of important Zn–metalloproteins has been proved by our findings. The estimation of Zn content in the purified enzyme not only showed a drastic reduction in the amount of Zn but also traces of Cd (Fig. 7), as recorded by flame atomic absorption spectrometry. Our experiments showed a reduction of 73% in Zn content and its substitution by Cd impaired the structure as well as the activity of CA. This will lead to non-functioning of CA and hence the associated photosynthetic processes also will be impaired. In many cases it has been reported that removal of Zn from CA leads to irreversible loss of catalytic activity,[42] which corroborates our results. Zn being catalytically associated with CA, maintains the spatial relationships with the different amino acid residues in the polypeptide backbone and maintains the protein conformation intact.[27] In such a case even a slight change in the protein conformation by substitution of catalytically inactive Cd would cause distorted unfoldings to form a totally impaired structure.[2]

Redox active metals like Cu and Cd are known to primarily affect the enzymes and proteins through its interaction with the -SH groups and induce redox cycling.[43] Probably, the Zn-thiolate bonds of the enzyme are broken and replaced by disulfide groups by an inactive and toxic metal like Cd.

The CA purified from Cd-treated plants with supplemented Zn showed a higher recovery as well as highly active CA (Table 3, Figs. 4 and 5). The Zn content estimated was also slightly higher than that of the control, accounting for the increased uptake of Zn for proper functioning of CA (Fig. 7). Zn-alone treatments also showed an active enzyme purified with Zn content closely comparable with that of the control (Table 4, Figs. 2, 3). The presence of sufficient levels of Zn in Cd-treated plants with supplemented Zn strengthens the Zn-metalloprotein interaction by protection of the -SH groups from thiol oxidation and intramolecular disulfide formation, a function primarily associated with Zn.[44] Zn, by associating with the -SH groups, renders them inactive by steric hindrance and hence their susceptibility towards disulfide formation.[45] Zn is a versatile interactant with the enzymes and has a variable coordination sphere and stereochemical adaptation to assume multiple coordination geometries, contributing to its unique and efficient functioning in the metalloenzymes.[39,46] In conclusion, Zn restored the Cd impaired CA in *C. demersum*.

Acknowledgements

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Zinc protects chloroplasts and associated photochemical functions in cadmium exposed *Ceratophyllum demersum* L., a freshwater macrophyte

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Abstract

Cadmium (Cd) is a non-essential and toxic element, without any metabolic significance whereas, zinc (Zn) is an essential element required by many vital enzymes, and plays a significant structural role as stabilizer of proteins, membranes and DNA-binding proteins (Zn-fingers). Therefore, the interactive functions of Zn and Cd on photosynthetic apparatus in *Ceratophyllum demersum* were investigated. *C. demersum* was treated with Cd 10 μM alone, and Cd along with Zn (10, 50, 100 and 200 μM). Treatments with Zn only (10, 50, 100 and 200 μM) were also given for comparison. Cd not only exhibited pronounced toxicity on the over all photosynthetic machinery, but also the pigment biosynthesis. Chlorophyll-a, chlorophyll-b, and carotenoids reduced due to Cd toxicity. Cd-induced severe destruction of chloroplast membrane structure as estimated by the intactness of isolated chloroplasts. As a result, the rate of photosynthesis, electron transport processes, and activity of photosystems (PS I and PS II) altered. Zn supplementation showed complete protection of chloroplasts and associated photochemical functions. Treatments with Zn alone did not show significant differences in the chosen parameters of investigation indicating, the non-toxic nature of the chosen Zn concentrations. These findings indicate that Zn alleviates Cd-induced toxicity in upholding the normal photochemical processes in *C. demersum*.

