PHYSIOLOGICAL AND BIOCHEMICAL SCREENING OF DIFFERENT TURKISH LENTIL (LENS CULINARIS M.) CULTIVARS UNDER DROUGHT STRESS CONDITION

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Approval of the thesis:

PHYSIOLOGICAL AND BIOCHEMICAL SCREENING OF DIFFERENT TURKISH LENTIL (*Lens culinaris* M.) CULTIVARS UNDER DROUGHT STRESS CONDITION

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ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL SCREENING OF DIFFERENT TURKISH LENTIL (*Lens culinaris* M.) CULTIVARS UNDER DROUGHT STRESS CONDITION

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September 2012, 80 Pages

Legumes being the most important crops worldwide are limited in terms of adaptability and productivity mainly by the abiotic stresses. In this study, the aim was to understand tolerance mechanisms of lentil cultivars under drought stress by physiological and biochemical analyses. This study was carried out with six Turkish Lentil cultivars (Seyran, Kafkas, Malazgirt, Çağıl, Çiftçi, Özbek) subjected to drought stresses (10% and 15% PEG) and their physiological and biochemical properties were examined to select drought-tolerant and drought-sensitive cultivars. Drought stress was applied for 5 days to 7 days-grown lentil plants. 12-days old, stressed and control plant shoots and roots were analyzed in terms of physiological and biochemical parameters (length, fresh weight, ion leakage, proline, MDA and H_2O_2 content). According to these analyses, Seyran and Çağıl cultivars were selected as drought-tolerant and drought-sensitive, respectively. The responses of tolerant and sensitive cultivars were compared via analyzes of antioxidative enzyme activities (APX, CAT, GR and SOD) and protein profiles.

Keywords: Lentil, *Lens culinaris*, drought stress, antioxidative enzyme, drought-tolerant, drought-sensitive

ÖZ

KURAKLIK STRESİ ALTINDA TÜRK MERCİMEK (*Lens culinaris* M.)
ÇEŞİTLERİNİN FİZYOLOJİK VE BİYOKİMYASAL TARAMASI

Gökçay, Derya
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Eylül 2012, 80 Sayfa

Bu çalışma kuraklık stresine maruz kalmış altı çeşit mercimek tohumunun fizyolojik ve biyokimyasal özellikleri incelenerek kuraklığa dayanıklı ve hassas tohum seçilmesi için yürütülmüşütür. 7 gün büyümüş mercimekler, 5 gün boyunca kuraklık stresine maruz bırakılmıştır. 12 gün büyütülmüş stres ve kontrol bitkilerinin gövde ve kök örneklerinin fizyolojik ve biyokimyasal parametreleri (boy, yaş ağırlık, prolin miktarı, iyon geçirgenliği, MDA ve H_2O_2 miktarı) incelenmiştir. Bu analizlere gore Seyran ve Çağıl tohumları sırasıyla kurağa dayanıklı ve hassas tohumlar olarak belirlenmiştir. Dayanıklı ve hassas bitkilerin kuraklık stresi altında gösterdikleri farklı tepkiler, Seyran ve Çağıl tohumlarının antioksidatif enzim sistemleri (APX, CAT, GR, SOD) ve protein profilleri incelenerek karşılaştırılmıştır.

Anahtar Kelimeler: Mercimek, *Lens culinaris*, kuraklık stresi, antioksidatif enzim, kuraklığa dayanıklı, kuraklığa hassas

To my family,

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LIST OF ABBREVIATIONS

AsA	Absicis Acid
APX	Ascorbate peroxidase
CAT	Catalase
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
ETC	Electron transport chain
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
NaCl	Sodium cloride
O_2	Molecular Oxygen
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide radical
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
ROS	Reactive oxygen species
SEM	Standard error of mean
SDS	Sodium dodecly sulfate
SOD	Superoxide dismutase

CHAPTER 1

INTRODUCTION

1.1 Lentil

Lentil (*Lens culinaris* M.) is a diploid (2n=14), self-pollinating, annual grain legume. Warm temperate, subtropical and high altitude tropical regions are suitable for Lentil cultivation (Muehlbauer *et al*., 1995). According to the Andrews and McKenzie (2007), on around 4 million hectares from more than 40 countries Lentil is under cultivation.

Lens culinaris, which is one of the oldest grain legumes having remains dated to 11,000 BC from Greece's Franchthi cave, is originated from Near East and Central Asia (Sandhu and Singh, 2007).

1.1.1 Nutritional Value and Use

Lentil is one of the first foods that have been cultivated and it has been an important food since prehistoric times. It is an important dietary source of energy, protein, carbohydrates, fiber, minerals, vitamins and antioxidant compounds as well as diverse non-nutritional components like protease inhibitors, tannins, α-galactoside oligosaccharides and phytic acid (Urbano *et al*., 2007).

Lentils are low in fat and sodium, high in protein and are an excellent source of both soluble and insoluble fiber, complex carbohydrates, vitamins and minerals, especially B vitamins, potassium and phosphorus (Yadav *et al.,* 2007). With about 25% protein Lentils are the vegetable with the highest protein level after soybeans (Bhattacharya *et al.,* 2005) and they are also a very good source of cholesterol-lowering fiber (Yadav *et al*., 2007). 100 g of dried seeds contain 340-346 g calories, 12% moisture, 20.2 g protein, 0,6 g fat, 65.0 g total carbohydrates, 4 g fiber, 68 mg Ca, 325 mg P, 7.0 mg Fe, 29 mg Na, 780 mg K, 0.46 mg

thiamine, 0.33 mg riboflavin and 1.3 mg niacin (Adsule *et al.,* 1989; Muehlbauer *et al.,* 1985).

Lentils are mainly used as a food. Only a small amount of low quality lentils are used for livestock feed when degrading factors make them undesirable for human food (Market Outlook Report, 2010). As a food, they are used in soups, salads, snack food and vegetarian dishes.

1.1.2 Global Production

Lentils are categorized based on cotyledon and seed coat color. Red and green lentils are grown and consumed predominantly. Around 75% of world production is constituted by red lentils. Green lentils have yellow cotyledon and pale green seed while red lentils have an orange cotyledon and dark seed coat (McNeil *et al.,* 2007).

The lentil production is dominated by three countries, Canada, India and Turkey with around 70% of world production. According to the Market Outlook Report (2010), for the major lentil producing countries lentil production has been trending upwards since 2002. However, some of the top producers including Turkey have been highly variable and trending down. The sharp reduce in lentil production and the crop yield was as a result of the severe drought in 2007 and 2008 in Turkey (Figure 1.1 and Figure 1.2).

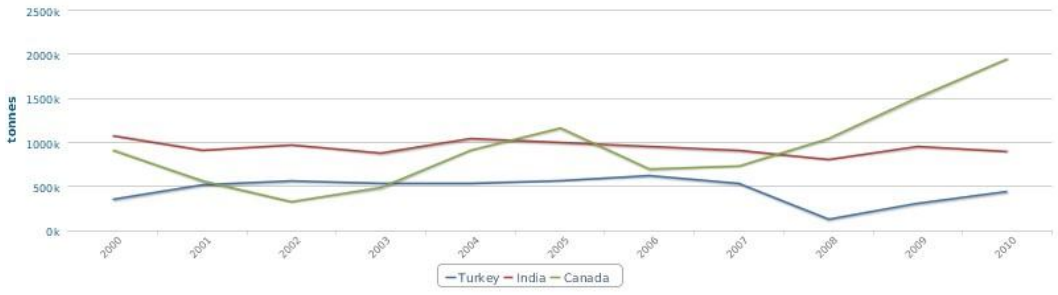

Figure 1.1 Top tree lentil producers in the world (2000-2010) (M=million, K=thousand) (FAOSTAT)

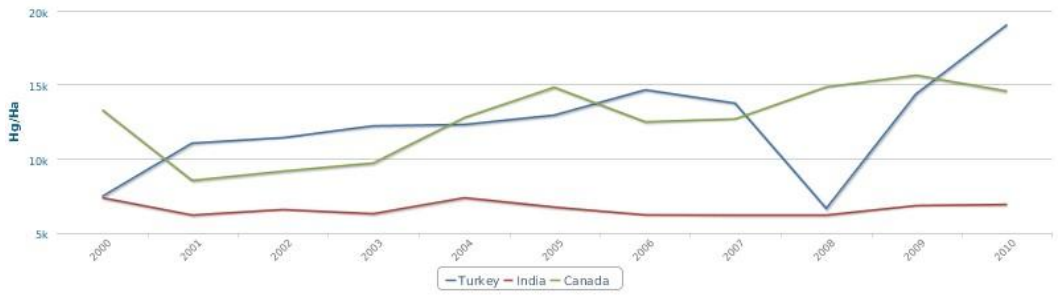

Figure 1.2 Lentil Yield (200-2010) (M=million, K=thousand) (FAOSTAT)

1.2 Environmental Stress

In physical terms, stress is defined as the average amount of force exerted per unit area. The shape and dimension of an object, which is exposed to a stress, changes as a response. On the other hand, in plants, it is hard to measure the exact force applied by stress and also a condition could be a stress factor for one plant while it is an optimum condition for another plant. Thus, it is difficult to define stress in biological terms (Mahajan and Tuteya, 2005). Biological stress can be defined as an overpowering pressure of some adverse force or condition that inhibits normal functions, growth and well-being of biological systems (Jones *et al.,* 1989).

Environmental stress is mainly divided into two groups. Biotic stress that occurs as a result of damages done by living organisms, and abiotic stress which is the negative impact of non-living factors on living organisms.

Throughout their lives, plants are subjected to several environmental stresses. They are frequently exposed to a number of abiotic stresses such as heat, salinity, flooding, heavy metals, radiation and soil structure as well as biotic stresses including pathogens, weeds and herbivores. Since plants are sessile, they are vulnerable to these environmental stress factors that adversely affect normal growth and metabolism of plants and cause reduction in crop productivity worldwide. (Aksoy, 2008; Mahajan and Tuteya, 2005).

Table 1.1 Various Biotic and Abiotic Stress Factors

BIOTIC STRESSES	ABIOTIC STRESSES
1. Viruses 2. Bacteria 3. Insects 4. Herbivores 5. Rodent 6. Weeds	1. Extreme temperatures (low & high) 2. Drought 3. Flooding 4. Salinity 5. Heavy metals 6. Pollutants 7. Oxidative stress 8. Soil structure (nutrient deprivation) 9. Extreme wind 10. Radiation

Legumes being the most important crops worldwide (Dita *et al.,* 2006) are limited in terms of adaptability and productivity mainly by the abiotic stresses. Only 10% of the arable land thought to be as non-stressed area and the other 90% of arable land are faced to at least one of the abiotic stresses (Blum, 1986). Abiotic stresses cause to lose hundreds of million dollars each year because of crop failure with a reduction of average yield by more than 50% for major crops (Mahajan and Tuteya, 2005). Among these abiotic stress factors, drought is the main limiting factor with its 26% followed by mineral stress with 20% and freezing stress with 15% (Blum, 1986).

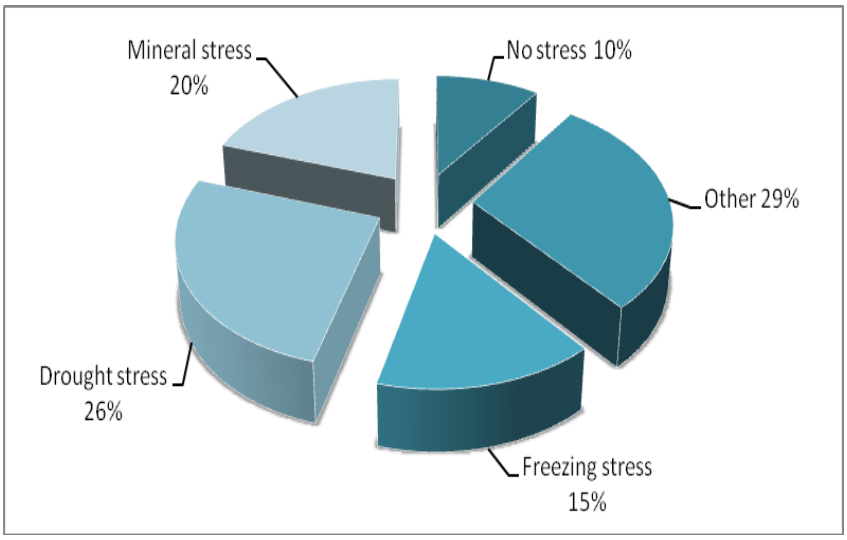

Figure 1.3 Effects of Abiotic Stress Factors on Crop Production

In response to these abiotic stress factors, plants have developed many stress tolerance mechanisms. These mechanisms may vary among species at different developmental stages (Ashraf, 1994), although basic responses to stress factors are conserved among most of the plant species (Zhu, 2001). In addition, different stress factors may lead to similar responsive adaptations like up-regulating the stress proteins and increasing compatible solute accumulation (Zhu, 2002).

All stress tolerance mechanisms are initiated by sensing the stress signals via the interaction of the extracellular materials with a plasma membrane protein. Following the perception of the signal, secondary signals are generated immediately (Agarwal and Zhu, 2005). Changes in the level of these secondary signals include calcium, inositolphospates (IPs) and reactice oxygen species (ROS), up-regulates further signals. Each secondary signal initiates a phosphorylation cascade, which triggers the expression of stress responsive genes and the transcription factors of these genes (Mahajan and Tuteya, 2005; Agarwal and Zhu, 2005). The stress responsive genes produce various osmolytes, antioxidants, proteins functioning in stress tolerance (Figure 1.2).

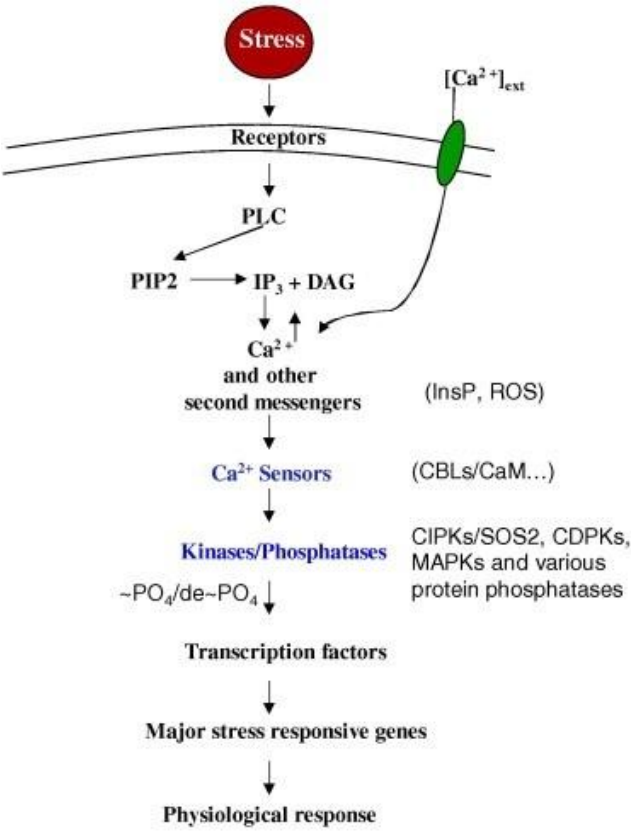

Figure 1.4 Signal Transduction Pathway in response to Abiotic Stress (Mahajan and Tuteya, 2005)

1.3 Reactive Oxygen Species (ROS)

Even though environmental stresses differ in damaging to plant metabolism, all of them have a common effect on plants that is increasing the amount of reactive oxygen species.

Although oxygen is required for the normal growth of plants, because of aerobic processes such as photosynthesis and cellular respiration, it leads to the production of reactive oxygen species (ROS) in mitochondria, chloroplast and peroxisomes. All ROS types have the capacity to cause oxidative damage to lipids, proteins and DNA. (Apel and Hirt, 2004).

In plants, ROS are produced continuously as byproducts of different metabolic pathways (Elster, 1991). Main source of ROS in plants is the photosynthetic electron transport system. There are two major processes involved in the

generation of ROS during photosynthesis. One is the direct photoreduction of oxygen to superoxide radical by photosystem I (PSI) electron transport chain. The other one is the oxygenase reaction of rubisco taken place in photorespiratory pathway (Arora *et al.,* 2002; Apel and Hirt, 2004). By these reactions, molecular oxygen is converted to superoxide by the removal of single electron. From this superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals are formed via series of reductions (Agarwal and Zhu, 2004).

Under nonstressful conditions, the production and removal of the ROS are controlled by various antioxidative defense mechanisms and plants are protected against harmful effects of these active oxygen molecules. However, the equilibrium between production and removal of ROS is disturbed by many abiotic stress factors resulting in rapid rising of the cellular level of ROS.

ROS are also thought to be functioning as signaling molecules in defense response pathways of plants. Among the reactive oxygen species, H_2O_2 is more likely to be a signaling molecule, since its half-life is longer than the other ROS, it is uncharged and able to diffuse through aqueous and lipid phases (Agarwal and Zhu, 2004).

1.3.1 Singlet Oxygen

Singlet oxygen is the electronically excited state of the molecular oxygen and less stable than the molecular oxygen. It destructs biological molecules by reacting with them.

The chlorophyll pigments, which are the components of photosynthetic reaction center, are the main source of the singlet oxygen ($^1O_2$). It is generated during the triplet chlorophyll production, in PSII.

1.3.2 Superoxide

A superoxide is formed when oxygen is reduced by a single electron, during the mitochondrial electron transport chain or during photosynthesis. During photosynthesis, ferredoxin or the electron carriers on the reducing side of PSI

donates their electrons to oxygen forming superoxide radical, $O_2^{\cdot -}$. It is thought that most of the superoxide anions are produced by the reduced ferredoxin (Arora *et al.,* 2002).

$$2O_2 + 2Fdred \rightarrow 2O_2^{\cdot -} + 2Fdox \qquad \textbf{Equation 1.1}$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \textbf{Equation 1.2}$$

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \textbf{Equation 1.3}$$

Throughout mitochondrial electron transport chain, molecular oxygen is reduced to superoxide anion either in the flavaprotein region of NADH dehydrogenase or in the ubiquinone-cytochrome region, as seen in the Figure 1.5 (Arora *et al.,* 2002)

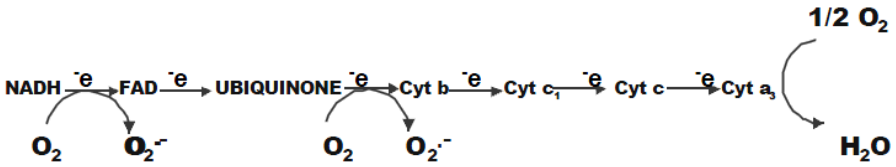

Figure 1.5 Superoxide Formation sites in mitochondrial electron transport chain. (Arora *et al.,* 2002)

Since its extra electron is unpaired, superoxide is a free radical and relatively unstable, so that it is either converted back to the molecular oxygen or further reduced to H_2O_2 ($2O_2^{\cdot -}$ **+ $2H^+$ → H_2O_2 + O_2**

Equation 1.2) (Desikan *et al.,* 2004).

1.3.3 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a product of normal metabolism taking place in peroxisome, chloroplast and electron transport chain in mitochondria. It acts both as an oxidant and as a reductant. Hydrogen peroxide is produced by the dismutation of superoxide and hdyroperoxy radical (HO_2^-) (Upadhyaya *et al.,* 2007; Aksoy, 2008).

Various environmental stresses induce hydrogen peroxide production via enzymes including NADPH oxidases localized on plasma membrane and cell wall peroxidases (Neill *et al*., 2002). Besides the normal metabolism, H_2O_2 can be generated by superoxide dismutases (SOD). Different types of SOD present at different locations in the cell, such as iron-containing SOD (FeSOd) being in chloroplast and managanase-containing SOD (MnSOD) being in mitochondria.

Besides being a toxic oxygen species, hydrogen peroxide functions as a signaling factor in stress signaling pathways. It initiates localized oxidative damage in leaf cells and changes the redox status of the surrounding to start antioxidative response.

1.3.4 Hydroxyl Radicals

Among the reactive oxygen species, hydroxyl radicals are the most damaging ones. Although hydrogen peroxide and superoxide radical do not directly destruct the vital cellular components like DNA, proteins and plasma membranes; they generate the damaging hydroxyl radicals. Hydroxyl radicals are produced according to the Haber-Weiss reaction in the presence of ferric ion, which is summarized as;

$$H_2O_2 + O_2^{\cdot -} \xrightarrow{Fe^{2}, Fe^{3+}} OH^{\cdot} + OH^{-} + O_2 \qquad \textbf{Equation 1.4}$$

Hydroxyl radicals destruct organic substances via oxidation, either by the addition of $OH^{\cdot}$ to the molecule or by the abstraction of a H atom from the molecule (Arora *et al.,* 2002).

1.4 ROS and Oxidative Damage to Biomolecules

Under normal conditions, production and removal of ROS are strictly controlled. When the level of ROS exceeds the defense mechanisms, organism is said to be under oxidative stress. Increased level of ROS cause various damages to biological molecules that can be seen in Figure 1.1 (Sharma *et al.,* 2012).

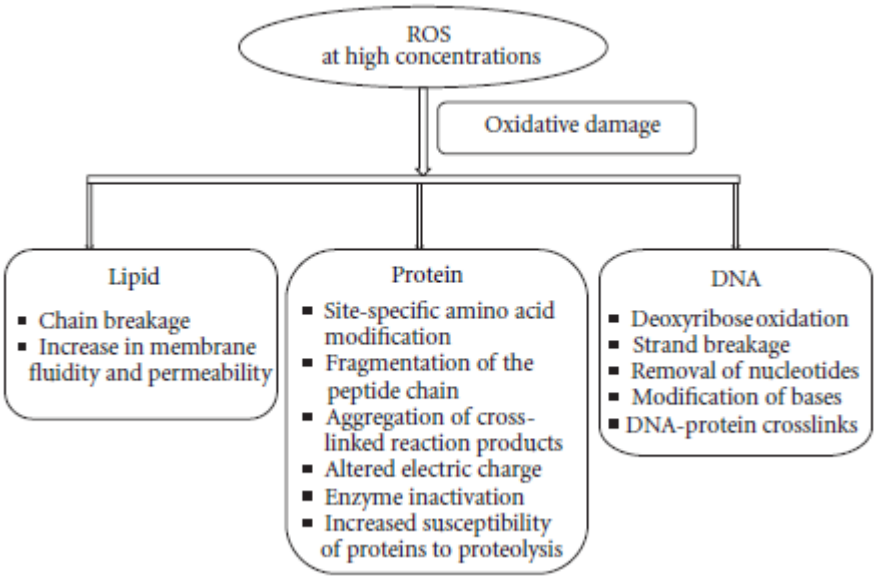

Figure 1.6 Oxidative damage of ROS on lipids, proteins and DNA (Sharma *et al.,* 2012).

