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Benchmarking of the Effects of Salinity on Antioxidant Enzymes Activities, Lipid Peroxidation and H_2O_2 Levels in the Leaves of Two Zinnia Species

Sara YASEMİN[1], Ayşin GÜZEL DEĞER[2]✉, Sertan ÇEVİK[3], Nezihe KOKSAL[4]
[1]Siirt University, Faculty of Agriculture, Department of Horticulture, Siirt, Turkey, [2]Mersin University, Vocational School of Technical Sciences, Department of Food Processing, Mersin, Turkey, [3]Vocational School of Mut, Mersin University, Mersin, Turkey, [4]Çukurova University, Faculty of Agriculture, Department of Horticulture, Adana, Turkey
[1]https://orcid.org/ 0000-0003-2193-6791, [2]https://orcid.org/0000-0001-6336-1872, [3]https://orcid.org/ 0000-0003-1259-7863
[4]https://orcid.org/ 0000-0002-5401-9730,
✉: agozel@mersin.edu.tr

ABSTRACT
In this study, it was aimed to investigate the effects of salinity, which is an important environmental problem, in the cultivation of ornamental plants (such as zinnia) and irrigation with high salt water, especially on the antioxidant defense mechanism. For this purpose, the two Zinnia species were irrigated by different concentrations of saline water (50, 100, 150, 200 mM NaCl); effects of salinity on superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2) in the leaves were determined. The results showed that salinity conspicuously increased SOD, CAT, GR, H_2O_2 and MDA content at two Zinnia species compared to the control groups. It was found that SOD and CAT enzyme activities increased remarkably with 150 mM NaCl in both Zinnia species, but decreased with 200 mM NaCl.The highest GR enzyme activity was observed in 200mM salt concentration at *Zinnia marylandica* 'Double Zahara Fire Improved'. MDA and H_2O_2 levels were observed higher in *Zinnia elegans* 'Zinnita Scarlet'.To conclude; it may be said that these two Zinnia varieties can tolerate salt concentration up to 150 mM.

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İki Zinnia Türünün Yapraklarında Tuzluluğun Antioksidan Enzim Aktiviteleri, Lipid Peroksidasyonu ve H_2O_2 Düzeyleri Üzerine Etkilerinin Karşılaştırılması

ÖZET
Bu çalışmada, önemli bir çevresel sorun olan tuzluluğun, süs bitkilerinin (zinnia gibi) yetiştirilmesinde ve yüksek tuzlu su ile sulamada, özellikle antioksidan savunma mekanizması üzerindeki etkilerinin araştırılması amaçlanmıştır. Bu amaçla, iki Zinnia türü farklı konsantrasyonlarda tuzlu su (50, 100, 150, 200 mM NaCl) ile sulanmıştır; tuzluluğun yapraklardaki süperoksit dismutaz (SOD), katalaz (CAT), glutatyon redüktaz (GR) lipit peroksidasyonu (MDA) ve hidrojen peroksit (H_2O_2) üzerindeki etkileri belirlenmiştir. Sonuçlar, tuzluluğun iki Zinnia türünde SOD, CAT, GR, H_2O_2 ve MDA içeriğini kontrol gruplarına kıyasla belirgin şekilde arttırdığını göstermiştir. SOD ve CAT enzim aktivitelerinin her iki Zinnia türünde 150 mM NaCl ile önemli ölçüde arttığı, ancak 200 mM NaCl ile azaldığı bulunmuştur. En yüksek GR enzim aktivitesi, *Zinnia marylandica* "Double Zahara Fire Improved" da 200mM tuz konsantrasyonunda gözlenmiştir. MDA ve H_2O_2 seviyeleri *Zinnia elegans* 'Zinnita Scarlet' da daha yüksek olarak gözlemlenmiştir. Sonuç olarak, bu iki Zinnia çeşidinin 150 mM'a kadar tuz konsantrasyonunu tolere edebildiği söylenebilir.

