# PHYSIOLOGICAL AND BIOCHEMICAL SCREENING OF DIFFERENT TURKISH LENTIL CULTIVARS UNDER SALT STRESS CONDITIONS

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**PHYSIOLOGICAL AND BIOCHEMICAL SCREENING OF DIFFERENT TURKISH LENTIL CULTIVARS UNDER SALT STRESS CONDITIONS**

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# ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL SCREENING OF DIFFERENT TURKISH LENTIL CULTIVARS UNDER SALT STRESS CONDITIONS

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Salinity is the 2nd major limiting abiotic factor on plant growth. As a result of this, soil salinity greatly reduce the yield of the crop production by dual action on plants which are ionic toxicity and water deficit. Therefore, improvement of stress tolerance is greatly concerned. This study was performed to screen and select a salt-resistant and a salt-sensitive cultivar among 6 Turkish lentil cultivars (*Lens culinaris* M.) which are Çağıl, Çiftçi, Kafkas, Malazgirt, Seyran and Özbek according to the physiological and biochemical properties. 12 days old lentil seedlings which were exposed to salt stress (100 mM NaCl and 150 mM NaCl) for 5 days as well as control groups analyzed physiologically by root-shoot fresh weights, and lengths; and biochemically by ion leakage, MDA, $H_2O_2$ and proline content determination. Seyran and Malazgirt were selected as salt-resistant and salt-sensitive, respectively. Selected two cultivars were also compared in terms of antioxidative defense system (SOD, CAT, APX and GR activity determination). Bioanalyzer and SDS-PAGE were performed to contrast total protein profile of salt-resistant and salt-sensitive cultivars.

Keywords: *Lens culinaris* M., lentil varieties, salt stress, antioxidant defense, total protein profile

# ÖZ

TUZ STRESİ ALTINDA TÜRK MERCİMEK ÇEŞİTLERİNİN FİZYOLOJİK VE BİYOKİMYASAL TARAMASI

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Eylül 2012, 88 Sayfa

Tuzluluk bitki büyümesini sınırlayan ikinci önemli abiyotik stres faktörüdür. Bu yüzden, toprak tuzluluğu ürün verimini ikili etki; iyon toksisitesi ve su eksikliği ile düşürür. Bunun sonucunda, strese karşı tolerans geliştirme geniş ölçüde düşünülmektedir. Bu çalışma 6 tane kırmızı mercimek çeşidi (Çağıl, Çiftçi, Kafkas, Malazgirt, Seyran ve Özbek) arasından gövde ve kök fizyolojik ve biyokimyasal analizlerin karşılaştırmalarına göre tuza dayanıklı ve tuza duyarlı çeşitleri belirlemek için yürütülmüştür. Kontrol grupları ile birlikte 100 mM ve 150 mM tuza (NaCl) maruz kalmış 12 günlük mercimek fideleri (*Lens culinaris* M.) fizyolojik olarak (ağırlık ve uzunluk) ve biyokimyasal olarak (iyon geçirgenliği, MDA, $H_2O_2$ ve prolin miktar tayini) incelendi. Seçilen 2 çeşit, tuz stresinin gövde ve kök antioksidant savunma sistemine (SOD, CAT, APX ve GR enzimleri aktivite tayini) etkisini gözlemlemek için karşılaştırıldı. Her çeşidi kendi içinde ve dayanıklı-duyarlı çeşitlerin total protein profillerini karşılaştırmak için Bioanalyzer ve SDS-PAGE uygulandı. Fizyolojik ve biyokimyasal analizler sonucu Seyran tuza dirençli ve Malazgirt tuza duyarlı mercimek çeşidi olarak seçildi.

Anahtar kelimeler: *Lens culinaris* M., mercimek çeşitleri, tuz stresi, antioksidant savunma, total protein profili

*To my family,*

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| | |
|---|---|
| ABA | Abscisic acid |
| APX | Ascorbate peroxidase |
| ASA | Ascorbate |
| CAT | Catalase |
| DHA | Dehydroascorbate |
| DHAR | Dehydroascorbate reductase |
| ETC | Electron transport chain |
| GR | Glutathione reducatse |
| GSH | Reduced glutathione |
| GSSG | Oxidized glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| LEA | Late embryogenesis-abundant |
| MDA | Malondialdehyde |
| MDHA | Monodehydroascorbate |
| MDHAR | Monodehydroascorbate reductase |
| MAPK | Mitogen-activated protein kinase |
| NaCl | Sodium chloride |
| $OH^{\bullet}$ | Hydroxyl radical |
| $O_2$ | Molcular oxygen |
| $^1O_2$ | Singlet oxygen |
| $O_2^{\bullet -}$ | Superoxide radical |
| ROS | Reactive oxygen species |
| SDS-PAGE | Sodium dodecyl polyacrylamide gel |
| SEM | Standard error of mean |
| SOS | Salt overly sensitive |

# CHAPTER 1

# INTRODUCTION

## 1.1 Lentil

Lentil (*Lens culinaris* Medik.) is a self-pollinated and annual legume plant. It is a diploid plant (2n=14) (Barulina, 1930). The seeds of lentil are round, small and disk-shaped. Lentil cotyledons are red, orange, yellow or green (Kay, 1979; Duke, 1981).

The name of the lentil species has been called *Lens culinaris* Medik. After Medikus published *Lens culinaris* in 1787.

### 1.1.1 History and Origin of Lentil

Lentil is an ancient grain legume species. The oldest (dated to 11,000 BC) carbonized lentil remnants were found at Greece in a cave and at Syria traced back to 8500-7500 BC (Van Zeist, 1971; Zohary, 1972; Hansen *et al.,* 1978). Lentil (*Lens culinaris*) is native to the near East and Central Asia. The ancestors of cultivated *Lens culinaris* subspecies *orientalis* was found in Turkey, Syria, Lebanon, Israel, Iraq and Uzbekistan. Archaelogical studies reveal that Turkey, Syria and Iraq are responsible for the spread of cultivated lentil to Central Europe, South Asia and America (Ladizinsky, 1979; Zohary, 1972).

### 1.1.2 Nutritional and Medicinal Value

Lentil is rich in protein, carbohydrate and calorie amount, besides it is cholesterol-free (Iqbal *et al.*, 2006; Muehlbauer, 1985). Because of this, it is an important component of human diet. It is also rich in soluble and insoluble fiber; vitamins like thiamin, riboflavin, Vitamin C, $B_6$, niacin, pantothenic acid and folic acid; and minerals such as potassium, calcium, magnesium, sodium and phosphorus.

Furthermore, people can easily afford this nutrient-rich food due to its being inexpensive. Therefore, its consumption is quite high especially in developing countries like India (Yadav *et al*., 2007).

Chemical composition of different anatomical parts of lentil seed is shown as gram / 100 gram Dry Matter (DM) in **Error! Reference source not found.** (Adsule *et al*., 1989). The energy of lentil varies between 1483 and 2010 kJ for 100 g DM (Yadav *et al*., 2007).

**Table 1.1** Chemical composition of lentil seed (Adsule *et al*., 1989)

| Component | Proportion to whole seed | Protein (g/100 g DM) | Fat (g/100 g DM) | Crude Fiber (g/100 g DM) | Ash (g/100 g DM) | Nitrogen free extract (g/100 g DM) |
|---|---|---|---|---|---|---|
| Seed Coat | 8.0–20 | 14.3 | 0.6 | 29.4 | 1.94 | 53.7 |
| Cotyledon | 80–90.0 | 26.5–30.1 | 3.0 | 1.0 | 2.45 | 63.4 |
| Embryo | 2.0 | 71.1 | 8.2 | 2.4 | 3.94 | 14.4 |
| Whole seed | 100.0 | 29.6 | 3.1 | 3.2 | 2.40 | 61.7 |

Sufficient amounts of magnesium provide proper blood flow, oxygen and nutrient transportation; lentil can fulfill this magnesium demand (Viadel *et al*., 2006). Due to its being cholesterol-free and fibered structure, consumption of lentil provides protection from heart diseases. In addition to this, fiber consumption facilitates digestion and contributes colon health, thus risk of colon cancer is some extent prevented (Anon,2006; Araya *et al*.,2002, Flight *et al*., 2006). Its glycemic index is lower than 55, it takes role in defense against Type-2 Diabetes.

### 1.1.3 Global Production

According to Canadian Market Outlook Report; lentil production in 2002-2003 production period was elevated from 2.1 million tons to 2.9 million tons in 2009-2010. The major lentil producing countries are Canada, India, Turkey, United States and Australia, orderly (**Error! Reference source not found.**).

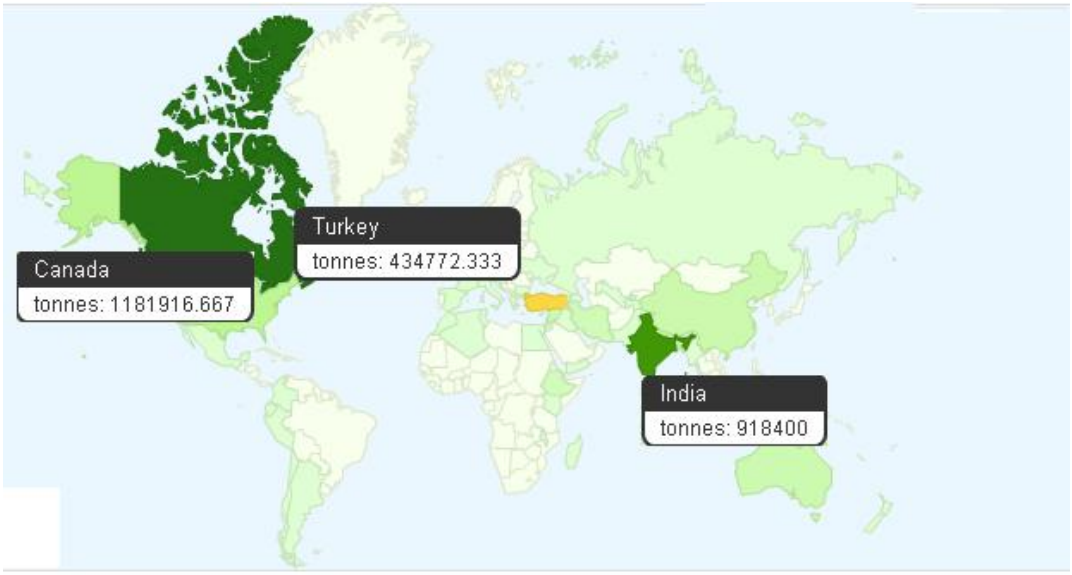


**Figure 1.1** Lentil production quantities of top 3 countries between 2005 and 2010 (FAOSTAT)

Red lentil is the highest ratio of production accounting for about 70% among other lentil types. Between 2006 and 2007, 26.6% and 23.9% of total 2.603 thousand tons of lentil production belongs to Canada and Turkey, respectively. However; in 2008-2009, worldwide 1.862 thousand tones of lentil was produced, 56% and 4.7% of them are belonging to Canada and Turkey, respectively. The alteration in the production trend is resulted from severe drought conditions in Turkey in 2007 and 2008. FAO Statistics between 2005 and 2010 reveals this sharp reduction in production trend in Figure 1.2.

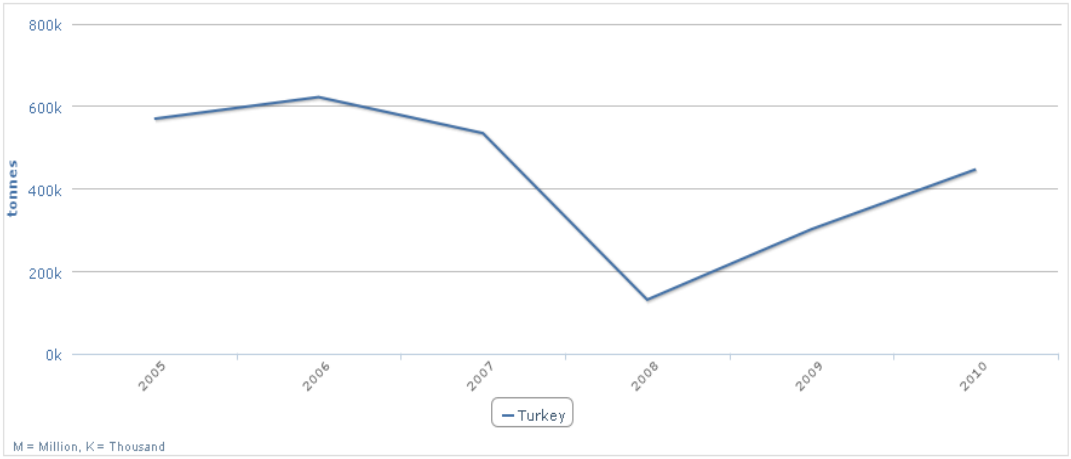


**Figure 1.2** Production of lentil in Turkey between 2005 and 2010 (FAOSTAT)

Canada, United States, Australia, Turkey and India are top lentil exporters. Although, India and Turkey are the second and third in terms of production, they fall behind in ranking of exporter countries, since lentil consumption is pretty much in these countries.

## 1.2 Environmental Stresses

Cells are sensitive to the environmental changes; these differences can be natural or man-made. Biologically, environmental stress can be defined as a detrimental condition, which prevents normal functions and metabolism of an organism such as plants (Gorham *et al.*, 1989).

Environmental stress can be categorized into two main groups; biotic and abiotic stress with respect to their origins. Former develops when plants are exposed to negative effects of other organisms like infection of pathogens and insects, herbivory and competition among organisms, whereas, abiotic stress emerges due to the negative impact of non-living factors on organisms. Types of abiotic stress are chemical stress such as salts, mechanical stress, temperature, water and radiation (Schulze *et al.*, 2005) (**Error! Reference source not found.**).

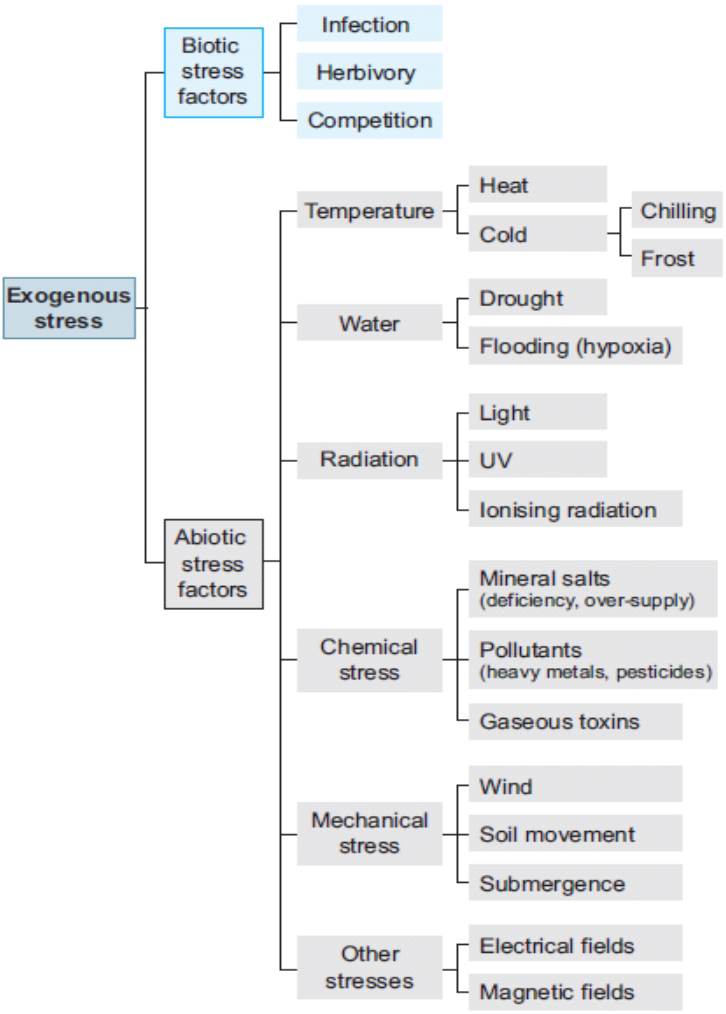


**Figure 1.3** Abiotic and biotic stress factors (Schulze *et al.*, 2005)

Organisms have to respond and additionally modify themselves to cope with environmental stress and also to survive. Unfortunately, plants are the most adversely affected organisms since they are sessile. Therefore, during evolutionary processes, plants develop systems to sense stress stimuli and then some genes are upregulated, which can diminish the effect of stress. Their tolerance to stress factors depends on the evolution level of adaptive features (Mahajan *et al.*, 2005). **Error! Reference source not found.** summarizes effects of main abiotic stresses and plant responses.

**Table 1.2** Abiotic stress effects on plants and responses of plants (Ciarmiello *et al.*, 2011)

| Stress | Consequences | Plant Responses |
|---|---|---|
| Heat stress | High temperature lead to high evaporation and water deficit. The consequent increased turnover of enzymes leads to plant death. | Efficient protein repair systems and general protein stability support survival, temperature can lead to acclimation. |
| Chilling and cold stress | Biochemical reactions proceed at slower rate, photosyntesis proceeds, carbon dioxide fixation lags, leading to oxigen radical damage. Indeed, freezing lead to ice crystal formation that can distrupt cells membranes. | Cessation of growth in adaptable species may be overcome by changes in metabolism. Ice crystal formation can be prevent by osmolyte accumulation and synthesis of hydrophilic proteins. |
| Drought | Inability to water transport to leaves leads to photosyntesis declines. | Leaf rolling and other morphological adaptations. Stoma closure reduces evaporative transpiration induced by ABA. Accumulation of metabolities, consequently lower internal water potential and water attracting. |
| Flooding and submergence | Generates anoxic or microaerobic conditions interfering with mitochondrial respiration. | Development of cavities mostly in the roots that facilitate the exchange of oxigen and ethylene between shoot and root (aerenchyma). |
| Heavy metal accumulation and metal stress | In excess, detoxification reactions may be insufficient or storage capacity may exceeded. | Excess of metal ions may be countered by export or vacuolar deposition but metal ions may also generate oxygen radicals. |
| High light stress | Excess light can lead to increased production of highly reactive intermediates and by-products that can potentially cause photo-oxidative damage and inhibit photosynthesis. | Exposure of a plant to light exceeding what is utilized in photochemistry leads to inactivation of photosynthetic functions and the production of reactive oxygen species (ROS). The effects of these ROS can be the oxidation of lipids, proteins, and enzymes necessary for the proper functioning of the chloroplast and the cell as a whole. |

Worldwide, abiotic stress is the major reason of decline in crop production. On one hand, average yield of most crops fall more than 50%. Therefore, loss of millions of dollars is inevitable each year. On account of this, stress threatens the sustainability of the agricultural sector (Bray, 2000). On the other hand, world population is increasing exponentially, and demand of nutrients cannot be supplied. As a result of this, improvement of stress tolerance of plants is greatly concerned by authorities (Mahajan *et al.*, 2005).

Among abiotic stress factors drought, salinity and extreme temperature conditions are the most limiting ones in terms of crop growth. When the plants encounter fluctuation in water availability, they have evolved some adaptive properties to overcome dehydration problem, such as improving root systems to facilitate water search and absorbance from the soil (Gupta, 2006).

Stress response of plants is highly complex and takes place in 3 steps which is shown in Figure 1.4. Firstly, stress signals are sensed, perceived and transduced. Then, control at transcriptional level takes place. Lastly, stress – responsive mechanisms are activated. Stress is first sensed through receptors on plant cell membrane, and results in formation of secondary messengers such as; calcium, reactive oxygen species and inositol phosphates. These secondary messengers further adjust intracellular calcium concentration and calcium ions bind to the calcium-binding proteins. This interaction causes the phosphorylation cascade and control of transcription factors of a stress-responsive gene. As a result of this, plants improve a defense mechanism against substantial stress factor. (Mahajan *et al.*, 2005).

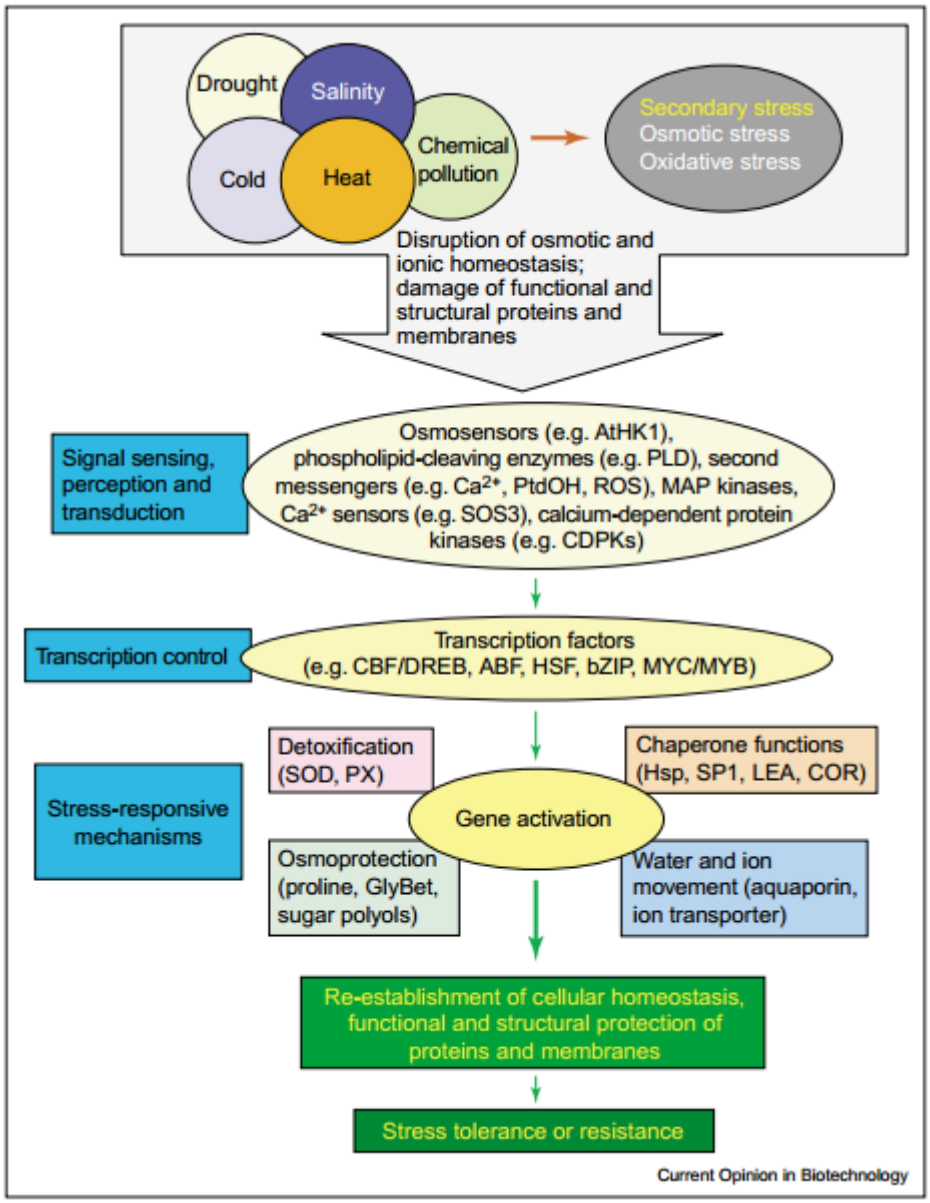


**Figure 1.4** Complex response mechanism of plants to the abiotic stress factors (Vinocur and Altman, 2005)

### 1.2.1 Reactive Oxygen Species

Environmental stress factors can affect the whole plant and decrease the productivity. In general, they cause the production of reactive oxygen species (ROS), which are highly autotoxic, so here first the generation of ROS will be explained and then protection mechanisms.

