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Heavy Metal Stress and Cellular Antioxidant Systems of Plants: A Review

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ABSTRACT

Heavy metal (HM) stress is one of the most important abiotic stresses affecting flora and fauna worldwide due to a rapid global increase in the urbanization and industrialization and the consequent rise of HM concentration in the atmosphere. Typically, plants have distinct mechanisms to cope up with HM stress. These mechanisms rendering tolerance to the plants by detoxifying the HMs. Additionally, a number of physiological and molecular changes occur in plant cells due to their exposure to HMs. This culminates in the generation of reactive oxygen species (ROS) that cause oxidative stress in plants, which significantly affects plant metabolism and disrupts normal vital cellular functions. However, in order to cope with such stresses, plants possess a strong antioxidant defence system to counteract increases in the ROS-induced stress. The enzymatic components of this system include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). The non-enzymatic components are ascorbate, glutathione and phenolic compounds along with lipid-soluble molecules such as carotenoids and tocopherols. This review makes an effort to collect and collate the currently available information on metal stress and cellular antioxidant systems of plants, emphasizing upon the role of enzymatic and non-enzymatic mechanisms for detoxification of HM-induced oxidative stress.

Key words: Antioxidative enzymes, Heavy metals, Plants, Stress Physiology.

The term ''heavy metal'' relates to any metallic element that has a comparatively high density and is toxic even at low concentrations. It is a general and collective term that applies to the group of metals and metalloids with atomic density greater than 5 g cm^{-3} or 5 times greater than water (Ansari *et al.,* 2012). However, compared to their density, the chemical properties of heavy metals (HMs) are more important for determining their interactions with plants (Gill, 2014). Of the naturally occurring nearly 98 elements (www.chemistry.about.com), 53 are regarded as heavy metals, of which 17 (*i.e*. Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Mo, Mn, Ni, Pb, Sb, U, V, W and Zn) are believed to important for organisms and/or ecosystems, based on their solubility in environmental devices (Lide, 2005). Globally, the HM pollution, which started to increase acutely since 1900, has become a serious problem for the biosphere and human health (Ansari *et al.,* 2021).

Sources of HMs

Metals are ubiquitous and found naturally in various concentrations in parent rocks, water, soil, air and biological matters. The major anthropogenic activities resulting in release of metal ions include smelting of metallic ores, industrial fabrication, use of agrochemical pesticides and burning of fossil fuels (Fig 1) (Mohammed *et al.,* 2011). Metals, which occur in various chemical forms, exhibit different physical and chemical behavior including chemical interactions, mobility, bioavailability and toxicity, depending on their chemical species (Ansari *et al.,* 2012). The solubility and mobility of metals are affected by adsorption, desorption and complexation processes, dependent largely on the soil type. Excessive HM concentrations can be toxic to plants as these metals disturb the physiological processes such as cell elongation, photosynthesis and respiration, and inhibit nitrogen metabolism and mineral nutrition (Ansari *et al.,* 2009; Manara, 2012). Additionally, HMs poses a long-term indirect risk to human health (Table 1) due to their potential to contaminate food chain

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and their ability to travel for long distances in the atmosphere posing a direct threat to soil and water bodies (Liu *et al.,* 2017).

HMs are non-degradable and are often accumulated in various plant parts and biologically magnified through trophic levels, causing deleterious biological effects (Ansari *et al.,* 2013). Even at relatively low concentrations, they may cause alteration of many cellular processes and structures. Among the pollution-producing heavy metals, arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) are regarded as non-essential elements, with no known physiological functions in plants (Ansari *et al.,* 2021). They are extremely toxic to plants and animals, have a long half-life and are persistent in the environment (Ansari *et al.,* 2009; 2013; 2023). In small quantities certain HMs such as copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) are nutritionally essential for plants, animals and human being important cofactors of many enzymes and constituents of cells (Ansari *et al.,* 2015). A major factor regulating the toxicity of metals in the soil is their bioavailability (Mittova *et al.,* 2004; Iqbal *et al.,* 2015). These are largely regulated by (i) the soil type - its physical and chemical characteristics such as soil pH and redox potential, (ii) metal speciation and (iii) the nature of microorganisms present (Mittova *et al.,* 2004). In addition, rainfall, evaporation and plant transpiration can change the trace-element concentrations in soil solution (Fischerov *et al.,* 2006). Elevated concentrations of HMs in cells may cause molecular damage in plants either directly or indirectly through the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide radicals (O_2•-) (Ansari *et al.,* 2009, 2013). The partial reduction of molecular oxygen occurs by addition of one, two and four electrons to O, which leads to the successive formation of intermediates - O_2•-, •OH and H_2O_2, which are highly reactive in comparison to molecular oxygen. Surplus concentrations of redox active HMs such as iron (Fe) and copper (Cu) result in oxidative injury to plants (Mittova *et al.,* 2004; Ansari *et al.,* 2021). The principal mechanism of Cu toxicity involves the Haber-Weiss reaction, characterized by a HM-catalysed production of hydroxyl radicals from hydrogen peroxide (Ansari *et al.,* 2012; Manara, 2012). This type of reaction has not been described for either Cd or Hg in plants (Ansari *et al.,* 2012), despite evidence of oxidative stress induction in different plants after exposure to Cd (Ansari *et al.,* 2012) and Hg (Ansari *et al.,* 2009). Unlike Cu and Fe, Cd are redox inactive metal and incapable of producing ROS directly, but can indirectly promote ROS generation by disrupting physiological processes (Ansari *et al.,* 2015b). Several studies have demonstrated that Cd treatment increases lipid peroxidation levels in common crops, indicating that oxidative stress is induced (Mittova *et al.,* 2004; Ansari *et al.,* 2009; Manara, 2012; Ansari *et al.,* 2016, 2018, 2019).

