CHAPTER 2

REVIEW OF LITERATURE
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The term heavy metals is usually used for any element that has metallic properties (ductility, conductivity, density, stability and ligand specificity, etc), atomic number greater than 20, density higher than 5.0 cm$^3$, generally excluding the alkali metals and alkali earth metals. The release of heavy metals in biologically available forms as a result of human activities, alters or damages both man-made and natural ecosystems (Tyler et al., 1989). When dispersed in the environment they do not degrade but accumulate in soils, which act as a sink (Eklund, 1995). They are toxic even at very low concentrations (Swamy, 1996), and are potential risk for human health when transferred from plant products to human diet (Rauser and Meuwly, 1995). The chemical form of heavy metals in soil solution is greatly dependent on the metal element concerned, and presence of other ions. Toxic actions of heavy metal ions are essentially exerted on the enzymes. Inhibition of enzymes may be due to alteration of catalytically active groups or protein denaturation (Das et al., 1997) as shown in table IV. Prolonged exposure of soils to heavy metals may result in marked decrease in soil enzyme activity. Impeded litter decomposition is common features of heavy metal polluted soils (Tyler et al., 1989).

Among the heavy metals, cadmium is of increased scientific interest. It is a non-essential heavy metal pollutant of the environment. It has been considered as an extremely significant pollutant due to its high toxicity and greater solubility in water. It does not simply end off the environment, but once used, it remains embedded in a product matrix, and hence, not directly bioavailable.

2.1 Physical and chemical characteristics of cadmium

Cadmium is a naturally occurring metallic element. It is one of the components of the earth's crust and present everywhere in our
## Table IV: Effect of Heavy metals on different metabolic processes

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environment. Existence of cadmium was revealed in 1817. It owes its name to 'cadmium fornacum', the "Zinc flowers" which formed on the walls of zinc distillation furnaces. Pure cadmium is a soft, silver-white metal, malleable, ductile and flexible. It belongs to class II group B of periodic table with atomic number 48 and atomic weight 112.41. Its density is 8.64g/cm³ and melting point 321°C. Pure cadmium is not usually found in the environment as a metal. It is usually found as a mineral combined with other element such as oxygen (cadmium oxide), chlorine (cadmium chloride) or sulfur (cadmium sulfate, cadmium sulfide). These compounds are solids that may dissolve in water, but do not evaporate or disappear from the environment.

2.2 Natural sources of cadmium in the environment

The natural occurrence of cadmium in the environment results mainly from gradual phenomena such as rock erosion and abrasion that estimates for 15,000mt per annum (OECD, 1994; WHO, 1992). The natural calamities such as volcanic eruptions add 820mt Cd per annum to the environment (OECD, 1994; WHO, 1992; Niragu, 1989; Niragu, 1980). Naturally existing concentration of Cd in atmosphere is 0.1-0.5ng/m³, in earth's crust is 0.1-0.5μg/g, but much higher levels may accumulate in sedimentary rocks, and marine phosphates. Phosphorites have been reported to contain levels as high as 500ppm (Cook and Morrow, 1995). Forest fires have also been reported as a natural source of cadmium air emissions with estimates from 1-70mt per annum (Niragu, 1980).

2.3 Anthropogenic sources of cadmium in the environment

Cadmium emissions into the environment started with the development of industrial applications during the first half of 20th century. The wide spread use of cadmium is based on its unique physical and chemical properties. It is highly resistant to chemicals, high temperatures and ultraviolet light
Cadmiun pigments are insoluble in water and inorganic solvents (Cook, 1994). They produce intense colorings such as yellow, orange, red, and are well known pigments in artistic colors, plastic, glasses, ceramics and enamels. For all these reasons Cd is widely used in special alloys, pigment coating stabilizers and above all (almost 70% of its use) in Ni-Cd batteries (Morrow, 1996). It can enter the air from the burning of coal, household waste, and metal mining as well as refining processes. It can enter water from disposal of wastewater from households or industries. Use of fertilizers makes its entry into soil. Spills and leaks from hazardous waste sites can also cause cadmium to enter soil and water. The Cd attached to small particles may get into the air and travel a long way before coming down to earth as dust or rain or snow. All these activities increase the level of Cd in the soil varying from 100-600mg/Kg dry wt (Ernst and Neilson, 2000; Lombi et al., 2000) or more (Meaghler, 2000) at sites that are often far away from naturally occurring ecosystems (Ernst, 2000). The general trend of metal enrichment appears to be urban > rural > remote location.

In the beginning of the 50's, the scientific community has drew its attention to the potential toxicity of Cd and to the risks presented by its accumulation in living organisms (plants as well as animals). Cadmium enters human body mainly through food chain. A bit of it is taken from water also. It has been found that our body rapidly takes in about one quarter of the Cd we breath and one twentieth of the Cd we eat. Cadmium concentration in food is rapidly becoming a quality parameter used to control food commodity marketing both nationally and internationally.

2.4 Cation exchange capacity and competitive cations

The most important factor regulating Cd-bioavailability is the cation exchange capacity (CEC) of the soil. Clay particles, called micelles, are negatively charged and reversibly bind (adsorb) positively charged particles...
Cations (cations) to their surface. Cation such as Cd may be exchanged for H⁺ on the micelle surface. Cations adsorbed to micelles are not available for plant uptake or ground water promoted migration. Conversely, cations not adsorbed to the micelle are available for uptake (Moore et al., 1995). Different metal cations adsorb to micelles with different affinity, and adsorption among clay soils varies by as much as 21% (Atanassova, 1999). Copper (Cu) binds more tightly than Zinc (Zn), which binds more tightly than Nickel (Ni) or Cd (Atanassova, 1999). Because of this, the mineral availability of the soil may increase or decrease depending on the presence of other metals. For example, a soil high in Cu would have more bioavailable Cd, since Cu inhibits Cd-adsorption to micelles (Atanassova, 1999).

Since Cu and Cd are preferentially bound to soil particles, higher Cu/Zn content is advantageous for Cd removal, because it often means Cd is more bioavailable. The addition of calcium (Ca²⁺) to soil reduces Cd bioavailability by increasing pH and competing for Cd on root absorption sites. Manganese (Mn) also competes with Cd for plant uptake (Kabata-Pendius and Pendius, 1992). Furthermore, Clijsters and Van Assche (1985) demonstrated that Mn could reverse the effect of Cd on plant metabolism. It has been suggested that Zn might compete with Cd for uptake into the plant, because of their structural similarity.

2.5 Effect of soil characteristics on cadmium uptake, transportation and bioavailability
Cadmium appears to be absorbed passively (Cutler and Raini, 1974) and translocated freely. The uptake of cadmium from soil to above ground parts depends upon many factors including (a) the portion of the total Cd that is available to the plant root system and (b) the pH. Factors like pH drastically affects the CEC of soil by limiting the available exchange sites. At low pH, H⁺ binds to soil particles tighter than other cations, thus, any metal bound to a soil particle will get booted off in the presence of excess H⁺ (Garcia-
Miragaya and Page, 1978). At high pH, cations are less bioavailable because they have less competition from H⁺ for available binding sites. Decrease in soil pH will increase cadmium bioavailability, and will usually increase plant uptake of cadmium unless the cadmium elicits a toxic response in the plant. Optimum cadmium mobility is achieved at pH 4.5-5.5 (Joner and Leyval, 1997; Bingham, 1980). The adsorption of cadmium is almost doubled for each increase of 0.5 units in the pH from 4-7 (Anderson, 1988).

2.6 Cadmium accumulation

Plants show a differing metal distribution and accumulation pattern among different parts. Most of the Cd that enters the plant system accumulates in the roots and only a small portion is translocated to stem, leaves, pods and seeds (Vitoria et al., 2001). Accumulation of Cd is directly proportional to the concentration of Cd supplied to the growing plant as well as the phenological stage at which Cd is supplied. However, the actual accumulations are influenced by the plant species and soil properties (Fediuc and Erdei, 2002; Jemal et al., 1998). Vassilev et al. (1998) observed 10 times higher Cd accumulation in roots than over ground parts. Shah and Dubey (1998) observed two and four fold accumulation of Cd in roots of two different cultivars of rice than shoots. In most of the plants, the maximum Cd in leaf at higher treatments is similar to plants or seedlings exposed to much lower Cd concentrations. This indicates an overall limitation in total leaf Cd uptake, irrespective of the Cd concentration administered to the plant (Haag-Kerwer et al., 1999; Leita et al., 1991). Leita et al. (1991) treated Phaseolus vulgaris with Cd(NO₃)₂ at a concentration of 1, 2 and 2.5mM and reported that as compared with the total Cd content of roots, stems of treated plants contained Cd lower by one order of magnitude; only a small portion of absorbed Cd was translocated from roots to leaves. Ten fold accumulation of Cd in roots of C. intybus and
five fold in the roots of *C. roseus* was found as compared to their shoots which suggest that these plants are Cd shoot excluders (Lozano-Rodriguez *et al.*, 1997; Florijn and Van Beusichem 1993). Cadmium retention in the root might be due to cross linking of Cd to carboxyl groups of cell wall proteins (Barcelo and Poschenrieder, 1990) and/or an interaction with the thiol groups of soluble proteins and non protein thiol operating as a tolerance mechanism in root cells (Chaoui *et al.*, 1997). There are some plants (e.g. *Alyssum spp, T. carrulescens*) that accumulate maximum Cd in shoots than roots. These are called hyper accumulators (Krämer *et al.*, 1996; Li *et al.*, 1996).

2.7 Morphological growth alterations

Plants respond not only to deficiencies, but excess availability of an element also exhibits its negative effects. The relationship between an element and plant growth shows a dose response curve. Under similar conditions different plants behave differently. Some may act as excluders, others indicators and some others as accumulators (Ouzounidou *et al.*, 1998). When present in excess, metals become stress factors that can reduce vigor, inhibit growth and cause death of plant. At the root-soil surface, interaction of Cd between other metals may be independent, antagonistic, additive or synergistic. Thus, the pattern of uptake of Cd and its effect on plant growth is governed by a large number of factors. But there is consensus on one thing that most primary effect of heavy metals (Cd) occurs on the root growth followed by leaf growth (di Cagno *et al.*, 1999). The toxicity is expressed in terms of inhibition of root elongation, root metabolic activities, decline in root biomass production, alteration in root architecture, impaired and reduced hair formation, reduction in initiation and development of lateral roots and formation of relatively compact and dense root system (Archambault *et al.*, 2001; Schutzendubel *et al.*, 2001; Peralta *et al.*, 2000; Mitra *et al.*, 1994; Kahle, 1993). Root length in sitka
spruce (*Picea sitchensis*) seedlings grown in acidic soil under greenhouse conditions for 100 days was significantly reduced when Cd in soil exceeded certain threshold concentration (Kahle, 1993). 10ppm Cd reduced root size by 6.0% as compared to control root elongation in alfalfa plant (Peralta *et al.*, 2000). Oncel *et al.* (2000) found similar effects of Cd in wheat seedlings.

Cadmium content also influences root volume. In response to 20 ppm CdCl$_2$ in sand cultures, roots of saplings were 50% smaller in volume than in control (Smith and Brennan, 1984). Radical elongation of soil grown maize seedlings was depressed by a concentration of 25µg Cd/g (Hasset *et al.*, 1976). Root growth and root weight gets significantly reduced in American sycamore by Cd treatment. (Carlsson and Bazzaz, 1977). Sandalio *et al.* (2001) observed a significant reduction of growth in roots as well as in leaves on treatment of 50µm CdCl$_2$. They reported a reduction of 20% and 90% in growth rates, when plants were exposed to a Cd concentration of 5 and 50µM, respectively within 12 hrs. Root elongation almost completely ceased after 5 days exposure to Cd at a concentration of 30 and 60 M (Kahle, 1993).

