# Reactive oxygen species and environmental stresses

**Usha Chakraborty**

*Plant Biochemistry Laboratory, Department of Botany, University of North Bengal Siliguri-734013; e-mail: chakrabortyusha@hotmail.com*

Reactive oxygen species (ROS) include oxygen ions, free radicals and peroxides both inorganic and organic. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. ROSs form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. While ROS have the potential to cause oxidative damage to cells during environmental stress, recent studies have shown that ROS play a key role in plants as signal transduction molecules involved in mediating responses to pathogen infection, environmental stresses, programmed cell death and developmental stimuli (Mittler *et al.* 2004, Torres and Dangl 2005). The rapid increase in ROS production, referred to as 'the oxidative burst', was shown to be essential for many of these processes, and genetic studies have shown that respiratory burst oxidase homolog (Rboh) genes, encoding NADPH oxidases, are the main producers of signal transduction-associated ROS in cells during these processes (Mittler *et al.* 2004, Torres and Dangl 2005).

However, during times of environmental stress ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases and catalases. Small molecule antioxidants such as ascorbic acid (vitamin-C), uric acid, and glutathione also play important roles as cellular antioxidants. Similarly, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals. In contrast, the antioxidant ability of the extracellular space is relatively less.

**Why ROSs are formed**

Oxygen is an enigmatic element in the biological system: on the one hand , aerobic life cannot go on without oxygen, and, on the other, it can be easily converted to the toxic reactive oxygen species which can damage cellular systems. It constitutes 21% of the atmosphere and is the most abundant chemical in and near the earth's crust. The concentration of oxygen in the atmosphere has risen dramatically since the beginning of life due to the evolution of oxygen during photosynthesis. Because of its chemical properties as an electron acceptor whereby each molecule of dioxygen can accept four electrons producing water, oxygen is fundamentally essential for energy metabolism in the predominantly aerobic modern biosphere (Levine 2004).

The evolution of aerobic life led to the production of reactive oxygen species in mitochondria, chloroplasts and peroxisomes (Apel and Hirt 2004). Molecular oxygen, itself relatively unreactive and non-toxic, becomes reactive and, in some cases, dangerously so, to biological systems when its electron structure is altered. The different forms of reactive oxygen species are all capable of causing oxidative damage to proteins, DNA and lipids. Oxygen, in its ground state, can be regarded as a radical ( with two unpaired electrons of parallel spin) but is the most stable form of oxygen. The parallel spin causes the oxygen to be limited to accepting one electron at a time and reacting with non-radicals only slowly because the valence electrons

of non-radicals are paired and anti-parallel. Singlet oxygen ($^1O_2$) is produced by input of energy without the addition of an electron. The spin is anti-parallel and therefore the oxidizing ability is considerably enhanced. Addition of an electron to ground state oxygen yields the superoxide radical ($O_2^{\cdot}$) while an additional electron gives the peroxide ion ($O^-_2$) in the form of hydrogen peroxide ($H_2O_2$). Another oxygen derived radical is the highly reaction hydroxyl radical ($OH^{\cdot}$).

**ROS Formation**

Active oxygen species are natural by-products of metabolism. These are formed during normal metabolic processes involving energy transfer like respiration and photosynthesis. The continuous flow of electrons in the cell – especially, but not exclusively, in the energy producing organelles such as mitochondria or chloroplasts –creates a constant leakage of electrons to oxygen, forming superoxide and other derivatives.

During photosynthesis, these are formed due to excess energy input into the photosynthetic electron transport system. When the light trapping systems of the chloroplast receive excess light, energy can flow. It can be released though thermal dissipation and fluorescence. The energy may be absorbed by the electron transport chain if it is running maximally, that it is not inhibited. It actually acts as a sink for excess energy. In mitochondrial respiration, it has been estimated that one to two percent of all the electrons travelling down the respiratory chain never make it to the end, but instead, form superoxide. The active oxygen species may also accumulate to toxic levels during a wide range of environmental stresses.

**Types of ROS**

Reactive oxygen species exist in several forms, some more toxic than the others.

