EFFECT OF SALT STRESS ON THE ANTIOXIDANT DEFENSE SYSTEM IN LENTIL (*Lens Culinaris* M.) SEEDLINGS

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EBRU BANDEOĞLU

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Prof. Dr. Tayfur Öztürk
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Faruk Bozoğlu
Head of the Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope of quality, as a thesis for the degree of Master of Science.

Prof. Dr. Meral Yücel
Co-Supervisor

Prof. Dr Hüseyin Avni Öktem
Supervisor

Examining Committee Members

Prof. Dr. Cumhur Çökmüş

Prof. Dr. Faruk Bozoğlu

Prof. Dr. Tülin Güray

Prof. Dr. Meral Yücel

Prof. Dr. Hüseyin A. Öktem

ABSTRACT

EFFECT OF SALT STRESS ON THE ANTIOXIDANT DEFENSE SYSTEM IN LENTIL (*Lens Culinaris* M.) SEEDLINGS

Bandeoğlu, Ebru

M.S., Department of Biology
Supervisor: Prof. Dr. Hüseyin Avni Öktem
Co-supervisor: Prof. Dr. Meral Yücel

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In this study 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), cell membrane stability, lipid peroxidation in terms of malondialdehyde (MDA) content, H_2O_2 content, proline content and physiological changes (as wet-dry weights, root-shoot lengths) of 14 days old lentil (*Lens culinaris*, Medik. cv. Sultan) seedlings subjected to 100 mM and 200 mM NaCl stress for 5 days were studied.

Four SOD isozymes were differentiated in lentil namely Cu/ZnSOD1, Cu/ZnSOD2, FeSOD and MnSOD, FeSOD being only in leaves. Salinity enhanced the activity of Cu/ZnSOD isozymes and the increase was much higher in roots when compared to leaves. MnSOD activity increased only at 200 mM NaCl stress while FeSOD activity decreased at both stress concentrations. Total SOD activity enhanced in both tissues but the increase was more pronounced in roots. APX and GR activity was induced under both salt treatments however a slight decrease in CAT activity was detected in both tissues.

Root-shoot lengths and wet-dry weights significantly decreased by salt treatments. A higher level of lipid peroxidation (MDA content) was observed in leaves when compared to roots at both NaCl stresses. This finding was matched with electrolyte leakage test results in which a significant increase was observed in leaves, while in roots no change was detected.

Proline content increased in both tissues in correlation with increased salt stress. A higher increase was observed in H_2O_2 content in leaves when compared to roots.

The results obtained suggest that leaves are more susceptible to salt stress. In roots Cu/ZnSOD, APX and proline seem to play an important role in defense against salt stress by effectively removing reactive oxygen species.

Key words: Lentil, salt stress, oxidative stress, SOD, APX, GR, CAT, proline, H_2O_2, lipid peroxidation, MDA.

ÖZ

TUZ STRESİNİN MERCİMEK (*Lens culinaris* M.) FİDELERİNDEKİ ANTİOKSİDAN SİSTEM ÜZERİNE ETKİSİ

Bandeoğlu, Ebru

Yüksek Lisans, Biyoloji Bölümü
Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem
Ortak Tez Yöneticisi: Prof. Dr. Meral Yücel

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Bu çalışmada, 5 gün süreyle 100 ve 200 mM NaCl stresine maruz kalmış 14 günlük mercimek fidelerinin kök ve yaprak dokularındaki süperoksit dismutaz (SOD: EC 1.15.1.1), katalaz (CAT: EC 1.11.1.6), askorbat peroksidaz (APX: EC 1.11.1.11) ve glutatiyon redüktaz (GR: EC 1.6.4.2) gibi antioxidan enzimlerin aktiviteleri, lipit peroksidasyonu (malondialdehid miktarı olarak), H_2O_2 ve prolin miktarları ile fizyolojik değişimler (yaş-kuru ağırlık ve kök-yaprak uzunlukları) normal şartlarda büyütülen mercimek fideleriyle karşılaştırılarak incelendi.

Mercimek fidelerinde Cu/ZnSOD1, Cu/ZnSOD2, MnSOD ve FeSOD olmak üzere 4 farklı SOD izoenzimi saptandı. Tuz stresi her iki Cu/ZnSOD izoenziminde artışa neden olurken, kök dokusunda bu artış daha belirgin bir biçimde gözlendi. MnSOD aktivitesi sadece 200 mM stress altında artış gösterirken, FeSOD aktivitesi her iki tuz stresinde de azaldı. Her iki dokuda da APX ve GR aktiviteleri tuz stresiyle paralel olarak arttı, ancak tuz stresi CAT aktivitesinde bir miktar düşüşe yol açtı.

Kök-yaprak uzunlukları ve yaş-kuru ağırlıklar tuz stresi altında önemli ölçüde düştü. Tuz stresi yaprakta lipit peroksidasyonunu (MDA konsantrasyonu) ve hücre zarı geçirgenliğini önemli miktarda arttırırken, kök dokusunda belirgin bir değişim gözlenmedi.

Prolin miktarı her iki dokuda da tuz stresiyle paralel olarak arttı. Tuz stresi her iki dokuda da H_2O_2 miktarının artmasına neden olurken, yapraktaki artış köke göre çok daha fazla gerçekleşti.

Elde edilen sonuçlar yaprağın tuz stresine daha fazla duyarlı olduğunu göstermektedir. Kökte Cu/ZnSOD, APX ve prolinin tuz stresine karşı direnç göstermede önemli bir rol oynadığı gözlenmiştir.

Anahtar Kelimeler: Mercimek, tuz stresi, oksidatif stres, SOD, APX, GR, CAT, proline, H_2O_2, lipit peroksidasyonu, MDA.

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$^{\cdot}OOH$	Perhydroxyl radical
$^1O_2$	Singlet oxygen
AOS	Active oxygen species
APX	Ascorbate peroxidase
AsA	Ascorbate
CAT	Catalase
DHAR	Dehydroascorbate reductase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O	Hydrogen peroxide
KCN	Potassium cyanide
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
NaCl	Sodium chloride
$O_2^{\cdot-}$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical

PAGE	Polyacrylamide gel electrophoresis
POD	Peroxidase
ROOH	Lipid hydroperoxide
SOD	Superoxide dismutase

CHAPTER 1

INTRODUCTION

1.1 Lentil

Lentils, one of the Man's oldest food crops, originated in the Fertile Crescent of the Near East, and date back to the beginning of agriculture itself. As a food, lentils provide a valuable protein source, which coupled with their ability to thrive on relatively poor soils and under adverse environmental conditions, has ensured their survival as crop species to the present day.

Lens is a Latin word that describes exactly the shape of seed of a cultivated legume, which nowadays botanists call *Lens culinaris*, following the name given to it by Medikus, a German botanist-physician, in 1787.

Lens culinaris Medik. belongs to the division Anthrophyta, sub-division Dicotyledonae, order Rosales, sub-order Rosinae, family Leguminosae and sub-family Papilionaceae. Within Papilionaceae, *Lens* holds an intermediate position between *Vicia* and *Lathyrus,* but is closer to *Vicia* (Williams et al,

1974). All *Lens* species are diploid with 2n=14 chromosomes (Sharma and Mukhopaday, 1963; Ladizinsky, 1979).

Turkey is one of the major lentil producing countries in the world with an area of over 700,000 ha next only to India. Lentil accounts for as much as 34% of total legume production. In 2001, the Food Legume Improvement Program of Central Field Crops Research Institute released five lentil varieties (ICARDA). Furthermore, with 2-3 kg/person rate of annual consumption, lentil plays an important role in the diet of especially rural populations of Turkey.

The protein contents of lentils are comparable with that of the faba bean; higher than chickpeas, and more than double that of wheat. 100 g of dried seeds contain 340-346 calories, 12% moisture, 20.2 g protein, 0.6 g fat, 65.0g total carbohydrate, about 4 g fiber, 2.1 g ash, 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). Among the cool season legume crops, lentil is the richest in the important amino acids (lysine, arginine, leucine, and sulfur containing amino acids) (Williams et al., 1994).

Lentil is a cool season crop with a restricted root system, which is only moderately resistant to high temperature and drought. Lentil does best in soil with pH levels 6.0 – 8.0 and will not tolerate waterlogging, flooding or soil with high salinity.

1.2 Biotic and Abiotic Stress Conditions Affecting Plant Growth and Productivity

All stresses are similar, in the sense that they all may induce potentially injurious strains in the plant, either reversibly by inhibiting metabolism and growth, or irreversibly by injuring or killing the cells.

World crop production is limited by environmental stresses. About 20% of the land is limited by mineral stress, 26% by drought stress and 15% by cold stress (Blum, 1986).

Environmental stresses are of two main types; biotic stresses including infection or competition by other organisms, and abiotic stresses. Six abiotic stresses have long been known to give rise to resistance adaptations (Table1.1).

Table 1.1 Environmental Stresses

A. Biotic: Infection and/or competition by other organisms.
B. Abiotic (Physicochemical Stress):
   - Light: High intensity, low intensity
   - Temperature: High, low (chilling, freezing)
   - Water: Deficit (drought), excess (flooding)
   - Radiation: IR, visible, UV, ionizing (X-ray and γ-ray)
   - Chemicals: Salts, ions, gases, herbicides, heavy metals
   - Mechanical factors: Wind, pressure

1.3 Salt Stress

Salinity can be defined as the presence of excessive concentrations of soluble salts in the soil that suppress plant growth. The major cations contributing to salinity are Na^+, Ca^{+2}, Mg^{+2}, K^+ and anions are Cl^-, SO_4^{-2}, HCO_3^-, CO_3^{-2}, and NO_3^{-2}. There exist also trace ions including B, Sr, Li, Rb, F, Mo, Ba, and Al (Tanji, 1990). Salinity is used in this thesis to mean Na^+ content of the growth solution. Na^+ is the main constituent of saline and sodic soils, and has been shown to be toxic independent of its accompanying anion and osmotic effect.

Salinity is a serious problem all around the world. It is one of the major limiting factors in agriculture. Course estimates show that only 10% of the agricultural area is in non-stress conditions. Soil affected with mineral stress conditions occupies an area of 30% of the world land area (Epstein, 1980).

Plants are stressed in two ways in a high salt environment. In addition to the water stress imposed by the decrease in osmotic potential of the rooting medium as a result of high solute content, there is the toxic effect of high concentration of ions (Hale and Orcutt, 1987, Pasternak, 1987).

The low osmotic potential of the soil salt solution makes it necessary for plants exposed to these media to maintain a lower intracellular osmotic potential, otherwise they would experience osmotic desiccation because water would move osmotically from the cells into the soil. The response of all plants to decreased osmotic potential is turgor loss, which results in stomatal closure, followed by reduction in gas exchange (i.e. transpiration and photosynthesis). Thus, the decreased turgor is the major cause of inhibition of plant growth under saline conditions (Ashraf, 1994).

In addition to osmotic effects, total ion activity and the relative proportion of ions in the external environment have considerable adverse effects on plant growth. It has been noted that uptake and translocation of major nutrients such as K^+ and Ca^{2+} are greatly reduced by salt stress. Hu *et al.* (1997) showed that salinity significantly increased sodium and chloride concentration in leaves and stems of wheat, while the concentration of K^+, Ca^{2+}, Mg^{2+} and NO_3^- decreased. Both K^+ and Ca^{2+} are required in the external growth medium to maintain the selectivity and integrity of the cell membrane. Ca^{2+} and K^+ play similar roles for selective transport of ions across the membranes. As a consequence, high Na^+/K^+ and Na^+/Ca^{2+} ratios in the saline environments may impair the selectivity of root membranes and account for passive accumulation of Na^+ in the roots and shoots. Other physiological mechanisms such as stomatal movement, photosynthesis, and transpiration are also affected by the high Na^+/K^+ ratio.

Salinity is the big problem in Turkey, especially in dry areas and in irrigated agriculture, and it affects lentil production adversely by decreasing the yield. Salinity is often encountered in irrigated agriculture in areas where the water table is shallow (0.5-3 m) or the irrigation water has a high salt content (>1000 ppm). Lentil is highly sensitive to salinity and suffers from salinity stress when the electrical conductivity of the saturation extract of the soil is >4.0 mmhos/cm. Under excessively high salinity there is failure of germination or, if germination occurs, there is stunted growth with plants developing yellowish discoloration followed by development of bright reddish pigmentation. Nodulation is poor or absent. When salinity develops because of the rise of water table after the crop establishment, the crop stops growing, shows moisture stress by drooping of leaves and after a few days the plants defoliate and die.

1.3.1 Salt Stress Conditions

Plants are exposed to salinity in two major ecosystems: i) when they are grown on inherently saline soils or, ii) when they are grown on heavily irrigated soils. Irrigation water contains dissolved salts that are concentrated as the water evaporates and build up in the soil over time (Özalp, 1993; Hale and Orcutt, 1987).

In arid and semiarid regions, insufficient precipitation results in extensive reliance on irrigation and a concentration of (mainly sodium) salts in soils water supplies that is high enough to impair the growth of plants. High salt concentration in the soil solution create high osmotic pressures, reducing the availability of soil water to plants, and specific ions such as sodium and chloride may prove toxic at high concentrations (Epstein, 1980).

High salinity is also characteristics of salt marshes and inland deserts. In desert soils, evaporation (which causes an increase in salt concentration) exceeds precipitation (which causes a decrease in salt concentration) and salts accumulate in the soil (Hopkins, 1995).

1.3.2 Uptake and Accumulation of NaCl

Sodium and chloride occur in soil solution as univalent ions. The role of these elements in plant metabolism is still uncertain. The observation that chloride is essential for production of oxygen by isolated chloroplasts has led to the view that chloride acts as an electron-transporting agent in photophosphorylation. Sodium is an activator of transport ATP-ases in animal and possibly also in plant. There is evidence that sodium can replace potassium partially in some of its functions (Sutcliffe & Baker, 1976).

It is suggested that the main route of Na^+ uptake is via the plasma membrane of cortical and/or epidermal root cells and that the transfer of Na^+ into the cytosol is essentially a passive process: the negative electrical potential difference at the plasma membrane and low cytosolic Na^+ concentrations strongly favor the movement of Na^+ into the cell. Although the mechanisms for Na^+ influx across the plasma membranes have not yet been established, it is evident that Na^+ ions can be transported into the cell through K^+ carriers due to the similarity between the hydrated radii of sodium and potassium, which makes difficult the discrimination between the two ions by transport proteins and this the basis of Na^+ toxicity (Blumwald, 2000).

In contrast, Na^+ extrusion and compartmentalization are active processes. Na^+/H^+ antiporters mediate the compartmentalization of Na^+ within the vacuole and the extrusion of the Na^+ from the cell. The H^+-ATPase found on the plasma membrane uses the energy of ATP hydrolysis to pump H^+ out of the cell, generating an electrochemical H^+ gradient. This protonmotive force generated by the H^+-ATPase allows the operation of plasma membrane Na^+/H^+ antipoters that couple the downhill movement of H^+ into the cell along its electrochemical gradient to the extrusion of Na^+ against the electrochemical gradient.

Intracellular Na^+ compartmentalization into the vacuoles provides an efficient mechanism to avert the deleterious effects of Na^+ in the cytosol. Moreover, the compartmentalization of Na^+ (and chloride) into the vacuole allows the plants to use NaCl as an osmoticum, maintaining the osmotic potential that drives water into the cells. The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter that is driven by electrochemical gradient of protons generated by the vacuolar H^+-transporting enzymes, the

H^+-ATPase and the H^+-PPiase. While the salt-sensitive plants depend mainly on the exclusion of Na^+ ions at the plasma membrane, salt-tolerant species accumulate large amounts of Na^+ in the vacuoles (Blumwald, 2000).

In the stele, active transport process are involved in loading solutes into the xylem stream. This offers another site of selectivity. Conversely, active transport out of the xylem transport cells may further regulate the properties and concentrations of different ions supplied to the shoots. In some of the most sensitive crop plants, sodium may be retransported from the xylem and exported from the roots. This has been reported from *Phaseolus vulgaris* and squash (Gorham, 1985).

