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RESPONSE OF CITRUS ROOTSTOCKS TO DIFFERENT SALINITY LEVELS FOR MORPHOLOGICAL AND ANTIOXIDATIVE ENZYME ACTIVITES

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ABSTRACT

Citrus is the most produced fruit crop and often constrained by salinity due to their geographic distribution. We investigated morphological and antioxidave enzyme responses of six different citrus rootstocks under various NaCl concentrations, including Cleopatra mandarin (*Citrus reshni* Tan.), sour orange (*C. aurantium* L.), rough lemon (*C. jambhiri* Lush.), Volkamer lemon (*C. volkameriana* Tan & Pasq.), Carrizo citrange (*Poncirus trifoliata* L. Raf. X *C. sinensis* L. Osbeck) and trifoliata orange (*P. trifoliata* Raf.). Morphological and antioxidative enzyme responses of plants grown in hydroponic media containing 0, 45, 90 and 135 mM of NaCl were assessed at day 10, 20, 30, and 40. The highest shoot growth was observed in Cleopatra mandarin whereas trifoliata orange had the lowest shoot growth. Carrizo citrange showed the lowest visible leaf symptoms among the rootstocks and rough lemon produced the highest level of root biomass. A rootstock x NaCl dose x exposure time interaction was detected for enzymatic responses. In general, ascorbate peroxidase (APX; EC: 1.11.1.11) and guaiacol peroxidase (GP; EC: 1.11.1.7) activity in the rootstocks increased with the elevated salt level. Superoxide dismutase (SOD; EC: 1.15.1.1) activity was lowered in leaves of the rootstocks subjected to higher NaCl concentrations. In conclusion, this study indicated complexity of salt tolerance mechanism probably due to complex genetic make-up of the citrus rootstocks used and importance of APX and GP in salt tolerance of *Citrus*.

Keywords: antioxidative enzyme; *Citrus*; oxidative stress; salinity; salt stress.

INTRODUCTION

Citrus fruits are the most grown crops in the world with over 120 million tons per year (FAO 2010). Citrus production spreads between 35^{o} N and S of the equator, but the most production is seen between 20^{o} N and S where moderate climate preveals. Citrus fruits are used as food and flavoring, for perfume and religious ceremonies. Their trees are subjected to biotic and abiotic stresses. Salt injury is one of the most important abiotic stresses, particularly under dry and semi-dry conditions, which limits cultivation of citrus fruits (Storey and Walker 1999).

Salt stress has two effects on plants, an initial osmotic shock followed by a toxic phase due to ion accumulation (Fernandez-Crespo *et al.* 2012). The first symptom of plants exposed to salt stress is a progressive burn of the leaf tips and borders, and the final stage of this process ends up as leaf abscission in plants (Banuls and Primo-Millo, 1995). Plants have evolved few mechanisms to overcome this problem. They may respond to salinity by overproducing compatible osmolites such as proline and absorbing ions from root medium which decrease water potential in leaf tissue, thus creating a negative pump effect to maintain water flux through xylem sap (Garcia-Sanches and Syvertsen, 2006).

Scavengers of free radicals during stress conditions may also play important roles to overcome saline conditions (Arbona *et al*. 2003). In the ascorbate-glutathione cycle, two key enzymes, ascorbate peroxidase (APX) and guaiacol peroxidase (GP), are assumed to deal with the detoxification of reactive oxygen species in plants. Another scavenger enzyme is superoxide dismutase (SOD) which converts superoxide to hydrogen peroxide. This toxic product is eliminated by APX at the expense of oxidizing ascorbate to monodehydroascorbate. Both enzymes exist as several isozymes and are active in chloroplasts and cytosol (Gueta-Dahan *et al.* 1997). The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide. Several researchers suggested that salt tolerance may not be explained only by antioxidant enzymes due to its polygenic characteristics (Gulsen *et al.* 2007; Hassine and Lutts 2010).

