Oxidative damage caused by Reactive Oxygen Species under drought stress in *Gossypium hirsutum*

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Abstract: Cotton (*Gossypium hirsutum* L*)* is an important fibre crop. It is very sensitive to drought stress. Water stress reduces yield in cotton by reducing number of bolls per plant, reduced boll size, and increased flower shedding. Drought stress severely affects the process of photosynthesis. Water stress results in production of Reactive oxygen species (ROS) to lethal a level that causes oxidative damage to lipids, protein and DNA. ROS includes superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2), alkoxy radical ($RO^{\cdot}$), peroxy radical ($ROO^{\cdot}$), singlet oxygen ($^1O_2$). ROS are produced in various organelles of the cell that includes chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum (ER), and plasma membranes. Plants have natural defence ROS scavenging enzymes like CAT (catalase), GR (glutathione reductase), superoxide dismutase (SOD), APX (Ascorbate peroxidase) etc. The extent of damage from ROS depends on the delicate balance between ROS production and ROS scavangers. This review paper highlights the sites of ROS production, their antioxidant enzymes, and certain genes identified in cotton that can enhance the production of ROS scavenging enzymes thus can enable cotton plant to tolerate drought stress.
[Bhutta MA, Muhammad KQ, Muhammad KS, Mahmood A, Malook SU Ali Q. **Oxidative damage caused by Reactive Oxygen Species under drought stress in** *Gossypium hirsutum***.** *Life Sci J* 2015;12(4s):51-59]. (ISSN:1097-8135). http://www.lifesciencesite.com. 7

Key words: *Gossypium hirsutum* L. Photosynthesis, Drought stress, Reactive oxygen species, Trehalose-6-phosphate-synthase

Introduction:

Cotton (*Gossypium hirsutum* L., 2n = 52) is the important fibre crop that provide natural fibre for the textile industry. It accounts for 7.8 percent of value added in agriculture and 1.6 percent of GDP. The covered an area of 2835 thousand hectares during 2011-12 (Anonymous 2011-12). The genus *Gossypium* contains at least 45 diploid and five allotetraploid species (Endrizzi *et al*., 1985). Among the various factors that reduces cotton yield and productivity, biotic and abiotic stresses appears to be most important. Biotic constraints involve various insect pests that attack cotton plant and diseases that cause severe losses in cotton production (Ahmad *et al*, 2011). Among various diseases, Cotton leaf curl virus (CLCuV) is the most destructive, causing huge losses to cotton production (Khan and Ahmad, 2005). Among the abiotic constraints, low plant available water is major limiting factor that affects crop productivity in many regions of the world (Sinclair, 2005). Drought stress is one of the major stresses that disrupts growth and development of the plant, reduces production and plant performance than any other abiotic factor (Shao *et al*., 2009). Pakistan's economy largely depends on agriculture. Its 35 million acres land is irrigated by canals and tube wells. With increasing population and depleting water resources, Pakistan is fast heading towards a situation of water shortage and threat of famine. There is a need to address and plan to overcome this upcoming threat. Water stress is the major factor that limits the cotton production in world including Pakistan (Anonymous, 2005). Drought stress effects membrane structure, pigments contents and photosynthesis of the plant (Benjamin and Nielsen, 2006; Praba *et al*., 2009; Azam *et al*., 2013). Degree of drought susceptibility of a crop plant depends upon severity of the drought, plant species and stage of plant development (Demirevska *et al*., 2009). Adaptability of plant to water deficiency is the cumulative result of different biochemical and physiological processes that causes changes in plant growth, structure, and antioxidant defences (Duan *et al*., 2007; Shan *et al*., 2015; Zameer *et al*., 2015). According to Mitra (2001) the mechanism of drought tolerance in plants is classified into three categories, i.e., drought escape, drought avoidance and drought tolerance. In case of drought escape plant completes its life cycle before the start of drought season. Whereas drought avoidance is ability of plant to efficiently uptake water by increasing its root length and maintaining its tissue water potential by reduced