Keywords: Cadmium; *Ceratophyllum demersum;* Freshwater macrophyte; Photosynthetic functions; Pigments; Zinc

1. Introduction

Trace metal contamination of aquatic ecosystems is of a serious environmental concern for the health of biota. Aquatic plants like *Ceratophyllum demersum* are known to accumulate industrial radionuclides and also heavy metals [1-3] especially Cd at low concentrations (0.1–0.5 ppm). Cadmium (Cd) is a non-essential and toxic element, without any metabolic significance whereas, zinc (Zn) is an important component of many vital enzymes having catalytic, co-catalytic or structural role as stabilizer of proteins, membrane and DNA-binding proteins (Zn-fingers) [4], but toxic in high concentrations [5]. Since both Cd and Zn belong to group II transition elements with similar electronic configuration and valency, they have similar geochemical and environmental properties [6]. The release of Zn to the environment from zinc production processes in smelters and wastewaters from Zn mines is normally accompanied by Cd environmental pollution because of zinc ores (ZnS) generally containing 0.1-5% and sometimes even higher cadmium [5,7,8]. Cadmium and Zn are available to the plants from a variety of other sources also, mainly from sewage sludge leaching, industrial wastewater irrigation and from fertilizers and pesticides [9,10]. Thus, this association of Cd and Zn in the environment and their chemical similarity, and interactive functions are of considerable importance [11,12]. Interactions between Cd and Zn and their transfer in soil–crop system under field conditions, in

Abbreviations: HEPES, *N*-{2-hydroxyethyl}piperazine-*N*′-2-ethanesulphonic acid; MV, Methyl Viologen; PS I, Photosystem 1; PS II, Photosystem II

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solution culture experiments have been reported recently [11,13,14]. However, the influence of Zn–Cd interaction on the photochemical processes has not been investigated.

Cd^{2+} ions are known to affect the structure and function of chloroplasts in many plant systems such as *Triticum aestivum* [15,16], *Beta vulgaris* [17], *Vigna Radiata* [18], *Spinacea oleracea* [19], *Zea mays* [20] and *Phaseolus vulgaris* [21]. The primary site of action by any heavy metal has been reported to be the photosynthetic pigments especially the biosynthesis of chlorophyll and carotenoids [22,23]. Cd inhibiting chlorophyll biosynthesis and proper development of chloroplast structure has been reported in *Pennisetum typhoideum* [24] and *T. aestivum* [25]. Cd ions directly affect the structure of the thylakoid membrane through peroxidation and oxidative stress and lead to disorganization [26] and changes in the lipid composition of the thylakoid membranes [27]. This ultimately leads to the inactivation of oxygen-evolving centers and impaired electron transport [28]. Zinc is known to have a stabilizing and protective effect on the biomembranes against oxidative and peroxidative damage, loss of membrane integrity [13,29,30] and also alteration of the permeability of the membrane [31]. Although there have been many reports on the biochemical events occurring in the chloroplast during heavy metal toxicity with regard to different metals in different systems, there are no reports concerning the influence of Zn on pigment biosynthesis and photosynthetic processes in a Cd and Zn coexisting system, especially in *C. demersum*. The study reported in this paper is therefore mainly focused on the precise mechanism by which Zn influences the photosynthetic processes and electron transport systems, to overcome Cd toxicity to the chloroplast in *C. demersum*.

2. Materials and methods

2.1. Plant material

C. demersum L. plants were collected from local ponds, washed thoroughly and maintained in multiple sets of aquaria at a temperature of 26-28 °C in diffuse light. The plants were supplied with 1/10 Hoaglands solution. After the plants were acclimatized to laboratory conditions for more than one month they were used for experiments.

2.2. Cadmium and zinc treatment

Plant material (2 g) was transferred to a 250 ml glass beaker with 200ml of 1/10 Hoaglands solution (containing 0.764n mol Zn) and the required concentration of the metals. Cd treatment of 10 μM concentration was given to the plant material using $CdCl_2$. Zn supplements (10, 50, 100, and 200 μM) were given to the plant as $ZnCl_2$ along with the Cd treatments. Treatments with Zn only (10, 50, 100, and 200 u,M) were also given to the plant. The plants were kept under the natural day and night cycle 1 week.

2.3. Photosynthetic pigments quantification

An amount of 200 mg of the treated plant material w taken and ground in 80% acetone. The extract was c trifuged at 8000 x *g* for 10 min and the supernatant was ft mated for chlorophyll-a, chlorophyll-b [32] and caroteno [33].

