EFFECT OF DROUGHT AND SALT STRESSES ON ANTIOXIDANT DEFENSE SYSTEM AND PHYSIOLOGY OF LENTIL (*Lens culinaris* M.) SEEDLINGS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

FEBRUARY 2008

Approval of the Thesis

EFFECT OF DROUGHT AND SALT STRESSES ON ANTIOXIDANT DEFENSE SYSTEM AND PHYSIOLOGY OF LENTIL (*Lens culinaris* M.) SEEDLINGS

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ABSTRACT

EFFECT OF DROUGHT AND SALT STRESSES ON ANTIOXIDANT DEFENSE SYSTEM AND PHYSIOLOGY OF LENTIL (*Lens culinaris* M.) SEEDLINGS

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February 2008, 107 pages

In this study, 14 days old lentil seedlings (*Lens culinaris* Medik cv. Sultan), which were subjected to 7 days of drought (20% PEG 6000), and salt (150 mM NaCl) stress , were examined in a comparative manner for the effects of drought and salt stress treatments.

In shoot and root tissues physiological parameters such as wet-dry weight, relative water content, root-shoot lengths, membrane electrolyte leakage, and lipid peroxidation in terms of malondialdehyde (MDA) were determined. H_2O_2 content, proline accumulation and chlorophyll fluorescence analysis were also performed.

Changes in the activity of antioxidant enzymes such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6) ascorbate peroxidase (APX: EC 1.11.1.11) and glutathione reductase (GR: EC 1.6.4.2) were observed upon stress treatments.

In salt treated lentil seedlings, significant decreases in wet-dry weight, RWC, shoot-root length and chlorophyll fluorescence measurements indicated a sensitivity, when

compared to drought treated plants. Higher MDA concentration and higher electrolyte leakage amounts are supported these results.

APX, GR and proline seem to play important roles in antioxidant defense against salt stress for both tissues by removing reactive oxygen species and protecting macromolecules and membranes. GR and proline are also maintains the main protective mechanism against drought stress effects. SOD is active in drought stressed roots and salt stressed shoots, where the H_2O_2 contents are also observed to be increased.

Key words: Lentil, *Lens culinaris*, drought stress, salt stress, antioxidant defense

ÖZ

KURAKLIK VE TUZ STRESLERİNİN MERCİMEK (*Lens culinaris* M.) FİDELERİNİN ANTİOKSİDAN SAVUNMA SİSTEMİ VE FİZYOLOJİSİ ÜZERİNE ETKİLERİ

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Şubat 2008, 107 sayfa

Bu çalışmada 7 gün süreyle kuraklık (% 20 PEG 6000) ve tuz (150 mM NaCl) stresine maruz bırakılan 14 günlük mercimek (*Lens culinaris* Medik cv. Sultan) fidelerinde kuraklık ve tuzluluk streslerinin etkileri karşılaştırmalı olarak incelenmiştir.

Gövde ve kök dokularında kuru-yaş ağırlık, nispi su içeriği, uzunluk, hücre zarının elektrolit geçirgenliği ve lipid peroksidasyonu (malondialdehit miktarı olarak) gibi fizyolojik ölçümlerin yanı sıra, H_2O_2 miktarı ve prolin birikiminin belirlenmesi ile klorofil fluoresans analizleri gerçekleştirilmiştir.

Kuraklık ve tuz streslerine bağlı olarak, süperoksit dismutaz (SOD: EC 1.15.1.1), katalaz (CAT: EC 1.11.1.6) askorbat peroksidaz (APX: EC 1.11.1.11) ve glutatyon redüktaz (GR: EC 1.6.4.2) gibi antioksidant enzimlerinin aktivitelerindeki değişimler incelenmiştir.

Kuraklık stresindeki bitkilerle karşılaştırıldığında, tuz stresi altındaki bitkilerde kuru-yaş ağırlık, nispi su içeriği, uzunluk ve klorofil fluoresans ölçümlerindeki anlamlı

düşüşler duyarlılıklarını işaret etmektedir. Yüksek MDA miktarları ve hücre zarlarının elektrolit geçirgenliklerindeki artışlar da bu durumu desteklemektedir.

APX, GR ve prolinin tuz stresi altındaki bitkilerde hem gövde hem de kök dokularda, reaktif oksijen türlerinin bertaraf edilip moleküllerin ve membranların korunmasıyla antioksidant savunmada önemli rolleri olduğu görülmektedir. GR ve prolinin aynı zamanda kuraklığın oluşturduğu etkilere karşı da temel bir koruma mekanizması olarak görev yaptığı gözlenmiştir. H_2O_2 miktarlarında artış gözlemlenen kuraklık stresi altındaki kök dokularda ve tuz stresi altındaki gövdelerde SOD enziminin aktif olduğu görülmüştür.

Anahtar kelimeler: Mercimek, *Lens culinaris*, kuraklık stresi, tuz stresi, antioksidant savunma

To my family,

ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor Prof. Dr. Huseyin Avni Öktem for his guidance, valuable comments and endless supports. I would also like to express my gratitude to Prof. Dr. Meral Yücel for her encouragement, valuable supports and precious remarks. I am pride of being a member of their laboratory.

I would like to thank to all my thesis committee for their suggestions and criticism.

I am grateful to all of my lab mates Ufuk Çelikkol Akçay, Ebru Özgür, Hamdi Kamçı, Beray Gençsoy, Tahir Bayraç, Musa Kavas, Gülsüm Kalemtaş, Cengiz Baloğlu, Taner Tuncer, Ceyhun Kayıhan, Nilüfer Afşar, Ferhunde Aysin, Murat Kavruk, Sena Cansız, Fatma Kılbacak and Ceren Karakaş one by one for their splendid technical and moral supports, valuable comments and sincere friendships.

I appreciate Emre Aksoy, Özge Şenyurt and Tufan Öz for their critical views throughout my study and writing the thesis; endless patience and valuable friendships whenever I needed. I am also thankful to Taylan Akça for his patience, encouragements and all of precious helps throughout this study.

I would like to thank to Firdevs Yalçın from METU Central Laboratory for her helps in chlorophyll fluorescence measurements.

I owe my sincere gratitude to my family and my grandmother for their endless love, encouragement and support dedicated to me throughout my life.

This study was supported by METU ÖYP BAP- 08-11-DPT-2002K120510

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
CAT	Catalase
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
GR	Glutathion reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
KCN	Potassium cyanide
MDA	Malondialdehit
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
NaCl	Sodium chloride
OH.	Hydroxyl radical
O_2^1	Singlet oxygen
$O2^{\cdot -}$	Superoxide radical
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
ROOH	Lipid hydroperoxide
ROS	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase

CHAPTER 1

INTRODUCTION

1.1 Lentil

Lentil (*Lens culinaris* Medik.) is an annual diploid grain legume which is one of the oldest known food crops. The crop originated from Near East, including Southern Turkey, more than 8,500 years ago and then spread to the Mediterranean regions, Asia, Europe and finally the Western hemisphere (Oplinger *et al.,* 1990).

'Lens' is a Latin word for a disc-shaped object, and the word 'culinaris' reflects its culinary use and edibility which had been firstly used by Tournefort. After a long taxonomical history lentil was eventually placed in the genus *Lens Miller* which belongs to the family Leguminosae, Subfamily Papilionaceae and is in the tribe Vicieae. Since *Lens culinaris* had been validly published in 1787 by Medikus who is a German botanist-physician, it has been called *Lens culinaris* Medik. All species in the genus are diploid with 2n=14 chromosomes and have similar karyotypes (Robertson and Erksine 1997).

Lentil is a valuable component in human diet with its high protein content ranging from 20 to 30%. It also includes high amounts of folic acid and fiber. Lentil differs from the animal originated proteins with its low calorie,fat and cholesterol amounts. The seeds are also rich sources of vitamins and minerals. 100 g of dried seeds contains 68 mg Ca, 325 mg P, 7.0 mg Fe, 29 mg Na, 780 mg K, 0,46 mg thiamine, 0,33 mg riboflavin, 1,3 mg niacin (Adsule *et al.,* 1989; Muehlbauer *et al.,* 1985).

Cultivation of lentil improves soil nitrogen, carbon and organic matter status and provides sustainable crop production systems and reduces the requirement for nitrogen fertilizer (Sarker and Erksine, 2006).

It can be used to diversify and lengthen the crop rotation, which reduces disease pressure in other crops and has weed control advantages. Also to grow lentils in rotation with other crops has the advantage of fixing dinitrogen when effectively nodulated, thus reducing the demand for nitrogen fertilizers and depletion of inorganic nitrogen from soil (Muehlbauer *et al*. 1985).

Lentil plants are typically short, but with variations in environmental conditions and variety, height can range from 20 to 75 cm. They are semierect annuals in which flowers are self-pollinated so they do not require insects for pollination or seed formation. Seedpods are small, usually less than 2.5 cm, and generally contain 1 or 2 seeds. Although lentil plants somewhat drought and high temperature tolerant, they require at least moderate moisture during the growing season to produce a full seed set. They do not tolerate waterlogging, flooding and salinity. The crop performs best on level or slightly rolling land with a soil pH of 6.0 to 8.0 (Saskatchewan Pulse Growers Production Manual, 2000). A large proportion of the lentil crop is grown in semiarid regions throughout the world without irrigation. In most of these regions, agriculture depends on water conserved in the soil after fall and winter rains.

Lentils can be identified as red or green commonly. Hovewer, they are also varying in the colour of the seed coat and cotyledon. Red lentils have reddish orange coloured cotyledons, small seed (2.5-4.5 mm) and a reddish grey coloured seed coat. They are also known as Persian type lentils. Green lentils have yellow to light brown coloured cotyledons, large seed (4-8 mm) and a pale green to olive green seed coat. Green lentils are also known as brown, yellow, Chilean or Continental lentils. Internationally, small-seeded, red cotyledon lentil is dominated by Australia, Canada and Turkey, on the other hand the market in the large-seeded, green lentil is held by Canada and USA. Countries in the Indian Subcontinent, West Asia and North Africa are the major importers of red lentil. Southern Europe and South America import large-seeded green lentils.

Afghanistan, Bangladesh, India, Nepal, Pakistan, Ethiopia, Morocco, Tunisia, Sudan, Iran, Syria, Turkey, Egypt and Iraq having lentil as an important dietary component, many of these countries are also major producers. During the last two decades Australia, Canada and the USA has been growing the crop and lentil has become an important agricultural export commodity. India, Canada and Turkey are sharing the 70% of the lentil production throughout the world as being three major producing countries (Sarker & Erksine 2002, 2006).

Turkey is one of the major lentil producing countries and had produced 555,000 tones by the year 2005 (IGEME,2005). Lentil is an important component of the Turkish diet, especially in rural areas, and has been widely cultivated nationally for many centuries. It provides an excellent balance together with cereals for human nutrition and the average consumption in Turkey is 2-3 kg / yr / person (Bayaner *et al.* 1997).

In Turkey lentil is cultivated in Eastern, South-Eastern and Central Anatolian Regions. Both green lentil for summer and red type for winter are present. Red lentil production is achieved in South-Eastern region, especially in the cities; Şanlıurfa, Diyarbakır, Mardin, Gaziantep, Adıyaman while the green lentil production is going on Central Anatolia and transition regions especially in Yozgat, Çorum, Konya and Eskişehir. In Turkey while the 43% of red lentil and 59,1% of green lentil is consumed in the country, 31% and 11% is exported respectively (IGEME, 2005).

1.2 Environmental Stress

Stress can be defined in biological terms as an adverse force or a condition that inhibits the normal functioning and well being of a biological system (Jones *et al.,* 1989).

Plants have experience various environmental stress conditions and factors during their life cycle. These factors can be classified according to their origins as biotic and

abiotic. The term “biotic” includes the living factors like microorganisms, insects or rodents and the drifts in the environmental parameters like temperature, water, radiation or chemicals cause the formation of abiotic stress factors (Gürel and Avcıoğlu, 2001). These stress factors are shown below in Fig.1.1.

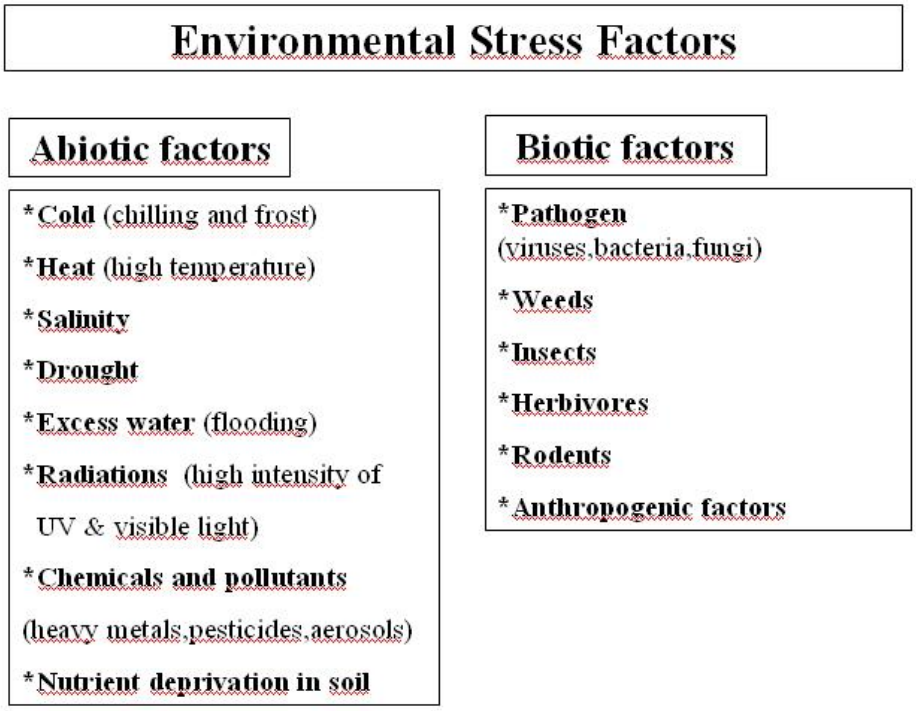

Figure 1.1. Environmental stress factors including abiotic and biotic origins

As plants are sessile, they endure adverse environmental conditions and consequently evolve a variety of responses to cope with environmental stresses. During evolution, plants have developed mechanisms to sense the changes of growth conditions, and trigger signal transduction cascades, which in turn activate stres responsive genes and ultimately lead to changes at the physiological and biochemical levels (Gao *et al*., 2007).

World population is increasing day by day and expected to reach about six billion by the end of year 2050. On the other hand due to the effect of various abiotic stresses, food productivity is decreasing. Abiotic stresses cause losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure. In fact these stresses, threaten the sustainability of agricultural industry (Mahajan and Tuteja, 2005).

In the limitations of world crop production drought stress has the highest percentage with 26% when the usable areas on the earth are classified in view of stress factors. Mineral and cold stress follows drought with the percentages of 20 and 15, respectively. On the whole other stresses get 29% whereas only 10% area is not exposed to any stress factors (Blum, 1986).

Plant growth, development and productivity is greatly affected by drought and soil salinity, thus posing a serious threat to agriculture in many parts of the world. It is estimated that up to 50% of agricultural yield is lost due to environmental stresses such as soil salinity and dehydration, compared to about 10 to 20% crop loss caused by pathogens (Kreps *et al*., 2002).

Several reactions in plants are triggered by exposure to drought or salt stress. Both stresses lead to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm into the extracellular space resulting in a reduction of the cytosolic and vacuolar volumes. Except for the ionic component, early responses to water and salt stress are largely identical and the similarities include metabolic processes such as decrease of photosynthesis or hormonal processes like rising levels of the plant hormone ABA. High intracellular concentrations of sodium and chloride ions are an additional problem of salinity stress. Adaptation to salinity and drought involves numerous changes including attenuated growth, increased expression or induction of genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways (Bartels and Sunkar, 2005). Another common feature of

different stress factors is their potential to increase the production of reactive oxygen species in plant tissues which negatively affects cellular structures and metabolism.

1.2.1 Drought Stress

Plant survival productivity is greatly affected by drought, accompanying with high temperature and radiation on a global basis. Recent changes in global climate make this conditions more threatable for the survival of agricultural productivity which is connected with sociological and economical issues. According to the definition of FAO Land and Water Development Division, agricultural drought occurs when water supply is insufficient for soil and plant development. Drought is also a meteorological term that means a lack of precipitation over a prolonged period of time.

Drought is also related with several concepts like salt, cold and high temperature stresses, pathological reactions, senescence, growth, development, cell circle, UV-B damage, wounding, embryogenesis, flowering and signal transduction (HongBo *et al.,* 2005).

1.2.2 Physiological and Biochemical Effects of Drought on Plants

In water deficit conditions, normal bilayer structure of the membrane is disrupted with the removal of water and it results in the membrane becoming substantially porous when desiccated. Loss of membrane integrity, selectivity, disruption of cellular compartmentalization and a loss of activity of membrane-based enzymes are the results of displacement of membrane proteins which is primarily caused by the stress within the lipid bilayer. When dehydration occurs, reduced activity or complete denaturation in cytosolic and organelle proteins may be also exhibited in addition to membrane damage. Cellular metabolism can be disrupted with the high concentration of cellular electrolytes due to the dehydration of protoplasm. Metabolical decompositions may also cause the degradation of nucleic acids which is

also a result of oxidative stress occured by ROS which will be described further in details.

Drought has profound effects on growth, yield, and plant quality. The loss of turgor is the first effect of drought stress which affects the rate of cell expansion and ultimate cell size. The result is a decrease of growth rate, stem elongation, leaf expansion and stomatal aperture. Reduced cell expansion as a primary response to water deficit serves to reduce plant water use. If the reduction in total plant water use is not sufficient to sustain turgor, then transpiration is further reduced by stomatal closure. Leaf growth is generally more sensitive than the root growth. As less leaf area means less transpiration, reduced leaf expansion is beneficial to plants under water deficit conditions. Abscission of the older leaves is another strategy against drought which is known as leaf area adjustment. On the other hand, the relative root growth may undergo enhancement in drought conditions to extract more water from deeper soil layers. Growth arrest can be considered as a possibility to preserve carbohydrates for sustained metabolism, prolonged energy supply, and for better recovery after stress relief. Nutrient uptake by the roots and its transportation to the other organs are also restricted by drought stress with restricted transpiration rates and impaired membrane permeability. Leaf scorch, undersized leaves, premature or abnormal fruit, leaf dropping, thinning of canopy and wilting are the visual symptoms of drought (Mahajan and Tuteja, 2005, Bartels and Sunkar, 2005).

Photosynthesis is an essential process for the growth and survival of virtually all plants on earth in which the plants combine water and CO_2 into oxygen and energy in the presence of light. The first response of virtually all the plants to acute water deficit is the closure of their stomata to prevent the transpirational water loss. Despite the stomatal closure, radiant energy continues to be intercepted and absorbed by leaves. Reducing substances are produced that ordinarily would be used in reducing the carbon of CO_2 but with stomates closed, CO_2 entrance into the leaf is limited and decline in the rate of photosynthesis is observed. With limited CO_2, the reducing compounds must either be stored or used. The subsequent use of carbohydrate

reserves results in a deficiency of carbon compounds for both growth and maintenance processes.