1.3.3 Tolerance Mechanisms

Salt tolerance means the ability of plants to grow satisfactorily in saline soils. Among the tolerance mechanisms, salt inclusion or salt exclusion has long been recognized in different plants in response to salinity. Salt excluders have the ability to restrict the uptake of salts into the shoot by reabsorption of toxic ions (especially Na^+) from the roots or shoots and either storage or retranslocation to the soil. By contrast, salt includers take up large quantities of salt and store it in the shoot. Many salt includers carry out compartmentalization of the salts into the vacuole and become succulent. Other salt includers possess special glands on their leaf surface to excrete high concentration of salts (Ashraf, 1994).

Plants respond to elevated Na^+ concentrations by maintaining the low cytosolic Na^+ concentration and a high cytosolic K^+/Na^+ ratio. The strategies for maintaining high K^+/Na^+ ratio in the cytosol include Na^+ extrusion and/or the cellular compartmentalization of Na^+ (mainly in the plant vacuole).

Besides the ion compartmentalization in vacuoles, plants also concentrate the ions in various specialized or nonspecialized organs such as roots, old leaves, leaf petioles and stems, or tracheids. Ion compartmentalization in these sites prevents, or at least delays, damage to more sensitive, essential organs, like blades of young active leaves or the meristematic tissues (Pasternak, 1987).

The synthesis and accumulation of organic solutes (also called osmoprotectants or compatible solutes) reduce the cell osmotic potential to a level that provides high turgor potential to maintain the growth. Compatible solutes also protect cellular enzymes and cell membranes against destabilization (Pasternak, 1987). These include sugars/polyols (sucrose, fructans, D-ononitol, manitol, trehalose), organic acids (malate, oxalate), amino acids (proline, ectoine), and their N-methylated derivatives (Ashraf, 1994; Nuccido, 1999). Polyamines (spermine and spermidine), which bind to enzymes and directly modulate their activity, were also shown to increase under salt stress in various plants (Bouchereau, 1999). They also function in ion balance and chromatin protection. Accumulation of K^+ in the cytoplasm also provides osmotic adjustment, macronutrient requisition and sodium exclusion and export. It is also known that pigments and caroteniods confer resistance to plants under osmotic stress by protecting the photoinhibition (Bohnert & Jensen, 1996).

Recently, it was shown that increased cytosolic Ca^{2+} concentration could also confer resistance to plant cells. In maize root protoplasts, external NaCl induced an increase in cytosolic Ca^{2+} concentration and increases in the external Ca^{2+} concentration can ameliorate the effects of NaCl on plant growth (Blumwald, 2000). Also, in the presence of Ca^{2+} in growth medium, K^+ concentration in the cytosol does not decrease, which is observed under

salinity stress. In yeast, there is evidence that salinity increases the intracellular Ca^{2+} concentration, which acts as a second messenger and interacts with a Ca^{2+}-dependent component in the cell to regulate ion homeostasis and mediate the salt tolerance (Bressan, 1998).

1.4 Oxygen Free Radicals

Responses by plants to extreme temperature and drought stress correlate with responses typically observed from increased oxidative stress. Oxidative stress may be initiated by the generation of organic free radicals that results in self-propagating autooxidation reactions. These radicals form in response to either direct excitation from incident radiation or secondarily from reactions with oxygen radicals or metastable forms of reduced oxygen, i.e., hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and superoxide radical ($O_2^{\cdot-}$). The damage to biological systems resides in the formation of other organic peroxides propagated through typical radical chain reaction (Alscher & Hess, 1993).

Atmospheric oxygen in its ground (triplet) state is distinctive among the gaseous elements because it is a biradical, or in other words it has two unpaired electrons. Although it does possess two unpaired electrons, they are in parallel spins. A pair of electrons could not be added without spin inversion. For spin reversal to take place, energy input is necessary. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins. This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction) (McKersie, 1996).

The second mechanism of activation is by the stepwise monovalent reduction of oxygen to form superoxide ($O^{\cdot}_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and finally the water. It is these intermediate oxyradical species, which are highly reactive, since they can react with proteins, nucleic acids, and/or lipids, potentially causing denaturation, mutagenesis and/or lipid peroxidation.

Superoxide can act as either an oxidant or reductant; it can oxidize sulphur, ascorbic acid or NADPH; it can reduce cytochrome C and metal ions. A dismutation reaction leading to the formation of hydrogen peroxide and oxygen can occur spontaneously or is catalyzed by the enzyme superoxide dismutase. In its protonated form (pH=4,8), superoxide forms the perhydroxyl radical ($^{\cdot}OOH$) which is a powerful oxidant (Gebicki & Bielski, 1981), but its biological relevance is probably minor because of its low concentration at physiological pH (McKersie, 1996).

The univalent reduction of superoxide produces hydrogen peroxide, which is not a free radical because all of its electrons are paired. Hydrogen peroxide readily permeates membranes and it is therefore not compartmentalized in the cell. Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecules. The well-known reactivity of hydrogen peroxide is not due to its reactivity per se, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical, which is the strongest oxidizing agent known and reacts with organic molecules at diffusion-limited rates (McKersie, 1996). In the late nineteenth century, Fenton described the oxidizing potential of hydrogen peroxide mixed with ferrous salts (Fenton, 1894, 1899). In the presence of trace amount of iron, the reaction of superoxide and hydrogen peroxide will form the destructive

hydroxyl radical and initiate the oxidation of organic substrates (Haber and Weiss, 1934).

$$O_2^- + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2 \quad \text{(Haber- Weiss Reaction)}$$

1.4.1 Sites of Activated Oxygen Production

The reduction of oxygen to form superoxide, hydrogen peroxide and hydroxyl radicals is the principle mechanism of oxygen activation in most biological systems. However in most photosynthetic plants, the formation of singlet oxygen by the photosystems has importance. Activated oxygen is often formed as a component of metabolism to enable complex chemical reactions or by the dysfunctioning of enzymes or electron transport systems as a result of perturbations in metabolism caused by chemical or environmental stress (McKersie, 1996). Chloroplasts, mitochondria, endoplasmic reticulum, microbodies, plasma membranes and cell walls are the major sites of activated oxygen production in a plant cell.

As described by Elstner (1991), there are at least four sites within the chloroplast that can activate oxygen. (1) The reducing side of PSI is thought to contribute significantly to the monovalent reduction of oxygen under conditions where NADP is limiting. (2) Photoactivated chlorophyll normally transfers its excitation energy to the PS reaction centers, but under conditions that prevent the captured light energy from being utilized in the electron transport systems, this energy can excite oxygen from the triplet to singlet form. These conditions include stomatal closure caused by drought, damage to the membrane transport systems, lack of specific nutrient, or the presence of xenobiotics. (3) The leaks of electrons from the oxidizing side of PSII or

the release of partially reduced oxygen products from this site contribute to activated oxygen production. (4) Photorespiration.

Reduced ubiquinoes in the electron transport system of the mitochondrial membrane is the possible site of superoxide production. Several conditions, which increase the reduction of ubiquinone, favor reduction of oxygen in the ubiquinone-cytochrome b region of the chain (Rich and Boner, 1978). The various Fe-S proteins and NADH dehydrogenase have also been implicated as possible sites of superoxide and hydrogen peroxide formation in the mitochondria (Turrens *et al.*, 1982)

Various oxidative processes occurring on the smooth endoplasmic reticulum involves the transfer of oxygen into an organic substrate using NAD(P)H as the electron donor. Superoxide is produced by microsomal NAD(P)H dependent electron transport involving cytochrome P_{450} (Winston and Cederbaum, 1983) Cytochrome P_{450} reacts with its organic substrate (RH) and forms a radical intermediate ($cytP_{450}$-$R^{\cdot}$) that can readily react with triplet oxygen (forming $cytP_{450}$-$ROO^{\cdot}$) because each has one unpaired electron. This oxygenated complex may be reduced by cytochrome b or occasionally the complex may decompose releasing superoxide.

Peroxisomes and glyoxysomes contain enzymes involved in β-oxidation of fatty acids and the glyoxylic acid cycle including glycolate oxidase, catalase, and various peroxidases. Glycolate oxidase produces H_2O_2 in a two-electron transfer from glycolate to oxygen (Lindqvist *et al.*, 1991). Xanthine oxidase, urate oxidase and NADH oxidase generate superoxide as a consequence of oxidation of their substrates.

A superoxide-generating NAD(P)H oxidase activity is also found in plasmalemma. Wounding, heat shock and xenobiotics transiently activate this superoxide generating enzyme, and consequently, it has been proposed that these superoxide generating reactions may serve as a signal in plant cells to elicit responses to biological, physical or chemical stress (Doke *et al.*, 1991). Also, in the cell wall such a mechanism is thought to be present. Some biosynthetic reactions (such as lignin biosynthesis) and oxidative enzymes (diamine oxidase, NADH oxidase) also lead to production of activated oxygen on the cell wall (McKersie, 1996) which may be signal for oxidative stress.

1.4.2 Free Radical Dependent Damage

Environmental stress in plants leads to production of activated forms of oxygen, which can cause the cell death. Active oxygen species can damage membrane lipids and degrade proteins and nucleic acids.

The effect of oxygen free radicals on the lipid peroxidation has been extensively studied. 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 propagates.

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 alkoxy radicals. Among the degradation products of ROOH are aldehydes, such as malondialdehyde, and hydrocarbons, such as ethane and ethylene that are commonly measured end products of lipid peroxidation. On the other hand, superoxide attack on phospholipid bilayer occurs at ester bonds, which results in the production of free fatty acids by deesterification reaction (McKersie, 1996).

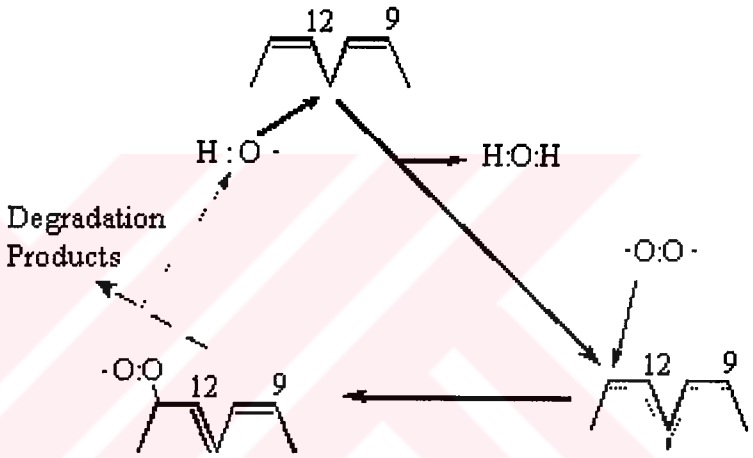

Fig. 1.1 The peroxidation of linoleic acid. The hydroxyl radical abstracts a H atom from carbon-11 of the fatty acid between the two double bonds forming water. The electron deficiency is shared among carbons 9 to 13 in a resonance structure. Triplet oxygen that has two unpaired electrons may attach to this structure at either carbon -9 or -13 forming a peroxy radical. This peroxy radical will abstract another hydrogen atom from a second linoleic acid molecule in a propagation reaction forming a lipid hydroperoxide. Chain breakage and cross-linkage reactions subsequently occur to produce aldehydes, hydrocarbons, alcohols and cross-linked dimers.

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of the cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. Sulphur containing amino acids and thiol groups specifically are very susceptible sites. Some of the free radical attacks on proteins are not reversible. For example, the oxidation of iron-sulphur centers by superoxide destroys enzymatic function (Gardner and Fridovich, 1991). The oxidative degradation of proteins is enhanced in the presence of metal cofactors that are capable of redox cycling, such as iron. In these cases, the metal binds to 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 oxidizes 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 radicals. 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 induces numerous lesions in DNA that cause deletions, mutations, and other lethal effects. Both sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986).

1.4.3 Effect of Salt Stress on Production of Active Oxygen Species

One of the major abiotic stress effecting plant growth and productivity is water stress resulting from drought or salinity. Plants may react to the salt damage by ion compartmentalization, ion exclusion or osmotic adjustment.

There are evidences that salt excess results in oxidative stress by inducing water deficit and, consequently, stomatal closure, a process which decreases CO_2 availability and photosynthesis, thus increasing the likelihood of reactive oxygen species formation in chloroplasts. As soon as the CO_2 concentration decreases inside the chloroplast, as a result of stomata closure, there is also lower availability of NADP to accept electrons from PSI, thus initiating O_2 reduction with the concomitant generation of activated oxygen species (Sudhakar et al., 2001). A large body of evidence has accumulated from various plant systems showing that environmental stresses, especially drought and salt stress, alter the amounts and the activities of enzymes involved in scavenging oxygen radicals (Gueta-Dahan, 1997).

Unspecific osmotic effects may determine partial stomatal closure, while specific ion toxicity may alter membrane structure and functionality, so allowing electron leakage from the electron transport chain. As a consequence, an improved production of activated oxygen species may drive (Meneguzzo, 1999).

Resistance to oxidative stress has been implicated in several studies involving NaCl stress. It has been suggested that H_2O_2 and $O_2^{\cdot-}$ might play an important role in the mechanism of NaCl injury, and there is strong relationship between NaCl tolerance and antioxidant capacity in several plants. Studies with pea (*Pisum sativum*) plants demonstrated that chloroplast and mitochondrion metabolism under NaCl stress favored the formation of $O_2^{\cdot-}$ radicals and H_2O_2 in two cultivars differing in sensitivity to NaCl; and tolerant plants responded to NaCl stress with increased activity of some antioxidant enzymes (Hernandez *et al.,* 1995).

1.5 Antioxidant Defense Systems

Because of the deleterious effects of active oxygen species, plants have evolved various protective mechanisms involving antioxidative enzymes and molecules to protect the cell. Increases in activities and concentrations of these enzymes and molecules under oxidative stress confer resistance to plants.

1.5.1 Non-enzymatic Systems

The non-enzymatic defense mechanisms include glutathione, ascorbate, carotenoids, vitamin E (α-tocopherol) and various phenylpropanoid derivatives (phenolic compounds), for example, flavonoids, lignans, tannins and lignins.

The tripeptide glutathione (γ-Glu- Cys-Gly, GSH) is the major low molecular weight thiol in most plants. Some legumes contain the homologous peptide homoglutathine (γ-Glu-Cys-Ala, hGSH), either exclusively or in combination with glutathione. GSH is found in most tissues, cells and subcellular compartments of higher plants. At subcellular level, GSH concentration is highest in the chloroplasts, but significant quantities also accumulate in the cytosol. The antioxidant function of GSH is mediated by sulfhydryl group of cysteine, which, upon oxidation, forms a disulfide bond with a second molecule of GSH to form oxidized glutathione (GSSG). GSH has a redox potential of –340 mV that enables GSH to reduce dehydroascorbate to ascorbate or to reduce the disulphide bond of proteins. The reduction of GSSG to GSG 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. GSH may stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price *et al*, 1990, McKersie, 1996, Hausladen and Alscher, 1993).

L-ascorbic acid (vitamin C) is an important antioxidant in both animal and plant tissue. Ascorbate is synthesized in the cytosol of higher plants primarily form the conversion of D-glucose to ascorbate. It is abundant in plant protoplasts, chloroplast and certain fruits. Ascorbate has been shown to have an essential role in several physiological processes in plants, including growth, differentiation and metabolism. Ascorbate functions as a reductant for many free radicals, thereby minimizing the damage caused by oxidative stress. In the light, the chloroplast of higher plants produce several destructive oxygen-derived species, including superoxide ($O_2^{\cdot-}$), H_2O_2, and the hydroxyl radical ($OH^{\cdot}$). Ascorbate is the terminal electron donor in the processes, which scavenge these free radicals in the hydrophilic environments of plant cells. Ascorbate scavenges hydroxyl radicals at diffusion-controlled rates (McKersie, 1996). The reaction with superoxide may serve a physiologically similar role to Superoxide scavenging enzyme, superoxide dismutase (SOD):

$$2O_2^{\cdot-} + 2H^+ + \text{ascorbate} \rightarrow 2H_2O_2 + \text{dehydroascorbate}$$

Ascorbate also reacts non-enzymatically with H_2O_2 at a significant rate, producing water and monodehydroascorbate. The reaction is catalyzed by ascorbate peroxidase in the chloroplast and cytosol of higher plants. In plants, H_2O_2 detoxification in chloroplasts is extremely important because photosynthesis is highly sensitive to very low concentrations of H_2O_2. Because chloroplasts lack catalase, which scavenges hydrogen peroxide in

the peroxisomes, ascorbate has a central importance in eliminating the H_2O_2 from chloroplasts. In addition to its role as a primary antioxidant, ascorbate has a significant secondary antioxidant function. The ascorbate pool represents a reservoir of antioxidant potential that is used to regenerate other membrane-bound antioxidants such as α-tocopherol and zexanthin. These scavenge lipid peroxide and singlet oxygen, respectively (Foyer, 1993).

