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A Review on The Role of Antioxidant Defence Machinery in Plants in Response to Zinc and Cadmium Stress

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Abstract
Being sessile in nature, plants are exposed to different elements present in the environment. Among these, several micronutrients like Zn, Fe, Cu, Mg, Mn, etc. are absorbed from the soil and utilized for their proper nutrition. These elements are essential for plant growth. However, excess amounts of these elements can become toxic for plants. On the other hand, elements like Cd, Hg, Cr, Pb, Ni, etc. have no known biological function in plants. They are also absorbed into the plant species and damage normal metabolic processes. Some plant species show extreme tolerance to one or more elements and can accumulate significant amount of heavy metals into their aerial parts. These plants are called hyperaccumulators. They have developed major strategies to resist high heavy metal exposure. In this review, we have prepared a detailed and comprehensive account on the toxicity of an essential element, namely zinc and a non-essential element, namely cadmium as well as the oxidative biomarkers of stress and the detoxification mechanisms present in plants.

Keywords
Zinc, cadmium, antioxidants, non-protein thiols.

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INTRODUCTION
Elements are essential components of the environment. But all the elements are not useful for the growth and development of biological organisms. Some elements are required for the proper nutrition of plants, deficiency or absence of these elements may cause malfunctioning of the living system. These are termed as 'essential' elements [1], cobalt, copper, iron, molybdenum, manganese, zinc etc. are some of these essential elements. On the other hand, there are some elements, which do not have any beneficial role in the biological system, rather they can induce toxicity even in very minute concentrations. These are termed as 'non-essential' elements [2] and arsenic, cadmium, chromium, lead, mercury, tin, etc. are a few examples of those. Both essential as well as non-essential elements can have harmful or deleterious effects depending upon their concentrations. Essential elements can induce toxicity beyond a threshold value [3], which is quite high. On the contrary, non-essential elements can cause it at a very low threshold concentration. These elements can disrupt the redox homeostasis of the cell [4] and cause oxidative stress. Plants respond to this kind of stress by activating their defence mechanism [Figure 1]. In this review, we are going to

discuss the toxicity of zinc and cadmium on plants and summarize our current knowledge of the various antioxidant defense mechanisms that plants employ to cope with heavy metals.

Cadmium and zinc are also designated as heavy metals. They are produced mainly from mining, industrial, agricultural and geochemical sources [5]. Cadmium is a toxic metal which can interfere with the uptake of essential micronutrients [6]. It is highly mobile and so, it can readily travel from the roots to the aerial parts [7]. On the other hand, zinc is an essential micronutrient which is required for the proper functioning of many enzymes [8] and for the stabilization of lipid membranes [9]. Zinc atoms bind to different protein domains which in turn, interacts with other proteins, RNA or DNA. One of the most important DNA binding proteins is the transcription factor TFIIIA, having zinc finger motiff [10].

CADMIUM TOXICITY AND ITS ENVIRONMENTAL IMPACTS

Cadmium has been designated as 'one of the ten leading metals of concern' by WHO (2006) [11]. Cadmium exists naturally in cadmium ores and as a minor component in zinc ores. Hence it is released as a by-product of refining zinc. It is a common component of commercial fertilizers used in agriculture (insecticides and fungicides), nickel-cadmium batteries, paints, industrial wastewater, plastics and neutron absorbent in neutron reactors [12]. The safe limits for cadmium in agricultural soils is 3 µg g^{-1} (European Union Standards, 2002) [13].

ZINC TOXICITY AND ITS ENVIRONMENTAL IMPACTS

Zinc has been found in at least 985 of the 1,662 current or former National Priorities List (NPL) sites [14]. Zinc occurs naturally in sphalerite in the form of zinc sulfide. The major anthropogenic sources of zinc come from discharges of smelter slags and wastes, mine tailings, coal and bottom fly ash, sewage sludge and utilization of commercial products such as fertilizers and wood preservatives. When zinc is consumed in large amounts, several harmful health effects can be seen in humans too. These range from stomach cramps, nausea, vomiting, anemia and even pancreatic damage [15]. Though several regulatory steps have been implemented to restrict the release of pollutants in the soil, they are not sufficient for checking the contamination. The safe limits for zinc in agricultural soils is 300 µg g^{-1} (European Union Standards, 2002) [13]. Keeping in mind the environmental impact and toxicity of zinc and cadmium, these two metals are the focus of this review.

