Botany Research International, 2 (1): 11-20, 2009
ISSN 2221-3635

Generation of ROS and Non-Enzymatic Antioxidants During Abiotic Stress in Plants

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Abstract: Abiotic stresses are major adverse environmental factors determining plant productivity. Under these stress conditions, reactive oxygen species (ROS) derived from molecular oxygen can accumulate in leaves, resulting in the oxidation of cellular components including proteins, chlorophyll, lipids, nucleic acids, carbohydrates etc. Sublethal amounts of ROS acclimate plants to various abiotic and biotic stresses and reduce plant growth probably as apart of the adaptational mechanism. Plants possess to a variable extent antioxidant, enzymes and non-enzymes that have the ability to detoxify ROS. The present review throws light on the generation of ROS and role of different non-enzymatic antioxidants in plants defense against oxidative stress caused by abiotic stress.

Key words: Abiotic stress · Reactive oxygen species · Non-enzymatic antioxidants

INTRODUCTION

Plants are subjected to various abiotic stresses because of unavoidable environmental conditions which adversely affect their growth and development and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Approximately 22% of the world agricultural land is saline [1] and areas under drought are already expanding and this is expected to increase further [2]. Abiotic stress environment can induce a wide number of responses in plants ranging from readjustments of transport and metabolic processes leading to growth inhibition [3-6].

The primary effect of abiotic stress is ion imbalance and hyperosmotic stresses. A direct result of these primary effects is the enhanced accumulation of reactive oxygen species (ROS) that are harmful to plant cells at high concentrations. Oxidative stress occurs when there is a serious imbalance in any cell compartment between the production of ROS and antioxidant defence, leading to significant physiological challenges [6-8]. Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$) and singlet oxygen ($^1O_2$), is an unavoidable consequence of aerobic metabolism [9] These excess ROS cause damage to proteins, lipids, carbohydrates, DNA and ultimately results in cell death [10, 11, 6, 8].

The polyunsaturated fatty acids (PUFAs) are particularly susceptible to attack by $^1O_2$ and $HO^{\bullet}$, giving rise to complex mixtures of lipid hydroperoxides [12]. Extensive PUFA peroxidation decreases the fluidity of the membrane, increases leakiness and causes secondary damage to membrane proteins [13]. Aldehydes formed in the mitochondria may be involved in causing cytoplasmic male sterility in maize because a restorer gene in this species encodes a mitochondrial aldehyde dehydrogenase [14, 15].

DNA can be modified by ROS in many different ways. $HO^{\bullet}$ is the most reactive, $^1O_2$ primarily attacks guanine and H_2O_2 and $O_2^{\bullet-}$ do not react at all [16]. 8-Hydroxyguanine is the most commonly observed modification. ROS damage to both mtDNA and nDNA is not completely random as mutation clusters at hot spots have been observed [9, 8]. So far, no gene has been identified, particularly susceptible to ROS damage. In addition to direct DNA oxidation, ROS can also indirectly

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modify DNA. A common type of damage involves conjugation of the PUFA breakdown product MDA with guanine [17]. In addition to mutations, oxidative DNA modifications can lead to changes in the methylation of cytosines, which is important for regulating gene expression [13].

The oxidation of sugars with $HO^{\bullet}$ often releases formic acid as the main breakdown product [18]. This may be the long-sought-after source of substrate for the enigmatic enzyme, formate dehydrogenase [19].

Protein oxidation is defined here as covalent modification of a protein induced by ROS or byproducts of oxidative stress. Most types of protein oxidations are essentially irreversible, whereas, a few involving sulfur containing amino acids are reversible [20]. Protein oxidation is widespread and often used as a diagnostic marker for oxidative stress.

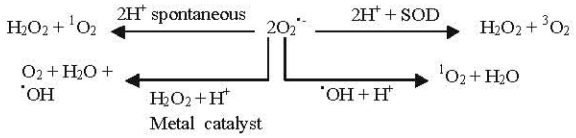
The toxic effects of ROS are counteracted by enzymatic as well as non-enzymatic antioxidative system such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid (AsA), tocopherol, glutathione and phenolic compounds etc [6, 21-23]. Normally, each cellular compartment contains more than one enzymatic activity that detoxifies a particular ROS. For example, the cytosol contains at least three different enzymatic activities that scavenge H_2O_2: APX, GPX and PrxR [24]. Development of genetically engineered plants by the introduction and/or overexpression of selected genes seems to be a viable option to generate abiotic stress tolerant plants [25].

