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*Full Length Research Paper*

# The effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidative enzymes of pumpkin seedling

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**Salinity is a major abiotic stress reducing the yield of a wide variety of crops all over the world. In order to investigate the antioxidant enzymes activity of four pumpkin genotypes (Iskenderun-4, AB-44, CU-7 and A-24) in response to salinity grown in hydroponic culture, 4 to 5 true leaf stages of pumpkin seedlings were subjected to 100 mM NaCl for 7 days. Salt stress induced changes in antioxidant enzymes, SOD, CAT, GR and APX, total chlorophyll content, lipid peroxidation and root and shoot fresh weight were measured. Salt treatment decreased root and shoots weight, chlorophyll content in salt sensitive genotypes more than salt tolerant genotypes. The four genotypes showed an increase in malondialdehyde (MDA) content under salt condition, but the increase in sensitive genotypes (CU-7 and A-24) were higher than that in salt tolerant genotypes (Iskenderun-4, AB-44). SOD, CAT, GR and APX activities increased salt stress. However these increases were higher in salt tolerant Iskenderun-4, AB-44 than salt sensitive CU-7 and A-24. These results indicate that pumpkin genotypes respond to salt induced oxidative stress by enzymatic defense systems.**

**Key words:** Pumpkin, salinity, antioxidant enzyme, oxidative stress, malondialdehyde (MDA).

## INTRODUCTION

Salinity is one of the most important environmental factors that cause reduction in plant growth, development and productivity worldwide. Salt stress changes the morphological, physiological and biochemical responses of plants (Amirjani, 2010; Siringam et al., 2011). There is evidence that high salt concentrations cause an imbalance in cellular ions, resulting in ion toxicity and osmotic stress, leading to the generation of reactive oxygen species (ROS) which cause damage to DNA, lipids and proteins (Yasar et al., 2006). At the same time ROS causes chlorophyll degradation and membrane lipid peroxidation, decreasing membrane fluidity and selectivity. Plants possess several anti-oxidant enzyme systems that protect their cells from the negative effects of ROS. These include non-enzymatic anti-oxidants such as ascorbic acid, glutathione and carotenoids, as well as antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR). SOD catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen. Hydrogen peroxide which is also toxic to cells is detoxified by CAT peroxidases to water and oxygen (Zhu et al., 2004). APX reduces $H_2O_2$ using ascorbate as an electron donor in the ascorbate- glutathione cycle. Oxidized ascorbate is then reduced by GSH generated from GSSG catalyzed by GR at the expense of NADPH (Kusvuran et al., 2007). Previous studies showed that the level of anti- oxidative enzymes increases when plants are exposed to oxidative stress including salinity (Yasar, 2007; Kusvuran et al., 2007; Dolatabadian et al., 2008; Li, 2009; Chookhampaeng, 2011).

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Pumpkins and squash (*Cucurbita* spp.) are important crops and are grown in almost all arable regions of the world. There are three economically important *Cucurbita* species, namely *C. pepo*, *C. maxima* and *C. moschata*, which have different climatic adaptations and are widely distributed in agricultural regions worldwide (Sarı et al., 2008).

Mediterranean countries, Turkey, Italy and Egypt meet one-third of world production of pumpkin (Paris, 1996). Turkey has an important place in terms of genetic diversity of Cucurbitaceae (Sarı et al., 2008). Local squash and pumpkin varieties grown in Turkey in terms of salt tolerance between the genetic variations were found (Sevengör, 2010). In many provinces (region), pumpkin can be grown on unproductive land without irrigation. Therefore, pumpkin can be considered as a good point alternative for the problem salinity or drought in areas

The aim of this study was to determine the effects of salt stress on the activity of antioxidant enzymes, lipid membrane peroxidation and chlorophyll content in pumpkin genotypes.