Stomatal closure can be initiated by two ways referred as hydropassive and hydroactive. Direct evaporation of water from the guard cells with no metabolic involvement causes hydropassive closure. Hydroactive stomatal closure may also be metabolically dependent and involve processes that result in reversal of the ion fluxes that cause stomatal opening and it is ABA regulated (Hale, 1998; Mahajan and Tuteja, 2005; Bartels and Sunkar, 2005). The pH of xylem sap increases and promotes the loading of ABA into the root xylem and its transport to the shoot, under the water deficit conditions (Hartung *et al*., 2002). ABA, synthesized in roots and transported to the guard cells via the transpiration stream causes the stomatal closure under drought stress conditions by binding to the ABA receptors on guard cells (Taiz and Zeiger, 2002). Efflux of K^+ ions from the guard cells is promoted by ABA, which results in the loss of turgor pressure leading to stomatal closure which responds directly to the soil desiccation even before there is any significant reduction in leaf mesophyll turgor pressure (Mahajan and Tuteja, 2005).

Over-reduction of components within the electron transport chain occurs by the decline in intracellular CO_2 levels and the electrons get transferred to oxygen at photosystem I (PS I). By these process, reactive oxygen species (ROS); including superoxide, hydrogen peroxide and hydroxyl radicals are produced which should be scavenged by the plant as they may lead to oxidative stress (Mahajan and Tuteja,2005).

1.2.3 Salt Stress

Salinity is the accumulation of excessive amounts of dissolved salts in the soil which inhibits plant growth and productivity occuring by natural or anthropogenic processes. Naturally, plants are exposed to high salinity near coastal regions and deltas. In more inner regions the salt leakages from geological sea accumulates

makes the area unsuitable for agriculture. Weathering of rocks also releases soluble salts of various types in which sodium chloride (NaCl) is the most common (Taiz and Zeiger, 2002). Salinity in a given land area depends upon various factors like amount of evaporation or the amount of precipitation. Inland deserts are marked by high salinity as the rate of evaporation far exceeds the rate of precipitation.

Drier areas in particular need intense irrigation, hovewer agricultural lands that have been heavily irrigated are highly saline because of the extensive water loss through a combination of both evaporation as well as transpiration. This process is known as evapotranspiration and as a result, the salt delivered along with the irrigation water gets concentrated, year-by-year in the soil. If not removed by dreinage systems it will damage crops. As most of the economically important crop species are very sensitive to soil salinity, it leads to huge losses in terms of arable land and productivity (Mahajan and Tuteja, 2005). Grain legumes are generally considered as salt sensitive (Katerji *et al*., 2001).

Anthropogenic cycles of salinization or sodication are caused by irrigation mismanagement, poor land levelling, dry season fallow practices in the presence of shallow watertable, misuse of heavy machinery and soil compaction, excessive leaching with insufficient drainage, and use of improper cropping patterns and rotations. Major salt-affected soils in Turkey are located as follows: Konya-Ereğli, Aksaray Plains of the Central Anatolia and alluvial plains of lower Seyhan, Iğdır, Menemen, Bafra, Söke, Acıpayam and Salihli. The last soil survey indicated that 1.5 million ha of land have some degree of salinity and sodicity problems and 2.8 ha of land have both salinity and waterlogging problems. Climatic, geochemical and hydrological conditions of Turkey often promote salt accumulation in the groundwater and the soil profile, particularly in arid and semi-arid lowlands where groundwater contains considerable amounts of soluble salts. In general, poor water management and agronomic practices without sufficient drainage systems are the major causes of salinity. Over 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or the associated condition of

sodicity (434 million ha) (FAO, Land and Plant Nutrition Management Service, 2007) .

Increased salinization of arable land is expected to have destroying global effects, resulting in 30% land loss within next 25 years and up to 50% by the middle of 21st century (Wang *et al.,* 2003).

Disruption of intracellular ion homeostasis, membrane dysfunction and inhibition of metabolic activity, resulting in inhibition of growth and yield reduction are the results of high concentrations of Na^+ in the soil (Hasegawa *et al.* 2000). Salt effects are the combined result of the complex interaction among different morphological, physiological, and biochemical processes. Salinity imposes two stresses on plant tissues, one of them being water-deficit stress that results from the relatively high solute concentrations in the soil, and the other is ion-specific stress resulting from altered K^+/Na^+ ratios and Na^+ and Cl^- concentrations (Apse and Blumwald, 2002).

1.2.4 Physiological and Biochemical Effects of Salt Stress on Plants

Reduction in the rate of leaf surface expansion, considerable decrease in the fresh and dry weights of leaves, stems, and roots, formation of necrotic leaves and wilting are the some common symptoms of salinity on plants. Leading to stoppage of expansion as salt concentration increases is the immediate response of salt stress.

Nutrient imbalance and/or ion toxicity is one of the deleterious effects of salt stres conditions. High salt (NaCl) uptake competes with the uptake of other nutrient ions, especially K^+, leading to K^+ deficiency. Increased treatment of NaCl induces increase in Na^+ and Cl^- and decrease in Ca^{2+}, K^+, and Mg^{2+} levels in a number of plants while they are necessary for metabolic activities (Khan *et al.,* 1999, 2000; Khan, 2001). K^+ is an important element to maintain the osmotic balance and also has a role in opening and closing of stomata. It is an essential co-factor for many enzymes like the

pyruvate kinase, whereas Na^+ is not. Ion transporters are responsible in salt tolerance, by mediating the osmotic homeostasis after salt stress. It is important to prevent Na^+ accumulation to a high level in the cytoplasm or in organelles other than the vacuole, because of its ability to inhibit many enzymes (Mahajan and Tuteja, 2005). Ca^{2+} has role in providing salt tolerance to plant, initiates the stress signal transduction which leads to the salt adaptation. Externally supplied Ca^{2+} reduces the toxic effects of NaCl, presumably by facilitating higher K^+/Na^+ selectivity (Cramer *et al.,* 1987; Liu *et al.,* 1998).

Environmental stresses affecting growth also affect photosynthesis because plant growth as biomass production is a measure of net photosynthesis (Parida and Das, 2005). Dehydration of cell membranes which reduce their permeability to CO_2, reduction of CO_2 supply because of hydroactive closure of stomata, salt toxicity, enhanced senescence induced by salinity, changes of enzyme activity induced by changes in cytoplasmic structure decrease photosynthetic rate (Iyengar and Reddy, 1996). Under salt stress the chlorophyll and carotenoid contents of leaves decrease. The oldest leaves start to develop chlorosis and fall with prolonged period of salt stress. It was observed with the electron microscopy that the thylakoidal structure of the chloroplasts becomes disorganized and starch content decreases in plants treated with NaCl (Hernandez *et al.,* 1999; Gadallah, 1999; Agastian *et al*., 2000).

Salt stress causes water deficit as a result of osmotic effects on a wide variety of metabolic activities of plants and this water deficit results in oxidative stress because of the formation of ROS which include singlet oxygen, superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide as it will be discussed in following part (Parida and Das, 2005).

1.3 Reactive Oxygen Species

Production of reactive oxygen species (ROS) in chloroplasts, mitochondria, and peroxisomes is an unavoidable result of the evolution of aerobic metabolic processes such as photosynthesis and respiration (Apel and Hirt, 2004).

Oxidative stress caused by ROS is a regulated process, the equilibrium between the oxidative and antioxidative capacities determining the fate of the plant. The antioxidant defence system provides efficient protection against ROS under nonstressful conditions. Several stress situations enhance the production of toxic oxygen derivatives (Arora *et al.,* 2002).

1.3.1 Generation of ROS

Oxygen in the air we breathe is in its ground, not energetically excited state and is symbolized as $^3O_2$. It is a free radical, as it has two unpaired electrons. Molecules whose outermost pair of electrons have parallel spins are in the "triplet" state; molecules whose outermost pair of electrons have antiparallel spins are in the "singlet" state. Ground-state oxygen is in the triplet state, its two unpaired electrons have parallel spins, a characteristic that, according to rules of physical chemistry, does not allow them to react with most molecules. Thus, ground-state or triplet oxygen is not very reactive. However, triplet oxygen can be activated by the addition of energy, and transformed into reactive oxygen species (Fig.1.2.) (Christophersen *et al*., 1991).

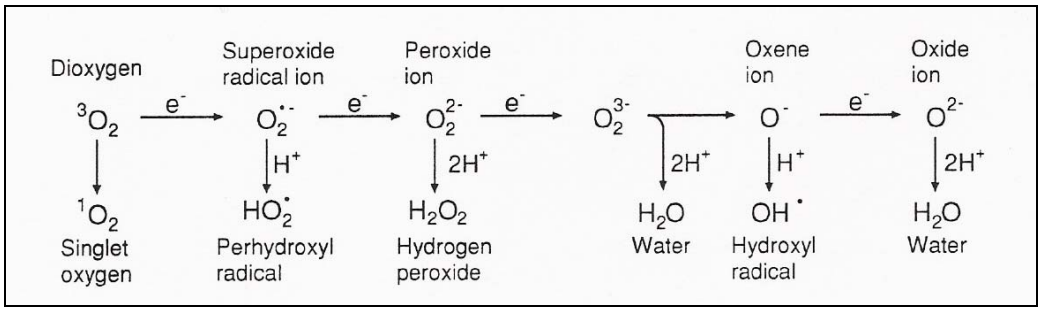

Figure 1.2. Oxidation level of reactive oxygen species

ROS types include free radicals as well as non-radicals. The high reactivity of ROS comes from the specificity of the electronic configuration. Thus, free radicals, containing unpaired electrons in the outer orbitals, easily form pairs with another electrons having antiparallel spin. A chain of free radical reactions are produced by the interactions with non radical compounds and transforming them into radicals (Edreva, 2005).

During environmental stress conditions the enhanced production of ROS can pose a threat to cells however ROS also act as important signals for the activation of stress-response and defense pathways. Stress-response signal transduction pathways include ROS as cellular indicators of stress and secondary messengers (Mittler, 2002).

Chloroplasts maintains ROS generation in plants as being one of the major sites and harbor ROS-producing centers (triplet chlorophyll, ETC in PSI and PSII) and a diversified ROS-scavenging network (antioxidants; SOD, APX–glutathione cycle, and a thioredoxin system) (Asada,2006; Edreva, 2005).

During photorespiration and fatty acid oxidation, peroxisomes and glyoxysomes are other major sites of ROS generation, respectively. Also, some cellular processes

including stress adaptation and programmed cell death are regulated by mitochondrial ROS (Rio *et al*., 2006, Robson and Vanlerberghe, 2002).

NADPH oxidases, amine oxidases and cell-wall-bound peroxidases are also the sources of ROS. They are tightly regulated and participate in the production of ROS during processes such as programmed cell death and pathogen defense (Dat *et al*., 2000; Grant and Loake, 2000).

1.3.2 Types of ROS

Singlet oxygen

If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it forms the singlet state. (Fig. 1.3) Singlet oxygen, $^1O_2$, has a pair of electrons with opposite spins. It is not a free radical but it is highly reactive (Christophersen *et al*., 1991).

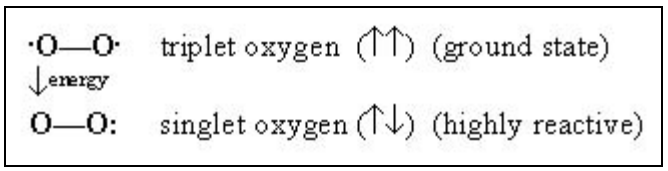

Figure 1.3. Formation of singlet oxygen

Absorption of light energy by chlorophyll molecule causes the formation of active triplet chlorophyll molecule (3 Chl*). The absorbed energy is transformed to the molecular oxygen by the activated chlorophyll molecule and the singlet oxygen production occurs. Sharing the role with tocopherols carotenoids can quench $^1O_2$

directly or prevent $^1O_2$ formation by quenching the excited triplet state chlorophyll (Asada, 2006). When $^1O_2$ is not deactivated, it can damage other molecules by transferring its energy. Nucleic acids and proteins can be damaged, also peroxidation of polyunsaturated fatty acids occurs which will then effect the membrane integrity.

Superoxide

Superoxide, $O_2^{\cdot -}$, is produced by photosynthetic electron transfer chains under conditions leading to overenergization of the electron transfer chains (Dat *et al*., 2000). Electron leakage from Fe-S centers of photosystem I or reduced ferredoxin to O_2 (Mehler reaction) are the main sources of $O_2^{\cdot -}$ formation (Gechev *et al.,*2006). Another process leading to superoxide formation is mitochondrial respiration (Moller, 2001).

Plasmalemma bound NADPH oxidases and cell wall associated peroxidases are the main $O_2^{\cdot -}$ producing enzymes (Sagi *et al*., 2006). $O_2^{\cdot -}$ is produced by xanthine oxidase during purine catabolism, also by ribonucleotide reductase during deoxyribonucleotide synthesis, and various other oxidases induced by biotic and abiotic stresses (Dat *et al.,* 2000).

$O_2^{\cdot -}$ is a free radical because it has an extra unpaired electron in the outer orbital. The biological toxicity of $O_2^{\cdot -}$ is due to its capacity to inactivate Fe-S cluster containing enzymes. They are critical in various metabolic pathways, thereby liberating free iron in the cell, which can undergo Fenton and Haber-Weiss reactions and causes the generation of the highly reactive hydroxyl radicals (Holleman and Wiberg, 2001).

Hydrogen peroxide

Within the plant cells during photosynthesis and photorespiration, and particularly in respiration processes hydrogen peroxide (H_2O_2) is produced. The superoxide radical

is either converted back to O_2, or in a reaction with a proton, to H_2O_2, either spontaneously or in a reaction catalysed by the enzyme superoxide dismutase. H_2O_2 belongs to non-radical ROS in contrast to the superoxide and carries no net charge. It is a long-distance signalling molecule in various physiological processes because of the longer half-life and non-radical formation. Basic acclimatory, defence and developmental processes in plants are also regulated by H_2O_2 (Slesak *et al*., 2007; Halliwell, 2006; Vranová *et al*., 2002).

Following the oxidation of respective substrates glycolate oxidases, glucose oxidases, amino-acid oxidases, and sulfite oxidases release H_2O_2 (Asada,1999). Cell wall-bound peroxidases, oxalate, amine and plasma membrane NADPH oxidases have been also identified as other enzymatic sources of $O_2^{\cdot-}$ and H_2O_2 (Bolwell *et al*., 2002; Svedruzic *et al*., 2005).

The H_2O_2 production in chloroplasts is catalyzed by superoxide dismutase forms containing in the active site copper/zinc (Cu/Zn-SOD) or iron (Fe-SOD). H_2O_2 can be generated in the peroxisome via β-oxidation of fatty acids and oxidation of other substrates (Dat *et al*., 2000). In plant mitochondria superoxide anion radical production results in the formation of H_2O_2, primarily through the action of a mitochondrion-specific manganese SOD (Mn-SOD) (Rhoads *et al*., 2006).

As H_2O_2 degraded by catalase without any reducing power, this enzyme provides plants with an energy-efficient way to remove H_2O_2. However, catalase is active only at relatively high H_2O_2 concentrations. APX and other peroxidases eliminate the lower H_2O_2 levels with the aid of various reductants like ascorbate and glutathione (Gechev *et al*., 2006).

The locally present antioxidants scavenge ROS at the sites of their production. However, when this local antioxidant capacity cannot cope with ROS production, maybe during stress or temporarily reduced antioxidant levels due to developmental signals, H_2O_2 can leak into the cytosol and diffuse to other compartments. Plants can

also cope with excess H_2O_2 by transporting it into vacuoles for detoxification, which are very rich in flavonoids, powerful antioxidants that can scavenge various ROS (Bienert *et al.,* 2006, Gould *et al.,* 2002, Tsuda *et al*., 2000).

Hydroxyl radical

Hydrogen peroxide and superoxide radicals are relatively less damaging than the species that they can form such as hydroxyl radicals ($OH^{\cdot}$) which are damaging the essential cellular components. They can initiate lipid peroxidation and also attack DNA, proteins and many small molecules.

Fenton, in the late nineteenth century described the oxidizing potential of hydrogen peroxide with ferrous salts. Forty years later, Haber and Weiss identified the destructive hydroxyl radical formation, which will then initiate the oxidation of organic substrates, in the presence of trace amount of iron with the reaction of superoxide and hydrogen peroxide (Arora *et al*., 2002).

$$Fe^{2+} + H_2O_2 \ldots\ldots..> Fe^{3+} + OH^{\cdot} + OH^{-} \quad \text{(Fenton Reaction)} \qquad (1.1)$$

$$H_2O_2 + O_2^{\cdot -} \ldots\ldots\ldots> OH^{\cdot} + OH^{-} + O_2 \quad \text{(Haber-Weiss Reaction)} \qquad (1.2)$$

1.3.3 ROS Dependent Damage

ROS can damage membrane lipids with peroxidation reactions and also degrade proteins and nucleic acids, thus the reactions can be lethal. Lipid peroxidation is the oxidative damage of membranes by free oxygen radicals. Several lipid molecules containing double bonds can be peroxidized under appropriate conditions. There are two common sites of oxygen free radical attack on the phospholipid molecule; the unsaturated double bonds of the fatty acid and the ester linkage between glycerol and the fatty acid. Hydroxyl radicals attack on the double bonds and initiate the peroxidation reaction by abstracting a single H atom. This creates a carbon radical product that is capable of reacting with the ground state oxygen in a chain reaction. The resulting molecule is ready to react with another phospholipid, and the reaction propogates. (Fig.1.4.) The basis for the hydroxyl radical's extreme reactivity in lipid systems is that at very low concentrations it initiates a chain reaction involving triplet oxygen, the most abundant form of oxygen in the cell (McKersie, 1996).

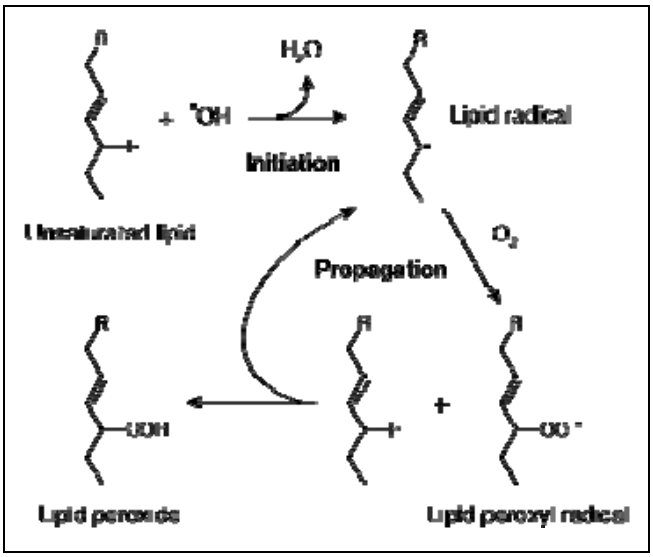

Figure 1.4. Mechanism of lipid peroxidation

A lipid hydroperoxide (ROOH) is unstable in the presence of Fe or other metal catalyst because ROOH will participate in a Fenton reaction leading to formation of reactive alkoxyl radicals. Among the degradation products of ROOH are aldehydes, such as malondialdehyde (MDA), and hydrocarbons, such as ethane and ethylene, that are commonly measured end products of lipid peroxidation. Measuring the end products lipid peroxidation such as MDA, a good marker for stress injury, is one of the most widely accepted assays for oxidative damage (McKersie, 1996).

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. Sulphur containing amino acids, and thiol groups specifically, are very susceptible sites. Altough the oxidation of thiol groups can be reversed by various enzymes, some forms of free radical attack on proteins are not reversible. For example, the oxidation of iron-sulphur centres by superoxide destroys enzymatic function (Gardner and Fridovich, 1991). The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. In these cases, the metal binds to a divalent cation binding site on the protein. The metal then reacts with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical that rapidly oxidises an amino acid residue at or near the cation binding site of the protein (Stadtman, 1986).