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INTRODUCTION

Quality water resources are used especially in the cultivation of agricultural products consumed as human food and these resources are decreasing daily. Therefore, it is necessary to irrigate ornamental and landscape plants with lower quality waters. Salinity in soil or water diminish the water uptake of the plants, so negatively affect the plant growth (Cassaniti et al., 2012). Salinity is one of the important abiotic stress factors that affect vital parameters such as growth, development, reproduction and crop yield in many plants (Läuchli and Grattan 2007; Waqas et al., 2019). Also, salinity affects the visual quality of ornamental plants that are as well of commercial value (Cassaniti et al. 2009; Koksal et al., 2016; Yasemin et al., 2017). In addition, it was reported that growth parameters were negatively affected in the case of salt stress in ornamental plants (Yasemin et al., 2017). So, it is important to determine salt tolerance mechanisms in ornamental plants. Zinnia, *Asteraceae* family, is cultivated worldwide for use as annual bedding plants and some of Zinnia could be used as cut flowers. Flower and leaf morphology, ray floret colors differ from each other. For this reason, these plants have a big interest for being used in gardens or landscaping (Stimart and Boyle, 2007). In particular, the determination of salinity defenses in such ornamental plants can be an important step for selection and use in these areas.

There are two basic questions to understand the impact of salt stress and how to deal with it. Firstly, which changes does the plant experience when it is exposed to salt stress? Second, what kinds of defense responses are given to these changes? Salinity is first detected by the root system and many signal paths are actived. Salinity primarily causes cell dehydration and a decrease in water potential in plants. Under salinity, nutrient imbalance and reduced water availability cause osmotic and ionic stress. After these effects, as in water shortage; it continues by reduced cellular and metabolic effects, closure of stomata, inhibition of photosynthesis, leaf fall, change in carbon distribution, production of reactive oxygen species (ROS) and cell death. Salinity stress also causes protein denaturation and the membranes to become unstable. As a result of all these, growth and development in plants decline (Munns 2005; Pang and Wang 2008; Acosta-Motos et al., 2017; Isayenkov and Maathuis, 2019).

Plants activate several complex mechanisms in the control of genes to deal with salt stress. And so, stomatal control, ion secretion, osmotic adjustment and antioxidant systems come into play (Munns 2005; Abogadallah, 2010). Abiotic stresses induce the formation of ROS ($O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$) in plants and oxidative damage occurs. Antioxidant enzymes and nonenzymatic compounds, which are antioxidant metabolism components, have an important role in detoxifying ROS caused by salt stress (Hasanuzzaman et al., 2012; Gill et al., 2013; Gupta and Huang 2014; Parvin et al., 2019).

Increasing in the activities of the antioxidant defense system under environmental stresses is generally correlated with the stress tolerance of plants (Zandalinas et al., 2017; Laxa et al., 2019). SOD, CAT, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), GR, glutathione peroxidase (GPX), and glutathione S-transferase (GST) are key enzymes in the enzymatic system that regulate the content of ROS, such as H_2O_2, $O_2^{\cdot-}$ and OH radicals (Asada, 1999; Liang et al., 2018; Parvin et al., 2019). Also lipid peroxidation, identified as MDA concentration is one of the remarkable markers of oxidative damage during salinity stress (Hernandez et al., 2000; Khan and Panda, 2008).

Salt stress can reduce growth and biomass as a result of a nutritional imbalance in ornamental plants as in many other plant groups. Salinity conditions can alter water relationships and photosynthetic capacity in these plants. Antioxidant mechanisms as well can use counteract these negative effects (García-Caparrós and Lao, 2018). Since the salinity problem affects both soil and water, ornamental plant research one of the main objectives is thought to be to determining tolerant plants (Cassaniti et al., 2013).

This article focuses on analyzing antioxidant defense in plant stress tolerance by watering ornamental plants with salty water (mimic low quality water), especially considering limited water resources. For this purpose, antioxidant enzyme activities (SOD, CAT and GR), lipid peroxidation (MDA) and H_2O_2 contents were measured in two different Zinnia species under saline conditions.