## MATERIALS AND METHODS

As the plant material, two salt tolerant (Iskenderun$^{-4}$ and AB-44) and two salt sensitive (CU-7 and A-24) local Turkish pumpkin varieties, whose salt tolerance characteristics were determined in previous study (Sevengör, 2010), were used. Iskenderun-4 and CU-7 genotypes belong to *C. pepo*, AB-44 and A-24 genotypes belong to *C. moschata*.

All plants were grown under 280 µmol $m^{-2}$ $s^{-1}$ of cool white fluorescent light with a 16 h photoperiod in a controlled climate room at 28 and 25°C day and night temperatures, and 70% relative humidity. Seeds were germinated in vermiculite moistened with distilled water. After the emergence of the first true leaves, they were transferred to hydroponic culture supported by air. For hydroponic culture, plastic dishes filled with the Hoagland nutrient solution were used. The nutrient solution was renewed once a week. When seedlings reached to 4 to 5 leaf stages salt-treatment started and the NaCl concentration was increased by 50 mM $d^{-1}$ until a final concentration of 100 mM was achieved. Non-salt-treated plants were kept under controls. Seven days after salt treatment, growing of seedlings was observed and the shoot and root fresh weight of plants were determined. The leaf samples were kept at -80°C for further analyses.

### Chlorophyll determination

Leaf segments (200 mg), either fresh or frozen at -40°C, were placed in 5 ml of 80% ethanol and heated in a water bath at 80°C for 20 min. Total chlorophyll was evaluated in the alcohol extracts from absorbance readings, using the appropriate extinction coefficient. Chlorophyll content (mg/g fr wt) was calculated as 1000 × A654/(39.8 × sample fr wt) according to Luna et al. (2000).

### Malondialdehyde content

Lipid peroxidation was measured as the amount of MDA determined by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Frozen samples were homogenized with a prechilled mortar and pestle with two volumes of ice-cold 0.1% (w/v) TCA and centrifuged for 15 min at 15 000 *g*. Assay mixture containing 1 ml of the supernatant and 2 ml of 0.5 % (w/v) TBA in 20% (w/v) TCA was heated at 95°C for 30 min and then rapidly cooled in an ice bath. After centrifugation (10 000 *g* for 10 min at 4°C), the supernatant absorbance was read at 532 nm, and the values corresponding to nonspecific absorption (600 nm) were subtracted. Lipid peroxidation products were measured as the content of TBA-reactive substances. The MDA content was calculated according to the molar extinction coefficient of 155/(mM cm).

### Enzyme extraction and assay

Fresh leaf samples were submersed for 5 min in liquid nitrogen. The frozen leaves were kept at -80°C for further analyses. Enzymes were extracted from 0.5 g leaf tissue using a mortar and pestle with 5 ml extraction buffer containing 50 mM potassium phosphate buffer pH 7.6 and 0.1 mM Na-EDTA. The homogenate was centrifuged at 15,000 *g* for 15 min and the supernatant fraction was used to assay for the various enzymes. All steps in the preparation of enzyme extracts were performed at 4°C.

SOD was assayed according to Karanlık (2001), by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm. One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of the photochemical reduction of NBT.

Catalase activity was determined by monitoring the disappearance of $H_2O_2$ according to the method of Cakmak and Marschner (1992).

APX activity was determined by measuring the consumption of ascorbate by following absorbance at 290 nm. One unit of APX activity was defined as the amount of enzyme required to consume 1 µmol ascorbate $min^{-1}$ (Cakmak and Marschner, 1992).

GR activity was determined by measuring the enzyme-dependent oxidation of NADPH by following absorbance at 340 nm. One unit of GR activity was defined as the amount of enzyme that oxidized 1 µmol NADPH $min^{-1}$ (Cakmak and Marschner, 1992).

The experiment was designed as completely randomized plot with three replicates. Data were analyzed statistically, and the means of each treatment were analyzed by Duncan's multiple range test using SAS software (1985).

