Heavy Metal-Induced Toxicity Responses in Plants: An Overview from Physicochemical to Molecular Level

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1 Introduction

Extensive industrialization coupled with unsustainable development approach has generated wastes and pollutants that have long-term detrimental effects on both terrestrial and aquatic ecosystems. In the name of development, reckless anthropogenic activities have exposed our environment to a range of organic and inorganic pollutants. Out of these, the intractable and persistent nature of heavy metals (HMs) along with their tendency to bioaccumulate makes them a pollutant of worldwide concern. HMs are loosely defined group of elements having atomic mass >20

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(excluding alkali metals) and specific gravity >5, exhibiting metallic properties (Rascio and Navari-Izzo 2011). Out of the 118 known chemical elements, 91 are metals, of which 53 are HMs. Some HMs such as Zn, Cu, Ni, Mn, Co, and Mo serve as essential micronutrients and are required for vital physiological pathways (Shahid et al. 2015). But others such as As, Pb, Cd, Hg, and Cr have no known biological role and prove to be toxic if their accumulation surpasses optimal concentrations (Pierart et al. 2015). The bioavailability of HMs is limited due to their strong affinity to soil particles and low solubility in water. However, the exudation of carboxylates and acidification of the rhizosphere lead to enhanced HM bioavailability (Clemens et al. 2002). Further, the extent of uptake of HMs by plants is also governed by the concentration of organic and inorganic matter, soil pH, temperature, and redox potential (Benavides et al. 2005).

Since enzymes are the key targets of HMs, their presence in soil can disrupt soil enzyme activity markedly. The toxicity resulting from HM exposure in plants encompasses a range of interactions at cellular level such as protein inactivity or enzyme denaturation (Hall 2002). All plant species modulate mechanisms such as uptake/efflux, transport/sequestration of HM in vacuoles, chelation to phytochelatins/metallothioneins, and actuation of antioxidants that allocate HM tolerance at basal level (Viehweger 2014; Shahid et al. 2015).

Heavy metal toxicity is known to disrupt the redox status of cells and leads to enhanced accumulation of reactive oxygen species (ROS) followed by oxidative damage. ROS comprises both free radical, i.e., superoxide ($O_2^{\bullet -}$), $OH^{\bullet}$, hydroxyl, $HO_2^{\bullet}$, perhydroxy and $RO^{\bullet}$, alkoxy, and molecular (non-radical) forms of O_2. ROS are also produced continuously as a result of various physiological reactions localized in intracellular compartments such as chloroplast, mitochondria, and peroxisomes (Gill and Tuteja 2010). Generally, there exists a balance between ROS production and detoxification by virtue of various antioxidative defense mechanisms. But in conditions of various abiotic or biotic stress factors such as temperature, drought, salinity, HMs, and pathogen attacks, this equilibrium gets disturbed leading to ROS accumulation which causes damage to intracellular machinery.

Apart from the detrimental effects on flora and fauna, the presence of HMs in environment has deleterious impact on soil health by disturbing pH, organic carbon, and cation-exchange capacity (Tiwari and Lata 2018) which further leads to imbalances in ecological systems such as habitat destruction, loss of biodiversity, and poor vegetation development (Prakash et al. 2019).

2 Effect of Toxic HMs on Growth and Physiology of Plants

HMs tend to accumulate and affect physiological and molecular reactions in plants adversely, leading to decline in crop productivity (Tiwari and Lata 2018). The physiological and biochemical effects of HM exposure are under scrutiny due to their tendency to bioaccumulate and enter food chain (Shahid et al. 2014). Several

studies have been carried out recently to examine HM toxicity, uptake, sequestration, detoxification, and tolerance at physiological and molecular levels. Excessive accumulation of HMs is known to affect seed germination, plant growth, biosynthesis of chlorophyll, photosynthesis, respiration, and overall metabolism adversely in plants (Singh et al. 2010).

ATSDR (Agency for Toxic Substances and Disease Registry) has ranked As, Pb, Hg, and Cd as first, second, third, and seventh, respectively, in its substance priority list 2017 as the most toxic HMs, based on the frequency of occurrence and severity of toxicity. Arsenic (As) is a naturally occurring metalloid originating via volcanic action, erosion of rocks, and anthropogenic activities such as mining, smelting, and use of pesticides (Neumann et al. 2010). In the environment, As exists in two forms (inorganic arsenate As(V) and arsenite As(III)), both of which are extremely toxic. However, As(III) is considered to be more toxic than As(V) since it interrupts biological functioning, disturbs metabolism, and generates ROS in plants, whereas As(V) interferes with oxidative phosphorylation and ATP synthesis during energy metabolism (Verma et al. 2016).

Lead (Pb) is one of the most widely present trace metals which is evenly distributed in natural sources. Pb occurs in many forms in which Pb^{2+} is extremely toxic to environment due to its nonbiodegradable nature. The use of leaded fuels in transport, plumbing, and painting elements contributes to anthropogenic sources of Pb pollution. Pb hampers basic metabolic processes in plants such as seed germination and development of seedling, elongation of root and cell division, photosynthesis, and transpiration (Pourrut et al. 2011). Pb is highly phytotoxic due to its ability to block active sites of enzymes and replace essential ions leading to changes in cell membrane permeability. Pb stress leads to overproduction of ROS and may inhibit ATP production and induce lipid peroxidation and DNA damage (Pourrut et al. 2011).

