# EFFECT OF SALT STRESS ON ANTIOXIDANT DEFENSE SYSTEMS OF SENSITIVE AND RESISTANT CULTIVARS OF LENTIL (*Lens culinaris* M.)

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Işın Nur Cicerali

## ABSTRACT

### EFFECT OF SALT STRESS ON ANTIOXIDANT DEFENSE SYSTEMS OF SENSITIVE AND RESISTANT CULTIVARS OF LENTIL

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In this study, two lentil cultivars (*Lens culinaris*, Medik.) (ILL5582-salt tolerant and ILL590) were characterized and compared due to their NaCl susceptibility and antioxidant mechanism was examined under laboratory conditions. Physiological parameters such as wet-dry weight, root-shoot lengths, cell membrane stability, lipid peroxidation in terms of malondialdehyde (MDA), $H_2O_2$, proline contents were determined. The activities of antioxidant enzymes such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), ascorbate peroxidase (APX: EC 1.11.1.11) and glutathione reductase (GR: EC 1.6.4.2) were examined and analyzed in 14 days old plant seedlings after 9 days of normal growth and 5 days of 100mM and 200mM NaCl stress conditions.

Shoot-root length and wet-dry weight percent decrease were more in ILL590. Especially shoot tissues were affected more from the stress conditions when compared to root tissues.

According to malondialdehyde (MDA) content and membrane stability results, lipid peoxidation was higher in ILL590 and significant increases were observed in shoot tissues.

Proline concentration showed a remarkable increase in salt concentration dependent manner. Higher concentrations of proline in ILL5582 might be the reason of higher salt tolerance when compared to ILL590.

Among the antioxidant enzymes SOD was the one which showed highest activity increase. At organ level roots showed highest activity when compared to leaves. In the organelle higher activity percent contribution was achieved by cytosolic Cu/ZnSOD isozyme. Higher percent increase of this isozyme was observed in ILL5582. This might be one of the tolerance mechanisms that get activated against NaCl stress. APX activity showed similar alterations in both cultivars. In leaf tissues significant increase was observed but in root tissues ascorbate peroxidase activity did not change significantly. Glutathione Reductase activity increase was significant in both cultivars leaf tissues but although ILL5582 showed a stress concentration dependent increase, ILL590 did not. The activity of CAT enzyme in leaf and root tissues of both cultivars did not significantly change under increasing salt stress conditions.

The results suggested that the leaves were more susceptible to salt stress. Also when two cultivars were compared ILL5582 was found to be more tolerant against salt stress than ILL590 under laboratory conditions and SOD enzyme seemed to be the most active component of the salt tolerant mechanism.

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

# ÖZ

## TUZ STRESİNİN, DİRENÇLİ VE HASSAS MERCİMEK ÇEŞİTLERİNİN ANTİOKSİDAN SİSTEMLERİ ÜZERİNE ETKİSİ

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Bu çalışmada, iki mercimek (*Lens culinaris*, Medik.) çeşidi ILL5582 ve ILL590, NaCl stresi altında karakterize edilerek karşılaştırılmış ve antioksidan mekanizmaları laboratuvar koşulları altında incelenip analize tabi tutulmuştur. Yaş-kuru ağırlık, kök-gövde uzunluğu gibi fizyolojik parametreler, lipid peroxidasyonu (malondialdehit (MDA) miktarı olarak), $H_2O_2$ ve proline miktarları, antioksidan enzimlerinden superoksit dismutaz (SOD: EC 1.15.1.1), katalaz (CAT: EC 1.11.1.6), askorbat peroksidaz (APX: EC 1.11.1.11), glutatyon redüktaz (GR: EC 1.6.4.2) aktiviteleri, 14 günlük fidelerde (9 gün normal büyüme ve 5 gün 100mM ve 200mMlık NaCl stresi altında incelenmiş ve analiz edilmiştir.

ILL590'da kök-gövde uzunluğu ve yaş-kuru ağırlık düşüş yüzdeleri daha yüksekken yaprak dokularının bu stres durumlarından, kök dokularına oranla daha çok etkilendikleri gözlenmiştir.

Malondialdehit (MDA) miktarlarına göre, ILL590'da lipid peroksidasyonunun daha yüksek olduğu ve yaprak dokularında anlamlı artışlar saptanmıştır.

Prolin konsantrasyonunda tuz konsantrasyonuna bağlı olarak dikkate değer bir artış olmuştur. ILL590'a oranla ILL5582'de ki yüksek artış tuza direncin bir göstergesi olabilir.

Antioksidan enzimleri arasında, SOD en yüksek aktivite artışını göstermiştir. SOD izozimleri iki çeşidinde kök dokularında, yaprak dokularına oranla daha yüksek aktivite göstermiştir. Bu enzimin artışındaki en yüksek katkı sitosolde bulunan Cu/ZnSOD izozimi tarafından yapılmaktadır. ILL5582'de Cu/ZnSOD'un artışının daha yüksek olduğu gözlenmiştir. Bu durum NaCl stresi altında aktive olan bir direnç mekanizması olarak düşünülebilir. APX enzim aktivitesi iki çeşidinde de yaprak dokularında anlamlı artışlar gözlenmiştir. Aynı şekilde GR enzimi iki çeşidin yaprak dokularında da anlamlı artış gostermiştir fakat bu artış ILL5582'de tuz stresi derişimine bağlı, ILL590'da ise değildir. CAT enzim aktivitesinde her iki dokuda da anlamlı değişiklik gözlenmemiştir.

Sonuçlara göre, tuz stresi sebebiyle yaprak dokusunda oluşan zarar, kök dokusundakinden daha fazladır. Ayrıca iki çeşit karşılaştırıldığında, laboratuvar koşulları altında ILL5582'nin ILL590'a oranla, tuza karşı daha yüksek bir toleransının olduğu ve SOD enziminin bu mekanizmanın en aktif parçası olduğu anlaşılmıştır.

Anahtar kelimeler: Mercimek, tuz stresi, oksidatif stres, SOD, APX, GR, CAT, proline, $H_2O_2$, lipit peroksidation, MDA

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| | |
|---|---|
| OOH | Perhydroxyl radical |
| $^1O_2$ | Singlet oxygen |
| ROS | Reactive oxygen species |
| APX | Ascorbate peroxidase |
| AsA | Ascorbate |
| CAT | Catalase |
| DHAR | Dehydroascorbate reductase |
| GR | Glutathione reductase |
| GSH | Reduced glutathione |
| GSSG | Oxidized glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| MDA | Malondialdehyde |
| MDHA | Monodehydroascorbate |
| NaC1 | Sodium chloride |
| $O_2^{\cdot -}$ | Superoxide radical |
| OH | Hydroxyl radical |
| PAGE | Polyacrylamide Gel Electrophoresis |
| POD | Peroxidase |
| ROOH | Lipid Hydroperoxide |
| SOD | Superoxide Dismutase |

# CHAPTER 1

# INTRODUCTION

## 1.1 Lentil

*Lens* is a Latin word that describes exactly the shape of the 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.

Lentil is a valuable crop for humanity with its high protein content (20.2 g /100g dried seed) which comparable with faba bean; higher than chickpeas, and more than double that of wheat. It has also high concentration of folic acid, fiber (4g/100g dried seed) and iron. In addition to these it is beneficial because of its low calorie (340 cal/ 100g dried seed), fat (0.6 g/100g dried seed) and cholesterol content. It has a remarkable vitamin and mineral value with 68mg Ca, 325mg P, 7.0 mg Fe, 29mg Na, 780mg K, 0.46 mg thiamine, 0.33 mg riboflavin, 1.3 mg niacine (Adsule et al.,1989; Muehlbauer et al.,1985). Besides its nutritive value, it has an agricultural value of nitrogen fixation. Lentil has been one of the oldest food crops of man which is originated in the Fertile Crescent of the Near East and date back to the beginning of the agriculture itself. It has the ability to thrieve on relatively poor soils and under adverse environmental conditions especially high temperature and drought, has ensured their survival as crop species to the present day.

*Lens culinaris* Medik. Belongs to the division Arthrophyta, subdivision Dicotyledonae, order Roseles, sub-order Rosinae, family Leguminosae and sub-family 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 *et al.*, 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. 34% of total legume production accounts for lentil.

Lentil is a cool season crop which is best in soil with pH 6.0-8.0 and can not tolerate waterlogging, flooding or high soil salinity. 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.

### 1.2 Biotic and Abiotic Stress Conditions and Their Effect on Plant Growth and Productivity

All stress conditions induce potential injury on plant species. This injury can be either a reversible inhibition of metabolism and growth, or irreversible injury by 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).

The two types of environmental stresses are biotic, caused by other organisms and abiotic, caused by non-living effects of environment. In Table1.1 list of the general stress elements are given.

**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 $\gamma$ -ray) |
| Chemicals: salts, ions, gases, herbicides, heavy metals |
| Mechanical factors: wind, pressure |

### 1.3 Salt Stress

Salinity is the presence of the 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). In this study salinity is used to mean $Na^{+}$ concentration of the growth solution, which is the main constituent of the saline soils. High concentrations of the $Na^{+}$ cation gives damage to the plant by two means; osmotic stress and toxic effect

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 to 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).

Also stomatal closure is a process, which decreases $CO_2$ availability and photosynthesis thus increasing the likelihood of ROS formation. As soon as the $CO_2$ concentration decreased in the chloroplast as a result of stomatal closure; there is also a 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).

Secondly 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 $Ca^{++}$ 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 $K^+$, $Ca^{++}$, $Mg^{++}$ and $NO_3^-$ decreased. Both $K^+$ and $Ca^{++}$ are required in the external growth medium to maintain the selectivity and integrity of the cell membrane. $Ca^{++}$ and $K^+$ play similar roles for selective transport of ions across the membranes. As a consequence, high $Na^+/K^+$ and $Na^+/Ca^{++}$ 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.

Grain legumes generally are considered as salt sensitive (Maas *et al*., 1977; Katerji *et al*.,2000). The salinity effect on bacterial activity with respect to nitrogen fixation is one of the hypothesis for explaining its salt sensitivity (Pessarakli *et al*., 1989; Katerji *et al.*, 2001a). Lentil under saline conditions systematically shows lower values of nitrogen fixation during the whole growing season (van Hoorn *et al.*, 2001)

Salinity is a serious problem all around the world. It is one of the major limiting factors in agriculture. Coarse estimates show that only 10% of the agricultural area is in

non-stress conditions (Epstein, 1980). In Turkey, especially in dry areas and in irrigated agriculture, 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). 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. Selection of salt-tolerant varieties would allow one to cultivate this crop on saline soils of with saline waters which occur frequently in Mediterranian area (Katerji *et al.,* 2000).

### 1.3.1 Salt Stress Conditions

The productivity of plants is greatly affected by various environmental stresses. Soil salinity affects plant growth and development by way of osmotic stress, injurious effects of toxic $Na^+$ and $Cl^-$ ions and to some extend $Cl^-$ and $SO_4^{2-}$ of $Mg^{+2}$ and nutrient imbalance caused by excess $Na^+$ and $Cl^-$ ions (Sairam *et al*., 2004).

When plants are grown on inherently saline soils or heavily irrigated soils they are exposed to salinity. 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. (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 $H^+$ transport ATPases 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).

$Na^+$ 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 $Na^+/H^+$ antiporters 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 ($H^+$-ATPase and $H^+$-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 Salt Tolerance Mechanisms

Salt tolerance is 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).

Salt tolerant plants differ from salt sensitive ones in having a low rate of $Na^+$ and $Cl^-$ transport to leaves and the ability to compartmentalize these ions in vacuoles to prevent their build up in cytoplasm or cell walls and thus avoid salt toxicity (Munns, 2002).

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, mannitol, 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).

One of the mechanisms of activation is by the stepwise monovalent reduction of oxygen to form superoxide radical ($O^{2\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide ($H_2O_2$) and finally the water. It is these intermediate oxyradical species, which are highly reactive, that can react with proteins, nucleic acids and/or lipids, potentially

causing denaturation, mutagenesis, and/or lipid peroxidation (McKersie, 1996).

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 (QOH), which is a powerful oxidant, but its biological relevance is probably minor because of its low concentration at physiological pH (as reviewed in 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. In the late nineteenth century, Fenton described the oxidizing potential of hydrogen peroxide mixed with ferrous salts. Also, as Haber and Weiss described, in the presence of a trace amount of iron, the reaction of superoxide and hydrogen peroxide will form the destructive hydroxyl radical and initiate the oxidation of organic substrates (as quoted in McKersie, 1996):

$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{\cdot} + OH^{-}$ (Fenton reaction)

$H_20_2 + O_2^{\cdot -} \rightarrow OH^{-} + O_2 + OH^{\cdot}$ (Haber-Weiss reaction)

In recent years $H_2O_2$ has been described as a diffusible transduction signal in different physiological processes and plant stress, leading to the function of genes encoding different cellular protectants (del Rio *et al*., 2002).

### 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 disfunctioning of enzymes or electron transport systems as a result of perturbations in metabolism caused by chemical or environmental stress. Chloroplasts, mitochondria, endoplasmic reticulum, microbodies, plasma membranes, and cell walls are the major sites of activated oxygen production in a plant cell (McKersie, 1996).

As described by Elstner (1991), there are at least four sites within the chloroplast that can activate oxygen. Firstly, the reducing side of PSI is thought to contribute significantly to the monovalent reduction of oxygen under conditions where NADP is limiting. Secondly, under conditions that prevent the captured light energy from being utilized in the electron transport systems, the excitation energy of photoactivated chlorophyll can excite oxygen from the triplet to singlet form. Thirdly, leaks of electrons from the oxidizing side of PSH to molecular oxygen, or release of partially reduced oxygen products contribute to activated oxygen production. Lastly, due to photorespiration glycolate is formed, whose subsequent metabolism in the peroxisomes leads to the generation of activated oxygen.

The main superoxide generators in the mitochondria are the ubiquinone radical and NADH dehydrogenases. Superoxide is formed by the autooxidation of the reduced components of the respiratory chain (Dat *et al.,* 2000). The various Fe-S proteins have also been implicated as possible sites of superoxide and hydrogen peroxide formation in mitochondria (Turrens *et al.,* 1982).

Various oxidative processes occuring 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 $cytP_{45}o$-$ROO^{\cdot}$) since each has one unpaired electron. This oxygenated complex may be reduced by cytochrome b or occasionally the complex may decompose releasing superoxide (McKersie, 1996).

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 $H_20_2$ in a reaction involving two electron transfer from glycolate to oxygen (Lindqvist *et al.*, 1991). Xanthine oxidase, urate oxidase, and NAD(P)H oxidase generate superoxide as a consequence of oxidation of their substrates (McKersie, 1996).

A superoxide-generating NAD(P)H oxidase activity has been clearly identified in plasmalemma enriched fractions. 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). In the cell wall, such a mechanism is also 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 which may be signal for oxidative stress (McKersie, 1996).

### 1.4.2 Free Radical Dependent Damage

The reactions of activated oxygen with organic substrates are complex in biological systems due to the surface properties of membranes, electrical charges, binding properties of macromolecules, and compartmentalization of enzymes, substrates, and catalysts. Thus, various sites even within a single cell differ in the nature and extent of the reactions with oxygen (McKersie, 1996).

The nature of oxidative injury that causes cell death is not always obvious. The mechanisms by which oxygen radicals damage membrane lipids are well accepted, and consequently oxidative damage is often exclusively associated with these peroxidation reactions in membrane lipids. However, activated forms of oxygen also degrade proteins and nucleic acids, reactions which can also be very lethal (McKersie, 1996).