2.4. Isolation of chloroplasts

Metal treated plants were homogenized in a prechil mortar and pestle in ice-cold isolation buffer, which c< tained 400 mM sucrose, 10 mM NaCl and 20 mM Tric (pH 7.8). The slurry was filtered through five layers cheesecloth and the chloroplasts were sedimented at 3000 for 5 min at 4 °C. The supernatant was carefully discarc and the pellet retained. The pellet was washed and res' pended in a small volume of chilled suspension buffer t contained 100 mM sucrose, 10 mM NaCl, 2 mM $MgCl_2$ a 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-2-ethanesulpho acid (HEPES) pH 7.5. Care was taken that the whole pro dure was completely done in ice-cold conditions as quid as possible to inactivate and prevent the degradation chloroplast by proteolytic enzymes [15,34]. Chlorophyll w extracted in chilled 80% acetone and estimated spectropl tometrically [32].

2.5. Intactness of isolated chloroplasts

The percentage intactness of the isolated chloroplasts v determined by the ferricyanide assay. This assay utilizes property that chloroplasts with intact envelopes will not ca out reactions like reduction of exogenous oxidants such ferricyanide or NADP at a faster rate. In this assay isola chloroplasts were given an osmotic shock and NH_4Cl the uncoupling agent, which rendered them 100% envel< free. The rate of O_2 evolution from intact chloroplasts envelope free uncoupled chloroplasts were measured us an oxygen electrode (YSI model 5300 biological oxyj monitor, Gilson med. Elec., Middleton, WI). The percenti intactness was calculated from the difference in the ra of O_2 evolution from intact chloroplasts and envelope f uncoupled chloroplasts [35].

2.6. Assay of photosynthesis

The photosynthetic light mediated evolution of oxy of the isolated chloroplasts was measured polarograp cally using oxygen electrode (YSI model 5300 biol cal oxygen monitor, Gilson med. Elec, Middleton, W The assay medium contained 0.5 M sorbitol, 10 mM K 0.5 mM $MgCl_2$, 0.05% (w/v) BSA, 10 mM $NaHCO_3$, HEPES-KOH (pH 7.6) [17].

2.7. Activities of photosystems II, I and whole chain electron transport

2.7.1. PS II

The reaction mixture for assaying PS II contained 20 mM HEPES (pH 7.5), 100 mM sucrose, 10 mM NaCl, 2mM $MgCl_2$, 1 mM K_4Fe (CN)6 and 0.1 mM Phenylenediamine [15].

2.7.2. PS I

The assay mixture contained 20 mM HEPES (pH 7.5), 100mM sucrose, 10 mM NaCl, 2 mM MgCh, 0.6 mM ascorbate, 1 mM sodium azide, 0.5 mM Methyl viologen and 5 μM DCMU [3-(3,4 dichloro diphenyl, 1,1, dimethyl urea)] and 100 μM DCPIP (2,6-dichlorophenol indophenol) as the electron donor [15].

2.7.3. Whole chain electron transport

The reaction mixture for whole chain electron transport, contained 20 mM HEPES (pH 7.5), 100 mM sucrose, 10 mM NaCl, 2 mM $MgCl_2$, 0.5 mM Methyl viologen and 1 mM sodium azide [15]. All the photochemical assays were carried out at a saturating intensity of white light (800 μE m^{-2} S^{-1}) at 25 °C and chloroplasts equivalent to 30 μg of Chl were used for all the assays.

2.8. Statistics

The values are means of three individual experiments with duplicates (value averaged to 1) for each experiment and the results were subjected to statistical analysis by student's *T*-test (n = 3). The level of significance (a) was set at 0.01 ($P < 0.01$)

3. Results

Cd-10 μM was found to be toxic to *C. demersum* as seen in the form of chlorosis and tissue dissolution of leaves as well as the stems of the plant. The survival of the Cd-treated plants was only up to a week. In contrast Cd-treated plants with supplemented Zn (10, 50, 100 and 200 μM) did not show any of the above symptoms and were healthy up to 3 weeks. Concentrations of Zn above 200 μM were also found to be toxic to the plant.

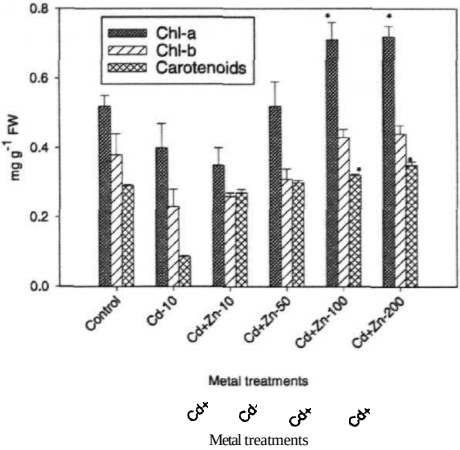

Fig. 1. Influence of Cd and Zn on the levels of chlorophyll and carotenoids in *C. demersum*. Values are means of three individual experiments with duplicates. Values in parenthesis indicate standard deviation (n = 3) and 'asterisk' indicates significant difference from Cd treatments.

