academicJournals

Vol. 10(23), pp. 2389-2398, 4 June, 2015
DOI: 10.5897/AJAR2014.9479
Article Number: CC9159E53345
ISSN 1991-637X

http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Protective effects of Ca^{2+} against NaCl induced salt stress in two lentil (*Lens culinaris*) cultivars

Hafize Dilek Tepe[1]* and Tülin Aydemir[2]

[1]Graduate School of Natural and Applied Sciences, Departments of Sciences, Celal Bayar University, Chemistry Manisa/Turkey.
[2]Chemistry Department, Faculty of Science and Letters, Celal Bayar University, Turkey.

Received 29 December, 2014; Accepted 7 April, 2015

Salinity affects ~ 950 million ha of the world's land area. More importantly, this worldwide problem is gradually increasing and limiting productivity. The aim of the present study was to investigate the protective effects of Ca^{2+} against NaCl induced salt stress in *Lens culinaris*. We comparatively analyzed growth, oxidative stress, photosynthetic potential and antioxidant enzyme activities in red and green lentils. Plants were allowed to germinate and then treated with or without NaCl (50, 200 mM) and/or $CaCl_2$ (5, 10 mM) for seven days. NaCl treatment decreased growth, chlorophyll content, carotenoid content and the activities of CAT and Ascorbate peroxidase (APX in both tested plants. Moreover MDA, H_2O_2 and proline levels were increased by NaCl treatment in red and green lentils, indicating that antioxidant system was disrupted by salinity. This study indicated that Ca^{2+} ameliorated the inhibitory effects of NaCl on growth and photosynthesis by regulating the activities of pivotal antioxidant enzymes such as superoxide dismutase (SOD), APX and catalase (CAT) in red and green lentils. Further studies are needed to investigate the underlying molecular mechanisms of Ca^{2+} dependent protective effects under salt stress.

Key words: *Lens culinaris*, calcium, salt stress, oxidative stress, antioxidant enzymes, lipid peroxidation.

INTRODUCTION

Salinity can be defined as the presence of soluble salts with excessive levels in soils or waters. If these salts contain a high proportion of sodium ions, it is called sodicity. Over six percent of the world's land and approximately one-third of agricultural areas are affected by these two increasing environmental problems (Laohavisit et al., 2013; Yadav et al., 2011). Decreasing in crop productivity is one of the most harmful consequences of salinity in particularly arid and semi-arid areas of the world.

Several metabolical and physiologica processes regulating plant growth can be damaged by salinity and sodicity. At first, cellular ionic balance is disrupted. The most common ions associated with salinity are Na^+, Mg^{2+}, Cl^- and HCO_3^-. Because of their dominant toxicity to plants, Na^+ and Cl^- are considered the most important. Increased concentrations of cellular Na^+ and Cl^- inhibit most enzymes and interfere RNA binding (Serrano et al., 1999). It is also shown that excessive uptake of Na^+ disrupts Na^+/K^+ homeostasis and may cause cellular injury and even cell death (Lockhart., 2013). The main phenotypic response of plants to salinity is inhibition

*Corresponding author. E-mail: hafize.dilek@hotmail.com

of growth (Romero et al., 2001; Hilal et al., 1998). The mechanism underlying this response directly depends on absorption of water. When dissolved salt concentration in the soil is higher than inside the plant roots, water tends to move from roots to soil via osmosis, as a result, absorption of water to plant is reduced. Moreover, salt accumulation causes premature senescence in leaves, reduces the photosynthesis rate and directly affects vital processes by generating reactive oxygen species (ROS) such as hydrogen peroxide, nitric oxide, superoxide and hydroxyl radicals. Despite low levels of ROS can mediate salinity tolerance, excessive levels mostly damage to lipids, proteins, and nucleic acids (Xue et al., 2008). Conversely, plant cells have non-enzymatic antioxidants such as proline and antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) which are protect cell from oxidative damage. Therefore, the balance between ROS and antioxidants is crucial for plant survival and is needed to keep on the side of antioxidants (Munns, 2002; Tsuganea et al., 1999).

Unlike Na^+ and Cl^-, it is well known that some ions such as Ca^{+2} increase the adaptation of plant to salt stress by mainly regulating ion transport and exchange mechanisms. Moreover, calcium can also act as a second messenger in stress signaling, stabilizes cell wall structure and restores photosynthesis under NaCl stress. The underlying molecular mechanism of plant cell response to salt stress is regulated by salt overly sensitive (SOS) signaling pathway. Salt stress induces a cytosolic calcium-signal that activates the calcium sensor protein SOS3. SOS3 binds and activates SOS2, which is a member of serine/threonine kinase family. Activated SOS2 regulates the activities of Na^+/H^+ antiporters localized in both plasma membrane and vacuole. This results in Na^+ efflux out of cytosol or vacuole (Hadia and Karimib, 2012).

Lens culinaris is one of the first cultivated annual plants in Middle East and Europe. The wild subspecies, L. culinaris orientalis, is found in particularly Middle East including Turkey. Since this ancient plant has high protein content and is a good source of vitamin B, iron and phosphorus, it is widely consumed as food (Faris et al., 2013). The aim of the present study is to investigate the protective effects of Ca^{2+} against NaCl induced salt stress in red and green lentils.

MATERIALS AND METHODS

Plants Material and Metal Treatment

In this study, the red (yerli) and green (kışlık pul 11) lentil (L culinaris L.cv) cultivars seeds were provided by Field Crops Central Research Institute, Ankara, Turkey. Seeds were surface disinfected with sodium hypochloride (30%) for 10 min and washed with sterile water thoroughly. After imbibition, approximately 15 to 20 seeds were planted onto plastic trays covered with filter paper and cotton containing half-strength Hoagland's solution. They were grown for 10 days in a growth chamber at 23±2°C with 16-h light: 8-h dark photo-cycle at a light intensity of 40 mmol m^{-2} s^{-1}. At the 7th day of growth, salt stress treatment was initiated by applying half-strength Hoagland's solution containing 1.02 g/L KNO_3, 0.492 g/L $Ca(NO_3)_2$, 0.23 g/L KH_2PO_4, 0.49 g/L $MgSO_4.7H_2O$, 1.81 mg/L $MnCl_2.4H_2O$, 0.08 mg/L $CuSO_4.5H_2O$, 0.22 mg/L $ZnSO_4.7H_2O$, 0.09 mg/L $NaMoO_4.H_2O$ and $FeSO_4.7H_2O$ (0.5%) contained 0.6 ml/L (0.4%) tartaric acid to seedlings. Control plants, salt stressed (50, 200 mM NaCl) and NaCl+$CaCl_2$ treated (50 mM NaCl+5 mM Ca, 50 mM NaCl+10 mM Ca, 200 mM NaCl+5 mM Ca, 200 mM NaCl+10 mM Ca) and plants were grown in the growth chamber with the same physical parameters for another 7 days. The shoot and root tissues of 7 days old seedlings were then freshly used in the experiments.

