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NACL STRESİNE KARŞI *LENS CULİNARS'İN* BİYOKİMYASAL VE FİZYOLOJİK CEVABI

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Özet: Bu çalışmada, farklı NaCl konsantrasyonlarının (50-400 mM) varlığında ve yokluğunda (kontrol) *Lens culinaris* bitkisindeki büyüme geriliği ile ilişkili olarak köklerde ve gövdede antioksidatif cevabın karşılaştırılmasını amaçladık. Köklerde ve gövdede büyüme parametreleri (kuru ve taze ağırlık) Na^+ içeriği, LPO seviyeleri, prolin, H_2O_2 içeriği, membran hasarı ve SOD, APX, CAT, GPX aktiviteleri incelendi. Kontrolle karşılaştırıldığında, 100-400 mM NaCl uygulaması, kök ve gövde ağırlığında ve yapraklardaki toplam klorofil ve karotenoid içeriğinde azalmaya sebep oldu. Buna karşın, LPO seviyeleri, H_2O_2 ve Na^+ içeriği, elektrolitik geçirgenlik ve prolin seviyelerinde her iki bitki dokusunda da doza bağlı olarak artışa neden oldu. Bu artışlar gövde ile karsılaştırıldığında köklerde anlamlı olarak daha yüksek bulundu. Bu çalışmada elde edilen sonuçlar ve literatür bilgileri H_2O_2 ve Na^+ birikiminin büyümenin düzenlenmesinde önemli rol oynadığını göstermektedir. Tuzluluğun antioksidan enzim aktiviteleri üzerindeki etkisi her iki bitki dokusunda da benzer şekilde bulundu. Tuz stresi köklerde ve gövdede SOD ve GPX enzim aktivitelerinde artışa sebep olurken, CAT ve APX aktivitelerini baskıladı. Sonuç olarak, *Lens culinaris'* in tuzluluk stresine olan hassasiyeti yüksek Na^+ seviyesi ve oksijen radikali indirgeme kapasitesinin düşmesiyle artan lipid peroksidasyonu ile açıklanabilir.

Anahtar Kelimeler: *Lens culinaris*, tuz stresi, büyüme, antioksidant enzimler, prolin, lipid peroksidasyon.

PHYSIOLOGICAL AND BIOCHEMICAL RESPONE TO NaCl STRESS IN *LENS CULINARIS*

Abstract: In this study we aimed to compare some antioxidative responses of shoots and roots associated to growth reduction in *Lens culinaris* in the absence (control) and presence of different NaCl concentrations (50-400 mM). Growth parameters (dry-fresh weight) Na^+ contents, LPO levels, proline, H_2O_2 contents, membrane damage and activities of SOD, APX, CAT and GPX was investigated in roots and shoots. Compared with controls, 100-400 mM NaCl treatment resulted in a reduction in roots and shoots weight, total chlorophyll and carotenoid in leaves. On contrary, LPO levels, H_2O_2 and Na^+ contents, proline levels, electrolytic leakage exhibited a dose dependent increase in both tissues. These increases were significantly higher in roots as compared to shoots. Our results and literature data suggest that the accumulation H_2O_2 and Na^+ plays an important role in regulating growth. The effect of salinity on antioxidant enzyme activities was similar trends in both tissues. Salt stress caused an increase on the activity of SOD and GPX in roots and shoots, while the activities of CAT and APX was inhibited. A higher Na^+ level and lipid peroxidation combined with a lower capacity for oxygen radical scavenging could probably explain the sensitivity of *Lens culinaris* to salt stress.

Key words: *Lens culinaris*, salt stress, growth, antioxidant enzymes, proline, lipid peroxidation.

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1. INTRODUCTION

NaCl stress is a major factor limiting crop production because it affects almost all plant functions. Plants exposed to high salt concentrations must withstand both water deficit and ion imbalance imposed by salt excess. Although water deficit always has a negative effect, many crop plants are primarily sensitive to Na^+ excess (Greenway and Munns, 1980) due to its adverse effects on K^+ nutrition, cytosolic enzyme activities, photosynthesis and metabolism (Wenxue et al., 2003). The depressive effect of salt on the growth is, according to Hajji et al. (1999), the results of (i) a reduction in the osmotic potential of the soil solution around the roots, (ii) an increase in the accumulation of some ions in harmful concentrations in tissues and (iii) a modification of the nutritional statute of the essential ions to the growth and the development. It has been generally observed that plants exposed to saline environment (NaCl), take up high amounts of Na^+, whereas the uptake of K^+ and Ca^{2+} is significantly reduced. However, reasonable amounts of both K^+ and Ca^{2+} are required by plants to maintain the integrity and functioning of cell membranes (Wenxue et al., 2003).

Many studies indicate that salt stress leads to increased production of active oxygen species (AOS) such as superoxide (O_2^-), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). The AOS are also produced during normal aerobic metabolism by the interaction between O_2 and electrons leaked from electron transport chains in the chloroplasts and mitochondria (Halliwell and Gutteridge, 1999). These cytotoxic activated oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Halliwell and Gutteridge, 1999). This lead to change in selective permeability of bio-membranes and thereby membrane leakage and change in activity of enzymes bound to membrane occurred (Lester and Stein, 1993).