Molecular oxygen ($O_2$) is essential for aerobic organisms. $O_2$ is reduced by four electrons to generate water molecule during electron transport chain (ETC) in mitochondria. $O_2$ in its ground state is not reactive due to its being paramagnetic. On the other hand, when adequate energy is absorbed or reduction is occurred in a step-wise manner; formation of reactive oxygen species (ROS) is initiated. ROS is synthesized by either enzymatically or ETC as shown in Figure 1.5. ROS has both benefits and hazards on plant depending on their amounts within the cell. During normal growth conditions, ROS elements are found at low concentrations within plant cells and they behave as secondary messengers in signal transduction process, which provide responses of plants such as stomatal closure, gravitropism and programmed cell death to the changing environmental conditions. Besides, removal of ROS is performed by an effective antioxidant defense mechanism. However, under stressed conditions, formation of photosynthetic ROS is accelerated. As a result of this, the balance between generation and elimination is disturbed and ROS accumulation occurs which leads to oxidative stress (Sharma *et al*., 2012). Oxidative stress causes damages in the structures of critical macromolecules including lipids, proteins and DNA (Mittler, 2002; Desikan *et al*., 2004; Miller *et al.*, 2008).

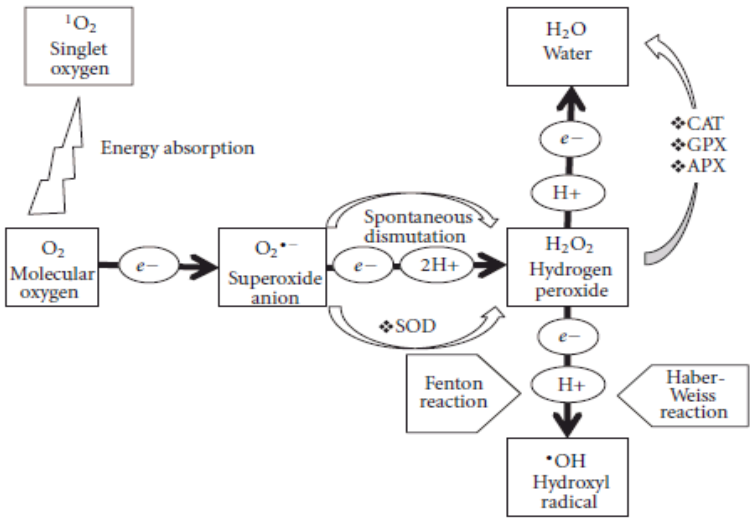


**Figure 1.5** ROS formation and elimination from plant cell (Sharma *et al*., 2012)

### 1.2.1.1 Types of ROS

Approximately 1% of consumed $O_2$ is converted to ROS in many subcellular sites such as chloroplast, mitochondria, endoplasmic reticulum and peroxisome (Figure 1.6) (Asada *et al.,* 1987; Navrot *et al*., 2007; Sharma *et al*., 2012). Superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\bullet}$) are the main ROS elements.

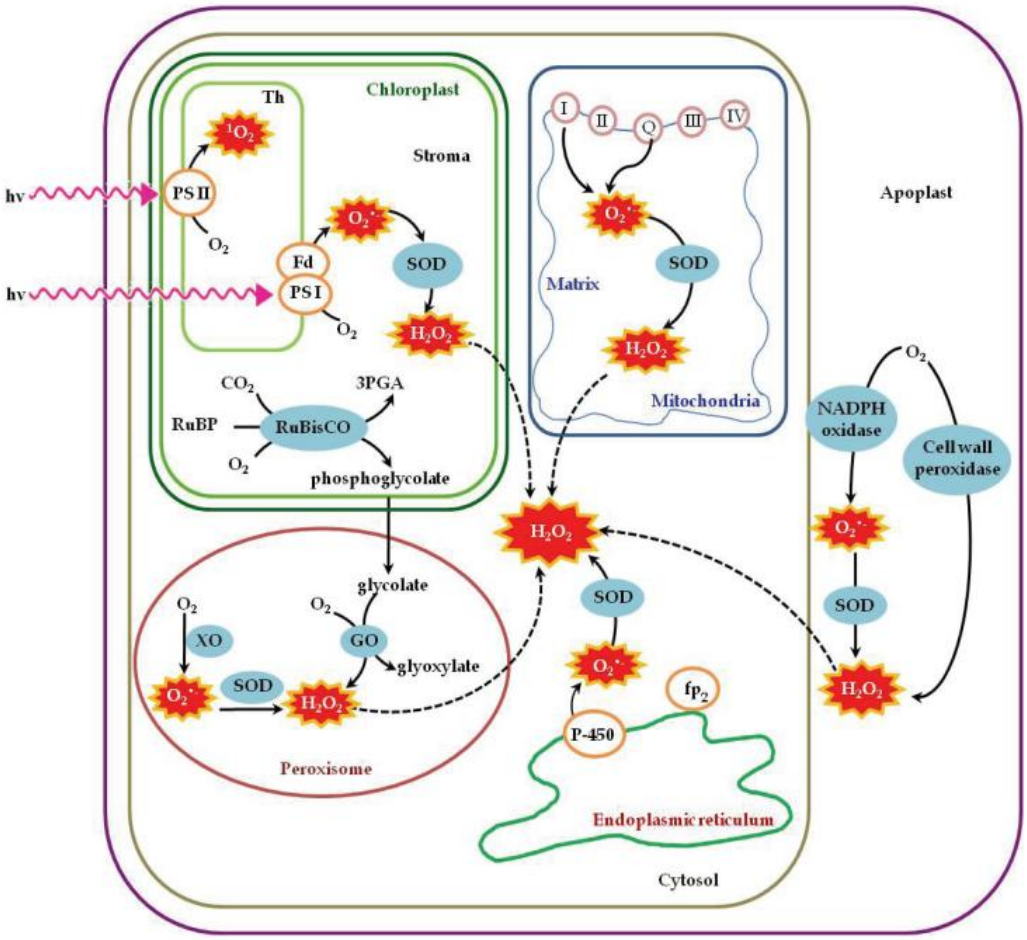


**Figure 1.6** Production sites of ROS (Hossain *et al*., 2011)

***Superoxide Radical***

Superoxide radical ($O_2^{\bullet-}$) generates when $O_2$ is reduced with single electron mostly in thylakoid membrane bound photosystem I (PSI) via Mehler reaction (**Error! Reference source not found.**). $O_2^{\bullet-}$ is moderate ROS that have been produced in first place. Superoxide dismutase (SOD) is further reduced $O_2^{\bullet-}$ and the reaction ends up with $H_2O_2$ (**Error! Reference source not found.**). Moreover $O_2^{\bullet-}$ give rise to $^1O_2$ by giving an electron to $Fe^{3+}$ (**Error! Reference source not found.**) (Gill *et al*., 2010).

$$O_2 + e^- \rightarrow O_2^{\bullet-} \qquad \textbf{Equation 1.1}$$

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

$$O_2^{\bullet-} + Fe^{3+} \rightarrow {}^1O_2 + Fe^{2+} \qquad \textbf{Equation 1.3}$$

***Singlet Oxygen***

Singlet oxygen ($^1O_2$) is the first formed ROS by the light-induced generation of triplet chlorophyll in antenna system and reaction center of photosystem II (PSII). Moreover, its production is induced in the absence of sufficient $CO_2$ because of stomatal closure. It takes role in the oxidation process of proteins, fatty acids and most importantly DNA. Unless it is inactivated by β-carotene or α-tocopherol, programmed cell death is indispensible (Wagner *et al*., 2004; Sharma *et al*., 2012; Gill *et al*., 2010).

$$^3Chl + {}^3O_2 \rightarrow Chl + {}^1O_2 \qquad \textbf{Equation 1.4}$$

***Hydrogen Peroxide***

Hydrogen peroxide ($H_2O_2$) is formed either spontaneously or SOD-catalyzed pathway. Spontaneously, glyoxylate cycle, β-oxidation and photorespiraton end up with $H_2O_2$ in peroxisomes. Moreover, ETC of mitochondria, chloroplast and endoplasmic reticulum are the other $H_2O_2$ sources. It is a neutral moecule which facilitates its diffusion through membranes. Therefore, at low concentrations $H_2O_2$ takes role as a signalling molecule of both biotic and abiotic stress. On the contrary, at high concentrations, programmed cell death is triggered (Bhattachrjee, 2005; Desikan *et al*., 2004; Quan *et al*., 2008).

***Hydroxyl Radicals***

Hydroxyl radical ($OH^{\bullet}$) is formed from $O_2^{\bullet-}$ and $H_2O_2$ by Haber-Weiss reaction (**Error! Reference source not found.**). It composes of two reactions. $O_2^{\bullet-}$ reduces $Fe^{3+}$ (**Error! Reference source not found.**) and formed $Fe^{2+}$ oxidizes $H_2O_2$, leads to the formation of hyrdoxyl radical via Fenton reaction (**Error! Reference source not found.**). $OH^{\bullet}$ is the most reactive ROS due to having a single unpaired electron. It has high affinity to interact all biological macromolecules, these interactions result in lipid peroxidation, destruction of protein structure, membrane disintegrity (Foyer *et al.*,1997). Moreover accumulation of $O_2^{\bullet-}$ causes programmed cell death (Vranova *et al*., 2002). Since there is no enzymatic elimination mechanism for hydroxyl radical, removal of $H_2O_2$ and $O_2^{\bullet-}$ is crucially important by catalase and superoxide dismutase, respectively (Pinto *et al*., 2003).

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \qquad \textbf{Equation 1.5}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad \textbf{Equation 1.6}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + O_2 \qquad \textbf{Equation 1.7}$$

### 1.2.1.2 Effects of ROS on Biological Macromolecules

Equilibrium between generation and annihilation is disturbed under various stressful conditions including salinity, drought, high light, metallic toxicity and invasion of pathogen. ROS in elevated levels has detrimental effects on important cellular macromolecules including lipids, proteins and most importantly DNA (Figure 1.7).

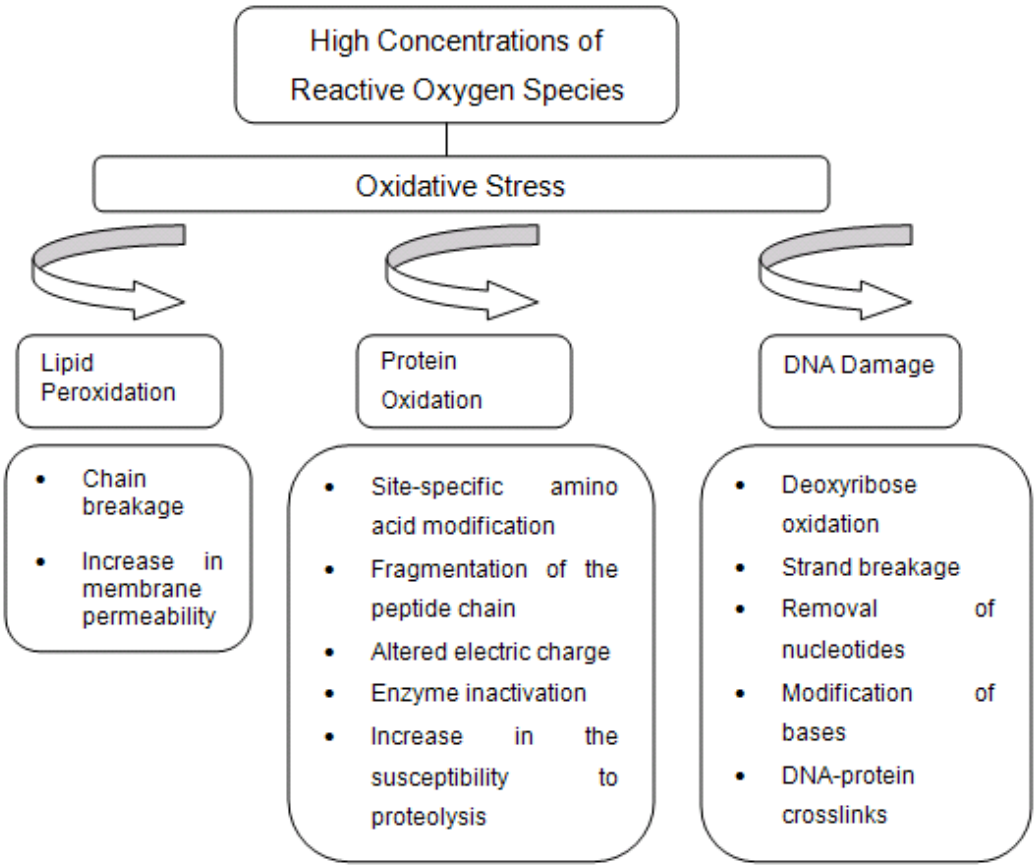


**Figure 1.7** ROS effects on critical macromolecules (Sharma *et al*., 2012)

### *Lipids*

Lipid peroxidation in plasma membrane and organelle membrane results from high concentrations of ROS due to a stressful condition. Lipid peroxidation level indicates damage of membrane composition in a stress-dosage manner (Sharma and Dubey, 2005; Tanou *et al*., 2009). End products of this metabolism can be used as a marker of membrane disintegrity; malondialdehyde (MDA) is one of these (Halliwell and Gutteridge, 1989). Lipid peroxidation pathway occurs in three steps; initiation, propagation and termination. Polyunsaturated fatty acid peroxidation results in rigidity of membrane and harm to the membrane proteins (Moller *et al.*, 2007; Smirnoff, 1995).

***Proteins***

ROS elements directly impair protein structure by chemical modifications and modifications are mostly irreversible. According to the studies, it was realized that protein modification is triggered by hydroxyl radical ($OH^{\bullet}$) but whole protein oxidation process depends on the presence of superoxide radical ($O_2^{\bullet-}$) or its protonated form ($HO_2^{\bullet}$) (Swallow, 1960; Garrison, 1962, 1987; Scheussler *et al*., 1984). Protein inactivation is resulted from oxidation of either backbone or side chains of amino acids. Former one occurs when ROS abstracts α-hydrogen atom of amino acid (Berlett *et al*., 1997). Nitrosylation, disulphide bridge formation, carbonylation and glutathionylation are some types of protein oxidation (Yamauchi *et al.*, 2008).

***DNA***

Nuclear, mitochondrial and chloroplastic DNA are damaged by ROS overaccumulation. Since proteins are encoded from DNA sequence, any change in the genetic code directly causes malfunction or complete inactivation of the proteins. $OH^{\bullet}$ is the most damaging ROS among others, since it attacks to the both nitrogenous bases and deoxyribose backbone (Halliwell *et al.*, 1989). As a result of this attack, protein synthesis reduction, plasma membrane destruction and metabolic proteins modification are observed, which adversely affect growth and development of whole organism (Gill *et al*., 2010; Britt, 1999).

#### 1.2.1.3 Antioxidative Defense System

In plant cells, antioxidative defense system is found in mitochondria, chloroplast and peroxisome as well as ROS production mechanisms (**Error! Reference source not found.**). Antioxidative defense system composes many enzymes including catalase (CAT), superoxide dismutase (SOD), and ascorbate-glutathione cycle enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Ascorbate, glutathione, tocopherols, carotenoids and phenolic compounds are non-enzymatic components of the antioxidative system (Figure 1.8). Under normal circumstances, there is a balance between ROS production and elimination. However; when plant cells face environmental stress factors

especially abiotic ones, generation of ROS overweighs and antioxidative system falls short of the production. As a result of this, ROS accumulates within the cell and plant cells suffer from oxidative stress (Sharma *et al*.,2012; Gill *et al*., 2010).

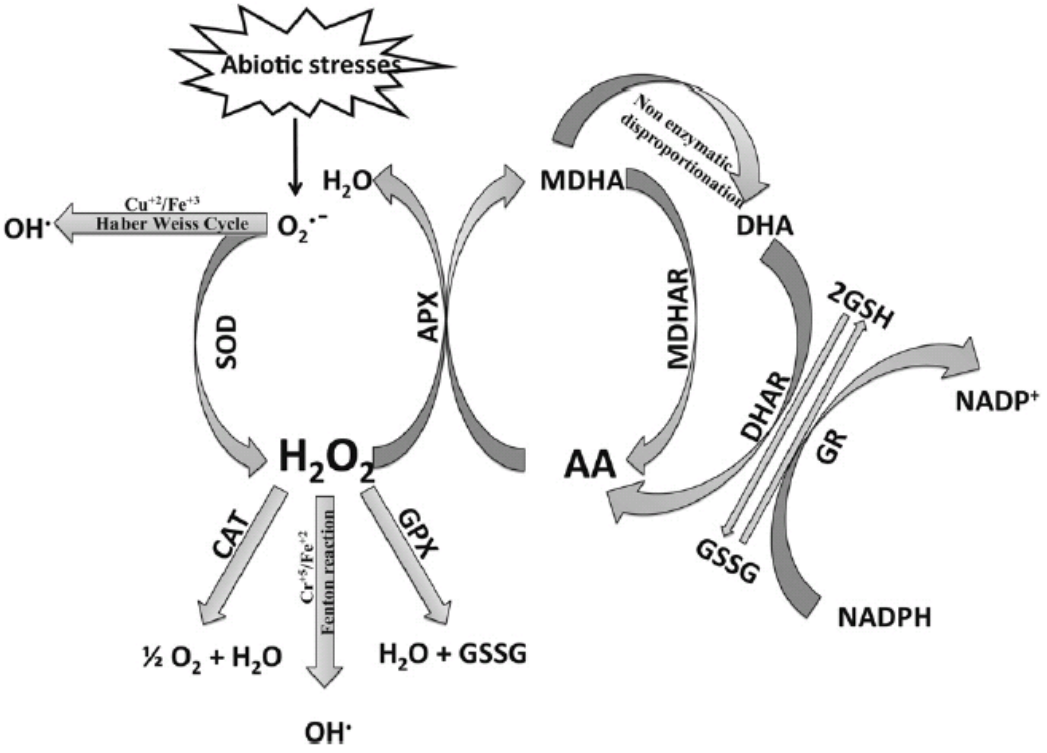


**Figure 1.8** Antioxidative defense system (Gill *et al*., 2010)

#### 1.2.1.3.1 Non-enzymatic Components of Antioxidative Defense System

***Ascorbate (Vitamin C)***

Ascorbate (ASA) is most abundant and influential antioxidant due to its being electron donor of many antioxidative reactions (Smirnoff,2005; Athar *et al.*,2008). It directly reacts with $O_2^{\bullet -}$ and $H_2O_2$ and regenerates α-tocopherol which brings membrane protection to the plant cell. Mitochondria is the center of ascorbic acid metabolism, it is the location of both synthesis and regeneration from oxidized form. Regeneration of ASA is crucially important since half-life of fully oxidized dehydroascorbic acid (DHA) is too short and if it is not reduced back, it is disappeared (Smirnoff,2000; Szarka *et al.*,2007).

***Glutathione (GSH)***

Tripeptide glutathione (γ-glutamyl-cysteinyl-glycine,GSH) is one of the most important scavenger of ROS elements in plant cells. It is synthesized in cytosol and chloroplast, but it takes antioxidative defense role in many cellular compartments such as endoplasmic reticulum, mitochondria and vacuoles (Foyer *et al.*, 2003). It can react with $^1O_2$, $OH^{\bullet}$ and $H_2O_2$ and also takes role in regeneration of ASA which is an important element of ASH-GSH cycle (Noctor *et al.,*1998; Foyer *et al.,*1997; Larson, 1988). GSH can protect critical macromolecules by either glutathiolation or formation of glutathione disulfide (GSSG-oxidized glutathione) (Asada, 1999).

***Tocopherols***

Tocopherols are lipophilic antioxidants, which interact with $^1O_2$ and lipid peroxy radicals (Diplock *et al*, 1989). α-tocopherols have the highest antioxidative activity among other types. They are found in thylakoid membrane of chloroplast and protect photosystem II by reacting with $O_2$ (Gill *et al.,* 2010; Ivanov *et al.*, 2003).

***Carotenoids***

Carotenoids are common pigments found in plants, also lipophilic antioxidants. They protect plant from oxidative stress by means of two ways. Firstly, they quench $Chl^3$ (triplet sensitizer) and $^1O_2$ which are formed during photosynthesis, as a result of this photosynthetic system is preserved and secondly they provide stability of light harvesting complexes of PSI (Gill *et al.,*2010; Collins,2001; Niyogi *et al*.,2001).

