M. Iqbal R. Khan
Nafees A. Khan *Editors*

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Preface

The increase in abiotic stress factors has become a major threat to the sustainability of crop production. This situation has led to thinking of ways which can help to come up with potential measures. It is, therefore, necessary to understand the influence of abiotic stress factors on crop performance and the mechanisms by which these factors impact plants. It has now become evident that abiotic stress impacts negatively on plant growth and development at every stage of the plant's life. Plants adapt to the changing environment with the adjustment at physiological, biochemical and molecular levels. The primary possible mechanism involved in the negative effects of abiotic stress factors is the excess production of reactive oxygen species (ROS). They alter physiological and molecular mechanisms leading to poor performance of plants. Plants, however, are able to cope with these adverse effects by inducing antioxidant systems as the priority. Nevertheless, the dual role of ROS has now also been ascertained which provides an evidence for the regulation of plant metabolism positively on a concentration-dependent manner. Under conditions of high ROS production, the antioxidant system plays a major role in diminishing the ROS effects. Thus, ROS production and antioxidant system are interwoven with abiotic stress conditions. The antioxidants have the capacity to hold the stability in metabolism in order to avoid disruption due to environmental disturbances.

The present edited book is an attempt to update the state of art of the knowledge on metabolism of ROS and antioxidants and their relationship in plant adaptation to abiotic stresses involving physiological, biochemical and molecular processes. The chapters are also focused on the current climate issues and how ROS metabolism can interact with the antioxidant system to accelerate detoxification mechanism. It will enhance the mechanistic understanding on ROS and antioxidant systems and will pave the path for agricultural scientists in developing tolerant crops to achieve sustainability under the changing environmental conditions. Additionally, the present book could provide an excellent material for undergraduate, postgraduate and doctoral students to understand fundamentals of ROS metabolism and antioxidant system under both optimal and stressful environments.

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An Introduction to Antioxidants and Their Roles in Plant Stress Tolerance

1

Neeti Mehla, Vinita Sindhi, Deepti Josula, Pooja Bisht, and Shabir H. Wani

Abstract

Various abiotic stresses lead to the formation of reactive oxygen species (ROS) in plants which are highly reactive and toxic in plant cell. The ROS comprises both free radical (superoxide radicals, O_2^-; hydroxyl radical, OH^-; perhydroxyl radical, HO_2^-; and alkoxy radicals, RO^-) and non-radical (molecular) forms such as hydrogen peroxide (H_2O_2) and singlet oxygen (O_2). Chloroplasts and mitochondria are the major sites for the generation of O_2^-. Plant's abiotic stress tolerance requires a number of physiological and biochemical mechanisms which includes enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaiacol peroxidase, GPOX; and glutathione-S-transferase, GST) and non-enzymatic (ascorbic acid, ASH; glutathione, GSH; phenolic compounds; alkaloids; flavonoids; carotenoids; non-protein amino acids; and α-tocopherols). Increased environmental stresses imbalance the production of reactive oxygen species and thereby quench the activity of antioxidants and thus

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resulting in oxidative damage. ROS can cause damage to cell structures, nucleic acids, lipids and proteins. Certain ROS like OH- ions are said to react with all components of DNA and damage the purines and pyrimidines. The increased production of antioxidants thus helps the plant to withstand the environmental stress. This chapter focuses on the description of antioxidant defence system under abiotic stress in plants and its involvement in the removal of reactive oxygen species. Hence various types of antioxidants, their types and role will be discussed in detail.

Keywords
Abiotic stress • Antioxidant defence system • Reactive oxygen species (ROS) • Oxidative stress

Abbreviations

$_1O^2$	Singlet oxygen
ABA	Abscisic acid
APX	Ascorbate peroxidase
AsA	Ascorbate
AsA-GSH	Ascorbate-glutathione
ASH	Ascorbic acid
ATP	Adenosine triphosphate
Ca2+	Calcium ions
Car	Carotene
Cars	Carotenoids
CAT	Catalase
Cd	Cadmium
Cu/ZnSOD	Copper/zinc superoxide dismutase
Cys	Cysteine
DHAR	Dehydroascorbate reductase
DNA	Deoxyribonucleic acid
FeSOD	Iron superoxide dismutase
GB	Glycine betaine
GDH	Glutamate dehydrogenase
GPOX	Guaiacol peroxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase

H_2O_2	Hydrogen peroxide
HO_2^-	Perhydroxyl radical
LPO	Lipid peroxidation
LDL	Low density lipoprotein
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase
Mg^{2+}	Magnesium ion
mM	Millimolar
MnSOD	Manganese superoxide dismutase
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NO	Reactive nitric oxide
O_2^-	Superoxide radical
OH	Hydroxyl radicals
POX	Peroxidases
Pro	Proline
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
SODs	Superoxide dismutases

1.1 Introduction

Plants produce excessive reactive oxygen radicals in response to stress caused as a result of environmental changes (Dabrowska et al. 2007; Khan and Khan 2014; Khan et al. 2014, 2015, 2016a, b). Reactive oxygen species (ROS) accumulate as a result of various abiotic stress factors such as salinity, UV radiations, heavy metals, extreme temperature changes, drought, air pollution, herbicides, nutrient deficiency, etc. (Wang et al. 2014; Feigl et al. 2015; Silveira et al. 2015; Thao et al. 2015; Farnese et al. 2016).