Mercury (Hg) is naturally present in earth's crust but its accumulation in natural resources is due to anthropogenic activities (Montero-Palmero et al. 2014). Hg exists in many forms in the environment such as elemental or metallic (Hg^0), organic (CH_3-Hg), inorganic (Hg_2Cl_2), and ionic (Hg^{2+}), of which ionic form is the most prevalent (Zhou et al. 2008). Though it may not cause significant harm at lower concentrations, it is highly phytotoxic if accumulated in higher concentrations. It can hinder water flow in plants by binding with water channel proteins leading to stomatal closure (Zhou et al. 2008). Besides, it has also been reported to induce oxidative stress, disrupt membrane lipids, and interfere with mitochondrial activity (Zhou et al. 2007).

Due to its high solubility in water, Cd is regarded to be the most phytotoxic HM. Since it is a commonly discharged pollutant in agricultural lands, it can be readily taken up and accumulated by plants leading to entry into food chain. It is a potent carcinogen and crop plants have been reported to be the main source of Cd exposure in humans (Gill and Tuteja 2011). Cd is known to hinder activities of several enzymes participating in basic metabolic reactions such as photosynthesis and growth, disrupt antioxidant machinery, and induce oxidative stress (Gill and Tuteja 2011).

3 Generation of ROS

Plants are known to produce increased quantities of reactive oxygen species (ROS) at some stage as a consequence of abiotic/biotic stress exposure. Even though molecular oxygen is fairly nonreactive, its consecutive reduction to water during cellular metabolism yields toxic intermediates which include (a) oxygen-derived free radicals such as hydroxyl (OH^-), superoxide anion ($O_2^{\bullet -}$), peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals or (b) oxygen-derived non-radical species such as hydrogen peroxide (H_2O_2), organic hydroperoxide (ROOH), and singlet oxygen ($^1O_2$) (Scandalios 2005; Shahid et al. 2014). Further, the presence of transition metals (such as Cu, Cr, and Fe) enables Haber-Weiss mechanism or Fenton reaction to yield $OH^{\bullet}$, considered to be the most reactive species biochemically (Gill and Tuteja 2010). Figure 1 depicts ROS generation from molecular oxygen.

Triplet oxygen ($^3O_2$) or dioxygen or molecular oxygen is in the electronic ground state and hence most stable and common allotrope of oxygen. Out of the total O_2 consumed by plants, around 1–2% is digressed to generate ROS in various organelles (Bhattachrjee 2005). As shown in Fig. 1, O_2 upon reduction yields $O_2^{\bullet -}$ and O_2^{2-}, which cannot pass through biological membranes and readily dismutate at low pH to yield H_2O_2. Singlet oxygen ($^1O_2$) is the first excited electronic state of $^3O_2$, formed by the reaction between photoexcited (triplet) state of chlorophyll and $^3O_2$. Its formation is also favored during conditions of abiotic stresses when the intracellular concentration of CO_2 is low due to stomatal closure. Due to its very reactive nature, it possesses very serious damaging effect on photosynthetic machinery including photosystem (PS) I and II. (Gill and Tuteja 2010). Superoxide radicals ($O_2^{\bullet -}$) are formed perpetually during photosynthesis as a result of partial reduction of O_2 during noncyclic pathway in thylakoid membrane. Their formation is also inevitable during aerobic respiration wherein O_2 may react with the components of electron transport chain (ETC) to yield $O_2^{\bullet -}$. Though $O_2^{\bullet -}$ is moderately reactive, short lived (half-life: 2–4 μs), and usually the first ROS to be generated, they can trigger the formation of more reactive ROS as shown in Fig. 1.

The univalent reduction of $O_2^{\bullet -}$ yields H_2O_2 which is also moderately reactive but possesses a relatively longer half-life (1 ms). It is a potent inducer of oxidative stress in plants and is capable of inactivating enzymes by oxidizing thiol groups. Though at low concentrations H_2O_2 acts as a signaling molecule during stress and is being

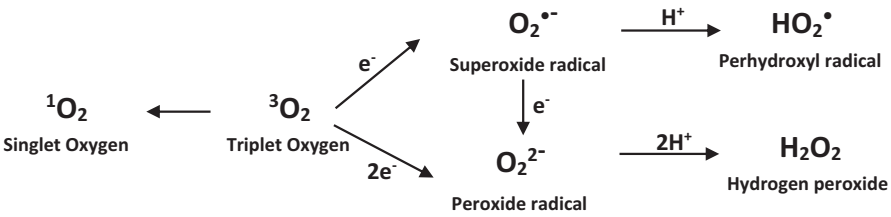

Fig. 1 ROS generation from molecular oxygen

regarded as second messenger due to its relatively longer half-life and permeability across membranes (Quan et al. 2008), it can trigger programmed cell death at high concentrations. Hydroxyl radicals ($OH^{\bullet}$) are one of the most reactive ROS known. As shown in Fig. 2, transition metals can lead to the generation of $OH^{\bullet}$ from $O_2^{\bullet-}$ and H_2O_2 via Fenton reaction. Overproduction of $OH^{\bullet}$ can induce cell death since it is potentially capable of reacting with all biological molecules and cellular machinery leading to oxygen toxicity.

HMs lacking redox capacity (Pb^{2+},Cd^{2+}, Hg^{2+}) are able to enhance the prooxidant status by reducing glutathione pool, activate Ca^{2+}-dependent systems, and affect Fe-mediated processes (Pinto et al. 2003). They can also lead to the production of by $O^{2\bullet-}$ and $^1O_2$ by disrupting the photosynthetic electron chain. ROS possess unpaired electrons in valence shell and are unstable and short lived but very reactive molecules chemically (Wang et al. 2010). The equilibrium between steady-state levels of ROS is regulated by the reciprocity between ROS production and detoxification mechanisms, which is ultimately guided by the physiological, biochemical, developmental, and environmental stimuli (Benavides et al. 2005). A pictorial representation of different ROS-generating and -detoxifying mechanisms has been illustrated in Fig. 2.