***Phenolic Compounds***

Phenolic compounds are secondary metabolites, which take role in antioxidative mechanism. Flavonoids, tannins, hydroxycinnamate esters and lignin are phenolic compounds and highly abundant in the plant cells. Polyphenols protect plant by chelating transition metal ions, eliminating ROS and inhibiting lipid peroxidation (Sharma *et al*.,2012).

***Proline***

According to the studies of Smirnoff in 1989, it was realized that proline scavenges $OH^{\bullet}$ and $^1O_2$ as well as being an osmoprotectant and it is now considered as an inhibitor agent of programmed cell death.

#### 1.2.1.3.2 Enzymatic Antioxidative Defense Mechanism

***Superoxide Dismutase (SOD)***

SOD is responsible for the dismutation of the $O_2^{\bullet -}$ to $O_2$ and $H_2O_2$ (**Error! Reference source not found.**), it is found in many compartments of the cell. Studies revealed that there are three types of SOD; copper/zinc SOD (Cu/Zn SOD), manganese SOD (Mn-SOD) and iron SOD (Fe-SOD). All forms are coded in nucleus and targeted to their destination (Fridovich, 1989; Racchi *et al*., 2001).

***Catalase (CAT)***

CAT catalyzes the dismutation of $H_2O_2$ to $H_2O$ and $O_2$ (**Error! Reference source not found.**). $H_2O_2$ is produced in peroxisomes by photorespiration, β-oxidation of fatty acids and purine catabolism. CAT is found in peroxisomes, therefore excess $H_2O_2$ can be removed before leakage into other compartments of the cell (Del Rio *et al*., 2006; Scandalios *et al.*, 1997; Corpas *et al.*, 2008).

$$\mathbf{2H_2O_2 \rightarrow 2H_2O + O_2} \qquad \textbf{Equation 1.8}$$

***Enzymes involved in Ascorbate-Glutathione Cycle***

Plant cells are sensitive to the alteration in the ratio of AsA to DHA and GSH to GSSG. Halliwell-Asada cycle regenerates AsA and GSH; and their presence is crucially important in the antioxidative defense mechanism. Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are the enzymes catalyzing redox reactions of AsA, GSH and NADPH (Pallanca *et al.*, 2000; Sharma *et al*., 2005).

APX catalyzes the reduction reaction of $H_2O_2$ to $H_2O$ by the help of AsA (**Error! Reference source not found.**). APX isoenzymes are found in cytosol, mitochondria, peroxisome, and chloroplast. Affinity of APX isoforms for $H_2O_2$ is

higher than that of CAT, therefore, APX is more efficient in the removal of $H_2O_2$ (Jimenez *et al.*, 1997; Madhusudhan *et al.*, 2003; Wang *et al*., 1999).

MDHAR regenerates AsA from monodehydroascorbate (MDHA) by using NADPH as electron donor (Figure 1.9). The activity of MDHAR is crucially important since the half-life of MDHA is too short (Hossain *et al.*, 1985; Ushimaru *et al*., 1997).

DHAR takes role in the regeneration of AsA from DHA by using GSH as reducing agent (Figure 1.9). Therefore, this enzyme is pivotal to maintain AsA concentration within the plant cell (Ushimaru *et al*., 1997).

GR reduces GSSG to GSH by using NADPH as electron donor (Figure 1.9). The activity of GR is elevated the ratio of GSH/GSSG, high levels of which is required for an efficient defense against oxidative stress (Reddy *et al*., 2006 and 2008). Majority of the enzyme is found in chloroplast, minority found in cytosol and mitochondria (Edwards *et al*., 1990; Creissen *et al*., 1994).

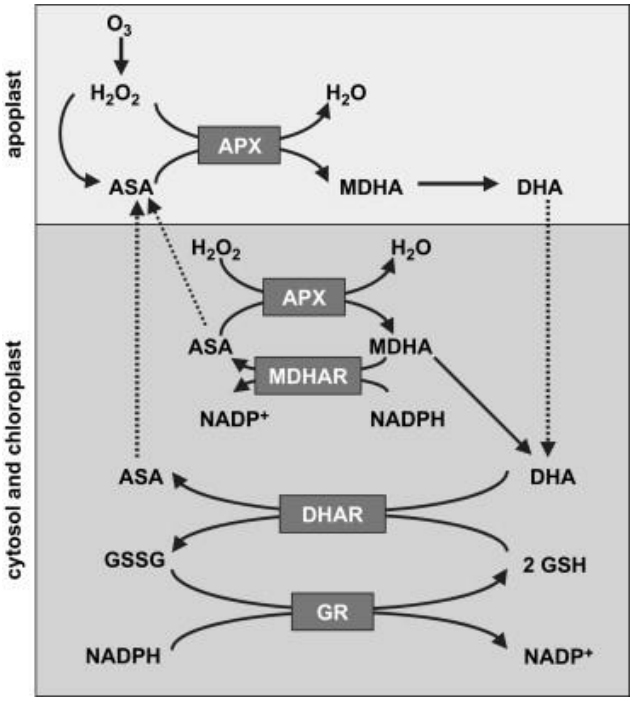


**Figure 1.9** Halliwell – Asada cycle (Castagna and Ranieri, 2008)

### 1.2.2 Salt Stress

Soil salinity is one of the major agricultural problems, which restricts crop production. According to the statistics of Food and Agricultural Organization (FAO), total salt – affected area has been predicted as approximately 800 million ha in 2005. Studies show that, in our planet there is no salt – resistant continent. Wang *et al*. mentioned in 2003 that increased salinity of cultivable area would have devastating impact on Earth; approximately 1/3 of arable land would be lost due to high salinization within next 25 years. As a result of this, plants face salinity problem during their growth period and defense mechanisms are crucially important for normal metabolism, growth and survival of plant (Pessarakli, 2011).

Soil features have influence on growth properties of plants, especially on crops. For efficient crop cultivation, appropriate conditions should be comprised like sufficient water amounts and nutrient supply (Bauder *et al.*, 1992). Basically, soil salinity results from the presence of excessive amounts of soluble salts in the soil. $Na^+$, $Mg^{2+}$, $Ca^{2+}$ and $K^+$ and $SO_4^{-2}$, $HCO_3^-$, $CO_3^{-2}$, $NO_3^{-2}$ and $Cl^-$ are the major cationic and anionic salts respectively (Tanji, 1990). High concentrations of sodium chloride have prohibitive effect on cell growth and survival, whereas potassium and calcium ions mitigate this ionic toxicity. Thus these ions have crucial role in defense mechanism of ionic toxicity of NaCl. Indeed, $Na^+$, $Ca^+$, $H^+$ and $K^+$ ions take role in stress signal transduction mechanism and when $Na^+$ disturbed the ionic homeostasis, other ions endeavor to reform ionic balance (Mahajan, 2005).

Salinization usually comprises in arid and semi – arid regions since salt concentration in soil goes up due to the lack of sufficient amount of water in soil. In addition to this, there are other reasons caused salinity which are natural soil salinity, interference of seawater to the land, usage of salty water as irrigation of cultivated area, low precipitation and high ratios of evaporation and transpiration of plant (Pessarakli, 2011).

Secondary salt-affected lands form due to the wrong attitudes of human beings. For example, wrong irrigation techniques applied by farmers. As reported by FAO

and UNESCO, 10 million ha of irrigated area become functionless due to the water logging or salinity. Moreover, not only old irrigated lands, but areas newly started to be irrigated also are confronted with this issue. According to a study in Pakistan performed by Ahmad, 1965 approximately 16 million ha area is irrigated and 15% of total irrigated land becomes salinized after a couple of years. Other causes of formation of secondary salt-affected lands can be listed as overgrazing, deforestation and contamination with chemicals (Pessarakli, 2011).

### 1.2.3 Effect of Salt Stress on Plant Growth

Most plants cannot tolerate salt stress and these plants are called glycophytes. On the contrary, halophytes are able to grow on salinized soils. Halophytes can survive under 300-400 mM NaCl conditions, whereas glycophytes cannot endure even 100-200 mM NaCl (Zhu,2007). Halophytes can cope with extreme salt conditions by means of tolerant mechanisms.

High salinity constitutes dual action on plants, which are water deficiency and ionic toxicity. As a result of these primary stresses, secondary stresses appear such as formation of reactive oxygen species, indicator of oxidative stress (Zhu, 2001). These abnormal conditions all together disturb the physiological and biochemical steady state Therefore, plant development, growth and survival are reduced. Munns, 1995 proposed a two-phase model which illustrates the osmotic and ionic effects of salinity on growth (Figure 1.10).

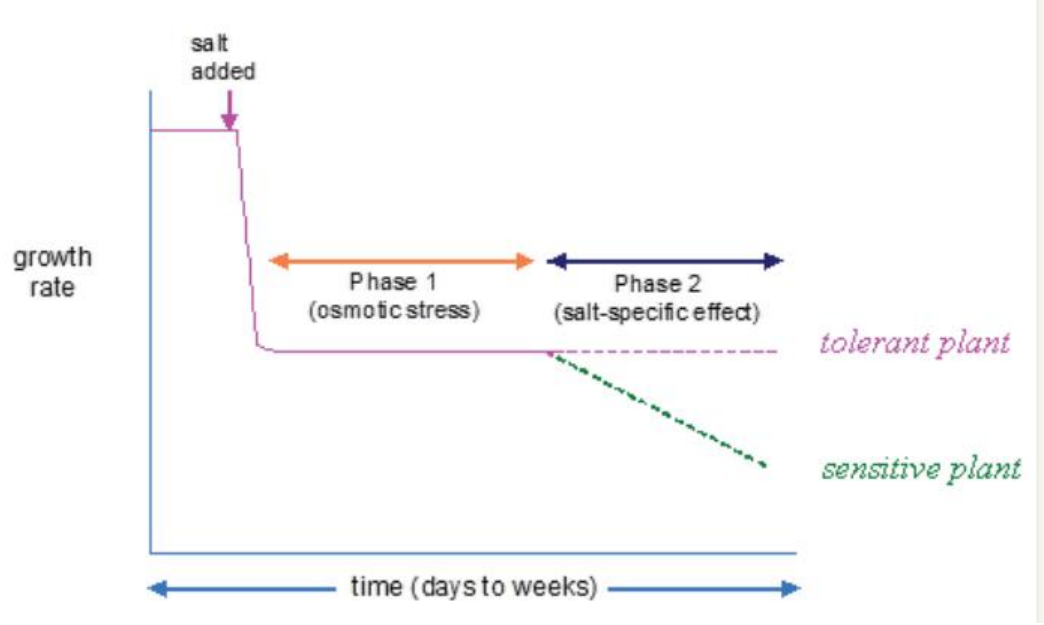


**Figure 1.10** Two-phase growth response to salinity (Munns, 1995)

During Phase 1, growth of both sensitive and resistant plants is declined due to the fact that root have difficulty in reaching and absorbing of water. High concentrations of salts lower water potential and result in loss of turgor, which directly bring stomatal closure. During Phase 2, ions especially $Na^+$ accumulates within plant cells and mostly in leaves. This accumulation is toxic, since excess $Na^+$ affect the photosynthetic components including enzymes and chlorophylls (Davenport *et al.*, 2005). Reduced photosynthetic rate induce the generation of ROS. Since normally, rubisco enzyme binds to carbon dioxide and Calvin – Benson cycle starts. However, in the absence of internal carbon dioxide due to the stomatal closure, rubisco binds to the oxygen molecule and this interaction results in the generation of ROS (Hasagawa *et al.*, 2000). These radicals should be detoxified immediately. Otherwise, they block cellular metabolism and eventually, programmed cell death can take place.

The first response of plant to salt stress is increasing cytosolic $Ca^{2+}$ concentration (Zhu,2007). Abscisic acid (ABA) is crucially important for plants to respond salt stress. Studies showed that mutants which are deprived of ABA, cannot cope properly with salt stress (Xiong *et al.*, 2001). $Ca^{2+}$ and ABA, together mediate the

expression of late embryogenesis-abundant (LEA) type genes. LEA type genes take role in damage repair mechanisms (Xiong *et al*.,2002).

### 1.2.4 Plant Response to Salinity

Countries especially, whose economies rely on agricultural industry are worried about stress factors (Pessarakli *et al*., 2011). Different techniques are considered to eliminate stress factor from soil, since under stress conditions, effective production of plants are overshadowed. Enhancement by chemicals and biotic approaches come into prominence. However; the latter has economic advantage over the former. Therefore, cultivation of salt-tolerant plants or crops is more applicable (Adcock *et al*., 2007; Afzal *et al*., 2006; Ahmad *et al*.,1992). Plants become more and more resistant to salt by traditional breeding methods or biotechnological approaches (Hasagawa *et al*., 2000).

Excess $Na^+$ and $Cl^-$ ions and change in turgor pressure act as inputs of salt signal transduction. Ion channels/transporters and ion binding proteins on plasma membrane sense the change in the concentration of ions. Excess $Na^+$ results in the depolarization of plasma membrane, which activates $Ca^{2+}$ channels (Zhu, 2002; Sanders *et al*., 1999).

$Ca^{2+}$ acts as secondary messenger in abiotic stress response mechanisms. Calmodulins, calcium dependent protein kinases (CDPKs) and salt overly sensitive – 3 (SOS3) and SOS3-like calcium binding proteins are the major calcium binding proteins (Liu and Zhu,1998; Guo *et al*.,2001).

Mitogen activated protein kinase (MAPK) comprises MAPK kinase kinase (MAPKKK) and that activates MAPK kinase (MAPKK). Plant MAPKs take role in signaling mechanism of development, cell division and environmental stresses. Salt stresses activates MAPKs in tobacco and *Arabidopsis* (Mikolajczyk *et al*.,2000; Mizoguchi *et al*.,1996).

ABA accumulation is induced by salt stress and amount of accumulation changes from tissue to tissue. In a study of maize 1-fold and 10-fold of ABA accumulation were observed in roots and leaves, respectively. May be the difference is resulted

from only osmotic stress in leaves and both osmotic and ionic stresses in roots (Jia *et al.*,2002).

Liu and Zhu came up with that to combat with ionic toxicity of salt stress salt overly sensitive (SOS) signaling pathway activation is required. First response to elevated NaCl concentration is the increase in cytosolic calcium concentration. Calcium-binding protein expressed by *SOS3* gene senses this calcium signal and activates SOS2 which is a serine/theronine protein kinase. Together with SOS2 and SOS3 regulate the expression of *SOS1* gene. SOS1 is a $Na^+/H^+$ antiporter located on plasma membrane. SOS1 takes role in efflux of excess $Na^+$ ions (Qiu *et al*., 2002). SOS3 and SOS2 complex also have function in compartmentalization of $Na^+$ ions into the vacuole. Ion accumulation in vacuole minimizes the hazardous influence on cytosolic and organellar metabolism (Niu *et al*., 1995; Hasegawa *et al*., 2000; Zhu, 2003). This mechanism not only overcomes ionic toxicity, but also copes with the water deficit due to hypersalinity. SOS3 might also activate absisic acid synthesis (ABA) (Zhu, 2002).

Soil salinity leads to decrease in water availability, which causes osmotic stress. Decrease in turgor pressure encourages ion uptake and compartmentalization in the vacuole and also synthesis of compatible solutes such as proline, glycine betaine and polyphenols. Compartmentalization of $Na^+$ is important to sustain low levels of $Na^+$ in cytosol, since if $Na^+$ accumulates within the cell, metabolic pathways are going to be inhibited. $Na^+$ is stored in vacuole by active transport. The process depends on the expression and activity level of $Na^+/H^+$ antiporter, as well as vacuolar $H^+$-ATPases and $H^+$-pyrophosphotase which are responsible for the generation of electrochemical gradient (Chinnusamy and Zhu,2003).

Plants try to keep cytosolic $K^+/ Na^+$ ratio high, since $K^+$ is a key element for the response of plant to salt-stress. $K^+$ facilitate the water uptake by plant cells and it is involved in the process of stomatal opening and closure. Also, it is essential for metabolic pathways because it is an important co-factor of many enzymes such as pyruvate kinase However, under saline conditions it competes with $Na^+$ for influx into the cell and to maintain ionic balance within the cell. (Mahajan *et al*., 2005; Chinnusamy and Zhu,2003).

Another struggling way of water loss from the cell is the synthesis of compatible solutes (osmoprotectants, osmolytes) (Ford, 1984; Breesan *et al*., 1998). Main compatible solutes are sugars like sucrose and fructose; and charged metabolites such as proline, ectoine and glycine betaine (McCue *et al*., 1990). The osmoprotectant solutes synthesized when plants meet stress factor, while osmoprotectant ions such as $K^+$, $Na^+$ and $Cl^-$ accumulated within vacuole of the plant cell. The most important advantage of osmolytes is that their accumulation within the cell does not inhibit ordinary cellular metabolism, rather protect enzymes and membranes from destructive damage of hypersalinity by osmotic adjustment (Gupta, 2006). Without any damage to the cell, they adjust water potential of the cell. With the accumulation of these non-toxic solutes, cells become hypertonic, and water uptake into the cell is triggered. Furthermore, osmolytes take role in defense against oxidative damage by detoxification of ROS. Shen *et al*. put forward that mannitol brings oxidative stress tolerance to tobacco according to the studies in 1997.

Proline is one of the osmolytes which protect plants against deleterious effects of dehydration due to osmotic stress like drought and salinity. According to a study on four potato species, proline contents rose with the increasing salt stress. This result reveals that there is a direct relationship between proline accumulation and defense of osmotic stress aspect of hypersalinity (Martinez *et al*.,1996).

Another important osmoprotectant used by plants is glycine-betaine (*N,N,N*-trimethylglycine-betaine) for protection against $H_2O$ unavailability (Rhodes *et al*.,1993; Hanson,1994). Gorham performed in vitro studies and found that glycine-betaine prevent from deleterious influences of excessive amounts of salts by enzyme structure stabilization and activity protection and cell integrity protection. Then, in vivo studies of Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*) and tomato (*Lycopersicon esculntum*) were performed. Gene responsible for the expression of glycinebetaine was transformed to the plants. Studies showed that transgenic plants could synthesize glycine betaine from choline and provide resistance to salt-stress (Hayashi *et al*., 1997, 1998; Alia *et al*., 1998, 1999; Sakamoto *et al*., 2001; Chen *et al*., 2002).

Late-embryogenesis-abundant (LEA) proteins help plant cells to cope with dehydration problem. Transgenic rice plants overexpressing barley *LEA* gene showed better adaptation to salt stress than wild type (Xu *et al*.,1996; Chinnusamy and Zhu,2003).

Sanan-Mishra *et al.*,2005 asserted that pea DNA helicase 45 (PDH45) facilitated the defense of plant against excessive salts. PDH 45 overexpressed transgenics were able to grow and produce viable seeds under hypersalinity conditions which indicates PDH45 counteracts the hazardous effects of salt stress without any reduction in the weight of seed.

### 1.3 Lab-on-a-Chip Technology for Protein Analysis

In spite of the developments in protein analysis technologies, still sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used as standard method for protein size determination. However; microfluidic or lab-on-a-chip technology is a good alternative to this conventional method. The major aim of this new technology is to lessen the processes to small dimensions, which allows to cope with very little volumes. Besides, this technology greatly reduces analysis time and minimize the errors by automating the process (Goetz *et al.*, 2004).

This chip-based protein analysis is based on a fluidic system in channels which are only a few micrometers and there is no moving part in this system. With this technology, many experimental steps including sample handling, separation, staining and analysis are integrated into one process (Kuschel *et al.*,2002).

The Agilent 2100 Bioanalyzer is the first commercialized application of microfluidics technology (Bousse *et al*., 2001). The instrument uses disposable chips composed of 2 glass plates bound together which is shown in Figure 1.11. One glass layer includes microfabricated channels and another layer composed of through wholes which function as reservoir of sample and buffer (Kuschel *et al*., 2002).

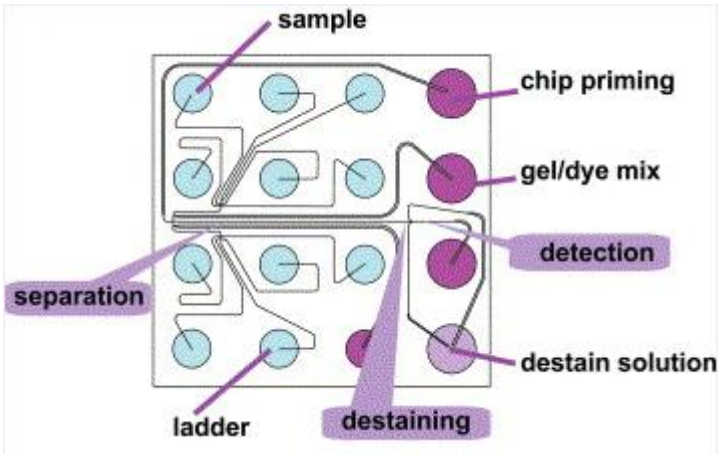


**Figure 1.11** Microfluidic chip system for protein analysis (Goetz *et al.,* 2004)

The principle of the chip-based protein assay is an electrophoretic analysis; denatured proteins interact with SDS molecules and move towards channels. These channels are filled with polymers, which separate proteins with respect to their molecular size. While proteins are walking through these channels, intercalating fluorescent dye stains them. Therefore, manual staining procedure is eliminated. A ladder is also run on each chip to increase accuracy of sizing of sample proteins. The software automatically forms a standard curve to determine the molecular weights of unknown proteins. Each chip can be analyzed ten protein samples between 5 and 250 kDa with a resolution of 5-10% in approximately 30 minutes. Whereas, it takes at least 2-3 hours to obtain comparable gel results for SDS-PAGE with a sizing precision of 0.5-5%. (Goetz *et al.*, 2004; Kuschel *et al*., 2002).