Growth analysis

After 0 and 7 days of metal treatment, 1 g plants for each group were taken at random and divided into separate shoot and root fractions. The fresh weights of shoots and roots were weighed, and root lengths were measured. The samples were then dried in a forced draft oven at 70°C for 24 h, and the dry weights (g/g FW) were determined.

Determination of proline and H_2O_2 content

Proline content was determined according to the modified method of Bates et al. (1973). 0.5 g of shoot and root tissues from control and NaCl, Ca^{2+} treated plants were homogenized 1 ml of 5 % sulfosalicylic acid solution using homogenizer. The homogenate was then centrifuged at 13.000 g for 10 min. 1 ml of the supernatant was then added into a test tube to which 1 ml of glacial acetic acid and 1 ml of freshly prepared acid ninhydrin solution were added (1.25 g ninhydrin dissolved in 30 ml of glacial acetic acid and 20 ml of 6 M orthophosphoric acid). Tubes were incubated in a water bath for 1 h at 95°C and then allowed to cool to room temperature. 2 ml of toluene was added and mixed on a vortex mixture for 20 s in a fume hood. The test tubes were allowed to stand for at least 10 min to allow the separation of toluene and aqueous phase. The toluene phase was carefully pipetted out into a glass test tube and the absorbance was measured at 520 nm in a spectrophotometer. The concentration of proline was calculated from a proline standard curve. The concentration of proline was expressed as µmol/g FW.

The hydrogen peroxide content was determined according to Jana and Choudhuri (1981). Aliguats of fresh shoots and roots were homogenized in 50 mM potassium phosphate, pH 6.5 and centrifuged at 10 000 g for 25 min. The solution was mixed with 1% titanium chloride (in concentrated HCl) and then centrifuged at 10 000 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.

MDA content

Lipid peroxidation was evaluated by measuring the amount of MDA amounts according to Heath and Packer (1968). 500 mg plant material was homogenized with 3 ml of 0.5% TBA in 20% TCA (W/V). The homogenized was incubated at 95°C for 30 min and the reaction was stopped in ice. The plant samples were centrifuged at 10 000 g for 15 min and absorbance of the resulting supernatant was recorded at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance.

Chlorophyll and carotenoid contents

Concentration of chlorophylls and carotenoids were determined in

Table 1. Effect of Ca^{2+} enrichment on the fresh shoot weight, fresh root weight, shoot lenght root lenght and electrolyte leakage of 7-day red lentil seedling treated with or without 5.0-10 mol L−1 $CaCl_2$, 50 to 200 mmol L^{-1} NaCl. (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

Red lentil	Shoot length(cm)	Root length(cm)	Shoot weight(g)	Root weight(g)	Electrolyte Leakage (µS/cm)	
					Shoot	Root
Control	19.85	8.78	0.1426	0.0522	48.00	57.53
50 NaCl	18.44 [a]	8.28 [a]	0.1360 [a]	0.0470 [a]	75.14 [a]	83.12 [a]
50 NaCl +5 Ca	18.87 [a]	8.90 [b]	0.1410 [b]	0.0526 [b]	60.98 [ab]	72.11 [ab]
50 NaCl + 10 Ca	19.71 [b]	9.35 [ab]	0.1544 [ab]	0.0639 [ab]	50.76 [ab]	64.45 [ab]
200 NaCl	16.28 [a]	5.05 [a]	0.0698 [a]	0.0417 [a]	120.45 [a]	133.17 [a]
200 NaCl + 5 Ca	17.30 [ac]	5.42 [ac]	0.0801 [ac]	0.0462 [ac]	110.66 [ac]	112.10 [ac]
200 NaCl + 10 Ca	18.78 [ac]	6.81 [ac]	0.1057 [ac]	0.0676 [ac]	98.70 [ac]	107.11 [ac]
RSD	0.36	0.06	0.004	0.003	1.25	3.38

DMSO extract of the young fully expanded leaf by the method of Lichtenthaler and Wellburn (1983). The homogenate was centrifuged at 4000×g for 10 min to remove the residue. The color intensity of clear supernatant was measured at 665, 645 and 470 nm for chlorophyll a, chlorophyll b and carotenoids, respectively. Results have been expressed as mg chlorophyll or carotenoids mg/g fresh weight.

Enzyme extracted and enzyme activities assays

Fresh shoot and root samples weighting about 1 g were homogenized using chilled mortar and pestle in 5 ml of cold 20 mM potassium phosphate buffer (pH 7.0) containing 1.0% insoluble polyvinyl pyrolidone (PVP) in ice bath. The homogenates were centrifuged at 12 000 g for 30 min. The supernatant was stored at 4°C and used for enzyme assays.

Ascorbate peroxidase (EC. 1.11.1.11) activity was measured immediately in fresh extracts and was assayed as describes by Nakano and Asada (1981) using a reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of as was followed by a decrease in the absorbance at 290 nm. The activity of APX was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Catalase (EC 1.11.1.6) activity was determined by monitoring the described of H_2O_2 which was carried out by measuring the decrease in absorbance at 240 nm of a reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract (Aebi, 1984).

SOD (EC 1.15.1.1) activity was measured spectrophotometrically as described by Beyer and Fridovich (1987). In this assay, 1 unit of SOD is defined as the amount required to inhibit the photo reduction of nitroblue tetrazolium by 50%. The activity of SOD was expresed as Unit/g fresh weight.

Statistical analysis

All experiments were carried out in triplicate and results were expressed as mean ± S.D. Data analysis and graphs were done by Graphpad Prism 6.0 software (La Jolla, CA, USA). Differences were analyzed by using two-way ANOVA at level of significance of $p<0.05$.