4 Sites of ROS Production in Plants

Green plants are particularly at the peril of oxidative damage due to oxygenic conditions and composition of chloroplast envelope (Gill and Tuteja 2010). ROS production is the outcome of interactions between HMs and ETC (electron transport chain), operating in chloroplast and mitochondrial membranes. Chloroplast and peroxisomes are the main sites of ROS generation under light conditions, whereas mitochondria are the main organelle involved during dark conditions. Besides these, ROS are also generated in cytoplasm and endoplasmic reticulum during detoxification reactions involving cytochrome P450. Cell wall peroxidases, germin-like oxalate oxidases, and polyamine oxidases are all sources of H_2O_2 in apoplasts. ROS are also generated in plasma membrane by virtue of NADPH-dependent oxidases. The NADPH oxidase generates $O_2^{\bullet-}$ by transferring electrons from cytosolic NADPH to O_2, which then dismutates to H_2O_2 (Das and Roychoudhury 2014).

Chloroplast consists of well-regulated thylakoid membranes which sheathes light harvesting machinery and encompasses anatomy for optimal light harvesting (Pfannschmidt 2003). During photosynthesis, O_2 generated can readily accept electrons passing through PSI and PSII (via ETC) to yield $O_2^{\bullet-}$. PSII also accounts for generation of $^1O_2$ when the ETC is over-reduced (Asada 2006). Moreover, the reaction between photoexcited/triplet state of chlorophyll ($^3chl^*$) and $^3O_2$ also generates $^1O_2$ in PSII (Karuppanapandian et al. 2011). Abiotic stress conditions leading to overloading of ETC also generate $O_2^{\bullet-}$ via Mehler reaction (Das and Roychoudhury 2014). Research has shown that even under low-light conditions, $^1O_2$ is a natural by-product of photosynthesis mainly formed at PSII (Buchert and Forreiter 2010).

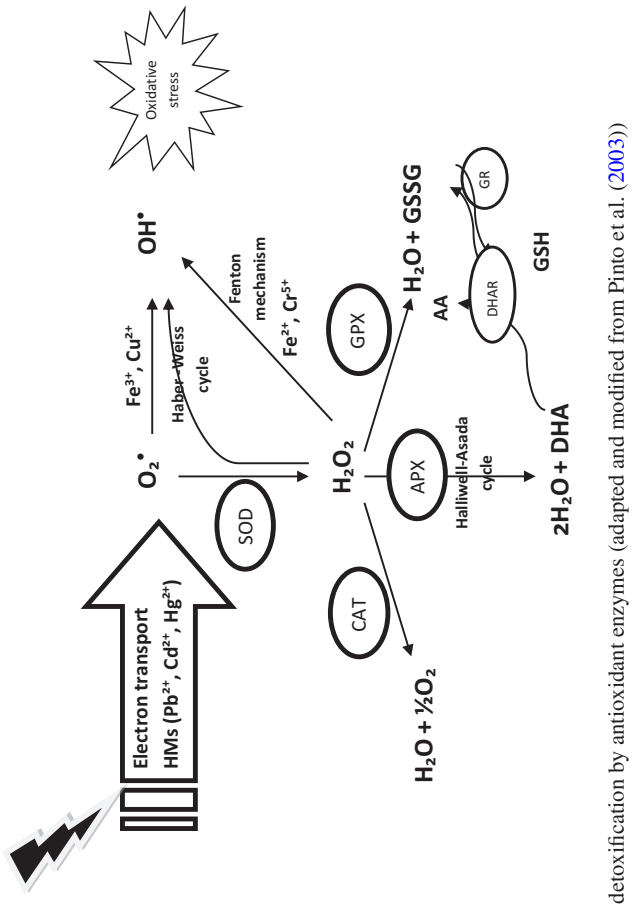

Fig. 2 ROS generation and detoxification by antioxidant enzymes (adapted and modified from Pinto et al. (2003))

Subsequently, on the stromal surface, a membrane-bound Cu/ZnSOD keeps on converting $O_2^{\bullet-}$ into H_2O_2 (Miller et al. 2010) and more toxic ROS like $OH^{\bullet}$ via H_2O_2 intermediate by the Fenton reaction at the Fe-S centers. Though chloroplast is the major source of ROS generation in plant cells, $^1O_2$ accumulating in it can lead to protein damage and peroxidation of its integral lipids and fatty acids, ultimately leading to cell death.

Mitochondria or the powerhouses are also potential sites of ROS generation such as H_2O_2 and $O_2^{\bullet-}$. Presence of specific ETC components, role in photorespiration, and an environment rich in O_2 and carbohydrates (due to photosynthesis) are key features that make plant mitochondria distinct from their animal counterparts (Noctor et al. 2007). The mitochondrial ETC (complexes I and III) abodes electrons with ample free energy and potential to reduce O_2 directly to $O_2^{\bullet-}$, which can be further dismutated to H_2O_2 by SOD. Around 1–5% of O_2 consumed is involved in H_2O_2 production in isolated mitochondria (Moller 2001). H_2O_2 upon reaction with reduced Fe^{2+} and Cu^{+} can lead to production of highly toxic $OH^{\bullet}$, which is capable of penetrating membranes and leaving the mitochondrion (Rhoads et al. 2006). A common outcome of $OH^{\bullet}$ generation is lipid peroxidation leading to formation of cytotoxic products capable of reacting with proteins, lipids, and nucleic acids, and ultimately causing cellular damage. ROS generation by mitochondrion is an unavoidable adjunct to aerobic respiration under normal conditions, which gets accelerated due to over-reduction of electron carriers during conditions of stress (Pastore et al. 2007). To combat oxidative stress and control ROS generation, plant mitochondria may employ energy-dissipating systems. Further, mitochondria are also equipped with pivotal enzymes, namely mitochondrial alternative oxidase (AOX) and mitochondrial SOD (Mn-SOD), which help in trimming down ROS generation (Das and Roychoudhury 2014).