Shoot growth also showed the same trend under heavy metal applications. Decrease in growth is concentration dependent. Usually low concentration did not show much differences. Leaf length also gets reduced by Cd treatment (Setia *et al.*, 1993). Even leaf area per plant also decreased in response to heavy metal treatment. Setia *et al.* (1993) found that Cd$^{2+}$ at a concentration of 8mM caused 23% reduction in diameter increment of new stems. New stem growth and foliage growth was studied by Carlson and Bazaaz (1977) in American sycamore (*Plantanus occidentalis*) using a concentration range from 10-100 µg/g Cd as CdCl$_2$. Cadmium added as chloride salt or oxide in a concentration of 5µg/g progressively reduced the yield of shoot. The symptoms were visible in terms of chlorosis on
interveinal areas of leaves. The chlorotic areas produced necrotic patches with advance in growth (Vassilev et al., 1998).

On a dry weight basis, a similar pattern of plant response to heavy metals (Cd) has been observed. Generally, an increase in metal supply results in inhibition of plant dry matter production. Reduction of plant dry matter (root) production was 10-30% in seedlings of American sycamore (*Plantanus occidentalis*) induced by a concentration of 10-100 ppm Cd in the growth medium (Kahle, 1993). Similarly in shoot dry weight reduction reached up to 37-48% with a wide range of Cd treatments (Huang et al., 1974). Bhattacharya and Choudhuri (1994) reported a 33.6% reduction in biomass of *Vigna* seedlings with a Cd concentration of $10^{-5}$M. Vassilev et al. (1998) observed a decrease in the dry mass accumulation in barley plants grown in pots. The inhibitory effect was 32-35% at the first harvest (the stage of tillering in control) decreasing to 10-73% at the fourth harvest (the stage of full ripeness in control), with 45mg Cd/Kg soil. Malan and Farrant (1998) observed a significant decrease in number of pods (83%) and seed mass (16%) with 0.05 mg/L Cd in soybean.

The overall reduction in growth could be due to repression of the elongation growth rates of cells, because of an irreversible inhibition exerted by Cd on proton pump responsible for the process (Aidid and Okamotto, 1993, 1992). However, lower doses of Cd may have a stimulatory effect on growth as observed in tomato and eggplant (Khan and Khan, 1983), tobacco cells (Hirt et al., 1989) and seedlings of alfalfa (Peralta et al., 2000).

2.8 Physiochemical alterations

Although some heavy metals, including Zn and Cu are required by plants as micronutrients, most of them virtually show some degree of toxicity in all types of organisms. The higher the heavy metal dosage, the greater the
apparent stress response in terms of alterations in physiochemical processes going on in the cell.

2.8.1 Pigment concentration

Pigments of photosynthesis, the chlorophylls and carotenoids, are the most abundant biological pigments known so far (Hall and Rao, 1999). The green color of this planet is due to these pigments. These pigments are soluble in organic solvents and insoluble in water. The presence of these pigments in plants reflects the nutritional status, growth as well as crop productivity (Seyyedi et al., 1999). Better is the status of these pigments, better will be the capability of the plant to fix carbon dioxide and hence, better will be the light harvesting capability. In most of the cases this status is also useful for the plant to acclimatize in adverse environmental conditions.

2.8.1.1 Chlorophyll

Chlorophylls, the light harvesting and photoactive pigments of all photosynthetic organisms, are the members of a large family of tetrapyrrole molecules that contain Mg as a central atom (Kannangari, 1991). Chlorophylls are present on the surface of thylakoid membranes, enclosed in double membrane bound organelle called chloroplasts. Chlorophylls are of many types; the most prominent of them is chl 'a' and chl 'b'. Both chl 'a' and chl 'b', are present in the vascular plants in the ratio of 2:1 or 3:1. A decrease in chlorophyll content upon treatment of plants with Cd has been reported by many workers (Larsson et al., 1998; Skorzynska-Polit and Baszynski, 1997; Rascio et al., 1993; Schlegel et al., 1987). During the time of Cd action chlorophyll concentration in leaves gradually decreases and chlorotic and necrotic changes occurred. Necrosis of leaf tissues may be the reason of Cd mobilization and its transport to above ground parts of plant (Skrzynska-Polit and Baszynski, 1997). Bhattacharya and Choudhuri
(1995) observed a decline in total chlorophyll and carotenoids in seedlings of *Vigna catjang* at a Cd concentration of $10^{-5}$ M CdCl$_2$.

Ralph and Burchett (1998) reported a decrease in chlorophyll after 5 hrs exposure to Cd at a concentration of 1, 5 and 10 Kg/L. This decline in chlorophyll in plant systems in response to Cd treatment is used as a marker to identify metal toxicity. It affects the biosynthesis of δ-aminolevulinic acid (ALA). The chlorophylls are derived from δ-aminolevulinic acid, which is the first specific precursor in chlorophyll synthesis for ALA-dehydratase (a metal sensitive enzyme). Two molecules of ALA are condensed to porphobilinogen (PBG) by Mg$^{2+}$ or Zn$^{2+}$ dependent ALA dehydratase (Sasa and Sugahara, 1976). This enzyme requires the presence of thiol group at the binding sites for the biosynthesis of all tetrapyrroles (Nandi and Shamim, 1968) and production of protochlorophyllide. ALA synthesis is thus the limiting step for chlorophyll production (Dahlin *et al.*, 2000; Boddi *et al.*, 1996; Masuda *et al.*, 1996; McEven *et al.*, 1996; Virgin and Ewen, 1995). Disorganization of lamellar structures mainly the stroma, is also one of the reason for inhibition of chlorophyll biosynthesis De *et al.* (1985) reported that enzymatic degradation is the reason for chlorophyll degradation since higher activity of chlorophyllase under heavy metal treated plants was reported. The decrease may also be attributed to reduce uptake of Mg and Fe ions due to presence of heavy metal in growth medium. Horvath *et al.* (1996) reported a decline in chlorophyll content upon treatment with Cd, which was due to disturbance in the integration of chlorophyll molecules into stable complexes rather than to the retarded biosynthesis of chlorophyll molecules. Ouzounidou *et al.* (1997) observed the inhibition of chlorophyll content in wheat leaves treated with 1mM Cd primarily due to indirect effect of cadmium on the content of essential nutrients. Erdei *et al.* (2002) reported a decline in chlorophyll content of barley seedlings treated with varied concentrations of cadmium.
2.8.1.2 Carotenoids

Carotenoids are the secondary light absorbing pigments called the accessory pigments. These are present on the thylakoid membrane. They are 40-C molecules, dimers of symmetrically joined polyisoprenes whose array of double bonds makes them particularly effective at quenching free radicals (Collins, 2001). They constitute the most diverse and widespread group of pigments found in nature and are synthesized by all photosynthetic and many non-photosynthetic organisms (Nishio, 2000). They impart yellow color to leaves after disintegration of chlorophyll. But the color becomes visible only when the plant approaches to senescence (naturally or under stress conditions). They provide essential photo protective mechanism, blocking the formation of ROS (Young and Britton, 1990). They are important source of scavenging the singlet oxygen. In this protective mechanism, energy is transferred from the excited chlorophyll and singlet oxygen to the carotenoid, which absorbs and dissipates it without chemical change (Young and Britton, 1990; Bartley and Scolnik, 1994). Carotenoid content is less affected (Clijsters and Van Assche, 1985) or is generally increased by the heavy metal exposure (Foyer and Harbinson, 1994; Ralph and Burchelt, 1998).

2.8.2 Protein levels

Proteins are the most abundant molecules in the cell, making up 50% or more of the dry weight. They are found in all cellular components forming the basis of the cell structure and function. Each kind of protein is specialized for its biological function. They operate as enzymes, transporting and regulatory proteins, and also serve as structure and storage of compounds. Many of them act in that part of metabolism, which participates in providing resistance against external stress conditions. Significant alteration in proteins, metabolism under heavy metal stress has been reported by components workers. Some of them observed an increase
in protein synthesis (Shah and Dubey, 1997; Chakravarty and Srivastava, 1997; Brune et al., 1995), while others observed a decrease (Costa and Spitz, 1997). In majority of plant cells, proteins are synthesized in cytoplasmic compartments. Most proteins have a lifetime less than that of cell and therefore are degraded and, if necessary, resynthesised (Nwokolo and Smartt, 1996; Nozaki, 1986).

Plants appear to contain a diversity of metal binding metallothioneins (MTs) with the potential to perform distinct roles in the metabolism of different metal ions. The change in the biochemical characterization for metal tolerance involved the de novo synthesis of metal binding proteins. Liu-kim and Rauser (1986) reported an induction of Cd-binding protein from crude extracts of roots of tomato with an apparent molecular weight 31,000 Da in high ionic strength and 21,500 Da at low ionic strength. Increase in soluble protein was also reported by Vogel-Lange and Wagner (1990) in tobacco leaves on Cd exposure at a concentration of 20\( \mu \text{m} \). Lozana-Rodriguez et al. (1997) observed an increase in the soluble protein content of pea root and shoot upon treatment of Cd at a concentration of 0.05mM, where as the same had no effect on maize. Ali et al. (1998) have observed an increase in the protein content of Bacopa monniera plantlets on exposure to cadmium stress. Hirt et al. (1989) reported stimulation of the protein and RNA synthesis in suspension cells of Nicotiana tabacccum on exposure to Cd stress at a concentration of 100\( \mu \text{M} \). The observed increase in protein content was probably due to the synthesis of new proteins to detoxify the intracellular Cd by binding with the same and rendering the internal concentration of free Cd low enough to minimize the toxic effect and allow stimulation of RNA synthesis.

Gil et al. (1995) reported that total soluble protein as well as rubisco decreased with time at Cd concentrations of 15 and 30 mg/L. Rubisco constitutes more than 50% of the leaf soluble protein; and is the key
enzyme in photosynthesis (Woolhouse, 1974), hence any decline in leaf soluble protein including rubisco will have an adverse impact on rubisco activity and ultimately on photosynthesis. Kevresen et al. (1998) observed a decrease in total soluble protein and rubisco activity in sugar beet plant with decreasing leaf water status and generation of ROS.

2.8.3 Protease activity

in a bewildering diversity of forms, proteins are both components of cell structure and the machinery of living organisms. The accumulation and action of a particular protein is determined by a combination of synthesis and degradation, often occurring simultaneously. Central to the process of protein destruction are proteases, the enzymes that degrade proteins. Peptide bonds, chemical links between the individual amino acids that make up a protein, are normally so stable that boiling in strong acid or alkali, for several hours, is often necessary to degrade a protein. Remarkably the same results can be achieved very quickly by a protease at room temperature and physiological pH. Proteases are, in fact, a major constituent of the cellular toolbox and they have a diversity of functions, from the breakdown of proteins into shorter lengths, to the subtle modifications that convert precursor proteins into functional enzymes with biological activity.

The degradation of proteins is important for normal plant growth as well as leaf development. Leaf development is characterized by a relatively short phase of expansion, followed by maturation, which gradually turns, into senescence. Senescence in leaves is particularly characterized by a pronounced decrease in the levels of proteins, chlorophylls and RNA, often to a level less than 20% of initial value and degradation of membrane lipids. Despite many years of measurements of both proteases and protein levels and turn over, the mechanisms of protein breakdown during senescence are still unknown. Oxidative changes of proteins in animal cells have long
been recognized as a post-translational modification that can result in protein becoming eligible for degradation by existing proteases. Although the oxidative state of proteins in plants has been given only little attention, there are reports showing that protein turnover is regulated or facilitated by the oxidative stress (Buchanan-Wollaston et al., 1997).

In addition to the programmed type of leaf senescence, the stress conditions like temperature, drought, poor light and nutrition supply, pathogen attack and metal availability have also been found to induce leaf senescence. It is assumed that the biochemical degradation process during leaf senescence or different kinds of stress take place through the mechanisms, which are probably similar. However a difference from the natural and programmed senescence is that, in these cases the process may be reversible if the stress conditions are relieved before senescence has progressed beyond a certain point (Stoddart and Thomas, 1982).