THE BIOLOGICAL ROLE AND EFFECTS OF ZINC AND CADMIUM ON PLANTS AND HUMAN HEALTH

Non-essential metals like cadmium and essential metals like zinc at high concentrations are extremely toxic for plants. However, zinc at normal concentrations have numerous biochemical and physiological functions in plants and animals. Two major characteristics of essential heavy metals are the following: (a) Participation in redox reaction, and (b) Being an integral part of several enzymes [16]. Several enzymes such as carboxypeptidases, pyruvate carboxylase, glyoxalase and superoxide dismutase contain zinc. Zinc is required to maintain the integrity of ribosome. Zinc finger proteins are a diverse family of proteins that serve for a wide range of biological functions. These proteins require coordination by at least one zinc ion. These zinc ions serve to stabilize the integration of the protein. Zinc also provides a structural role in many transcription factors and is a cofactor of RNA polymerase. The linking of zinc fingers to the corresponding sites on DNA initiates the process of transcription. It takes part in the formation of carbohydrates and catalyzes the oxidation processes in plants. This essential trace metal nutrient takes part in redox reactions, electron transfer and structural functions in nucleic acid metabolism [2].

Heavy metals such as cadmium, a toxic non-essential element, is strongly poisonous for metal-sensitive enzymes. The threshold value for cadmium in soils for plant toxicity is 3 mg kg^{-1} [17]. The plants grown in cadmium poisoned soils showed visible symptoms of injury which is revealed in terms of chlorosis [18], browning of root tips [19] and eventually death [20]. Photosynthesis is greatly harmed in cadmium treated plants. There are several reports that show that cadmium interferes with the uptake, transport and use of several elements (calcium, magnesium, phosphorus and potassium) by plants. Metal toxicity can affect the plasma membrane permeability, and thus makes them susceptible to peroxidation of membrane lipids [2]. Cadmium accumulates in the liver and kidneys and has a long biological half-life of 10-30 years in humans [21]. The International Agency for Research on Cancer of USA has classified cadmium as a #1 category human carcinogen [22].

The phytotoxic concentration of zinc has been measured to be in the range of 150-300 mg kg^{-1} of soil [3]. Zinc is an essential micronutrient that affects several metabolic processes of plants and has a long biological half-life i.e., 280 days [16]. Excess zinc concentrations inhibited growth in *Vigna mungo* [23], *Spartina densiflora* [24] and *Lablab purpureus* [25]. The symptoms of zinc toxicity were typically

displayed as a yellow colour on the lower leaves starting from the tips and spreading toward the bases of the leaves, which became more severe as the experiment continued. The dry weight of shoot and root of sugar beet (*Beta vulgaris* L.) decreased as the concentration of zinc in the nutrient solution increased [26].

ORIGIN AND GENERATION OF ROS

In order to fully understand the role of antioxidative defense in plants, we should have a clear idea of all the sources that can activate the antioxidant system. The presence of heavy metals can induce oxidative stress as well as induce the activation of several defense factors in plants. An inevitable result of membrane-linked electron transport in mitochondria and chloroplast is the spilling of electrons onto molecular oxygen in plant cells and thus, the generation of highly toxic reactive oxygen species (ROS). The ground state of oxygen or the energetically favorable state ($^3O_2$) does not react with organic molecules easily. This triplet state of oxygen is unable to react because it has two unpaired electrons with opposite spins. That is why activation is necessary for oxygen to react. This activation occurs by any of the following ways: a) If molecular oxygen absorbs sufficient light energy, the spin on any one of the electrons is reversed forming singlet oxygen, which is a very hazardous ROS. This happens only when the excess light energy absorbed by the photosynthetic machinery cannot be successfully dissipated. b) Another form of activation is via the partial reduction of oxygen by adding one, two and three electrons forming superoxide radical, hydrogen peroxide and hydroxyl radical respectively [10].

Approximately 1% of O_2 taken up by plants is channeled to produce ROS in various subcellular compartments. The major ROS capable of causing oxidative damage include superoxide ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$).

MAJOR TYPES OF ROS

Superoxide ($O^{\cdot-}_2$) is a moderately reactive radical, with a half-life of approximately 2-4 µs and mobility of 30 nm [27]. It cannot pass through biological membranes as it is readily dismutated to hydrogen peroxide [28]. It is normally formed in the Mehler reactions, mitochondrial electron transport chain reactions, glyoxisomal photorespiration and in NADPH oxidases, xanthine oxidases, membrane polypeptides, in peroxisomes and plasma membranes. It can either transfer its excitation energy to other biological molecules or continue with them, thus forming endoperoxides or hydroperoxides [29]. It reacts with the double bond containing iron-sulphur proteins and reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$) [30].

Hydroxyl radical ($OH^{\cdot}$) is a highly reactive radical, with a half-life of 1 ns and mobility of 1 nm. It is formed by the reaction of hydrogen peroxide with a) superoxide radical (Haber-Weiss reaction) and b) Fe^{+2} (Fenton reaction) [31]. Hydroxyl radicals can affect the integrity of pigments, proteins, lipids, nucleic acids and macromolecules by reacting with them [32].

Hydrogen peroxide (H_2O_2) is a highly reactive radical, with a half-life of approximately 1 ms and mobility of 1 µm [32]. It is formed in the electron transport complexes of mitochondria, endoplasmic reticulum, chloroplasts and plasma membrane and during photorespiration and fatty acid oxidation. NADPH oxidase, pH-dependent cell wall-peroxidases, oxalate oxidases and amine oxidases have been proposed as a source of H_2O_2 in apoplast of plant cells [31]. At low concentrations, it serves as a messenger in the stress signaling response as it freely travels through membranes. However, at supra-optimal concentrations, it results in programmed cell death [33].