The ROS are free radical and non-radical molecules (Sharma et al. 2012) and are the key components of the signalling pathways' network, which act as primary regulators of cellular responses and cell physiology of plant in response to environmental factors (Das and Roychoudhury 2014). However, sudden rise in intracellular levels of ROS is caused due to the imbalance between production and scavenging of ROS under stress conditions (Mittler et al. 2004, Miller et al. 2010; Srivastava and Dubey 2011). In plant tissues, a variety of reactions which consume 1–2% oxygen can lead to excess ROS production which results in cell structure damage (Bhattacharjee 2005). The ROS are by-products of various metabolic activities which take place in the mitochondria, chloroplast and peroxisomes of the plant cell (Navrot et al. 2007; Luis 2015). Plants utilize ROS, which are both free radical like perhydroxyl radical (HO_2^-), superoxide radical (O_2^-), alkoxy radical (RO^-)

and hydroxyl radical (OH^-) and non-radical molecular forms like singlet oxygen (O_2) and hydrogen peroxide (H_2O_2), in various metabolic processes like lignin formation in cell wall (Inzé and Van Montagu 1995; Denness et al. 2011), the abscission of flower and leaf, cell senescence, ripening of fruit and flowering (Mehlhorn et al. 1996; Bhattacharjee 2005). Increased levels of ROS however result in loss of crop productivity (Mittler 2002; Apel and Hirt 2004; Mahajan and Tuteja 2005; Zlatev et al. 2006; Khan et al. 2007; Tuteja 2010; Gill et al. 2011). It is interesting to note that increased levels of ROS are highly reactive and affecting various cellular, biochemical and physiological functions such as disruption of the cell membrane through lipid peroxidation; damage to DNA, pigments and enzymes; carbohydrate deoxidation; and protein denaturation (Noctor and Foyer 1998; Bose et al. 2013). In addition, ROS have the ability to initiate new gene expression and at times can even damage a cell (oxidize proteins, damage nucleic acids or cause lipid peroxidation (LPO) (Foyer and Noctor 2005).

The role of ROS as a protective signalling factor or a damaging factor is dependent on the equilibrium between ROS production and scavenging (Gratão et al. 2005). Plants have their own defence mechanism (antioxidant system) that includes both enzymatic and non-enzymatic systems to regulate the oxidative stress. The enzymatic system includes CAT, peroxidase (POX), SOD, glutathione reductase (GR), ascorbate peroxidase (APX), polyphenol oxidase (PPO), etc., and non-enzymatic system includes carotenes, ascorbic acid (vitamin C), α-tocopherols, etc. When plants become senesced, the antioxidant system does not work well, with some antioxidants increasing and others decreasing as a result of which the ROS may accumulate in a large amount. This is often indicated by enhanced lipid peroxidation and decreased levels of antioxidant enzymes leading to programmed cell death (PCD), particularly under severe abiotic stress conditions (Dhindsa and Matowe 1981; Hodges and Forney 2000; Gill et al. 2011; Duarte et al. 2013). There are some natural antioxidants like vitamins, phenols, flavonoids, carotenoids, glutathione and endogenous metabolites which also help in scavenging ROS (Larson 1988) (Fig. 1.1). The antioxidant system scavenges toxic radicals by functioning as singlet and triplet oxygen quenchers, synergists, enzyme inhibitors and peroxide decomposers (Manach et al. 1998). In this chapter we will try to highlight the antioxidant defence system during abiotic stress in plants and its association with the removal of reactive oxygen species.

1.1.1 Antioxidant Defence Mechanism

The atoms or molecules having unpaired electrons are known as free radicals and are considered as a major source of ageing. These free radicals are stabilized by special category of substances known as antioxidants which prevent and protect the cell from the damage caused by free radicals. Few examples of such substances include beta-carotene; lycopene; vitamins C, E and A; catalase; etc. which help in sustaining the rate of oxidation reactions in a cell (Sies 1997). The process of transfer of electrons from one substance to another with low reduction potential is