When ROS levels increases, lipid peroxidation is triggered in cellular and organellar membranes. Lipid-derived radicals, which are produced as a result of lipid peroxidation, increases oxidative stress via reacting with proteins and DNA (Han *et al*., 2009; Tanou *et al*., 2009; Mishra *et al*., 2011; Sharma *et al*., 2012). Malondialdehyde (MDA) being one of the end-products of phospholipid peroxidation is responsible for the membrane damage (Halliwell and Gutteridge 1989).

On phospholipid molecules there are two main sites for the ROS attack; the double (unsaturated) bond between two carbon atoms and the ester linkage. Thus, polyunsaturated fatty acids are more vulnerable to the ROS attacks. Lipid peroxidation process consists of three stages as initiation, progression and termination (Smirnoff, 1995). Peroxidation of phospholipids ends up with many reactive species such as aldehydes, lipid epoxides, alcohols, alkoxyl radicals and alkanes and leads to the increase in membrane permeability (Sharma *et al*., 2012).

Alterations of proteins upon the ROS attacks can be either direct or indirect. Protein activity modulation via carbonylation, nitrosylation or disulphide bond formation constitute direct alteration, while indirect modification occurs by the

interaction with end-products of lipid peroxidation (Yamauchi *et al*., 2008). High levels of ROS lead to the site-specific aminoacid modification, peptide-chain fragmentation, increase in proteolysis susceptibility and charge alterations (Moller and Kristensen, 2004).

Amino acids of a protein have different vulnerability to ROS attacks. Thiol groups and iron-sulphur centers of sulphur-containing amino acids are the most vulnerable sites for ROS attack. oxidized peptides increases the proteolytic digestions (Cabiscol *et al*., 2000).

ROS are also responsible for the DNA damages. They oxidatively damage all types of DNA; nuclear, mitochondrial and chloroplastic. Since mitochondrial and chloroplast DNA lack repair systems, they are more susceptible to oxidative damages than the nuclear DNA (Richter, 1992). Although nuclear DNA has repair system, excess ROS leads to permanent damages to DNA that mostly result in changes at protein level ending up with malfunctioning or complete inactivation of proteins. Some of the damages of ROS attack on DNA are strand breakage, deoxyribose oxidation, nucleotide removal and modifications or removal of nucleotides (organic base part) that further results in mismatches with the other strand (Sharma *et al*., 2012).

Oxidative attacks on bases of DNA occur via $^{\cdot}OH$ addition to the double bonds, while sugar damages occur as a result of hydrogen removal from the deoxyribose (Dizdaroğlu, 1993). $^1O_2$ reacts only with guanine base, on the other hand H_2O_2 and $O_2^{\cdot -}$ do not react any of the bases (Dizdaroğlu, 1993; Halliwell and Aruoma, 1991).

Oxidative damages on the DNA sugars results in single-strand breakage. Attack of ROS produces deoxyribose radical via removal of hydrogen atom from the C4' position of the sugar, which in turn generate strand breakage (Evans *et al*., 2004).

The hydroxyl radical attacks on the DNA and related proteins lead to the DNA-protein cross-links, which can be lethal if replication or transcription takes place before repair system activation.

1.5 Antioxidant Defense Systems in Plants

Under normal conditions, ROS generation occurs at a low level and its generation and removal are balanced. This balance is disturbed by increasing ROS level due to the environmental stress factors (Sharma *et al*., 2012). For the removal of excess ROS and reducing oxidative damages, plants have evolved antioxidative defense systems consisting of non-enzymatic and enzymatic mechanisms.

Non-enzymatic antioxidants include major cellular redox buffers ascorbate (vitamin A) and glutathione (Apel and Hirt, 2004). Tocopherol (vitamin E), flavonoids, caretonoids and phenolics are also components of non-enzymatic antioxidant system. They take place in defense systems as well as influence plant growth and development. Enzymatic ROS scavenging system consists of several antioxidants enzymes including superoxide dismutase (SOD), catalase (CAT), enzymes of ascorbate-glutathione cycle being ascorbate peroxide (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Sharma *et al*., 2012; Desikan *et al.,* 2003).

1.5.1 Non-enzymatic Antioxidants

Among the non-enzymatic antioxidants, ascorbate is the most abundant one. It buffers cell against oxidative damage of high ROS level. It is synthesized in mitochondria and transferred to other cellular compartments including chloroplast as well as in apoplast (Desikan *et al*., 2003). Due to the ability to donate electrons, ascorbate is a powerful antioxidant (Sharma *et al*., 2012). Ascorbate protects membrane by directly reacting with $O_2^{\cdot -}$ and H_2O_2 and also takes role in removal of H_2O_2 via ascorbate-glutathione cycle (Zaefyzadeh *et al*., 2009; Foyer *et al*., 1997) that is shown in Figure 1.7-c.

Under environmental stress factors, level of ascorbate depends on the balance between ascorbate synthesis rate and turnover related to antioxidant demand (Chaves *et al*., 2002). Stress tolerant plants induce overexpression of enzymes related ascorbate synthesis.

The tripeptide glutathione is one of the major redox buffers in aerobic cells (Foyer *et al*., 2001). It is a low molecular weight nonprotein thiol and an important part of the antioxidative defense system. GSH is synthesized in cytosol and chloroplast and it is transferred to different cellular compartments (Sharma *et al*., 2012). Due to its reducing power, GSH has many roles in different biological processes such as cell growth, signal transduction, enzymatic regulation, protein synthesis and expression of the stress-related genes (Foyer *et al*., 1997).

As an antioxidant, GSH takes part in ascorbate-glutathione cycle, as well as reacting with hydrogen peroxide to be oxidized to GSSG (Desikan *et al*., 2003). Maintenance of reduced GSH is vital for the cell. Under environmental stress conditions the GSH/GSSG ratio is altered, promoting the GSH synthesizing enzyme's activity (Vanacker *et al*., 2000).

Tocopherols are another type of antioxidant involved in ROS scavenging. They present only green parts of plants. Tocopherols protect membrane components including lipids via reacting with O_2 in chloroplast (Ivanov and Khorobrykh, 2003). They also avoid chain propagation of lipid autooxidation.

Carotenoids belonging to the group of lipophilic antioxidants, are able to detoxify ROS (Young, 1991). Carotenoids inhibit oxidative damage via removing $^1O_2$ and also prevent $^1O_2$ formation by quenching triplet and excited chlorophyll to protect photosynthetic system. Besides ROS scavenging roles, carotenoids take place in signaling to enhance stress responses.

Phenolic compounds, such as flavanoids, esters, lignin and tannins are secondary metabolites found in plant tissues. They have variety of functions as antioxidants including, removal of reactive oxygen species, preventing lipid peroxidation, chelating transition metal ions and decreasing membrane fluidity. These processes limit peroxidation via hindering the ROS diffusion into the cells (Arora *et al*., 2000).

1.5.2 Enzymatic Antioxidative Defense Systems

Enzymatic ROS scavenge systems consists of superoxide dismutase (SOD), catalase (CAT) and enzymes of ascorbate-glutathione cycle (APX, MDHAR, DHAR). Although these enzymes function in different cell compartments, they work in collaboration as responding to ROS damage.

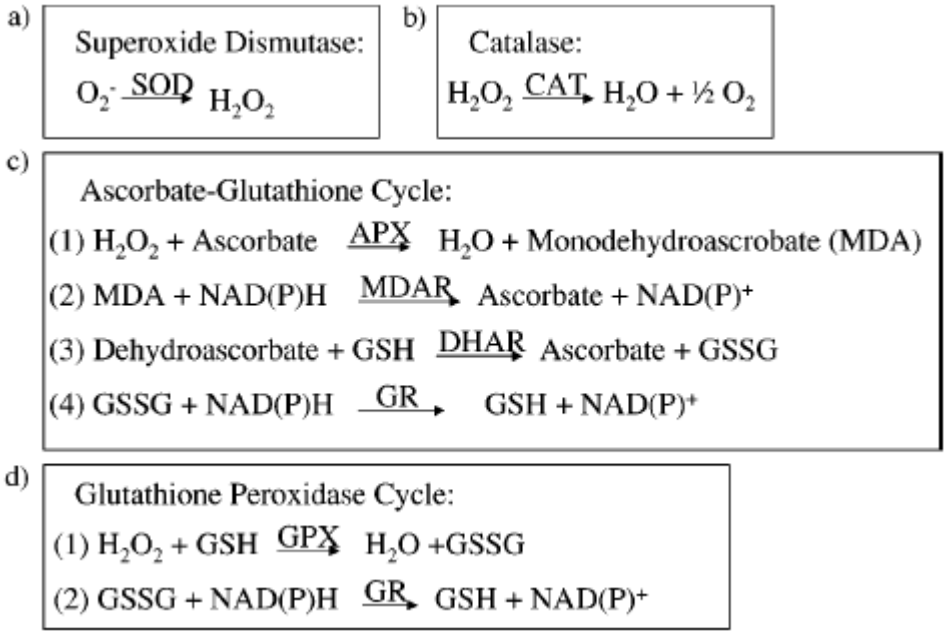

Figure 1.7 Enzymatic ROS scavenge mechanisms (Apel and Hirt, 2004)

SOD, being a metalloenzyme found mainly in three isoforms in plants. The isozymes are classified according to the metal co-factors of the enzyme and they operate at different parts of the cell. Manganese SOD functions in mitochondria, while iron SOD present in chloroplast and cupper/zinc SOD found in cytosol, chloroplast, peroxisome and mitochondria (Jackson *et al*., 1978). SOD catalyses the dismutation of superoxide to oxygen and hydrogen peroxide. As a result of environmental stresses, SOD activity of cells increases as a tolerance mechanism. High levels of SOD activity is an indicator of resistance to the stress factor (Zaefyzadeh *et al*., 2009).

Catalase is a ubiquitous, tetrameric, heme-containing enzyme (Sharma *et al*., 2012). It has high specificity for hydrogen peroxide and catalyzes the degradation of hydrogen peroxide to water and oxygen as shown in -b. Catalase is located

mainly in peroxisomes, where is the major cellular compartment of H_2O_2 synthesis via photorespiratory oxidation and β-oxidaiton of fatty acids (Scandalios *et al*., 1997; Corpas *et al*., 2008).

Ascorbate-glutathione cycle is an important regulator of the oxidative balance of cells (Nactor and Foyer, 1998). The AsA-GSH cycle consists of detoxification of H_2O_2 via the interactions of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Desikan *et al*., 2003).

Ascorbate peroxidase is a heme peroxidase and has an important role for balancing ROS level, as an AsA-GSH cycle member. It catalyzes the reduction of H_2O_2 to water by using two molecules of ascorbate. As an end product of this reaction, monodehydroascorbate (MDHA) is generated (Welinder, 1992; Patterson and Poulos, 1995). The MDHA radical is converted to ascorbate via MDHAR enzyme using NADPH as electron donor (Sakiham *et al*., 2000). Although ascorbate is regenerated from MDHA by enzymatic reactions, an amount of DHA is produced during the oxidation os ascorbate. This DHA is also reduced to ascorbate via DHAR enzyme oxidizing GSH to GSSG (Ushimaru *et al*., 1997). In order to maintain the cellular GSH/GSSG ratio, glutathione reductase, a flavoenzyme, regenerate GSH from GSSG.

To remove reactive oxygen species and eliminate their oxidative damage, the balance between the antioxidative enzymes is very important. Overexpression of one component could not be sufficient for the defense, while enhancing combination of enzymes has been shown to increase tolerance (Aono *et al*., 1995; Kwon *et al*., 2002).

1.6 Drought Stress

All living organisms have two fundamental natures, which are the cellular organization and requirement for liquid water (Wood, 2007). In plants, water has many functions. Water accounts for 80% - 95% of fresh weight of non-woody plants, being the main medium for transporting metabolites and nutrients. It is also the major solvent with its unique biophysical properties including high heat of

vaporization and high surface tension. Due to these properties water can remain liquid over a wide temperature range and solvate many molecules. Water has roles in a number of biochemical processes as a reactant like being electron donor. Besides these biochemical functions, water is the key component in maintaining cell turgor (Wood, 2007; Bartels and Souer, 2004).

Water stress may either develop due to excess of water or water deficit (Mahajan and Tuteja, 2005). Excess of water results in reduced oxygen in roots, which in turns results in disruption of root functions such as respiration and nutrient uptake. The more common water stress is water deficit, which is called as drought. Drought is the limitation of water over a prolonged period of time.

Water deficiency is the main limiting factor to crop production worldwide. Drought is a regular and severe constriction to crop yields in many areas of the world where lentils were grown (McWilliam, 1986).

1.6.1 Physiological and Biochemical Effects of Drought Stress on Plants

1.6.1.1 Effect of Drought Stress on Cell Integrity and Plant Growth

As a result of water removal from the cell membrane, lipid bilayer structure of the membrane is disrupted and membrane proteins is displaced. This leads to lose of membrane integrity, selectivity and interruption of cellular compartmentalization. Due to the intense water deficit, cells shrink and mechanical strain occurs on membranes. All these defects damage the functioning of transporters, ions and membrane based enzymes (Mahajan and Tuteja, 2005). As a consequence of cell shrinkage cellular volume decreases, resulting in viscous cellular content that increases protein aggregation and denaturation via protein-protein interaction (Hoekstra *et al*., 2001).

Another effect of water deficit is the reduction of vegetative growth. Under drought stress conditions cyclin-dependent kinase activity reduces, resulting in slower cell division and even inhibition of growth (Shuppler *et al*., 1998). Leaf growth is more sensitive than the root growth to water deficiency, as reducing leaf area is

advantageous for plants decreasing water loss through transpiration under drought conditions (Mahajan and Tuteja, 2005).

1.6.1.2 Effect of Drought Stress on Photosynthesis

The rate of photosynthesis decreases due to the stomatal closure, under water deficit conditions. Photosynthetic system in plants depends on the availability of CO_2, especially in photosystem II. Under drought stress, the primary reason of the decline in photosynthetic rate is the CO_2 deficiency (Meyer *et al*., 1998). The closure of stomata under drought stress leads to the decrease in intracellular CO_2 levels, which in turns results in over-reduction of electron transport chain components. Thus, the electrons are transferred to oxygen at photosystem I generating reactive oxygen species (Mahajan and Tutja, 2005).

Water deficiency also results in decreasing Rubisco, a carboxylating enzyme, activity thus limits photosynthesis (Bota *et al*., 2004). In plants, the amount of rubisco is controlled by the rate of synthesis and degradation (Reddy *et al*., 2004). Under drought stress conditions, synthesis of rubisco decreases.

Normally, rubisco activase regulates the active site conformation of rubisco, removes inhibitors allowing the enzyme to undergo carboxylation (Chaves *et al*., 2002). During water deficiency, rubisco activase activity decreases due to the reduced ATP concentrations. Thus, removal of inhibitors from rubisco active site is impaired (Tezara *et al*., 1999).

1.6.1.3 Overproduction of ROS under Drought Stress

Under drought stress conditions, production of reactive oxygen species is increased in several ways. Down regulation of photosystem II due to the water deficiency, results in an imbalance between generation and consumption of electrons. The changes occurring in photosystem II results in the dissipation of excess light energy generating reactive oxygen species including O_2^-, $^1O^+$, H_2O_2 and OH (Peltzer *et al*., 2002). Superoxide radicals are also generated due to the changes in the photosynthetic electron transport chain under drought stress.

Inhibition of CO_2 assimilation, coupled with the changes in photosystem I & II and electron transport chain result in enhanced ROS production (Asada, 1999). During water deficiency stomatal closure results in reduced CO_2 fixation that leads to reduction in $NADP^+$ production via Calvin cycle. Thus the lack of electron acceptor results in overproduction of electrons through photosynthetic electron transport chain and these electrons are trapped by O_2, generating ROS (Sharma *et al*., 2012).

Oxidative damages occurring during drought stress are due to the overproduction of reactive oxygen species. ROS attack the most important cellular components to disrupt their function. Some of the ROS dependent damages are amino acid and protein oxidation, DNA nicking and lipid peroxidation (Asada, 1999; Reddy *et al*., 2004). If the damaged components of the cell are not repaired, the cell death occurs, eventually.

1.6.2 Drought Avoidance and Drought Tolerance in Plants

Plants respond to drought stress and adapt themselves to drought conditions by many different anatomical, morphological, physiological and biochemical changes. Plants also develop strategies to cope and resist drought stress, including drought avoidance and drought tolerance (Reddy *et al*., 2004). Drought avoidance is the ability of plants to preserve water potential under water deficiency. It is mainly achieved via morphological changes like stomatal closure, decreased leaf area and development of extensive root systems that increases root/shoot ratio (Levitt 1980). On the other hand, drought tolerance is the ability to withstand water deficiency by utilizing adaptations to maintain normal metabolism at low water potentials (Wood, 2007). Drought tolerance strategies include cell and tissue specific physiological, biochemical and molecular mechanisms. Accumulation of specific proteins and stress metabolites, stress regulatory gene expressions, decline in photosynthetic rate, and upregulation of antioxidative enzymes are some of these mechanisms (Figure 1.8) (Reddy *et al*., 2004).

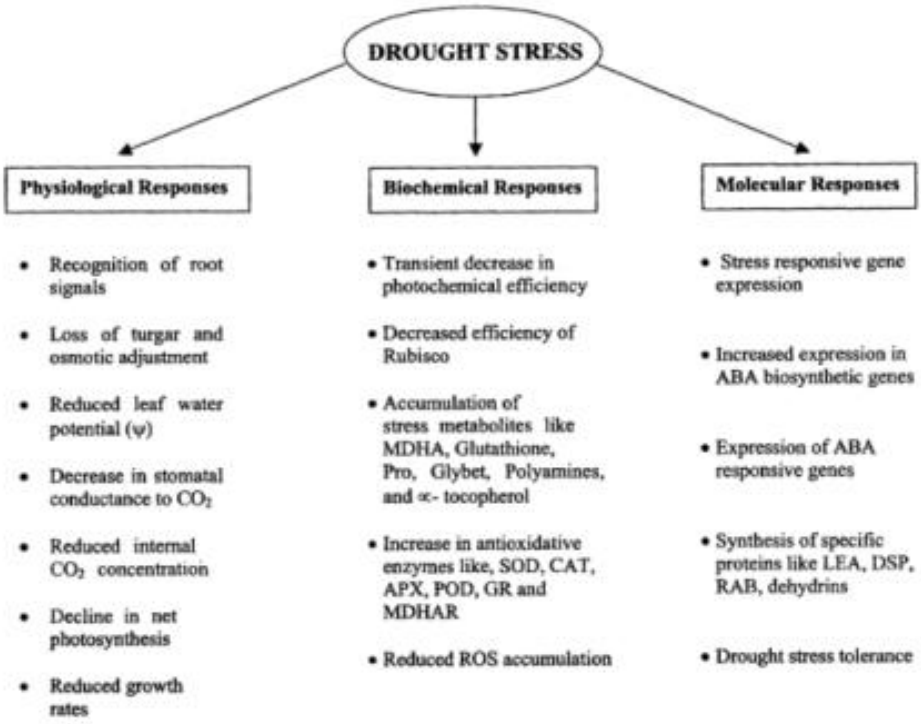

Figure 1.8 Drought stress responses of higher plants (Reddy *et al.,* 2004)

1.6.2.1 Response of Stomata to Drought Stress

Temperature increase and rapid drop in humidity result in water deficiency in plants. In addition, dry air mass leads to rapid water losses from plants. These kind of atmospheric changes lead to increase in the vapor pressure gradient between plant's leaves and the air, which causes an increase in the transpiration rate. Besides transpiration, water loss from soil is also enhanced due to the raise in the vapor pressure gradient (Mahajan and Tuteja, 2005).

As a response to the water deficiency, plants close their stomata to prevent water loss via transpiration (MansWeld and Atkinson, 1990). Closure of stomata can occur in two ways. It can be as hydropassive closure, which does not include any metabolic activity but occurs as the direct evaporation of water from the guard cells. On the other hand, the hydroactive closure of stomata requires ions and metabolites and results in reversal of the ion fluxes that is responsible for stomatal opening. This process is ABA regulated.

The transport of ABA into root xylem is regulated by factors like pH. The increase in pH of xylem sap due to the water deficiency enhances the ABA accumulation in the root xylem and its transport to shoots. At the same time, increasing transpiration rate leads to increase in leaf pH resulting in high ABA concentrations in leaves, which in turns promotes efflux of potassium ions from guard cells and results in stomatal closure (Mahajan and Tuteja, 2005).

1.6.2.2 Osmoprotectant Accumulation in response to Drought Stress

One of the main strategies of plants to cope with drought stress is osmotic adjustment. In this process, plants try to decrease their osmotic potential by overproduction of different types of solutes, known as compatible solutes or osmolytes (Smirnoff, 1998; Ashraf and Foolad, 2007; Mahajan and Tuteja, 2005). Compatible solutes are low molecular weight, highly soluble compounds. These solutes are nontoxic at high concentrations and most importantly compatible solutes do not get involve in normal metabolic processes of cells. Their primary function is turgor maintenance via cellular osmotic adjustment by increasing the number of particles in solution. Additionally they have other protective roles including detoxification of ROS, stabilization of protein structures and membrane integrity protection (Smirnoff, 1998; Bartels and Souer, 2003).

The compatible solutes tha accumulate during stress conditions include organic solutes such as proline and other amino acids, polyamines and quaternary ammonium compounds like betaines or ions such as K^+, Na^+ and Cl^- (Tamura *et al*., 2003). In addition, sucrose polyols, oligosaccharides and sugar alcohols such as mannitol and sorbitol are produced as osmolytes (Reddy *et al*., 2004; Bartels and Souer, 2003).