RESULTS

Growth analysis

For growth analysis, plants were allowed to germinate and then treated with or without NaCl (50, 200 mM) and/or $CaCl_2$ (5, 10 mM) for seven days in sand culture under glass house conditions. At the end of the day 7, growth of both red and green lentil was significantly reduced by 50 and 200 mM NaCl treatment (Tables 1 and 2).

In 50 and 200 mM NaCl treated red lentil, shoot length was decreased by 7.1 and 17%, while it was decreased by 4.65 and 12% in green lentil, respectively, as compared to untreated control. Combination of 200 mM NaCl and 10 mM Ca^{2+} treated resulted in an increased shoot length in red and green lentils by 15 and 9%, respectively as compared to 200 mM NaCl. Shoot weight was decreased by 62 and 39% in red and green lentils treated with 200 mM NaCl, respectively, as compared to untreated control. Combination of 200 mM NaCl and 10 mM Ca^{2+} treated resulted in increased shoot weight by 42 and 14% in red and green lentils, respectively as compared to 200 mM NaCl.

Chlorophyll (Chl) content

The presence of NaCl decreased total chlorophyll (Chl a + Chl b) and total carotenoid levels in a concentration dependent manner in the shoots of both plants. In red lentil, 200 mM NaCl treatment decreased Chl a, Chl b, and total carotenoid levels by 54, 58 and 61%, respectively, as compared to untreated control. Combination of 10 mM Ca^{2+} and 200 mM NaCl significantly decreased the inhibitory effects of NaCl on Chl a, Chl b and total carotenoid levels by 22, 76 and 34%, respectively, as compared to 200 mM NaCl (Table 3). In green lentil, 200 mM NaCl treatment decreased Chl a,

Table 2. Effect of Ca^{2+} enrichment on the fresh shoot weight, fresh root weight, shoot lenght root lenght and electrolyte leakage of 7- day green lentil seedling treated with or without 5.0 to 10 mol L^{-1} $CaCl_2$, 50-200 mmol L^{-1} NaCl. (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

Green lentil	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)	Electrolyte Leakage (µS/cm)	
					Shoot	Root
Control	21.04	8.24	0.2294	0.1422	73.02	86.26
50 NaCl	20.06 [a]	5.01 [a]	0.2029 [a]	0.0826 [a]	90.13 [a]	102.23 [a]
50 NaCl +5 Ca	24.85 [ab]	7.71 [ab]	0.2414 [ab]	0.1200 [ab]	72.33 [b]	97.12 [ab]
50 NaCl +10 Ca	26.20 [ab]	7.90 [ab]	0.2602 [ab]	0.1321 [ab]	63.16 [ab]	90.10 [ab]
200 NaCl	18.5 [a]	3.40 [a]	0.1393 [a]	0.0706 [a]	130.74 [a]	148.65 [a]
200 NaCl +5 Ca	19.35 [ac]	6.07 [ac]	0.1558 [ac]	0.0810 [ac]	122.23 [ac]	145.00 [a]
200 NaCl +10 Ca	20.21 [ac]	7.85 [ac]	0.1686 [ac]	0.0933 [ac]	111.39 [ac]	127.34 [ac]
RSD	0.32	0.05	0.007	0.004	2.26	4.28

Table 3. Effect of Ca^{2+} enrichment on the photosynthetic pigments of 7- day red lentil seedling treated with or without 5.0 to 10 mol L−1 $CaCl_2$, 50 to 200 mmol L^{-1} NaCl. (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

Red lentil	Chl a (mg/g fresh weight)	Chl b (mg/fresh weight)	Total Chl (mg/g fresh weight)	Chl a/b (mg/g fresh weight)	Total Carotenoid (mg/g fresh weight)
Control	6.31	1.97	8.28	3.20	1.96
50 NaCl	4.52 [a]	1.25 [a]	5.77 [a]	3.61 [a]	1.66 [a]
50 NaCl + 5 Ca	4.70 [ab]	1.60 [ab]	6.33 [ab]	2.95 [ab]	1.74 [ab]
50 NaCl + 10 Ca	5.14 [ab]	1.87 [ab]	7.01 [ab]	2.74 [ab]	1.87 [ab]
200 NaCl	2.90 [a]	0.82 [a]	3.72 [a]	3.53 [a]	0.75 [a]
200 NaCl + 5 Ca	3.14 [ac]	0.94 [ac]	4.08 [ac]	3.34 [ac]	0.88 [ac]
200 NaCl + 10 Ca	3.56 [ac]	1.45 [ac]	5.01 [ac]	2.45 [ac]	1.01 [ac]
RSD	0.08	0.04	0.09	0.04	0.03

Table 4. Effect of Ca2+ enrichment on the photosynthetic pigments of 7- day green lentil seedling treated with or without 5.0 to 10 mol L^{-1} $CaCl_2$, 50 to 200 mmol L^{-1} NaCl. (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

Green lentil	Chl a (mg/g fresh weight)	Chl b (mg/g fresh weight)	Total Chl (mg/g fresh weight)	Chl a/b (mg/g fresh weight)	Total Carotenoid (mg/g fresh weight)
Control	5.56	2.91	8.47	1.91	1.65
50 NaCl	4.65 [a]	1.31 [a]	5.96 [a]	3.54 [a]	1.21 [a]
50 NaCl + 5 Ca	5.67 [b]	2.84 [ab]	8.51 [b]	1.99 [ab]	1.55 [ab]
50 NaCl + 10 Ca	5.83 [ab]	2.98 [b]	8.81 [ab]	1.95 [b]	1.66 [b]
200 NaCl	2.58 [a]	0.76 [a]	3.34 [a]	3.39 [a]	0.86 [a]
200 NaCl + 5 Ca	2.79 [ac]	0.95 [ac]	3.74 [ac]	2.93 [ac]	0.99 [ac]
200 NaCl + 10 Ca	2.95 [ac]	1.13 [ac]	4.08 [ac]	2.61 [ac]	1.11 [ac]
RSD	0.01	0.04	0.08	0.05	0.04

Chl b, and total carotenoid levels by 53, 73 and 47%, respectively, as compared to untreated control. Combination of 10 mM Ca^{2+} and 200 mM NaCl significantly decreased the inhibitory effects of NaCl on Chl a, Chl b and total carotenoid levels by 14, 48 and 29%, respectively, as compared to 200 mM NaCl (Table 4).