3.1. Levels of photosynthetic pigments

The levels of chlorophyll also indicated the toxic nature of Cd to the plant system. Chlorophyll-a and chlorophyll-b showed 23.00 and 40.00% reduction in Cd-treated plants, respectively. Cd-treated plants with supplemented Zn however showed 44.44 and 47.72% restoration of the chlorophyll levels (Fig. 1).

Carotenoids were also equally affected as chlorophyll with reduction in carotenoids level in Cd-treated plants being a drastic 70% from the control. Cd-treated plants with supplemented Zn showed active restoration (75.14%) of the decreased carotenoid levels indicating its efficiency in protecting pigment levels in the system (Fig. 1). However, Zn alone treatments retained the levels of chlorophyll and carotenoids in the plant system (Table 1).

3.2. Intactness of chloroplasts

The intactness of isolated chloroplasts revealed the extensive membrane damage caused by Cd treatments. Cd-treated plants showed a 40% reduction in chloroplast intactness when compared to the chloroplasts isolated from control

Table 1
Influence of Zn alone treatments on the levels of chlorophyll, carotenoids, and intactness of chloroplast in C. *demersum*

Treatments (μM)	Chlorophyll-a (mg g^{-1} FW)	Chlorophyll-b (mg g^{-1} FW)	Carotenoids (mgg-^{1} FW)	Intactness of isolated chloroplasts (%)
Control	**0.51** (0.022)	0.38 (0.061)	0.3 (0.004)	77.73 (1.22)
Zn-10	**0.51** (0.048)	0.39 (0.060)	**0.29 (0.011)**	77.56(1.05)
Zn-50	0.52 (0.030)	0.40 (0.083)	**0.31** (0.020)	78.10(1.56)
Zn-100	0.55 (0.038)	0.41 (0.041)	0.32 (0.006)	78.60 (1.20)
Zn-200	0.55 (0.04)	**0.41** (0.067)	0.32 (0.015)	**79.23** (0.90)

Values are means of three individual experiments with duplicates. Values in parenthesis indicate standard deviation (n = 3).

plants without any metal treatment. Zn restored (41.18%) the intactness of the isolated chloroplasts in Cd-treated plants with supplemented Zn indicating its active role in protecting chloroplast thylakoid membranes from peroxidative damage (Fig. 2). Treatments with only Zn did not reflect much change in the chloroplast intactness indicating the non-toxic nature of Zn (Table 1).

3.3. Rates of photosynthesis and electron transport processes

Cd treatments showed a drastic reduction in the rate of photosynthesis and electron transport processes in the system. Rate of photosynthesis showed a 39% reduction in Cd-treated plants against control. This impairment was effectively controlled and restored completely by Zn (39.19%) in Cd-treated plants with supplemented Zn (Fig. 3). Whole chain electron transport ($H_2O \rightarrow$ MV), PS II and PS I showed 42.15, 45.83 and 42.22% reduction, respectively, in Cd-treated plants. The addition of Zn to Cd treatments

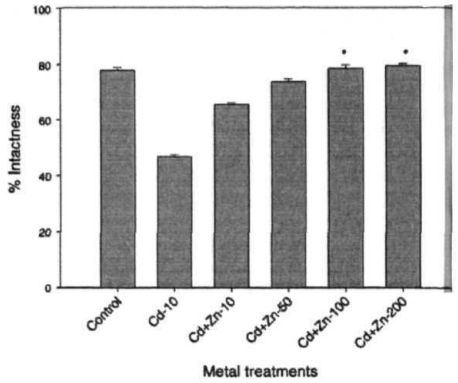

Fig. 2. Influence of Cd and Zn on the intactness of chloroplasts isol from *C. demersum*. Values are means of three individual experiments duplicates. Values in parenthesis indicate standard deviation (n = 3) 'asterisk' indicates significant difference from Cd treatments.