*Singlet oxygen*

Singlet oxygen can be formed chemically or photochemically. An example of chemical formation is as a by-product of lipoxygenase activity. It is formed photochemically by addition of energy from chlorophyll, specially when an excess of light energy is being received. The reaction center complex of PSII consists of cytochrome $b_{559}$ and the heterodimer of the D1 and D2 proteins. The heterodimer binds the reaction center's functional prosthetic groups including chlorophyll P680, phaeophytin, and the quinine electron acceptors $Q_A$ and $Q_B$. Excitation of the reaction center results in charge separation between P680 and phaeophytin and the subsequent sequential reduction of $Q_A$ and $Q_B$. When the redox state of the plastoquinone pool and $Q_A$ and $Q_B$ are over reduced because of excess light energy, charge separation cannot be completed and the oxidized P680 chlorophyll recombines with the reduced phaeophytin.Under these conditions, forming the triplet state of P680 is favoured, leading to the generation of singlet oxygen by energy transfer ( Apel and Hirt 2004). Excited singlet chlorophyll in the pigment beds of the light harvesting systems is converted to triplet chlorophyll if the excitation energy cannot be used or lost by electron transport, fluorescence or thermal dissipation.The excited triplet chlorophyll then converts ground oxygen to singlet oxygen by elevating the valence electrons into higher energy orbitals and subsequently inverting the spin to create an anti-parallel pair (Foyer *et al* 1994). The production of singlet oxygen is therefore dependent on the rate of thermal dissipation and electron transport.

*Superoxide*

The one-electron reduction resulting in the superoxide radical ($O_2^{\cdot-}$) requires a slight input of energy that is often provided by NAD(P)H in biological systems. This radical can be formed enzymatically by flavoprotein dehydrogenases. More importantly, it can be formed non-enzymatically by autooxidation of ferredoxins, hydroquinones, thiols and reduced hemeproteins. Ferredoxin, and other electron carriers on the reducing side of Photosystem I have sufficiently negative electrochemical potentials to donate an electron to oxygen. The majority of superoxide formation in this way is through ferredoxin and the Mehler reaction.

*Hydrogen peroxide*

Hydrogen peroxide may be formed during oxidation of glycolate in the peroxisome. More importantly, it is formed from superoxide by the activity of superoxide dismutase. Hydrogen peroxide is a relatively stable oxidant and is the most stable of active oxygen species. It is a strong nucleophilic oxidant but is relatively unreactive. $H_2O_2$ readily crosses the lipid bilayer of cell membranes. It can directly oxidize transition metals such as $Fe^{2+}$ or oxidize organic molecules, generally via peroxidases.

*Hydroxyl radical*

The hydroxyl radical is believed to be formed via the metal catalysed Fenton and Haber-Weiss reactions.The hydroxyl radical is highly reactive, oxidizing most organic compounds at almost diffusion controlled rates ( $K>10\ M^{-1}s^{-1}$).

**Formation of ROS and involvement of antioxidant systems during stresses**

The formation of reactive oxygen species is, therefore, a consequence of some of the normal metabolic processes in plants, which is enhanced during various stresses. ROS have the potential to cause deleterious effects on the cellular processes. However, aerobic organisms have a battery of enzymatic and non-enzymatic antioxidants that scavenge the ROS ( Fig.1). Thus, in the normal course the formation of ROS is closely related to its detoxification and the plants survive without much harm. However, under extreme environmental or other stresses, even such scavenging activities are not sufficient and the cells succumb to the damage.

*Enzymic ROS scavenging mechanisms*

Some of the most important antioxidant enzymes in plants are the superoxide dismutases, catalases, peroxidases, ascorbate peroxidases and glutathione peroxidases.