Carotenoids are C_{40} isoprenoids or tetraterpenes that are located in the plastids of both photosynthetic and non-photosynthetic plant tissues. In addition to their function as accessory pigments in light harvesting, they detoxify various forms of activated oxygen and triplet chlorophyll that are produced as a result of excitation of photosynthetic complexes by light. There are two classes of carotenoids; the carotenes are hydrocarbons, the xanthophyls are carotene derivatives that contain one or more oxygen atoms. In terms of their antioxidant properties, carotenoids can protect the photosystems in one of four ways: by reacting with lipid peroxidation products to terminate chain reactions (Burton and Ingold, 1984); by scavenging singlet oxygen and dissipating the energy as heat (Mathis and Kleo, 1973); by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen; or by the dissipation of excess excitation energy through the xanthophylls cycle (McKersie, 1996).

Tocopherol, another family of antioxidants, has been found in all higher plants, in both photosynthetic and non-photosynthetic tissues. Because of its hydrophobic nature, it is always located in membranes of the cell. α-tocopherol (vitamin E) is well established as a membrane-stabilizing agent. In addition to its influence in membrane lipid organization, it has the ability to complex with free fatty acids. Free fatty acids act as detergents in membranes causing disruption of the lipid bilayer, membrane aggregation

and fusion. The association of free fatty acids and tocopherol reduces this destabilization (McKersie, 1996). Vitamin E is considered to be an effective quenching agent for both singlet oxygen and for alkyl peroxides. It has been observed in six plant species that vitamin E biosynthetic capacity increases readily in response to the demands of oxidative stress associated with drought (Hess, 1993).

Terrestrial vascular plants synthesize a structurally, biogenetically diverse array of phenolic products that are compartmentalized or accumulate in specific tissues or organs. Plant phenolics have the potential to function as antioxidants by trapping free radicals generated in oxidative chemistry which then normally undergo coupling reactions leading eventually to (colored) polymeric or oligomeric products (Lewis, 1993).

1.5.2 Enzymatic Systems

The enzymatic defense against the reactive oxygen species is essential for plants under biotic or abiotic stress. The antioxidative enzymes, superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.1.1.11), glutathione reductase (GR; EC 1.6.4.2) and other ascorbate-glutathione cycle enzymes (monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1)), catalyze the synthesis, degradation and recycling of antioxidant molecules and can directly catalyze the removal of free radicals from the cells.

Superoxide dismutase, originally discovered by McCord and Fridovich (1969), catalyze the dismutation of superoxide to hydrogen peroxide and oxygen:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

This enzyme is unique in that its activity determines the concentrations of $O_2^{\cdot-}$ and H_2O_2 , the two Haber-Weiss reaction substrates, which generates the most reactive hydroxyl radicals. The importance of SOD has been established by the demonstration that SOD-deficient mutant of *Escherichia coli* (Carlioz and Touati, 1986) and yeast (Bilinski *et al.*, 1985; van Loon *et al.*, 1986) are hypersensitive to oxygen. Therefore the enzyme is likely to be central in defense mechanism. SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen. The three known types of SOD are classified by their metal cofactor: The copper/zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD) forms. Experimentally these three different types can be identified by their differential sensitivities to KCN and H_2O_2. Cu/Zn SOD is characterized as being sensitive to both H_2O_2 and KCN; FeSOD is sensitive only to H_2O_2, while MnSOD is resistant to both inhibitors. Subcellular fractionation studies have been performed in many plant species and in general plants contain a mithochondrial matrix localized MnSOD and a cytosolic Cu/ZnSOD, with FeSOD and/or Cu/ZnSOD present in the chloroplast stroma (Bowler *et al*, 1992). Moreover, there are findings that MnSOD has also glyoxysomal and peroxisomal isozymes in various plant species (Bowler, 1994). For each type, the mechanism of catalysis is thought to be the same, essentially involving a protein pocket bordered by positively charged amino acid residues that creates an electrostatic sink for attracting the superoxide anion radicals to the active site. The transition metal present at the active site then carries out a one-electron transfer between two superoxide radicals and undergoes alternating oxidation/reduction reactions.

Catalase is a heme-containing enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in β-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism (McKersie, 1996). Various forms of catalase have been described in many plants. In maize there are three isozymes localized separately in peroxisomes, cytosol and mitochondria (Scandalias, 1993). Catalase is very sensitive to light and has a rapid turnover rate. Regardless, stress conditions, which reduce the rate of protein turnover, such as salinity, heat shock or cold, cause the depletion of catalase activity (Hertwig et al., 1992). This may have significance in plant's ability to tolerate the oxidative components of these environmental stresses.

Detoxification of H_2O_2 is mediated by catalase, which is mostly localized in peroxisomes. However, catalase possesses a very low affinity for H_2O_2 and its activity is either extremely low or not detectable in the cytosol, mitochondria and chloroplast (Halliwell, 1981). In plant cells, an alternative and more effective detoxification mechanism against H_2O_2 also exists, operating both in chloroplast and cytosol, called the ascorbate-glutathione or Halliwell- Asada cycle (Asada and Takahashi, 1987; Foyer and Halliwell, 1976), (Fig.1.1). The pathway seems to be major H_2O_2 detoxification system both in cytosol and chloroplast, as well as in mitochondria. It is also important for the maintenance of ascorbate and glutathione pools in the reduced state.

The first enzyme of this cycle, ascorbate peroxidase (APX) catalyses the reduction of H_2O_2 to water and has high specificity and affinity for ascorbate as reductant (Asada, 1999). Its sequence is distinct from other peroxidases, and different forms of APX occur in the chloroplast, cytosol, mitochondria, peroxisomes and glyoxysomes (Jimenez et al., 1997; Leonardis et al., 2000). Membrane bound APXs occur on the peroxisome and thylakoid membranes. By the ascorbate-glutathione cycle, hydrogen peroxide is effectively scavenged while the ascorbate level is maintained at a constant level.

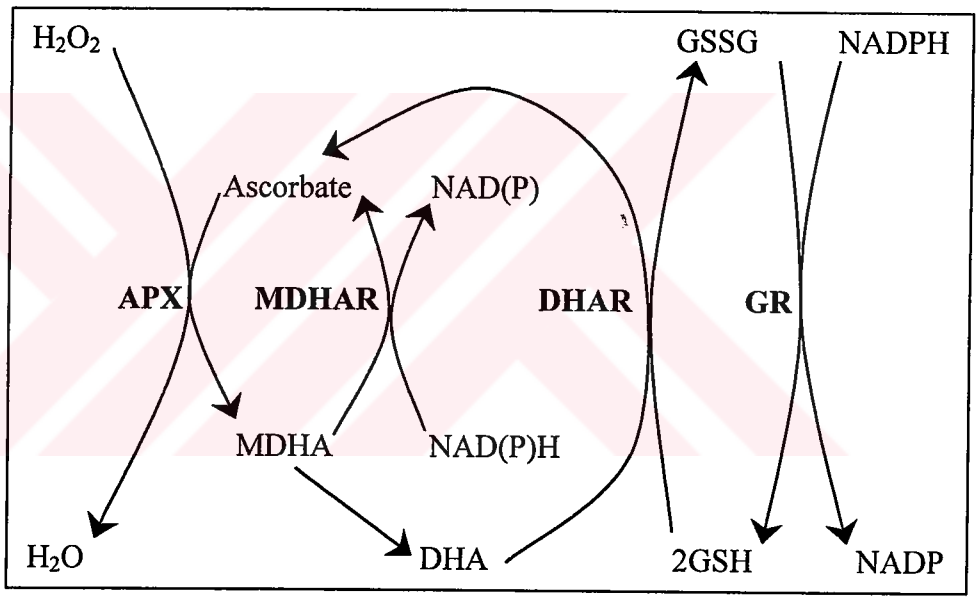

Fig.1.2. The ascorbate-glutathione cycle. Dehydroascorbate (DHA), monodehydroascorbate (MDHA), reduced glutathione (GSH), oxidized glutathione (GSSG), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR).

The oxidation of ascorbic acid occurs in two sequential steps, forming in the first instance, monodehydroascorbate, and subsequently dehydroascorbate. Monodehydroascorbate is the primary product of ascorbate peroxidase reaction. Monodehydroascorbate is either directly reduced to ascorbate by the action of NAD(P)H-dependent monodehydroascorbate reductase (MDHAR) or spontaneously disproportionates to dehydroascorbate. Dehydroascorbate is also very unstable at pH values greater than pH 6.0. The carbon chain is cleaved to products such as tartrate and oxalate, and may decompose to yield toxic derivatives. To prevent loss of ascorbate pool following oxidation, the chloroplast contains efficient mechanisms of recycling both monodehydroascorbate and dehydroascorbate, and these ensure that the ascorbate pool is maintained largely in reduced form. Reduced glutathione (GSH), which is present in chloroplast in milimolar concentrations, will nonenzymatically reduce dehydroascorbate back to ascorbate at pH values greater than pH 7.0, however, it accounts for only the 0.1% of total reduction. This reaction is catalyzed by dehydroascorbate reductase (DHAR), which is present at high activities in leaves, seeds and other tissues. DHAR uses reduced glutathione (GSH) as an electron donor for the reduction of dehydroascorbate to ascorbate. Thiol groups are involved in the catalysis, as SH-reagents deactivate the enzyme (Foyer, 1993)

The last enzyme of Halliwell-Asada cycle, glutathione reductase (GR), catalyses the NADPH-dependent reduction of oxidized glutathione. Several isozymes of GR are present in plant tissue. Subcellular fractionation studies have shown GR to occur in chloroplast, cytosol and also in mitochondria (Hausladen and Alscher, 1993). GR is the rate-limiting enzyme in H_2O_2 scavenging pathway and it is involved in the maintenance of high ratio of

GSH/GSSG, which is required for the regeneration of ascorbate. (Sudhakar et al, 2001).

The other group of enzyme that scavenges hydrogen peroxide is peroxidases (POD). PODs participate in lignin biosynthesis, IAA degradation, and convert hydrogen peroxide to water. They can use a wide range of electron donors, e.g. guaiacol, therefore, they are referred as guaiacol peroxidases (Hegedüs, *et al.*, 2001) Peroxidases are widely distributed in plant cells. They are classified as water soluble and cell wall peroxidases (Jbir et al., 2001).

The initial step in the catalytic mechanism of a peroxidase and a catalase is heterolysis of the oxygen-oxygen bond of hydrogen peroxide. This causes the release of one water molecule and coordination of second oxygen atom to the iron center, forming an intermediate, compound I. In catalase reaction, a second peroxide molecule is used as a reducing agent for compound I. On the other hand, in peroxidase reaction compound I is reduced by two electron transfer from the iron center or from the enzyme (Zamocky *et al.*, 2001).

1.6 Effect of Salt Stress on Antioxidant Defense Systems

Numerous studies have been carried out for the determination of the effect of salt stress on antioxidant system in many plants. The results show that changes in antioxidant enzymes and molecules under salt stress vary with plant species, different organs of the plant and intraorganeller distribution of antioxidative molecules and enzymes.

1.6.1 Effect on Non-enzymatic Systems

Among the specific protective mechanisms evolved by plants, the importance of ascorbate (AsA) and glutathione (GSH) is well known. Ascorbate is the major scavenger of oxygen radicals in biological systems. It scavenges hydroxyl radical, as well as superoxide and singlet oxygen, reduces thiyl radicals and dismutes hydrogen peroxide through the action ascorbate peroxidase. GSH can metabolize free radicals and can act in the protection of thiol status of proteins. Thus, the effective recycling of these antioxidants is required in plant cells under oxidative stress. The salt stress induced changes in ascorbate and glutathione has been shown in various studies. Under salt stress, the content of ascorbate and glutathione in roots and shoots of wheat has been shown to increase (Meneguzzo *et al*, 1999). In salt tolerant potato, both reduced glutathione and ascorbate significantly elevated, while in sensitive cultivar reduced glutathione content decreased by 35% and ascorbate increased by 35% (Benavides *et al.*, 2000). Comba *et al.* (1998) showed that in soybean root nodules, salt stress resulted in an increase in reduced glutathione content and it remained high after recovery from salt stress. In cucumber, salt stress increased the content of ascorbate and reduced glutathione (Lechno *et al.*, 1997). The salt tolerant genotype of wheat showed a higher ascorbic acid content when compared with the sensitive one under water deficit (Sairam *et al.*, 1998). In pea, NaCl stress was accompanied by losses in ascorbate and glutathione pool (Hernandez *et al.*, 1999). After 12 h of NaCl treatment of *Nicotiana plumbaginifolia* L., the antioxidants ascorbate and glutathione were found to be largely in oxidized form, suggesting an inability of the recycling process to cope with the stress (Savoure *et al.*, 1999). Although we cannot drive a single conclusion, it seems that ascorbate and glutathione levels increase in salt tolerant plants under stress and that it may confer resistance for oxidative stress.

α-tocopherol (vitamin E) and carotenoids are important in scavenging the damaging oxygen species, especially singlet oxygen, in the chloroplasts. In wheat, drought induced oxidative stress resulted in 2.4 fold increase in α-tocopherols and 2.6 fold increase in β-carotene (Bartoli et al., 1999).

1.6.2 Effect on Enzymatic Systems

The effect of salt stress on the antioxidant enzyme activities has been shown in many cases. It seems that salt stress strongly affects the oxidative defense mechanisms in plants but the change in the activities of antioxidant enzymes depends on plant species, isozymes of antioxidant enzymes and their subcellular distributions.

It has been shown in many studies that salt tolerant cultivar of a plant generally has enhanced antioxidant enzyme activities under salt stress when compared with the sensitive cultivars. This was observed in wheat (Meneguzzo et al., 1998), tomato (Gueta-Dahan et al., 1998; Rodriguez et al., 1999), soybean root nodules (Comba et al., 1997), tobacco (Benavides et al., 2000), Mulberry (Sudhakar et al., 2001), citrus (Gueta-Dahan et al., 1997), rice (Dionisio-Sese and Tobita, 1998), fox-tail millet (Sreenivasulu et al., 1998), potato (Mescht et al., 1998) and arabidopsis (Tsugane et al., 1999). It has been shown that salt stress increased the activity of antioxidant enzymes (SOD, GR, APX and CAT) of soybean root nodules in salt tolerant cultivar. In contrast, salt sensitive cultivar responded to NaCl stress by decreasing the activities all antioxidant enzymes, except SOD (Combo et al., 1997).

Responses of antioxidant enzymes to NaCl stress also differ with different subcellular locations of enzymes. In isolated chloroplasts and mitochondria of pea, under low NaCl stress the activities of all isozymes of SOD (chloroplastic and mitochondrial Cu/ZnSOD, FeSOD and MnSOD), APX, DHAR and GR elevated, however, chloroplastic DHAR, APX and GR as well as mitochondrial DHAR decreased to control levels under severe NaCl stress. On the other hand, mitochondrial APX and MDHAR, and chloroplastic Cu/ZnSOD strongly enhanced under severe NaCl stress (Gomez et al., 1999).

Impact of the salt stress on roots and shoots may also differ. This was observed in wheat (Meneguzzo et al., 1998). Although the activity of antioxidant enzymes significantly increased in wheat shoots, in roots, which firstly suffer stress, general decrease in the activities of all antioxidant enzymes was observed.

1.7 Aim of the Study

To our knowledge there is no literature data on behavior of antioxidant defense system of lentil under NaCl stress.