Each kit contains 25 microfluidic chips and the reagents required for analysis including sample buffer, dye concentrate, protein sizing standard and sieving matrix (Kuschel *et al*., 2002). Samples are prepared according to the kit protocol, this facilitates preparation step and eliminates time consuming reagent preparation.

The sensitivity of microfluidic chip protein analysis is reported to 80 ng. However; it is determined for 50 ng and 10 ng for standard Coomassie stain (R-250) and colloidal Coomassie stain (G-250), respectively. On the other hand, gel-to-gel comparisons are difficult for SDS-PAGE due to the low repeatability. Careful controlling of experimental conditions such as pH, temperature, gel compositions

and staining times can improve reproducibility. But, reproducibility of the chip-based protein assay is greatly higher than that of SDS-PAGE. Therefore, it is possible to make comparisons between the results of different chips.

## 1.4 Improvement of Stress Tolerance by Biotechnological Approches

Many genes and biochemical pathways are involved in the response of plants to the abiotic stress factors. Understanding the molecular mechanisms of the stress response is vital for the improvement of stress tolerance of plants especially crops (Avni Öktem *et al*.,2008).

Crop improvement through genetic engineering was performed by using either *Agrobacterium tumefaciens* as a vector or particle-bombardment (Gulati *et al*., 2002; Li *et al.*, 2004). The inserted DNA is either a specific gene having a biochemical function or regulatory gene. Studies showed that over-expression of vacuolar or cellular membrane $Na^+/H^+$ antiporter coding gene in tomato and *Arabidopsis* provide salt tolerance (Apse *et al*., 1999; Zhang *et al*., 2001).

*Medicago truncatula* and *Lotus japonicas* have used as model plant organisms to understand the molecular mechanisms underlying the stress resistance due to their short generation time and small genome (Cook,1999).

Most studies based on DNA or RNA levels, by assuming abiotic stresses can be explained by up or down-regulation of genes. However; many stress-related proteins are post-translationally modified. For example, Lee *et al*. stated that a novel salt stress signaling molecule of *Arabidopsis*, annexin 1 was upregulated upon stress.

Comprehensive study of –omics (transcriptomics, proteomics and metabolomics) as well as genetic approaches will be key factor of understanding and improving the tolerance of plants against stress factors (Vinocur an Altman,2005).

## 1.5 Studies Performed in METU – Plant Molecular Biology and Biotechnology Laboratory

Lentil (*Lens culinaris* M.) plant has been studied in large scale. Mehrzad Mahmoudian, 2000 studied tissue culture in her study of “Optimization of tissue culture conditions and gene transfer studies in lentil”. Ufuk Çelikkol, 2008 performed regeneration and Agrobacterium-mediated gene transfer studies in her PhD study titled “Regeneration of Lentil (*Lens culinaris*) and genetic transformation by using Agrobacterium tumefaciens-mediated gene transfer” study. Hamdi Kamçı, 2011 focused on genetic transformation in his study of “Genetic transformation of lentil with transcriptional factors and evaluation of abiotic stress tolerance”. Moreover, Ebru Bandeoğlu, 2001; Işın Nur Cicerali, 2004 and Oya Ercan,2008 focused on the effects of environmental stress factors on antioxidative defense system. Emre Aksoy, 2008 studied gene expression levels in his project of “Effect of drought and salt stresses on the gene expression levels of antioxidant enzymes in lentil (*Lens culinaris M*.) seedlings”.

However, there are still unknown mechanisms, therefore in this study 6 Turkish lentil cultivars were compared with respect to some physiological and biochemical parameters. According to screening, two cultivars were selected and contrasted in terms of antioxidative defense response and total protein profiles.

## 1.6 Aim of the Study

In this study, major aim was to screen 6 Turkish lentil varieties for salt tolerance using physiological and biochemical approaches. After screening, 2 cultivars were selected with respect to their behavior under salt stress conditions. For this purpose, following experiments were carried out:

i. Physiological analysis; fresh weight and length
ii. Biochemical analysis; ion leakage, MDA, $H_2O_2$, and proline content determination

Antioxidative enzyme activities (SOD, CAT, APX and GR) of selected two cultivars were analyzed for further comparison. Then total proteins were extracted to observe different protein bands between two cultivars. For this purpose, Bioanalyzer and SDS-PAGE were performed.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemical Materials

Chemicals required for this study were supplied from Sigma Chemical Company, Applichem Chemical Company and Merck Chemical Company. All solutions used during experiments were prepared with distilled water.

### 2.1.2 Plant Materials

In this study 6 Turkish lentil (*Lens culinaris* M.) cultivars were examined under salinity conditions, named as Seyran, Malazgirt, Çağıl, Çiftçi, Kafkas and Özbek. Meyveci cultivar was used and eliminated at the stage of preliminary studies of determination of stress dosage and duration. Lentil seeds were kindly provided by Field Crops Central Research Institute (TARM).

**Table 2.1** Turkish lentil cultivars used in this study

| Cultivar Names | Cotyledon color | Season | Weight of 1000 seeds (g) | Yield (kg/da) | Region of Cultivation |
|---|---|---|---|---|---|
| **Seyran 96** | Red | Winter | 30-36 | 150-200 | Southeastern Anatolia |
| **Malazgirt 89** | Red | Summer | 27-30 | No info | South Anatolia |
| **Çağıl** | Red | Winter | 31-40 | 165-237 | Southeastern Anatolia |
| **Çiftçi** | Red | Winter | 30-36 | 150-190 | Southeastern Anatolia |
| **Kafkas** | Red | Winter | 36.5 | 143.7 | Central Anatolia |
| **Özbek** | Red | Winter | 35.2 | 170-190 | Central Anatolia |
| **Meyveci 2001** | Yellow | Summer | 72 | 130-160 | Central Anatolia |

## 2.2 Methods

### 2.2.1 Growth of Plants

Lentil seeds were surface sterilized by using 20% ethanol for 30 seconds and then rinsed with distilled water for three times for removal of ethanol. After that, they were imbibed within distilled water overnight which makes them ready for germination.

Lentil seeds were grown via hydroponic culture with 50% strength of Hoagland's medium (Hoagland and Arnon, 1950). Plastic pots filled with 200 ml 0.5X Hoagland's medium were covered with sterile cheesecloth and approximately 10 seeds were placed onto each pot. Seeds were grown for 7 days with 0.5X Hoagland. Stress was initiated at the 7th day of germination. Salinity was generated by the application of 0.5X Hoagland's solution including 100 mM and 150 mM NaCl. Both control and stress-treated lentils were grown in growth

chamber, the conditions of which are 22±2 $^0$C, 45% humidity and 16 hours light (400 µmol $m^{-2}s^{-1}$) and 8 hours dark photo-cycle. Twelve days old seedlings of all cultivars were collected and stored at -80$^0$C for further analysis.

Stress dosage and duration of stress application of lentil varieties were determined by preliminary studies (data is given in appendix). 100 mM and 200 mM NaCl containing half-strength Hoagland's medium were applied to Meyveci, Malazgirt, Çağıl, Çiftçi, Kafkas and Özbek for 4 days and 5 days after 7 days of normal growth. Meyveci was mildewed during germination period, so it was eliminated at this stage. Instead of Meyveci, Seyran which is another red-lentil variety was preferred. 100 mM and 150 mM NaCl and 5 days of stress application was selected as stress dosage and duration, respectively.

### 2.2.2 Screening Process of Cultivars for Selection

Shoot and root tissues of both control and salt stress-treated lentil seedlings screened via physiological analysis like fresh weight and length, and biochemical parameters like ion leakage, proline, MDA and $H_2O_2$. The aim of this screening step is the selection of two cultivars responding different from each other like sensitive and tolerant under salinity conditions. Selected 2 cultivars were subjected to further analysis.

#### 2.2.2.1 Physiological Changes

At the twelfth day of germination, fresh weight and length of shoot and root samples of all cultivars were measured for both control and salt-stress treated groups. The photographs of all samples were taken in order to observe the level of wilting or aliveness.

#### 2.2.2.2 Analysis of Ion Leakage

Membrane permeability of shoot and root tissues was measured according to Nanjo *et al.,*(1999). Before placement of tissues into the falcon tubes, they were washed with deionized water to eliminate adhered ions onto the tissues. 5 ml of 0.4 M Mannitol solution was put onto the tissues. Samples were incubated for 3

hours at room temperature with continuous gentle mixing. Conductivities of the samples were measured via Mettler Toledo MPC 227 conductivity meter and initial conductivity recorded as C1. After that, samples were incubated within boiling water for 10 minutes. Samples were cooled by the help of ice until they reached to the room temperature. After this period, the conductances were recorded as C2. At that time, total ion concentration due to the complete membrane destruction was estimated. Conductivity was measured as percentage electrolyte leakage, (C1/C2)*100.

#### 2.2.2.3 Determination of MDA Amount

Determination of MDA concentration was carried out according to the Ohkawa *et al.*,1979. Shoot and root tissues were grinded into a fine powder in pre-cooled mortar and pestle with liquid nitrogen. 0.1 – 0.3 g powder was transferred into eppendorf and extracted with 2 ml 0.1% TCA. After centrifugation at 10000 rpm at 4°C for 15 minutes, 0.5 ml of supernatant was taken into new eppendorf tube and 0.5 ml of 0.1 M Tris/HCl buffer at pH 7.6 and 1 ml of Trichloroacetic acid-Thiobarbituric acid-HCl (TCA – TBA – HCl) were added. The tubes were incubated at 95°C for 45 minutes. After cooling step within ice, they were centrifuged at 10000 rpm for 5 minutes. The absorbances of supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbances at 600nm. The extinction coefficient of 155 $mM^{-1}cm^{-1}$ was used for the calculation of MDA amount.

#### 2.2.2.4 Determination of $H_2O_2$ Content

$H_2O_2$ amounts were estimated according to the method of Ohkawa *et al.,*1979 Shoot and root tissues were grinded into a fine powder in pre-cooled mortar and pestle with liquid nitrogen. 0.1 – 0.3 g powder was transferred into eppendorf and extracted with 2 ml 0.1% TCA. Then, extracts were centrifuged at 10000 rpm at 4°C for 15 minutes, and 0.5 ml of supernatant was taken into new eppendorf tube and 0.5 ml of 0.1 M Tris/HCl buffer at pH 7.6 and 1 ml KI were added onto the supernatant. The tubes were incubated for 1.5 hours in dark conditions. The absorbances of the samples were recorded at 390 nm. The amount of $H_2O_2$ MDA was calculated by using 39.4 $mM^{-1}cm^{-1}$ as extinction coefficient.

#### 2.2.2.5 Determination of Proline Content

Proline amounts were estimated according to the method of Bates *et al.,*(1973). Shoot and root tissues were grinded into a fine powder in pre-cooled mortar and pestle with liquid nitrogen. 0.1 – 0.3 g powder was transferred into eppendorf and suspended with 1 ml 3% sulphosalicilic acid, samples were centrifuged at 14000 rpm for 5 minutes at 4 °C. After centrifugation step, 0.2 ml acid ninhydrin, 0.2 ml 96% acetic acid, 0.1 ml 3% sulphosalicilic acid, 0.1 ml supernatant from each extract were put into a new eppendorf tube orderly. Before the incubation step, eppendorf tubes were mixed. Then, the tubes were incubated at 96°C for 60 minutes at heat block to hydrolyze proteins. 1 ml toluene was added to the eppendorf tubes and then the tubes were vortexed. After the centrifugation step at 14000 rpm for 5 minutes at 4°C, upper pinky colored phase was transferred to cuvettes and the absorbance of all samples were read at 520 nm, and toluene was used as blank. 18 $mM^{-1}cm^{-1}$ was used to calculate the proline concentrations, which is the slope of standard curve of proline.

According to the results of these analysis Seyran was selected as resistant and Malazgirt as tolerant cultivar. Following experiments were done with these 2 cultivars.

### 2.2.3 Determination of Enzymatic Activities

#### 2.2.3.1 Preparation of Shoot and Root Crude Extracts

Shoot and root tissues of Seyran and Malazgirt cultivars were grinded into a fine powder in pre-cooled mortar and pestle with liquid nitrogen. 0.1 – 0.3 g powder was transferred into eppendorf and then suspended with 1 ml 50 mM potassium phosphate buffer at pH 7.8 containing 1 mM EDTA and 2% PVP. After centrifugation of the suspension at 13000 g for 20 minutes at $4^0$C, supernatant phase was taken for protein amount determination and enzyme assay.

#### 2.2.3.2 Protein Amount Determination

The protein concentrations of shoot and root tissues were estimated as stated in Bradford method (Bradford, 1976). Bradford reagent was prepared by using Coomassie Brilliant Blue G-250. Then, it was filtered to remove impurities and kept at room temperature. 20 µl and 40 µl samples were used for shoot and root tissue, respectively. Each sample was worked as duplicate. Tissues were diluted to the 500 µl with distilled water. 5 ml Bradford reagent was added onto the samples and vortexed. After 10 minutes incubation at room temperature, the absorbance was read at 595 nm, mixture of 500 µl water and 5 ml Bradford reagent were used as a blank. Standard curve was created by using 10, 20, 40, 60, 100, 160 and 200 µg/ml of bovine serum albumin.

#### 2.2.3.3 Determination of Catalase Activity

Catalase activity of shoot and root tissues were measured according to the Chance *et al.,*(1995). 900 µl 50mM potassium phosphate buffer at pH 7, enzyme extract containing 100 µg soluble protein determined by Bradford method and 100 µl 100 mM $H_2O_2$ were mixed immediately since $H_2O_2$ is the substrate of catalase enzyme and the reaction was started when it was added to the enzyme assay. The mixture of 900 µl potassium phosphate buffer at pH 7 and 100 µl $H_2O_2$ were used as blank. The decrease in $H_2O_2$ concentration was measured and recorded by reading absorbance at 240 nm with Schimadzu double-beam spectrophotometer continuously for 120 seconds. The enzyme activity was determined by using the initial rate of the CAT enzyme. Extinction coefficient of $H_2O_2$ at 240 nm is 39.4 $mM^{-1}cm^{-1}$.

Hydrogen peroxide → Water + Oxygen (2.1)

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ (2.2)

#### 2.2.3.4 Determination of Ascorbate Peroxidase Activity

APX activity of shoot and root tissues were calculated according to the method of Wang *et al.,*(1991). 800 µl 50 mM potassium phosphate buffer at pH 6.6, 100 µl ascorbate, enzyme extract containing 100 µg soluble protein determined by

Bradford method and 100 µl 100 mM $H_2O_2$ were mixed and the absorbance reading was started immediately due to the fact that addition of $H_2O_2$ starts the reaction of catalase enzyme. The mixture of 800 µl potassium phosphate buffer at pH 6.6, 100 µl ascorbate and 100 µl 100 mM $H_2O_2$ were used as blank. The fall in $H_2O_2$ concentration was measured and recorded by reading absorbance at 290 nm with Schimadzu double-beam spectrophotometer continuously for 120 seconds. For the calculation of the APX concentration 2.8 $mM^{-1}cm^{-1}$ was used as extinction coefficient.

Ascorbate + Hydrogen peroxide → Dehydroascorbate + Water (2.3)

$C_6H_8O_6 + H_2O_2 \rightarrow C_6H_6O_6 + 2\ H_2O$ (2.4)

### 2.2.3.5 Determination of Glutathione Reductase Activity

GR activity of shoot and root tissues were measured by the means of method of Sgherri *et al.,*(1995). 800 µl 200 mM potassium phosphate buffer at pH 7.5, 100 µl GSSG, enzyme extract containing 100 µg soluble protein determined by Bradford method and 100 µl 0.5 mM NADPH were mixed and the decline in NADPH concentration was measured and recorded immediately due to the fact that NADPH is the substrate of the enzyme and the reaction was started by the time of addition of substrate. Enzyme assay without protein extract was used as blank Schimadzu double-beam spectrophotometer was used for the record of absorbances at 340 nm for 2 minutes. The amount of GR enzyme was measured with the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

Glutathione disulfide + NADPH → Glutathione + $NADP^+$ (2.5)

GSSG + NADPH → 2GSH + $NADP^+$ (2.6)

### 2.2.3.6 Determination of Superoxide Dismutase Activity

According to the method of Beuchamp and Fridovich (1971), one dimensional native polyacrylamide gel electrophoresis was used for determination of SOD isozyme activities of control and salt-treated lentil seedlings. Gel was stained by negative activity stain.

Superoxide + $2H^+$ → Hydrogen peroxide + Oxygen (2.7)

$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ (2.8)

Grinded shoot and root tissues of lentil seedlings were homogenized by 800 and 500 µl homogenization buffer composed of 9 mM Tris-HCl buffer at pH 6.8 and 13.6% glycerol. The suspension was vortexed well and centrifuged for 30 minutes at 10000 g. Supernatants were used for SOD assay.

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

According to the method of Laemmli (1970), 10 ml 12% separating gel and 2.5 ml 5% were prepared for 1-D PAGE. 50 µg and 20 µg proteins (determined by Bradford method) were loaded for shoot and root tissue, respectively. Electrophoresis was performed under constant current of 6 mA in stacking gel and 9 mA in separating gel for approximately 4 hours.

#### 2.2.3.6.2 Negative Activity Staining

Gel was dark incubated with negative activity stain including 50 mM potassium phosphate buffer at pH 7.5,0.1 mM EDTA, 0.2 %(v/v) N,N,N'N'- tetramethyl ethylene diamine (TEMED), 3 mM riboflavin and 0.25vmM nitroblue tetrazolium. Under gentle mixing, gel was stained for 45 minutes. Then, gel was washed with distilled water several times under illumination until color formation occurs.

### 2.2.4 Statistical Analysis

Physiological parameters and ion leakage analysis were studied with 12 replicates. Biochemical characteristics and enzyme activities were performed with 3 replicates. Statistical analysis was performed by using MINITAB 15 program (MINITAB Inc., USA). Data obtained from screening process were analyzed with one-way analysis of variance (ANOVA). Data obtained from enzyme assays were analyzed with one-way and two-way ANOVA. Differences with P value less than 0.05 ($p<0.05$) were considered as significant.

### 2.2.5 Identification of Total Protein

#### 2.2.5.1 Preparation of Protein Extracts of Shoot and Root Tissues

Total protein isolation were performed according to Wang *et al.,*(2006). Shoot and root tissues were grinded into a fine powder in pre-cooled mortar and pestle with liquid nitrogen. 0.1 – 0.3 g powder was transferred into eppendorf and 2 ml 10% w/v TCA/Acetone was added onto the powder and mixed well by using vortex. After centrifugation at 16000g for 3 minutes at 4 $^0$C, supernatant was removed and 2 ml 0.1 M ammonium acetate in 80% methanol was added, mixture was vortexed. After centrifugation at 16000 g for 3 minutes at 4 $^0$C, supernatant was removed and 2 ml 80% acetone was placed into the eppendorf. The mixture was vortexed until the pellet was dispersed. After centrifugation at 16000 g for 3 minutes at 4 $^0$C, supernatant was removed and pellet was incubated at 50 $^0$C for 10 minutes for air-drying in order to get rid of remaining acetone. After incubation, 0.8 ml Tris-buffered phenol at pH 8.0 and 0.8 ml dense SDS buffer (30% w/v sucrose, 2% SDS, 0.1 M Tris-HCl at pH 8.0, 5% β-mercaptoethanol) was added to the eppendorf and mixed thoroughly. After incubation for 5 minutes at room temperature, samples were centrifuged at 16000g for 5 minutes at 4 $^0$C. Upper phenol phase was transferred into new eppendorf tube and the tube was filled with four times of the volume of phenolic phase. The mixture was incubated at -20$^0$C overnight. After incubation, centrifugation at 16000g for 5 minutes at 4 $^0$C was performed, and supernatant was discarded, white pellet was remained. The pellet was washed with 100% methanol and centrifuged 16000g for 3 minutes at 4 $^0$C, then with 80% acetone again centrifuged under same conditions. After washing steps, acetone was removed and proteins were air-dried. Dried proteins were dissolved in 200 µl sample buffer (5 ml distilled water, 1 ml 0.5 M Tris- HCl at pH 6.8, 1.6 ml 10% SDS and 0.4 ml β-mercaptoethanol) and stored at -20 $^0$C.

#### 2.2.5.2 Determination of Total Protein Amount

Total protein amounts of both shoot and root tissues of all samples were determined by Bradford method (Bradford, 1976).

#### 2.2.5.3 Identification of Proteins by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared according to Laemmli (1970). Separating gel and stacking gel were prepared as 12% and 4.5%, respectively. Equal amounts of proteins (15µg) measured by Bradford experiment were loaded to the wells for each sample. Samples were prepared by means of sample and sample buffer ratio of 1:3. 12 µl unstained protein marker was also loaded to predict the protein content. Electrophoresis was performed with voltage of 60 V in stacking gel and 90 V in separating gel approximately 24 hours.

#### 2.2.5.4 Silver Staining of SDS-PAGE

Gel was silver stained by means of Blum *et al.,*(1987). Every step was performed with gentle shaking and since staining procedure is light sensitive, gel was treated under dark conditions. Gel was transferred into the fixation solution and incubated at room temperature overnight, then was washed with 50% ethanol three times for 20 minutes. After washing, pre-treatment was performed with sodium thio sulfate solution for 60 seconds. Gel was rinsed with distilled water three times for 20 seconds to remove remnants of pre-treatment solution. Then, gel was treated with silver nitrate solution for 20 minutes. After washing with distilled water twice for 20 seconds, gel was treated with developing solution until band formation was observed apparently. Then, distilled water was used two times for 120 seconds to get rid of excess staining of the gel. After that, gel was transferred into stop solution for short-term storage.