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 the 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. The basis for the hydroxyl radical's extreme reactivity in lipid systems is that at very low concentrations it initiates a chain reaction involving triplet oxygen, the most abundant form of oxygen in the cell (McKersie, 1996).

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).

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. Sulphur containing amino acids, and thiol groups specifically, are very susceptible sites. Although the oxidation of thiol groups can be reversed by various enzymes, some forms of free radical attack on proteins are not reversible. For example, the oxidation of iron-sulphur 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).

Although there are a number of promising selection criteria for salinity tolerance, the complex physiology of salt tolerance and the variation between species make it difficult to identify single criteria. Progress is more likely if biochemical indicators for individual species rather than generic indicators can be determined (Ashraf *et al.,* 2004).

### 1.5 Antioxidant Defense Systems

The presence of oxygen in the environment and various cellular locations where active oxygen species are produced render oxidant scavengers necessary for plant growth and survival. Plants have several antioxidant enzymes and metabolites located in different plant cell compartments to prevent the deleterious effects of active oxygen species (Dat *et al.,* 2000). Salinity stress response is multigenic, as a number of processes involved in the tolerance mechanism are affected, such as various compatible solutes/osmolytes, polyamines, reactive oxygen species and antioxidant defense mechanism, ion transport and compartmentalization of injurious ions (Sairam *et al.*, 2004). Elevation in activities and concentrations of these enzymes and molecules under abiotic stresses confer resistance to plants.

#### 1.5.1 Non-enzymatic Systems

The non-enzymatic defense mechanisms include glutathione, ascorbate (vitamin C), carotenoids, Q-tocopherol (vitamin E), and various phenylpropanoid derivatives (phenolic compounds) such as 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 homoglutathione (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 forms a disulfide bond with a second molecule of GSH to form oxidized glutathione (GSSG) upon oxidation (Hausladen and

Alscher, 1993). GSH has a redox potential of -340 mV that enables GSH to reduce dehydroascorbate to ascorbate or to reduce disulfide bond of proteins. The reduction of GSSG to GSH is catalyzed by the enzyme glutathione reductase (GR) (McKersie, 1996). GSH can function as an antioxidant in many ways. It can react chemically with singlet oxygen, superoxide, and hydroxyl radicals; therefore, it functions directly as a free radical scavenger. Also, GSH may stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price *et al.,* 1990).

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 from the conversion of D-glucose to ascorbate. It is abundant in plant protoplasts, chloroplasts and certain fruits. Ascorbate has been shown to have an essential role in several physiological processes in plants, including growth, differentiation, and metabolism. It functions as a reductant for many free radicals, thereby minimizing the damage caused by oxidative stress. Ascorbate is the terminal electron donor in the processes, which scavenge the free radicals in the hydrophilic environments of plant cells. It 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 (APX) in the chloroplast and cytosol of higher plants. In plants, hydrogen peroxide 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, which 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 m 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 xanthophyll cycle (McKersie, 1996).

Tocopherol, another family of antioxidants, has been found in all higher plants in both photosynthetic and non-photosynthetic tissues. α-tocopherol (vitamin E) is considered to be the most active form of tocopherol. Because of its hydrophobic nature, α -tocopherol is always located in membranes and it 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, which 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.

#### 1.5.2.1 Superoxide Dismutase

Superoxide dismutase, originally discovered by McCord and Fridovich (1969), catalyzes 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 $O_2$ 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 *et al*., 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.

#### 1.5.2.2 Catalase

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 (Scandalios, 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.

#### 1.5.2.3 Ascorbate Peroxidase

Ascorbate peroxidase (APX) is the first enzyme of this cycle and 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.

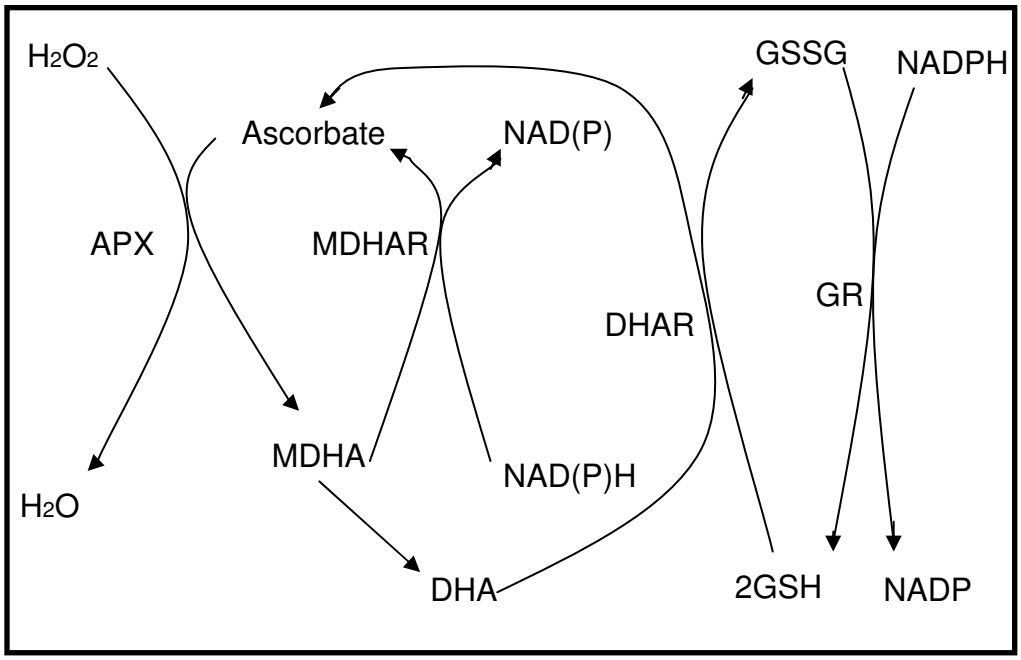


**Fig. 1.2.** The ascorbate-glutathione cycle. Dehydroascorbate (DHA), monodehydroascorbate (MDHA), reduced glutathione (GSH), oxidized glutathione (GSSG), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR).

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 pooi 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 0.100 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).

#### 1.5.2.4 Glutathione Reductase

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 $H_20_2$ 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 (Hegedus, *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 3500 and ascorbate increased by 3500 (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, NaC1 stress was accompanied by losses in aseorbate and glutathione pool (Hernandez *et al*., 1999). After 12 h of NaCI 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*., 1999), 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 (Comba 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 NaC1 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 NaC1 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  Although the activity of antioxidant enzymes significantly increased in wheat shoots a general decrease in the activities of all antioxidant enzymes was observed in roots, which firstly suffer stress (Meneguzzo *et al.,* 1999).

### 1.7 Aim of The Study

Lentil is, like other leguminous plants, sensitive to high salt concentrations. However in some field studies, it is found that there are some cultivars that show relatively tolerance to salt.

There is no literature data about the mechanism of salt tolerance in lentil plants. So in this study under laboratory conditions NaCl susceptibilities of two lentil cultivars (ILL5582-tolerant and ILL590) are compared and characterized to identify the salt

tolerance mechanism at molecular level. These results may enhance and direct further research on this subject.

For this purpose the following experiments are planned to study; (i) the antioxidant determining parameters ($H_2O_2$, proline, MDA contents) (ii) Activities of antioxidant enzymes (iii) physiological parameters related to phenotype.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemical Materials

In this study the chemicals used were obtained from Sigma Chemical Company (N.Y., USA) or MERCK Chemical Company (Deisenhofen, Deutschland). Distilled water was used during the preparation of all the solutions.

### 2.1.2 Plant Materials

In this study we used cultivar ILL (International Legume Lentil) 5582, named as salt tolerant by the greenhouse experiments of Ashraf and Waheed, 1990. For comparison, we chose a variety ILL 590, which we don't know the salt tolerance property. Both cultivars are from International Center of Agricultural Research and Development Association (ICARDA). Cultivar 5582 (commercialized as Idlib1) is also identified as Hirat, Afghanistan, 2000 and cultivar 590 as Cumra, Australia, 1998.

## 2.2 Methods

### 2.2.1 Growth of Plants

Seeds were surface sterilized with 10% Ethanol for 2 minutes, then washed with distilled water for several times and imbibed in distilled water for 1 day. Seeds then were transfered to plastic pots covered with cheesecloth so that each pot contains 8

seeds and 400 mL ½ strength Hoagland's solution (Hoagland and Arnon, 1950). Planted seeds were grown for 9 days in a growth chamber at 23±2ºC with 16 hours light and 8 hours dark photo-cycle.

### 2.2.2 Application of Salt Stress

Salt treatment was achieved on the nineth day of normal growth. Hoagland's solution (½ strength) containing 100 mM and 200 mM NaCl was used to give the stress. All control and salt-treated seedlings were kept in the growth chamber with the same physical parameters. Forteen days old seedlings were subjected to analysis of SOD isozymes, APX, CAT and GR activities; proline, MDA and $H_2O_2$ contents; conductivity and other physiological parameters like wet-dry weight and shoot-root lengths.

### 2.2.3 Wet-Dry Weight Analysis and Physiological Changes

After 14 days of growth, root and shoot tissues were weighed and then they were let to dry in 70ºC oven for 2 days and weighed. Lengths of the shoots and roots were measured. The photographs of 14 day old control and treated plants were taken to observe necrosis and wilting symptoms.

### 2.2.4 Determination of Proline Content

Proline amount was determined according to the modified method of Bates *et al*., (1973). 0.2 g of root and shoot tissues from control and treated plants were weighed. In 2 ml of 3 %sulphosalicylic acid, 0.2 g of shoot samples were homogenized with glass-glass homogenizer, root samples were homogenized with mortar and pestle.

Homogenates were transfered into eppendorf tubes and centrifuged with Thermo IEC Micromax RF microcentrifuge at 14000 rpm at 4 ºC for 5 minutes.

Into an eppendorf tube, 0.2 ml acid ninhydrin (0.31 g ninhydrin, 7.5 ml acetic acid, 5 ml 6M phosphoric acid), 0.2 ml phosphoric acid, and 0.1 ml 3% sulphosalicilic acid were put. 0.1 ml supernatant from the homogenate was added into the eppendorf tube. This was done for each sample and eppendorf tubes were incubated at 96 ºC for 1hour for complete hydrolysis of the proteins. Tubes were vortexed and centrifuged at 14000 rpm for 5 minutes.1 ml of toluene was added to each tube. Upper phase was transfered into the quartz cuvette and absorbance was measured at 520 nm wavelength in Schimadzu double beam spectrophotometer. Toluene was used as blank.

Proline standard curve was constructed to determine the proline concentration in range 0.01µM – 1.5 mM proline. The extinction corefficient is 0.8 $\mu M^{-1}cm^{-1}$

### 2.2.5 Determination of MDA Content

MDA content was determined according to the method of Ohkawa *et al*., (1979). 0.1 g of fresh root and shoot tissues were taken. In 5 % trichloro acetic acid (TCA) shoot samples were homogenized with glass-glass homogenizer, root samples were homogenized with mortar and pestle. The homogenates were then transfered into eppendorf tubes and centrifuged at 12000 rpm for 15 minutes by Thermo IEC Micromax RF microcentrifuge at room temperature.

Thiobarbituric acid (0.5%) in 20% TCA (freshly prepared) and supernatant in equal volumes were put into eppendorf tubes and incubated for 25 minutes at 100ºC. The tubes were then transfered into ice and centrifuged at room temperature at 10000 rpm for 5 minutes. Absorbance of the supernatant at 532 nm was recorded and corrected

for non-specific turbidity by subtracting the absorbance at 600 nm. Extinction coefficient to determine the MDA content is 155 $mM^{-1}$ $cm^{-1}$ .

### 2.2.6 Determination of Peroxide Content

Root and shoot tissues (0.5 g) were homogenized by mortar-pestle and homogenizer respectively in 1.5 ml of 100 mM potassium phosphate buffer (pH 6.8). Homogenate was filtered through 2 layers of cheesecloth and then centrifuged at 18000 g for 20 min at 4ºC. Modified method of Bernt and Bergmeyer (1974) was used to determine the $H_2O_2$ content of the supernatant. Peroxidase enzyme was used and the reaction is started in an aliqout of 0.5 ml of supernatant and 2.5 ml of peroxidase reagent ( 83 mM potassium phosphate, pH 7.0, 0.005% (w/v) o-dianizidine, 40µg peroxidase/ml ) at 30ºC. After 10 minutes of incubation the reaction was stopped by adding 0.5 ml of 1 N perchloric acid and centrifuged with Sigma 3K30 centrifuge at 5000 g for 5 minutes. Absorbance was read at 436 nm. $H_2O_2$ content was determined using the extinction coefficient 39.4 $mM^{-1}$ $cm^{-1}$.

### 2.2.7 Electrolyte Leakage Test

Method of Nanjo et al., (1999) is used for determination of electrolyte leakage. For conductance of leaves, 6 leaves per plant; for conductance of roots total root tissue was put into separate plastic tubes. Mannitol solution (5 ml of 0.4 M) was added onto the samples and incubated for 3 hour at room temperature. Electrical conductances were measured and recorded by using Mettler Toledo MPC 227 conductimeter as C1. Then the plastic tubes containing the samples were put into boiling water for 10 minutes and the conductances were measured and recorded as C2, which indicates the total ion concentration by complete membrane disintegration. By using the formula

(C1/C2)*100, percent ion leakage was determined and indicates the proportional membrane damage to control vials.

### 2.2.8 Protein Determination

Bradford method (Bradford, 1976) was used to determine the protein amount in the root and shoot crude extracts. Bradford reagent was prepared by using 500 mg Coomasie Brillant Blue G-250 in 250 ml 95 % ethanol and 500 ml 85 % phosphoric acid. Finally the solution was diluted to 1 L and filtered. The prepared reagent was 5X. It was diluted to 1X before each use.

From each sample 20 µl was taken and put into a test tube. 480 µl of distilled water was added onto the extract. 5 ml of 1X Bradford reagent was put onto the diluted sample. After 10 minutes of incubation at room temperature the absorbances were measured at 595 nm by Schimadzu UV-1201 spectrophotometer against blank of 500 µl water and 5 ml Bradford reagent. For the protein standard curve Bovine Serum Albumin (BSA) was used at concentrations of 10, 20, 30, 40, 50, 60 µg/ml.

### 2.2.9 Determination of SOD Isozyme Activities

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

#### 2.2.9.1 Shoot Crude Extract Preparation

Shoot tissues of 150 mg were weighed and homogenized in 800 µl homogenization buffer on ice for few minutes. Homogenization buffer was composed of 9 mM Tris-HCl, pH 6.8 and 13.6 % glycerol. The homogenates were then transfered into an eppendorf tubes and centrifuged at 14000 rpm for 5 minutes at 4ºC. Supernatants were used for superoxide dismutase assay.

#### 2.2.9.2 Root Crude Extract Preperation

Root tissues were washed with distilled water and then dried with tissue paper. About 1g of tissue was weighed and ground in 2ml of grinding solution with cold mortar and pestle on ice for few minutes. Grinding solution contained 0.2 M sodium phosphate buffer and 2 mM EDTA (pH 7.8). The suspensions were filtered through 2 layer cheesecloth into eppendorf tubes and centrifuged at 10000 rpm for 30 minutes at 4 ºC in centrifuge. Supernatants were used for SOD assay.