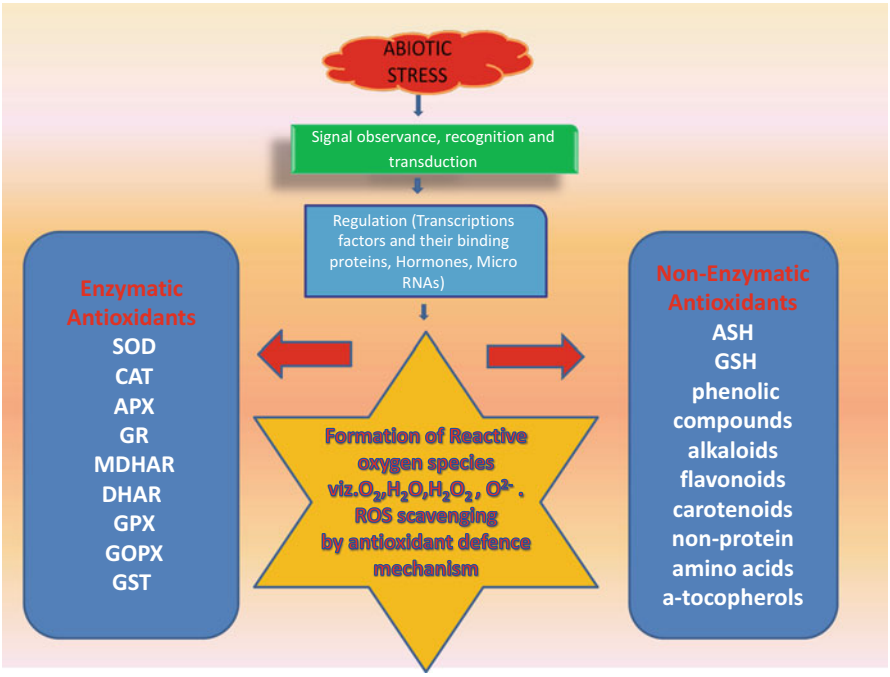

Fig. 1.1 Abiotic stress responses in plants

termed as oxidation, and these oxidation reactions are responsible for the generation of free radicals, which can cause injury to the cells by an order of chain reactions. Antioxidants play a very crucial role in mitigating or preventing the process of oxidation of other molecules (Ahamadi et al. 2015).

In order to prevent damage by oxidation, plants and animals have a complex systems of antioxidants like catalase, superoxide dismutase, peroxidises, glutathione, vitamin C; etc. which act as reducing agents that terminate oxidative reactions by removing free radicals. The production of these antioxidant molecules may, at first, be inhibited to allow the occurrence of the oxidative burst (Luis 2015), but once the signal is initiated, these mechanisms are activated and perform function in a united way (Viehweger 2014; Farnese et al. 2016).

There are two mechanisms by which the antioxidants act on ROS:

1. Chain-breaking mechanism – Electrons are donated to the free radicals by primary antioxidants (e.g. lipid radical)
2. Removal of ROS – Quenching chain initiator catalyst removes ROS and RNS (reactive nitrogen species)

Fats give rise to unsaturated fatty acids in presence of oxygen which in return generates free radicals. These free radicals are then neutralized by the antioxidants (Borek 1991).

1.1.2 Types of Antioxidants

Antioxidants can be classified into two main groups, namely, (1) primary or natural antioxidants and (2) secondary or synthetic antioxidants.

1.1.2.1 Primary or Natural Antioxidants

Primary antioxidants are also known as chain-breaking antioxidants as they convert lipid radicals into more stable products. They are mainly phenolic in structure and include the following (Hurrell 2003):

1. Antioxidant enzymes – Catalase, superoxide dismutase, peroxidase, glutathione reductase, etc., are major enzymes that help in scavenging ROS.
2. Antioxidant vitamins – Vitamin B, vitamin E and vitamin C are important for metabolic activities that take place inside the body.
3. Antioxidant minerals – Copper, iron and zinc act as cofactors of antioxidant enzymes and if absent can affect the metabolism of several macromolecules like carbohydrates.
4. Phytochemicals – These are phenolic compounds and include flavonoids.

1.1.2.2 Secondary or Synthetic Antioxidants

Phenolic compounds that capture free radicals and stop the chain reactions are called secondary antioxidants. These include (Hurrell 2003):

1. Nordihydroguaiaretic acid (NDGA)
2. Butylated hydroxyl anisole (BHA)
3. Tertiary butyl hydroquinone (TBHQ)
4. Butylated hydroxytoluene (BHT)
5. Propyl gallate (PG) and metal-chelating agent (EDTA)

1.2 Natural Antioxidants

1.2.1 Antioxidant Enzymes

Plants have a unique and incredible defence mechanism for detoxification of the free radicals. Basically these mechanisms include catalysis of various antioxidant enzymes like superoxide dismutase, glutathione peroxidase and catalase followed by a metastasis of superoxide to hydrogen peroxide and further conversion into water and oxygen. The ratio of GSSG: GSH regulates the catalysis reactions of glutathione peroxidase where GSH is required as a substrate. The detoxification of

Table 1.1 Antioxidant defence mechanism (antioxidant enzymes) in various plant species in response to abiotic stress

Antioxidant enzymes	Type of stress	Plant species	References
Superoxide dismutase	Salt	*Mulberry*	Harinasut et al. (2003)
	Salt	*C. arietinum*	Kukreja et al. (2005) and Eyidogan and Öz (2007)
	Salt and drought	*Glycyrrhiza uralensis*	Pan et al. (2006)
	Metal (Cd)	*Brassica juncea*	Mobin and Khan (2007)
		Vigna mungo	Singh et al. (2008)
	Aluminium	*H. vulgare*	Šimonovičová et al. (2004)
	Drought	*O. sativa*	Sharma and Dubey (2005)
		P. vulgaris	Zlatev et al. (2006)
		Alternanthera philoxeroides	Wang et al. (2008b)
	Water	*Lycopersicum esculentum*	Sánchez-Rodríguez et al. (2016)
	Salt	Maize	Hamada AbdElgawad et al. (2016)
Catalase	Salt+copper	*A. dolium*	Srivastava et al. (2005)
	Salt+drought	*Glycyrrhiza uralensis*	Pan et al. (2006)
	Ultraviolet-B rays	*C. auriculata*	Agarwal (2007)
	Drought	Wheat	Simova-Stoilova et al. (2010)
	Salt	Maize	Hamada AbdElgawad et al. (2016)
	Water	*Lycopersicum esculentum*	Sánchez-Rodríguez et al. (2016)
Peroxidase	Metal (Fe)	Bean	Pekker et al. (2002)
	Metal (Cd)	*A. thaliana*	Cho and Seo (2005)
	Water	*P. vulgaris*	Zlatev et al. (2006)
		P. asperata	Yang et al. (2008)
	Heat +drought	*Arabidopsis thaliana*	Koussevitzky et al. (2008)
	Salt+osmotic stress	*O. sativa*	Cunha et al. (2016)
	Salt	*Eurya emarginata*	Zheng et al. (2016)
Glutathione reductase	Light	*P. asperata*	Yang et al. (2008)
	Cold	*W. somnifera*	(Mir et al. 2015)
	Water	*Arachis hypogaea*	Chakraborty et al. (2015)
	Salt	Chickpea	Shankar et al. (2016)
	Water	*O. sativa*	Kundur et al. (2016)
	Drought	Maize	Ahmad et al. (2016)

