Chapter 4
Antioxidant Defense System in Plants Exposed to Metal Toxicity

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Abstract Heavy metals are abiotic pollutants and plants are susceptible to heavy metal toxicity. There is a two way relationships between the abundance of heavy metals and manifestation of their toxicity. Thus on the one hand, heavy metals compete with essential mineral nutrients for uptake thereby disturbing the mineral nutrition of plants and on the other hand, after uptake by the plant, they accumulate in plant tissue and cell compartments and hampers the general growth and metabolism of the plant. Many heavy metals like Fe, Cu, Cd, Cr, Zn, Ni etc. have been shown to cause oxidative damage in various higher plants by production of free toxic oxygen radicals. In order to cope with highly toxic metals or to maintain the level of essential metals within the physiological range, plants have developed a variety of complex mechanisms for metals tolerance. While major step is to accumulate and compartmentalize the metals in plant tissue, antioxidative sytem comprising of non-enzymatic and enzymatic components have also been observed. These include antioxidants like ascorbate (ASC), glutathione (GSH), carotenoids, flavonoids and enzymes of the water-water and ASC-GSH cycle via the enzyme superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) for breakdown of hydrogen peroxide and in the regeneration of ascorbate (ASA) from monodehydro-ascorbate reductase (MDHAR) and dehydro-ascorbate reductase (DHAR) and GSH is oxidized to glutathione disulphide (GSSG) by glutathione reductase (GR). This chapter throws light on the studies carried out on oxidative stress which indicates that antioxidative defense mechanism has an important role to play in overcoming oxidative damage in heavy metal stressed stress plants.

Keywords Heavy metals · ROS · Oxidative stress · Antioxidants · Antioxidative enzymes

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4.1 Introduction

All metals having a density higher than 5 g cm^3 are known as heavy metals. Depending on their availability under physiological conditions 17 heavy metals may be available and have significance, for the plant and animal communities within various ecosystems. Of these most heavy metals are toxic to living forms but on the contrary certain metals like iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) etc. are not only required by plants in very small trace quantity but also play essential roles which are specific to the metals. A decrease in the economic yield of the crops is observed in agricultural fields which have high amount of the heavy (Nellessen and Fletcher 1993; Akinola and Ekiyoyo 2006). The availability of heavy metals in soils is dependent on natural procedure, especially lithogenic and pedogenic soils (Angelone and Bini 1992. Besides natural, manmade or anthropogenic activities like mining, plating, excessive use of fertilizers, dumping of household waste and sewage sludge result in anomalies in the soil (Alloway 1995) which present a major threat to sustainable agriculture.

Plants differ in their response to various types and concentration of heavy metals (Al-Hellal 1995). Across a range of plant species and experimental conditions, the phytotoxicity of the heavy metals followed the trend (from most to least toxic): Pb > Hg >Cu >Cd > As >Co > Ni > Zn >Mn (Kopittke et al. 2010). The toxicity is dependent on a number of factors which contribute to the phytotoxicity of the metals. These maybe exposure time, age of plant, the status of other nutrients in the soil, interaction between metals, and infection by mycorrhiza (Påhlsson 1989). The toxic levels of heavy metals interfere with the growth and physiological function of plants by forming complexes with O, N and S ligands (Van Assche and Clijsters 1990). Heavy metals interfere with seed germination (Soudek et al. 2010), mineral uptake (Drazic et al. 2004), protein metabolism (Tamas et al. 1997), membrane functioning (Quariti et al. 1997; Gratão et al. 2005) and water relations (Kastori et al. 1992; Kevrasan et al. 2001). Many heavy metals cause oxidative damage in various higher plants by accumulation of reactive oxygen species (Weckx and Clijsters 1997; Panda and Patra 2000).

Production of reactive oxygen species (ROS) is an unavoidable consequence of normal aerobic metabolism in plants. The four major types of ROS are singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). Metals like Fe, Cu, Zn, etc. accentuate the production of free radicals in biological systems (Dietz et al. 1999). Reactive Oxygen Species damage biological molecules including DNA, RNA, protein and lipid by peroxidation (Shah et al. 2001). Due to excessive levels of ROS peroxidation of the lipids takes place and results in breakdown of tissues which affects growth and metabolism. Lipid peroxidation occurs when excessive ROS cannot be scavenged immediately and effectively (Yang et al. 2005). The concentration of malondialdehyde, (a byproduct of lipid peroxidation) is a direct indication of the extent of peroxidation in plants (Bailly et al. 2006). Therefore MDA content is a powerful tool for assessing the extent of oxidative damage of plants under stress.

Oxidative stress is produced when there is an imbalance in the pro-oxidant and antioxidant status of the cell. The level of ROS in plant tissues is controlled by an antioxidative defense system which include antioxidative enzymes and non-enzymatic low molecular weight antioxidants like ascorbate (ASA), carotenoid (Car), glutathione (GSH), tocopherols, flavanoids etc. Superoxide dismutase (SOD, EC 1.15.1.1) is a key antioxidative enzyme that catalyzes dismutation of superoxide anion ($O_2^{\bullet-}$) to H_2O_2 and O_2. Catalase (CAT, EC 1.11.1.6) scavenges H_2O_2 by converting it to H_2O and O_2. Peroxidase (POD, EC 1.11.1.7.) reduces H_2O_2 using several reductants, e.g. phenolic compounds. In order to overcome effects of heavy metal toxicity plants have developed different mechanisms for tolerance. Amongst these accumulation and detoxification appear to be the main strategies. Thus defense system that reduce oxidative stress may play an important role in heavy metal tolerance.

4.2 Reactive Oxygen Species

Molecular oxygen arising from photosynthetic processes is pivotal to almost all organisms. Although indispensable for life it may also become toxic for it in the form of the activated or partially reduced derivatives of oxygen, the reactive oxygen species (ROS), which are the highly reactive by-products of aerobic metabolism. They are called ROS because they readily partake reactions than molecular oxygen (O_2). The term ROS is generic, and embraces not only free radicals such as superoxide ($O_2^{\bullet-}$), and hydroxyl radicals (•OH) but also H_2O_2 and singlet oxygen ($^1O_2$). Superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) are formed during the course of metabolic processes chiefly during electron transport in the mitochondria and the photosystems in chloroplast (Elstner 1991; Asada 1994). Singlet oxygen ($^1O_2$) is another species of oxygen formed in the thylakoid membrane under excess light due to the transfer of excessive energy from excited triplet state of chlorophyll to the oxygen (Demmig-Adams et al. 1998). Although all ROS are harmful for living cells however $^1O_2$ and $OH^{\bullet}$ Are most detrimental (Fridovich 1997; Asada 1994; Apel and Hirt 2004).

Reduction of molecular O_2 proceeds through four steps, generating several O_2 radical species. The first product of O_2 reduction is the superoxide radical ($O_2^{\bullet-}$). Addition of one electron to the ground state O_2 molecule, results in the formation of $O_2^{\bullet-}$. With only one unpaired electron, $O_2^{\bullet-}$ is less of a radical than O_2, but is more reactive because it readily undergoes a one-electron redox reaction. In aqueous solution $O_2^{\bullet-}$ dismutates slowly to H_2O_2 and O_2 through a dismutation reaction:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction is in itself slow but is catalyzed by the enzyme superoxide dismutase (SOD).

Hydrogen peroxide is the two electron reduction product of O_2. It is a reactive oxygen species, however it is not a free radical (Cheeseman 2006). As compared with superoxide, $O_2^{-\bullet}$, and the hydroxyl radical (•OH), H_2O_2 is relatively less harmful It is stable and unreactive in the absence of transition metals, even at very high concentrations which is possible to be generated by the biological system. Functionally, this makes it mobile within the tissues serving as a substrate for biological reaction as well as a newly found role of a ROS signaling molecule. However, H_2O_2 is fairly reactive with molecules containing Fe^{2+} or other transition metals, through the Fenton reaction (Becana et al. 1998) which causes the homolysis of H_2O_2 to •OH the most reactive of the ROS:

$$H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + HO^{\bullet} + HO^{-}$$

The reaction is called the Haber-Weiss reaction. It is a Fe catalysed reaction (Cadenas 1989; Elstner 1991; Scandalios 1993) and proceeds via the following steps:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$
$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + HO + HO^{-}$$

The second step of the reaction is called the Fenton reaction. The greater the generation of the $O_2^{\bullet -}$, the higher will be the chance of •OH• formation, and in turn, the greater will be the chance of peroxidative damage of the membrane lipid. Chloroplasts contain as much as 80% of the Fe in a plant, and are a good source of ROS. Similar Fenton reaction mechanisms have been associated with H_2O_2 (or •OH) sensitivity of Fe SOD (Bhattacharya et al. 2004). Excessive H_2O_2 is reduced enzymatically by catalase or ascorbate peroxidase, or by complexing Fe(III) and Fe(II) with compounds such as tannic acid and proanthocyanidins, thus preventing •OH generation (Andrade et al. 2006).

The highly toxichydroxyl radical (•OH) and singlet oxygen $^1O_2$, are not formed as a result of direct addition of an electron to ground state oxygen molecule. While ·OH is formed as a result of reaction of $O_2^{\bullet -}$ and H_2O_2 and $^1O_2$ is generated by input of energy readily obtained from light quanta provided by chloroplastic photosensory molecules (Foote et al. 1985; Elstner 1991). $^1O_2$ ($^1\Delta gO_2$) is even more reactive than •OH, reacting with most biological molecules at near diffusion control rates (Knox and Dodge 1985; Cadenas 1989; Apel and Hirt 2004).

4.2.1 Generation of ROS in Plants

The main sources of ROS are photosystems I and II in chloroplast (Asada and Takahashi 1987), respiratory chain of mitochondria (Moller 2001), electron transport chains in microsomes, peroxisomes and nuclear envelope (Gille and Sigler 1995), superoxide generating enzymes (Xanthine oxidase, aldehyde oxidase, galactose oxidase, cellobiose oxidase, NADPH oxidase), hydrogen peroxide generating oxidases (Aldehyde oxidase, D-amino acid opxidase, xanthine oxidase), hemoproteins (Halliwell and Gutteridge 2007); photosensitized reaction (Flavins, cercosporin) (Duab and Hangarter 1983) and autooxidation of reduced flavins, thiols, catechol amines, reducing sugars (Sun and Leopold 1995).