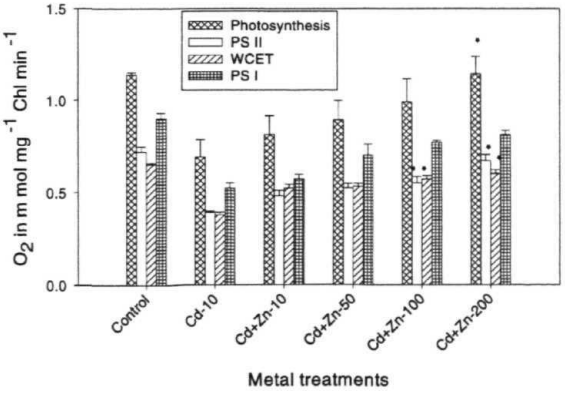

Fig. 3. Influence of Cd and Zn on the rate of photosynthesis, PS II, whole chain electron transport and PS I activity in *C. demersum*. Values are m of three individual experiments with duplicates. Values in parenthesis indicate standard deviation (n = 3) and asterisk' indicates significant differ from Cd-treatments

Table 2
Influence of Zn alone treatments on the rate of photosynthesis and electron transport activities of WCET, PS II and PS 1 in *C. demersum*

Treatments (MM)	Rate of photosynthesis amount of O_2 in (mmol mg^{-1} Chl rain"')	Whole chain electron transport activity amount of O_2 in (m mol mg^{-1} Chl min^{-1})	PS II activity amount of O_2 in (m mol mg^{-1} Chl min^{-1})	PS I activity amount 0: O_2 in (m mol mg^{-1} Chl min^{-1})
Control	1.11 (0.035)	0.72 (0.023)	0.71 (0.018)	0.92 (0.072)
Zn-10	1.14 (0.032)	0.70 (0.046)	0.65 (0.025)	0.86 (0.141)
Zn-50	1.09 (0.025)	0.68 (0.002)	0.65 (0.025)	0.86 (0.141)
Zn-100	1.02 (0.007)	0.68 (0.002)	0.62 (0.089)	0.85 (0.032)
Zn-200	1.01 (0.016)	0.67 (0.007)	0.62 (0.072)	0.85 (0.056)

Values are means of three individual experiments with duplicates. Values in parenthesis indicate standard deviation (n = 3).

was effective in restoring the electron transport processes almost completely (Fig. 3) [37.33% for ($H_2O \rightarrow$ MV) and 41.79% for PS II, and 35.80% for PS I] indicating complete protection of electron transport processes from Cd toxicity. Treatments with only Zn showed very little differences in all the above parameters (Table 2).

4. Discussion

This study was undertaken to identify the mechanisms and the influence of Zn on the photosynthetic processes in a Cd poisoned plant system. The interaction between two metals especially a heavy metal and a nutrient leads to a better understanding of the metabolic pathways and improving the defense strategies through various parameters. We have earlier reported that Zn alleviates Cd-induced oxidative stress by its antioxidative capacity [13] and protects macromolecules like proteins and enzymes [14] from Cd toxicity in *C. demersum*. Further it was demonstrated that in Zn supplemented Cd treatments, Cd uptake ($105\ \mu g\ g^{-1}$ DW) was lowered by Zn (Zn $270.56\ \mu g\ g^{-1}$ DW) compared to only Cd-treated plants ($143.89\ \mu g\ g^{-1}$ DW) [13]. The present findings reconfirmed the protective role of Zn to plant chloroplast functions in Cd-treated *C. demersum*. Our studies showed that chlorophyll-a, b and carotenoids were severely reduced in Cd-treated plants in the absence of Zn supplementation (Fig. 1). Cadmium is known to specifically inhibit chlorophyll biosynthesis through δ-aminolevulinic acid dehydratase (ALA dehydratase) [36] and protochlorophyllide reductase [22,37-39] by its interference with the sulphydryl site [40] leading to the lower production of 5-aminolevulinic acid (ALA), the first common precursor for all the tetrapyrroles. In Cd-treated plants with supplemented Zn, we observed that there is full protection and restoration of the chlorophyll levels. Zn probably maintains chlorophyll synthesis through sulphydryl group protection, a function primarily associated with Zn [29]. Zn always prefers binding to the -SH groups of the protein moiety and protects them from thiol oxidation and disulphide formation [41] either by direct binding or to a site close to the sulphydryl group or by conformational change resulting in apparent stability of the enzymes, membrane proteins as well as the lipid structure [30]. Since ALA dehydratase catalyzing the conversion of ALA to porphobilinogen requires Mg^{2+} or Zn^{2+} for its efficient functioning [42], Zn possibly plays a role in activating this enzyme, and hence protochlorophyllide to chlorophyllide conversion facilitating the formation of complete chlorophyll moiety [43]. 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.