DNA is an obvious weak link in a cell's ability to tolerate oxygen free radical attack. First, it seems that DNA is effective in binding metals that are involved in Fenton reactions, and secondly less damage can be tolerated in DNA than other macromolecules. Activated oxygen and agents that generate oxygen free radicals, such as ionising radiation, induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects. Characterisation of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986; Beyer *et al*., 1991).

1.4 Drought and Salt Tolerance in Plants

Drought and salt stress triggers many common reactions in plants. Both stresses lead to cellular dehydration, which causes osmotic stress. Removal of water from the cytoplasm into the extracellular space results in reduction of the cytosolic and vacuolar volumes. High intracellular concentrations of sodium and chloride ions are an additional problem of salinity stress. Reactive oxygen species which affects cellular structures and metabolism negatively, are produced during stress conditions. Early responses to water and salt stress are largely identical except for the ionic component. These similarities include metabolic processes such as a decrease of photosynthesis or hormonal processes like rising levels of the plant hormone ABA, production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes (Bartels and Sunkar, 2005).

The first step in the regulation of stress response and tolerance pathways is the recognition of the stress. The initial stress signals like osmotic and ionic effects, or membrane fluidity changes, initiate the downstream signaling process and transcription controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes (Wang *et al.,* 2003).

After sensing and perception of the stress, signalling mechanisms are actively transducing the signals to induce the specific genes. Products of the genes induced during stress conditions are classified into two groups. The first group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response: protein kinases, transcription factors , phospholipase C and phosphatases. In addition, the phytohormone absisic acid (ABA) also plays important roles in stress response mechanisms. The second group includes proteins that probably function in stress tolerance: water channel proteins, the enzymes required for the biosynthesis of various osmoprotectants (sugars, proline, glycine-betaine), proteins that may protect macromolecules and

membranes (late embryogenesis abundant (LEA) proteins, chaperons), proteases, Na^+/H^+ transporters and detoxification enzymes. (Shinozaki and Yamaguchi-Shinozaki, 1997)

1.4.1 Group I : Stress Response

Protein kinases

One of the major mechanisms for controlling cellular functions in response to external signals is protein phosphorylation. The mitogen-activated protein kinase (MAPK) cascades are common signalling modules in eukaryotic cells including plants. MAPK pathways can mediate signalling of an extracellular stimulus and bring about specific responses (Bartels and Sunkar, 2005).

The MAPK cascade consists of three functionally interlinked protein kinases: MAPKKK, MAPKK, and MAPK. In this phosphorylation module, a MAPKKK is phosphorylated directly downstream of the stimulus. The activated MAPKKK then phosphorylates and activates a particular MAPKK, which in turn phosphorylates and activates a MAPK. Activated MAPK is imported into the nucleus, where it phosphorylates and activates specific downstream signalling components, such as transcription factors or serine/threonine residues on the target protein substrate to induce cellular responses (Agrawal *et al.,* 2003; Xiong and Yang, 2003). These kinases are suggested to be widely responsible for osmolarity signalling molecules. Their transcript levels increase in response to osmotic stres and results in the accumulation of osmolytes. Osmolytes help reestablishing the osmotic balance, protection from stress damage or repair mechanisms. The diverse and multiple stress responses of MAPKs suggest that there is a fundamental difference in functional specificity of MAPKs with respect to drought/salt response. Understanding of the MAPK cascade can provide insight to understanding and solving the problem of drought and salt stress in agricultural crops (Kaur and Gupta, 2005; Mikolajczyk *et al.,* 2000; Mahajan and Tuteja, 2005).

One early response to drought and salinity stress in plant cells is a transient increase in cytosolic Ca^{2+}, derived from either influx from the apoplastic space or release from internal stores (Knight, 2000; Sanders *et al.,* 1999). Transient increases in cytosolic Ca^{2+} are perceived by various Ca^{2+} binding proteins. In the case of abiotic stress signaling, evidence suggests that Ca^{2+} dependent protein kinases (CDPKs) and the SOS3 family of Ca^{2+} sensors are major players in coupling this universal inorganic signal to specific protein phosphorylation cascades (Xiong *et al.,* 2002). The CDPKs have roles in the activation of transcription factors which induce gene expression of LEA-like proteins. Another group of calcium sensors in plants is the SOS3 family of calcium-binding proteins. Their role has been implicated in ion homeostasis (Kaur and Gupta, 2005; Bartels and Sunkar, 2005).

Transcription factors

Stress responses primarily include transcriptional regulation of gene expression by transcription factors (TFs). This process relies on interactions between sequence specific DNA-binding protein with cis-elements located in the promoter and enhancer regions of the corresponding genes (Bartels and Sunkar, 2005).

Multiple signaling pathways regulate plant stress responses are activating gene transcription and its downstream machinery. Plant genomes contain a large number of transcription factors; Most of these TFs belong to a few large multigene families, e.g. MYB, AP2/EREBP, bZIP (Wang *et al.,* 2003). The dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) bind to DRE and CRT cis-acting elements. Members of the CBF/DREB1 family are themselves stress-inducible, such as CBF1, CBF2, and CBF3 (or DREB1B, DREB1C, and DREB1A, respectively). They are encoded by AP2/EREBP multigene families. These transcription factor genes are induced early and transiently by cold stress, and activate the expression of target genes. Similar transcription factors DREB2A and DREB2B are activated by osmotic stress and responsible for osmotic stress induction of target stress-responsive genes (Stockinger *et al.* 1997; Gilmour *et al.* 1998; Liu *et al.* 1998; Seki *et al*. 2001; Thomashow *et al.* 2001).

As evidenced by the fact that many of the drought-inducible genes studied are also induced by ABA, its signaling plays a vital role in plant stress responses. Two TF families bZIP and MYB, are involved in ABA signaling and its gene activation (Wang *et al.,* 2003).

Several basic leucine zipper (bZIP) transcription factors (named ABF/AREB) that can bind to ABRE and activate the expression of ABRE driven reporter genes also have been isolated (Choi *et al.,* 2000; Uno *et al.,* 2000). On the other hand, Abe *et al*. (2003) showed that the Arabidopsis MYB transcription factor proteins AtMYC2 and AtMYB2 function as transcriptional activators in ABA-inducible gene expression, suggesting a novel regulatory system for gene expression in response to ABA, other than the ABRE-bZIP regulatory system.

Phospholipase C

As the selective barrier between living cells and their environments, the plasma membrane plays a key role in the perception and transmission of external information. Upon osmotic stress, changes in phospholipid composition are detected (Munnik *et al.,* 1998). Membrane phospholipids constitute a dynamic system that generates a multitude of signaling molecules like inositol 1,4,5-triphosphate (IP3), diacylglycerol (DAG), phosphatidic acid (PA), etc. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into IP3 and DAG which act as second messengers, is catalyzed by phospholipase C, (PLC). IP3 releases Ca^{2+} from internal stores (Zhu, 2002; DeWald, 2001; Takahashi, 2001).

Phosphatases

Phosphatases provide modulation and reversibility of the phosphoregulatory mechanism. They counteract the action of the protein kinases. It is also suggested that protein tyrosine dephosphorylation is involved mostly downstream of the Ca^{2+} signalling which is responsible for stomatal closure (MacRobbie, 2002; Bartels and Sunkar, 2005).

Absisic acid and abiotic stress signalling

The phytohormone abscisic acid (ABA) plays a central role in response to various stress factors, as well as seed development, dormancy, germination and stomatal closure. The main function of ABA seems to be the regulation of plant water balance and osmotic stress tolerance (Mahajan and Tuteja, 2005). Studies suggest that osmotic stress imposed by high salt or drought is transmitted through at least two pathways; one is ABA-dependent and the other ABA-independent. Cold exerts its effects on gene expression largely through an ABA-independent pathway (Finkelstein et al., 2002). ABA-inducible genes have the ABA-responsive element (ABRE) (C/T)ACGTGGC in their promoters. Basic leucine zipper factors (bZIP) function in signal transduction by binding to the ABRE element in stress-inducible genes (Kaur and Gupta, 2005). Several ABA deficient mutants namely *aba1*, *aba2* and *aba3* have been reported for *Arabidopsis* (Koornneef, 1998). Without any stress treatment the growth of these mutants is comparable to wild type plants. Under drought stress, ABA deficient mutants readily wilt and die if the stress persists. Under salt stress also ABA deficient mutants show poor growth (Xiong et al., 2001).

1.4.2 Group II Proteins: Stress Tolerance

Water channel proteins

Drought and salt stress require changes in water flow for the adaptation of cells and tissues to the stress situation. Water potential gradient acts as the driving force for transport and the water permeability of the membrane. Aquaporins are central components in plant water relations. In plants, they are localized in the tonoplast and plasma membrane (Bartels and Sunkar, 2005).

Enzymes for osmoprotectants and accumulation of solutes

In response to several environmental stress factors, certain metabolic processes, which increase the net solute concentration in the cell, thereby helping the movement of water into the cell are triggered. Plants tend to cope with water deficit stress by this process, known as osmotic adjustment. Large numbers of compounds, which play a key role in maintaining the osmotic equilibrium and in the protection of membranes as well as macromolecules are synthesized. They are also responsible for scavenging reactive oxygen species. These compounds include proline, glutamate, glycine-betaine, carnitine, mannitol, sorbitol, fructans, polyols, trehalose, sucrose, oligosaccharides and inorganic ions like K+ (Mahajan and Tuteja, 2005). Inside the cells, these organic compounds exist in stable form and are not easily metabolized, neither do they have any effect on cell functions, even when they have accumulated in high concentrations (Iba, 2002).

Compatible solutes fall into three major groups: amino acids (e.g. proline), quaternary amines (e.g. glycine betaine, dimethylsulfoniopropionate) and polyol/sugars (e.g. mannitol, trehalose). Overexpression of compatible solutes in transgenic plants can result in improved stress tolerance (Wang *et al.,* 2003).

Several physiological studies suggested that under stress conditions nonstructural carbohydrates (sucrose, hexoses, trehalose and sugar alcohols) accumulate in different plant species. It is suggested that, sugars either act as osmotica and/or protect specific macromolecules and contribute to the stabilization of membrane structures (Bartels and Sunkar, 2005). The hydroxyl group of sugar alcohols substitutes the OH group of water to maintain the hydrophilic interactions with the membrane lipids and proteins. Thus, these molecules, like mannitol and sorbitol, help to maintain the structural integrity of the membranes (Mahajan and Tuteja, 2005).

Glycine betaine (*N*,*N*,*N*-trimethylglycine-betaine) is a major osmolyte and thought to protect the plant by maintaning the water balance between the plant cell and the

environment by stabilizing macromolecules. It is synthesized by many plants in response to abiotic stresses (Chen and Murata, 2002; Rontein *et al.*, 2002; Bartels and Sunkar, 2005).

Proline is the most common osmolyte and has several roles in response to various environmental stresses. It is responsible for osmotic adjustment, protection of plasma membrane integrity and free radical scavenenge. Proline is synthesized from glutamate via glutamic-γ-semialdehyde (GSA) and D1-pyrroline-5-carboxylate (P5C). P5C synthase (P5CS) catalyzes the conversion of glutamate to P5C, followed by P5C reductase (P5CR), which reduces P5C to proline. In the reverse reaction, proline is metabolized to glutamate in a feed-back manner, via P5C and GSA with the aid of proline dehydrogenase (PDH) followed by P5C dehydrogenase (P5CDH). Overexpressing P5CS genes, removing feedback inhibition on P5CS genes, and reducing PDH level via antisense technology are the transgenic approaches which are the direct evidences for the role of proline during osmotic stress. All approaches resulted in elevated proline pools and improved osmotic stress tolerance (Wang *et al.,* 2003).

Proteins that may protect macromolecules and membranes

Heat-shock proteins (Hsps) and late embryogenesis abundant (LEA)-type proteins are two major types of stress-induced proteins that accumulate upon water, salinity, and extreme temperature stress (Wang *et al.,* 2003). The heat shock protein family encompasses many chaperones, which have an important role in the folding and assembly of proteins during synthesis, and in the removal and disposal of nonfunctional and degraded proteins. They prevent the aggregation of denaturated proteins caused by drought, heat and other stresses. They also maintain the renaturation of aggregated proteins. LEA proteins are protective molecules, which enable the cells to survive protoplasmic water depletion. It is also suggested that they act as water-binding molecules, in ion sequestration and in macromolecule and membrane stabilization (Ingram and Bartels, 1996; Iba, 2002; Bartels and Sunkar, 2005).

Proteases

Proteolysis is an important cellular activity to maintain protein homeostasis. Proteases destroy previously synthesized proteins and enzymes to maintain protein turnover. Increased proteolysis in response to stress is frequently observed and can be interpreted as a way to get rid of damaged proteins or to mobilize nitrogen (Shinozaki and Yamaguchi-Shinozaki, 1997).

Na^+/H^+ transporters

In salt tolerance ion transporters play important roles, mediating the osmotic homeostasis after salt stress. It is important to prevent Na^+ accumulating to a high level in the cytoplasm or in organelles other than the vacuole, since it inhibits many enzymes. Na^+ toxicity is not only due to toxic effects of Na^+ in the cytosol, but also because K^+ homeostasis is disrupted possibly due to the ability of Na^+ competing for K^+ binding sites.

In principle, three mechanisms exist to prevent excess Na^+ accumulation in the plant cells;

1. Restricting the Na^+ permeation and entry into plants by Na^+ transporters
2. Compartmentalizing the Na^+ in the vacuole.
3. Extruding Na^+; cytosolic Na^+ can be transported back to the external medium or the apoplast *via* plasma membrane Na^+/H^+ antiporter activity (Bartels and Sunkar, 2005; Zhu, 2001).

DuPont (1992) listed the membrane proteins involved in cation selectivity and redistribution of Na^+ and K^+. These proteins are: (1) primary H^+-ATPases which generate the H^+ electrochemical gradient that drives ion transport, (2) Na^+/H^+ antiports in the plasma membrane for pumping excess Na^+ out of the cell, (3) Na^+/H^+ antiports in the tonoplast for extruding Na^+ into the vacuole and (4) cation channels with high selectivity for K^+ over Na^+. Removal of sodium from the cytoplasm or compartmentalization in the vacuoles is done by a salt-inducible Na^+/H^+ antiporter.

The energy for these reactions is provided by H^+-ATPases that serve as primary pumps. Three distinct proton pumps are responsible for the generation of the proton electrochemical gradients: (i) the plasma membrane H-ATPase pump (PM H-ATPase) which extrudes H^+ from the cell and thus generates a proton motive force; (ii) the vacuolar-type H-ATPase pump (V-ATPase); (iii) the vacuolar H-pumping pyrophosphatase pump (H-PPase) (Apse *et al.,* 1999; Dietz *et al*., 2001).

The A. thaliana plasma membrane Na^+/H^+ antiporter (AtNHX1), encoded by the SOS1 gene, was observed to be essential for salt tolerance. Overexpression of SOS1 improves salt tolerance in transgenic Arabidopsis plants. It is also observed that the increased salt tolerance was correlated with reduced Na^+ accumulation (Shi *et al*.,2002). Also, Na^+ can be used as an osmolyte in the vacuole to help to maintain the osmotic homeostasis so that Na^+ compartmentation is an economical way of preventing Na^+ toxicity in the cell (Zhu, 2001).

1.5 Antioxidant Defense Systems

Plants have a multi-level antioxidant system consisting of both enzymatic and non-enzymatic components against ROS damage.

1.5.1 Non-enzymatic Systems

Non-enzymatic antioxidants include ascorbate and glutathione, as well as tocopherol, phenolics and carotenoids.

The tripeptide glutathione (γ-glutamylcysteinylglycine), (GSH) is the major low molecular weight thiol compound in most plants. Glutathione reacts with singlet oxygen and hydroxyl radicals and regenerates ascorbate (Arora *et al*., 2002). It has been found in all cell compartments: cytosol, endoplasmic reticulum, vacuole, apoplast, peroxisome, chloroplast and mitochondria (Dat *et al.,* 2000).

The antioxidant function of GSH is mediated by sulfhydryl group of cysteine, which forms a disulfide bond with a second molecule of GSH to form oxidized glutathione (GSSG) upon oxidation. The reduction of GSSG to GSH is catalyzed by the enzyme glutathione reductase (GR). GSH can function as an antioxidant in many ways. It can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger. Also GSH can stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price *et al.,* 1990; McKersie, 1996; Hausladen and Alscher, 1993). It also participates in the regeneration of ascorbate from dehydro-ascorbate via the enzyme dehydro-ascorbate reductase (Arora *et al,* 2002). Glutathione levels, redox status and biosynthesis can regulate the expression of a large number of genes, among them are components of the antioxidant defence system and the pathogen related 1 (*PR1*) gene (Ball *et al*., 2004; Mateo *et al*., 2006).

L-Ascorbic acid (Vit C) is present in chloroplasts, cytosol, vacuole, apoplastic space, mitochondrium and peroxisomes (Asada, 2006). It is an important antioxidant molecule in plant tissues having effective roles in stres physiology as well as growth and development.

Ascorbate has the main activities of being an enzyme cofactor and antioxidant molecule, and oxidant/reductant factor in the electron transportation in chloroplasts or plasma membranes, as all related with oxidative stress resistance (Conklin, 2001) Ascorbate reacts directly with hydroxyl radicals, superoxide, singlet oxygen and lipid peroxides and indirectly eliminates H_2O_2 through the activity of ascorbate peroxidase (Asada 1992). Antioxidants such as ascorbic acid and glutathione, which are found at high concentrations in chloroplasts and other cellular compartments (5–20 mM ascorbic acid and 1–5 mM glutathione) are crucial for plant defense against oxidative stress. Maintaining a high reduced per oxidized ratio of ascorbic acid and glutathione is essential for the effective scavenging of ROS in cells. This ratio is maintained by glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) using NADPH as reducing power (Noctor and Foyer, 1998; Asada, 1999).

Carotenoids (carotens and xanthophylls) which are lipid soluble antioxidants are yellow, orange, and red pigments present in many plants. Several of them are precursors of vitamin A (i.e. *β*-carotene, *γ*-carotene, and *β*-cryptoxanthin), and they are both radical scavengers and quenchers of singlet oxygen due to conjugated double bonds (Podsedek, 2005).

Carotenoids are lipophylic isoprenoid compounds synthesized by all photosynthetic organisms. Carotenes such as β– carotene are linear hydrocarbons and there are also oxygenated derivatives of carotenes such as lutein, violaxanthin, neoxanthin, and zeaxanthin which are generally called xanthophylls. Most carotenoids are located in functional pigment-binding protein structures embedded in photosynthetic (thylakoid) membranes (Pavia and Conception, 2006). Carotenoids have critical roles as photoprotective compounds by quenching triplet chlorophyll and singlet oxygen derived from excess light energy. By the way they limit membrane damage (Howitt and Pogson, 2006).

Tocopherol (vitamin E) is also from the group of lipid-soluble antioxidants like the carotenoids. They have ability to bring an end to the free radical reactions, by the way ending lipid peroxidation. They can efficiently quench singlet oxygen and scavenge various radicals. The biological activity of vitamin E exhibit tocopherols (α–, β–, γ-, and δ-tocopherols) and tocotrienols, especially α-tocopherol. They are important components of the biological membranes (Slesak et al., 2007; Maeda and DellaPenna, 2007; Podsedek, 2005). Tocopherol levels increase in response to a variety of abiotic stresses, including high-intensity light, drought, toxic metals, and high and low temperatures in photosynthetic organisms (Bosch *et al*., 1999; Luis *et al*, 2006).