ROS largely depends on the concentration of these reactants and their ratio. The concentration of redox-active metals such as iron should be monitored by binding to storage and transport proteins (e.g. ferritin, transferrin, lactoferrin) so as to control the formation of reactive oxygen species (ROS).

Different antioxidant enzymes play different roles (Table 1.1). Some enzymes like superoxide dismutase, catalase and peroxidase eliminate ROS, while others like glutathione peroxidase eliminate internal lipid peroxidation products. Glutathione S-transferase plays an important role in eliminating toxic secondary oxidation radicals (Timofeyev and Steinberg 2006).

1.2.1.1 Superoxide Dismutase

Superoxide dismutase (SOD, EC 1.15.1.1) catalyses the destruction of the O^{2-} free radical. They act as the first line of defence against harm caused by ROS (Das and Roychoudhury 2014):

$$2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$$

It protects oxygen-metabolizing cells against harmful effects of superoxide free radicals. Three types of superoxide dismutase are characterized by different metal contents (Worthington-biochem):

1. A blue-green Cu(II)-Zn(II) enzyme comes from human and bovine erythrocytes.
2. A wine-red Mn(III) protein is found in *E. coli* and in chicken and rat (Peeters-Joris et al. 1975) liver mitochondria (Tyler 1975).
3. A yellow Fe (III) enzyme from *E. coli* (Villafranca et al. 1974).

In higher plants, SOD isozymes are localized in various cell organelles (Mn-SOD in mitochondria and peroxisomes, Fe-SOD in chloroplasts and peroxisomes and CuZn-SOD in chloroplasts, cytosol, apoplast and peroxisomes) and protect them from being oxidized by reactive oxygen species (ROS) (Corpas et al. 2001, 2006; Alscher et al. 2002). A substantial increase in SOD activity in response to salt stress has been observed in various plants, viz. *C. arietinum* (Kukreja et al. 2005), mulberry (Harinasut et al. 2003) and *Lycopersicon esculentum* (Gapińska et al. 2007). Eyidogan and Oz found three SOD activity bands (MnSOD, FeSOD and Cu/ZnSOD) in *C. arietinum* with significantly high activities of Cu/ZnSOD and MnSOD isozymes under salt stress (Eyidogan and Öz 2007). Pan et al. studied drought and salt stress effects on *Glycyrrhiza uralensis* and found a significant increase in SOD activity and presence of an additional MnSOD at the time of salt stress (Pan et al. 2006). On treating *C. arietinum* (Hasan et al. 2008), *Hordeum vulgare* (Guo et al. 2004), *O. sativa* (Hsu and Kao 2004), *Triticum aestivum* (Khan et al. 2007), *Brassica juncea* (Mobin and Khan 2007), *Vigna mungo* (Singh et al. 2008) and *A. thaliana* (Skórzyńska-Polit et al. 2003) with cadmium, SOD activity was found to be high. Increase in SOD activity following drought stress was noted in three cultivars of *O. sativa* (Sharma and Dubey 2005), *P. vulgaris* (Zlatev et al. 2006) and *Alternanthera philoxeroides* (Gao et al. 2008).

The effects of water stress on the activities of total leaf SOD and chloroplast SOD in *Trifolium repens* were studied, and it was found that with the increase in water stress, the activity of SOD also increased (Wang et al. 2008a). An increase in SOD activity in *H. vulgare* root tips was seen under Al stress at 72 h (Šimonovičová et al. 2004). Yang et al. (2008) demonstrated the effect on *Picea asperata* when a combination of drought and low light were used.