This review throws light on the involvement of ROS in damaging cellular structures in plant system and the role of anioxidants in overcoming the deleterious effects of these oxidants.

ROS Production: In plants chloroplasts and peroxisomes are the main source of ROS production through photorespiration during light [26] and mitochondria during darkness [27]. Chloroplast is a major producer of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in plants. In chloroplast thylakoids, the reaction centers of PSI and PSII are the major generation sites of ROS [28].

The mitochondria produce $O_2^{\bullet-}$ at complexes I and III, as byproducts. An estimated 1–5% of the oxygen consumption of isolated mitochondria results in ROS production [27]. The peroxisomes produce $O_2^{\bullet-}$ and H_2O_2 in several key metabolic reactions. And, finally, the NADPH oxidase in the plasma membrane produces $O2^{\bullet-}$, which participates in several physiological processes [29].

The most important free radicals in biological systems are derivatives of oxygen [30, 8]. The complete reduction of O_2 by univalent pathway results in the formation of superoxide anion hydrogen peroxide and other products such as triplet O_2 ($^3O_2$), singlet O_2 ($^1O_2$), hydroxyl radical ($^{\bullet}OH$) and hydrogen radical ($H^{\bullet}$), as shown below:



Hydrogen peroxide is an oxidizing agent but not especially reactive. Its main significance lies in it being a source of hydroxyl radicals. In the absence of metal catalysts, superoxide and hydrogen peroxide are readily removed and are virtually harmless. The hydroxyl radical is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion controlled rates. Hydroxyl radicals are known to be produced by Harber Weiss reaction [31].

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{\text{Cu and/or Fe}} O_2^{\cdot-} + {}^{\cdot}OH + {}^{\cdot}OH$$

Hydroxyl radicals are also formed during exposure of high energy radiations like X-rays or gamma-rays to the living tissues. Most of the energy is absorbed by the cell sap having very high water content. It may result in splitting of one of the covalent bonds of water.

$$H_2O \xrightarrow{\text{Radiation}} \underset{\text{steps}}{\text{Intermediate}} \longrightarrow {}^{\cdot}H + {}^{\cdot}OH$$

The hydroxyl radicals are most reactive free radicals in living system known so far. They can damage almost every type of molecule found in a cell. Although it is very highly reactive, it has very short life (micro-seconds). Generally, free radicals in biological systems are extremely reactive and unstable. Most of these radicals exist only at a low concentration and they do not move far from their site of formation.

H_2O_2 can give rise to $HO^{\bullet}$ through the Fenton reaction, which is catalyzed mainly by free transition metal ions. $HO^{\bullet}$ reacts rapidly with all types of cellular components, $O2^{\bullet-}$ reacts primarily with protein Fe-S centers and $^1O_2$ is particularly reactive with conjugated

double bonds as found in polyunsaturated fatty acids (PUFAs). This means that they leave different footprints in the cell in the form of different oxidatively modified components [32].

Non-Enzymatic Antioxidants

Ascorbic Acid (Vitamin C): Most eukaryotic organisms produce ascorbic acid (AsA or vitamin C), a powerful, water-soluble antioxidant as scavenger of ROS [33] to prevent or at least alleviate deleterious effects caused by ROS. It occurs in all plant tissues, usually being higher in photosynthetic cells and meristems (and some fruits). About 30 to 40% of the total ascorbate is in the chloroplast and stromal concentrations as high as 50 mM have been reported [11]. It is highest in the mature leaf, where the chloroplasts are fully developed and the chlorophyll levels are highest. Under normal physiological conditions, AsA is available mostly in the reduced form, in leaves and chloroplasts [33]. AsA is considered as the most popular and powerful ROS detoxifying compound because of its ability to donate electrons in a number of enzymatic and non-enzymatic reactions. AsA can directly scavenge $^1O_2$, $O_2^{\bullet-}$ and $OH^{\bullet}$ and regenerate tocopherol from tocopheroxyl radical, thus providing membrane protection [34]. In chloroplast, AsA acts as a cofactor of violaxantin de-epoxidase thus sustaining dissipation of excess excitation energy [33]. Vitamin C cooperates with Vitamin E to regenerate α-tocopherol radicals in membranes and lipoproteins [35]. AsA plays a great role in minimizing the damage caused by oxidative process [36, 37]. In addition to the importance of ascorbate in the Ascorbate-Glutathione cycle, it plays a role in preserving the activities of enzymes that contain prosthetic transition metal ions [38]. The ascorbate redox system consists of L-ascorbic acid, MDHA and DHA. Both oxidized forms of ascorbate are relatively unstable in aqueous environments while DHA can be chemically reduced by GSH to ascorbate [39]. Evidence to support the actual role of DHAR, GSH and GR in maintaining the foliar ascorbate pool has been observed in transformed plants overexpressing GR [40]. *Nicotiana tabacum* and *Populus* × *Canescens* plants have higher foliar ascorbate contents and improved tolerance to oxidative stress [40]. Yang *et al.* [41] reported that high light condition and drought significantly increased the ascorbic acid content in *Picea asperata* Mast. Seedlings. Agarwal [42] reported that the AsA and DHA content as well as the GSH/GSSG content ratio were significantly increased by the UV-B stress in *Cassia auriculata* seedlings. A decrease in ascorbate content under Cd stress have been observed in the roots and nodules of *Glycine max* [43]. Cadmium also decreases the ascorbate content in *Cucumis sativus* chloroplast and in the leaves of *Arabidopsis thaliana*, *Pisum sativum* and *Brassica campestris* [44-46], whereas, it remained unaffected in *Populus* × *Canescens* roots [47]. Kukreja *et al.* [48] noted significant decrease in AsA content under salinity stress in *Cicer arietinum* roots.