Differences in the relative tolerance of citrus rootstocks to salt stress have been previously reported. Cleopatra mandarin was notified as salt tolerant while rough lemon, trifoliata orange and Carrizo citrange were reported as sensitive to high salt concentration (Arbona *et al.* 2003). On the other hand, Volkamer lemon was reported as moderate in salt tolerance (Anjum *et al.*, 2000). In general, the decreasing order of salinity

tolerance is as follows: Rangpur lime = Cleopatra mandarin (*C. reshni* Hort. Ex Tan.) > sour orange (*C. aurantium* L.) > sweet orange = Swingle citrumelo > rough lemon (*C. jambhiri* Lush.) > trifoliata orange (*P. trifoliata* Raf.) (Al-Yassin 2004). On the other hand, Hassan and Galal (1989) reported that salt tolerance of citrus rootstocks decreased in following order: Rangpur, Volkamer lemon (*C. volkameriana* Ten. and Pasq.), *C. amblycarpa*, sour orange, and Cleopatra mandarin. It can be concluded that there is no agreement in the order of salt tolerance of the citrus rootstocks. This may be caused by experimental materials and conditions such as scion, climate and soil condition, age of the plant, grafted or non-grafted trees (Storey and Walker, 1999). In this study morphological and biochemical responses of six common citrus rootstocks were assessed to elucidate mechanism involved in salt tolerance. To date, this study was the most comprehensive because of the higher number of treatments used.

MATERIALS AND METHODS

Plant materials, growing conditions and experimental design: Six different citrus rootstocks used were; sour orange, trifoliata orange, Carrizo citrange, rough lemon, Volkamer lemon, and Cleopatra mandarin. They produce high ratio of apomictic embryos or nucellar seedlings, which allowed us to propagate them asexually for this study. The source trees were located at Alata Horticultural Research Institute, Erdemli, Mersin, Turkey. Seeds were collected from the trees in harvest season, and treated with benomyl to avoid fungal diseases, and sown into washed-sand as seedbed in greenhouse. Seedlings indicating similar growth pattern (plant height, leaf shape, color, etc.) were transferred to 8-liter pots included in hydroponic culture (Hoagland solution) (Hoagland and Arnon, 1950) where the rest of the study was carried out.

Treatment design was factorial [six rootstocks, four concentrations (0, 45, 90, and 135 mM NaCl), and four time intervals (10, 20, 30, and 40 days after treatment) with three replications. Experimental design was split-plot and the main plot was salt concentration. Growth media was Hoagland solution (Hoagland and Arnon 1950), and refreshed every ten days with fresh Hoagland solution. The solution was circulated through a water pump and aerated using air pump. pH of the solutions was adjusted to 6.5 ± 0.1 by using 1N H_2SO4 or KOH. During the experimental period, temperature was between 22 and 30 ^oC and relative humidity was between 65 and 80%. When seedlings reached a height of 60 cm, salt treatments (0, 45, 90, and 135 mM) were initiated. The experiment was carried out under day light conditions in a semi-controlled glasshouse from May to September.

Assessment for morphological responses: Observations for plant height, dry root weight, and level of salt injury were made every ten days. Dry root weights were measured after exposing fresh root tissues at 70 ^oC for 72 h. Salt injury was assessed according to Kusvuran *et al.* (2007) with some modifications using a scale of 0 to 5 where 0: no injury, 1: slowing down in growth and local chlorosis, 2: chlorosis and 25% of leaf necrotic spot, 3: 25-50% of leaf necrotic spot and fall of leaf, 4: 50-75% of leaf necrotic spot, and 5: 75-100% injury or loss of plant. Intermediate leaves of each plant were collected at day 0, 10, 20, 30, 40 and stored at –80 ^oC to assess antioxidative enzyme responses.

Enzyme extraction and assays: Soluble proteins were extracted from 20 mg of plant tissue using a standard sap extraction method described by Gulsen *et al.* (2010a) with little modifications. Plant tissues were placed between two rollers of a sap extraction apparatus (Ravenel Specialities Co., Seneca, SC, USA). One and a half mL of 20 mM HEPES buffer at pH 7.2, containing a protease inhibitor cocktail [0.3 g/1 g of tissue of 4-(2-aminoethyl) benzenesulfonyl fluoride, bestatin, pepstatin A, E-64, leupeptin, 1,10-phenanthroline (Sigma, St. Louis, MO, USA)] and 1% polyvinylpyrrolidone (PVP) was dropped on the top of the roller. The homogenate was collected from the bottom of the roller and centrifuged at 15.000 rpm for 15 min at 4 °C. The supernatant was collected and placed at 4 °C for further analysis.

