REVIEW ARTICLE

Antioxidants and Antioxidative Enzymes as Potential Biomarkers for Assessing Stress in Plants

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Abstract

Reactive oxygen species (ROS) are an inevitable part of normal cellular metabolism in almost every known living organism. But the excess accumulation of these radicals disturbs cellular homeostasis which can be harmful to the plant. Unlike animals who can migrate themselves away from the stress conditions, plants that are sedentary in nature have developed certain defence mechanisms to cope with the same. These mechanisms include a plethora of enzymatic and non-enzymatic antioxidants that help in scavenging free radicals. The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione-s-transferase, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and the non-enzymatic antioxidants include ascorbic acid (ASA), glutathione (GSH), tocopherols, carotenoids, etc. All these antioxidants help in maintaining the balance between ROS generation and scavenging by keeping their concentration below the threshold level. Numerous earlier studies have reported that only certain enzymatic antioxidants have shown increased activity in response to particular stress and likely these enzymes can be utilized as biomarkers against a multitude of biotic and abiotic stresses. In this review, we have discussed certain enzymatic and a few non-enzymatic antioxidants which can be used for assessing stress in plants.

Keywords: Antioxidants, Biomarkers, Enzymes, Stress.

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Introduction

The term "biomarker" has been borrowed from epidemiology to specify the alteration in biomolecules that have resulted from their reaction with reactive oxygen species as well as halide or nitrogen species (Offord 2000). The definition applies equally to by-products derived from the reaction of lipids, DNA, protein, etc with reactive species (Griffiths *et al.,* 2002). For a molecule to be regarded as a biomarker it has to bear certain criteria-

- It should be a considerable by-product of oxidative induction and directly involved in stress development;
- It should be a stable molecule not prone to loss during storage;
- It could be quantified through a specific assay technique;
- It should be measurable within the specific limits of detection.

Biomarkers can also be defined as quantitative changes that are generally restricted to cellular, physiological, biochemical, or molecular changes in cells, tissues, or within an organism (Lam and Gray, 2003). The usage of the term "biomarkers" is usually restricted to the sub-organismic level (rather than at the population level) as the early warning signals of biological changes are generally sub-organismic (cellular, physiological, biochemical, or molecular) and anticipate those that manifest at the population level (Lam and Gray, 2003). Biochemical markers are of significant toxicological importance because of their quick and reliable response to any kind of contamination even at the simplest organizational level (Goncalves *et al.,* 2021). Biomarkers are usually categorized into two groups- exposure biomarkers and effect biomarkers. Exposure biomarkers are those chemical metabolites or responses of an organism that can be measured on exposure to a certain environmental condition or stress, even if it doesn't depict any direct toxicological effects on the organism (Conti, 2008). Those responses which include both the exposure and its toxicological influence can be regarded as effect biomarkers.

Most organisms in standard conditions allocate energy and resources toward growth, metabolism, and reproduction (Goncalves *et al.,* 2021). In stressed conditions, like ozone stress, the energy allocation pattern between above and below ground parts is altered (Jeon *et al.,* 2013; Bendis and Relyea 2014) resulting in an increased transfer of energy to a specific mechanism that maximizes the organism's chance of survival (Sancho *et al.,* 2009). Oxidative stress induced by a multitude of factors favors the production of reactive oxygen species, thereby leading to biochemical, physiological, and molecular changes (Goncalves *et al.,* 2021). Free radical species impair cellular metabolism through lipid peroxidation, damage to the membrane organization, and structural wreckage of DNA and proteins (Kelly *et al.,* 1998). Nevertheless, almost every cell of an organism has a certain defensive mechanism against various stresses, intended at maintaining cellular homeostasis against ROS, a process that is commonly carried out with the help of antioxidant enzymes (Goncalves *et al.,* 2021). Enzymes that have the potential of scavenging ROS generally serve as biomarkers

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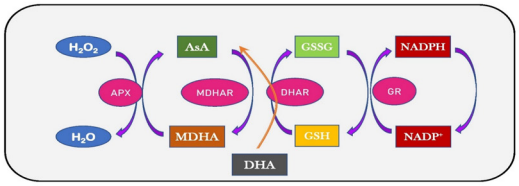

Fig. 1: The illustrative portrayal of the ASA-GSH pathway. The first step is the reduction of H_2O_2 by APX catalyzed peroxidation of ASA which generates monodehydroascorbate. MDHA is either converted back to ASA or DHA. GSH is again generated from the GSSG by GR. (Modified from Prachi *et al.,* 2015).

of environmental pollution (Somashekariah *et al.,* 1992; Vitoria *et al.,* 2001) and a direct proportionality with the stress levels further enhances the usage of biomarkers. The practice of using enzymes as biomarkers of various environmental stresses has been established by several scientists (Ahmad *et al.,* 2000; Geret *et al.,* 2002). Numerous experiments done over the years depict the effect of enzyme activity concerning the different environmental stresses, from stimulating the antioxidant enzyme pool in order to maintain the redox potential inside the cell, to the disruption of enzyme-mediated processes (Goncalves *et al.,* 2021).