One common event in plants during these two physiological conditions is the development of oxidative processes mediated by active oxygen (AOS) species. During foliar senescence or under any unfavorable environmental conditions (Heavy metal pollution), the concentration of AOS rise to toxic levels causing several cellular injuries such as LPO, inactivation/denaturation of enzymes and DNA damage. It is generally postulated that AOS treatment can increase the hydrophobicity of the protein, modify certain amino acid residues and produce intra and intermolecular cross linking and protein fragmentation. In addition an increase in the susceptibility to proteolysis has also been reported. The impact of oxidative stress on protease activity is well studied. Programmed cell death (PCD) that is a natural phenomenon is initiated and propagated through the generation of ROS. Compelling evidences points to the active participation of ROS in plant PCD (Schraudner et al., 1997).
Solomon et al. (1999) reported that oxidative stress induced the activity of a number of proteases by post-translational mechanisms in soybean cells. Metal treatment has a well and distinguished impact on protease activity of plants. Bhattacharyya and Choudhuri (1994) reported an increase in protease activity in the leaves of terrestrial (Vigna) as well as in aquatic (Hydrilla) plants with Cd treatment at a concentration of 1.124 ppm. However, the increase was more in Hydrilla than in Vigna. Shah and Dubey (1997) reported that Cd\(^{2+}\), Cd(NO\(_3\))\(_2\) at a concentration of 500 \(\mu\)M reduces protease activity in root and shoot of rice seedling. Protease activity in general decreased in both embryo axes and endosperms due to Cd treatment in two varieties of rice. However, in vitro studies showed an enhancement in protease activity with low Cd levels (50-100 \(\mu\)M), whereas concentration above this caused inhibition in enzyme activity. It is suggested that there is a possible suppression of protease and peptidase activities due to Cd treatments in germinating rice seeds leading to altered levels of protein and amino acids (Shah et al., 1998). Nagoor (1999) treated maize seeds with CuCl\(_2\), CdCl\(_2\) or HgCl\(_2\) at a concentration of 50-300 \(\mu\)g/ml and reported that lower concentration of Cu stimulated protease activity, particularly at 50 \(\mu\)g/ml in the early stages of germination. Cadmium and mercury generally decreased the protease activity. Alizada (2001) reported a decrease in protease activity of one cultivar while increase in the other cultivar of rice, with Al stress at the same concentration. This difference may be related to the genotypic variability.

2.8.4 Soluble amino acids and proline

The amount of amino acids in plants tissue are carefully regulated to just meet the requirements for biosynthesis of proteins, nucleic acid, and a few other molecules needed to support growth. Amino acid catabolism in plants is generally concerned with the production of metabolites for other biosynthetic pathway. They serve as precursor of many kinds of small
molecules like glutathione and proline that have important and diverse biological roles. Amino acid in excess of those needed for the synthesis of protein and other biomolecules cannot be stored and are used as metabolic fuels. Organisms always have a free amino acid pool in the cell. Costa and Morel (1994) reported that total amino acid levels and incorporation of 14-C into amino acid increases at low cadmium concentration in both roots and leaves in seedlings of lettuce, but high concentration (10 μM) of Cd in the medium decreased both amino acid content and 14C incorporation into amino acids, suggesting a decrease in plant metabolism. Increase in amino acid pool was observed by heavy metal stress in maize germinating seed (Nagoor, 1999). Bhattacharya and Choudhuri (1995) also reported an increase in free amino acid content under Cd stress in hydrila and Vigna. Shah and Dubey (1997) reported about 20-40% increase over control in free amino acid content of roots and 40-80 % over control in shoots of rice seedlings on Cd treatment with 500 M Cd(NO₃)₂.

Proline is an imino acid with aliphatic side chain, but differs from other members of the set of 20 amino acids in that its side chain is bonded to both the nitrogen and the α carbon atoms.

The resulting cyclic structure markedly influences protein architecture. Usually, glutamate is the precursor of proline. Proline is the only imino acid that is found to accumulate fastly and frequently than any other
amino acid under unfavorable environmental conditions. It has been shown to play an important role in ameliorating such conditions as drought, salinity and heavy metal stress (Andrade et al., 1995; Kishor et al., 1995). It has been used as a single parameter to measure physiological dryness (Latts et al., 1999; Hervieu et al., 1994). It oxidizes in turgid tissues rapidly, and also gets affected with the duration of stress conditions (Jäger and Meyer, 1977). Cadmium has a strong and positive relation with proline accumulation. A number of workers reported an increase in proline content under cadmium stress (Nagoor, 1999; Wu et al., 1995). Chakravarty and Srivastava (1997) reported an increase in proline accumulation in linseed under Cd stress. Bhattacharya and Choudhuri (1994) observed a marked increase in proline content in *Hydrilla* and *Vigna* under cadmium stress at a concentration of $10^{-5}$M. Wu et al. (1995) studied the impact of Cu$^{2+}$ and Cd$^{2+}$ on intracellular proline level in four species of algae and reported that proline accumulation is the general response of algal cells to Cd stress.

It may be argued that proline acts as a sink for nitrogenous compounds resulting from the degradation of proteins and protects cell metabolism from the harmful nitrogenous compounds (Aspinall and Paleg, 1981; Yancey et al., 1982). It is often considered to be involved in stress resistance mechanisms by acting as an osmoprotectant thereby facilitating osmoregulation, protection of enzymes, stabilization of the of protein synthesis machinery and regulation of cytosolic acidity, etc (Alia and Saradhi, 1991).

### 2.8.5 DNA damage

Free radicals, highly reactive chemical species are produced in cells by ionizing radiation, a variety of chemicals, and by normal metabolic processes during the reduction of molecular-oxygen to provide energy. However, oxygen reduction is a mixed blessing, because incompletely reduced oxygen species are more reactive and can when out of control,
damage biological molecules, viz. lipids, proteins, RNA and DNA as well as organelles like mitochondria and chloroplasts.

About 50% of the DNA damage produced by ionizing radiations occurs from the radiolysis of water (Ward, 1988), hydroxyl radical being the principal damaging species (Kuwabara, 1991; Breen and Murphy, 1995). If a hydroxyl radical is formed in the vicinity of DNA, potentially mutagenic or lethal lesions can be produced. Hydroxyl radical or ROS, being the principal player in inducing oxidative damage as stated earlier, attacks either at the deoxyribose sugar at a base (purines and pyrimidines) or cleaves the phosphodiester DNA backbone. This results in the strand fragmentation or a chemically modified base. DNA strand breaks produced by the hydroxyl radical generally occurs by its interaction with the C4 of the deoxyribose sugar, leading to the cleavage to the phospho diester back bone (Breen and Murphy, 1995). Strand-breaks formed in this manner usually have 3'-blocked termini with phosphoglycolate or phosphate groups derived from remnants of the deoxyribose moiety (Janicek et al., 1985; Breen and Murphy, 1995), the 5' terminus is usually normal. Sites of base loss are produced by hydroxyl radical attack on the C1 and C4 of the purine and pyrimidine base. In this case the DNA is left with the intact backbone, but with a disrupted deoxyribose moiety lacking its attached purine or pyrimidine base. Hydroxyl radical has been found to attack on the π-bonds of the bases at C5 and C6 of pyrimidines and C4 of purines (Steenkens, 1989). All these changes result in the formation of a large number of modified bases, some of which are stable and the others are unstable, the unstable ones are further broken down to more stable product. Among the large number of these stable and unstable products of DNA damage, 8-oxoguanine and 8-oxoadenine (the damaged products of purine) are common, stable, oxidised bases that have received considerable attention recently. Crosslinks, DNA and protein (Mee and Adelstein, 1981; Olenick et
al., 1986), and DNA-DNA, are also produced by ionizing radiation attack on DNA (Ward, 1988), but little is known about their biological processing.

Cadmium is classified as probable human carcinogen by IARC (1993). It has been found to induce DNA damage in a number of living organisms including plants (Fatur and Filipic, 2002; Mouron et al., 2001; Stohs et al., 2000; Bagchi et al., 1996; Koppen and Verchaeve, 1996). The genotoxic potential of cadmium is rather weak and restricted to high cellular concentration. However, at lower concentrations, cadmium enhances genotoxicity of other DNA damaging agents (Hartig, 1995). It has been shown that cadmium interferes with nucleotide excision repair (NER) by inhibiting DNA damage recognition and incision step of NER (Hartig et al., 1996). Fatur and Filipic (2002) reported a dose dependent increase in DNA damage caused by CdCl₂ in human hepatonic cells (Hep G2).

### 2.8.6 Fatty acid composition

Fatty acids are carboxylic acids with hydrocarbon chains of 4 to 36. Biological systems contain 16-18 carbon fatty acids most commonly. In some fatty acids, this chain is fully saturated and unbranched, others contain one or more double bonds and a few contain 3-carbon rings or hydroxyl groups. These are physiologically important as components of phospholipids and glycolipids, lipophilic modifiers of protein, fuel molecules and hormones and intracellular messengers. Fatty acids are synthesized in the cytosol. A reaction cycle based on the formation and cleavage of citrate carries acetyl groups from mitochondria to cytosol. NADPH needed for the synthesis of fatty acids is generated by the pentose phosphate pathway and in the transfer of reducing equivalents from mitochondria by malate pyruvate shuttle. Fatty acids are stored in adipose tissue as triacylglycerols (neutral fat), which can be mobilized, by the hydroxylic action of lipases that are under hormonal control. Fatty acids are activated to acyl-CoAs, transported across the inner mitochondrial membrane by carnitine, and
degraded in mitochondrial matrix. They are elongated and desaturated by enzyme systems in the endoplasmic reticulum membrane. The desaturation requires NADH and O$_2$. A complex consisting of a flavoprotein, a cytochrome, and a non-heme iron protein carries it out.

Adverse environmental conditions like high and low temperature (Pleines et al., 1987; Tremolieres et al., 1982), salinity (Allakhverdiev et al., 1999, Elenkov et al., 1996) and heavy metals (Jemal et al., 2000; Howlett and Avery, 1997; Fodor et al., 1995; Frostegard et al., 1993; Krupa and Baszynski, 1989) change the composition of fatty acids in plants. High temperature has been found to result in a significant increase of C$_{18:1}$ content and a decrease in C$_{18:3}$ content. Low temperature increases C$_{18:1}$ and C$_{18:2}$ desaturation, resulting in a higher C$_{18:3}$ (Pleines et al., 1987; Tremolieres et al., 1982). In a study carried out by Ouarti et al. (1997), Cadmium stress has been found to increase the proportion of C$_{16:0}$, and decrease in the C$_{18:2}$ and C$_{18:3}$, in 17 day old tomato seedlings. The result of this study suggests that metal treatment has induced an alteration in the fatty acid desaturation processes. Furthermore, the accumulation of C$_{16:0}$ rather than C$_{18:0}$ indicated an alteration in the ratio of products from the fatty acid synthase. Similarly, Krupa and Baszynski (1989) reported that thylakoids from 4 week old tomato seedling grown for 14 days in nutrient solutions containing Cd, showed a decrease in the content of all individual glyco-and phospholipids to approximately 75% of control. The greatest decrease was in the phosphatidylcholine content. The fatty acid composition of the acyl lipids extracted from the thylakoids was characterized by a significant decrease in the trans-6-3 hexadecanoic acid component of the phosphatidylglycerol and by a tendency for the linolenic acid content in all lipids to fall.
2.9 Cadmium-induced oxidative stress

Cadmium is the fifth most toxic metal to vertebrates and the forth most toxic metal to vascular plants (Oberlunder and Roth, 1978). Even at low concentration, it may adversely affect the plant reproduction by inhibiting pollen germination and tube growth (Xiong and Peng, 2001). Various authors reported the toxic effect of cadmium on biological systems (Baryla et al., 2001; Sanita di Toppi and Gabbrielli, 1999; Mehindirata et al., 1999; Bhattacharya and Chaudhuri, 1995; Sharma et al., 1985; Mukherjee et al., 1984). In plants, the symptoms of cadmium toxicity are easily identifiable ranging from slight injury to lethality or crop failure. Most of the physiological research on the mechanism of Cd toxicity has involved a single plant or species or variety. The most general symptoms are stunting, chlorosis and alteration of anatomical, morphological, physiological and biochemical properties of leaf, stem and roots (Liu et al., 2000; Viswanath, 1999; Zak et al., 1996). Cadmium toxicity appeared to induce mineral deficiency, interferes with the transport and use of several elements (Ca, Mg, Mn, P, K), and alters water balance and mineral nutrition in plants (Rubio et al., 1994; Trivedi and Erdei, 1992; Godbold and Huttermann, 1985). In some cases toxicity depends on even temperatures (Nasu and Kugimoto, 1981).

