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).

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

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

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

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

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

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 $\cdot OH$ 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),