#### 2.2.5.5 Identification of Total Proteins by Bioanalyzer

Analysis was performed with Agilent 2100 Analyzer. Protein extracts of control and salt-treated lentil seedlings including 20 µg proteins were analyzed via Agilent Protein 230 Kit. 12 µl of gel-dye mix was loaded to the channels of the chip (through the well marked as G) by application of pressure for 1 minute with a syringe. The remaining solution was discarded in the well marked G and 12 µl of gel-dye mix was pipetted into 4 wells which are marked as G. Then, 12 µl of destaining solution was loaded into the well marked as DS. 4 µl of protein extracts

and 2 µl of denaturing solution were mixed. 6 µl of ladder and sample containing tubes were incubated at 95º C for 5 minutes within heat block. After heating, 84 µl of deionized water was added and mixed by vortex. 6µl of diluted samples and ladder were pipetted into the wells. After proper placement of chip into the Agilent 2100 Bioanalyzer, chip run was initiated through software. The bioanalyzer contains 16 high-voltage power supplies connected to platinum electrode. When chip is placed into the instrument, the electrodes can touch to the liquids in the wells, thus electric circuit forms. With this electric circuit, samples were moved from wells towards channels. Samples were sequentially separated in the separation channel and detected by laser-induced fluorescence detection. The data are displayed by software as an electropherogram as well as gel-like image.

# CHAPTER 3

# RESULTS

## 3.1 Screening Process of Cultivars

First of all, 6 red lentil cultivars (Çağıl, Çiftçi, Kafkas, Malazgirt, Seyran and Özbek) were screened to select one salt-tolerant and one salt-sensitive cultivar. During screening period physiological parameters (fresh weight and length of root and shoot tissues) and biochemical parameters such as proline, $H_2O_2$, MDA content and ion leakage were analyzed. Selected two cultivars were further compared with respect to their antioxidative enzyme activities (CAT, GR and APX).

### 3.1.1 Effect of Salt Stress on Physiological Parameters of Lentil Seedlings

Alterations of both shoot and root tissues in the physiological parameters were observed by fresh weight and length measurements under salt-stress conditions. Photographs of 12 day-old seedlings of control and salt-treated shoot and root tissues were shown in Figure 3.1. Decreased leaf area, puny shoots and wilted leaves were observed in salt-treated samples, especially Malazgirt.

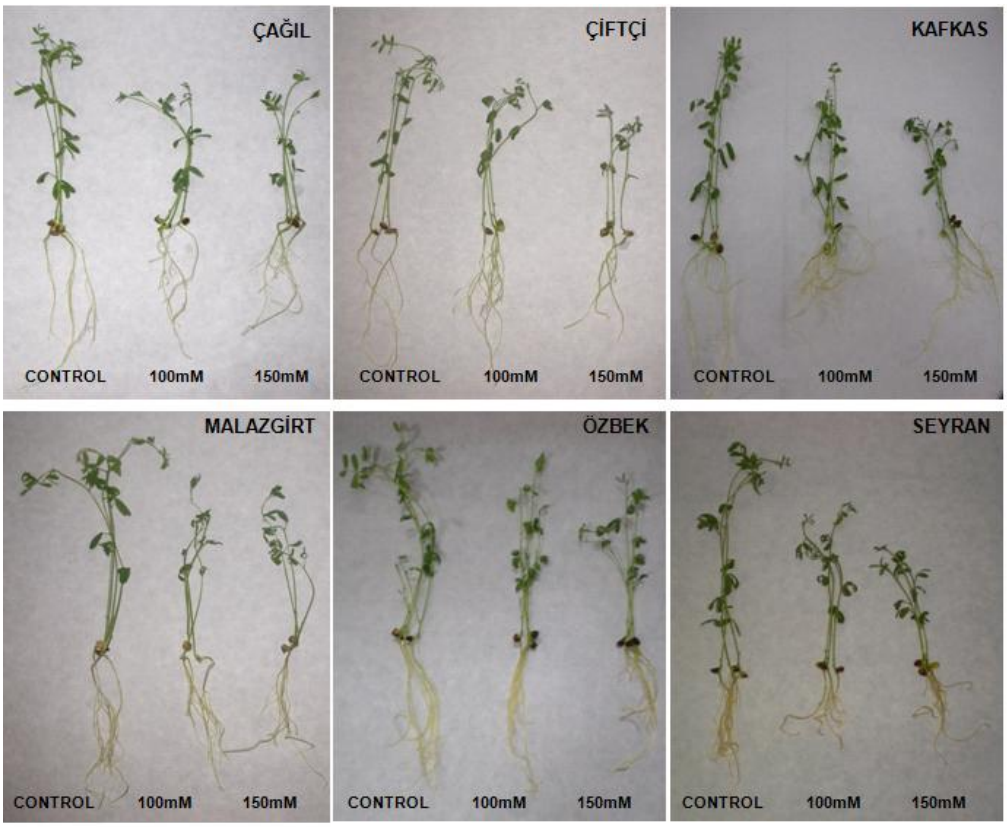


**Figure 3.1** The physical appearance of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

Shoot fresh weights of all cultivars decreased as a dosage-dependent manner which is shown in Figure **3.2**. Salt-treated shoot tissues of Malazgirt and Seyran were significantly different from control group. Changes in root fresh weight was shown in Figure 3.3. Özbek root weight 20% increased and 47% decreased at 100 mM and 150 mM NaCl, respectively. Kafkas showed 22% and 27% decline for 100 mM and 150 mM. 100 mM NaCl treated root tissues of all cultivars were significantly different from control.

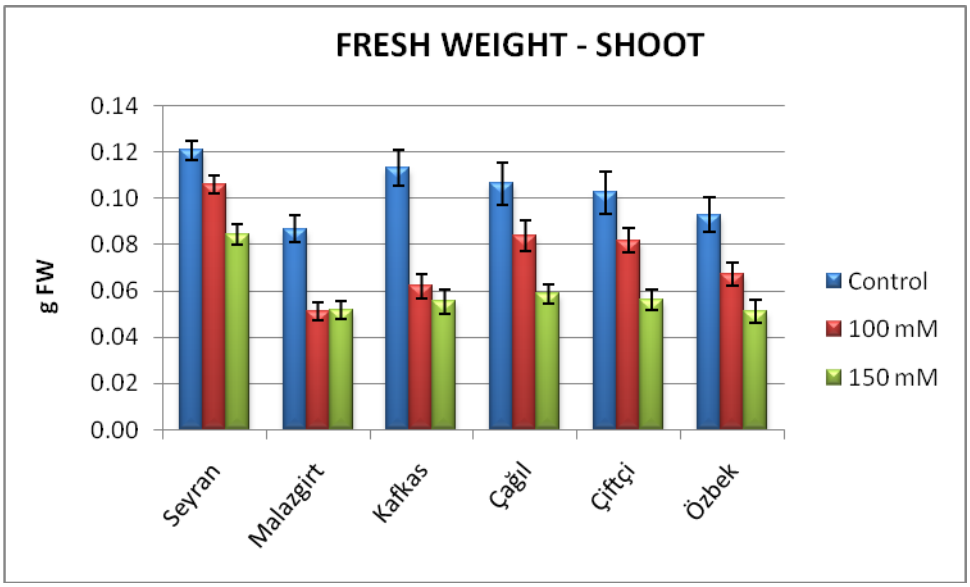


**Figure** 3.2 Shoot fresh weights of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth). Vertical bars indicate the mean values ± S.E.M. values.

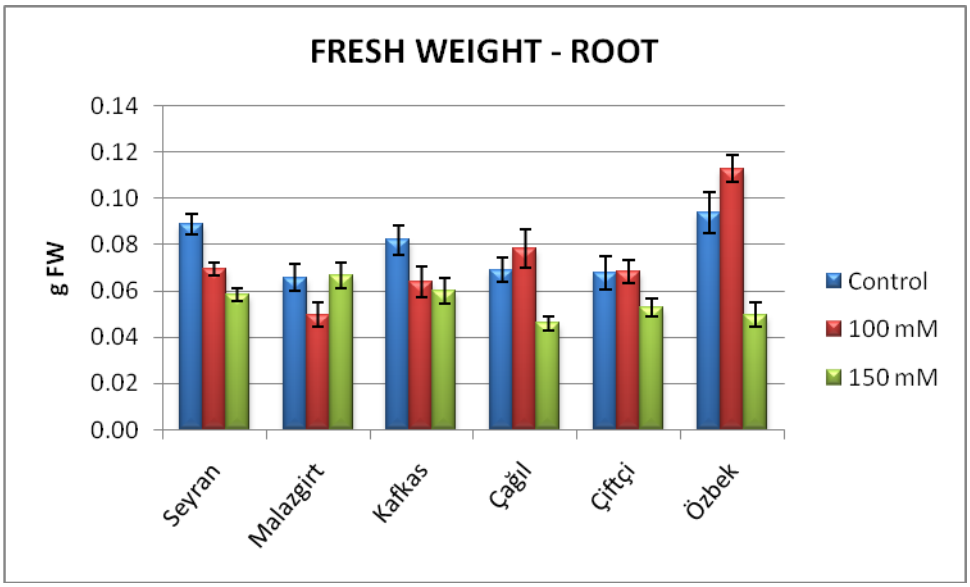


**Figure 3.3** Root fresh weights of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

Shoot lengths of all cultivars except Seyran were decreased and given in Figure 3.4. The shoot length of Seyran increased by 5% under 100 mM NaCl and decreased by 14% under 150 mM NaCl compared to the control. Shoot length of 150 mM treated Seyran showed significant difference from control and 100 mM. Lengths of salt-treated shoots of Malazgirt were significantly different from control. Root lengths of other cultivars showed in Figure 3.5 and decreased when the dose of stress increased. Salt-treated root tissues of Malazgirt, Seyran and Kafkas showed significant decrease from control.

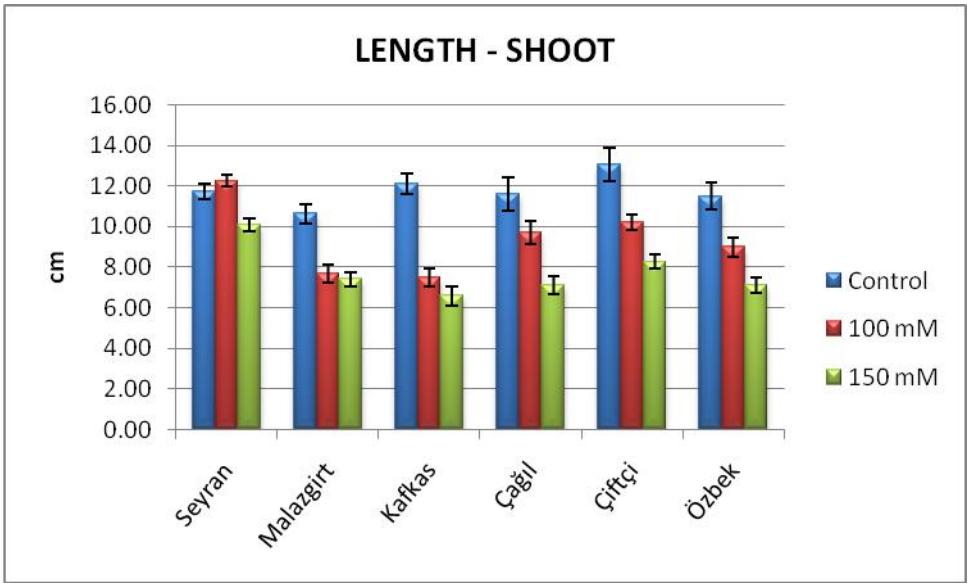


**Figure 3.4** Shoot lengths of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

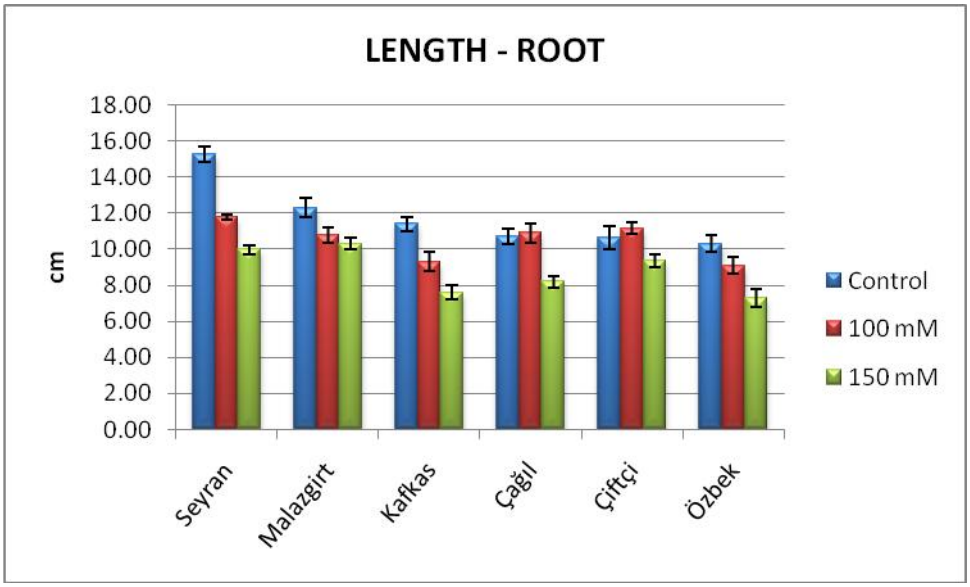


**Figure 3.5** Root lengths of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

### 3.1.2 Effect of Salinity on Biochemical Parameters of Lentil Cultivars

***Ion Leakage***

For all cultivars, increase in shoot ion leakage was observed when salt concentration increased and given in Figure 3.6. Ion leakage levels of shoot tissues of Seyran, Malazgirt, Çiftçi and Çağıl showed significant increase when salt stress was applied. Increase in root ion leakage level was observed in Figure 3.7 as dose-dependent manner except Çiftçi and Özbek. Control and stress-treated root tissues of Malazgirt and Kafkas showed significant difference from each other. Seyran root control were significanlty different from salt-treated ones.

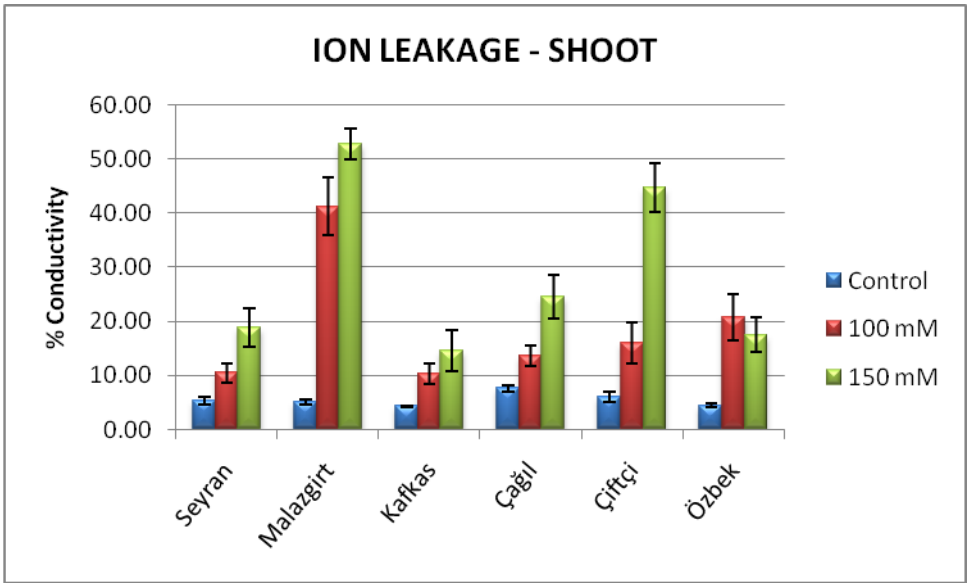


**Figure 3.6** Shoot ion leakage of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

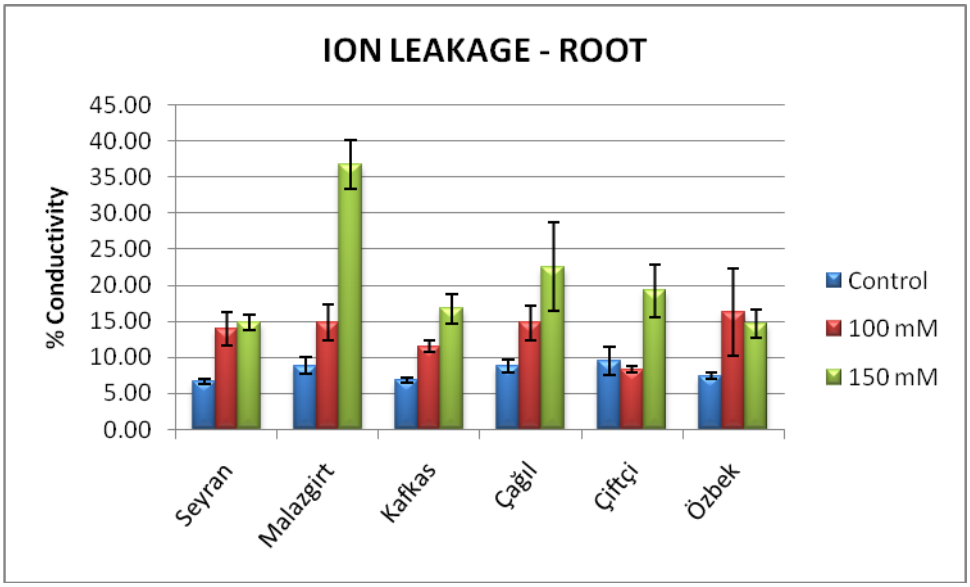


**Figure 3.7** Root ion leakage of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

***Malondialdehyde(MDA)***

All shoot MDA contents increased in a salt concentration dependent manner except Seyran and given in Figure 3.8. Decline of 26% and 36% were observed for Seyran shoot samples under 100 mM and 150 mM salt stress conditions. However; 76% and 194% elevation were observed for Malazgirt; and 4% and 245% increase for Kafkas shoot samples. Root tissue of cultivars respond in a different manner in terms of MDA content with the application of salt stress in Figure 3.9. Seyran has 3% and 15 % decrease for 100 mM and 150 mM NaCl treatment, respectively. Çiftçi decreased by 6% and increased by 13% under 100 mM and 150 mM when compared to control samples.

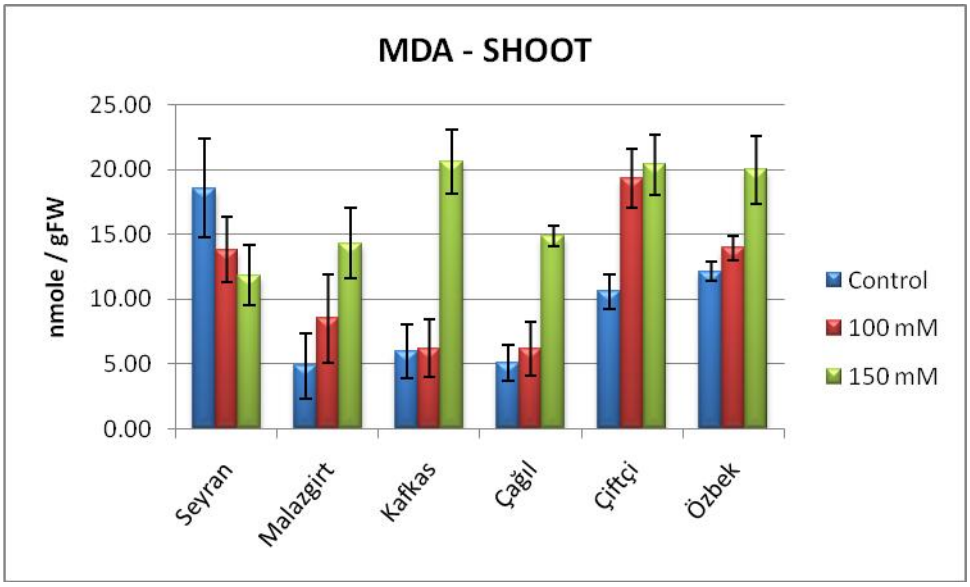


**Figure 3.8** Shoot MDA contents of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

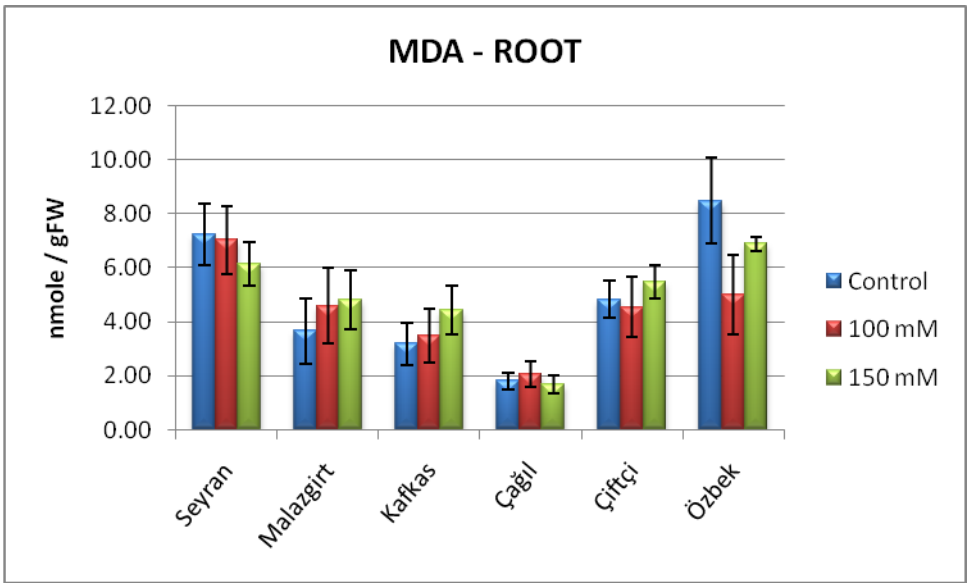


**Figure 3.9** Root MDA contents of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

### *Hydrogen Peroxide ($H_2O_2$)*

Changes in the level of shoot $H_2O_2$ were shown in Figure 3.10 and all cultivars except Seyran have uptrend for shoot $H_2O_2$ amounts when salt stress is applied. Seyran has 34% decline under 100 mM NaCl and 39% decline under 150 mM NaCl conditions. There is no similar trend between cultivars for $H_2O_2$ content in root samples and was shown in Figure 3.11. On one hand, Malazgirt decreased by 19% and 5% under 100 mM and 150 mM NaCl conditions, respectively. On the other hand, Kafkas increased by 40% and 77% when the concentration of NaCl was increased.