MDA, H_2O_2 and proline contents

The presence of NaCl led to an increase in MDA levels in a concentration dependent manner in the shoots of both plants. 200 mM NaCl treatment increased MDA levels by 73 and 66% in the shoots of red and green lentils,

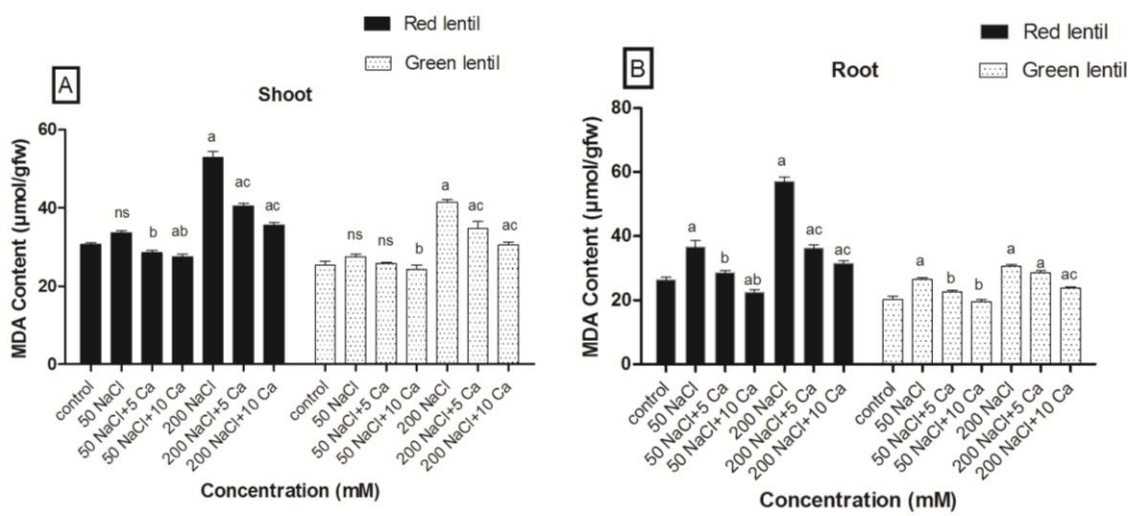

Figure 1. Effect of Ca^{2+} enrichment on the MDA content (mol/gFW) of 7-day old red and green lentil seedlings treated with or without 5,10 mM $CaCl_2$, 50, 200 mM NaCl. (A=Shoot, B=Root) (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

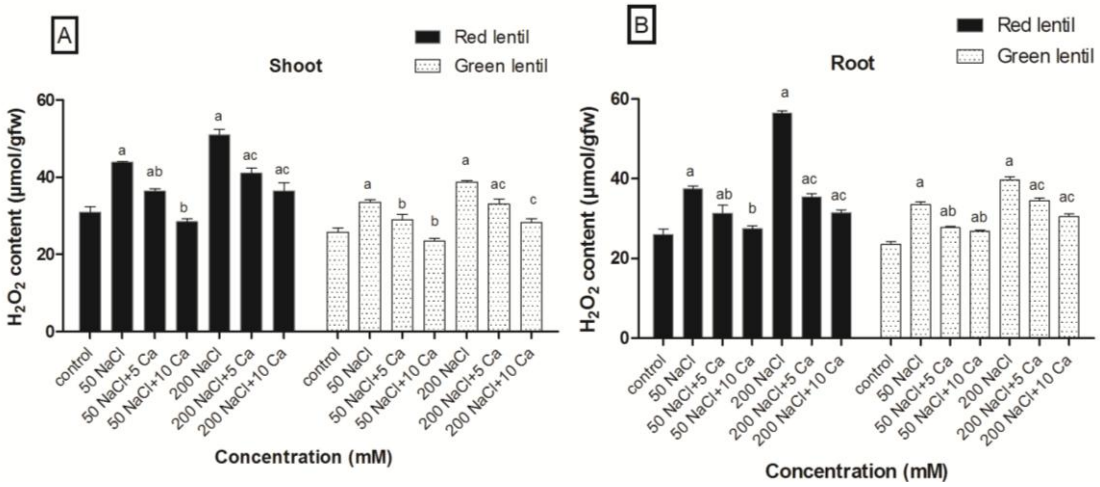

Figure 2. Effect of Ca^{2+} enrichment on the H_2O_2 content (mol/gFW) of 7-day old red and green lentil seedlings treated with or without 5,10 mM $CaCl_2$, 50, 200 mM NaCl. (A=Shoot, B=Root) (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

respectively, as compared to untreated control. Combination of 10 mM Ca^{2+} and 200 mM NaCl solution reduced MDA levels by 33 and 26% in red and green lentils, respectively, as compared to 200 mM NaCl (Figure 1A). Same results were observed in the roots of both plants. Combination of 10 mM Ca^{2+} and 200 mM NaCl decreased MDA content by 44 and 20% in the shoots of red and green lentils, respectively, as compared to 200 mM NaCl (Figure 1B). H_2O_2 content also increased in a concentration dependent manner by NaCl treatment in the shoots of both plants. In the shoots of red lentil treated with 50 and 200 mM NaCl, H_2O_2 content was increased by 41 and 64%, respectively, while it was increased by 29 and 50% in the leaves of green lentil, as compared to untreated controls (Figure 2A). Combination of 10 mM Ca^{2+} and 200 mM NaCl decreased H_2O_2 content by 28 and 27% in the shoots of red and green lentils, as compared to 200 mM NaCl (Figure 2B).

It was observed that proline content in both shoots and roots were significantly increased by NaCl treatment in a concentration dependent manner in red and green lentils