This study was aimed to characterize the physiological and biochemical responses of a Turkish lentil variety, Sultan 1, under salt stress. The analyses listed were used to determine the antioxidant defense system of the lentil under salt stress.

i) Determination of antioxidant enzyme activities (SOD, APX, CAT and GR)
ii) Determination of SOD isozymes
iii) Lipid peroxidation through MDA and membrane leakage tests
iv) Proline content determination
v) Determination hydrogen peroxide content
vi) Wet-dry weight and length measurements of roots and shoots

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical Materials

The chemicals used in this study were obtained from Sigma Chemical Company (N.Y., USA) or Merck Chemical Company (Deisenhofen, Deutschland). All of the solutions were prepared by using distilled water.

2.1.2 Plant Materials

In this study a Turkish cultivar of lentil (*Lens culinaris*), Sultan-1, were used. It is among the most widely sown and available Turkish cultivar. The seeds were originally obtained from the Exporter Unions Seed and Research Company, as well as the Ministry of Agriculture and Rular Affairs.

Sultan-1 is the summer sown, drought resistant cultivar. Its seeds are 6-7 mm in diameter and the weight of 1000 of them is 60-65 g.

2.2 Methods

2.2.1 Growth of Plants

Seeds were washed and imbibed in distilled water for one day. After one day of imbibitions, they were transferred to plastic trays covered with cheesecloth containing 300 ml of ½ strength Hoagland's solution (Hoagland and Arnon, 1950). Approximately 15-20 seeds were grown per tray. Planted seeds were grown for 9 days in a growth chamber at 23±2 °C with 16 hours light and 8 hours dark photo- cycle.

2.2.2 Application of Salt Stress

At the 9^{th} day of normal growth salt stress treatment was initiated by giving ½ strength Hoagland's solution containing100 mM and 200 mM NaCl. Salt-stressed plants and control plants were grown in the growth chamber with the same physical parameters for another 5 days. Fourteen days old seedlings were then analyzed for SOD isozymes, APX, CAT and GR activities; proline, MDA and H_2O_2 contents; conductivity and physiological parameters like wet-dry weight and root-shoot lengths.

2.2.3 Wet-Dry Weight Analysis and Physiological Changes

The root and shoot tissues of 14 day old control and treated plants were weighed and then they were let to dry in an oven. After one day the dry weights were measured.

The photographs of 14-day-old control and treated plants were taken to observe necrosis and wilting symptoms. The shoot and root lengths of the same samples were taken to determine the percent changes under salt stress.

2.2.4 Determination of Proline Content

The amount of proline was determined according to a modified method of Bates *et al.*, (1973). Root and shoot tissues from control and treated plants were weighed (≈ 0.2 g). Shoot samples were cut into small pieces and homogenized by addition of 2 ml of 3% sulphosalicylic acid in a glass-glass homogenizer. Root samples were cut into small pieces and homogenized by the addition of 2 ml of 3% sulphosalicylic acid in a mortar. Homogenates were transferred into eppendorf tubes and centrifuged by using a microcentrifuge at 14000 rpm at 4°C for 5 minutes.

For each sample 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% phosphoric acid and 0.1 ml 3% sulphosalicylic acid were prepared. 0.1 ml supernatant from each homogenate was added to the eppendorf tubes. Tubes were incubated at 96 °C for 1 hour in a water bath for complete hydrolysis of proteins. 1 ml toluene was added to each tube. Tubes were vortexed and centrifuged at 14000 rpm for 5 minutes. A pink-red upper phase was transferred to the quartz cuvette and absorbance at 520 nm was measured, blank being toluene.

A standard curve for proline containing the range 0.01 μM - 1.5 mM was constructed to determine the proline concentration in each sample.

2.2.5 Determination of MDA Content

The amount of MDA was determined according to the method of Ohkawa *et al.*, (1979). Fresh leaf and root tissues from control and treated plants were weighed (≈0.1g). Shoot tissues were cut into small pieces and homogenized in 5% trichloro acetic acid (TCA) solution in ice bath by using a glass-glass homogenizer. Root samples were cut into small pieces and homogenized by the addition of 5% TCA solution with cold mortar and pestle in ice bath. The homogenates were transferred into eppendorf tubes and centrifuged at 12000 rpm for 15 minutes at room temperature by using an eppendorf microcentrifuge.

Equal volumes of supernatant and 0.5% thiobarbituric acid in 20% TCA (freshly prepared) were added into a new eppendorf tube and incubated at 100 °C for 25 minutes in a water bath. The tubes were transferred into ice bath and than centrifuged at 10000 rpm for 5 minutes. The absorbance of the supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. MDA content was determined using the extinction coefficient 155 $mM^{-1}cm^{-1}$.

2.2.6 Determination of Peroxide Content

For assay of H_2O_2 content, 0.5 g tissue form control and treated plants were homogenized in 1.5 ml of 100 mM potassium phosphate buffer (pH 6.8). To remove cellular debris, the homogenate was filtered through two layers of cheesecloth and then centrifuged at 18000 g for 20 min at 4 °C. The supernatant was collected for assay of H_2O_2 content.

Measurement of H_2O_2 content was performed according to the modified method of Bernt and Bergmeyer (1974) using peroxidase enzyme. To initiate the enzyme reaction an aliquot of 0.5 ml of supernatant was mixed with 2.5 ml of peroxidase reagent, consisting of 83 mM potassium phosphate, pH 7.0, 0.005% (w/v) o-dianizidine, 40 µg peroxidase/ml and incubated for 10 minutes at 30 °C in a water bath. The reaction was stopped by adding 0.5 ml of 1 N perchloric acid and centrifuged at 5000 g for 5 minutes. The resultant supernatant was read at 436 nm and its absorbance was compared to the extinction of an H_2O_2 standard.

2.2.7 Electrolyte Leakage Test

Ion leakage of root and shoot tissues were measured according to the method of Nanjo *et al.* (1999) For the leaf conductance, six leaves of control and treated plants were shaken gently in 5 ml of 0.4 M mannitol solution for 3 hours in plastic vials at room temperature and electrical conductances were measured (C_1). Then, the vials were boiled in boiling water bath for 10 minutes to kill the tissue completely and the second conductances (C_2) were taken to measure the total ion concentration after complete membrane disintegration. The NaCl injury to the membranes were measured by the formula $(C_1/C_2)*100$, and expressed as % of control vials.

2.2.8 Protein Determination

The protein concentration in root and shoot crude extracts were determined according to Bradford method (Bradford, 1976). Bradford reagent was prepared by mixing 500 mg of Coomassie Brilliant Blue G-250 in 250 ml 95% ethanol. 500 ml 85% phosphoric acid was added to this solution. The

resultant solution was diluted to 1 liter with distilled water and filtered. Before use this concentrated Bradford reagent (5X) was diluted 5 times.

Twenty µl of sample was diluted with 480 µl distilled water in a test tube and 5 ml 1X Bradford reagent was added. The tube was mixed, and left at room temperature for at least 10 minutes. The absorbance at 595 nm was measured with Schimadzu UV-1201 spectrophotometer against a blank solution containing 500 µl distilled water and 5 ml Bradford reagent. Bovine Serum Albumin (BSA) was used as a standard at following concentrations: 10, 20, 30, 40, 50, 60 µg/ml.

2.2.9 Determination of SOD Isozyme Activities

SOD activity and SOD isozyme patterns of treated and control plants were determined by running one dimensional native polyacrylamide gel electrophoresis. Gels were further stainied for SOD activity by negative activity staining technique according to Beauchamp and Fridovich (1971).

2.2.9.1 Preparation of Shoot Crude Extracts

The shoot tissues from treated and control plants were weighed (≈150 mg) and cut into small pieces. Each sample was homogenized by a glass-glass homogenizer in 800 µl homogenization buffer containing 9 mM Tris- HCl, pH 6.8 and 13.6% glycerol on ice (0-4°C) for several minutes. The homogenate was than transferred into an eppendorf tube. The homogenization procedure was carried out for each sample separately. The homogenates in eppendorf tubes were centrifuged at 14000 rpm for 5 minutes in an eppendorf microcentrifuge. Supernatants were used for the SOD assay.

2.2.9.2 Preparation of Root Crude Extracts

The roots of control and treated plants were washed with distilled water and dried with filter papers for a few minutes. About 1 g from each sample was weighed and cut into small pieces. Samples were ground in 2 ml of grinding solution containing 0.2 M sodium phosphate buffer and 2 mM EDTA (pH 7.8) by using cold mortar and pestle. The suspensions were then filtered through cheesecloth and filtrates were collected in centrifuge tubes. They were centrifuged at 10000 rpm for 30 minutes at 4°C and supernatants were used for the SOD activity assay.

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

Five ml 12% separating gel and 2.5 ml 5% stacking gel were prepared to carry out 1-D PAGE according to Laemmli (1970). Gels were polymerized in Bio Rad Minigel Apparatus. Equal amounts of proteins (determined by Bradford, 1976) were loaded per well. Electrophoresis was carried out under constant current of 6 mA in stacking gel and 10 mA in separating gel for approximately 2 hours.

2.2.9.4 Negative Activity Staining

At the end of electrophoresis, gel was transferred into a glass container. 20 ml of negative activity stain containing 50 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.2 % (v/v) N,N,N'N'-tetramethyl ethylene diamine (TEMED), 3 mM riboflavin and 0.25 mM nitroblue tetrazolium was added and gel was incubated in dark for 45 minutes by shaking. After staining, gel was washed with distilled water and exposed to

light approximately for 15 minutes to observe the bands. The gel was photographed in Vilber gel imager and densitometric analysis was carried out by Bio-Profil V99 software program. The percent changes in SOD isozymes with respect to control 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 25 °C in a 3 ml reaction medium.

2.2.9.5 SOD Isozyme Determination

The root and shoot extracts of control plants were loaded into the 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 10 minutes in separate 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.

2.2.10 Determination of APX Activity

APX activity in shoots and roots tissues of treated and control plants were determined by a spectrophotometric assay (Wang, 1991). 0.5 g of fresh tissue was homogenized with liquid nitrogen and the powder were suspended in 1 ml of suspension solution containing 50 mM Tris-HCl (pH 7.2), 2% PVP, 1 mM EDTA and 2mM ascorbate. The suspension was centrifuged at 12100 g for 20 minutes at 4 °C and supernatant was used for the enzyme assay.

Assay solution contained 50 mM potassium phosphate buffer (pH 6.6), 0.25mM ascorbate, 1 mM H_2O_2 and enzyme extract containing 100 µg proteins, determined by Bradford Method (1976). The reaction was initiated by the addition of peroxide. The decrease in ascorbate concentration was recorded at 290 nm with Schimadzu double-beam spectrophotometer for 3 minutes. The enzyme activity was calculated from the initial rate of the enzyme. A standard curve covering the range of 0-0.5 mM ascorbate was used. (Extinction coefficient of ascorbate = 2.8 $mM^{-1}cm^{-1}$ at 290 nm). Corrections were made for the non-enzymatic reduction of peroxide with ascorbate.

2.2.11 Determination of CAT Activity

Catalase activity was determined according to the method of Chance *et al* (1995). 0.5 g of fresh leaf and root tissues of control and treated plants were ground with liquid nitrogen by using cold mortar and pestle. The powders were than suspended in suspension solution containing 50 mM Tris-HCl buffer (pH 7.8). The suspensions were filtered through 2 layers of cheesecloth into centrifuge tubes and centrifuged at 12000 g for 20 minutes at 4 °C. The supernatant parts were used for the enzyme assay.

Assay medium contained 50 mM potassium phosphate buffer (pH 7.0), 25 mM H_2O_2, and the enzyme extract containing 100 µg proteins determined by Bradford method (1976). The reaction was initiated by the addition of enzyme extract. The decrease in absorbance was recorded at 240 nm for 2 minutes by using Schimadzu double-beam spectrophotometer. The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of H_2O_2 = 40 mM^{-1} cm^{-1}).

2.2.12 Determination of GR Activity

Glutathione reductase activity was determined according to the method of Sgherri *et al* (1994). 0.5 g of fresh leaf and root tissues of control and treated plants were homogenized with liquid nitrogen and the powder was suspended in suspension solution containing 100 mM potassium phosphate (pH 7.0), 1 mM Na_2EDTA and 2% insoluble PVP. The GSSG-dependent oxidation of NADPH was monitored by the decrease in absorbance at 340 nm at 30 °C.

The assay mixture contained 200 mM potassium phosphate (pH 7.5), 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.25 mM GSSG, 25 µM NADPH and enzyme extract, containing 100 µg proteins (determined by Bradford method, 1976), in a final volume of 1 ml. The reaction was initiated by NADPH addition. Corrections were made for the background absorbance at 340 nm, without NADPH. (Extinction coefficient of NADPH = 6.2 $mM^{-1}cm^{-1}$).

2.2.13 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 were grown for 9 days under normal environmental conditions and then subjected to 100 mM and 200 mM NaCl stress for 5 days. The physiological changes, antioxidant enzyme activities, electrolyte leakage tests, as well as hydrogen peroxide and proline concentrations were measured in salt treated plants and compared to the controls, which were grown under normal environmental conditions for 14 days.

Salt stress resulted in decreased growth and increased membrane damage. Proline and hydrogen peroxide concentrations were significantly elevated under salt stress. The results we presented here show that salt stress causes oxidative stress as evident from the increased antioxidant enzyme activities.

The responses of lentil roots and leaves to NaCl stress differed in that they contained distinct antioxidant enzyme activities and proline content.

3.1 Physiological Changes Under Salt Stress

The physiological changes under salt stress of 100 mM and 200 mM NaCl were evaluated by measuring the wet-dry weights and lengths of roots and shoots. The photographs of 14 days old control and salt treated plants were shown in Fig. 3.1.

After 5 days of salt treatment, the plants were wilted, especially the old leaves. 200 mM NaCl stress resulted in necrosis in leaves. Decreased leaf area was observable at both stress treatments.

Salt stress strongly inhibited the growth of lentil seedlings. As it is shown in Fig. 3.2, root and shoots lengths of 100 mM and 200 mM salt stressed plants were significantly different than the control plants ($p < 0.05$). Growth inhibition was correlated with the increased salt stress. When compared with the control, shoot lengths were 27 % and 43 % shorter at 100 mM and 200 mM NaCl stress, respectively. Root lengths were 20 % and 38% shorter than the control plants at 100 mM and 200 mM NaCl stress, respectively. At 200 mM salt stress, the growth of seedlings was totally inhibited.

100 mM Control 200 mM

Fig. 3.1. 14 days old lentil seedling subjected to 100 mM and 200 mM NaCl stress for 5 days, and the control plant.

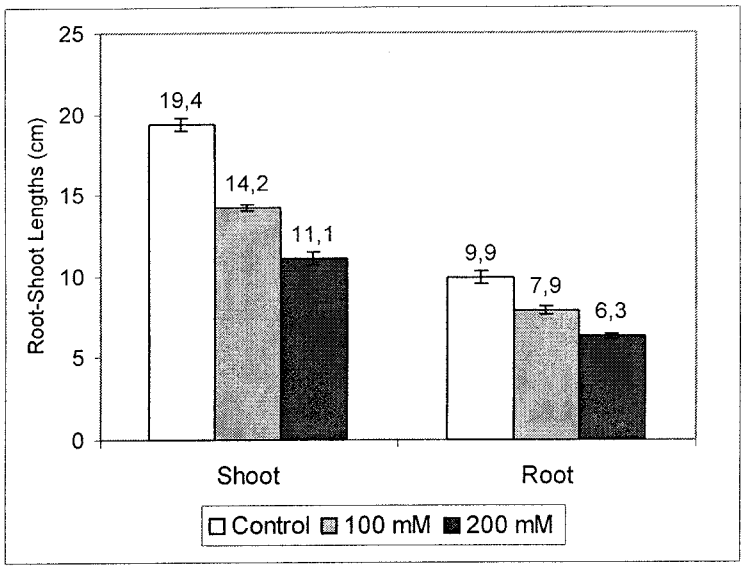

Fig 3.2 Root and shoot lenghts (cm) of control and NaCl treated plants. Bars indicate the mean lenghts ± S.E.M.