1.2.1.2 Catalase

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric haem-containing enzyme which acts on H_2O_2 and degrades it at a rapid rate. It is found in all aerobic organisms and is unique as it does not require a reducing equivalent (Das and Roychoudhury 2014). It has been reported that one molecule of CAT can convert 26 million molecules of H_2O_2 to H_2O and O_2 per minute and depending on the concentration of H_2O_2, it exerts a dual function (Deisseroth and Dounce 1970). At low concentrations ($<10^{-6}$ M) of H_2O_2, it acts "peroxidatically", where a variety of hydrogen donors (e.g. ethanol, ascorbic acid) can be oxidized in the following manner:

$$RH_2 + H_2O_2 \rightarrow R + 2H_2O$$

When the substrate concentrations are high, catalase decomposes toxic H_2O_2 at an extremely rapid rate using the "catalytic" reaction in which H_2O_2 acts as both acceptor and donor of hydrogen molecules (Tausz et al. 2004):

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

A decrease in CAT activity was seen in *A. doliolum* under NaCl and Cu stress (Srivastava et al. 2005). In wheat plants, CAT activity was seen to be enhanced under drought stress, and its levels were higher especially in sensitive varieties (Simova-Stoilova et al. 2010). However, Sharma and Dubey reported a decrease in CAT activity under drought stress in rice seedlings (Sharma and Dubey 2005). Increased levels of CAT activity were seen in *P. asperata* under drought stress and high light conditions (Yang et al. 2008). The UV-B stress increased CAT activity in *C. auriculata* seedlings (Agarwal 2007). The combined effect of salt and drought stress decreased the CAT act in *Glycyrrhiza uralensis* seedlings (Pan et al. 2006).

1.2.1.3 Peroxidases

Guaiacol Peroxidase

Guaiacol peroxidise (GPOX) comprises of 40–50 kDa monomers and uses aromatic electron donors such as guaiacol and pyrogallol and decomposes indole-3-acetic acid (IAA) (Das and Roychoudhury 2014). It consumes H_2O_2 and is important for the biosynthesis of lignin. The activity of GPOX is different in different plant species and with varying stress conditions. It increases in Cd-exposed plants of *T. aestivum* (Milone et al. 2003), *A. thaliana* (Cho and Seo 2005) and *C. demersum*

(Aravind and Prasad 2003). On subjecting spruce needles to Cd stress, it was observed that GPOX activity initially increased and later decreased as the stress was further increased (Radotić et al. 2000). The leaf and root tissues of *Vigna radiata* (Panda 2001) and *O. sativa* (Yamane et al. 2009) showed an increase in the levels of CAT under salinity stress.

Ascorbate Peroxidase (APX)

Ascorbate peroxidases (APXs) are haem-containing proteins with iron present at the catalytic site. The substrate used for reducing hydrogen peroxide is the ascorbate, and its concentration defines the sensitivity of the enzyme. Lower concentrations (lower than 20 μM) make the enzymes become less stable with decreased activity (Shigeoka et al. 2002). They are mainly found in higher plants, algae and some cyanobacteria (Miyake et al. 1991; Sano et al. 2001; Sharma and Dubey 2004; Shigeoka et al. 1980a, b; Takeda et al. 1992, 1998, 2000) but are also found in tissues rich in ascorbate (Boveris et al. 1980; Wada et al. 1998), in insects (Mathews et al. 1997), in *Trypanosoma cruzi* (Mathews et al. 1997) and in the choroid and iris epithelium of the bovine eye. Plants contain five isoforms of APX: mitochondria isoforms, cytosolic isoforms, chloroplastic isoforms and peroxisomal/glyoxysomal isoforms. They function as scavengers of H_2O_2 (Miyake and Asada 1996). When plants are exposed to various stress conditions, APX activity generally increases. Enhanced leaf APX activity under cadmium stress has been reported in *B. juncea* (Mobin and Khan 2007), *V. mungo* (Singh et al. 2008), *T. aestivum* (Khan et al. 2007) and *Ceratophyllum demersum* (Aravind and Prasad 2003). Rice seedlings showed increased APX activity with pretreatment of H_2O_2 under non-heat shock conditions (Hsu and Kao 2007). Salt-stressed *A. doliolum* showed high APX activity (Srivastava et al. 2005). Significant increase in APX activity was noted under water stress in three cultivars of *P. vulgaris* (Zlatev et al. 2006) and *P. asperata* (Yang et al. 2008). Sharma and Dubey reported higher chloroplast APX activity in plants under mild drought stress than control-grown plants, but the activity later decreased with the increasing drought stress (Sharma and Dubey 2005). Pekker et al. found an increase in cAPX levels in the leaves of de-rooted bean plants in response to Fe stress (Pekker et al. 2002). Cytosolic APX1 is important in protecting plants against heat and drought stress (Koussevitzky et al. 2008). Simonovicova et al. found an increase in APX activity in *H. vulgare* L. cv. Alfor root tips under aluminium stress at 72 h (Šimonovičová et al. 2004).

1.3 Halliwell-Asada Cycle

Halliwell-Asada cycle (ascorbate-glutathione cycle) is one of the most important antioxidant systems in plants. It is a set of reactions catalysed by glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR). ATP and NAPH are utilized to convert H_2O_2 with the help of glutathione and ascorbate (Foyer and Noctor 2005). The highly energetic reactions of photosynthesis that take place in chloroplasts are responsible for the organelle to

act as a major source of superoxide and H_2O_2. The ascorbate-glutathione cycle plays an important role in chloroplast redox reactions because of non-residence of catalase enzyme.