Peroxisomes are single lipid bilayer membrane-bound subcellular organelles, possessing oxidative metabolism. Peroxisomes produce $O_2^{\bullet-}$ as a part of their routine metabolism, similar to mitochondria and chloroplasts. $O_2^{\bullet-}$ is generated in the peroxisomal membrane ETC as well as in the matrix. Several metabolic reactions, namely β-oxidation of fatty acids, photorespiratory glycolate oxidase reaction, flavin oxidase pathway, and disproportionation of $O_2^{\bullet-}$ radicals, are responsible for the generation of H_2O_2 in peroxisomes (Gill and Tuteja 2010). Under conditions of high temperature or low water availability, the concentration of CO_2:O_2 reduces considerably and causes increased photorespiration. This leads to formation of glycolate, which is oxidized by glycolate oxidase in peroxisomes, releasing H_2O_2 ultimately and making peroxisomes the leading producer of H_2O_2 during photorespiration (Noctor et al. 2002). Though overproduction of ROS leads to oxidative damage and cell death in plants, some research also shows that small concentrations of $O_2^{\bullet-}$ and H_2O_2 are engaged as signaling molecules in plants (McDowell and Dangl 2000). Hence peroxisomes can regarded as organelles capable of contributing to a better consolidated communication system among cellular compartments by generating and releasing vital signaling molecules such as H_2O_2, $O_2^{\bullet-}$, and $NO^{\bullet}$ into the cytosol (Corpas et al. 2001).

5 Targets of ROS Generated in Plants

ROS generation is known to damage vital biomolecules, namely lipids, proteins, and nucleic acids, which hampers cellular functioning, ultimately leading to cell death.

Lipids are the integral part of plasma membrane and play a vital role in cellular integrity and metabolism. Lipid peroxidation is a single, sufficient parameter tested to estimate the amount of membrane damage occurring due to stress. ROS upon crossing a certain threshold level lead to peroxidation of lipids and formation of cytotoxic products capable of exacerbating cellular damage. The ester linkage between glycerol and fatty acids and the double bond between C atoms (C=C) are the two main sites prone to ROS attack in membrane phospholipids. Further, $OH^{\bullet}$ can trigger a cyclic chain reaction to peroxidate the polyunsaturated fatty acids present in membranes leading to membrane damage. Lipid peroxidation affects membrane fluidity, renders the membrane leaky to molecules which would have otherwise been unable to cross it except by using specific transporters, and causes damage to the membrane proteins, disband membrane receptors, ion channels, and membrane-localized enzymes (Gill and Tuteja 2010).

Proteins are prone to reversible or irreversible covalent modifications induced by ROS (Ghezzi and Bonetto 2003). ROS, irrespective of their location of generation, probably target proteins which in turn respond with different susceptibilities based on their composition. Proteins composed of amino acids like lysine, arginine, proline, threonine, and tryptophan are susceptible to site-specific modification and proteolytic degradation (Møller et al. 2007). Proteins composed of thiol groups and sulfur-containing amino acids (cysteine and methionine) are most vulnerable since they fairly reactive $^1O_2$ and $OH^{\bullet}$. Proteins containing Fe-S centers upon oxidation with $O_2^{\bullet-}$ get irreversibly inactivated. Proteins can undergo direct or indirect modifications; direct modification involves a chemical modification (carboxylation, disulfide bond formation, nitrosylation) to alter protein activity, whereas damage upon reaction with products of lipid peroxidation in oxygenic conditions is an indirect modification.

DNA: Chloroplast and mitochondrial DNA are more prone to oxidative damage than plant nuclear DNA, due to the proximity to ROS generation machinery. ROS can initiate endogenous or spontaneous DNA damage in many ways including base deletion, formation of pyrimidine dimers, strand breaks, cross-links, and modification of bases by alkylation and oxidation (Gill and Tuteja 2010). Different nucleotide bases respond differently to ROS; for example guanine is predominantly attacked by $^1O_2$ whereas not at all by $O_2^{\bullet-}$ and H_2O_2. $OH^{\bullet}$ is highly reactive and can damage all four nucleotide bases along with the deoxyribose backbone. It can also react with DNA or associated proteins to create DNA-protein cross-links which cannot be repaired easily and prove to be lethal for the plant cells. DNA damage can result in errors during replication, arrest or induction of transcription, and reduction in protein synthesis and signal transduction pathways leading to genomic instability besides affecting overall growth and development.

6 HM Tolerance Mechanisms in Plants

All plants presumptively exhibit elementary HM tolerance by regulating a nexus of uptake/efflux, transport/sequester, and chelation (Viehweger 2014). These pivotal elements play the decisive role in determining the hyperaccumulating, hypertolerating, or non-accumulating nature of plants. While hyperaccumulating plants are able to translocate and accumulate high concentration of HMs in aerial parts without suffering phytotoxicity, hypertolerant plants are capable of excluding HMs accumulating, especially in aerial parts. Since efflux and sequestration are the key elements leading to basal tolerance (Clemens 2001), they do happen in specific plant structures, namely cuticle, epidermis, and trichomes (Shahid et al. 2014), where they may cause damage to photosynthetic machinery, if not detoxified. In order to cope up with stress, plants are equipped with mechanisms at every level. While some of these mechanisms may either altogether prohibit the entry of HMs into plants or increase the excretion of HMs by roots, others may lead to binding of HMs to the cell wall, or chelation of HMs by organic molecules followed by sequestration in vacuoles (Tang et al. 2010).