During photosynthesis the major sites of formation of ROS are the direct photoreduction of O_2 to the $O_2^{\bullet-}$ by reduced electron transport components of PSI. Secondly the reactions associated with the photorespiratory cycle-like Rubisco in the chloroplast and glycolate-oxidase and CAT-peroxidase reactions in the peroxisome lead to the generation of H_2O_2 (Apel and Hirt 2004). In photosystem I, the main generation site of $O_2^{\bullet-}$ is at the level of ferrodoxin $NADP^+$ reductase; while in photosystem II P680, pheophytin and protein Q_A are the probable sites proposed for $O_2^{\bullet-}$ generation (Barber 1998). Under conditions that inhibit regeneration of $NADP^+$, e.g. inhibited transport of photosynthetic products out of the chloroplasts, low CO_2 availability, water stress etc. O_2 reduction at photosystem I may take place leading to $O_2^{\bullet-}$ formation (Furbank et al. 1983). Reduced ferredoxin may also react directly with molecular oxygen forming $O_2^{\bullet-}$ (Asada 1994).

In mitochondria, complex I (NADH-coenzyme reductase complex) is the main sites of leakage of electron from the electron transport chain to molecular oxygen (Halliwell and Guteridge 1984; Moller 2001; Blokhina et al. 2003). The reason for this is because the Complex I is at a more negative redox potential than that of O_2/$O_2^{\bullet-}$ (−0.33 V). The reduced form of Co-enzyme Q is another site for reduction. Like quinoid compounds, electrophiles when reduced to semiquinones can subsequently reduce O_2 via one–electron transfer within a process termed redox cycling (Cadenas 1989; Purvis 1997).

Apart from these, peroxisomes, that serve important functions like photorespiration, fatty acid β-oxidation, the glyoxlate cycle and the metabolism of ureides, have also been recognized as source of ROS (Corpas et al. 2001). Similarly, in microsomes, NADPH-cytochrome-P450–reductase (NCR) a flavoprotein and a component of the microsomal cytochrome P450 enzyme system, which is normally reduced by NADPH and transfers electrons to cytochrome P450 (Serioukova and Peterson 1995), may also transfer electrons directly to molecular oxygen thus generating ROS.

Plants also generate ROS by activating various oxidases and peroxidases that produce ROS in response to environmental cues (Baker and Orlandi 1995; Allan and Fluhr 1997; Bolwell et al. 2002). Enzymes like xanthine oxidase, aldehyde oxidase and flavin dehydrogenases dehydrogenases can also produce $O_2^{\bullet-}$ as a catalytic byproduct (Fridovich 1995).

4.2.2 ROS Detoxication

The generation of ROS is a continuous process which is an outcome of the different biochemical pathways in the cell system (Foyer and Harbinson 1994). Under normal condition the ROS are scavenged by efficient antioxidative defense components which are localized in specific compartments (Alscher et al. 1997). The term antioxidant is designated for a compound which can efficiently quench ROS without itself forming a destructive radical. Plants have evolved elaborate system for ROS scavenging which include enzymatic and non enzymatic antioxidants. Antioxidant enzymes are those that either catalyze such reactions or are involved in the direct detoxification of ROS. Enzymes showing antioxidative response include SOD, APX and CAT (Willekens et al. 1995). The redox reactions are catalyzed by these enzymes many of which rely on electrons supplied by reductants of low molecular weight antioxidants like ascorbate and glutathione. They fulfill many roles in defense mechanism. The major ROS scavenging pathways of plants include SOD, found in almost all cellular compartments, the water cycle in chloroplast, the ascorbate glutathione cycle in chloroplast, cytosol, mitochondria, apoplast and peroxisome, glutathione peroxidase and CAT in peroxisomes.

4.2.3 Non-enzymatic Antioxidants

Non-enzymatic antioxidants include the ascorbate (ASA) and glutathione (GSH), as well as tocopherol, flavonoids, alkaloids and carotenoids (Table 4.1). Creissen et al. (1994) reported that mutants with decreased ascorbic acid levels or altered GSH content are hypersensitive to stress. GSH is oxidized by ROS forming oxidized glutathione (GSSG) and ASA is oxidized to monodehydroascorbate (MDHA) and dehydroascorbate (DHA). Through the ascorbate-glutathione cycle, GSSG, MDHA and DHA are reduced back to GSH and ASA. A high ratio of reduced to oxidized ascorbate and GSH is essential for ROS scavenging in cells. Reduced states of the antioxidants are maintained by GR, MDAR, and DHAR, using NADPH as reducing power (Asada and Takahashi 1987).

Ascorbic acid is localized mainly in chloroplast, cytosol, mitochondria, peroxisomes and apoplast (Mittler 2002). Ascorbate is mainly known to regulate cell wall

Table 4.1 Sub cellular localization of antioxidants

Non-enzymatic antioxidant molecules	Subcellular location
Acorbate (ASA)	Plastid; apoplast; cytosol; mitochondria, peroxisomes
β-Carotene	Plastid
Glutathione (GSH)	Plastid; mitochondria; cytosol
Polyamines (e.g., putrescine, spermine)	Nucleus; plastid; mitochondria; cytosol
α-Tocopherol (vitamin E)	Cell and plastid membranes
Zeaxanthin	Chloroplast

associated enzymes. Both ASA and GSH are indispensible for plant defense against oxidative stress and are mostly found in high concentrations in chloroplasts and other cellular compartments (5–20 mM ASA and 1–5 mM GSH), (Noctor and Foyer 1998). Ascorbic acid is one of the most powerful antioxidant (Smirnoff 2000) because of its ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions (Blokhina et al. 2003). It is generally believed that a high ASA/DHA and GSH/GSSG ratio is essential for removing excessive ROS in cells (Mittler 2002). Ascorbic acid can directly scavenge $^1O_2$, $O_2^{\bullet-}$ and $^{\cdot}OH$ radicals and can reduce H_2O_2 to water via APX reaction (Foyer and Noctor 2011). Ascorbate content was found to be high on polluted area in comparison to unpolluted region (Pukacka and Pukacki 2000). The concentration in chloroplast is high and DHA which is oxidized form of ASA, is taken up more rapidly than ASA. Uptake of ascorbate into chloroplast is inhibited by dehydroascorbate (Anderson et al. 1983). Ascorbate also regenerates the lipophilic antioxidants α- tocopherol from α-chromanoxyl radical (Asada 1994). It is a co factor for a range of hydroxylase enzymes. It is powerful antioxidants (Smirnoff 2000) and it removes H_2O_2 from chloroplast as chloroplast lacks catalase (Miyake and Asada 1992; Mehlhorn et al. 1996).

Glutathione (GSH) plays a significant role in plant defense against biotic and abiotic stress and helps to balance the cellular redox status (Kopriva and Koprivova 2005). Glutathione is a tripeptide (γ- glutamylcysteinyl glycine) and abundant in plant tissues. It plays a crucial role in cellular defense against heavy metal stress and oxidative stress as well as in plant growth and development. GSH is a key component of the ASA/GSH cycle, associated with H_2O_2 scavenging (Noctor and Foyer 1998; Goraya and Asthir 2016). The role of GSH in to heavy metal tolerance via ROS scavenging has been demonstrated in various transgenic plants by increased endogenous GSH levels (Jozefczak et al. 2012 and references therein). It is found in cytosol, endoplasmic reticulum, vacuole and mitochondria (Jimenez et al. 1997). Together with its oxidized form (GSSG) GSH maintains a redox balance in the cellular compartments. The ability of GSH to regenerate another powerful water soluble antioxidant, ASA via the ascorbate-glutathione cycles a major role of GSH in the antioxidative defense system (Noctor and Foyer 1998). GSH, can participate in the regulation of cell cycle due to redox properties of its reduced SH- group and the high GSH/GSSG pair. GSH is the predominant non-protein thiol (Rennenberg 1982) and is a major reservoir of non-protein reduced sulfur for the chelation of heavy metal ions and as a precursor of phytochelatin (PC) (Cobbett and Goldsbrough 2002; Yadav 2010) and thus prevents ROS formation in plants. It has also been demonstrated that overexpression of γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS), the essential enzymes for GSH biosynthesis, resulted in improved tolerance against various heavy metal toxicity in transgenic plants. The high GSH levels aided metal sequestration into vacuoles resulting in HM accumulation (Reisinger et al. 2008). However a difference was observed in tolerance. Thus on one hand overexpression of plant *E. coli* γ-GCS and GS increased the accumulation of Cd and Zn (Zhu et al. 1999a, b, c; Bittsánszky et al. 2005), overexpression of γ-GCS or GS in *Brassica juncea* and *A. thaliana* decreased the accumulation of As and Hg (Li et al. 2006). The functionality of GSH in heavy

metal tolerance by directly applying GSH in the presence of heavy metals have also been studied. However it was the plant species and type of metal which determined the effect of exogenous GSH on heavy metal tolerance and accumulation (Chen et al. 2010; Schmöger et al. 2000; Saxena and Saxena 2012; Sun et al. 2013).

The Carotenoids and α-tocopherol (vitamin E) are important membrane-bound quenchers or antioxidants. Carotenoids are the non-enzymatic antioxidants that can be synthetized during unfavorable stress conditions. One of their roles is scavenging and deactivating free radicals (Safafar et al. 2015). Carotenoids include the xanthophylls, which contain oxygen, and carotenes, which are purely hydrocarbons (Safafar et al. 2015). The membrane bound carotenoids not only quench $^1O_2$, but also prevent the formation of $^1O_2$. This is done by quenching the triplet excited state of chlorophylls that otherwise would lead to the formation of $^1O_2$. For example, β-carotene, and also other higher plant carotenoids, reacts with $^1O_2$, rather than the chlorophylls and proteins of photosystems, quenching the energy of excitation. They also quench the energy of triplet chlorophyll, preventing the formation of $^1O_2$ (di Mascio et al. 1989). Subsequently, the excited carotenoid (*Car) undergoes radiation less decay to ground state (Car).

$$\mathrm{Car} + {}^{*}\mathrm{Chl}\left(\mathrm{or}\,{}^{1}\Delta\mathrm{O}_2\right) \rightarrow {}^{*}\mathrm{Car} + \mathrm{Chl}\left(\mathrm{or}\left({}^{1}\Delta\mathrm{gO}_2\right)_2\right)$$

Plant phenolic compounds such as flavonoids and lignin precursors have recently been assigned a role as beneficial antioxidants which can scavenge harmful active oxygen species (Appel 1993). Phytophenolics can act as antioxidants by donating electrons to guaiacol type peroxidases (GuPXs) for detoxification of H_2O_2 produced under stress conditions (Sakihama et al. 2002). The electron spin resonance signals of phenoxyl radicals are eliminated by monodehydroascorbate (MDHA) reductase, suggesting that phenoxyl radicals, like the ASA radical, are enzymatically recycled back to phenolics. Thus, phenolics in plant cells can form an antioxidant system equivalent to that of ASA. In contrast to their antioxidant activity, phytophenolics also act as pro-oxidants under certain conditions. Zn, Ca, Mg and Cd have been found to stimulate phenoxyl radical-induced lipid peroxidation (Sakihama et al. 2002). It was shown in experiment with *Raphanus sativus* that phenolic acids as well as the total and reduced ASA content increased with increase in Cu toxicity along with GSH oxidation and lipid peroxidation. Accumulations of phenolic compounds were also observed in roots of maize (Shemet and Fedenko 2005) and scot pine (Schützendübel et al. 2001) under Cd treatment. Increase of phenylalanine ammonia lyase activity, which is observed under stress conditions (Schützendübel et al. 2001) is probably responsible for the induction of phenolic metabolism In maize roots it was due to the increase in content of cyanidin-3-glucoside with its chromophore group modified by binding to Cd ions (Shemet and Fedenko 2005). Zagoskina et al. (2007), observed that phenolic metabolism in tea (*Camellia sinensis* L.) callus culture was dependent on Cd dose and that the lignin content in root and stem calli increased, but it did not change in the leaf calli.