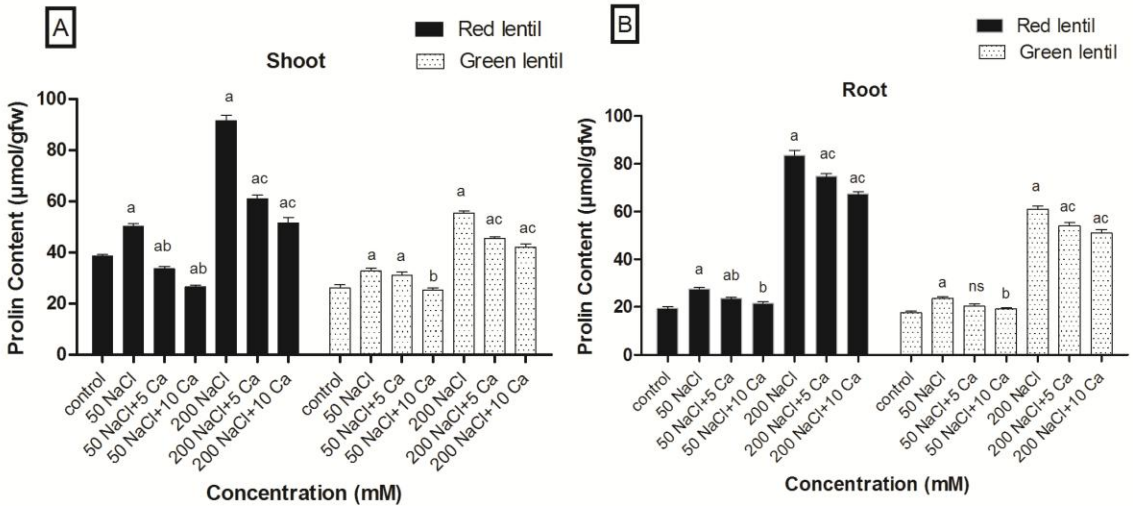

Figure 3. Effect of Ca^{2+} enrichment on the Prolin content (mol/gFW) of 7-day old red and green lentil seedlings treated with or without 5,10 mM $CaCl_2$, 50, 200 mM NaCl. (A=Shoot, B=Root) (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

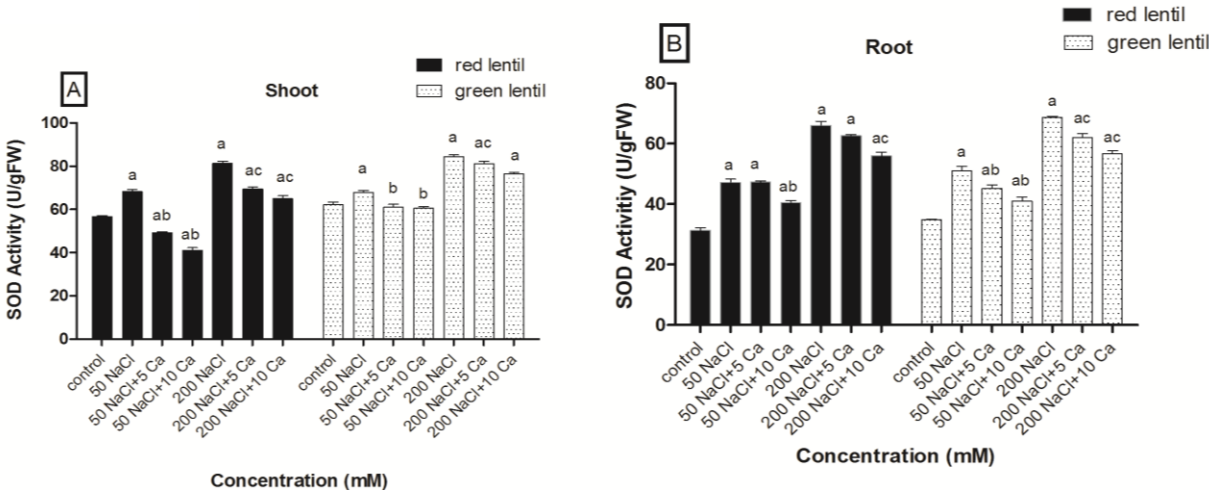

Figure 4. Effect of Ca^{2+} enrichment on the Superoxide Dismutase (SOD) Activity (U/gFW) of 7-day old red and green lentil seedlings treated with or without 5,10 mM $CaCl_2$, 50, 200 mM NaCl. (A=Shoot, B=Root) (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

(Figure 3A). In the shoots of red and green lentils, combination of 10 mM Ca^{2+} and 200 mM NaCl significantly decreased proline content by 43 and 24%, respectively, as compared to 200 mM NaCl while in the roots, proline content was decreased by 19 and 16%, respectively, as compared to 200 mM NaCl (Figure 3B).

Activities of antioxidant enzymes

Activities of SOD, CAT and APX were measured by using spectrophotometric methods.

Superoxide dismutase (SOD) activity

SOD activity was increased by 20 and 9% in the shoots of green and red lentils treated with 50 mM NaCl, respectively. Combination of 50 mM NaCl and 10 mM Ca^{+2} resulted in an increased SOD activity by 39 and 10%, respectively (Figure 4A). 200 mM NaCl treatment increased SOD activity by 43 and 35%, respectively. In the root of both red and green lentils, combination of 200 mM NaCl and 10 mM Ca^{2+} resulted in an increased SOD activity by 15 and 17.5%, respectively, as compared to 200 mM NaCl (Figure 4B).

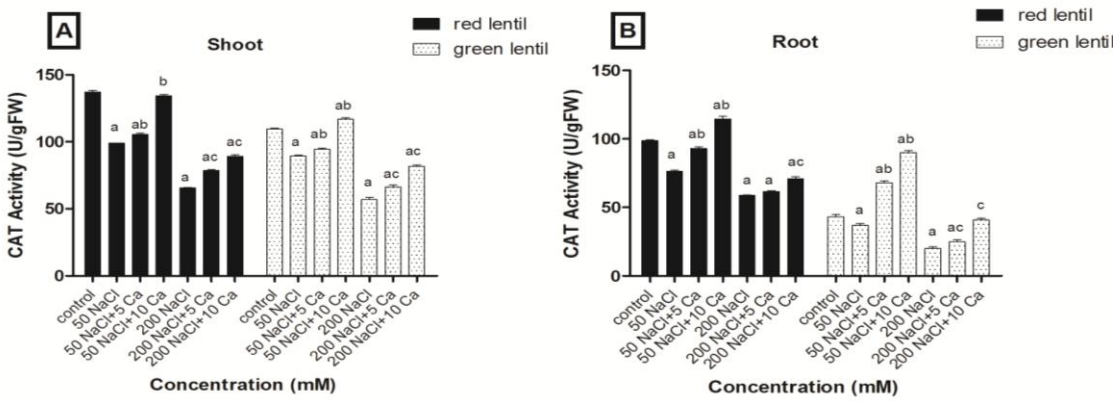

Figure 5. Effect of Ca^{2+} enrichment on the Catalase (CAT) Activity (U/gFW) of 7-day old red and green lentil seedlings treated with or without 5,10 mM $CaCl_2$, 50, 200 mM NaCl. (A=Shoot, B=Root) (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