6.1 Primary-Level Mechanisms of HM Tolerance

HMs primarily gain entry into plants through roots. At entry level, the cell wall and plasma membrane are the first structures that encounter HM stress. Immobilization of HMs by the root cell wall and extracellular carbohydrates serves as the first barrier against HM toxicity. The thickness of roots may increase in order to adsorb HMs onto the surface and reduce its absorption as a response to HM toxicity. Further the selective permeability of plasma membrane excludes many HMs from gaining entry into the cytosol. However, the efficiency of these structures is governed by the intensity of exposure along with species involved. In order to restrict the translocation of HMs absorbed by roots to aerial parts, HMs are either detoxified (complexed with organic acids or amino acids) or sequestered into vacuoles (Shahid et al. 2014). Increased sequestration of HMs in root cells can be achieved by precipitation of HMs as insoluble salts in intercellular spaces, accumulation in plasma membranes, immobilization of HMs by negatively charged pectins within the cell wall, or sequestration in the vacuoles of rhizodermal and cortical cells (Shahid et al. 2014).

6.2 Secondary-Level Mechanisms of HM Tolerance

Plants exhibit homeostatic cellular mechanisms in order to minimize the possible damage caused due to HM exposure. After absorption of HMs, toxicity can be evaded by plants if they possess efficient sinks to store HMs. Vacuoles are such

multifunctional organelles that function for metal homeostasis and detoxification by sequestering HMs. This takes place either as a result of ligand binding or by vacuolar entrapment using transporters. Several families of transporters involved in HM homeostasis have been identified using genome sequencing in plants, namely heavy metal ATPases (HMAs), ATP-binding cassettes (ABC), Zrt/Irt-like protein (ZIP), natural resistance-associated macrophage (NRAMP), cation exchangers (CAXs), and cation diffusion facilitators (CDF). Of these, ABC, CDF, and NRAMP have been identified as being crucial for HM tolerance (Chaffai and Koyama 2011).

Metallothioneins (MTs) and phytochelatins (PCs) are crucial and the best characterized HM-binding ligands in plants. The responsiveness of plants to HMs is determined by an allied system of physiological and molecular mechanisms comprising uptake and acquisition of HMs via binding and chelation to polypeptides, namely MTs and PCs; induction of defense metabolites; and alteration of plant metabolic pathways to provide rapid defense and repair (Benavides et al. 2005). HM accumulation in plants is generally a function of uptake capacity and intracellular binding sites. The concentration and affinity of phytochelatins along with the presence and specificity of transporters govern the uptake kinetics (Clemens et al. 2002).

Chelation of HMs by ligand has been a regular mechanism for HM detoxification in organisms, which can be followed by subsequent compartmentalization of ligand-HM complex in vacuoles to prevent free circulation of ions in cytosol. MTs are small gene-encoded, cysteine-rich polypeptides which are classified on the basis of arrangement of cys residues (Cobbett and Goldsbrough 2002). Class I MTs are widespread in vertebrates whereas class II MTs are found in invertebrates, fungi, and plants. PCs have been confusingly described as class III MTs in this system of classification. PCs are small, enzymatically synthesized cysteine-rich peptides with the structure (g-glu-cys)n-gly, (g-glu-cys)n-b-ala, (g-glu-cys)n-ser, (g-glu-cys)n-glu, (g-glu-cys)n-gln, or (g-glu-cys)n, where n varies from 2 to 11. The biosynthesis of PCs requires glutathione (γ-Glu-CysGly) as substrate and phytochelatin synthase (PCS) (EC 2.3.2.15) as enzyme. PCS is a constitutive enzyme that gets activated only in the presence of HMs post-translationally (Cobbett 2000). Cd along with PCs has been shown to accumulate in vacuoles via ABC transporters (Hall 2002). HM tolerance has also been attributed to extracellular chelation via organic acids, namely malate and citrate.

6.3 HM Transport and Signaling in Plants

The advancement in molecular techniques has led to the identification of several cation transporters in recent years, which are able to transport different HMs across biological membranes. Of these, ZIP and Nramp are the major families of transporters involved in micronutrient uptake (Williams et al. 2000). It is unlikely that specific transporters for HMs occur in organisms and hence HMs are likely to enter cells via transporters with broad specificity (Clemens 2001). Cation transporters

that show affinity for both Zn and Cd have also been identified suggesting that inessential HMs are taken up along with essential micronutrients. *Arabidopsis halleri* is known to hyperaccumulate both Zn and Cd (Bert et al. 2003). Further, Cd transport has also been shown by AtNramp3 which is involved in Fe transport in *Arabidopsis thaliana* (Thomine et al. 2000). Transcriptomic studies have shown that at least 30 candidate genes are overexpressed in hyperaccumulator *A. halleri* than non-accumulator *A. thaliana.*