There are a plethora of molecules both enzymatic and non-enzymatic which have been used lately as biomarkers against several environmental stresses are- ascorbic acid (ASA), Tocopherols, Glutathione (GSH), Superoxide dismutase (SOD), Catalase (CAT), Glutathione reductase (GR), Guaiacol peroxidase (GPX), Ascorbate peroxidase (APX), Glutathione-s-transferase, Monodehydroascorbate reductase (MDHAR), Dehydroascorbate reductase (DHAR), etc. However, out of these SOD, CAT, GR, and GPX are the most pivotal enzymes that scavenge the oxidative-induced ROS inside the cell. The objective of this review is to discuss the role of several antioxidants and antioxidative enzymes under different abiotic stresses in plants.

Potential Biomarkers

Ascorbic Acid

ASA, a low molecular weight compound, is one of the most common antioxidants that play a pivotal role in defense against

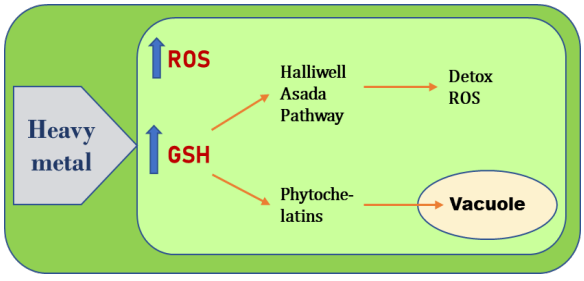

Fig. 2: Glutathione-mediated detoxification of heavy metal stress response in plants. Exposures of plants to surplus heavy metals generate ROS. GSH neutralizes ROS through the Halliwell-Asada pathway. Secondly, GSH is used in the synthesis of phytochelatins (PCs). PCs form complexes with the metal ions in the cytosol and are transported to the vacuole.

the ROS generated due to oxidative stresses (Fatima *et al.,* 2019; Sharma *et al.,* 2012). It derives its virtue as a robust antioxidant from the ability to donate electrons in a wide range of reactions. ASA concentration in plants is mainly maintained by the Smirnoff-Wheeler pathway which includes GDP-D-mannose, GDP-P-galactose, and L-galactose-1,4-lactone (Wheeler *et al.,* 1998) as well as a uronic acid pathway which includes D-galacturonic acid (Isherwood *et al.,* 1954). It is found in almost all cells of the plant along with the apoplastic region and organelles (Shao *et al.,* 2008), with a particularly high concentration in photosynthetic tissues. Almost 90% of the ASA pool is localized in cytoplasmic regions with a considerable amount at the apoplast, where it serves as the first line of defense against all the ROS (Barnes *et al.,* 2002; Sharma *et al.,* 2012). ASA protects macromolecules (proteins, lipids, nucleic acids, and carbohydrates) against oxidatively induced ROS, which are crucial in maintaining normal cell metabolism (Sharma *et al.,* 2012). ASA concentration has been revealed to vary in response to different environmental stresses (Hernandez et al. 2001; Sharma and Dubey 2005; Maheshwari and Dubey, 2009; Radyuk *et al.,* 2009; Mishra *et al.,* 2011; Srivastava and Dubey, 2011) and its level depends upon the equilibrium between rate of biosynthesis and usage as an antioxidant (Chaves *et al.,* 2002).

Ascorbate pool is known to fluctuate in response to various biotic and abiotic stresses (Hernandez *et al.,* 2001; Sharma and Dubey, 2005; Maheshwari and Dubey, 2009). Zhang *et al.* (2011) reported that upregulation of several genes of the GDP-Mannose 3, 5-epimerase (GME) gene family, those involved in the biosynthesis of ASA, increased environmental stress tolerance in tomato plants. Another overexpression of the gene encoding D-galacturonic acid which is also an integral part of the ASA biosynthesis pathway improved the tolerance potential of *Solanum tuberosum* plants (Upadhyaya *et al.,* 2009). In *vtc-1* mutants (for GDP-mannose pyrophosphorylase, an enzyme involved in AsA biosynthesis) of *Arabidopsis thaliana* plants were more sensitive to UV-B treatment than the wild-type plants (Gao and Zhang, 2008). Wang *et al.* (2010) also observed similar results in which a high ASA pool conferred increased tolerance against various biotic and abiotic stresses. All the above findings suggest the possibility of utilization of ASA as a biomarker against environmental stresses.

Glutathione

Glutathione (GSH), a tripeptide (γ-glutamyl-cysteinyl-glycine) is another very common thiol molecule that acts as an antioxidant and plays a crucial role in intracellular defense against oxidants (Sharma *et al.,* 2012). It has been localized in almost every corner of the cell such as chloroplast, mitochondria, cytosol, endoplasmic reticulum, vacuoles, etc (Foyer and Noctor, 2003). GSH is specifically synthesized in cytosol and chloroplast via, Glutathione synthetase (GS) and γ-glutamyl-cysteinyl synthetase (γ-ECS). GSH is synthesized mainly with the help of two enzymes i.e, γ-glutamylcysteine synthetase (GSH-1, E.C. 6.3.2.2) and glutathione synthetase (GSH-2, E.C. 6.3.2.3), with both the reactions being energy demanding (Yadav, 2010). The fundamental factor behind maintaining intracellular homeostasis is the balance between GSH and its oxidized form glutathione disulfide (GSSG). The regulation of the GSH: GSSG