Glutathione (GSH): GSH may be the most important intracellular defense against damage by ROS. The tripeptide (γ-GluCysGly) glutathione GSH is one of the crucial metabolites in plants. It occurs abundantly in reduced form in plant tissues and is localized in all cell compartments like cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes as well as apoplast [49]. It plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics [50] and the expression of stress-responsive genes [51]. GSH has also been associated with several growth and development related events in plants, including cell differentiation, cell death and senescence, pathogen resistance and enzymatic regulation [52]. The reduced form of glutathione is necessary to maintain the normal reduced state of cells so as to offset all the injurious effects of stress induced oxidative stress. It can potentially scavenge $^1O_2$ and H_2O_2 [53, 38] as well as other ROS like $OH^{\bullet}$ [54]. In addition, GSH plays a key role in the antioxidative defense system by regenerating another potential water soluble antioxidant, ascorbic acid, *via* the Ascorbate–Glutathione cycle [39, 55]. GSH is the substrate of glutathione-*S*-transferase (GST), which plays an important role in the detoxification of dehydroascorbate reductase (DHAR) and xenobiotics [56, 57, 58]. In combination with its oxidized form (GSSG), GSH maintains redox equilibrium in the cellular compartments. This property is of considerable biological importance for maintaining the cellular redox system normal under normal or stressful conditions. It also plays an indirect role in protecting membranes by maintaining α-tocopherol and zeaxanthin in the reduced state. It has been reported that when the intensity of a stress increases, glutathione concentrations usually decline and redox state becomes more oxidized, leading to deterioration of the system [59].

GSH is a precursor of PCs, (Phytochelatins) which are crucial in controlling cellular heavy metal concentrations. GSH and its oxidized form, GSSG maintains a redox balance in the cellular compartments. This property of

GSH is of great biological importance since it allows fine-tuning of the cellular redox environment under normal conditions and upon onset of stress and provides the basis for GSH stress signaling. A central nucleophilic Cys residue is responsible for higher reductive potential of GSH. It scavenges cytotoxic H_2O_2 and reacts non-enzymatically with other ROS i.e. $O_2^{\bullet-}$, $OH^{\bullet}$ and $^1O_2$ [54].

The central role of GSH in the antioxidative defence system is due to its ability to regenerate another water soluble antioxidant, ascorbate, in ascorbate-glutathione cycle [39, 38]. The role of GSH in the antioxidant defence system provides a strong basis for its use as a stress marker. However, the concentration of cellular GSH has a major effect on its antioxidant function and it varies considerably under Cd stress. Furthermore, strong evidence has indicated that an elevated GSH concentration is correlated with the ability of plants to withstand metal-induced oxidative stress [60]. Xiang *et al.* [50] observed that plants with low levels of glutathione were highly sensitive to even low levels of Cd^{2+} due to limited PC synthesis. The increased demand for GSH can be met by the activation of pathways involved in sulfur assimilation and cysteine biosynthesis. Its concentration is controlled by a complex homeostatic mechanism where the availability of sulfur seems to be required [56]. Manipulation of GSH biosynthesis increases resistance to oxidative stress [61]. It has been observed that upon Cd exposure, one of the main responses observed was the induction of genes involved in sulfur assimilation–reduction and glutathione metabolism in the roots of *Arabidopsis* [62].