Application of various proteomics techniques such as MALDI-TOF and LC-MS have enabled identification of target proteins that participate in HM detoxification in several plants (Tiwari and Lata 2018). Likewise, several amino acids, organic acids, and secondary metabolites (phenols, α-tocopherol) have been traced to play major roles in HM detoxification (Singh et al. 2016). Receptors/ion channels percept HM stress and along with nonprotein messengers (Ca^{2+}, H^{+}, cyclic nucleotides) they initiate stress signal transduction. These stress signals are relayed by various kinases and phosphatases leading to gene expression of transcription factors (TFs) synthesizing metal-detoxifying peptides (Kumar and Trivedi 2016). Distinct signaling pathways, namely mitogen-activated protein kinase (MAPK), ROS signaling, hormone signaling, and calcium-dependent signaling, are activated by HMs and enhance the expression of stress-responsive genes (Kumar and Trivedi 2016). Numerous TFs can be phosphorylated by MAPK signaling cascade as a response to HM stress. Likewise, alterations in cytosolic Ca^{2+} concentrations are sensed by numerous Ca^{2+} sensors like Ca^{2+}-dependent protein kinases (CDPKs), calmodulins (CaMs), CaM-like proteins, and calcineurin B-like proteins (CBLs) and conveyed to induce stress response (Steinhorst and Kudla 2014). Phytohormone signaling pathways like auxin, ethylene, and jasmonic acid (JA) are also key mechanisms to counter HM stress as variation in the levels of phytohormones affects plant response to HM stress. Exposure to phytohormones can improve antioxidant response in plants during HM stress (Singh and Shah 2014).

6.4 ROS-Induced Defense Responses in Plants

ROS overproduction can distort the redox status of plant cells resulting in oxidative damage that leads to degeneration of biomolecules, dismantling of membranes, lipid peroxidation, ion leakage, and DNA strand cleavage (Shahid et al. 2014). In order to combat oxidative damage occurring during stress conditions, plants have evolved an array of defense mechanisms to transform ROS into less toxic products. These mechanisms help plants to sustain their cellular redox state and mitigate the damage caused by oxidative stress. Majority of these mechanisms rely on synthesis of metabolic intermediaries comprising two arms: (1) nonenzymatic and (2) enzymatic components. Records of HM-induced increase in nonenzymatic and enzymatic antioxidants have been summarized in Table 1.

Table 1 Upregulation of enzymatic and nonenzymatic antioxidants upon exposure to HMs in plants

Antioxidant	HM	Plant species	Reference
Nonenzymatic			
Tocopherol (Vit. E)	Cu	*Anabaena doliolum*	Srivastava et al. (2005)
Ascorbic acid (Vit. C)	Cd, Hg	*Hordeum vulgare, Medicago sativa*	Demirevska-Kepova et al. (2006), Zhou et al. (2007)
Glutathione	Cd, Hg	*Pisum sativum, Sedum alfredii, Vigna mungo, Medicago sativa*	Metwally et al. (2005), Sun et al. (2007), Molina et al. (2008), Zhou et al. (2007)
Phenolics	Cd, Zn	*Kandelia obovata*	Chen et al. (2019)
Carotenoids	Pb	*Arabidopsis thaliana*	Baek et al. (2012)
Proline	Cd, Ni	Microalga (*Chlamydomonas reinhardtii*), *Pisum sativum*	Siripornadulsil et al. (2002), Gajewska and Skłodowska (2005)
Enzymatic			
CAT	Cd	*Oryza sativa, Brassica juncea, Triticum aestivum, Cicer arietinum, and Vigna mungo*	Hsu and Kao (2004), Mobin and Khan (2007), Khan et al. (2007), Hasan et al. (2008), Singh et al. (2008)
	Pb	*Eichhornia crassipes, Acalypha indica*	Malar et al. (2014), Venkatachalam et al. (2017)
	Hg	*Sesbania grandiflora*	Malar et al. (2015)
SOD	Pb	*Eichhornia crassipes, Acalypha indica*	Malar et al. (2014), Venkatachalam et al. (2017)
	Cd	*Hordeum vulgare, Arabidopsis thaliana, Oryza sativa, Brassica juncea, Triticum aestivum, Cicer arietinum, Vigna mungo, Hibiscus cannabinus*	Guo et al. (2004), Skorzynska-Polit et al. (2003), Hsu and Kao (2004), Mobin and Khan (2007), Khan et al. (2007), Hasan et al. (2008), Singh et al. (2008), Feng-tao et al. (2013)
	Hg	*Sesbania grandiflora*	Malar et al. (2015)
APX	Pb	*Eichhornia crassipes, Acalypha indica*	Malar et al. (2014), Venkatachalam et al. (2017)
	Cd	*Brassica juncea, Triticum aestivum, Vigna mungo, Ceratophyllum demersum, Hibiscus cannabinus*	Mobin and Khan (2007), Khan et al. (2007), Singh et al. (2008), Arvind and Prasad (2003), Feng-tao et al. (2013)
	Hg	*Sesbania grandiflora*	Malar et al. (2015)
POX	Hg	*Sesbania grandiflora*	Malar et al. (2015)
GPOX	Cd	*Arabidopsis thaliana, Triticum aestivum, Ceratophyllum demersum*	Skorzynska-Polit et al. (2003), Khan et al. (2007), Arvind and Prasad (2003)
GR	Cd	*Capsicum annuum, Arabidopsis thaliana, Vigna mungo, Triticum aestivum, Brassica juncea*	Leon et al. (2002), Skorzynska-Polit et al. (2003), Singh et al. (2008), Khan et al. (2007), Mobin and Khan (2007)

6.5 Nonenzymatic Components

These include various groups of bioactive molecules, namely tocopherols, ascorbic acid (AA), reduced glutathione (GSH), phenolics, carotenoids, proline, etc. Besides protecting cellular components from damage, they play key roles in plant growth and development (de Pinto and De Gara 2004).