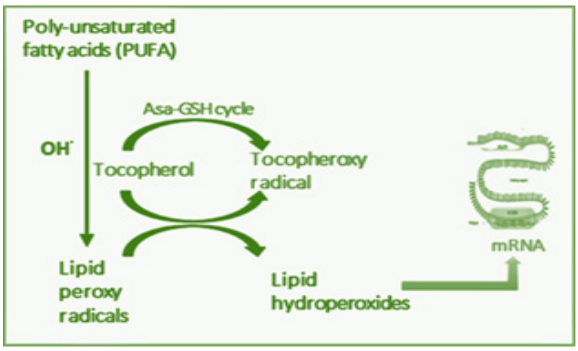

Fig. 3: Tocopherols contribute to controlling redox homeostasis and gene expression by regulating the extent of lipid peroxidation in leaves. Tocopherols inhibit the magnitude of lipid peroxidation by scavenging lipid peroxyl radicals and prevent lipid peroxidation by reacting with other reactive oxygen species, such as singlet oxygen.

ratio mainly depends upon four factors i.e, GSH pool, pH, GSH synthesis, and its catabolism (Mullineaux and Rausch 2005). Due to the virtue of this reducing capacity, GSH participates in diverse cellular proceedings such as nucleic acid and proteins synthesis, signal transduction, enzymatic regulation, cell division, and growth, increased expression of stress factor genes, metal chelation through the synthesis of phytochelatins, detoxification of xenobiotics, etc (Foyer *et al.,* 1997). GSH also takes part in the formation of ASA, through the ASA-GSH cycle where it converts ASA from its oxidized to reduced form with the help of the enzyme DHAR (Loewus 1988). When the aspect of Hydrogen peroxide (H_2O_2) is taken into consideration, Guaiacol peroxidase catalyzes the conversion of GSH into its oxidized form GSSG, which further can be reduced to GSH, a reaction catalyzed by GR (Goncalves *et al.,* 2021). The reduced form of GSH then scavenges the oxidatively induced ROS by acting as an electron donor to electrophilic species (Pompella *et al.,* 2003). Being an antioxidant, GSH can be utilized as a biomarker in many ways because it reacts with superoxide ($O2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) and neutralizes them. Exposures of plants to surplus heavy metals generate ROS. GSH neutralizes ROS through the Halliwell-Asada pathway (Fig. 2). Secondly, GSH is used in the synthesis of phytochelatins (PCs). PCs form complexes with the metal ions in the cytosol and are transported to the vacuole (Sharma *et al.,* 2012). The involvement of GSH in stress management makes it a worthy biomarker against various environmental stresses. As previously described by many scientists, GSH concentration in plants has shown a significant reduction in heavy metal exposure (Yadav 2010; Schutzendubel *et al.,* 2001). GSH has been found to be especially effective against Cadmium (Cd) stress where it exerts significantly improved defence when present generously and vice-versa (Hediji *et al.,* 2021). Under extended drought conditions, Malus sp. trees showed an initial drop in GSH concentration but eventually regained their initial concentration (Tausz *et al.,* 2004). GSH showed higher concentration by several folds on exposure to oxidative stress in transgenic potatoes (Eltayeb *et al.,* 2010). Alteration of GSH: GSSG ratio has also been reported in plants on introduction to a multitude of environmental stresses i.e, chilling (Radyuk *et al.,* 2009); salting, and heavy metal exposure (Maheshwari and Dubey 2009). The nucleophilic aspect of GSH, in particular of the non-protein thiol group, is of prime importance in the formation of mercaptide bonds with heavy metals and their reaction with certain electrophiles. The high stability of GSH along with its diverse reactive potential makes it one of the exemplar biomarkers which is used against oxidative stress, heavy metal stress, and the presence of xenobiotics (Foyer and Noctor 2005; Rausch *et al.,* 2007).

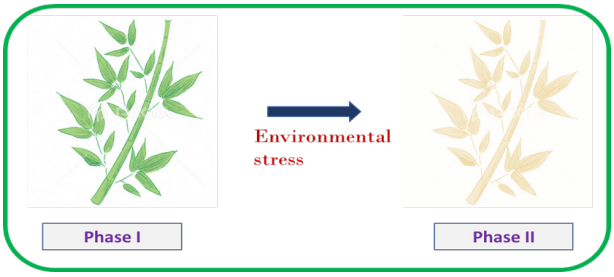

Fig. 4: Chlorophyll degradation and typical changes in *a*-tocopherol occur in plant responses to several biotic and abiotic stresses. Stress-induced changes in *a*-tocopherol levels, like those shown in the inlet diagram, are characterized by two phases (modified from Munne-Bosch 2005).

Tocopherols

Tocopherols, mainly of four types α, β, γ, and δ, are major vitamin E compounds (lipophilic) that help in defence against oxidative stress, singlet oxygen ($^1O_2$), and peroxy radicals (Diplock *et al.,* 1989). The relative antioxidant activity of the four isomers is grouped on the phenyl ring, and by virtue of its three methyl groups, α-tocopherol has been found with the highest antioxidant activity (Kamal-Eldin and Appelqvist 1996). They are found mainly in plastids with a presence strictly prohibited to photosynthetic organisms (plants, algae, and certain cyanobacteria) (Munne-Bosch 2005). Their existence in α > β > γ > δ and the trend is based on the pattern of methylation and the number of methyl cyanobacteria can be speculated that they might have evolved to neutralize the photosynthetically generated ROS (Munne-Bosch 2005). They are synthesized in plastids with two compounds as precursors i.e, homogentisic acid (HGA) and phytyl diphosphate (PDP) (Sharma *et al.,* 2012). Tocopherols are known to protect cell components, especially lipids, through quenching and also safeguard PSII by reacting with $^1O_2$ in plastids (Ivanov and Khorobrykh 2003). They limit lipid peroxidation by trimming the peroxy radicals and preventing them from converting into hydroperoxides (Fig. 3). Tocopherols are one the most efficient singlet oxygen scavengers inside the cell as a single α-tocopherol molecule has the potential to subdue around 220 molecules of $^1O_2$ through the phenomenon of resonance energy transfer (Fukuzawa *et al.,* 1982). Hence α-tocopherol mainly assists in defense against various environmental stresses protects thylakoid membrane structure as well as maintains a redox state in plastids (Munné-Bosch and Alegre 2002; Sattler *et al.,* 2004).