To mitigate and repair damage initiated by reactive oxygen species, plants have developed a complex antioxidant system (Van Breusegem et al., 2001) including low molecular mass antioxidants as well as antioxidative enzymes, such as superoxide (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). SOD catalyzes the dismutation of two molecules of superoxide into oxygen and H_2O_2. Catalase (EC 1.11.1.6) and variety of peroxidases catalyze the breakdown of H_2O_2 (Asada 1992). Although catalase is apparently absent in the chloroplasts, H_2O_2 could be detoxified through the ascorbate-glutathione cycle (Asada 1992) in a reaction catalyzed by an ascorbate specific peroxidase (EC 1.11.1.11), which is often present in high levels in this organelle (Asada 1992). GPX, which is less specific to electron donor substrate, decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or ascorbate. H_2O_2 is an endogenous signaling molecule involved in plant responses to abiotic and biotic stresses such as extremes of temperature, light intensity, drought, pathogen, salinity, as well as stimuli such as plant hormones and gravity (Dionisio-Sese and Tobita, 1998). Accumulation of H_2O_2 will also lead to enhance potential for production of hydroxyl radicals, which leads to lipid peroxidation (LPO) and membrane deterioration. Under saline environments, the plant lipit metabolism is interrupted because of oxidative damage to membrane lipids by active oxygen species (AOS) and LPO (Misra and Gupta, 2006). LPO can also be initiated enzymatically by lipoxigenases (LOX), and this enzyme incorporates molecular oxygen into linoleic and linolenic acids, to form lipid hydroperoxides (Elkahoui et al, 2005). LOX are also responsible for membrane degradation because they catalyze the dioxygenation of polyunsaturated fatty acids producing hydroperoxy fatty acids that toxic to the cell (Elkahoui et al, 2005).

The loss of chlorophyll is often considered as a marker of a cellular component of salt stress. Also, reductions in carotenoid contents under salinity stress have also been reported (Rout and Shaw, 2001). Carotenoids are responsible for quenching singlet oxygen, and hence their comparative levels in a cultivar may determine relative stress tolerance (Sairam et al., 2002).

Accumulation of proline has been widely advocated for use as parameter of selection for salt stress response (tolerance) (Ashraf and Haris, 2004). However, proline accumulation cannot be regarded as marker for salt tolerance, as it accumulate under various condition of stresses such as temperature, drought, and starvation, where as in many salt stressed plants its levels decreases (Lee and Liu, 1999; Lacerda et al., 2005).

In the present experiment an attempt was made to study the growth responses, ion accumulation and changes in activity of enzymes in response to added NaCl in *Lens culinaris* shoots and roots.

2.MATERIALS AND METHODS

Plant materials culture conditions

Seeds were surface sterilized with 0.1 % (w/v) sodium hypochlorite solution for 5 min. and thoroughly rinsed with distilled water and allowed to germinate in petri plates lined with filter papers (Whatman no:1). 10 Days-old seedlings were transferred to containing grated half strength Hougland nutrient solution in the absence (control) and presence of varying concentrations(50-400 mM) NaCl (salt stressed).

The environmental conditions in the growth chamber were 60-70 % humidity, 25 ºC and light intensity of 350 $\mu molm^{-2}sec^{-1}$with a 16 h photoperiod. The pH of the medium varied from 5.8 to 6.0. Shoot and root samples were harvested after 1 and 7 d of NaCl treatment from different sets of plants for assay of various antioxidants and antioxidative enzymes.

Growth parameters

After 1 and 7 days of NaCl treatment and control, 10 plants for each group were taken at random and divided in to separate shoot and root fractions. The fresh weights of shoots and roots were weighed. The samples were then dried in a forced draft oven at 70 ºC for 48 h, and the dry weights were determined.

Ion concentrations

The shoots and roots of plants were separated manually, dried in an oven 80 ºC for four day. The dried material was wet-ashed in 2 ml HNO_3 and Na^+ and K^+ were assayed by flame emission photometry (Jenway PFPF) and Ca by atomic absorption spectrophotometer (Varian FS 2000).

Proline Determination

The amount of proline was determined according to a modified method of Bates et al., (1973). Approximately 0.5 g of shoot and root tissues from control and treated plants were cut into small pieces and homogenized by the addition of 1 ml of 3 % 5-sulphosalicylic acid solution in ice bath. Shoot tissues were homogenized with glass homogenizer and root tissues were grounded by using cold mortar and pestle. The homogenates were centrifuged at 14 000 x g for 5 min at 4 ºC. For each sample, an eppendorf tube containing 0.2 ml acid ninhydrin (0.31 g ninhydrin, 7.5 ml acetic acid, and 5 ml 6 M phosphoric acid), 0.2 ml 96 % acetic acid and 0.1 ml 3 % 5-sulphosalicylic acid were prepared. Supernatant (0.1 ml) from each homogenate was added to the tubes. Tubes were incubated at 96 ºC for 1 h in a hot block and then 1 ml of toluene was added to each tube. The tubes were mixed and centrifuged at 14 000 x g for 5 min at 4 ºC. Absorbance of the pink-red upper phase was recorded at 520 nm against toluene blank. A standard curve for proline in

the range 0.01 µM-1.5 mM was constructed to determine the proline concentration in each sample.