The decreased carotenoid content observed in Cd-treated plants would increase the membrane damage due to loss of ROS scavenging function. Carotenoids are known to quench the oxidizing species and triplet state of the chlorophyll and other excited molecules in the pigment bed, which are seriously involved in disrupting metabolism through oxidative damage to cellular components [44]. In Cd-treated plants with supplemented Zn, the carotenoid levels were well maintained (Fig. 1). Perhaps Zn is able to increase or restore the biosynthesis of carotenoids. From our findings it can be deduced that Zn antagonizes Cd toxicity by counteracting the inhibition of photosynthetic pigments synthesis by maintaining the levels of pigments. Thus, Zn influences not only the proper development of the chloroplasts, but also the maintenance of the photosynthetic activity of the system.

In plants treated with toxic Cd alone, isolated chloroplasts showed a significant loss of photosynthesis, water oxidation capacity (Whole chain transport), PS II and PS I catalyzed electron transport system (Fig. 3). Metal stress impairs electron transport activity by inducing peroxidation and loss of thylakoid membrane integrity. It is very clear from our results of chloroplast intactness that chloroplasts isolated from Cd-treated *C. demersum* showed a very high loss of intactness accounting for the ROS mediated membrane damage and integrity (Fig. 2). It has been reported that Cd affects the lipid structure around PS II especially the light harvesting Chl a/b protein complex II [45,23] leading to loss of major fatty acids [46] and production of lipid hydroperoxides. This induces release of several Chl a-protein complexes and thylakoid membrane proteins including manganese stabilizing protein (MSP) and plastocyanin (PC) [47]. An impaired PC remains as a block in further electron transport process. This severe extent of peroxidative protein loss is ultimately responsible for the crippled photosynthetic process. Zn on the other hand shows clear restoration of the lost photosynthetic activity indicating its action against the toxic nature of Cd (Fig. 3). Zn is known to have a stabilizing and protective effect on the biomembranes by its antioxidative capacity inducing active antioxidant enzyme system to control the destructive oxygen species [13]. This in addition with increased carotenoid synthesis triggered by Zn protects the thylakoid membrane from ROS-mediated peroxidative damage and hence the loss of thylakoid proteins also. This is conclusively proved by our results of chloroplast intactness where Cd-treated plants with supplemented Zn showed higher intactness of isolated chloroplasts indicating intact thylakoid membrane.

In aquatic plants growing in a heavy metal contaminated environment the metabolically active metal ions of chloroplasts has been reported to be substituted by heavy metals [40], Cd^{2+} ions have been reported to replace Mn^{2+} ions at the oxygen-evolving centers, the primary source of electrons from water to PS II, thereby inhibiting the reactions of PS II [22,23] and associated proteins of the PS II reaction centers especially the D1 polypeptide. Cd complexes with aromatic amino acid residues like tryptophan [19] and PS II–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 an impaired PS II activity [48]. This dysfunction propagates throughout

the electron transport chain in the system. Zn by controlling the levels of Cd entering into the system [13] not only controls its intracellular levels, but also replaces the toxic metal, Cd, maintains the integrity at the oxygen-evolving centers and prevents oxidative burst at antenna Chl molecules and probably prevents binding of Cd to the major thylakoid proteins like D1 of the PS II. An active PS II initiates a proper electron transport and hence an active functioning of PS I and complete photophosphorylation process. In conclusion our experimental results indicate that in Cd-treated plants with supplemented Zn there is active protection of photosynthetic apparatus and hence restoration of photosynthetic as well as electron transport activities. Zn also enhanced the biosynthesis of photosynthetic pigments, viz. chlorophyll and carotenoids ultimately proving beneficial for the photosynthetic machinery of the plant system.

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