transpiration (Agbicodo *et al*., 2009). In drought tolerance, plant avoids injuries caused by drought through maintaining its physiological and biochemical parameters (Vazifedous *et al*., 2008; Zafar *et al*., 2015). Root characters, physiological traits (gaseous exchange, osmotic adjustment) plant water status measurement (leaf water potential, relative water contents, cell membrane stability) are such attributes that are considered as main component of drought tolerance in cotton. One of the parameter of drought tolerance by Plants is their ability to water loss by closing stomata and its morphological leaf attributes like waxines of leaf (Franca *et al*., 2000; Muhammad et al., 2015; Butt *et al*., 2015).

Morphological Responses of cotton plant to Drought:

The initial effect of drought is poor germination of seed and dire crop establishment (Harris *et al*., 2002). Severe drought stress inhibits cell elongation because of restricted flow of water from xylem to nearby cells (Nonami, 1998). Drought stress reduces photosynthesis due to suppressed leaf expansion (Rucker *et al*., 1995). Water stress reduces plant height due to reduction in cell enlargement and leaf senescence (Manivannan *et al*., 2007). Even slight moisture stress reduces plant growth and yield. Drought stress reduces the fibre yield and deteriorates quality of lint in cotton (McWillium, 2003). It reduces cotton crop production due to reduced boll weight, less number of bolls per plant, reduced plant height and less seed cotton yield. Water stress reduces number of bolls per plant and lint yield (Saranga *et al*., 1998). Water stress at the time of flowering significantly reduces yield. In cotton it results in less number of nodes and reduced dry weight of stem and leaves. Water stress condition reduces shoot-to-root ratios of cotton plant. There exists a great variation for root length in cotton germplasm (Basal *et al*., 2005; Ullah *et al*., 2008). Mechanism of deep rooting, indeterminate growth habbit of cotton plant help to minimize the losses due to flower shedding during water stress and allows cotton plant to adapted to semi-arid regions. Under drought stress the length of root increases as compared to normal. This enables the cotton plant to survive under water deficient condition by getting moisture from the deeper soil profile.

Metabolic rearrangements induced by drought stress in plants:

Plants subjected to drought stress, accumulates different osmolytes like proline, soluble carbohydrates, glycinebetaine and sucrose. These osmolytes protect the cell membrane from degradation by maintaining turgor pressure through osmotic adjustments. Plants exposed to stress shows amino acid accumulation (Lugan *et al*., 2010). Proline accumulation in plants has been associated with stresses. Proline protects the cell from damage caused by stress through scavenging of reactive oxygen species (ROS), stabilizing protein structure. Proline plays role in cell proliferation, cell death and initiates specific gene expressions that are essential for recovery after stress (Szabados and Savoure, 2009). Whereas GB is accumulated in chloroplast under drought stress and protects PSII from damage (Ben *et al.* 2008).

Osmotic adjustment:

Accumulation of solutes in the ell due to loss of water under water stress condition is termed as osmotic adjustment.The accumulated compatible solutes have low molecular weight, highly soluble and helps in water absorption by lowering osmotic potential of the cell. These compatible solutes includes sugars, such as trehalose, fructose and sucrose; polyols such as mannitol, pinitol and sorbitol; amino acids like proline; quaternary ammonium compounds including glycine betain; ions such as potassium; and organic acids such as malate and citrate (Ashraf and Iram, 2005). Accumulation of these solutes helps to maintain metabolic activity and tugor pressure of the cell (Kiani *et al*., 2007). Osmotic adjustment is one of the major mechanisms that makes a plant adaptive to water deficit condition. Cotton plant has more ability of osmotic adjustment as compared to other crops. This enables the cotton plant to tolerate moisture stress condition (Saranga *et al*., 2001).