## RESULTS

### Plant growth

Salt stress significantly decreased shoot and root fresh weights of the pumpkin genotypes in comparison with the control without salt. Tolerant genotypes (Iskenderun-4, AB-44) protected their growth performances under saline stress (Table 1), while the genotypes CU-7 and A-24 (salt sensitive) had high reductions in their shoot and root fresh weights. Salt stress caused 24.56 and 42.75% reductions in tolerant genotypes and 50.41 and 59.66% reductions in sensitive genotypes, respectively. The weight of fresh shoot was drastically reduced from 22.30 and 52.98% in tolerant genotypes and 65.10 and 79.52% in sensitive genotypes.

**Table 1.** The effects of salt stress on the root and shoot fresh weight in four pumpkin genotypes.

| Genotypes | Root fresh weight (g) | | | Shoot fresh weight (g) | | |
|---|---|---|---|---|---|---|
| | Control | Salt | Decrease (%) | Control | Salt | Decrease (%) |
| Iskenderun-4 | 1.14$^{a}$ | 0.86$^{ab}$ | 24.56 | 1.39$^{a}$ | 1.08$^{a}$ | 22.30 |
| AB-44 | 1.56$^{a}$ | 1.05$^{a}$ | 42.75 | 1.68$^{a}$ | 0.79$^{ab}$ | 52.98 |
| CU-7 | 1.21$^{a}$ | 0.60$^{b}$ | 50.41 | 1.49$^{a}$ | 0.52$^{bc}$ | 65.10 |
| A-24 | 1.19$^{a}$ | 0.48$^{b}$ | 59.66 | 1.66$^{a}$ | 0.34$^{c}$ | 79.52 |

Mean values indicated by the same letter are not significant different ($p \leq 0.01$).

**Table 2.** The effect of salt stress on the content of MDA and chlorophyll in four pumpkin genotypes.

| Genotypes | MDA (µ mol/g F.W.) | | | Chlorophyll (µ g/mg F.W.) | | |
|---|---|---|---|---|---|---|
| | Control | Salt | Increase (%) | Control | Salt | Loss (%) |
| Iskenderun-4 | 5.11$^{a}$ | 7.82$^{c}$ | 53.03 | 0.183$^{b}$ | 0.162$^{a}$ | 11.48 |
| AB-44 | 4.82$^{a}$ | 6.49$^{c}$ | 34.65 | 0.274$^{a}$ | 0.159$^{a}$ | 41.97 |
| CU-7 | 5.41$^{a}$ | 13.01$^{a}$ | 140.48 | 0.141$^{b}$ | 0.107$^{b}$ | 24.11 |
| A-24 | 5.24$^{a}$ | 12.20$^{ab}$ | 132.82 | 0.243$^{a}$ | 0.098$^{b}$ | 59.67 |

Mean values indicated by the same letter are not significant different ($p \leq 0.01$).

**Table 3.** Antioxidant enzymes (SOD and CAT) activities in leaves of salt stressed pumpkin genotypes.

| Genotypes | Superoxyde dismutase (µmol/min/mg F.W.) | | | Catalase (µmol/min/mg F.W.) | | |
|---|---|---|---|---|---|---|
| | Control | Salt | Increase (%) | Control | Salt | Increase (%) |
| Iskenderun-4 | 166.3$^{c}$ | 430.8$^{b}$ | 159.05 | 88.4$^{ab}$ | 331.0$^{a}$ | 274.43 |
| AB-44 | 201.6$^{b}$ | 546.4$^{a}$ | 171.03 | 81.0$^{b}$ | 371.7$^{a}$ | 358.89 |
| CU-7 | 217.1$^{a}$ | 198.9$^{d}$ | - 8.38 | 91.5$^{a}$ | 198.3$^{b}$ | 116.72 |
| A-24 | 159.4$^{c}$ | 240.2$^{c}$ | 50.69 | 98.3$^{a}$ | 205.5$^{b}$ | 109.05 |

Mean values indicated by the same letter are not significant different ($p \leq 0.01$).