The ROS are produced by all aerobically respiring cells. As they are generated in significant quantities in the subcellular compartments or organelles, each organelle has some potential targets for oxidative stress as well as mechanisms for eliminating the oxyradicals (Ansari *et al.*, 2009). Being the potentially destructive chemical species, ROS may cause oxidative damage to biomolecules such as proteins, membrane lipids and nucleic acids (Halliwell and Gutteridge, 2007) and disrupt the cellular metabolism (Ansari *et al.,* 2009). It is therefore essential for aerobic organisms to regulate the ROS levels and activities in order to protect themselves against toxicity. Rather than avoiding stressful conditions, as mobile organisms may, plants have developed, due to their sessile nature, sophisticated metabolic responses to cope and survive the stress (Madlung and Comai, 2004). As a response to exposure to toxic metals, various protective mechanisms have evolved in plants (Kleizaite *et al.,* 2004). The control of oxidant levels is achieved through a defense system composed of

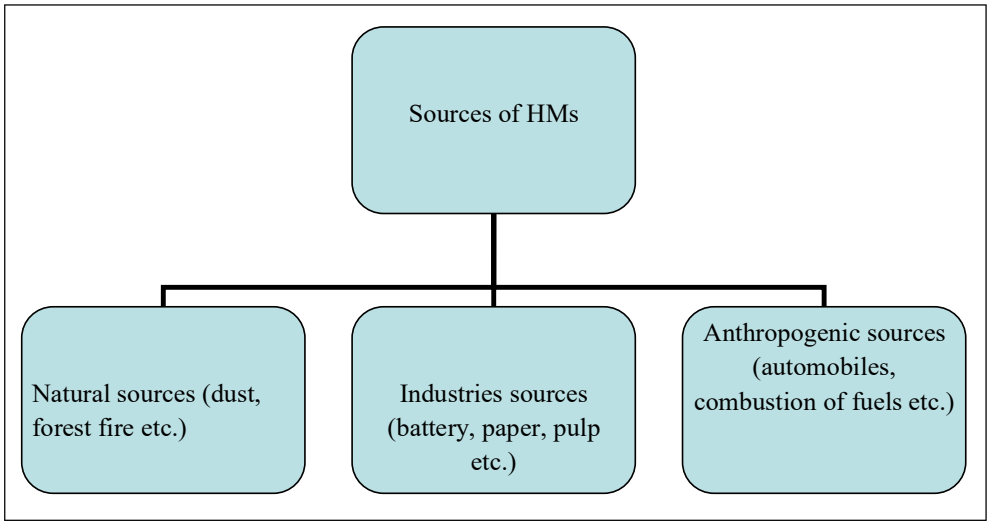

Fig 1: Sources of heavy metals.

enzymatic scavengers and non-enzymatic metabolites (Ansari *et al.,* 2009).

Plant capacity for HM tolerance varies with species. HM-tolerant plants can be classified into (i) metal excluders, (ii) metal indicators and (iii) hyperaccumulators. The last group has enormous importance for phytoremediation processes, which involve utilization of the ability of plants to take up, extract, sequester, accumulate and detoxify different HMs to clean up the soil or water environments (Ansari *et al.,* 2013, 2015; Iqbal *et al.,* 2015). When HM ions reach the cytosol of the cell, toxic HMs become a strong threat to the plant survival. Several strategies have been developed by plants to uptake and accumulate HMs. The HM tolerance mechanisms in plants include metal binding to the cell wall, reduced transport via the cell membrane, active efflux and compartmentalization of metals, chelation and sequestration of HMs (Iqbal *et al.,* 2015). Some plants, such as *Anthyllis vulnenaria* and *Biscutella leavigata*, have the ability to transport excess metals to aging organs and the apoplast of leaves and hence remove them seasonally (Siedlecka *et al.,* 2001). Another strategy which contributes to HM tolerance is the chelation of metals by different ligands/chelators that include phytochelatins (PCs), metallothioneins (MTs), organic acids and amino acids (Ansari *et al.,* 2012). PCs, which are sulfhydryl (SH)-containing peptides with basic structure of (g-Glu-Cys) n-Gly (n is generally in the range of 2 to 11), can firmly bind and sequester metals (Ansari *et al*., 2015b). The PCs are rapidly induced by a wide range of HM ions and enzymatically synthesized from GSH by the enzyme called PC synthase (Ansari *et al.,* 2012). Clemens *et al.,* (1999) reported the presence of PC synthase in a variety of different higher plants. PCs, as metal-binding proteins, chelate and inactivate all toxic metal ions entering the cytosol before they can inactivate the enzymes of important metabolic pathways. The metal-PC complex is subsequently actively transported from the cytosol to the vacuole (Salt and Rauser, 1995). During the oxidative stress, depletion of GSH concentrations in some plants has been attributed to HM induced PCs synthesis (Ansari *et al.,* 2012).