Superoxide dismutases (SOD), which catalyze the dismutation of superoxides to hydrogen peroxide are a family of metal containing enzymes present in the cytosol, chloroplasts and mitochondria. The hydrogen peroxide formed through this reaction is further scavenged by other enzymes. SOD is generally viewed as one of the most important antioxidants. Hydrogen peroxide, in turn, is scavenged by catalase in the peroxisomes and by ascorbate peroxidase in the chloroplast. Catalase, which converts hydrogen peroxide into water and molecular oxygen is a tetrameric heme-containing enzyme found in all aerobic organisms.

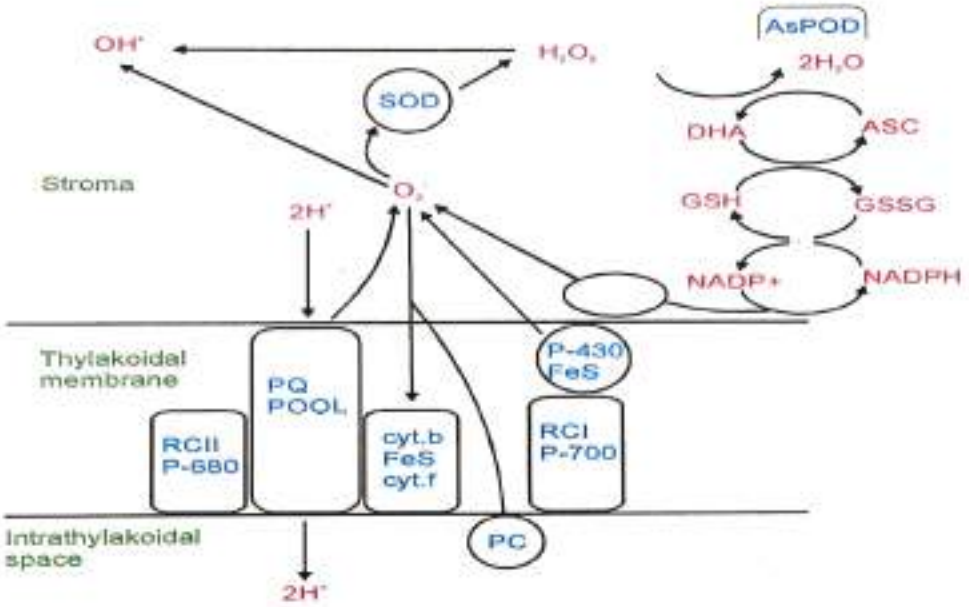


Fig.1 Oxy-intermediate generation by chloroplasts and endogenous protective systems

In plants, catalase is primarily located in peroxisomes and is involved in the detoxification of active oxygen species, which are generated during cellular processes such as photorespiration and β-oxidation of fatty acids or by different environmental stresses. In general, plant catalases contain several subunits that are encoded by a small gene family, the number varying among different genera (Suzuki *et al.* 1994 ). However, due to its high $K_m$, catalase is very inefficient in scavenging low levels of $H_2O_2$ produced in cells. Other scavenging systems are therefore considered more important in scavenging $H_2O_2$.

An important scavenger of $H_2O_2$, particularly in the chloroplasts, is the ascorbate peroxidase which uses ascorbic acid as a hydrogen donor to break down hydrogen peroxide, forming dehydroascorbate and water ( Asada 1994). The enzyme has two cytosolic forms with a purely defensive role and a membrane bound form with a functional role in addition to hydrogen peroxide scavenging. It is involved in modulation quantum efficiency and control of electron transport in conjunction with the ascorbate glutathione cycle.

Antioxidant systems play an important role in the protection of chloroplasts from oxidative damage and under normal conditions, adequate protection is provided by the anti-oxidant pathway of plants. Superoxide is scavenged by superoxide dismutase (SOD), and hydrogen peroxide by catalase (CAT), ascorbate peroxidase(AsPOD) and ascorbate glutathione cycle, whereas hydoxyl radicals and singlet oxygen non-enzymatically with ascorbate, carotenoids and α-tocopherol The anti-oxidant system is implicated in regulation of electron transport as it is important in modulating the decrease in quantum efficiency of the photosystems. This is achieved through the generation of trans-thylakoidal proton gradients when coupled with pseudocyclic flow.