Glycine betaine (GB) is the most abundantly produced quaternary ammonium compound as a response to drought stress. GB is found in chloroplasts, where it protects thylakoid membrane preserving photosynthetic machinery. It is synthesised from choline via betaine aldehyde using cholinemonooxygenase and betaine aldehyde dehyrogenase (Figure 1.9).

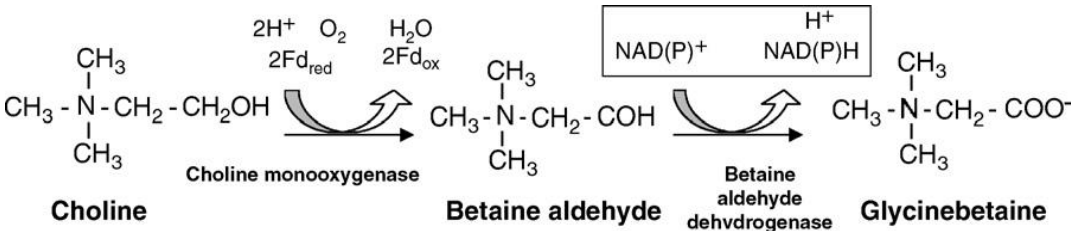

Figure 1.9 Glycine betaine synthesis (Ashraf and Folad, 2007)

Proline is one of the amino acids, accumulates in large quantities as a response to many environmental stresses including drought. During drought stress, proline amount increases in cytosol where it provides osmotic adjustment. Besides osmotic adjustment proline also stabilize proteins and membranes, removes reactive oxygen species and maintains cellular redox potential.

Proline is synthesized from L-glutamic acid via pyroline-5-carboxylate synthetase and pyroline-5-carboxylate reductase enzymes (Figure 1.10). Proline accumulation during dehydration is enhanced not only by the activation of proline synthesis but also by the inactivation of proline degradation.

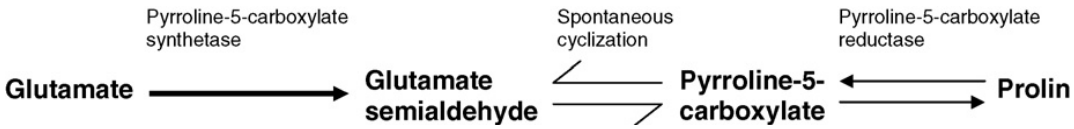

Figure 1.10 Proline synthesis (Ashraf and Folad, 2007)

1.6.2.3 Response of Abcisic Acid to Drought Stress

Abscisic acid (ABA), a plant hormone, is normally produced for proper development of plants. Many studies suggest that osmotic stress caused by salt and drought stresses is transmitted through ABA-dependent or ABA-independent pathways. Studies have demonstrated that ABA and environmental stresses including salinity and drought result in increased Calcium levels, which is an important component of signaling pathway.

Under drought stress, ABA production is enhanced as a response and tolerance to dehydration. The biosynthesis of ABA is a side-branch of the carotenoid pathway and many enzymes of this biosynthetic pathway is upregulated during dehydration (Seo and Koshiba, 2002). After exceeding a certain threshold level ABA leads to the stomatal closure and induces the expression of many genes related to defense against drought stress (Hirt and Shinozaki, 2004; Bartels and Souer, 2003).

ABA also allows seeds to surpass the stress condition and germinate only when the conditions are suitable for germination and the growth of the seed (Mahajan and Tuteja, 2005).

1.6.3 Lab-on-a-chip Technologies for Protein Analysis

Lab-on-a-chip or microfluidic technologies shrink processes to very small dimensions. They allowing very little sample volumes, to shorten analysis time and to automate the analysis process (Goetz *et al*., 2004). Microfluidics also, allows the active control of fluids in microfabricated channels, which are a few micrometers and have no moving parts. In these chips many functional elements are combined such as, emulation of pumps, valves, dispensers for sample handling, a separation column, a reaction system and detection. The recent developments of lab-on-a-chip or microfluidic systems offers an alternative for protein analysis (Kuschel *et al*., 2002).

Protein purification, quantitation and identification are the main tasks of protein characterization (Goetz *et al*., 2004). The first commercial lab-on-a-chip analysis system for protein sizing and quantitation is the Agilent 2100 bioanalyzer (Agilent Technologies Deutschland). This system provides a rapid and automated electrophoretic protein separation. It integrates sample handling, separation, staining, detection and analysis (Kuschel *et al*., 2002).

The principle of analysis with Agilent 2100 bioanalyzer is an electrophoretic process. The microchannels in the chips are filled with polymerizing gel and the proteins are separated according to their molecular weight. A fluorescent dye stains the proteins during separation process. At the end of the separation

fluorescence is detected with laser. The results are analyzed aoutomatically with the software of the system. Besides the protein samples, a sizing ladder is also run on the chip in order to generate a standard curve for determining size of the unknown proteins. The Agilent 2100 bioanalyzer software also provides the relative concentration of different proteins. The determination of relative concentrations is achieved by one-point calibration with the upper marker. The peak area of the upper marker with known concentration is compared to the peak area of unknown sample (Goetz *et al*., 2004).

The chip-based analysis of proteins is comparable to SDS-PAGE analysis, the current standard method for protein sizing, in terms of sensitivity, sizing accuracy and reproducibility. In the study of Kuschel *et al.* (2002), it is stated that the resolution of the chip-based separation is comparable and even better than the SDS-PAGE analysis. According to this study, the resolution of the chip-based separation improves when molecular weight is increasing. While SDS-PAGE has an optimal resolution for specific, narrower size ranges, the chip-based analysis provides high resolution across a large size range. This is due to the linear polymer gel with dynamic pores used in the chip-based analysis, while SDS-PAGE uses cross-linked gel separation depends on pore size.

Although the sensitivity of the chip-based process is comparable to the standard SDS-PAGE gels, SDS-PAGE allows larger sample to be loaded, increasing total protein amount and removing preconcentration steps. The sensitivity of chip-based system depends on the ionic strength of the sample buffer due to the electrokinetic injection. The sensitivity is enhanced by lowering salt concentration in both chip-based analysis and SDS-PAGE analysis, while SDS-PAGE is only slightly affected by the ionic strength.

The sizing accuracy of chip-based and SDS-PAGE analysis depends on the protein characteristics like isoelectric focusing, amino acid sequence, structure and the presence of side chains. Besides sizing, chip-based analysis also provides relative and absolute protein quantitation.

The lab-on-a-chip system is comparable to SDS-PAGE in terms of sizing and sensitivity but its resolution is higer and analysis time is greatly reduced. Lab-on-

a-chip system has additional advantages such as, reduced manual labor, ease of use, automated separation, detection and data analysis, good reproducibility and reduction of harmful wastes (Goetz *et al*., 2004).

1.6.4 Stress Tolerance Enhancement by Genetic Approaches

For crop improvement, it is possible to transform many grain legumes, although the rate of recovery of transgenic lines may be low in some cases (Chandra and Pental, 2003; Somers *et al,*. 2003; Dita *et al,*. 2005). Both particle bombardment and *Agrobacterium*-mediated transformation have been used for DNA delivery into either embryogenic or organogenic cultures (Dita *et al*., 2005). Transformation has been mainly based on *A. tumafaciens* infection. The inserted DNA can be a specific gene that has a specific biochemical function, or a regulatory gene or multiple genes to generate long-term resistance.

Agrobacterium transformation of legumes has been described as difficult to perform due to the poor susceptibility of regenerable legume tissues to *Agrobacterium* strains (Akçay *et al*., 2003). In recent years, with the identification of more virulent strains *Agrobacterium*-mediated transformation was improved for many legume species (Öktem *et al*., 2008). A number of legumes have been transformed to enhance tolerance against biotic stresses, including insects and viruses (Walker *et al*., 2000; aragao *et al*., 2002). On the other hand, to enhance abiotic stress resistance is not as easy as in the case of biotic stress since abiotic stresses disrupt various cellular functions and activates complex metabolic pathways (Dita *et al*., 2005). Therefore, for the successful transformation a better physiological and molecular understanding of abiotic stresses are required.

1.7 Studies Done in Plant Molecular Biology and Biotechnology Laboratory

Lentil (*Lens culinaris* M.) plant have been studied in many aspects in Plant Molecular Biology and Biotechnology Laboratory, in METU. Tissue culture studies were performed by Mehrzad Mahmoudian in her study of “Optimization of tissue culture conditions and gene transfer studies in lentil” in 2000. Regeneration and transformation of lentil was studied by Ufuk Çelikkol (2002) and Hamdi Kamçı

(2011) in “Regeneration of Lentil (*Lens culinaris*) & genetic transformation by using Agrobacterium tumefaciens-mediated gene transfer” and “Genetic transformation of lentil with transcriptional factors and evaluation of abiotic stress tolerance” projects, respectively. Also, effects of different environmental stresses on antioxidative defense systems of lentil cultivars was studied by Ebru Bandeoğlu (2001), Işın Nur Cicerali (2004) and Oya Ercan (2008). Lastly, gene expression of Lentil under stress conditions was studied by Emre Aksoy in his study of “Effect of drought and salt stresses on the gene expression levels of antioxidant enzymes in lentil (*Lens culinaris M.)* seedlings” in 2008.

1.8 Aim of the Study

Crop plants, which are the important components of human diets, are exposed to many environmental stresses throughout their growing period. In most of the time these environmental stresses causes reduction in crop yield and leads to loss of million dollars each year. To overcome this reduction, it is essential to generate stress-tolerant crop lines. For this purpose, understanding the defense mechanisms of plants under stress conditions is very important.

In this study most cultivated Turkish lentil (*Lens culinaris* Medik.) cultivars (Seyran, Malazgirt, Çağıl, Çiftçi, Özbek, Kafkas) have been exposed to drought stress by applying 10% and 15% PEG to 7 days old seedlings and 12 days old plants were screened for determining drought-tolerant and drought-sensitive cultivars with respect to certain physiological and biochemical parameters under drought stress. Drought-tolerant and –sensitive cultivars have been further analyzed to observe their different responses under stress condition. The analyses listed below were performed to determine the effects of drought stress on the different lentil cultivars in a comparative manner.

i. Fresh weight and length measurements
ii. Proline content determination
iii. Lipid peroxidaiton through MDA content and ion leakage tests
iv. Hydrogen peroxide content determination
v. Determination of antioxidant enzyme activities (APX, CAT, GR)
vi. Total protein analyses through SDS-PAGE and Bioanalyzer

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Materials

For this study, 7 Turkish Lentil (*Lens culinaris* M.) cultivars, named as Seyran, Çiftçi, Malazgirt, Çağıl, Kafkas, Özbek and Meyveci, were used. These cultivars were supplied by Central Research Institute for Field Crops (TARM). Among these cultivars only Meyveci was a green type cultivar and the others were all red type cultivars. After preliminary studies, Meyveci cultivar was excluded.

According to the information obtained from TARM, Meyveci and Malazgirt cultivars are summer-type cultivars. Meyveci are grown mainly in Ankara with a 130-160 kg/da yield. Other five cultivars, Seyran, Kafkas, Özbek, Çağıl and Çiftçi are winter-type cultivars. Among these Kafkas has not much economical value, since cultivation of Kafkas is spreading newly. Çiftçi and Özbek cultivars are cultivated in central Anatolia region with 150-195 kg/da and 170-200 kg/da yields respectively. The remaining two cultivars Seyran and Çağıl are cultivated in southeastern Anatolia region, where about 90% of red lentils cultivated. Seyran are grown mainly in Diyarbakır and around of it, while Çağıl cultivar can be grown in whole southeastern Anatolia region. Both of them have high yields; Seyran has a yield of 150-200 kg/da and Çağıl has 165-240 kg/da.

2.1.2 Chemicals

The chemicals used in this study were obtained from Merck Chemical Company, Sigma Chemical Company or Applichem Chemical Company. The solutions used in experiments were all prepared with dH_2O.

2.2 Methods

2.2.1 Growth of Plants

Seeds were surface sterilized with 20% ethanol 3 times and after each time they were washed with distilled water. After sterilization, seeds were put in falcon tubes filled with dH_2O and left at dark for overnight to be imbibed. The imbibed seeds were distributed to cheesecloth covered plastic pots (250 mL) and filled completely with ½ x Hoagland's solution (Hoagland and Arnon 1950). Each pot contained 8-10 seeds. Before stress application, seeds were grown for 7 days in the controlled growth chamber at 22±2 °C and 45% humidity with 18 h light – 6 h dark photocycle.

2.2.2 Drought Stress Application

At the 7^{th} day of growth, drought stress was applied via Polyethylene Glycol (PEG 6000) treatment. ½ x Hoagland's solution containing 10% (w/v) and 15% (w/v) PEG 6000 was used to generate drought stress condition, which are decided by preliminary studies. Besides these drought-treated groups, there was also control group containing ½ X Hoagland's solution without PEG 6000. After stress application all seedlings were grown another 5 days in the growth chamber with the same physical conditions. At the end of the 12^{th} day shoots and roots of the seedlings were collected for further analysis including physiological parameters such as fresh weight, lengths of shoot and root tissues; membrane permeability; proline, MDA, H_2O_2 contents; enzyme activities (APX, CAT, GR and SOD); SDS-Page analysis and Bioanalyzer.

2.2.3 Fresh Weight and Physiological Analysis

Both shoot and root tissues of the 12 days-old control and drought-treated plants were weighed immediately after they were collected. Lengths of the both tissues were measured. All plants were photographed to observe the effects of drought stress on growth of the plants.

2.2.4 Measurement of Membrane Permeability

Membrane permeability was determined according to the method of Nanjo *et al.* (1999). For conductance of shoots and roots total tissues were separately put into falcon tubes and filled with 5 ml of 0.4 M Mannitol solution. Samples were incubated in a shaker for 3 hours. After incubation the initial conductivities were measured by conductivity meter, Mettler Toledo MPC 227 and recorded as C_1. Then, samples were incubated at boiling water for 10 min and after they reach to RT, total conductivities by complete membrane disruption were measured and recorded as C_2. The final conductivity was calculated as percent ion leakage, (C_1/ C_2)*100.

2.2.5 Determination of Proline Content

Proline content was determined according to the method of Bates *et al*. (1977). Around 0.1-0.3 g of shoot and root tissues from control and treated samples were used. Samples were homogenized in liquid nitrogen with mortar and pestle and then extracted in 1 ml 3% sulphosalicilic acid. The extracts transferred into eppendorf tubes were centrifuged with MPV centrifuge at 14000 rpm for 5 min. at 4°C. For each sample, 0.2 ml acid ninhydrin, 0.2 ml 96% acetic acid, 0.1 ml 3% sulphosalicilic acid and 0.1 ml supernatant were put in a new eppendorf tube and incubated at 96°C for 1 hour for the complete protein hydrolysis. After incubation, 1 ml toluene was added in each eppendorf tube. The tubes were vortexed and centrifuged at 14000 rpm for 5 min at 4°C. The red-colored upper phase was taken to measure absorbance at 520 nm wavelength as toluene being blank. To determine the proline concentration in the range of 5-500µm, a standard curve was constructed.

2.2.6 Determination of MDA & H_2O_2 Content

MDA and hydrogen peroxide contents were estimated according to the Okhawa *et al*. (1979). 0.1-0.3 g of shoot and root tissues were homogenized in liquid nitrogen by using mortar and pestle. Homogenized tissues were suspended in 2 ml 0.1% TCA solution and centrifuged by MPV centrifuge at 10000 rpm at for 15 min.

For determination of MDA amount, from each sample 0.5 ml supernatant were taken and 0.5 ml 0.1M Tris/HCl buffer at pH 7.6 and 1 ml TCA-TBA-HCl solution were added in a new eppendorf tube. Samples were incubated at 95°C for 45 min. After incubation they were put into ice until reaching to room temperature and centrifuged at 10000 rpm for 5 min. Absorbance of supernatant was measured at 532 nm wavelength and to correct the non-specific turbidity absorbance at 600 nm was measured and subtracted. The amount of MDA was estimated using the extinction coefficient 155 $M^{-1}.cm^{-1}$.

For determination of hydrogen peroxide content, 0.5 ml supernatant was taken from each sample. For each of them 0.5 ml 0.1 M Tris/HCL buffer at pH 7.6 and 1 ml KI were added. Samples were incubated for 90 min at dark conditions. After incubation absorbance at 390 nm was measured. The H_2O_2, amount was determined with the extinction coefficient 39.4 $mM^{-1}.cm^{-1}$.

2.2.7 Protein Determination

For protein amount determination of shoot and root extracts, Bradford method (Bradford, 1976) was used. 500 mg of Commassie Brilliant Blue G-250, 250 ml of 95% EtOH and 500 ml of 85% (w/v) phosphoric acid were used to prepare 5X Bradford reagent. The solution was completed to 1 L with dH_2O and filter-sterilized. Before each experiment the reagent was diluted to 1X.

20 µl extracts from shoots and 40 µl extracts from roots were taken and diluted with 480 µl and 460 µl of dH_2O in test tubes, respectively. 5 ml of 1X Bradford reagent (Bradford, 1976) was added on the tubes and incubated for 10 min. After incubation, absorbances were measured at 595 nm. Mixture of 500 µl water and 5 ml Bradford reagent was used as blank.

Protein amounts were determined according to the Bradford standard curve that is constituted using Bovine Serum Albumin (BSA) with concentrations 0.01, 0.02, 0.04, 0.06, 0.10,0.16 and 0.20 mg/mL.

2.2.8 Determination of APX Activity

The Ascorbate Peroxidase activity was measured according to the Wang *et al.* (1991). 0.2-0.5 g tissue were grinded in mortar and pestle with liquid nitrogen and suspended in 1 ml 50 mM potassium phosphate buffer at pH 7.8 including 1 mM EDTA and 2% PVP. The suspensions were centrifuged at 13000 g for 20 min at 4°C by using MPV centrifuge. In an assay medium containing 50 mM potassium phosphate buffer at pH 6.6 and 2.5 mM ascorbate, the enzyme extract containing 100 µg of protein, which is determined by Bradford method, was added. With the addition of hydrogen peroxide the reaction was started and the decline in the concentration of ascorbate was measured at 290 nm with Schimadzu double-beam spectrophotometer continuously for 2 minutes, using assay medium without enzyme as blank. From the initial rate, the enzyme activity was calculated. (Extinction coefficient of ascorbate = 2.8 $mM^{-1}.cm^{-1}$)

$$H_2O_2 + \text{Ascorbate} \xrightarrow{\text{APX}} H_2O + \text{Monodehydroascorbate} \qquad \textbf{Equation 2.1}$$

2.2.9 Determination of CAT Activity

To determine Catalase activity method of Chance and Maehly (1995) was used. 0.2-0.5 g tissue were grinded in mortar and pestle with liquid nitrogen and suspended in 1 ml suspension solution composed of 50 mM potassium phosphate buffer at pH 7.8, 1 mM EDTA and 2% PVP. The suspensions were centrifuged at 13000 g for 20 min at 4°C. Enzyme extract containing 100 µg of soluble protein determined by Bradford method and 50 mM potassium phosphate buffer at pH 7.0 were mixed and reaction was started with the addition of hydrogen peroxide. The decrease in H_2O_2 concentration was recorded for 2 min by measuring the absorbance at 240 nm wavelength with Schimadzu double beam spectrophotometer. The initial rate of the enzyme was used to calculate enzyme activity. (Extinction coefficient of H_2O_2 = 39 $mM^{-1}.cm^{-1}$)

$$H_2O_2 \xrightarrow{\text{CAT}} H_2O + \tfrac{1}{2}\,O \qquad \textbf{Equation 2.2}$$

2.2.10 Determination of GR Activity

Method of Sgherri *et al*. (1994) was used to determine the glutathione reductase activity. 2-0.5 g tissue were grinded in mortar and pestle with liquid nitrogen and suspended in 1 ml suspension solution composed of 50 mM potassium phosphate buffer at pH 7.8, 1 mM EDTA and 2% PVP. The suspensions were centrifuged at 13000 g for 20 min at 4°C by using. Enzyme extract containing 100 µg protein determined by Bradford method was added into an assay medium containing Buffer-EDTA-$MgCl_2$ solution (200 mM potassium phosphate buffer at pH 7.5, 0.25 mM Na_2EDTA and 1.875 mM $MgCl_2$) and 5 mM GSSG. By adding 0.5 mM NADPH the reaction was started and oxidation of NADPH was recorded by measuring the absorbance at 340 nm continuously for 2 min. from the initial rate, the enzyme activity was calculated. (Extinction coefficient of NADPH = 6.2 $mM^{-l}.cm^{-l}$)

Equation 2.3

2.2.11 Determination of SOD Activity

One dimensional native polyacrylamide gel electrophoresis was used to determine the SOD acitivity of control and drought treated plants. Staining of the gels was carried out by negative activity staining according to the method of Beauchamp and Fridovich (1971).

2.2.11.1 Sample Preparation

Shoot and root tissues (~0.2 g) were grinded with cold mortar and pestle in liquid nitrogen and grinded samples were homogenized in 800 µl homogenization buffer, which was composed of 9 mM Tris-HCl, pH 6.8 and 13.6 % glycerol. The homogenates were centrifuged at 10000 rpm for 30 minutes at 4°C. Supernatants were used for SOD assay.

2.2.11.2 One Dimensional Native Polyacrylamide Gel Electrophoresis (1-D PAGE)

To carry out 1-D PAGE, separating gel (5 ml 12%) and stacking gel (2.5 ml 5%) were prepared according to Laemmli (1970). Gels were polymerized in Cleaver Minigel Apparatus. Equal amounts of proteins (50 µg) determined by Bradford method (Bradford, 1976) were loaded to each well. Electrophoresis was carried out for about 3 hours under constant current of 6 mA for stacking gel and 9 mA for separating gel.

2.2.11.3 Negative Activity Staining

50 ml of negative activity stain, composed of 50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 0.2 % (v/v) N,N,N'N'-tetramethyl ethylene diamine (TEMED), 3 mM riboflavin and 0.25 mM nitroblue tetrazolium (NBT), was prepared. Separating gel was cut and incubated in staining solution in dark conditions for 45 minutes with gentle shaking. After incubation, gel was washed with dH_2O several times under illumination, until the color development occurred.

2.2.12 Total Protein Analysis

SDS-PAGE method and Bioanalyzer were used to analyze total protein profiles of control and stress-treated plants of different cultivars.