Flavonoids are plant secondary metabolites and also act as antioxidants. Antioxidants such as anthocyanins (in vacuole) and tocopherol (membrane associated) are major components found in the plants and protect them against oxidative stresses (Hernandez et al. 2009). The ability to capture free radical ions by donation of phenolic hydrogen atoms results in the antioxidant activity of flavanoids which helps to protect plants cells from abiotic stresses (Hernandez et al. 2009). It is reported that in maize seedlings cyanidin and anthocyanins is localized at external parts of root's parenchyma and this increases with HM accumulation (Yamasaki et al. 1997).

α-Tocopherol is a lipophilic antioxidant capable of quenching singlet oxygen. A free radical, α-tocopherylquinone is the product of the reaction of α-tocopherol with singlet oxygen which can be reduced back by ascorbic acid which has high concentration in the stroma (Halliwell and Gutteridge 1984). It also quenches the reducing peroxidised membrane lipid (ROO•), thus breaking the lipid peroxidation cascades (Fryer 1992). a-Tocopherol is chemically the most active form of vitamin E and is capable of scavenging free oxygen radicals and lipid peroxides (Lushchak and Semchuk 2012). a-Tocopherol may be involved in the protection of the plant tissues against oxidative stress and can physically quench O_2 in chloroplast. It has been estimated that before being degraded, one molecule of a-tocopherol can deactivate up to 220 O_2 molecules by resonance energy transfer. In addition, the α-tocopherol can chemically scavenge O_2 and lipid peroxyl radicals (Munne-Bosch 1998). It has been reported that HMs, might alter the different form of a-tocopherol levels in plant tissues (Collin et al. 2008; Yusuf et al. 2010; Lushchak and Semchuk 2012). *A. thaliana* treated with 75 lM Cd and 75 lM Cu showed increase in a-tocopherol content in comparison with control. Collin et al. (2008) Observed that the increased concentration of a-tocopherol in Cd- or Cu-treated *Arabidopsisα* leaves was associated with the up regulation of a number of genes related to tocopherol biosynthesis. Experiment with wild-type and transgenic *B. juncea* plants showed Cd-induced increased accumulation of α-tocopherol in both plants compared with control (Yusuf et al. 2010). An increase in tocopherol content in the shoots was associated with the enhancement of lipid peroxidation was also observed in wheat exposed to Ni (Gajewska and Skłodowska 2007).

4.2.4 Enzymatic Component of Antioxidative Defense

Besides the non-enzymatic antioxidants the scavenging of ROS requires the action of antioxidant enzymes (Table 4.2). Superoxide dismutase (SOD), dismutates $O_2^{\bullet-}$ to H_2O_2 and is the first enzyme involved. The $O_2^{\bullet-}$ produced in the different plant cell compartments is immediately converted reduced to H_2O_2 by SOD (Bowler et al. 1992). However the dismutation of $O_2^{\bullet-}$ results in the production of another ROS. H_2O_2 is a strong oxidant and oxidizes thiol groups, therefore its accumulation is harmful for organelles which show thiol – regulated enzyme activity. Catalase (CAT), peroxidases (POD), ascorbate peroxidase (APX) and glutathione peroxidase

Table 4.2 Antioxidant enzymes and the reactions catalyzed by them

Enzyme	EC number	Reaction catalysed
Superoxide dismutase	1.15.1.1	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \Leftrightarrow 2H_2O_2 + O_2$
Catalase	1.11.1.6	$2H_2O_2 \Leftrightarrow O_2 + 2H_2O$
Glutathione peroxidase	1.11.1.12	2GSH + PUFA–OOH ⇔ GSSG + PUFA + $2H_2O$
Glutathione *S*-transferases	2.5.1.18	RX + GSH ⇔ HX + R-S-GSH
Phospholipid-hydroperoxide glutathione peroxidase	1.11.1.12	2GSH + PUFAHOOH (H_2O_2) ⇔ GSSG + PUFA + $2H_2O$
Ascorbate peroxidase	1.11.1.11	AA + H_2O_2 ⇔ DHA + $2H_2O$
Guaiacol peroxidase	1.11.1.7	Donor + H_2O_2 ⇔ oxidized donor + $2H_2O$
Monodehydroascorbate reductase	1.6.5.4	NADH + 2MDHA ⇔ NAD^+ + 2AA
Dehydroascorbate reductase	1.8.5.1	2GSH + DHA ⇔ GSSG + ASA
Glutathione reductase	1.6.4.2	NADPH + GSSG ⇔ $NADP^+$ + 2GSH

(GPX) assist in detoxification of H_2O_2. Catalases (CAT) convert H_2O_2 to water and molecular oxygen (Willekens et al. 1995). Due to requirement of two H_2O_2 molecules CAT has a high catalytic rates but low substrate affinities. It is unavailable for the thiol-regulated enzymes of Calvin cycle in the chloroplast. The H_2O_2 destruction is thus dependent on peroxidases, which are adequately present in the cell system and also have a much higher affinity for H_2O_2 than CAT. Specific roles for antioxidant enzymes have been explored examine based on transgenic approaches. Catalase was found to be indispensable for oxidative stress tolerance for abiotic and biotic stresses and transgenic tobacco plants with low CAT exhibited enhanced ROS levels (Willekens et al. 1997). The extent of oxidative stress in a cell is determined by the amounts of superoxide, H_2O_2 and hydroxyl radicals. Therefore, the balance of SOD, APX, and CAT activities is critical for managing toxic ROS levels in a cell. Thus whenever CAT activity was reduced in plants, scavenging enzymes such as APX and GPX were upregulated (Apel and Hirt 2004). Studies have also shown the upregulation of these antioxidant enzymes in heavy metal tolerant plants (Table 4.3).

Peroxidases, can be considered to be one of the key enzymes that are involved in the removal of ROS because both its extra- and intra-cellular forms participate in the reduction of H_2O_2 and unlike CAT it require a reductant along with H_2O_2 (Jimenez et al. 1997). An increase in POD activity is regarded as a reliable indicator of heavy metal toxicity because it is expressed in response to oxidative damage and is associated with increase in peroxides, membrane disruption due to lipid peoxidation along with free radical production (MacFarlane and Burchett 2001).

The most important reducing substrate for H_2O_2 detoxification is ascorbate by APX. Ascorbate peroxidase requires an ascorbate and GSH regeneration system which is obtained from the ascorbate-glutathione cycle. Ascorbate peroxidase uses two molecules of ASA to reduce H_2O_2 to water, with the concomitant generation of two molecules of monodehydroascorbate, which in turn can be regenerated by MDA reductase (MDAR) using NADPH as reductant (Asada 1997).

Table 4.3 Antioxidant defense system developed in some heavy metal tolerant plants

Organism	Phenotype	Antioxidant trait for tolerance	References
Algae	Cu tolerant	High APX activity	Nikookar et al. (2005)
Dunaliella tertiolecta			
Higher plants	Zn and Cu hyperaccumulator	Upregulation of APX and MDAR expression; high APX and class III peroxidases activity	Chiang et al. (2006)
Arabidopsis halleri, A. thaliana			
Salix viminalis	Metal (Cu, Zn, Cd) tolerant genotype	High SOD activity	Landberg and Greger (2002)
Pisum sativum	Cu tolerant	High peroxisomal Mn-SOD and CAT activity	Palma et al. (1987) and Maheshwari and Dubey (2009)
	Ni tolerant	High SOD, GPX and APX	
Oryza sativa	As tolerant	SOD, GPX and APX	Mishra et al. (2011)

Monodehydroascorbate can spontaneously dismutate into dehydroascorbate. Ascorbate regeneration is by dehydro ascorbate reductase (DHAR) which oxidizes GSH to GSSG. Glutathione reductase (GR) can regenerate GSH from GSSG using NADPH as reductant (Foyer and Halliwell 1976). Like APX, glutathione peroxidase (GPX) also detoxifies H_2O_2 to H_2O but uses GSH directly as reductant. GPX cycle is closed by regeneration of GSH from GSSG by GR.

4.3 Heavy Metal Toxicity

Heavy metals accumulation in environment, is due to its disposal from industrial units of breweries, tanneries, plating etc., the sewage-sludge and phosphate fertilizers which are transferred to plants and ultimately enter the food chain (Alloway 1995). The reduction in plant growth during metal toxicity is due to low water potential, hampered nutrient uptake and oxidative stress. This also results in leaf chlorosis and alteration in the activity of many key enzymes of various metabolic pathways (Bhatacharya et al. 2010). The toxic dose depends on the type of ion, ion concentration, plant species, and stage of plant growth.

Since heavy metals mostly accumulate in soil and water, they are a threat to plants which are rooted to soil/water and hence get maximum exposure. Heavy metals like Fe, Mn, Cu, Zn and Ni are essential for plant functions but they are toxic at high concentrations. Some metals like mercury (Hg), cadmium (Cd), arsenic (As) and chromium (Cr), have no known biological roles and are toxic even at very low concentration (Salt et al. 1995). Since heavy metals are not biodegradable they are toxic for plants and study of plant's exposure to heavy metals particularly at biochemical level deserves priority. Exposure of plants to heavy metal toxicity stimulates generation of free radicals leading to oxidative stress (De Vos et al. 1991; Weckx and Clijsters 1996, 1997; Dietz et al. 1999). The role of oxidative stress in

metal toxicity has been assessed by measuring alterations in the antioxidative components in plants (Schützendübel and Polle 2002; Pandey et al. 2009). Enhanced activity of the antioxidant defense system due to heavy metals have been observed by a number of workers (Sharma and Dietz 2009 and references therein). This has been done by comparing metal tolerant genotypes with their non-tolerant relatives (Srivastava et al. 2005; Singh et al. 2006a). Use of transgenic plants that over express these antioxidant components also provides an understanding of the relationship between toxicity of heavy metals and cellular redox imbalance. In the following sections we discuss the antioxidant responses of both redox heavy metals like Fe, Cu, Mn and Cr and non-redox heavy metals like Zn, Cd, Ni, As and Hg.