Studies done by scientist report α-tocopherol-induced tolerance in several plant species like Arabidopsis thaliana, Oryza sativa, and Medicago sativa against various environmental stresses like drought, salinity, and chilling conditions (Guo 2006;

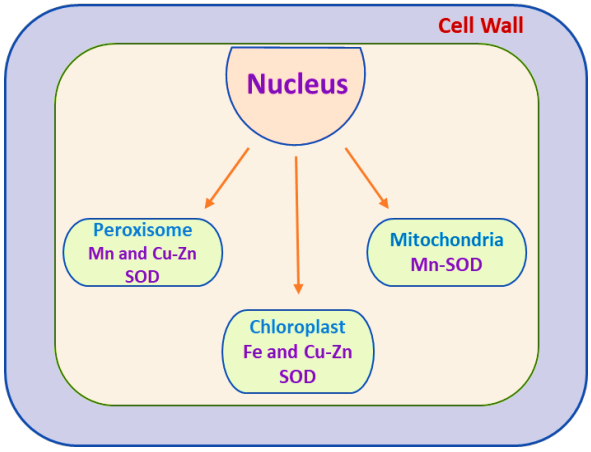

Fig. 5: Localization of isoforms of SOD (modified from Alscher *et al.,* 2002).

Bafeel and Ibrahim 2008). A robust interdependence between salicylic acid and α-tocopherol has been reported in Phillyrea angustifolia plants under water deficit conditions, which further suggests that the synthesis of these two compounds is coupled under stress (Munne´-Bosch and Penuelas 2003). Yamauchi and Matsushita (1979) were the pioneers who found that plastids when exposed to light stress result in higher production of α-tocopherolquinone, which in turn facilitates singlet oxygen formation in chloroplasts and oxidation of α-tocopherol to α-tocopherol quinone present a double role in stressed plants (Munne-Bosch 2005). A multitude of environmental stresses is usually correlated with higher oxidatively derived ROS, which simultaneously results in increased α-tocopherol levels and makes it a worthy biomarker (Munne-Bosch 2005). The stressed-induced changes in α-tocopherol concentration in plants can be portrayed in two ways- firstly there is a rise in α-tocopherol levels (due to high ROS levels) and the second phase due to extreme stress conditions the rate of degradation exceeds the rate of synthesis which results in net loss (Munne-Bosch 2005) (Fig. 4).