2.2.12.1 Total Protein Extraction

Proteins from shoots and roots of control and treated samples were extracted according to the method of Wang *et al*. (2006). 0.1-0.3 g grinded samples were put into eppendorf tubes and 10% (w/v) TCA/Acetone solution was added. Tubes were mixed well and centrifuged at 16000 g for 3 min at 4°C. The pellet was methanol-washed by adding 2 ml 0.1 M ammonium acetate in 80% MetOH. Samples were mixed by vortex and centrifuged at 16000 g for 3 min at 4°C. After centrifuge, the pellet was washed with 2 ml 80% Acetone and vortexed until the pellet was fully dispersed, than they were centrifuged at 16000 g for 3 min at 4°C. The pellet was incubated at 50°C for 10 min to remove remaining acetone. After incubation, 0.8 ml Trsi-buffered phenol at pH 8.0 and 0.8 ml dense SDS buffer

were added for protein extraction. Samples were mixed and incubated for 5 min, and centrifuged at 16000 g for 3 min at 4 °C. The upper phenol phase, which is containing proteins, was transferred into a new 2 ml eppendorf tube and filled with MetOH containing 0.1 M ammonium acetate. The Proteins were incubated at -20°C for overnight to precipitate. A white pellet should was visible after samples were centrifuged at 16000 g for 5 min at 4°C. The Proteins were washed first with 100% MetOH and than with 80% acetone. In each step they were mixed and centrifuged at 16000 g for 3 min at 4°C. Proteins were allowed to air-dry and dissolved in sample buffer containing 5ml dH_2O, 1ml 0.5 M Tris-HCl buffer at pH 6.8, 1.6 ml %10 SDS and 0.4 ml β-mercaptoethanol.

2.2.12.2 SDS-PAGE Analysis

SDS-PAGE was performed according to the method of Laemmli (1970). Stacking gel (4.5%) and separating gel (12%) were prepared and polymerized in gel apparatus. From each sample, equal amounts of proteins (15. µg), which is determined by Bradford, 1976, were taken and were pre mixed with sample buffer with 3:1 ratio, respectively. Diluted samples were heated at 90°C for 10 min. Also, a molecular marker (Unstained Protein MW Marker) was prepared by heating at 90°C for 5 min. 28 µl of each sample and 12 µl of marker were loaded to wells. Gel was run about 1 hour at 60V through stacking gel and continues at 90V overnight through separating gel.

2.2.12.3 Silver Staining

Gels were stained according to the silver staining method of Blum *et al*. 1987. After SDS-PAGE, gels were fixed in the fixation solution for overnight. Fixed gels were washed 3 X 20 min with 50% EtOH. After washing, they were put in pretreatment solution for exactly 1 min and washed 3 X 20 sec with distilled water. For impregnation, gels were incubated in silver nitrate solution for 20 min and washed 2 X 20 sec with distilled water. Then, gels were transferred to developing solution for about 10 min for color development. When color development was observed, developing was terminated by washing gels with 50% stop solution for 2 X 2 min and with stop solution for at least 10 min. After staining photographs of gels were taken.

2.2.12.4 Bioanalyzer

For protein analysis, Agilent 2100 Bioanalyzer and Agilent Protein 230 kit were used. The Agilent 2100 Bioanalyzer is a microfluidic system for the electrophoresis-based analysis of biomolecules. The Protein 230 kit, that is used, is for general protein analysis up to 230 kDa. After setting up the assay equipment and the bioanalyzer, Gel-dye mix, destaining solution, denaturing solution and samples were prepared according to the Agilent protein 230 assay protocol. Onto a new protein chip, 12 µl gel-dye mix, 6 µl from each sample and ladder were loaded. The sample loaded chip was put into bioanalyzer and chip run was started. Results were analyzed with 2100 expert software.

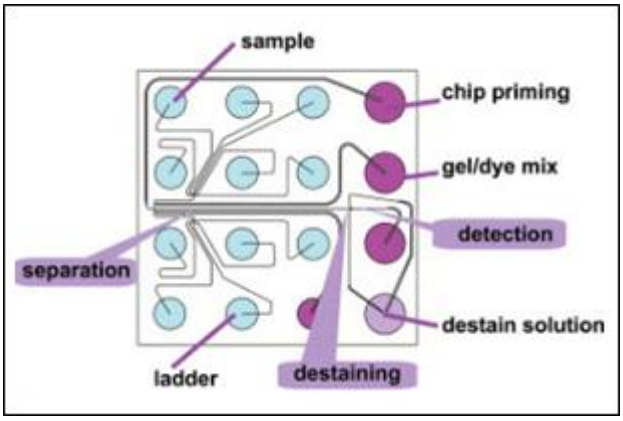

Figure 2.1 The channel layout of microfluid protein chip (Goetz et al. 2004)

2.2.13 Statistical Analysis

The physiological analyses including fresh weight and length measurements, and ıon leakage test were performed with 12 replicates. Biochemical Analyses including proline, H_2O_2 and MDA contents and enzyme activity measurements were performed 3 times. Data obtained in the study were analyzed with one-way analysis of variance (ANOVA) or two-way ANOVA, where necessary by using MINITAB 13 program (MINITAB Inc., USA). Differences were considered significant where P value was less than 0.005 ($p<0.05$).

CHAPTER 3

RESULTS

3.1 Screening Analysis for Cultivar Selection

In the screening step of this study six lentil cultivars (*Lens culinaris,* Medik.) were used to select one tolerant and one sensitive cultivar to the drought stress. Lentil seedlings of each cultivar (Seyran, Malazgirt, Çiftçi, Çağıl, Özbek and Kafkas) grown for 7 days under normal conditions were subjected to different polyethylene glycol (PEG) concentrations for 5 days. 10% and 15% PEG containing Hoagland's solutions were used to induce drought stress. After drought stress treatment 12 days old shoot and root tissues of both stress treated and control plants were analyzed in terms of physiological parameters including length and fresh weight, biochemical parameters like proline content, H_2O_2 content, MDA content and ion lekage.

3.1.1 Physiological Effects of Drought on Lentil Cultivars

The physiological effects of drought stress were evaluated by measuring lengths and fresh weights of shoot and root tissues. 12 days old seedlings of control and stress treated plants were photographed to observe physical changes occurred under stress condition, as shown in Figure 3.1.

Under drought stress, growth inhibition is observed in all cultivars except Seyran and Malazgirt cultivars. The increases in the growth inhibition of the plants become more significant at high PEG concentration.

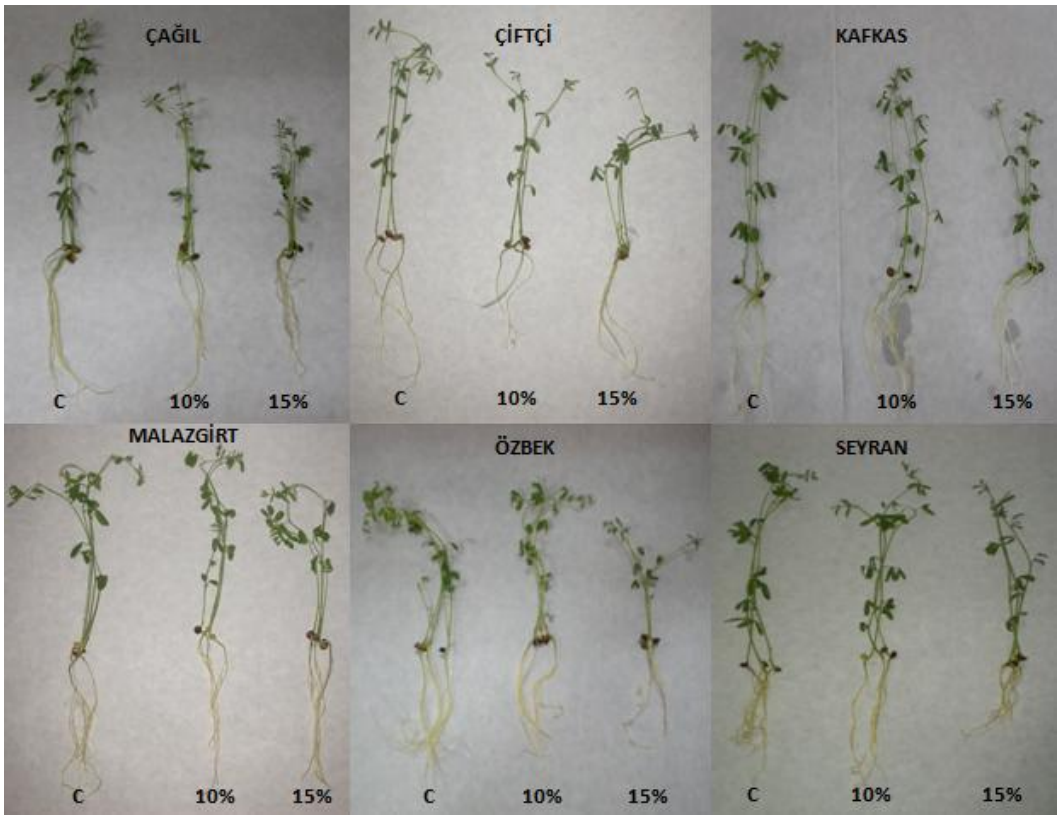

Figure 3.1 The appearance of 12 days old (7 days grown + 5 days treated) lentil seedlings of control, 10% PEG and 15% PEG treatment

3.1.1.1 Effect of Drought Stress on Shoot and Root Length of the Lentil Cultivars

As the PEG concentration increased shoot length of all cultivars except Seyran decreased (Figure 3.2). Kafkas shoot length showed the highest decrease by 21% under 15% PEG while Seyran shoot length remained almost unchanged by 2% under 15% PEG (Figure 3.2). Root lengths of cultivars were not changed much under drought treatment. The highest decrease in root length was observed in Seyran cultivar (Figure 3.3).

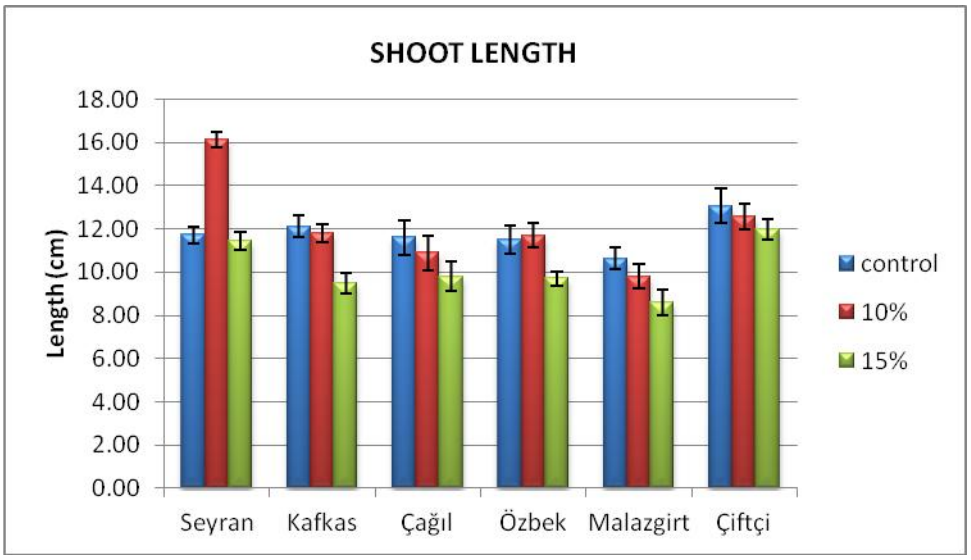

Figure 3.2 Shoot lengths (cm) of control and treated plants of all cultivars. Bars indicate the mean values ± S.E.M.

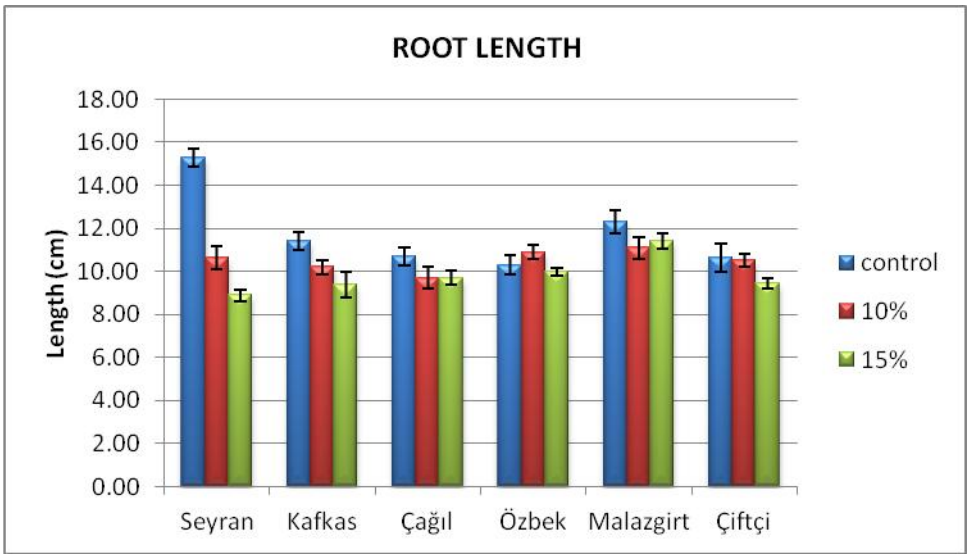

Figure 3.3 Root lengths (cm) of control and PEG treated plants of all cultivars. Bars indicate the mean values ± S.E.M.

3.1.1.2 Effect of Drought Stress on Fresh Weight of Lentil Cultivars

Shoot weight decreased significantly in all cultivars. The decreases were observed at 15% PEG concentrations much apparent. Seyran fresh weight decreased by 35% under 15% PEG and remained unchanged under 10% PEG. All other cultivars decreased under both 10% and 15% PEG (Figure 3.4). Root

weight changes were not as apparent as shoot weight changes in all cultivars. Seyran and Kafkas root weight of treated plants were similar as the root weight of control ones. In the other four cultivars, nonsignificant increases were observed (Figure 3.5).

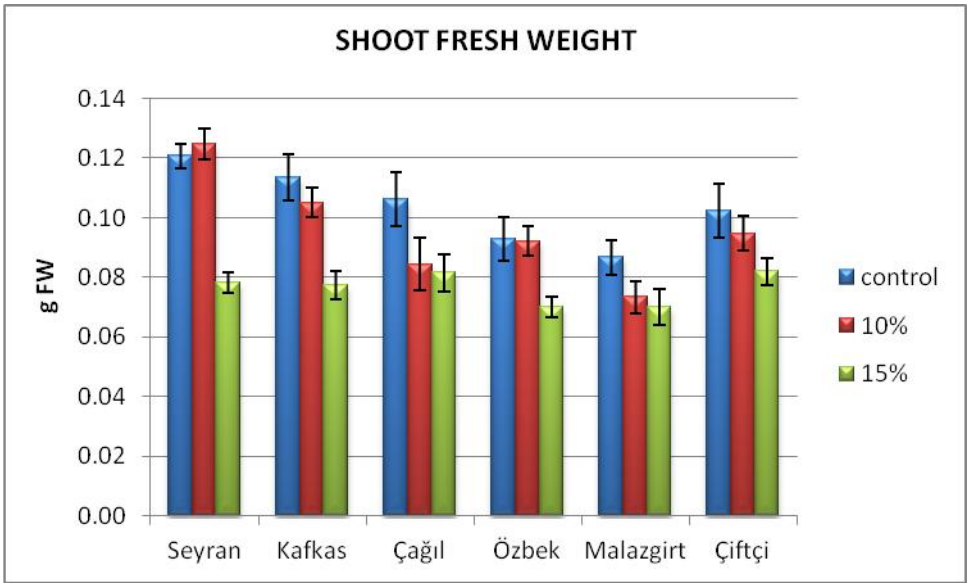

Figure 3.4 Shoot fresh weights of control and PEG treated plants. Bars indicate the mean values ± S.E.M.

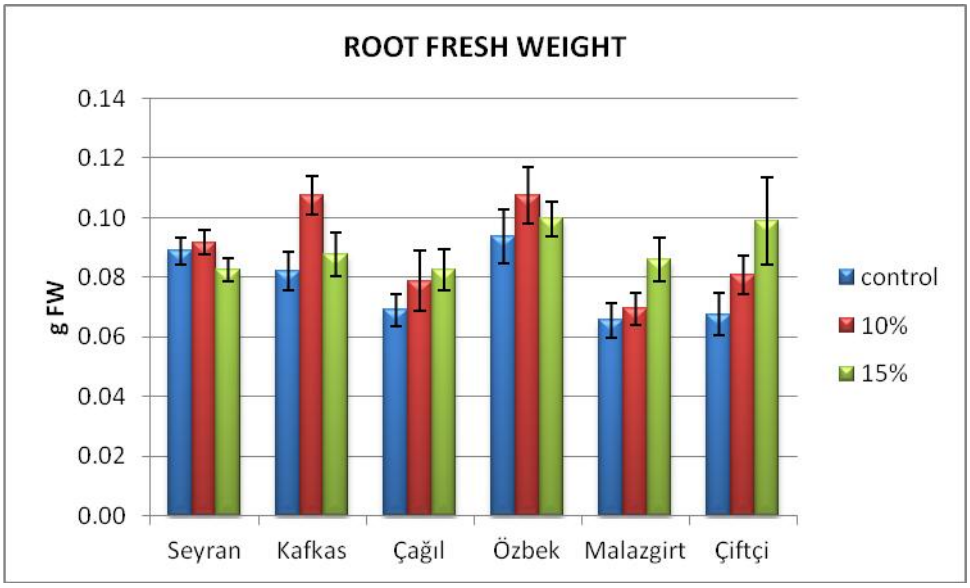

Figure 3.5 Shoot fresh weights of control and PEG treated plants. Bars indicate the mean values ± S.E.M.

3.1.2 Biochemical Effects of Drought Stress on Lentil Cultivars

3.1.2.1 Proline Content

In both shoot and root tissues of all cultivars, proline concentration increased significantly as the PEG concentration increased. Proline content increase was much noticeable under 15% PEG treatment in shoot tissues.

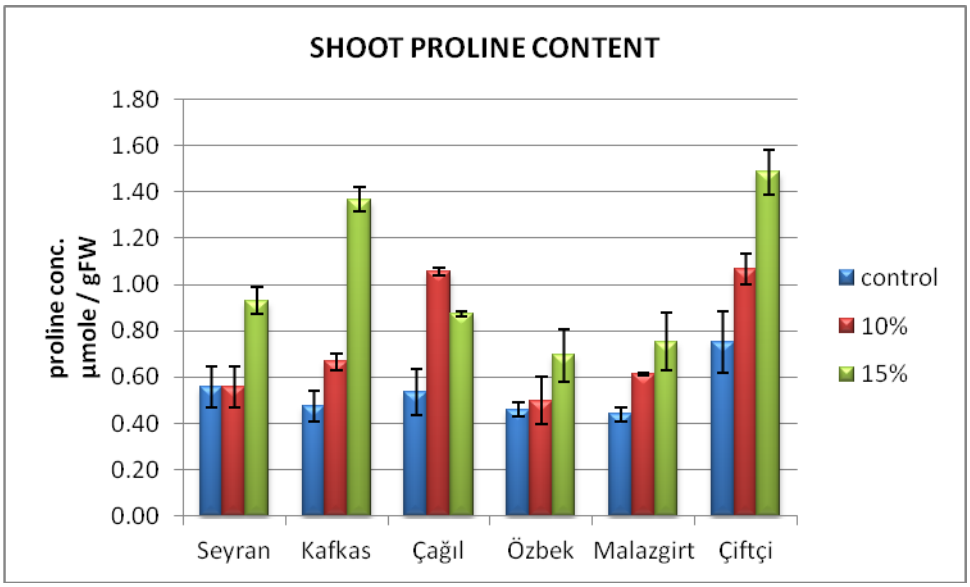

Figure 3.6 Effect of PEG on proline conc. in shoots of control and drought treated lentil cultivars. Bars indicate the mean values ± S.E.M.

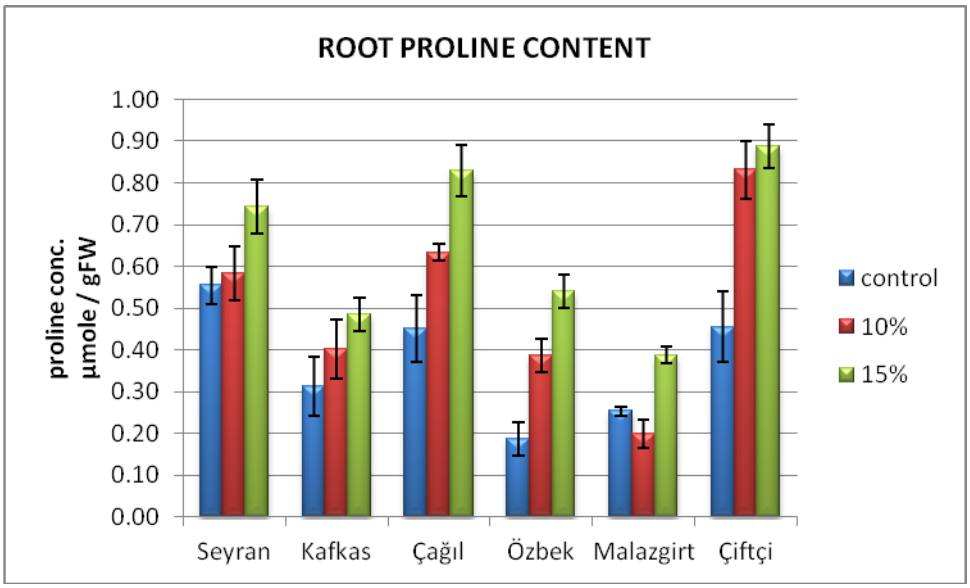

Figure 3.7 Effect of PEG on proline conc. in roots of control and drought treated lentil cultivars. Bars indicate the mean values ± S.E.M.