MTs are low molecular weight cystein-rich metal-binding proteins (Robinson *et al.,* 1993). In addition to their role in detoxification by sequestering metals (Cu in particular) in plant cells, they also participate in the maintenance of essential transition metals' homeostasis and protect against intracellular oxidative damage (Ansari

Table 1: Effect of HMs on human health (Singh *et al*., 2018).

HMs	EPA regulatory limit (ppm)	Effects on human health	References
As	0.01	Affects essential cellular processes such as oxidative phosphorylation and ATP synthesis	Tripathi *et al*., 2007
Ag	0.10	Exposure may cause skin and other body tissues to turn gray or blue-gray, breathing problems, lung and throat irritation and stomach pain	ATSDR, 1990
Cd	5.0	Carcinogenic, mutagenic, endocrine disruptor, lung damage and fragile bones, affects calcium regulation in biological systems	Degraeve, 1981
Cr	0.1	Hair loss	Salem *et al*., 2000
Cu	1.3	Brain and kidney damage, elevated levels result in liver cirrhosis and chronic anemia, stomach and intestine irritation	Wuana and Okieimen, 2011
Hg	2.0	Autoimmune diseases, depression, drowsiness, fatigue, hair loss, insomnia, loss of memory, restlessness, disturbance of vision, tremors, temper outbursts, brain damage, lung and kidney failure	Gulati *et al*., 2010
Ni	0.2 (WHO permissible limit)	Allergic skin diseases such as itching, cancer of the lungs, nose, sinuses, throat through continuous inhalation, immunotoxic, neurotoxic, genotoxic, affects fertility, hair loss	Duda *et al*., 2008
Pb	15	Excess exposure in children causes impaired development, reduced intelligence, short-term memory loss, disabilities in learning and coordination problems,a risk of cardiovascular disease	Wuana and Okieimen, 2011
Se	50	Dietary exposure of around 300 µg/day affects endocrine function, impairment of natural killer cells activity, hepatotoxicity and gastrointestinal disturbances	Vinceti *et al*., 2001

et al., 2012). While the first plant MT identified was the wheat Ec (eraly cystein labelled) protein (Lane *et al.,* 1987), many gene encoding MT-like proteins have been found in plants (Iqbal *et al.,* 2015). Among the HM-binding ligands, PCs and MTs are the most studied and best characterized groups in plant cells. However, there are also other candidate ligands for cellular chelators, including some low molecular weight organic acids, such as citrate, malate and oxalate (Ansari *et al.,* 2012) and amino acids, such as nicotinamine, histidine and proline (Kerkeb and Kramer, 2003). Redox active HMs exhibit toxic effects by ROS generation through auto-oxidation and Fenton reactions. According to Ahmad *et al*. (2008), oxidative stress is introduced to plant cells in several ways by the actions of reactive HMs. Like transition metals (such as Fe and Cu), reactive HMs can accept or donate a single electron, which promotes the ROS interconversion (Ansari *et al.,* 2012); metabolic pathways may be disturbed by metals that again cause enhanced ROS production (Richards *et al.,* 1998). For example, zinc treatment of *Phaseolus vulgaris* increased the levels of H_2O_2 in roots and lipid peroxidation in primary leaves (Weckx and Clijsters, 1997). Also, HMs generally inactivate the antioxidant enzyme activities and hence HM accumulation results in the depletion of some well-known antioxidants, such as GSH, which is used for PCs production (Ansari *et al.,* 2012). This severe depletion of GSH is accepted as a very common response of plant exposure to HMs (Ansari *et al.,* 2015b). Thus, this review article explores the heavy metal stress and role of the antioxidative defense enzymes against HM stress in plants.