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

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

#### 2.2.9.4 Negative Activity Staining

In a glass container, 20 ml of negative activity stain, composed of 50 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.2 % (v / v) N,N,N'N'-tetramethyl ethylene diamine (TEMED), 3 mM riboflavin and 0.25 mM nitroblue tetrazolium, was prepared. Separating gel was cut and incubated in staining solution and kept in dark for 45 minutes with gentle shaking. After staining, gel was washed with distilled water and exposed to light approximately 15 minutes to observe the bands. The photograph of the gel was taken by Vilber gel imager and densitometric analysis was carried out by Bio-Profil V99 software program. The SOD isozyme percent changes with respect to control were evaluated from band intensities.

The unit of SOD activity was determined by running a SOD standard from bovine erythrocyte. One unit of standard SOD activity inhibits the rate of reduction of Cyt c by 50% in coupled system with xanthine oxidase at pH 7.8 at 25ºC in a 3 ml reaction medium.

### 2.2.10 Determination of APX Activity

Wang spectrometric assay (Wang *et al*., 1991) was used to determine the APX activity. Tissue of 0.5 g was weighed and powdered with liquid nitrogen in a mortar. Powder was homogenized with 1 ml of suspension solution containing 50 mM Tris-HCl (pH 7.2), 2% PVP, 1 mM EDTA and 2 mM ascorbate. The suspension was centrifuged at 12000 g for 20 minutes at 4 ºC and supernatant was used for the enzyme assay.

Enzyme extract containing 100 µg protein, determined by Bradford Method (1976), was added into assay medium containing 50 mM potassium phosphate buffer (pH 6.6), 0.25 mM ascorbate, 1 mM $H_2O_2$. The reaction was started by the addition of peroxide. The decrease in the 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. Standard curve of range 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 the 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 tissue was ground with liquid nitrogen by using cold mortar and pestle and then suspended with suspension solution containing 50 mM Tris-HCl (pH 7.8). After filtering through 2 layers of cheesecloth, the suspensions were centrifuged at 12000 g for 20 minutes at 4ºC. Supernatant was taken for the enzyme assay.

Enzyme extract containing 100 µg protein, determined by Bradford Method (1976), was added into assay medium containing 50 mM potassium phosphate buffer (pH 7.0), 25 mM $H_2O_2$. The reaction was started by the addition of enzyme extract. The decrease in absorbance was recorded at 240 nm with Schimadzu double-beam spectrophotometer for 2 minutes. The enzyme activity was calculated from the initial rate of the enzyme. ( Extinction coefficient of $H_2O_2$ = 40 $mM^{-1}cm^{-1}$ at 240 nm ).

### 2.2.12 Determination of GR Activity

Glutathione reductase activity was determined according to the method of Sgherri *et al*. (1994). 1 g (shoot/root) tissue was ground with liquid nitrogen and the powder was suspended in 1.5 mL of suspension solution containing 100 mM potassium phosphate buffer (pH 7.0), 1 mM $Na_2EDTA$ and 2% insoluble PVP. The GSSG dependent oxidation of NADPH was monitored by the decrase in absorbance at 340 nm at 30ºC.

Enzyme extract containing 100 µg protein, determined by Bradford Method (1976), was added into assay solution containing 200 mM potassium phosphate buffer (pH 7.5), 0.2 mM $Na_2EDTA$, 1.5 mM $MgCl_2$, 0.25 mM GSSG, 25µM NADPH, in a final volume of 1 mL. The reaction was started by the addition of NADPH. Corrections were made for the background absorbance at 340nm , without NADPH. The enzyme activity was calculated from the initial rate of enzyme after subtrating the non-enzymatic oxidation. (Extinction coefficient of NADPH = 6.2 $mM^{-1}cm^{-1}$).

### 2.2.13 Statistical Analysis

The significance of the 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

In this study two lentil cultivars (*Lens culinaris*, Medik.) were used. Both cultivars, 5582 and 590 were subjected to salt stress of 100 mM and 200 mM for 5 days.

Physiological parameters (length, wet-dry weight), biochemical parameters (proline content, $H_2O_2$ content, MDA content, ion leakage amount), antioxidant enzyme alterations (SOD, GR, CAT, APX) were analyzed and results were presented.

## 3.1 Effect of NaCl Stress on Physiological Parameters of Seedlings

The physiological changes under salt stress were evaluated by measuring the lengths and wet-dry weight of shoot and root tissues. The photograph of 14 days old control and salt treated plants were shown in Fig. 3.1. For ease of comparison both cultivars and salt treated ones were presented in one photograph.

After 5 days of salt treatment, the plants were wilted, especially the old leaves. In both cultivars under stress conditions growth inhibition is observed and these differences become more significant as the salt concentration increases. In the root tissues of both cultivars decrease in lateral roots was determined.

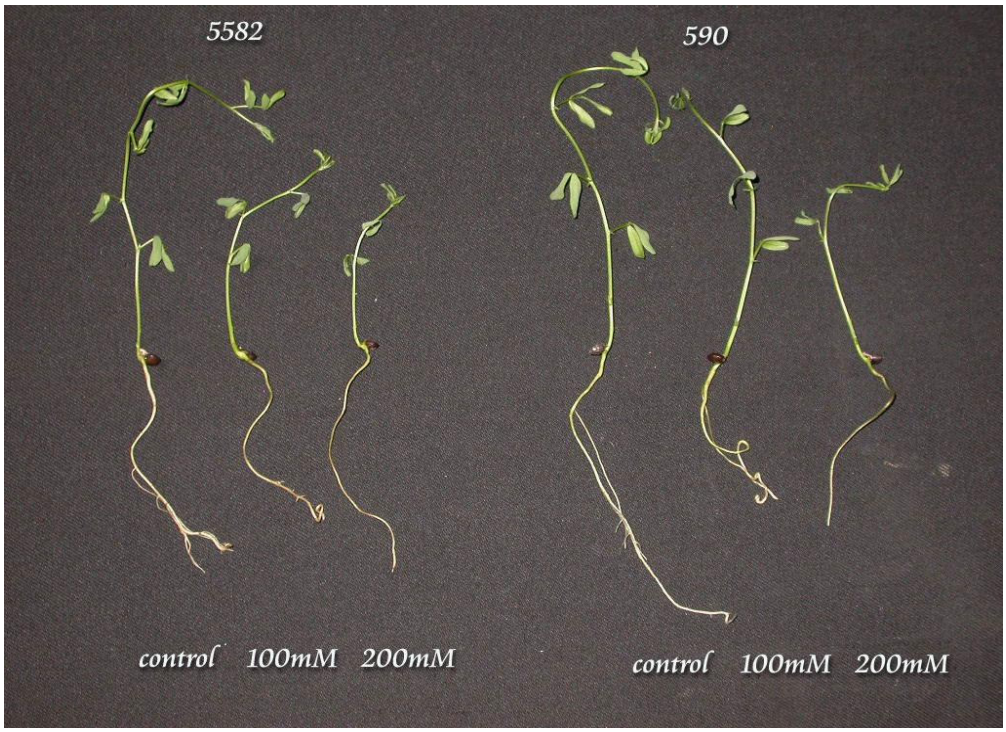


**Fig. 3.1.** The phenotypic appearance of 9 days old lentil seedlings subjected to 5 days of 100 mM and 200 mM NaCl stress and the control plant.

### 3.1.1 Effect of Salt Stress on Shoot and Root Length of the Lentil Cultivars

As concentration of salt treatment increased shoot length of both cultivars decreased significantly (Fig 3.2, Fig 3.3). Cultivar ILL5582 shoot length decreased by 34% under 100 mM NaCl and 39% under 200 mM NaCl. ILL590 shoot length decreases by 36% under 100 mM NaCl, 58% under 200 mM NaCl. Root lengths of both cultivars were not changed much. The root length of 5582 decreased by 2% and root length of 590 decreased by 16% under 200 mM NaCl (Figure 3.2). The decreases in length were much evident in the tissues of cultivar 590.

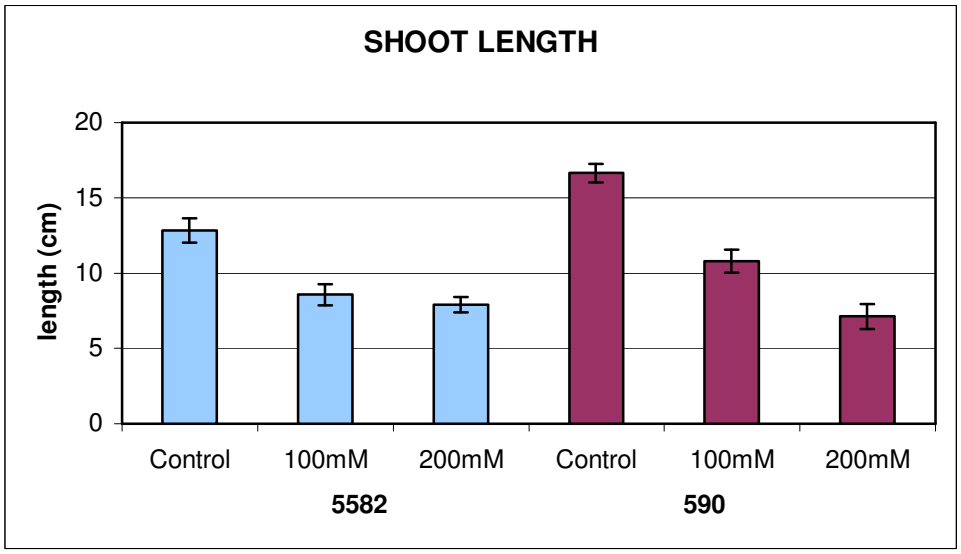


**Fig. 3.2**. Shoot lengths (cm) of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

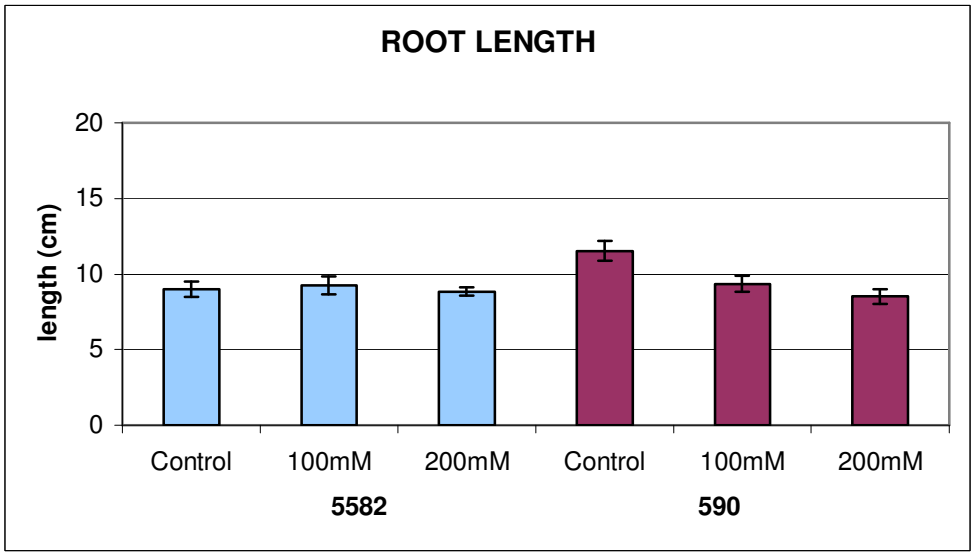


**Fig. 3.3**. Root lengths (cm) of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

### 3.1.2 Effect of Salt Stress on Wet Weight of Seedlings

Significant decreases in shoot weight of both cultivars were observed and these became much more evident as salt concentration increased. Decreases were more apparent in shoots than in the roots. 5582 shoot wet weight decreased by 40% under 100 mM NaCl, 59% under 200 mM NaCl. 590 shoot wet weight decreased by 45% under 100 mM NaCl, 69% under 200 mM NaCl. 5582 root wet (Fig. 3.4) weight decreased by 16% under 100 mM NaCl, 30% under 200 mM NaCl. 590 root wet weight decreased by 33% under 100 mM NaCl, 54% under 200 mM NaCl (Fig. 3.5).

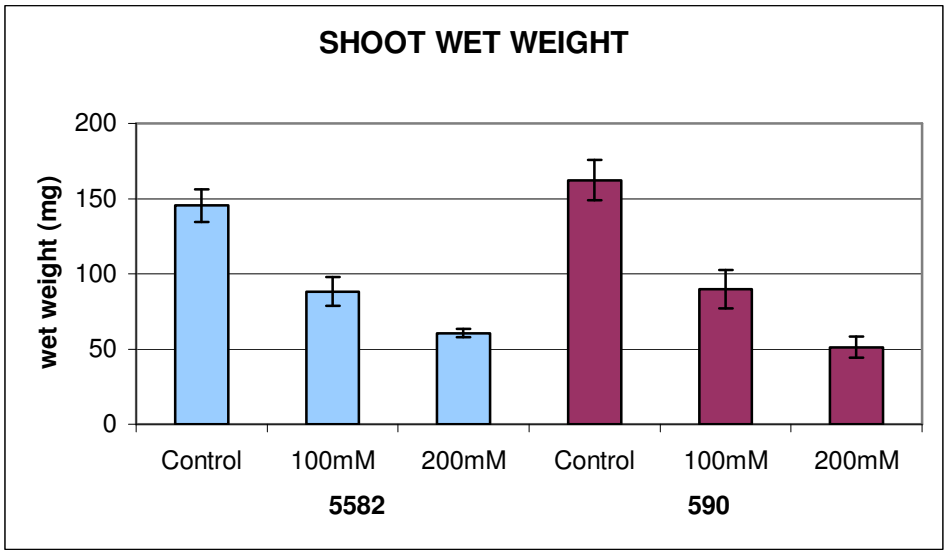


**Fig. 3.4.** Shoot wet weights of control and NaCl treated plants. Bars indicate the mean weights ± S.E.M. (n=30)

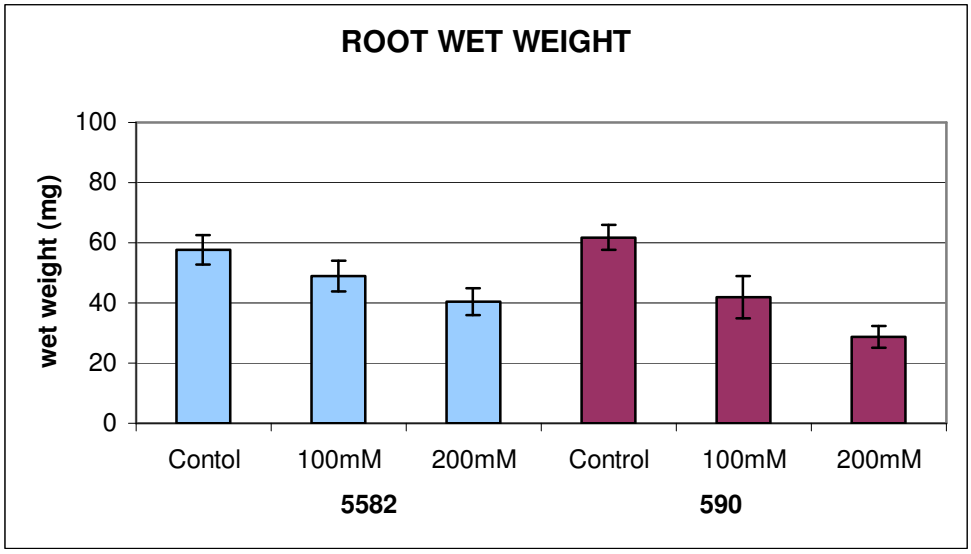


**Fig.3.5**. Root wet weights of control and treated plants. Bars indicate the mean weights S.E.M. (n=30)

### 3.1.3 Effect of Salt Stress on Dry Weight of Seedlings

A similar result in the dry weights of the shoot tissues of both cultivars was observed. In the root tissues dry weight values did not show significant change although the salt concentration increased. In cultivar ILL5582 shoot dry weight decreased by 15% under 100 mM NaCl, 32% under 200 mM NaCl. 590 shoot dry weight decreased by 27% under 100 mM NaCl, 50% under 200 mM NaCl (Fig. 3.6). Root dry weight of 5582 increased by 7% under 100 mM NaCl, decreased 6% under 200 mM NaCl (Fig. 3.7). In cultivar 590 root length decreased by 11% under 100 mM NaCl, 13% under 200 mM NaCl. The dry weight alteration in the shoot tissues of both cultivars and the root tissues of 590 were found to be significant.