Phenolic compounds like flavonoids, tannins and lignin, are a large group of the secondary metabolites widespread in plant kingdom. Phenolics are able to scavenge reactive oxygen species due to their electron donating properties. Their antioxidant

effectiveness depends on the stability in different systems, as well as number and location of hydroxyl groups.

1.5.2 Enzymatic System

The enzymatic defense against the ROS is essential for plants under stress conditions. The main antioxidative enzymes are superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.1.1.11), glutathion reductase (GR; EC 1.6.4.2) and other ascorbate-glutathion cycle enzymes (monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1)). They catalyze the synthesis, degradation and recycling of antioxidant molecules and can directly sustain the removal of ros from the cells.

Catalase is a heme containing enzyme that catalyzes the dismutaion of hydrogen peroxide into water and oxygen thus protecting the cell from the deleterious effects of H_2O_2. Catalase is localized in mitochondria, glyoxysomes, and mostly in peroxisomes and detoxifies H_2O_2 which is mainly generated by oxidases involved in β-oxidation of fatty acids and glyoxylate cycle (photorespiration) in peroxisomes with no requirement for a reductor (Gechev *et al*., 2006). It is also suggested that the peroxisomal catalase is photosensitive, can be inactivated in moderate light (Dat *et al.,* 2000). Catalase provides plants with an energy-efficient way to remove H_2O_2 as it degrades H_2O_2 without any reducing power. However, catalase possesses a very low affinity for H_2O_2 and only active at relatively high H_2O_2 concentrations (Gechev *et al.,* 2006).

There is also a more efficient process for H_2O_2 removal, which operates in almost all cellular compartments, called the ascorbate-glutathion or Halliwell-Asada cycle (Fig.1.5). It was experienced that the ascorbate–glutathione cycle presents in almost all cellular compartments as cytosol, chloroplasts and mitochondria, and the high affinity of APX for H_2O_2 was also observed. These data shows that this cycle plays a

crucial role in controlling the level of ROS in these compartments. It is also important for the maintenance of ascorbate and glutathion pools in the reduced state (Asada, 1999; Mittler, 2002).

Maintaining a high reduced per oxidized ratio of ascorbic acid and glutathione is essential for the proper scavenging of ROS in cells. This ratio is maintained by glutathione reductase (GR), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) which are the ascorbate-glutathion cycle enzymes using NADPH as reducing power (Asada, 1999; Noctor and Foyer,1998).

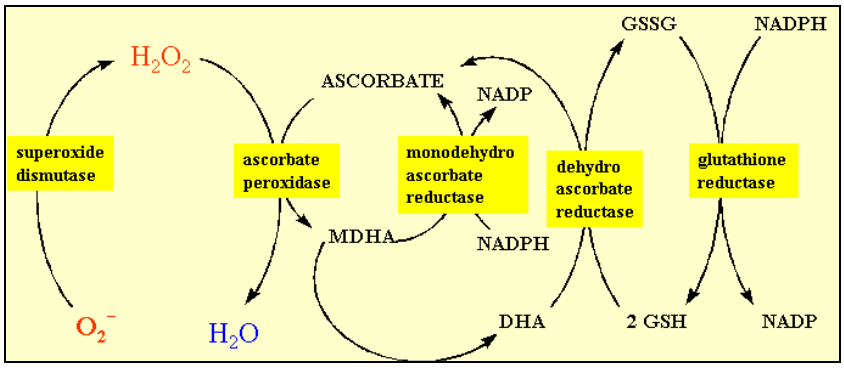

Figure 1.5. Asada- Halliwell pathway of hydrogen peroxide scavenging and ascorbic acid regeneration involving various antioxidant enzymes.

The first enzyme of this cycle, ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to water and has high specificity and affinity for ascorbate as reductant (Asada, 1999).

It is suggested that APX and CAT belong to two different classes of H_2O_2 scavenging enzymes from their difference in affinities for H_2O_2. APX and CAT has

affinities for H_2O_2 in micromolar and milimolar ranges, respectively. APX might be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during stress (Mittler, 2002). Different forms of APX is found in cytosol, chloroplast, mitochondria and peroxisomes (Asada, 2006).

In the ascorbate glutathion cycle the H_2O_2 generated by SOD is reduced to water by ascorbate with the reaction catalyzed by APX, and ascorbate is oxidized to monodehydroascorbate. Subsequently, monodehydroascorbate is directly reduced to ascorbate by either reduced ferredoxin or NADPH with the reaction catalyzed by with chloroplastic monodehydroascorbate reductase (Sano *et al.,* 2005; Miyake and Asada, 1994). If monodehydroascorbate fails to reduce directly to ascorbate, it is spontaneously disproportionated to dehydroascorbate and ascorbate. Dehydroascorbate is then reduced to ascorbate by reduced glutathione (GSH) catalyzed with dehydroascorbate reductase (Shimaoka *et al*., 2003). Monodehydroascorbate reductase and dehydroascorbate reductase are found in cytosol, chloroplast and mitochondria (Mittler *et al.,* 2004).

Ascorbate-glutathione cycle is completed by glutathione reductase with regenerating the glutathione pool with NADPH as the electron donor (Apel & Hirt, 2004). Glutathione reductase maintains a high ratio of GSH/GSSG, which is required for the regeneration of ascorbate and it is localized in cytosol, chloroplast, mitochondria and peroxisomes (Mittler *et al.,* 2004).

The superoxide dismutase enzymes (SODs) remove superoxide, $O_2^{\cdot -}$, by catalyzing its dismutation, one $O_2^{\cdot -}$ being reduced to H_2O_2 and another oxidized to O_2 (Halliwell, 2006).

Within a cell superoxide dismutases constitute the first line of defence against ROS. The enzyme is present in all aerobic organisms and in all subcellular compartments susceptible of oxidative stress (Mittler *et al.,* 2004). Its activity determines the

concentrations of $O_2^{\cdot-}$ and H_2O_2 , the two Haber-Weiss reaction substrates, which generate the most reactive hydroxyl radicals.

Based on the metal co-factor used by the enzyme, SODs are classified into three groups: Iron SOD (Fe SOD), Manganase SOD (Mn SOD) and copper-zinc SOD (Cu-Zn SOD) and they are located in different compartments of the cell. Fe SODs are located in chloroplasts, Mn SODs are in the mitochondrion and peroxisomes, and Cu-Zn SODs in the chloroplast, the cytosol and possibly the extracellular space. Experimentally different types of SOD can be identified by their differential sensitivities to KCN and H_2O_2. Cu/Zn SOD is characterized as being sensitive to both KCN and H_2O_2; Fe SOD is sensitive only to H_2O_2, while Mn SOD is resistant to both inhibitors. The mechanism of catalysis involves attraction of negatively charged superoxide radicals to the active site of the enzyme where positively charged amino acid residues are present for each type of SOD. The transition metal at the active site then carries an electron to the superoxide radical and produces hydrogen peroxide (Alscher *et al.,* 2002).

1.6 Aim of the Study

In this study a Turkish lentil (*Lens culinaris* Medik., cv Sultan-1) cultivar has been characterized with respect to certain physiological and biochemical parameters under salt and drought stresses. The analyses listed below were performed to determine the effects of these stresses on antioxidant defense system and physiology of lentil in a comparative manner.

i) Determination of antioxidant enzyme activities (SOD, APX, CAT, GR)
ii) Lipid peroxidation through MDA and electrolyte leakage tests
iii) Proline content determination
iv) Detection of H_2O_2 content
v) Chlorophyll fluorescence analysis
vi) Wet-dry weight, relative water content and length measurements.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical Materials

In this study the chemical materials were provided from Applichem Chemical Company, Merck Chemical Company or Sigma Chemical Company. All of the solutions were prepared with distilled water.

2.1.2 Plant Materials

Sultan-1, a Turkish cultivar of lentil (*Lens culinaris*) was used in this study. The seeds were obtained from Eskişehir Anadolu Agricultural Research Institute (ATAEM). Sultan-1 is a summer sown large-seeded green lentil cultivar and the weight of 1000 seeds is 59.1-62.3 g. Sultan-1 is cultivated in Central Anatolia and transition regions in Turkey.

2.2 Methods

2.2.1 Seed Surface Sterilization

Seeds were sterilized with 20% ethanol for 30 seconds and then washed with distilled water. After sterilization seeds were imbibed in distilled water for one day.

2.2.2 Growth of Plants

Imbibed seeds were transferred to plastic pots covered with cheesecloth containing ½ strength Hoagland's Solution (Hoagland and Arnon, 1950), and each pot contains eight seeds. Planted seeds were grown for 7 days in a plant growth incubator at 25 ± 2 º C with 16 hours light and 8 hours dark photo cycle.

2.2.3 Application of Drought and Salt Stresses

Experiments were initiated with 10, 20, 30 % PEG 6000 and 100, 150, 200 mM NaCl treatment. Sampling was done on the first, third, fifth and seventh days. 20% PEG 6000 and 150 mM NaCl treatment during 7 days were chosen as the experimental conditions. Drought and salt stress treatments were initiated on the seventh day of normal growth. ½ strength Hoagland's Solution in the pots were replaced with ½ Hoagland's Solution containing 20% PEG 6000 or 150 mM NaCl. Control and stressed plants were grown in the growth chamber with the same physical parameters for another 7 days. Fourteen days old seedlings were then analyzed for APX, CAT, GR and SOD activities; proline, MDA and H_2O_2 contents; chlorophyll fluorescence and physiological parameters like wet-dry weight, relative water content and root-shoot lengths.

2.2.4 Wet -Dry Weight Analysis and Physiological Changes

At the fourteenth day of growth the weight of shoot and root tissues from control and stress treated groups were recorded as wet weight (WW). The tissues were let to dry in an oven at 60º C. After one day the dry weights (DW) were taken. The lenghts of shoots and roots were measured and the seedlings were photographed to observe the symptoms caused by the stresses and determine the changes in growth.

2.2.5 Relative Water Content

The leaf samples which were collected at the seventh day of stress treatments were used for relative water content determinations. After wet weight determinations, the tissues were floated on distilled water for 24 hours at room temperature. The hydrated shoot tissues were weighed to have the turgid weights (TW) and the tissues were dried in an 60ºC oven for one day and weighed again to determine the dry weights. Relative water content was calculated according to the Smart and Bingham, using the following formula.

Relative water content (%) = { WW-DW}/ {TW-DW} × 100 (2.1)

2.2.6 Chlorophyll Fluorescence Analysis

Two different tests were performed by using OS5-FL Modulated Fluorometer. In dark adapted leaf tissue, "Fv/Fm" test were performed which indicates the maximum photochemical yield of PSII. "Yield" (Y) of photosynthetic efficiency measurements of light adapted leaf tissues were also done which determines the effective (actual) PSII photochemical yield.

Fv/Fm= (Fm-Fo)/Fm; Yield (Y)= (Fms-Fs)/Fms (2.2)

Fo and Fm are the minimal and maximal fluorescence yields of a "dark adapted" sample, with all PSII reaction centerrs fully open and closed, respectively. Fv is the variable fluorescence. Fs and Fms are the steady state and maximal fluorescence values of a sample during exposure to a source of light driving photosynthesis (Opti-Sciences, 2003).

2.2.7 Determination of Malondialdehyde Content

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to the method of Ohkawa *et al*., (1979) . Fresh shoot and root

tissues were weighed as 0.2 g. The fresh tissues were homogenized with liquid nitrogen by using cold mortar and pestle and then suspended in 1 ml of 5 % Trichloroacetic acid (TCA). The homogenates were transferred to eppendorf tubes and centrifuged at 12000 rpm for 15 minutes at room temperature (Hettich microcentrifuge) Freshly prepared 0.5% Thiobarbituric acid (TBA) in 20 % TCA and supernatant in equal volumes were put into eppendorf tubes and incubated for 25 minutes at 96º C by using heat block. The tubes were put into ice after incubation until they reach to room temperature and then they were centrifuged at 10000 rpm for 5 mins. Absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. 0.5% TBA in 20% TCA was used as blank and the MDA contents were calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.2.8 Measurement of Membrane Leakage

Membrane leakage was estimated by measurement of electrolyte leaked from leaves and roots according to the method of Nanjo *et al*. (1999). For conductance of leaves, 6 leaves per plant; for conductance of roots total root tissues were put into seperate 15 ml falcon tubes and immersed in 5 ml of 0.4 M mannitol at room temperature with gentle shaking for 3 h. Electrical conductances were measured and recorded by using Mettler Toledo MPC 227 conductivity meter as C1, initial conductivity. Then the tubes containing the samples were put into boiling water for 10 minutes. After this period the tubes were put into ice until they reach to the room temperature. The conductances were measured and recorded as C2 which indicates the total conductivity by complete membrane disintegration. The conductivity due to leakage is expressed as the percentage of the initial conductivity over the total conductivity [(C1/C2)* 100].

2.2.9 Determination of Proline Content

Proline amount determination was carried out according to the method of Bates *et al*., (1973). 0.2 g of shoot and root tissues from control and treated plants were homogenized with liquid nitrogen by using mortar and pestle and then suspended in 1 ml of 3% sulphosalicylic acid. The extracts were then transferred into eppendorf tubes and centrifuged (MPV centrifuge) at 14000 rpm for 5 minutes at 4ºC. An eppendorf tube containing 0.2 ml acid ninhydrin (0.31 g ninhydrin, 7.5 ml acetic acid and 5 ml 6M phosphoric acid), 0.2 ml 96% acetic acid and 0.1 ml 3% sulphosalicylic acid was prepared for each sample. 0.1 ml supernatant from each extract was added to the eppendorf tubes. After a gentle shaking the tubes were incubated at 96ºC for 1 hour by using heat block (Techne), for the hydrolysis of proteins. 1 ml toluene was added after the incubation and the tubes were vortexed. After centrifugation at 14000 rpm for 5 minutes, the pink-red colored upper phase was transferred to the cuvettes and the absorbances were determined at 520 nm as blank being toluene. A standard curve for proline was constructed to estimate the the proline concentration between the range of 5µm-500µm.

2.2.10 Measurement of H_2O_2 Content

H_20_2 content was estimated according to the method of Bernt and Bergmeyer (1974), using peroxidase enzyme. 0.5 g shoot and root tissues from control and treatment groups were grinded using a cold mortar and pestle with liquid nitrogen and the powders were suspended in 1.5 ml of 100 mM potassium phosphate buffer at pH 6.8. The homogenates were then transferred to centrifuge tubes and centrifuged at 18000 g for 20 min at 4ºC. To start the enzyme reaction 0.25 ml supernatant and 1.25 ml peroxidase reagent consisting of 83 mM potassium phosphate buffer at pH 7.0, 0.005% (w/v) o-dianizidine, 40 µg peroxidase/ml, were put in an eppendorf tube and incubated for 10 min at 30ºC in a waterbath. The reaction was stopped by adding

0.25 ml of 1N perchloric acid and the samples were centrifuged at 5000 g for 5 minutes. The absorbance of the supernatant was read at 436 nm and the amount of hydrogen peroxide determined by using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$.

2.2.11 Protein Determination

The protein amounts in the root and shoot crude extracts were determined by Bradford method (Bradford, 1976). Bradford reagent was prepared by dissolving 500 mg of Coomassie Brillant Blue G-250 in 250 ml of 95% ethanol. 500 ml of 85% phosphoric acid was added to the mixture and the solution was diluted to 1L with dH_20 and filtered. As the reagent had been prepared as 5X, it was diluted to 1X before each use. 20µl from shoot and 40µl from root samples were taken and diluted to 500µl with dH_20. 5 ml of 1X Bradford reagent was added to the diluted sample. After an incubation of 10 minutes at room temperature the absorbances were read at 595 nm by using Schimadzu UV-1201 spectrophotometer against a blank of 500 µl water and 5 ml bradford reagent. Bovine Serum Albumin (BSA) with concentrations 10, 20, 30, 40, 50, 60 µg/ml was used for preparation of standard curve.

2.2.12 Determination of APX Activity

Ascorbate peroxidase activity determination was done according to the spectrophotometric method of Wang *et al*., (1991). 0.5 g of shoot and root tissues were homogenized with liquid nitrogen and the powders were suspended in 1 ml of suspension solution containing 50 mM Tris-HCl (pH 7,2), 2% PVP, 1 mM EDTA and 2mM ascorbate. After the centrifugation at 12100 g for 20 minutes at 4ºC the supernatants were used for enzyme assay. Enzyme extract containing 100 µg protein, determined by Bradford method, was added into the assay medium consisting of 50 mM potassium phosphate buffer (pH 6.6) , 0,25 mM ascorbate and 1mM H_2O_2. Addition of hydrogen peroxide initiated the reaction. The decrease in the ascorbate concentration was recorded at 290 nm with Schimadzu double-beam spectrophotometer for 1.5 minutes. Assay medium without enzyme extract was used as blank.

The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of ascorbate = 2.8 $mM^{-1}cm^{-1}$ at 290 nm).

Ascorbate + Hydrogen peroxide → Dehydroascorbate + Water (2.3)

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

2.2.13 Determination of CAT Activity

Activity of catalase was determined according to the method of Chance *et al*. (1955). 0.5 g of tissue from shoots and roots were ground in a cold mortar and pestle and then suspended in 1 ml of 50 mM Tris-HCl suspension solution at pH 7.8. The homogenates were centrifuged at 12000 g for 20 min at 4ºC and the supernatants were used for the enzyme assay. Enzyme extract containing 50 µg protein, determined by Bradford method (1976), was added into assay medium containing 50 mM potassium phosphate buffer at pH 7 and 10 mM H_2O_2. By the additon of the enzyme extract the reaction was started. The decrease of the H_2O_2 absorbance was recorded at 240 nm with Schimadzu double-beam spectrophotometer for 1.5 minutes. The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of H_2O_2 = 39,4 $mM^{-1}cm^{-1}$.)

Hydrogen peroxide (H_2O_2) → Water (H_2O) + Oxygen (O_2) (2.5)

2.2.14 Determination of GR Activity

Glutathion reductase activity was determined according to the method of Sgherri *et al.,* (1994), with minor modifications. 0.5 g of tissue was homogenized with liquid nitrogen by using cold mortar and pestle. The powder was suspended in 0,75 ml of suspension solution containing 100 mM potassium phosphate buffer (pH 7.0) , 1 mM Na_2EDTA and 2% insoluble PVP. The suspensions were centrifuged at 18000 g for 20 min at 4ºC and the supernatants were used for the enzyme assay. Enzyme extract containing 75µg protein, determined by Bradford method (1976), was added into

assay medium containing 200 mM potassium phosphate buffer (pH 7.5), 0,2 mM Na_2EDTA, 1,5 mM $MgCl_2$, 0,50 mM GSSG, 50 µM NADPH, in a final volume of 1 ml. By the addition of NADPH the reaction was started. Assay medium without enzyme extract was used as blank. The decrease in the NADPH concentration was recorded at 340 nm with Schimadzu double-beam spectrophotometer during 1.5 minutes The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of NADPH = 6.2 $mM^{-1}cm^{-1}$ at 340 nm)

Glutathione disulfide (GSSG) + NADPH → Glutathione (2GSH) + $NADP^+$ (2.6)

2.2.15 Determination of SOD Isozyme Activities

One dimensional native polyacrylamide gel electrophoresis was used to estimate the SOD activity and SOD isozyme patterns of stress treated and control plants. Gels were stained by negative activity staining technique according to the method of Beuchamp and Fridovich (1971).