Cellular antioxidant system of plant

The term “antioxidant” is used to describe the compound capable of quenching reactive oxygen species (ROS) without itself undergoing conversion to a destructive radical. The determinants of the efficiency of the antioxidant system include several factors such as compartmentalization of ROS, synthesis, transport and/or localization of antioxidants, plant’s ability to induce the antioxidant defence and cooperation (and/or compensation) between different antioxidant systems (Blokhina *et al.,* 2003). Pernicious changes in the activity of antioxidant isozymes in different cell compartments are considered more important than the overall enzymatic activities. The delicate balance between ROS production and scavenging that allows this duality in function to exist in plants is thought to be directed by a large network of genes termed as the ‘ROS gene network’, which includes more than 152 genes in *Arabidopsis*, which tightly regulate the ROS production and scavenging. The ROS signaling is therefore controlled predominantly by production and scavenging (Mittler *et al.,* 2004). The common ROS-scavenging enzymes are SOD, CAT, POXs and glutathione peroxidases (Glu-POX, EC 1.11.1.9). The multiple (three) isoforms of the upstream enzyme SOD (mitochondrial Mn-, chloroplastic Fe- and the cytosolic, chloroplast, or paroxysmal-Cu/Zn-SODs) catalyzes the dismutation or disproportionation of two superoxide radicals to H_2O_2 and oxygen, thus maintaining superoxide radicals to steady state levels (Mittova *et al.,* 2004). Since CAT does not require an additional substrate, it can decompose H_2O_2 formed into H_2O and O_2 within the peroxisome. The scavenging of H_2O_2 in other cell compartments (cytosol, vacuole, cell wall and extracellular space) depends on distinct POXs, such as guaiacol peroxidases (GPX, EC 1.11.1.7) and ascorbate peroxidases (APX, EC 1.11.1.11) that use a substrate for their activity (Noctor and Foyer, 1998). While CAT is primarily localized in peroxisomes, isoforms of POXs and SODs are distributed throughout the cell and can be found in cytosolic, mitochondrial and chloroplastic compartments. The CAT functions through an intermediate CAT-H_2O_2 complex and produces water and dioxygen (Mittova *et al.,* 2004). In the presence of an appropriate substrate, CAT-H_2O_2 complex drives the peroxidatic reaction, which is a much more effective antioxidant than H_2O_2 itself. Thus the reaction of CAT-H_2O_2 complex with another H_2O_2 molecule (CAT action) represents a one-electron transfer, which splits peroxide and produces another strong antioxidant, the hydroxyl radical, which is capable of initiating radical chain reactions with organic molecules, particularly with polyunsaturated fatty acids (PUFA) in membrane lipids (Ansari *et al.,* 2015b). Apart from ROS-scavenging enzymes, there are also additional enzymes like glutathione S-transferases (GST, EC 2.5.1.18), phospholipid hydroperoxide Glu-POX and APX, which can detoxify lipid peroxy (LP) products and other electrophilic xenobiotics. GST catalyses the conjugation of electrophilic toxic molecules with GSH, which are then transported out of the cytosol by glutathione pumps. In addition, a whole array of enzymes is needed for the regeneration of the reduced forms of antioxidants, like monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2) (Ansari *et al.,* 2012; Yadav *et al.,* 2015).

The non-enzymatic antioxidant components involve a network of low molecular mass antioxidants with high reducing potentials, such as ascorbic acid (AA), cysteine, non-protein thiols, glutathione, tocopherols, β-carotene, polyamines, phenolic compounds, flavonoids, tannins and lignin precursors. Interactions between AA and glutathione or AA and phenolic compounds are well known (Blokhina *et al.,* 2003). AA and glutathione are considered the two main information-rich redox cell buffers and redox sensors (Noctor and Foyer, 1998). A decrease in their redox status leads to a loss of cell redox homeostasis (Noctor and Foyer 1998). They together are coupled or work in concert with enzymes such as APX, DHAR and GR to scavenge H_2O_2 generated in the chloroplasts and modulate the oxidation state of the cell (Creissen *et al.,* 1994), where the corresponding biochemical pathway is called the ascorbate-glutathione cycle (AGC) or Halliwell-Asada pathway (Chew *et al.,* 2003). Fig 2 depicts free radical formation as well as scavenging in plant cells. In this review,

we limit our discussion to AA and glutathione, together with the enzymatic components of AGC, with particular emphasis on perspectives of their regulatory expression, activity and protective role in response to HM stress.

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. These are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis (Mittova *et al.,* 2004; Ansari *et al.,* 2021). However, during environmental stress, ROS levels can increase dramatically and cause significant damage to cell structures (Bashir *et al.,* 2007). When a plant recognizes an attacking pathogen, one of the first induced reactions is to rapidly produce superoxides (O_2^-) or hydrogen peroxide (H_2O_2) to strengthen the cell wall. This prevents the spread of pathogen to other parts of the plants, essentially forming a net around the pathogen to restrict its movement and reproduction (Yadav *et al.,* 2015). In aerobic organisms the energy needed to fuel biological functions is produced in the mitochondria *via* the electron-transport chain. In addition to energy, ROS are produced, which can damage DNA, RNA and proteins and this contributes to physiological ageing. ROS are constantly generated and eliminated in the biological system and are essential to drive regulatory pathways (Ansari *et al.,* 2009).

Production of ROS in cells

During the course of normal metabolic processes all organisms produce a range of ROS, including superoxides (O_2^-), hydroxyl radical (-OH) and hydrogen peroxide (H_2O_2).