MATERIALS and METHODS

Plant Growth Condition and Salt Stress Treatment

This study was conducted in the greenhouse at the Department of Horticulture, Cukurova University in Adana/Turkey (32.9/19.7°C day/night, relative humidity 54 %). In this study seeds of *Zinnia elegans* 'Zinnita Scarlet' and *Zinnia marylandica* 'Double Zahara Fire Improved' species (Tasaco Farm,Turkey) were used as plant material. These two cultivars were determined to be relatively sensitive ('Zinnita Scarlet') and tolerant ('Double Zahara Fire Improved') to salt stress in a screening study conducted among twenty Zinnia cultivars (Yasemin, 2020). Seeds of Zinnia cultivars were germinated in tray plugs containing peat. After germination, plantlets were transferred into 2 L plastic pots which included peat: perlite (2:1). 5 days after the transfer, the plants were irrigated with solution included 0, 50, 100, 150 and 200 mM

NaCl concentrations one-day interval. Salinity treatments were continued for three weeks. Treatment was terminated, as soon as the first visual symptoms were seen on the plants.

Extraction for Antioxidant Enzyme Assays

SOD, CAT, GR extractions

Fresh leaves of two Zinnia species (1g) were homogenized with 5 mL of potassium phosphate buffer (0.1 M, pH 6.8) including 100 mg of PVP and EDTA (0.1 mM). The homogenate was centrifuged at +4 °C 16 000 g for 5 min and the supernatant was collected for enzyme analysis (Beyer and Fridowich, 1987).

Measurement of Antioxidant Enzyme Activity

Superoxide dismutase (SOD) activity

SOD activity (EC 1.15.1.1) was determined according to the modified method of Beyer and Fridowich, 1987. Reaction mixtures composed 200 µL of enzyme extract, 100 µL of 5 mM nitro blue tetrazolium (NBT), 150 µL of 0.1 mM riboflavin, 200 µL of 0.25 M methionine, and 1 mL of 200 mM sodium carbonate. These reagents except for riboflavin wasprepared with 0.1 M potassium phosphate buffer (pH 7.5). SOD activity was determined as the amount of enzyme causing 50% inhibition of NBT measured at 560 nm spectrophotometer.

Catalase (CAT) activity

CAT activity (EC 1.11.1.6) was detected by measuring the rate of decomposition of H_2O_2 at 240 nm, according to Aebi, (1974). The reaction mixture contained 120 µL of enzyme extract, 2.8 mL of 50 mM potassium phosphate buffer (pH 7 without EDTA), and 80 µL of 0.5 M H_2O_2.

Glutathione reductase (GR) Activity

GR (EC 1.6.4.2) activity was determined as described by Carlberg and Mannervik (1985). The reaction mixture consisted of 1.5 mL 0.1 M phosphate buffer, 150 µL of 200 mM oxidized glutathione, 150 µL of 2 mM NADPH, 1 mL pure water, 200 µL enzyme extract. The oxidation of NADPH at 340 nm was defined as GR activity.

Measurement of Soluble Protein Content

The amount of soluble protein was carried out using the working solution according to the Bradford method. Bovine serum albumin was used to generate a standard curve; samples were read and recorded on the Elisa instrument at 595 nm (Bradford, 1976). The datum of soluble protein content was used to estimate specific activities of SOD, CAT, and GR.

Malonyldialdehyde (MDA) assay

Lipid peroxidation was detected by measuring the MDA level. Fresh leaf tissue (0.5 g) of Zinnia plants was homogenized in 1 mL (5%) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at room temperature for 15 min at 16.000g. The supernatant was transferred to the tubes by taking equal volumes of 0.5% thiobarbituric acid (TBA) and 20% TCA solutions. The tubes were incubated at 96°C for 25 min. Then, the tubes were transferred to an ice bath and centrifuged at 12.000 g for 5 min. The supernatant was measured at 532 and 600 nm. 0.5% TBA in 20% TCA solution was used as a blank sample. The MDA content was calculated using the extinction coefficient (Ohkawa et al., 1979).

Hydrogen peroxide (H2O2) assay

H_2O_2 level was determined according to Loreto and Velikova, (2001) with some modifications. Firstly, 0.5g leaf tissue was homogenized in the ice bath with 5 mL, 1% (v:v) TCA, the homogenate was centrifuged at 12.000 g and 0.75 mL supernatant was added to 0.75 mL phosphate buffer and 1.5 ml KI. H_2O_2 level was evaluated by comparing its absorbance at 390 nm to a standard calibration.

Statistical Analysis

Stress treatment was carried out completely randomized experimental design with two factors. Treatments had five replications with five plants each. Data were subjected to ANOVA and the means were separated using the LSD multiple range test at $P<0.05$. Student-T analysis test was used to compare the groups. All the statistical analyses were performed using the JMP8 Software package.