Superoxide ($2O_2^-$) + $2H^+$ → Hydrogen peroxide (H_2O_2) + Oxygen (O_2) (2.7)

2.2.15.1 Preparation of Shoot Crude Extracts

Shoot tissues from control and treated plants were weighed as 0,2 g and homogenized by using cold mortar and pestle with liquid nitrogen. The powder was suspended in 800 µl of homogenization buffer consisting of 9 mM Tris-HCl at pH 6.8 and 13.6% glycerol. The susupensions were then transferred into eppendorf tubes and centrifuged at 14000 rpm for 5 minutes by using Hettich microcentrifuge. Supernatants were used for the SOD assay.

2.2.15.2 Preparation of Root Crude Extracts

Root tissues from control and treated plants were weighed as 0,5 g and homogenized by using cold mortar and pestle with liquid nitrogen. The powder was suspended in 750 µl of grinding buffer consisting of 0.2 M sodium phosphate buffer and 2 mM EDTA at pH 7.5. The suspensions were then centrifuged at 10000 g for 30 minutes at 4ºC and supernatants were used for the SOD activity assay.

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

Seperating gel (5 ml 12%) and stacking gel (2,5 ml 5%) were prepared to carry-out 1-D PAGE according to the method of Laemmli (1970). Gels were polymerized in Cleaver Minigel Apparatus. Equal amounts of proteins determined by Bradford method (30 µg/ well) were loaded to the wells. 5µl standard SOD protein was also applied in one of the wells to determine the intensities. (0,1 unit/ µl) Electrophoresis was carried out under constant current of 6 mA in stacking gel and 10 mA in seperating gel for approximately 4 hours.

2.2.15.4 Negative Activity Staining

Gels were transferred into glass containers including 25 ml 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), 3mM riboflavin and 0,25 mM nitroblue tetrazolium. After a dark incubation period for 45 minutes with occasional shaking, gels were washed with dH_2O several times under illumination, until the colour development occurs. The gels were photographed in Vilber gel imager and densitometric analysis was carried out by Obitek 2006 ImageMaster software program. The changes in SOD isozymes were evaluated from band intensities. The gels were air dried between two cellophane sheets and stored. The unit SOD activity was determined by running a SOD Standard from bovine erythrocyte. One unit of Standard SOD activity inhibits

the rate of reduction of Cyt c by 50% in coupled system with xanthine oxidase at ph 7.8 at 25ºC in a 3 ml reaction medium.

2.2.15.5 SOD Isozyme Determination

The root and shoot extracts of control and treated plants were loaded into wells as three duplicates and gel was run as previously described. At the end of electrophoresis, the gel was cut into three, each containing one shoot and one root sample. Two of them were incubated for 15 minutes in seperate inhibition solutions; one containing 50 mM potassium phosphate buffer (pH 7.8), 0,5 mM EDTA and 5 mM KCN, and the other containing 5 mM H_2O_2. The other gel was incubated in 50 mM potassium phosphate buffer (pH 7.8) and 0,5 mM EDTA as control. Then the gels were treated with negative activity stain as described above. MnSOD is resistant to both H_2O_2 and KCN, FeSOD is resistant only to KCN and Cu/ZnSOD is sensitive to both inhibition solutions.

2.2.16 Statistical Analysis

The significance of difference between mean values obtained from at least 5 independent experiments was determined by one-way analysis of variance at 95% confidence interval. The standard deviations among means were calculated by descriptive statistics test on Minitab software program.

CHAPTER 3

RESULTS

Lentil seedlings grown for 7 days under normal environmental conditions were subjected to different concentrations of polyethylene glycol (PEG 6000) and sodium chloride during 7 days and observed to determine the most effective stress condition and duration. The seedlings were treated with 10 %, 20% and 30 % PEG and 100 mM, 150 mM, 200 mM NaCl to induce drought and salinity stresses, respectively. The sampling were done on the first, third, fifth and seventh days for the determination of malondialdehyde content which is one of the end products of lipid peroxidation by free radicals and marker of the injury caused by stress. The results are shown in Figures 3.1, 3.2, 3.3 and 3.4. After these preliminary observations, 20% PEG and 150 mm NaCl treatment during 7 days were chosen for experimental process. After the treatment with 20% PEG and 150 mM NaCl seperately for 7 days, the 14 days old shoot and root tissues of both control and stress treated plants were analyzed for the parameters like; body length, wet and dry weight, relative water content, electrolyte leakage and chlorophyll fluorescence (Fv/Fm, Yield); MDA, proline and H_20_2 contents; APX, CAT, GR and SOD activities.

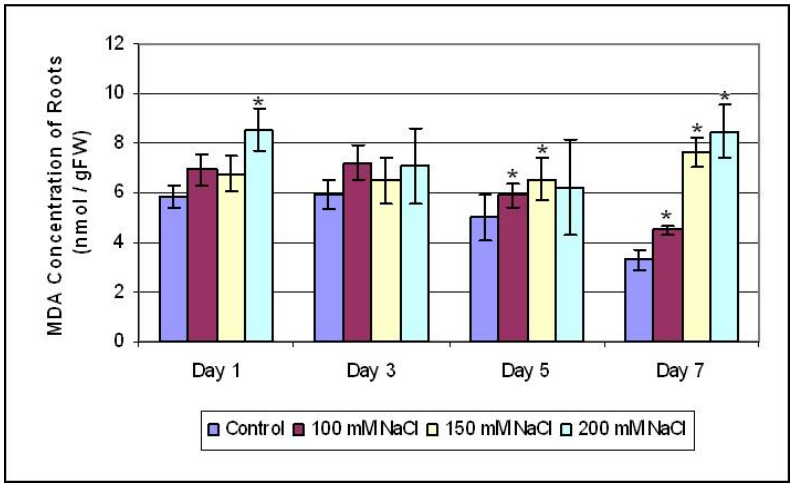

Figure 3.1. Root MDA content of control and 1, 3, 5, 7 days of 100 mM, 150 mM and 200 mM salt stress. Vertical bars indicate the mean ± S.E.M values. * Asterisks show significant difference ($p < 0.05$) with respect to control.

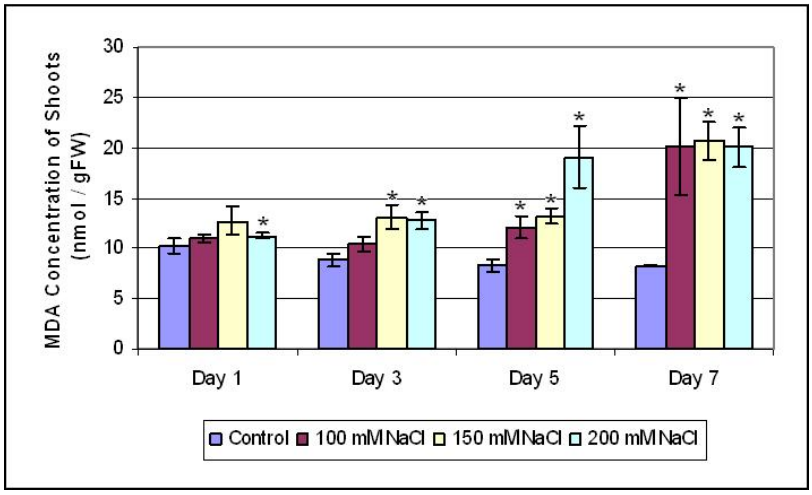

Figure 3.2. Shoot MDA content of control and 1, 3, 5, 7 days of 100 mM, 150 mM and 200 mM salt stress. Vertical bars indicate the mean ± S.E.M values. * Asterisks show significant difference ($p < 0.05$) with respect to control.

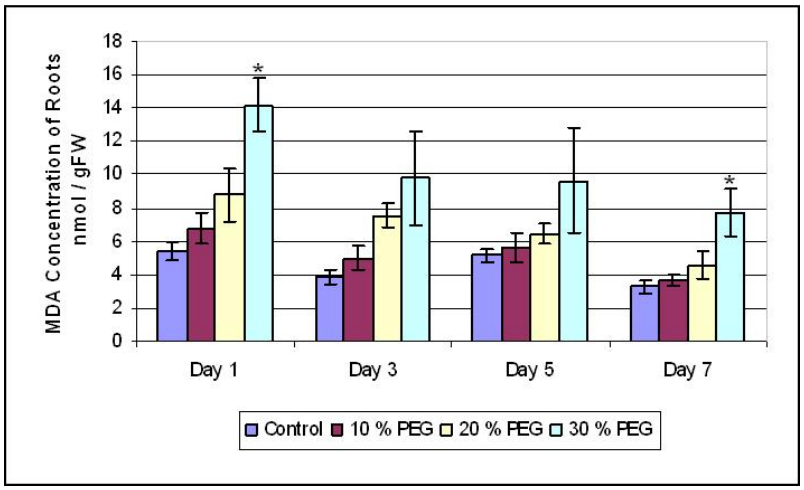

Figure 3.3. Root MDA content of control and 1, 3, 5, 7 days of 10 % PEG, 20 % PEG and 30 % PEG drought stress. Vertical bars indicate the mean ± S.E.M values. * Asterisks show significant difference ($p < 0.05$) with respect to control.

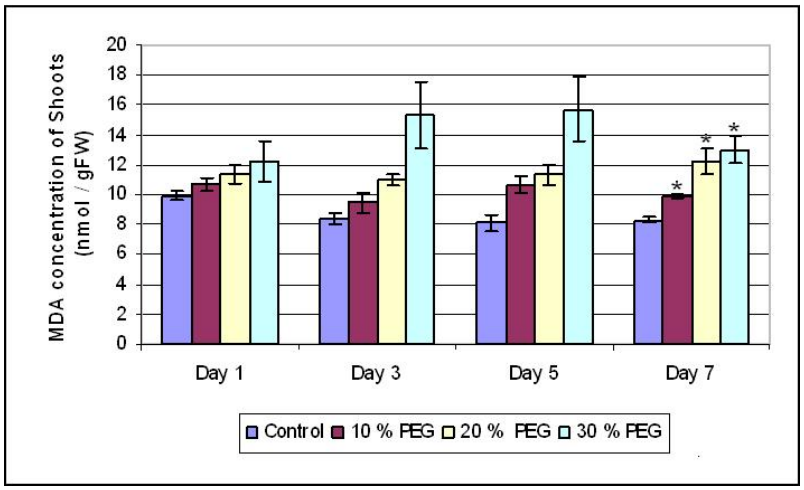

Figure 3.4. Shoot MDA content of control and 1, 3, 5, 7 days of 10 % PEG, 20 % PEG and 30 % PEG drought stress. Vertical bars indicate the mean ± S.E.M values. *Asterisks show significant difference ($p < 0.05$) with respect to control.

3.1 Physiological Changes Under Drought and Salt Stress

The physiological changes in both shoot and root tissues under drought (20 % PEG) and salt (150 mM NaCl) stresses were evaluated by measuring the wet-dry weights, lengths and relative water contents. The photographs of 14 days old control and stress treated plants were shown in Fig. 3.5.

After 7 days of salt treatment, the plants were wilted and stunted. Decreased leaf area was observed in both drought and salt treatments but it is also accompanied with necrosis in salt effected plants.

Figure 3.5. 14 days old lentil seedlings. a, b and c show control, 20 % PEG treated and 150 mM NaCl treated plants both in pots and individually as seedlings.

3.1.1 Length

Salt stress strongly inhibited the growth of lentil seedlings. Shoot and root lengths of NaCl treated plants were significantly different than the control plants ($p < 0.05$). Shoot and root lengths of salt treated plants were 46% and 32% shorter than the control groups, respectively. Shoot lengths were also decreased significantly by drought stress treatment, with the ratio of 35 % when compared to control plants. The root lengths of drought effected plants were not changed significantly, they were slightly shorter than the control groups with a 14 % decrease. Root lengths of salt treated plants were significantly shorter than drought treated plants, hovewer there was no significant change in shoot lenghts as shown in Fig.3.6.

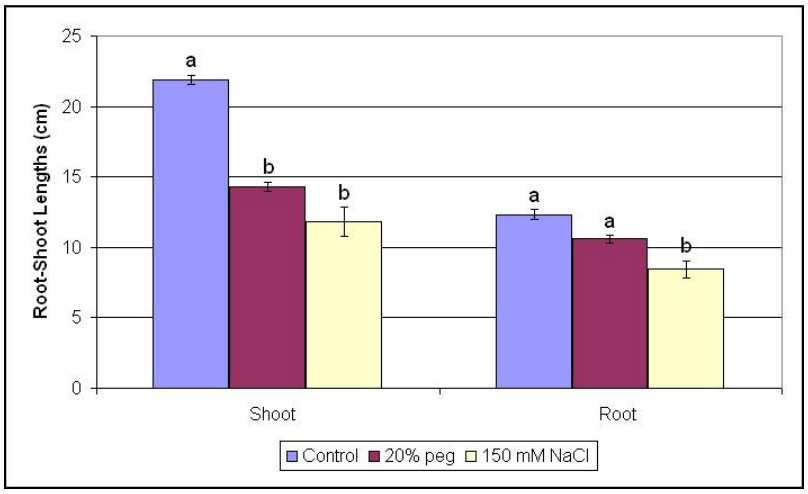

Figure 3.6. Shoot and root lengths (cm) of control, 20 % PEG and 150 mM NaCl treated plants. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups.

3.1.2 Wet and Dry Weight Analysis

The wet-dry weight of the lentil seedlings were decreased significantly with the salt stress application and the decreases were more apparent in shoots than in the roots. The wet weights of shoots and roots of salt treated plants were decreased by 80 % and 61 %, respectively. Dry weights of shoots decreased by 57 % while the decrease in roots was about 30 %. The wet and dry weights of drought treated shoots were significantly decreased by 46 % and 17 %, respectively. There was no significant change in the wet weights of drought stressed roots, however a significant increase was observed in the dry weights by about 45 % when compared to control groups. The wet and dry weights of shoot and root tissues of drought treated plants are significantly higher than that of salt treated plants. (Fig. 3.7; Fig. 3.8)

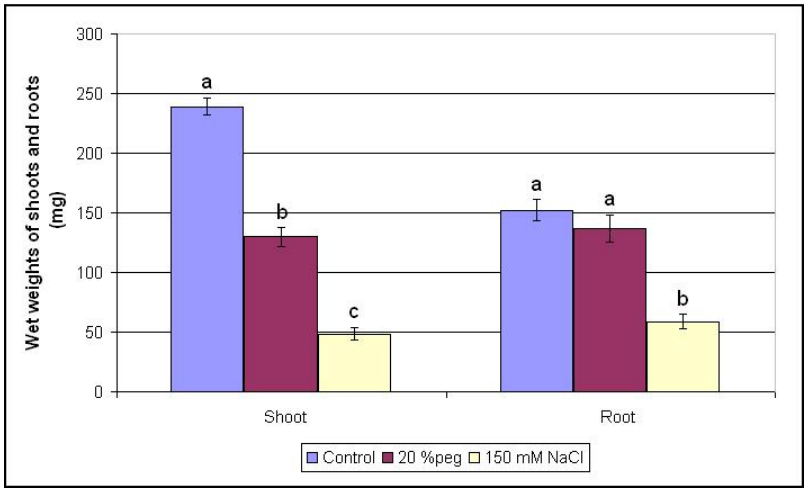

Figure 3.7. Shoot and root wet weights of control, 20 % PEG and 150 mM NaCl treated plants. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups.

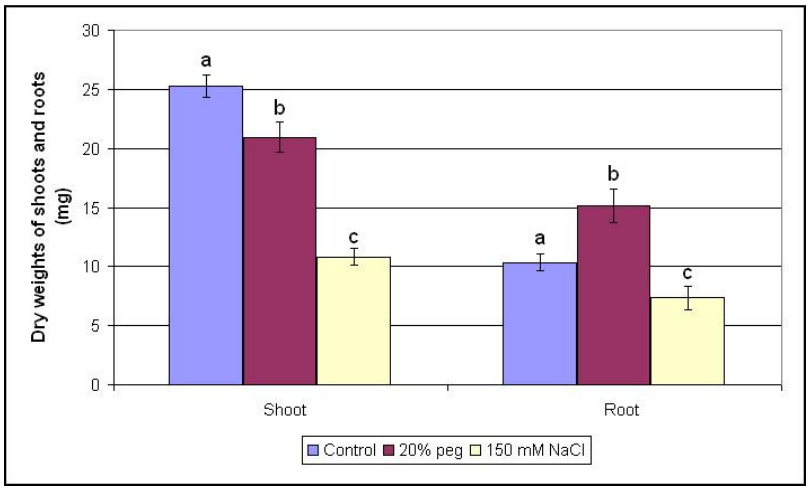

Figure 3.8. Shoot and root dry weights of control, 20 % PEG and 150 mM NaCl treated plants. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups.

3.1.3 Relative Water Content

Relative water content of shoots were decreased significantly in both drought and salt stress treatments, when compared to control groups, by about 15 % and 38 %, respectively as shown in Fig. 3.9. Moreover, relative water content of salt stressed shoots is significantly lower than drought stressed shoots.

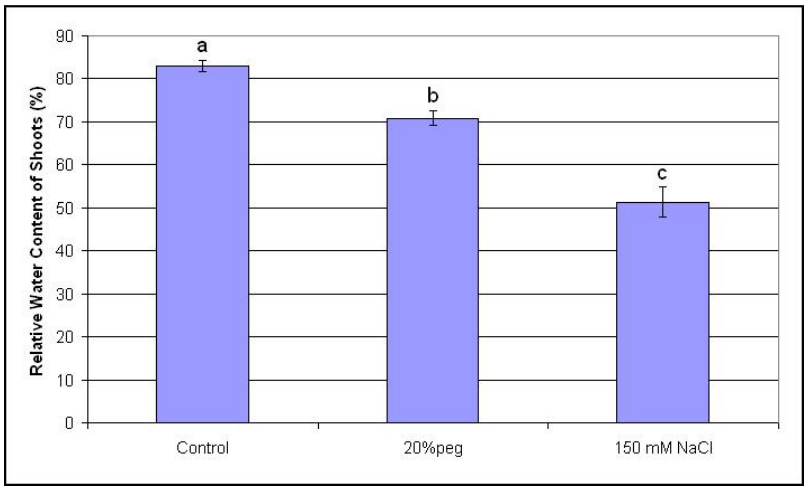

Figure 3.9. Shoot relative water contents of control, 20 % PEG and 150 mM NaCl treated plants. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups.