Singlet oxygen ($^1O_2$) is highly reactive radical, with a half-life of 1 µs and a mobility of 1 nm [32]. It is primarily formed during the electron transfer reactions at photosystem II in the chloroplast. It may also result from lipoxygenase activity. It can interact with other target biomolecules either by transferring its excitation energy or by chemical reactions [27]. It prefers conjugated double bonds in polyunsaturated fatty acids (PUFAs) or guanine bases in DNA as their target for oxidative attack [32].

Peroxynitrite ($ONOO^-$) is a highly reactive radical, with a half-life of approximately 1 s. It is formed when nitric oxide diffuses into a cellular compartment containing superoxide. It readily oxidizes target molecules which are close to it. It is a powerful oxidizing agent which can initiate lipid peroxidation, oxidize sulfhydryls, and nitrate the aromatic residues of proteins [30,34]. However, if no oxidizable targets are present, peroxynitrite will decompose harmlessly. It also affects mitochondria function and triggers cell death via oxidation and nitration [35].

Besides the above listed types of ROS, other types of reactive intermediates are also produced like per hydroxy, alkoxyl and peroxyl radicals. These toxic intermediates pose a serious threat to several macromolecules.

Under steady state conditions, these ROS molecules are scavenged by various antioxidative defense mechanisms. However, when stress is imposed, the equilibrium between the production and scavenging of ROS is disturbed. The increased concentration of reactive oxygen species causes redox imbalances and damages cell structures. To protect themselves against these toxic intermediates, plant organelles like mitochondria, chloroplast and peroxisomes employ a number of antioxidant defense mechanisms. These mechanisms involve a vast number of the ascorbate-glutathione cycle enzymes and several non-enzymatic compounds.

NON-ENZYMATIC ANTIOXIDANT DEFENSE MECHANISMS IN PLANTS.

The non-enzymatic compounds which play a major defense strategy against excess ROS include ascorbic acid, glutathione, carotenoids, tocopherols, proline, phenols, flavonoids and non-protein thiols.

ASCORBIC ACID (AsA)

Ascorbic acid is one of the very potent water-soluble antioxidants presents in high concentration in all plant cells [32]. It is present in millimolar concentrations in leaves and even in fruits. Ascorbic acid, also known as vitamin C, exists mostly in the reduced form (90% of the ascorbate pool) in chloroplasts [27]. Ascorbic acid is an efficient electron donor and participates in many enzymatic and non-enzymatic reactions. This makes ascorbic acid the main ROS-detoxifying compound. It reacts directly with singlet oxygen, hydrogen peroxide, hydroxyl radical and lipid hydroperoxides [27]. Ascorbate also acts a substrate to reduce hydrogen peroxide to water which is catalyzed by ascorbate peroxidase [28].

Cadmium treatment (10, 50 and 100 µM) in *Helianthus annuus* L.cv. Vedoc hybrid seedlings led to increased ascorbate production [36]. The ascorbate levels increased slightly in 5 µM zinc stressed *Hibiscus esculentus* plants which were treated for 4 weeks [37]. *Vigna mungo* L. (var. DPU_88_31) plants showed significantly higher production of ascorbate when they were treated with zinc sulphate solution for 50 days after seed germination [23].

GLUTATHIONE (GSH)

Glutathione is a tripeptide containing glutamate, cysteine and glycine. It is one of the most crucial intracellular defense mechanisms against ROS induced oxidative damage. It is localized in all cell compartments like cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes as well as in apoplast [40,41]. It exists in two principal forms: the reduced form, represented by GSH and the oxidized form, represented by GSSG. The two primary physiological functions of glutathione can be categorized as: - a) It is involved in the sulphur metabolism and b) in defense reactions i.e., detoxification of xenobiotics against oxidative stress. It induces the expression of stress responsive genes [32]. A normal concentration of glutathione is necessary to resist the inhibitory effects of ROS induced oxidative stress. It is a potential scavenger of $^1O_2$, H_2O_2 and a very dangerous ROS, hydroxyl radical ($OH^{\cdot -}$) [27,32].

The leaves of *Camellia sinensis* plants, treated for 24 hours with 100 µM Cd induced the expression of glutathione metabolic genes [19]. Glutathione accumulation was observed in response to the effect of cadmium stress in sunflower plants (*Helianthus annuus* L.cv. Vedoc hybrid) [36]. The roots of safflower (*Carthamus tinctorius)* seedlings when subjected to cadmium stress showed enhanced glutathione levels [38]. Glutathione levels were high in the leaves of hyacinth bean (*Lablab purpureus)* stressed with 100 and 600 µM zinc chloride after 48 hours but decreased after 72 hours [25].