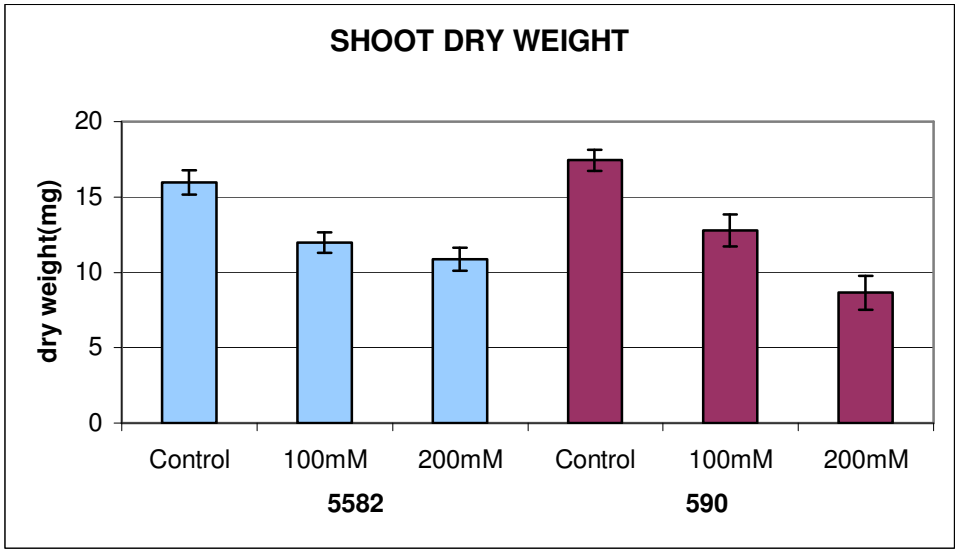


**Fig. 3.6.** Shoot dry weights of control and salt treated plants. Bars indicate the mean weights ± S.E.M. (n=30)

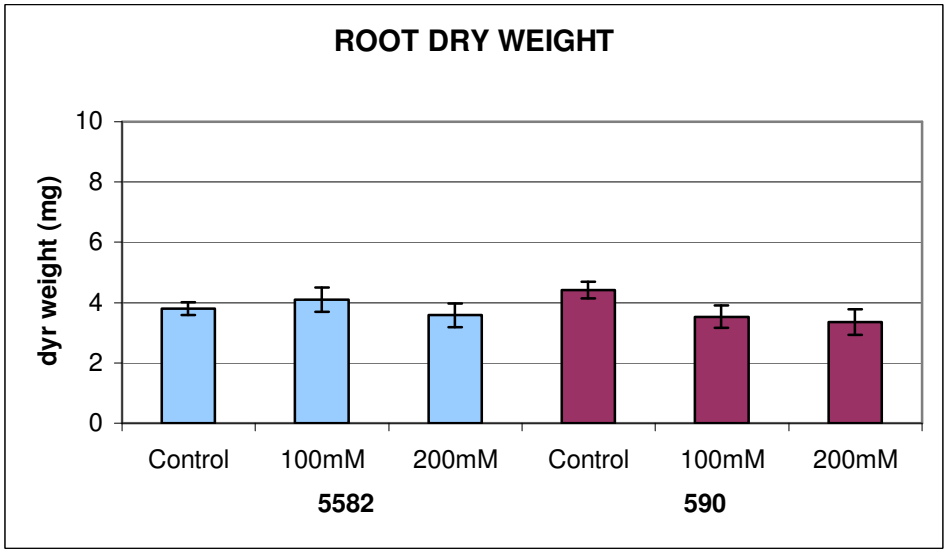


**Fig. 3.7.** Root dry weights of control and salt treated plants. Bars indicate the mean weights ± S.E.M. (n=30)

### 3.2 Measurement of oxidative stress parameters

#### 3.2.1 Lipid Peroxidation

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

In roots of both cultivars MDA content did not show significant changes under increased salt concentrations (Fig. 3.9). However leaf tissues, especially cultivar 590 showed a significantly increased malondialdehyde content, which correspond to higher lipid peroxidation (Fig. 3.8).

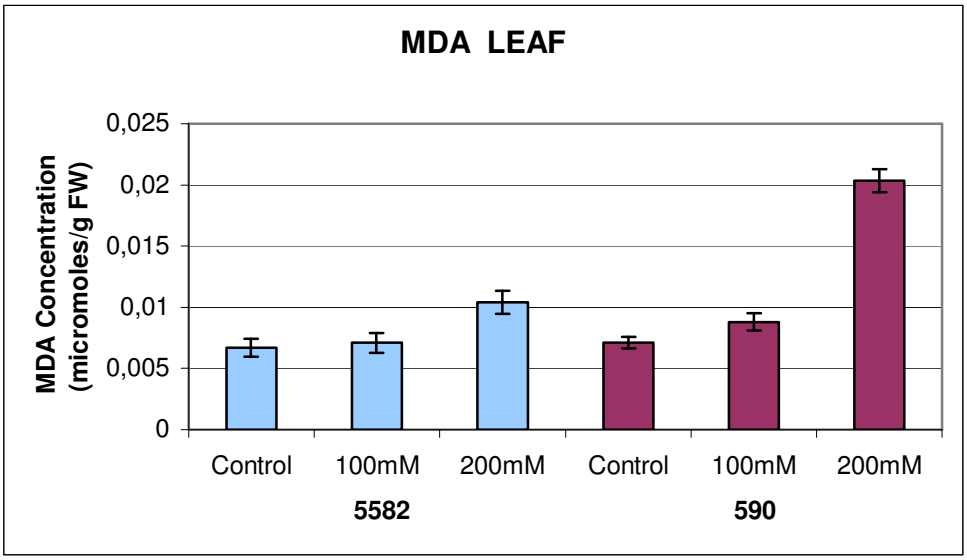


**Fig. 3.8.** Leaf MDA concentrations of control and salt treated plants. Bars indicate the mean weights ± S.E.M. (n=30)

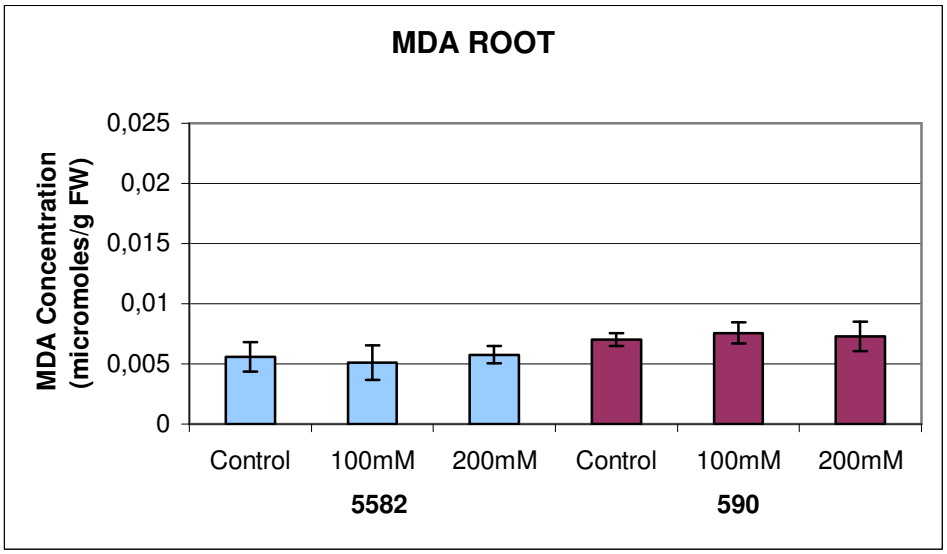


**Fig. 3.9.** Root MDA concentrations of control and salt treated plants. Bars indicate the mean weights ± S.E.M. (n=30)

### 3.2.2 Electrolyte Leakage Test

Membrane permeabilities of leaf and root tissues showed different responses against NaCl stress. As it can be seen from the data, leaf ion leakage amount was very much higher from the root tissues of both cultivars. Leaf tissues showed almost 2.5 and 3.5 fold increase under 200 mM salt stress in 5582 and 590, respectively (Fig. 3.10, Fig. 3.11).

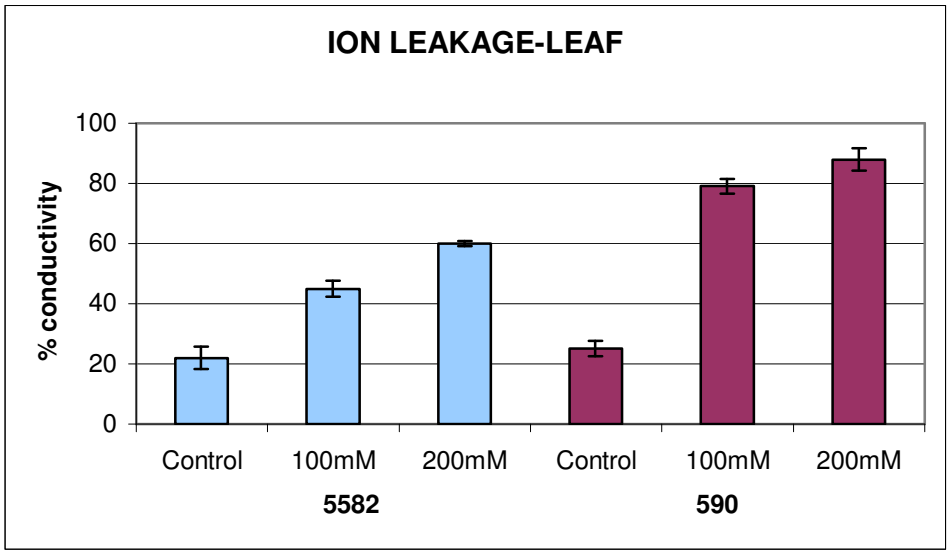


**Fig.3.10** Leaf membrane permeability of control and salt treated plants. Bars indicate the mean weights ± S.E.M. (n=30)

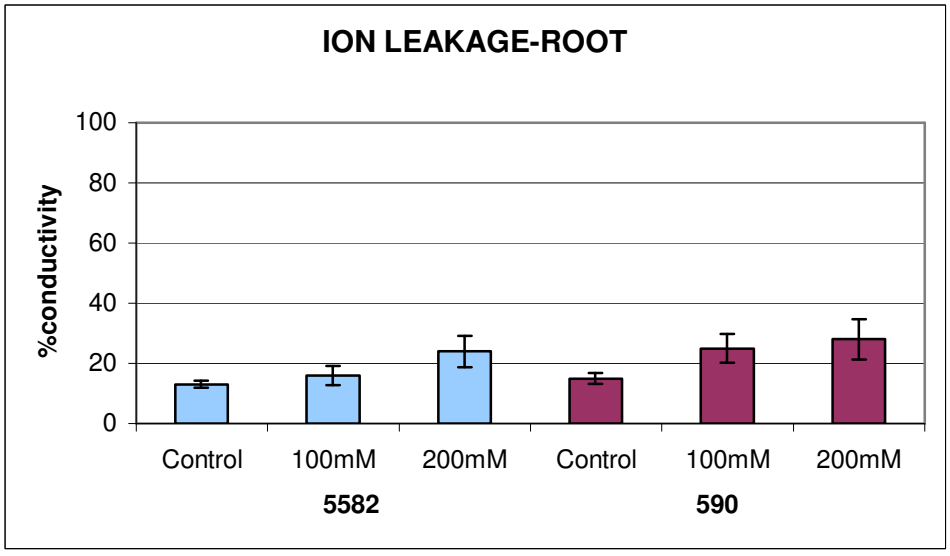


**Fig.3.11** Root membrane permeability of control and salt treated plants. Bars indicate the mean weights ± S.E.M. (n=30)

### 3.2.3 Proline Content

In both tissues of both cultivars, proline content increased significantly as the salt concentration increased. The leaf tissues gave a higher response to the salt stress by means of proline content. The significant increase in the leaf tissues of 5582 was 147% under 100 mM salt and 320% under 200 mM salt stress. Lower but significant values for the leaves of 590 were obtained; 57% and 232% under 100 mM and 200 mM salt stress respectively (Fig. 3.12). Proline level response of the root tissues of 5582 was 47% and 117% increase under 100 and 200 mM salt stress respectively. For root tissues of 590, 28% and 195% increase were observed (Fig. 3.13)

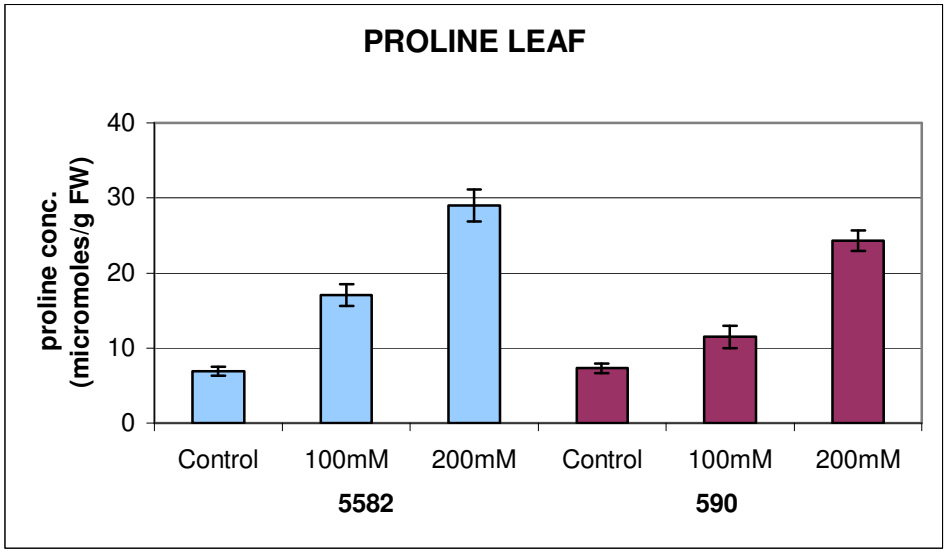


**Fig. 3.12** Effect of NaCl on proline concentration in shoots of control and salt treated plants of both cultivars. Bars indicate the mean weights ± S.E.M. (n=30)

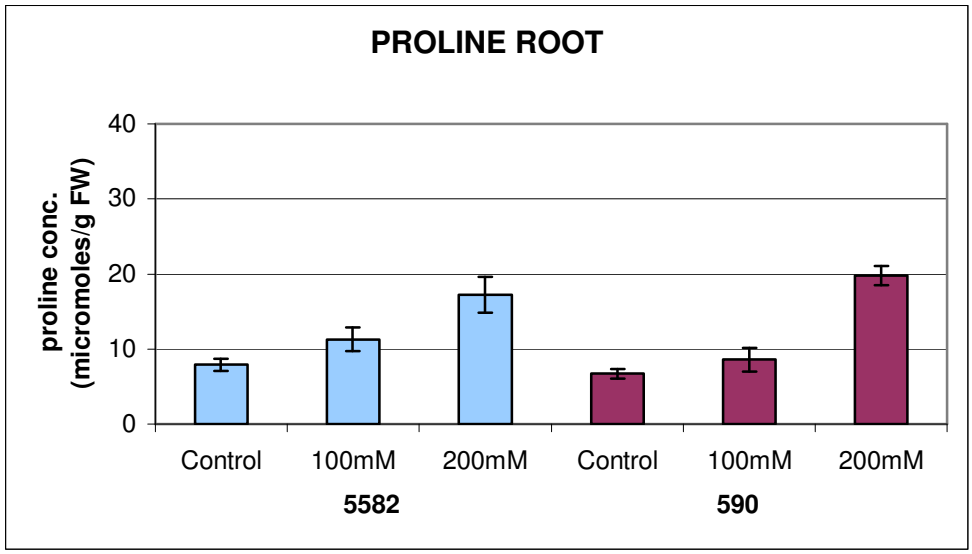


**Fig. 3.13** Effect of NaCl on proline concentration in roots of control and treated plants of both cultivars. Bars indicate the mean weights ± S.E.M. (n=30)

### 3.2.4 $H_2O_2$ Content

Under salt stress, root tissues of both cultivars showed increasing $H_2O_2$ content in a salt concentration dependant manner. For cultivar 590 root tissues 80% and 269% significant increase; for 5582 root tissues 11% and 45% significant increase was observed under 100 and 200 mM NaCl stress respectively (Fig. 3.15). However in the leaf tissues of 5582 no significant change could be observed (Fig. 3.14).