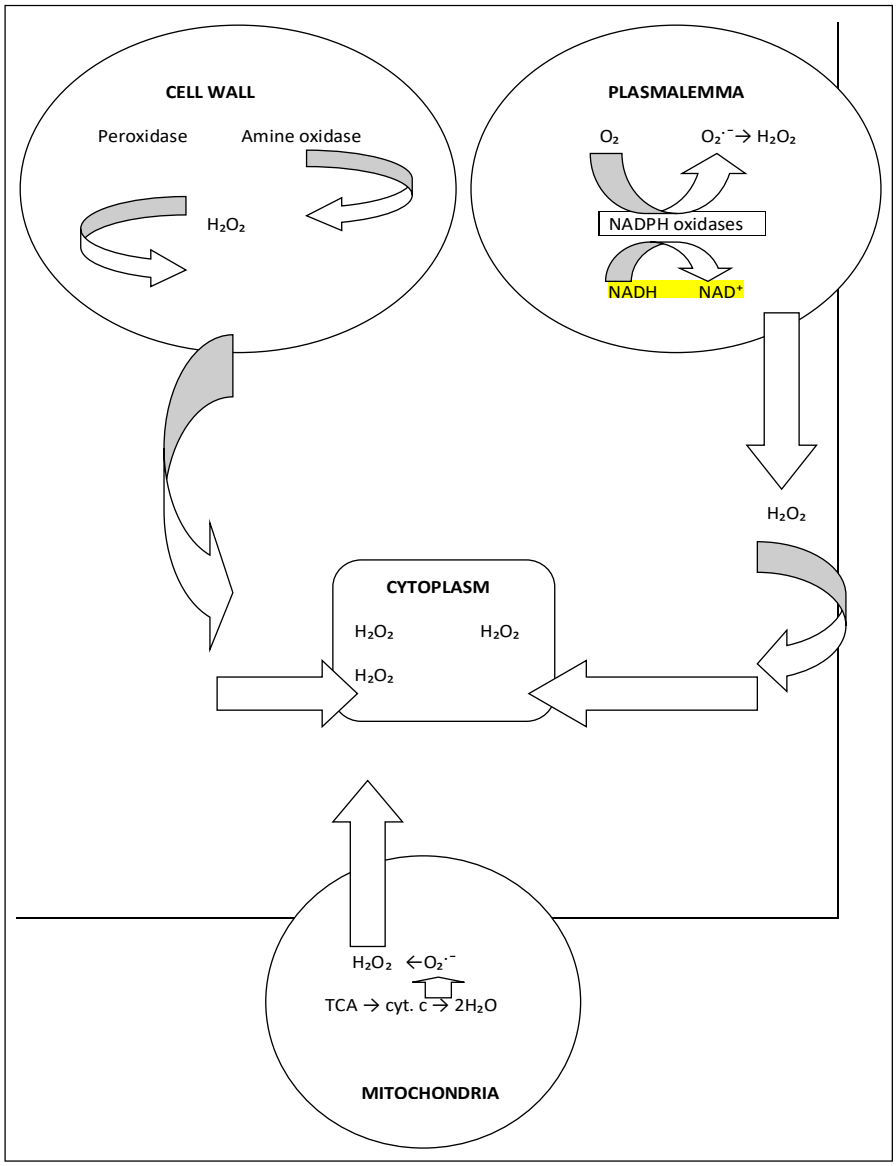

Fig 2: Production of reactive oxygen species at different sites within the plant cell.

If not effectively and rapidly removed from cells, ROS can damage a wide range of macromolecules, possibly leading to cell death (Ansari *et al.,* 2012). The scavenging systems include antioxidants such as ascorbate and glutathione, as well as enzymes such as SOD, CAT and peroxidase. Mitochondria are the major source of ROS in eukaryotic cells. Superoxides, which are rapidly converted to H, O, through the action of SOD, are produced during respiration, primarily by the autoxidation of reduced mitochondrial electron-transport components (Bashir *et al.,* 2007). In plants, ROS are the normal byproducts of various metabolic pathways, but these are produced in excess under stress conditions in different cellular compartments including chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum (ER) and plasma membrane (Creissen *et al.,* 1994). At very high temperature, severe cellular injury and even cell death may occur, which could be attributed to a catastrophic collapse of the cellular organization (Creissen *et al.,* 1994). At a moderately high temperature, injuries or death may occur only after a long-term exposure.

Enzymatic antioxidants

The complexity and diversity of oxygen radical scavenging systems parallel the complexity of their sites of production. Enzymatic antioxidants typically work in tandem to detoxify ROS. For example, superoxide dismutase converts the superoxide anion to hydrogen peroxide (Yadav *et al.,* 2015), which must also be subsequently scavenged in order to avoid both its direct oxidizing capacity and propensity to react with the superoxide anion in the presence of a divalent iron ion to form the highly reactive hydroxyl radical (Ansari *et al.,* 2012). Detoxification of H_2O_2 can be accomplished with peroxidase, ascorbate peroxidase, glutathione peroxidase, thioredoxin peroxidase and catalase. If the enzyme is ascorbate peroxidase, ascorbate acts as a scavenger of peroxide and the oxidized ascorbate is subsequently recycled *via* the action of monodehydroascorbate reductase (MDHAR) (Arora *et al.,* 2002). Glutathione peroxidase and glutathione reductase can also operate as a fully regenerative detoxification agent. Oxidase can also provide an alternate electron flow, which can reduce the production of ROS in a damaged or inhibited electron-transport chain (Ansari *et al.,* 2021). However, when the production of ROS exceeds the capacity of these self-regenerating systems, a decline in many or all of the antioxidants may occur.