3.1.2.2 Ion Leakage

Membrane permeabilities of the plants were observed by measuring ion leakage amounts. Shoot tissue of Çiftçi cultivar increased its permeability more than 3-fold under 15% PEG treatment. Membrane permeability of Çağıl and Özbek shoot tissues also increased as drought stress increased, but not significantly. Seyran and Kafkas cultivars kept their ion leakage levels almost constant under 10% and 15% PEG (Figure 3.8). In root tissues membrane permeability of Seyran cultivar was constant as in shoot tissue. In other cultivars root tissues shows different responses from shoot tissues (Figure 3.9).

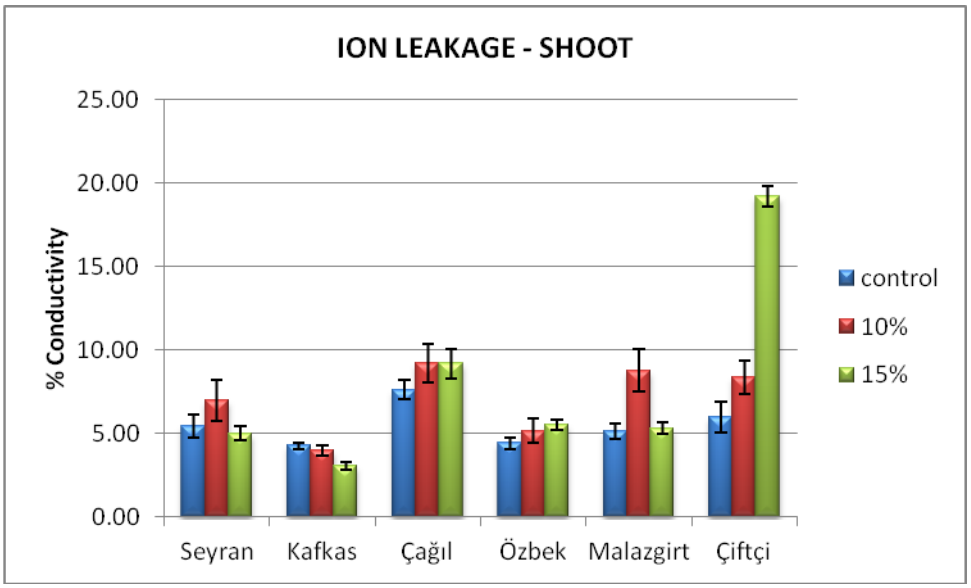

Figure 3.8 Membrane permeability of shoots of control and drought treated plants. Bars indicate the mean values ± S.E.M.

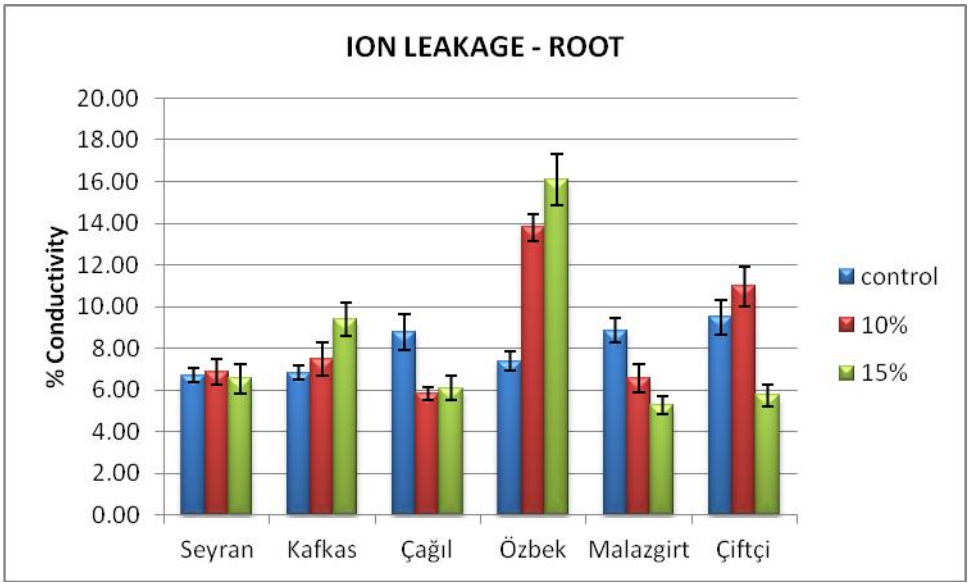

Figure 3.9 Membrane permeability of shoots of control and drought treated plants. Bars indicate the mean values ± S.E.M.

3.1.2.3 MDA Content

Being one of the end products of lipid peroxidation, malondialdehyde (MDA) content was measured to determine oxidative effect of drought stress on lentil cultivars.

In shoot tissues of Kafkas, Çağıl and Özbek cultivars MDA content increased significantly as at 15% PEG by 84%, 135% and 70% respectively. Malazgirt and Çiftçi cultivars showed insignificant changes in MDA content under drought stress. MDA content of Seyran cultivar shoot tissue decreased by 42% under 10% PEG and did not change under 15% PEG (Figure 3.10). Çağıl and Kafkas root tissues increased under drought stress but not as significant as shoot tissues. Seyran root tissue decreases by 37% and 22% under 10% and 15% PEG, repectively.

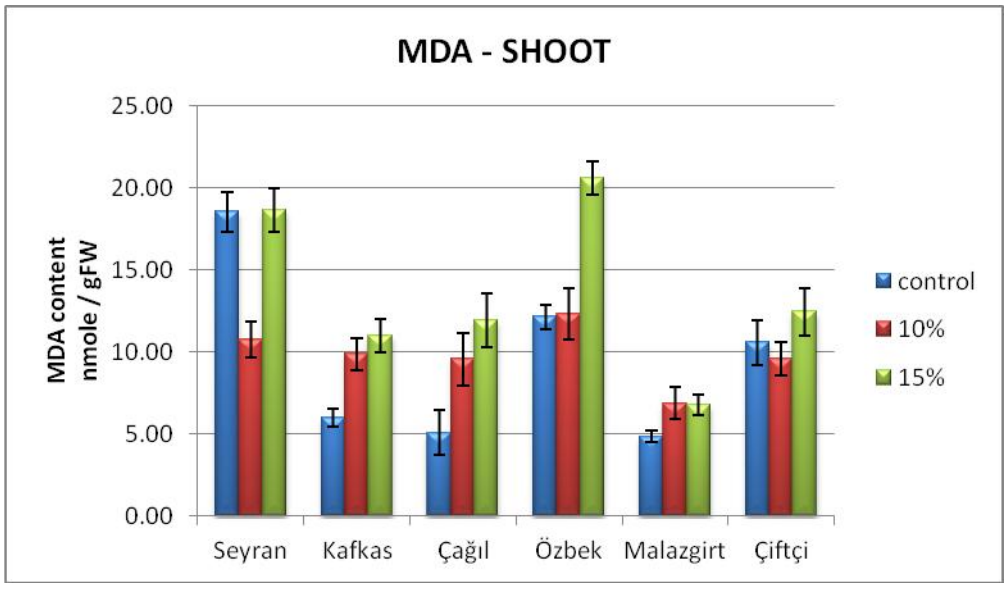

Figure 3.10 Shoot MDA content of control and drought treated lentil cultivars. Bars indicate the mean values ± S.E.M.

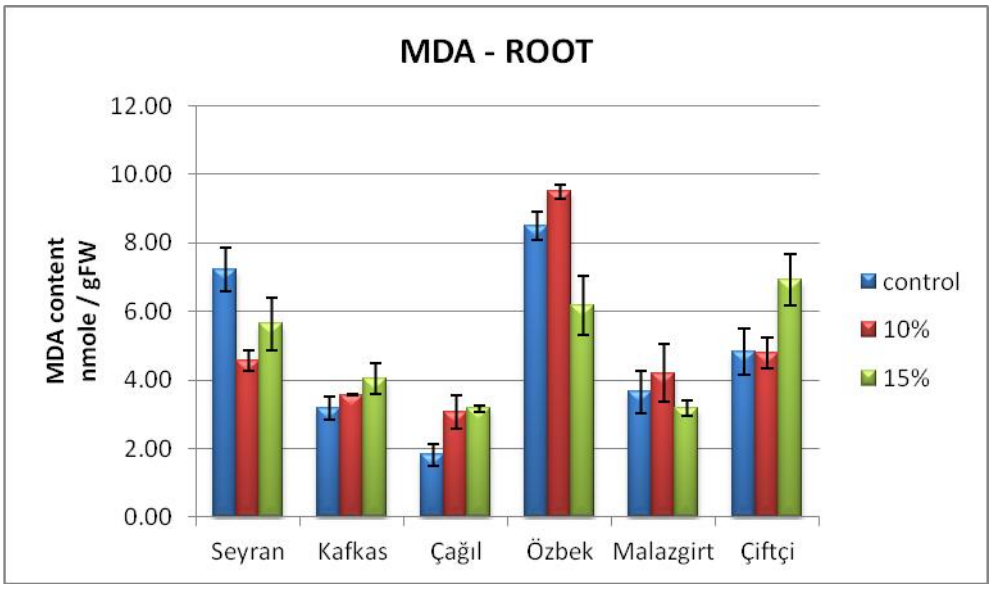

Figure 3.11 Root MDA content of control and drought treated lentil cultivars. Bars indicate the mean values ± S.E.M.

3.1.2.4 H_2O_2 Content

Under drought stress, Çağıl cultivar showed significantly decreasing H_2O_2 concentrations in shoot tissues, while Seyran, Kafkas, Özbek and Malazgirt cultivars showed no change or slight increases. In root tissues, H_2O_2 concentrations are lower than that of the shoot tissues for all cultivars. Among all cultivars only Özbek shows a significant increase in H_2O_2 amount under drought stress conditions, in roots.

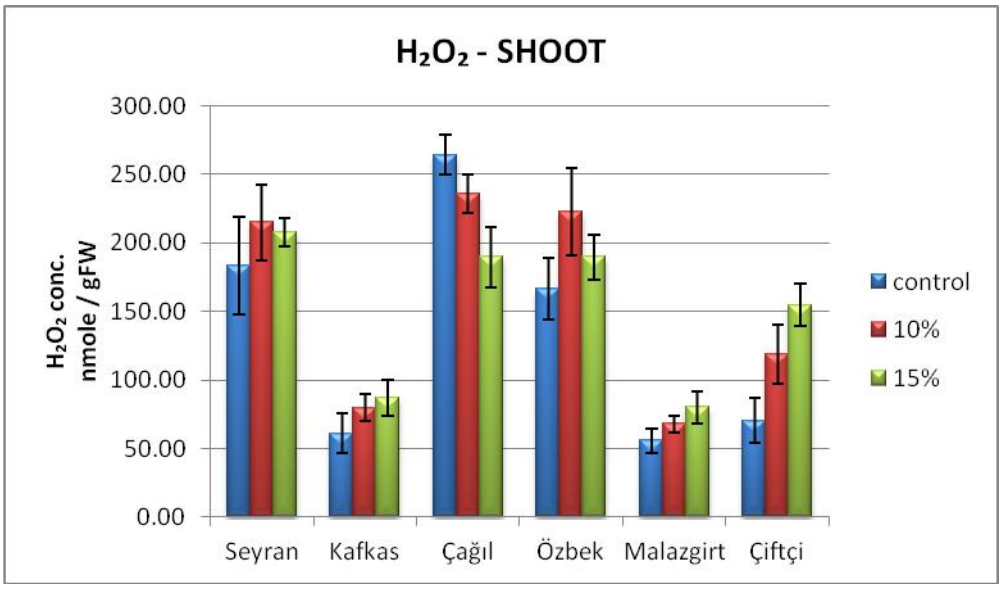

Figure 3.12 H_2O_2 concentrations of shoots of control and drought treated lentil cultivars. Bars indicate the mean values ± S.E.M.

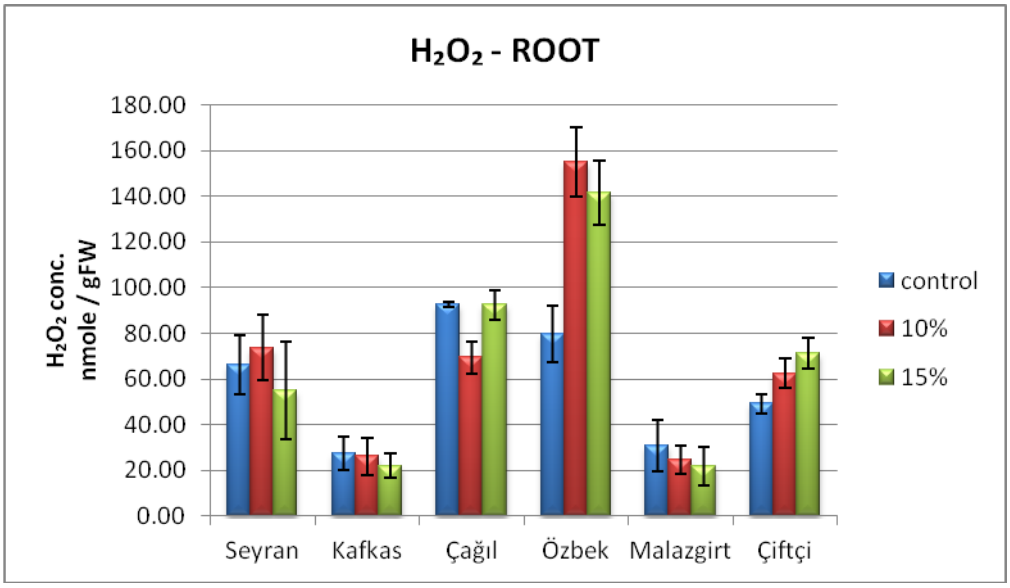

Figure 3.13 H_2O_2 concentrations of shoots of control and drought treated lentil cultivars. Bars indicate the mean values ± S.E.M.

3.2 Effect of Drought Stress on Antioxidative Defense Systems of Tolerant and Sensitive Lentil Cultivars

After screening studies, Seyran and Çağıl cultivars were selected as tolerant and sensitive to drought stress respectively. The selected cultivars were further analyzed in terms of antioxidative enzyme activities and protein profiles to compare their behaviors under drought stress conditions.

3.2.1 Ascorbate Peroxidase Activity

Under drought stress ascorbate peroxidase enzyme activity of Seyran cultivar shoot tissue decreased and of root tissue increased. Enzyme activity increased in both shoot and root tissues of Çağıl cultivar (Figure 3.14 and Figure 3.15). Significantly higher values of the APX activity were observed in both shoot and root tissues of Seyran cultivar than the Çağıl cultivar under both normal and treatment conditions.

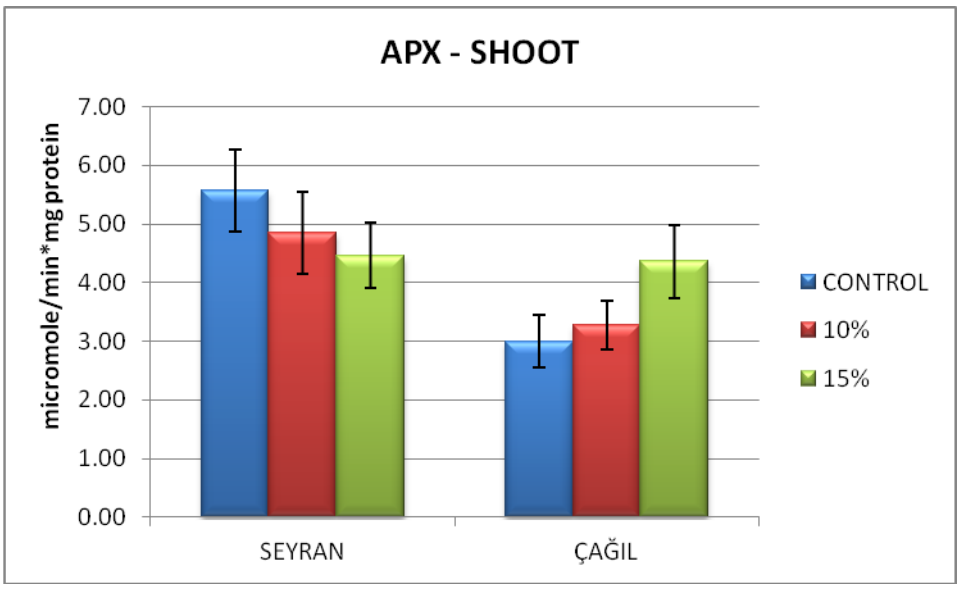

Figure 3.14 APX activity in shoot tissues of control and drought treated plants of Seyran and Çağıl cultivars. Bars indicate the mean values ± S.E.M.

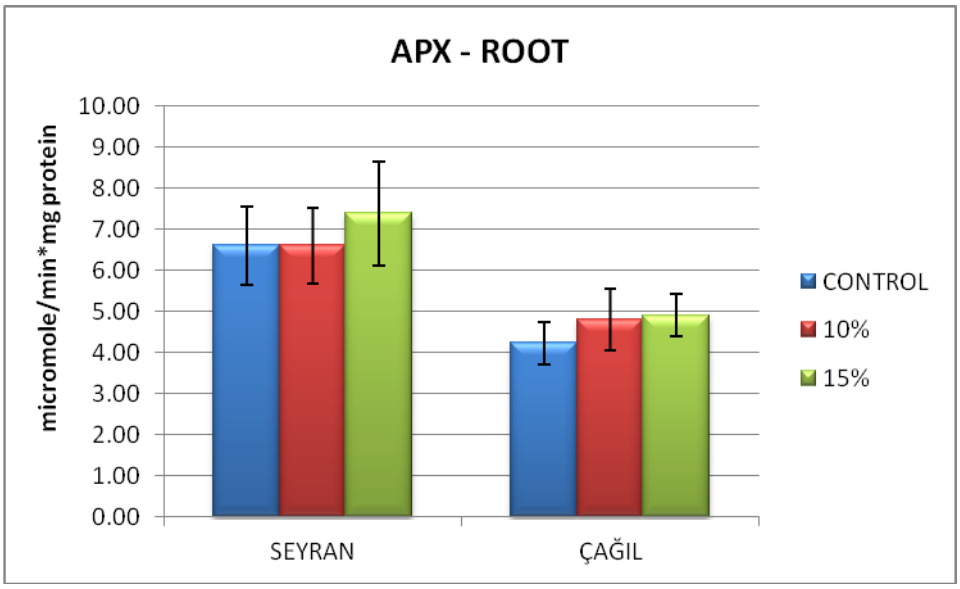

Figure 3.15 APX activity in root tissues of control and drought treated plants of Seyran and Çağıl cultivars. Bars indicate the mean values ± S.E.M.

3.2.2 Catalase Activity

Activity of the catalase enzyme did not change significantly in shoot and root tissues of both cultivars. However CAT activity differs significantly between two cultivars. The enzyme activity was about 2 fold higher in Seyran cultivar than Çağıl cultivar both in shoot and root tissues (Figure 3.16 and Figure 3.17).

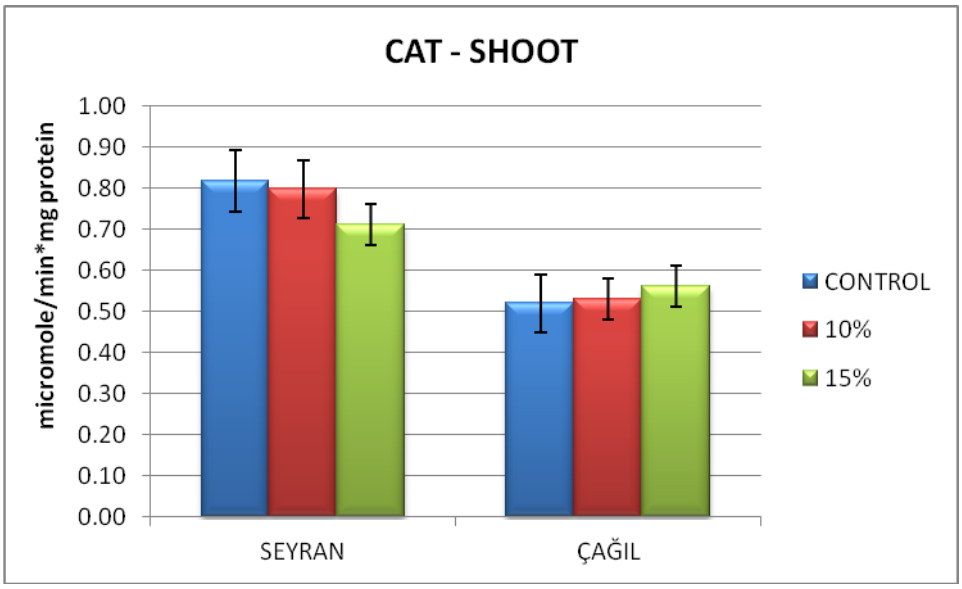

Figure 3.16 CAT activity in shoot tissues of control and drought treated plants of Seyran and Çağıl cultivars. Bars indicate the mean values ± S.E.M.

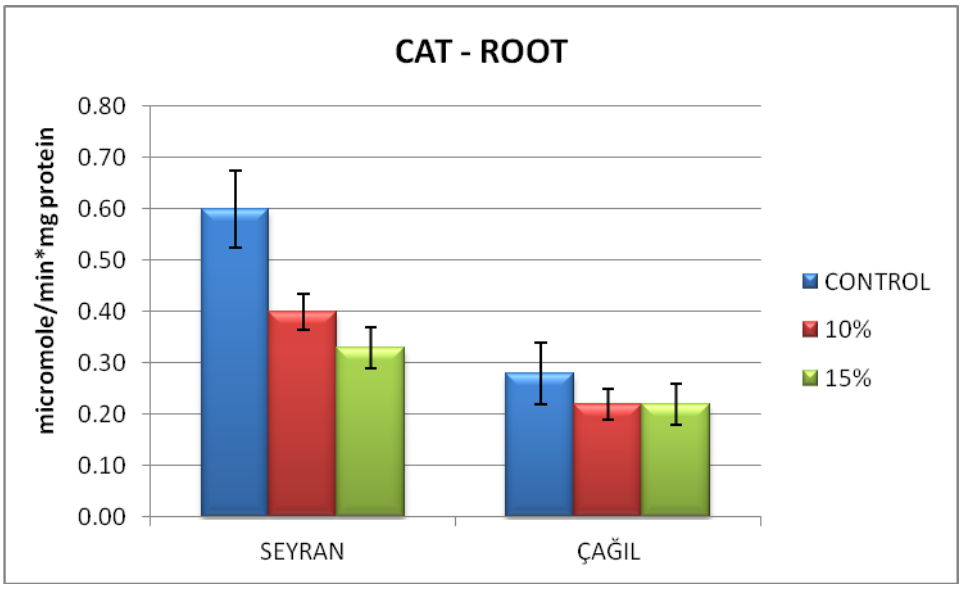

Figure 3.17 CAT activity in root tissues of control and drought treated plants of Seyran and Çağıl cultivars. Bars indicate the mean values ± S.E.M.