Salt stress significantly decreased the wet-dry weight of lentil seedlings, at both NaCl concentrations (Fig. 3.3; Fig. 3.4). The decrease in wet-dry weights was more apparent in shoots than in the roots. At 100 mM NaCl stress wet weights of shoots and roots were decreased by about 40 % and 30%, respectively. At 200 mM NaCl stress, wet weight of shoots and roots decreased about 60% and 40% respectively, when compared with control.

A similar result was also observed in dry weights analysis of control and treated plants. Dry weights of tissues were decreased by about 25% at 100 mM NaCl stress. The decrease was correlated with increased salt stress. At 200 mM NaCl stress, 40 % decrease in dry matter was observed in both tissues.

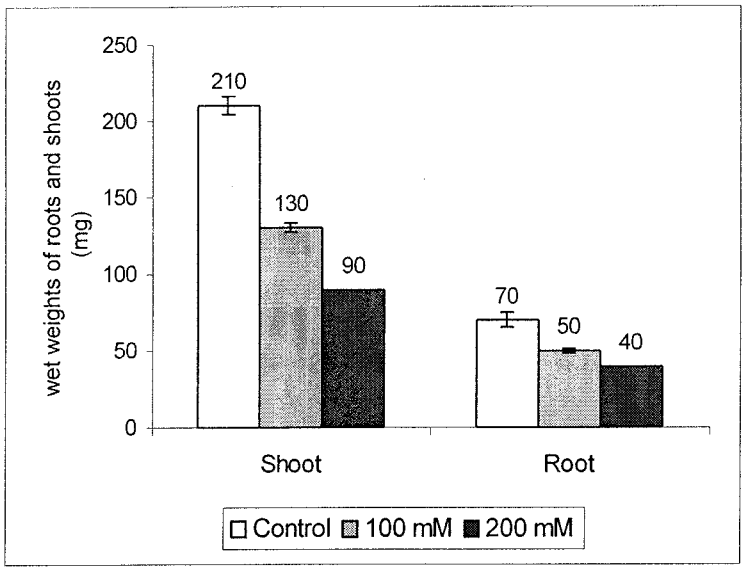

Fig. 3.3 Shoot and root wet weights of control and salt treated plants. Bars indicate the mean weights ± S.E.M.

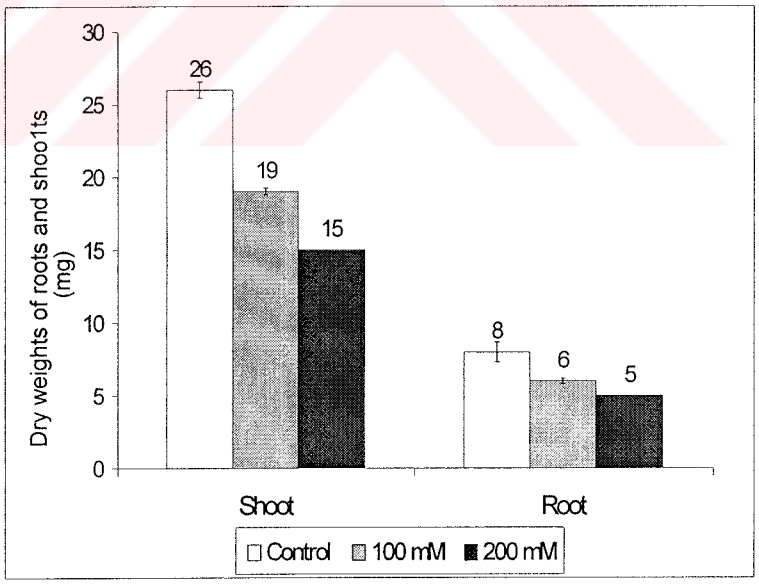

Fig. 3.4 Shoot and root dry weights of control and salt treated plants. Bars indicate the mean weights ± S.E.M.

3.2 Effect of Salt Stress on Proline Content

Effect of salt stress on proline concentration was assessed in roots and leaves of 14 days old control and treated plants. In both tissues, proline concentration increased significantly under salt stress in a concentration dependent manner (Fig. 3.5). 100 mM salt stress caused 55 – 60 % increase in proline concentration in both tissues. The degree of increase at 200 mM NaCl stress was higher in roots than in shoots, with respect to control. Proline content in leaves increased by 115 % in roots at 200 mM NaCl stress, whereas in leaves 67 % increase was observed at the same treatment. The experiments were repeated 5 times with separately grown control and stressed lentil seedlings. The data is given as µmoles proline / g fresh weight.

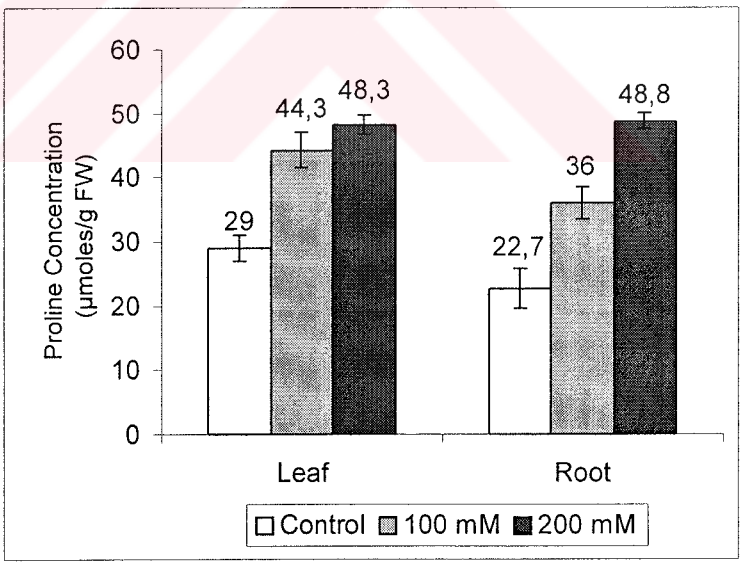

Fig 3.5 Effect of NaCl on proline concentration (µmoles proline / g fresh weight) in shoots and roots. Bars indicate mean concentration ± S.E.M.

3.3 Effect of Salt Stress on Lipid Peroxidation

Effect of salt stress on lipid peroxidation in leaf and root tissues was determined by measuring the malondialdehyde (MDA) content, which is the product of lipid peroxidation.

Effect of salt stress on MDA content was evaluated in roots and shoots of control and treated plants. Salt stress caused different responses in the MDA content of leaves and roots (Fig. 3.6). In roots, MDA concentration did not increase significantly under both NaCl concentrations. In leaves, however, MDA level increased by 113 % at 200 mM NaCl stress when compared to control. At 100 mM NaCl stress, MDA content also increased in leaves, but it was not significantly different than the control plants. Experiment was repeated for 5 times with independently grown control and treated lentil seedlings. The data is given as nmoles of MDA / g fresh weight.

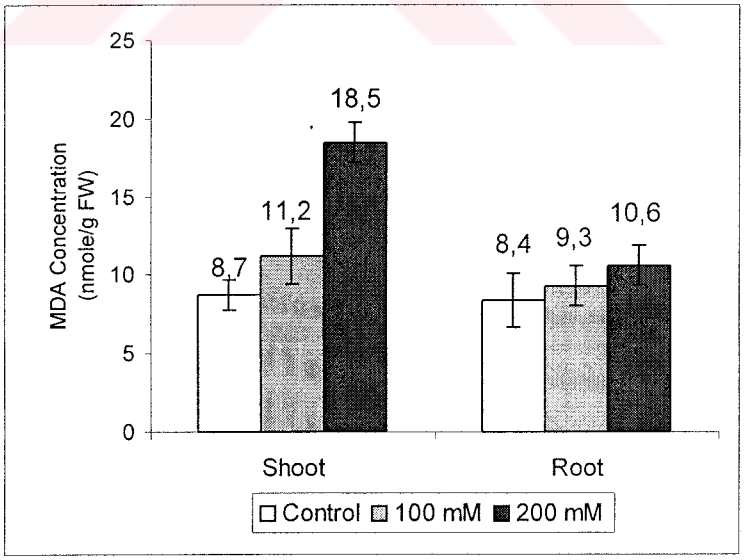

Fig.3.6 Effect of salt stress on the MDA concentration in leaves and roots. Bars indicate the mean ± S.E.M.

3.4 Electrolyte Leakage

For the determination of effect of NaCl on membranes of lentil seedlings, electrolyte leakage test was performed on roots and shoots of control and treated plants. Experiment was repeated 5 times with independently grown seedlings, each set containing 15 plants. The NaCl damage to membrane was calculated as percent of complete injury. At 100 mM NaCl stress, no change was observed in membrane permeability of both tissues. Root tissue was also resisted increasing NaCl concentration and membrane permeability was not changed at 200 mM NaCl stress, as well. However, shoots are strongly affected from extreme NaCl stress. Membrane permeability of shoots was 4 fold higher than the control at 200 mM NaCl stress (Fig. 3.7).

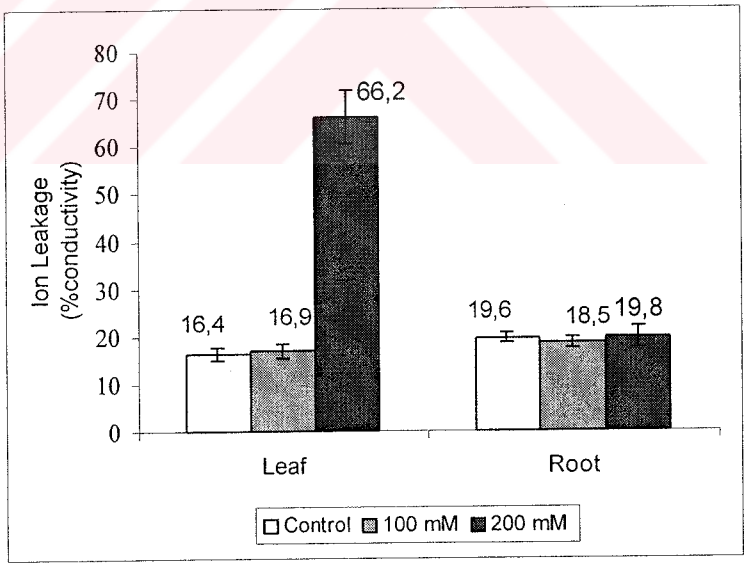

Fig. 3.7 Effect of NaCl on membrane permeability. Bars indicate the mean leakage ± S.E.M.

3.5 Effect of Salt Stress on Hydrogen Peroxide Concentration

Salt stress significantly ($p<0.05$) elevated the hydrogen peroxide (H_2O_2) concentration in roots and shoots, and the increase was correlated with the increasing NaCl concentration (Fig. 3.8). The increment was much higher in shoots when compared the control plants. H_2O_2 concentration increased by about 165 % and 340 % in shoots at 100 mM and 200 mM NaCl concentrations, respectively. In roots, about 35 % and 60 % increase was detected at 100 mM and 200 mM NaCl concentrations, respectively.

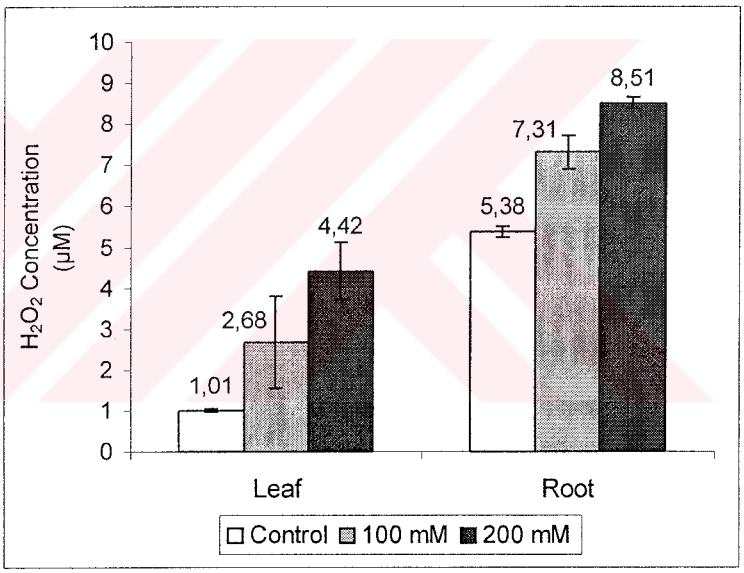

Fig. 3.8 Effect of NaCl stress on H_2O_2 content of leaves and roots of lentil seedlings. Bars indicate the mean of $[H_2O_2] \pm$ S.E.M.

3.6 Identification of SOD Isozymes

For the identification of SOD isozymes in lentil, leaf and root crude extracts containing 5.2 mg/ml and 1.1 mg/ml proteins, respectively, were loaded to the native PAGE with increasing protein concentrations. After running, the gel was stained for SOD activity by negative activity staining.

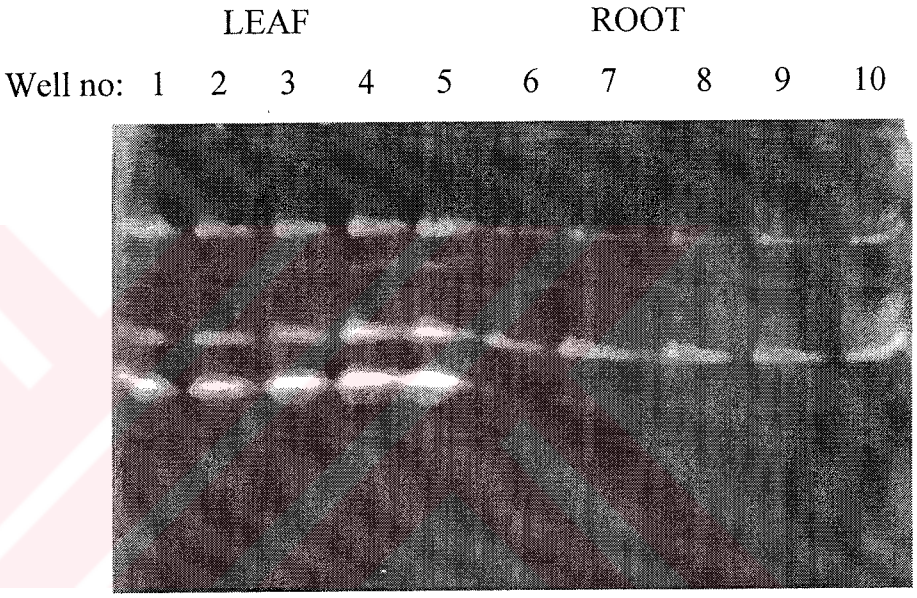

Fig. 3.9 SOD isozymes in leaves (well no:1-5 with protein concentrations 0.1, 0.13, 0.16, 0.18, 0.2 μg/well, respectively) and in roots (well no: 6-10 with proten concentrations 0.022, 0.027, 0.033, 0.038, 0.044 μg/well, respectively) of lentil.

Four SOD isozymes were identified in leaves and three isozymes were identified in roots of lentil seedlings. All isozymes were visible at lowest and highest protein concentrations applied (Fig.3.9).

3.7 Determination of SOD Isozymes

SOD isozymes in roots and leaves of lentil were determined in gels of native PAGE with negative activity staining and inhibition tests. In leaves and roots, mitochondrial MnSOD, and cytosolic Cu/ZnSOD-1 and Cu/ZnSOD-2 isozymes were present. In leaves a chloroplastic FeSOD isozyme was also detected (Fig. 3.10).