1.3.1 Glutathione Reductase

Glutathione reductase (GR, EC 1.6.4.2) is a ubiquitous enzyme, which catalyses the reduction of oxidized glutathione (GSSG) to glutathione (GSH). Glutathione redox cycle is needed to maintain the required amount of reduced GSH in the cell and GSH functions as an antioxidant as it acts upon organic peroxides and free radicals in amino acid transport. Glutathione reductase is needed for glutathione redox cycle, and it also serves as a substrate for glutathione S-transferases and glutathione peroxidises. It is mainly found in chloroplasts, but small amount is also present in cytosol and mitochondria (Creissen et al. 1994; Edwards et al. 1990). GR and GSH determine the tolerance of a plant under various stresses (Rao and Reddy 2008). GR activity showed an increase in the presence of Cd in *C. annuum* (León et al. 2002), *A. thaliana* (Skórzyńska-Polit et al. 2003), *V. mungo* (Singh et al. 2008), *T. aestivum* (Khan et al. 2007) and *B. juncea* (Mobin and Khan 2007). Eyidogan and Oz observed that under salt stress, the GR activity increased in the leaf tissue of *C. arietinum* L. cv. Gokce (Eyidogan and Öz 2007). An increased GR activity was seen in *C. arietinum* roots following salt stress (Kukreja et al. 2005). Decrease in GR activity has been observed on exposing *A. doliolum* to Cu stress but showed to be escalated when subjected under salt stress (Srivastava et al. 2005). A significant increase in GR activity is reported when *O. sativa* seedlings were exposed to drought stress (Sharma and Dubey 2005).GR activity increases in *P. asperata* seedlings under high light intensity, but no change was observed in low light (Yang et al. 2008).

1.3.2 Monodehydroascorbate Reductase (MDHAR)

MDHAR is a flavin adenine dinucleotide (FAD) enzyme having chloroplastic and cytosolic isozymes. Monodehydroascorbate (MDHA) is an electron acceptor specific to MDHAR. It uses NADH rather than NADPH as the electron donor (Asada 1999). APX and MDHAR scavenge H_2O_2 in the mitochondria and peroxisomes. A decrease in MDHAR activity in Cd-exposed poplar hybrids (*Populus* and *Canescens*) and an increase in Cd-exposed *Pinus sylvestris* were observed by Schutzendubel et al. (2001). The enzymes responsible for regeneration of ASH were found by Sharma and Dubey (2005). DHAR, GR and MDHAR were higher in rice seedlings under drought stress. Increased MDHAR activity develops a tolerance in tomato plant against chill stress (Stevens et al. 2008). Overexpression of MDHAR in transgenic tobacco resulted with an increase in tolerance against salt and osmotic stresses (Eltayeb et al. 2007).

1.3.3 Dehydroascorbate Reductase (DHAR)

DHAR plays an important role in regulating and regenerating ASH. This role of DHAR has been studied in transgenic tobacco plants overexpressing *A. thaliana* cytosolic DHAR when exposed to Al stress. It was seen that plants overexpressing DHAR, by maintaining high ASH level, showed tolerance to aluminium stress (Yin et al. 2010). Higher levels of DHAR help in protecting tobacco plants against ozone toxicity (Chen and Gallie 2005) and drought and ozone stress tolerance (Eltayeb et al. 2006) and increased salt tolerance in *Arabidopsis* (Ushimaru et al. 2006).

1.3.4 Glutathione Peroxidase

GPXs (EC 1.11.1.9) are a family of selenium-containing heterogeneous isozymes that shield plant cells against oxidative damage by reducing H_2O_2 and organic and lipid hydroperoxides utilizing GSH (Noctor et al. 2002). Selenium acts as scavenger of peroxides from cytosol and cell membrane (Mahapatra et al. 2013). Millar et al. identified a family of seven related proteins named from GPX1 to GPX7 in various cellular organelles including mitochondria cytosol, endoplasmic reticulum and chloroplast *Arabidopsis thaliana* (Millar et al. 2003). Stress increases GPX activity in cultivars of *C. annuum* plants (León et al. 2002) but is reduced in roots and shows no significant change in the leaves of cadmium-exposed *P. sativum* plants (Dixit et al. 2001). Gapinska et al. (2007) reported that 150 mM NaCl stress resulted in higher activity of GPX in *L. esculentum* roots.

1.3.5 Glutathione S-Transferases (GST)

Glutathione S-transferases (GST, EC 2.5.1.18) or the plant glutathione transferases are large group of enzymes that catalyse tripeptide glutathione and electrophilic xenobiotic substrate amalgamation (GSH; g-gluecysegly). Plant GSTs help in herbicide detoxification, vacuolar sequestration of anthocyanin, hormone homeostasis, hydroxyperoxide detoxification, tyrosine metabolism and regulation of apoptosis and affect plant responses to biotic and abiotic stresses (Dixon et al. 2010). It has been observed that GSTs can eliminate cytotoxic or genotoxic compounds, which can damage the DNA, RNA and proteins (Noctor et al. 2002). They use GSH to decrease peroxides. GSTs represent one of the exuberant protein and in few instances exemplifying more than 1% of soluble protein in plant cells (Edwards et al. 2000). An increased GST activity was observed in roots of *Phragmites australis* plants (Moons 2003) and leaves and roots of cadmium-exposed *P. sativum* plants (Dixit et al. 2001). Gapinska et al. (2007) observed GST activity to be enhanced in *L. esculentum* roots when subjected under salinity stress. When two contrasting sorghum varieties, drought sensitive (SPV-839) and drought tolerant (M35-1), were exposed to 150 mM NaCl for 72 h, it was observed that the

tolerant variety showed better response in terms of efficient H_2O_2 scavenging mechanisms with high GST and CAT activities (Jogeswar et al. 2006).