3.2.3 Glutathione Reductase Activity

Glutathione Reductase activity of Seyran shoot tissue increased under 15% PEG treatment while in root tissue it remained constant under both 10% and 15% PEG treatments. The enzyme activity change was insignificant for Çağıl cultivar shoot and root tissues. In both shoot and root tissues Seyran cultivar had a significantly higher GR activity than the Çağıl cultivar under normal and treatment conditions.

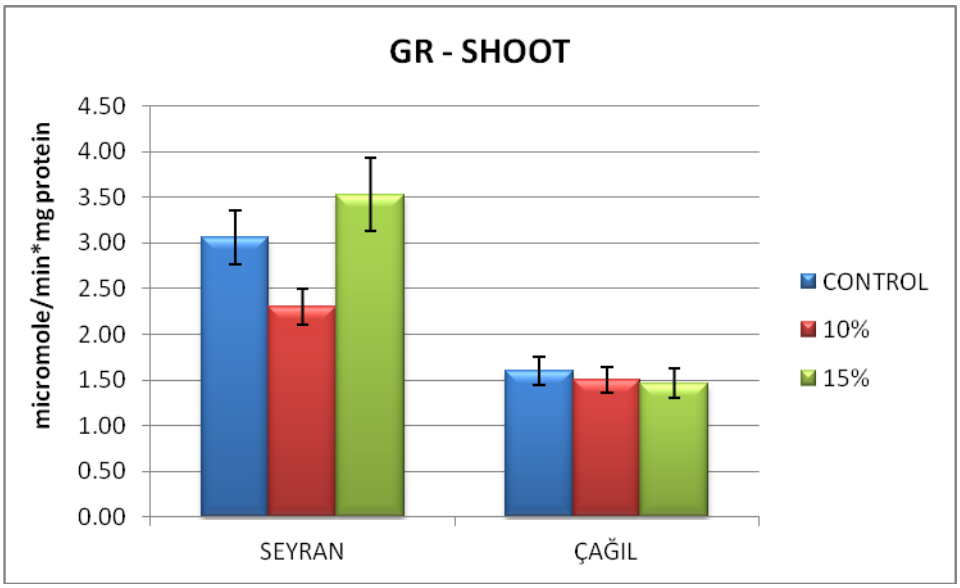

Figure 3.18 GR activity in shoot tissues of control and drought treated plants of Seyran and Çağıl cultivars. Bars indicate the mean values ± S.E.M.

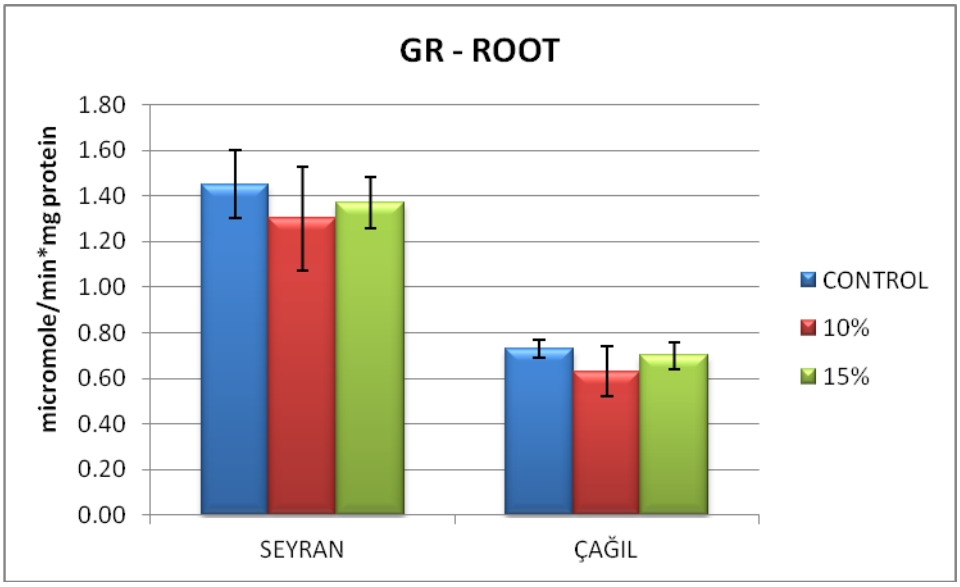

Figure 3.19 GR activity in root tissues of control and drought treated plants of Seyran and Çağıl cultivars. Bars indicate the mean values ± S.E.M.

3.2.4 Superoxide Dismutase Activity

SOD isozymes in shoot and root tissues of lentil plants were determined in native PAGE gel with negative activity staining. In both shoot and root tissues Mn SOD, Cu/Zn SOD1 and Cu/Zn SOD2 isozymes were identified. Also, in shoot tissues chloroplastic Fe SOD isozyme was observed (Figure 3.20). A significant decrease was observed in all SOD isozymes under 15% PEG treatment of Çağıl cultivar. Seyran cultivar did not show any significant change in SOD isozymes under drought stress conditions. In root tissues SOD isozyme amount was lower than the shoot SOD isozymes and their activity did not show any significant change under drought stress conditions. It is observed that, in shoot tissues Cu/Zn SOD1 and Cu/Zn SOD2 isozymes were predominant, while in root tissues only Cu/Zn SOD1 isozyme was the main component.

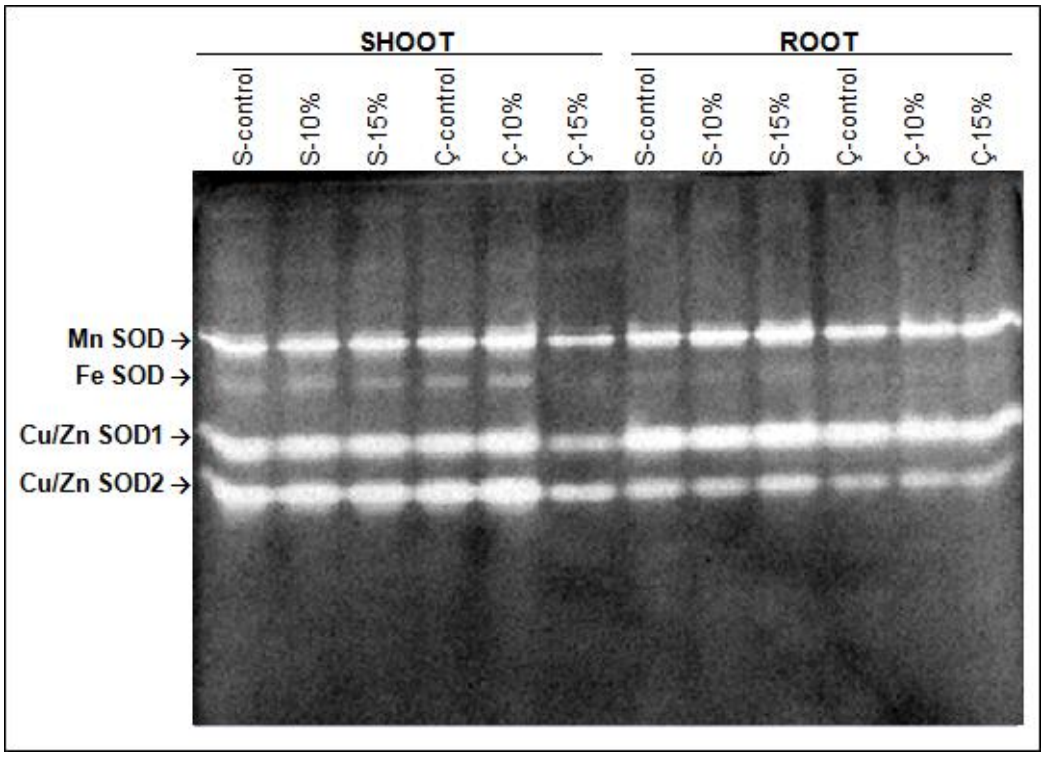

Figure 3.20 Activities of SOD isozymes in shoots and roots of control and drought-treated plants of Seyran and Çağıl cultivars.

3.3 Protein Profiles of Tolerant and Sensitive cultivars of Lentil

In order to compare total protein profiles of tolerant and sensitive cultivars of lentil SDS-PAGE and Bioanalyzer studies were performed and the results are shown in Figures 3.20, 3.21 and 3.22.

3.3.1 SDS-PAGE

According to the SDS-PAGE result of the total proteins of both cultivars, root tissues have less proteins than the shoots. Seyran and Çağıl cultivars showed some differences in the protein bands as seen in Figure 3.21-a.

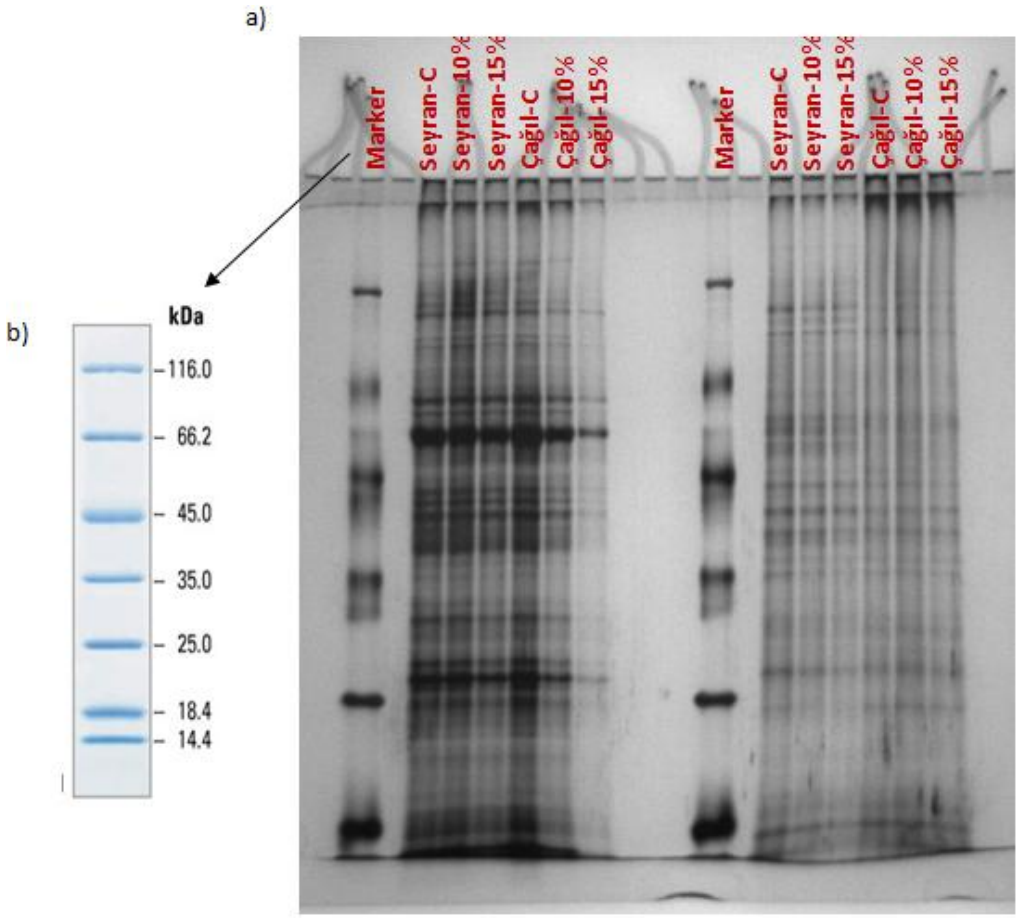

Figure 3.21 a) SDS-PAGE results of the total proteins of Seyran and Çağıl cultivars under both normal (C=control) and treatment (10% PEG and 15% PEG) conditions b) Thermo Scientific Unstained Protein Molecular Weight Marker

3.3.2 Bioanalyzer

According to the electropherogram images and electrophoresis run summaries of bioanalyzer there were many proteins observed, at different peak levels under control and drought stress conditions. Also, differences in protein peaks were observed between Seyran and Çağıl cultivars under normal and stress treatment conditions as shown in Figure 3.22 and Figure 3.23.

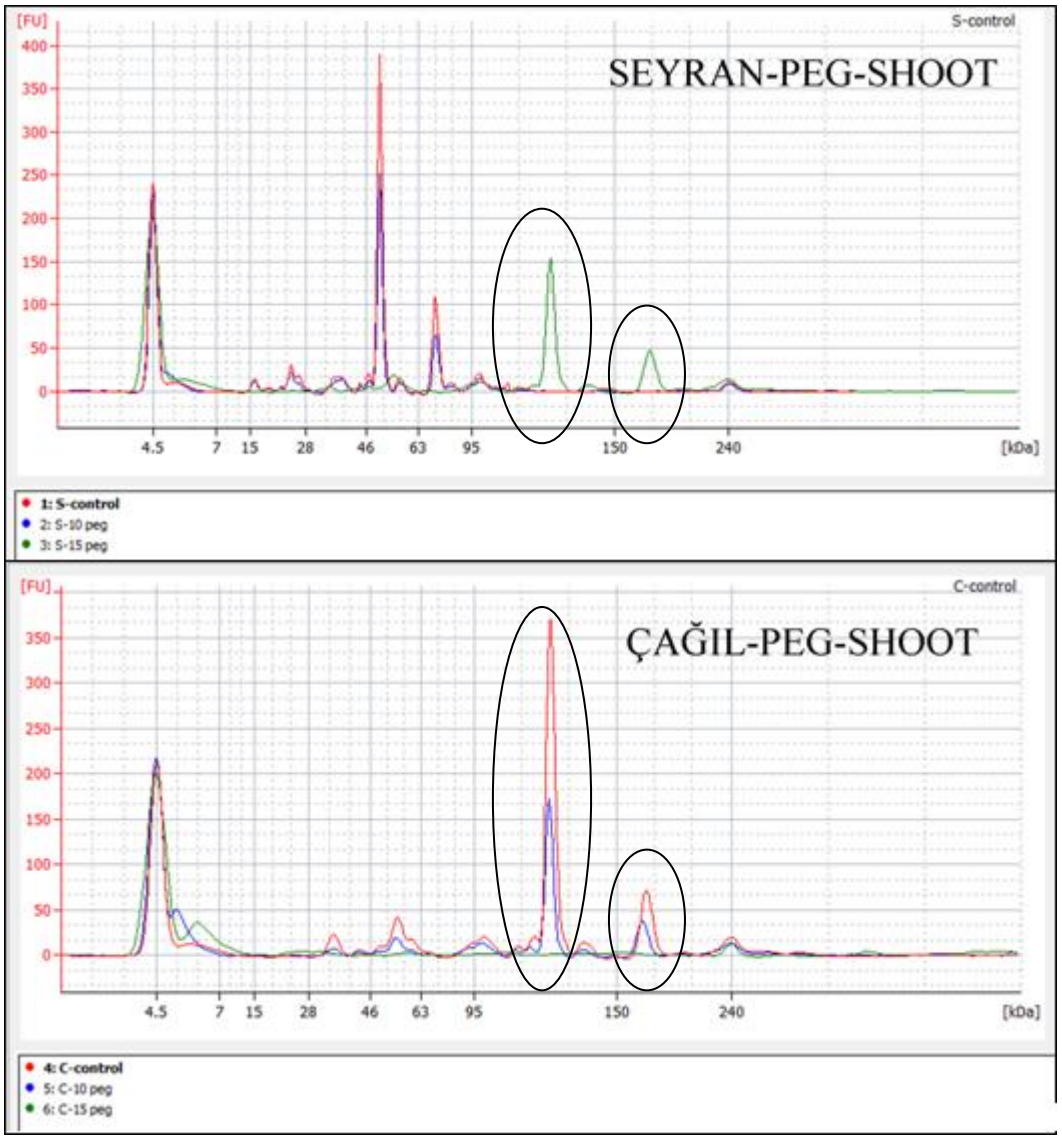

Figure 3.22 Electropherogram images of control and stress treated shoot tissues of Seyran and Çağıl cultivars under normal and drought stress conditions

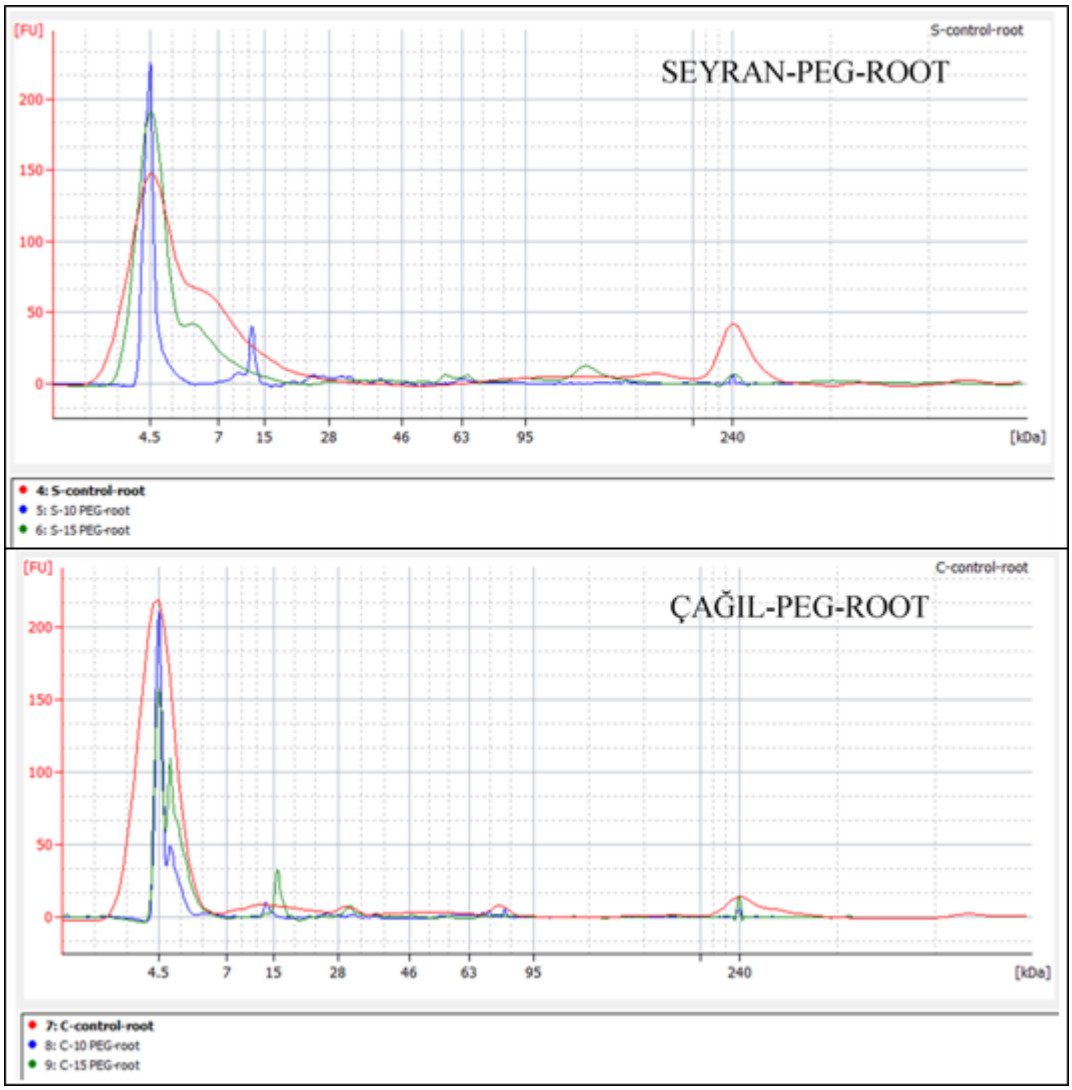

Figure 3.23 Electropherogram images of control and stress treated root tissues of Seyran and Çağıl cultivars under normal and drought stress conditions

CHAPTER 4

DISCUSSION

Lentil (*Lens culinaris)* is an important crop plant with its high-quality protein amount (high protein, low fat) for human diet and its value as animal fodder. Drought is considered one of the most devastating among the environmental stresses (Vinocur and Altman, 2005). All agricultural regions will experience drought and some areas experience predictable dry seasons as some others exposed to unpredictable drought periods. Drought stress decreases the crop quality and yield up to 50% or more (Wood, 2007). Due to the need of developing and identifying drought tolerant crop lines, understanding the functioning capacity of drought tolerant plants under water deficit conditions is inevitable (Bhardwaj and Yadav, 2012).

In this study two lentil cultivars (Seyran and Çağıl) selected by the preliminary studies (physiological biochemical studies) and behaving differently under drought stress conditions were compared by their physiological and biochemical changes, antioxidative defense systems and protein profiles.

4.1 Effect of Drought Stress on Physiological and Biochemical Parameters of Lentil Cultivars

5 days application of PEG treatment to induce drought stress decreased the fresh weights of shoots significantly. The leaf areas of PEG treated shoot tissues was observed as decreased which is the one of the first responses of the plants to reduce water loss through transpiration under water deficiency (Ercan, 2008). Shoot length was increased only in Seyran shoot tissue under 10% PEG and it did not change under 15% PEG, while other 5 cultivars showed decrease in shoot length as PEG concentration increasing in addition to the leaf area decreasing against water loss. It is stated by Bartels and Sunker (2005) that growth arrest might allow plants to preserve carbohydrates for prolonged energy supply and for

sustained metabolism. The lengths of root tissues were not changed significantly, only in seyran cultivar a significant decrease of root length observed. Although its root length decreases, Seyran has kept the root fresh weight constant under stress conditions. That shows lateral root growth enhanced in this cultivar to reach more water, as it was mentioned by Sarker *et al.* (2003) that lateral root number is a very important trait for the plants to cope with drought environments.

