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Reactive Oxygen Species, Oxidative stress and ROS scavenging system in plants

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ABSTRACT

Formation of small and highly reactive oxygen species (ROS) is normal phenomena in living cells. The Reactive Oxygen Species cover free radical such as superoxide (O_2^-), hydroxyl (OH), perhydroxy (HO_2^-) and alkoxy (RO) and non-radicals like, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Their formation and reactivity is well established and found to be tightly regulated. ROS level exceeding the antioxidative capacity of cells lead to oxidative stress which may result in malfunctioning and ultimately death of the cell. Singlet oxygen, Superoxide, hydroxyl and hydrogen peroxide tends to react easily with most biomolecules of the cell, causing their degradation and destruction, contributing to cellular stress. In plant cells, environmental changes and developmental transitions such as seed germination undergoes ROS generation. To avert the oxidative stress and excess of ROS, plant cells are equipped with antioxidative machinery comprised of both enzymatic and non-enzymatic compounds of low molecular weight. While Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX), Glutathione peroxidase (GPX), Glutathione-S- Transferase (GST), Monodehydroascorbate reductase (MDHAR) and Dehydroascorbate reductase (DHAR) forms the enzymatic part, Ascorbate, phenolic compounds, carotenoids and tocopherols contribute in the non-enzymatic arm of the antioxidative defense of the cell. The present review focuses upon the sources of ROS, their characteristics and contribution to oxidative stress and means of antioxidative machinery to deal with oxidative stress.

Keywords- Reactive Oxygen Species, Oxidative stress, enzymatic antioxidants, non-enzymatic antioxidants

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INTRODUCTION

Plant life exclusively depends upon light energy known as Photosynthetic Active Radiation (PAR), wavelength ranging from 400 to 700 nm. Light intensity more or less than this wavelength range leads to reduced plant productivity [1]. High light intensity influences the generation and accumulation of ROS due to altered antioxidative system. ROS are generated in normal cellular metabolism under tight regulation but sometimes it can turn into oxidative stress [2]. There is an increase in antioxidative activity when plants are transferred from low to high sun light. Evidence for an involvement of ROS during high light treatment is provided by the degradation of glutamine synthetase, phosphoglycolate phosphatase, large subunit of Rubisco and an increase content of carbonyl groups in stromal proteins of pea [3]. Drought stress induces inhibition of photosynthesis leading to accumulation of ROS [4]. Accumulation during such conditions originates mainly from the decline in CO_2 fixation, which causes higher leakage of electrons to O_2. Changes in antioxidant level are also correlated with water deficit [5]. Plant responses to Salinity often include drought-mediated symptoms due to excessive uptake of ions (Na^+ and Cl^-) [6]. Consequently, it reduces the soil water potential leading to limit the water uptake generating conditions similar to drought.