CAROTENOIDS

Carotenoids are a ubiquitous group of isoprenoid pigments. They are important precursors of retinol (vitamin A). They have an important protective role during photosynthesis as these molecules can quench the excited states of chlorophyll. In this way, they avoid the production of singlet oxygen [39]. Thus, they are efficient physical and chemical quenchers of singlet oxygen and scavengers of reactive oxygen species. The triplet energy levels of carotenoids are very close to that of $^1O_2$. That is why they can efficiently quench singlet oxygen ($^1O_2$), both *in vitro* and *in vivo* [40].

Cadmium enhanced the carotenoid concentration in the leaves and shoots of *Jatropha curcas* L. [41]. The levels of carotenoids in zinc stressed *Vigna mungo* L. (var. DPU_88_31) plants increased after 20 and 40 days of treatment [23]. Carotenoid content in zinc treated seedlings of *Sesuvium portulacastrum* L. was the highest only at 200 mg kg^{-1} zinc stress [42]. [60].

Zinc stressed seedlings of *Phaseolus vulgaris* L. showed a steady increase in carotenoid production when treated with zinc for 10 days [43].

TOCOPHEROLS

Tocopherols are a class of lipid soluble compounds synthesized by photosynthetic organisms. The major isomer of Vitamin E is α-tocopherol. Plants synthesize tocopherols by certain enzymes localized in the chloroplast membrane [44]. This antioxidant is a scavenger of ROS produced by the triplet state of chlorophyll in PSII reaction center [28,44]. Besides, it can terminate chain reactions occurring during lipid peroxidation [33]. A large amount of tocopherols are needed to stop the auto-oxidation of chloroplast membranes.

In *Phaseolus vulgaris* L. seedlings, treated with 0.05, 0.06- and 0.08-mM cadmium, α-tocopherol increased by 14.4, 14.8 and 15.1%, respectively [45]. After 30 days of Cd exposure to 1.0, 5.0, and 10.0 mg kg^{-1}, a 21, 35, and 48 % increase in α-tocopherol content was noticed respectively, compared to the control in young leaves of alfalfa. 20 day-olds hydroponically grown (*Phaseolus vulgaris* L. cv. Limburgse vroege) seedlings showed a marked increase (5.9%, 9.1% and 12.2%) at 1.5, 2.0- and 2.5-mM zinc concentrations [46].

PROLINE

Proline is an amino acid of low molecular weight and high solubility. It is a compatible solute that functions as an osmolyte. It has been recently reported to provide plant resistance to oxidative stress by scavenging reactive oxygen species like singlet oxygen and hydroxyl radicals [47]. It is also involved in the protection of membrane integrity and enzyme stabilization. Also, it increases the activity of antioxidative enzymes while maintaining redox homeostasis [48]. Although the concentration of proline has been shown to increase under heavy metal stress in certain plants, its exact role in heavy metal detoxification is unclear [49].

Proline accumulation in the roots of hybrid poplar increased significantly in the roots compared to that of shoots when treated with cadmium (10^{-4} M) [50]. Proline accumulated in the leaves of *Arachis hypogaea* L. with increasing cadmium concentrations [51]. Cadmium stress (10^{-3} M Cd) caused accumulation of proline in the shoots and roots of 1-week old hydroponically grown wheat seedlings (*Triticum aestivum* L. cv. Alföld-90) [52]. Zinc stressed leaves of 10-day old *Lablab purpureus seedlings* produced a higher concentration of proline after 72 hours of 100 mM, 300 mM and 500 mM Zn exposure [25]. In the 500 µM $ZnCl_2$ treated *Brassica oleracea var botrytis* seedlings, there appears to be a nearly two-fold increase in proline accumulated after 15 hours, compared to control [53].

FLAVONOIDS

Flavonoids are a group of diverse hydroxylated polyphenolic compounds. The functional hydroxyl groups of flavonoids are responsible for detoxifying free radicals and/or chelating metal ions [54,55]. These compounds can chelate many ions of metals and form different complexes. Metal-flavonoid complexes have a much stronger free radical scavenging properties than the free flavonoids. They donate electrons and hydrogen to hydroxyl, peroxyl and peroxynitrite radicals and stabilizes them [56].

It was reported that the total flavonoid content increased in 5 µg g^{-1} Cd, but it decreased at 50 µg g^{-1} Cd in *Erica andevalensis* plants after 5 days of Cd treatment [57]. The flavonoid levels in the leaves of two cultivars of *Ocimum basilicum* L. (Ardestan and Isfahan) [58] showed a gradual increase in response to increasing cadmium treatments after 18 days of treatment.

PHENOLICS

Phenolics are characterized by at least one aromatic ring (C_6) bearing one or more hydroxyl groups [55]. Phenolics possess hydroxyl and carboxyl groups, which are able to bind to iron and copper. They may inactivate iron ions by chelating and additionally inhibiting the Fenton reaction, which is believed to be the most important source of ROS. They terminate lipid peroxidation by inhibiting the lipid peroxyl radical. They are able to alter peroxidation kinetics by modifying the lipid packing order. They stabilize membranes by decreasing membrane fluidity and hinders the diffusion of free radicals [56,57].