RESULTS and DISCUSSION

In this study, the effects of salt stress on the antioxidant enzymes activities (SOD, CAT, GR), H_2O_2 and MDA levels in the leaves of two Zinnia species grown under different salt (0, 50, 100, 150, 200 mM) concentrations were comparatively investigated. It was determined that salinity notably caused increases the SOD, CAT, GR, H_2O_2 and MDA content at two Zinnia species compared to the control groups(Table 1).

Cultivar effects in terms of activities of three antioxidant enzymes, lipid peroxidation and H_2O_2 levels were statistically significant, except of SOD (Table 2). MDA and H_2O_2, stress indicators, were higher level in *Z. elegans* (Zi.S). was identified as a sensitive cultivar to salinity in previous studies (Yasemin, 2020; Yasemin et al., 2020 in press). When considering antioxidant enzyme activity, GR and CAT activities were higher in *Z. marylandica* (D.Za.F.I) relatively tolerant cultivar than in *Z. elegans* (Zi.S) sensitive cultivar.

Table 1. Treatment effect on SOD, CAT, GR, MDA and H_2O_2 levels.
Tablo 1. SOD, CAT, GR, MDA ve H_2O_2 seviyeleri üzerine uygulamaların etkisi.

NaCl (mM)	*SOD*	*CAT*	*GR*	*H_2O_2*	*MDA*
0	8.354d	10.339e	0.094c	0.676d	1.961d
50	10.117c	14.278d	0.139b	0.840c	2.386c
100	11.267bc	20.690b	0.150ab	0.956b	2.424c
150	14.203a	27.940a	0.163ab	0.978b	2.833b
200	11.764b	17.656c	0.170a	1.372a	3.086a
LSD	1.435***	2.466***	0.026***	0.106***	0.234***

(***p<0.001)

Table 2. Cultivars effect on SOD, CAT, GR, MDA and H_2O_2 levels.
Tablo 2. Çeşitlerin SOD, CAT, GR, MDA ve H_2O_2 seviyeleri üzerine etkisi.

Parameters Parametreler	*Z. elegans Zi.S*	*Z. marylandica D.Za.F.I*	*t test*
SOD	11.436	10.847	0.1908^{NS}
GR	0.127	0.159	0.0006*
CAT	16.389	19.972	0.0001*
H_2O_2	1.100	0.828	<0001*
MDA	2.712	2.363	<0001*

According to results, the effects of salinity on SOD activity were found important, statistically (Figure 1). The highest SOD activity was observed at 150 mM salt concentration in both species. As shown in Figure 1, SOD activity decreased in 200 mM salinity partly, compared with 150 mM salinity, in both species. In the study, we detected similar increasing trends in both species in terms of SOD activity which has an important role in defense mechanisms of cells against ROS and is one of the ubiquitous enzymes in aerobic organisms (Bowler et al., 1992). It is well known that low concentration ROS act as signal molecules, while higher levels of ROS damage cellular components (Choudhury et al., 2013; Liu et al., 2019). In various studies, many researchers have been reported that increases in SOD enzyme activities occur in plants exposed to salt stress, similar to obtain the results (Mittova et al. 2002; Bor et al., 2003; Ahmad, 2010; Özkoku et al., 2019). It was stated, SOD activity increased importantly in the leaves of *Olea europaea* L. which exposed to 200 mM NaCl (Valderrama et al., 2006), in this study similar result observed on 150 mM salt concentration. Manivannan et al. (2015) also reported that 50 mM NaCl treatment increases SOD enzyme activity in *Z. elegans* 'Dreamland Yellow'. This means that SOD enzyme activity varies depending on plant species and salt concentration.