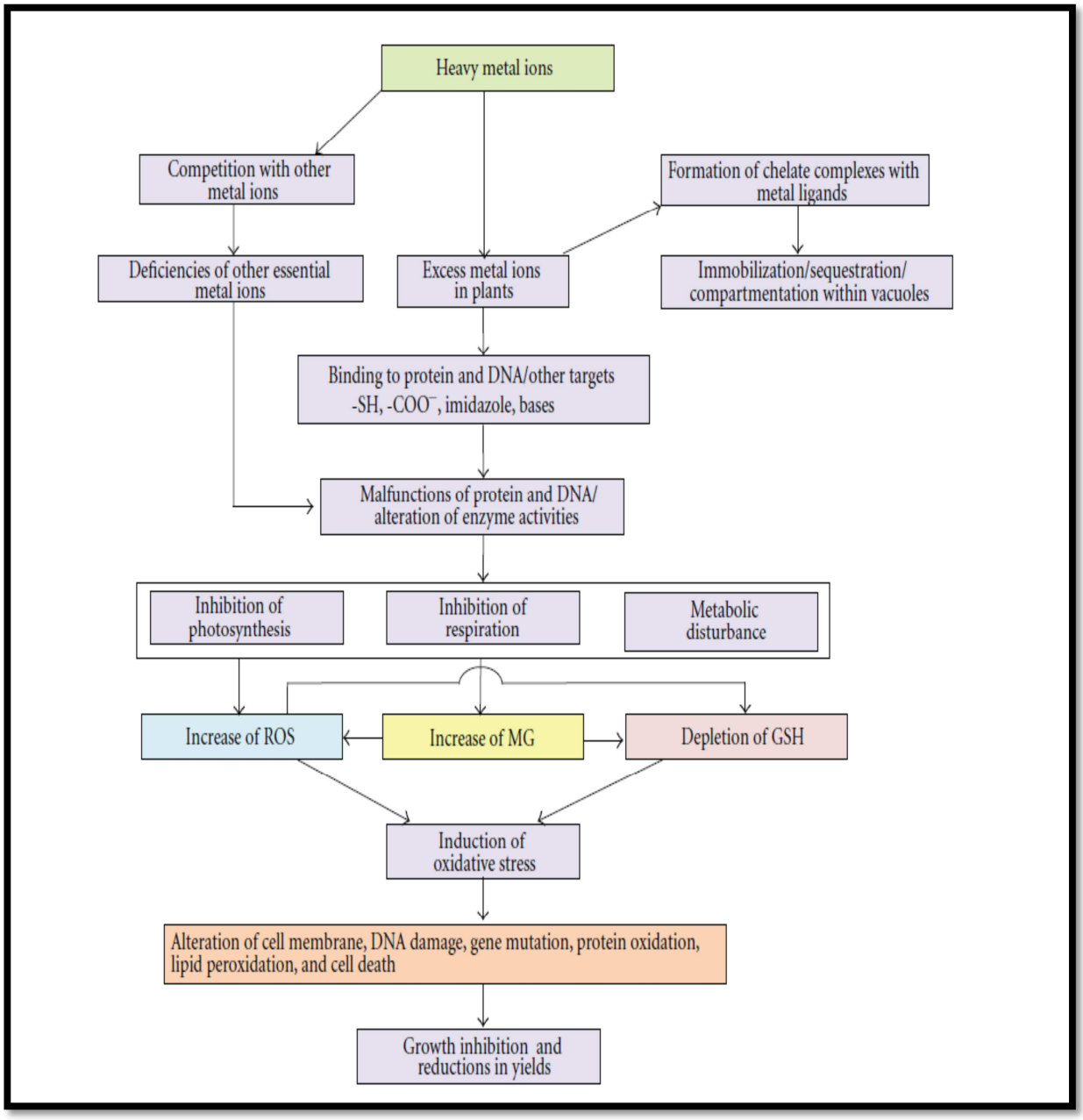

Figure 1: Possible biochemical and molecular mechanisms of heavy metal-mediated ROS induction and damage to the development of higher plants [11]

Therefore, the generation of ROS during salt stress is almost similar to that during drought. It mainly leads to the generation of superoxide and H_2O_2 [7]. Heavy metals (HMs) are natural constituents of soils and occur naturally in the environment but in the present world scenario, contamination of soils by toxic metals and metalloids is of major concern worldwide [8]. HMs are known to disturb redox homeostasis by stimulating the formation of free radicals and ROS such as singlet oxygen ($^1O_2$), superoxide radicals ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) [9]. Plants which are exposed to heavy metals shift the balance of ROS metabolism towards accumulation of ROS [10]. Recently, methylglyoxal (MG), a cytotoxic compound, was also found to increase in response to various stresses including HMs (Figure 1) [11]. An increase in MG level in plant cells further intensifies the production of ROS by interfering with different plant physiological and metabolic processes such as inactivation of the antioxidant

defense system and interfering with vital plant physiological processes such as photosynthesis [11]. One of the most deleterious effects induced by heavy metals exposure in plants is lipid peroxidation which can directly cause biomembrane deterioration [12] (Figure 1).

1. Derivatives of Oxygen and other ROS

The metabolic pathways in the univalent reduction of O_2 to water lead to generation of ROS due to the high energy time window. The spin restriction makes O_2 prefer to accept its e- one at a time, leading to ROS. The ground state O_2 is inactive until it accepts electrons and become activated. The activation leads to singlet oxygen participating in reactions involving the simultaneous transfer of two electrons. The single e- reduction of O_2 results in the generation of the superoxide radical ($O_2^{\cdot-}$). At low pH, dismutation of $O_2^{\cdot-}$ is unavoidable, with one $O_2^{\cdot-}$ giving up its added electron to another $O_2^{\cdot-}$, followed by protonation to generate of hydrogen peroxide (H_2O_2). Furthermore, $O_2^{\cdot-}$ can be protonated to form hydroperoxy radical ($HO_2^{\cdot}$). Additionally, $O_2^{\cdot-}$ can also donate an electron to iron (Fe^{3+}) to yield a reduced form of iron (Fe^{2+}) which can then reduce H_2O_2 to hydroxyl radical ($OH^{\cdot}$). The reduction through which $O_2^{\cdot-}$, H_2O_2 and iron rapidly generate $OH^{\cdot}$ is called the Haber-Weiss reaction whereas the final step which involves the oxidation of Fe^{2+} by H_2O_2 is referred to as the Fenton's reaction (Figure 2). H_2O is formed when $OH^{\cdot}$ is further reduced. Details of different ROS are summarized in Table 1.