The ascorbate-glutathione cycle ( Fig.2) maintains a high level of ascorbate in the chloroplast stroma. Monodehydroascorbate (MDHA) is formed by oxidation of ascorbate and is reconverted to ascorbate via protonation by MDHA reductase and NAD(P)H. This reaction also forms

dehydroascorbate (DHA), which in turn is reduced to ascorbate by DHA reductase while oxidizing glutathione.

The oxidized glutathione is reduced by glutathione reductase, using NADPH as electron donor. The utilization of NADPH acts as an energy sink which may indirectly have an impact on the efficiency of the electron transport system. It also causes the production of a trans-thylakoidal proton gradient, which is involved in the control of electron transport.

*Non-enzymic ROS scavenging mechanisms*

Besides the enzymes , there are several small molecule antioxidants including the lipid soluble α-tocopherol ( vitamin E), carotenoids and the water soluble ascorbate (vitamin C) and glutathione.

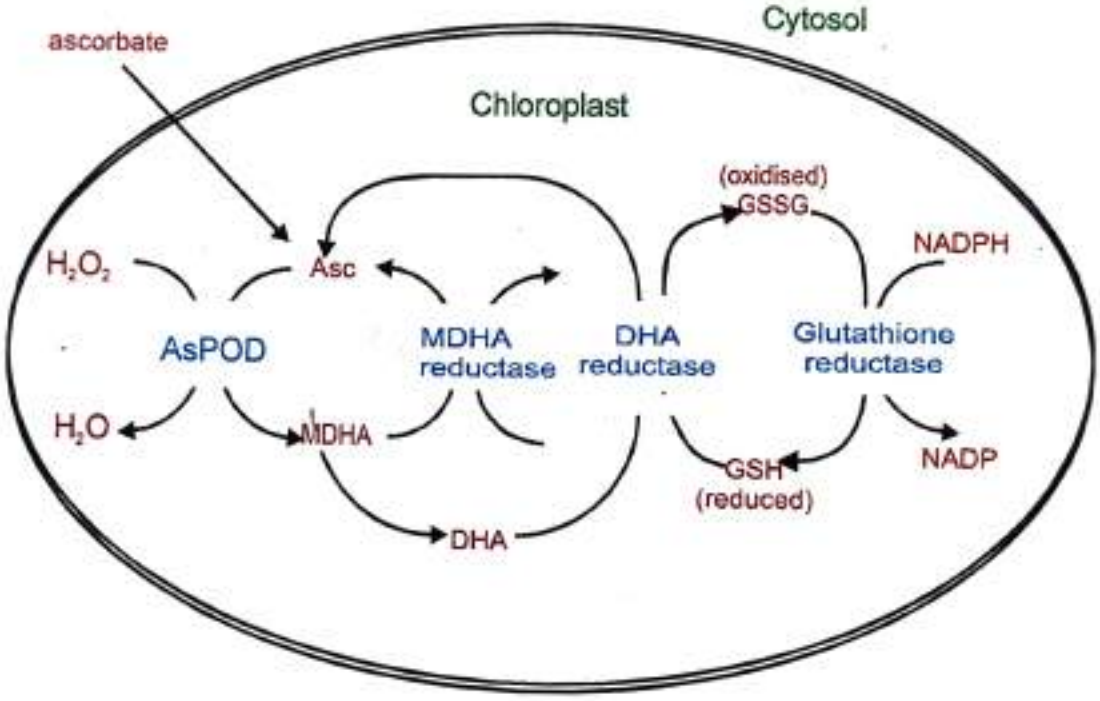


Fig.2 Ascorbate-glutathione cycle (adapted from Foyer *et al.* 1994)

In plants, mutants with decreased ascorbic acid levels or altered GSH contents are hyper sensistive to stress. Whereas GSH is oxidized by ROS forming oxidized glutathione, ascorbate is oxidized to monodehydro ascorbate and dehydroascorbate. Through the ascorbate-glutathione cycle, GSSG, MDA and DHA can be reduced , reforming GSH and ascorbate. A high ratio of reduced to oxidized ascorbate and GSH is essential for ROS scavenging in cells.

