Chapter 3

Oxidative Stress Studies in Plant Tissue Culture

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Additional information is available at the end of the chapter

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1. Introduction

Higher plants are sessile therefore are continuously exposed to different environmental stress factors, such as drought, salinity, heavy metals, nutritional disorders, radiation without any protection. Most of these stresses produce certain common effects on plants, like induced oxidative stress by overproduction of reactive oxygen species (ROS), besides their own specific effects (Rao, 2006). Thus, plants have developed their own specific response(s) against each of these stresses as well as cross-stress response(s). Investigating these responses is difficult under field conditions, but plant tissue culture techniques are performed under aseptic and controlled environmental conditions. These advantages of plant tissue culture allow various opportunities for researcher to study the unique and complex responses of plants against environmental stresses (Sakthivelu et al., 2008, Lokhande et al., 2011).

ROS have inevitably been factors for aerobic life since the introduction of molecular oxygen (O_2) into our atmosphere by O_2-evolving photosynthetic organisms. ROS can simply be described highly reactive and partially reduced-oxygen forms. ROS, including the superoxide radical ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) like that, are produced not only during metabolic pathway in several compartments of plants, including chloroplasts, mitochondria, peroxisomes, plasma membrane, apoplast, endoplasmic reticulum, and cell-wall but also as a result of induced environmental stress factors. When exposing of environmental stress factors, ROS levels can dramatically increase and this increase, in the later stage, leads to oxidative stress. Oxidative stress is defined a serious imbalance between the production of ROS and antioxidant defense and this situation can cause damage to cellular macromolecules, including proteins, lipids, carbohydrates and DNA (Mittler et al., 2004; Gill and Tuteja, 2010). Under steady-state conditions, the ROS are scavenged by various antioxidant defense systems: both enzymatic antioxidant (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate

reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaiacol peroxidase, POX and glutathione-S- transferase, GST) and non-enzymatic (ascorbate, glutathione, carotenoids, phenolic compounds, proline, glycine betain, sugar, and polyamines) defense systems (Foyer and Noctor, 2005; Desikan et al., 2005; Ahmad et al., 2008; Gill and Tuteja, 2010).

Plant tissue culture techniques are used to grow plants under aseptic and controlled environment for the purpose of both commercial (like mass production) and scientific (like germplasm preservation, plant breeding, physiological, and genetic) studies (www.kitchenculturekit.com). Two of these application areas are important to study ROS homeostasis in plants. The first one of these techniques is used as a model to induce oxidative stress under controlled conditions via different stressor agents for researching *in vitro* screening in plants against abiotic stress, studying and observing morphological, physiological and biochemical changes in both unorganized cellular (i.e. suspension cultures and callus cultures) and organized tissue (i.e. axillary shoot, shoot tip, mature embryo, whole plant) levels (Sivritepe et al., 2008; Cui et al., 2010; Shehab et al., 2010; Patada et al., 2012). Additionally, plant tissue culture techniques also allow opportunities for the researcher to improve plants against abiotic stress factors with the *in vitro* selection method (Jain, 2001). The purpose of this study is to compile the recent studies about ROS and oxidative stress, how to maintain ROS homeostasis in plants, plant tissue culture, the effects of induced-oxidative stress on antioxidant defense system in plant tissue culture and antioxidant defense systems of *in vitro* selected-plant against abiotic stresses.

2. Oxidative stress and Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS), is also sometimes called Active Oxygen Species (AOS), or Reactive Oxygen Intermediates (ROI), or Reactive Oxygen Derivatives (ROD),is the term used to describe highly reactive and partially reduced-oxygen forms (Desikan et al., 2005). ROS are produced in many ways in several cellular compartments, including mitochondria, chloroplast, peroxisomes, endoplasmic reticulum, cytoplasm, plasma membrane and apoplast, during normal metabolic processes and due to induction of environmental perturbations, such as drought, salinity, radiation, heavy metals, and herbicides (Desikan et al., 2005). ROS are highly reactive due to the presence of unpaired valence shell electrons and high concentration of ROS can result in non-controlled oxidation in cells, which is defined as oxidative stress, as a result of ROS-attack, cellular compartments, including DNA, protein, membrane lipids may damage (Cassells and Curry, 2001; Desikan et al., 2005).

ROS include a wide range of oxygen-radicals, such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), perhydroxyl radical ($HO_2^{\cdot}$) and hydrogen peroxide (H_2O_2), they become the sequential reduction of molecular oxygen. Singlet oxygen ($^1O_2$), another form of ROS, can be produced by excited-chlorophyll formation in the photosystem II (PSII) reaction center and in the antennae systems. This is the major formation mechanism of $^1O_2$ in plant cells. Insufficient energy dissipations during the photosynthesis, the chlorophylls are excited,

which then can lead the formation of chlorophyll (Chl) triplet state. Chl triplet state can react with $^3O_2$ to give up the very reactive $^1O_2$ (Arora et al., 2002; Reddy and Raghavendra, 2006; Gill and Tuteja, 2010). $^1O_2$ has powerful damaging effect on the whole photosynthetic machinery, including chloroplast membrane lipids, proteins and nucleic acids. The primary means of defense within the chloroplast are the carotenolds (CARs) and a-tocopherol (vitamin E), which are located within the thylakold membranes. They are a quencher against damages of $^1O_2$ (Knox and Dodge, 1985). Hossain et al. (2006) and Helaley and El-Hosieny (2011) reported that carotenoid contents increase under salinity stress in various plant species. $O_2^{\cdot-}$, which is generally known as the first ROS to be generated, usually generate with the single electron reduction of O_2. The major site of $O_2^{\cdot-}$ production is in the photosystem I (PSI) by Mehler Reaction. The generation of $O_2^{\cdot-}$ may lead to formation of $OH^{\cdot}$ and $^1O_2$. The reaction of $O_2^{\cdot-}$ with Fe^{+3} may become $^1O_2$ (1), and reduced-form of Fe^{+2}. $O_2^{\cdot-}$ can also reduce to H_2O_2 by SOD (2). $HO_2^{\cdot}$ is formed from $O_2^{\cdot-}$ by protonation in aqueous solutions. $HO_2^{\cdot}$ can cross biological membranes and subtract hydrogen atoms from polyunsaturated fatty acids (PUFAs) and lipid hydroperoxides, thus initiating lipid auto-oxidation (Halliwell and Gutteridge, 2000). Additionally, complex I, ubiquinone, and complex III in mitochondrial electron transfer chain (ETC), the other major ROS (H_2O_2 and $O_2^{\cdot-}$) producing sites in cells (Reddy and Raghavendra, 2006; Gill and Tuteja, 2010). Xanthine oxidase generates $O_2^{\cdot-}$ during the catabolism of purines in the peroxisomes, and an increasing production of $O_2^{\cdot-}$ is caused certain herbicides, like paraquat, which is known photosynthetic inhibitors. Paraquat (also called methyl violeng) prevents the transfer of electrons from ferredoxin (Fd) in PSI, afterwards increase generation of $O_2^{\cdot-}$ with the transfer of electrons from molecular oxygen (Peixoto et al., 2007; Gill and Tuteja, 2010). It is also clear that environmental stress induced the production of $O_2^{\cdot-}$ and the other ROS (Arora et al., 2002; Reddy and Raghavendra, 2006; Gill and Tuteja, 2010). H_2O_2 is produced as a result of dismutation reaction of $O_2^{\cdot}$. This reaction mostly catalyzed by SOD (Arora et al., 2002). H_2O_2 is formed in the peroxisomes as part of photorespiratory, and also produced from β-oxidation of fatty acids as a by-product. H_2O_2 is not a free radical, but is participates as an oxidant or a reductant in several cellular metabolic pathways (Reddy and Raghavendra, 2006). By means of transition metals, such as Fe and Cu, further reduction of H_2O_2 take place OH^- and $OH^{\cdot}$, which are mentioned below as Haber-Weiss/Fenton Reaction (3, 4). $OH^{\cdot}$ is extremely reactive and will potentially react with all biological molecules, such as DNA, proteins, and lipids. If productions of hydroxyl radicals are not eliminated by any enzymatic and non-enzymatic defense mechanisms, overproduction of its ultimately leads to cell death (Desikan et al., 2005; Gill and Tuteja, 2010). As a result of the measurement of ROS using spectrophotometric, fluorescent dye probe and electron spin resonance (ESR) methods showed that various abiotic stress factors induced ROS formation in a wide range of plant species under *in vitro* conditions (Mohamed and Aly, 2004; Chakrabarty et al. 2005; Gallego et al., 2005; Reddy and Raghavendra, 2006; Azevedo et al., 2009; Shehab et al., 2010; Yang et al., 2010; El-Beltagi et al., 2011).