Superoxide Dismutase

SOD (EC 1.15.1.1) is an antioxidant enzyme that is involved in the first line of detoxification and is considered the most powerful inside the cell (Ighodaro and Akinloye 2018). Being endogenous in origin, it neutralizes the superoxide ($O_2^{\bullet-}$) anions by converting them into hydrogen peroxide(H_2O_2) and oxygen (O_2) (Ighodaro and Akinloye 2018). SOD reduces the chance of formation of hydroxyl radical ($OH^{\cdot}$) by discarding $O_2^{\bullet-}$ radical via Haber and Weiss reaction and the reaction being 10,000 times faster than spontaneous dismutation (Gill and Tuteja 2010). SOD is a metalloenzyme and has been categorized based on metals used as a cofactor. The various isoforms of SOD are iron (Fe)-SOD zinc (Zn)-SOD, copper (Cu)-SOD, and manganese (Mn)-SOD. The localization of these isoforms of SOD in plant cells (Dringen *et al.,* 2005) is shown in (Fig. 5). Fe-SOD is usually found in chloroplasts of plants and also in prokaryotes. Cu/Zn-SOD is largely localized in eukaryotes and also in both cytosol and certain organelles like peroxisomes and chloroplasts. And lastly, Mn-SOD is predominant in the mitochondria of eukaryotes and prokaryotes (Gill and Tuteja, 2010; Karuppanapandian *et al.,* 2011). Several earlier inferences suggest that SOD can be utilized as a biomarker against various environmental stresses in plants. High SOD activity was reported in plants under heavy metal stress and severe water deficit conditions (Sharma and Dubey 2005; Mishra *et al.,* 2011). A direct correlation has been found between SOD activity and stress tolerance in plants exposed to environmental stresses (Sharma *et al.,* 2012). Elevated SOD activity in three cultivars of *Phaseolus vulgaris* (Zlatev *et al.,* 2006), *Oryza sativa* (Sharma and Dubey 2005), and *Alternanthera philoxeroides* (Wang et al. 2008) was reported under severe drought conditions. A significant increment in SOD activity was observed in two varieties of *Brassica campestris* exposed to high Cu stress (Li *et al.,* 2009). Eyidogan and Oz (2007) observed three kinds of SOD activity bands (all three isoforms) in *Cicer arietinum* exposed to high salt concentration along with higher activities of Mn-SOD and Cu/Zn-SOD isoforms. Tropospheric ozone (O_3) enters the plant tissues through the leaf stomata and exerts its most negative effect in the apoplastic fluid, but the overexpression of Mn-SOD in chloroplasts restricts the O3-induced ROS to aggravate the damage (Alscher *et al.,* 2002). When a Fe-SOD gene extracted from *Arabidopsis thaliana* was inserted into chloroplasts of *Nicotiana tabacum* cv. In Petit Havana, the scavenging potential against superoxide radicals in the plasma membrane and photosystem II was significantly increased (Van Camp *et al.,* 1996). Wang *et al.* (2004) reported significantly increased salt tolerance and activity of all isozymes of SOD in *Arabidopsis thaliana* ecotype Columbia under salt stress. Out of all the antioxidant enzymes, catalase (CAT, EC 1.11.1.6) was the first to be characterized (Sharma *et al.,* 2012). It is ubiquitous in nature i.e, present in almost all living beings ranging from monera to advanced eukaryotes (Sharma and Ahmad, 2014). It is a tetrameric heme-containing enzyme that plays a pivotal role in checking cellular damage by disrupting hydrogen peroxide (H_2O_2) into water and oxygen (Parvaiz and Ahmad, 2011). CAT is usually confined to peroxisomes, but its trace has also been found in chloroplast and mitochondria (Sharma and Ahmad, 2014). Various studies have shown a direct correlation between increased catalase activity as a part of improved defense response against a multitude of abiotic stresses (Leung, 2018). The diverse catalase genes express differentially under different environmental conditions (Fig. 5). Mhamdi *et al.,* (2010) reported three types of catalase proteins namely, CAT 1, 2, and 3. He did a knockout experiment in which he found out that the knockout of the CAT 2 gene resulted in a higher reduction of catalase activity than the knockouts of the other two catalases. In an experiment done by Zhou *et al.,* (2017), he observed a significant increase in catalase activity in *Cucumis sativus* plants under different stress conditions i.e, heat, salinity, and osmotic. High catalase activity was also reported in the roots of *Oryza sativa* L. seedlings exposed to acidic conditions (pH < 4) (Ju *et al.,* 2017). Zong *et al.,* (2017) also reported similar results where they found significantly increased catalase activity in leaves of *Brassica napa* L. plants under cadmium stress conditions. High catalase activity was observed in *Citrus sinensis* [L.] Osbeck 9 and *Poncirus trifoliata* [L.] Raf plants are exposed to water deficit conditions (Oliviera *et al.,* 2017). The pivotal role of the catalase enzyme during photosynthetic processes in C^3 plants has been well documented via antisense technology

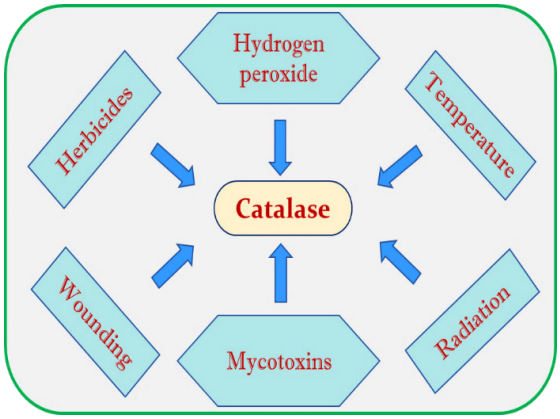

Fig. 6: Factors affecting Catalase activity.

as lesion development and stress conditions were observed in cat-deficient plants (Willekens *et al.,* 1997; Chamnongpol *et al.,* 1998). All these studies make the catalase enzyme a worthy biomarker against several environmental stresses.

Catalase

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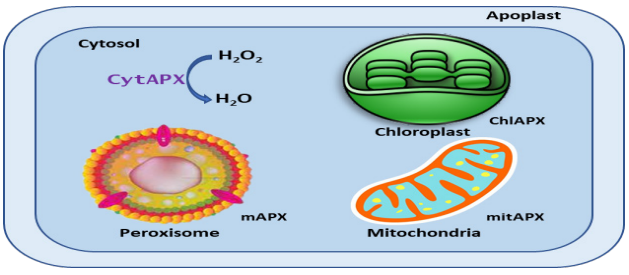

Fig. 7: Localization of APX isozymes in various subcellular compartments (modified from Caverzan *et al.,* 2012).

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Ascorbate Peroxidase (APX)

APX (EC 1.1.11.1) is an enzyme of paramount importance involved in the ASA-GSH cycle (Fig. 1) and in maintaining ROS levels inside the cell (Sharma *et al.,* 2012). It is a type of heme-I peroxidases and is found in a wide range of organisms starting from higher plants to some protists (Wilkinson *et al.,* 2002). Based on amino acid sequence, five different isozymes are found in diverse sub-cellular localizations i.e, cytosol, stroma, mitochondria, thylakoid, and peroxisome (Sharma and Dubey, 2004) (Fig. 7). APX reduces H_2O_2 to H_2O using two ASA molecules and in the process, two molecules of MDHA are generated (Sharma *et al.,* 2012) stresses enhance H_2O_2 and chlAPX (Chloroplast), mAPX (Peroxisomal), cAPX (Cytosol), and mitAPX (Mitochondria) enzymes which can eliminate ROS excess in different subcellular compartments (Fig. 7). It is one of the most extensively dispersed antioxidant enzymes in plants that has a greater affinity for H_2O_2 as compared to Catalase, making APX one of the potent H_2O_2 scavengers under stress (Wang *et al.,* 1999). Organelle localized isoforms of APX neutralize H_2O_2 generated inside various organelles, while the cytosolic isoforms of APX scavenge H_2O_2 generated in cytosol and apoplast (Mittler and Zilinskas 1992). Teixeira *et al.* (2006) reported three APX genes in *Oryza sativa* L. plants (OsAPX 2, OsAPX 7, and OsAPX8) whose expression was significantly higher when exposed to high NaCl treatment. Similar results were observed by Shi *et al.* (2001) in barley plants in which the expression of mAPX genes was significantly increased under high salt concentration. After a multitude of studies, Lin and Pu (2010) concluded that the expression of three APX genes (cAPX, mAPX, and chlAPX) were tissue-specific