In plants, the singlet oxygen formed in the pigment harvesting complexes is quenched by α-tocopherol and carotenoids The carotenoids are embedded in the pigment bed and so are able to react immediately with singlet oxygen as soon as it is formed, because the singlet oxygen molecule will react with the first molecule it encounters. Over expression of β-carotene hydroxylase in *Arabidopsis* leads to increased amounts of xanthophylls in chloroplats and

results in enhanced tolerance towards oxidative stress in high light. Phenolics in plants can also form an antioxidant system equivalent that of ascorbate.

The extent of oxidative stress in a cell is determined by the amounts of superoxide, $H_2O_2$, and hydroxyl radicals.Therefore, the balance of SOD, APX and CAT activities will be crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms.

**Antioxidant systems during environmental stresses**

One of the earliest responses of plants to pathogens, wounding, drought, salinity, extremes of temperature or physical and chemical shocks is the accumulation of active oxygen species such as superoxide, hydroxyl radicals, hydrogen peroxide and singlet oxygen. When ROS production surpasses the antioxidant system capacity, oxidative stress occurs, resulting in cytotoxic protein damage, DNA damage, and lipid peroxidation. When plants are subjected to environmental stresses such as drought, temperature, air pollution, heavy metals, pesticides, soil pH, and pathogen attack, the balance between the production of ROS and the antioxidant quenching systems is likewise shifted in favour of reactive oxygen species accumulation or enhanced scavenging, depending on the type of stress. Response to biotic and abiotic stresses vary.

*Biotic stresses*

During plant pathogen interactions, plant defense, including programmed cell death (PCD) may be activated by suppressing the levels of ROS detoxifying enzymes APX and CAT by salicylic acid and nitrous oxide.Therefore, during plant pathogen defense response, the plant simultaneously produces more ROS while decreasing its ROS scavenging capacities, and accumulation of ROS leading to PCD occurs. The suppression of ROS scavenging enzymes is crucial for PCD. ROS production at the apoplast alone without suppression of ROS detoxification does not result in the induction of PCD. Hence, there is an absolute requirement for the coordinated production of ROS and downregulation of ROS scavenging mechanisms. Induction of PCD potentially limits the spread of disease from the infection point. In this case, production of $H_2O_2$ occurs in a biphasic manner.The initial and very rapid accumulation of $H_2O_2$ is followed by a second and prolonged burst of $H_2O_2$ production. During compatible interactions, when a pathogen overcomes the defense and systemically infects the host, only the first peak of $H_2O_2$ .
accumulation occurs.Although the oxidative burst is a primary response to pathogen challenge that leads to PCD, and $H_2O_2$ induces PCD in various systems, in some cases, $H_2O_2$ is not required for PCD induction .

*Abiotic stresses*

Abiotic stresses also, in some cases, cause PCD, similar to biotic stresses, an example being the response to ozone. However, the role that ROS play during abiotic stresses is opposite to the role that ROS play during pathogen defense.

High temperature is one of the most important environmental stresses that a plant encounters and it is also a major factor limiting the growth of cool season plant species (Paulsen 1994). Physiological injury due to heat stress has been associated with increases in oxidative damage