Tocopherols and tocotrienols, together known as tocochromanols, are lipophilic antioxidants belonging to group of vitamin E, known to scavenge ROS and lipid radicals (Falk and Munné-Bosch 2010). Out of the four isomers (α-, β-, γ-, δ-) of tocopherols identified in plants on the basis of the number and position of chromanol ring system, α-tocopherol possesses the highest antioxidant activity as it consists of three methyl groups. Tocopherols can only be synthesized by photosynthetic plants and hence localized in green tissues only. Since chloroplasts of higher plants contain significant amount of α-tocopherol, they are secure against photooxidation since they can react with O_2 and quench its excess energy (Das and Roychoudhury 2014). Tocopherols are also known to protect thylakoid membranes against lipid peroxidation similar to carotenoids (Moucheshi et al. 2014). By halting the chain propagation step and reducing the lipid radicals ($RO^{\bullet}$, $ROO^{\bullet}$), tocopherols themselves get oxidized as tocopheroxyl radical ($TOH^{\bullet}$) which later reacts with GSH and AA to get recycled into its reduced form (Igamberdiev et al. 2004).

AA (vitamin C) is the most widely present and studied antioxidant. Because of its tendency to act as a reducing agent in a number of biological reactions, it is considered to be a potent antioxidant. It is water soluble and synthesized by Smirnoff-Wheeler pathway in plant mitochondria. It acts as a first line of defense against ROS because of its substantial presence in cytosol and apoplast (Barnes et al. 2002) in its reduced form (ascorbate) under normal physiological conditions. The regeneration of ascorbate from fully oxidized dehydroascorbic acid is crucial because it has a short half-life and would be bygone unless it is reduced back. AA can directly reduce $O_2^{\bullet -}$, $^1O_2$, $OH^{\bullet}$, and H_2O_2 and regenerate α-tocopherol from $TOH^{\bullet}$, in order to protect membranes from oxidative stress.

GSH is a cysteine-containing, low-molecular-weight thiol tripeptide involved in various cellular processes like cell growth, division, differentiation, synthesis, and transport of biomolecules. It is also water soluble like AA and found in almost all cell organelles in its reduced form abundantly. Its elementary role is in thiol-disulfide interactions, where GSH is continuously oxidized to its disulfide form (GSSG) which is recycled back to GSH either de novo or enzymatically in the presence of NADPH-dependent glutathione reductase (GR), ultimately replenishing the cellular GSH pool. GSH is involved in the synthesis of phytochelatins which chelate HMs and aid in detoxification. Both GSH and GSSG play a pivotal role in actuating secondary metabolism, ROS signaling, and antioxidant defense mechanism by regenerating AA via ascorbate-glutathione (ASH-GSH) cycle. The intricate equilibrium between GSH and GSSG significantly conserves the normal redox system of the cell under normal and stress conditions (Moucheshi et al. 2014).

Phenolic antioxidants are of particular importance due to their expression of antioxidant activity in both in vitro and in vivo studies (Trchounian et al. 2016). Out of the five major groups classified (phenolic acids, flavonoids, lignans, tannins, and stilbenes) flavonoids and phenolic acids constitute the widest classes of plant phenolics biosynthesized majorly from phenylalanine, an aromatic amino acid synthesized from shikimic acid pathway. Flavonoids are water-soluble N-deficient plant pigments possessing a three-ring chemical structure (C6-C3-C6). On the basis of their structure, flavonoids can be classified into four classes: anthocyanins (red-purple pigments), flavonols (colorless-pale yellow pigments), flavanols (colorless pigments that become brown upon oxidation), and proanthocyanidins (PAs) or condensed tannins (Petrussa et al. 2013). Flavonoids show varied concentrations in plants depending upon the species, growth stage, and environment conditions. They serve as secondary ROS scavengers and are known to shield photosynthetic apparatus (Das and Roychoudhury 2014). Flavonoids show synergistic amplification in activities of some antioxidants (tocopherol, ascorbate) by interacting with them (Kasote et al. 2015). They also prevent lipid peroxidation by inhibiting enzyme lipoxygenase (Moucheshi et al. 2014).

Carotenoids, the most common tetraterpenoids, are organic lipophilic pigments localized in plastids of plants and other photosynthetic organisms. They are antennae molecules that absorb visible light (450–570 nm) and pass it on to chlorophyll. There are different types of carotenoids in plants but β-carotenes and xanthophylls are the most abundant and commonly studied. Carotenoids serve as antioxidants and protect the photosynthetic machinery in either of four ways: (1) avoiding the formation of $^1O_2$ by quenching $^3chl^*$ (Moucheshi et al. 2014), (2) scavenging $^1O_2$ and generating heat at by-product, (3) involving xanthophyll cycle to dissipate excess excitation energy, and (4) reacting with lipid peroxidation products to terminate the chain reaction (Das and Roychoudhury 2014).

Proline, besides being an osmolyte, is also a potent ROS scavenger and is known to inhibit the damage caused by lipid peroxidation. The accumulation of proline in considerable amounts in plants during stress can be attributed to either increased synthesis or decreased degradation (Verbruggen and Hermans 2008).