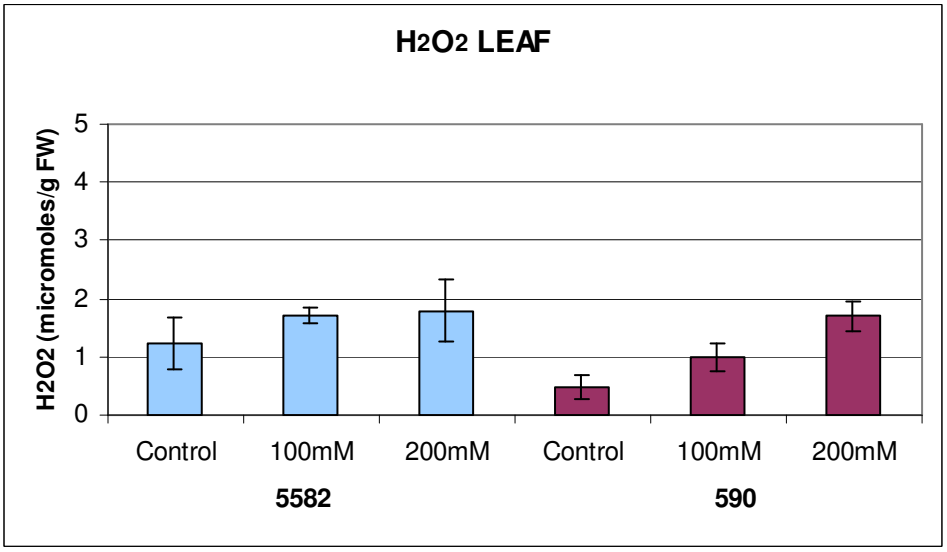


**Fig.3.14** Effect of NaCl on $H_2O_2$ contents of shoots of control and treated plants of both cultivars. Bars indicate the mean weights ± S.E.M. (n=30)

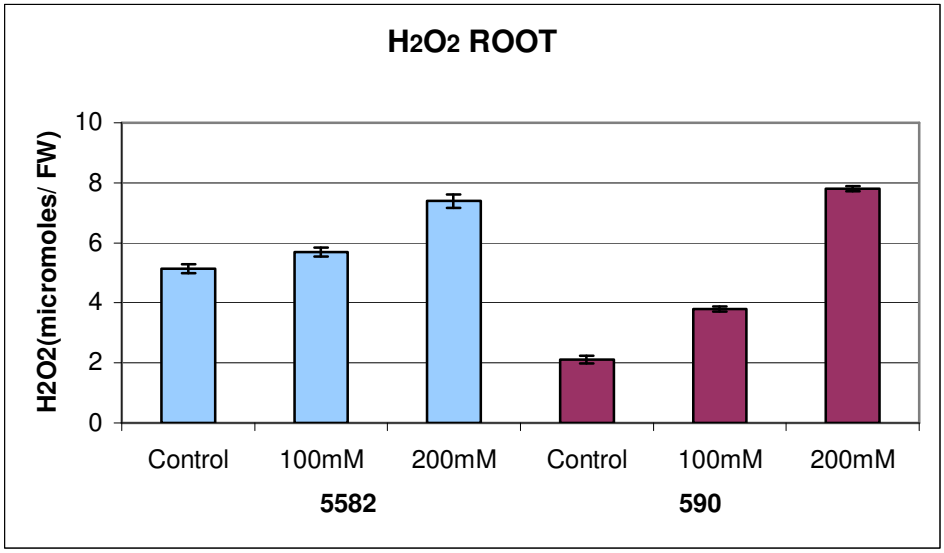


**Fig.3.15** Effect of NaCl on $H_2O_2$ contents of shoots of control and treated plants of both cultivars. Bars indicate the mean weights ± S.E.M. (n=30)

### 3.3 Effect of Salt Stress on Antioxidant Enzyme Activities

#### 3.3.1 Ascorbate Peroxidase Activity

For both cultivars in leaf and root tissues, ascorbate peroxidase enzyme activity responses under increased salt concentration were similar. In leaf tissues significant increase under salt stress was observed but for both cultivars in root tissues ascorbate peroxidase activity did not change significantly (Fig. 3.16, Fig. 3.17). Higher values of the enzyme were detected in root tissues.

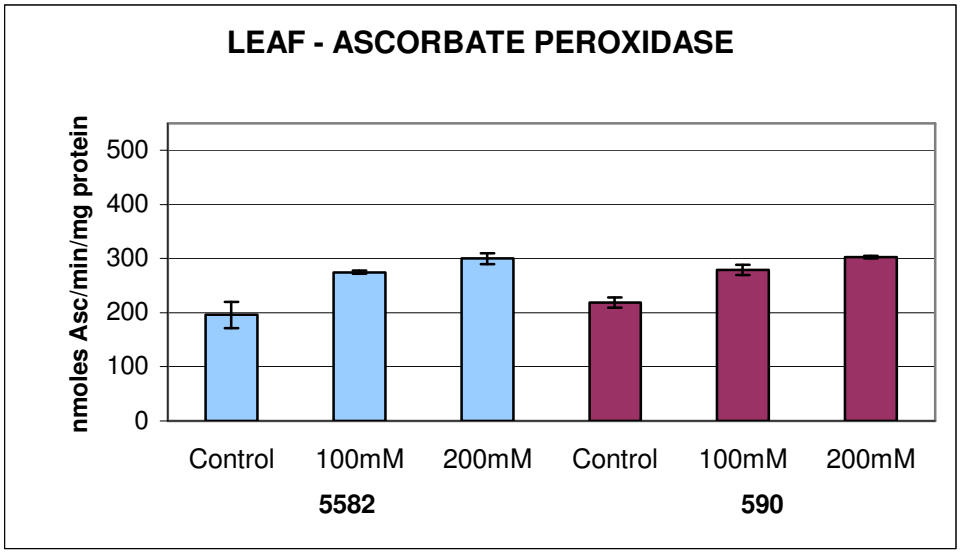


**Fig.3.16** APX activity in leaves of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

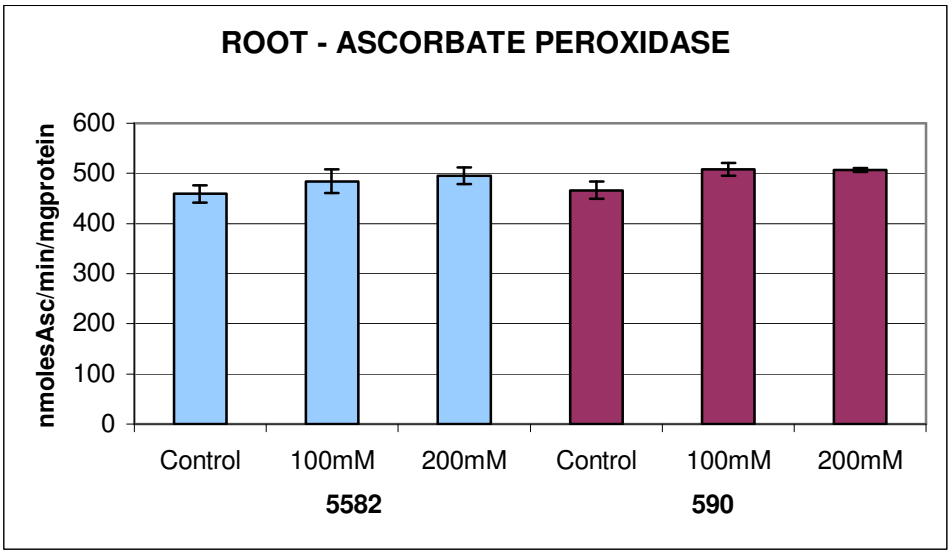


**Fig. 3.17** APX activity in roots of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

### 3.3.2 Catalase Activity

The activity of catalase enzyme in leaf and root tissues of both cultivars did not significantly change under increasing salt stress conditions. In the leaf tissues of both cultivars higher catalase activity than roots was observed (Fig. 3.18, Fig. 3.19).

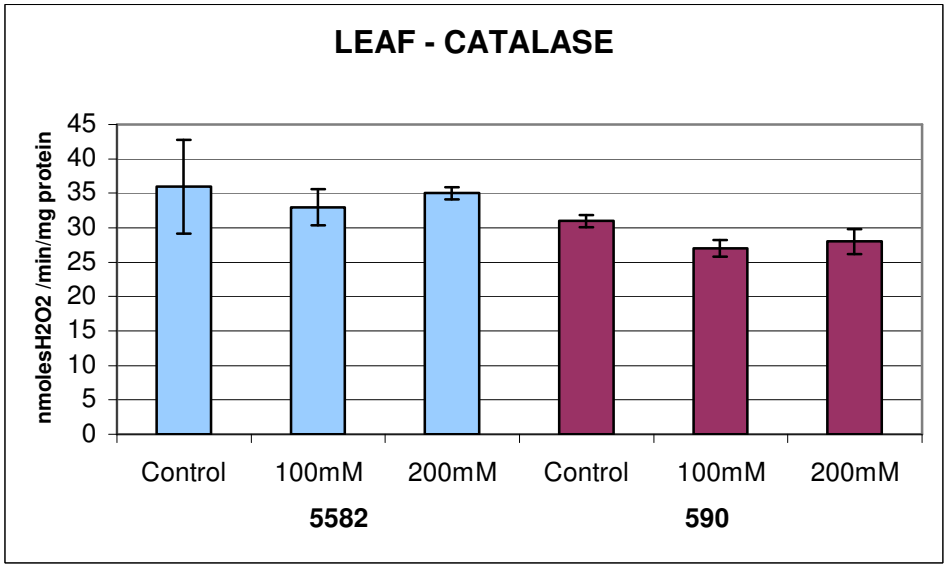


**Fig.3.18** CAT activity in leaves of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

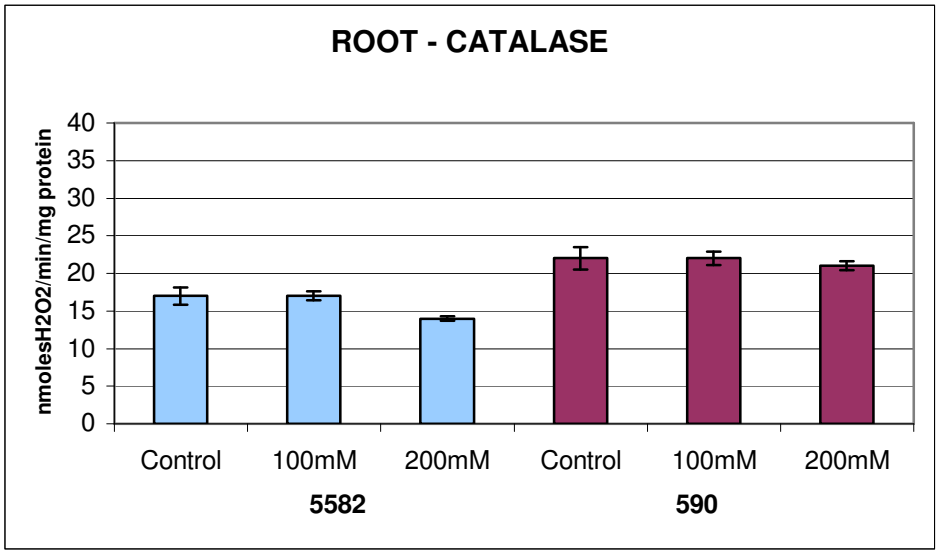


**Fig.3.19** CAT activity in roots of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

### 3.3.3 Glutathione Reductase Activity

The increase in the glutathion reductase activities of leaf tissues of both cultivars were significant. Higher activity of GR was observed in the root tissues of both cultivars under 200 mM NaCl stress. In the leaf tissues of cultivar 5582, the observed GR activity was 24 % and 61 % higher than the control under 100 mM and 200 mM NaCl stress. In cultivar 590, the values were 32 % and 34 % under same conditions. It can be clearly seen that increased salt stress did not cause a dependent increase in 590 leaf tissues. The activity increases in the root tissues were not significant (Fig. 3.20, Fig. 3.21).