## Chlorophyll content

To investigate the effect of salt stress on the chlorophyll content in pumpkin genotypes, chlorophyll content decreased by salinity (Table 2). In sensitive genotypes (CU-7 and A-24) chlorophyll content decreased by 24.11 and 59.67%, respectively, when compared to control plants. On the other hand, tolerant genotypes protected their chlorophyll content.

## Lipid peroxidation

With regard to malondialdehyde (MDA), significant differences were found between genotypes in salt treatments (Table 2). Salinity led to a gradual increase in the levels of MDA in genotypes. MDA accumulation in sensitive genotypes was higher (132.82 and 140.48 %) than tolerant genotypes (Iskenderun-4: 53.03%, and AB-44: 34.65 %).

## Antioxidant enzyme activities

To determine the response of pumpkin to salt induced oxidative stress, SOD, CAT, APX and GR activities were measured in leaves of seedlings grown with or without 100 mM NaCl.

SOD activities of four pumpkin genotypes under the effect of salt stress and control are shown in Table 3. The SOD activity was increased by salinity. The SOD activity was the highest in tolerant genotypes, Iskenderun-4, AB-44, 430.8 and 546.4 µmol/min/mg T.A, respectively. These genotypes had increased SOD activity by 159.05" and "171.03%. On the other hand, CU-7 exhibited significant decrease in SOD activity with salt stress by -8.38%. Under non-salt control conditions, CU-7 and A-24 had the highest activity of CAT among all genotypes. Salt treatment increased CAT activity in all genotypes, when compared with their control groups. The increases in CAT activities in AB-44 and Iskenderun-4 were higher than salt sensitive genotypes CU-7 and A-24. CU- and A-24

**Table 4.** Antioxidant enzymes (GR and APX) activities in leaves of salt stressed pumpkin genotypes.

| Genotypes | Glutathion reductase (μmol/ min/mg F.W.) | | | Ascorbat peroxidase(μmol/ min/mg F.W.) | | |
|---|---|---|---|---|---|---|
| | Control | Salt | Increase (%) | Control | Salt | Increase (%) |
| Iskenderun-4 | 167.2$^{bc}$ | 973.2$^{b}$ | 482.06 | 843.5$^{a}$ | 5746.0$^{a}$ | 581.21 |
| AB-44 | 234.4$^{a}$ | 1012.2$^{a}$ | 331.83 | 805.5$^{a}$ | 5823.8$^{a}$ | 623.00 |
| CU-7 | 158.3$^{c}$ | 563.0$^{d}$ | 255.65 | 622.2$^{b}$ | 3100.7$^{b}$ | 398.34 |
| A-24 | 173.6$^{b}$ | 613.5$^{c}$ | 253.40 | 656.3$^{b}$ | 2678.5$^{c}$ | 308.12 |

Mean values indicated by the same letter are not significant different ($p \leq 0.01$).

under salt stress were increased by 274.43 and 358.89%, respectively, in comparison with that of the non-salinized control plants. Salt sensitive genotypes (CU- and A-24) were only increased 116.72 and 109.05%, respectively (Table 3).

A significant increase in GR activity was observed in all four genotypes under salt stress. However, CU-7 and A-24 had the lowest GR activity under saline conditions. In contrast, Iskenderun-4 and AB-44 were the highest in GR activities of all cultivars under salt stress (Table 4). In response to the salt treatment, the GR activity was found to be enhanced in both genotypes reaching 973.2 and 1012.2 μmol/min/mgT.A in Iskenderun-4 and AB-44, respectively.

A significant increase in APX activity was observed in all four cultivars under salt stress ($P \leq 0.01$). The APX activities of four cultivars were increased significantly with salt treatment (Table 4). The APX activities of Iskenderun-4 and AB-44 were significantly higher than that of CU-7 and A-24. APX activities of the tolerant genotypes Iskenderun-4 and AB-44 under salt stress were increased by 581.21 to 623% in comparison with that of the non-salinized control plants while that of the salt sensitive genotypes A-24 and CU-7 were increased by 308.12 to 398.34%.