4.3.1 Cellular Antioxidative Defense and Redox Heavy Metals (Fe, Cu, Mn and Cr)

4.3.1.1 Iron

Fetoxicity is the most widely reported nutritional disorder due to the easy reduction of insoluble Fe^{3+} to soluble Fe^{2+} by microbes in the soil. The latter is taken up by roots and translocated to leaves by transpiration. Free radical formation takes place due to excess Fe^{2+} taken up resulting in irreparable impairment of cell structure and damage to membrane, DNA and proteins. Several workers have reported an increase in H_2O_2 and phenolics and decrease in chlorophyll and soluble protein by oxidative stress (Caro and Putarulo 1996; Sinha et al. 1997). Kampfenkel et al. (1995) found that excess Fe increased APX, reduced ASA and GSH levels and increased DHA and GSSG, resulting in a redox imbalance in *Nicotiana* plants. Iron excess was found to increase APX, CAT and GR activities and ascorbate content in *Phaseolus vulgaris* plants (Shainberg et al. 2000). Enhancement of POD activity and induction of POD isozymes were seen in rice leaves treated with excess Fe (Fang and Kao 2000). Up regulation of antioxidative enzymes was also observed in black gram plants subjected to Fe excess (Kumar and Prasad 1999). They also observed induction of ferritin, an Fe storage protein which acts as an antioxidant by sequestering the free Fe and thus preventing generation of the free hydroxyl radicals. Thus exposure to Fe led to increased ascorbyl/ascorbate in *Chlorella* cells (Estevez et al. 2001).

Lipid peroxidation is an indicator of oxidative stress and is estimated by production of malondialdehyde (Azevedo Neto et al. 2006). Lipid peroxidation has been reported to be increased with increasing Fe concentration and therefore indicates the production and accumulation of reactive oxygen species (ROS) due to Fe toxicity. Enhanced lipid peroxidation under Fe toxicity has been reported for different species (Sinha et al. 1997; Souza-Santos et al. 2001; Sinhá and Saxena 2006; Kuki et al. 2008; Kumar et al. 2008; Stein et al. 2008; Xing et al. 2010). The intensity of the oxidative damage, however, differs among species and/or genotypes, and the plant response depends on the duration and intensity of the applied stress (Sgherri

et al. 2000). In *Euglena uniflora* L., antioxidative enzymes (e.g., SOD, POX, GR, GPX and CAT) and low molecular weight antioxidants such as ASA and GSH were observed to be involved in the scavenging of the ROS (Reddy et al. 2005; Singh et al. 2006a). Superoxide dismutase (SOD), was also enhanced by Fe toxicity. Enhanced SOD activity due to Fe toxicity has also been observed in plant species, such as *Nicotiana plumbaginifolia* (Kampfenkel et al. 1995), *Hydrilla verticillata* (Sinhá et al. 1997), *Clusia hilariana* (Pereira et al. 2009), and rice (Stein et al. 2008). Neves et al. (2009) observed a decrease in SOD activity in the leaves of *E. uniflora* exposed to Fe dust deposition and to treatment with 5.0 mM FeEDTA. However this could be due to the extremely high concentration of Fe. Infact it has been observed that at pronounced stage of Fe toxicity the antidefense system is down regulated. The resultant H_2O_2 produced is detrimental for plants and, therefore should be removed. Catalse is an important enzyme which removes H_2O_2 in plants. The activity of this enzyme increases with increase in Fe concentration, especially in the leaves, indicating an increase in the production of H_2O_2. The increase in CAT activity due to Fe stress has been observed in other plant species (Kuki et al. 2008; Stein et al. 2008; Xing et al. 2010). Neves et al. (2009) observed a decreased CAT activity in *E. uniflora* after treatment with 5.0 mM FeEDTA for 2 months. Reductions in the activities of the enzymes SOD and CAT, as observed by Neves et al. (2009), are indicative of a much stronger oxidative stress, affecting enzyme biosynthesis and/or the assembly of enzyme subunits (Singh et al. 2006a). Jucoskzi et al. (2013) observed an increase in POD and APX activities in both the leaves and, particularly, the roots of the *E. uniflora* plants with increasing Fe concentration. An increase in the activity of these antioxidative enzymes implicates their role in the detoxification of ROS accumulated due to Fe stress. This was observed in *Schinus terebinthifolius* (Kuki et al. 2008), *Oryza sativa* (Stein et al. 2008), *Elodea nuttallii* (Plach) H. St. John (Xing et al. 2010) and wheat (Verma and Pandey 2016).

Glutathione peroxidase (GPX) also detoxifies H_2O_2 to H_2O but utilizes GSH as reducing agent. Jucoskzi et al. (2013) observed a decreased GPX activity in roots and leaves with increasing Fe concentration and exposure time. This reduction may be result of a direct attack by toxic ROS, as suggested by Wang et al. (2004) or a reduced supply of GSH. This is less possible since the GR activity was decreased and the GSH content were enhanced under increasing Fe concentration in the leaves. However they also made contrary observations in roots. This indicates a higher utilization of GSH in the roots, probably to provide substrate for phytochelatin biosynthesis, a Cys-rich polypeptide involved in plant metal detoxification or other consuming –SH process.

An increase in the AA content in plants treated with Fe was observed in *Bacopa monnieri* (Sinha and Saxena 2006) and in *Spartia densiflora* (Domínguez et al. 2009). Ascorbate is consumed by the enhanced APX in Fe toxic plants and to eliminate the H_2O_2 an increase in MDHA would be expected. Thus, enzymes such as DHAR and MDHAR are activated to partially regenerate AA consumed during H_2O_2 detoxification (Drazkiewicz et al. 2003; Aravind and Prasad 2005). Jucoskzi et al. (2013) observed in *Euglena uniflora* L that the ASA and GSH contents and the

ASA/DHA and GSH/GSSG ratios, in general, increased with increasing Fe concentration and treatment exposure. Their results indicate that under toxic levels of Fe, young *E. uniflora* plants suffer increased oxidative stress, which is ameliorated through changes in the activities of antioxidative enzymes and in the contents of the antioxidants AA and GSH. The GSH/GSSG ratio is considered to be an important redox sensor in different signaling processes which helps to maintain a positive balance between GSH-producing enzymes, such as GR and consumer enzymes, such as GPX, suggesting an important role for this system in the defense against oxidative stress.

4.3.1.2 Manganese

Mn is an essential micronutrient that plays a pivotal part in many metabolic and growth processes in plants including photosynthesis, respiration, and the biosynthesis of enzymes (Millaleo et al. 2010). It is also a cofactor required for multiple plant enzymes, for example, Mn dependent superoxide dismutase (MnSOD). The contribution of Mn in photosystem II (PSII) especially during the course of splitting of water molecules into oxygen and protection of PSII from photo damage are of significant importance (Hou and Hou 2013). Mn^{2+} is the most stable and soluble form of Mn in the soil environment but lower soil pH, less soil organic matter, and decreased redox potential increase the availability or toxicity of Mn^{2+} to plants. The physiological mechanisms to manage Mn toxicity are not very clear but reports suggest a role for excess Mn in the induction of oxidative stress. In pea del Rio et al. (1985) observed high Mn-SOD in plants exposed to toxicity of Zn and Mn. Leidi et al. (1987a, b) found high Mn-SOD activity in soybean genotypes grown under excess Mn. Panda et al. (1986) reported lipid peroxidation in aging, isolated chloroplasts treated with excess Mn. Gonzalez et al. (1998) observed that SOD and APX activity increased in Mn toxic bean plants and the increase was less in the tolerant cv CALIMA than in the susceptible cv ZPV-292. The cv CALIMA had less ascorbate oxidation due to Mn-toxicity and showed low ascorbate levels even before chlorosis was observed in Mn-stressed plants, especially in cv ZPV-292. They suggested that higher ascorbate levels are maintained in tolerant than in sensitive genotypes.

Fecht-Christoferrs et al. (2003) studied the differential response of *Vigna unguiculata* to oxidative stress between Mn-sensitive cv. TVu 91 and Mn-tolerant cv. TVu 1987. They observed that apoplastic POD was enhanced by increasing Mn concentration and was significantly higher in leaves of cv. TVu 91 than in cv. TVu 1987. At toxic Mn supply, the activities of the POD isoenzymes increased more in the Mn-sensitive cultivar. Levels of ascorbic acid in the apoplast and cytoplasm of the Mn-sensitive cv. TVu 91 decreased with increasing leaf Mn supply, whereas Mn-tolerant cv. TVu 1987 was not affected. They also observed that Mn treatment lead to a stimulation of the enzymes of the ascorbic acid regeneration system (MDHAR and GR) in both cultivars, but the activation of GR was more enhanced in

the Mn-tolerant cultivar TVu 1987. The results indicate the involvement of antioxidative system in the expression of Mn toxicity and genotypic Mn tolerance.

Oxidative stress caused by Mn excess has been recorded in several plant species (Shi et al. 2006). Mora et al. (2009) observed that APX and guaiacol peroxidase (G-POD) activities increased with Mn excess in ryegrass. Mn-tolerant Jumbo and Kingston had high activity of these enzymes and relatively low lipid peroxidation. Kingston was highly tolerant to high Mn concentrations and had the highest SOD activity. Thus increased activity of antioxidative enzymes in Mn-tolerant cultivars could protect their tissues against oxidative stress triggered by Mn excess as also reported by Srivastava and Dubey (2011). Sytar et al. (2013) reported that excess Mn^{2+} enhances the activity of SOD and CAT enzymes, but low Mn decreased their activities. Activities of APX and POD were inversely related to SOD and CAT activities. Increases of CAT activities together with SOD provided a partial protection against oxidative damage and caused lower peroxidation levels in *Mentha.pulegium* under Mn^{2+} toxicity than Cu^{2+} and Zn^{2+}. Candan and Tarhan (2003a, b) also reported the potential of Mn, Zn and Cu to cause oxidative damage in the following order Mn<Zn<Cu.