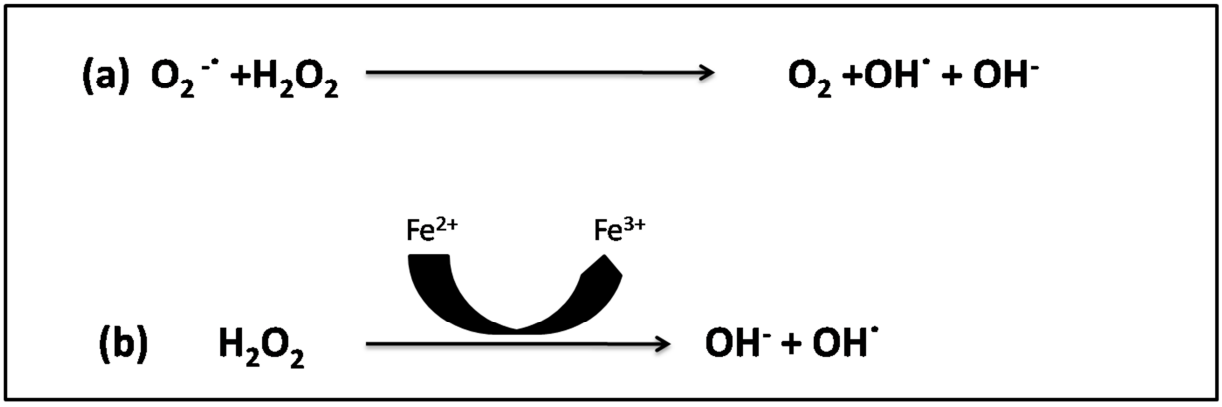

Figure 2: Equations depicting Haber-Weiss Reaction and Fenton Reaction

3.1 Singlet Oxygen ($^1O_2$)

Significant production of singlet O_2 can occur in all living organisms through various mechanisms but is particularly prominent in the chloroplast because of the routine formation of excited pigments in photosynthesis. Abiotic stress factors lead to closing of stomata and results in low intracellular CO_2 concentration in the chloroplast, favoring the formation of $^1O_2$. $^1O_2$ can last for nearly 4 μs in water and 100 μs in polar organic solvents. The lifetime of $^1O_2$ in a cell has been measured to be approximately 3 μs [13]. In this small time, a fraction of $^1O_2$ may be able to diffuse over considerable distances of several 100nm. However, recent measurements show that its lifetime is much longer (6 μs) than stated earlier [14]. $^1O_2$ can react with proteins, pigments and lipids and is thought to be the most important species responsible for light-induced loss of PSII activity, the degradation of the D1 protein (protein of the reaction centre of PSII) and for pigment bleaching.

3.2 Superoxide radicals ($O_2^{\cdot-}$)

Superoxide radical is considered as the primary ROS to be generated. In plant tissues about 1-2 % of O_2 consumption leads to the generation of $O_2^{\cdot-}$. $O_2^{\cdot-}$ is produced by the single electron reduction of O_2 during e^- transport along the non-cyclic pathway in the Electron Transport Chain of chloroplast at the level of PSI [15] while in non-green plant parts or in darkness, the mitochondria appear to be the main ROS producers [16]. $O_2^{\cdot-}$ is also produced in peroxisomes [17] and in the plasma membrane by NADPH oxidase [18]. Its half-life is approximately 2-4 μs; therefore it cannot cross biomembranes and easily get dismutated to H_2O_2.

Table 1: Key reactive oxygen species (ROS), their properties, and main scavenging systems in plant cell

S.No.	ROS	Half-life and mobility	Mode of Action	Main scavenging systems
1.	Superoxide radical ($O_2^{\cdot-}$)	1 μs, 30 nm	Reacts with double bond-containing compounds such as iron-sulphur (Fe-S) clusters of proteins; reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$)	Superoxide dismutases (SODs)
2.	Hydroxyl radical ($OH^{\cdot}$)	1 ns, 1 nm	Extremely reactive with protein, lipids, DNA, and other macromolecules	Flavonoids, prevention of $OH^{\cdot}$ formation by sequencing Fe
3.	Hydrogen peroxide (H_2O_2)	1 ms, 1 μm	Oxidizes proteins; reacts with $O2^{\cdot-}$ in a Fe-catalyzed reaction to form $OH^{\cdot}$	Catalases, various Peroxidases, peroxiredozins, and flavonoids
4.	Singlet oxygen ($^1O_2$)	1 μs, 30 nm	Directly oxidizes protein, polyunsaturated fatty acids, and DNA	Carotenoids and α-tocopherols