## DISCUSSION

Salinity stress induced lower fresh weight and chlorophyll concentration of pumpkin genotypes. It has been reported that the typical symptom of salinity injury to the plant is the growth retardation due to the inhibition of cell elongation (Yasar et al., 2008). Salt tolerance genotypes like Iskenderun-4 and AB-44 protected their weight than salt sensitive genotypes (CU-7 and A-24) under salt conditions. This is in accordance with the previous reports in melon, eggplant, bean and tomato (Kusvuran et al., 2007; Yasar et al., 2006; Kaya et al., 2007; Dasgan and Koc, 2009).

In the present study, photosynthetic pigments, chlorophyll content decreased significantly in all pumpkin genotypes due to salt stress. The decrease in chlorophyll content in the pumpkin genotypes might have been due to salt-induced increase in the activity of the chlorophyll degrading enzyme, chlorophylase (Rao and Rao, 1981; Noreen and Ashraf, 2009). The decrease in chlorophyll content under salinity conditions is reported by Yasar et al. (2008), Kusvuran (2010), and Nazarbeygi et al. (2011). In the salt tolerant genotypes (Iskenderun-4 and AB-44), the chlorophyll content was protected probably because of the high antioxidant enzyme activities that prevented degradation of leaf chlorophyll.

Radical oxygen species cause membrane lipid peroxidation, reducing membrane fluidity and selectivity. Lipid peroxidation measured as MDA content is considered to be indicator of oxidative damage from stress. MDA content was increased by salinity in all the genotypes. Tolerant genotypes, Iskenderun-4 and AB-44, showing better growth under salt stress had less MDA than sensitive genotypes, CU-7 and A-24. This result shows that salt tolerant genotypes have induced capability of plant protection against oxidative damage caused by salt stress In addition, antioxidant enzymes are the most effective in preventing cell damage (Yu and Zengel, 1999; Türkan et al., 2005; Yasar et al., 2008; Kusvuran, 2010).

Salinity stress caused generation of excessive reactive oxygen species (ROS), which leads to cell toxicity, membrane dysfunction and cell death (Chookhampaeng, 2011). Plants have developed enzymatic and non-enzymatic mechanism to scavenge ROS (Asada, 1999; Yasar et al., 2006). Among the active oxygen species superoxide is converted by SOD enzyme to $H_2O_2$, which is further scavenged by CAT and APX. Overexpression of the APX gene in plants has showed improvement in protection against oxidative stress (Yasar et al., 2008). Our results showed that, these enzyme activities in all genotypes were increased by salinity and were higher in salt tolerant genotypes Iskenderun -4 and AB-44 than salt sensitive genotypes (CU-7 and A-24). These enzymes were also reported to be important in salt tolerance in melon (Yasar et al., 2006; Kusvuran et al., 2007), green bean (Yasar et al., 2008), and soybean (Amirjani, 2010).

GR also plays a key role by reducing H2O2 (Wang et al., 2011). Therefore increases in GR activity have been reported to play a role in tolerance to salt stress. Kusvuran et al. (2007) in melon, Li (2009) in tomato and Wang et al. (2011) in alfalfa also observed that salinity increased GR activity in salt tolerant genotypes. In this

study, GR activity was increased in tolerant genotypes (Iskenderun-4 and AB-44) compared to salt sensitive genotypes. This result suggested that, salt tolerant genotypes were more active in exhibiting ascorbate-glutathione cycle and reducing $H_2O_2$.

In conclusion, tolerant and sensitive genotypes showed different responses under salinity. Anti-oxidative enzyme activities played a protective role against salt stress, and that antioxidative defense mechanisms were effective in providing resistance to stress in pumpkin. The result of this study is that salt tolerant genotypes Iskenderun-4 and AB-44 may have a better protection against stress by increasing the activity of antioxidant enzyme in salinity.

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