The cadmium stressed plants of *Erica andevalensis* also showed enhanced phenolic content [57]. Two cultivars (Ardestan and Isfahan) of *Ocimum basilicum* L. showed an increase in the total phenolic content when they were treated with 75 µM Cd concentration for 18 days [58]. There was a steady rise in the total phenol content of 30-day old *Brassica rapa* seedlings subjected to 0.2, 0.4- and 0.6-mM Cd treatments [59]. The phenol content in 1-year old *Citrus reticulata* plants, treated with zinc sulphate solutions for 14 weeks, increased with increasing zinc concentration up to 5 mM and thereafter, the phenolic content was reduced in both leaves and roots [60].

NON-PROTEIN THIOLS

Glutathione (GSH) is the most abundant non-protein thiol, present in the millimolar range in most cells [61]. These low molecular weight thiols containing compounds have an essential role because they are easily oxidized and rapidly regenerated [62]. All plants contain GSH or GSH homologues, where the *C*-terminal amino acid glycine is replaced by another amino acid, such as β-alanine, serine or glutamate [63]. Glutathione is involved in the scavenging of ROS. It is usually synthesized in the cytosol, chloroplasts and mitochondria [64,65]. It is also essential for the synthesis of cadmium-binding peptides such as phytochelatins, which inactivate and sequesters cadmium by forming stable cadmium-complexes in the vacuole [66].

Non-protein thiol compounds were markedly increased in the wheat variety Balcali-85 with increasing cadmium supply [67]. In response to increasing cadmium treatments (5, 100, 200 µM), *Pisum sativum* cv. *Swati* plants accumulated a significant amount of non-protein thiols [68]. Toxicity induced by zinc in hydroponically grown *Glycine max* L. seedlings produced increased levels of non-protein thiols [69]. The non-protein thiols increased in zinc treatments of 400 and 600 µM in *Zea mays* L. treated for 12 days [70].

ENZYMATIC ANTIOXIDANT DEFENSE MECHANISMS IN PLANTS

The enzymatic components of the antioxidative defense mechanism constitute several antioxidant enzymes, some of which are listed as follows:

SUPEROXIDE DISMUTASE

The enzyme superoxide dismutase is a very crucial factor for defense against oxidative stress. It catalyzes the dismutation of superoxide molecules into hydrogen peroxide and oxygen [71]. This hydrogen peroxide is subsequently converted to water by peroxidases and catalases. This reduces the chance of hydroxyl formation via the metal catalyzed Haber-Weiss reaction. Based on the metal co-factor used by the enzyme, SODs can be classified into three groups: - copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD) and iron SOD (Fe-SOD) [32]. These three forms are encoded in the nucleus and targeted to their respective locations via a amino acid targeting sequence [72]. Cu/Zn SOD are localized in the cytosol, chloroplast, peroxisome and mitochondria. Fe-SODs are found in the chloroplasts, whereas Mn-SODs are found in the mitochondria [73].

SOD activities enhanced progressively in *Cucurbita maxima* when they were treated with cadmium for 24 hours and was the highest at 250 ppm Cd [74]. SOD activities increased significantly when *Cleome gynandra* plants were treated with 100 and 200 mg kg^{-1} cadmium chloride for 4 weeks [75]. *Oryza sativa* L. Xiushui 110 seedlings exposed to high cadmium concentrations (0.5 mM Cd) for 24 hours showed an increase in SOD activity [76]. Zinc treatments (5 µM) in *Ceratophyllum demersum* L. produced a 2.6-fold increase in SOD activities [77]. The root cells of soyabean showed increasing SOD activities with increasing Zn concentrations (500, 1000 and 2000 µM) for 5 days [69].

CATALASE

Catalase is a tetrameric heme-containing enzyme [27] which catalyzes the dismutation of hydrogen peroxide into water and oxygen. These enzymes are unique because unlike most other enzymatic antioxidants, they do not require a reducing substrate. Catalase breaks down the H_2O_2 formed by the glycolate oxidase in the C2 photorespiratory pathway and during β-oxidation of fatty acids [16]. This breakdown occurs by a two-step mechanism in which the hydrogen peroxide alternately reduces and oxidizes the heme iron at the active site. In the first step, one hydrogen peroxide molecule oxidizes the heme to an oxyferryl species. In the second step, a second hydrogen peroxide molecule is used as a reductant to regenerate the enzyme, producing water and oxygen. Significant catalase activity was also reported in the cytosol, chloroplast and mitochondria. All angiospermic species contain three CAT genes. Class I CAT genes are expressed in the green photosynthetic tissues. Class II and Class III CAT genes are highly expressed in vascular tissues and in young seedlings, respectively [78].