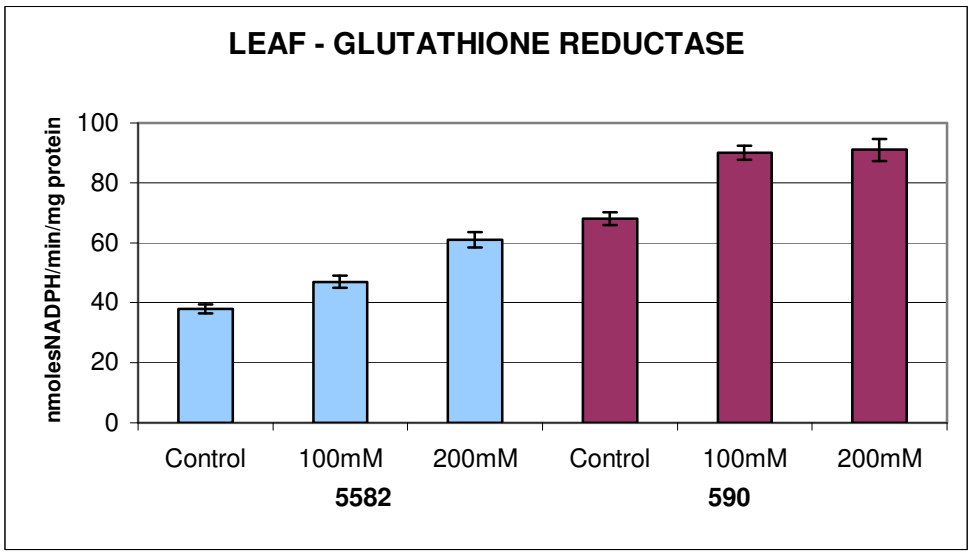


**Fig.3.20** GR activity in leaves of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

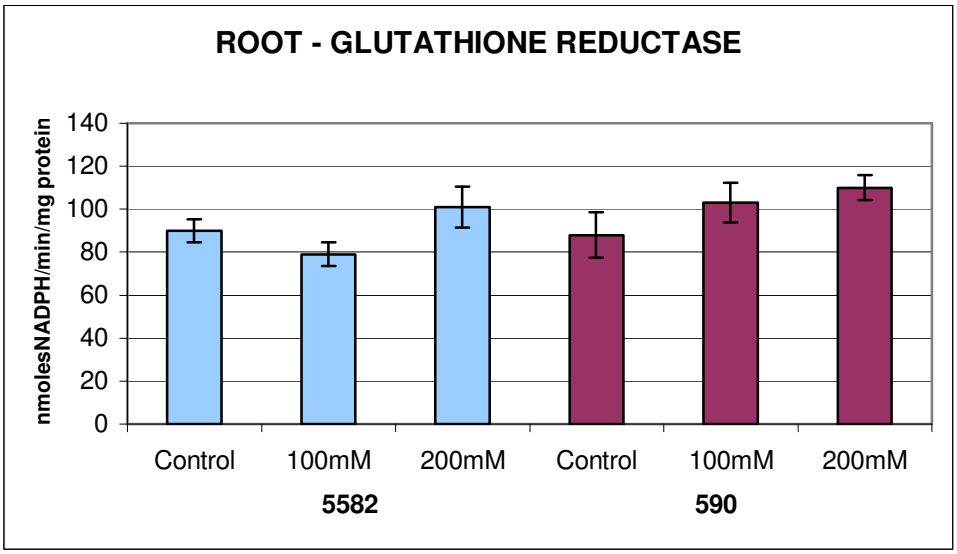


**Fig.3.21** GR activity in roots of control and treated plants of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

### 3.3.4 Superoxide Dismutase Activity

Four SOD isozymes were identified in lentil seedlings namely MnSOD, Cu/Zn SOD-1, Cu/Zn SOD-2 (according to increased mobility), FeSOD was only identified in leaves indicating a chloroplastic localization (Bandeoğlu, 2001).

MnSOD isozyme increased significantly under 200 mM NaCl concentration for the leaf tissues of both cultivars. Among the root tissues, only 5582 showed significant increase under 100 mM whereas under 200 mM both cultivars showed significant increase (Fig. 3.22). It seems MnSOD isozyme is inducible under higher salt concentration.

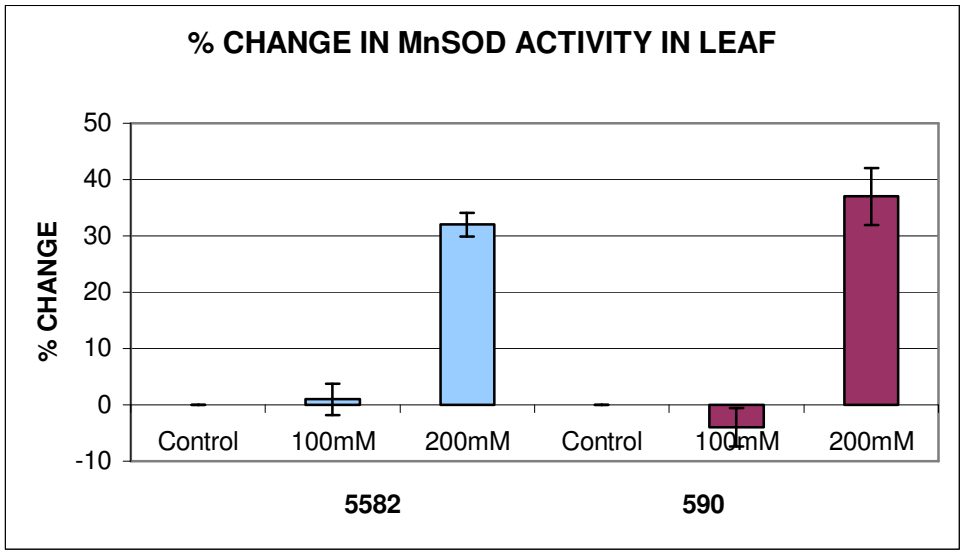


**Fig.3.22** Percent change in the activity of MnSOD in leaf tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

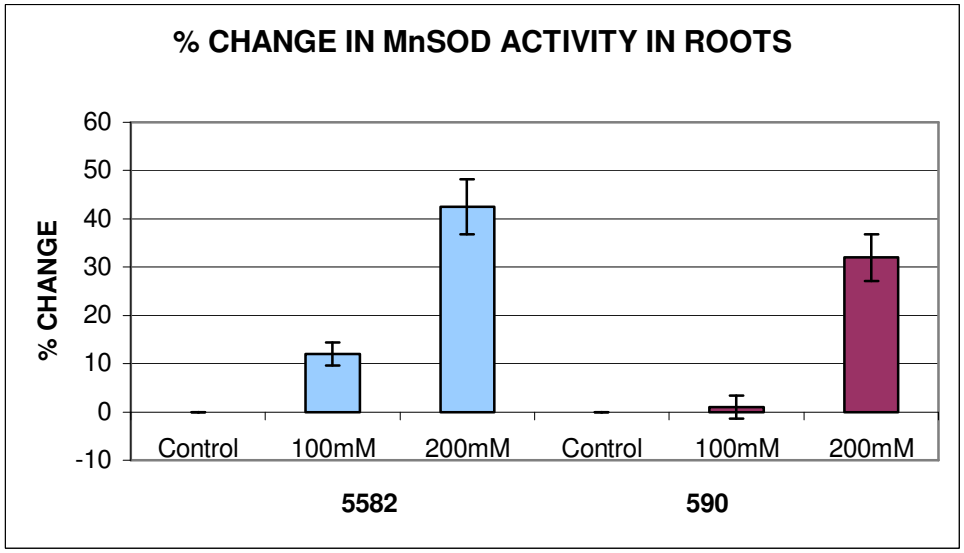


**Fig.3.23** Percent change in the activity of MnSOD in root tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

Chloroplastic FeSOD activity did not show a significant change under 100 mM salt stress in the leaf tissues of both cultivars. However in comparison with the control plants a remarkable decrease in this isozyme with the increasing salt concentration was observed (Fig. 3.24).

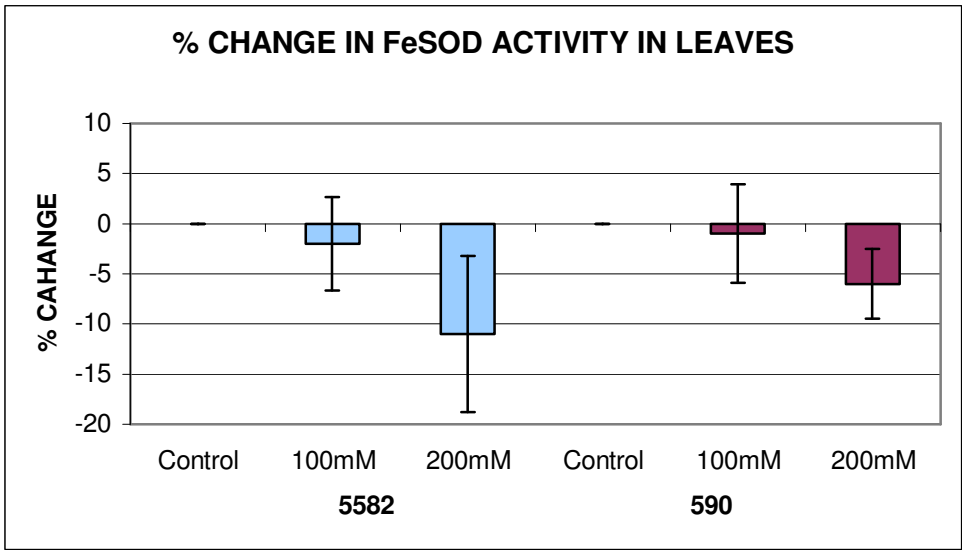


**Fig.3.24** Percent change in the activity of FeSOD in leaf tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

Cytosolic Cu/ZnSOD-1 isozyme activity change in the leaf tissues was significant for both cultivars under 200 mM salt stress but activity percent change was higher in cultivar 5582 (Fig. 3.25). In the root tissues, the case was similar but the percent increase was significant under 100 mM NaCl and higher in comparison with the leaf tissues of the same cultivar under both stress conditions (Fig. 3.26).

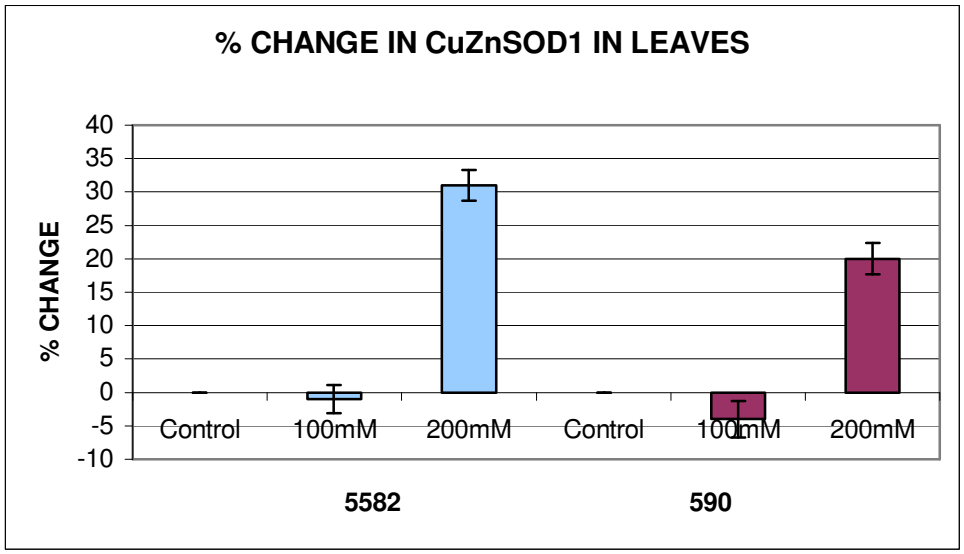


**Fig.3.25** Percent change in the activity of Cu/Zn SOD1 in leaf tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

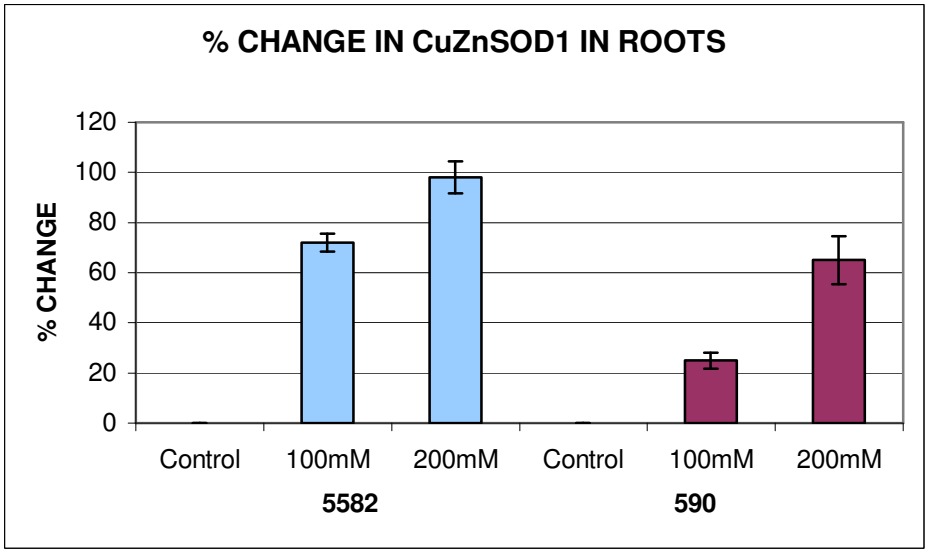


**Fig.3.26** Percent change in the activity of Cu/Zn SOD 1 in root tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

Under 100 mM salt stress the cytosolic isozyme Cu/ZnSOD-2 percent increased in leaf tissues of 5582 and root tissues of both cultivars were significant. Under 200 mM salt stress both cultivars showed significant increase in comparison with control plants. Higher percent increase was detected in the root tissues of both cultivars (Fig. 3.27, Fig. 3.28).

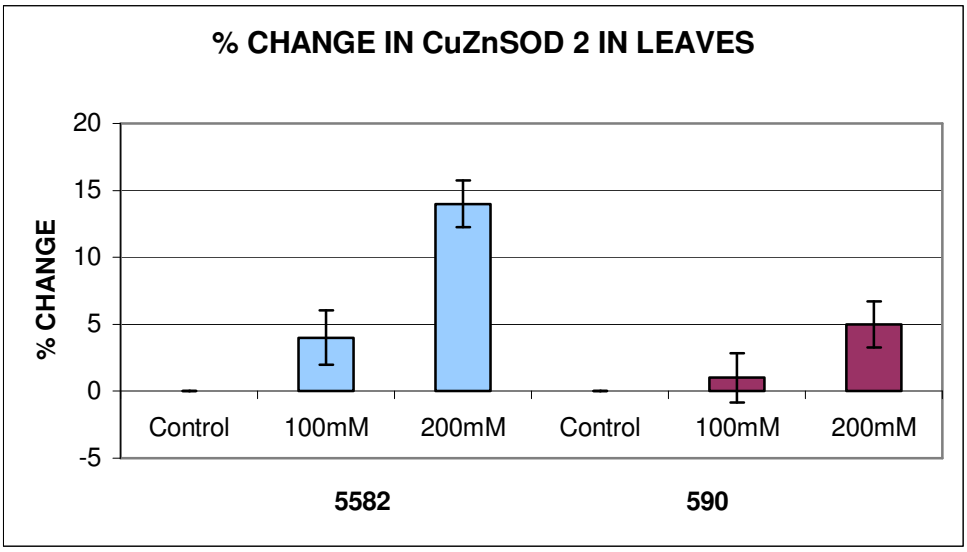


**Fig.3.27** Percent change in the activity of Cu/Zn SOD 2 in leaf tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

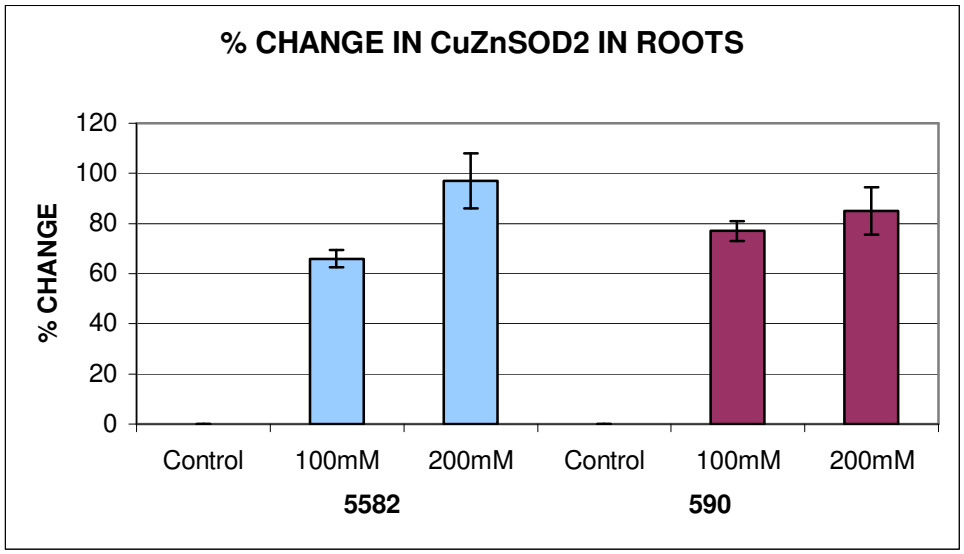


**Fig.3.28** Percent change in the activity of Cu/Zn SOD 2 in root tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

The activity of each isozyme was also calculated as percent of total SOD activity in Table 3.1. The results showed that for cultivar 5582, in leaves Cu/ZnSOD-2 by 42%, is the highest contributing isozyme, whereas in roots Cu/ZnSOD-1 contributes by 48% to the total SOD activity. The total Cu/ZnSOD isozymes makes about 68% and 74% of the total SOD activity in leaves and roots, respectively. MnSOD contributes 27% and 26% to the total SOD activity of the leaves and the roots, respectively, while FeSOD contributes only 5%.