Metal ions have been categorized into two classes on the basis of their ligand preferences (Nieboer and Richardson, 1980). Class A contains Al, which forms the most stable complexes with ligands containing 'O', while class B metal ions, including Zn^{2+} and Cd^{2+} forms the most stable compounds with 'S' and 'N' centers. This association of essential metal ions with such ligand is necessary for their biological activity, but is also the reason of toxicity of non-essential metal ions.

Cadmium has the ability to replace the metal cofactor of the metalloenzymes. Cadmium has an outer shell filled with electrons. It tends
to form tight covalent bonds with positively charged molecules such as proteins and DNA. It readily binds to proteins with sulphydryl groups, and may inactivate enzymes in this way (Scidlecka et al., 1997; Van-Assche and Clijsters, 1990). It may also directly damage DNA. In pea plants chromatin alterations have been reported (Hadwiger et al., 1973). It is reasonable to hypothesize that excessive amounts of Cd usually not present in the environment, replaces Zn ions in the “Zinc fingers” and consequently Cd interferes with the transcription mechanisms (Sanita di Toppi and Gabbrieli, 1999).

Cadmium was found to induce oxidative stress (Okamoto et al., 2001; Piquerers et al., 1999; Hendry et al., 1992; Somashekaraiah et al., 1992), but in contrast with other heavy metals, such as Cu, it does not seem to act directly on the production of ROS through Fenton-type reactions or Haber-Weiss reactions (Salin, 1988). Evidence that Cd causes the production of ROS (Poyer et al., 1997) in plants, came from observation that new isozymes of peroxidases were detectable in both root and leaves of Phaseolus vulgaris (Van Assche and Clijsters, 1990). Further evidence of the Cd-induced oxidative stress comes from the detection of lipid peroxidation, increased lipogenase activity, chlorophyll degradation and inhibition or stimulation of the activity of several antioxidant enzymes, viz. SOD, GR, DHAR, CAT, APX and Guaiacol peroxidase (Schutzendubel et al., 2001; Dalurzo et al., 1997; Chaoui et al., 1997; Lozano-Rodriguez et al., 1997; Gallego et al., 1996a). Cadmium induces varying responses in plants in relation with these enzymes. The varying responses are most probably related both to the levels of Cd supplied and to concentration of thiol groups already present or induced upon treatment. All these changes lead to the alteration and the production of ROS in the plants. Cadmium provokes significant disturbances in the structural organization and functional activity of photosynthetic apparatus (Dahlin et al., 2000; Krupa and Baszynski, 1995; Vassilev et al., 1995; Baszynski, 1986). The main
targets of toxic Cd effects are the pigment apparatus and photosynthetic gas exchange systems (Lang et al., 1995; Tukendrof and Baszynski, 1991; Clijsters and Van Assche, 1985).

2.9.1 Malondialdehyde

Acrobic life is threatened by the oxygen toxicity that is its inherent feature. Photosynthetic plants are especially at the risk of oxidative damage, because of their oxygenic conditions and the abundance of the photosensitizers and polyunsaturated fatty acids in the chloroplast envelope. It is reported that 1% of the oxygen consumed by the plants is diverted to produce activated oxygen species like hydroxyl radical (OH$^\bullet$), singlet oxygen ($^1$O$_2$) and super oxide radical (O$_2^\bullet$) also called ROS (Asada and Takahashi, 1987).

Free radicals and other derivatives of oxygen are inevitable by-products of biological redox reactions. Their production is considered to be a universal or common feature of living world under natural condition as a by-product of respiration and photosynthesis during electron transport systems of mitochondria and chloroplast. The concentration increases under unfavorable conditions. Intracellular structures like membranes and biomolecules like proteins, enzymes, lipids and DNA have a high degree of organization that is at the risk of being destructed by these oxidative radicals.

Lipids are most prone to oxidative damage. The peroxidation of lipids is considered as the most damaging process known to occur in every living organism (Hung and Koa, 1997; Zhang and Kirkham, 1996). The most well studied situation that initiated lipid peroxidation is that in which an external oxidant usually oxygen centered free radical such as OH$^\bullet$ or ROO$^\bullet$ attacks an allylic methylene group and converts it to a new carbon centered free radical.
or a singlet oxygen reacts with unsaturated lipids and undergoes an "ene reaction" that leads to incorporation of oxygen into the lipid chain and migration of a double bond.

This ensures oxidative stress as the defense mechanism of plants is overwhelmed by the formation of these free radicals and other pro-oxidants (del Rio et al., 1996). Lipid peroxidation (LPO) results in membrane damage (Vaughan et al., 1982) because the geometry of the alkyl chain becomes greatly altered and the packing order in the bilayer is disrupted. Membrane damage is sometimes taken as a single parameter to determine the level of lipid destruction i.e. membrane damage is synonymous to lipid peroxidation. For many years it has been recognized that during lipid peroxidation, products are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, malondialdehyde (MDA), etc and compounds related to them (Reddy et al., 1998; Weckx and Clijsters, 1996; de Vos et al., 1993; Bird et al., 1983). Some of these compounds react with thiobarbituric acid (TBA) to form colored products hence, called thiobarbituric acid reactive substances (TBARS) that can be measured by monitoring their absorption at around 530 nm (Gray, 1978). MDA is usually used to assess the impact of adverse conditions on the organism (Bennicelli et al., 1998).

Plants exposed to heavy metal stress exhibited an increase in LPO due to generation of free radicals (Vanaja et al., 2000; Lozano-Rodriguez et
Somashekaraiah et al. (1992) reported an increase in TBA reacting substances with increasing concentration of cadmium in germinating seedlings of mung bean. This was related to blockage of electron flow in PSII by metal ions that lead to the formation of excited chlorophyll which in turn causes the production of free radicals (Kato and Simizu, 1985).

In *Phaseolus vulgaris*, roots and leaves exposed to a Cd concentration of 5 μM showed an enhancement in the activities of guaiacol and ascorbate peroxidase (Chaoui et al., 1997). Cadmium treatment notably increased lipid peroxidation in pea plants (Lozano Rodriguez et al., 1997), whereas no peroxidation was noticed in cadmium exposed plants of hairy roots of *Daucus carota* (Sanita di Toppi et al., 1998).

Piqueras et al. (1999) reported an increased peroxidation of lipids in BY2 cell cultures of tomato exposed with 5 mM Cd. The increase was related to a rapid generation of H2O2 followed by an alteration of the antioxidant enzymatic system. Dixit et al. (2001) reported an increase in lipid peroxidation in both roots and leaves of pea plants exposed to varying range of Cd in hydroponic system. Okamoto et al. (2001) observed oxidative damage to lipids in isolated chloroplasts of unicellular alga *Gonyaulax polyedra* under Cd stress. An increase in the accumulation of lipid peroxides was also found in growing pea plants treated with CdCl2 at a concentration of 0-5 μM. The increase was related to severe disturbances in chloroplast structure leading to the alteration of activated oxygen metabolism. However, the increase was concentration (CdCl2) dependent (Sandario et al., 2001).
2.10 Modulation of antioxidant systems by Cd stress

Plants exposed to Cd stress invariably showed marked alteration in the electron transport in both chloroplast and mitochondria. This results in the production of toxic oxygen species such as $\cdot$O$_2$, OH and H$_2$O$_2$ (Foyer et al., 1997). Due to the multiplicity of the processes that produce ROS during metabolism, including photosynthesis and photorespiration (Nocter and Foyer, 1998), fatty acid oxidation, response to pathogen attack and senescence, plants have developed a series of detoxification reaction mechanisms (Vitoria et al., 2001). These detoxification mechanisms are usually divided into three classes:

1. Lipid soluble, membrane associated antioxidants (e.g. α-tocopherol, β-carotene, etc.)

2. Water soluble, reductants (e.g. ascorbate and glutathione)

3. Enzymatic antioxidant e.g., SOD, EC 1.15.1.1; CAT, EC 1.11.1.6; POD, EC 1.11.1.7 and enzymes of ascorbate glutathione cycle (Schutzendubel et al., 2001; Noctor and Foyer, 1998; Smirnoff, 1993).

Given the above-cited mechanisms utilized by plants to detoxify ROS, it is clearly important to establish whether exposing plants to Cd causes a detrimental or stimulatory effect on the enzymes involved in these detoxification processes. Somewhat surprisingly, the experiments that have been carried out on the subject have frequently produced contradictory results (Vitoria et al., 2001). These varying response to Cd induced antioxidative response are probably related both to levels of Cd supplied and to the concentration of thiolic groups already present or induced by Cd stress (Pichorner et al., 1993).

2.10.1 Superoxide dismutase (SOD)

Superoxide dismutase is a group of metalloenzymes virtually present in all living organisms. It is a highly efficient catalyst, involved as a first line of
defense among the enzymatic mechanisms against the removal of superoxide, with rates being almost diffusion inhibited. Superoxide (O$_2^-$) can be formed enzymatically by flavoprotein dehydrogenases. More importantly it can be formed non-enzymatically by autoxidation of ferredoxins, hydroquinones, thiols and reduced hemoproteins in electron transport systems (chloroplast and mitochondria) under normal conditions (Fig 4). Ferredoxin and other electron carriers on the reducing side of PSI have sufficiently negative electrochemical potentials to donate an electron to oxygen (Asada and Takahashi, 1987). The majority of superoxide production in this way is through ferredoxin (Furbank and Badger, 1983) and the Mehler reaction and their rate enhances under oxidative stress. Superoxide is not as toxic as other oxygen species but is possibly involved in lipid peroxidation, membrane damage, cellular toxicity and single strand breaks in DNA (Fridovich, 1986). It may react with hydrogen peroxide to produce the hydroxyl radical in the metal catalysed Haber-Weiss reaction. It also causes the inactivation of catalase and glutathione peroxidase as well as NAD(P)H and epinephrine oxidation (Fridovich, 1986). It is implicated in sunscald damage in high light and heat. Superoxide is converted to hydrogen peroxide via a dismutation reaction catalysed by the enzyme family, superoxide dismutase. SOD plays a pivotal role in the antioxidant pathway (Foyer et al., 1994; Salin, 1988). SOD catalyses a reaction in which two identical substrates have different metabolic fates in this case one molecule of superoxide is oxidized and the other is reduced.

\[ O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2. \]

McCord and Fridovich, (1969) reported an enzymatic role for a copper containing protein isolated from bovine albumin serum named erythrocuperin. They were able to show that erythrocuperin can dismute twosuperoxide anion radical to hydrogen peroxide and diatomic oxygen. They named the enzyme SOD. An SOD isoform was found to be in the
chloroplasts and shown to act in concert with APX, MDAR and GR (Dalton 1995; Salin and Bridges, 1981). In this scheme APX reduces the H$_2$O$_2$, generated by SOD activity, into water.

SODs are classified according to their metal factors present at their active sites, as FeSOD, MnSOD, or Cu-Zn SOD. In plants, unlike other organisms, there appear to be multiple forms or isoenzymes of SOD, which are encoded by multiple genes. Chloroplast generally contains Cu-ZnSOD, but FeSOD is also present in a number of plant species (Van Camp et al., 1994).