GR enzyme showed high activity from the lowest NaCl concentration (50 mM) to highest one (200 mM) in *Z. marylandica* D.Za.F.I which is a relatively tolerant cultivar. The highest GR activity in *Z. elegans* Zi.S was determined at salt level ≥ 100 mM (Figure 1). Omari and Nhiri, (2015) reported that GR enzyme activity in the leaves increased by approximately 90% with 150 mM salt application. Similarly, Saı Kachout et al., (2013) reported that GR activity increased steadily with NaCl concentration and it nearly doubled in response to 90 mM NaCl in *Atriplex hortensis* var. rubra (red). GR is an important enzyme that plays a role in protecting against various abiotic stresses such as salinity (Romero-Puertas et al., 2006; Saı Kachout et al. 2013). Moradi and Ismail, (2007) reported that increasing salt stress in salt-tolerant rice line (IR651) caused GR activity increased in leaves of the tolerant lines. However, salinity did not affect GR activity in leaves of in salt-intolerant rice line (IR29). In light of this information, it can be concluded that the GR level may vary according to the tolerance degree of genotype and salt concentration (Moradi and Ismail 2007; Çekiç and Ünyayar, 2006). Many researchers showed that increases in GR activity could mean tolerance to salt stress. Owing to increasing of GR activity in salt tolerant genotypes, it could be thought that, salt tolerant genotypes were more active in reducing H_2O_2 compared to salt sensitive genotypes (Kusvuran et al., 2007; Li, 2009; Sevengor et al., 2011).

CAT activity was affected important from salinity, statistically (Figure 1). CAT activity increased with stress in both species. While the highest enzyme activity was found in 150 mM NaCl level, CAT activity decreased at 200 mM NaCl in both species. The CAT decreased at 200mM NaCl might be resulted from the prevention of new enzyme synthesis or catalase photo-inactivation (Basu et al., 2010; Çevik et al.,2015). The highest value of enzyme (CAT) activity was obtained from 150 mM NaCl concentration in the *Z. marylandica* D.Za.F.I plants. Manivannan et al. (2015) reported that CAT activity did not differ significantly between control and 50mM NaCl treatments in *Z. elegans* 'Dreamland Yellow', similar to this result.