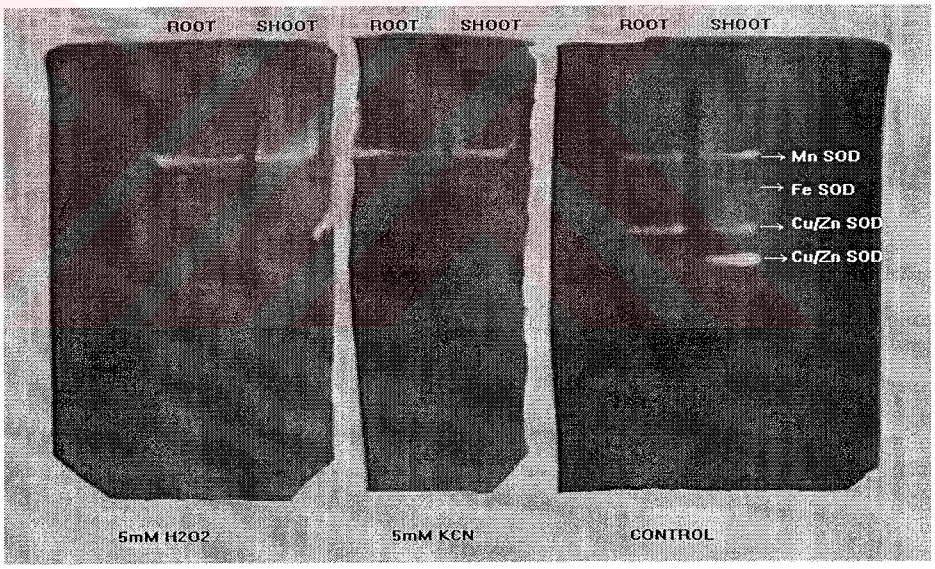

Fig. 3.10 SOD isozymes determined by specific inhibitions by H_2O_2 and KCN. MnSOD, 2 isozymes of Cu/ZnSOD (named 1 and 2, according to increased relative mobility), and FeSOD were detected

3.8 Effect of NaCl on SOD Activity

The activities of SOD isozymes were detected by in gel enzyme activity staining assay according to Beauchamp and Fridovich. (1971). Crude extracts containing equal amounts of protein (80 µg/well) were run on a native PAGE and then stained for SOD activity by negative activity staining method (Fig. 3.11). Gels were photographed in Vilber gel imager and the enzyme activities were analyzed by Bio Profile V99 software program. Experiment was repeated 8 times and the percent change in SOD isozyme activities with respect to controls were calculated.

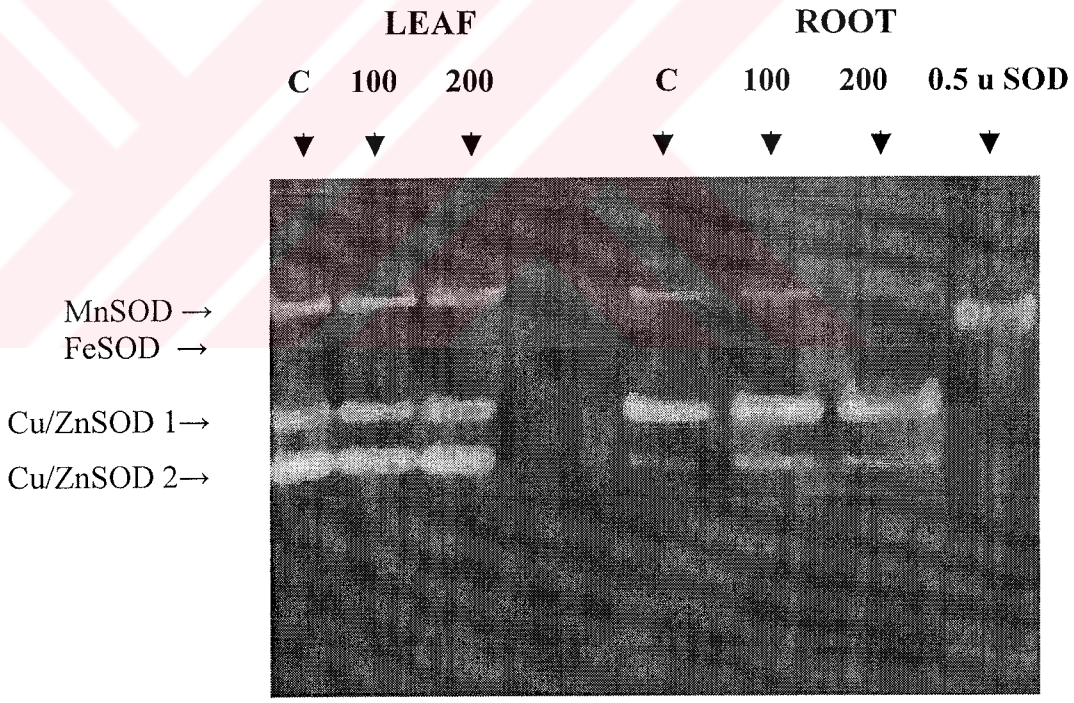

Fig. 3.11 Activities of SOD isozymes in root and leaf tissues of control and NaCl treated lentil seedlings. Intensity of the bands show the activity of each isozyme and tabulated in Table 3.1.

Total SOD activity was calculated by applying known activity of a SOD standard from bovine erythrocyte into one well of the gel (Fig. 3.11). The activity (or, intensity) of the standard was used as a reference for the activities of other isozymes of SOD. Table 3.1 shows the total activity, as well as the activity of each isozymes of SOD in leaves and roots of lentil seedlings.

The activity of each isozyme was also calculated as percent of total SOD activity as tabulated in Table 3.1. The results show that in leaves Cu/ZnSOD2 makes the highest contribution (39 %) to the total SOD activity, whereas in leaves Cu/ZnSOD1 contributes significantly (58 %) to the total SOD activity. The total Cu/ZnSOD isozymes makes about 67 % and 78 % of the total SOD activity in leaves and roots, respectively. MnSOD contributes 25 % and 22 % of the total SOD activity in leaves and roots, respectively, while FeSOD has only the 8 % of total SOD activity in leaves.

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

	SOD Activity (units/mg protein)		**Activity of each isozymes as % of total SOD activity**	
	LEAF	ROOT	LEAF	ROOT
MnSOD	3.5	3.2	25	22
FeSOD	1.1		8	
Cu/ZnSOD1	4	8.4	28	58
Cu/ZnSOD2	5.5	2.8	39	20
TOTAL SOD	14.1	14.4	100	100

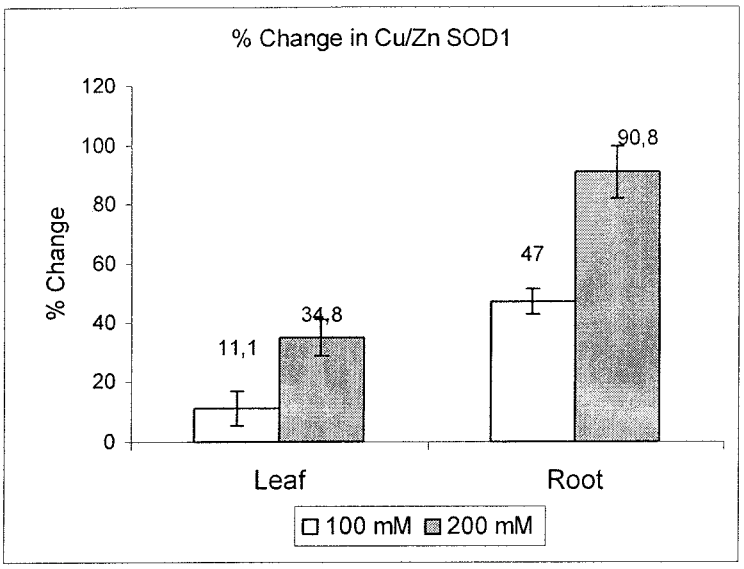

Fig. 3.12 Percent change in the activity of Cu/ZnSOD 1 in root and leaf of lentil seedlings subjected to 100 mM and 200 mM NaCl stress. Bars indicate the mean of percent change with respect to control ± S.E.M.

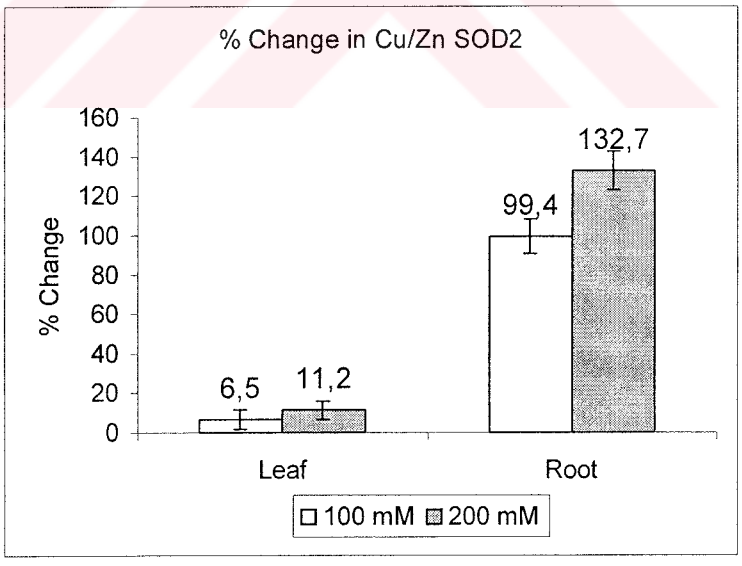

Fig. 3.13 Percent change in the activity of Cu/ZnSOD 2 in roots and leaves of lentil seedlings subjected to 100 mM and 200 mM NaCl stress. Bars indicate the % change with respect to control ± S.E.M.

As it is shown in Fig. 3.12, Cu/ZnSOD1 activity was induced by salt stress. The increase in the activity of Cu/ZnSOD1 in roots was much higher than in the leaves. The elevation in the activity of the enzyme was also correlated with the increase in salt stress.

Cu/ZnSOD2 activity was significantly increased by NaCl treatment in roots. Cu/ZnSOD2 activity in roots increased by about 100 % and 130 % at 100 mM and 200 mM NaCl concentrations, respectively (Fig. 3.13). However, in leaves salt treatment did not cause a significant change in the activity of Cu/ZnSOD2 at neither NaCl concentrations.

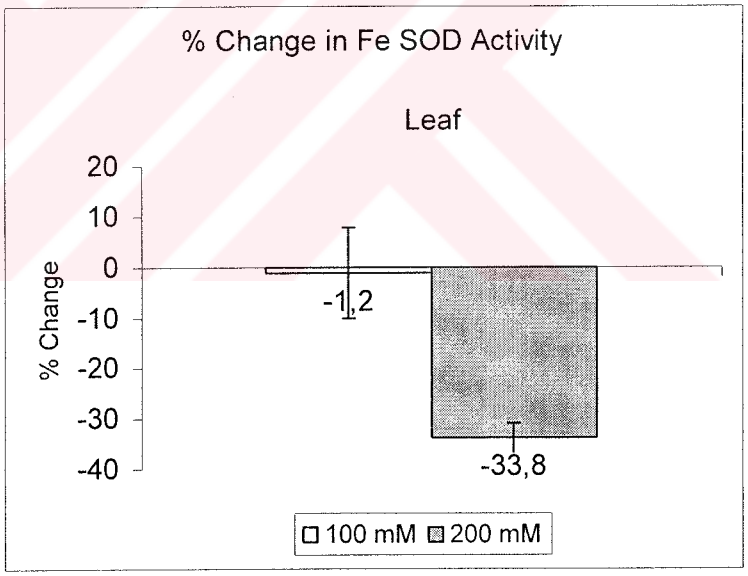

Fig. 3.14 Percent change in the activity of chloroplastic FeSOD in leaves of 100 mM and 200 mM NaCl treated lentil seedlings. Bars indicate the mean of % change with respect to control ± S.E.M.

Chloroplastic FeSOD activity was not affected by 100 mM NaCl stress, yet, at 200 mM NaCl stress its activity decreased by about 34 % with respect to control (Fig. 3.14). In addition, as it is seen in negative activity stained native gels, the activity of FeSOD isozyme in control plants was very low when compared with other isozymes (Fig. 3.11).

MnSOD activity did not affected significantly from 100 mM NaCl stress in neither leaves nor roots. Leaf MnSOD activity did not change significantly at 200 mM NaCl stress, as well. On the other hand, root MnSOD activity increased significantly at 200 mM NaCl stress (Fig 3.15). It seems that, MnSOD is only inducible by extreme salt concentrations in roots.

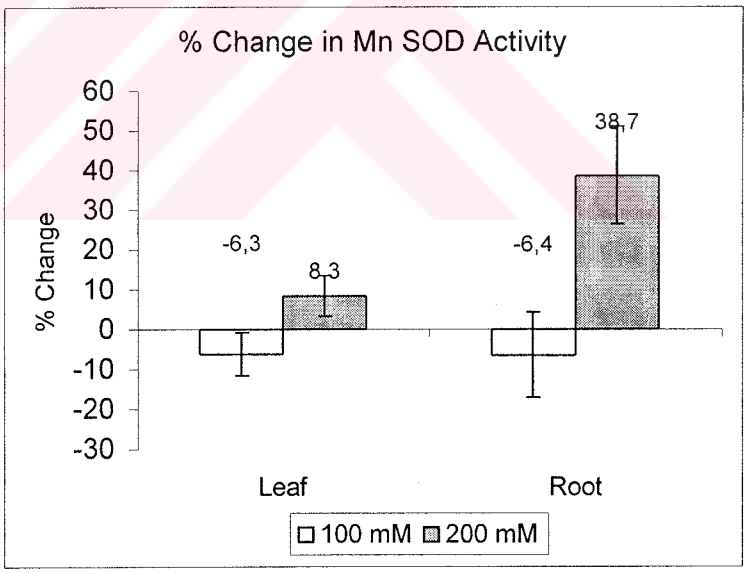

Fig. 3.15 Percent change in MnSOD activity in leaves and roots of 100 mM and 200 mM NaCl treated plants. Bars indicate the mean of percent changes with respect to control ± S.E.M.

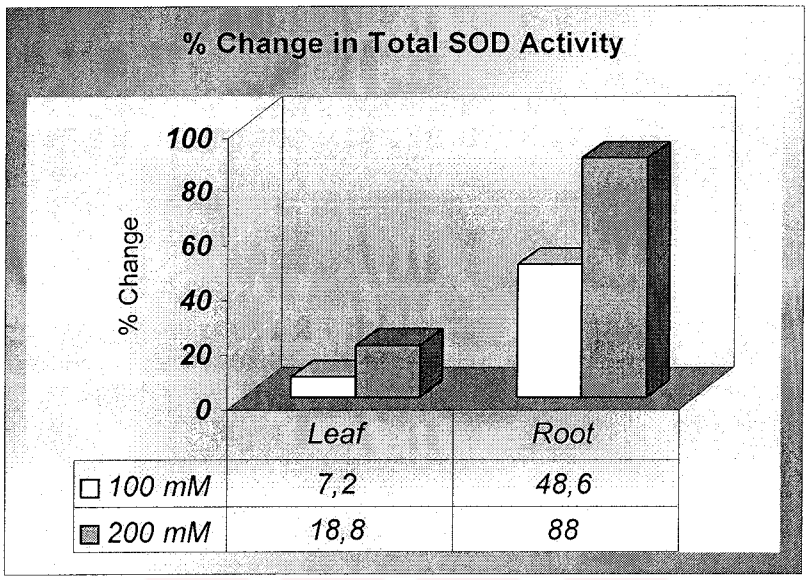

Fig. 3.16 % Change in total SOD activity under 100 mM and 200 mM NaCl stress with respect to controls.

When compared to the controls, total SOD activity increased in leaves by 7.2 % and 18.8 % at 100 mM and 200 mM NaCl stress, respectively. In roots, much higher increase was observed when compared to the leaves. 100 mM and 200 mM NaCl stress caused 48.6 % and 88 % increase in total SOD activity, respectively.

Major increase in total SOD activity was resulted from increases in Cu/ZnSOD isozymes in both tissues.

3.9 Effect of NaCl on Catalase Activity

The activity of catalase (CAT) slightly decreased in shoots and roots of lentil seedlings subjected to salt stress (Fig. 3.17). The decrease in CAT activity in roots at 200 mM NaCl stress was significant at $p<0.05$. Inhibition of CAT activity was correlated with increased salt stress in both tissues. On the other hand, CAT activity was significantly higher in shoots than in roots, at all salt treatments and control.

The experiment was repeated for 5 times with independently grown lentil seedlings and the data is given as the nmoles H_2O_2 turned into water / min / mg protein.