3.3 Hydrogen Peroxide (H_2O_2)

Major pathways producing H_2O_2 are photorespiration, β- oxidation, proton-induced decomposition of $O_2^{\cdot-}$ anion and defense against pathogens and via enzymes like cytochrome P-450, D-amino acid oxidase, acetyl coenzyme A oxidase and uric acid oxidase [19, 20]. Further, the oxidation of sarcosine in the pahway of glycine metabolism leads to H_2O_2 formation [21]**.** It is moderately reactive and relatively long-lived molecule with a half-life of 1ms. Its stability and ability to cross membranes makes H_2O_2 a good signalling molecule [22]. It's not a free-radical but it plays a radical forming role as an intermediate in the production of more reactive free radicals.

3.4 Hydroxyl Radical ($OH^{\cdot}$)

$OH^{\cdot}$ is considered as most reactive and damaging free radical with a half-life of 1 ns [23]. It is highly reactive with proteins, lipids, DNA and other macromolecules. The major pathways producing hydroxyl are decomposition of ozone in the presence of H^+ in apoplastic space and during the pathogen response [24]. In the presence of suitable transition metals, especially Fe, $OH^{\cdot}$ can also be produced from $O_2^{\cdot-}$ and H_2O_2 at neutral pH through Fenton reaction (Figure 2). $OH^{\cdot}$ are thought to be largely responsible for mediating oxygen toxicity in vivo. Plants are unable to scavenge this highly reactive ROS. Hydroxyl is not considered to have signaling function but the products of its reactions can elicit signaling responses [25].

2. ROS and Oxidative damage

During metabolic processes there is continuous production of ROS which induces oxidative stress and can result in damage to cell membranes, inactivation of enzymes, damage to genetic material and to other vital cell components. There is equilibrium between production and removal of ROS which can be perturbed by various biotic and abiotic factors. The main cellular modifications are discussed below.

4.1 Modifications to PUFA (Poly Unsaturated Fatty Acids)

Lipids are one of the essential biomolecules required for cells and cell organelles. It is of utmost importance to maintain the integrity of the membrane made of phospholipids, sphingolipids and cholesterol. PUFA are very important to maintain the membrane's property but they are also most susceptible to the damaging effects of ROS, whereas fatty acids having one or no double bonds are more resistant to peroxidation than PUFA. Due to high oxidizing potential, $OH^{\cdot}$, $HO_2^{\cdot}$, $RO^{\cdot}$ and $RO_2^{\cdot}$ are capable of doing lipid oxidation. Several aldehydes, like 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) as well as hydroxyl and keto fatty acids are formed as a result of PUFA peroxidation [26, 27]. The occurrence of MDA is considered as a useful index of general lipid peroxidation.

Due to lipid peroxidation, membrane properties are altered, which include the composition and organization of lipids inside the bilayer, degree of PUFA unsaturation, mobility of lipids within the bilayer, localization of the peroxidative process in a particular membrane and the preventive antioxidative process including ROS scavenging and lipid product detoxification [28]. Lipid peroxidation causes decreased membrane fluidity, makes it easier for phospholipids to get exchanged between the two halves of the bilayer, increases the leakiness of membrane to substances that normally don't cross the bilayer other than through specific channels and damage membrane proteins, inactivating receptors, enzymes, and ion channels [29].

4.2 Modifications to DNA

$HO^{\cdot}$ is considered to be the most reactive ROS in the context of damaging DNA, the genetic material of the cell. $^1O_2$ primarly attacks guanine while hydrogen peroxide and superoxide does not react at all [26]. Very high levels of UV-B radiation can also induce oxidative damage in DNA. ROS can react with DNA in three distinct ways – First is the chemical predominat reaction which involve the breakage of double bonds in the DNA bases resulting in the loss of UV absorption at 260 nm. Second is the liberation and detection of all 4 bases either treated or untrearted with hydrogen peroxide. Third is the breakage of the sugar- phosphate backbone due to an indirect result of prior base alteration and removal. DNA damage results in various physiological effects, such as reduced protein synthesis, cell membrane destruction and damage to photosynthetic proteins which affects growth and development of the whole organism [30]. Indirectly, ROS can also modify DNA which involves conjugation of the PUFA breakdown product MDA with guanine, which creates an extra ring [31].