H_2O_2 Content

The hydrogen peroxide content was determined according to Jana and Choudhuri (1981). Aliguats of fresh shoots and roots were homogenized in 25 mM potassium phosphate, pH 6.5 and centrifuged at 10 000 x g for 25 min. The solution was mixed with 1 % titanium chloride (in concentrated HCl) and then centrifuged at 10 000 x g for 15 min. The absorbance of the supernatant was measured at 410 nm and the H_2O_2 content calculated using 0.28 $\mu M^{-1}cm^{-1}$ as extinction coefficient.

Electrolyte Leakage

To determine electrolyte leakage, fresh samples were cut and placed in the test tubes containing 10 ml distilled deionized water. The tubes were covered with plastic caps and placed in a water bath maintained at the constant temperature of 32 °C. After 2 h the initial electrical conductivity of the medium (EC_1) was measured using an electrical conductivity meter (Hanna Germany). The samples were heated at 121 °C for 20 min to completely kill the tissue all electrolytes. Samples were cooled to 25 °C and the final electrical conductivity (EC_2) was measured (Devi and Prasad, 1998). The electrolyte leakage (EL) was expressed following the formula

$$\% EL = (EC_1 / EC_2) \times 100.$$

Enzyme assays

Shoot and root tissues were homogenized with 20 mM phosphate buffer (pH 7.0) containing 1 % (w/v) polyvinylpyrolidone in a chilled mortar and pestle. The homogenate was filtered and centrifuged in a refrigerated centrifuge at 13 000xg for 15 min. The supernatant fraction was used as crude extract for enzyme activity. All operations were carried out at 4°C

CAT activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption according to Aebi (1974). The reaction volume and reaction time were 3 ml and 1 min, respectively. The reaction mixture contained 25 mM phosphate buffer, pH 7.0 and 10 mM H_2O_2.

The assay for total SOD (EC 1.15.1.1) activity was based on the method described by Beauchamp and Fridovich (1971) which measures inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm using a spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of NBT by 50 %.

The activity of APX (EC 1.11.1.11) was determined according to Asada (1992) measuring the decrease in A_{290} due to ascorbate oxidation (ε=2.8 mM-1 cm-1). The enzyme activity was calculated in terms of µmol of ascorbate oxidized per minute at 25 ^oC.

GPX activity was measured spectrophotometrically at 25 °C by following the method of Tatiana et al (1999). The reaction mixture (3ml) consisted of 25 mM potassium phosphate (pH 7.0) buffer, 10 mM H_2O_2 and 0.05 % guaiacol. The reaction was started by the addition of an enzyme extract. The formation of tetraguaiacol was measured at 470 nm (ε =26.6 mM^{-1} cm^{-1}).

LOX activity was measured according to Minguez- Mosquera et al., (1993) using 50mM K-phosphate buffer (pH 6.0) for extraction. Neither the addition of Triton X-100 to improve the solubility of the enzyme nor the addition of dithiothreitol (DTT) to protect SH groups from oxidation improved the assay results. The reaction mixture consisted of 20 µl crude extract and 0.5 mM linoleic acid in 50 mM K-phosphate buffer (pH 6.0). LOX activity was calculated following the rise in the extinction at 234 nm using an extinction coefficient of 25,000 M^{-1} cm^{-1}.

GSH-Px (EC, 1.11.1.9) activity was assayed by the oxidation of NADPH at 340 nm (ε: 6.22 mM^{-1} cm^{-1}) (Gunzler and Flohe, 1985). The reaction mixture consisted of 50mMK-phosphate buffer (pH 7.0) containing 1mM EDTA, 0.24 unit GR (EC 1.6.4.2; Sigma-Aldrich, St. Louis, USA), 10 mM GSH, 0.20 mM NADPH, and 1 mM sodium azide. After addition of enzyme eluate, test tubes were incubated at 37 ℃ for 10 min. The reaction was initiated by addition of 1 Mm H_2O_2.

Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) formation using the thiobarbituric acid method described by Heat and Packer (1968)**.** The extracts preparation was mixed with the same volume of 0.5 % (w/v) thiobarbuturic acid solution containing 20 % (w/v) trichloroacetic acid. The mixture was heated at 65 ^{o}C 30 min and the reaction was stopped by quickly placing in an ice-bath. The cooled mixture was centrifuged at 10 000xg for 10 min and the absorbance of the supernatant at 532 and 600 nm was read. After subtracting the non-specific absorbance at 600 nm the MDA concentration was determined by its extinction coefficient of 155 mM^{-1} cm^{-1}.

Photosynthetic pigments

Chlorophyll and carotenoid contents were estimated by extracting 0.05 g of the leaf material in 10 ml dimethylsulfoxide. The samples were heated at 65 ℃ for 4 h and than the absorbance of extract recorded at 645, 665 and 470 nm. Chlorophyll and carotenoid content were calculated as per standard methods (Lichtenthaler, 1983).

Statistical analysis

Data were obtained as means ± standard deviations (SD). Analysis of variance and least significant difference test were conducted to identify differences among means. Statistical significance was declared at $P < 0.01$.