Feed back inhibition of γ-glutamylcysteine synthase (γ-ECS) by GSH has been considered as a fundamental central point for GSH synthesis. *In vitro* studies with the enzymes from tobacco and parsley cells showed that the plant γ-ECS was inhibited by GSH [38]. Oxidation of GSH to GSSG decreases GSH levels and allows increased γ-ECS activity under stressed conditions [38].

Environmental stresses trigger an increase in ROS levels in plants and the response of glutathione can be crucial for adaptive responses. Antioxidant activity in leaves and chloroplast of *Phragmites australis* Trin. (cav.) ex Steudel was associated with a large pool of GSH, protecting the activity of many photosynthetic enzymes against the thiophilic bursting of Cd exerting a direct important protective role in the presence of Cd [63]. Increased concentration of GSH has been observed with the increasing Cd concentration in *Brassica juncea* [64], *Pisum sativum* [65] and *Sedum alfredii* [66]. However, decay in GSH content in *Pinus sylvestris* roots [47], *Cucumis sativus* chloroplast [229], *Populus* × *Canescens* roots [67] and *Oryza sativa* leaves [68] has been reported under Cd stress. Cadmium-induced depletion of GSH has been mainly attributed to phytochelatin synthesis [69]. PC-heavy metal complexes have been reported to be accumulated in the vacuole of tobacco leaves [70] and in *Avena sativa*. These complexes have been shown to be transported across the tonoplast [71]. The decline in the levels of GSH might also be attributed to an increased utilization for ascorbate synthesis or for a direct interaction with Cd [63]. The variety of response to Cd-induced oxidative stress is probably related not only to the levels of Cd supplied, but also to the plant species, the age of the plant and duration of the treatment. Srivastava *et al.* [72] reported an appreciable decline in GR activity and GSH pool under copper stress and significant increase under NaCl stress. Sumithra *et al.* [73] also reported that the activities of ROS scavenging enzymes and GSH concentration were found to be higher in the leaves of Pusa Bold than in CO 4 cvs. of *Vigna radiata*, whereas, GSSG concentration was found to be higher in the leaves of CO 4 compared to those in Pusa Bold. It indicates that Pusa Bold has efficient antioxidative characteristics which could provide better protection against oxidative damage in leaves under salt-stressed conditions.

Vitamin E (α-Tocopherols): Tocopherols, a lipid soluble antioxidant found in all plant parts and are a potential scavengers of ROS and lipid radicals [74]. Kagan [75] have reported that tocopherols (a membrane bound compound) are essential components of biological membranes, where they play both antioxidant and non-antioxidant functions. Out of four isomers of tocopherols (α-, β-, γ-, δ-) found in plants [76], α-tocopherol has the highest antioxidative activity due to the presence of three methyl groups in its molecular structure. Tocopherols prevent the chain propagation step in lipid autooxidation and this makes it an effective free radical trap [77]. In addition, tocopherols act as scavengers of oxygen radicals, especially $^1O_2$ [78]. According to an estimate, one molecule of α-tocopherol can scavenge up to 120 $^1O^2$ molecules by resonance energy transfer [79].

It is well established that oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants [80]. Antioxidants including α-tocopherol and AsA have been reported to increase following triazole treatment in tomato and these may have a role in protecting membranes from oxidative damage,

thus contributing to chilling tolerance in tomato plants [81]. Increase in tocopherol during water stress in plants has also been reported by many workers [80, 81]. α-tocopherol is synthesized from γ-tocopherol in chloroplasts by γ-tocopherolmethyltransferase (γ-TMT; VTE4). Leaves of many plant species including *Arabidopsis* contain high levels of α-tocopherol, but are low in γ-tocopherol. Nitration of γ-tocopherol has been suggested to be an important mechanism for the regulation and detoxification of reactive nitrogen oxide species in animal tissues. To investigate whether this reaction does also occur in plants, *in vivo* 5-nitro-γ-tocopherol (5-NγT) was identified in leaves of the *Arabidopsis* mutant line (*vte*4), which has insertion in the gene encoding γ-tocopherol methyltransferase and consequently lacks α-tocopherol and accumulates high levels of γ-tocopherol [82]. Quantification of NOx in leaves revealed that the *vte*4 mutant in comparison to wild type and the mutant *vte*1, which does not contain any tocopherol, has a reduced NOx concentration. This 5-NγT was also detectable in germinating seeds of *Brassica napus, Nicotiana tabacum* and *Arabidopsis thaliana*. It has been suggested that γ-tocopherol or its respective derivative, 5-NγT, may prolong early development by reducing the level of NOx [82].