1.3.6 Low Molecular Weight Antioxidants

These antioxidants can be lipid soluble and membrane associated like α-tocopherol and β-carotene or water-soluble reductants like ascorbate and glutathione (Ahmad et al. 2008). Non-enzymatic antioxidants can also be divided into nutrient antioxidants and metabolic antioxidants. Metabolic antioxidants are produced by the body like bilirubin, metal-chelating proteins and uric acid. Nutrient antioxidants are provided through diet or supplements like vitamins E and C and trace metals (Madhavi et al. 1995). Radical-scavenging antioxidants (e.g. vitamin E) capture the radical, thus interrupting chain reactions; the vitamin E radical is relatively stable and can be converted back to its non-radical form. These ROS defence mechanisms are valuable for the whole body and subcellular distribution of the different components. Concentrations of the enzymes (SOD, catalase and GSH peroxidase), vitamin E and substrates (GSH) are higher in areas where ROS damage is more likely (ROS 1997).

1.3.6.1 Ascorbic Acid (ASH)

Ascorbic acid commonly known as vitamin C has been reported to be the most opulent, strong and water-soluble antioxidant which checks or neutralizes the damage done by the ROS in plants (Smirnoff 2000; Khan and Ashraf 2008). It is mostly found in all plant tissues but is abundant in meristematic regions, photosynthetic cells and some fruits. ASH is considered as an efficient scavenger of reactive oxygen species as it has the potential to donate electrons through enzymatic or non-enzymatic reactions.

It either regenerates a-tocopherol from tocopheroxyl radical or directly scavenges the OH^- and O_2 radicals. In chloroplast, the excess excitation energy is dissipated by ASH acting as a cofactor of violaxanthin de-epoxidase (Smirnoff 2000). In addition, it also plays a vital role in preserving the activities of enzymes that contain transition metal ions as a prosthetic group (Noctor and Foyer 1998). The ASH redox system comprises of L-ascorbic acid, MDHA and DHA. Both the oxidized forms of ASH are comparatively unstable in aqueous environments, while DHA can be chemically reduced by GSH to ASH (Foyer and Halliwell 1976).

1.3.6.2 Glutathione (GSH)

Glutathione acts as an intracellular defence in plants against oxidative damage. It is localized in reduced state in the cell compartments like mitochondria, peroxisomes, cytosol, vacuole, apoplasts and even endoplasmic reticulum (Mittler and Zilinskas 1992; Jiménez et al. 1998) where it is involved in several physiological processes, including signal transduction, regulation of sulphate transport, detoxification of xenobiotics, conjugation of metabolites (Xiang et al. 2001) and the expression of genes responsive during stress conditions (Mullineaux and Rausch 2005). Growth

and development-related events in plants, including cell senescence and death, cell differentiation, enzymatic regulation and pathogen resistance, also require GSH (Rausch and Wachter 2005). In many cellular reactions, GSH acts as a substrate and produces GSSG, which are two glutathione molecules linked by a disulphide bond. The redox state of a cell can be maintained with the help of this equilibrium between GSH and GSSG (Zlatev et al. 2006). GSH counteracts the inhibitory effects of ROS-induced oxidative stress by retaining the reduced state of the cells (Meyer 2008). It acts as an efficient scavenger of O_2, H_2O_2 (Briviba et al. 1997; Noctor and Foyer 1998) and most treacherous ROS like $OH^{\cdot}$ (Larson 1988). Furthermore, GSH plays an important role in the anti-oxidative defence system by regenerating another adequate water-soluble antioxidant like ASH, through the ASH-GSH cycle (Foyer and Halliwell 1976). GSH also detoxifies many inhaled pollutants like ozone, NO_2, etc. (Mahapatra et al. 2013). Glutathione, a major non-protein thiol, has long been reported to be essential for recycling of antioxidants like vitamin C and vitamin E (Kayang 2007).

1.3.6.3 Proline (Pro)

Besides being an osmolyte, proline is a non-enzymatic antioxidant that is useful for microbes, animals and plants to reduce the adverse effects of ROS. Pro accumulates under salt, drought and metal stress as a result of increase in synthesis or decrease in degradation. Free Pro acts as a protein stabilizer, an osmo-protectant, a metal chelator, an OH^- and O_2 scavenger and an inhibitor of LPO (Ashraf and Foolad 2007; Trovato et al. 2008). Proline, mannitol, sorbitol and myo-inositol have been tested, and proline has been found to possess excellent OH^--scavenging ability (Smirnoff and Cumbes 1989). Hence in case of the reactive oxygen species produced as a result of metal, salt and dehydrated stress, proline acts as an effective quencher along with a redox-signalling molecule (Alia and Sardhi 1991).