![Figure 4. The Mehler-peroxidase reaction sequence associated with the thylakoid membrane. Adapted from Foyer, Lefebvre and Kunert 1994](image)

The activity of SOD is altered under different environmental conditions. Various workers have reported alteration in SOD activity under metal stress. Somashekaraoiah et al. (1992) reported decrease in SOD activity in germinating seedlings of mung bean treated with different concentrations of cadmium acetate. The decrease was related to the lipogenase- mediated accumulation of lipid peroxides and inhibition of free radical scavenging system. The same results were also reported by many
investigators also reported (Sandalo et al., 2001; Groppa et al., 2001; Mishra and Choudhuri, 1996). Okamoto et al. (2001) observed a slight increase in SOD activity in isolated chloroplast of unicellular alga Gonyaulax polyedra exposed to acute Cd stress and high SOD activity in the same cells when exposed to chronic Cd stress. These results indicate that heavy metals are able to induce oxidative stress particularly under acute conditions and the cell follow high antioxidant capacity during acclimation to chronic metal stress. Piqueras et al. (1999) treated BY2 cell cultures of tobacco with 5 mM Cd and observed a significant SOD activity after 10 minutes of treatment. The results suggest that plasma membrane is the primary target for short-term production of AOS in response to Cd in BY2 tobacco cells followed by a coordinated activation of the antioxidant enzymatic system. Many other workers also reported increase in SOD activity during stress conditions (Schutzendubel et al., 2001; Dixit et al., 2001; Vitoria et al., 2001; Prasad et al., 1999).

2.10.2 Catalase (CAT)

In both prokaryotes and eukaryotes the catalases are present. They catalyse the dismutation of $H_2O_2$ to water and oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The sources of $H_2O_2$ generation are electron transport chain of mitochondria, PS II, components of chloroplasts or the P450 oxidases of the endoplasmic reticulum. Intercellular sources of $H_2O_2$ generation include peroxisome and reactions catalysed by their associated oxidases and dehydrogenases. $H_2O_2$ can readily permeate cell membranes, and put DNA to a major hazard by generation of highly reactive hydroxyl radicals ($OH^\cdot$) through the interaction of $H_2O_2$ with transitional metal ions such as $Fe^{2+}$ in Fenton-type reactions.
Catalases are mainly located in peroxisomes and degrade H₂O₂ without consuming cellular reducing equivalents i.e., it is a very efficient means of removing H₂O₂ from cells. Plants have isozymes of catalases. CAT-1 and CAT-2 are associated with peroxisomes, whereas CAT-3 is associated with mitochondria. Alteration in CAT activity under heavy metal (Cd) is reported depending upon the duration, dose of the treatment and the plant organism under study. Increase in CAT activity is reported by a number of workers (Vitoria et al., 2001; Prasad et al., 1999; Mishra and Choudhuri, 1996). Dixit et al. (2001) treated pea plants with varied Cd concentrations and observed a prominent increase in CAT activity of leaves. The increase was attributed to its additive function for providing metal tolerance.

Schutzenedubel et al. (2001) raised Scots pine (Pinus sylvestris) in a hydroponic solution treated with 50 µM CdCl₂. The treatment initially (after 6 hrs) inhibits CAT activity, after 24 hrs it was stimulated by a factor 1.5 and after 48 hrs the activity decreased. This response was related to Cd induced imbalance of redox systems of cell due to increase in H₂O₂.

Chaoui et al. (1997) treated bean plants (Phaseolus vulgaris) with 5 µM Cd for 96 hrs and observed a decrease in CAT activity of roots and leaves but not in stems. They suggested that some antioxidant enzymes could be activated, notably in upper plant parts, in response to oxidative stress induced by cadmium. Fediuc and Erdei (2002) studied physiological and biochemical aspects of cadmium stress in common reed (Phragmites australis) and cattail (Typha latifolia). The cadmium treatment was applied as a concentration series between 0.1-100 µM/L. They observed that CAT activity decreased in leaves of common reed but had no effect in the roots after 4 weeks of Cd treatment. In case of cattail, CAT activity increased in shoots but roots showed a gradual inhibition at the same duration. Since roots rely partly on shoots for the supply of reduced sulfur which binds the cadmium (Meuwly and Rauser, 1992), the response of defense mechanism
are different in the two plant organs. Sandalio et al. (2001) and Somashekeriarh et al. (1992) also reported decrease in CAT activity on Cd treatment.

2.10.3 Glutathione levels

Glutathione (γ-glutamyl-cysteinyl-glycine) is the most abundant form of organic sulphur in plants, forming a major source of non-protein thiol, apart from that incorporated into proteins (Dixon et al., 1998; May et al., 1998; Bergmann and Rennenberg, 1993). The chemical reactivity of the thiol group of glutathione makes it particularly suitable to serve a broad range of biochemical functions in all organisms. It has an oxidative reduction potential of -0.23V that allows it to act as an effective electron acceptor and donor for numerous biological reactions. In addition to function as a translocatable store of organic sulphur (Hell, 1997), it has multiple functions in living systems. It appears to function as an intracellular signaling agent, responsive to changes in the extra cellular environment (Sanchez- Fernandez et al., 1997). The nucleophilic nature of the thiol groups is also important for the formation of mercaptide bonds with metals and for reacting with a variety of compounds containing electrophilic cites (Xiang et al., 2001). GSH has been also implicated in the regulation of enzyme activities, in DNA synthesis, in maintaining the viability of chloroplasts and mitochondria, in the protection of nitrogenase from inhibition by oxygen, and in the regulation of gene expression possibly via thiol sensitive transcription factors (Wingsle and Karpinski, 1996; Kulik and Storz, 1994).

Reduced glutathione is one of the most efficient scavengers of peroxides arising as by-product of cellular metabolism or during oxidative stress (Noctor and Foyer, 1998). As an antioxidant, glutathione together with ascorbate and other enzymes, SOD and APX, controls the cellular concentration of H₂O₂ and O²⁻. Here, it protects cell against damage from
free radicals. The reactivation of these antioxidants requires adequate amounts of reduced glutathione. The efficiency with which the oxidized dithiol (GSSG) can be converted back to GSH during the reductive inactivation of peroxides contributes to the centrality of glutathione (May et al., 1998).

Under normal conditions, glutathione is predominantly present in its reduced form (GSH), with only a small portion present in its fully oxidized state (GSSG). The size of reduced glutathione pool shows marked alteration in response to a number of biotic and abiotic environmental conditions. Under some stress conditions, oxidation of GSH is accompanied by net glutathione degradation (Foyer et al., 1997). But most of the studies have shown that glutathione accumulates in response to increased AOS generation or is constitutively higher in plant adapted to exacting conditions (Arora et al., 2002; Willekens et al., 1997; Madamanchi et al., 1994; May and Leaver, 1993). Differences in GSH content may be wholly or partly due to modulated rates of GSH biosynthesis. The pathway of synthesis appears to be common to all organisms that contain GSH. Two ATP-dependant steps, catalysed by \( \gamma \) glutamyl cysteine synthetase (\( \gamma \)-ECS) and glutathione synthetase (GS) lead to the sequential formation of \( \gamma \)EC and GSH (Noctor and Foyer, 1998). Glutathione can be synthesized in the cytosol and the chloroplast. Both \( \gamma \)-ECS and GS have been detected in chloroplastic and extrachloroplastic fractions (Ruegsegger and Brunold, 1993). Over expression of \( \gamma \)-ECS in either compartment led to a three-fold increase in foliar GSH contents.

Estimation of chloroplastic GSH concentration has yielded values 1 to 1.45 mM (Bielawski and Joy, 1986). Glutathione transport at the plasmalemma has been investigated in tobacco cells (Schneider et al., 1992) and broad beans (Jamai et al., 1996), but no studies on possible transporters have yet appeared. Further investigation is required to
establish whether glutathione associated with GR activity in the mitochondria and peroxisomes is produced in situ or must be imported. Knowledge of one pathway of GSH degradation in plant tissue has come from work on tobacco cells (Romer et al., 1992). This route is confirmed to the cytosol and with the possible exception of first step proceeds through the same sequence as the \( \gamma \)-glutamyl cycle in animals (Meister, 1988).

Some studies have shown initial decrease in GSH accumulation in oxidizing conditions (Sen Gupta, 1991; Smith, 1985) as mentioned above. In other studies such decrease were less evident. Moreover the decrease was sometimes reversed well before accumulation of total glutathione had ceased (Sen Gupta, 1991). Schutzendubel et al.(2001) observed great fluctuations in glutathione pool in scots pine roots with Cd treatment. Upon exposure to cadmium at a concentration of 50\( \mu \)m, the glutathione pool (GSH+GSSG) was almost completely depleted within 6h and the remaining glutathione was oxidized. In cadmium treated roots, the increase in GSH was initially accelerated as compared with controls at 24hrs. After 96 hrs, Cd treated roots contained nearly four times higher concentration of GSH than controls.

Haung and Kyung (2000) exposed two-week-old seedlings of tomato to various Cd concentrations (0-100\( \mu \)M) in the nutrient solution for 9 days. They reported that under these conditions, the content of acid-soluble thiol and cysteins in leaves and roots, the GSH pool in the leaves and the ratio of oxidized/reduced glutathione in both leaves and roots increased. While investigating the adaptive responses to metal stress in isolated chloroplasts of unicellular alga Gonyaulax polyedra, Okomoto et al. (2001) reported that under chronic conditions there was an increase in glutathione content in isolated chloroplasts of this alga, under acute metal stress there was a decrease in reduced glutathione pool. Prasad et al.(1999) also observed a significant increase in GSH content in seedlings of Brassica juncea L. on
treatment with toxic levels of Zn. This increase was attributed to the presence of high levels of cellular oxidants under Zn toxicity that promotes the synthesis of glutathione (Foyer et al., 1997). Bousama et al. (1999) treated maize cv. Ex 74 seedling to increasing cadmium concentration for 12 days and observed a significant and dose dependent increase in the level of glutamate parallel with the increased content of γ-glutamylcysteine and glutathione. They suggested that cadmium induced a substantial shift in the operative pathway of ammonia assimilation, and the induction of NADH-glutamate dehydrogenase activity under cadmium stress may provide glutamate, required for enhancing the synthesis of proline, γ-glutamylcysteine and glutathione, the common response to cadmium stress.

2.10.4 ATP-Sulphurylase

Sulphur in its reduced form plays an important role in plants being utilized in the primary and secondary metabolites in the synthesis of coenzymes. Even oxidized sulphur metabolites are necessary to the synthesis of plant sulpholipids in the intact chloroplast membrane. Thus sulphur plays an important role in plant growth and in the regulation of plant development (Downei, 1989; Jakoby, 1981).

Sulphate uptake increases after prolonged sulphur starvation (Lee and Leustek, 1999; Lappartient et al., 1999; Bieldlingmaier and Schmidt, 1987). However, it is unclear, if a new sulphate permease is incorporated, into the membrane or if only the “normal” permease increases leading to a lower apparent $K_m$ for sulphate uptake. Evidences from cyanobacteria suggest that a second permease is formed during sulphur starvation (Laudenbach and Grossman, 1991). Further, AS and AR activity have long been known to increase during sulphur starvation (Brunold, 1990). The mRNAs for AR and serine acetyl transferase (The O-acetyl-L-serine producing enzyme) but not AS, have recently been shown to accumulate in
sulphur starved *Arabidopsis thaliana* along with the mRNA for sulphate permease (Takahashi *et al.*, 1997). Thus it appears that the regulation of sulphate at least some of sulphur assimilation enzymes are at the level of mRNA expression. Sulphate uptake is regulated by two other ways also. Some evidences from bacteria suggest cysteine as a regulatory feedback signal controlling sulphate uptake, thereby proposing cysteine molecule itself or a closely related metabolite as its regulatory signal (Ostrowski and Kredich, 1989). But there are many other evidences from both algae as well as cyanobacteria that regard the sulphate pool to be an important factor to govern the sulphate uptake (Biedlingmaier and Schmidt, 1989). Higher plants also seem to follow the same regulatory mechanisms, since the sulphate pool of plants increase after feeding of reduced sulphur compound. Therefore the size of sulphate pool could regulate sulphate uptake in higher plants as well.