In the leaves of *Pfaffia glomerata treated with* 90 µM Cd L^{-1} after 24 hours, the maximum increase in CAT activity was of 54% [79] in relation to the control. However, at this same concentration a maximum reduction was observed, down to 34%, after 20 days. Phytotoxic concentrations of cadmium led to increased catalase activity in *Cucurbita maxima* Duchesne seedlings [74]. Significant catalase activity was recorded in the leaves of *Jatropha curcas* L. on treatment with 50 mg kg^{-1} cadmium for one month [41]. Catalase activity in the hypocotyls and radicles of *Glycyrrhiza uralensis* seedlings increased significantly with the increasing cadmium concentrations up to 0.2- and 0.1-mM L^{-1}, representing 35.1% and 89.9% increments, respectively [80]. Leaf CAT activity of wheat (*Triticum*

aestivum L., cv Xihan 3) seedlings significantly enhanced in response to different Zn concentrations (0.5, 1- and 3-mM Zn) [81]. In contrast, root CAT activity was not changed by 0.5 mM Zn concentration, but exposure to 1- and 3-mM Zn resulted in distinctly increased CAT activity. Wheat seedlings exposed to different concentrations of zinc showed increased catalase activity. Rice (*Oryza sativa* L. cv. Tarom Hashemi) seedlings exposed to zinc treatments showed an increase in catalase activity at 5 µM Zn but decreased at higher Zn concentrations (50 and 100 µM [82].

GUAIACOL PEROXIDASE

Guaiacol peroxidase is a heme-containing protein [32] which oxidizes certain substrates like ascorbate at the expense of hydrogen peroxide. They prefer electron donors such as guaiacol and pyrogallol [83]. They are important in biotic and abiotic stresses by consuming H_2O_2. They have been widely accepted as a 'stress' enzyme. Isozymes of guaiacol peroxide are localized in the cytosol, vacuoles and cell wall [32]. It is an effective quencher of reactive intermediary forms of oxygen and peroxy radicals under stressed conditions [84,16].

GPx activity was 144% higher than control in *Ceratophyllum demersum L*. with cadmium treatment [77]. Guaiacol peroxidase activity increased in the leaves of *Pfaffia glomerata* to a maximum of 183% and 95% (at 90 µM L^{-1} after 12 and 20 days, respectively) [79]. The activity of GPx increased at all concentrations (100, 300 and 600 µM $ZnCl_2$) after 48 hours of stress in the leaves of *Lablab purpureus*[26].

GLUATHIONE PEROXIDASE

They are a family of multiple enzymes which catalyze the reduction of hydrogen peroxide, organic hydroperoxides and lipid hydroperoxides by reduced glutathione [85]. Interestingly, this is one of the rare proteins which contain the amino acid selenocysteine which is encoded by TGA- a stop codon. Since this amino acid has a selenium in its structure, this amino acid has a strong nucleophilic power. In this way, glutathione peroxidase proves to be a powerful redox agent towards its substrates [32].

Activity of glutathione peroxidase was higher in 0.2- and 0.6-mM Cd treated *Brassica juncea* seedlings treated for 30 days [86]. Treatment with Zn at 5, 10, 20 and 40 mM for 4 weeks in *Hibiscus esculentus* cv. *Hassawi* plants showed decreased glutathione peroxidase levels [37].

POLYPHENOL OXIDASE

Polyphenol oxidase is an oxidoreductase enzyme that catalyzes the oxidation of monophenols to o-diophenols and o-dihydroxyphenols to o-quinones. The quinine products then polymerize and reacts with amino acid groups of cellular proteins, resulting in black and brown deposits. This enzyme requires copper atoms for its proper functioning. It has also been associated to a catalase-like activity and could thus have a role in direct hydrogen peroxide removal [87].

In the cotyledons and radicles of *Glycyrrhiza uralensis* treated with cadmium (0.2 mmol), PPO activity was highly affected. It had increased by 114.8% and 44.4% compared to the control, respectively [80]. Polyphenol oxidase activity was increased in cadmium treated (20, 30 and 50 mg kg^{-1} Cd) roots of *Jatropha curcas* L. for one month, in relation to control [41]. Zinc stress (100, 300 and 600 µM) enhanced the activity of polyphenol oxidase in the leaves of Hyacinth bean (*Lablab purpureus* L.), when treated for 24, 48 and 72 hours [25].

Besides the enzymes mentioned above, the enzymes of the ascorbate-glutathione cycle are involved in ROS detoxification. The change in the ratio of AsA to DHA and GSH to GSSG is crucial for the cell to sense oxidative stress and respond accordingly [88]. It occurs in several cell compartments, like chloroplasts, cytosol, mitochondria, peroxisomes and apoplast. It uses ascorbate peroxidase and glutathione peroxidase as well as other enzymes like glutathione reductase, which is important to maintain the pool of reduced glutathione. Thus, AsA-GSH cycle involves successive oxidation and reduction of AsA, GSH and NADPH catalyzed by the enzymes APX, MDHAR, DHAR, and GR. These enzymes are discussed in detail below.