$$O_2^{\cdot-} + Fe^{+3} \rightarrow Fe^{+2} + {}^1O_2 \quad (1)$$

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (2)$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2 \text{ and}$$

$$H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + O_2^{\bullet -} + H^+ \quad (\text{Haber} - \text{Wiess Reaction}) \tag{3}$$

$$H_2O_2 + Fe^{+2}\left(Cu^{+}\right) \rightarrow Fe^{+3}\left(Cu^{+2}\right) + {}^{\cdot}OH + HO^- \left(\text{Fenton Reaction}\right) \tag{4}$$

As I mentioned above, an overproduction of ROS can result in non-controlled oxidation in cells, resulting in ROS-attack, which may damage several cellular macromolecules, such as lipid membranes, proteins and DNA (Cassells and Curry, 2001; Desikan et al., 2005). The peroxidation of membrane lipids both cellular and organelles are known as the most damaging factors in all living organisms, including plants. As a result of lipid peroxidation (LPO) some products are formed by PUFAs. One of them is malondialdehyde (MDA). The reactions of MDA with thiobarbituric acid (TBA) produces color product, which is called thiobarbituric acid reactive substances (TBARS). The spectrophotometric measurement of TBARS or MDA generally used as oxidative stress biomarker and also to assess the degree of LPO. Many researchers reported that MDA content increased under several abiotic stress factors, which were induced *in vitro* conditions (Gallego et al., 2005; Erturk et al., 2007; Sivritepe et al., 2008; Shri et al., 2009; Azevedo et al., 2009; Cui et al., 2010; Shehab et al., 2010; El-Beltagi et al., 2011; Ghanaya et al., 2011). Another way to detect LPO is determination of Lipoxygenase (LOX; EC 1.13.11.12) activity. LOX catalyze the hydroperoxidation of PUFAs, with the further degradation reactions of these reactions produce free radicals and thus initiating the chain reactions of LPO (Blokhina et. al., 2003). Dewir et al., (2006) and Basu et al., (2010) reported that LOX activities and MDA contents increased in *Euphorbia millii* and all rice varieties under hypehydric conditions and PEG induced drought stress in tissue culture, respectively. It is also clear that all LPO-products are highly cytotoxic and as a result of reaction in biological molecules, including proteins, and DNA damage to them (Gill and Tuteja, 2010).

Another result of ROS-attack in cells is an increase in protein oxidations. Site specific amino acid modifications, fragmentation of the peptide chain, and aggregation of cross linked reaction products occur in plants as consequence of protein oxidations induced by ROS or by-products of oxidative stress. These reactions are mostly irreversible (Ahmad et al., 2008; Gill and Tuteja, 2010). Various mechanisms can cause protein oxidation, such as the formation of disulfide cross-links and glycoxidation adducts nitration of tyrosine residues, and carbonylation of specific amino acid residues (Oracz et al., 2007). The spectrophotometric measurement of protein carbonyl with dinitrophenylhydrazine (DNPH) method is widely used marker for detection of protein oxidation in biological organisms. Basu et al., (2010) reported that an increasing ratio of protein oxidations were measured in all rice varieties induced drought conditions in tissue culture.

ROS-induced genotoxic damage can induce structural changes in DNA, such as chromosomal rearrangement, strand breaks, base deletions, prymidine dimers, cross-links and base modifications, mutations and other lethal genetic effects (Cassells and Curry, 2001; Ahmad et al., 2008; Gill and Tuteja, 2010). When DNA-lesions are endogenously generated

mostly via ROS, it is called spontaneous DNA damage (Ahmad et al., 2008; Gill and Tuteja, 2010). Oxidative stress, as well as effects of damaging which were referred above, also has a great potential creating variability in the plant genome by activating transposons, inducing chromosome breakage/rearrangement, and base mutation and these situations are one of the main reasons of spontaneous mutations in cells (Cassells and Curry, 2001; Gaspar et al., 2002). As I mentioned below, spontaneous mutations are one of the key factors of plant breeding.

Additionally, low concentrations of ROS are key factors to maintain intercellular signal transductions in plants. Further information about ROS, there is an excellent review (Ahmad et al., 2008; Gill and Tuteja, 2010; Karuppanapandian et al., 2011) about this subjects books (Smirnoff, 2005; Rao et al., 2006; Del Rio and Puppo, 2009) published in recent years.

3. Antioxidant defence system

ROS are generated in plant cells by normal cellular metabolism or due to unfavorable environmental conditions such as drought, salinity, heavy metals, drought, herbicides, nutrient deficiency, or radiation. Their productions are controlled by various enzymatic and non-enzymatic antioxidant defense systems. Enzymatic antioxidant defense systems, including CAT, APX, POX, SOD, MDHAR, DHAR and GR and non-enzymatic antioxidant defense systems, including ascorbate, glutathione, carotenoids, phenolic compounds, proline, glycine betain, sugar, and polyamines (Ahmad et al., 2008; Gill and Tuteja, 2010; Karuppanapandian et al., 2011).

3.1. Enzymatic antioxidants

3.1.1. Superoxide dismutase (SOD; EC 1.15.1.1)

Superoxide dismutase, as a metalloenzyme, is the first enzyme of the detoxification processes, which catalyzes $O_2^{\cdot-}$ to H_2O_2 and O_2. SODs are classified into three types based on their metal cofactor: Fe-SOD (localized in chloroplasts), Mn-SOD (localized in mitochondria), and Cu/Zn-SOD (localized in chloroplasts, peroxisomes, and cytosol). The activity of SOD isozymes can be detected by negative staining and can be identified on the basis of their sensitivity to KCN and H_2O_2. The Mn-SOD is resistant to both inhibitors; Cu/Zn-SOD is sensitive to both inhibitors whereas; Fe-SOD is resistant to KCN and sensitive to H_2O_2 (Ahmad et al., 2008; Gill and Tuteja, 2010; Karuppanapandian et al., 2011). There have been many reports of the increased activities of SOD under abiotic stresses induced with tissue culture techniques in a wide range of plant species, including heavy metals, such as Al, Cd, Cr, and Cu, hyperhydricity, salinity, gamma radiation, and drought (Gallego et al., 2002; Saher et al., 2004; Dewir et al., 2006; Israr et al., 2006; Erturk et al., 2007; Dasgupta et al., 2008; Sivritepe et al., 2008; Gupta and Prasad, 2010; Shehab et al., 2010; Yang et al., 2010; El-Beltagi et al., 2011; Helaly and El-Hosieny , 2011; Lokhande et al., 2011; Sen and Alikamanoglu, 2011; Xu et al., 2011; Patada et al., 2012) on the other hand, Fe-deficiency stress reduced activity of SOD (Lombardi et al., 2003). Advanced-antioxidant defense

systems play an important role in plants not only to tolerate environmental stress but also to improve plants against these stresses. Enhanced activities of SOD were observed in various plants to improve tolerance against salinity (Hossain et al., 2006; Hossain et al., 2007; Chen et al., 2011; Helaly and El-Hosieny , 2011), and S-(2-aminoethy)-cysteine AEC (Kim et al., 2004) using *in vitro* selection method.