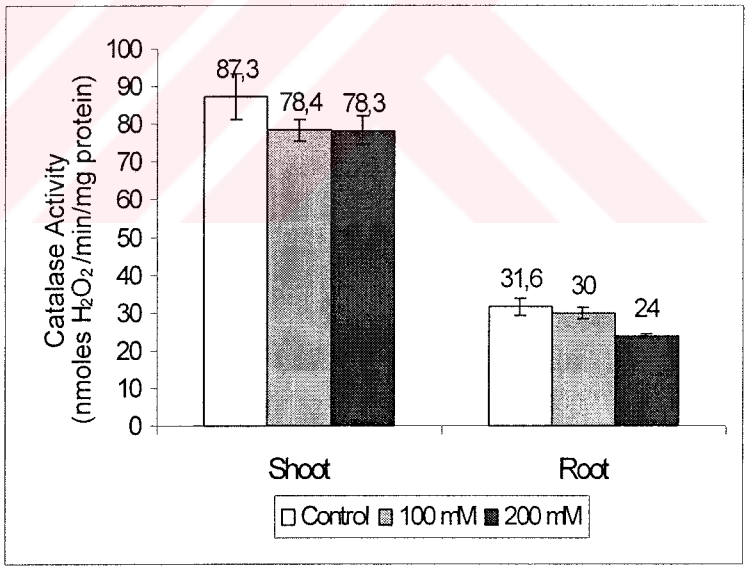

Fig. 3.17 Effect of NaCl stress on the activity of CAT in roots and shoots of lentil seedlings. Bars indicate means of CAT activity (nM H_2O_2/min/µg protein) ± S.E.M.

3.10 Effect of NaCl on Ascorbate Peroxidase Activity

NaCl stress increased the activity of ascorbate peroxidase (APX) both in roots and shoots (Fig. 3.18). In shoots, APX activity increased significantly at 100 mM NaCl stress; however, further increase in stress concentration did not alter the activity of the enzyme. In roots, salt treatment increased the APX activity; yet, the increase was not significant (at $p<0.05$) at both NaCl treatments when compared with the control plant. The activity of APX was found to be much higher in roots than in shoots, in contrast to the case in CAT activity. The experiment was repeated for 5 times with independently grown lentil seedlings. Data is given as nmoles ascorbate converted to monodehydroascorbate (product) / min / mg protein.

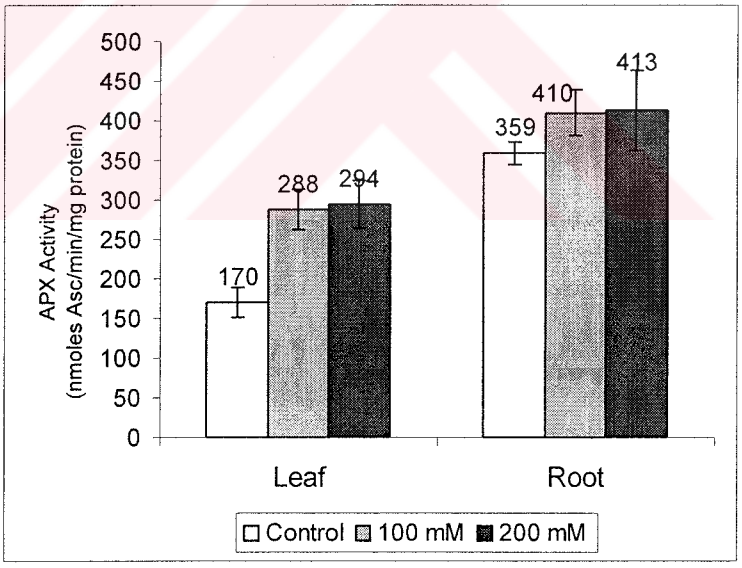

Fig. 3.18 Effect of NaCl on the activity of APX in shoots and roots of lentil seedlings. Bars indicate the mean of APX activity ± S.E.M.

3.11 Effect of NaCl on Glutathione Reductase Activity

When compared with control, glutathione reductase (GR) activity increased by 50 % and 100 % in shoots at 100 mM and 200 mM NaCl stress, respectively. However, in roots, the activity of GR did not change significantly ($p<0.05$) with respect to control (Fig. 3.19). At 100 mM NaCl stress, a slight increase was observed, yet, the activity of the enzyme decreased to control levels at 200 mM NaCl stress. The experiments were repeated 5 times with independently grown lentil seedlings. Data is given as nmoles NADPH converted to $NADP^+$ / min / mg protein.

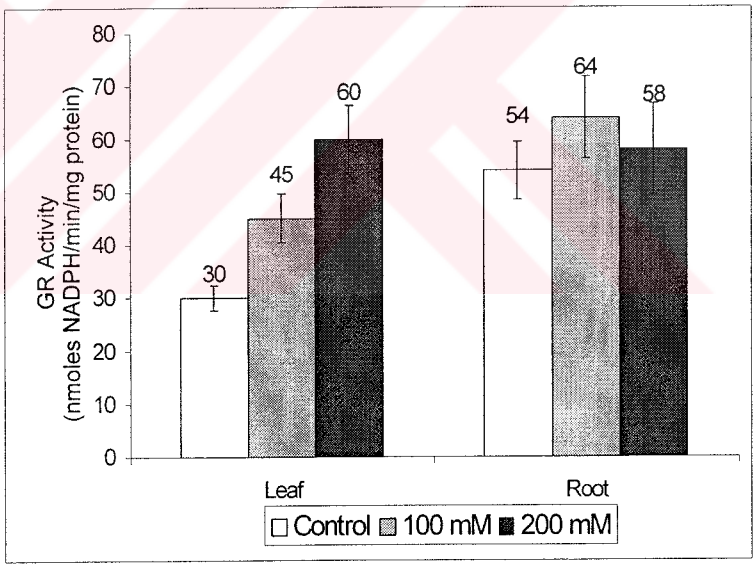

Fig. 3.19 Effect of NaCl on the activity of GR in shoots and roots of the lentil seedlings. Bars indicate mean GR activity of 5 independent experiments ± S.E.M.

Table 3.2. Percent changes in all parameters at 100 mM and 200 mM NaCl stress in shoots and roots of lentil seedlings with respect to controls.

	SHOOTS		ROOTS	
	100 mM	200 mM	100 mM	200 mM
LENGHTS	27 % ↓	43 % ↓	20 % ↓	36 % ↓
WET WEIGHTS	38 % ↓	57 % ↓	28 % ↓	43 % ↓
DRY WEIGHTS	27 % ↓	42 % ↓	25 % ↓	38 % ↓
LEAKAGE	3 % ↑	304 % ↑	6 % ↓	1 % ↑
MDA	29 % ↑	113 % ↑	11 % ↑	26 % ↑
PROLINE	53 % ↑	67 % ↑	59 % ↑	115 % ↑
$[H_2O_2]$	165 % ↑	338 % ↑	36 % ↑	58 % ↑
Cu/ZnSOD1	11 % ↑	35 % ↑	47 % ↑	91 % ↑
Cu/ZnSOD2	7 % ↑	11 % ↑	99 % ↑	133 % ↑
MnSOD	6 % ↓	8 % ↑	7 % ↓	39 % ↑
FeSOD	1 % ↓	34 % ↓		
CAT	10 % ↓	10 % ↓	5 % ↓	25 % ↓
APX	69 % ↑	73 % ↑	14 % ↑	15 % ↑
GR	50 % ↑	100 % ↑	19 % ↑	7 % ↑

CHAPTER 4

DISCUSSION

Lentil is an important crop grown in Turkey. It accounts for as much as 34 % of total legume production, and Turkey is the only country to export lentil. With a high nutritive value, lentil plays an important role in diet of especially rural populations of Turkey. However there are some factors effecting growth and grain yield of lentil. One of these factors is the salinity.

Salinity is one of the environmental adverse conditions imposed on plants. Arid and semiarid regions, in which agriculture depends on irrigation water, are the important source of salinity. It decreases the crop quality and yield by imposing toxic ion effect and water stress. Lentil is very sensitive to salinity, much more than other legumes such as broadbean and soybean and it can only be grown in non-saline soil (Katerji *et al.*, 2001).

In addition to osmotic stress and toxic effects of ions, salt stress results in oxidative stress by interfering with the electron transport and other oxidative processes taking place in the cells. Salt stress causes oxidative stress by inducing water deficit and, consequently, stomatal closure, a process which decreases CO_2 availability and photosynthesis, thus increasing

the likelihood of active oxygen species (AOS) formation in chloroplasts. As soon as the CO_2 concentration decreases inside the chloroplasts, as a result of stomata closure, there is a lower availability of NADP to accept electrons from PS I, thus initiating O_2 reduction with the concomitant generation of AOS. On the other hand, toxic concentrations of ions (especially Cl^- and Na^+) disrupts normal electron flow and result in excess electron leakage, which in turn increases the generation of AOS and induce oxidative stress.

It is well documented that salt stress enhances AOS production which results in improved antioxidant defense in plants, especially in the salt tolerant cultivars. In this study, we have investigated the effect of salt stress on the antioxidant defense system of lentil. In addition, physiological changes, membrane damage and osmoprotective responses of lentil seedlings were examined under increasing NaCl stress.

4.1 Physiological Changes Under Salt Stress

Salt stress resulted in wilting of leaves and necrosis. It significantly prevented the growth of lentil seedlings, in parallel with the increased salt concentration. At 200 mM NaCl stress, the growth was almost totally stopped and leaves wilted.

According to our observations, younger leaves were affected to a lesser extend when compared to the older ones due to the longer period available for the older leaves to accumulate salt. Or, it may be hypothesized that the plant preferentially accumulated salt to the older leaves to maintain growth and protect the actively growing meristematic tissues from damage.

Wet and dry weights also decreased by increasing salt stress in both tissues. Shoot wet weight decreased by 38 % and 57 % at 100 mM and 200 mM NaCl stress, respectively. In roots, a similar, but a bit lesser decrease in wet weights was observed under salt stress. Root wet weight decreased by 28% and 43 % at 100 mM and 200 mM NaCl stress, respectively.

Dry weight of shoots and roots affected similarly with salt stress. About 25 % and 40 % decrease were observed under 100 mM and 200 mM NaCl stress, respectively.

Decreased growth under salt stress has been shown in many plant species. Hernandez *et al* (1999) have shown that increased salt stress resulted in decreased leaf area as well as decreased dry and wet weight in both roots and leaves of pea. Similarly, in narrow-leafed lupins, salt stress caused decreased wet and dry weights (Yu and Rengel, 1999). In rice, growth rate was decreased in salt sensitive cultivar more, when compared to the salt tolerant cultivar (Dionisio-Sese and Tobita, 1998).

4.2. Effect of NaCl on Lipid Peroxidation and Electrolyte Leakage

Salt stress adversely affects the membranes by accumulating toxic ions and AOS. Accumulation of Na^+ and Cl^- under salt stress changes the ion balance of membranes by replacing with the K^+ and Ca^{2+} that have important roles in the functioning of membrane proteins. Thus, the membrane permeability adversely affected. On the other hand, AOS, especially the hydrogen peroxide and hydroxyl radicals, damage the membrane lipids and result in lipid peroxidation, damaging the membrane structure and integrity.

The effect of salt stress on membrane integrity has been extensively studied in many plant species by electrolyte leakage tests and malondialdehyde (MDA) content measurements. MDA is one of the products of lipid peroxidation and is generally used as the indicator of membrane damage. The results show that the extent of damage to membranes under salt stress depends on the plant species and the degree of tolerance to the salt stress.

In rice, NaCl stress resulted in no increase lipid peroxidation and conductivity (Lin and Kao, 2000). Dionisio-Sese (1998) used four different varities of rice differentially sentivite to NaCl and observed that in salt-tolerant cultivars MDA content and electrolyte leakage did not change significantly, while sensitive ones show a significant increase in MDA content and electrolyte leakage. In mulberry and fox-tail millet, it was shown that MDA content increased significantly in sensitive cultivar, while no change was observed in tolerant one (Sreenivasulu *et al.*, 1998; Sudhakar *et al.*, 2001). Salt stress also resulted in increased MDA content in citrus but no difference in the response of salt-sensitive and salt-tolerant cultivars was observed (Gueta-Dahan *et al.*, 1997)

To our knowledge, there is no literature for the effect of salt stress on the membranes of lentil. We investigated the salt induced damage on membranes by MDA content measurements and electrolyte leakage tests. According to our results, lentil roots tolerate salt stress much more effectively than the shoots. The conductivity of root membranes did not change significantly under both NaCl treatments. Shoots also did not affected from 100 mM NaCl concentration. However, conductivity increased to 304 % of the control in shoots at 200 mM NaCl stress. This result was well correlated with the MDA content measurements. All salt treatments increased the MDA

content of tissues, but the increase in shoots at 200 mM NaCl stress was 113 %, which was well above the others. In roots, MDA concentration increased by 11 % and 26 % at 100 mM and 200 mM NaCl concentrations respectively, when compared with the control. The results indicate that, roots are much more tolerant to NaCl stress than the shoots, in terms electrolyte leakage and lipid peroxidation. A supporting result comes from another legume, soybean. Comba *et al.* (1998) showed that salt stress did not affected the MDA content of soybean root nodules.

4.3 Effect of NaCl on Proline Concentration

The amino acid proline is the most widely distributed compatible osmolyte. In organisms from bacteria to plants, there is a strong correlation between increased cellular proline levels and the capacity to survive both water deficit and the effect of high environmental salinity. Many other environmental stresses have also been reported to increase the level of proline in plants. The compound has been attributed to a variety of functions, such as an osmoticum, a protective agent for cytoplasmic enzymes, a reservoir of nitrogen and carbon source, a stabilizer of membranes and the machinery of protein synthesis, a scavenger of free radicals and a sink for energy to regulate redox potential (Rout and Shaw, 1998).

The effect of salt stress on proline concentration has been extensively studied in many plants. In some legumes (alfalfa, soybean and pea) it has been shown that salt stress resulted in extensive proline accumulation (Tramontano and Jouve, 1997). In tomato salt tolerance has been attributed to the degree of plant to accumulate osmoprotectants, like proline (Santa-Cruz *et al.*, 1998).

Supporting the researches on proline accumulation under salt stress, we observed a significant increase in proline concentration in both roots and shoots. Accumulation of proline was correlated with the increased NaCl stress. However, in roots increase in proline concentration at 200 mM NaCl stress was much higher than the other conditions. In shoots, 200 mM NaCl stress resulted in 67 % increase in proline, while in roots it was 115 %. Owing to the protective role of proline on membrane stability we can hypothesize that extensive increase in proline concentration in roots helped for the stabilization and integrity of the root membranes at 200 mM NaCl stress. Mansour (1998) showed that exposure of onion epidermal cells to proline before or during the application of salt stress lower the injurious effect of NaCl on plasma membranes, supporting our hypothesis. Furthermore, free radical scavenger role of proline also correlates with the MDA results. Increased proline content may help detoxification of free radicals, which may be resulted in decreased membrane damage.

4.4. Effect of NaCl on Hydrogen Peroxide Content

Hydrogen peroxide (H_2O_2) is an active oxygen species and produced by the action of superoxide dismutase (SOD) in various compartments of the cell. H_2O_2 is highly toxic and involved in the generation of the potent oxidant hydroxyl radical ($OH^{\cdot}$). Thus, it should be effectively removed from the cell. The detoxification of H_2O_2 is mediated by catalases and various peroxidases. On the other hand, H_2O_2 has been proposed as part of signaling cascade leading to protection from abiotic stresses.

Hydrogen peroxide content has been shown to increase under NaCl stress in a number of studies in various plants. In salt susceptible wheat H_2O_2 content has been found to increase more obviously than in the salt tolerant

wheat exposed to NaCl stress (Tang *et al.*, 1999). In pea mitochondria and chloroplasts salt stress significantly increased the H_2O_2 content (Gomez *et al.*, 1999). A contrasting result has been shown in detached rice leaves that NaCl stress did not cause accumulation of H_2O_2 (Lin and Kao, 2000), which may be attributed to the relatively tolerant nature of this rice species and effective detoxification mechanisms.

We measured the H_2O_2 content of roots and leaves of lentil seedlings. When compared with the controls, in roots, H_2O_2 concentration did not increase significantly at 100 mM NaCl stress, but at 200 mM NaCl stress the H_2O_2 content increased significantly ($p<0.05$), which was about 58 % of the control. The elevation in H_2O_2 concentration was much more higher in leaves. It increased by about 165% and 340% of the control at 100 mM and 200 mM NaCl stress, respectively.