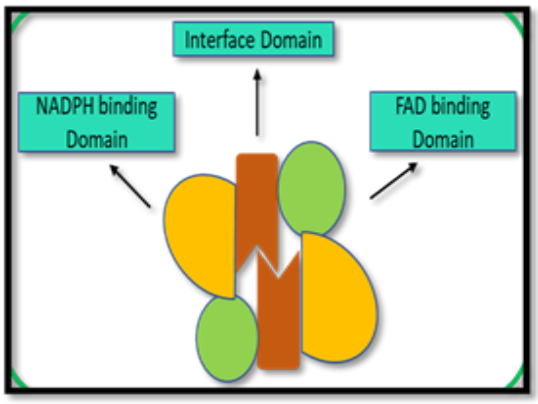

Fig. 8: Structure of GR (Modified from Gill *et al.,* 2013).

Table 1: Up-regulation of different antioxidant enzymes in response to environmental stresses.

Antioxidant Enzyme	*Type of Stress*	*Plant*	*References*
Cu/Zn SOD	Oxidative, drought, and salinity	*Oryza sativa* Pusa Basmati-1	Prashanth *et al.* 2008
Mn-SOD	SO2 pollution	*Brassica campestris L.*	Tseng *et al.* 2007
Fe SOD	Mild water stress	*Medicago sativa L.*	Rubio *et al.* 2002
Catalase	Biotic stress (Mite *Tetranychus cinnabarinus*)	*Manihot esculenta*	Lu *et al.* 2017
Catalase	Biotic stress (Fungus *Odium lini*)	*Linum usitatissimum (Linseed)*	Ashry and Mohamed 2012
Catalase	UV-B treatment	*Picea asperata*	Han *et al.* 2009
APX	Heat, drought, chilling	*Lycopersicon esculentum*	Wang *et al.* 2006
APX	Salinity	*Brassica juncea*	Saxena *et al.* 2020
GPX	Drought, chilling, salinity	*Arabidopsis thaliana*	Gaber *et al.* 2006
GPX	Heavy metal (Ni)	*Oryza sativa*	Maheshwari *et al.* 2009
GPX	Heavy metal (As)	*Oryza sativa*	Mishra *et al.* 2011
GR	Chilling	*Zea mays*	Fryer *et al.* 1998
GR	Heavy metal (Aluminium)	*Arabidopsis thaliana*	Yin *et al.* 2017
MDHAR	Ozone and salinity	*Nicotiana tabacum*	Eltayeb *et al.* 2007
MDHAR	Salt	*Arabidopsis thaliana*	Li *et al.* 2020
MDHAR	Drought	*Oryza sativa*	Sharma and Dubey 2005
DHAR	Ozone and Drought	*Nicotiana tabacum*	Ushimaru *et al.* 2006
DHAR	Salt	*Arabidopsis thaliana*	Liu *et al.* 2016

and dependent upon the duration of stress. In coffee plants, Gomes-Junior *et al.* (2006b) observed high APX activity under cadmium stress. In two cultivars of *Vigna unguiculata* (one drought tolerant and the other drought-sensitive) significantly higher APX activity (specifically cAPX) was observed in the tolerant species in comparison to sensitive species. The potential of these enzymes to neutralize the ROS at their generation site makes them a worthy biomarker against various stresses (D'Arcy-Lameta *et al.,* 2006). A significantly high APX activity (all isoforms) was recorded in many plants under heavy metal stress such as Aluminium (Sharma and Dubey, 2007).

Guaiacol Peroxidase (GPX)

GPX (EC 1.11.1.7), a heme-bearing protein, is a class III peroxidase which at the cost of H_2O_2 oxidizes aromatic electron-rich compounds like guaiacol and pyrogallol (Sharma *et al.,* 2012). The amino acid sequences of these enzymes show strong variability within the plant peroxidases family, with less than 20% similarity (Jouili *et al.,* 2010). GPX is mainly localized subcellular in vacuoles and apoplast (Takabe *et al.,* 2001; Andrews *et al.,* 2002). It is found in a wide range of organisms such as plants, animals, and microbes (*Sharma et al.,* 2012). A myriad of isoforms of GPX is found in plants that are confined to the cell walls, cytosol, and vacuoles (Asada, 1992). GPX enzymes two calcium ions and contains four conserved disulfide bridges (Schuller *et al.,* 1996). They are universally regarded as "stress enzymes" because it is involved in several processes i.e, IAA degradation, defense against environmental stresses, ethylene biosynthesis, and healing of wound in plants (Sharma *et al.,* 2012). Under stressful conditions, GPX plays a very important role in scavenging peroxy radicals and different ROS (Vangronsveld and Clijsters, 1994). The stimulatory effect on GPX activity was observed in roots of *Phaseolus vulgaris* plants under heavy metal exposure (50 µM $CuSO_4$) (Bouazizi *et al.,* 2008). Metwally *et al.* (2005) also reported increased GPX activity in the roots of garden pea plants when given the treatment of 5µM $CdCl_2$. As shown by Hosseini *et al.* (2007), leaves of *Brassica napus* plants showed high GPX activity when exposed to heavy metal stress (200 µM $Pb(NO_3)_2$). When water deficit conditions were maintained in the growth period of *Beta vulgaris* plants, GPX activity was found to be significantly increased (Sayfzadeh and Rashidi, 2011).