(i) Superoxide dismutase (SOD)

SODs are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2). As such, they impart a great defense in nearly all cells exposed to oxygen. The SOD-catalyzed dismutation of superoxide may be represented by the two following two half-reactions:

$$M^{(n+1)}\_SOD + O_2^- \rightarrow M^{n+}\_SOD + O_2$$

$$M^{n+}\_SOD + O_2 + 2H^+ \rightarrow M^{(n+1)+}\_SOD + H_2O_2$$

In this reaction the oxidation state of the metal cation (M) oscillates between n and n+1.

(ii) Catalase (CAT)

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of H_2O_2 to water and oxygen (Chelikani *et al.,* 2004). It has a big role in protecting the cell from oxidative damage by ROS and has a very high turnover rate; one catalase molecule can convert approximately 5 million molecules of H_2O_2 to water and oxygen per second (Morana *et al.,* 2012).

H_2O_2 is a harmful byproduct of many normal metabolic processes and needs to be converted quickly into other, less dangerous substances in order to prevent damage to cells/tissues. To this end, catalase is frequently used by cells to rapidly catalyze the H_2O_2 decomposition forming the less-reactive gaseous oxygen and water molecules (Ansari *et al.,* 2012). It is located usually in a cellular, bipolar organelle called the peroxisome, which is involved in photorespiration (Chelikani *et al.,* 2004).

Ascorbate peroxidase (APX)

Ascorbate peroxide (APX) detoxifies peroxides such as H_2O_2 using ascorbate as a substrate. The enzyme catalyzes the reaction involving the transfer of electrons from ascorbate to peroxide, which produces dehydroascorbate and water as the byproducts (Ansari *et al.,* 2012).

$$\text{Ascorbate} + H_2O_2 \rightarrow \text{Dehydroascorbate} + H_2O$$

$$C_6H_8O_8 + H_2O_2 \rightarrow C_6H_6O_6 + 2H_2O$$

APX is an integral component of the glutathione-ascorbate cycle (Noctor and Foyer, 1998). Peroxidases, of which APX is the most common, are all proteins containing the heme prosthetic group in which iron plays an important role in the catalytic site (Caverzan *et al.,* 2012). Ascorbate, the electron donor to which the enzyme exhibits high affinity, is the substrate most commonly utilized in the reduction of H_2O_2. APX occur mainly in higher plants, algae and some cyanobacteria (Chelikani *et al.,* 2004).

(iii) Glutathione-S-Transferase (GST)

Glutathione-S-transferases (GSTs) constitute a family of cytosolic multifunctional enzymes, which catalyze the conjugation of glutathione with a variety of reactive electrophilic compounds, thereby neutralizing their active electrophilic sites and subsequently making the parent compound more water soluble (Chelikani *et al.,* 2004). Glutathione peroxidases are substantially more efficient on a molar basis than most other enzymes. They also act as a radical scavenger, membrane stabilizer and the precursor of HM-binding peptides (Ansari *et al.,* 2015a).

Non-enzymatic antioxidants

Non-enzymatic antioxidants include the major cellular redox buffers, *viz*. ascorbate and glutathione (GSH), as well as tocopherol, flavonoids, alkaloids and carotenoids. Mutants

with decreased ascorbic acid levels (Conklin *et al.,* 1996) or altered GSH content (Creissen *et al*., 1999) are hypersensitive to stress. Whereas GSH is oxidized by ROS, forming oxidized glutathione (GSSG), ascorbate is oxidized to monodehydroascorbate (MDA) and dehydroascorbate (DHA). In response to chilling, heat shock, pathogen attack, heavy metals and drought stress, plants increase the activity of GSH biosynthetic enzymes (Ansari *et al.,* 2012) and the GSH levels (Chelikani *et al.,* 2004).

Ascorbate-glutathione (AsA-GSH) cycle

The role of ROS has received special attention because a variety of environmental factors such as drought, cold, heat, salinity, herbicides, pesticides and heavy metals tend to harm the cell by raising the oxidative level and causing the loss of cellular structure and function, which then requires availability of detoxification agents including the non-enzymatic antioxidants.

The ascorbate-dependent detoxification of H_2O_2 is associated with guaiacol-type peroxidases also, thus suggesting that ascorbate is the natural substrate for many types of peroxidase *in situ* and not just for the ascorbate-specific peroxidases (Chew *et al.,* 2003). The ascorbate-dependent destruction of H, O, in the more acidic cellular compartments, such as the vacuole, may be an important function of such non-specific peroxidaes. In plants, the ascorbate-glutathione (AsA-GSH) cycle constitutes one of the most important antioxidant systems, where ascorbate and glutathione are utilized as reducers and recycled through consumption of ATP and NADPH (Noctor and Foyer, 1998), in a process which involves four enzymes (APX, MDHAR, DHAR and GR) (Fig 3). The AsA-GSH cycle enzymes are activated to dissipate the excess excitation energy created during photosynthesis (Morana *et al.,* 2012).