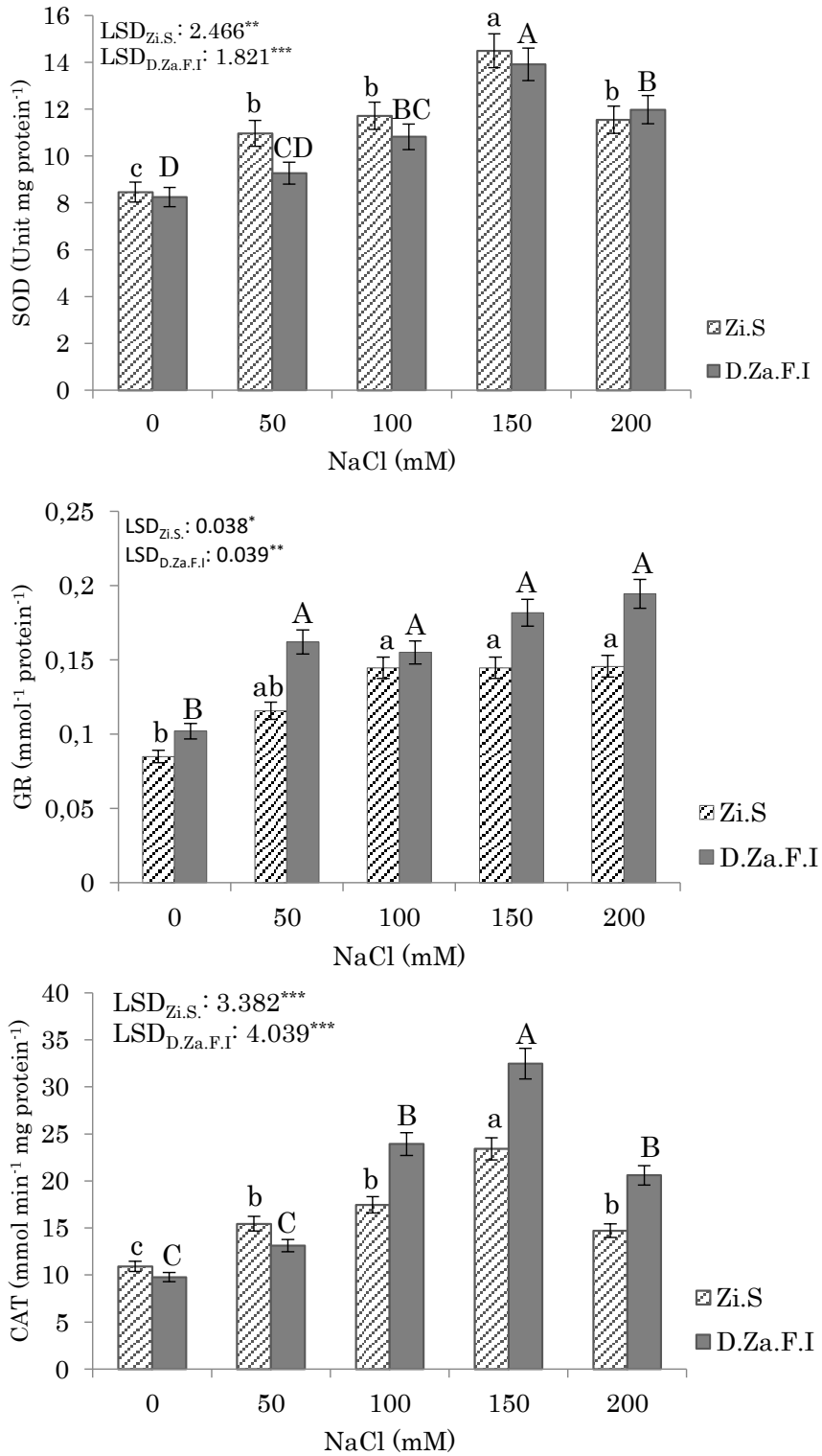

Figure 1. SOD, GR, CAT enzyme activities in two Zinnia species exposed to salinity. The data followed by the same letters in the figure show that there is no significant difference between groups (*p <0.05, **p <0.01,***p <0.001).

*Şekil 1. Tuzluluğa maruz kalan iki Zinnia türündeki SOD, GR, CAT enzim aktiviteleri. Şekilde aynı harflerin izlediği veriler, gruplar arasında anlamlı bir fark olmadığını göstermektedir (*p <0.05, **p <0.01, ***p <0.001).*

Sevengor et al., (2011) reported that salt treatment increased CAT activity in all genotypes (salt tolerant and salt sensitive), when compared to their control groups in local Turkish pumpkin varieties. However, they determined that these increases were higher in

salt tolerant species than that of salt sensitive ones. Valderrama et al. (2006) were reported that the leaves of olive (*Olea europea* L.) treated with 200 mM NaCl increased CAT activities, significantly. Findings in this study support the report of Bor et al., (2003) who also found high CAT and GR activity intolerant beet under salt stress. Also, CAT is the most effective antioxidant enzyme in preventing cellular damage by H_2O_2. Yaşar and Ellialtıoğlu, (2013) declared that significant differences in CAT enzyme activities at 150 mM salt concentration in a study on eggplant genotypes.

While there was a systematic increase in SOD and CAT enzyme activities up to 150 mM salt concentration, there was a sudden decrease in the 200 mM salt concentration in both species (Figure 1, Table 1). According to these results, the question of whether the 200 mM salt concentration can be an overdose or not for these species comes to mind!

As salinity increased, H_2O_2 and MDA content in the leaves of two cultivars increased, in parallel (Figure 2). These increases were found important, statistically. The highest H_2O_2 and MDA content in both species were observed at 200mM salt concentration. Manivannan et al., (2015) reported that 50mM NaCl treatment significantly increased the H_2O_2 and MDA content in *Z. elegans* 'Dreamland Yellow'. A significant increase in the level of H_2O_2 (3.6-fold) and MDA (2.2 fold) was seen in cumin seedlings subjected to 100 mM salt stress for 7 days concerning 0 mM NaCl (Pandey et al., 2015). Also, it was reported that MDA content increased significantly under salt stress in rice (Shobbar et al., 2012). Similar to findings results in this study, Sevengor et al., (2011) found an increase in MDA content in four pumpkin genotypes at 100mM salt concentration, this increase was found higher in sensitive genotypes. Lipid peroxidation increased in salt stressed leaves of the salt-sensitive maize genotypes, whereas salt-tolerant plants were better protected from oxidative damage under salt stress (Neto et al., 2006). In these results, a significant difference was observed in the MDA content between two species at 200mM salt concentration. It is known that abiotic stresses, especially salinity and drought, cause a significant increase in H_2O_2 content that causes lipid peroxidation of cell membranes. (Møller et al.,2007).