As a result of native polyacrylamide gel electrophoresis (native–PAGE), Chakrabarty et al., (2005) and Dewir et al., (2006) detected that Mn-SOD and Cu/Zn-SOD isoenzymes seem to play a major role in response to hyperhydricity. Additionally, Rahnama and Ebrahimzadeh (2006) and Roy et al., (2006) also reported that against salinity and gamma radiation Mn-SOD and Cu/Zn-SOD seem to play a major role in the potato and *Vigna radiate* calli, respectively. In *Malus domestica* Borkh. rootstock MM 106, NaCl and KCl treatment induced Mn-SOD isoenzyme form in leaves (Molassiotis et al., 2006). Shri et al., (2009) observed that during the As-stress Cu/Zn-SOD isoenzyme band induced. NaCl stress induced new SOD isoenzyme bands in Agria and Kennebec potato cultivar (50 mM) and in *Jatropha curcas* callus (40, 60, and 80 mM), respectively (Rahnama and Ebrahimzadeh, 2005; Kumar et al., 2008).

3.1.2. Catalase (CAT, EC 1.11.7.6)

CAT is a tetrameric heme-containing enzyme that catalyzes dismutation reactions of H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stress conditions. CAT is also important in the removal of H_2O_2 generated in peroxisomes during the β-oxidation of fatty acids, photorespiration, and purine catabolism (Ahmad et al., 2008; Gill and Tuteja, 2010; Karuppanapandian et al., 2011). Various abiotic stresses induced CAT activities under *in vitro* conditions in different plants, including hyperhydricity, salinity, drought, and gamma radiation (Saher et al., 2004; Chakrabarty et al., 2005; Rahnama and Ebrahimzadeh, 2005; Dewir et al., 2006 Niknam et al., 2006; Erturk et al., 2007; Dasgupta et al., 2008; Sivritepe et al., 2008; Shehab et al., 2010; Yang et al., 2010; Zamora et al., 2010; El-Beltagi et al., 2011; Sen and Alikamanoglu, 2011; Helaly and El-Hosieny, 2011; Patade et al., 2012) in contrast, Fe-deficiency stress reduced activity of CAT (Lombardi et al., 2003; Mohamed and Aly, 2004). CAT activities also induced in *Medicago sative* clones, which were improved with *in vitro* selection method, under PEG-treatment (Safarnejad, 2004). Additionally, CAT activities were detected with native–PAGE analysis besides spectrophotometric measurements. Chakrabarty et al., (2005) reported that as a result of native–PAGE analysis, three CAT (CAT-1, CAT-2, and CAT-3) isoenzyme bands were observed on the gels. Two of them (CAT-1 and CAT-3) were strongly induced in hyperhydric apple leaves compared healthy leaves. Sen and Alikamanoglu, (2011) reported that under NaCl stress conditions one, one and two CAT isoenzyme bands were visualized on the native-PAGE in Tekirdag, Pehlivan and Flamura-85 wheat varieties tissue cultures.

3.1.3. Guaiacol Peroxidase (POX, EC 1.11.1.7)

POX is a heme-containing enzyme, like CAT. POX prefers aromatic electron donors such as guaiacol and pyragallol to catalyze H_2O_2, and many researchers reported that excess POX

activities were measured in a wide range of plant varieties under abiotic stress conditions induced with *in vitro* culture techniques (Saher et al., 2004; Chakrabarty et al., 2005; Rahnama and Ebrahimzadeh, 2005; Dewir et al., 2006; Niknam et al., 2006; Erturk et al., 2007; Dasgupta et al., 2008; Kumar et al., 2008; Sivritepe et al., 2008; Zamora et al., 2010; Sen and Alikamanoglu, 2011, ; Helaly and El-Hosieny, 2011). POX also decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defense against biotic stresses by consuming H_2O_2 in the cytosol, vacuole, and cell wall as well as in extracellular space (Gill and Tuteja, 2010; Karuppanapandian et al., 2011).

There have been many reports of the changes in POX isoenzymes depending considerably upon plant species and abiotic stresses under tissue culture conditions. NaCl stress stimulated new POX isoenzyme band in Agria and Kennebec potato cultivar (50 mM) and in *Jatropha curcas* callus (40, and 60 mM), respectively (Rahnama and Ebrahimzadeh, 2005; Kumar et al., 2008). In *Prunus cerasus* cv. CAB-6P rootstock leaves, POX-3 isoenzyme band appeared under different concentrations of NaCl and $CaCl_2$, but POX-4 isoenzyme band were detected highest in NaCl concentration (60 mM), and both 30 and 60 mM $CaCl_2$ concentrations (Chatzissavvidis et al., 2008). Radić et al., (2006) reported that in *Centaurea regusina* L., all NaCl and mannitol treatments induced POX-3 and POX-4 isoenzymes but POX-9 appeared only in response to high NaCl concentration. A new POX isoenzyme band (Rf 0.34) was also detected in *Chrysanthemum* salt-tolerant strain, which was improved using *in vitro* selection method (Hossain et al., 2006). In *Malus domestica* Borkh. rootstock MM 106, NaCl and KCl treatment induced new POX isoenzyme form in leaves and stems (Molassiotis et al., 2006). Additionally, at the highest Zn concentration induced new POX isoenzyme bands in *Jatropha curcas* cotyledons (POX IV), hypocotyls (POX V) and radicles (POX IV) (Luo et al., 2010). On the other hand, mild Fe deficiency was caused to disappearance of one POX band with Rf value 0.85 (Mohamed and Aly, 2004). After the electrophoretic analysis, four, four and five POX isoenzymes were detected in *Luffa cylindrica* cotyledons, hypocotyls and radicles under Pb-induced oxidative stress (Jiang et al., 2010). Similar results were obtained under Cd-stress in *Glycyrrhiza uralensis* cotyledons, hypocotyls and radicles, five, five and three POX isoenzyme bands were visualized, respectively (Zheng et al., 2010). Sen and Alikamanoglu, (2011) reported that under NaCl stress conditions two, two and five POX isoenzyme bands were detected on the native-PAGE in Tekirdag, Pehlivan and Flamura-85 wheat varieties tissue cultures.

3.1.4. Halliwell-Asada Cycles' Enzymes (Ascorbate peroxidase (APX, EC 1.11.1.1), Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), Dehydroascorbate reductase (DHAR, EC 1.8.5.1) and Glutathione reductase (GR, EC 1.6.4.2))

The Ascorbate-Glutathione Cycle, sometimes called Halliwell-Asada Cycle, is another metabolic pathway that detoxifies H_2O_2. This is located in the cytosol, mitochondria, chloroplasts and peroxisomes in plants and it may have a more crucial role in the management of ROS during stress (Noctor and Foyer, 1998). The cycle involves the antioxidant metabolites: ascorbate, glutathione and NADPH and the enzymes linking these metabolites, involving APX, MDHAR, DHAR and GR. In the first step of this pathway, H_2O_2

is reduced to H_2O and monodehydroascorbate (MDHA) by APX using ascorbate as the electron donor. The oxidized ascorbate (MDHA) is regenerated by MDHAR. MDHAR is a flavin adenin dinucleotide (FAD) enzyme which uses NAD(P)H directly to recycle ascorbate, and dehydroascorbate (DHA). After, DHA is reduced to ascorbate by dehydroascorbate reductase (DHAR) using of glutathione (GSH) as the electron donor. As a result of this reaction oxidized glutathione (GSSG) occur. Finally GSSG is reduced to GSH by glutathione reductase (GR) using NADPH as electron donor (Noctor and Foyer, 1998; Ahmad et al., 2008; Gill and Tuteja, 2010; Karuppanapandian et al., 2011). Enhanced expression of Halliwell-Asada Cycles' enzymes in plants has been demonstrated during different stress conditions. Saher et al., (2004) reported that hyperhydric stress increased Halliwell-Asada Cycle's enzyme activities (APX, MDHAR, DHAR and GR) in *Dianthus caryophyllus*. A further study by hyperhydration, Chakrabarty et al., (2005) reported that APX, MDHAR and GR activities increased but DHAR activity decreased in apple. In a wide range of plant species were observed increase in GR and APX activities under different abiotic stress conditions-induced with tissue culture (Israr et al., 2006; Erturk et al., 2007; Sivritepe et al., 2008; Shehab et al., 2010; Zamora et al., 2010; Helaly and El-Hosieny, 2011). Generally known that APX has a higher affinity for H_2O_2 (μM range) than CAT and POX (mM range) and it may have a more crucial role in the management of ROS during stress (Gill and Tuteja, 2010; Karuppanapandian et al., 2011). Lokhande et al., (2011) observed that under NaCl-induced oxidative stress conditions, APX enzyme activities increased but CAT enzyme activities decreased in *Sesuvium portulacastrum* tissue cultures. Mohamed and Aly, (2004) reported that Fe-deficiency stress reduced activity of APX in *Borage officinalis* tissue culture. Peixoto et al., (2007) reported that different types of herbicides (paraquat, 2.4-D and dicamba) induced GR activities in potato tuber calli. In increase activities of some enzymes belonging to Halliwell-Asada Cycle's, such as APX, GR, and DHAR (Kopyra and Gwozdz, 2003; Kim et al., 2004; Hossain et al., 2006; Hossain et al., 2007; Bittsanszky et al., 2008; El-Beltagi et al., 2011; Helaly and El-Hosieny, 2011) were observed in various plants improved tolerance against abiotic stresses with *in vitro* selection method.