During the ascorbate-glutathione cycle, H_2O_2 is converted to harmless water (Fig 3). The reducing agent in the first reaction, which is catalyzed by ascorbate peroxidase (APx), is ascorbate, which oxidizes into monodehydroascorbate (MDA). Monodehydroascorbate reductase (MDHAR) then reduces MDA to ascorbate with the help of NADPH. Dehydroascorbate (DHA) is produced spontaneously by MDA and can be reduced to ascorbate by dehydroascorbate reductase (DHAR) with the help of GSH that is oxidized to form the oxidized glutathione (GSSG). The cycle closes with glutathione reductase (GR) converting GSSG back to GSH with the reducing agent NADPH (Yadav *et al.,* 2015).

APX utilizes AsA as an electron donor to reduce H_2O_2 to water with the concomitant generation of MDA, a univalent oxidant of AsA. MDA is spontaneously disproportionate to AsA and DHA. MDA is directly reduced to AsA by the action of NADPH-dependent MDA reductase. DHA reductase utilizes glutathione (GSH) to reduce DHA and thereby regenerate AsA (Mittova *et al.,* 2004). The oxidized GSH is then regenerated by GSH reductase, utilizing the reducing equivalents from NADPH. Thus, APx in combination with an effective AsA-GSH cycle, functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms (Asada, 1992; Yadav *et al.,* 2015). In the chloroplasts of higher plants, in addition to the AsA-GSH cycle located in the stroma, the water-water cycle, which is the photoreduction of oxygen to water in PS-I by the electrons derived from water in PS-II, participates in the detoxification of active oxygen species and the dissipation of energy photons (Asada, 1999).

Components of AsA-GSH cycle

(i) Monodehydroascorbate reductase (MDHAR

In plants, monodehydroascorbate reductase (MDHAR) is an enzymatic component of the ascorbate-glutathione cycle, which is one of the major antioxidant systems operating within plant cells for protection against the ROS damage. MDHAR activity has been observed in several cell compartments, including chloroplasts, cytosol, mitochondria, glyoxysomes and peroxisomes (Halliwell and Gutteridge, 2007).

Primarily MDHAR catalyzes a chemical reaction involving three substrates (NADH, H^+ and MDA) to produce two products (NAD^+ and ascorbate).

$$NADH + H^+ + 2MDA \leftrightarrow NAD^+ + 2 \text{ ascorbate}$$

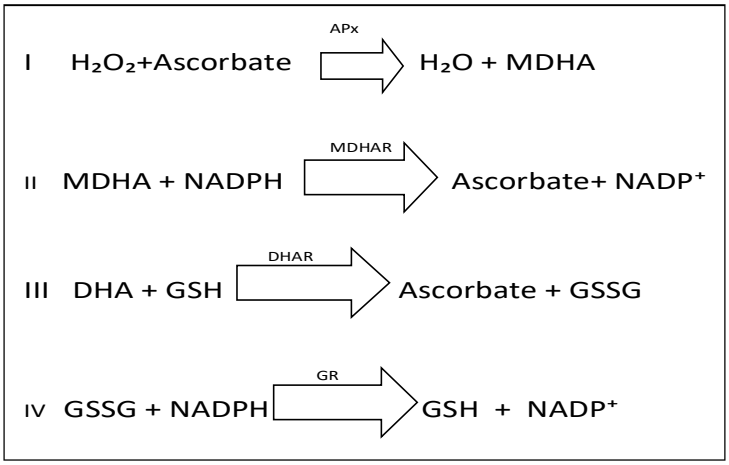

Fig 3: The ascorbate-glutathione (AsA-GSH) cycle.

(ii) Dehydroascorbate reductase (DHAR)

Dehydroascorbate reductase (DHAR) is a physiologically important reducing enzyme of the ascorbate-glutathione cycle reaction in higher plants. It plays important roles in plant adaptation to environmental stresses and in the regulation of growth, differentiation and various metabolisms (Asada, 1992). It affects the growth of plant cells by controlling the concentration and redox state of ascorbate (Asada, 1992). If ascorbate and its derivatives participate in cell growth, it is likely that DHAR and MDHAR have different physiological roles (Yadav *et al.,* 2015).