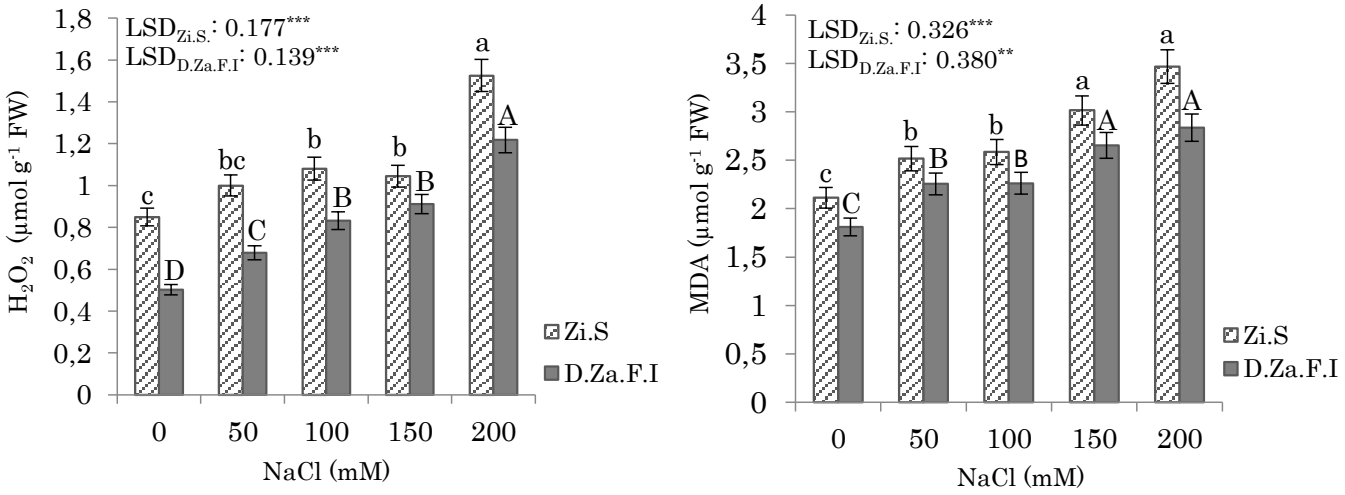

Figure 2. H_2O_2 and MDA content in two Zinnia species exposed to salinity. The data followed by the same letters in the figure show that there is no significant difference between groups (**p <0.01, ***p <0.001).

*Şekil 2. Tuzluluğa maruz kalan iki Zinnia türünde H_2O_2 ve MDA içeriği. Şekilde aynı harflerin izlediği veriler, gruplar arasında anlamlı bir fark olmadığını göstermektedir (*p <0.05, **p <0.01, ***p <0.001).*

When plants are exposed to salt stress, it activates the antioxidant defense system to eliminate the damaging effect of increased reactive oxygen (Ahmad, 2010; Acosta-Motos et al., 2017; García-Caparrós and Lao, 2018; Parvin et al., 2019; Özkoku et al., 2019). In various studies, it was shown that antioxidative defense responses differ in the plants under salt or other abiotic stress conditions (Acosta-Motos et al., 2017; García-Caparrós and Lao, 2018). The increase of SOD enzyme activity under salinity conditions indicates that SOD is good oxidative stress scavenge enzyme (Panda and Khan, 2004). It was found that 100 mM NaCl application in corn increased SOD, APX, GPX and GR activities compared to control and was more pronounced in tolerant genotypes (Neto et al., 2006). Moreover, the intracellular H_2O_2 concentration also determines the activity of antioxidant enzymes (Mittler, 2002). In several plants, salinity increased the peroxidation of lipids, implying that in the cellular membranes damages as the result of oxidative damage (Gong et al., 2005). These results indicated that the ability of plants defense against oxidative damage caused by salt stress may change (Ashraf, 2009).

CONCLUSION

Scientists who work on ornamental and landscape plants, try to understand how plants perceive and get used to stressful conditions and find out resistance mechanisms. In various studies, it has been shown by many researchers that tolerant and sensitive genotypes respond differently under salinity. Antioxidative enzyme activities play a protective role against salinity. Antioxidative defense mechanisms are effective in providing resistance to stress in Zinnia plants. It has been shown in the current study that there may be differences between Zinnia species.

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Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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