3.RESULTS

Effects of NaCl on the growth of *Lens culinaris* assessed by fresh and dry weight were studied after 7 day of exposure to varying concentrations of NaCl (Table 1). Salinity effect on growth was not uniform in all plant parts (Figure 1). The growth of plants was greatly influenced by high salinity, the root being more detrimentally affected than the shoot. But root and shoot fresh and dry weights were not affected after exposing the plants
to 50 mM NaCl. In shoot dry weight reduced by 21 and 42 % in 100 mM and 400 mM NaCl, respectively, whereas in roots it decreased by 25 and 48 % in response to low and high salinity. In addition to growth reduction, the roots become yellow and the leaves appeared dark-green at 200 and 400 mM NaCl.

The results show that increase in NaCl concentrations steadily increased Na^{+} in shoots and roots of *Lens culinaris* and the accumulation pattern between shoots

Table 1: The fresh-dry matter, electrolytic leakage and proline contents in different plant parts of *Lens culinaris* after 7 days of NaCl treatment.

NaCl (mM)	Fresh Matter of Plant (mg plant^{-1})		Dry Matter of Plant (mg plant^{-1})		RLR %		Proline (µmol g fw^{-1})	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
0	127.7 ± 3	76.8 ± 2	14.0 ± 1.3	10.3 ± 1.4	18 ± 1.1	22 ± 1.8	39 ± 3.1	18 ± 2.2
50	128.0 ± 4*	77.0 ± 5*	14.3 ± 2.1*	10.0 ± 2.0*	20 ± 1.9*	23 ± 2.1*	50 ± 2.5	29 ± 2.7
100	110.5 ± 3	64.3± 6	11.0 ± 1.5	7.7 ± 1.8	26 ± 2.0	31 ± 2.8	64 ± 3.0	32 ± 2.9
150	102.0 ± 5	61.1 ± 2	9.9 ± 2.2	7.1± 1.5	31 ± 2.3	35 ± 1.5	75 ± 1.8	35 ± 3.8
200	98.9 ± 1	58.1 ± 3	8.8 ± 1.8	6.3 ± 1.7	35 ± 3.0	42 ± 2.7	100 ± 3.7	36 ± 2.2
400	97.7 ± 4	55.3 ± 3	8.1 ± 1.5	5.4 ± 2.1	41 ± 2.5	52 ± 3.0	112 ± 5.9	42 ± 3.4

The values are mean ± SD (n = 5), Students-t-test revealed that the NaCl treatments are significantly different from control ($p<0.01$), *Not significantly ($p>0.05$) differed from control,

Figure 1: Effect of NaCl on the growth of *Lens culinaris* after 7 days of treatment.

Table 2: Effect of NaCl on ion content in different plant parts of *Lens culinaris* after 7 days of NaCl treatment,

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	K^+ mg/g DW		Ca^{+2} mg/g DW		Na^+ mg/g DW		K^+/Na^+		Ca^{2+}/Na^+	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	62.2 ± 3.1	51.3 ± 3.1	8.5 ± 0.3	11.6 ± 0.6	12.1 ± 2.1	17.2 ± 1.7	5.14	2.98	0.70	0.67
50 mM	57.1 ± 3.6	42.2 ± 2.5	7.9 ± 0.2	10.0 ± 0.4	16.1 ± 2.3	24.5 ± 2.0	3.54	1.72	0.49	0.41
100 mM	37.1 ± 2.5	32.5 ± 2.9	7.4 ± 0.7	9.8 ± 0.7	18.8 ± 1.9	35.3 ± 2.3	1.97	0.92	0.39	0.27
150 mM	31.3 ± 2.1	26.6 ± 1.4	6.6 ± 0.9	9.1 ± 0.2	33.2 ± 1.4	45.9 ± 3.3	0.94	0.58	0.20	0.20
200 mM	20.7 ± 1.6	17.1 ± 1.6	6.3 ± 0.4	8.5 ± 0.5	37.7 ± 2.1	62.1 ± 1.9	0.55	0.27	0.17	0.14
400 mM	11.5 ± 0.9	9.2± 1.1	5.9 ± 0.5	7.9 ± 0.3	45.1 ± 2.3	101.0 ± 2.4	0.25	0.09	0.13	0.08

and roots of salt treated plants differed considerably (Table 2). The root Na^+ concentration values were higher than in shoot of *Lens culinaris*. At 400 mM NaCl, the concentration of Na^+ in shoot and root was 45.1 and 101.0 mg/g dry weight, respectively as compared to controls plants on 7 day of treated. On the other hand, a progressive decrease in concentrations of K^+ and Ca^{2+} in both shoots and roots of *Lens culinaris* was found with increase in concentration of NaCl in the growth medium (Table 2). There was a consistent decrease in K^+/Na^+ and Ca^{2+}/Na^+ ratios in the shoots and roots of *Lens culinaris* with increase in salt level of the growth medium.

High salinity (150-400 mM) caused significant decreases in chl a, ($P < 0.01$) chl b ($P < 0.01$) and carotenoid ($P < 0.01$) in leaves of Lens culinaris as compared to control plants (Table 3). In the leaves, the reduction in chl a and chl b concentrations were found to be 45 % and 26 % respectively at 400 mM NaCl. Chl a / b was decreased at high NaCl concentrations.