3.2. Chlorophyll Fluorescence Analysis

Chlorophyll fluoresecence analysis includes 'Fv/Fm' and 'Yield' tests in the study. In dark adapted leaf tissue Fv/Fm (maximum photochemical yield of PSII) value decreased significantly in salt treated plants when compared to the controls and drought treated plants. Drought stress did not cause any significant change in the Fv/Fm values. Salt stress also decreased the effective (actual) PSII photochemical yield significantly but no significant change was observed in drought stressed plants when compared to control plants. Effective (actual) PSII photochemical yield is significantly lower in salt stressed plants than that of drought stressed ones. (Fig. 3.10; Fig. 3.11)

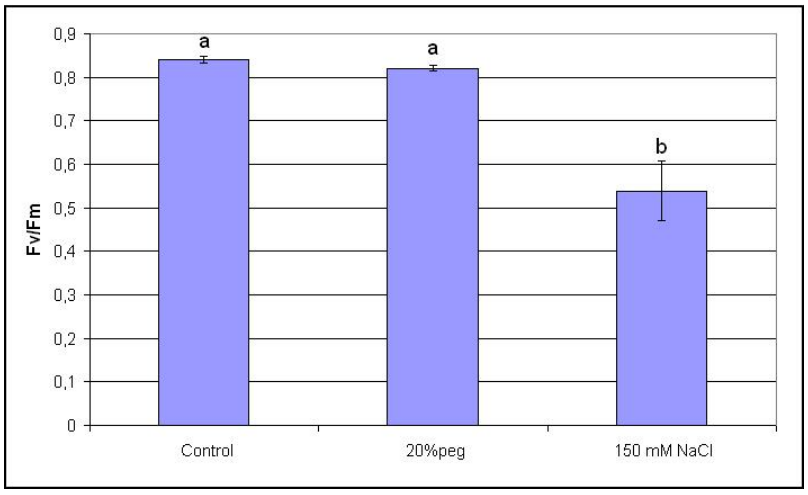

Figure 3.10. Leaf "Fv/Fm" values of control, 20 % PEG and 150 mM NaCl treated plants. Bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$

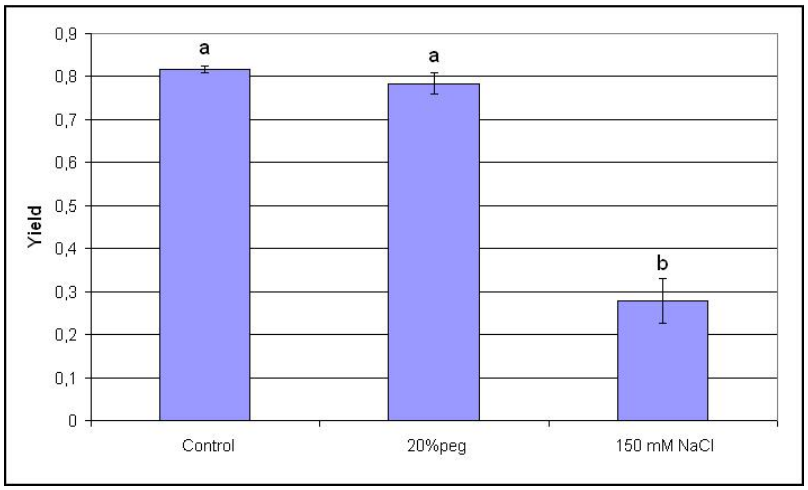

Figure 3.11. Leaf "Yield" values of control, 20 % PEG and 150 mM NaCl treated plants. Bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$

3.3. Measurement of Oxidative Stress Parameters

3.3.1 Malondialdehyde (MDA) content

Effect of drought and salt treatments on lipid peroxidation was determined by measuring the MDA contents of shoot and root tissues. MDA amount showed significant increase in shoot tissues by 47 % for drought stress and 150 % for salt stress treatments. MDA amounts are lower in roots than shoots which corresponds to lower lipid peroxidation. Salt stress significantly affected the MDA contents of roots with an 130 % increase compared to controls. MDA amounts of drought stressed roots was not significantly different than the control groups. For both root and shoot tissues MDA contents were significantly higher in salt treated plants than drought treated plants. (Fig. 3.12)

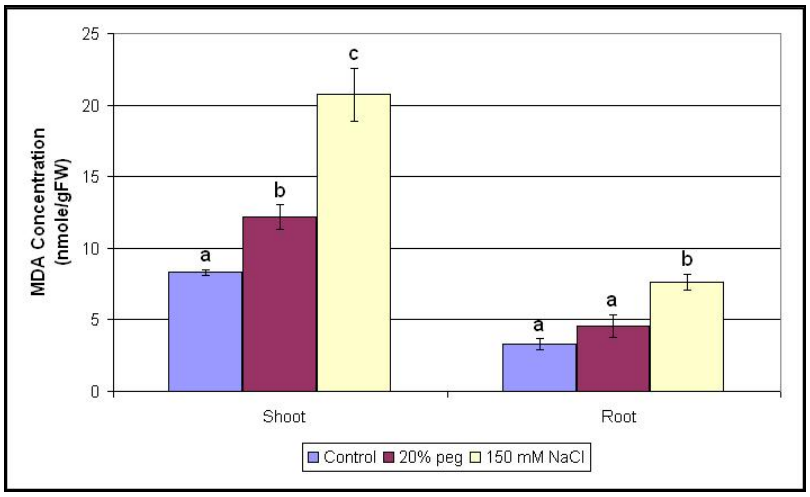

Figure 3.12. Effect of 20 % PEG and 150 mM NaCl treatment on the MDA concentration. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups.

3.3.2. Electrolyte Leakage Test

Drought stress did not cause any significant changes in electrolyte leakage of membranes. Salt stressed shoot and root tissues showed significant increase when compared to controls, by almost 5 fold and 2,5 fold, respectively. (Fig. 3.13)

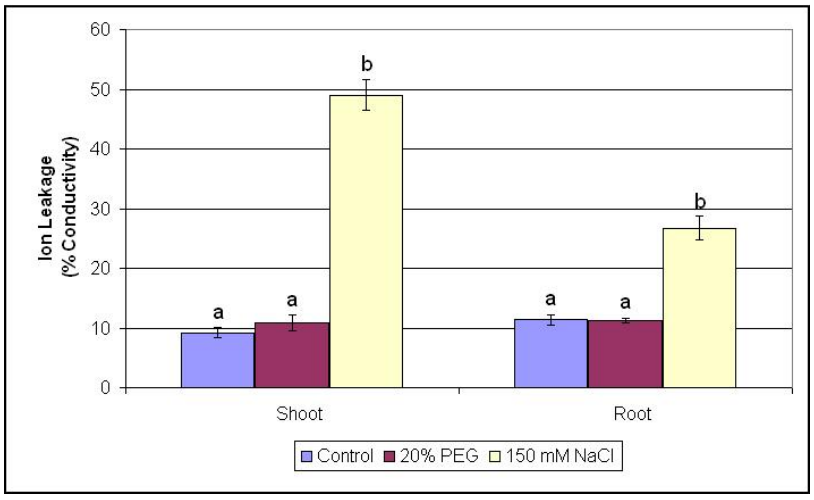

Figure 3.13. Effect of 20 % PEG and 150 mM NaCl treatment on membrane permeability. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups.

3.3.3. Proline Content

Proline contents of shoot and root tissues from drought and salt stressed plants were significantly higher than control plants as shown in Fig. 3.14. Drought and salt treated shoot tissues had approximately 5,2 and 6,5 fold higher proline contents than control plants, respectively. They had also significantly different values within themselves. Proline contents of roots were also higher than controls in drought group with approximately 9 fold and, 6 fold in salt group. They had significantly different values within themselves, too.

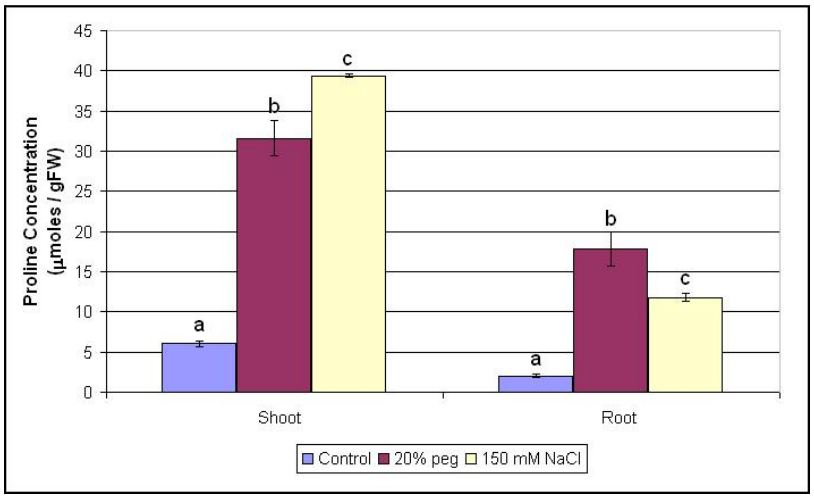

Figure 3.14. Effect of 20 % PEG and 150 mM NaCl treatment on proline concentration. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups.

3.3.4. H_2O_2 Content

H_2O_2 content increased significantly in salt stressed shoot tissues by almost 64 %, while a nonsignificant increase was observed under drought stressed ones. In drought stressed root tissues H_2O_2 content was found to be 67 % higher than control groups. This significant increase was also observed under salt stressed roots with a lower value of 21 % when compared to controls. (Fig. 3.15)

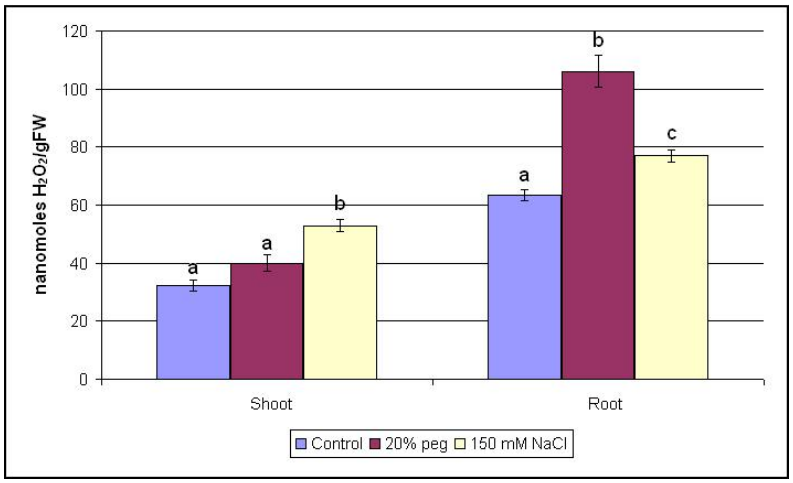

Figure 3.15. Effect of 20 % PEG and 150 mM NaCl treatment on H_2O_2 content. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p< 0.05$ among shoot and root groups.

3.4 Effect of Drought and Salt Stresses on Antioxidant Enzyme Activities

3.4.1 Ascorbate Peroxidase Activity

Salt stress application increased the APX activity significantly in both shoot and root tissues by almost 23% and 94.5%, respectively. There was a very slight nonsignificant decrease in the activity of APX in drought stressed shoot tissues while a significant increase was observed in root tissues. APX activity is significantly higher in salt stressed plants than drought stressed ones (Fig. 3.16).

3.4.2 Catalase Activity

Under drought stress conditions, catalase activity was not changed significantly in shoots however a significant decrease was observed in roots. Salt stress application caused significant decreases in CAT activity by approximately 60 % in shoots and 48

% in roots. CAT activity was significantly lower in salt stressed plants than drought stressed ones (Fig.3.17).

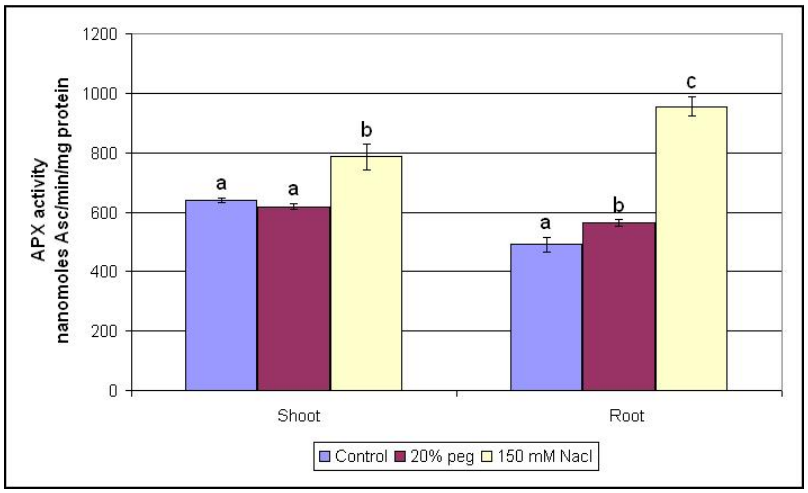

Figure 3.16. Effect of 20 % PEG and 150 mM NaCl treatment on APX activity. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p< 0.05$ among shoot and root groups.

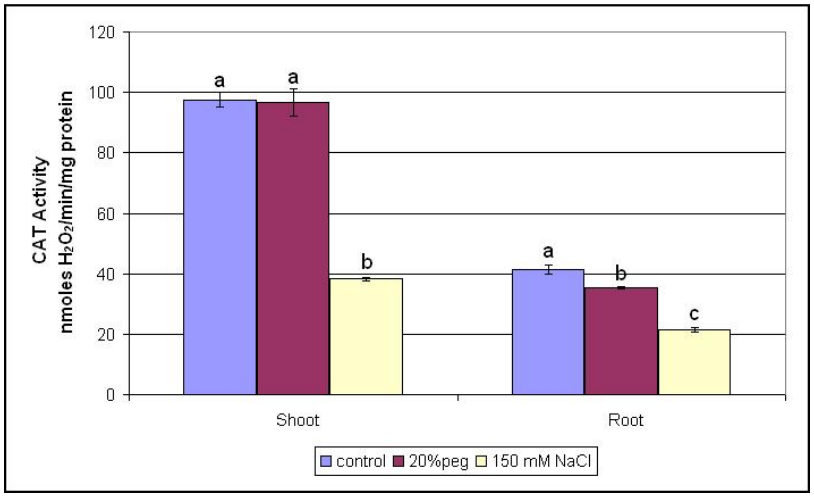

Figure 3.17. Effect of 20 % PEG and 150 mM NaCl treatment on CAT activity. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p< 0.05$ among shoot and root groups.

3.4.3 Glutathione Reductase Activity

Glutathione reductase activity significantly increased in both drought and salt stressed plants. Activity increased in shoots compared to control groups by 43 % and 67 % with drought and salt stress application, respectively. When compared to controls, there was also significant increases observed in roots by drought and salt treatments but they do not have important differences between each other (Fig. 3.18).

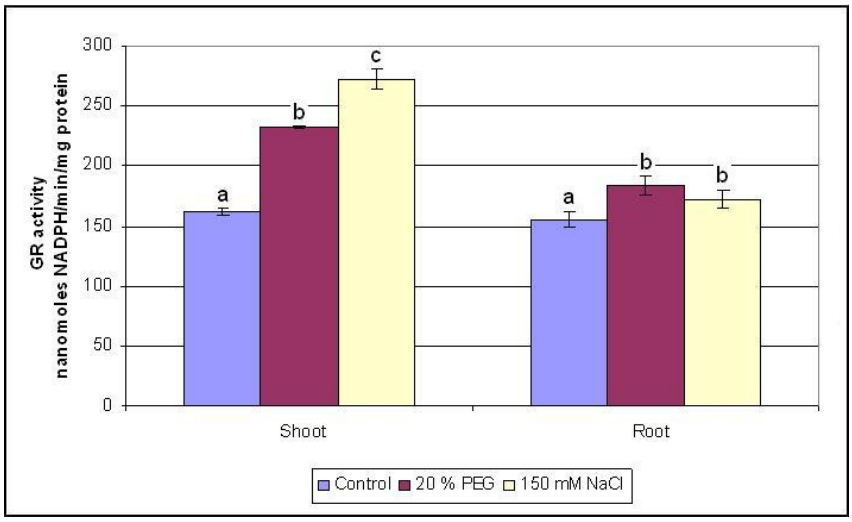

Figure 3.18. Effect of 20 % PEG and 150 mM NaCl treatment on GR activity. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p < 0.05$ among shoot and root groups

3.4.4 Superoxide Dismutase Activity

SOD isozymes in roots and shoots of lentil were determined in gels of native PAGE with negative activity staining and inhibition tests. In shoots and roots Mn SOD, Cu-Zn SOD1 and Cu-Zn SOD2 were identified. Chloroplastic Fe SOD was not determined in shoot tissues under both drought and salt stress applications. (Fig. 3.19)

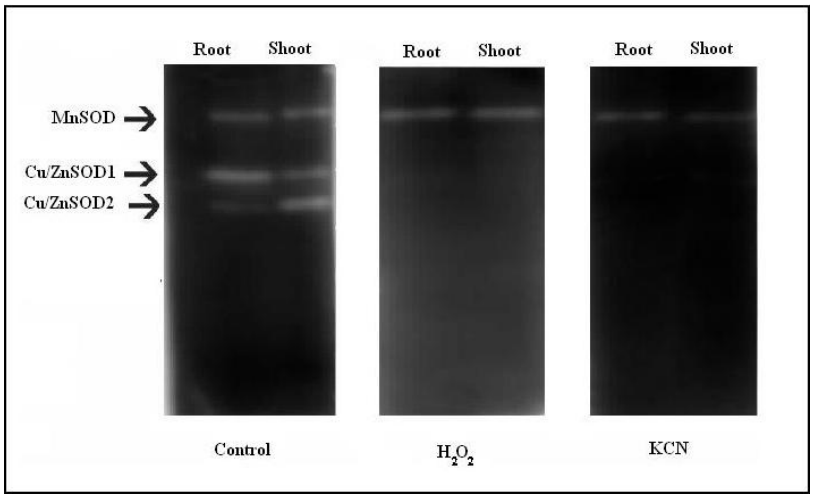

Figure 3.19. SOD isozymes determined by specific inhibitions by H_2O_2 and KCN. MnSOD and 2 isozymes of Cu/ZnSOD (named 1 and 2, according to increased relative mobility) were detected.

SOD activity was calculated by applying known activity of a SOD standard from bovine erythrocyte into one well of the gel. The activity (or, intensity of the Standard was used as a reference for the activities of other isozymes of SOD. (Fig.3.20)

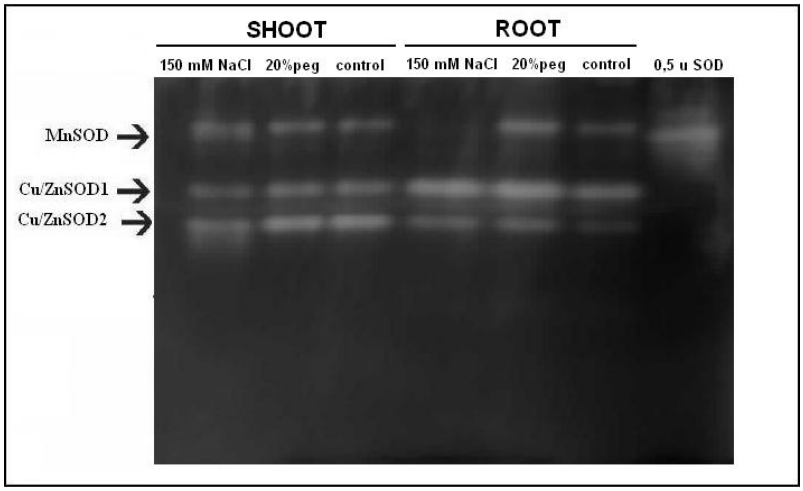

Figure 3.20. Activities of SOD isozymes in shoots and roots of 20 % PEG and 150 mM NaCl treated lentil seedlings.

Manganase SOD (Mn SOD) alteration showed significant increase in roots upon drought stress. Hovewer the activity significantly decreased under salt stressed roots. There were slight but non-significant increases of SOD activity in shoot tissues under both drought and salt treatments (Fig. 3.21). In shoots, Mn SOD had the lowest percentage among other isozymes in total SOD.