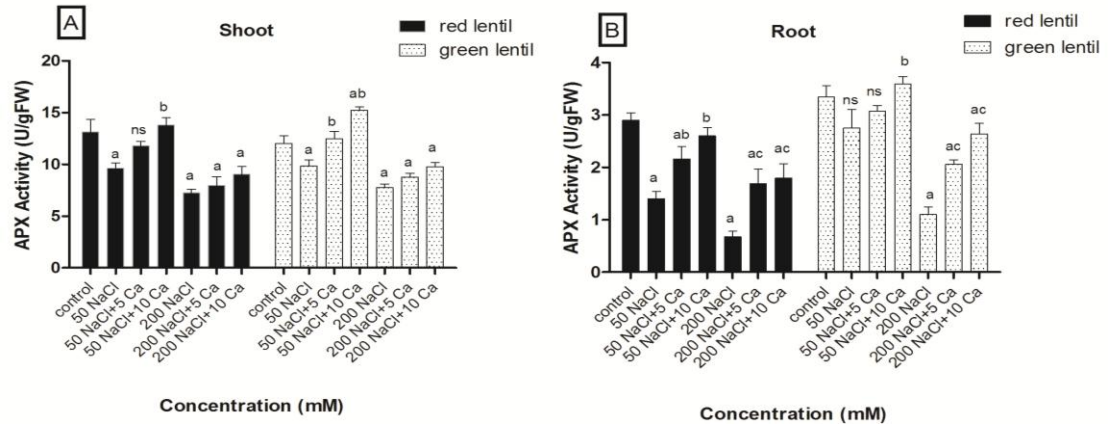

Figure 6. Effect of Ca^{2+} enrichment on the Ascorbat Peroxidase (APX) Activity (U/gFW) of 7-day old red and green lentil seedlings treated with or without 5,10 mM $CaCl_2$, 50, 200 mM NaCl. (A=Shoot, B=Root) (a= compared to the control, b= compared to the 50 mM NaCl , c= compared to the 200 mM NaCl).

Catalase (CAT) activity

In the shoot of red lentil after treatment with 50 and 200 mM NaCl, CAT activity was reduced by 27 and 52%, while in the stem of green lentil, it was reduced by 18 and 47%, respectively, as compared to untreated control. Combination of 200 mM NaCl and 5 mM Ca^{2+} resulted in an increased CAT activity in the shoot of red and green lentils by 19 and 15%, respectively, as compared to 200 mM NaCl (Figure 5A).

Ascorbate peroxidase (APX) activity

It was found that APX activity was reduced in the shoots and roots of both green and red lentils treated with NaCl in a concentration dependent manner (Figure 6A). In the shoot of red lentil treated with 50 mM and 200 mM NaCl, APX activity was reduced by 26 and 44%, while in the shoot of green lentil it was reduced by 18 and 35%, respectively, as compared to untreated control. Moreover, in the root of red lentil treated with 50 mM and 200 mM NaCl, APX activity was reduced by 51 and 76.9%, while in the root of green lentil it was reduced by 17 and 67%, respectively, as compared to untreated control. Combination of 50 mM NaCl and 5 mM Ca^{+2} resulted in an increased APX activity in the root of red and green lentils by 54 and 16%, respectively (Figure 6B).

DISCUSSION

Salinity affects approximately 950 million ha of the world's land area. More importantly, this worldwide problem is

gradually increasing and limiting plant growth and productivity. Currently, saline soil defined as having an electrical conductivity of the saturation extract (ECe) of 4 dS m^{-1} or more, and soils with ECe's exceeding 15 dS m^{-1} are considered strongly saline (Munns, 2002). From bacteria to plants, organisms develop biochemical and molecular mechanisms to adapt saline stress. In plants, many cellular processes including photosynthesis, membrane transport and protein synthesis are mainly affected during development. Salt tolerance can be defined as the ability of plant to complete its life cycle in soil which contains high concentrations of soluble salt (Parida and Das., 2005). It is well known that significant differences are found between salt tolerant plant species (Munns, 2002). Thus, every species and even subspecies should be assessed individually. Lentil species are considered extremely sensitive to salinity (ECe < 2 dS/m) as compared to other legumes such as soybean and broadbean (Sidari et al., 2008; Katerji et al., 2001). In this study, we investigated the protective effects of Ca^{+2} against NaCl induced salt stress in *Lens culinaris*, which is widely consumed as food in Middle East and Europe. We compared red and green lentils in terms of several physiological parameters including growth, chlorophyll content and antioxidant systems which are affected by NaCl stress. Moreover, aside from the effects of NaCl stress, ameliorative effects of Ca^{2+} in both plants were also investigated in this study.

The most common observed phenotypic response of plants to salinity is reduction of growth (Romero et al., 2001; Hilal et al., 1998). We found that NaCl (50 and 200 mM) treatment reduced growth parameters such as length and weight of shoots and roots in both tested plants in a dose dependent manner. Moreover, it was interesting to find that red lentil was more sensitive than green lentil to salt stress, according to our growth analysis. In parallel with our study, Bandeoğlu et al. (2004) reported a dose dependent reduction in the growth of lentil plant treated with 100 and 200 mM NaCl. Kökten et al. (2010) were investigated the effects of salinity on five lentil genotypes and they found that increasing concentrations of NaCl (50 to 200 mg/L) resulted in a significant decrease in length and weight of shoots and roots.

Nutrient enrichment is one of the most useful approaches to minimize the inhibitory effects of salinity (Manaa et al., 2013). N, P, K, Mg and Ca are widely used to reduce Na^{+} and Cl^{-} dependent injuries in plants (Kaya et al., 2003). In the present study, we examined the effects of Ca^{2+} to cope with NaCl dependent salinity in red and green lentils.