Lei et al. (2007) observed oxidative stress (accumulation of H_2O_2 and MDA) due to excess Mn on two *Populus cathayana* populations which were from the wet and dry climate regions in western China. They observed that compared with the dry climate population, the wet climate population accumulated more Mn in plant tissues especially in leaves. It showed lower activities of SOD and APX, thus suffering more serious oxidative damage than dry climate population. Thus the antioxidative status of the two populations indicated the susceptibility of the population to high Mn stress. Boojar and Goodarzi (2008) investigated the high Mn effect on levels of Mn, antioxidative enzymes and oxidative damage biomarkers in *Datura stramonium, Alhagi camelthorn* and *Chenopodium ambrosioides*. They observed high Mn levels associated with higher MDA and lower antioxidative enzymes as compared to that in the leaves and stem in plants grown in mines rich in Mn as compared to those in non mine zones. Accordingly, antioxidative enzymatic response to Mn-stress in the three genera and possibly accumulation of Mn in leaf vacuoles of *A. camelthorn*, protected to evade Mn toxicity, Mn was excluded into cell wall or cell vacuole. They also observed that the total phenolic compounds and polyamine (putrescine and spermidine) were enhanced to quench the toxic oxygen radicals. Recently Sieprawska et al. (2016) observed in wheat that the increase of lipid peroxidation induced by ROS generation, was lower in the tolerant ('Parabola') than in the sensitive cultivar ('Raweta'). They also corroborated the works of earlier authors that activation of antioxidative enzymes was more effective in the cells of tolerant wheat, which showed less accumulation of Mn.

4.3.1.3 Copper

Copper is an essential element but similar to Fe its redox activity also leads to formation of ROS and, consequently to oxidative damage to different cell constituents (Gallego et al. 1996). A net decrease in photosynthesis has been observed under Cu toxicity due to photosynthetic metabolism and photosynthetic electron transport (Vinit-Dunand et al. 2002). The impaired photosynthetic metabolism leads to overproduction of ROS such as $O_2^{\bullet -}$, •OH, and H_2O_2. Thus, Cu can become extremely toxic exhibiting symptoms such as chlorosis and necrosis, stunting, leaf discoloration and inhibition of root growth (van Assche and Clijsters 1990; Marschner 2012). At the cellular level, toxicity may result from (i) inhibition of protein function and enzyme activity due to binding between –SH groups (ii) induction of a deficiency of other essential ions; (iii) impaired cell transport processes; (iv) oxidative damage (van Assche and Clijsters 1990; Meharg 1994). Hence, excess Cu can cause oxidative damage in plants which subsequently increases the antioxidant responses The injurious effect of Cu toxicity could thus be alleviated by enzymatic systems scavenging ROS by SOD, CAT, APX, MDHAR, DHAR, and GR (Luna et al. 1994; Navari-Izzo et al. 1998; Gupta et al. 1999; Drazkiewicz et al. 2003; Wang et al. 2004). Leaves and roots both exhibited antioxidant responses in a Cu-concentration dependent and time-dependent manner (Lombardi and Sebastiani 2005; Tanyolaç et al. 2007; Panda 2008). The ascorbate-glutathione cycle has been reported to be involved in response to excess Cu (Gupta et al. 1999; Drazkiewicz et al. 2003). Jouili and Ferjani (2004) observed enhanced antioxidant enzyme activities of SOD and peroxidases as also reported by Van Assche and Clijsters (1990) and Mazhoudi et al. (1997). They also observed a decrease in CAT activity probably due to deterioration of its enzymatic structure by ROS mediated by Cu toxicity. Their studies also suggest that cupric stress modulates lignifying peroxidase activities which could be related to the plant growth inhibition.

Ali et al. (2006) reported that excess Cu induced oxidative damage by increasing $O_2^{\bullet -}$ and H_2O_2 contents and also induced antioxidant defense by enhancing activities of SOD, POD, APX and DHAR. Moreover, they also found decrease in GSH and oxidation of AsA into DHA at toxic Cu levels reflecting a disturbed redox balance in Cu-stressed roots. Khatun et al. (2008) observed that APX, MDHAR, DHAR, GST and G-POD activities were increased in leaves of Cu toxic *Withania somnifera* plants as compared to control indicating that antioxidant enzymes played an important role in protecting the plant from Cu toxicity. Six APX and four G-POD isoforms were detected and significantly induced in metal-treated Cu toxic plants, by Native PAGE as compared to control plants. However a marked decrease in SOD, CAT, GR and GPX activities seems to reflect its inability for eliminating the ROS resulting from Cu-induced oxidative stress. They also observed that total phenolic contents increased with increasing concentration of Cu. Their study revealed that plants have the ability to grow in Cu polluted areas by various physiological changes. Phenolic compounds which could also be substrates for different peroxidases were increased in response to Cu excess and were reported to be the most active enzymes in Cu-exposed red cabbage seedlings (Posmyk et al.

2009). Zhao et al. (2010) demonstrated strong inhibition of root growth along with enhanced MDA content indicating lipid peroxidation under Cu stress in *Festuca arundinacea* L. and *Lolium perenne* L. The higher SOD and POD activity enhanced the tolerance of *F. arundinacea* roots to copper stress. The lower SOD and POD activity in *L. perenne* roots under Cu exposure was correlated with high Cu content in roots. Their results suggested that the tolerance of the turf grass cultivars to Cu toxicity was related to their antioxidant systems. Lower SOD and higher Cu concentration in leaves and roots of maize were also observed in leaves and roots of Cu sensitive maize varieties (Tanyolaç et al. 2007; Madejón et al. 2009). Chen et al. (2015) reported that Cu toxicity in bamboo plants increased SOD and POD to protect it from oxidative damage, but extremely high Cu levels (>25 μM) produced large amount of ROS which inhibited the enzyme activity.

4.3.1.4 Zinc

Zinc is an essential nutrient, but it is toxic if accumulated in high concentration in plant. Zinc is a widespread toxic element in agro-ecosystems (Pahlsson 1989; Di Baccio et al. 2003). Organic ligands and hard cations such as Ca^{2+} reduces its availability but the bioavailability of Zn in soil solution increases at low pH, by industrial and agricultural activities, such as smelter and incinerator emissions, dispersal from mine wastes, excessive applications of Zn-containing fertilizers or pesticides and use of Zn-contaminated sewage sludges, manures or industrial wastes as fertilizers. Excess Zn ions may disrupt the function of proteins by binding to their functional groups. Excess Zn ion can also displace other essential metal ions from their binding sites (Tennsledt et al. 2009).

Zinc toxicity in plants can disturb the homeostasis in plants by interfering with the uptake, transport, osmotic potential and regulation of essential ions which disrupts the vital growth processes such as transpiration (Rout and Das 2003), photosynthesis and chlorophyll biosynthesis (Bonnet et al. 2000), membrane integrity (DeVos et al. 1991) and enzyme activities related to metabolism (Zlatimira and Doncheva 2002), disintegration of cell organelles, condensation of chromatin material and increase in number of nucleoli in cells (Jain et al. 2010).

Zinc is required for stabilization of biomembranes due to its interaction with phospholipids and sulfhydryl groups of membrane proteins. According to Von Glos and Boursnell (1981), phospholipids are essential components of the membrane binding sites of Zn. Similar to its role as a structural component in biomembranes like Ca, Zn plays a significant role in generation as well as detoxification of free oxygen radicals, which are potentially damaging to membrane lipids and sulfhydryl groups. Zinc prevents membrane damage catalyzed by $O_2^{\bullet-}$ – generating NADPH oxidase, in higher plants. Higher levels of $O_2^{\bullet-}$ found in roots of Zn-deficient plants (Cakmak and Marschner 1988) are attributed to enhanced activity of an $O_2^{\bullet-}$ generating NADPH oxidase as well as decrease in superoxide dismutase (SOD) activity. Excess Zn concentration may stimulate the formation of ROS resulting in oxidative damage in various plant species (Weckx and Clijsters 1997; Chaoui et al. 1997;

Prasad et al. 1999; Dietz et al. 1999; Pandey et al. 2002; Panda et al. 2003; Li et al. 2008; Pathak et al. 2009). Excess Zn and oxygen metabolism are closely linked to the redox control of cells (Foyer et al. 1994a, b; Schutzendubel and Polle 2002). Weckx and Clijsters (1997), di Baccio et al. (2005), and Khan (2007) reported that phytotoxic concentration of Zn increased lipoxygenase activity and induced lipid peroxidation.

Weckx and Clijsters (1997) reported that Zn toxicity enhanced the levels of hydrogen peroxide, which could not be efficiently removed by the antioxidative system. They also reported that the Zn treatment had no effect on activity of catalase and SOD while the activity of APX was increased. Bonnet et al. (2000) reported that Zn toxicity increased the activity of SOD and APX in rye grass. Bittsánszky et al. (2005) observed that elevated levels of GSH in transgenic poplars helped to tolerate Zn toxicity. Li et al. (2008) observed enhanced activities of SOD, CAT, APX and GPX at excess Zn in *Sedum alfredi* Hance. Wang et al. (2009) observed that compared with control, NADH oxidase and POD activity increased in leaves and roots of plants under high Zn, but SOD, CAT and APX activities decreased in rape seed seedlings.

4.3.1.5 Chromium

It is well documented that Cr is a toxic agent for the growth and development of plants (Singh et al. 2013a, b) and is known to cause environmental pollution (Oleiveira 2012). In plants, Cr is found in the forms of trivalent Cr^{3+} and hexavalent Cr^{6+} species (Mohanty and Patra 2013). Under reducing condition, Cr^{6+} is converted to its more toxic form Cr^{3+}, which can indirectly influence and change soil pH to both alkalinity or acidity extremes, and this phenomenon disturbs the nutrients bioavailability and their absorption by plants. Panda and Patra (2000) studied the relationship between the toxicity of Cr(III) ions and oxidative reactions in wheat. They observed the breakdown of chlorophyll, carotenoid and increase in membrane permeability and lipid peroxidation at higher concentrations of Cr(III) ions. Cr toxicity increased the CAT activity in younger leaves while the activity decreased in older ones. POD activity decreased with increasing Cr(III) ion concentration. An increase in SOD activity was seen in younger and older leaves at lower Cr(III) level while it decreased at higher concentrations. Free radical scavengers such as mannitol and sodium benzoate prevented the increase in the senescence parameters and protected these enzymes against inactivation.