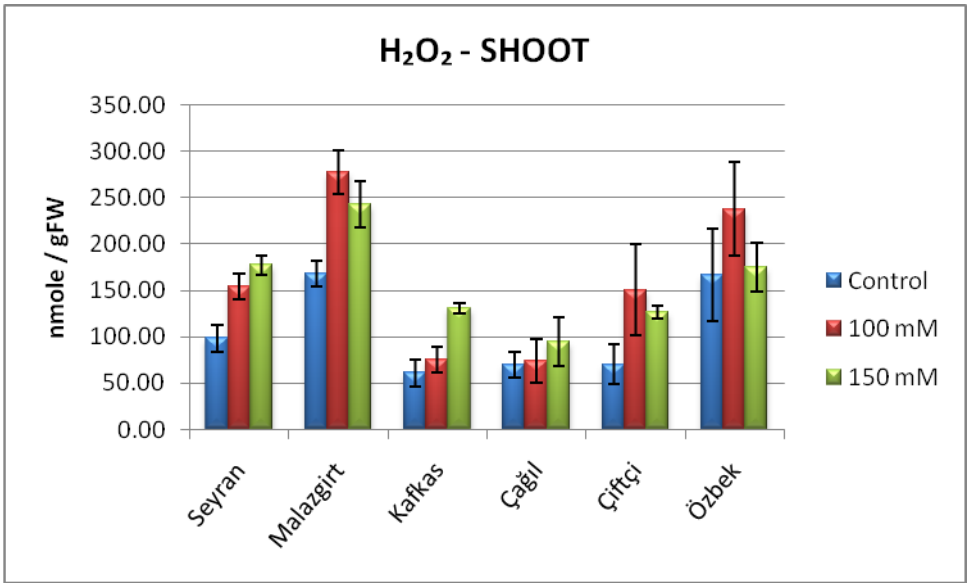


**Figure 3.10** Shoot $H_2O_2$ contents of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

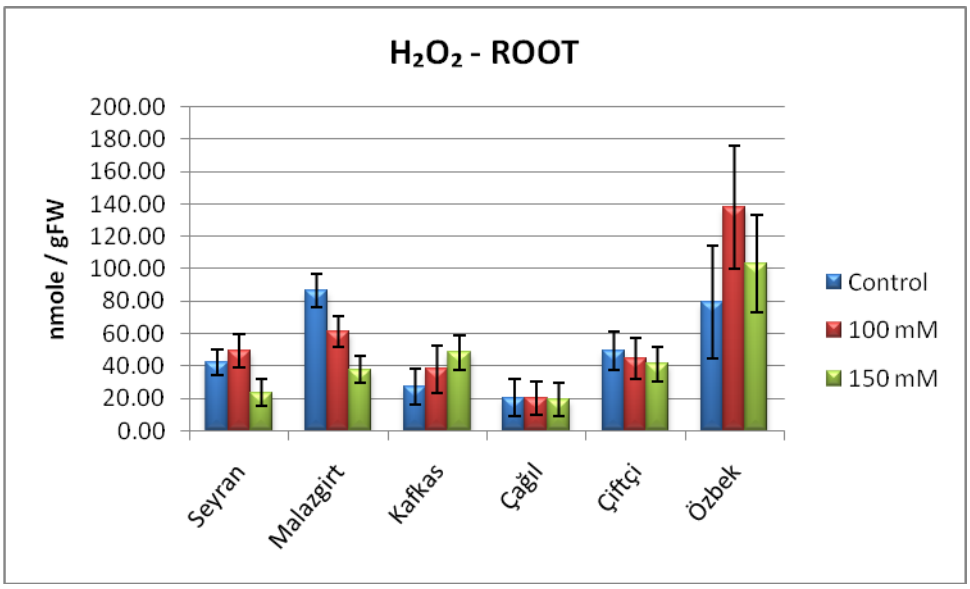


**Figure 3.11** Root $H_2O_2$ contents of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

***Proline***

Increase in the proline amounts of shoot tissue of all cultivars were shown in Figure 3.12. Malazgirt shoot proline level showed 79% and 63% increase, for 10 mM and 150 mM, respectively. Çiftçi increased by 129% under 100 mM and 423% under 150 mM. Changes in the proline level of root tissues were shown in Figure 3.13. Roots of Seyran and Malazgirt showed significant increase under 100 mM and 150 mM salt stress conditions.

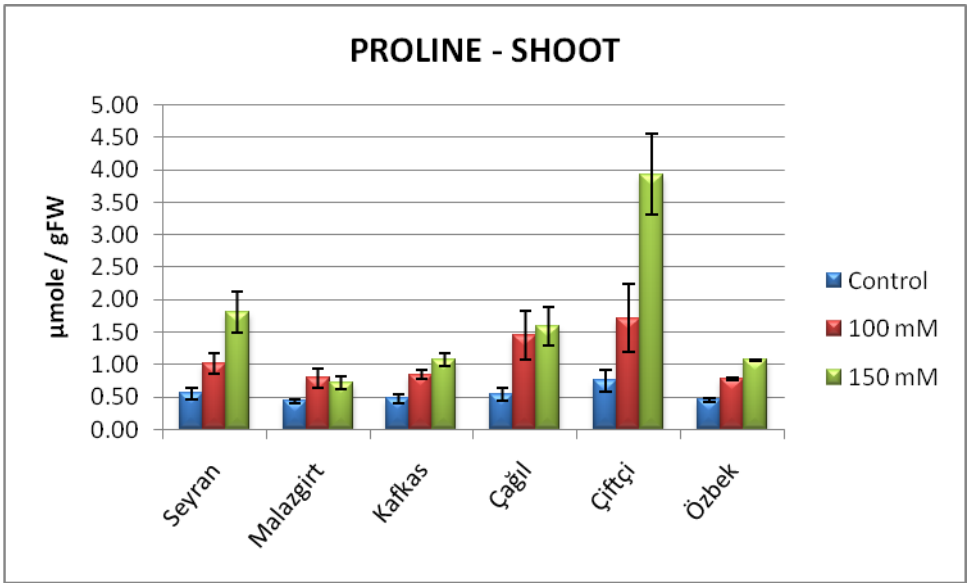


**Figure 3.12** Shoot proline contents of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

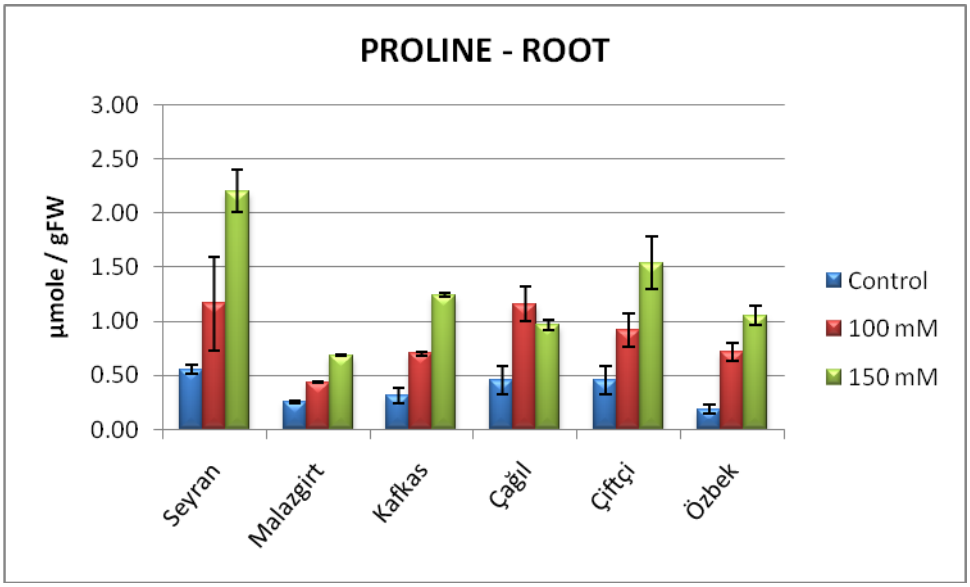


**Figure 3.13** Root proline contents of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

### 3.1.3 Effect of Salt Stress on Antioxidative Enzyme Defense System

After selection process, catalase, ascorbate peroxidase and glutathione reductase activities of Seyran and Malazgirt cultivars were analyzed to compare cultivars within themselves and also contrast the selected resistant and sensitive cultivars under salt stress conditions.

***Glutathione Reductase***

GR activities of Seyran shoot tissue showed increase under salt stress treatment when compared to control (Figure 3.14). However; there was no such change in shoot tissue of Malazgirt. Also, shoot GR activities of Seyran was approximately double of Malazgirt. In Figure 3.15 GR activities of root tissues for both cultivars were shown. Seyran root tissues showed slight decrease and Malazgirt root tissues showed slight increase for GR activities under salt stress conditions.

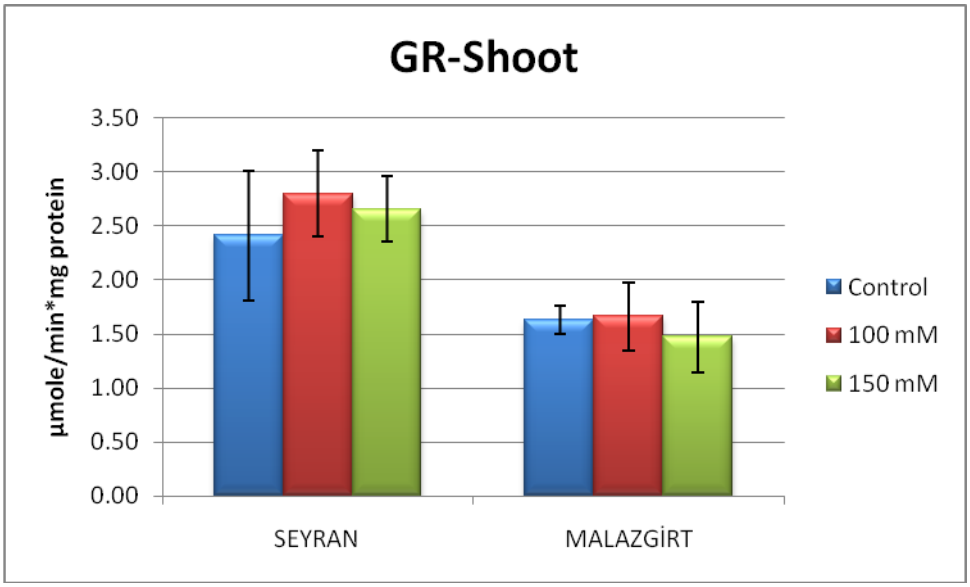


**Figure 3.14** GR activity in shoots of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

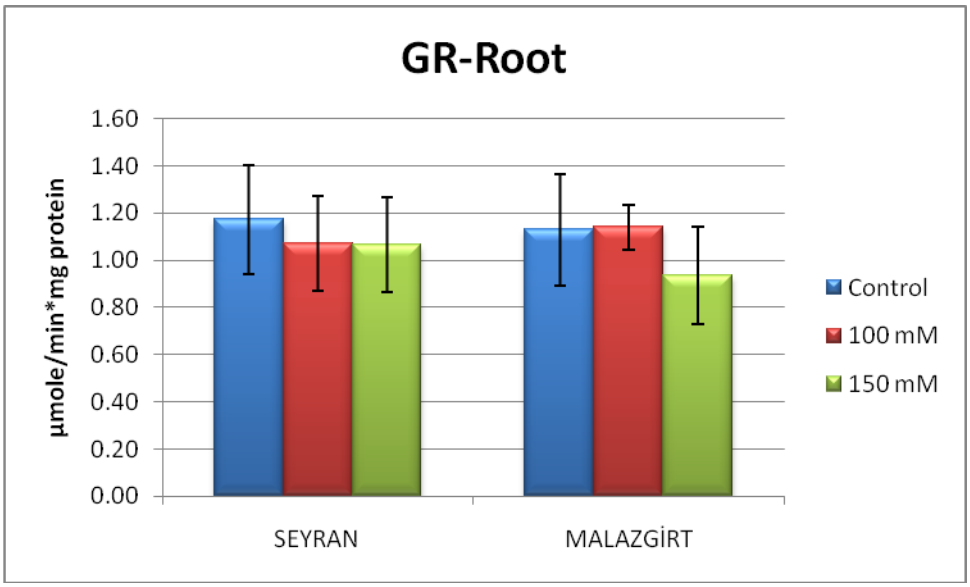


**Figure 3.15** GR activity in roots of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

***Ascorbate Peroxidase (APX)***

APX activities of shoot tissues were shown in Figure 3.16. APX activities of salt-treated Seyran shoots showed increase with respect to control whereas, salt-treated Malazgirt shoot tissues had a declining profile compared to control. APX activities of root tissues were shown in Figure 3.17. APX activity of 100 mM NaCl-treated Seyran root tissue increased when compared to control. On the other hand, Malazgirt roots showed decrease as dose-dependent manner in APX activities. Root results of Seyran showed significant difference from that of Malazgirt for APX activities.

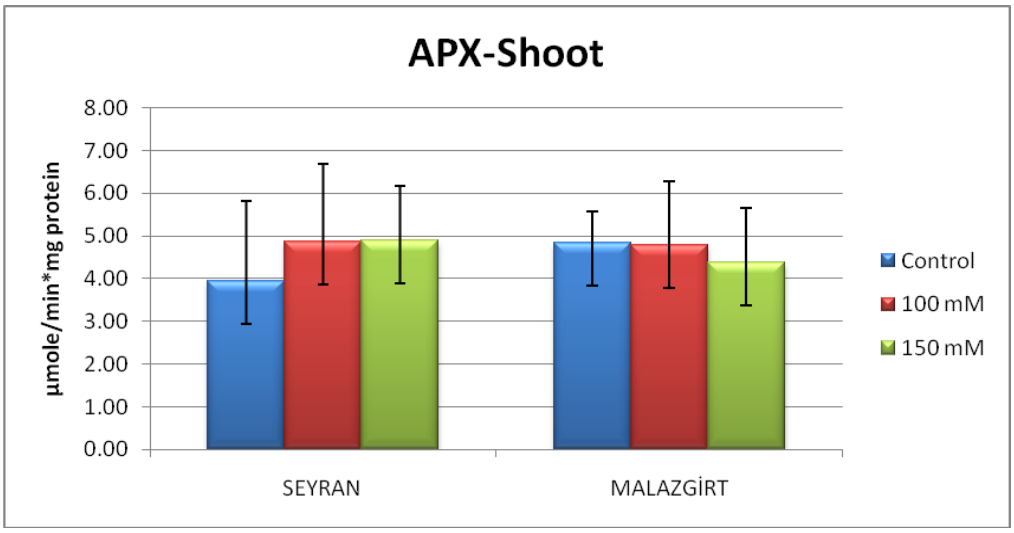


**Figure 3.16** APX activity in shoots of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

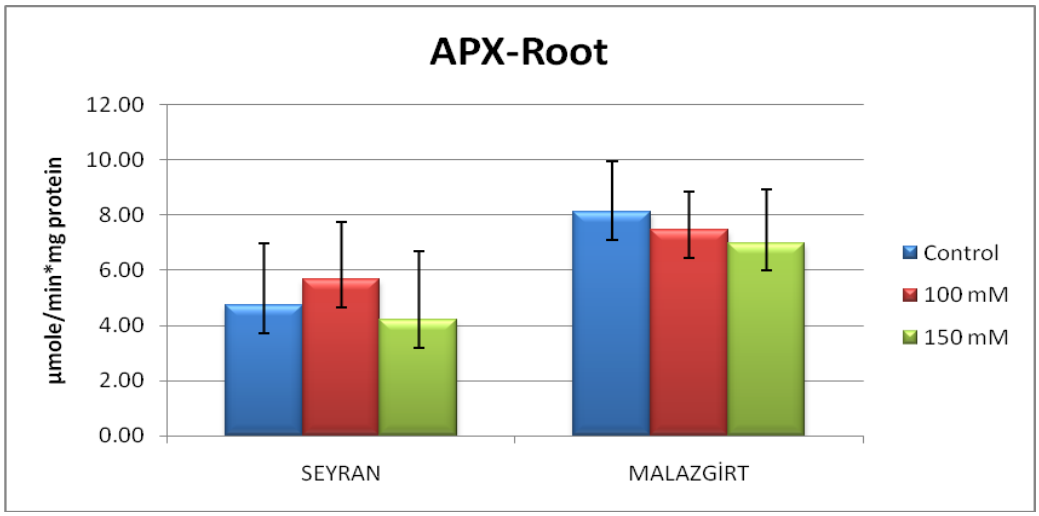


**Figure 3.17** APX activity in roots of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

### *Catalase (CAT)*

Alterations in the activity of catalase enzyme for shoot tissue were shown in Figure 3.18. Shoot CAT enzyme activities remained almost same in Seyran, whereas Malazgirt CAT activities had a declining profile when salt stress was applied. The behavior of root tissues of two cultivars were opposite of the shoot tissue (Figure 3.19). Seyran root CAT activities showed significant decrease when lentils were treated with NaCl but still higher than Malazgirt CAT activity levels.

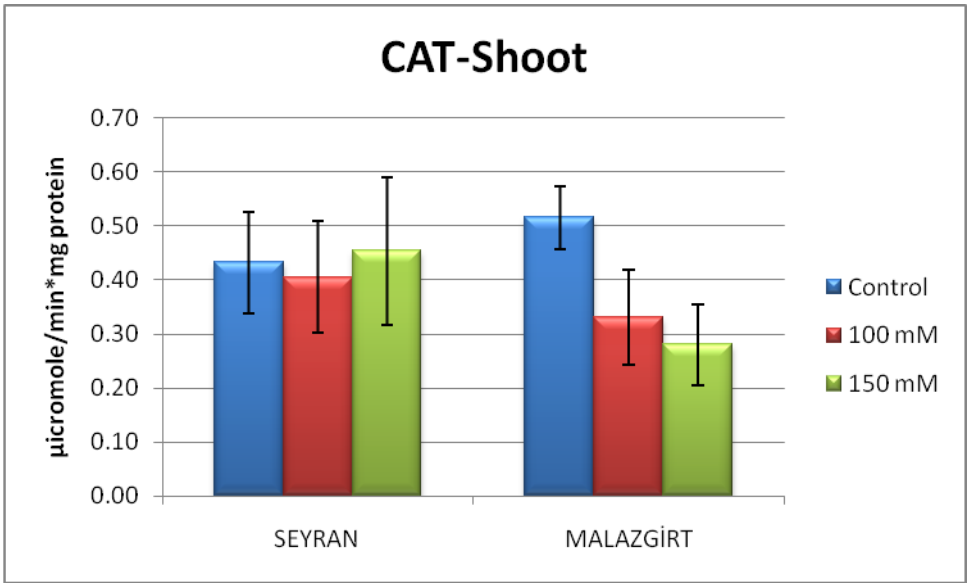


**Figure 3.18** CAT activity in shoots of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

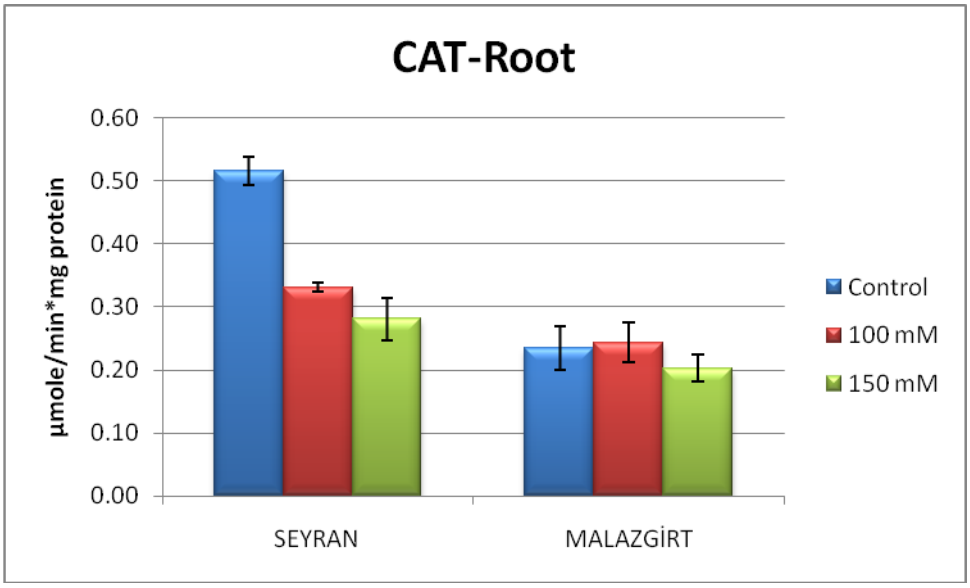


**Figure 3.19** CAT activity in roots of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

***Superoxide Dismutase (SOD)***

SOD isozymes in shoot and root tissues of Seyran and Malazgirt cultivar were determined in native PAGE gel stained by negative activity stain in Figure 3.20. Three SOD isozymes were identified in both of the tissues of Seyran and Malazgirt cultivars which are mitochondrial Mn SOD, cytosolic Cu/Zn SOD-1 and Cu/Zn SOD-2 in Figure 3.20. Chloroplastic Fe SOD could not be observed. In general manganase SOD (Mn SOD) did not show variation in the intensity of protein bands with the salt stress treatment for both tissue. Only change was observed in 150 mM NaCl treated Malazgirt shoot tissue when compared to control group. Seyran cultivar did not show any sharp change in the Cu/Zn SOD-1 and Cu/Zn SOD-2 activities with the salt stress treatment for shoot tissue. Whereas, the activities of Cu/Zn SOD-1 and Cu/Zn SOD-2 showed increase with salt stress treatment in Malazgirt shoot tissue. For root tissue, no change in the activities of SOD isozymes was observed with salt stress treatment for both of the cultivar.