Calcium is one of the essential elements for growth and development of several organisms including plants. At first, plants use calcium as a seconder messenger to control numerous cellular processes including cell expansion, elongation, proliferation, circadian rhythms and fertilization. A lot of evidence suggest that calcium plays a crucial role in the adaptation of plants to different kinds of stresses including salt stress. It stabilizes cell wall structure, induces proline synthesis, activates antioxidant enzymes and restores photosynthesis under NaCl stress (Reddy and Reddy., 2004; Yang and Poovaiah., 2002). We showed that Ca^{2+} significantly reduced the growth inhibitory effects of NaCl in red and green lentils. Combination of 10 mM Ca^{+2} and 200 mM NaCl increased length and weight of shoots and roots in both tested plants as compared to 200 mM NaCl. Xue et al. (2008) found that Ca^{2+} enrichment (10 mol L^{-1}) significantly alleviated the inhibitory effect of NaCl on growth of the Jerusalem artichoke. Cha-um et al. (2012) were observed parallel results in *Oryza sativa* by using 1.98 mM $CaCl_2$ transferred to 200 mM NaCl solution. Protective effects of calcium against NaCl stress were also shown in soybean, *Withania somnifera*, linseed and *Rumex sp*. (Arshi et al., 2010; Khan et al., 2010). After the growth analysis, we measured chlorophyll and carotenoid contents to determine the effects of NaCl on the photosynthetic potential of red and green lentils. We found that NaCl treatment significantly decreased Chl a, Chl b, and total carotenoid levels in red and green lentils. Combination of 10 mM Ca^{2+} and 200 mM NaCl reduced the inhibitory effects of NaCl on Chl a, Chl b and total carotenoid levels in red and green lentils. Xue et al. (2008) found that NaCl-treated Jerusalem artichoke showed 12% loss in leaf chlorophyll content after 5 days of treatment. Moreover, they reported that addition of calcium significantly decreased the chlorophyll loss in NaCl-treated plants. Since salt stress is strongly correlated with the generation of reactive oxygen species and Ca^{+2} activates the molecules of antioxidant system, we measured the activities of three antioxidant enzymes (SOD, CAT and APX) and three well known indicators (MDA, proline and H_2O_2) of ROS generation by using spectrophotometric methods. In shoots and roots of both plants, NaCl treatment significantly increased MDA, proline and H_2O_2 levels in a concentration dependent manner. In parallel with the growth results of this study, we observed that red lentil was more sensitive than green lentil to salt stress dependent ROS generation. Combination of 10 mM Ca^{2+} and 200 mM NaCl reduced the effects of NaCl on MDA, proline and H_2O_2 levels in roots and shoots of both tested plants. H_2O_2 is one of the most common ROS which generated in the cell under the normal as well as stressed conditions (Sharma et al., 2012). While it acts as a seconder messenger involved in tolerance to various stresses at low concentrations, it is highly toxic and leads to the programmed cell death at high concentrations (Gill and Tuteja.,2010). Valderrama et al. (2006) and Saha et al. (2010) showed that H_2O_2 content was increased in *Olea europaea* and *Vigna radiata* under the salt stress. We have shown that Ca^{2+} alleviated NaCl dependent excessive H_2O_2 generation in red and green lentils. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acid

peroxidation in the cell. Evaluation of MDA is commonly used as a marker of oxidative stress (Sharma et al., 2012). In parallel with this study, Tavallali et al. (2010) and Ashraf et al. (2010) reported that MDA levels were significantly increased in *Pistacia vera* and *Triticum aestivum* under the salt stress. We also showed that Ca^{2+} reduced NaCl dependent increased MDA levels in red and green lentils. Proline, a non-enzymatic antioxidant, is a scavenger of ROS under various stresses including salinity in plants (Gill and Tuteja., 2010). Moreover, accumulation of proline is an indicator of oxidative stress (Hare et al., 1998). We found that increased proline content under NaCl stressed red and green lentils was attenuated by Ca^{2+} treatment.

It was reported that antioxidant enzymes can be activated or inactivated under saline conditions. Xue et al. (2008) also suggested that Ca^{2+} controlled the activities of antioxidant enzymes in stressed plants. We have shown that NaCl treatment increased SOD activity in both tested lentils. However, CAT and APX activities were found to be decreased in both NaCl treated plants. We also found that in red and green lentils, Ca^{2+} regulated these enzymes under saline conditions. SOD is the most effective antioxidant enzyme and it provides the first line of defense against ROS generation under various environmental stresses including salt stress (Gill and Tuteja., 2010). Significant increase in SOD activity under saline stress has been reported in several plants such as *Lycopersicon esculentum* (Gapinska et al., 2008), *Jerusalem artichoke* ((Xue et al., 2008)), *Chrysanthemum morifolium* (Hossain et al., 2004) and *L. culinaris* (Bandeoğlu et al., 2004). We found that Ca boosted SOD activity in red and green lentils under saline stress. APX plays an important role in scavenging particularly H_2O_2 in various organisms including plants. It has been shown that increased APX levels enhanced salt tolerance in many plants including *L. culinaris* (Bandeoğlu et al., 2004; Gill and Tuteja ., 2010). We have shown that Ca^{2+} significantly increased APX activity which was reduced by NaCl treatment in red and green lentils. Another important H_2O_2 scavenger is CAT, which has one of the highest turnover rates in all enzymes. It can convert approximately 6 million molecules of H_2O_2 to H_2 and O_2 per minute (Gill and Tuteja., 2010). We found that Ca^{2+} increased the activity of CAT which was reduced by NaCl treatment in red and green lentils. Our results suggest that in salt stressed red and green lentils, inhibition of MDA, H_2O_2 and proline contents may be due to the stimulatory effects of Ca^{2+} on the activities of SOD, APX and CAT.

In conclusion, this study indicated that Ca^{2+} ameliorated the inhibitory effects of NaCl on growth and photosynthesis by regulating the activities of pivotal antioxidant enzymes such as SOD, APX and CAT in red and green lentils. Further studies are needed to investigate the underlying molecular mechanisms of Ca^{2+} dependent protective effects under salt stress.

Conflict of Interest

The authors have not declared any conflict of interest.

REFERENCES

Ashraf MA, Ashraf M, Ali Q (2010). Response of two genetically diverse wheat cultivars to salt stress at different growth stages: leaf lipid peroxidation and phenolic contents. Pak. J. Bot. 42:559-56.

Arshi A, Ahmad A, Aref IM, Iqbal M (2010). Calcium interaction with salinity-induced effects on growth and metabolism of soybean (*Glycine max* L.) cultivars. J. Environ. Biol. 31:795-801.

Bandeoğlu E, Eyidoğan F, Yücel M, Öktem HA (2004). Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. Plant Growth Regulat. 42:69-77.

Bates L (1973). Rapid determination in free proline of water stress studies. Plant Soil 39:205.

Beyer WF, Fridovich I (1987). Assaying for superoxide dismutase activity some large consequences of minor changes in conditions. Anal. Biochem. 161:559-566.