Samantary (2002) observed that the activity of antioxidant enzymes, POD, CAT and SOD) increased in case of Cr-sensitive of mungbean exposed to different Cr concentrations. However, the level of antioxidant enzymes decreased in Cr-tolerant cultivars (Samantary 2002). SOD and CAT activities decreased in roots and shoots of *T. aestivum* L. grown in the presence of $K_2Cr_2O_7$ but POD decreased in roots and increased in shoots (Dey et al. 2009). CAT activity also decreased in *A. viridis* L. exposed to Cr(VI) but an increase in SOD and guaiacol peroxidase (POD) activity was observed with increase of Cr(VI) concentration (Liu et al. 2008). Zaimoglu

et al. (2011) studied the antioxidant responses of *Brassica juncea* and *Brassica oleracea* to soils enriched with Cr (VI) and found that total enzymatic activity was higher in *B. oleracea* than in *B. juncea* with a decrease in CAT activity in both species. They concluded that cellular antioxidants play an important role in protecting *Brassica* sp. to Cr-induced oxidative stress. According to them a high activity of antioxidant enzymes and consequent detoxification of ROS contributes to relative tolerance of these species to Cr (VI). Exogenous application of GSH in rice plants has shown to alleviate Cr toxicity (Zheng et al. 2012).

4.3.2 Cellular Antioxidative Defense and Non-redox Heavy Metals Ni, Cd, As and Hg

4.3.2.1 Nickel

As a component of the enzyme urease, Ni is essential nutrient as it catalyzes the hydrolysis of urea in plant tissues. Inhibitory effect of toxic Ni on plant growth has been reported by many authors (Parida et al. 2003; Vinterhalter and Vinterhalter 2005). Due to certain characteristics similar to Ca, Mg, Mn, Fe, Cu, and Zn, Ni may compete with these metals in absorption and transpiration processes (Estew et al. 1983). Thus at high concentrations Ni may inhibit the absorption of these metals, decrease their concentration and even lead to their deficiency in plants subsequently, affecting important physiological processes (Van Assche and Clijsters 1990; Gajewska et al. 2006; Ahmad and Ashraf 2011). Therefore, Ni toxicity in plants is partly due to interference with other essential metal ions via competition and/or the formation of chelate complexes with metal ligands. Enzymes, such as SOD and CAT, contain Fe, Cu, Zn, or Mn in their prosthetic groups. Since excess Ni has been shown to decrease the contents of Fe (Pandey and Sharma 2002), Cu and Zn (Parida et al. 2003) in plant tissues, it is possible that Ni can reduce the biosynthesis of these metalloenzymes by causing deficiencies of these metal ions (Chen et al. 2009; Sreekanth et al. 2013). Studies have shown that Ni can competitively remove Ca ions from the Ca-binding site in the oxygen evolution complex (Boisvert et al. 2007) and replace the Mg ion of chlorophyll (Küpper et al. 1996; Souza and Rauser 2003; Solymosi 2004), which may eventually inhibit the PSII electron transport chain leading to oxidative damage.

Increasing evidence suggests that Ni toxicity is associated with oxidative stress in plants (Rao and Sresty 2000; Gonnelli et al. 2001; Boominathan and Doran 2002; Gajewska et al. 2006). Excessive Ni leads to increases in the concentration of $^{\bullet}OH$, $O_2^{\bullet-}$, NO and H_2O_2 (Boominathan and Doran 2002; Gajewska and Sklodowska 2008; Stohs et al. 2001; Hao et al. 2006). Since Ni is not a redox-active metal, it cannot directly generate these ROS, but it interferes indirectly with a number of antioxidant enzymes such as, SOD, CAT, GPX, GR, POD, guaiacol peroxidase (GOPX), and APX (Pandey and Sharma 2002; Hao et al. 2006; Pandolfini et al. 1992; Baccouch et al. 1998; Baccouch et al. 2001; Gajewska and Sklodowska 2005;

Pandey and Pathak 2006). Exposure of plants to Ni increase the activities of SOD, POD, GR, and GPX leading to activation of other antioxidant defenses and removal of ROS (Freeman et al. 2004; Gajewska and Sklodowska 2005; Schickler and Caspi 1999; Gomes-Juniora et al. 2006). However, excess Ni has also been found to reduce the activity of many cellular antioxidant enzymes, both *in vitr*o and *in vivo*, and plant's capability to scavenge ROS, leading to ROS accumulation and finally oxidative stress in plants (Zhao et al. 2008; Gajewska and Sklodowska 2008). Duration of stress, concentration of Ni treatment, and the plant species as well as the plant parts involved greatly influence the antioxidant enzymes. Thus in experiments conducted by Gajewska and Sklodowska (2007) SOD and CAT activities decreased significantly in the leaves of wheat plants in response to 100 μM Ni treatment for 3, 6 and 9 days, whereas GPX, GPOX and APX activities were increased. However earlier Gajewska and Sklodowska (2005) found that exposure of 14 day old pea plants to 10, 100 200 μM Ni for 1, 3, 6 and 9 days resulted in reductions in SOD activities in both leaves and roots, and APX activity in roots, together with increases in APX activity in leaves, increases in GST in both leaves and roots (most pronounced in roots), while CAT activity generally remained unchanged. Rao and Sresty (2000) observed that Ni at 0.5 mM concentration increased the activities of SOD, GR and POD and decreased the activity of CAT in 6 day old seedlings of pigeonpea whereas CAT and POD activities in leaves decreased significantly in cabbage treated with 0.5 mM Ni for 8 days (Pandey and Sharma 2002). The same trend was observed in SOD, CAT and POD activities in leaves of *Hydrocharis dubia* in response to 0.5, 1, 2, 3, 4 mM Ni treatments for 3 days (Papadopoulos 2007).

Treatment of wheat plants with higher Ni concentration decreased the activities of SOD and CAT. This decrease has also been found in hairy roots of *Alyssum bertolonii* and *Nicotiana tabacum* (Boominathan and Doran 2002) but the opposite effect has been observed in shoots of *Zea mays* (Baccouch et al. 1998). Decrease in antioxidative enzyme activities could be due to damage to the enzyme molecules by toxic ROS (Gallego et al. 1996; Sandalio et al. 2001). Decline in SOD activity might lead to enhancement of $O_2^{\bullet -}$ level in wheat shoots. However, despite the inhibition of CAT activity, accumulation of its substrate, H_2O_2 might not occur since it could be utilized in POD catalyzed reaction. Sun et al. (2009) reported a dose dependent accumulation of ROS and MDA in *Thuidium cymbifolium* grown under Ni toxicity. They also reported that SOD and CAT activities decreased while the peroxidase (POD) activity increased in Ni toxic plants. Bhattacharya et al. (2010) reported high POD activity and synthesis of new protein bands at high Ni. Induction of this enzyme activity after treatment of plants with heavy metals including Ni has previously been reported (Pandolfini et al. 1992; Gabbrielli et al. 1991). Enhancement of POD activity under metal stress is explained by its role in building up physical barrier against toxic metals entering the cell as well as in scavenging H_2O_2. Since POD which also contains Fe as a cofactor was activated in response to Ni stress, mechanisms different from Ni-induced disturbance in iron-porphyrin synthesis were probably involved in the inhibition of CAT activity in wheat shoots. Catalase molecule might be cleaved by proteases induced by oxidative stress, which has been found by Distefano et al. (1999) in senescent pea leaves.

Nickel has also been shown to increase the plasma membrane (PM) NADPH oxidase, which was shown to be involved in Ni induced ROS generation in roots of 5 day old wheat seedlings (Hao et al. 2006). In Ni toxic plants ROS have also been shown to cause damage to cell membrane, proteins, lipids and resulting in lipid peroxidation (Boominathan and Doran 2002; Baccouch et al. 1998, 2001; Schickler and Caspi 1999), developmental defects and genetic instability in plant species (Papazoglou et al. 2005). For example, malondialdehyde (MDA, a lipid peroxidation product increased in Ni toxic (Rao and Sresty 2000; Boominathan and Doran 2002; Baccouch et al. 2001; Schickler and Caspi 1999; Dietz et al. 1999). Rao and Sresty (2000) reported Ni induced depletion of low molecular weight proteins, such as GSH, contributed to the induction of oxidative stress in plants.

4.3.2.2 Cadmium

Cadmium is a non-essential heavy metal, extremely water soluble and widespread element which is also extremely toxic (Romero-Puertas et al. 2004). Cd is rapidly taken up by plants due to its solubility in water and it is therefore much more toxic than other metals. It is toxic to plant cells even at low concentrations because it can be easily absorbed by plants from soil or water (Gallego et al. 2012). Symptoms of toxicity of Cd toxicity are leaf chlorosis, growth and photosynthesis inhibition and disturbance in water balance (Aghaz et al. 2013). Similar to other heavy metals, it is known to induce oxidative stress (Li et al. 2016; Wang et al. 2008). As a non redox-metal, Cd is not able to generate ROS directly through Haber–Weiss reactions. The mechanisms include interacting with the antioxidant system (Srivastava et al. 2004), disrupting the electron transport chain (Qadir et al. 2004), disturbing the metabolism of essential elements (Dong et al. 2006) and activation of lipoxygenase with resulting lipid peroxidation (Somashenkaraiah et al. 1992; Smeets et al. 2005). In addition, it is able to induce ROS indirectly, through activation of NADPH oxidase in membranes (Gallego et al. 2012). One of the most deleterious effects induced by Cd is lipid peroxidation which can directly cause biomembrane deterioration. MDA, one of the decomposition products of polyunsaturated fatty acids of membrane, is regarded as a reliable indicator of oxidative stress (Demiral and Türkan 2005).

Apart from the damage they cause, Cd-induced ROS also exert a positive role. ROS accumulation, in fact, has been suggested as part of the signalling cascade leading to stress protection (Mittler 2002; Sharma and Dietz 2009). Besides enzymatic and non-enzymatic mechanisms, compatible solutes, such as proline, protect plants from stress through the detoxification of ROS, cellular osmotic adjustment, protection of membrane integrity and stabilisation of enzymes/proteins, (Szabados and Savoure 2009). Romero-Puertas et al. (2004) studied the involvement of H_2O_2 and $O_2^{\bullet -}$ in the signalling events that lead to the variation of the transcript levels of CAT, GR and Cu/Zn-SOD in pea plants under Cd stress.

Cadmium induced oxidative stress has been reported by many workers (Stohs et al. 2001; Schutzendubel and Polle 2002; Pandey et al. 2009; Panda et al. 2011; Zhao 2011; Chen et al. 2011). Cadmium produces lipid peroxidation due to

malondialdehyde accumulation in sunflower seedlings and leaf discs (Gallego et al. 1996), leaves of *Phaseolus vulgaris* (Chaoui et al. 1997), *Helianthus annuus* (Gallego et al. 2002), *Oryza Sativa* (Panda et al. 2011), *Abelmoschus esculentus* (Sharma et al. 2010), *Medicago truncatula* have (Xu et al. 2010) and *Vicia faba* (Cabala et al. 2011). Treatment with Cd stimulated accumulation of lipid peroxides in *Pisum sativum* (Dixit et al. 2001; Metwally et al. 2005), different barley genotypes (Wua et al. 2003), *Arabidopsis thaliana* seedlings (Cho and Seo 2004) and soybean nodules (Balestrasse et al. 2004), but decreased the rate of lipid peroxidation in peroxisomes of pea plants (Romero-Puertas et al. 2004). In pea Cd increased lipid peroxidation due to H_2O_2 accumulation and enhanced antioxidative defence system (Pandey and Singh 2012).