Chakrabarty et al., (2005) reported that after the native–PAGE analysis, five APX isoenzyme bands were observed on the gels in hyperhydric apple leaves. Three of them (APX-1, APX-4 and APX-5) only appeared in hyperhydric apple leaves. New APX and GR isoenzyme bands were also induced during the As-stress both shoots and roots, for APX, and only roots, for GR, in rice tissue culture, respectively (Shri et al., 2009).

3.1.5. Glutathione Peroxidases (GPX, EC 1.11.1.9)

GPXs are a large family of diverse isozymes that use GSH to reduce H_2O_2, besides this situation GPX also has more crucial role for lipid peroxidation process, and therefore helps plant cells from oxidative stress (Gill and Tuteja, 2010). Millar et al., (2003) reported that GPX includes a family of seven related proteins in cytosol, chloroplast, mitochondria and endoplasmic reticulum. Hyperhydric stress increased GPX activity in *Prunus avium* and apple, respectively (Franck et al., 2004; Chakrabarty et al., 2005).

3.1.6. Glutathione S-transferases (GST, EC 2.5.1.18)

GSTs catalyse the conjugation of electrophilic xenobiotic substrates with the tripeptide glutathione (GSH; γ-glu-cys-gly). Plant GST gene families are large and highly diverse, like GPXs. GSTs are generally cytoplasmic proteins, but microsomal, plastidic, nuclear and apoplastic isoforms has also been reported. They are known to function in herbicide detoxification, hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification, regulation of apoptosis and in plant responses to biotic and abiotic stresses. GSTs have the potential to remove cytotoxic or genotoxic compounds, which can react or damage the DNA, RNA and proteins (Gill and Tuteja, 2010). Enhanced activities of GST in potato tuber callus was demonstrated during 2,4-D and Dicamba treatments (Peixoto et al., 2007) and also in paraquat- tolerant poplar clones, which were improved using *in vitro* selection technique (Bittsanszky et al., 2008).

3.2. Non-enzymatic antioxidants

Apart from the enzymatic defense system, several non-enzymatic antioxidant defense mechanisms also play an important role in the response of plant stress tolerance, such as ascorbate, glutathione, carotenoids, phenolic compounds, proline, glycinebetain, sugar, and polyamines.

Two of them, ascorbate and glutathione are crucial metabolites in plants which are considered as most important intracellular defense against ROS induced oxidative damage. Ascorbate can directly scavenge $^1O_2$, $O_2^{\cdot-}$ and ·OH and by regenerate a-tocopherol from tocopheroxyl radical. It also acts as co-factor of violaxanthin de-epoxidase, thus sustaining dissipation of excess excitation energy. Glutathione, like ascorbate, plays a pivotal role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics and the expression of stress-responsive genes. Both of them are also main components of the Halliwell-Asada Cycle (Gill and Tuteja, 2010). Shehab et al., (2010) and El-Beltagi et al., (2011) reported that ascorbate and glutathione contents were increased under PEG-induced drought stress and low doses gamma radiation in rice and *Rosmarinus officinalis* L callus culture, respectively. Glutathione contents increased in *Sesbania drummondii* callus under Cd-induced oxidative stress (Israr et al., 2006). Additionally, increasing ascorbate and glutathione contents were observed in salt tolerant *Chrysanthemum morifolium* strain and paraquat-tolerant poplar clones, which were improved using *in vitro* selection technique (Hossain et al., 2006; Bittsanszky et al., 2008).

Carotenoids are a lipid soluble antioxidant, which are considered as potential scavengers of ROS and lipid radicals. They are known major antioxidants in biological membranes for protection of membrane stability against lipid peroxidation, including quenching or scavenging ROS like $^1O_2$. Carotenoids have several major functions such as preventing membranes for lipid peroxidation. One of them, they act as energetic antenna, absorb light at wavelength between 400 and 550 nm and transfer it to the Chl. Second, they protect the photosynthetic apparatus by quenching a triplet sensitizer (Chl^3), $^1O_2$ and other harmful free radicals which are naturally formed during photosynthesis. Third, they are important for

the PSI assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization (Gill and Tuteja, 2010). Helaly and El-Hosieny, (2011) reported that carotenoid contents increased in *Citrus lemon* shoots under different oxidative stress conditions. Carotenoid content also increased in salt tolerant *Chrysanthemum morifolium* strain, which was improved *using in vitro* selection technique (Hossain et al., 2006).

Accumulating osmotic adjustment, sometimes is called osmoprotectant, in their structures is another crucial mechanism in many plant species in response to environmental stress, including proline (amino acids), glycinebetain (quaternary ammonium compounds) and sugars (mannitol, D-ononitil, trehalose, sucrose, fructan). Proline and glycinebetain act as osmoprotectants by stabilizing both the quaternary structure of proteins and the structure of membranes, Proline also acts a metal chelator, an inhibitor of LPO, and $OH^{\cdot}$ and $^1O_2$ scavenger (Arshaf and Harris, 2004). Enhanced osmoprotectant contents have been demonstrated in plants during different stress conditions by many researchers. Patada et al. (2012) reported that glycinebetain, proline and reduced sugar contents increased in embryonic sugarcane callus under PEG and NaCl treatment. In another study, Lokhande et al. (2010) observed that glycinebetain, proline and soluble sugar contents enhansed in *Sesuvium portulacastrum* callus under NaCl treatment. Also, in *Salicarnia persica* and *S. europaea* callus culture the increasing amounts of proline were observed under Mannitol and NaCl induced stresses (Torabi and Niknam, 2011). Cui et al., (2010) reported that proline and glucose contends were increased under sucrose-induced osmotic stress in *Hypericum perfortum* root suspension cultures. Proline contents also increased in hyperhydric *Prunus avium* shoots (Franck et al., 2004). Increasing ratios of proline and soluble sugar contents were observed in drought tolerant *Tagetes minuta* clones and salt tolerant sugarcane (*Saccharum* sp.) callus, respectively (Mohamed et al., 2000; Gandonou et al., 2006). Additionally, increasing proline, reduced-sugar and disaccharide-sugar contents were observed in drought-tolerant callus line of sunflower (Hassan et al., 2004). Drought tolerant *Tagetes minuta* clones, sunflower callus lines, and salt tolerant sugarcane (*Saccharum sp*.) callus were improved using *in vitro* selection technique (Mohamed et al., 2000; Hassan et al., 2004; Gandonou et al., 2006). NaCl and gamma radiation-induced oxidative stress conditions increased proline, total sugar, glycinebetain and total soluble phenol contents in *Citrus lemon* shoots (Helaly and El-Hosieny, 2011).