Physiological responses of plants to Drought:

Drought Signalling through Roots:

Root-shoot ratio of the plant increases in response to moisture stress because of less sensitivity of the roots to growth inhibition as compared to shoots (Wu and Cosgrove, 2000). Guerrero and Mullet, (1986) reported increased level of ABA in case of plant dehydration due to loss in cell turgor pressure. During water stress condition ABA is produced in roots and translocated to xylem and controls stomatal opening and leaf growth. Moreover Cytokinins produced in roots cause nutrient depletion in plant but helps the roots to respond against drought. Drought stress causes disruption in pigment and protein structure in plants (Schachtman and Shin, 2007).

Effect on Photosynthetic apparatus and gas exchange attributes:

Cotton plant has efficient photosynthetic activity but reduction in photosynthetic activity has been noticed under water stress condition (Pettigrew, 2004). In cotton several researchers have reported reduction in photosynthetic activity could be due to reduced stomatal opening or non-stomata mechanism

that includes ribulose 1,5-bisphosphate (RuBP) regeneration that could be a limiting factor in photosynthesis under water-deficit condition (Samarah *et al*., 2009). The major component of chloroplast is chlorophyll and is essential element for photosynthesis. Both chlorophyll *a* and *b* are susceptible to drought stress. Drought severely affects the photosynthetic machinery of plant by limiting the CO2 availability, disruption of lipids, thylakoid electron transport and limiting water availability (Allen and Ort., 2001). Under drought ATP synthesis could be reserved leading to the decrease in photosynthesis rate (Tezara *et al*., 1999). Due to soil water deficit condition the net photosynthetic rate, stomatal conductance and transpiration rate decreases in cotton. Selection based upon high photosynthetic rate under optimum growing condition has not resulted in yield improvement (Ullah *et al*. 2008). However such type of selection under drought condition could be a decisive element for improved cotton yield (Lopez *et al*., 1995).

Biochemical responses of plant to drought stress:

Reactive oxygen species (ROS) production is the major biochemical response of plants against stress conditions. ROS are normal byproducts of metabolic pathways and are also produced under drought condition (Corpas *et al*., 2001; Mittler, 2002; Asada, 2006; Navrot *et al*., 2007). These ROS includes superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2), alkoxy radical ($RO^{\cdot}$), peroxy radical ($ROO^{\cdot}$), singlet oxygen ($^1O_2$) (Dismukes *et al*., 2001; Karuppanapandian *et al*., 2011; Vellosillo *et al*., 2010). ROS is produced from O_2 by absorption of high energy and transfer of excited electron (Mittler, 2002; Halliwell, 2006). Hydroxyl radical ($OH^{\cdot}$) is produced from (H_2O_2) by Haber-Weiss/Fenton reactions and this ($OH^{\cdot}$) is responsible for lipid peroxidation (Lee *et al*., 2007). Hydroxyl radical ($OH^{\cdot}$) is the key radical among ROS that can react with all biochemical molecules like pigments, proteins, lipids and DNA and its excess production causes programmed cell death PCD (Manoharan *et al*., 2005; Karuppanapandian *et al*., 2011). An overview of production and function of diffrent type of ROS is given in the table 1.

Sites of ROS production:

ROS production exceeds under stress condition in various organelles of the plant cell, that includes chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum (ER), and plasma membranes (Corpas *et al*., 2001; Mittler, 2002; Asada, 2006; Navrot *et al*., 2007).

Chloroplast:

Photosystem 1 (PSI) and PSII in chloroplast thylakoids are the main sources of ROS production. ROS production increases when light intensity exceeds, that is required for assimilation of CO_2. In case of drought stress condition superoxide radical ($O_2^{\cdot-}$) are produced by Mahler reaction in which direct electron transfer to molecular oxygen takes place thus generating superoxide ions at PSI due to reduced available CO_2 that results from excess light and stomata closure (Asada 2006). H_2O_2 is produced from conversion of superoxide radical by membrane attached copper/zinc superoxide (Cu/ZnSOD) at PSI and this H_2O_2 is converted into water by thylakoidascorbate peroxidase (tylAPX) (Rizhsky, Liang & Mittler 2003). Controlling ROS production in chloroplast is a main focus in transgenic plants that can confer drought tolerance (Hernandez *et al.* 2001; Mittler & Berkowitz 2001; Tseng, Liu & Yiu 2007).