4.3 Modifications to Proteins

Proteins can be damaged by direct attack of ROS/RNS upon them or by secondary damage involving attack by end products of lipid peroxidation, such as MDA or HNE. Most types of protein oxidations are essentially irreversible whereas a few involving sulfur-containing amino acids are reversible [32]. Generally, whatever is the location of ROS synthesis and action, proteins having sulfur containing amino acids or thiol groups are always the target of ROS [33]. There are approximately four modes of protein oxidation: metal catalyzed oxidation, amino acid oxidation, oxidation induced cleavage and the conjugation of lipid peroxidation products [34]. Metal catalyzed oxidation is one of the most prevalent forms of protein oxidation. Enzymes such as NADH and NADPH oxidase are required for this system, which catalyzes the reduction and oxidation of metal ions such as Fe (III) /Fe (II), and Cu (II)/Cu (I) and generate H_2O_2. Fe (II) and Cu (I) ions then bind to a specific metal binding site within the protein and react with H_2O_2 to generate $OH^{\cdot}$, which then attacks the amino acid residues near the metal binding site [35]. One of the consequences is the oxidation induced cleavage of peptide bonds. This can be either achieved by $OH^{\cdot}$ which reacts with proteins and forms alkyl radicals that from cross-links with other similar alkyl-radicals to form protein aggregates or reacts with O_2 to generate an alkylperoxide radical. The cleavage of the peptide bond can also be a consequence of the reaction of a free radical such as $OH^{\cdot}$ with the glutamyl, prolyl and aspartyl residues of the protein chain [36].

Another oxidative pathway is the direct modification of amino acids. The most sensitive amino acid residues are those having aromatic side chains or those containing sulfydryl groups. For example, oxidation of phenylalanine residues leads to a formation of mono and di-hydroxy derivatives whereas tryptophan residues are converted to several hydroxyl-derivatives to formylkynurenine or to nitrotryptophan [33, 37]. Tyrosine residues gets converted to a diydroxy derivative, nitrotyrosine, chlorotyrosine and a dityrosine derivative upon oxidation while basic amino acids like histidine residues can be oxidized to 2-oxohistidine and 4-hydroxyglutamate [38]. They can be used as markers to determine the amount of cellular oxidative damage.

The oxidation of sulfur-containing amino acids is reversible and also plays important roles in redox control mechanism [39]. Cys and Met react specifically with $^1O_2$ and $OH^{\cdot}$. The thiols of cysteine can be modified to a disulfide (PSSP), sulphenic acid (PSOH), sulphinic acid (PSO_2H) or sulphonic acid (PSO_3H) [40]. The reduced forms can be regenerated by the thioredoxin (Trx) or glutaredoxin systems [26, 35]. Like cysteine, methionine is also one of the most readily oxidized amino acids, owing to the presence of sulphur and is susceptible to attack by most reactive oxygen or nitrogen species. Oxidation of methionine usually yields methionine sulphoxide ($R\text{-}SOCH_3$) and a higher level of modification leads to sulphone ($R\text{-}SO_2CH_3$). Met sulphoxide can be reversed by chemical reduction or by the action of methionine sulphoxide reductase, whereas methionine sulphone is thought to be irreversible and damaging to protein [40].

The major products of protein oxidation, such as peptide bond cleavage and amino acid oxidation are protein carbonyls. Carbonyl groups can also be formed by secondary reaction with the lipid peroxidation product like HNE or with reducing sugars or their oxidation products [26]. Protein Carbonylation is found to be higher in the mitochondria than in chloroplasts and peroxisomes [41], indicating that the mitochondria are more susceptible to oxidative damage or the removal of modified proteins is less efficient in the mitochondria. Oxidized proteins cause cellular dysfunction making their removal necessary. They are normally recognized and degraded by proteasomal complexes. If oxidized proteins are not efficiently removed, they get accumulated and alter the cell functioning and promote toxicity.

3. Antioxidant System

The effective control and rapid elimination of ROS is essential to the proper functioning and survival of organisms. This is performed by a vigorous antioxidant defense system (Table 2). Antioxidants are interdependent in nature and subject to variations due to intrinsic biological cycles, ambient physico-chemical environment and anthropogenic pollutants [42]. When ROS levels rise due to rapid metabolism, antioxidants keep active oxygen species under control and function as a reductant for many free radicals.

The antioxidative system is comprised of two components – antioxidant enzymes and low-molecular weight components. Antioxidant enzymes include SOD, CAT, POD, APX and GR while low-molecular weight antioxidants can be water soluble like ascorbic acid and GSH or lipid soluble like tocopherol, carotenoids, quinines and some polyphenols.