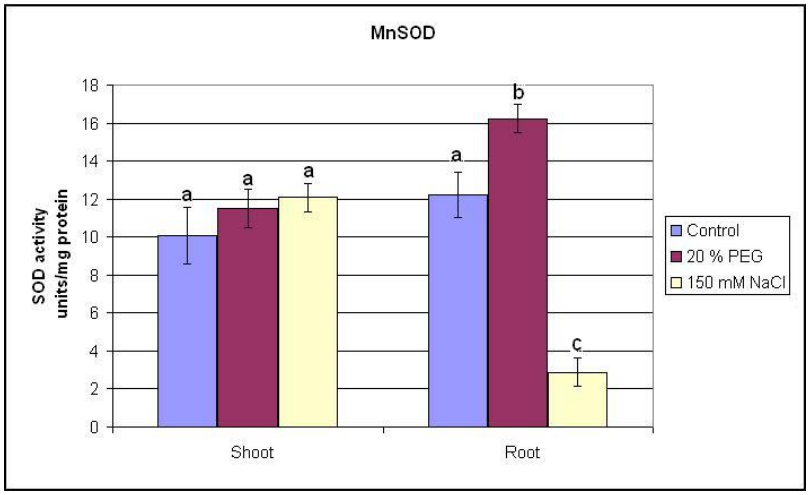

Figure 3.21. Effect of 20 % PEG and 150 mM NaCl treatment on MnSOD activity. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p< 0.05$ among shoot and root groups.

Upon two of the stress treatments, Cu-Zn SOD1 and Cu-Zn SOD2 had slight but non-significant increases in their activities in shoot and root tissues. (Fig 3.22; Fig. 3.23) Cu-Zn SOD1 had the highest percentage among the others in total SOD activity in shoots and it is also true for Cu-Zn SOD2 in roots.

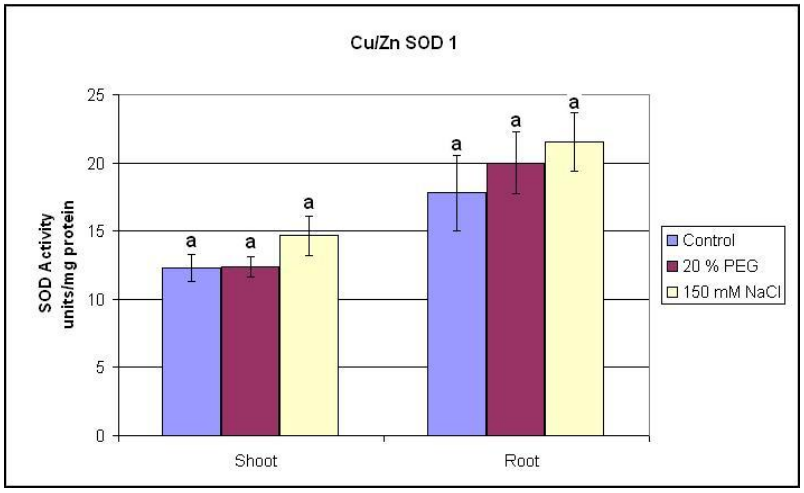

Figure 3.22. Effect of 20 % PEG and 150 mM NaCl treatment on Cu/ZnSOD1 activity. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p< 0.05$ among shoot and root groups.

Figure 3.23. Effect of 20 % PEG and 150 mM NaCl treatment on Cu/ZnSOD2 activity. Vertical bars indicate the mean ± S.E.M values. Different letters indicate the significant difference at $p< 0.05$ among shoot and root groups.

Total SOD and SOD isozyme activities in shoots and roots of lentil seedlings in both control and stress treated plants are given in Fig.3.24 and Table 3.1, 3.2, and 3.3. The results show that in shoots Cu/ZnSOD-2 makes the highest contribution to the total SOD activity, whereas in roots Cu/ZnSOD-1 contributes significantly to the total SOD activity in control, drought and salt groups.

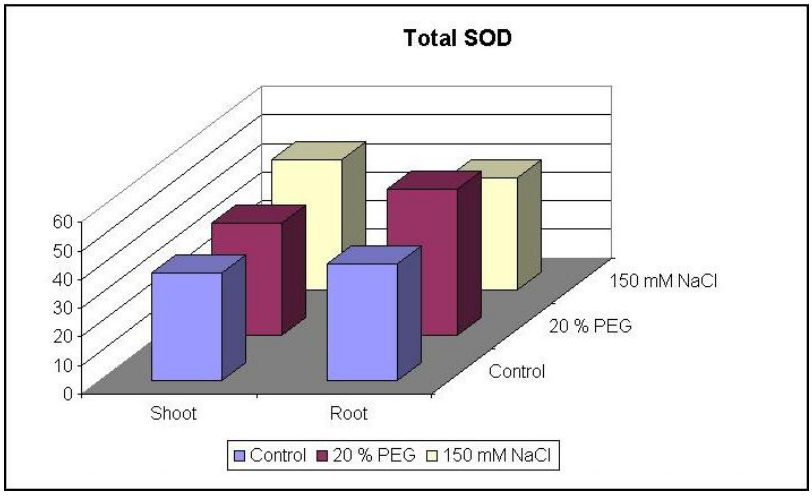

Figure 3.24. Total SOD activities in shoots and roots of 20 % PEG and 150 mM NaCl treated lentil seedlings.

Table 3.1 Total SOD and SOD isozyme activities (determined from the band intensities) in shoots and roots of lentil seedlings grown under normal conditions.

Control	**SOD Activity units/mg protein**		**Activity of each isozyme as % of total SOD Activity**	
	SHOOT	*ROOT*	*SHOOT*	*ROOT*
MnSOD	10,1	12,2	26,9	30
Cu/ZnSOD-1	12,3	17,8	32,8	43,8
Cu/ZnSOD-2	15,1	10,6	40,2	26,1
Total SOD	37,5	40,6	100	100

Table 3.2 Total SOD and SOD isozyme activities (determined from the band intensities) in shoots and roots of lentil seedlings grown under 20 % PEG treatment.

20 % PEG	**SOD Activity units/mg protein**		**Activity of each isozyme as % of total SOD Activity**	
	SHOOT	*ROOT*	*SHOOT*	*ROOT*
MnSOD	11,5	16,2	29,5	31,7
Cu/ZnSOD-1	12,4	20	31,8	39,2
Cu/ZnSOD-2	15	14,8	38,5	29
Total SOD	38,9	51	100	100

Table 3.3 Total SOD and SOD isozyme activities (determined from the band intensities) in shoots and roots of lentil seedlings grown under 150 mM NaCl treatment

150 mM NaCl	**SOD Activity units/mg protein**		**Activity of each isozyme as % of total SOD Activity**	
	SHOOT	*ROOT*	*SHOOT*	*ROOT*
MnSOD	12,1	2,9	26,7	7,45
Cu/ZnSOD-1	14,7	21,5	32,4	55,2
Cu/ZnSOD-2	18,5	14,5	40,8	37,2
Total SOD	45,3	38,9	100	100

CHAPTER 4

DISCUSSION

4.1 Effects of Salt and Drought Stress on Physiological Parameters

After 7 days of salt treatment with 150 mM NaCl, the plants were wilted and nearly stopped growing, necrotic and yellowish leaves were also observed with decreasing areas. Shoot and roots of NaCl treated plants were significantly shorter than the control plants. Mature leaves were observed as more wilted when compared to the younger leaves which may be due to the longer period of exposure to salt toxicity and osmotic stress. Higher accumulation of salt in mature leaves connecting with time period may also be another explanation for this purpose. The wet-dry weight of the lentil seedlings were also decreased significantly with the salt stress application and the decreases were more apparent in shoots than in the roots. Growth retardation was more obvious in leaf tissues when compared to root tissues.

Decreased growth under salt stress has been shown in many leguminous plants. In *Lotus tenuis* and *Lotus corniculatus* which are perennial legumes, NaCl treatment reduced the shoot and root dry mass (Teakle *et al.,* 2006). In chickpea, 100, 200 and 500 mM salt treatment during 4 days caused significant decreases in the parameters of wet-dry weight and length in shoot and root tissues (Eyidoğan and Öz, 2007). The same cultivar of lentil (cv. Sultan1) showed similar results under 100 and 200 mM NaCl application. Shoot-root length and fresh-dry weight, were significantly reduced by NaCl treatments in a dose-dependent manner. Growth retardation was also observed in leaf tissues more apparently when compared to root tissues (Bandeoğlu *et al.,* 2004). In *plantago* which is from order *Lamiales*, growth rate was decreased in salt sensitive cultivar more, when compared to the salt tolerant cultivar (Sekmen *et al.,* 2007).

PEG 6000 treatment for 7 days to induce drought stress decreased lengths and wet-dry weights of shoots significantly. Decreased leaf area was also observed similar with the results of salt treatment which is a beneficial strategy for plants under water deficit conditions to reduce water loss with transpiration. The lengths and wet weights of drought stressed roots were not changed significantly while a significant increase was observed in the dry weights by about 45 % when compared to control groups. Enhancement of root growth in drought conditions provides plant to extract more water from deeper zones. It was also mentioned by Sarker *et al*. (2003) that tap root length and lateral root number are the most important root traits associated with lentil performance under drought conditions in Mediterranean-type environments. Similar with salt stress results, growth retardation was more obvious in leaf tissues when compared to root tissues in drought application. Bartels and Sunkar (2005), suggest that growth arrest can be considered as a possibility to preserve carbohydrates for sustained metabolism, prolonged energy supply, and for better recovery after stress relief.

Effects of drought on plant growth was reported in many species. Similar with our results, drought stress decreased water usage and consequently shoot dry matter production in a leguminous plant, faba bean (Khan *et al*., 2007). PEG 6000 treatment caused more decrease in vegetative growth in drought- sensitive legume *Phaseolus vulgaris* than in drought- tolerant legume *Phaseolus acutifolius.* Root length and wet-dry weight increased upon PEG treatment in drought-tolerant cultivar. Wet-dry weight of roots decreased in drought-sensitive one, while there were no change in root length (Türkan *et al.,* 2005). Nayyar and Gupta (2006) observed the differential sensitivity of wheat and maize to water deficit stress which are the representatives of C_3 and C_4 plants, respectively. Wheat experienced significantly more injury than maize. Relatively, leaves had more injury than roots and their growth was inhibited more than roots in both the plant types similar to our results.

In this study, root lengths of drought treated plants were significantly higher than salt treated plants, hovewer there was no significant change in shoot lenghts. The wet and dry weights of shoot and root tissues of drought treated plants are significantly higher than that of salt treated plants. In drought stress conditions, enhancement of root growth is clearly seen different from salt stress application, which provides plant to extract more water. This result may also shows the relative tolerance of lentil against drought conditions. Shoot growth retardation in two different stress conditions may be due to the decline in photosynthesis.

Relative water content (RWC) estimation is used to evaluate the water status of plant during the stress period. RWC represents a physiological measure of plant water deficit. In this study RWC of shoots were decreased significantly in both drought and salt stress treatments, when compared to control groups, by about 15 % and 38 %, respectively. RWC of salt stressed shoots is significantly lower than drought stressed shoots. It has been shown in many studies that the RWC decreases with the increased sensitivity to a given stress. PEG 6000 treatment to induce drought stress, had no effects on RWC in tolerant *P. acutifolius* while RWC in sensitive *P. vulgaris* decreased (Türkan *et al.,* 2005). In wheat, the relative water content declined significantly with induction and duration of salt stress. However, decline in RWC was lower in salt-tolerant cultivar as compared to salt-sensitive one (Mandhania *et al*., 2006). It is also suggested that reduced leaf RWC seems to suppress the growth of *Plantago media*, a salt sensitive cultivar, under salt stress as it implies the water status in the plant (Sekmen *et al.,* 2007).

4.2 Effects of Salt and Drought Stress on Lipid Peroxidation and Electrolyte Leakage

When plants are subjected to environmental stresses involving salinity and drought, ROS production overcomes the antioxidant system capacity and oxidative stress occurs which results in cytotoxic protein damage, DNA damage, and lipid peroxidation (Yazıcı *et al*., 2007). Lipid peroxidation generates a complex variety of

products, many of which are reactive electrophiles. Malondialdehyde which is marker of the injury caused by stress, is one of the end products of lipid peroxidation by free radicals. Its reaction with thiobarbituric acid to form an intensely colored chromogen makes it a convenient biomarker for determination of lipid peroxidation (Marnett, 1999). The effects of various abiotic stresses on membrane integrity has been studied in many plant species by electrolyte leakage tests and malondialdehyde content measurements.

We investigated the drought and salt induced damage on membranes by MDA content measurements and electrolyte leakage tests. According to our results lentil roots tolerate both drought and salt stress much more effectively than shoots. In drought stress application, MDA amounts showed significant increase in shoot tissues by 47 % while there were no significant change in root tissues. Salt stress significantly affected the MDA contents of shoots and roots by 150 % and 130 %, respectively. For both root and shoot tissues MDA contents were significantly higher in salt treated plants than drought treated plants which corresponds to higher lipid peroxidation. Drought stress did not cause any significant changes in electrolyte leakage of membranes. From this result it can be suggested that significant increase in lipid peroxidation by 47 % in drought stressed shoots may not contribute to the disruption of membrane integrity at all. Protective role of a common osmolyte, proline may also be integrated with the conservation of membrane integrity. Being not fully but partially the 47 % increase may also come from age dependent manners. Salt stressed shoot and root tissues showed significant increase in electrolyte leakage of membranes when compared to controls, by almost 5 fold and 2,5 fold, respectively. This result was well correlated with the MDA content measurements.

A supporting result comes from the same cultivar for salt stress application. Bandeoğlu *et al.* (2004) showed the reduced membrane damage of root tissues compared to shoots, under salinity stress with MDA content measurements and electrolyte leakage test.

In chickpea, another leguminous plant, MDA contents were found higher in shoots than that of roots under salinity stress (Eyidoğan and Öz, 2007). Also, in wheat and maize, drought stress application increased electrolyte leakage and MDA contents in shoots more than in roots (Nayyar and Gupta, 2006). *Phaseolus vulgaris,* a drought sensitive legume, showed increased lipid peroxidation in leaves when compared to drought tolerant cultivar*, Phaseolus acutifolius* (Türkan *et al.,* 2005). In a recent study (Eraslan *et al.,* 2007) it was shown that membrane permeability of the lettuce leaves increased in the NaCl treatment, parallel to the significant increase in lipid peroxidation (MDA). The reducing effects of drought acclimation on lipid peroxidation was also shown in wheat seedlings under drought stress conditions (Selote *et al.,* 2004). Our results indicate that lentil seedlings are much more tolerant to drought stress than the salinity stres, in terms of electrolyte leakage and lipid peroxidation.

4.3 Effects of Salt and Drought Stress on Chlorophyll Fluorescence

In this study chlorophyll fluoresecence analysis included 'Fv/Fm' and 'Yield' parameters. 'Fv/ Fm' is the maximum photochemical yield of PSII while the 'Yield' is the effective (actual) PSII photochemical yield. Since abiotic stress affects the function of PSII, directly or indirectly, chlorophyll fluorescence can represent a useful tool to get rapid evidence of stress conditions affecting the photosynthetic activity and to get a quantification of the effect of stress on the productivity (Torzillo *et al*.).

The Fv/ Fm ratio indicates the maximum (potential) quantum yield of photosystem II in dark adapted leaf tissues and its value decreased significantly in salt treated plants when compared to the controls and drought treated plants in our study. 'Yield' has fundamental importance since it provides data on the effective or actual efficiency of the overall photosynthetic process under steady-state illumination. Salt stress also decreased the yield of photosynthetic efficiency of photosystem II significantly. Drought stress did not cause any significant changes

in the Fv/Fm and Yield values, except for the very slight decreases. A supporting result comes from another legume, *Phaseolus vulgaris*. The Fv/ Fm ratio did not increase in three different genotypes, only showed a small tendency to decrease (Zlatev and Yordanov, 2004). It was also shown in *Arabidopsis* that *F*v/*F*m ratio was unaffected by drought stress in young leaves and slightly declined in drought-stressed mature leaves. A considerable drop in PSII photochemical yield was detected with drought treatment in mature leaves of *Arabidopsis,* but a slight drop in drought-stressed young leaves . Upon rewatering for 24 h, the photosynthetic parameters of mature leaves recovered rapidly to those of controls (Jung, 2004). In our study decreases in the Fv/Fm and Yield values under salinity may indicate the limitations on photosynthesis. It was suggested by Sekmen *et al.* (2007), that the decrease in chlorophyll fluorescence can be attributed to the oxidation of chlorophyll. This decrease may be also due to the ionic toxicity effect of salt stress. Supporting our results, a dose-dependent decrease was observed in Fv/Fm and quantum yield of photosynthesis under salt stress was observed in chickpea seedlings under salinity (Eyidoğan and Öz, 2007).

4.4 Effects of Salt and Drought Stress on Proline Content

Plants cope with osmotic stress by synthesizing and accumulating some compatible solutes, which are termed as osmoprotectants or osmolytes, and the process is known as 'osmotic adjustment' or osmoregulation. Proline is one of the most common osmolyte, accumulating in response to various environmental stresses in plants. It maintains cell turgor, stabilizes membranes and maintain protein conformation at low leaf water potentials. It can also be inferred as a free radical scavenager. Proline is also known to be involved in reducing the photodamage in the thylakoid membranes by scavenging and/or reducing the production of singlet oxygen ($^1O_2$) (Reddy *et al.,* 2004).

Accumulation of proline under stress in many plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher

in stress-tolerant than in stress-sensitive plants (Ashraf, 2007). In our study significant changes in extremely higher values were observed in proline contents of shoot and root tissues from drought and salt stressed plants. Proline content of drought stressed roots was 8,9 fold and salt stressed roots was 5,9 fold higher than control groups. Proline accumulation is significantly higher in drought stressed roots than that of salt stressed ones. These results are correlated with MDA contents and electrolyte leakage tests. Electrolyte leakage and MDA content results were not significantly different in drought stressed roots than control plants while significant increases were observed in salt treated roots which may be correlated with the lower proline accumulation. Drought and salt treated shoot tissues had approximately 5,2 and 6,5 fold higher proline contents than control plants, respectively. Proline accumulation of salt stressed shoots were also significantly higher than drought stressed shoots. In drought stressed shoots these results are correlated with the non-changed electrolyte leakage test compared to control and lower MDA contents. A more common explanation for the accumulation of proline is that it confers advantages by protecting membranes and proteins when RWC decreases (Reddy *et al*., 2004). Our results are also supported by this explanation as the drought stressed plants have adequate physiological parameters under a significant decrease in RWC. Proline contents and RWC values of drought and salt stressed shoots were also in a good correlation within themselves. In salt treated shoots proline concentrations are extremely higher than controls. Hovewer the electrolyte leakage and MDA estimations are higher than controls, too. It can be suggested that proline accumulation was increased with the significant decrease in RWC but can not cope to maintain membrane integrity at all. It has been shown in many studies (lettuce: Eraslan *et al.,* 2007; purslane: Yazıcı *et al*., 2007; lentil: Bandeoğlu *et al.,* 2004; chickpea: Eyidoğan and Öz, 2007) that salinity causes increases in proline content. Drought stress application resulted in an increase in proline contents of 5 different sunflower varieties both in their root and shoot tissues (Manivannan *et al.,* 2007) .

4.5 Effects of Salt and Drought Stress on Hydrogen Peroxide Content

It is the most stable of the reactive oxygen species, and therefore plays a crucial role as a signalling molecule in various physiological processes under environmental stress conditions. On the other hand its uncontrolled accumulation is toxic and can initiate various deteriorations within the organism. Removing of hydogen peroxide is maintained by catalases and several peroxidases (Slesak *et al.,* 2007).