in perennial grasses (Jiang and Huang 2001) and other plant species (Larkindale and Knight 2002). Oxidative protection is an important component in determining the survival of a plant during heat stress. Heat stress was shown to cause impairments in mitochondrial functions and result in the induction of oxidative damage that manifested in lipid peroxidation (Davidson and Schiestl 2001, Larkindale and Knight 2002; Vacca *et al.* 2004). The steady-state transcript and protein level of many ROS-scavenging enzymes were found to be elevated by heat stress (Vacca *et al.* 2004). In addition, acquired thermotolerance, i.e. the ability of plants to develop heat tolerance following a mild heat pretreatment, was shown to be mediated in part by enhancing cellular mechanisms that prevented oxidative damage under heat stress (Bergmüller *et al.* 2003, Larkindale and Huang 2004). Heat stress-response signal transduction pathways and defense mechanisms, involving heat shock transcription factors (HSFs) and heat shock proteins (HSPs), are thought to be intimately associated with ROS (Pnueli et al. 2003). Several studies have indicated that HSFs are involved in the sensing of ROS. The works of Mittler and Zilinskas (1992) and Storozhenko *et al.* (1998) have revealed the presence of a HSF-binding sequence at the promoter region of the gene encoding the $H_2O_2$-scavenging enzyme APX1. Transgenic *Arabidopsis* over-expressing HSF3 showed higher activity of APX during postheat-stress recovery and had a much stronger induction of *Apx2* than wild type plants (Panchuk et al. 2002). HSF21 was elevated during early stages of light stress in knockout *Apx1* plants that accumulate $H_2O_2$ under moderate light stress (Davletova *et al.* 2005a, Pnueli *et al.* 2003). Transcripts encoding HSF21 were also found to accumulate in wild-type cells treated with $H_2O_2$ (Davletova *et al.* 2005b). Transgenic plants expressing a dominant negative variant of HSF21 showed suppressed expression of Zat12, a $H_2O_2$-responsive zinc finger protein required for expression of APX1, and APX1 (Davletova *et al.*2005 a).

Cold stress was shown to enhance the transcript, protein, and activity of different ROS-scavenging enzymes (O'Kane *et al.* 1996, Prasad *et al.* 1994, Saruyama and Tanida 1995, Sato *et al.* 2001). Low temperature stress was also shown to induce $H_2O_2$ accumulation in cells (O'Kane *et al.* 1996). In *Arabidopsis*, a number of cold responsive genes such as RD29A, KIN1, KIN2, COR15A, COR47, DREB1A, DREB2A, and ERD10 have been identified (Seki *et al.* 2002, Thomashow 1999). The contribution of some cold-responsive genes to controlling ROS under cold stress was suggested by Lee *et al.* (2002). *Arabidopsis* frostbite1 (fro1) mutant displayed reduced expression of cold-responsive genes such as RD29A, KIN1 COR15A, and COR47, and accumulated ROS constitutively. The *FRO1* gene was shown to encode a mitochondrial complex I protein, suggesting that expression of the cold-responsive genes and ROS accumulation might be modulated by the disruption of a mitochondrial function.DNA regulatory elements in the promoters of cold-responsive genes such as C-repeat (CRT)-related – and dehydration responsive element (DRE) – motifs have been identified (Yamaguchi-Shinozaki and Shinozaki 1994). Hsieh et al. (2002) showed that transgenic expression of the transcriptional activator, CRT/DRE-binding factor 1 (CBF1), enhanced the cold tolerance of tomato plants. Enhanced expression and enzymatic activity of CAT were also detected in transgenic plants, and the level of $H_2O_2$ in transgenic plants was lower than that of wild-type plants. In *Arabidopsis*, overexpression of NDP kinase 2 (NDPK2) enhanced cold tolerance (Moon *et al.* 2003).NDP kinase 2 was shown to interact with two oxidative stress-related mitogen- activated protein kinases (MAPKs), AtMPK3, and AtMPK6 (Moon *et al.* 2003). Transgenic plants overexpressing NDPK2 showed lower levels of $H_2O_2$ compared with wildtype, and a mutant lacking AtNDPK2

displayed an enhanced accumulation of $H_2O_2$. Zat12, an ROS-response zinc finger protein (Davletova *et al.* 2005a,) was shown to regulate cold-induced genes. Microarray analysis demonstrated that cold-responsive genes were upregulated by overexpression of Zat12 (Vogel *et al.* 2005), and Zat12 downregulated CBF transcript expression suggesting a role for Zat12 in suppressing the CBF cold-response pathway. These studies demonstrate a close link between ROS, ROS signaling, and the cold stress response.