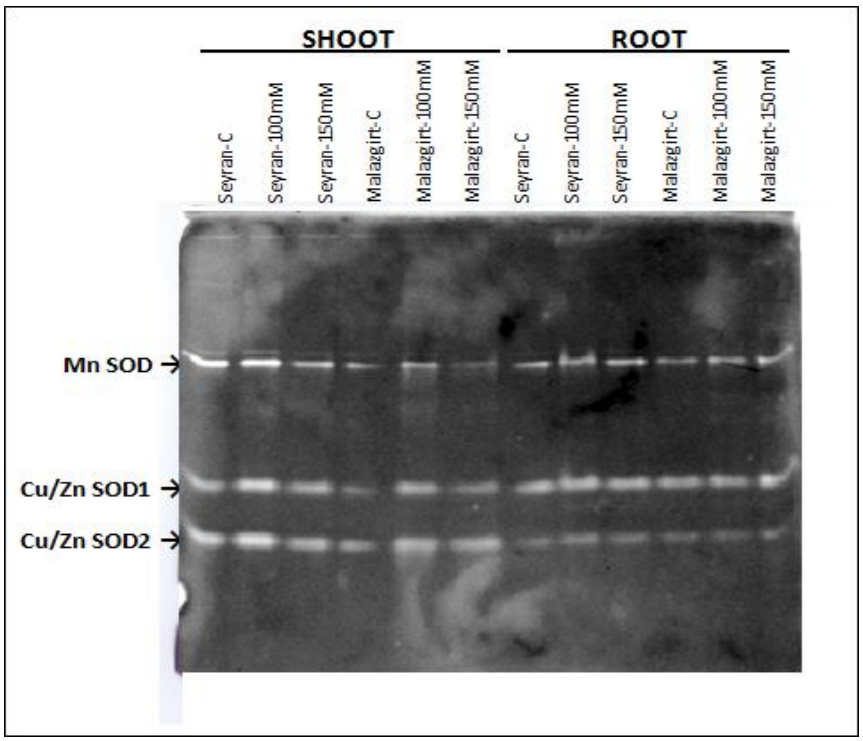


**Figure 3.20** Activities of SOD isozymes in shoot and root tissue of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

### 3.1.4 Total Protein Identification

Total proteins were isolated and analyzed with Bioanalyzer and SDS-PAGE to observe the differences in the total protein profiles of the two cultivars with salt-stress treatment.

SDS-PAGE was performed to both ensure that proteins were extracted properly and observe different protein bands between resistant and sensitive cultivar; and also within each cultivar. Gel was silver stained to visualize protein bands in the range of 14 and 116 kDa. However; any difference in the intensity of protein bands could not be observed for both of tissue in salt-treated samples when compared to control group (Figure 3.21).

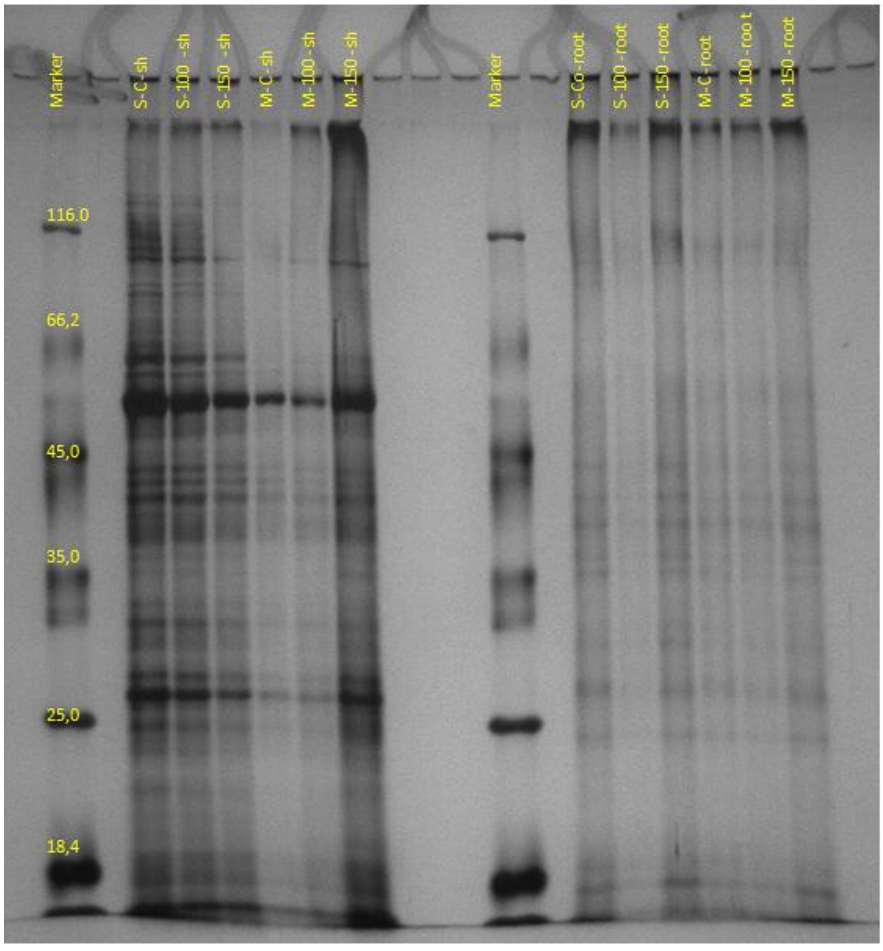


**Figure 3.21** Silver stained SDS-PAGE (12% separating, 4% stacking) gel image of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

In Figure 3.22 and Figure 3.23, the Bioanalyzer results of shoot and root tissues of both cultivars were shown, respectively. Different protein peaks were observed both within different treatments of same cultivar and between Seyran and Malazgirt cultivar. Blue, green and red colors represent control, 100 mM and 150 mM, orderly.

At 46 kDa, a distinctive protein peak was observed in control group of Seyran shoot tissue, which was disappeared upon NaCl stress treatment for Seyran shoot tissue, and also this protein band was missing in both control and salt-stress treated shoot tissues of Malazgirt.

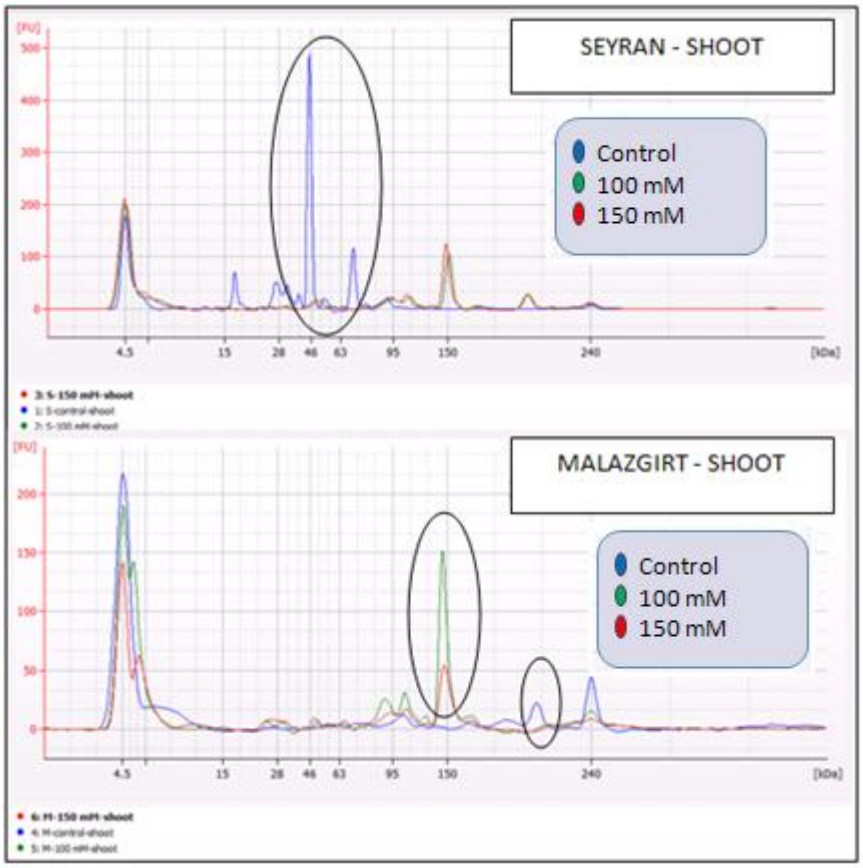


**Figure 3.22** Electropherogram images of shoot tissue of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

Variation was observed in root tissues of Seyran cultivar, whereas no such change could be identified for root tissue of Malazgirt cultivar. New peaks were observed 100 mM NaCl and 150 mM NaCl treated Seyran root tissue as shown in Figure 3.23.

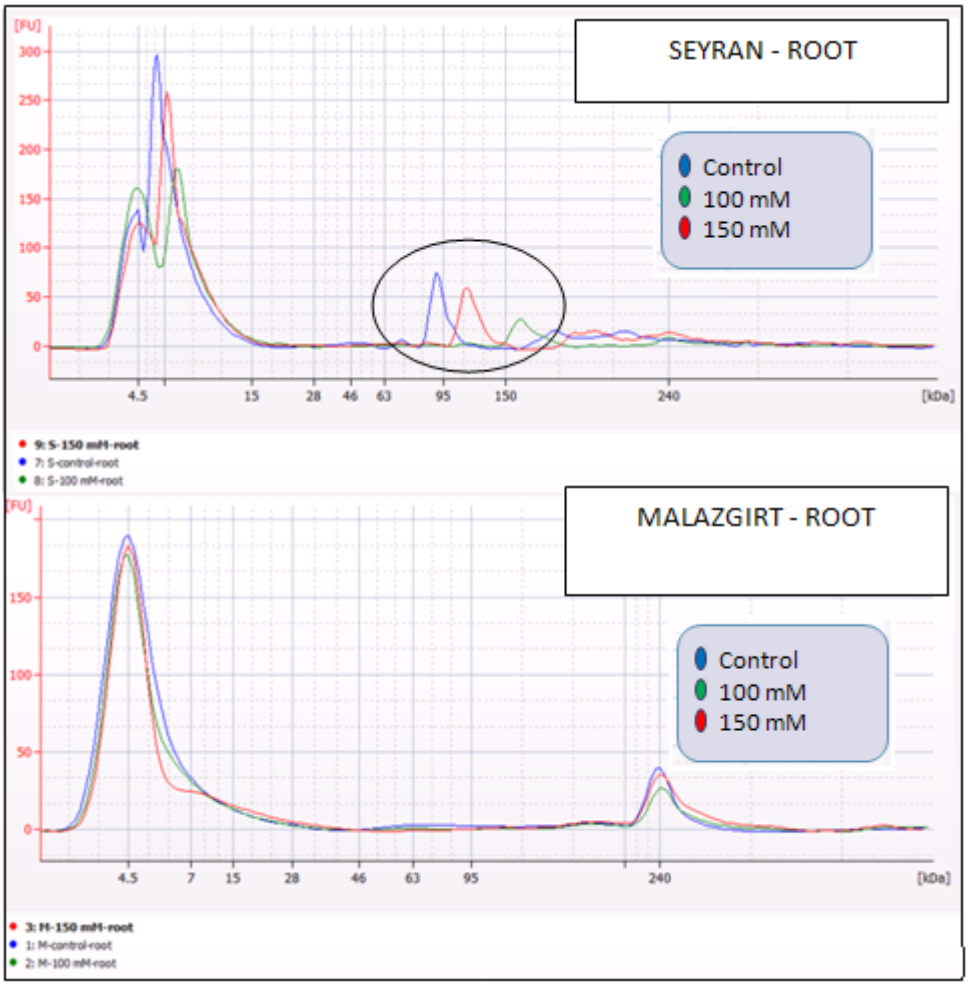


**Figure 3.23** Electropherogram images of root tissue of 12 days old lentil seedlings of control, 100 mM NaCl and 150 mM NaCl stress (5 days of salt-stress treatment after 7 days of normal growth)

# CHAPTER 4

# DISCUSSION

Lentil is an important nutrient of human diet since its protein content is high and it is cholesterol-free. Turkey is one of the major lentil producing countries. However; worldwide human population is increasing exponentially, and sources are going to be depleted. Major cause of this depletion is that organisms are exposed to abiotic stresses, and salt stress is one of the major limiting factors of plant growth and crop yield.

## 4.1 Effect of Salinity on Physiological Growth Parameters

According to a study on sugar beet plants in 2001 by Ghoulam *et al*., it was realized that salinity led to progressive decrease in the growth parameters with the increase in the stress dosage. Studies with Turkish lentil cultivar (Sultan 1) exhibited that shoot and root tissues of length and fresh weights were significantly diminished at hypersalinity conditions (Bandeoğlu,2004; Ercan,2008). In this study, after 5 days of salt stress application to the 7 days old seedlings, fresh weights of 6 lentil cultivars decreased as dose-dependent manner, however; an increase was observed in the fresh weights of roots for Özbek and Çağıl under 100 mM NaCl conditions. Among length results of shoot tissues of all cultivars, only Seyran showed an increase at 100 mM NaCl conditions. Shoots of most crop species are more sensitive to hypersalinity than root tissues (Bernstein *et al*., 2004). In this study, an increase in the lengths of roots for Çiftçi and Çağıl under 100 mM NaCl conditions was observed, this result was supported by the study of Bernstein. On the other hand, Gupta, 2006 mentioned that plants respond different from each other even under same stress conditions. For example, some plants improve root systems to facilitate water absorbance. In this sense, Çağıl, Özbek and Çiftçi behave different from other cultivars with respect to their root growth parameters. Ashraf and Waheed, 1990 examined the salt effect on

germination of 133 lentil varieties, and only 4 % of them showed significant increase in the physiological parameters.

Decreased leaf area, puny shoots and wilted leaves were realized in all cultivars especially Malazgirt. Under salt stress, Malazgirt lentil seedlings nearly stopped growing. Ercan,2008 stated that leaves of Turkish lentil cultivar (Sultan 1) became small to prevent high rates of transpiration when lentil plant faced with water deficiency problem.

## 4.2 Effects of Salinity on Biochemical Parameters

### *Lipid Peroxidation and Ion Leakage*

Under normal growth conditions, ROS generation and scavenge is at equilibrium, however when plant is exposed to environmental stress, this balance is disturbed, since antioxidative system do not eliminate as fast as the generation rate. Under oxidative stress plants suffer from lipid peroxidation, protein oxidation and most importantly DNA damage. MDA is one of the end-products of lipid peroxidation mechanism, therefore it is an important marker of oxidative stress on plants.

MDA results in this study showed that salt treatment induced damage on membranes in all cultivars except Seyran. MDA levels of Seyran were alleviated by the treatment of stress in both shoots and roots. Probably, Seyran possesed more efficient antioxidative defense system than others, therefore it could scavenge ROS elements before high levels of LPO occurred. This inference was supported by the study of Khan and Panda, 2008. This study focused on the response of *Oryza sativa* cultivars under salinity conditions, lipid peroxidation (LPO) levels of one cultivar was higher than other cultivar, although both had elevated LPO ratios. They concluded that cultivar with lower LPO had more efficient antioxidative system than the other, so that it could some extent cope with the oxidative damage. Malazgirt has 76% and 194% increase of MDA results for the shoots of 100 mM and 150 mM, respectively. For root tissues of Malazgirt 25% and 32% of increase was observed in the 100 mM and 150mM NaCl treated samples, respectively. Ercan, 2008 mentioned that MDA levels of Sultan 1 lentil cultivar showed significant decrease in both shoot and root tissues as the drought

stress was applied. Cicerali,2004 also mentioned that membrane destruction level of shoot tissues of lentil cultivars were higher than the root tissues. In this study also shoot MDA levels were higher than root MDA levels. Maybe, this is because of leaves are more susceptible to the oxidative stress, since leaves are the sites of photosynthesis.

All cultivars had an increase in ion leakage levels with the salt stress treatment in both shoot and root tissues. Malazgirt was the most affected one among all cultivars. Since it is 8-fold and 10-fold of the control for shoot tissue; and 1.5 and 4 fold for root tissue with the 100mM and 150mM NaCl treatment, respectively. It seemed that Malazgirt was more vulnerable to oxidative stress resulted from hypersalinity and, it could not improve an efficient scavenger mechanism. Cicerali, 2004 also mentioned that ILL 590 lentil cultivar seemed to be more sensitive to NaCl stress. In the study of Ercan, 2008, both of the tissues showed significant increase in electrolyte leakage under salinity conditions. Another study with sugar beet by Jamil *et al*., 2012 was exhibited that conductivity of membrane was increased when salt stress was applied to sugar beet plants, which also supported our ion leakage results.

***Hydrogen Peroxide Content***

Stomatal closure directly leads to low chloroplastic ratio of $CO_2$ / $O_2$, since $CO_2$ could not be taken into the cell from outside (Hernandez *et al*.,2000). Therefore photorespiration rate overweighs to the rate of photosynthesis. As a result of high levels of photorespiration, ROS production induced such as $H_2O_2$. Since $H_2O_2$ is the least reactive of ROS, it takes important role in signaling mechanism under stress conditions. Therefore, $H_2O_2$ content is also used as a marker of oxidative stress (Slesak *et al*., 2007).

All cultivars showed an increase in $H_2O_2$ levels with the stress treatment. Çağıl shoot tissue showed least increase in $H_2O_2$ levels when faced with salinity problem among all cultivars. Çiftçi showed mitigation in the shoot $H_2O_2$ levels with the increase of the concentration of salt treatment. From this result it can be suggested that defense system of shoot tissue operate better under severe conditions but still not as much as control group. Malazgirt showed higher

elevation in $H_2O_2$ levels for shoot tissue under salt stress condition when compared to Seyran shoot tissue. Superoxide dismutase is the major $H_2O_2$ producing enzyme and the activities of SOD might explain the increase of $H_2O_2$. Malazgirt shoot SOD activities showed increase while, Seyran shoots remained almost same with salt stress application.

In general, shoot $H_2O_2$ levels were higher than the roots which was also observed in the study of Ercan, 2008. This might be resulted from that shoot tissues are exposed to oxidative stress much more than roots. Bandeoğlu *et al*., 2004 and Ercan 2008 were studied same cultivar of lentil and they found that $H_2O_2$ levels went up under NaCl stress conditions.

***Proline***

Proline is an important osmoprotectant synthesized by plants, which provide osmotic adjustments to plants under stressful conditions. Accumulation of proline do not inhibit normal cellular metabolism therefore, over accumulation bring plants overprotection against environmental stresses. Proline protects membrane stability and three-dimensional protein structure when plant suffer from water deficiency. Moreover, studies show that proline takes role also in antioxidative system by scavenging singlet oxygen (Smirnoff 1989; Reddy *et al*.,2004).

In this study, Malazgirt had least elevation in proline amount among all cultivars for both tissues when NaCl stress was applied. Çiftçi cultivar showed highest elevation among all cultivars. Seyran could not show that much of increase under salt stress, since may be Seyran started to reduce proline levels at the $5^{th}$ day of stress treatment due to its being more resistant to salt stress than other 5 Turkish cultivars. This suggestion was supported by a study of chickpea performed by Eyidoğan and Öz, 2007. Study exhibited that proline content significantly increased at the 2th day of salt treatment both root and shoot tissue. However, at the $4^{th}$ day of application, proline amounts decreased significantly.

Although every cultivar showed decrease in fresh weight and length for both shoot and root tissue, only Seyran cultivar had a declinining profile of $H_2O_2$ and MDA content under salinity conditions for both tissue. Seyran could cope with lipid

peroxidation and ROS producing factors, which could imply that Seyran behaves as if it is salt-resistant among 6 red lentil cultivars. Ion leakage percentage greatly increased in Malazgirt cultivar for both shoot and root tissue when tissues were salt stress treated. Increases in the MDA and $H_2O_2$ contents also supported the electrolyte leakage results. Based upon these results, Malazgirt cultivar was selected as salt-sensitive cultivar.

### 4.3 Effect of Salinity on Antioxidative Enzymatic Defense System on Selected two Lentil Cultivars

ROS generation and elimination is balanced under normal growth conditions. However, when plant encounters an environmental stress factor, this balance is destroyed and ROS elements start to accumulate within the cell, which then results in oxidative stress.

SOD reduces $O_2^{\bullet-}$ and the reaction ends up with $H_2O_2$. Therefore this enzyme is responsible for the production of $H_2O_2$. SOD has three and four isozymes for root and shoot tissues. Native PAGE and negative activity staining was used for SOD activity determination.

APX and GR are crucially important enzymes of Halliwell-Asada cycle. Former is responsible for elimination of $H_2O_2$ by using ascorbate as electron donor. Latter is pivotal for the the regeneration of GSH (reduced glutathione), which is required for the activity of DHAR enzyme. CAT is responsible for the dismutation of hydrogen peroxide to water and oxygen. Nagamiya 2007 *et al.* indicated that overexpressed *katE* gene which encodes *Escherichia coli* CAT, provides transgenic rice plants tolerance to salt stress.

According to the comparative studies of *Plantago maritima* and *Plantago media,* it was found that salt- tolerant *P.maritima* had lower levels of MDA resulted from better antioxidative protection by increasing SOD, GR , APX and CAT activities compared to the salt-tolerant *P.media* (Sekmen *et al*.,2007). Salt-tolerant plants (pea, mulberry and tomato) increases the activity of APX implying the importance

as an antioxidant enzyme (Hernandez et al., 1999; Sudhakar *et al.*, 2001; Rodriguez-Rosales *et al.*,1999).