5.1 Enzymatic antioxidants

5.1.1 Superoxide dismutase (SOD)

Metalloenzyme SOD catalyzes the disproportionation of $O_2^{\cdot-}$ into H_2O_2 and O_2. This reaction has a 10,000 fold faster rate than spontaneous dismutation. It is present in all aerobic organism and subcellular components susceptible to oxidative stress. SOD are classified into three types based on their metal cofactor, two of which are similar i.e., Fe-SOD (localized in chloroplast) and Mn- SOD (localized in mitochondria) and one of which is structurally unrelated i.e., Cu/Zn –SOD (localized in chloroplast, peroxisome and cytosol) [43]. Recently a new type of SOD with Ni in the active centre has been described in Streptomyces [28].

5.1.2 Catalase (CAT)

Catalase also known as H_2O_2 Oxidoreductase is a heme containing tetrameric enzyme. The enzyme occurs in all aerobic eukaryotes and its function is to remove the H_2O_2 generated in the peroxisome during β-oxidation of fatty acids, photorespiration, purine catabolism and during oxidative stress. Catalase controls peroxisomal H_2O_2 without limiting its production. Catalase has one of the highest turnover rate similar to that of D1 protein of PSII but unlike APX, it doesn't require reducing power wheras APX requires a reductant (Ascorbate) and has a higher affinity for H_2O_2, allowing for the scavenging of small amounts of H_2O_2 in more specific locations. There are three main isoforms of catalase- CAT1, CAT2 and CAT3. All the isoforms are suddivided into classes I, II and III. CAT1 and CAT2 are localised in peroxisomes and the cytosol whereas CAT3 is mitochondrial [44].

5.1.3 Peroxidase

Peroxidases are a group of enzymes which use H_2O_2 to oxidize another substrate. Peroxidases can be specific for a particular substrate (such as GSH for glutathione peroxidase) but most have broader substrate specificity. Peroxidases include cytochrome C peroxidase, NADH peroxidase, Ascorbate peroxidase etc. There are some 'Non-specific peroxidases' include Guaiacol peroxidase, Horseradish peroxidase etc.

Table 2: Major ROS scavenging enzymatic antioxidants

S.No.	Enzymatic antioxidant	Enzyme code	Reactions catalyzed
1.	Superoxide dismutase (SOD)	EC 1.15.1.1 O2	$\mathbf{O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow 2H_2O_2 + O_2}$
2.	Catalase (CAT)	EC 1.11.1.6	$\mathbf{H_2O_2 \rightarrow H_2O + 1/2O_2}$
3.	Glutathione reductase (GR)	EC 1.6.4.2	$\mathbf{GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+}$
4.	Ascorbate peroxidase (APX)	EC 1.11.1.11	$\mathbf{H_2O_2 + AA \rightarrow 2H_2O + DHA}$
5.	Guaicol peroxidase (GPX)	EC 1.11.1.7	$\mathbf{H_2O_2 + GSH \rightarrow H_2O + GSSG}$
6.	Monodehydroascorbate reductase (MDHAR)	EC 1.6.5.4	$\mathbf{MDHA + NAD(P)H \rightarrow AA + NAD(P)^+}$
7.	Dehydroascorbate reductase (DHAR)	EC 1.8.5.1	**DHA + 2GSH→AA + GSSG**

5.1.3.1 Ascorbate peroxidase (APX)

APX plays an essential role in scavenging ROS and protecting cells in higher plants, algae, Euglena and other organisms. Ascorbate is found in the chloroplast, cytosol, vacuole and extra-cellular compartments of the cell [45]. Whereas CAT reduces H_2O_2 levels in peroxisome, APX performs this function in chloroplast and cytosol of plant cells. APX uses ascorbate as a hydrogen donor to break down H_2O_2 forming H_2O and Monodehydroascorbate (MDHA) in the process. It is a very important enzyme in the ascorbate-glutathione cycle. The APX family consists of at least five different isoforms including thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) [27]. These enzymes are haem proteins inhibited

by cyanide and azide and operate by the 'classical' peroxidase mechanism where they form an ascorbyl radical, which disproportinates into ascorbate and Dehydroascorbate.

5.1.3.2 Guaiacol Peroxidase (GPoX)

GPoX is a heme-containing protein, which are monomers of approximately 40-50 kDa, oxidize certain substrates at the expense of H_2O_2 and rid the cell of excess peroxide produced by metabolism under both normal and stress conditions**.** GPX differs from APX in terms of differences in sequences and physiological functions. GPX decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defence against biotic stresses by consuming H_2O_2. GPoX and other peroxidase bound to plant cell walls and oxidize the phenols like aromatic e- donors into phenoxyl radicals which polymerize to form lignin. It has been considered as a "stress enzyme" because both of its extra and intracellular forms participate in the breakdown of H_2O_2.