Sulphur is relatively inert; it must be activated for further metabolism. Activation is achieved in all cases studied by the enzyme ATP-sulphurylase and APS kinase. Green algae and higher plants possess multiple ATP-sulphurylase (Li *et al.*, 1991). ATP-sulphurylase catalyses the first step of sulphate assimilation in all cells. It involves the reaction of sulphate (SO4^2-) with ATP forming adenosine 5'-phosphosulphate (APS). The sulphur of APS is reduced and sulphide resulting from reduction of APS does not accumulate, as it is rapidly converted into organic sulphur compounds especially cysteine and methionine.

Heavy metal exposure induces a significant alteration in sulphur metabolism of some higher plants that accumulate heavy metal binding peptides termed as PCs (Tukendorf and Rauser, 1990). PCs are related to glutathione, a cysteine containing tripeptide, formed enzymatically by PC synthase, from glutathione or related peptides, the γ-glutamyl cysteine moiety being the repeated unit (Chen *et al.*, 1997). During cadmium
induced PC formation the cellular glutathione levels may decline, at least transiently (Steffens, 1990). However, enzymes of glutathione synthesis i.e. γECS and GS may show an increase in activity, indicating a cellular response to transient glutathione depletion (Klepheck et al., 1995; Ruesegger and Brunold, 1992; Ruesegger et al., 1990).

As cysteine is the major limiting substrate for glutathione synthesis (Arisi et al., 1997; Noctor et al., 1996), it is therefore expected that an increased glutathione synthesis also require an increased assimilatory flux of sulphur. Cysteine is synthesized by the condensation of O-acetyl-L serine and sulphide in the pathway that proceeds through three enzymatic steps involving ATP-sulphurylase, APS reductase and ferridoxin dependent sulphite reductase (Schmidt and Jäger, 1992). Several studies have analyzed single aspects of sulphur assimilatory flux and glutathione synthesis in response to heavy metal stress, but a comprehensive study comparing changes of gene expression for the sulphur assimilation pathway and glutathione synthesis in roots and shoots from the same species has not yet been performed (Heiss et al., 1999). Nussbaum et al. (1988) showed that AS and AR activities are induced in cadmium exposed maize seedlings. Similarly Ruesegger et al. (1990) reported that AR activity is induced co-coordinately with glutathione synthetase in cadmium treated pea plants. Lee and Leustek (1999) treated 20 days Brassica juncea L.cv.426308 seedlings with various concentration of CdSO₄, ranging from 1-200 μM in Hoagland's solution for 9 hrs and reported that both AS and AR mRNA increase after cadmium treatment, but that the rate of enzyme accumulation lags far behind the mRNA or does not correlate with the level of mRNA. They suggested disparity between mRNA and enzyme and that the cadmium may impede the accumulation of enzymes necessary for its detoxification.
2.10.5 Ascorbate (As)

Ascorbate is a hydrophilic antioxidant found in all plants, where it can accumulate to mM concentrations in both photosynthetic as well as in non photosynthetic tissues (Foyer et al., 1983). It is often claimed to be an important antioxidant in vivo. Its ability to show antioxidant properties is related to the fact that the dehydrogenase radical is much less reactive than are many of the radicals (Rose and Bode, 1993). Enzymatic systems exist in vivo to reduce this radical to ascorbate using NADH or GSH (Fig. 5) as source of reducing power. Leaves often contain more ascorbate than chlorophyll, with the ascorbate pool representing over 10% of the soluble carbohydrate. Of the many functions ascribed to As, relatively few are well characterized. It is clear however, that As is a major primary antioxidant (Nijs and Kelley, 1991), reacting directly with hydroxyl radicals, superoxide and singlet oxygen (Buettner and Jurkiewicz, 1996). In addition to its importance in photoprotection and regulation of photosynthesis (Forti and Elli, 1995; Foyer and Harbinson, 1994). Ascorbate plays an important role in preserving the activities of enzymes that contain prosthetic transition metal ion (Padh, 1990). As is also a powerful secondary antioxidant, reducing the oxidized form of α-tocopherol an important antioxidant in aqueous phase (Padh, 1990).

Recent years have witnessed a large number of reports correlating increase in one or more of antioxidant systems while combating with stress conditions. Several workers have observed the fate of As under various environmental conditions, differential responses have frequently been observed. (Schutzendubel et al., 2001; Prasad et al., 1999; Kubo et al., 1995; Malan et al., 1990). Prasad et al. (1999) observed that when Brassica juncea L. seedlings were exposed to toxic concentration of heavy metals (Zn), a significant increase in the accumulation of As occurs. Schutzendubel et al. (2001) observed that total ascorbate (As+DAs) increased significantly after 12 hrs exposure of root tip to Cd at 5 and 50
µM compared with controls. However a depletion of total ascorbate was observed in root tips of plant treated with the higher Cd concentration over control.

2.10.6. Ascorbate-glutathione cycle

In plant cells, the most important reducing substrate for H₂O₂ is ascorbate (Mehlhorn et al., 1996; Smirnoff, 1993; Alscher, 1989).

![Ascorbate-glutathione cycle](image)

Fig. 5: The ascorbate-glutathione cycle. Adapted from Foyer, Lelandais and Kunert, 1994.

H₂O₂ can be detoxified by the action of APX, DHAR and GR through ascorbate-glutathione cycle. Although it is well established that ascorbate-glutathione cycle is localized in chloroplast, but it is now becoming clear that the enzymes of this cycle are also found in mitochondria and peroxisomes and may represent an important antioxidant protection system against H₂O₂ generated in these organelles (Jimenez et al., 1997).
enzymes of As-GSH cycle have also been reported in the apoplast, which may play an important role in the plant-pathogen interaction. The As-GSH cycle may also play an important role in detoxifying AOS in roots during re-aeration following hypoxia or anoxia. In legumes, homoglutathione may substitute for glutathione in the ascorbate-glutathione (homo) H₂O₂-scapenging system of root nodule.

2.10.7 Ascorbate peroxidase (APX)

APX, scavenging peroxidase, is a heme protein, its prosthetic group is protoporphyrin IX. Its molecular size is similar to the classic plant peroxidase GuPX and primary function is rapid removal of H₂O₂ at the site of generation (Asada, 1992). APX isoenzymes are distributed in at least four distinct cell compartments, the stromal (sAPX), thylakoid membrane (tAPX), the mitochondria (mAPX), and the cytosol (cAPX) (Ishikawa et al., 1998; Asada, 1992; Miyake and Asada, 1992). cDNA encoding the different isoforms of APX have been isolated and characterized (Ishikawa et al., 1998). The two chloroplastic APX (Chl APX) isoenzymes have been found to be encoded by only one gene (ApxII) and their mRNAs are regulated by the alternative splicing of its two 3'terminal exons (Yoshimura et al., 1999). Different isoform of APX behave differently under different type of stress. The enzyme has two cytosolic forms with a purely defensive role and a membrane bound (27 kDa) form, which has a functional role in addition to hydrogen peroxide scavenging. Stromal form is slightly larger. The cytosolic form is present in leaf and in non-photosynthetic tissue.

Ascorbate can be regenerated from MDHA by the reaction catalyzed by MDHAR. MDHAR are flavin nucleotide containing enzymes found in chloroplasts (55 kDa) and in the cytosol (47 kDa), as well as in mitochondria and peroxisomes. They catalyze the reduction of MDHA to As by NAD (P) H:

\[
2\text{MDHA} + \text{NAD} \rightarrow 2\text{As} + \text{NAD} \text{H}^+
\]
MDHA radical can also be reduced to As by photoreduced ferredoxin in the chloroplast PSI. Alternatively it can spontaneously disproportionate to As and DHA, which can subsequently be reduced by another enzyme DHA reductase which regenerates As. DHA reductase, present in chloroplast stroma, reduces DHA to As by the ubiquitous cellular peptide, glutathione (GSH).

\[
\text{DHA + } 2\text{GSH} \rightarrow \text{As} + \text{GSSG}
\]

2.10.8 Glutathione reductase (GR)

Usually APX operates in cycle with glutathione reductase. GR uses reducing equivalents derived from glucose through the pentose phosphate pathway and NADPH to generate the reduced form of glutathione (GSH) from the oxidized disulphide form (GSSG) that results by the action of APX.

\[
\text{GSSG + NADPH + H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

GR are mainly found in chloroplast, cytoplasm and mitochondria of the plants. They range in size from around 90 to 140 KDa and usually contain two protein subunits, each with a flavin dinucleotide (FAD) at its active site. It appears that NADPH reduces the flavin nucleotide, which then transfers its electrons onto a disulphide bridge (-S-S-) in the enzyme. The two-sulphhydryl groups (-SH) that result then interact with GSSG and reduce it to GSH. The activity of GR suggests that the GSH/GSSG ratio in normal cell is kept high. The utilization of NADPH acts as an energy sink, which may impact indirectly on the efficiency of the electron transport system. It also causes the production of a trans-thylakoidal proton gradient that is involved in control of electron transport (Foyer et al., 1994).

2.10.9 Fate of the enzymes of ascorbate-glutathione cycle during cadmium stress

Several workers reported alteration in the activities of enzymes involved in the glutathione ascorbate cycle following exposure to heavy metal stress.
Some of them reported an increase in their activities whereas others reported a decrease (Schut zendusel et al., 2000; Stroinski et al., 1999; Schickler and Caspi, 1999; Stevens et al., 1997; Chaoui et al., 1997).

Erdei et al. (2002) treated seven day-old barley seedlings (Hordeum vulgare L.cv. Triangle) with different concentrations of CdCl₂ (0.1, 0.3 and 1mM) and observed that at all Cd concentrations the APX activity was dramatically reduced after the third day of treatment. In leaves the APX activity exhibited similar changes as those found in roots but no inhibition could be detected at the highest (1mM) Cd concentration. It was suggested that Cd induce drastic changes in plant metabolism that are reflected in the alteration of antioxidant enzymes. Schutzendubel et al. (2001) reported that in case of scot pines, Cd at a concentration of 50µM caused a significant inhibition after 6hrs and 12hrs of treatment. Recovery occurred within 24hrs and after 96hrs the activity was approximately 1.5 fold higher over the control. Groppa et al. (2001) reported that Cd induces alteration in enzymatic antioxidant system (APX and GR activity) in Helianthus annuus treated with CdCl₂ at a concentration 0.5mM.

Vitoria et al. (2001) treated radish seedlings with varied concentrations of Cd (0.25, 0.5 and 1.0 mM) and observed that GR activity increased considerably in the roots and leaves. However the increase was more in roots than in leaves. They suggested that the glutathione-ascorbate cycle is operating at high rate in order to detoxify the ROS formed in the root and that it is essential to keep the glutathione in reduced form prior to its incorporation into PCs (Cobbet, 2000).

Chaoui et al. (1997) treated ten day old bean (Phaseolus vulgaris L.cv. Morgan) plants with with 5µM Cd for 96 hrs and reported that the exposure to Cd did not modify the activity of ascorbate- specific peroxidase either in roots or in stems, where as in leaves there was increase in APX as well as in
GR activity, suggesting that antioxidant enzymes can be activated notably in upper parts, in response to oxidative stress induced by Cd.

Gallego et al. (1996b) reported that in *Helianthus annuus* leaves, treated with Cd, a decrease in the activity of APX, GR and DHAR is observed. These changes in the enzyme activities clearly reflect the role of Cd in inducing oxidative stress. Hence, the development or degeneration of antioxidative system of the cell depends upon the dose, duration as well as on the plant species studied.

### 2.10.10 Overexpression of antioxidant enzymes and plant stress tolerance

From the functions of several enzymes responding against abiotic stress in plants, it is highly emphasized that they provide them stress tolerance. Several experiments have shown the overexpression of these enzymes in stress conditions. Overexpression of HFA-cycle enzymes is under extensive research. Till date experiments showed positive results due to which a revolution has been made in the field of genetic engineering to develop stress tolerant transgenic plants.