Phenolic compounds, which are often referred to as secondary metabolites and functions of most of them have still poorly understood, including flavonoids, tannins, anthocyanins, hydroxycinnamate esters, and lignin, are abundant in plant tissues. Many secondary metabolites play widely important role from as defensive agents against pathogens to general protection against oxidative stress using as electron donors for free radical scavenging (Grace, 2005). Phenylalanine ammonia lyase (PAL) activity is one of the main enzymes in the synthesis of phenolic compounds, and phenolic contents were increased under PEG-induced drought stress in rice callus culture (Shehab et al., 2010). PAL activities also increased in *Glycyrrhiza uralensis* and *Luffa cylindrica* cotyledons under Cd and Pb treatments in tissue culture conditions, respectively (Zheng et al., 2010; Jiang et al.,2010). It was observed that under hyperhydric conditions PAL and lignin-concentrations reduced (Saher et al., 2004). In another study with rice cultivars were detected that under PEG induced drought stress conditions,

anthocyanins, flavonoids and phenolics contents increased (Basu et al., 2010). Under sucrose-induced osmotic stress total flavonoids and phenolics contends were increased in *Hypericum perfortum* root suspension cultures (Cui et al., 2010). Phenol oxidases (PPO) activities, another important enzyme which plays important role for oxidation of phenolic compounds, was changed under NaCl induced stress conditions in callus and seedlings of *Trigonella* species (Niknam et al., 2006). Low doses gamma radiation induced total phenol, flavonoid, soluble sugar and PAL activity in *Rosmarinus officinalis* L. (El-Beltagi et al., 2011).

Franck et al., (2004) and Ghnaya et al. (2011) reported that polyamine contents increased in hyperhydric *Prunus avium* shoots and *Brasica napus* cv. Jumbo under Zn-induced oxidative stress, respectively. Polyamines (spermidine, putresine and spermine) are among the important non-enzymatic antioxidants, which act to protect nucleic acids against enzymatic or oxidative denaturation and to prevent lipid peroxidation (Kaur-Sawhney et al., 2003).

Additionally, measuring free radical scavenging or quenching capacities in cells are the other techniques the detection of total non-enzymatic antioxidant activity. Various methods have been used for measuring total antioxidant activities in biological systems. The increasing ratios of total antioxidant capacity were measured under different abiotic stress conditions induced with tissue culture techniques using 2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) (Cui et al., 2010); 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Hossain et al., 2006; Cui et al., 2010; Basu et al., 2010; Zamora et al., 2010) and ferric reducing antioxidant power (FRAP) (Sotiropoulos et al., 2006; Chatzissavvidis et al., 2008) methods, respectively.

4. Plant tissue culture

Plant tissue culture, as an alternatively known cell, tissue and organ culture or *in vitro* culture, refers to growing and multiplication of cells, tissues and organs of plant outside of an intact plant on solid or into liquid media under aseptic and controlled environment. This technique is one of the key tools of plant biotechnology, especially, after the understanding the totipotency nature of plant cells. It has also been used to describe various pathways of cells and tissue in culture depending on starting plant materials, such as shoot-tip and meristem-tip cultures, nodal or axillary bud cultures, cell suspension and callus cultures. Starting plant materials of this techniques, which are commonly called explant, can be taken from any part of a plant, i.e. shoot tips, axillary buds, nodes, immature or mature embryos and generally can be obtained from the environment. Therefore, they are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms. For this reason, surface sterilization of explants in chemical solutions (usually sodium or calcium hypochlorite or mercuric chloride) is required. Explants are then usually placed on a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts. The most well-known of these inorganic salts is MS (Murashige and Skoog, 1962), Gamborg B5 (Gamborg et al, 1968), LS (Linsmaier and Skoog, 1965), SH (Schenk and Hilderbrandt, 1972). Synthetic media do not include only inorganic salts, it also includes a few organic nutrients, energy sources (such as sucrose, glucose, maltose and raffinose),

vitamins and plant growth regulators (i.e. auxins such as 2,4-Dichlorophenoxyacetic acid (2,4-D), 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), Naphtaleneacetic acid (NAA); Indole-3-acetic acid (IAA), and/or cytokinins such as 6-benzylaminopurine (BAP), 6-Furfurylaminopurine (Kinetin). Solid medium is prepared from liquid medium with the addition of a gelling agent, usually purified agar. The composition of the medium, particularly the plant growth regulators and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots. A balance of both auxin and cytokinin will frequently produce an unorganized growth of cells, which is called callus. Synthetic medium compositions are generally prepared to be based on purpose and explant-source (IAEA, 2004; George at al., 2008). As I previously mentioned plant tissue culture techniques are performed under aseptic and controlled environmental conditions. In addition, this technique allows for the study of large plant population, stress treatment of large population in a limited space and short period of time, and homogeneity of stressor application (Sakthivelu et al., 2008, Lokhande et al., 2011). Plant tissue culture techniques, because of these advantages, have vast potential for various applications both plant science and commercially, such as producing large numbers of identical individuals via micropropagation using meristem and shoot cultures, producing secondary products in liquid cultures, crossing distantly related species by protoplast fusion and regeneration of the novel hybrid, production of dihaploid plants from haploid cultures to achieve homozygous lines more rapidly in breeding programs, using tissue cultures as a model for inducing oxidative stress via different stressor agents and improving plants against abiotic stresses using *in vitro* selection techniques (www.liv.ac.uk). There are also several excellent books about plant tissue culture techniques for those who want further information (Jha and Ghosha, 2005; Yadav and Tyagi, 2006; Kumar and Singh, 2009; Nuemann et al., 2009).

4.1. Induced-oxidative stress conditions in plant tissue culture and antioxidant defense systems

Nowadays, one of the most serious problems is the influence of environmental stress factors on plants which are exacerbated day-by-day through anthropogenic effects. Thus, plant growth, development and the yield performance of plants is adversely affected. These negative results forced humans to find new solutions to minimize these problems.

In recent years, *in vitro* techniques have been extensively used not only *in vitro* screening in plants against abiotic stress but also creating *in vitro* models for studying and observing morphological, physiological and biochemical changes of both unorganized cellular (such as suspension cultures and callus cultures) and organized tissue (such as axillary shoot, shoot tip, mature embryo, and whole plant) levels against abiotic stresses. As the name suggests, *in vitro* tissue culture techniques is performed under aseptic and controlled environment using artificial solid or liquid media for growing and multiplication of explants. Because of these characteristics *in vitro* techniques are suitable for researching both specific and common response to stress factors in plants. As it is known, oxidative stress is secondary

effect of these stress factors and several techniques have been used to induce oxidative stress under tissue culture conditions. Between table 1 and 4 were summarized in recent studies by screening against abiotic stresses in a wide range of plants, including cereals, vegetables, fruits and other commercially important plant. If I summarize in a few sentences of these studies which are referred in these tables, polyethylene glycol (PEG), mannitol, and sucrose generally used as osmotic stress agents in *in vitro* culture conditions to stimulate drought stress in plants. Adding NaCl or any kind of specific metals, such as Cd, Pb, Zn, Cu, Ni, Hg, Al, Cr and As in MS culture media also widely used techniques to induced salt or heavy metal stress conditions. Generating nutritional disorders, sometimes researchers prepared missing media contents or adding some chemicals (such as, $NaHCO_3$, $KHCO_3$, $CaCO_3$) in artificial medium to imply calcareous conditions. In addition, for researching hyperhydricity, researchers generally prefer changing agar concentrations or gelrit agents in media compositions and/or using bioreactors. The general method for investigating the biological effects of gamma radiation, researching-material is irradiated with different doses of gamma radiation.If these studies are also carefully examined, it will be seen that investigated-oxidative stress parameters, which were detected various methods, by inducing under stress conditions, varied from species to species and also in plant organs with respect to different stressor treatments.