Proline

Proline is one of the most important organic solutes that maintain the water content under environmental stress conditions in plants as osmoprotectant for membrane stabilization (Kavi Kishor *et al*., 2005). Plants enhance their synthesis and accumulation against osmotic stress, which is called as osmotic adjustment (Ercan, 2008). Besides its osmoprotectant role, proline also stabilizes membranes and proteins, maintains cell turgor and removes free radicals. By Ashraf and Foolad, it has been stated that proline accumulation under stress conditions is corolated with stress tolerance in many species and the concentartion of proline has been shown to be higher in tolerant plants than the sensitive ones. Increase in proline concentration was observed in many plants under water deficit. For example, Hsu *et al*. (2003) found that under water deficiency rice plants increases the proline amount in leaf tissues. In another study, higher proline accumulation was reported under dry habitat in the shoots and roots of *Tephrosia purpurea* Pers and Ragi (*Eleusine coracana*) by Erakar and Murumkar (1995). This high proline content was related with the stress tolerant nature and survival capability of these plants. Increase in proline accumulation has been reported not only under drought stress, but also under other abiotic stress conditions. In a study of Misra and Gupta (2006), increased proline content under salt stress in preen gram plants was reported.

In this study, an increase in proline accumulation was observed in both shoot and root tissues of all lentil cultivars. Proline content increased in shoot tissues by 2,3 folds under drought stress. In Seyran cultivar porline accumulation increased only at 15% PEG treatment and at 10% PEG concentration Seyran did not need to enhance proline accumulation to be able to survive, indicating its tolerant nature.

In root tissues of Seyran the same pattern observed too. Seyran root proline content showed little increase when compared to other cultivars.

Malondialdehyde and Membrane Permeability

MDA is by product of peroxidation of unsaturated fatty acids in phospholipids of membranes and the level of peroxiddaiton has been used as an indicator of ROS damage under stress conditions (Bhardwaj and Yadav, 2012). When ROS are overproduced, lipid peroxidaiton is induced and the lipid-derivative radicals produced, which damage the DNA and proteins. MDA is widely used in studies as an indicator of membrane integrity of many plant species under environmental stresses (Ercan, 2008).

Simova-Stoilova *et al*. stated that membrane integrity weakening and lipid oxidation were more pronounced in sensitive varieties under drought conditions. Also, it is reported by Pan et al. in liquorice seedlings MDA content increased under both salt and drought stress conditions. It has been stated that lower MDA level in the salt tolerant maize cultivar suggests its stress tolerance (Azoz *et al.,* 2009). Bhardwaj and Yadav (2012), reported that under drought stress conditions the increase was more significant for HPKC 2 (drought-sensitive horesgram variety) than for HPK 4 (dought-tolerant horsegram variety) suggesting that the relatively lower degree of MDA content increase in tolerant variety under drought stress supported its tolerant nature.

In this study, the MDA content of Seyran shoots remained as constant under 15% PEG treatment and decreased in roots under both 10% and 15% PEG treatments, showing that Seyran cultivar might be tolerant to drought stress. The possible reason for this might be that ROS production and membrane damage would be low in tolerant plants leading to lower MDA contents. This result was also supported by increasing proline content of both shoots and roots of the Seyran cultivar.

The effects of abiotic stresses on membrane integrity of the cells have been studied in plant species by MDA content measurement and ion leakage test together (Ercan 2008).

Also in this study, MDA results were supported with ion leakage tests and electrolyte leakage changes of Seyran cultivar showed its drought-tolerant nature, again. Ion lekage amounts under drought stress was not change significantly in shoots and roots of Seyran, while other cultivars showed increases in their shoot tissues.

Hydrogen peroxide

Toxic H_2O_2 is a product of peroxisomal and choloroplast oxidative reactions and act both as an oxidant and reductant. Being the most stable reactive oxygen species and the capability of rapid membrane diffusion, has important roles in signaling under stress conditions (Ercan, 2008; del Rio *et al*., 1992). It is known that drought, salinity, metal toxicity and other environmental stress conditions enhanced endogenous H_2O_2 accumulation. The protein and pigment degradations in senescing leaves could be as a result of the cytotoxic effect of H_2O_2 (Upadhyaya *et al*., 2005).

In this study, Çağıl cultivar showed significantly decreasing H_2O_2 concentrations in shoot tissues, while Seyran, Kafkas, Özbek and Malazgirt cultivars showed no change or slight increases. This decrease observed in H_2O_2 concentration might be the result of the inactivation of SOD enzyme, which is an important part of the antioxidant defense system. It converts reavtive oxygen species such as singlet oxygen or hydroxyl radicals to the less reactive H_2O_2, thereby decreasing the harmful effects of ROS.

Although fresh weight and length of the Seyran shoots and roots showed decreases as drought stress increases, accumulation of MDA and H_2O_2 concentration and ion leakage amount, which are the important parameters of oxidative damage, decreased under drought stress. These analysis resulted that Seyran cultivar behaves as drought-tolerant among our six lentil cultivars. The

other five cultivars (Çağıl, Çiftçi, Malazgirt, Kafkas and Özbek) showed very similar responses to drought stress. Their lengths and fresh weights decreased in both shoot and root tissues. Besides, increasing drought stress resulted in high oxidative damage in these cultivars that are shown by increasing MDA content, hydrogen peroxide accumulation and electrolyte leakage results in these cultivars. Among these five cultivars, Çağıl was the only one with decreasing H_2O_2 amount, that showing an impairment in the antioxidative ezyme system. When comparing the lentil cultivars, their annual yields was also considered. Since the Çağıl cultivar has higher yield among other cultivars, it was selected as behaving drought-sensitive. However, selection based on these physiological and biochemical analyses was not sufficient. That's why to support these results enzyme activity and protein analyses were also performed.

4.2 Effect of Drought Stress on Antioxidative Enzymes of two Lentil Cultivars

Under normal conditions, the production of ROS in plants is low. However, under environmental stresses like drought, ROS accumulation is increased drastically and disturbs the balance of $O_2^{\cdot-}$, OH and H_2O_2 in the cell (Sharma *et al*., 2010). To cope with the damages posed by ROS, plants produce different scavenging enzymes. Enhanced activity of several antioxidant enzymes has been reported under drought stress in many plant species (Sharma *et al*., 2012).

Sariam *et al*. (1998) reported in the comparative study of the antioxidant reponses in drought tolerant and drought sensitive genotypes, that tolerant genotypes have higher antioxidant capacity. In contrast to the sensitive wheat genotype, tolerant genotype had higher APX and CAT activity, while having lower H_2O_2 and MDA content. In another study, two apple rootstocks *Malus prunifolia* (drought-tolerant) and *Malus hupehensis* (drought-sensitive) were analyzed. Larger increase of $O_2^{\cdot-}$, H_2O_2 and MDA in *Malus hupehensis* than in *Malus prunifolia* is reported. Also it is stated that APX and GR activities of the *Malus prunifolia* increased more than of the *Malus hupehensis* due to the drought stress.

For the antioxidative defense system the balance between antioxidative enzymes activities are very crucial while coping with ROS damaging effects. Changing the balance of the enzymes will generally induce compensatory mechanisms. When one enzymes activity was reduced plants upregulate other oxidative enzymes (Apel and Hirt, 2004).

In our study, CAT activity of both cultivars (Seyran and Çağıl) showed almost no change in shoot tissues and decreased in root tissues. However, when Seyran and Çağıl cultivar were compared in terms of CAT activities, it is observed that Seyran CAT activity is about 2 fold of Çağıl CAT activity in both control and treatment plants. Also, the MDA content of Seyran cultivar was lower than that of the Çağıl cultivar as similar as the study of Sariam *et al*. (1998), in which tolerant genotype showed high CAT activity and lower MDA amounts compared to the sensitive one. The APX activity of Seyran cultivar did not change significantly in both shoot and root tissues, while Çağıl cultivar showed slight increases in shoot tissue. According to the comparison of two cultivars, it has seen that Seyran APX activity levels were always higher than that of the Çağıl. Another antioxidative enzyme GR was also analyzed. In Seyran shoot tissues increasing GR activity under 15% PEG was observed. In Seyran root tissue and Çağıl shoot and root tissues no significant changes were observed. As the CAT and APX activities, GR activity level was also higher in Seyran cultivat than the Çağıl cultivar. In root tissues of Seyran cultivar a decrease was observed in CAT activity, showing that the CAT enzyme in Seyran roots is not as effective as in shoots.

The SOD enzymes convert superoxide radicals to hydrogen peroxide and it constitutes the first line defense against ROS in the cell (Halliwell, 2006). Based on the metal cofactor used by the enzyme, SOD's are classified in three groups; Fe SOD, Mn SOD and Cu/Zn SOD. In this study, Mn SOD, Cu/Zn SOD1 and Cu/Zn SOD2 were identified in both shoot and root tissues, whereas the chloroplastic Fe SOD was identified only in shoot tissues. In all SOD isozymes, a significant decrease was observed for Çağıl cultivar under 15% PEG treatment, which is correlated with the H_2O_2 parameter in drought stress conditions. In shoot tissues of Çağıl cultivar H_2O_2 content showed decrease as in the case of SOD activity. Since the SOD is the main converter of reactive radicals to H_2O_2, the

decreased activity of SOD leads accumulation of free radicals and increases membrane damages. The increasing MD content of Çağıl cultivar under drought stress is also support these results. In Seyran cultivar the SOD activity did not changed, correlating with the H_2O_2 results.

Although there wasn't observed much increase in the activity of antioxidative enzymes of Seyran cultivar under drought stress, when compared with Çağıl enzyme activity Seyran enzyme activities were much higher in both control and treated plants. Also, SOD activity of Çağıl cultivar decreases significantly under drought stress conditions. Thus, these results supported our selection that Seyran cultivar behaves as tolerant to drought stress as compared Çiftçi cultivar.

To verify our results and to understand the underlying mechanism of defense systems of lentils under drought stress, protein analyses were performed.

4.3 Effects of Drought Stress on Total Protein Profiles of two Lentil Cultivars

One of the strategies of plants that are capable of surviving under drought conditions is to enhance synthesis of proteins with protective function to withstand dehydration (Bartels and Sauer, 2003).

Many genes that are expressed as a response to dehydration have been isolated from many species via differential screening of dehydrated and non-stressed plant tissues. Seeds of various plants have been rich in genes that are expressed due to dehydration and involved in resistance to water deficit (Bartels *et al.,* 1990). The expression of *late embryogenesis abundant* (*lea*) genes is correlated with dehydration (Galau *et al*., 1986). The related transcripts accumulate to high levels in vegetative tissues of drought-tolerant plants upon dehydration.

In this study the changes in the protein profiles of drought-tolerant and drought sensitive lentils upon dehydration was compared with bioanalyzer system. With bioanalyzer, the selected proteins are analyzed in terms of their abundance or purity in many studies. In this study, bioanalyzer was used for total protein analysis for the comparision of selected lentil cultivars. Under drought stress, in

shoot tissues of both cultivars some upregulated and some downregulated proteins were detected. These proteins were different in Seyran and Çağıl cultivars, which are drought-tolerant and drought-sensitive respectively. The upregulated proteins of tolerant cultivar might be the heat shock proteins or chaperons, while the downregulated proteins of sensitive cultivar might be part of the defense system.

In future studies, the identification of these up- and down-regulated proteins by 2D-electophoresis and LC-MS/MS will be aimed.

CHAPTER 5

CONCLUSION

In this study six Turkish lentil (*Lens culinaris* Medik.) cultivars (Seyran, Malazgirt, Çağıl, Çiftçi, Özbek, and Kafkas) were screened concerning certain physiological and biochemical parameters under drought stress condition to determine a drought-tolerant and a drought-sensitive cultivar. Selected cultivars were further analyzed in terms of antioxidant enzyme activities and protein profiles to compare their defense mechanisms under drought stress conditions.

According to the physiological and biochemical parameters, Seyran and Çağıl cultivars were selected as drought tolerant and drought sensitive, respectively. Although its fresh weights and lengths showed little change, high level of proline concentration as a strong osmoprotectant, and the decreased H_2O_2 and MDA contents under stress conditions supported its drought tolerant nature. On the other hand, Çağıl cultivar showed increasing levels of H_2O_2 and MDA content, which are the indicators of oxidative damage, as stress increases. Also, decreasing shoot length and fresh weight was a result of sensitivity against drought stress.

Antioxidative enzymes are important components of defense systems for plants under stress conditions. They take role in scavenging of reactive oxygen species thus reducing their harmful effects. In this study, APX, CAT, GR and SOD activities of drought sensitive and drought tolerant cultivars were analyzed. For both cultivars significant changes in enzyme activities were not observed. However; as two cultivars compared, it has been seen that for three antioxidative enzymes (APX, CAT and GR) Seyran cultivar had much higher enzyme activities than that of the Çağıl cultivar and for SOD activity a significant decrease was observed in Çağıl cultivar under drought stress conditions, while Seyran cultivar did not show any change in SOD activity.

Effect of drought on the protein levels of drought sensitive and drought tolerant lentil cultivars were also analyzed by SDS-PAGE and Bioanalyzer. Both of these analyses showed that drought-tolerant and drought sensitive cultivars up- and down-regulated different proteins due to the water deficiency. these proteins might be heatshock proteins, chaperons or any other plant defense mechanism proteins. Identification of these proteins will be very important for understanding resistance mechanisms of crop plants under drought conditions and enhancing tolerance via genetic approaches.

REFERENCES

Adsule R, N., S. S. Kadam, et al. (1989). Nutritional Chemistry, Processing Technology, and Utilization. Lentil in Handbook of World Food Legumes. D. K. Salunkhe, S. S. Kadam, eds. CRC Press, Inc, Boca Raton, Florida, Vol II: 131–152.

Aksoy, E. (2008). “Effect of drought and salt stresses on the gene expression levels of antioxidant enzymes in lentil (*Lens culinaris M.) s*eedlings”. MSc Thesis. Middle East Technical University, Ankara Turkey.

Andrews, M. and B. A. McKenzie (2007). Adaptation and Ecology
Lentil. S. S. Yadav, D. L. McNeil and P. C. Stevenson, Springer Netherlands**:** 23-32.

Aono, M., H. Saji, et al. (1995). "Paraquat Tolerance of Transgenic Nicotiana tabacum with Enhanced Activities of Glutathione Reductase and Superoxide Dismutase." Plant and Cell Physiology **36**(8): 1687-1691.

Apel, K. and H. Hirt (2004). "Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction." Annual Review of Plant Biology **55**(1): 373-399.

Arora, A., T. M. Byrem, et al. (2000). "Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids." Arch Biochem Biophys **373**(1): 102-109.

Arora, A., R. K. Sairam, et al. (2002). Oxidative stress and antioxidative system in plants, Current Science Association.

Asada, K. (1999). “The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons.” Ann Rev Plant Physiol Plant Mol Biol **50:** 601–39.

Ashraf, M. and M. R. Foolad (2007). "Roles of glycine betaine and proline in improving plant abiotic stress resistance." Environmental and Experimental Botany **59**(2): 206-216.

Ashraf, M. and L. Wu (1994). "Breeding for Salinity Tolerance in Plants." Critical Reviews in Plant Sciences **13**(1): 17-42.

Bandeoğlu, E. (2001). ""Effect of salt stress on antioxidative defense system in Lentil". MSc Thesis. Middle East Technical University, Ankara Turkey.

Bandeoğlu, E., F. Eyidoğan, et al. (2004). "Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress." Plant Growth Regulation **42**(1): 69-77.

Bartels, D., R. Sunkar (2005). "Drought and salt tolerance in plants." Criti. Rev. Plant Sci. **24**: 23–58.

Bartels, D. and E. Souer (2004). Molecular responses of higher plants to dehydration. Plant Responses to Abiotic Stress. H. Hirt and K. Shinozaki, Springer Berlin / Heidelberg. **4:** 9-38.

Bates, L.S., R. P. Waldren, et al. (1977). "Rapid determination of free proline for water stress studies". Plant Soil **39:** 205-207.

Bhattacharya, S., H. V. Narasimha, et al. (2005). "The moisture dependent physical and mechanical properties of whole lentil pulse and split cotyledon." International Journal of Food Science & Technology **40**(2): 213-221.

Bhardwaj, J. and S. K. Yadav (2012). "Comparative study on biochemical parameters and antioxidant enzymes in a drought tolerant and a sensitive variety of Horsegram (*Macrotyloma uniflorum*) under drought stress." American journal of Plant Physiology **7**(1): 17-29.

Blum, A., R. Munns, et al. (1996). “Genetically engineered plants resistant to soil drying and salt stress. How to interpret osmotic relations?” Plant Physio **110:** 1051.

Bota, J., H. Medrano, et al. (2004). "Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress?" New Phytologist **162**(3): 671-681.

Bradford, M.M. (1976). “A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding.” Analytical Biochemistry **72:** 248-254.

Cabiscol, E., E. Piulats, et al. (2000). "Oxidative Stress Promotes Specific Protein Damage inSaccharomyces cerevisiae." Journal of Biological Chemistry **275**(35): 27393-27398.

Celikkol Akcay, U., O. Ercan, et al. (2010). "Drought-induced oxidative damage and antioxidant responses in peanut seedlings." Plant Growth Regulation **61**(1): 21-28.

Chance, B., and A. C. Maehly (1995). “Assay of catalases and peroxidases” Methods in Enzymology **2:** 764-817.

Chandra, A. and D. Pental (2003). “Regeneration and genetic transformation of grain legumes: An overview.” Curr Sci **84:** 381–387.

Chaves, M. M., J. S. Pereira, et al. (2002). "How Plants Cope with Water Stress in the Field? Photosynthesis and Growth." Annals of Botany **89**(7): 907-916.

Chen, T. H. and N. Murata (2002). "Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes." Curr Opin Plant Biol **5**(3): 250-257.

Cicerali, I.N. (2004). “Effect of salt stress on antioxidant defense systems of sensitive and resistant cultivars of Lentil (*Lens culinaris* M.)”. MSc Thesis. Middle East Technical University, Ankara Turkey.

Corpas, F. J., J. M. Palma, et al. (2008). "Peroxisomal xanthine oxidoreductase: characterization of the enzyme from pea (Pisum sativum L.) leaves." J Plant Physiol **165**(13): 1319-1330.

Del Río, L. A., L. M. Sandalio, et al. (1992). “Metabolism of oxygen radicals in peroxisomes and cellular implications.” Free Radical Biology and Medicine **13**: 557–580.

Desikan, R., J. Hancock, et al. (2004). Oxidative stress signalling
Plant Responses to Abiotic Stress. H. Hirt and K. Shinozaki, Springer Berlin / Heidelberg. **4:** 121-149.

Dita, M., N. Rispail, et al. (2006). "Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes." Euphytica **147**(1): 1-24.

Dizdaroglu, M. (1993). Chemistry of free radical damage to DNA and nucleoproteins. DNA and Free Radicals, B. Halliwell and O. I. Aruoma, Eds. Ellis Horwood, London, UK: 19–39.

Elstner, E. F. (1991). Active Oxygen/Oxidative Stress in Plant Metabolis.

Erakar, S. and C. Murumkar (1995). "Proline accumulation in *Tephrosia purpurea* pers." Biologia Plantarum **37**(2): 301-304.

Ercan, O. (2008). “Effect of drought and salt stress on antioxidant defense system and physiology of Lentil (*Lens culinaris* M.) seedlings”. MSc Thesis. Middle East Technical University, Ankara Turkey.

Evans, M. D., M. Dizdaroglu, et al. (2004). "Oxidative DNA damage and disease: induction, repair and significance." Mutation research **567**(1): 1-61.

Foyer, C. H. and J. C. Harbinson (1994). Causes of Photooxidative Stress and Amelioration of Defense Systems in Plant.

Foyer, C. H., H. Lopez-Delgado, et al. (1997). "Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling." Physiologia Plantarum **100**(2): 241-254.

Foyer, C. H. (2001). “Prospects for enhancement the soluble antioxidants ascorbate and glutathione.” Biol Fac **15**:75–8.

Goetz, H., M. Kuschel, et al. (2004). "Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination." J Biochem Biophys Methods **60**(3): 281-293.

Goodwin, S. M. and M. A. Jenks (2007). Plant Cuticle Function as a Barrier to Water Loss. Plant Abiotic Stress, Blackwell Publishing Ltd**:** 14-36.

Halliwell, B. and O. I. Aruoma (1991). "DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems." FEBS letters **281**(1-2): 9-19.

Halliwell, B. and J. M. C. Gutteridge (1989). Free radicals in biology and medicine. Oxford, Clarendon Press.

Han, C., Q. Liu, et al. (2009) “Short-term effects of experimental warming and enhanced ultraviolet-B radiation on photosynthesis and antioxidant defense of *Picea asperata* Seedlings.” Plant Growth Regulation **58**(2):153–162.

Hirt, H. and K. Shinozaki (2003). Plant Responses To Abiotic Stress. Topics in Current Genetics, Vol. 4, Springer-Verlag Berlin Heidelberg.

Hoagland, D. R. and D. I. Arnon (1938). The water-culture method for growing plants without soil. Berkeley, Calif., University of California, College of Agriculture, Agricultural Experiment Station.

Hoekstra, F. A., E. A. Golovina, et al. (2001). "Mechanisms of plant desiccation tolerance." Trends in plant science **6**(9): 431-438.

Hossain, M. A. and K. Asada (1985). "Monodehydroascorbate reductase from cucumber is a flavin adenine dinucleotide enzyme." J Biol Chem **260**(24): 12920-12926.

Hsu, S. Y., Y. T. Hsu, et al. (2003). "The Effect of Polyethylene Glycol on Proline Accumulation in Rice Leaves." Biologia Plantarum **46**(1): 73-78.

Ivanov, B. N. and S. Khorobrykh (2003). “Participation of photosynthetic electron transport in production and scavenging of reactive oxygen species.” Antioxidants and Redox Signaling **5**(1): 43–53.

Jackson, C., J. Dench, et al. (1978). "Subcellular Localisation and Identification of Superoxide Dismutase in the Leaves of Higher Plants." European Journal of Biochemistry **91**(2): 339-344.

Jones , H.G. and M.B. Jones (1989). Introduction: some terminology and common Mechanisms. Plants Under Stress, H.G. Jones, T.J. Flowers, and M.B. Jones (Eds.), Cambridge university Press, Cambridge: 1–10.

Kavi-Kishor, P.B., S. Sangam, et al. (2005). “Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance.” Curr. Sci. **88**(3): 424-438.