The extent of membrane damage was assessed indirectly by conductometric measurements of solute leakage from cells. Low values of electrolyte leakage were recorded in the controls. In the shoots and roots salt stressed plants increased ($P < 0.01$) lipid peroxidation, H_2O_2 content and electrolyte leakage ratio were significantly observed with increasing NaCl concentration when compared to control plants (Table 4-5). These increases were significantly higher ($P < 0.01$) in roots as compared to shoots at high NaCl concentrations. These results suggest that oxidative stress induced by salt stress in the roots may be significantly higher than in the shoots. In addition, after 7 day of exposure, we found the harmful effect of excessive accumulation of Na^+ under saline condition to be a companied by increasing lipid peroxidation (Na concentration-LPO levels for roots $r = 0.95$, $P < 0.01$; for shoots $r = 0.96$, $P < 0.01$)

Table 3: Effects of NaCl stress on photosynthetic pigments in leaf of *Lens culinaris*, after 7 day NaCl treatment,

Concentration (µM)	Chl a (µg/g FW)	Chl b (µg/g FW)	Chl a/b	Total Chl (µg/g FW)	Carotenoid (µg/g FW)
Control	5.85 ± 0.19	1.72 ± 0.1	3.40	7.57 ± 0.25	1.78 ± 0.08
50	5.68 ± 0.21*	1.68 ± 0.2*	3.38	7.36 ± 0.19*	1.62 ± 0.09*
100	4.96 ± 0.32	1.53 ± 0.3	3.22	6.49 ± 0.22	1.04 ± 0.07
150	4.56 ± 0.35	1.49 ± 0.3	3.06	6.05 ± 0.15	0.85 ± 0.04
200	3.97 ± 0.25	1.37 ± 0.1	2.89	5.34 ± 0.27	0.82 ± 0.05
400	3.22 ± 015	1.27 ± 0.2	2.53	4.49 ± 0.30	0.80 ± 0.04

The values are mean ± SD (n=5), Students-t-test revealed that the NaCl treatments are significantly different from control ($p<0,01$), * Not significantly ($p>0,5$) differed from control,

Proline is an osmoprotectant that has been shown to accumulate in plants in response to salinity stress. After 7 day of exposure, the proline content increased by 2.9 and 2.3 fold in the shoots and roots of 400 mM NaCl treated plants as compared to control plants respectively (Table 1).

Table 4 and 5, shows the change in the activities of SOD, CAT, APX, GPX, GSH-Px and LOX of *Lens culinaris* in shoots and roots during exposure to varying concentrations of NaCl. The effect of salinity on some antioxidant enzymes activities was similar in shoots and roots of *Lens culinaris*. In shoots and roots of salt-stressed plants, superoxide dismutase ($P < 0.01$),

Table 4: Changes some antioxidant enzyme activities, LPO and H_2O_2 levels in crude extracts obtained from shoots of *Lens culinaris* treated for 1 and 7 days with five concentrations of NaCl

NaCl (mM)	Day	SOD (U g fw^{-1})	CAT (U g fw^{-1})	APX (U g fw^{-1})	GPX (U g fw^{-1})	LOX (U g fw^{-1})	GSH-PX (U g fw^{-1})	MDA (nmol gfw^{-1})	H_2O_2 (µmol g fw^{-1})
Control	1	67.5 ± 3.2	126.2 ± 7.3	11.0 ± 0.8	29.8 ± 2.2	18.8 ± 2.4	63.3 ± 4.3	15.4 ± 1.0	5.2 ± 0.17
	7	68.0 ± 2.9	123.7 ± 6.6	11.8 ± 0.9	31.4 ± 2.7	20.9 ± 1.9	65.4 ± 3.6	16.0± 1.4	5.3 ± 0.17
50	1	66.6 ± 3.7	125.4 ± 7.4	11.5 ± 0.7	30.4 ± 3.9	20.2 ± 2.6	65.6 ± 4.7	18.5 ± 1.2	4.8 ± 0.30
	7	72.0 ± 2.5	106.6 ± 6.2	11.3 ± 0.4	39.6 ± 3.1	39.6 ± 2.2	76.8 ± 5.1	26.0 ± 1.5	6.5 ± 0.23
100	1	64.0 ± 2.7	122.1 ± 6.6	11.3 ± 0.6	30.8 ± 2.4	22.3 ± 2.1	65.3 ± 3.9	23.1 ± 1.0	5.8 ± 0.24
	7	76.9 ± 2.9	96.8 ± 5.7	10.4 ± 0.6	46.4 ± 2.8	44.4 ± 3.8	79.8 ± 4.3	34.0 ± 1.8	7.2 ± 0.22
150	1	64.0 ± 3.7	108.3 ± 5.3	10.8 ± 0.5	31.8 ± 3.1	24.6 ± 3.2	67.9 ± 4.1	27.0 ± 1.3	5.3 ± 0.21
	7	80.0 ± 3.3	67.7 ± 4.5	9.4 ± 0.4	50.0 ± 2.5	49.7 ± 4.3	84.4 ± 6.7	40.1 ± 1.8	7.1 ± 0.15
200	1	68.0 ± 3.9	105.8 ± 6.1	10.5 ± 0.7	32.3 ± 2.7	25.8 ± 2.5	69.6 ± 3.6	33.0 ± 1.5	5.2 ± 0.25
	7	86.0 ± 3.8	55.4 ± 3.9	8.4 ± 0.5	53.2 ± 2.1	53.5 ± 5.1	86.2 ± 5.4	45.6 ± 2.1	7.5 ± 0.30
400	1	69.5 ± 3.5	104.2 ±5.5	10.1 ± 0.6	32.9 ± 3.1	26.4 ± 2.6	70.9 ± 3.9	36.0 ± 2.1	6.2 ± 0.25
	7	92.0 ± 4.1	46.4 ±2.1	8.0 ± 0.5	60.4 ± 2.6	64.6 ± 4.9	92.7 ± 5.9	50.1 ± 2.5	9.5 ± 0.31

Table 5: Changes some antioxidant enzyme activities. LPO and H_2O_2 levels in crude extracts obtained from roots of *Lens culinaris* treated for 1 and 7 days with five concentrations of NaCl.