High light enhances the production of ROS and has the potential to damage the photosynthetic apparatus. High-light stress could therefore enhance ROS-mediated damage during temperature stresses. Compared with dark conditions, temperature stress-induced damage to cells was shown to be enhanced by light (Jeong et al. 2002, Larkindale and Knight 2002). In cucumber, the primary target of cold stress combined with high light is Cu/Zn SOD, followed by inactivation of PSI by ROS (Choi *et al.* 2002). Transgenic plants over-expressing Cu/Zn SOD, APX, and glutathione reductase, GPX, or thylakoid-bound APX were found to be more tolerant than wild-type plants to a combination of temperature and high-light stress (Allen 1995, Payton *et al.* 2001, Yabuta *et al.* 2002, Yoshimura *et al.* 2004). Mutants deficient in ascorbate, zeaxanthinin or glutathione were subjected to a lethal heat stress in the presence or absence of high light (Larkindale *et al.* 2005). These mutants showed a dramatic decrease in survival rate under a combination of high light and temperature stress. In addition, transgenic plants overexpressing β-carotene hydroxylase, an enzyme catalyzing the conversion of β-carotene to zeaxanthin, showed enhanced tolerance to a combination of heat and high-light stress (Davison *et al.* 2002), indicating a role for zeaxanthin in enhancing the tolerance of plants to a combination of heat and high-light stress. These results indicate that ROS-scavenging enzymes play an important role in preventing photooxidative damage under a combination of temperature and high-light stress

A putative model for the role of ROS in temperature stress was suggested by Suzuki and Mittler (2006). They have shown two converging operative pathways during stress: the temperature stress-response pathway and the ROS-response pathway. Temperature stress is shown to result in the enhanced production of ROS in cells by the disruption of cellular homeostasis and the uncoupling of metabolic processes (stress- generated ROS box). In addition, the sensing of temperature stress by the temperature sensor could lead to the enhanced production of ROS by NADPH oxidases (ROS generation by Rboh box). The ROS sensor would sense ROS produced by these processes and activate the ROS defense mechanisms that include ROS-scavenging enzymes (ROS defense box) or further enhance ROS production by Rboh (ROS generation by Rboh box) to enhance the ROS signal (Mittler *et al.* 2004). Both sensors, for ROS or temperature stress, could activate the temperature defense pathway that includes HSPs and other protective mechanisms (temperature defense box) and/or the ROS-scavenging pathways (ROS defense box), resulting in the suppression of stress- associated ROS production. It was proposed that the pathways would be activated upon temperature stress; however, their converging nature would cause them to suppress each other when the stress subsides or when the cell achieves a new state of homeostasis that enables it to survive the temperature stress. The latter could be achieved, for example, once the uncoupling of pathways and the enhanced production of ROS would be put under control by different adjustments to cellular homeostasis and the function of HSPs and/or other cellular protectants.

AOS are key components contributing to cellular redox poise. They participate in all processes controlled by redox reactions. These include signal transduction, gene expression, protein synthesis and turn-over, thiol-disulpide exchange reactions and regulation of metabolism. AOS accumulation is sensed as an 'alarm' signal that initiates pre-emptive defense responses. Common and linked signal transduction pahways are activated that can lead either to stress acclimation or to cell death depending on the degree of oxidative stress (Foyer *et al.* 2001).Plant responses to stress are therefore directed to acclimate and repair damage (Fig.3).