Overproduction of chloroplastic SOD has been found to result in enhanced oxidative stress tolerance in transgenic tobacco (Foyer et al., 1994; Van Camp et al., 1994; Bowler et al., 1991), alfalfa (McKersie et al., 1993), and potatoes (Perl et al., 1993). In addition an enhanced tolerance to freezing stress in transgenic alfalfa overproducing SOD in the chloroplast has been reported (McKersie et al., 1993). The transgenic tobacco overproducing SOD in the chloroplast exhibit enhanced tolerance to chilling in the dark (Foyer et al., 1994). The overexpression of plant mitochondrial MnSOD in chloroplast of transgenic tabacco reduced cellular damage generated with methyl viologen (Bowler et al., 1991; Slooten et al., 1995) or ozone (Van Camp et al., 1994). The transgenic plants overproducing either FeSOD or MnSOD in the chloroplasts indicate FeSOD provides better
protection against methyl viologen (herbicide) induced oxidative stress than MnSOD. This may be due to higher membrane affinity of FeSOD allowing this enzyme to scavenge super oxide radicals at the site of their formation i.e. at PSI. This difference between MnSOD and FeSOD is probably connected to the original sub cellular localization of these enzymes. Further, more functional differences between FeSOD and MnSOD were also observed. MnSOD is more effective than FeSOD in protecting DNA.

2.11 Mechanism of metal tolerance

All organisms must possess mechanisms that regulate metal ion accumulation and thus, avoid their toxicity (Ybarra and Webb, 1999). The mechanisms that provide tolerance in plants are largely unknown (Kärenlampi et al., 2000). Since plants generally have no choice where they germinate and grow, they must develop some specific physiology that collectively enables them to adapt to unfavorable environmental conditions to survive (Cobbett, 2000; Brune et al., 1994b). However for Zn and Cd, there is circumstantial evidence of increased vacuolar transport (Ortiz et al., 1995; Verkleij et al., 1991, 1990). The other strategies adapted by plants against Cd stress involve:

2.11.1 Binding of metal to cell wall

In nature, the root systems of plants act usually as the first barrier to heavy metals in the soil. In spite of differential mobility of metals in plants, the root system accumulates them to a significantly higher extent (Vassilev et al., 1998). At the first barrier, Cd can be immobilized by means of cell wall (Nishizono et al., 1989; Ernst 1998) and extra cellular carbohydrates like mucilage and callose (Wagner 1993; Verkleij and Schat, 1990). In some cases, Cd ions seem to be mostly bound by pectic sites and histidyl groups of cell wall (Leita et al., 1996). The tolerances imparted by these
mechanisms are primarily dependent on the concentration of Cd supplied (Sanita di Toppi and Gabbrielli, 1999).

2.11.2 Reduced transport across cell membrane or exclusion

Little is known about transport mechanisms for most metals. It appears that transporters mediate transport of cations across cell membranes. Recently Cu, Zn and Fe transporters were cloned from Arabidopsis thaliana (Salt et al., 1998). Preventing Cd ions from entering the cytosol through the action of plasma membrane could theoretically represent the best defense mechanism (Sanita di Toppi and Gabbrielli, 1999). Cd seems to enter the cells through Ca-channels in the plasma membrane (Rivet et al., 1997).

2.11.3 Immobilisation of metals through phytochelatin

All living cells are confronted with the dilemma that on one side they need a certain amount of free heavy metal ions (such as Zn$^{2+}$, Cu$^{2+}$, etc.) for their normal metabolic function and on the other side, they have to protect themselves from an intracellular excess of heavy metal ions that would lead to cell death. This dilemma can be overcome only by a stringent regulation of free metal ion concentration within the cell. Plant cells have developed one general mechanism to achieve this goal i.e. the synthesis of phytochelatins. The process involves the chelation of metal ions by specific-high affinity ligands that reduces the solution concentration of free metal ions, and consequently thereby reduces their phyto-toxicity. A number of metal binding ligands have now been recognized in plants (Cobbett, 2000). Extracellular chelation by organic acids, such as citrate and malate is an important mechanism of heavy metal (aluminum) tolerance.

2.11.3.1. Phytochelatins

Cadmium has a high affinity towards the activation of peptide ligands. Once cadmium has entered the cytosol a system strictly related to sulfur
metabolism is promptly activated, finally resulting in the production of important complexing agents termed as 'phytochelatins' by (Kubota et al., 2000; Grill, 1989; Grill et al., 1989). Phytochelatins were isolated and purified for the first time by Grill et al. (1985). They form a family of structures with increasing repetition of the (γ-Glu- Cys)_n-Gly, where n has been reported to be as high as 11, but is generally in the range of 2-5. Phytochelatins form various complexes with Cd (with molecular masses of about 2500 or 3600), due to the presence of thiolic groups of Cys, which chelate Cd, and as a result prevent it from circulating as free Cd\(^{2+}\) inside the cytosol (Grill et al., 1985). The production of phytochelatins is a widespread mechanism of cadmium detoxification in higher plants (Hall 2002; Vatamaniuk et al., 2001; Escarre et al., 2000; Mejare and Bulow, 2001; Verkleij et al 1990; Gregkler et al., 1989). The use of phytochelatins as biomarkers for Cd toxicity was recently proposed by Keltiens and Van Beusichem, (1998).

Phytochelatins are synthesized from glutathione by means of enzyme phytochelatin synthetase, a specific γ-glutamyl cysteine dipeptidyl transpeptidase (Grill et al., 1989). This enzyme is self regulated, since its reaction products (phytochelatins) chelate cadmium and the reaction ends unless further cadmium is supplied (Loefler et al., 1989). Numerous Physiological, biochemical and genetic studies have confirmed that glutathione or in other cases related compounds is the substrate for PC biosynthesis (Rauser, 1995; 1999, Zenk, 1996) as well as for other complexes collectively known as "iso-phytochelatins" and homophytochelatins". Iso -phytochelatins contains Glu instead of Gly as reported in Zea mays. (Meuwly et al., 1993). Homo-phytochelatins are characterized by the presence of β-Ala instead of Gly as a terminal amino acid as reported in few members of the Fabales (Grill et al., 1986). A strong correlation was found between phytochelatins content and cadmium
tolerance in tomato cells and *A. thaliana* (Howden *et al.*, 1995). PC biosynthetic pathway (Fig. 6) is under the regulation of a number of factors.

![Diagram showing the synthesis of PCs and sequestration of cadmium in the plant cell vacuole.](image)

**Fig. 6:** Synthesis of PCs and sequestration of cadmium in the plant cell vacuole. CAD1 and CAD2 are in *Arabidopsis*; hmt1 and hmt2, ade2, ade6, ade7 and ade8 are in fission yeast; hem2 in *Candida*

The first of these is likely to be regulation of GSH biosynthesis. When the cell detects cadmium, Glutamine and Cysteine molecules are changed by the enzyme γ-glutamyl cysteine synthase, coded for by the CAD 2 gene, with an end product of γ-glutamyl cysteine. The γ-glutamyl cysteine is then transformed into glutathione (GSH) by the enzyme glutathione synthase. Once at the stage of glutathione, two steps can occur the first is the binding of two GSH molecules to a cadmium molecule and the passage of this compound into a storage vacuole, the other option is that the GSH is acted upon by the enzyme phytochelatins synthase, coded for by the gene CAD1,
resulting in functional phytochelatins. These proteins then bind with cadmium ions and create low molecular weight PC-Cd complexes, which pass into a storage vacuole, and react with sulphides to make high molecular weight CdS-PC complexes. Transgenic Indian mustard plants, in which the increase in the expression of the enzymes of the GSH biosynthetic pathway was studied by overexpression of the concerned gene, has shown that PC biosynthesis and Cd tolerance can also be increased (Yong et al., 1999; Zhu et al., 1999a; Chen and Goldsbrough, 1994). Wild-type Indian mustard plants respond to exposure to Cd with increased levels of GCS transcript (Schäfer et al., 1998). Cadmium tolerance and accumulation has been found to increase by overexpression of γ-glutamylcysteine synthetase (γECS) in Indian mustard (Zhu et al., 1999a).

The cytosolic O-acetyl serine (thiol) lyase gene that is supposed to function in cadmium tolerance is regulated by heavy metals. Similarly, exposure of Arabidopsis plants to Cd and Cu causes an increase in transcript levels of the two genes in the GSH biosynthetic pathway and of GSH reductase (Xiang and Oliver, 1998). The signal molecule, jasmonate, mediated a similar effect in the absence of heavy metal exposure although it has not been demonstrated that the effect of heavy metal stress on gene expression is mediated via jasmonate. There is also circumstantial evidence supporting post-transcriptional regulation of GCS expression in addition to the well-recognized regulation of GCS activity through GSH feedback inhibition (May et al., 1998; Noctor and Foyer, 1998). Regulation of PC synthase activity is expected to be the primary point at which PC synthesis is regulated. This has been demonstrated by in vitro studies of PC synthase expressed in E. coli or in Brewer's yeast where the enzyme was activated to varying extents by Cd, Cu, Ag, Hg, Zn and Pb ions (Clemens et al., 1999; Ha et al., 1999, Vatamaniuk et al., 1999). One model for the function of PC synthase enzymes is that the conserved N-terminal domains possess the
catalytic activity. Activation probably arises from metal ions interacting with residues in this domain, possibly Cys or His residues. Five Cys (two of which are adjacent) and a single His residue are conserved in this domain. This model is supported by the molecular, cad1-5, has a nonsense mutation that would result in premature termination of translation downstream of the conserved domain (Ha et al., 1999). The truncated polypeptide is predicted to lack nine of the 10 Cys residues in the C-terminal domain. That this mutant enzyme is the least affected (as measured by in vivo PC levels and sensitivity to Cd) and mutant activity is expressed only in the presence of Cd (Howden et al., 1995a) confirms that the C-terminal domain is not absolutely required for either catalysis or activation.

Previous studies indicated PC synthase is expressed constitutively and levels of enzyme are generally unaffected by exposure of cell cultures or intact plants to cadmium. This suggests the induction of PC synthase gene expression is unlikely to play a significant role in regulating PC biosynthesis. This is supported by northern or reverse transcriptase-PCR analysis of the expression of AtPCS1/CAD1 which showed that levels of mRNA were not influenced by exposure of plants to Cd, even under conditions of severe stress, thus suggesting an absence of regulation at the level of transcription (Ha et al., 1999; Vatamaniuk et al., 1999). Interestingly, however, reverse transcriptase-PCR analysis of TaPCS1 expression in roots indicated increased levels of mRNA on exposure to cadmium (Clemens et al., 1999). This suggests that, in some species, PC synthase activity may be regulated at both the transcriptional and post-transcriptional levels. The importance of sulfide in the function of PCs has been obtained from the analysis of Cd-sensitive mutants of fission yeast that are deficient in PC-Cd complexes. In one case the gene mutated in the Cd-sensitive derivative, when cloned, was identified as a gene involved in adenine biosynthesis. Subsequent genetic analysis demonstrated that
different single or double mutant deficient in steps in the adenine biosynthetic pathway lack high molecular weight (HMW) complexes (Speiser et al., 1992).

Biochemical characterization of the enzymes encoded by these genes indicated that this pathway, in addition to catalyzing the conversion of Asp to intermediates in adenine biosynthesis could also utilize a sulfur-containing compound Cys sulfinate. These are believed to be intermediates or carriers in the pathway of sulfide incorporation into HMW complexes. More recently, Cd-sensitive mutants isolated in fission yeast and Candida glabrata have identified additional functions that are probably also important in sulfide metabolism. In fission yeast the hmt2 mutant hyperaccumulates sulfide in both the presence and absence of cadmium (Vande-Weghe and Ow, 1999). The HMT2 gene encodes a mitochondrial sulfide/quinone oxidoreductase, which was suggested to function in the detoxification of endogenous sulfide. The role of HMT2 in Cd tolerance is uncertain, but one possibility is to detoxify excess sulfide generated during the formation of HMW PC-Cd complexes after cadmium exposure. In C. glabrata the hem2 mutant is deficient in porphobilinogen synthase involved in siroheme biosynthesis (Hunter and Mehra, 1998). Siroheme is a cofactor for sulfite reductase required for sulfide biosynthesis. This deficiency may contribute to the Cd-sensitive phenotype. These studies suggest that sulfur is important in alleviating toxic effects of Cd and that sulfur metabolism and Cd detoxification could be closely related. However, additional studies are required to establish the precise influence of this pathway on PC function.