ASCORBATE PEROXIDASE (APX)

Ascorbate peroxidase is the principal enzyme of the ascorbate-glutathione cycle. Ascorbate peroxidase uses ascorbate as its specific electron donor to reduce hydrogen peroxide to water with the concomitant generation of monodehydroascorbate, a univalent oxidant of ascorbate [32]. It belongs to Class I heme-peroxidases that is found in higher plants [16]. The heme cofactor serves as the site for the redox reaction shown below: -

$$\mathbf{C_6H_8O_6 + H_2O_2 \rightarrow C_6H_6O_6 + 2H_2O}$$

Five isoforms of ascorbate peroxidase have been reported: - cytosolic, mitochondrial, stromal, thylakoidal and peroxisomal isoforms [32]. The

organeller isozymes work within their compartments and the cytosolic isozyme removes any excess peroxide from the cytoplasm and even the apoplast [88]. The chloroplast and mitochondrial isozymes have a high specificity for ascorbate as their electron donor. In addition to ascorbate, these chloroplast and mitochondrial isozymes can also oxidize guaiacol or pyrogallol. APx has a higher affinity for H_2O_2 as compared to catalases and other peroxidases. However, when the concentration of ascorbate is less than 20 µM inside the cell, APX activity is rapidly lost [88]. Peroxidases can be divided into three forms based on their electron donors: guaiacol peroxidase; uses mainly phenolic donors, ascorbate peroxidase; uses ascorbic acid and glutathione peroxidase; uses glutathione [32].

Increase in the concentrations of cadmium resulted in an increase of the APx activity in the leaves of *Phaseolus vulgaris* L. seedlings, when treated with varying concentrations of Cd for 15, 30 and 45 days [45]. APx activities increased in response to zinc stress (500, 1000 and 2000 µm) in the seedlings of *Glycine max* L. treated for 5 days [69].

MONODEHYDROASCORBATE REDUCTASE (MDHAR)

This is a flavin adenin dinucleotide (FAD) enzyme whose main function is to regenerate ascorbate using NAD(P)H as the electron donor [32]. Asada [83] studied the multi-step reduction of this enzyme in detail. The first step is the reduction of enzyme-FAD to form a charge transfer complex. In the second step, the reduced enzyme donates electrons successively to MDHA. This results in two molecules of ascorbate. The thylakoidal, mitochondrial, cytosolic and peroxisomal isozymes of MDHAR are reported till date [88]. In the chloroplast, the thylakoid bound isozymes serve two purposes: a) In presence of monodehydroascorbate, ascorbate regeneration takes place. b) In absence of monodehydroascorbate, this enzyme mediates the photoreduction of dioxygen to singlet oxygen [32]. Cadmium treated *Spartina densiflora* plants showed an increase in MDHAR activity [24].

DEHYDROASCORBATE REDUCTASE (DHAR)

It is a monomeric thiol enzyme and has been purified and characterized from rice [89], potato [90] and spinach plants [91]. This enzyme catalyzes the regeneration of ascorbate from dehydroascorbate (the oxidized state of ascorbate) and oxidizes reduced glutathione (GSH) to produce oxidized glutathione (GSSG). In the Asada-Halliwell pathway, ascorbate is oxidized to monodehydroascorbate, which is again converted to dehydroascorbate via spontaneous disproportionation or further oxidation [32]. For maintaining a proper level of ascorbate, the proper functioning of this particular enzyme is very important. Isoforms of DHAR have been reported in the cytosolic and chloroplast in *Arabidopsis sp.* [32]. At 1000 µM Cd treatment for 27 days, DHAR activity had increased in the leaves of *Spartina densiflora* [25]. DHAR activity in the shoots of *Hibiscus esculentus* cv. *Hassawi*[37] had increased with increasing Zn concentrations (5, 10, 20 and 40 mM Zn for 30 days).

GLUTATHIONE REDUCTASE

Glutathione reductase is a NAD(P)H-dependent enzyme and belongs to a group of flavoenzymes [87]. It contains an essential disulfide group. It catalyzes the conversion of oxidized glutathione disulfide (GSSG) to the reduced glutathione form (GSH)[33]. This enzyme is critical in combating oxidative stress for sustaining a reducing environment. It can act as a scavenger for hydroxyl radicals, singlet oxygen, and various electrophiles [85].

The leaves of *Phaseolus vulgaris* L. seedlings treated with increasing cadmium concentrations from 1.5 g kg to 2.5 g kg^{-1} showed a gradual rise in GR activity [45]. GR activity also increased in the leaves of *Lablab purpureus* L. treated with different concentrations of zinc chloride (100, 300 and 600 µM Zn for 72 hours) [26]. Increase in GR activity was observed with increasing zinc concentrations (500, 1000 and 2000 µM) in the shoots and roots of *Glycine max* L. [69]. *Triticum aestivum* L. cv. *Xihan 3* plants treated with Zn (0.5, 1 and 3 mM) for 6 days showed significant increase in leaf GR activity. However, in the roots, GR activity was markedly inhibited [81].