In this study salt stress significantly increased the hydrogen peroxide concentration in shoots and roots. The rise was much higher in shoots than roots when compared to controls. Activity of the superoxide dismutase, major H_2O_2 generating enzyme, was also higher in shoots. Approximately 64 % increase was shown in shoots while it is 21 % in roots. These results are well correlated with the study of NaCl stress with the same cultivar (Bandeoğlu *et al.,* 2004). The conditions in the elevations of hydrogen peroxide concentrations in different tissues under salt stres treatments are also supporting the results of MDA contents, electrolyte leakage tests and RWC, thus the shoots suffer more from stress. Lipid peroxidation (MDA) rate may also be contributed to the ability of H_2O_2 to produce $OH^{\cdot}$. The results of Mandhania *et al.* (2006) also showed that higher accumulation of H_2O_2 accompanies the higher lipid peroxidation in wheat seedlings under NaCl stress.

Under drought stress conditions shoots had a non-significant increase in H_2O_2 concentration, as well as there were no differences in the activities of SOD and APX, which are the hydrogen peroxide generating and detoxifying enzymes, respectively. Electrolyte leakage was also observed as non-changed in drought stressed shoots meaning that, lipid peroxidation in drought stressed shoots may not contribute to the disruption of membrane integrity at all. Ability of H_2O_2 to produce $OH^{\cdot}$ may also be contributed with the lipid peroxidation and the protective role of a common osmolyte, proline may also be integrated with the conservation of membrane integrity. The H_2O_2 content of root tissues was found significantly higher than controls. As supporting this SOD activities were also higher. The increment was also

higher than shoots. The enhanced accumulation of H_2O_2 does not correlate with the non-changed MDA contents and electrolyte leakage tests compared to controls. This condition may be explained with the protective role of proline which showed an extreme increase in drought stressed roots by almost 8,9 fold. On the other hand, elevated production of H_2O_2 as a signalling molecule can be contributed with the induction of antioxidative systems and contributing this, APX and GR showed increased activities. Hovewer, in wheat and maize water deficit stress caused H_2O_2 content elevation in shoots more than in roots (Nayyar and Gupta, 2005). Drought stress effects on H_2O_2 content in plant tissues was also shown in two populations in *Populus*, which were from wet and dry climate regions. The levels of H_2O_2 were significantly increased by drought, additionally wet climate population exhibited higher level of H_2O_2 than the dry climate population (Lei *et al.,* 2006).

4.6 Effects of Salt and Drought Stress on Catalase Activity

Catalase is a heme containing enzyme that catalyzes the dismutaion of hydrogen peroxide into water and oxygen thus protecting the cell from the deleterious effects of H_2O_2. It is localized in mitochondria, glyoxysomes, and mostly in peroxisomes. Catalase possesses a very low affinity for H_2O_2 and only active at relatively high H_2O_2 concentrations (Gechev *et al.,* 2006).

In this study, CAT activity was significantly higher in drought treatments than salt treatments in both shoot and root tissues. Under drought stress conditions, catalase activity was not changed significantly in shoots hovewer a significant decrease was observed in roots. These results are well corelated with the H_2O_2 concentrations. Similarly in shoots, CAT activity and H_2O_2 concentrations did not change. In roots, it was observed that CAT activity significantly decreased, and H_2O_2 concentrations significantly increased. A supporting result comes from another legume, drought tolerant *Phaseolus acutifolius* and drought sensitive *Phaseolus vulgaris.* CAT activity did not increase in the leaves of both cultivars upon PEG mediated water stress. Hovewer, constitutive levels were higher in drought-tolerant cultivar than

drought-sensitive one (Türkan *et al.,* 2005). In another study, the drought induced reductions in CAT activities were observed in young leaves of *Arabidopsis thaliana*, whereas CAT activities increased in drought stressed mature leaves (Jung, 2004).

150 mM salt stress application caused significant decreases in CAT activity by approximately 60 % in shoots and 48 % in roots. These results are correlated with the increases of H_2O_2 concentrations in both tissues. In a study with the same cultivar salt treatment at 100 mM NaCl or 200 mM NaCl did not cause a significant change in CAT activity of leaf and roots of lentil seedlings (Bandeoğlu *et al*., 2004). But supporting our results, the activity was found to be much higher in leaves than in roots of lentil under both control and stress conditions. In another study, salt stress in wheat seedlings caused an increase in CAT activity (Mandhania *et al.,* 2006). In chickpea, salinity caused a decrease in CAT activity in leaf tissues whereas there was also an increase in root tissues (Eyidoğan and Öz, 2007). In maize, salt stress did not affect the leaf CAT activity of salt-tolerant cultivar but reduce the activity significantly in roots of tolerant cultivar and in both tissues of salt-sensitive cultivar (de Azevedo Neto *et al*., 2006).

4.7 Effects of Salt and Drought Stress on Ascorbate Peroxidase Activity

Ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to water and has high specificity and affinity for ascorbate as reductant which is the first enzyme of the Halliwell-Asada cycle. It is predominantly found in cytosol, mitochondria, and chloroplasts. (Mittler, 2002)

In our results, there was a very slight nonsignificant decrease in the activity of APX in drought stressed shoot tissues while a significant increase was observed in root tissues. The CAT activity was also not changed in drought stressed shoots compared to control, just like the APX, and the unchanged concentration of H_2O_2 compared to control, is in a good correlation with these results. The different affinities of APX (μM range) and CAT (mM range) for H_2O_2 suggest that they belong to two different

classes of H_2O_2-scavenging enzymes. APX might be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during stress (Mittler, 2002). Whereas in drought stressed roots APX activity increased but there was also a significant increment in H_2O_2 concentration. These may be explained by the decreasing activity of catalase and inefficient activity of APX. Contributing the increase in H_2O_2 concentration, SOD activity also increased which is a H_2O_2 generating enzyme. Differing from our results, several studies showed the increased activities of APX in shoot tissues under drought stress conditions. APX actvity was shown to be increased in drought stressed leaves of *Phaseolus acutifolius*, which is also a leguminous plant (Türkan *et al.,* 2005). The different cultivars of *Populus* which are from wet and dry climate regions, showed significantly increased APX activities in their leaves under drought stress conditions (Lei *et al.,* 2006). At high stress levels, activity of APX was shown to be decreased in both shoot and root tissues of maize and wheat (Nayyar and Gupta, 2006).

Salt stress application increased the APX activity significantly in both shoot and root tissues by almost 23 % and 94,5 %, respectively. APX activity is significantly higher in salt stressed plants than drought stressed ones. Despite the increased APX activity in shoots, which is also significantly higher than the activity of drought stressed ones, the H_2O_2 concentration is significantly higher than control and drought treated plants.

This may be due to the lower activity of CAT to remove H_2O_2. As a contributing result, in salt treated roots APX activity is significantly higher than drought treated roots and the H_2O_2 concentrations are significantly lower. As supporting our results APX activity was found to be increased in leaves under salt stress in lettuce (Eraslan *et al.,* 2007). In rice APX activity increased in roots of salt tolerant cultivar but remained unchanged in salt sensitive one (Demiral and Türkan., 2005). In maize, APX activity in leaves of both tolerant and sensitive genotypes was remarkably increased as a result of salt stress, not affected in roots of salt-tolerant and slightly decreased in roots of salt-sensitive (de Azevedo Neto *et al*., 2006). APX activity in leaves of chickpea increased upon salt stress but remain unchanged in roots (Eyidoğan and Öz, 2007).

4.8 Effects of Salt and Drought Stress on Glutathione Reductase Activity

Glutathion reductase (GR) is found in cytosol, chloroplast, mitochondria and peroxisomes and catalyzes the last step of Halliwell-Asada cycle. It maintains the reduced form of glutathione which is itself a free radical scavenger in a NADPH consuming reaction.

Effect of salt and drought stress on GR activity has been studied in several plants. In rice roots, GR activity was shown to be significantly decreased in both tolerant and sensitive cultivars under salt stress (Demiral and Türkan, 2005). As supporting our results, leaves and roots of two maize cultivars, differing in their salt tolerance, showed increased GR activity (de Azevedo Neto *et al.,* 2006). Upon drought stress application GR activity was found to be increased in wheat and maize (Nayyar and Gupta, 2006), populus (Lei *et al*., 2006), common bean (Türkan *et al.,* 2005).

Glutathione reductase activity was significantly increased in both drought and salt stressed plants in this study. In drought stressed shoots GR activity significantly increased, contrary to the APX and CAT activities. It can be suggested that, accompanying with each other GR and proline, are the important elements against oxidative stress in shoot tissues, under drought stress. Its activity also increased in roots under drought treatment similar with APX and proline. Upon salt stress treatment GR activity increased in shoots, and it was also significantly higher than that of drought treated ones, similar with APX. GR, APX and proline are shown to be responsible for antioxidative defense in shoots under salt stress conditions according to these results. Also in roots, upon salt treatment, GR activity increased significantly, and showed no difference from drought treated ones. Again in the salt stressed roots GR and APX activities are shown to be responsible for defense against oxidative stress.

4.9 Effects of Salt and Drought Stress on Superoxide Dismutase Activity

The superoxide dismutase enzymes (SOD) remove superoxide, $O_2^{\cdot -}$, by catalyzing its dismutation, one $O_2^{\cdot -}$ being reduced to H_2O_2 and another oxidized to O_2. Superoxide dismutases constitute the first line of defence against ROS within a cell. (Halliwell, 2006) Based on the metal co-factor used by the enzyme SOD's are classified into three groups. Iron SOD (Fe SOD), manganase SOD (Mn SOD) and copper-zinc SOD (Cu-Zn SOD). In this study, in shoots and roots for both stress applications; Mn SOD, Cu-Zn SOD1 and Cu-Zn SOD2 were identified. Chloroplastic iron SOD was not determined in shoot tissues under both applications. Manganase SOD (Mn SOD) alteration showed significant increase in roots upon drought stress. Hovewer the activity significantly decreased under salt stressed roots. There were slight but non-significant increases of SOD activity in shoot tissues under both drought and salt treatments. In shoots, Mn SOD had the lowest percentage among other isozymes in total SOD quantification. Upon two of the stress treatments, Cu-Zn SOD1 and Cu-Zn SOD2 had slight but non-significant increases in their activities in shoot and root tissues (Fig. 3.22; Fig. 3.23). Cu-Zn SOD1 had the highest percentage among the others in total SOD activity in shoots and it is also true for Cu-Zn SOD2 in roots.

When total SOD activity was considered, the results are correlated with the previous parameters in drought stress conditions. In shoots SOD activity did not change like the H_2O_2 content and APX activity. Roots showed increased SOD activity with the increased H_2O_2 content. Elevations in the H_2O_2 detoxifying enzyme, APX, also contributes with the non- changed MDA and electrolyte leakage results. Under salt stress conditions SOD activity increased in shoots, also resulting an increase in H_2O_2 contents. Despite the increases in APX and GR activities, and proline contents, increased membrane damage was observed from MDA and electrolyte leakage results. This can be due to the deleterious effects of ionic stress on membranes and altered but not sufficient antioxidative activities.

SOD activity changes have been studied in many other plants under different stress conditions. In shoots of purslane, 140 mM NaCl treatment for 18 days caused a decrease in superoxide dismutase activity (Yazıcı *et al*., 2007). In liquorice seedlings, which is a widely used herbal medicine and shows high stress-resistance, the activities of SOD and POD were upregulated by salt and drought stress, while CAT activity decreased (Pan *et al*., 2006).

CHAPTER 5

CONCLUSION

In this study a Turkish lentil (*Lens culinaris* Medik., cv Sultan-1) cultivar has been characterized with respect to certain physiological and biochemical parameters under salt and drought stress. Alterations in antioxidant and osmoprotective mechanisms, as well as the visible symptoms of salt and drought stress were observed.

The results presented here suggest that lentil is quite tolerant to drought but sensitive against NaCl. In salt stress conditions, decreases in wet-dry weight, RWC, shoot-root length and chlorophyll fluorescence measurements are indicating the sensitivity. Higher MDA concentration and higher electrolyte leakage amounts also supported this conclusion.

Shoot and root tissues respond differently towards stress conditions. Roots have better protective mechanisms against membrane damage than shoots.

Proline, which is a strong osmoprotectant, is a protective component in lentil seedlings under both salt and drought stresses.

APX, GR and proline are important components of protection for both tissues under salt stress conditions. GR and proline are also maintaining the main protective mechanism against drought stress effects. SOD is active in drought stressed roots and salt stressed shoots, where the H_2O_2 contents were also observed to be increased.

Effects of drought and salt stress on antioxidant defense systems of lentil were investigated in a comparative manner and the study may be used as a base for further analysis of differential effects and relative responses of lentil against various environmental stresses. However, for a better understanding of the comparisons

between different stress factors, it is of importance to study with cultivars having differential sensitivities.

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APPENDIX A

OVERALL CHANGES IN ALL PARAMETERS UNDER DROUGHT AND SALT STRESS

A. Physiological changes under 20 % PEG and 150 mm NaCl stress. Numbers indicate the means ±S.E.M. Given in paranthesis are the percent control values and the significant differences from control values at $p < 0.05$ are shown with * asterisks.

	SHOOT			***ROOT***		
	Control	**20 %PEG**	**150 mM NaCl**	**Control**	**20 %PEG**	**150 mM NaCl**
Length (cm)	21,88± 0,29	14,28± 0,34 (65,2)*	11,81±1,05 (54,1)*	12,33± 0,37	10,58± 0,30 (85,8)*	8,43± 0,58 (68,3)*
Wet Weight (mg)	238,94±6,99	129,74±8,39 (54,2)*	48,32±5,22 (20,2)*	152,3±9,19	136,94± 11 (89,9)	58,74± 5,97 (38,5)*
Dry Weight (mg)	25,24±0,956	20,94± 1,27 (82,9)*	10,8±0,705 (42,7)*	10,36±0,719	15,12± 1,42 (145,9)*	7,34±0,981 (70,8)*
RWC (%)	83,08± 1,33	70,88± 1,64 (85.3)*	51,32±3,58 (61,7)*			

B. Changes in electrolyte leakage, MDA, proline, H_2O_2 and chlorophyll fluorescence under 20 % PEG and 150 mm NaCl stress. Numbers indicate the means ±S.E.M. Given in paranthesis are the percent control values and the significant differences from control values at $p< 0.05$ are shown with * asterisks.

	SHOOT			***ROOT***		
	Control	**20 %PEG**	**150 mM NaCl**	**Control**	**20 %PEG**	**150 mM NaCl**
Ion leakage (%)	9,15± 0,851	10,87± 1,31 (118,7)	49,02± 2,64 (535,7)*	11,36± 0,89	11,26± 0,37 (99,1)	26,7± 2,02 (235)*
MDA (nmole/gFW)	8,29± 0,18	12,2± 0,87 (147,1)*	20,72±1,84 (249,9)*	3,29± 0,38	4,56± 0,82 (138,6)	7,62± 0,58 (231,6)*
Proline (µmol/gFW)	7,73±0,495	40,59±2,83 (525)*	50,63±0,29 (654,9)*	2,61±0,266	22,93±2,71 (878,5)*	15,17±0,69 (581,2)*
H_2O_2 (nmole/gFW)	32,34± 1,88	40,02± 2,95 (123,7)	52,9± 2,23 (163,5)*	63,47± 1,78	106,1±5,61 (167,1)*	76,99±2,02 (121,3)*
Fv/Fm	0,84± 0,01	0,82±0,01 (97,6)	0,54± 0,07 (64,2)*			
Yield	0,82± 0,01	0,78± 0,02 (95,1)	0,28± 0,05 (34,1)*			

C. Alterations in antioxidant enzyme activities under 20 % PEG and 150 mm NaCl stress. Numbers indicate the means ±S.E.M. Given in paranthesis are the percent control values and the significant differences from control values at $p< 0.05$ are shown with * asterisks.

	SHOOT			***ROOT***		
	Control	**20 %PEG**	**150 mM NaCl**	**Control**	**20 %PEG**	**150 mM NaCl**
APX (nmoles Asc/min/mg protein)	639,1±24,8	618,5± 13,2 (96,7)	786,4±32,1 (123)*	491±7,35	564±9,11 (114,8)*	955±43,1 (194,5)*
CAT (nmoles H_2O_2/min/mg protein)	97,6± 2,46	96,56± 4,57 (98,9)	38,31±0,556 (39,2)*	41,37±1,42	35,3±0,424 (85,3)*	21,42±0,68 (51,7)*
GR (nmoles NADPH/min/mg protein)	162,2±6,69	232,3±8,28 (143,2)*	272,3± 7,65 (167,9)*	155,5±2,85	183,7±1,59 (118)*	172±8,37 (110,3)*
Cu/ZnSOD1 (units/mgprotein)	12,29±1	12,37±0,752 (100,6)	14,66±1,46 (119,2)	17,79±2,78	19,98±2,28 (112,3)	21,55±2,12 (121,1)
Cu/ZnSOD2 (units/mgprotein)	15,11±1,86	15,04±1,28 (99,5)	18,55±1,86 (122,7)	10,60±2,39	14,79±1,67 (139,5)	14,51±1,51 (136,8)
MnSOD (units/mgprotein)	10,09±1,49	11,50±1,02 (113,9)	12,09±0,747 (119,8)	12,20±1,19	16,24±0,749 (133,1)*	2,88±0,747 (23,6)*
TotalSOD (units/mgprotein)	37,5	38,9 (103,7)	45,3 (12,08)	40,6	51 (125,6)	38,9 (95,8)

APPENDIX B

BASIS OF NEGATIVE ACTIVITY STAINING

O_2 can be generated by photochemical processes and that $O_2^{\cdot -}$ can reduce tetrazolium dyes to the insoluble formazans. These facts can be used for devising assays for SOD, which could be applied in the free solutions or acrylamide gels. Illuminations of solutions containing riboflavin lead to the photooxydation of TEMED and photoreduction of riboflavin. The reduced flavin interacts with oxygen to generate $O_2^{\cdot -}$. If NBT is also present, it will be reduced to the blue formazan, and SOD, if present, will prevent this blueing by intercepting the $O_2^{\cdot -}$. When applied to the acrylamide gels, zones containing SOD remain achromatic, while the rest of the gel turn blue.

APPENDIX C

1-D PAGE STOCK SOLUTIONS AND GEL FORMATION

I. Acrylamide Stock (30 % T, 2,67 % C)

Acrylamide 73 g
N'N'-bis-methylene-acrylamide 2 g
Dissolved in 150 ml distilled water, completed to 250 ml with distilled water, filtered and stored at 4º C in dark.

II. Seperating Gel Buffer Stock (1,5 M Tris-Hcl pH 8.8)

Trizma base 54,45 g
Dissolved in 150 ml distilled water, adjusted the pH 8.8 and completed to 300 ml with distilled water. Filtered and stored at 4º C in dark.

III. Stacking Gel Buffer Stock (0,5 M Tris-Hcl pH 6.8)

Trizma base 6 g
Dissolved in 60 ml distilled water, adjusted the pH 6.8 and completed to 100 ml with distilled water. Filtered and stored at 4º C in dark.

IV. Running Buffer 5X (25 mM Tris, 192 mM Glycine

Trizma base 30 g
Glycine 144 g
Dissolved in sufficient amount of water and raised to 2 L with distilled water. Diluted to 1X before electrophoresis.

V. Gel Formation

Seperating gel formation (for 5 ml):

1,65 ml dH_2O

1.25 ml seperating gel buffer

2 ml acrylamide-bis solution (30% T)

35 µl APS (10%)

3.5 µl TEMED

Stacking gel formation (for 2.5 ml):

1,7ml dH_2O

0.315 ml stacking gel buffer

0.415 ml acrylamide-bis solution (30% T)

15 µl APS (10%) 3.75 µl TEMED