Cha-um S, Singh HP, Samphumphuang T, Kirdmanee C (2012). Calcium-alleviated salt tolerance in indica rice (Oryza sativa L. spp. indica): Physiological and morphological changes. AJCS 6”176-182.

Faris MAE, Takruri HR, Issa AY (2013). Role of lentils (Lens culinaris L.) in human healthand nutrition: a review. Mediterr J. Nutr. Metab. 6:3–16.

Gapinska M, Sk1odowska M, Gabara B (2008). Effect of short- and long-term salinity on the activities of antioxidative enzymes and lipid peroxidation in tomato roots, Acta Physiol. Plant, 30:11-18.

Gill SS, Tuteja N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48:909-930.

Hadia MR, Karimib N (2012). The role of calcium in plants' salt tolerance. J. Plant Nutr. 35:2037-2054.

Hare P, Cress WA Van SJ (1998). Dissecting the roles of osmolyte accumulation during stress. Plant Cell. Environ. 21:535-553.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplasts. Arch Biochem. Biophys. 125:189–198.

Hilal M, Zenoff AM, Ponessa G, Moreno H, Massa EM (1998). Saline stress alters the temporal patterns of xylem differentiation and alternative oxidase expression in developing soybean roots. Plant Physiol. 117:695–701.

Hossain Z, Mandal AKA, Shukla R, Datta SK (2004). NaCl stress- its chromotoxic effects and antioxidant behaviour in roots of Chrysanthemum morifolium Rama. Plant Sci. 166:215-220.

Jana S, Choudhuri MA (1981). Glycolate metabolism of three submerged aquatic angiosperms during aging. Aquat. Bot. 12:345–354.

Katerji N, Hoorn JWV, Hamdy A, Mastrorilli M, Oweis T, Erskine W (2001). Response of two varieties of lentil to soil salinity. Agric. Water Manage. 47:179–190.

Kaya C, Ak BE, Higgs D (2003). Response of salt-stressed strawberry plants to supplementary calcium nitrate and/or potassium nitrate. J. Plant Nutr. 26:543–560.

Khan MN, Siddiqui MH, Mohammad F, Naeem M, Masroor M, Khan A (2010). Calcium chloride and gibberellic acid protect linseed (*Linum usitatissimum* L.) from NaCl stress by inducing antioxidative defense system and osmoprotectant accumulation. Acta Physiol. Plant 32:121-132.

Kökten K, Karaköy T, Bakoğlu A, Akçura M (2010). Determination of salinity tolerance of some lentil (*Lens culinaris* M.) varieties. J. Food, Agric. Environ. 8:140-143.

Laohavisit A, Siân LR, Shabala L, Chen C, Renato Colaço DDR, Swarbreck SM, Shaw E, Dark A, Shabala S, Shang Z, Davies JM (2013). Salinity-Induced Calcium Signaling and Root Adaptation in Arabidopsis Require the Calcium Regulatory Protein Annexin1. Plant Physiol. 163:253-262.

Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Transact. 11:591-592.

Lockhart J (2013). Salt of the earth: ethylene promotes salt tolerance by enhancing Na/K homeostasis. Plant Cell 25:3150. doi: 10.1105/tpc.113.250911. Epub.Sep 24

Manaa A, Faurobert M, Valot B, Bouchet JP, Grasselly D, Causse M, Ahmed HB (2013). Effect of salinity and calcium on tomato fruit proteome. OMICS. 17:338-352.

Munns R (2002). Comparative physiology of salt and water stress. Plant Cell Environ. 25:239–250.

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 221:867-880.

Parida AK Das AB (2005). Salt tolerance and salinity effects on plants: A review. Ecotoxicol. Environ. Safety 60:324–349.

Reddy VS, Reddy ASN (2004). Proteomics of calcium-signaling components in plants. Phytochemistry 65:1745–1776.

Romero-Aranda R, Soria T, Cuartero J (2001). Tomato plant-water uptake and plant-water relationships under saline growth conditions. Plant Sci. 160:265-272.

Serrano R, Mulet JM, Ríos JAG, Márquez de Larriona IF, Leube MP, Mendizabal I, Pascual-Ahuir A, Proft MRR, Montesinos C (1999). A glimpse of the mechanism of ion homeostasis during salt stress. J. Exp. Bot. 50:1023-1036.

Saha P, Chatterjee P, Biswas AK (2010). NaCl pretreatment alleviates salt stress by enhancement of antioxidant defense system and osmolyte accumulation in mungbean (*Vigna radiata* L. Wilczek). Indian. J. Exp. Biol. 48:593-600.

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. J. Bot. Article ID 217037, P. 26.

Sidari M, Santonoceto C, Anastasi U, Preiti G, Muscolo A (2008). Variations in four genotypes of lentil under NaCl-salinity stress. American J. Agric. Biol. Sci. 3:410-416.

Tavallali V, Rahemi M, Eshghi S, Kholdebarin B, Ramezanian A (2010). Zinc alleviates salt stress and increases antioxidant enzyme activity in the leaves of pistachio (*Pistacia vera* L. 'Badami') seedlings. Turk. J. Agric. For. 34:349-359 © TÜBİTAK doi:10.3906/tar-0905-10

Tsuganea K, Kobayashia K, Niwaa Y, Ohbab Y, Wadab K, Kobayashia H (1999). A Recessive Arabidopsis Mutant That Grows Photoautotrophically under Salt Stress Shows Enhanced Active Oxygen Detoxification. Plant Cell 11:1195-1206

Valderrama R, Corpas FJ, Carreras A, Gómez-Rodríguez MV, Chaki M, Pedrajas JR, Fernández-Ocaña A, Del Río LA, Barroso JB (2006). The dehydrogenase-mediated recycling of NADPH is a key antioxidant system against salt-induced oxidative stress in olive plants. Plant, Cell. Environ. 29:1449–1459.

Yadav S, Irfan M, Ahmad A, Hayat S (2011). Causes of salinity and plant manifestations to salt stress: a review. J. Environ. Biol. 32:667-685

Yang T, Poovaiah BW (2002). Hydrogen peroxide homeostasis: Activation of plant catalase by calcium/calmodulin. Proc. Natl. Acad. Sci. U.S.A. 99:4097–4102.

Xue YF, Liu L, Liu ZP, Mehta SK, Zhao GM (2008). Protective role of Ca against NaCl toxicity in Jerusalem artichoke by up-regulation of antioxidant enzymes. Pedosphere 18:766–774.