(iii) Glutathione reductase (GR)

Glutathione reductase (GR), also known as glutathione-disulfide reductase (GSR), is an enzyme that catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form of glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell (Noctor and Foyer, 1998). GR functions as a dimeric disulfide oxidoreductase and utilizes NADPH to reduce one mole of GSSG to two moles of GSH.

$$GSSG + NADPH \rightarrow 2GSH + NADP (+)$$

Glutathione plays a key role in maintaining proper function and preventing oxidative stress by acting as a scavenger for hydroxyl radicals, singlet oxygen and various electrophiles. Reduced glutathione reduces the oxidized form of the enzyme glutathione peroxidase, which in turn reduces H_2O_2 a dangerously reactive species within the cell. In addition, GSH plays a key role in the metabolism and clearance of xenobiotics, acts as a cofactor in certain detoxifying enzymes (Mittler *et al.,* 2004; Ansari *et al.,* 2012) and regenerates antioxidants such as vitamins C and E to their reactive forms. The ratio of GSSH/GSH present in the cell is a key factor in maintaining the oxidative cell balance where it is critical that a cell maintains high levels of the reduced glutathione and a low level of oxidized glutathione disulfide. This narrow balance is "buffered" by GR, which catalyzes the reduction of GSSG to GSH (Gill, 2014). In plants, reduced glutathione participates in the glutathione-ascorbate cycle in which reduced glutathione reduces dehydroascorbate, a reactive byproduct of the reduction of H_2O_2. In particular, GR contributes significantly to the plant response to abiotic stress, where the enzyme activity has been shown to be modulated in response to metals, metalloids, salinity, drought, UV radiation and heat-induced stress (Gill *et al.,* 2014).

(iv) Glutathione (GSH)

In plants, glutathione (GSH) is an important antioxidant, which prevents the ROS-caused damage to important cellular components (Ahmad *et al.,* 2008). Once oxidized, GSH can be reduced back by glutathione reductase, using NADPH as an electron donor (Noctor and Foyer, 1998). The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity (Ahmad *et al.,* 2008). GSHt is also a major endogenous antioxidant produced by cells, participating directly in the neutralization of free radicals and ROS. Glutathione, a major non-protein thiol in living organisms, has a pivotal role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. It is a key component of the ascorbate-glutathione cycle, a system that reduces poisonous hydrogen peroxide (Noctor and Foyer, 1998; Ansari *et al.,* 2012, 2015a).

(v) Ascorbate (AsA)

Ascorbate (AsA), an enzyme cofactor, is perhaps the best known antioxidant. Its participation in photosynthesis and photorespiration, in defence against oxidative stresses and speculations about its role in cell expansion and cell division are well known. Ascorbate occurs in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall. Its high concentration in chloroplasts is possibly related to its central role in photosynthesis (Asada, 1992). Dehydroascorbate (DHA), the oxidized form of ascorbate, is taken up by plants more rapidly than ascorbate. As an antioxidant, ascorbate reacts rapidly with superoxide, singlet oxygen, ozone and hydrogen peroxide (Caverzan *et al.,* 2012). It thus participates in the removal of these reactive forms of oxygen, which are generated during aerobic metabolism and during exposure to certain pollutants and herbicides. MDA and DHA are reduced to ascorbate by the ascorbate-glutathione cycle, thus ensuring a capacity to regenerate significant concentrations of AsA in cellular defense (Ansari *et al.,* 2021; 2023).

Ascorbate reacts with ROS-generating monodehy droascorbate (MDA) and dehydroascorbate (DHA). The DHA is decomposed into tartiaric acid and ketosuccinic acid. The MDA and DHA are reduced to ascorbate by monodehydroascorbate reductase enzyme, using NADPH or glutathione as reducer. Thus, ROS are consumed through this ascorbate-glutathione cycle (Mohammed *et al.,* 2011).

(vi) Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)

In photosynthetic organisms, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) is produced by ferredoxin-$NADP^+$ reductase in the last step of photosynthesis, where reducing power is utilized in the biosynthetic reactions of the Calvin cycle to assimilate CO_2 and convert CO_2 into glucose (Bashir *et al.,* 2015). It is also needed in the reduction of nitrate into ammonia during N assimilation in plants via the nitrogen cycle. NADPH provides reducing equivalents for biosynthetic reactions and for the oxidation-reduction involved in protection against the ROS toxicity, allowing for regeneration of reduced glutathione (GSH). NADPH is also used in anabolic pathways such as lipid synthesis, cholesterol synthesis and fatty acid chain elongation (Asada, 1992). It also acts as a source of reducing equivalents for cytochrome P450 hydroxylation of aromatic compounds, steroids, alcohol and

drugs (Asada, 1992). The NADPH system is also responsible for generating free radicals in immune cells where the generated radicals are used to destroy pathogens in a process termed respiratory burst (Ahmad *et al*., 2008).

CONCLUSION

In plants under stress, a wide variety of ROS are produced as byproducts of common metabolic reactions, such as photosynthesis and respiration due to HMs stress. Oxidative stress occurs when there is a serious imbalance between the production of ROS and the antioxidant-defense. Generation of ROS causes rapid cell damage by triggering a chain reaction. Cells have evolved an elaborate system of enzymatic and non-enzymatic antioxidants which help to scavenge these indigenously generated ROS. Various enzymes involved in ROS-scavenging have been manipulated, over-expressed or down-regulated and a dynamic monitoring of concentrations of such enzymes significantly adds to our present knowledge and understanding of their role in antioxidant systems.

Conflict of interest

The authors declare no potential conflict of interest related to this study.

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