CONCLUSION

From this review, it is quite clear that above threshold levels of zinc and very minute concentrations of cadmium can induce a state of oxidative stress and activate the antioxidant defense system in plants. Hyperaccumulators are defined as a group of plants which are capable of accumulating large amounts of heavy metals and transporting it to the above-ground tissues. Till date, about 450 angiospermic species have been classified as heavy metal hyperaccumulators [92]. These plants are known to chelate metal ions and confine them to the cell walls or sequester them into vacuoles. These plants also have excellent antioxidant defense mechanisms to detoxify ROS and maintain redox homeostasis. The antioxidant defenses could be either non-enzymatic (e.g. glutathione, ascorbate, proline, α-tocopherols, carotenoids, phenols and

flavonoids) or enzymatic (e.g. superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase). Overexpression of ROS scavenging enzymes like isoforms of superoxide dismutase (Mn-SOD, Cu/Zn-SOD, Fe-SOD), catalase, guaiacol peroxidase, glutathione peroxidase, polyphenol oxidase, ascorbate peroxidase, mono dehydro reductase, dehydro reductase and glutathione reductase resulted in heavy metal stress tolerance in various plants due to efficient ROS scavenging capacity. In our earlier publication [93], we have already reported that young castor seedlings exposed to excess zinc and cadmium stress have produced enhanced levels of antioxidants namely, carotenoids, proline, non-protein thiols, phenolic compounds, flavonoids and several enzymatic antioxidants like superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase. Castor is one such hyperaccumulator which has already been reported to accumulate large amounts of lead [94]. Therefore, in the future, plants with the ability to tolerate such toxic heavy metals can be used for any further investigations about the antioxidative defense system in plants.

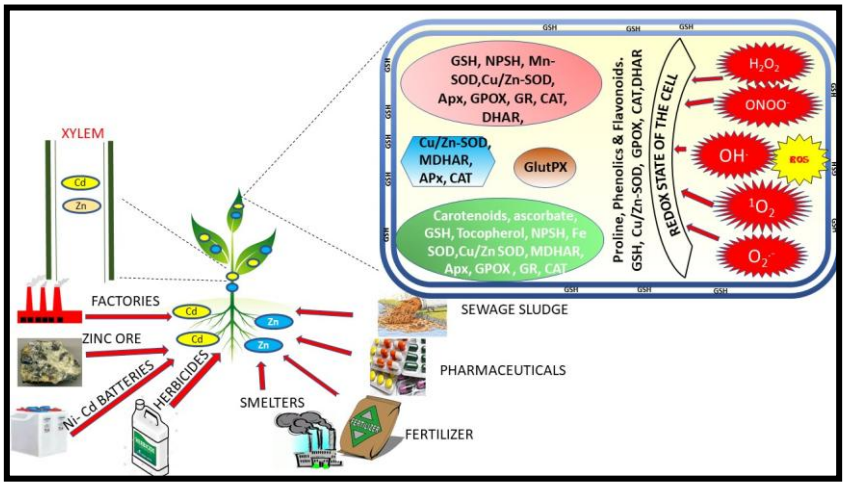

Figure1: Heavy metal uptake, transport and detoxification in plants. Heavy metals such as cadmium (marked as yellow) and zinc (red) are produced from various natural and anthropogenic sources. They are translocated from roots to the leaves via xylem. Cadmium in low concentrations and zinc at higher above threshold concentrations can activate the excess production of reactive oxygen species (ROS). The common ROS found in plants are H_2O_2-Hydrogen peroxide, $ONOO^-$-Peroxynitrite, $^1O_2$-Singlet oxygen, $OH^{\cdot}$ -Hydroxyl, $O^{\cdot-}_2$-Superoxide. As a result, the redox state of the cell is disrupted. In response to this stress, plants activate their antioxidative enzymes. Antioxdative enzymes, both enzymatic and non-enzymatic, are located in different organelles in the cell. The mitochondria (pink oval shape) houses GSH, NPSH, Mn-SOD, Cu/Zn-SOD, Apx, GPOX, GR, CAT. In the chloroplast (green oval shape), carotenoids, ascorbate, GSH, tocopherol, NPSH, Fe-SOD, MDHAR, GPOX, GR are stored. The peroxisome (blue heaxagon) provides Cu/Zn-SOD, MDHAR, APx, CAT and finally, glutathione peroxidase can be found in the endoplasmic reticulum (brown oval shape). GSH can also be seen confined in the cell walls (apoplast).[Abbreviations:Mn-SOD-Manganese superoxide dismutase, Fe-SOD-Iron superoxide dismutase, Cu/Zn-SOD-Copper/Zinc superoxide dismutase, MDHAR-Monodehydroreductase, GlutPX-glutathione peroxidase, Apx-Ascorbate peroxidase, GPOX-Guaiacol peroxidase, GR- Glutathione reductase, CAT-Catalase, GSH- glutathione,NPSH- non-protein thiols]

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