Mitochondria:

Mitochondria are also known as a source of ROS production such as H_2O_2 and $O_2^{\cdot-}$ (Jezek and Hlavata, 2005; Navrot *et al*., 2007). ROS production increases during elevated mitochondrial respiration under water stress by transport of electron to O_2 from cytochrome electron transport system (Norman *et al.* 2004). Increased demand for mitochondrial ATP enhances respiration in order to overcome the ATP deficiency in chloroplast causes enhance ROS production under severe drought stress (Atkin & Macherel, 2009). Ubiquinone cytochrome *b* reduces (O) to $O_2^{\cdot-}$ which is readily converted to H_2O_2 by reduction through SOD (Moller, 2001). This H_2O_2 generates highly toxic $OH^{\cdot}$ radical by reacting with reduced Fe^{2+} and Cu^{+}. This toxic uncharged $OH^{\cdot}$ has the ability to cross membranes (Moller, 2001; Rhodas *et al*., 2006).

Peroxisomes:

Plant peroxisomes produces $O_2^{\cdot-}$ as a part of their normal metabolism. In peroxisomes matrix O_2^{-} is produced by an enzyme xanthine oxidase (XOD) that catalyses the oxidation of xanthine, and produces $O_2^{\cdot-}$ which is readily converted to O_2 and H_2O_2 by SOD (Corpas *et al.* 2001). Under water stress condition stomata closes that reduces ratio of CO_2 to O_2 in mesophyll cells which results in production of glycolate. This glycolate produces H_2O_2 by oxidation through glycolate-oxidase during process of photorespiration in peroxisomes (Noctor *et al.* 2002; Karpinski *et al.* 2003).

Apoplast:

In apoplast H_2O_2 is produced under drought stress and in response to abscisic acid (ABA) (Hernandez *et al.* 2001; Hu *et al.* 2006; Jubany-Marí *et al.* 2009). Cell wall-associated oxidases, pH-dependent cell wall POXs, germin-like oxalate oxidases and polyamine oxidases are the enzymes

that produce ROS in apoplast (Mittler, 2002). Accumulation of H_2O_2 in apoplast is responsible for acclimation responses of plants such as growth rate modulation and strengthening of cell wall to drought (Hernandez *et al*., 2001; Rodriguez *et al*., 2004).

ROS scavenging system in plants:

Plants have internal defence system that protects the plant from damaging effect of ROS and makes a plant able to carry out its normal cellular functions (Horváth *et al*., 2007). A delicate balance between ROS production and ROS scavenging determines the occurrence and level of damage (Moeller *et al*., 2007). Plant contains a variety of enzymatic and non-enzymatic antioxidant defence system (Appal and Hirt, 2004).

Enzymatic ROS scavenging systems:

Plants have several antioxidant enzymes that scavenge ROS such as CAT (catalase) removes H_2O_2 that is formed in peroxisomes during photorespiration and oxidation of fatty acids by oxidases (Vellosillo *et al*., 2010). H_2O_2 diffused from the cytosol is scavenged by CAT that proliferates from peroxisomes (Lopez-Huertas *et al*., 2000). Salinity and drought however reduces the CAT activity due to reduced rate of protein turnover (Chen *et al*., 2010; Hojati *et al*., 2010). Ascorbate peroxidase (APX) reduces H_2O_2 level in cytosol and chloroplast, ascorbate of APX donates electron which breaks H_2O_2 into H_2O and monodehydroascorbate. In addition to the H_2O_2 scavanging ability of APX, it has two cytosolic forms and a membrane bound form that are involved in controlling transport of electron in combination with ascorbate-glutathione (AsA-GSH) cycle (Foyer and Noctor, 2005). APX is present in soluble and thylakoid bounded form in chloroplast thus $O_2^{\cdot-}$ that is generated at membrane site is converted into H_2O_2 which is readily scavenged by APX (Asada, 2006). Superoxide dismutase (SOD) belongs to the metalloenzymes family that catalyses the conversion of $O_2^{\cdot-}$ into H_2O_2 and O_2 (Moussa and Abdel-Aziz, 2008; Chen *et al*., 2010). Guaiacol peroxidase (GPX) is involved in breakdown of H_2O_2 .An augmentation GPX activity suggesting that this enzyme serves as a defense tool to oppose stress-induced oxidative damage in plants (Cavalcanti *et al*., 2007; Koji *et al*., 2009). An overview of production sites and functions of enzymatic ROS scavenging enzymes is given in the table 1.