1.3.6.4 α-Tocopherols (Vitamin E)

Tocopherols commonly known as vitamin E are known to be powerful scavengers of ROS and lipid radicals. In plants they are found in thylakoid membrane and are lipid soluble in nature. There are four different isomers of tocopherols which are present in the plant system (α, β, γ, δ). However, α-tocopherol shows the maximum antioxidant activity due to presence of three methyl groups in its molecular structure. They also stabilize polysaturated fatty acids within lipid bilayers and low density lipoprotein (LDL) by protecting them from lipoxygenase attack (Sathishkumar et al. 2010; Mahapatra et al. 2013).

1.3.6.5 Carotenoids (Car)

In every photosynthetic organisms, b-carotene, zeaxanthin, carotenoids and tocopherols play a vital photoprotective role, by dissipating excess excitation energy as heat or by scavenging ROS and suppressing LPO. B-carotene is an excellent singlet oxygen scavenger (Mahapatra et al. 2013). Carotenoids are pigments that are soluble in lipid and are generally found in plants and microorganisms. There are more than 600 carotenoids found in plants that have

several metabolic functions like providing plant tolerance to oxidative stress. There are predominantly three major functions that are carried out by Car:

1. An accessory light-harvesting role – they absorb light (wavelength between 400 and 550 nm) and transfer to the chlorophyll (Siefermann-Harms 1987).
2. An antioxidant – they quench naturally formed triplet sensitizer (Chl3), $O_2^{\cdot}$ and other harmful free radicals during photosynthesis (Collins 2001).
3. Structural role – they provide stability to thylakoid membrane and light-harvesting complex proteins and important for the PSI assembly (Niyogi et al. 2001).

1.3.6.6 Flavonoids

Flavonoids are mostly found in the floral parts, leaves and pollens. They can also be seen accumulated in aerial parts of the plant, in leaf surface and in the plant vacuole as glycosides. Flavonoid concentration in a plant cell is predominantly maintained over 1 Mm (Vierstra et al. 1982). Flavonoids can be categorized into different types depending on their structural forms like flavonols, flavones, isoflavones and anthocyanin. Flavonoids have many functions like acting as signal molecules in plant-microbe interactions; protection against UV light; flowers, fruits and seed pigmentation; role in plant fertility; germination of pollen; and defence against pathogenic microorganisms, insects and animals (phytopathogens) (Olsen et al. 2010). They are considered to be the most bioactive plant secondary metabolites and can perform better than certain well-known antioxidants, like ASH and α-tocopherol (Hernandez et al. 2009). They prevent cell damage in plants under environmental stress by locating and neutralizing radicals (Løvdal et al. 2010). Flavonoids inhibit lipid peroxidation and lipoxygenases and are functionally dependent on the number and alignment of their hydroxyl groups attached to ring structures (Mahapatra et al. 2013). The antioxidant behaviour of flavonoids gets influenced by the reduction potentials and the accessibility of the radicals. Plants that produce flavonoids and other phenolic compounds develop a tolerance to high UV irradiation as these compounds can absorb UV light (Cle et al. 2008). Stress conditions may induce various flavonoid biosynthetic genes. The biotic and abiotic stresses such as wounding, drought, metal toxicity and nutrient deprivation can increase the levels of flavonoids in the plants as a part of their defence strategy (Winkel-Shirley 2002). Phenolic compounds also act as antioxidants because of their ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers. They are classified into two groups – polyphenols and simple phenols (Prakash et al. 2001). They are generally known to be present in both edible and non-edible plants. Phenolic compounds are also used as metal chelators (Oboh and Rocha 2007).

1.3.6.7 Secondary Antioxidants

Secondary antioxidants are preventives that lower the rate of chain initiation through various mechanisms. They slow down the rate of auto-oxidation of lipids by various processes like decomposing hydro peroxides to non-radical products, scavenging oxygen and binding metal ions (Gordon 1990). Primary antioxidants are

generated by secondary antioxidants by donating electron or hydrogen to primary antioxidant radicals. Chelating agents remove pro-oxidant metals and prevent metal-catalysed oxidation by stabilizing the oxide form of metals which have reduced redox potential. Metal chelating is an example of secondary antioxidant mechanism (Madhavi et al. 1995).

1.4 Conclusion

Oxidative stress is caused due to overproduction of ROS generated by different types of abiotic stresses in plants. These ROS or free radicals, dwelling in either extracellular or intracellular environment, impose their detrimental effects to the cells. The cells have antioxidant defence mechanisms which prevent oxidative damage to proteins, lipids and nucleic acids. The antioxidant defence mechanisms could be enzymatic such as superoxide dismutase, glutathione peroxidise, catalase and glutathione reductase or non-enzymatic including proline, a-tocopherols, glutathione, carotenoids and flavonoids. The ROS, though being key regulatory molecules, can harm cells when generated in abundance or when the antioxidant defence system is not working properly. The free radicals have the ability to interact with each other and with antioxidant systems. ROS, as previously explained in pathogenesis, is known to play a dual role in its mechanism of action. Plant cell and organelles have cellular antioxidant machinery for controlling the concentration of ROS in the cell. ROS-scavenging enzymes like catalase, peroxidases, superoxide dismutase, glutathione reductases, etc. are useful in helping plants develop tolerance against various abiotic stresses. Therefore, to tolerate extreme environmental conditions, plants with higher ability to scavenge and/or control the level of cellular ROS may be useful in future.

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