**Table 3.1** Total SOD and SOD isozyme activities of cv.5582 (determined from the band intensities) in leaves and roots of lentil seedlings grown under normal environmental conditions.

| **Cultivar ILL 5582** | | | | |
|---|---|---|---|---|
| | **SOD Activity (units/mg protein)** | | **Activity of each isozyme as % of total SOD activity** | |
| | **LEAF** | **ROOT** | **LEAF** | **ROOT** |
| **MnSOD** | 7.1 | 8.2 | 27 | 26 |
| **FeSOD** | 1.3 | - | 5 | - |
| **Cu/ZnSOD-1** | 6.6 | 14.8 | 26 | 48 |
| **Cu/ZnSOD-2** | 10.9 | 7.9 | 42 | 26 |
| **TOTAL SOD** | 25.9 | 30.9 | 100 | 100 |

**Table 3.2** Total SOD and SOD isozyme activities of cv.590 (determined from the band intensities) in leaves and roots of lentil seedlings grown under normal environmental conditions.

| **Cultivar ILL 590** | | | | |
|---|---|---|---|---|
| | **SOD Activity (units/mg protein)** | | **Activity of each isozyme as % of total SOD activity** | |
| | **LEAF** | **ROOT** | **LEAF** | **ROOT** |
| **MnSOD** | 5.6 | 8.9 | 23 | 30 |
| **FeSOD** | 2.1 | - | 9 | - |
| **Cu/ZnSOD-1** | 4.7 | 9.9 | 20 | 34 |
| **Cu/ZnSOD-2** | 11.6 | 10.7 | 48 | 36 |
| **TOTAL SOD** | 24.0 | 29.5 | 100 | 100 |

For cultivar 590, in leaves Cu/ZnSOD-2 by 48% is the highest contributing isozyme (Table 3.2). In roots Cu/ZnSOD-1 and Cu/ZnSOD-2 contribute very close to each other by 34% and 36% to the total SOD activity. The total Cu/ZnSOD isozymes makes about 68% and 70% of the total SOD activity in leaves and roots, respectively. MnSOD contributes 23% and 30% to the total SOD activity of the leaves and the roots, respectively, while FeSOD contributes only 9%.

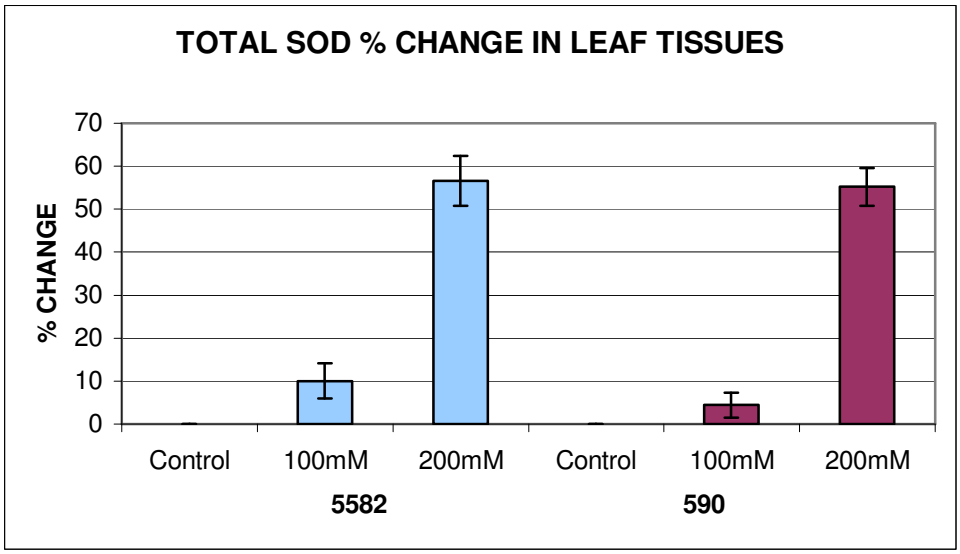


**Fig.3.29** Total SOD activity percent change in leaf tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

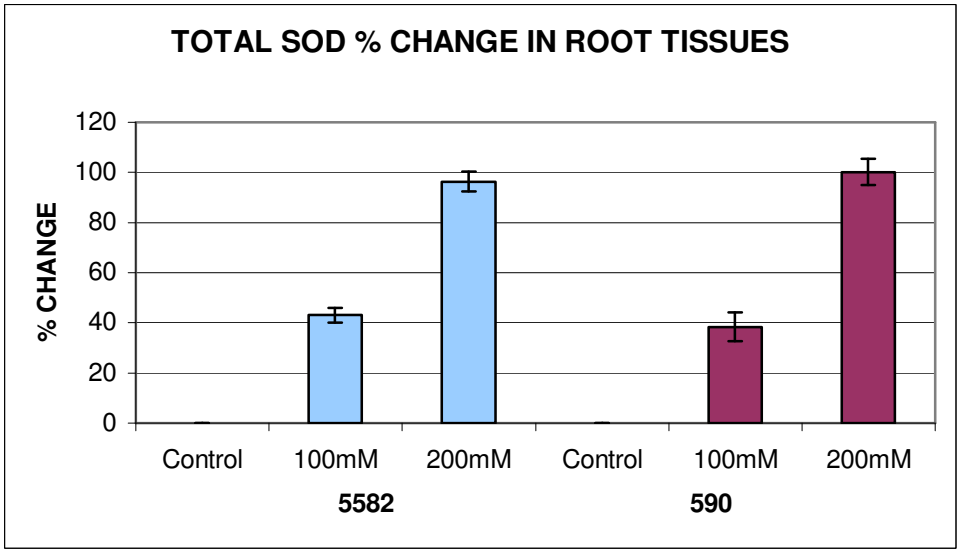


**Fig.3.30** Total SOD activity percent change in leaf tissues of both cultivars. Bars indicate the mean values ± S.E.M. (n=30)

For both cultivars the total SOD activity percent change in the roots are almost twice the leaf total SOD percent changes. Also for both cultivars under 200 mM salt stress the enzyme activity responses are higher and increase with salt concentration dependent manner (Fig. 3.30, Fig. 3.31).

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

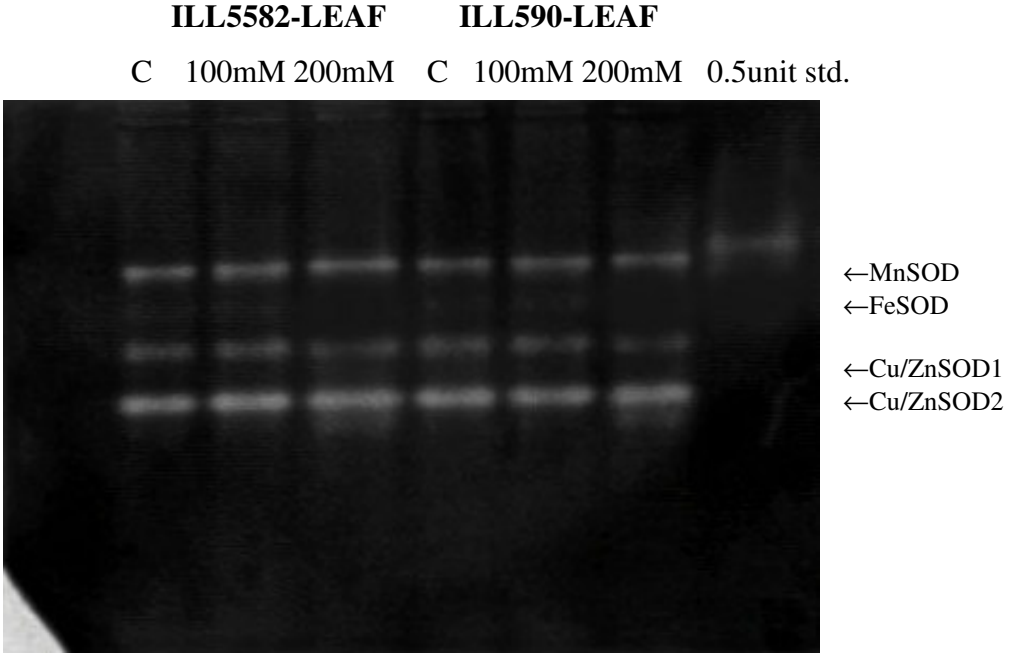


**Fig.3.31** Activities of SOD isozymes in leaf tissues of 5582 and 590. (C: Control, 100 mM NaCl stress, 200 mM NaCl stress, 0.5u Std: 0.5 unit standard SOD activity, one unit of standars SOD activity inhibits the rate of reduction of cytochrome c by 50% in coupled system with xanthin oxidase at pH 7.8 at 25°C in a 3ml reaction medium)

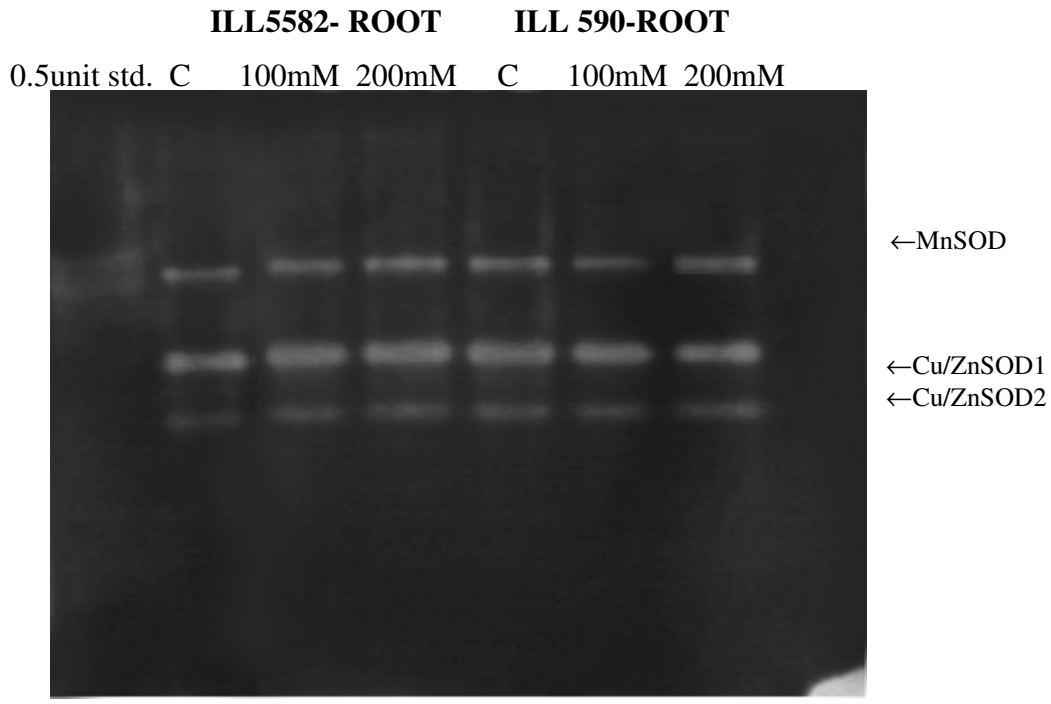


**Fig.3.32** Activities of SOD isozymes in root tissues of cultivars 5582 and 590. (C: Control, 100: 100 mM NaCl stress, 200: 200 mM NaCl stress, 0.5u Std: 0.5 unit standard SOD activity, one unit of standars SOD activity inhibits the rate of reduction of cytochrome c by 50% in coupled system with xanthin oxidase at pH 7.8 at 25°C in a 3ml reaction medium)

Percent changes in all parameters under salt stress in two different lentil cultivars (ILL5582 and ILL590) in shoots and roots were given in Table 3.3 and Table 3.4

**Table: 3.3.** percent changes with respect to control in all parameters at 100 mM and 200 mM NaCl concentration in shoots of both cultivars.

| Shoot | **ILL5582** | | ILL590 | |
|---|---|---|---|---|
| | 100 mM | 200 mM | 100 mM | 200 mM |
| **LENGTH** | 34%↓* | 39%↓* | 36%↓* | 58%↓* |
| **WET WEIGHT** | 40%↓* | 59%↓* | 45%↓* | 69%↓* |
| **DRY WEIGHT** | 25%↓* | 27%↓* | 32%↓* | 50%↓* |
| **LEAKAGE** | 104↑* | 172↑* | 215↑* | 250↑* |
| **MDA*** | 5%↑ | 55%↑* | 23%↑* | 185%↑* |
| **PROLINE*** | 147%↑* | 320%↑* | 57%↑* | 232%↑* |
| **$H_2O_2$** | 41%↑ | 48%↑ | 103%↑ | 261%↑ |
| **Cu/ZnSOD1*** | 1%↓ | 31%↑* | 4%↓ | 20%↑* |
| **Cu/ZnSOD2*** | 4%↑ | 14%↑* | 1%↑ | 5%↑* |
| **MnSOD** | 1%↑ | 32%↑* | 4%↓ | 37%↑* |
| **FeSOD** | 2%↓ | 11%↓ | 1%↓ | 6%↓ |
| **CAT** | 9%↓ | 3%↓ | 13%↓* | 10%↓ |
| **APX*** | 40%↑* | 53%↑* | 27%↑* | 38%↑* |
| **GR*** | 24%↑* | 61%↑* | 32%↑* | 34%↑* |

* Values show significant difference when compared to control

**Table: 3.4.** Percent changes with respect to control in all parameters at 100 mM and 200 mM NaCl concentration in roots of both cultivars.

| Root | **ILL5582** | | ILL590 | |
|---|---|---|---|---|
| | 100 mM | 200 mM | 100 mM | 200 mM |
| LENGTH | 2%↑ | 2%↓ | 19%↓* | 26%↓* |
| **WET WEIGHT** | 16%↓ | 30%↓ | 33%↓* | 54%↓* |
| **DRY WEIGHT** | 7%↑ | 6%↓ | 21%↓* | 23%↓* |
| **LEAKAGE*** | 23↑ | 84↑* | 66↑* | 86↑* |
| **MDA** | 9↓ | 3↑ | 8↑ | 4↑ |
| **PROLINE*** | 43%↑* | 117%↑* | 28%↑ | 195%↑* |
| **H2O2*** | 11%↑* | 45%↑* | 80%↑* | 269%↑* |
| **Cu/ZnSOD1*** | 72%↑* | 98%↑* | 25%↑* | 65%↑* |
| **Cu/ZnSOD2*** | 66%↑* | 97%↑* | 77%↑* | 85%↑* |
| **MnSOD*** | 12%↑* | 43%↑* | 1%↑ | 32%↑* |
| **FeSOD** | - | - | - | - |
| **CAT** | 0 | 18%↓ | 0 | 5%↓ |
| **APX** | 5%↑ | 7%↑ | 9%↑ | 8%↑ |
| **GR** | 13%↓ | 12%↑ | 17%↑ | 25%↑ |

* Values show significant difference when compared to control

# CHAPTER 4

# DISCUSSION

Lentil (*Lens culinaris*) is an important legume in the farming systems of the Mediterranean area because lentil seed is a source of high-quality protein for human consumption and its straw is highly valued animal fodder. The crop is salt sensitive (Ashraf and Waheed, 1990) like many other leguminous crops. Salinity is one of the environmental adverse conditions imposed on plants. Arid and semi arid 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. Selection of salt-tolerant varieties would allow one to cultivate this crop on saline soils or with saline waters, which occur frequently in the Mediterranean area (Katerji et al., 2001).