4.2. Antioxidant activities of *in vitro* selected plants under abiotic stress conditions

In vitro selection is another technique, which is widely used in plant tissue culture. This technique conventionally defines selection of desired genotypes after the induction of genetic variation among cells, tissues and/or organs in cultured and regenerated plants. These genetic variations may occur spontaneously or may be induced by any kind of agents (such as physical (i.e. gamma radiation, X-ray) or chemical agents (i.e. Ethyl methanesulfonate (EMS)) which are called mutagen) in culture conditions (Rai et al., 2011). Oxidative stress is one of the main reasons for spontaneous mutations in genome because of hyperactive ROS. Oxidative stress also induced to be indirect effects of physical or chemical mutagens. But the main effects of these mutagens directly trigger to DNA-lesions, such as transposons activation, inducing chromosome breakage and/or rearrangement, polyploidy, epigenetic variations, point mutations. Hereby, genotypic and phenotypic variations created in the progeny of plants regenerated from plant tissue culture. If these variations are created in somatic cells or tissues, they are called somaclonal variations which are useful in crop improvement (Joyse et al., 2003). After the creating genetically stable variations, for selection of desired genotypes, as referred to some studies, which were published in recent years, in the table 5, explants are exposed to various kinds of selective agents, such as NaCl (for salt-tolerance), PEG or mannitol (for drought-tolerance), Cd (for Cd-tolerance), paraquat or atrazine (for herbicide-resistance), these selective agents added to the culture media. In plants screening for variations depending on the ability to tolerate relatively high levels of stressors in media, various systems, such as callus, cell suspensions, embryonic callus, shoot cultures, nodal cultures, have been used in tissue culture conditions. It is assumed that *in vitro* selection is an efficient, a rapid and a low cost breeding method (Lu et al., 2007; Rai et al.,

Plant Species	Type of Explants	Stressors and Concentration	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Saccharum officinarum L. cv. Co 86032	embryogenic callus	0, and 20% (w/v)PEG 8000	SOD, CAT, APX, TBARS, TSS and TRS, Pro., PC, and GB	spectrophotometric	Patada et al. (2012)
Salicornia persica and *S.europaea*	callus	0,500,and 1000 mM Mannitol	SOD, CAT, PPO, H_2O_2, POX, MDA, PC, CARs, and Pro.	spectrophotometric	Torabi and Niknam (2011)
Hypericum perforum L.	adventitious roots	0, 1, 3, 5, 7, and 9% (w/v) Sucrose	DPPH, ABTS, Pro., H_2O_2, MDA, Flavonoid, Phenol and chlorogenic acid, Hypericin and Residual Sugars	spectrophotometric	Cui et al. (2010)
Oryza stiva L. (cv. IR-29, Pokkali, and PB)	seedlings	0, and 20% (w/v) PEG-6000	SOD, CAT, POX, H_2O_2, LOX, MDA, PP PO, DPPH, Antocyanin Flavonoid, and Phenol	spectrophotometric	Basu et al. (2010)
Deschampia antarctica	shoots	PEG-8000	DPPH, APX, CAT, POX, GR, Proline, H_2O_2, MDA, Ascorbate, Flavonoid, PP, and Phenol	spectrophotometric	Zamora et al. (2010)
Oryza sativa L.	callus	0, 5, 10, 15, and 20% PEG	H_2O_2, MDA, GSH, AsA, SOD, APX, CAT GR, PAL, TSS and AA, and Phenol	spectrophotometric	Shehab et al. (2010)
Prunus cerasus x P. canecens	shoot tips	0, 1, 2, and 4% PEG 8000	SOD, CAT, POX, APX, GR, MDA, PP, and Pro.	spectrophotometric	Sivritepe et al. (2008)
Malus domestica Borkh. rootstock MM 106	shoot tips	0, and 576 mM Mannitol 0, and 562.5 mM Sorbitol	SOD, CAT, POX, FRAP, H_2O_2, Pro., PP, and MDA	spectrophotometric and isoenzyme variations	Molassiotis et al. (2006)
Centaurea ragusina L.	shoots	0, and 300 mM Mannitol	POX, MDA, and H_2O_2	spectrophotometric and isoenzyme variations	Radić et al.(2006)
Musa AAA 'Berangan' and *Musa* AA 'Mas'	shoot tips	0, and 40% PEG 6000	SOD, CAT, APX, GR, and MDA	spectrophotometric	Chai et al. (2005)

Abbreviations: AA: Amino Acid; CARs: Carotenoids; GB: Glycinebetaine; Pro.: Proline; PC: Protein Content; PP: Photosynthetic Pigments TSS: Total Soluble Sugar; TRS: Total Reducing Sugar.

Table 1. *In vitro* studies concerning drought stress

2011). As for the characterizations of selected abiotic stress tolerant plants, several methods have been used. One of them is based on antioxidant defense systems. Additionally, there is known to be a strong correlation between stress tolerance and antioxidant capacity in plant species. As also shown in the table 5, *in vitro* selected abiotic stress tolerant plantlets have been characterized by detections of enzymatic antioxidants (SOD, APX, CAT, POX, GR etc.) and/or non-enzymatic antioxidant (proline, ascorbate, glycinebetaine etc.) as well as the other oxidative stress biomarkers (H_2O_2, MDA, etc.). All of them also agreed with advanced-antioxidant capacity increase tolerance against stress factors in plants.

Plant Species	**Type of Explants**	**NaCl Concentrations**	**Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers**	**Detection Methods**	**References**
Cucumis melo L. (cv. Besni, Yuva, Midyat, Semame and Galia C8)	callus	100 mM	SOD, and CAT	spectrophotometric	Kusvuran et al. (2012)
Saccharum officinarum L. cv. Co 86032	callus	150 mM	SOD, CAT, APX, TBARS, TSS and TRS, Pro., PC, and GB	spectrophotometric	Patada et al. (2012)
Salicornia persica and *S.europaea*	callus	0, 100, 300, and 600 mM	SOD, CAT, PPO, H_2O_2, POX, MDA, PC, CARs, and Pro.	spectrophotometric	Torabi and Niknam, (2011)
Triticum aestivum L. (cv. Tekirdag, Pehlivan and Flamura-85)	mature embryos	0, 50, 100, 150, 200, and 250mM	PP, SOD, POX, and CAT	spectrophotometric and isoenzyme variations	Sen and Alikaman oglu, (2011)
Sesuvium portulacastrum L.	axillary shoots	0, 200, 400, and 600 mM	SOD, CAT, APX, TSS, Pro., GB, and MDA	spectrophotometric	Lokhande et al. (2011)
Sesuvium portulacastrum L.	callus	0, 100, 200 and 400 mM	SOD, CAT, APX, TSS, Pro., and GB	spectrophotometric	Lokhande et al. (2010)
Nitraria tangutorum Bobr.	callus	0, 50, 100 and 200 mM	SOD, POX, CAT, APX, H_2O_2, and NADPH-oxidase	spectrophotometric	Yang et al. (2010)
Paulownia imperialis (Seibold and Zuccarini) and *P. fortune* (Seemann and Hemsley)	seedlings	0, 20, 40, 60, 80 and 160 mM	MDA, PP, CARs, PC, and Proline,	spectrophotometric	Ayala Astorga and Alcarez-Melendez, (2010)

Plant Species	Type of Explants	NaCl Concentrations	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Catharantus reseus L. cv. Rosea and Alba	shoots	0, 15, 30, 45, 60, 75, and 100 mM	SOD, CAT, POX, MDA, Pro., Phenol, PP, and Sugar	spectrophotometric	Garg, (2010)
Pinus pinaster	suspension cells	0, 50, 100, and 150 mM	MDA, SOD, and ROS mes.	isoenzyme variations and transcription analysis	Azevedo et al. (2009)
Thellungiella halophila and *Arabidopsis thaliana*	callus	0, 50, 100, 150, 200, and 250mM	Sucrose, Treholase Pro., Total Flavonoid and GB	spectrophotometric	Zhao et al. (2009)
Solanum tuberosum L. cv. Cardinal and Desiree	shoot apices and callus	0, 20, 40, 60, 80, 100, 120 and 140 mM	SOD, CAT, POX, and PC	spectrophotometric	Sajid and Aftab,(2009)
Jatropha curcas	callus	0, 20, 40, 60, 80, 100 mM	SOD, POX, PC, and Pro.	spectrophotometric and isoenzyme variations	Kumar et al. (2008)
Impomoea batatas L.	shoot apexes	0, 0.5, and 1%	SOD, POX and CAT	spectrophotometric	Dasguptan et al. (2008)
Prunus cerasus L. Rootstock CAB-6P	shoot tips	0, 30, and 60 mM (NaCl and $CaCl_2$)	FRAP, CAT, POX, and PP	spectrophotometric and isoenzyme variations	Chatzissavvidis et al. (2008)
Malus domestica Borkh. Rootstock M 4	shoots	0, 35, 100, and 200 mM (NaCl) 0, 5, and 10 mM ($CaCl_2$)	Sugar, and Proline	spectrophotometric	Sotropoulos, (2007)
Sweet chery rootstock Gisela 5 *Prunus cerasus* x *P.canescens*	shoot tips	0, 50, 100, and 150 mM	SOD, POX, CAT, APX, GR, MDA and Proline	spectrophotometric	Erturk et al. (2007)
Olea europea L. cv. Manzanillo	seedlings	0, 25, 50, 100, and 200 mM	H_2O_2, GSH, Ascorbate, SOD, CAT, GR, PP, G6PHD, NADP-ICDH, and FNR	spectrophotometric,immunofluores., isoenz. and transcript.	Valderrama et al. (2006)