6.6 Enzymatic Components

Catalase (CAT; E.C.1.11.1.6) is tetrameric heme-containing enzyme with the potential to dismutate H_2O_2 into H_2O and O_2 directly ($2H_2O_2 \rightarrow O_2 + 2H_2O$). It possesses a very high affinity as well as turnover rate (~six million molecules min^{-1}) for H_2O_2. The unnecessity of reducing agent in reactions catalyzed makes catalases distinctive among other antioxidants. Generally H_2O_2 is generated in peroxisomes due to oxidative stress resulting from β-oxidation of fatty acids, photorespiration, and purine catabolism (Gill and Tuteja 2010). But catalases are also present in cytosol and organelles such as chloroplast and mitochondria (Mhamdi et al. 2010). Several isoforms of *CAT* genes have been reported in higher plants (up to 12 in

Brassica) of which the 3 isoforms in *Zea mays* are found to be differentially localized and independently expressed (i.e., although both *CAT 1* and *CAT 2* are localized in peroxisomes and cytosol *CAT 1* is expressed in pollen and seeds whereas *CAT 2* is expressed in photosynthetic tissues, roots, and seeds; *CAT 3* is localized in mitochondria of leaves and vascular tissues):

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

Superoxide dismutase (SOD; E.C.1.15.1.1) is a multimeric metalloprotein and the most effective intracellular antioxidant known to detoxify $O_2^{\bullet -}$ and provide first line of defense against oxidative stress (Gill and Tuteja 2010). Based on the metal cofactor present at the active site, protein folds, and subcellular distribution, SOD isoforms occurring in plants can be characterized as Cu/Zn-SOD (localized in cytosol, peroxisomes, and chloroplasts), Mn-SOD (localized in mitochondria), and Fe-SOD (localized in chloroplasts) (Das and Roychoudhury 2014). SODs catalyze the dismutation of $O_2^{\bullet -}$; that is, one $O_2^{\bullet -}$ is reduced to H_2O_2 and the other $O_2^{\bullet -}$ is oxidized to O_2, henceforth decreasing the risk of Haber-Weiss-catalyzed $OH^{\bullet}$ formation (Gill and Tuteja 2010). Under abiotic stress conditions, the activity of SOD has been found to be upregulated in plants (Boguszewska et al. 2010):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Ascorbate peroxidase (APX; E.C.1.1.11.1) is an intrinsic constituent of ASH-GSH cycle. Using ascorbic acid as a reducing agent, APX transforms H_2O_2 into H_2O and DHA (dehydroascorbate) in water-water and ASH-GSH cycle. While it primarily scavenges H_2O_2 in cytosol and chloroplast, CAT executes the same function in peroxisomes (Das and Roychoudhury 2014). Based on the amino acid composition and subcellular localization, five isoforms originating from alternative splicing (contributing to the differential regulation of expression of various isoforms) have been characterized in plants. Soluble isoforms are found in cytosol (cAPX), mitochondria (mitAPX), and chloroplast stroma (sAPX), while membrane-bound isoforms are found in microbody (including peroxisome and glyoxysome) (mAPX) and chloroplast thylakoids (tAPX) (Caverzan et al. 2012). Since APX possesses a higher affinity for H_2O_2 (μM range) than CAT (mM range) and is widely distributed, it is bound to play a crucial role in H_2O_2 scavenging during stress (Gill and Tuteja 2010):

$$H_2O_2 + AA \rightarrow 2H_2O + DHA$$

Guaiacol peroxidase (GPOX; E.C.1.11.1.7) is a heme-containing enzyme that scavenges excess H_2O_2 under normal conditions and stress as well. Plant-derived GPX is different from APX both in sequences and its physiological role. In addition to being active in cell wall, GPX is active both intracellularly (cytosol, vacuoles) and extracellularly (Das and Roychoudhury 2014). GPX prefers aromatic compounds (namely guaiacol and pyrogallol) usually as electron donors, oxidizing

ascorbate at a rate of around 1% to that of guaiacol (Gill and Tuteja 2010). Besides playing a pivotal role in the biosynthesis of lignin, GPOX also decomposes indole-3-acetic acid (IAA) and provides defense against biotic stresses by consuming H_2O_2:

$$\mathbf{H_2O_2 + GSH \rightarrow H_2O + GSSG}$$

Glutathione reductase (GR; E.C.1.6.4.2) is a flavoprotein oxidoreductase, playing a key role in ROS detoxification by maintaining the reduced status of GSH from GSSG using NADPH as reductant. It is localized mostly in chloroplasts, but also present in mitochondria and cytosol in small amounts. GSH is a compound with low molecular weight that acts as a reducing agent in preventing thiol groups from getting oxidized, and reacts with detrimental ROS members like $^1O_2$ and $OH^{\bullet}$ (Das and Roychoudhury 2014). Since GSH is continuously used up in ASH-GSH cycle to regenerate AA, it gets converted into its oxidized form GSSG. GR is a pivotal enzyme of this cycle as it catalyzes the formation of disulfide bond in GSSG and maintains GSH pool:

$$\mathbf{GSSG + NADPH \rightarrow 2GSH + NADP^+}$$

7 Conclusions and Future Perspectives

The presence of heavy metals in environment is known to exert genotoxic and clastogenic effects on plants. Subsequently plants are equipped with various defense mechanisms which are imperative for their survival. Various omics approaches, namely transcriptomics, proteomics, metabolomics, and ionomics, are being employed to encode regulatory mechanisms involved in HM tolerance in plants. The induction of genes central to HM stress signaling points to a composite cross talk between plant and HM during stress response and tolerance. Therefore, a precise interpretation of the intricate HM stress signaling pathways is of key requirement to elucidate stress response network in plants. Functional genomics techniques can be synergized with omics technologies for the development of improved varieties with enhanced abiotic stress tolerance. This strategy can also be employed to raise genetically engineered plants with enhanced accumulation which can be used not only for phytomining, but also for biofortification.

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