It is well documented that salt stress enhances ROS production, which results in improved antioxidant defense in plants, especially in the salt tolerant cultivars.

The effect of NaCl on germination and emergence of 133 lentil varieties was determined recently (Ashraf and Waheed, 1990) in a greenhouse study with nutritive solutions. According to this study, only five varieties produced significantly greater fresh and dry biomass than the others. The authors, however, felt that this first selection should be confirmed by a study covering the entire growth period (Katerji et al., 2001). In the study of Katerji and coworkers, two lentil varieties, one of which previously determined as salt sensitive and the other as salt tolerant by Ashraf and Waheed, were selected to be examined. When the results of the latter study are compared with the former one, it can be seen that greenhouse experiment and lysimeter experiment do not support each other by means of the tolerance to salt.

In this study the salt tolerant cultivar ILL 5582 (as identified by Ashraf and Waheed, 1990) and ILL 590 were compared by their physiological changes, membrane damage and osmoprotective responses under salt stress.

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

Under salt stress, lentil seedlings nearly stopped growing, wilted and yellowish leaves were observed. Significant decreases in shoot lengths of both cultivars were detected.

Although cultivar 590 leaf tissue length, wet-dry weight values were higher than cultivar 5582 under normal growth conditions, higher effect of salt stress so higher percentage of decrease in length was seen on 590.

Old leaves became more wilted when compared to the young leaves. This might be because of the salt toxicity and osmotic stress affecting the old leaves more due to the higher accumulation of the salt in the old leaves or by longer time period of exposure to salt. For another stress condition, Jung (2004) stated that, the drought-stressed mature leaves suffer more stress than drought-stressed young leaves which suggests that developmental stages of leaves might contribute to the differential prevention of oxidative damage in plants exposed to drought.

Different variety of lentil (cv. Sultan1) showed similar results; shoot-root length and fresh-dry weight was significantly reduced by NaCl treatments in a dose-dependant manner. Under 200 mM salinity stress more growth retardation was observed in leaf tissues when compared to root tissues (Bandeoğlu, 2004). In most crop species growth of the shoot is more sensitive to salt stress than root growth. (Bernstein *et al.,* 2004) 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 cause decreased wet and dry weights (Yu and Rengel, 1999). Also in roots of *Chrysanthemum morifolium*, with the increase in NaCl concentration there was an increase in the mitotic inhibition (Hossain *et al*., 2004).

### 4.2 Lipid Peroxidation and Ion Leakage

The adverse effects of the salt on membranes are results of the accumulating toxic ions and ROS. The ion balance of membranes changes by $Na^+$ and $Cl^-$ accumulation. These ions replace $K^+$ and $Ca^{2+}$ ions, which have important roles in the functioning of the membrane proteins, under salt stress. On the other hand, ROS, especially the hydrogen peroxide and hydroxyl radicals, damage the membrane lipids and result in lipid peroxidation, damaging the membrane structure and integrity.

According to measured MDA and ion leakage data, ILL590 seemed to be more vulnerable to increasing NaCl stress. Also root tissues showed lower values of membrane damage, which indicates higher protection. This might be because of the higher increase in the total SOD enzyme activity in the root tissues.

The effect of salt stress on membrane integrity has been studied in many plant species by electrolyte leakage tests and MDA contents. The extent of membrane damages varies due to the tolerance due to the tolerance of plant species against salt. For example, in rice, NaCl stress resulted in no increase in lipid peroxidation and conductivity (Lin and Kao, 2000). 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., 1999; Sudhakar et al., 2001).

### 4.3 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 attribute to a variety of functions, such as an osmoticum, 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)

In leaf and root tissues, the percent increases of proline under increasing salt stress were significant and very high for both cultivars. The highest percent increase was, 320% with 5582-leaf tissue under 200 mM salt stress, with 590-leaf tissue the increase was 232% increase under same stress conditions.

Proline, which is a scavenger of free radicals and a protective agent of plasma membrane, can be identified as a component of mechanism in the build up of the salt tolerance. Higher concentration of proline in cultivar 5582 might be the reason of higher salt tolerance when compared to cultivar 590.

According to Bandeoğlu, 2004, lentil variety Sultan 1 showed similar results such that, under stress, proline levels in shoot and root tissues increased significantly with increasing NaCl concentration. The effect of salt stress on proline concentration has been extensively studied in many other plants. In some legumes (alfalfa, soybean and pea) it has been shown that salt stress resulted in extensive proline accumulation (Tramontano and Jouve, 1997). Salt stress resulted in a significant accumulation of free proline in leaves of both salt sensitive and tolerant mulberry cultivars under salt stress the magnitude of increase in free proline accumulation was higher in the tolerant cultivar than in the sensitive cultivar (Kumar *et al.,* 2003).

### 4.4 Hydrogen Peroxide Concentration

Hydrogen peroxide ($H_2O_2$) is one of the active oxygen species and produced by the action of 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 stress.

In our study the results showed that level of $H_2O_2$ in the root tissues is higher when compared to leaf tissues for both cultivars. Also when seedlings were exposed to increased salt stress, the $H_2O_2$ concentration increase in roots was significant. SOD enzyme percent changes, when examined; give an idea for the reason of this increase in root tissues. The increasing total SOD activity in roots was almost twice the activity increase in leaves. Besides this, the hydrogen peroxide detoxifying enzymes catalase and ascorbate peroxidase did not show a significant change in root tissues under salt stress conditions. The results of three enzyme support our $H_2O_2$ data and we observed a higher increase in roots. In cultivar 590 root tissues, the percent increase was higher and this might lead to the generation of hydroxyl radical by reacting with superoxide radical, and to increased lipid peroxidation and electrolyte leakage. The significant increase in the leaves of the cultivar 590 might be the reason of the high membrane damage, high MDA concentration. Although total SOD changes for both cultivars were similar in leaf tissues, the other $H_2O_2$ producing enzymes may be the reason for the significant increase in leaves of cultivar 590. The higher percent increases of 590 leaf and root tissues might be the indication of sensitivity towards NaCl.

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). 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. Both peroxide content and lipid peroxidation level increased with the salt treatment from the control of *Oryza sativa* L. (Khan *et al.*, 2002)

### 4.5 Ascorbate Peroxidase

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

In our study, it was observed that in leaf tissues of both cultivars, there were significant increases of ascorbate peroxidase under salt stress. However root tissues did not respond significantly by means of ascorbate peroxidase. Activity of the enzyme in the roots was much higher than in the leaves. This might be one of the factors that remove the disruptive effect of $H_2O_2$ in the root tissues. In this case the significant elevation of APX activity in leaves under salt stress suggested that leaf APX was salt inducible.

APX activity has been studied in many other plants. Guetha-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) under salt stress suggesting its role in salt tolerance mechanism.

### 4.6 Catalase

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

In our study, we found that CAT activity was higher in leaf tissues when compared with root tissues under all conditions. The reason for the leaf tissues bearing low $H_2O_2$ content might be this higher activity of catalase enzyme in the leaf tissues.

In spite of the slight decrease in both of the tissues of both cultivars under salt stress, those changes were not found significant. CAT enzyme probably did not get activated in lentil seedlings under salt stress. This enzyme may not contribute to the tolerance of the lentil cultivar against salt stress.

Catalase activity changes have been studied in many other plants. CAT activity has been found to increase under salt stress in tomato (Rodriguez-Rosales *et al*., 1999), soybean (Comba *et al*.,1998), tobacco (Bueano *et al*.,,1998; Savoure *et al*.,1999), cucumber (Lechno *et al*.,1997) and mulberry (Sudhakar *et al*., 2001).However it has been found that potato(Benavides *et al*., 2000) and rice (Lin and Kao, 2000) CAT activity does not change under salt stress.

### 4.7 Glutathion Reductase

Glutathione reductase (GR) is found in chloroplasts as well as in mitochondria and cytoplasm and catalyzes the rate-limiting step of the ascorbate-glutathione pathway.

The enzyme is important for the maintenance of reduced form of the glutathione in the cell at high levels, because reduced glutathione is itself a 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 (Sudhakar *et al*., 2001).

In our study, we observed a significant increase in leaf tissues of both cultivars. However it was evident that for the cultivar 5582 leaf tissues, there was a salt concentration dependant increase in the activity of GR but for 590, there was no significant change in GR activity between 100 and 200 mM salt stress. For the root tissues we observed see a higher activity of GR when compared to leaves but for both cultivars significant increase was not observed. The enzyme may behave as salt inducible in the leaf tissues but not in the root tissues.

Effect of salt stress on GR activity has been studied in several plants. In rice ((Lin and Kao, 2000), soybean (Comba et al., 1997) and mulberry (Sudhakar et al., 2001) increase activity of GR was indicated under salt stress. Also GR activity was found to be higher in salt tolerant cultivars of mulberry, tomato (Guetha-Dahan et al., 1998) and soybean. In roots of Chrysanthemum morifolium, with the increase in NaCl concentration there was an increase in the glutathione reductase activity (Hossain *et al*., 2004). In tobacco (Bueano et al., 1998) salt stress increased the activity of all antioxidant enzymes except GR, which remained unchanged.

### 4.8 Superoxide Dismutase

Superoxide dismutase (SOD) responds to a variety of environmental stresses and plants acquire tolerance to them by increasing the SOD activity, as well as the activity

of $H_2O_2$ detoxifying enzymes. SOD has four and three isozymes identified in leaf and root tissue extracts of lentil, respectively. These isozymes are differentially affected from the salt treatment (Bandeoğlu et al., 2004). By native PAGE and negative activity staining those isozymes were examined separately.

In our study, mitochondrial MnSOD alteration showed significant increases under 200 mM salt stress. This isozyme was salt inducible for both tissues and showed higher activity in root tissues of 5582.

Chloroplastic FeSOD has very low contribution to the total SOD enzyme alteration (5% for 5582 and 9% for 590) so the inhibition of the isozyme under 200 mM salt stress did not significantly affect the total SOD.

Cytosolic CuZnSOD-1 and CuZnSOD-2 isozymes showed the highest contribution to the total SOD enzyme change. Although both cultivars leaf tissues showed significant changes under 200 mM salt stress, in both tissues 5582 has higher activity change of both CuZnSOD isozymes. Leading the significant and higher increase of this isozyme more effective detoxification of the superoxide radicals takes place in 5582. This may contribute to the higher tolerance of 5582 when compared with 590.

When total SOD activity was considered, root tissues of both cultivars showed almost twice the activity in the leaf tissues. Also CAT and APX enzymes did not show significant changes in root tissues. From these data, what we expected is what we encountered; $H_2O_2$ concentration in root tissues is higher than the concentration in leaf tissues.

The higher concentrations of CuZnSOD isozymes in 5582 may contribute to the removal of superoxide radical efficiently. In spite of the similar values of $H_2O_2$ concentrations of both cultivars under 200 mM salinity, high SOD and so that low

superoxide radical inhibit the hydroxyl radical to be formed. In this way, cultivar 5582 might be protected from higher membrane damage.

Lower concentrations of CuZnSOD isozymes in cultivar 590 could not support the removal of large amount of the superoxide radical. The immediate percent increase of $H_2O_2$, which was considered to be produced by other peroxide generating enzymes, caused membrane damage. In addition to this the superoxide radical that could not be removed, might react with $H_2O_2$ and form hydroxyl radical that leads to high membrane damage in the tissues of 590.

SOD activity changes have been studied in many other plants under different stress conditions. Increased SOD activity has been shown to confer resistance to various environmental stresses in plants. In roots of Chrysanthemum morifolium, with the increase in NaCl concentration there was an increase in the superoxide dismutase activity (Hossain *et al*., 2004). In maize, activity of superoxide dismutase was stimulated by the deficiency of each of the macronutrients; N, P, K, Ca, Mg, S (Tewari *et al.,* 2004). Superoxide dismutase substantially increased only in drought stressed mature leaves of Arabidopsis (Jung, 2004).

# CHAPTER 5

# CONCLUSION

In this study, two lentil cultivars (*Lens culinaris*) were characterized and compared due to their NaCl susceptibility (cultivar 5582, named as salt tolerant by the green house experiments of Ashraf and Waheed, 1990 and cultivar 590 which we have no information about its salt tolerance property) under laboratory conditions. Physiological parameters, oxidative stress evaluating parameters and antioxidant enzyme activities were examined and analysed under 100 mM and 200 mM NaCl stress conditions.

When compared with salt tolerant ILL5582, ILL590 was found to be sensitive against NaCl. Besides the percent decreases in wet-dry weight and length measurements indicating the sensitivity, higher MDA concentration and higher electrolyte leakage amounts supported this conclusion.

According to our results, superoxide dismutase enzyme was the main antioxidant enzyme that leads to the protection of the plant tissues against NaCl stress. Among the isozymes CuZnSOD was the most contributing and remarkable one. It was concluded that the higher increase in SOD enzyme activity in cultivar ILL5582 might be the main determining factor in protective mechanism against salt stress.

Leaf and Root tissues respond differently towards NaCl stress. Antioxidant enzyme activities were higher in root tissues. It could be also seen from the small percentage alterations of root physiological parameters that roots were better-protected tissues when compared with leaves.

Also proline, which is a strong osmoprotectant, is a protective component in lentil seedlings under salt stress. It was concluded from very high concentration increases of proline in a stress concentration dependant manner, in both tissues.

Lentil is salt sensitive like many other leguminous crops. Further studies and determinations about antioxidant defense mechanisms might lead to the development of salt tolerant varieties and cultivation of this crop on saline soils.

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## APPENDIX A

## l-D PAGE STOCK SOLUTIONS AND GEL FORMATION

I. Acrylamide Stock (30 % T, 2.67 % C)

Acrylamide 73 g

N,N bis-methylene acrylamide 2g

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-HC1 pH 8.8)

Trizma base 54.45 g

Dissolved in 150 ml distilled water, adjusted to pH 8.8 with concentrated HC1 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 6g

Dissolved in 60 ml distilled water, adjusted to pH 6.8 with concentrated HC1 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 lx before electrophoresis.

V. Gel Formation

| | Separating Gel | Stacking Gel |
|---|---|---|
| Acrylamide/bis stock | 2m1 | 0.4 15 ml |
| Separating Buffer | 1.25ml | - |
| Stacking Buffer | - | |
| $dH_2O$ | 1.65 ml | 1.7ml |
| 10%APS | 25 µl | 10µl |
| TEMED | 2.5 µl | 2.5 µl |

# APPENDIX B

## BASIS OF NEGATIVE ACTIVITY STAINING

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