Three SOD isozymes were identified in both of the tissues of Seyran and Malazgirt cultivars which are Mn SOD, Cu/Zn SOD-1 and Cu/Zn SOD-2. In general mitochondrial Mn SOD did not show variation in the intensity of protein bands with the salt stress treatment for both tissue. Only decrease in the activity of Mn SOD was observed in 150 mM NaCl treated Malazgirt shoot tissue, it can be suggested that enzyme structure could be destroyed under hypersalinity conditions. While Cicerali, 2004 observed significant increase at 200 mM NaCl for lentil cultivars. Seyran cultivar did not show any sharp change in the cytosolic Cu/Zn SOD-1 and Cu/Zn SOD-2 activities with the salt stress treatment for shoot tissue. Whereas, the activities of Cu/Zn SOD-1 and Cu/Zn SOD-2 showed increase with salt stress treatment in Malazgirt shoot tissue. These results were correlated with $H_2O_2$ results. Malazgirt $H_2O_2$ levels for shoot tissue were higher than that of Seyran shoot tissue. Ercan, 2008 found that lentil seedlings showed slight increase in cytosolic SOD activities for both tissues. However; in this study for root tissue,no change in the activities of SOD isozymes was observed with salt stress treatment for both of the cultivar.

In this study, GR activities of Seyran shoot tissue showed increase under salt stress treatment when compared to control whereas; no such change was observed in shoot tissue of Malazgirt. Shoot GR activities of Seyran was approximately double of Malazgirt which indicated that Seyran cultivar used GR enzyme more efficiently than Malazgirt cultivar.

Apel and Hirt, 2004 stated that under stressful conditions some of the antioxidative enzymes were mostly preferred and overexpressed, while activities of other enzymes was decreased. Cicerali, 2004 also mentioned that CAT enzyme may not contribute tolerance to the lentil plant. However; GR and APX activities of both cultivars increased significantly as a salt concentration dependant manner.

In this study, CAT activities of Seyran shoot slightly increased under severe salt concentrations, whereas, Malazgirt CAT activities decreased as the concentration of salt was increased. APX activities of all samples were higher than any activities of CAT. Sharma *et al.*,2012 mentioned that APX has higher affinity to $H_2O_2$ than CAT enzyme, as also shown in our results. Malazgirt APX activities for both tissue showed slight decrease. On the other hand, Seyran had slight increase in shoot tissue and diminished activity for root tissue under severe salt conditions. Also, root results of Seyran showed significant difference from that of Malazgirt for APX activities. It has seen that Malazgirt could not use both of the scavenger enzyme of $H_2O_2$ properly under salt stress. These results are supported by $H_2O_2$ results. Besides, Seyran could cope with $H_2O_2$ generation by slightly increasing CAT and APX activities, and achieve to alleviate $H_2O_2$ levels as the salt concentration increased.

GR activities of Seyran shoots showed an increase, while Malazgirt remained almost same. It was observed that root tissues of Seyran preferred APX, while shoot tissues of Seyran preferred both APX and GR to defend against oxidative stress. On the contrary, there was no distinctive antioxidant enzyme preference for Malazgirt cultivar. Enzyme activity results also supported the changes in biochemical parameters of both cultivar. Malazgirt had significant increase in lipid peroxidation and related to this electrolyte leakage. Conversely, Seyran cultivar succeeds to decrease MDA levels as the salt concentration was increased.

## 4.4 Effect of Salinity on Total Protein Profile of Selected two Lentil Cultivars

In the study of Parida *et al*.,2008, mangrove *Bruguiera parviflora* were exposed to different concentrations NaCl and intensity of the many protein bands decreased due to the salt stress treatment. In this study, no difference in the intensity of protein bands could be observed in SDS-PAGE results. Electropherograms of Bioanalyzer showed different protein patterns with salt stress treatment. There were distinctive peaks at 46 and between 63 and 93 kDa in shoot tissues of Seyran control which were disappeared in salt-treated Seyran and all samples of Malazgirt. This protein might not be crucial for the response to ionic toxicity and

dehydration or oxidative stress caused by hypersalinity. Therefore Seyran cultivar might suppress the synthesis of this protein and give priority to the others to defense against hypersalinity. Besides, at 150 kDa differences in the protein intensity was observed when salt stress was applied. This protein might be salt-induced proteins to cope with salinity problem. However, characterization of down-regulated and up-regulated proteins in the salt-resistant (Seyran) and salt-sensitive (Malazgirt) cultivars is not possible with Bioanalyzer and SDS-PAGE, therefore in future studies 2-D electrophoresis and LC- MS/MS are going to be performed.

# CHAPTER 5

# CONCLUSION

In this study, 2 cultivars were selected for salt stress treatment among 6 red lentil cultivars (Malazgirt, Çağıl, Çiftçi, Kafkas and Özbek) through a wide range screening process including physiological analysis such as fresh weight and fresh length measurement and biochemical analysis like determination of proline, MDA and $H_2O_2$ content and measurement of electrolyte leakage. Selected cultivars were further compared with respect to their enzymatic antioxidant defense systems.

Seyran and Malazgirt cultivars was found to be resistant and sensitive against salt stress among 6 cultivars especially, with respect to the changes in the MDA and $H_2O_2$ content and percentages of ion leakage. Malazgirt increased the level of these stress indicator biochemical parameters. On the contrary, Seyran had a declining profile of the same parameters.

Enzyme activity analysis also showed that Malazgirt could not use any of the enzyme efficiently against salt stress. However; slight increase in the enzyme acitivites of Seyran could be adequate to overcome damaging effects of salt stress.

To compare total protein profile SDS-PAGE and Bioanalyzer were carried out. The intensity of some proteins were decreased within each cultivar by stress treatment and also between resistant and sensitive one. On the other hand, root tissue protein amount did not show such change. However; for better identification of different protein spots, 2-D electrophoresis and then LC-MS/MS analysis are required.

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# APPENDIX A

## HOAGLAND'S MEDIUM PREPARATION

**Table A**

| 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 B: 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 C: Preparation of FeEDTA Stock Solution**

| ADDITION | STOCK SOLUTION |
|---|---|
| $FeCl_{3.}6H_2O$ | 0.121 g / 250 ml |
| EDTA | 0.375 g / 250 ml |

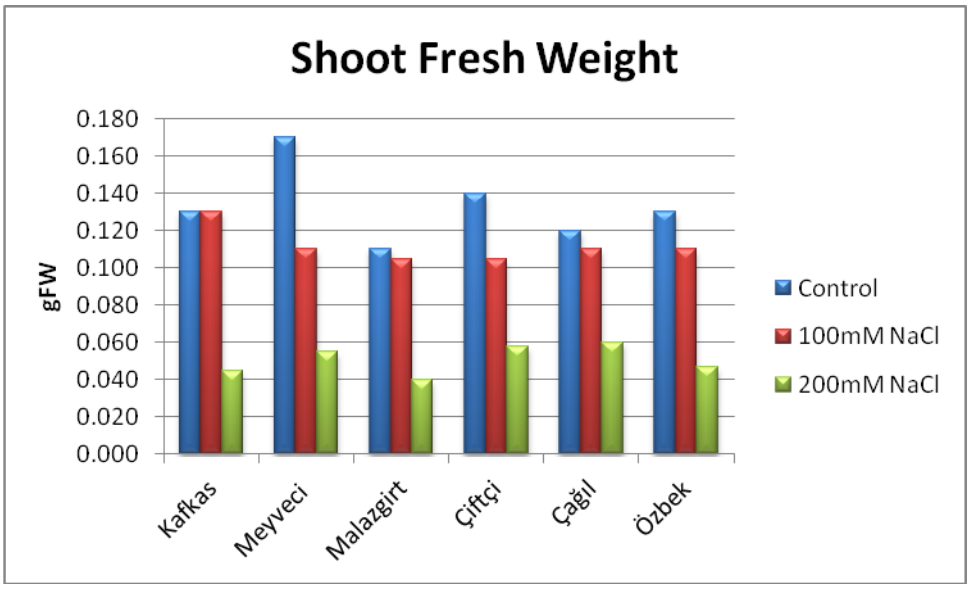
After addition of proper amounts of solutions as given in Table A, pH is adjustedto the 5.8.

# APPENDIX B

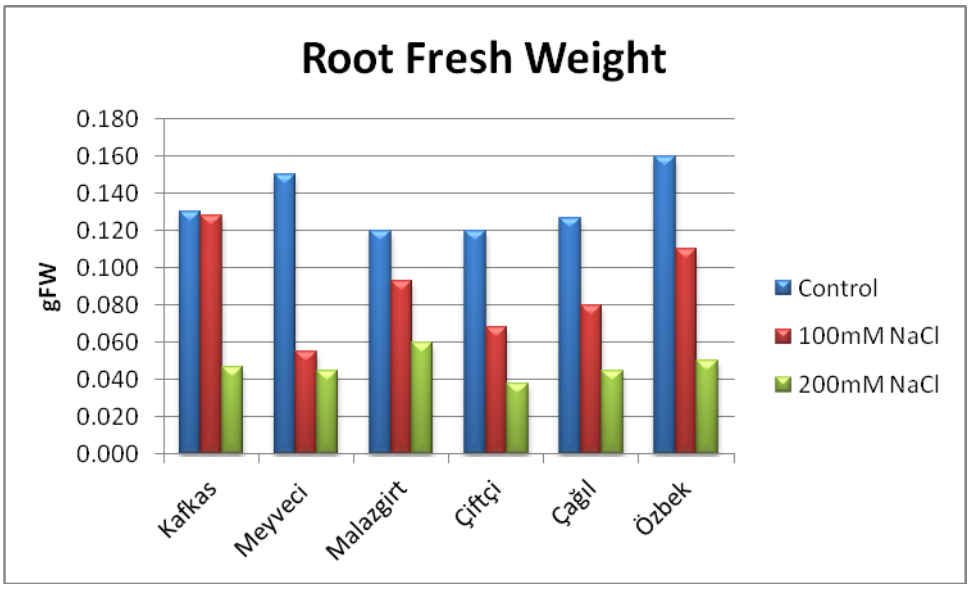
## PRELIMINARY STUDIES FOR DETERMINATION OF STRESS DURATION AND STRESS DOSAGE

Stress dosage and stress duration of lentil varieties were determined by fresh weight analysis of Meyveci, Malazgirt, Çağıl, Çiftçi, Kafkas and Özbek. 100 mM and 200 mM NaCl containing half-strength Hoagland's medium were applied for 4 days and 5 days after 7 days of normal growth. Meyveci was mildewed during germination period, so it was eliminated at this stage. Instead of Meyveci, Seyran that is another red-lentil variety was preferred. 100 mM and 150 mM NaCl and 5 days of stress application was selected as stress dosage and duration, respectively.

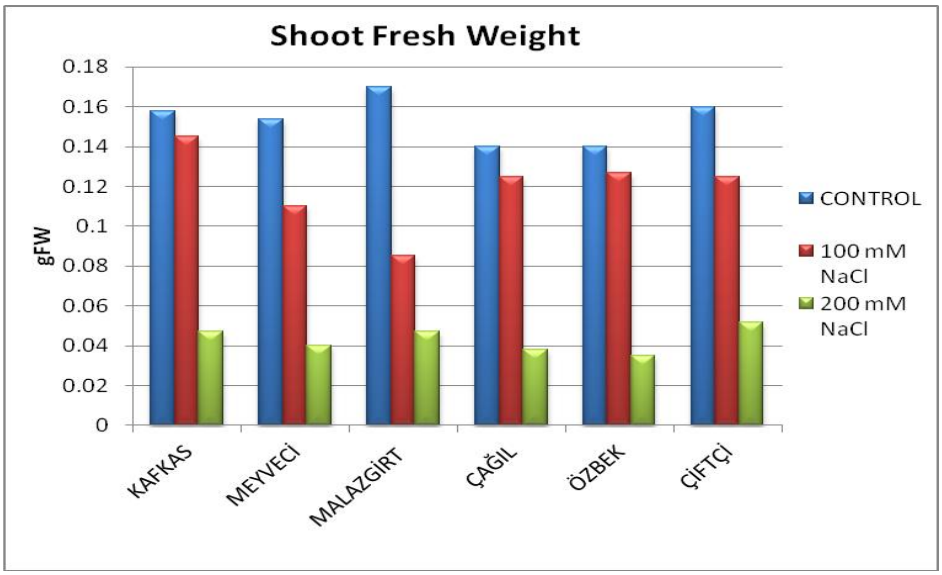
A. Changes in shoot fresh weights under 100 mM NaCl and 150 mM NaCl for 4 days of stress treatment after 7 days of normal growth



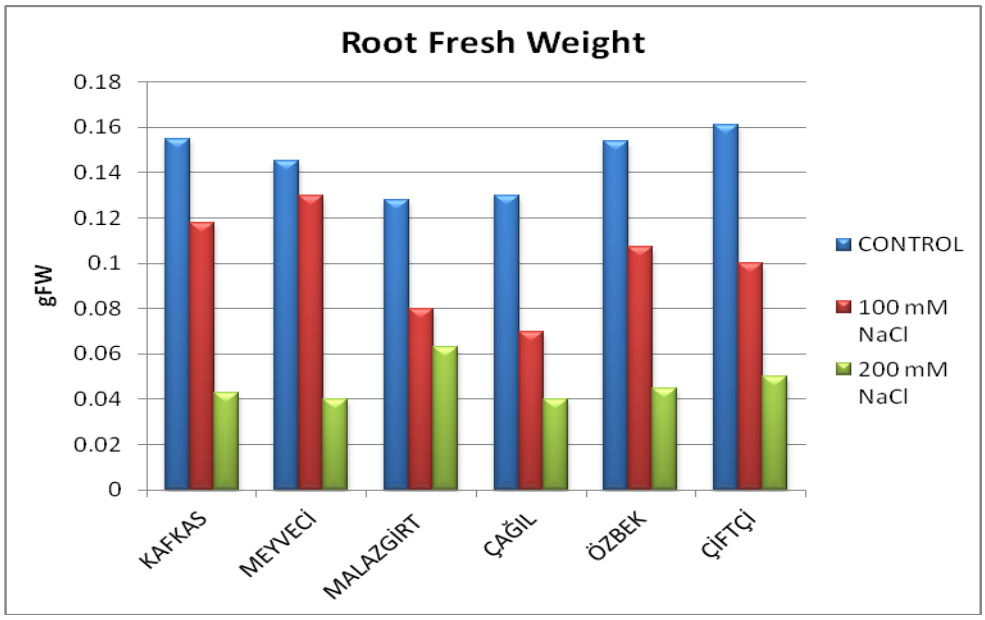
B. Alterations in root fresh weights under 100 mM NaCl and 150 mM NaCl for 4 days of stress treatment after 7 days of normal growth




C. Shoot fresh weight changes under 100 mM NaCl and 150 mM NaCl for 5 days of stress treatment after 7 days of normal growth



D. Changes in root fresh weights under 100 mM NaCl and 150 mM NaCl for 4 days of stress treatment after 7 days of normal growth



# APPENDIX C

## BRADFORD METHOD FOR PROTEIN DETERMINATION

### 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 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-2** Preparation of Samples

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

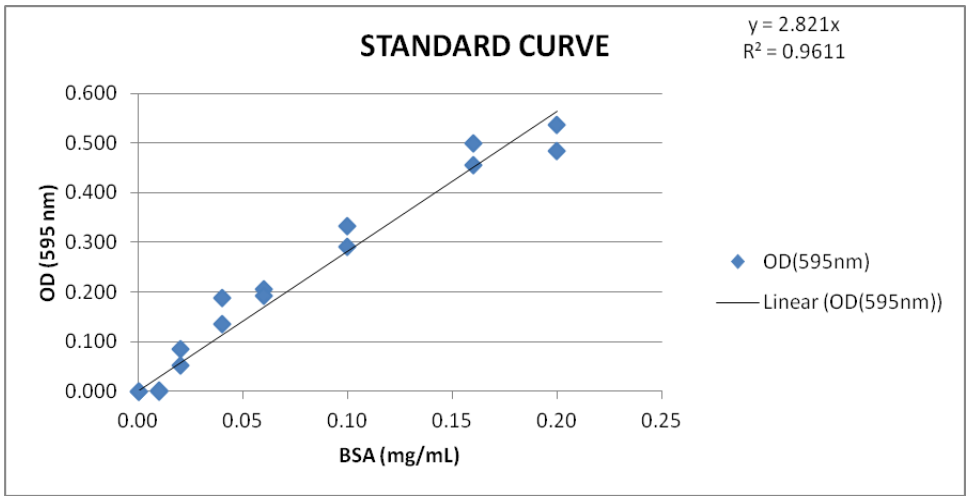
2. Read the absorbances of the tubes against the blank (1-1' of standard curve) at 595 nm.
3. Calculate the concentrations of the samples in each tube and make necessary calculations in order to load the equivalent amounts of samples.

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

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

   500/40 for roots

A. Standard curve obtained by Bradford method



# APPENDIX D

## CHANGES IN PHYSILOGICAL & BIOCHEMICAL PARAMETERS OF SALT-TREATED SAMPLES WITH RESPECT TO CONTROL GROUPS

A. Fresh weight changes under 100 mM NaCl and 150 mM NaCl stress of all cultivars. Numbers indicate the changes with respect to control as percentages. The values are measured with the formula of stress-treated/control*100

| | | CHANGES IN FRESH WEIGHT (%) FROM CONTROL | | | | | |
|---|---|---|---|---|---|---|---|
| | | **SEYRAN** | **MALAZGİRT** | **KAFKAS** | **ÇAĞIL** | **ÇİFTÇİ** | **ÖZBEK** |
| **SHOOT** | **100 mM NaCl** | 88 | 59 | 55 | 79 | 80 | 72 |
| | **150 mM NaCl** | 70 | 60 | 49 | 55 | 55 | 55 |
| **ROOT** | **100 mM NaCl** | 78 | 76 | 78 | 113 | 101 | 120 |
| | **150 mM NaCl** | 66 | 102 | 73 | 67 | 78 | 53 |

B. Length changes under 100 mM NaCl and 150 mM NaCl stress of all cultivars. Numbers indicate the changes with respect to control as percentages. The values are measured with the formula of stress-treated/control*100

| | | CHANGES IN LENGTH (%) FROM CONTROL | | | | | |
|---|---|---|---|---|---|---|---|
| | | **SEYRAN** | **MALAZGİRT** | **KAFKAS** | **ÇAĞIL** | **ÇİFTÇİ** | **ÖZBEK** |
| **SHOOT** | **100 mM NaCl** | 105 | 72 | 62 | 84 | 78 | 78 |
| | **150 mM NaCl** | 86 | 70 | 55 | 61 | 63 | 62 |
| **ROOT** | **100 mM NaCl** | 77 | 88 | 82 | 102 | 105 | 88 |
| | **150 mM NaCl** | 65 | 84 | 67 | 77 | 88 | 71 |

C. Ion leakage changes under 100 mM NaCl and 150 mM NaCl stress of all cultivars. Numbers indicate the changes with respect to control as percentages. The values are measured with the formula of stress-treated/control*100

| | | CHANGES IN ION LEAKAGE (%) FROM CONTROL | | | | | |
|---|---|---|---|---|---|---|---|
| | | **SEYRAN** | **MALAZGİRT** | **KAFKAS** | **ÇAĞIL** | **ÇİFTÇİ** | **ÖZBEK** |
| **SHOOT** | **100 mM NaCl** | 193 | 803 | 243 | 179 | 268 | 470 |
| | **150 mM NaCl** | 348 | 1030 | 341 | 321 | 751 | 396 |
| **ROOT** | **100 mM NaCl** | 208 | 167 | 169 | 168 | 88 | 219 |
| | **150 mM NaCl** | 221 | 414 | 245 | 257 | 202 | 199 |

D. Changes in malondialdehyde (MDA) under 100 mM NaCl and 150 mM NaCl stress of all cultivars. Numbers indicate the changes with respect to control as percentages. The values are measured with the formula of stress-treated/control*100

| | | CHANGES IN MDA (%) FROM CONTROL | | | | | |
|---|---|---|---|---|---|---|---|
| | | **SEYRAN** | **MALAZGİRT** | **KAFKAS** | **ÇAĞIL** | **ÇİFTÇİ** | **ÖZBEK** |
| **SHOOT** | **100 mM NaCl** | 74 | 176 | 104 | 122 | 183 | 115 |
| | **150 mM NaCl** | 64 | 294 | 345 | 292 | 193 | 165 |
| **ROOT** | **100 mM NaCl** | 97 | 125 | 110 | 114 | 94 | 59 |
| | **150 mM NaCl** | 85 | 132 | 140 | 93 | 113 | 81 |

E. Changes in hydrogen peroxide ($H_2O_2$) under 100 mM NaCl and 150 mM NaCl stress of all cultivars. Numbers indicate the changes with respect to control as percentages. The values are measured with the formula of stress-treated/control*100

| | | CHANGES IN $H_2O_2$ (%) FROM CONTROL | | | | | |
|---|---|---|---|---|---|---|---|
| | | **SEYRAN** | **MALAZGİRT** | **KAFKAS** | **ÇAĞIL** | **ÇİFTÇİ** | **ÖZBEK** |
| **SHOOT** | **100 mM NaCl** | 157 | 165 | 124 | 106 | 214 | 143 |
| | **150 mM NaCl** | 181 | 144 | 214 | 136 | 180 | 105 |
| **ROOT** | **100 mM NaCl** | 117 | 71 | 140 | 98 | 91 | 173 |
| | **150 mM NaCl** | 56 | 44 | 177 | 84 | 84 | 130 |

F. Alterations in proline under 100 mM NaCl and 150 mM NaCl stress of all cultivars. Numbers indicate the changes with respect to control as percentages. The values are measured with the formula of stress-treated/control*100

| | | CHANGES IN PROLINE (%) FROM CONTROL | | | | | |
|---|---|---|---|---|---|---|---|
| | | **SEYRAN** | **MALAZGİRT** | **KAFKAS** | **ÇAĞIL** | **ÇİFTÇİ** | **ÖZBEK** |
| **SHOOT** | **100 mM NaCl** | 182 | 179 | 178 | 272 | 229 | 170 |
| | **150 mM NaCl** | 324 | 163 | 225 | 298 | 523 | 234 |
| **ROOT** | **100 mM NaCl** | 209 | 171 | 224 | 257 | 201 | 380 |
| | **150 mM NaCl** | 397 | 269 | 397 | 214 | 337 | 560 |