NaCl (mM)	Day	SOD (U g fw^{-1})	CAT (U g fw^{-1})	APX (U g fw^{-1})	GPX (U g fw^{-1})	LOX (U g fw^{-1})	GSH-PX (U g fw^{-1})	MDA (nmol gfw^{-1})	H_2O_2 (µmol gfw^{-1})
Control	1	30.0 ± 3.4	125.0 ± 6.1	2.2 ± 0.3	33.3 ± 2.3	36.2 ± 2.9	27.8 ± 2.1	18.0 ± 1.3	7.0 ± 0.41
	7	32.2 ± 3.1	121.2 ± 5.5	1.9 ± 0.2	34.0 ± 2.1	36.6 ± 2.4	28.3 ± 2.8	19.1 ± 1.6	6.9 ± 0.44
50	1	30.4 ± 2.9	123.5 ± 6.1	1.8 ± 0.1	34.5 ± 2.8	36.8 ± 3.2	28.9 ± 3.1	19.0 ± 1.3	7.0 ± 0.51
	7	44.9 ± 3.7	106.4 ± 5.4	1.5 ± 0.4	45.0 ± 3.1	40.1 ± 2.7	33.7 ± 3.6	24.0± 1.5	8.6 ± 0.45
100	1	31.2 ±3.3	118.4 ± 4.6	1.8 ± 0.2	35.1 ± 2.2	38.5 ± 2.4	31.8 ± 4.3	20.0 ± 1.3	6.8 ± 0.48
	7	47.2 ± 2.1	88.1 ± 4.4	1.1 ± 0.3	47.6 ± 2.5	41.2 ± 1.9	35.4 ± 2.7	33.6 ± 2.8	10.6 ± 0.53
150	1	31.5 ± 2.8	109.6 ± 5.1	1.8 ± 0.3	36.3 ± 2.0	37.6 ± 3.1	33.2 ± 3.5	23.5 ± 1.3	7.6 ± 0.48
	7	52.1 ± 3.5	71.5 ± 3.9	0.9 ± 0.1	54.2 ± 3.7	42.2 ± 2.5	38.6 ± 4.1	43.7 ± 2.2	10.4 ± 0.45
200	1	32.2 ± 2.2	101.9 ± 5.6	1.8 ± 0.3	37.1 ± 2.5	38.5 ± 1.7	35.8 ± 3.6	31.1 ± 1.3	8.8 ± 0.49
	7	59.4 ± 3.9	57.2 ± 3.4	0.9 ± 0.2	59.4 ± 3.1	44.6 ± 2.9	43.5 ± 3.7	58.2 ± 1.5	12.7 ± 0.91
400	1	33.5 ± 3.1	93.7 ± 4.7	1.6 ± 0.4	38.2 ± 2.8	37.9 ± 2.7	36.8 ± 2.7	35.6 ± 1.6	9.6 ± 0.51
	7	64.3 ± 3.6	38.6 ± 2.8	0.7 ± 0.1	74.3 ± 3.6	47.3 ± 3.0	47.5 ± 3.9	62.6 ± 2.5	15.9 ± 1.20

glutathione peroxidase ($P < 0.01$) and lipoxgenase ($P < 0.01$) activities increased with NaCl concentrations when compared to the controls.

The activities APX and CAT significantly decreased ($P < 0.01$) in *Lens culinaris* shoots and roots with increasing NaCl as compared to controls. At a high NaCl dose (400 mM), loss in APX activity was rapid and in shoots and roots the maximal extent of decline in activity after 7 days of exposure was 32 % and 63 % respectively while no loss in APX activity was observed in control plants during 7 day period of experimentation.

The activity of guaiacol peroxidase, which also decomposes H_2O_2 significantly increased ($P < 0.01$) in response to the NaCl treatment in *Lens culinaris*. 400 mM NaCl treatments caused, in shoots and roots respectively, 1.9 and 2.2 fold increase in GPX activity in comparison to the controls plants at the end of 7 days.

4.DISCUSSION

Although *Lens culinaris* is a commercially important plant, there is not much known about its salt stress responses, and the physiological consequences of salt stress.

In the present study, high salinity (150-400 mM NaCl) decreased *Lens culinaris* plant growth significantly. Growth reduction under saline condition has been reported in various plants by many researches (Lacerda, et al., 2005; Ashraf and Haris, 2004).