Plant Species	Type of Explants	NaCl Concentrations	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Malus domestica Borkh. rootstock MM 106	shoot tips	0, and 240 mM NaCl 0, and 220 mM KaCl	SOD, CAT, POX, FRAP, H_2O_2, FRAP, PP, Proline, and MDA	spectrophotometric and isoenyme variations	Molassiotis et al. (2006)
Trigonella foenum-graecum L. and *Trigonella aphanoneura* Rech.f.	seeds and callus	0, 50, 100, 150; and 200 mM	CAT, POX, PPO, PC, and Proline	spectrophotometric and isoenyme variations	Niknam et al. (2006)
Solanum tuberosum L. (cv. Agria, Kennebec, Diamant and Ajax)	internodes	0, 50, 100 and 150 mM	SOD and POX	spectrophotometric and isoenyme variations	Rahnama and Ebrahimzadeh (2006)
Citrus hybrid 'Carvalhal' and *C. sinensis* cv. 'Valencia late	cell suspension	0, 50, 100, 150, 200, 300 and 400 mM	SOD, POX, CAT, Proline and MDA	spectrophotometric	Ferreira and Lima-Costa (2006)
Centaurea ragusina L.	shoots	0, 150, 300, 450 and 600 mM	POX, MDA, and H_2O_2	spectrophotometric and isoenyme variations	Radić et al. (2006)
Solanum tuberosum L. (cv. Agria, Kennebec, Diamant and Ajax)	nodes	0, 50, 75 and 100 mM	SOD, CAT, POX and APX,	spectrophotometric and isoenyme variations	Rahnama and Ebrahimzadeh (2005)
Eucalyptus camadulensis Dehnh. clones	shoots	0, 50, 100 mM	Proline, PP	spectrophotometric	Woodward and Bennett (2005)

Abbreviations: AA: Amino Acid; CARs: Carotenoids; GB: Glycinebetaine; Pro.: Proline; PC: Protein Content; PP: Photosynthetic Pigments TSS: Total Soluble Sugar; TRS: Total Reducing Sugar
FNR: ferredoxin-NADP reductase; G6PHD: glucose-6-phosphate dehydrogenase; NADP-ICDH: NADP-isocitrate dehydrogenase; PC: Protein Content; PP: Photosynthetic Pigments:
Isoenz: Isoenzyme variation; Flour.: Fluorescent dying; Trascript.: Transcription analysis

Table 2. *In vitro* studies concerning NaCl stress

Plant Species	Type of Explants	Stressors	Concentrations	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Pinus nigra L. (clone Poli and 58-861)	callus	Cd	0, 150 and 250μM	APX, CAT, POX, Thiols, and Phytochelatins	spectrophotometric	Iori et al. (2012)
Brassica napus L. cv. Jumbo	thin cell layers	Zn	0-1 mM	POX, MDA, PP, CARs, and Polyamines	spectrophotometric	Ghnaya et al.(2011)
Alternanthera philoxeroides	callus	Cu	0, 0.05, 0.1, 0.2, 0.6, 0.8, and 1 mM	SOD, CAT, POX, MDA, PP, ROS mes. (H_2O_2 and $O_2^{-\bullet}$)	spectrophotometric	Xu et al. (2011)
Glycyrrhiza uralensis L.	seeds	Cd	0, 0.05, 0.1, 0.2 and 0.4 mM	SOD, CAT, POX, PPO, and PAL	spectrophotometric and isoenzyme variations	Zheng et al.(2010)
Luffa cylindrical L.	embryos	Pb	0, 100, 200, 400 and 800μM	SOD, CAT, POX, and PAL	spectrophotometric and isoenyme variations	Jiang et al. (2010)
Jatropha curcas L.	embryos	Zn	0, 0.25, 0.5, 1, 2 and 3 mM	SOD, CAT, POX, and PAL	spectrophotometric and isoenyme variations	Luo et al. (2010)
Oryza stiva L. cv. Lalat	seeds	As(III) and As(V)	0, 50 and 100μM As(III) 0, 100 and 500μM As(V)	SOD, APX, GR, GSSG, POX, and MDA	spectrophotometric and isoenyme variations	Shri et al. (2009)
Jatropha curcas L.	embryos	Ni	0, 100, 200, 400 and 800 μM	SOD, CAT, POX, and PAL	spectrophotometric and isoenyme variations	Yan et al. (2008)
Arachis hypogaea L. cv. JL-24	seeds	Cd	0, 50, 100, 200 and 300 μM	CAT, POX and MDA	spectrophotometric	Kumar et al.(2008)
Picea rubens Sarg.	suspension cultures	Cd and Zn	0, 12.5, 25, 50, 100 and	Thiol, AA, and Polyamines	spectrophotometric	

Plant Species	Type of Explants	Stressors	Concentrations	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
			200μM (Cd) 0, 50, 100, 200, 400 and 800μM (Zn)			Thangavel et al.(2007)
Medicago sativa L. cv. Aragon		Cd and Hg	0, 3, 10 and 30μM	H_2O_2 , Ascorbate, GSH, APX, and SOD,	spectrophotometric, isoenz., fluor., and transcrit.	Villasante et al.(2007)
Sesbania drummondii	callus	Cd	0, 10, 25, 50, 100 and 250μM	SOD, APX, GR, GSSG, and GSH	spectrophotometric	Israr et al. (2006)
Malus domestica Borkh. rootstock MM 111	shoots	B	0.1, 0.5, 1, 3, and 6 mM	SOD, CAT, POX , PP, and FRAP	spectrophotometric	Sotiropoulos et al. (2006)
Helianthus annuus L. cv. Mycosol	callus	Cd	150 μM	MDA, GSH, GSSG, Phytochelatins, and ROS mes.,	spectrophotometric and fluorescein dye	Gallego et al. (2005)
Helianthus annuus L.	callus	Cd^{+3}, Al^{+3}, Cr^{+3}	150 μM	POX, SOD, CAT, APX GR, TBARS, GSH, GSSG, Ascorbate, ROS mes, and Dehydroascorbate, Phytochelatins	spectrophotometric	Gallego et al. (2002)
Saccharum officinarum L.	callus	Cd	0,0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mM	SOD and CAT	spectrophotometric and isoenyme variations	Fornazier et al. (2002)

Abbreviations: AA: Amino Acid; PP: Photosynthetic Pigments; Isoenz: Isoenzyme variation; Flour.: Fluorescent dying; Trascript.: Transcription analysis.