The activity of the antioxidative enzymes SOD, CAT, APX, GR and DHAR decreased or increased depending on concentration of Cd, the organ used and the age of the plants (Gallego et al. 1996; Sandalio et al. 2001; Balestrasse et al. 2004). In *Phaseolus vulgaris* roots and leaves, 5 mM Cd enhanced activities of the peroxidases GPX and APX (Chaoui et al. 1997). In two species of *Alyssum*, GR activity increased at 0.02 mM Cd but decreased at 0.05 mM Cd (Schickler and Caspi 1999). Cadmium treatment induced lipoxygenase, with the simultaneous inhibition of the antioxidative enzymes, SOD and CAT (Somashekaraiah et al. 1992). In particular, CAT activity often decreased following exposure to elevated cadmium concentrations (Sandalio et al. 2001; Fornazier et al. 2002; Shim et al. 2003). However, Vitoria et al. (2001) reported that the activities of CAT, GR and specific isoenzymes of SOD increased in the leaves and roots of a resistant variety of radish, following exposure to increasing (between 0.25 and 1 mM) concentrations of Cd. Lipid peroxidation and H_2O_2 levels, SOD, CAT, APX and GR activities increased in pea roots and leaves under cadmium stress (Dixit et al. 2001), while APX and CAT decreased at high cadmium concentrations (Sandalio et al. 2001). A severe suppression of SOD and CAT, and almost complete loss of APX activities after 48 h of exposure to 50 μM Cd was observed in pine roots (Schützendubel et al. 2001). Schützendubel and Polle (2002) also reported inhibition of APX and CAT, H_2O_2 accumulation along with growth retardation in the poplar roots due to Cd toxicity.

Pereira et al. (2002) observed in *Crotalaria juncea* seedlings that although CAT activity did not exhibit any major variation in the roots following Cd treatment, 2 mM Cd induced a sixfold increase in activity in the leaves. They also observed that the results observed for SOD were different of those observed for CAT activity. The concentration of 2 mM Cd induced a small increase in the SOD activity in the roots after 48 h of exposure, whereas in leaves a sevenfold increase in GR activity was detected after 48 h exposure to 2 mM Cd. The results suggest that in *C. juncea* the ROS induced by Cd are metabolized by CAT in the peroxisomes. An increase in GR suggests that GR is also playing a role in the detoxification of Cd induced ROS possibly via the ASC-GSH cycle (Pereira et al. 2002). GR also plays a key role in the antioxidant defense processes, by reducing GSSG, thus allowing a high GSH/GSSG ratio to be maintained. Study on maize plantlets after 6 days action of low Cd concentration showed an increase in GR and GPX activity (Metawally et al. 2005). This was also observed by the analysis of leaves and roots of sugarcane plant which

showed that GR activity increases in response to Cd exposure to detoxify H_2O_2 or to produce glutathione for the synthesis of phytochelatins, whereas CAT exhibited considerable variability (Fornazier et al. 2002).

Milone et al. (2003) reported Cd induced antioxidative responses in wheat treated with up to 10 μM Cd. In this work, APX and CAT were inhibited in the roots of the most sensitive cultivar, *Adamello*, while SOD was scarcely affected in both roots and leaves of *Adamello* and the most tolerant cultivar, *Ofanto*. Studies on biomass, Cd accumulation and membrane lipid composition of a metal accumulator *Brassica juncea*, and a Cd sensitive *Brassica napus*, of similar families have indicated different responses of the two species to Cd supply (Nouairi et al. 2006). In contrast to *Brassica napus*, low production of MDA in the leaves of *Brassica juncea* was observed despite its high Cd accumulation (Nouairi et al. 2006). Moreover, it was also observed that fatty acid composition of membrane lipids remained unchanged in leaves of *Brassica juncea* plants treated with Cd (Nouairi et al. 2006). The low MDA content and the stability of membrane lipid composition observed in *Brassica juncea* leaves seem to be closely related to heavy metal tolerance in plants. The non-protein thiol content also showed an increase in leaves of *Brassica juncea* plants. *Brassica juncea* had higher concentrations of each compounds (PCs, NPT and GSH) in control and treated plants in comparison with *Brassica napus*. In contrast to *Brassica juncea* activities of antioxidative enzymes (SOD, CAT, APX and GR) were generally depressed at highly toxic Cd level in leaves of *Brassica napus* plants. Ann et al. (2011) studied oxidative damage and antioxidant defence response and observed that, the expression of genes of cytosolic Cu, Zn-SOD slightly decreased, while the Mn-SOD increased in the presence of excess Cd ions. Treatment of 15 M Cd to germinating *Solanum nigrum* showed that SOD and APX increased and CAT was decreased after 45 days (Fidalgo et al. 2011). It has also been reported that activities of SOD, CAT, APX, and DHAR increased upon Cd treatment in two varieties of rice (Iqbal et al. 2010).

Thiols are able to counteract oxidative stress due to their strong antioxidative properties (Pichorner et al. 1993; Noctor and Foyer 1998; Cabala et al. 2011). A number of reports of Cd induced depletion of GSH in several plant species exist (Dixit et al. 2001; Yang et al. 2011). The decline in the levels of GSH might also be attributed to a decreased GR activity (Dixit et al. 2001; Wu et al. 2004; Anjum et al. 2008), an increased utilization for ASC synthesis or for a direct interaction with Cd (Pietrini et al. 2003; Anjum et al. 2008, 2012). However, GSH increased in *Phragmites australis* roots and leaves, treated either with 50 μM Cd (Ianelli et al. 2002) and 100 μM Cd (Pietrini et al. 2003). According to Paradiso et al. (2008) the changes that occurred in the ASC–GSH cycle enzymes of the leaves also suggest that the whole plant improved its antioxidant defense, even in those parts which had not yet been reached by Cd. This increase in the enzymes of the ASC–GSH cycle further highlights the tight regulation and the relevance of this cycle in the defense against heavy metals. In maize seedlings Sun et al. (2013) observed an ameliorative effect of GSH application which helped to provide Cd tolerance.

It was observed that the overexpression of γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS), leading to biosynthesis of GSH also

helped in tolerance of Cd toxicity by enhancing its accumulation in transgenic plants (Zhu et al. 1999a, b, c; He et al. 2015). Heavy metal tolerance has been shown to be related to changes in heavy metal accumulation. Overexpression of plant or *E. coli* γ-GCS and GS provides protection from Cd and mercury (Hg) (Zhu et al. 1999a, b; Guo et al. 2008; Reisinger et al. 2008). Recently Zhou et al. (2017) studied Cd tolerance in four apple rootstocks and found that higher tolerance of Cd was due to elevated concentrations of free proline, soluble phenolics and ASC; higher concentrations of total thiols (T-SH) and GSH in bark and leaves; and enhanced activities of SOD, CAT, GPX, and APX in roots and wood, and SOD, CAT, and GR in leaves after Cd exposure.

4.3.2.3 Mercury

This is one of the most harmful heavy metals, even at extremely low concentrations, for plants. It is known to generate ROS and induce oxidative stress resulting in lipid peroxidation, membrane damage, enzyme inactivation, and DNA anomalies (Cargnelutti et al. 2006; Ortega-Villasante et al. 2007; Zhou et al. 2007; Shiyab et al. 2008). It is known to inhibit photosynthesis, transpiration, and nutrient uptake in plants (Patra and Sharma 2000). The mechanism of Hg tolerance and accumulation in plants is not worked out and needs more study. Hg treatment affected the amount of photosynthetic pigments, like chlorophylls and carotenoids through direct inhibition of enzymes involved in this process (Lenti et al. 2002; Puzon et al. 2014). As a result, the reduced chlorophyll levels led to lower photosynthetic capacity of plant exposed to Hg (Moreno-Jimenez et al. 2007). Smolinska and Leszczynska (2017) recently showed that increasing Hg concentration in plant shoots was correlated with enhanced activation of POD activity and changes in total carotenoid concentration. Zhou et al. (2007) reported that Hg caused oxidative damage to roots of alfa alfa due to peroxidation of membranes. By non-denaturing PAGE they observed that, activities of SOD, POD and APX were increased in roots after Hg treatment but GR activity was decreased. Treatments of seedlings with Hg decreased the ASA and GSH amounts but increased total non-protein thiols (Chen and Yang 2012). In *Juncus maritimus* grown with high Hg levels, Anjum et al. (2014) observed enhanced activity of non-glutathione-based H_2O_2-metabolizing enzymes such as CAT and APX, whereas the activity of glutathione-based H_2O_2-scavenging enzymes GPX was inhibited. They also observed enhanced GR activity and high GSH in toxic Hg shoots. According to them the GSH-based H_2O_2-scavenging enzyme system was more effective in overcoming oxidative damage under Hg toxicity.

According to Carrasco-Gil et al. (2011) and Sobrino-Plata et al. (2014), an important mechanism of Hg detoxification is due to the high affinity for SH groups to form the Hg-phytochelatin (Hg-PC) complexes. The role of exogenous GSH as an ROS scavenger during Hg stress is also supported by a marked reduction in membrane lipid peroxidation, as evidenced by a low MDA level, which is increased by Hg stress (Cargnelutti et al. 2006; Lomonte et al. 2010; Sahu et al. 2012). Recently Kim et al. (2017) showed that exogenous GSH application improved Hg

tolerance during seed germination and seedling growth in *Arabidopsis thaliana*, tobacco, and pepper. The effect of exogenous GSH was specific to Hg tolerance as it was observed in the presence of other heavy metals, such as Cd, Cu, and Zn, together with Hg. This is probably because GSH treatment increased chlorophyll content and significantly decreased H_2O_2 and $O_2^{\bullet -}$ levels and lipid peroxidation. They also observed that GSH treatment resulted in significantly less accumulation of Hg in *Arabidopsis* plants. Thin layer chromatography and NMR analysis also revealed that GSH had much stronger binding affinity to Hg than to Cd, Cu, or Zn, suggesting that tight binding of GSH to Hg inhibits Hg uptake, leading to low Hg accumulation in plant cells. Thus results show that exogenous GSH greatly reduces the levels of H_2O_2 and $O_2^{\bullet -}$ which otherwise rapidly accumulate following Hg exposure in *Arabidopsis* roots and leaves, implying that exogenous GSH functions as an efficient ROS scavenger. Probably exogenous GSH rescues cell vitality and plant growth from Hg stress by relieving Hg-induced oxidative stress. This is supported by the observation that *Arabidopsis* leaves maintain their green color and retain a high chlorophyll content under Hg stress following the application of exogenous GSH.