5.1.4 Glutathione reductase (GR)

GR is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes [17]. Its role is well-established in Halliwell-Asada pathway. It is a regulator of GSH redox state, catalyzing the NADPH dependent formation of a disulphide bond in GSSG pool and is important for maintaining the reduced pool of GSH. Hence, it plays a very important role in maintaining the redox poise of the cell. It is localized predominantly in chloroplast but small amount of this enzyme has also been reported in mitochondria and cytosol. It reduces GSSG in which two GSH are linked by a disulphide bridge which can be converted back to GSH by GR. GR and GSH play a crucial role in determining the tolerance of a plant to various stresses.

5.1.5 Monodehydroascorbate reductase (MDHAR)

MDHAR is a flavin adenine dinucleotide (FAD) enzyme found in chloroplast and cytosol where it regenerates the reduced ascorbate [46]. Accompanying APX, MDHAR is also located in peroxisome and mitochondria where it scavenges H_2O_2. It is an efficient e- acceptor and accepts e- preferentially from NADH rather than NADPH. It has been suggested that the activities of enzymes involved in regeneration of ascorbate i.e., MDHAR, Dehydroascorbate reductase (DHAR) and GR were higher in drought stressed rice seedlings**.** The mechanism of regeneration involves the reduction of the enzyme-FAD to form a charge –transfer complex. The reduced enzyme donates e- successively to MDHA, producing two molecules of ascorbate via a semiquinone form [E-FAD-$NADP^+$].

5.1.6 Dehydroascorbate reductase (DHAR)

DHAR regenerates ascorbate from the oxidized state and serves as an important regulator of Ascorbic acid (AA) recycling [47]. The univalent oxidation of ascorbic acid leads to the formation of MDHA, which is converted to the divalent oxidation product (DHA) via spontaneous disproportionation or further oxidation. DHA is then reduced to AA by DHAR in a reaction that requires GSH [47, 48].

5.1.7 Glutathione-s-transferase (GST)

GST is in fact a large and diverse group of enzymes which catalyze the conjugation of elctrophilic xenobiotic substrates with the tripeptide glutathione (GSH; Ɣ-glu-cys-gly). GST can reduce peroxides with the help of GSH and produce scavenging of cytotoxic and genotoxic compounds. Plant gene families are large and highly diverse with 25 numbers reported in soybean, 42 in maize and 54 in Arabidopsis [27]. Plant GSTs are known to function in herbicides detoxification, hormone homeostasis, vacuoler sequestration anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification, regulation of apoptosis and in plant responses to biotic and abiotic stresses.

5.1.8 Glutathione peroxide (GPX)

GPX are a large family of isozymes that use GSH to reduce hydrogen peroxide and organic and lipid hydroperoxides generated during oxidative stress [49]. A family of seven related proteins of GPX is identified in cytosol, chloroplast, mitochondria and endoplasmic reticulum named AtGPX1- AtGPX in Arabidopsis.

5.2 Non-enzymatic antioxidants

5.2.1 Ascorbic Acid

Ascorbic acid is one of the most powerful antioxidants and it is present in most plant cell types. All plants and animals except primates and guinea pig can synthesize ascorbic acid [50]. Ascorbic acid mostly remains unavailable in reduced form in leaves and chloroplast under normal physiological conditions but its intracellular concentration can build up to millimolar range i.e., 20 mM in the cytosol and 20-300 mM in the chloroplast stroma [51]. It helps in ROS scavenging and is also involved in a potent mechanism for preventing photo-oxidation. Although ascorbate is

an essential metabolite implicated in vital cell functions, but its synthetic pathway in plants remains to be established. The hypothesis that Ascorbate is synthesized from glucose is widely accepted but measured rates of conversion of labeled glucose into Ascorbate are very low. Still, two routes of Ascorbate biosynthesis are illustrated – inversion (found in animals) and non-inversion (found in plants) pathway [52].

Ascorbate is synthesized in both green and non-green tissues and its formation is not directly dependent on photosynthesis. For its transport in chloroplast, facilitated diffusion is required, while the thylakoid membrane has no Ascorbate transport system. There are also different mechanisms in plasma membrane for facilitating Ascorbate-mediated transport of reducing equivalents between the cytosol and apoplast-

1. A highly specific b-type cytochrome transferring e- from cytosolic Ascorbate to extracellular acceptors, including MDHA
2. A plasma membrane localized MDHAR
3. Ascorbate carriers selectively transporting Ascorbate and DHA between the cytosol and apoplast.