Table 3. *In vitro* studies concerning heavy metals stress

Plant Species	Type of Explants	Stressors	Treatments	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Rosmarinus officinalis L.	callus	gamma radiation (^{60}Co)	0, 5, 10, 15 and 20Gy	MDA, AsA, GSH, SOD, PAL, TSS, AA, Phenol, Flavonoid, H_2O_2 and $O_2^{\cdot-}$ mes. PAL, APX, CAT, and POX	spectrophotometric	El-Beltagi et al. (2011)
Gladiolus hybridus Hort.cv.Wedding Bouquet	callus	hyperhydricity	different culture systems	MDA, AsA, SOD, APX, CAT, and POX	spectrophotometric and isoenyme variations	Gupta and Prasad, (2010)
Solanum tuberosum L.	calli	herbicides	paraquat, 2,4-D and dicamba	SOD, CAT, GR and GST	spectrophotometric	Peixoto et al. (2007)
Vigna radiate L. Wilczek	callus	gamma radiation (^{60}Co)	0, 20, 50, 100 and 200 Gy	SOD, and POX	isoenyme variations	Roy et al. (2006)
Apple "M9 EMLA"	nodal segments	hyperhydricity	bioreactor culture	SOD, APX, GR, GPX, MDHAR, DHAR, and ROS mes.	spectrophotometric, flour. and isoenyme variations	Chakrabarty et al. (2006)
Euphorbia millii L.	inflorescences	hyperhydricity	bioreactor culture	SOD, APX, CAT, POX, GR, GST, GPX, MDHAR,DHAR, GSH, GSSG, LOX, and MDA	spectrophotometric and isoenyme variations	Dewir et al. (2006)
Prunus rootstocks (Barrier, Cadaman, Saint Julien 655/2 and GF-677)	shoots	Fe-deficiency	MS (+Fe) control, MS (-Fe), MS (5 mM $NaHCO_3$ + 0.5 gl^{-1} $CaCO_3$) (pH 6.9) and MS (10 mM $NaHCO_3$ + 0.5 gl^{-1} $CaCO_3$) (pH 7.3)	CAT, POX, H_2O_2 and FRAP	spectrophotometric and isoenyme variations	Molassiotis et al. (2005)

Plant Species	Type of Explants	Stressors	Treatments	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Dianthus caryophyllus L. (cv. Oslo, Killer, and Alister)	shoots	hyperhydricity	changing concentration of agar from 0.8% to 0.58%	SOD, CAT, APX, POX, GR, MDHAR, DHAR, LOX, PAL, H_2O_2 lignin, AsA,PP, Ethylen, and MDA	spectrophotometric	Saher et al. (2004)
Prunus avium L.	shoots	hyperhydricity	changing from 0.8% agar to 0.25% gelrit)	Proline, GPX, Ethylene, and Polyamines	spectrophotometric	Franck et al. (2004)
Borage officinalis L.	seeds	Fe-deficiency	0, 13 and 27.8 $mgl^{-1}FeSO_4$	APX, CAT, MDA, GSH, PP, and EPR	spectrophotometric and isoenyme variations	Mohamed and Aly, (2004)
Prunus cerasifera rootstocks Mr.S2/5	shoots	Fe-deficiency	control, MS (+Fe) and MS (+ 1mM $KHCO_3$)	PP,CARs, CAT, and SOD	spectrophotometric and transcript.	Lombardi et al. (2003)

Abbreviations: AA: Amino Acid; PP: Photosynthetic Pigments; Isoenz: Isoenzyme variation; Flour.: Fluorescent dying; Trascript.: Transcription analysis

Table 4. *In vitro* studies concerning the other abiotic stresses

Plant Species	Culture Techniques	Mutagens	Stressors	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Beta vulgaris L. cv. Felicita	shoot tips	gamma radiation (^{137}Cs)	drought (PEG)	SOD, APX, CAT and POX	spectrophotometric and isoenyme variations	Sen and Alikamanoglu, (2012)
Citrus limon L. Burm.f. cv. Feminello	protoplasts	gamma radiation (^{60}Co)	NaCl	SOD, APX, CAT, POX, GR, H_2O_2, MDA, Pro, PP, TS, GB, and Phenols	spectrophotometric	Helaly and El-Hosieny, (2011)
Solanum tuberosum L. cv. Agat and Konsul	nods	gamma radiation (^{137}Cs)	drought (PEG)	SOD, APX, CAT and POX	spectrophotometric	Alikamanoglu et al., (2011)

Plant Species	Culture Techniques	Mutagens	Stressors	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Zoysia matrella [L.] Merr.	embryogenic callus	gamma radiation (^{60}Co)	NaCl	SOD, CAT, POX and Pro.	spectrophotometric	Chen et al. (2011)
Solanum tuberosum L. cv. Granola	nodes	gamma radiation (^{137}Cs)	NaCl	SOD, APX, CAT and POX	spectrophotometric	Alikamanoğlu et al., (2009)
Arabidopsis thaliana (ecotype Colombia, Co10)	plantlets	sucrose-induced	atrazine	SOD, APX, CAT, DHAR, MDAR, GR, H_2O_2, $^1O_2$, $O_2^{\cdot-}$, CARs, PP, and ROS-scavenging systems	spectrophotometric, fluorescent dying, and transcriptomic analy.	Ramel et al., (2009)
Poplar clones *(Populus X Canescens)*	leaf petioles		paraquat	GR, APX, GST, and LOX	spectrophotometric and transcriptomic analy.	Bittsánszky et al. (2008)
Chrysanthemum morifolium Ramat.cv. Maghi Yellow	callus		NaCl	SOD, APX, GR, and Pro.	spectrophotometric	Hossain et al., (2007)
Cynodon transvaalensis x *C. dactylon* cv. Tifeagle	callus		NaCl and drought	SOD, CAT, and Pro.	spectrophotometric	Lu et al., (2007)
Chrysanthemum morifolium Ramat.cv. Regal Time	shoot	EMS	NaCl	SOD, APX, DHAR, MDAR, GR, H_2O_2, DPPH, PP, AsA PP, CARs, and Pro.	spectrophotometric and isoenzyme variations	Hossain et al., (2006)
Saccharum sp. cv. CP65-357	callus		NaCl	Pro., and TSS	spectrophotometric	Gandonou et al. (2006)
Helianthus annuus L. cv. Myak	callus		drought (PEG)	Pro., and Charbonhydrates	spectrophotometric	Hassan et al., (2004)
Oriza japonica L. cv. Donganbyeo	callus	gamma radiation (^{60}Co)	AEC res.	SOD, APX and AA	spectrophotometric and proteomic analysis	Kim et al. (2004)
Medicago sativa L. cv. CUF 101	seeds		drought (PEG)	CAT, GR and Pro.	spectrophotometric	Safarnejad, (2004)

Plant Species	Culture Techniques	Mutagens	Stressors	Antioxidant Enzymes, None-antioxidant Components and Oxidative Stress Biomarkers	Detection Methods	References
Armoracia rusticana Geart.	cell suspension		paraquat and Cd	SOD, APX, CAT and POX	spectrophoto metric and isoenzyme variations	Kopyra and Gwozdz, (2003)
Tagetes minuta	callus		drought (Mannitol)	Pro., and TSS	spectrophoto metric	Mohamed et al., (2000)

Abbreviations: GB: Glycinebetaine; Isoenz: Isoenzyme variation; Pro.: Proline; PP: Photosynthetic Pigments TS: Total Sugar; AA: Amino Acid; analy.: analysis; CARs: Carotenoids; TSS: Total Soluble Sugar; var.: variation.

Table 5. *In vitro* selected examples of against abiotic stresses, and the activities of antioxidants and oxidative stress indicators

5. Conclusion

The overproduction of ROS in plants is stimulated by environmental stressors as well as many metabolic reactions, such as photosynthesis, photorespiration, and respiration. All of these ROS are toxic to biological molecules and generally lead to non-controlled oxidation in cellular macromolecules, such as lipid autocatalytic peroxidation, protein oxidation or DNA-lesions. These irreversible damages of cellular macromolecules cause many cases in plants from mutations to cell death. Plants possess sophisticated-antioxidant defense mechanisms, including antioxidant enzymes and molecules that can protect cells from oxidative damage and maintain ROS homeostasis. Besides causing damage, ROS can also participate in signal transduction. Despite all these knowledge about ROS, how to maintain balance between these oxidant and antioxidant properties in plants have still poorly been understood. Plant tissue culture techniques are performed under aseptic and controllable environmental conditions for this reason allows various opportunities to study details of this balance. Controlled stress in *in vitro* may help to overcome the cross tolerance/cross responses. Additionally, improving crops against abiotic stress factors and isolating of cell/callus lines or plantlets using *in vitro* techniques are the other main usage of plant tissue cultures. Improving plants via *in vitro* selection methods generally based on spontaneous or induced mutations, and oxidative stress is one of the main reasons of both spontaneous and induced mutations which are caused DNA damages in various ways. Therefore, it is a driving force for improving crops. Although, *in vitro* selections will save time to improve crops, as suggested by Jain (2001), it should not be forgotten that these mutants need to be tested under field conditions to maintain genetic stability for desirable character/characters.

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