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,

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 occurring 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) 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 (Dey 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-

1.3. Zinc - an essential plant micronutrient

Zinc \((^{65,39}_{30}\text{Zn})\) 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 \(\text{CO}_2\) and \(\text{HCO}_3^-\) (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
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
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$^0$ 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, $\text{Zn}^{2+}$ 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 $\text{Zn}^{2+}$ state (Schutzendubel and Polle 2002). Hence Zn is stable in biological medium whose oxidoreductive potential is subjected to continuous flux (Vallee and Falchuk 1993, Cakmak 2000, Powell 2000, Zago and Oteiza 2001). This property forms the basis for the efficient functioning of Zn in biological systems. 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 adaptability 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 vulgaris* (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 II B 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 $\text{H}^+$ 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 salvia* (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$_2^-$), perhydroxyl radical (HOO$^-$), the protonated form of superoxide, hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH), alkoxyl radical (RO), peroxyl radical (ROO), organic hydroperoxide (ROOH), singlet oxygen (O$_2^*$) 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$_2$O$_2$ non-enzymatically proceeding through HOO$^-$ or in an enzyme catalyzed reaction (Gebicki and Bielski 1981). H$_2$O$_2$ is not a free radical in a real sense because all of its electrons are paired, but is capable of initiating reduction and OH$^+$ formation, thereby being classified as an intermediate reduction product of oxygen (Shaw et al. 2004). The well known reactivity of H$_2$O$_2$ is not due to its reactivity per se, but requires the presence of a metal reductant specifically Fe to form the highly reactive OH$^+$ radical, the reaction called Haber-Weiss Fenton reaction involving coupling of reduction of Fe (III) by O$_2^-$ and reoxidation to Fe (II) by H$_2$O$_2$ (Girotti 1985).

$$\text{Fe}^{3+} + O_2^- \rightarrow \text{Fe}^{2+} + O_2$$

$$\text{Fe}^{2+} + H_2O_2 \rightarrow \text{Fe}^{3+} + OH^+ + OH^-$$

Copper has also been reported to catalyze this reaction (Wardman and Cadeias, 1996). The damage caused by OH$^+$ would therefore be site-specific whereby iron catalyst attached to membrane lipids on OH$^+$ 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$^+$ 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'. 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\textsuperscript{–} 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 in turn abstracts another hydrogen atom from adjacent lipid molecule propagating the chain reaction further finally forming lipid hydroperoxides (ROOH). An alternative fate of ROO\textsuperscript{–} 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 alkoxy (RO) and peroxyl (ROO\textsuperscript{2–}) radical which in turn can further propagate lipid peroxidation by chain branching (Tadolini et al 1989)

Initiation: \[ \text{RH} \rightarrow \text{R} \]

Propagation: \[ \text{R} + \text{O}_2 \rightarrow \text{ROO} \]
\[ \text{ROO} + \text{RH} \rightarrow \text{ROOH} + \text{R} \]

Termination: \[ 2\text{R} \rightarrow \text{R-R} \]
\[ \text{R} + \text{ROO} \rightarrow \text{ROOR} \]
\[ 2\text{ROO} \rightarrow \text{ROOR} + \text{O}_2 \]

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

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* (Sershen 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 (Skorzynska 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 $\text{O}_2^-$ 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 (Cabisco 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 al 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$^-$ 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 $\text{H}_2\text{O}_2$ in a fenton reaction to form an 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 $\text{OH}^-$ 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$_2$O$_2$ 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' 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' radicals are far too reactive to be controlled easily, defense mechanisms are based on the elimination of its precursors (Of and H$_2$O$_2$). 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 \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-GCS), glutathione-S-transferase (GST), and glutathione peroxidase (GSH-PX). The endogenous non-enzymatic antioxidants include carotenoids, \(\alpha\)-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 \(O_2^-\) 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-chromanol radical (Asada 1994, Arrigoni and De Tullio 2000). Ascorbate-glutathione cycle, a major H₂O₂ scavenging pathway operates both in chloroplast as well as the cytosol (Zhang and Kirkham 1996). In this cycle H₂O₂ 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 up to 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 


1.9. *Ceratophyllum de* m*ersum* L. - an ideal aquatic macrophyte for toxic itv 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-1,3,5-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 CO₂ and HCO₃⁻, 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.