Its an effective antioxidant because of the ability to donate e- in a wide range of enzymatic and non-enzymatic reaction in the aqueous phase. It scavenges $O_2^{\cdot -}$, $^1O_2$ and $OH^{\cdot}$ and reduces H_2O_2 to water via Ascorbate-peroxidase reaction. It also acts as a cofactor of violaxanthin de-epoxidase in chloroplast and function in dissipation of excess excitation energy. It regenerates tocopherol from tocopheroxyl radical providing membrane protection [52]. Like GSH, it also plays non-antioxidative role as has been implicated in the regulation of cell division, cell cycle progression from G_1 to S Phase and cell elongation.

5.2.2 Tocopherols

Tocopherol is present in all phototropic organisms except for some cyanobacteria. It is a lipid soluble antioxidant and is considered as potential scavenger of ROS and lipid radicals. In addition to tocopherols, tocotrienols are also considered as an important antioxidant. There are four isomers of these – α, β, Y and δ, which structurally consist of a chroman head group and a phytyl side chain giving vitamin E its amphipathic character [53, 54]. Related antioxidative activity of the tocopherol isomers *in vivo* is α> β> Y> δ which is due to the methylation pattern and the amount of methyl groups attached to the phenolic ring of the polar head structure. So accordingly, α- tocopherol with its three methyl subsituents has the highest antioxidative capacity.

They are considered as general antioxidant for protection of membrane stability, including quenching or scavenging ROS like singlet oxygen. In case of protecting the membrane, tocopherols protect the lipids and other membrane components by physical quenching and react chemically with O_2 in chloroplast, thus protecting PSII structure and function. They act as physical deactivators of singlet oxygen by charge transfer mechanism. One molecule of α-tocopherol can scavenge up to 120 $^1O_2$ molecule by resonance energy transfer. Tocopherols react with $RO^{\cdot}$, $ROO^{\cdot}$ and $RO^{\cdot}$ derived from PUFA oxidation. The reaction between α- tocopherols and lipid radicals occurs in the membrane- water interface, where α- tocopherol donates hydrogen atom to lipid radicals with the consequent formation of $TOH^{\cdot}$ that can be recycled back to the corresponding α- tocopherols by reacting with ascorbic acid or other antoxidants. Regenration of $TOH^{\cdot}$ back to its reduced form can be achieved by vitamin C (ascorbate), reduced GSH or CoQ.

5.2.3 Carotenoids (CARs)

Carotenoids are lipophilic organic compound located in the plastids of both photosynthetic and non-photosynthetic tissues [55,56]. In all photosynthetic organisms, carotenoids, β- carotene, zeaxanthin and tocopherols serve important photoprotective role, either by dissipating excess excitation energy as heat or by scavenging ROS and suppressing lipid peroxidation. There are over 600 CARs occurring in nature. They play multitude of functions in plant metabolism including oxidative tolerance and also serve as antenna molecules for photosystems, as they absorb light in the region from 450-570nm of the visible spectrum [55].

They protect the photosynthetic apparatus by quenching triplet sensitizer chlorophyll (Chl^3), $^1O_2$ and other harmful free radicals which are naturally formed during photosynthesis. During quenching of Chl^3, energy is transferred from chlorophyll to CAR which subsequently dissipates the energy in a non-radiative form. Thus, CARs act as a competitive inhibitor of singlet formation and this is aided by their proximity to Chlorophyll in the light harvesting complex.

5.2.4 Phenolic compounds

Phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxycinnamte esters and lignin) abundant in plant tissues [57, 58]. One of the phenolic compound groups, flavonoids occur widely in the plant kingdom and are commonly found in leaves, floral parts and pollens. Flavonoids are suggested to have many functions like flowers, fruits and seed pigmentation, protection against UV light, defense against phytopathogens (pathogenic microorganism, insects and animals), plant fertility, germination of pollen and acting as signal molecules in plant-microbe interaction. Their ability to act as antioxidants depends on the reduction potential and accessibility of their radicals [59]. They have high reactivity as e- donors and are able to stabilize and delocalize unpaired e- (i.e., their chain- breaking function) and are able to chelate transition metal ions (by terminating the fenton reaction). They are also able to alter the peroxidation kinetics by modifying the lipid packing order to decrease fluidity of the membrane [60]. These changes could hinder the diffusion of free radicals and restrict peroxidative reaction.

ROS formation is the integral part of cells and has both positive and negative roles to play in plants and animals. While positive impact of ROS lead to growth and development, preventing the negative consequences of ROS formation is a necessity and antioxidant defense system must keep reactive oxygen under control for this. Other than reacting with biological macromolecules, ROS also influence the number of gene expressions and signal transduction pathways. ROS specifically react with some molecules and direct the plant response towards stress. Antioxidative capacity of the cell forms the major front towards plant stress response. They work in cooperation for better defense. Further investigation into ROS signaling, their specificity, antioxidant studies are required to explore the ROS impact on living organisms.

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