One of the harmful effects of salinity on plants, growth involves the excessive accumulation of Na^+ in the plant parts (Munns et al., 2000; Ashraf and Haris, 2004). This accumulation under saline conditions depends on the plant's capacity to limit the uptake of this element. The results obtained by us do support these claims and there were negative correlation between Na concentration and dry weight ($r = 0.93$ for shoot, $r = 0.91$ for root $P < 0.05$). Under high salt stress (150-400 mM NaCl), *Lens culinaris* accumulated more Na^+ ion in the roots than in the shoots. Most plants when exposed to saline medium accumulate some Na^+ in their roots and exclude it from the shoots (Ashraf and Haris, 2004). Such plants are referred to as Na^+ excluders. Regulation of Na^+ uptake by cells and long distance Na^+ transport seems to be a crucial adaptation of plants to salt stress (Munns et al. 2000). In contrast, some species efficiently accumulate high amounts of Na^+ in the shoots and thus are known as Na^+ includers (accumulators).

On the other hand, salinity affects the plant growth by inducing nutrient (notably K^+, Ca^{2+}, and Mg^{2+}) deficiency (Wenxue et al., 2003). This was also the case in *Lens culinaris* shoots and roots (Table 2). However, reasonable amounts of both K^+ and Ca^{2+} are required by plants to maintain the integrity and functioning of cell membranes (Wenxue et al., 2003). Concerning K^+/Na^+ selectivity, our data (Table 2) are consistent with previous studies, showing the presence of a strong relation between potassium and sodium uptake in plant performance under salinity (Zhu at al., 1998; Wenxue et al., 2003). It has been reported that K^+ takes part in many enzymatic activities in plant cells, and maintaining a high cytosolic K^+/Na^+ ratio is a key requirement for plant growth in highly salt conditions (Zhu et al., 1998; Wenxue et al., 2003). The low Ca^{2+} / Na^+ ratio of a saline medium plays a significant role in growth inhibition in addition to causing significant changes in morphology and anatomy of plants (Zhu et al., 1998). Although both K^+ / Na^+ and Ca^{2+} / Na^+ ratios decreased consistently in the shoots and roots of *Lens culinaris* with increase in salt level of the growth medium. At the highest salt level (150-400 mM NaCl) the shoots and roots K^+ / Na^+ ratio was lower than 1, a minimum level suggested for the normal functioning of most

mesophytes under saline conditions. However, high K^+ / Na^+ selectivity in plants under saline conditions is considered as one of the important selection criteria for salt tolerance (Wenxue et al., 2003; Ashraf and Harris, 2004). Furthermore, the maintenance of Ca acquisition and transport under salt stress is also an important determinant of salinity tolerance (Unno et al., 2002).

In *Lens culinaris* electrolyte leakage significantly increased as all NaCl concentrations. Salinity caused a disturbance in membrane permeability expressed by an increase in electrolyte leakage. The disturbance in membrane stability (Table 1) and the increase of lipid peroxidation levels in *Lens culinaris* (Table 4-5) may be considered an index of oxidative damage due to the in adequate response of antioxidative systems, as observed in several crops (Dionisio-Sese and Tobita 1998, Sreenivasulu et al., 2000,). These results agree with the finding of Lutts et al. (1996) where they reported that electrolyte leakage in rice cultivars was high for salt sensitive. Sreenivasulu et al. (2000) observed electrolyte leakage as a function of NaCl in salt stressed seedlings of foxtail millet.

It is now widely accepted that reactive oxygen species (ROS) are responsible for diverse stress-induced damage to macromolecules and ultimately to cell structure (Mittler, 2002). Dionisio-Sese and Tobita, (1998) demonstrated increased lipid peroxidation and electrolyte leakage as well as Na^+ accumulation in the leaves of rice plants under salinity stress.

The total chloropyll and carotenoid contents of leaves were decrease in *Lens culinaris* under salt stress. The loss of chlorophyll is often considered as a marker of a cellular component of salt stress. The decrease in chl content at salt stress might possible changes in the lipid protein ratio of pigment-protein complexes increased chlorophyllase activity (Iyengar and Reddy, 1996). Carotenoids are responsible for quenching singlet oxygen and thereby avoiding lipid peroxidation and consequent oxidative damage. (Sairam et al, 2002). The oldest leaves start to develop chlorosis and fall with prolonged period of salt stress (Hernandez et al. 1999). In leaves of tomato and rice plant the content of total chlorophyll (Chl a + b), Chl a and β-carotene decrease by NaCl stress (Parida et al, 2002). Rout and Shaw (2001) found that the ratio of Chl a/b decreased significantly in *H verticillata* treated with NaCl. Decrease in Chl a / b ratio is considered to be a symptom of oxidative stress conditions. In the present study also the decrease in the ratio upon NaCl treatment might be due to the enhanced generation of ROS's causing damage to Chl a. But, Juan et al (2005) reported that Chl a and Chl b would not be good indicators for salt stress tolerance as reflected by the simple-regression analysis between the pigments and foliar biomass production.

Proline is one of the most accumulated osmolytes found in plants with salinity and water deficit conditions (Ashraf and Haris, ,2004). Proline acts as osmoprotectant, it can also function as a protein stabilizer, it functions as a hydroxyl radical scavenger, stabilizes cell membranes by interacting with phospholipids, and serves as a source of carbon and nitrogen. Many plants, such as wheat, sorghum, *Pringlea antiscorbutica* accumulate proline in leaves, stems or roots as a nontoxic and protective osmolyte under saline conditions (Lee and Liu, 1999; Lacerda et al., 2005; Lutts et al, 1996).