4.3.2.4 Arsenic

Arsenic is a highly toxic metalloid for plants and humans. Its uptake and thus its toxicity is further increased since it is a phosphate analogue and enters the plants easily as a phosphate transporter. Most prevalent forms of As in plants are arsenite (As III) and arsenate (As V). Uptake of As disturbs the plant metabolism and leads to structural and biochemical disorders. Cell division, photosynthesis and redox status are adversely affected by As (V). As (III) can also react with thiol (–SH) groups of enzymes and inhibits various metabolic processes (Zhao et al. 2009). Although, arsenic is a non-redox active it is known to induce oxidative stress directly by generating reactive oxygen species (ROS) due to electron leakage during conversion of its valence forms As (V) to As (III) or indirectly by inactivating antioxidant molecules through binding with their –SH groups. This reduction may lead to methylation of inorganic As which is a redox driven reaction and can generate ROS (Zaman and Pardini 1996). These ROS stimulates the peroxidation of polyunsaturated fatty acids of lipid bilayer which increases the electrolyte leakage, TBARS and H_2O_2 content in plants like, *Holcus lanatus* (Hartley-Whitaker et al. 2001), red clover (Mascher et al. 2002), mung bean (Singh et al. 2007) and rice (Shri et al. 2009). Membrane damage of the chloroplast by As has also been observed leading to breakage and swelling of thylakoid membranes in *Pitters vittata* (Li et al. 2006). Arsenic induced oxidative stress generate many toxic effects like decrease in photosynthetic pigments (Mascher et al. 2002), photosynthetic rate (Stoeva and Bineva 2003), glutathione depletion (Hartley-Whitaker et al. 2001) and reduction in soluble protein content in plant (Stoeva et al. 2004, 2005).

Arsenic mediated oxidative stress causes activation of enzymatic antioxidants namely, SOD, APX, CAT, GR, GPX as well as non antioxidant compounds such as,

ASA, GSH. Carotenoids are reported to mitigate As mediated oxidative stress. During As stress the up regulation of Cu/Zn SOD has been reported in rice seedlings (Shri et al. 2009). The analysis of native PAGE SOD activity shows that one Mn-SOD and two major Cu/Zn SOD isozymes is increased in red clover exposed to arsenate (Mascher et al. 2002). By proteomic analysis of maize root Requejo and Tena (2005) observed that Cu/Zn SOD is one of the highly responsive enzymes to As which is involved in cellular homeostasis during redox disturbance. Mylona et al. (1998) demonstrated that SOD activity increased in response to low As concentration but high concentration of As inhibited the accumulation of SOD mRNA and leads to decline in its activity. Antioxidant-related genes coding for SOD and POD play a prominent role in response to arsenate. The microarray experiment revealed induction of chloroplast Cu/Zn superoxide dismutase (SOD) (at2g28190), Cu/Zn SOD (at1g08830), as well as an SOD copper chaperone (at1g12520) and strong suppression of Fe SOD in response to As (V) stress (Abercrombie et al. 2008). Higher activity of CAT was observed in As-tolerant *Pteris vittata* than As-sensitive *Ptries ensiformis* and *Nephrolepis exaltata* (Srivastava et al. 2005). CAT activity was enhanced in *Zea mays* due to As toxicity (Mylona et al. 1998). In contrast As decreased CAT activity in mung bean along with decrease in H_2O_2 content (Singh et al. 2007). However Singh et al. (2007) concluded that As causes a reduction in root elongation by inducing an oxidative stress that is related to enhanced lipid peroxidation, but not to H_2O_2 accumulation.

In the ASA-GSH cycle upregulation of APX activity has been reported in rice seedling (Shri et al. 2009) and mung bean (Singh et al. 2007). Stoeva et al. (2005) reported increased POD activity and lipid peroxidation in beans treated with As. An enhanced GR activity during As induced oxidative stress in higher plants was reported. The increased requirement of GSH during As induced oxidative stress was supported by the stimulation of GR in rice seedlings (Shri et al. 2009). Enhanced GR activity has also been observed in roots of *Pteris vittata, Pteris ensiformis* and, *Nephrolepis exaltata*, but GR activity in fronds and rhizome was higher in As-sensitive *P. ensiformis and N. exaltata* than in As-tolerant *Pteris vittata* (Srivastava et al. 2005). GPX activity increased in response to As-induced oxidative stress. Srivastva et al. (2005) has suggested that GPX serves as an intrinsic defense tool to resist oxidative damage in *P. ensiformis* and *N. exaltata*. An increase in GPX activity was reported upto 20 μg g^{-1} arsenic and then decrease at higher tested concentrations of As in *Pteris vittata* (Cao et al. 2004). Mylona et al. (1998) earlier observed increase in GST activity in maize plants. Singh et al. (2006a, b) observed significant increase in ASA (reduced) concentration and ratio of ASA/DHA in fronds of As-hyperaccumulator *Pteris vittata* as compared to As- sensitive *P. ensiformis* after arsenic exposure. Ascorbate concentration increased in hypocotyls, whereas decreased in roots of cucumber plants exposed to As (Czech et al. 2008). Contradictory reports have come forth on the levels of GSH in As toxic plants. Another report suggests that high As resulted in GSH depletion and phytochelatin production in *Holcus lanatus* (Hartley-Whitaker et al. 2001). Similarly decrease in GSH content was observed in red clover plant exposed to As (Mascher et al. 2002). However significant increase in GSH and PCs upon As exposure has been

demonstrated in tolerant plants like *Hydrilla verticillata* (Srivastava et al. 2005). Shri et al. (2009) have reported that GSH and cystine application resulted in overcoming oxidative stress. Examined the metabolic adaptations of *Pteris vittata* L, an As hyperaccumulator, under As stress as compared to *Pteris ensiformis*, a non-As hyperaccumulator. The levels of ASA and GSH, and their reduced/oxidized ratios in the fronds of *P. vittata* of the control were much greater than *P. ensiformis.* This indicates that *P. vittata* has an inherently greater antioxidant potential than *P. ensiformis*. The lower levels of antioxidant compounds (ASA, Car and GSH) in *P. ensiformis* than *P. vittata* are consistent with its greater exposure to ROS and lower scavenging ability. The results together indicate that protection from oxidative damage by a greater level of ascorbate–glutathione pool is involved in the As-tolerance in As-hyperaccumulator *P. vittata*.

Singh et al. (2013a, b) substantiated that the oxidative stress markers such as. $O_2^{\bullet-}$, H_2O_2 and MDA (lipid peroxidation) contents were enhanced, activities of SOD and CAT were stimulated, while the activities of APX and GST, and ASA content and ASA/DHA ratio were decreased. They further reported alleviation of As toxicity by nitric oxide (NO). Upon addition of sodium nitropruside (SNP- source of NO), they observed a further increase in SOD and CAT activity and APX and GST activity, and levels of ASA and ASA/DHA ratio were restored considerably. Results indicate that excess accumulation of As decreases growth, photosynthesis, APX and GST activities and ASA content, and therefore results in oxidative stress. However, the addition of SNP protected seedlings against As stress by regulating As accumulation, oxidative stress and antioxidant defense system. Recently Singh et al. (2017) investigated arsenic (As) accumulation, translocation and tolerance in *Vetiveria zizanoides* L. Nash, a suitable candidate for the phytoremediation of heavy metals. *V. zizanoides* plants were found effective in remediation of As, at 200 μM after 14 days of exposure. The up-regulation of antioxidant enzyme activities of SOD, APX, GPX, CAT and GST provided enhanced tolerance to plants against arsenic induced oxidative stress. Their results indicated that in vitro developed plants of *V. zizanoides* have the potential to remediate and tolerate varying levels of As.

4.4 Conclusions

Heavy metals are important environmental pollutants and plants are susceptible to heavy metal toxicity. Oxidative stress is induced by a wide range of environmental factors including high light intensity, UV radiations, drought, pathogen invasion, herbicide action and heavy metal toxicity. Of the ROS, hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) are both produced in a number of cellular reactions, including the Fe-catalysed Fenton reaction, and by various enzymes such as lipoxygenases, peroxidases, NADPH oxidase and xanthine oxidase. The main cellular components which are damaged by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates and nucleic acids. Though ROS are always regarded to impart negative impact on plants, some reports

consider them to be important in regulating key cellular functions. Such reports in plant are limited, never the less the specific function of ROS as signaling molecules and in activating signal transduction processes in response to various stresses is a matter of investigation.

Plants have their own antioxidant defense mechanisms to encounter ROS that is of enzymic and non-enzymic nature. Coordinated activities of these antioxidants regulate ROS detoxification and reduce oxidative damage in plants. The formation of ROS is prevented by an antioxidant system: low molecular mass antioxidants (ASA, GSH, tocopherols), enzymes such as SOD, CAT, POD and enzymes regenerating the reduced forms of antioxidants (APX, MDHAR, DHAR, GR, GPX). Antioxidants employ a series of redox reactions and interactions between ASA, GSH and phenolic compounds which are well known. However reports on molecular aspects of heavy metal on the redox state of GSH and ASA, and their redox couple GSH/GSSG, AsA/MDHA is lacking in plants. Mechanisms involved in oxidative stress are important because many metals such as Fe, Mn Cu and Zn are known to activate different antioxidant systems such as SOD, CAT, POD, APX and GPX. At the same time these metals (Cu, Fe, Mn and Zn) in excessive amounts cause uncontrolled redox reactions and transfer electrons directly from the metal ion to form free radicals, e.g. $O_2^{\bullet -}$. Although exhaustive work has been done on generation of ROS and response of defense molecules in plant system but more pertinent studies need to be carried out especially in relation to the activities of antioxidant enzymes and changes in their isoforms during heavy metal mediated stress in plant.

If mechanisms related to oxidative stress are involved in metal tolerance, these mechanisms should be differently expressed in plants that are resistant and sensitive to nutrient stress and need to be further explored for providing protection to plants. Thus, metals may activate a cascade of antioxidative systems. However there are variations in the antioxidant responses of plant to different heavy metal toxicities and over expression of antioxidant capacity need not always relate to the required level of protection. Therefore it is essential to correlate the compartmentalization of ROS formation and antioxidant localization, for an efficient antioxidative system. The ROS-induced responses and nutrient signaling cascades in plants need to be studied. In the future, it will be important to understand how these signals regulate changes in plant function and growth under metal toxicity and thus affect the crops and help in their sustainable productivity.

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