Table 1: An overview of production and function of ROS

ROS	Source of Generation	Function
Superoxide radical ($O_2^{\cdot-}$)	Formed by Mehler reaction in chloroplasts, mitochondria, peroxisomes	Reacts with iron-sulphur (Fe-S) clusters of proteins; reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$)
Hydroxyl radical ($OH^{\cdot}$)	Reaction of H_2O_2 with $O_2^{\cdot-}$ (Haber-Weiss reaction), reactions of H_2O_2 with Fe^{2+} (Fenton reaction).	Highly reactive with protein, lipids, DNA
Hydrogen peroxide (H_2O_2)	ETCs of mitochondria, chloroplasts, endoplasmic reticulum, and plasma membrane.	Oxidizes proteins; reacts with $O_2^{\cdot-}$ in a Fe-catalyzed reaction to form $OH^{\cdot}$
Singlet oxygen ($^1O_2$)	Electron transfer reactions in chloroplasts	Oxidizes protein, polyunsaturated fatty acids, and DNA

Non enzymatic ROS scavenging system:

Non enzymatic antioxidants like Ascorbic acid (AA) reduce H_20_2 to H_2O and also scavenge $O_2^{\cdot}$, $OH^{\cdot}$, and $^1O_2$ (Noctor and Foyer, 1998). Excess concentration of AA is necessary for plant to carry out normal metabolic processes and protect plant from oxidative stress. Under drought stress condition AA donates electron in a wide range of enzymatic and non enzymatic reaction and detoxifies ROS (Smirnoff, 2000). Tocopherols are the main component of biological membranes of all plants. Genes responsible for the formation of α-TOC are activated during oxidative stress , α-TOC functions as scavenger of ROS under drought especially of $^1O_2$ (Havaux *et al*., 2005). Tocopherols (TOC_S) protect PSII structure and function by reacting with O_2 in chloroplast and it also protects lipids and membrane components (Igamberdiev *et al*., 2004). Carotenoids are lipid loving organic compounds located in plastids of photosynthetic tissues. Under drought stress carotenoids protects the photosynthetic tissues from oxidative damage by preventing $^1O_2$ generation via quenching of excited triplet chlorophyll (^3Chl) (Collins, 2001). Flavonoids, tannins, hydroxycinnamate esters, and lignin, are the phenolic compounds abundantly found in plant tissue. Phenolics have the antioxidant property because their ability to donate electron, and chain breaking function under water stress (Jung *et al*., 2003).

ROS as messenger in plant hormonal responses:

Under normal concentration ROS acts as a messenger to various enzymes that are involved in plant responses leading to biotic and abiotic stress tolerance. The activity and expression of ROS

scavenging genes is enhanced by ABA (Hu *et al.* 2005). Salicylic acid (SA), Jasmonic acid (JA) and ethylene induces systemic acquired resistance (Pieterse & Van Loon 2004). The promoter regions of ROS-responsive genes such as *Zat7*, *Zat12, WRKY25* and *Apx1* contains SA, JA and ethylene responsive *cis* elements which shows their role in hormone-mediated response under stress condition (Rizhsky *et al.* 2004). Under drought stress condition (SA) reduces ROS production by increasing AOX activity (Norman *et al.* 2004). Jasmonic acid enhances H_2O_2 production in guard cells and causes the stomata to close under drought condition ultimately reducing water loss (Suhita *et al.* 2004). Whereas, ROS at high concentration causes oxidative damage to lipids, protein and DNA.