In addition, proline may act as a signaling / regulatory molecule able to activate multiple responses that are component of the adaptation to abiotic stresses including salt stress (Lacerda et al., 2005). In contrast, Hasegawa et al. (1986) suggested that proline accumulation does not lead to salinity adaptation, but it may be synthesized due to initiation of other responses to salt stress. In addition, Greenway and Munns (1980) were of the view that proline is related to survival rather than to growth maintenance.

Increased proline levels together with enhanced H_2O_2 contents are a common response of plant cells upon osmotic stress treatments. In our experiments, under salt stress significant increased were encountered in proline and H_2O_2 content of shoots and roots tissue. These results demonstrated that, salt stress was accompanied with osmotic stress. These observations were indirect evidence that, the membrane damage that was observed under salt stress was due to reactive oxygen species. The accumulation of H_2O_2 has been reported to function as an intercellular signal and it stimulates a number of genes and proteins involved in stress responses, such as catalase, peroxidase and alternative oxidase (Prasad et al., 1994). Our findings suggest that an increased H_2O_2 generation in the salt-stressed shoots and roots of *Lens culinaris* may be induced by the induction of SOD and it may function in the signaling of oxidative stress which leads to the induction of antioxidant enzymes associated with an H_2O_2 scavenging system, particularly an-ascorbate-glutathione cycle. Higher H_2O_2 accumulation and lipid peroxidation in sensitive cultivar of pea (Hernandez et al, 1999) and rice (Dionisio-Sese and Tobita, 1998) have been reported earlier.

Salt stress is known to result in extensive lipid peroxidation, which has often been used as indicator of salt-induced oxidative damage in membranes (Hernandez et al, 1999). An enhanced level of lipid peroxidation and LOX activity were observed in the roots and shoots of *Lens culinaris* in the present study, which may be due to the generation of AOS under salinity-stress as reported by other authors (Polkowska et al. 2004; Van Breusegem et al 2001; Hernandez et al, 1999). In our experiment, after 7 day of exposure, there was a statistically significant correlation between the lipid peroxidation and lipoxygenase activity ($r = 0.98$ for shoot, $r = 0.96$ for root $P < 0.01$).

Even under optimal conditions many metabolic processes produce active oxygen species. The production of toxic oxygen derivatives is increased as results of all types of environmental or non-made stresses. Plants process efficient systems for scavenging active oxygen species that protect them from destructive oxidative reactions (Van Breusegem et al 2001). As part of this system, antioxidative enzymes are key elements in the defense mechanisms. Many changes have been detected in the activities of antioxidant enzymes in plants under salinity. The activity of antioxidant enzymes was reported to increase under saline conditions in the case of cotton, (Meloni et al., 2003) shoot cultures of rice (Fadzilla et al., 1997), a cucumber (Lechno et al., 1997) wheat shoot (Meneguzzo et al, 1999) and pea (Hernandez, 1999), but decreased in wheat roots (Meneguzzo et al., 1999). Morever, membrane peroxidation can also be initiated enzymatically lipoxygenases, which convert 18:2 and 18:3 fatty acids to their corresponding hydroperoxidase (Elkahoui et al., 2005). In the present work, an increase in LOX activity was observed in high concentrations salt treated plants, mainly at 150 and 400 mM NaCl.

The decrease of growth parameters was reflected by enzyme response variability. Salt treatment increased the SOD enzyme in *Lens culinaris* roots and shoots that converts superoxide to H_2O_2. However, with regard to enzyme that converts H_2O_2 to water, GPX activity increased, whereas APX and CAT activities decreased. This suggests that GPX play important role to detoxify H_2O_2 under salt stress in *Lens culinaris*. Loss in catalase activity due to salt stress in cotton (Gossett et al., 1994), sunflower (Santos et al., 2001), and rice (Lee et al., 2001) as well as due to photo-oxidative stress in tobacco chloroplasts (Miyagawa et al., 2000), agrees with our results in *Lens culinaris.*

Some authors reported that H_2O_2 accumulated in leaves of *Vigna*, *Oryza* seedlings and cotton under salinity stress was related to a decrease in CAT activity (Singha and Choudhuri, 1990, Gosset et al., 1994). Decreased CAT activity, in turn, might have

promoted H_2O_2 accumulation in *Lens culinaris*. Which could results in a Haber-Weiss reaction to form hydroxyl radicals Since $OH^{\cdot}$ radicals are known to damage biological systems (Halliwell and Gutteridge, 1999) they might have hastened lipid peroxidation and membrane damage in *Lens culinaris*. However, regardless the sequence of reactions, the appearance of H_2O_2 seemed to be a crucial event associated with growth loss.

5.CONCLUSIONS

In conclusion, *Lens culinaris* exhibited highly root Na^+ accumulation resulting in symptoms of oxidative damage such as decreased CAT and APX activities, increase in lipid peroxidation, electrolyte leakage, proline levels, GSH-Px, GPX, LOX and SOD activities and decrease in growth rate. At the highest salt level (150-400 mM NaCl) the shoot and root K^+/Na^+ ratio was lower than 1, a minimum level suggested for the normal functioning of most mesophytes under saline condition.

In addition, this study showed that the difference in Na^+ content, electrolyte leakage, and H_2O_2 content of root and shoot of *Lens culinaris* is due to the difference in mechanisms underlying oxidative stress injury and subsequent sensitivity to high salinity.

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