Glutathione Reductase

GR (EC 1.8.1.7) is an enzyme present in both eukaryotic and prokaryotic organisms and belongs to the oxidoreductase group (Gill *et al.,* 2013). There are several isoforms of GR found in plants i.e, cytosolic, plastidic, and mitochondrial, and the majority of the enzyme (80%<) found in the photosynthetic organs is of plastidic nature (Ashraf, 2009). GR plays a pivotal

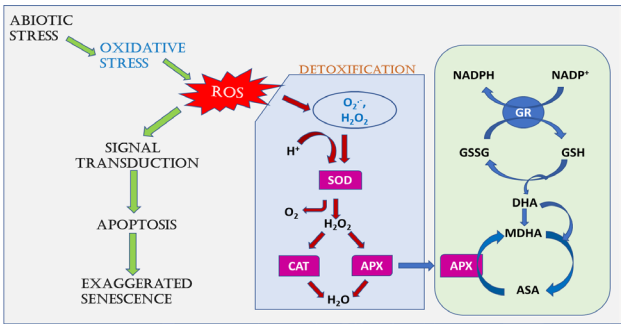

Fig. 9: Cross-talk between pathways showing adverse effects of ROS and its detoxification.

role in cellular armament against reactive oxygen species by preserving the intracellular reduced glutathione pool which is achieved through the GR-induced conversion of GSSG into GSH (Anjum *et al.,* 2010). Structurally, GR is a homodimer containing flavin adenine dinucleotide and a disulfide group (Gill *et al.,* 2013) (Fig. 8). The catalytic mechanism of the enzyme is completed in two steps: in the first step, NADPH reduces the flavin group and the second step is the reduction of GSSG, via disulfide-thiol reactions (Ghisla and Massey 1989). Increased activity of GR upon exposure to a multitude of environmental stresses has been previously reported by many researchers which makes it an essential biomarker (Hernandez *et al.,* 2001; Sharma and Dubey, 2005, 2007; Maheshwari and Dubey, 2009). A direct proportionality was found between GR activity and stress tolerance in plants and even found that $H_2O_2^-$ induced oxidative stress stimulated de novo synthesis of GR (Pastori and Trippi 1992). Antisense-mediated deletion of GR in tomato plants increased their sensitivity to chilling stress (Shu *et al.,* 2011). Aono *et al*. (1993) showed that transgenic *Nicotiana tabacum* plants with overexpressed cytosolic and plastidic GR decreased their sensitivity against air and herbicide pollution. Severe water deficit conditions led to the decline of cellular GR activity in *Oryza sativa* plants increased their susceptibility to drought (Sharma and Dubey, 2005). Salinity stress in *Olea europea* plants resulted in an increase in GR activity by several folds which improved its tolerance capacity against high salt exposure (Valderamma *et al.,* 2006). Heavy metal exposure in rice plants also showed a similar response with synchronous high activity of cultivars of wheat plants (H 168, Gimmeza 7, and Beni swif 1) showed that the H 168 cultivar showed the highest GR activity which in turn conferred the most tolerance against oxidative stress.

Monodehydroascorbate Reductase (MDHAR)

MDHAR (EC 1.6.5.4) is a FAD enzyme-containing thiol (SH) group in its catalytic domain and using NADPH as an electron donor it converts monodehydroascorbate (MDHA) into ASA (Hossain and Asada, 1985). MDHAR is the only known antioxidant enzyme that uses organic radicals as a substrate and also neutralizes the phenoxy radicals (Sakihama *et al.,* 2000). It is very widely present in plants as its different isoforms are localized in many organelles i.e, mitochondria, peroxisome, chloroplast, and cytosol. MDHAR activity upregulation has been reported by numerous researchers in response to various environmental stresses (Hernandez *et al.,* 2001; Sharma and Dubey, 2005, 2007; Maheshwari and Dubey 2009). Eltayeb *et al.* (2007) observed higher activity of the MDHAR gene in tobacco plants which in turn improved tolerance against salt stress. Increased activity of the plastidic MDHAR gene in Arabidopsis plants conferred enhanced tolerance against heat and oxidative stress (Li *et al.,* 2010). Multiple experiments were done by Shin *et al.* (2013) on *Brassica rapa* L. plants suggesting the pivotal role of the MDHAR enzyme in conferring tolerance against chilling and oxidative stress. MDHAR activity can be effectively utilized as a biomarker in *Triticum aestivum* leaves as a strong correlation between the number of grains and harvest index and foliar MDHAR activity (Shokat *et al.,* 2020).