ROS scavengers in cotton and their role:

Water stress results in higher ROS levels. This causes damage to cellular compartments and their function, whereas antioxidant enzymes protect the cell from this damage. *Kawakami* M (http://arkansasagnews.uark.edu/562-19.pdf) conducted a study to observe the effect of 1-Methylcyclopropene (1-MCP; a plant growth regulator) on level of antioxidant enzyme production. They observed increased level of GR (glutathione reductase) and superoxide dismutase (SOD) and reduced membrane leakage and high protein contents in cotton plants treated with (1-MCP) under water stress . Their study suggests that application of (1-MCP) to cotton plant can protect from ROS damages under water stress. A protein was isolated from cotton 3 metallothionein, designated GhMT3a and introduced into tobacco. This transgenic tobacco showing higher levels of the transcript showed increased tolerance to ROS stresses. This study showed that enhanced level of GhMT3a in cotton can also act as ROS scavenger (Xue *et al.,* 2009). A cotton cytosolic APX1 (Gh*APX1*) gene was identified that produces in response to H_2O_2 and ethylene during fiber development stage , Gh*APX1* scavenge H_2O_2 and reduces it to water hence detoxifying the effect of H_2O_2 and regulating fiber cell elongation (Qin *et al.,* 2008).

KC3

An alpha-crystalline heat shock protein gene (*GHSP26*) identified from *Gossypium arboreum* L is activated under water deficit condition. This gene confers drought tolerance by preventing protein denaturation and helps in protein folding (Maqbool *et al*., 2007). Another gene was identified from *Gossypium hirsutum* that was involved in improved drought tolerance. However full length identification and characterization of this gene is still in progress (Selvam *et al*., 2009). A gene TPS (trehalose-6-phosphate-synthase) was isolated from *Gossypium hirsutum* L. Trehalose (a-D glucopyranosyl-1,1-a-D-glucopyranoside) is a non-reducing disaccharide that act as an osmolyte and protect the cell membrane from denaturation . The increased level of expression was found in stressed leaves as compared to well-watered (Kosmas *et al*., 2006). Yue *et al.,* (2012) isolated a gene from *Arabidopsis thaliana (AtLOS5)* and transferred this gene in cotton. These transgenic cotton plants showed better drought tolerance by accumulating more ABA and proline thus regulating root and shoot growth, less membrane damage, reducing water losses through transpirtion resulting in less leaf wilting. A gene *IPT* (isopentenyltransferase gene) from *Agrobacterium tumefaciens* was used to transform cotton plants by Kuppu *et al* (2013). The transgenic cotton plants showed delayed leaf senescence, less fruit shedding by up-regulating cytokinin production, produced increased biomass of root and shoot and maintained chlorophyll contents under drought condition. A study was conducted by Shamim *et al* (2013) using trangenic approach. They transformed cotton plants with two genes *GUSP1* (Universal Stress Protein Gene), and *Phyto-B* (Phytochrome-B Gene). Transformed cotton plants showd enhanced drought tolerance at the vegetative, squaring, and boll formation stage. Transgenic plants produced more number of boll, increased boll weight and seed cotton yield under water defeciet condition.

Conclusion:

Drought stress is the main among abiotic stresses that disrupts plant growth and performance .It causes severe yield losses in cotton. Drought stress impairs many physiological and biochemical reactions in plant cell. ROS are normal byproducts of biochemical reactions of the cell. Under water stress condition ROS production exceeds the normal concentration that is toxic for the cell and degrades many cellular components and hamper their function, there should exist a balance between ROS production and ROS scavenging enzymes for a plant cell to carryout its normal functions. Certain genes in cotton like (Gh*APX1*), GhMT3a enhances the production of scavenging enzymes and protects the cotton plant from oxidative damage. So finding more such kind of genes can fulfil our future need for developing drought tolerant cotton with enhanced ROS scavenging and thus drought tolerance.

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8/31/2015