Kuschel, M., T. Neumann, et al. (2002). "Use of lab-on-a-chip technology for protein sizing and quantitation." J Biomol Tech **13**(3): 172-178.

Kwon, S. Y., Y. J. Jeong, et al. (2002). "Enhanced tolerances of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress." Plant, Cell & Environment **25**(7): 873-882.

Levitt, J. (1980). “Responses of plants to environmental stress: chilling, freezing and high temperature stresses.” 2nd ed.. New York: Academic Press.

Mahajan, S. and N. Tuteja (2005). "Cold, salinity and drought stresses: an overview." Arch Biochem Biophys **444**(2): 139-158.

MansWeld, T. J., and C.J. Atkinson (1990). Stomatal behaviour in water stressed plants. R.G. Alscher, J.R. Cumming (Eds.), Stress Responses in Plants: Adaptation and Acclimation Mechanisms, Wiley-Liss, New York: 241–264.

Materne, M., D. McNeil, et al. (2007). Abiotic Stresses
Lentil. S. S. Yadav, D. L. McNeil and P. C. Stevenson, Springer Netherlands**:** 315-329.

McNeil, D., G. Hill, et al. (2007). Global Production and World Trade
Lentil. S. S. Yadav, D. L. McNeil and P. C. Stevenson, Springer Netherlands**:** 95-105.

McWilliam, J. R. (1986). “The national and international importance of drought and salinity effects on agricultural production.” Australian Journal of Plant Physiology **13**: 1–13.

Meyer, S. and B. Genty (1998). "Mapping intercellular CO2 mole fraction (Ci) in rosa rubiginosa leaves fed with abscisic acid by using chlorophyll fluorescence imaging. Significance Of ci estimated from leaf gas exchange." Plant Physiol **116**(3): 947-957.

Mishra, S., A. B. Jha, et al. (2011). “Arsenite treatment induces oxidative stress, upregulates antioxidant system, and causes phytochelatin synthesis in rice seedlings.” Protoplasma **248**(3): 565–577.

Mittler, R. (2006). "Abiotic stress, the field environment and stress combination." Trends in plant science **11**(1): 15-19.

Moller, I. M. and B. K. Kristensen (2004). "Protein oxidation in plant mitochondria as a stress indicator." Photochemical & Photobiological Sciences **3**(8): 730-735.

Muehlbauer, F. J., W. J. Kaiser, et al. (1995). “Production and breeding of lentil.” Advances in Agronomy **54**: 283–332.

Nanjo, T., M. Kobayashi, et al. (1999). “Antisense suppression of the proline degradation improves tolerance to freezing and salinity in Arabidopsis thaliana” FEBS Letters **461:** 205-210.

Nayyar, H. and D. P. Walia (2003). "Water Stress Induced Proline Accumulation in Contrasting Wheat Genotypes as Affected by Calcium and Abscisic Acid." Biologia Plantarum **46**(2): 275-279.

Neill, S., R. Desikan, et al. (2002). “Hydrogen peroxide signalling,” Current Opinion in Plant Biology **5**(5): 388–395.

Ohkawa, H., N. Ohishi, et al. (1979). “Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction.” Analytical Biochemistry **95:** 51-358.

Pallavi, S., J. Ambuj, et al. (2010). Oxidative Stress and Antioxidative Defense Systems in Plants Growing under Abiotic Stresses. Handbook of Plant and Crop Stress,Third Edition, CRC Press**:** 89-138.

Patterson, W. R. and T. L. Poulos (1995). "Crystal structure of recombinant pea cytosolic ascorbate peroxidase." Biochemistry **34**(13): 4331-4341.

Peltzer, D., E. Dreyer, et al. (2002). “Temperature dependencies of antioxidative enzymes in two contrasting species.” Plant Physiol Biochem **40:**141–50.

Ramachandra Reddy, A., K. V. Chaitanya, et al. (2004). "Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants." J Plant Physiol **161**(11): 1189-1202.

Redden, B., N. Maxted, et al. (2007). Lens Biodiversity
Lentil. S. S. Yadav, D. L. McNeil and P. C. Stevenson, Springer Netherlands**:** 11-22.

Richter, C. (1992). "Reactive oxygen and DNA damage in mitochondria." Mutation research **275**(3-6): 249-255.

Sandhu, J. S. and S. Singh (2007). History and Origin
Lentil. S. S. Yadav, D. L. McNeil and P. C. Stevenson, Springer Netherlands**:** 1-9.

Sarker, A. and W. Erskine (2006). "Recent progress in the ancient lentil." The Journal of Agricultural Science **144**(01): 19-29.

Scandalios, G., L. Guan, et al. (1997). Catalases in plants: gene structure, properties, regulation and expression. Oxidative Stress and the Molecular Biology of Antioxidants Defenses, J. G. Scandalios, Ed., Cold Spring Harbor Laboratory Press, New York, NY, USA: 343–406.

Sharma, P. and A. B. Jha, et al. (2012). "Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions." Journal of Botany **2012**: 26.

Sharma, P., A. B. Jha, et al. (2010). “Oxidative stress and antioxidative defense system in plants growing under abiotic Stresses.” in *Handbook of Plant and Crop Stress*,M. Pessarakli, Ed., pp. 89–138, CRC Press, Taylor and Francis Publishing Company, Fla, USA, 3rd edition

Schuppler, U., P. H. He, et al. (1998). "Effect of water stress on cell division and cell-division-cycle 2-like cell-cycle kinase activity in wheat leaves." Plant Physiol **117**(2): 667-678.

Seo, M. and T. Koshiba (2002). “Complex regulation of ABA biosynthesis in plants.” Trends Plant Sci **7:**41-48

Sgherri, C.L.M., B. Liggini, et al. (1994). “Antioxidant system in Sporobolus stapfianus: changes in response to dessicationand rehydration.” Phytochem **35**(3):561-565.

Simova-Stoilova, L., K. Demirevska, et al.(2009). “Antioxidative protection and proteolytic activity in tolerant and sensitive wheat (*Triticum aestivum* L.) varieties subjected to longterm field drought.” Plant Growth Regul. **58**: 107-117.

Smirnoff, N. (1998). "Plant resistance to environmental stress." Curr Opin Biotechnol **9**(2): 214-219.

Smirnoff, N. (1995). Antioxidant systems and plant response to the environment. Environment and Plant Metabolism: Flexibility and Acclimation, N. Smirnoff, Ed, Bios Scientific Publishers, Oxford, UK: 217–243.

Somers, D.A., D.A. Samac, et al. (2003). “Recent advances in legume transformation.” Plant Physiol **131:** 892–899.

Tamura, T., K. Hara, et al. (2003). “Osmotic stress tolerance of transgenic tobacco expressing a gene encoding a membrane-located receptor-like protein from tobacco plants.” Plant Physiol **131:**454–62.

Tanou, G., A. Molassiotis, et al. (2009). “Induction of reactive oxygen species and necrotic death-like destruction in strawberry leaves by salinity.” Environmental and Experimental Botany **65**(2-3): 270–281.

Tezara, W., V. J. Mitchell, et al. (1999). “Stress inhibits plant photosynthesis by decreasing coupling factor and ATP.” Nature **1401**:914–7.

Upadhyaya, H., M. H. Khan, et al.(2007).” Hydrogen peroxide induces oxidative stress in detached leaves of *Oryza sativa* L.” Gen. Appl. Plant Physiology **33**(1-2): 83-95.

Urbano, G., J. Porres, et al. (2007). Nutritional Value. Lentil. S. Yadav, D. McNeil and P. Stevenson, Springer Netherlands**:** 47-93.

Ushimaru, T., Y. Maki, et al. (1997). "Induction of Enzymes Involved in the Ascorbate-Dependent Antioxidative System, Namely, Ascorbate Peroxidase, Monodehydroascorbate Reductase and Dehydroascorbate Reductase, after Exposure to Air of Rice (Oryza sativa) Seedlings Germinated under Water." Plant and Cell Physiology **38**(5): 541-549.

Vanacker, H., T. L. W. Carver, et al. (2000). “Early H2O2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction.” Plant Physiol. **123**:1289–300.

Vinocur, B. and A. Altman (2005). "Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations." Curr Opin Biotechnol **16**(2): 123-132.

Wang, S.Y., H. Jiao, et al. (1991). “Changes in ascorbate, glutathione and related enzyme activities during thiodiazuron-induced bud break of apple.” Plant Physiology **82:** 231-236.

Wang, W., F. Tai, et al. (2008). "Optimizing protein extraction from plant tissues for enhanced proteomics analysis." J Sep Sci **31**(11): 2032-2039.

Wang, W., R. Vignani, et al. (2006). "A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis." Electrophoresis **27**(13): 2782-2786.

Welinder, K. G. (1992). “Superfamily of plant, fungal and bacterial peroxidases.” Current Opinion in Structural Biology **2**(3): 388–393.

Whitmore, A. P. and W. R. Whalley (2009). "Physical effects of soil drying on roots and crop growth." Journal of Experimental Botany **60**(10): 2845-2857.

Wood, A. J. (2007). Eco-physiological Adaptations to Limited Water Enviornments. Plant Abiotic Stress, Blackwell Publishing Ltd**:** 1-13.

Yadav, S., P. Stevenson, et al. (2007). Uses and Consumption
Lentil. S. S. Yadav, D. L. McNeil and P. C. Stevenson, Springer Netherlands**:** 33-46.

Yamauchi, Y., A. Furutera, et al. (2008). "Malondialdehyde generated from peroxidized linolenic acid causes protein modification in heat-stressed plants." Plant Physiol Biochem **46**(8-9): 786-793.

Young, Y. (1991). “The photoprotective role of carotenoids in higher Plants.” Physiologia Plantarum **83**(4): 702–708.

Zhu, J. K. (2001). "Plant salt tolerance." Trends Plant Sci **6**(2): 66-71.

Zhu, J. K. (2002). "Salt and drought stress signal transduction in plants." Annual Review Plant Biology **53**: 247-273.

APPENDIX A

HOAGLAND'S E-MEDIUM PREPARATION

1. Add proper amounts of solutions as given in Table A.1.
2. Adjust the pH of the medium to 5.8 by using NaOH or HCl.
3. Sucrose may be added as 10 g/L if the culture is axenic.
4. Autoclave the medium. (FeEDTA addition to the medium should be performed after autoclaving.)

Table A. 1 Preparation of Hoagland's Medium

COMPOSITION	STOCK SOLUTION	USE (ml/L)
$MgSO_4.7H_2O$	24.6 g/100ml	1.0 ml
$Ca(NO_3)_{2.}4H_2O$	23.6 g/100ml	2.3 ml
KH_2PO_4	13.6 g/100ml	0.5 ml
KNO_3	10.1 g/100ml	2.5 ml
Micronutrients	See table B	0.5 ml
FeEDTA	See table C	20.0 ml

Table A. 2 Preparation of Micronutrient Stock Solutions

MICRONUTRIENT	STOCK SOLUTION
H_3BO_3	2.86 g/L
$MnCl_{2.}4H_2O$	1.82 g/L
$ZnSO_{4.}7H_2O$	0.22g/L
$Na_2MoO_{4.}2H_2O$	0.09 g/L
$CuSO_{4.}5H_2O$	0.09 g/L

Table A. 3 Preparation of FeEDTA Stock Solution

ADDITION	STOCK SOLUTION
$FeCl_3.6H_2O$	0.121 g / 250 ml
EDTA	0.375 g / 250 ml

Dissolve both $FeCl_3.6H_2O$ and EDTA in dH_2O and then complete the volume to 250 ml.

(Use 3.0 ml of FeEDTA stock to 150ml of Hoaglands.)

APPENDIX B

BRADFORD RESULTS

I. Preparation of Solutions:

1. 5X Bradford Reagent:

Dissolve 500 mg of Coomassie Brilliant Blue G-250 in 250 ml of 95% ethanol, add 500 ml of 85% (w/v) phosphoric acid to the mixture, dilute the solution to 1 L with dH_2O and filter before use.

2. 1 mg/ml BSA: freshly prepared

Dissolve 1 mg of BSA in 1 ml dH_2O.

II. Procedure:

For Standard Curve:

1. Make the necessary dilutions as indicated in the table (duplicates for each tube).

Table B. 1 Preparation of BSA Standards

	dH_2O (µl)	BSA (µl)	Bradford Reagent (ml)
1-1’	500	0	5
2-2’	495	5	5
3-3’	490	10	5
4-4’	480	20	5
5-5’	470	30	5
6-6’	450	50	5
7-7’	420	80	5
8-8’	400	100	5

2. Vortex the tubes and incubate them at RT for 10 mins.
3. Read the absorbances of the tubes against the blank (1-1') at 595 nm.
4. Plot OD (595nm) vs. concentration (mg/ml) of BSA as the standard curve.

For the Protein Determination of Samples:

1. Make the necessary dilutions for each sample as indicated in the table

Table B. 2 Preparation of Samples

	dH_2O (µl)	Sample (µl)	Bradford Reagent (ml)
Shoot	480	20	5
Root	460	40	5

2. Read the absorbances of the tubes against the blank (1-1' of standard curve) at 595 nm.
3. Calculate the concentrations of the samples in each tube and make necessary calculations in order to load the equivalent amounts of samples.

   Conc. = (Av. OD / slope) * DF

   where DF (dilution factor) = 500/20 for leaves

   500/40 for roots

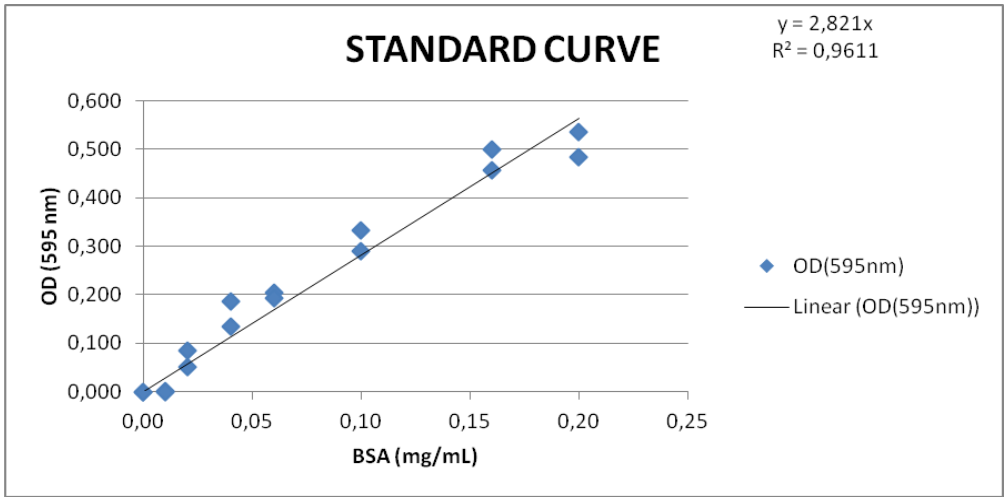

Figure B. 1 Standard curve of Bradford for enzyme and total protein analyses

APPENDIX C

RESULTS OF PRELIMINARY STUDY

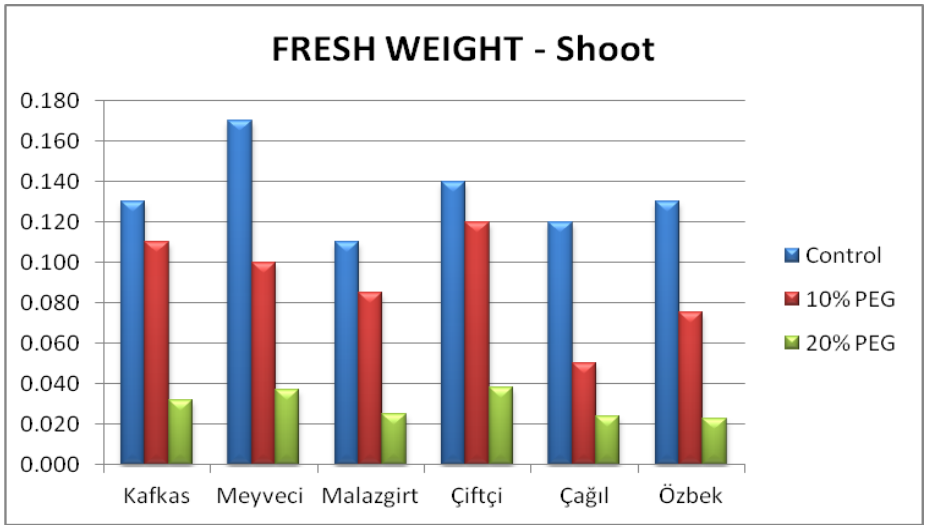

Figure C. 1 Shoot fresh weights of 7 days grown and 4 days treated (C, 10% PEG, 20% PEG) control and PEG treated plants

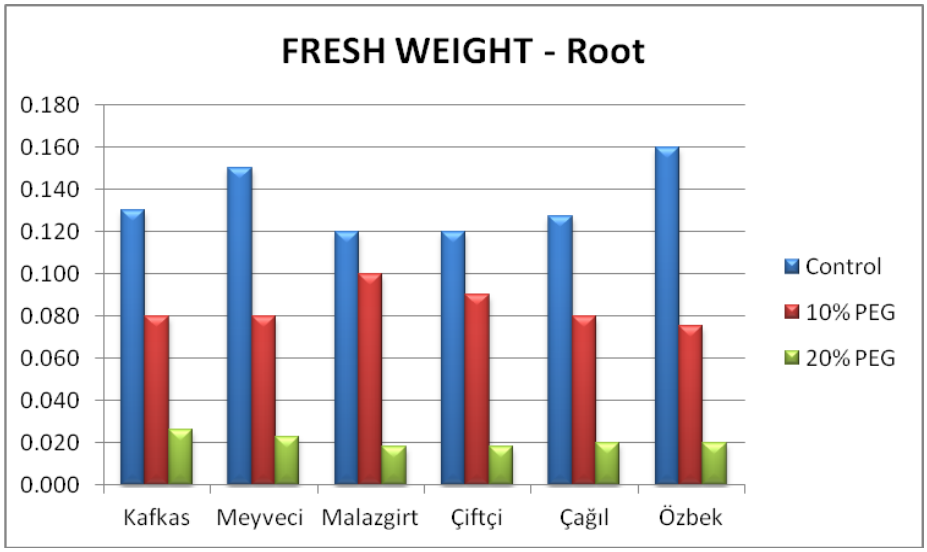

Figure C. 2 Root fresh weights of 7 days grown and 4 days treated (C, 10% PEG, 20% PEG) control and PEG treated plants

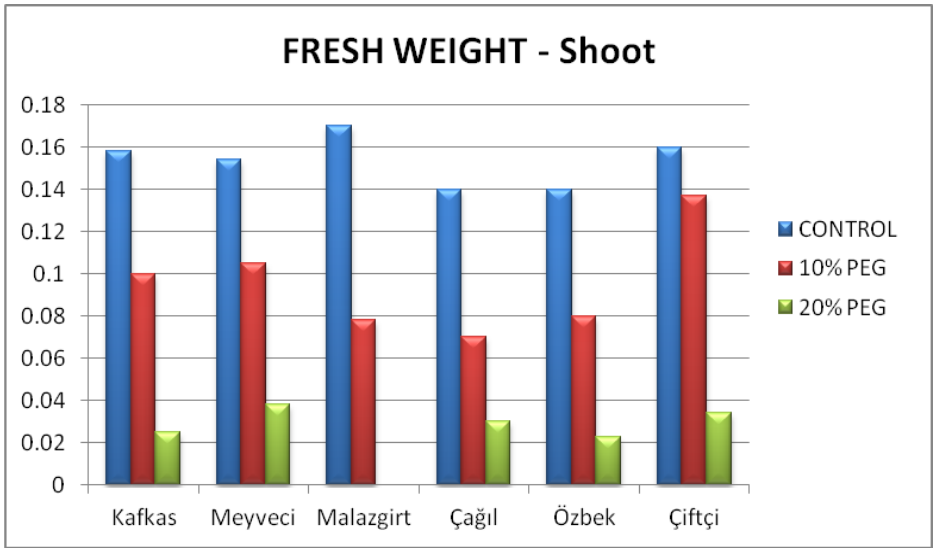

Figure C. 3 Shoot fresh weights of 7 days grown and 5 days treated (C, 10% PEG, 20% PEG) control and PEG treated plants

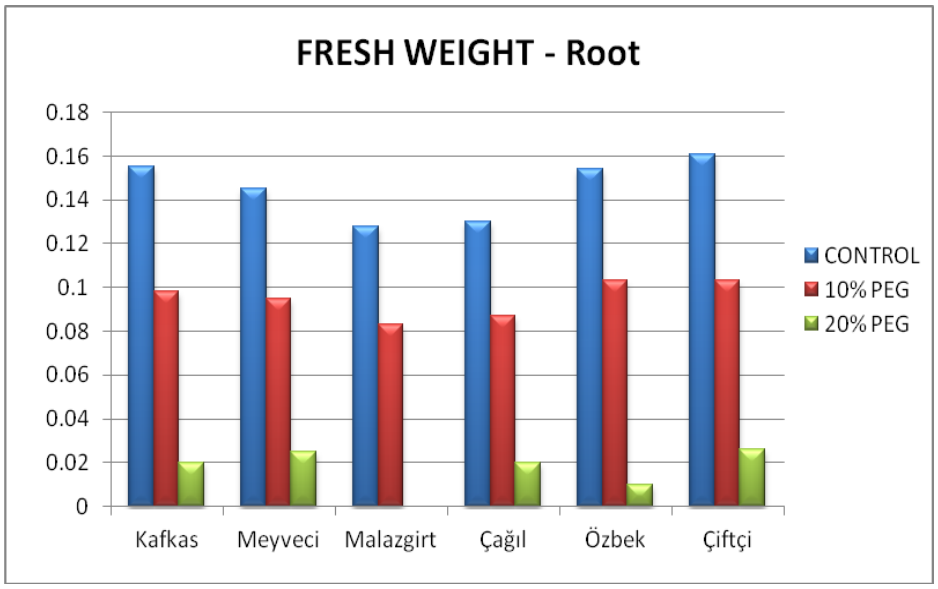

Figure C. 4 Root fresh weights of 7 days grown and 5 days treated (C, 10% PEG, 20% PEG) control and PEG treated plants