Dehydroascorbate Reductase (DHAR)

DHAR (EC 1.8.5.1) plays a vital role in maintaining a reduced state of Ascorbic acid (ASA) by catalyzing the conversion of Dehydroascorbate (DHA) into ASA using GSH as a substrate (Rajput *et al.,* 2021). Untimely and inefficient conversion of DHA results in its permanent hydrolysis to 2,3-diketogulonic acid, which is why DHAR activity is so critical in determining the ascorbate pool inside the cell (Deutsch, 2000). A higher significant amount of ASA as a result of overexpression of DHAR activity was observed in potato, maize, and tobacco leaves which recommends the pivotal role of DHAR in cytosolic DHAR in *L. japonicus* plants making them less sensitive against salt stress when grown among other legumes (Rubio *et al.,* 2009). Drought, herbicide, and salt stress tolerance were significantly higher in *Arabidopsis thaliana* plants that showed upregulation of the cytosolic DHAR gene (Eltayeb *et al.,* 2011). DHAR is generally used as a biomarker because its production is usually regulated in response to a multitude of environmental stresses i.e, heavy metal, chilling, drought, etc (Hernandez *et al.,* 2001; Sharma and Dubey, 2005, 2007; Maheshwari and Dubey, 2009). Maintaining the reduced ASA pool in the cell (Chen *et al.,* 2003; Qin *et al.,* 2011). Recently an isoform of DHAR enzyme was identified in *Liriodendron Chinese* trees (*Lc*DHAR) and higher activity of this isoenzyme conferred increased tolerance against salt and drought stress (Hao *et al.,* 2019). Over-expression of antioxidant enzymes in response to a multitude of environmental stresses is shown in Table 1.

Discussion

In the current scenario, the plants are exposed to different kinds of environmental stresses which makes this review meaningful. In present times, the deteriorative effects of ROS and the process outlining the function of antioxidants to neutralize these free radicals have ended up as a major issue for scientists. The production of reactive oxygen species is unavoidable as they are the by-products of regular cellular homeostasis. ROS has dual functions such that it helps in signal transduction at lower concentrations while they are deleterious at above threshold level. The extraneous generation of free radicals can be associated with severe environmental conditions like heat, tropospheric ozone, salinity, drought, heavy metal, chilling, UV-B, etc. To cope with the enhanced free radicals plants have developed their innate scavenging machinery comprising of both enzymatic and non-enzymatic antioxidants. Ascorbic acid (Asa) is one of the most pivotal antioxidants because of its competency to donate electrons in a multitude of biochemical processes. Asa plays a very significant role in different processes of plants including growth, development, and environmental stress physiology. An upsurge in Asa content in plants has shown improved UV-B and oxidative stress tolerance in many plants. Glutathione (GSH) is another most common antioxidant which protects intra-cellular components against reactive oxygen species. GSH regulates numerous cellular processes such as it serves as a precursor of phytochelatins and chelates harmful metal ions, inhibits the denaturation of proteins under oxidative conditions, and it preserves the integrity of cellular membranes by maintaining the reduced state of certain molecules like

α-tocopherol. Tocopherols can be recycled from tocopheroxyl radicals which makes them an effective non-enzymatic antioxidant. Tocopherols are very efficient antioxidants as they regulate the lifecycle of free radicals. Tocopherols control the measure of lipid peroxidation in plants and hence regulates the level of gene expression and membrane integrity. Superoxide dismutase (SOD) is an enzymatic antioxidant found in both plants and animals which helps in controlling the levels of reactive oxygen species inside cells. SOD helps in dismutating superoxide ($O2^{\cdot-}$) ions into oxygen and water and therefore its activity can be directly linked with the tolerance potential of any plant. Another elemental enzymatic antioxidant found in plants is catalase which also helps in converting hydrogen peroxide (H_2O_2) into water and oxygen. Another important antioxidant that takes part in plant defence machinery is Glutathione reductase (GR). It's unique in the way that it takes part in both the enzymatic and non-enzymatic redox processes of the cell. Along with GR, there are also a few more enzymes like (MDHAR and DHAR) that takes part in the most crucial Halliwell-Asada pathway. These enzymes have been found to vary in response to different environmental stresses and therefore can be utilized as potential biomarkers in the assessment of different stresses in plants (Fig. 9).

Conclusion

Reactive oxygen species (ROS) are those unstable and reactive derivatives of oxygen that though overproduced as a consequence of a plethora of environmental stresses are also generated as by-products of normal cellular metabolism. ROS concentration is usually low inside the cell and is produced from electron transport pathways of various cell organelles i.e, mitochondria, chloroplast, plasmalemma, etc. Surplus generation of ROS alters the balance between ROS generation and scavenging which can be perilous for the cell. To deal with such a condition, the cell has a piece of antioxidative machinery, consisting of both enzymatic and non-enzymatic antioxidants, which scavenge the excess ROS and protect the macromolecules of the cell i.e, nucleic acids, proteins, and lipids. These antioxidants (such as SOD for $O2^{\cdot-}$ and CAT and APX for H_2O_2) can be utilized as biomarkers for the assessment of various biotic and abiotic stresses. Biomarkers are those molecules that depict quantifiable changes at the biochemical, physiological, and molecular levels of an organism in response to stress exposure. In the above literature numerous enzymatic and non-enzymatic antioxidants have been discussed which illustrates their importance in stress response studies.

Declaration and Statements

There is no conflict of interest between the authors.

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