The significant increase in leaves indicates a very low H_2O_2 scavenging system, while in roots H_2O_2 was much more effectively detoxified. High H_2O_2 content in leaves may lead to generation of hydroxyl radicals by reacting with the superoxide radical, which may lead to increased lipid peroxidation and electrolyte leakage. H_2O_2 may itself lead to lipid peroxidation and generates lipid hydroperoxides. Thus, the extensive membrane damage in leaves under NaCl stress may be attributed to the high hydrogen peroxide content and relatively low detoxification system in this tissue.

As will be discussed later, the substantial increase in H_2O_2 content in leaves did not correlate with the SOD and APX activities, which are the hydrogen peroxide generating and detoxifying enzymes, respectively. However, there are other routes of H_2O_2 generation in plant cells. Aside from

SOD, H_2O_2 is generated by several oxidases in various organelles. In peroxisomes H_2O_2 is produced mainly in the photorespiration, glycolate pathway, in fatty acid β-oxidation, in the enzymatic reaction of flavin oxidases and in the disproportionation of $O_2^{\cdot-}$ radicals (Jimenez *et al.*, 1997; Lindqvist *et al.*, 1991; Rich *et al.*, 1976). NADH-oxidase on plasmalemma generates H_2O_2, which is used in lignin biosynthesis (Gross *et al.*, 1977). Increased salt stress may have been increased the activity of these oxidases and resulted in increase in H_2O_2 content in leaves. Inefficient H_2O_2 elimination in leaves may be due to low reaction rate of APX when compared to that of roots, although it increased with increased salt stress. Furthermore, the activity of CAT, which also detoxifies H_2O_2, decreased under salt stress.

4.5. SOD Isozymes Present in Lentil

SOD has several isozymes, namely MnSOD, Cu/ZnSOD and FeSOD, distributed in various organelles of a cell. To the best of our knowledge, this study will be the first showing the isozymes of SOD in lentil. According to the results obtained from inhibition tests for specific SOD isozymes, lentil leaves contain four different SODs, namely MnSOD, FeSOD and two isozymes of Cu/ZnSOD (named 1 and 2 in order of increased mobility). In roots, three isozymes differentiated as MnSOD, Cu/ZnSOD 1 and Cu/ZnSOD 2, were detected.

We did not determine the subcellular distribution of SOD isozymes, however, MnSOD is known to be associated with mitochondria, and FeSOD is chloroplastic because it was not detected in root tissue. Our results indicate that neither isozymes of Cu/ZnSOD are chloroplastic, but they are most probably confined in cytosol.

Native PAGE analysis of SOD isozymes indicated that in leaves FeSOD represents 8 %, while MnSOD and Cu/ZnSODs comprises 25 % and 67 % of the total leaf SOD activity, respectively. In roots, of the total SOD activity MnSOD and Cu/ZnSODs represents 22 % and 78 %, respectively.

These results agrees with the studies made on other leguminous plants, such as pea (Hernandez et al., 1999) reporting the presence same isozymes of SOD and high activity of Cu/ZnSODs.

Total SOD activity in roots and leaves, calculated from the intensity of band having 0.5 units SOD activity (from bovine erythrocyte), was found to 14.1 and 14.4 units / mg protein, respectively. No significant difference between roots and leaves was found in total SOD activity in control plants. The major SOD activity in both tissues comes from Cu/ZnSOD isozymes, being 9.5 units in leaves and 11.2 units in roots.

4.6 Effect of NaCl Stress on the Activity of SOD Isozymes

Active oxygen species cause cellular damage during a wide variety of environmental stresses. Superoxide dismutases (SOD) respond to a range of stresses and plants acquire tolerance to them by increasing the SOD activity, as well as the activity of H_2O_2 detoxifying enzymes.

Hernandez *et al.* (1999) showed that pea plants cope with oxidative stress by inducing antioxidant enzyme activity under salt stress. Breusegem *et al.* (1999) indicated that the overproduction of MnSOD in chloroplasts increases the antioxidant capacity of the maize leaves. It has also been shown that the transgenic plants overproducing SODs show enhanced tolerance to experienced stresses.

In our study, we carried out native PAGE and negative activity staining techniques for leaf and root crude extracts to determine the individual SOD isozyme activities. The intensity of bands is proportional to the activity of SOD isozymes present in tissues and it may also give an idea about the amount of each isozyme present.

Although the total SOD activity increased significantly in both tissues, our results indicate that SOD isozymes were differentially affected from salt stress. MnSOD was not affected from 100 mM NaCl stress, but it was induced slightly in both tissues when stress concentration was increased.

The chloroplastic FeSOD was inhibited by 200 mM salt stress, while its activity remained same at low NaCl stress. The activity of FeSOD comprises about the 8 % of total SOD activity in both tissues and decrease in the activity of this isozyme does not significantly affect the general increase in SOD activity.

In leaves, both isozymes of Cu/ZnSOD activity increased by salt stress and the increase was correlated with the stress concentration. However, it was not significantly different from the control plants at 100 mM NaCl stress. On the other hand, drastic inclines in the activity of both isozymes of Cu/ZnSOD were observed in roots under both stress conditions. Considering that Cu/ZnSODs comprise about the 70 to 80 % of the total SOD activity in both tissues, increase in the activity of this isozyme is very important for the effective detoxification of superoxide radicals.

We calculated the changes in total SOD activity in referring to the alterations of each SOD isozymes and their relative contributions to total SOD activity. The results show that total SOD activity in leaves increased only slightly at both salt treatments. Whereas, in roots total SOD activity increased in correlation to the applied salt stress, and it is significantly different from the controls. At 100 mM NaCl stress we observed 48.6 % increase in total SOD activity and at 200 mM NaCl stress it increased by 88 % when compared to control. As we pointed out before, Cu/ZnSOD isozymes made the major contribution to increase in SOD activity in both tissues.

Supporting results have been observed in lupins (Yu and Rengel, 1999) that salt treatment at all levels increased the activity of Cu/ZnSOD, without affecting the activities of other SOD forms. An increased Cu/ZnSOD activity, along with the simultaneously decreased MnSOD activity was found in cowpea plants (Hernandez *et al.,* 1993). The consistent response of Cu/ZnSOD among different plant species exposed to salt stress could indicate that Cu/ZnSOD was closely involved in the salt-induced oxidative stress events.

We suggest that significant increase in Cu/ZnSODs results in removal of superoxide from the roots, which significantly inhibited reaction of H_2O_2 with superoxide through Fenton reaction, and generation of highly reactive hydroxyl radical. This, in turn, decreased the lipid peroxidation significantly and prevented the membrane damage in roots. Thus, we observed a much lesser increase in MDA (a product of lipid peroxidation) contents in roots compared to leaf tissues.

3.7. Effect of NaCl Stress on Catalase Activity

In plants, catalase (CAT) is found predominantly in peroxisomes (and also in glyoxysomes) where it functions chiefly to remove the H_2O_2 formed during photorespiration or during β-oxidation of fatty acids in glyoxysomes. Increase in CAT activity is supposed to an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of hydrogen peroxide produced during cell metabolism.

A number of studies are available for the effect of salt stress on CAT activity in several plant species. CAT activity has been found to increase under salt stress in tomato (Rodriguez-Rosales *et al.*, 1999), soybean (Comba *et al.*, 1998), tobacco (Bueno *et al.*, 1998; Savoure *et al.*, 1999), cucumber (Lechno *et al.*, 1997) and mulberry (Sudhakar *et al.*, 2001). Furthermore, in salt tolerant cultivars of tomato and mulberry CAT activity has been found to be higher when compared with the salt sensitive cultivars. However, it has been shown that in potato (Benavides *et al.*, 2000) and rice (Lin and Kao, 2000) CAT activity does not change under salt stress.

We found a slight decrease in CAT activity in both leaves and roots of lentil seedlings under both salt treatments. However, it was not significantly different from the control at 95 % confidence interval.

In comparison to the roots, CAT activity was found to be much higher in leaves in both control and treated plants suggesting that it has a major role in scavenging of H_2O_2 in leaves. Also, a relatively higher decrease in CAT activity in roots could suggest that CAT activity decreased as a result of accumulation of toxic ions in roots, which are subjected to direct NaCl stress.

3.8. Effect of NaCl Stress on Ascorbate Peroxidase Activity

The role of ascorbate peroxidase (APX) in the H_2O_2 scavenging in plant cells has been well established in Halliwell-Asada enzyme pathway. It is predominantly found in cytosol, mitochondria and chloroplasts and utilizes ascorbate as an electron donor in the neutralization of H_2O_2.

It has been reported that APX activity may have an important role in the mechanism of salt tolerance in plants. Gueta-Dahan *et al.* (1997) indicated that APX seems to be a key enzyme in determining salt tolerance in citrus as its constitutive activity much higher in salt tolerant cultivar. APX activity has been shown to be higher in tolerant cultivars of pea (Hernandez *et al.*, 1999), mulberry (Sudhakar *et al.*, 2001), tomato (Rodriguez-Rosales *et al.*, 1999) and potato (Benavides *et al.*, 2000) under salt stress, suggesting its role in salt tolerance.

Our results show that salt stress caused a significant increase in APX activity in leaves of lentil seedlings at 100 mM NaCl stress but 200 mM salt treatment did not cause a further increase in APX activity. In roots, salt treatment resulted in only a slight increase in APX activity, which was not significant at 95 % confidence interval.

The activity of APX was much higher in roots in both control and treated plants, suggesting that it has an important role in the elimination of hydrogen peroxide in roots. However, significant elevation of APX activity in leaves under salt stress suggests that leaf APX isozyme is salt inducible.

3.9 Effect of NaCl Stress on Glutathione Reductase Activity

Glutathione reductase (GR) is found in chloroplasts as well as in mitochondria and cytoplasm and catalyzes rate-limiting last step of ascorbate-glutathione pathway. The enzyme is important for the maintenance of reduced form of glutathione in the cell at high levels, because reduced glutathione is itself free radical scavenger. Elevated levels of GR activity could increase the ratio of $NADP^+$/NADPH, thereby ensuring the availability $NADP^+$ to accept electrons from photosynthetic electron transport chain, and minimizing the reduction of oxygen and formation of superoxide radicals.

Effect of salt stress on GR activity has been studied in several cases including rice (Lin and Kao, 2000), soybean (Comba *et al.*, 1997), tomato (Gueta-Dahan *et al.*, 1998), tobacco (Bueno *et al.*, 1998), citrus (Gueta-Dahan *et al.*, 1997) and mulberry (Sudhakar *et al.*, 2001). It was evident that salt stress triggers differential alterations in GR activity in these plant species. In rice, soybean, and mulberry increased activity of GR was indicated under salt stress.

Furthermore, GR reductase activity was found to be higher in salt-tolerant cultivars of mulberry, tomato and soybean. However, in tomato GR activity decreased in both salt-tolerant and salt sensitive cultivars subjected to NaCl stress. In tobacco, salt stress increased the activity of all antioxidant enzymes except GR, which remained unchanged.

In our study, we observed a significant elevation in the GR activity in leaves of lentil seedlings and the increased activity was correlated with the concentration of salt stress. In roots, salt stress did not alter the GR activity at both salt concentrations. The constitutive activity of GR in roots was much

higher when compared to that of leaves. However, salt stress induced level of leaf GR activity exceeded the roots GR activity at 200 mM NaCl stress. This may suggests that leaf isozyme of GR is salt inducible.

CHAPTER 5

CONCLUSION

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

The results presented here suggest that salt stress is accompanied by oxidative stress in lentil, as seen by its effect on antioxidant enzymes tested. Another component of salt stress is the osmotic effect, which resulted in increased proline concentration for osmoprotection.

Salt stress resulted in decreased growth of lentil seedlings, which may give rise to low yield and quality in further developmental stages.

Our results show that roots and leaves of lentil seedling are affected from NaCl stress differentially. In roots, we observed a higher osmoprotection and antioxidant enzyme activities, except catalase.

Furthermore, the effect of salt stress on membranes is much higher in leaves when compared to the roots.

The results of this study are first to the effect of salt stress on the antioxidant defense system of lentil and may be used as a base for further analysis of lentil under different stress conditions. In this study salt stress resulted in an induction of antioxidative system in lentil. However, for a better understanding of the relationship between salt stress and oxidative stress, lentil varieties having different sensitivities to salt stress should be analyzed.

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APPENDIX A

OVERALL CHANGES IN ALL PARAMETERS UNDER NaCl STRESS

A. Physiological changes under 100 mM and 200 mM NaCl stress. Numbers indicate the means ± S.E.M.

	SHOOT			ROOT		
	Control	**100 mM**	**200 mM**	**Control**	**100 mM**	**200 mM**
Length (cm)	19.4±0.3	14.2±0.3	11.1±0.3	9.9±0.2	7.9±0.3	6.3±0.2
Wet Weight (mg)	210±0.0	130±0.0	90±0.0	70±0.0	50±0.0	40±0.0
Dry Weight (mg)	26±0.0	19±0.0	15±0.0	8±0.0	6±0.0	5±0.0

B. Changes in electrolyte leakage, MDA, proline and H_2O_2 concentration under NaCl stress. Numbers indicate the means ± S.E.M

	LEAF			ROOT		
	Control	**100 mM**	**200 mM**	**Control**	**100 mM**	**200 mM**
Leakage (% cond.)	16.4±1.4	16.9±1.4	66.2±5.7	19.6±1.0	18.5±1.1	19.8±2.2
MDA (nmoles/g FW)	8.7±2.3	11.2±3.9	18.5±3.1	8.4±4.2	9.3±2.8	10.6±3.1
Proline (µmole/g FW)	29±2.0	44.3±2.7	48.3±1.6	22.7±3.0	36±2.5	48.8±1.3
H_2O_2 (µM)	1.01±0.0	2.68±0.1	4.42±0.7	5.38±1.1	7.31±0.4	8.5±0.1

C. Alterations in antioxidant enzyme activities under NaCl stress. Numbers indicate the means ± S.E.M.

	LEAF			ROOT		
	Control	**100 mM**	**200 mM**	**Control**	**100 mM**	**200 mM**
Cu/ZnSOD1 (% Change)	100	111.1±5.8	134.8±6.0	100	147±4.4	191±8.8
Cu/ZnSOD2 (% Change)	100	106.5±4.7	111.2±4.8	100	199.4±8.9	232.7±10
MnSOD (% Change)	100	93.7±5.4	108.3±5.1	100	93.4±10.1	139±12.3
FeSOD (% Change)	100	98.8±9	66.1±2.9			
Total SOD (% Change)	100	107.2	118.8	100	148.6	188
CAT (nmoles H_2O_2min/mg protein)	87.3±6	78.4±2.9	78.3±3.7	31.6±2.2	30±1.5	24±0.5
APX (nmoles Asc/min/mg protein)	170±19	288±14	294±30	359±25	410±29	413±50
GR (nmoles NADPH/ min/mg protein)	30±2.3	45±4.6	60±6.4	54±5.5	64±7.8	58±8.6

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 an 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 4°C in dark bottle, used within on month.

II. Seperating Gel Buffer Stock 4x (1.5 M Tris-HCl pH 8.8)

Trizma base	54.45 g

Dissolved in 150 ml distilled water, adjusted to pH 8.8 with concentrated HCl and raised to 300 ml with distilled water. Filtered and stored at 4°C.

III. Stacking Gel Buffer Stock 4x (0.5 M Tris-HCl pH 6.8)

Trizma base	6 g

Dissolved in 60 ml distilled water, adjusted to pH 6.8 with concentrated HCl and raised to 100 ml. Filtered and stored at 4°C.

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

Trizma base	45 g
Glycine	216 g

Dissolved in sufficient amount of water and raised to 3 L with distilled water. Diluted to 1x before electrophoresis.

V. Gel Formation

	Separating Gel	Stacking Gel
Acrylamide/bis stock	2 ml	0.415 ml
Separating Buffer	1.25 ml	_
Stacking Buffer	_	0.315 ml
dH_2O	1.65 ml	1.7 ml
10 % APS	25 µl	10 µl
TEMED	2.5 µl	2.5 µl