The clearest evidence for the role of PCs in heavy metal detoxification comes from characterization of the PC synthase-deficient mutants of Arabidopsis and fission yeast. A comparison of the relative sensitivity of the Arabidopsis and fission yeast mutants to different heavy metals
revealed a similar, but not identical pattern (Ha et al., 1999). Phytochelatins appeared to play an important role in Cd and arsenate detoxification and no apparent role in the detoxification of Zn, Ni, and selenite ions. Minor differences between the two organisms were observed with respect to Cu, Hg and Ag.

Regarding the molecular-genetic basis of heavy metal tolerance, it has been pointed out that some minor genes (modifiers), not conferring tolerance on their own, can modify the major gene(s), perfecting and enhancing its (their) effects on tolerance (Macnair, 1990). Further efforts are urgently required in order to confirm whether the genetic control of heavy metal tolerance is polygenic, oligogenic or monogenic.

From the proceeding discussion it is apparent that the mechanism of metal detoxification is more complex than simply the chelation of the metal ion by PCs. The metal ion must activate PC synthase, be chelated by the PCs synthesized, and then presumably transported with, for example, sulfide or organic acids to the vacuole and possibly form a more complex aggregation in the vacuole.

2.11.3.2 Metallothioneins (MTs)

Metallothioneins were first discovered in Equine kidney and have since been broadly distributed amongst animals, eukaryotic micro-organisms, certain prokaryotes and plants (Kawashima et al., 1992, 1991; Lane et al., 1987). These are small gene-coded Cys rich polypeptides, generally lacking aromatic amino acids (Kagi, 1991), and have a high metal content in coordination of metal thiolate clusters. Their molecular weight varies from 8-14 kDa (Robinson et al., 1993) and is thought to be aggregates of phytochelatins. MTs behave similarly as PCs and often-metal complexation duties are shared between MTs and PCs as seen in datura and zea mays (Rivai et al., 1990). In fact, PCs were originally classified as class III MTs,
until they were deemed sufficiently different in structure and synthesis pathway to be classified as PCs. The classification of MTs places all animal MTs into class I, and all other MTs (including plant) into class II. Class II MTs, those identified in plants, are further classified into two types. Both types of MTs are characterized by having patterns of twelve cystein residues, but the amino acid composition and the arrangement of cystein residues varied in the proteins.

Type I MTs have 12 cystein residues, and they are arranged in such a way in the protein that there is 6cys-Xaa-6cys, with Xaa consisting of approximately 40 amino acids. Type II MTs are configured in either a 6cys-6cys or 6cys-Xaa-Xaa-6cys formation (Murphy et al., 1997). It is thought that these differences in structure and number of central amino acids dictates, which metal the MT, give tolerance to, or detoxifies. Each plant has multiple MT gene families, each producing proteins that give resistance to different metal MTs have highest affinity for Cu and are induced by exposure to it (Murphy et al., 1997; Rauser, 1999). Various MT genes, Mouse MT I, Human MTIA, MT II, Chinese hamster MTII, yeast cupl, pea PsMTA has been transferred to Nicotiana spp, Brassica spp, and A.thaliana (Hasegawa et al., 1997; Hattori et al., 1994; Maiti et al., 1989; Misra and Gedamu, 1989; Lefebvre et al., 1987). As a result varying degrees of constitutively enhanced tolerance was achieved, being maximally 20-fold compared with the control. Recently MT gene was also isolated from metal tolerant Silene vulgaris.

These studies suggest that MT gene may be useful in improving metal tolerance of plants (Kärenlampi et al., 2000). The actual function of the MTs is thought to be one of several hypothesized but as of yet unproven theories. One-theory states that MTs create ion storage pools for free excess heavy metal ions, which are chelated until the plant can use them, if the metals are essential. A second school of thought is that MTs are transport
proteins that are responsible for moving excess heavy metal from sites where they have built up to toxic levels to areas of the plant where they are needed, or at least where the ion levels are not toxic.

2.11.4 Vacuolar compartmentalization

A very significant role in detoxification and tolerance is played by vacuolar compartmentalization. It prevents the free circulation of Cd ions in the cytosol and forces them into a limited area (Sanita di Toppi and Gabbrielli, 1999). Cadmium, free and complexed, is sequestered in vacuole of root cells in most species. It is actively transported from the cytosol into the vacuole across the tonoplast via an H⁺/Cd²⁺ antiport or an ATP-dependent phytochelatin transporter (Gries and Wagner, 1998; Salt and Wagner, 1993). Cd-PC complexes as well as apo-phytochelatins are transported against the concentration gradient across the tonoplast by means of specific carriers. They accumulate inside tonoplast vesicles up to 38 times more than in the external solution (Salt and Rauser, 1995). In the vacuole because of acidic pH, these complexes dissociate and Cd can be complexed by vacuolar organic acids like citrate, oxalate and malate (Krutz et al., 1989) and possibly by amino acids. Apo-phytochelatins may be degraded by vacuolar hydrolases and or return to the cytosol, where they can continue to carry out their shuttle role. A gene, which codes for a PC-transporter in yeast, was recently isolated. When overexpressed in a plant, this gene (hmt 1) might allow for increased production of PC-transporters, which would boost the ability of a plant to sequester PC-Cd complexes in the vacuole (Oritz et al., 1995).

2.11.5 Stress proteins

Plants being subjected to Cadmium stress showed the induction of several classes of heat-shock proteins [(hsp) hsp100, hsp90, hsp70, hsp60] or stress proteins or hsp cognates (Vierling, 1991). It has been demonstrated
that the DNA of Cd stressed cells produces specific mRNA transcripts, which regulate the synthesis of stress proteins (Sanita di Toppi and Gabberila, 1999; Edelman et al., 1988; Czarnecka et al., 1984; Schoffl and Key, 1982). In several species, Cd exposure induced the synthesis of stress proteins with a molecular mass ranging from 10,000-70,000Da (Fenik et al., 1997; Prasad 1997; Urwin et al., 1996; Marchetti and Leita, 1995; Leita et al., 1991; Delhaize et al., 1989b). Interestingly Rivetta et al. (1997) demonstrated that Cd binds to calmodulin and competes with Ca in this binding. In deed, various Ca calmodulin dependent transduction signals are associated with variations in protein phosphorylation (Behra, 1993; Sharman and Golderg, 1993; Friedman and Poovaiah, 1991).

2.11.6 Stress ethylene and Stress triterpenes

Cd has been shown to stimulate ethylene biosynthesis through the MSEA (methione-S-adenosyl methionine, 1-aminocyclopropane-1-carboxylic acid, ethylene) pathway (Adams and Yang, 1979). Production of stress ethylene increased the activity of guaiacol peroxidases and accumulation of soluble and insoluble phenolics (Fuhrer, 1982b). It is hypothesized that this increase in stress ethylene production could be contributed to Cd sequestration, which diminishes the Cd stress. Recently triterpenes compounds have also been found to reduce the cadmium toxicity in HepG2 cells. Ten triterpene compounds were examined, among which the betulin was found to almost completely abolish the cytotoxicity of CdCl2 at lower concentration of 1\( \mu \)g/ml. When HepG2 cells were incubated with betulin and then again incubated in fresh betulin-free medium before the addition of CdCl2, the toxic effects of Cd were reduced. It has been suggested that betulin promoted the expression of several genes, synthesizing proteins that protect cells against the toxic effects of Cd (Miura et al., 1999).
2.12 Green clean technology for remediation of Cd in the environment

Since cadmium is easily incorporated into the human food chain, much attention has been paid on how to either clean up Cd in soil or to reduce the risk of its being incorporated into food chain. Earlier there used to be two methods that were followed for clean up strategy of the environment. One of it was to remediate areas contaminated with heavy metals through excavation and relocation of the contaminated sites to a hazardous waste landfill. Second was to form a concrete layer to prevent leaching of contaminant into ground water. Although these two traditional methods are still followed, but they are expensive. Nowadays a recent approach has been developed through scientific study to remediate contaminated sites popularly known as "Phytoremediation." Many other terms like Bioremediation, Green remediation, Greenclean, are also in use. The term Phytoremediation means the use of plants to cleanup contaminants from a substrate whether it is soil, air or water. More frequently it involves the use of plants to take up metals from soil, harvesting and removal of the plant material to dispose off sites, usually by burning to concentrate the metals (Chaney et al., 1997). The efficacy of phytoremediation as a viable remediation technology is still being explored, and so far the results are positive. Because the cost of growing a crop are minimal compared to those of soil removal and replacement, the use of plants to remediate hazardous soils is seen as having great promises. The idea of using rare plants that hyperaccumulate metals to selectively remove and recycle excessive soil metals was introduced in 1983 (Chaney, 1983), gained public exposure in 1990 (Anonymous, 1990) and has increasingly been examined as a potential practical and more cost-effective technology than the soil replacement, solidification and washing strategies (Salt et al., 1996; Cunningham et al., 1995). To be recognized as a hyper accumulator of one of the following metals, the minimum tissue concentration are 0.01% by dry
weight for Cd, 0.1% for Ni, Co, Cu, Cr and Pb and 1-3% of Zn and/or Mn (Chaudhry et al., 1998). Phytoremediation is currently divided into following areas:

2.12.1 Phytodetoxification- the use of plants to change the chemical species of contaminant to a less toxic form.

2.12.2 Phytodegradation- the use of plants and associated microorganisms to degrade organic pollutants.

2.12.3 Rhizofiltration- the use of plant roots to remove contaminants from water and aqueous waste streams (Dushenkov et al., 1995).

2.12.4 Phytostabilization- the use of plants to immobilize contaminants chemically and physically at the polluted sites and hence reduce the bioavailability of pollutants in the environment.

2.12.5 Phytoextraction- the use of pollutant accumulating plants to remove metals or organics from soil by concentrating them in harvestable part (Salt et al., 1998; Vassil et al., 1998).

The plants that are to be used for phytoremediation should possess three characters. The plant must be able to tolerate high levels of element in root and shoot cell. Hypertolerance is the backbone or key factor that makes hyper accumulation possible. Secondly they have must have the ability to translocate an element from root to shoots at high rates, and lastly, there must be a rapid uptake rate for the element at levels that occur in soil solution (Chaney et al., 1997). For the remediation of metal contaminated soils, phytoextraction and phytostabilization are applicable. Phytoextraction currently involves two basic strategies Chelate assisted phytoextraction, which is also termed as induced phytoremediation, and long-term continuous phytoextraction.

2.12.5.1 Chelate-assisted phytoextraction

Chelate-assisted phytoextraction involves the release of metals into soil solution combined with transport of metals to the harvestable part (Shoot).
Studies have shown that adding an artificial chelate enhances metal accumulation by plants (Vassil et al., 1998; Blaylock et al., 1997; Huang and Cunningham 1996; Jorgenson, 1993). Chelate assisted transport of metal to shoots appears to occur in the xylem (Huang; et al., 1997) via the transpiration stream (Bockers et al., 1994). The metal appears to move to shoots as a metal-chelate complex where water evaporates and metal-chelate complex remains. In this way, after chelate assisted induction the plant becomes a wick, which drives chelated metal from the soil solution into the leaves. The operation of the wick relies on a high surface area collection system provided by the roots and the efficient capillary plumbing system inside the plant.

2.12.5.2 Continuous phytoextraction

Continuous phytoextraction is purely a genetic character of plants. It involves the translocation and accumulation of metal in aerial parts through specialized physiological processes over the complete growth cycle. This idea was first introduced by Chaney (1983) and Baker et al. (1988). The ideal plant for continuous phytoextraction should grow on metal polluted soils to high biomass, accumulate and resist high concentrations of metals in shoots. Such plants called hyper accumulators grow happily on contaminated sites that contain metal levels far exceeding those present in the surrounding medium, or the non-hyperaccumulating species growing near by (Chaudhry et al., 1998).