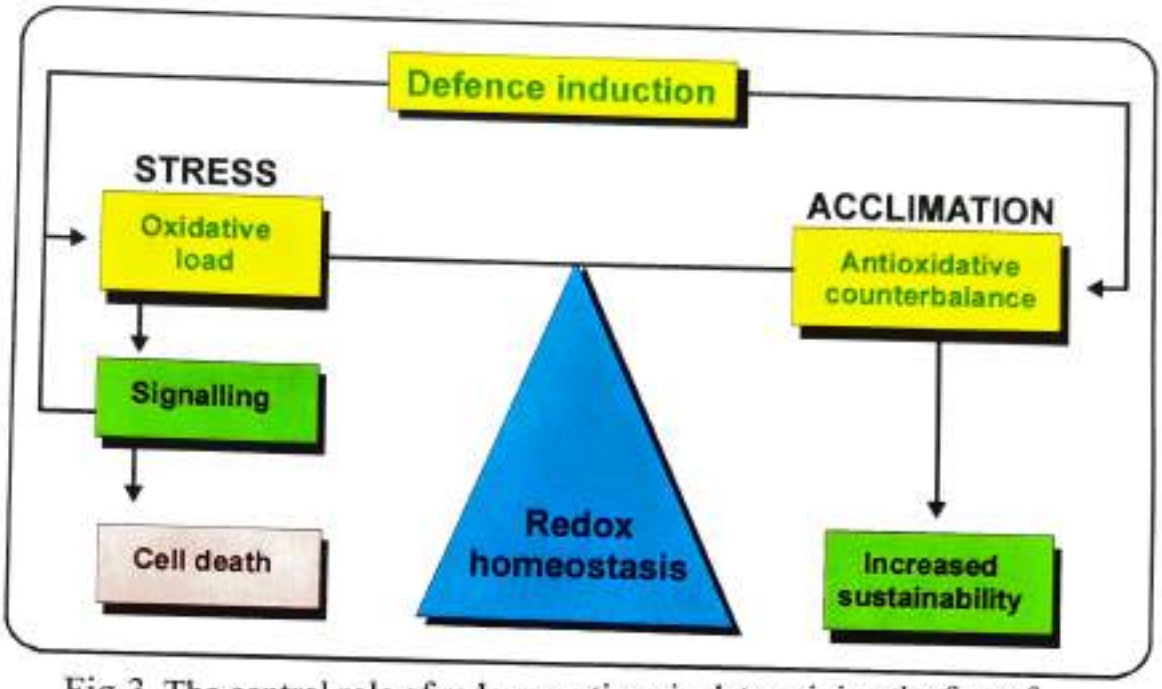


Fig.3 The central role of redox reactions in determining the fate of plant cells exposed to stress ( Foyer et al. 2001)

Plants with elevated levels of antioxidants, either constitutive or induced, have been shown to exhibit greater tolerance to a number of stresses, including salinity and drought. In cotton, it has been shown that when plants were exposed to NaCl , the tolerant cell lines displayed increased antioxidant activity when compared to salt-sensitive cell line (Rajguru *et al.* 1999). Similar results were also reported in mulberry (Harinasut *et al.* 2003). Transgenic maize lines overproducing MnSOD and FeSOD were more tolerant to the superoxide generating herbicide methyl viologen (paraquat), showing an increased antioxidant capacity in the chloroplasts (Van Bruesegem *et al.* 1999 a & b).

**Conclusion**

In conclusion, it might be stated that active oxygen species are an unfortunate consequence of metabolism, which are also formed during different environmental stresses. However, nature has determined means by which cells can tolerate or detoxify these ROS in the form of antioxidant systems which mainly include a number of enzymes.. ROS have also been shown to be involved in signaling mechanisms.One of the key areas of research today is enhancing stress tolerance in plants, and this can be achieved by manipulation of expression of genes of the enzymes in the antioxidant systems. Many of the compounds synthesized in response to environmental stress also participate in AOS detoxification in plants and may also function as antioxidants when

consumed in human diet. Reducing the rate of ROS production in cells is likely to be as important as active scavenging of ROS during stress (Mittler 2002).

This could be achieved, for example, by adjustments to cellular metabolism that reduce the rate of electron flow in particular compartments or by controlling the accumulation of particular compounds with a high redox potential. A good example for a defense enzyme that suppresses the potential of a charged electron transfer chain to form ROS is alternative oxidase (McIntosh 1994). Alternative oxidases are found in the mitochondria and chloroplast and reduce the formation of ROS during stress (Mittler 2002). Protection of different complexes and pathways using HSPs and/or other protective compounds such as sugars could also lower the rate of electron leakage from different apparatuses. To completely understand how plants cope with temperature stress, we should include in future research the study of these mechanisms and the manner by which they are coordinated with other, more active, defenses.

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