Bergmuller *et al*. [83] reported that during oxidative stress (high light, high temperature, cold treatment) the amounts of α-tocopherol and γ-tocopherol increased in wild type and γ-tocopherol in *vte4-1*. However, chlorophyll content and photosynthetic quantum yield were very similar in wild type and *vte4-1*, suggesting that α-tocopherol can be replaced by γ-tocopherol in *vte4-1* to protect the photosynthetic apparatus against oxidative stress. Giacomelli *et al*. [84] found that cellular concentrations of a-tocopherol, ascorbate and glutathione showed dramatic increase in response to high light (1,000 μmol photons m^{-2} s^{-1}) in all eight genotypes of *Arabidopsis* and the four ascorbate deficient *vtc2* genotypes accumulated more glutathione under control light (120 μmol photons m^{-2} s^{-1}) than the others. Tocopherol cyclase (VTE1, encoded by VTE1 gene) catalyzes the penultimate step of tocopherol synthesis [85]. In an experiment transgenic tobacco plants overexpressing VTE1 from *Arabidopsis* were exposed to drought conditions during which transgenic lines had decreased lipid peroxidation, electrolyte leakage and H_2O_2 content in comparison with the wild type. Thus, they concluded that VTE1 can be used to increase vitamin E content of plants and also to enhance tolerance to environmental stresses [85].

Carotenoids: Carotenoids (Car) are pigments that are found in plants and microorganisms. There are over 600 carotenoids occurring in nature. Car a lipid soluble antioxidants, plays a multitude of functions in plant metabolism including oxidative stress tolerance. Car are lipophilic organic compounds which occur in chloroplasts. Car carry out three major functions in plants. First, they absorb light at wavelength between 400 and 550 nm and transfer it to the Chl (an accessory light-harvesting role) [86]. Second, they protect the photosynthetic apparatus by quenching a triplet sensitizer (Chl^3), singlet oxygen and other harmful free radicals which are naturally formed during photosynthesis (an antioxidant function) [87, 88]. Third, they are important for the photosystem (PS) I assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization (a structural role) [86, 89].

Rai *et al*. [90] and Ekmekci *et al*. [91] reported decreased Car contents in *Phyllanthus amarus* and *Zea mays* cultivars respectively with increasing Cd concentration. Collin *et al*. [92] also observed decreased concentration of Car in *Arabidopsis* plants. An increase in Car content was reported by Foyer and Harbison [93] following Cd stress. It has been considered that some isoprenoids (including several carotenoids and tocopherols) play an effective role in photoprotection [94]. Furthermore, it has been proved that monoterpene improved thermotolerance at elevated temperatures [95] and that monoterpene had a protecting role against oxidative stress [96].

Flavonoids: Flavonoids also show antioxidant activity against a variety of oxidizable compounds [97]. They belong to a large category of organic compounds i.e., phenolics. Flavonoids occur widely in the plant kingdom and are commonly found in leaves, floral parts and pollens. Flavonoids usually accumulate in the plant vacuole as glycosides, but they also occur as exudates on the surface of leaves and other aerial plant parts. Flavonoid concentration in plant cells is often over 1 mM [98]. Several flavonoids act as the potential inhibitors of the enzyme lipoxygenase, which converts polyunsaturated fatty acids to oxygen containing derivatives [99]. One of the most actively studied properties of flavonoids is their protection against oxidative stress [100, 97] and these are ideal scavengers of H_2O_2 due to their favourable reduction potentials relative to alkyl peroxyl radicals and thus, in principle, they are effective inhibitors of lipid peroxidation.

CONCLUSION AND FUTURE PERSPECTIVE

Higher plants survive in a constantly fluctuating environment as they develop a series of pathways at different levels that combat with environmental stress, which produces more ROS. Increase in ROS causes damages to the metabolites such as proteins, lipids and nucleic acids etc. Plants possess specific mechanisms to detoxify the reactive oxygen species which include activation of antioxidant enzymes. During the last few decades, genetic engineering approach has given appreciable results in terms of improving tolerance to a multitude of abiotic stresses by enhanced activities of enzymatic and non-enzymatic antioxidant. Overexpression of antioxidant genes provides the opportunity to develop plants with enhanced tolerance to abiotic as well as biotic stress. The road to engineering such tolerance into sensitive species is still far from us. Much effort is still required to uncover in detail each product of genes induced by abiotic stress and signal transduction pathways.

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