Total protein content was determined using a commercially available (BCA) protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard as described by Gulsen *et al.* (2010a). Triplicate aliquots of each sample were measured using a semi-automated microplate reader, PowerWave (BIO-TEK Instruments, Inc., Winooski, VT, USA) for assessing total protein concentration and the following antioxidant enzyme activities.

Ascorbate peroxidase (APX) activity was estimated according to Nakano and Asada (1981) with some modifications. The reaction mixture contained 50 mM potassium-phosphate buffer (pH 7.6), 0.25 mM L (-) ascorbic acid, 12 mM H_2O_2 and 1 µg/mL protein extract. Volume of reaction mixture was adjusted to 200 µL and the reaction was read every 30 s at 2 min. Activity of APX was calculated from the oxidized ascorbate concentration by using the extinction coefficient of 2.8 mM^{-1} cm^{-1}. One enzyme unit was defined as mmol ml^{-1} oxidized ascorbate per min. Guaiacol peroxidase (GP) activity assay was performed as described by Gulsen *et al.* (2010a). Two µL of 30% hydrogen peroxide to wells of a 96- well microplate containing 60 µL of 18 mM guaiacol, 20 µL of 200 mM HEPES (pH 7.0), 117 µL of distilled water and 1 µL of 1 µg/mL enzyme extract. Increase in absorbance at 470 nm was monitored for 2 min. The specific activity of peroxidase was determined using the molar absorptivity of guaiacol at 470 nm (26,6 X 10^3 M^{-1} cm^{-1}).

Superoxide dismutase (SOD) activity was measured as performed by Cakmak and Marschner (1992) with some modifications. The final volume of reaction mixture was 200 µL containing 50 mM sodium phospate, 0.1 mM EDTA, 4 µL of 1 µg/mL enzyme extract, 12 mM L-methionine, 75 µM *p*-nitro blue tetrazolium chloride (NBT) and 10 µM riboflavine. Reaction was carried out under light for 15 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of p-nitro blue tetrazolium chloride reduction at 560 nm.

Statistical analyses: Statistical analyses were carried out using JMP v.5 software (SAS Institute, Cary, NC, USA) to detect differences in shoot growth, fresh and dry root weight and visible leaf injury of rootstocks exposed to different salinity levels. Means were separated and grouped (a, ab, abc, b, etc.) using Student's t multiple range test ($P < 0.05$). Antioxidant enzyme activity levels among treatments showed using graphics with standard errors bars.

RESULTS

Plant growth: Plant growth was significantly affected by increased salinity. During the experimental period, control plants continued to grow normally, while growth of salt treated plants decreased at 45, 90 and 135 mM salt doses (Table 1). However, shoot growths of Carrizo and Cleopatra were slightly higher at 135 mM salt treatment than 90 mM treatment. The maximum plant growth was observed in Cleopatra at control, 45 and 90 mM salt concentrations compared to the other rootstocks used. Trifoliata orange showed the least plant growth at all salt concentrations used. The shoot growth of Volkamer lemon, rough lemon and sour orange decreased at 135 mM NaCl to over 70% of that in the control. Some leaves showed yellow or brown necrotic tissue with varying levels depending on salt concentration and rootstock.

There were significant differences in root fresh and dry weights among rootstocks at all salt concentrations (Table 2). In control plants, shoot, root fresh and dry weights of rough lemon were the highest whereas those of Cleopatra were the lowest. Rough lemon also indicated the highest fresh and dry root weight at all salt concentrations except dry weights at 45 mM salt dose. Cleopatra and trifoliata orange had the lowest fresh and dry root weight at all NaCl treatments. Root biomass of rootstocks generally decreased with increased NaCl level. Fresh and dry weight of Carrizo citrange increased at 45 mM NaCl treatment compared to the control. At 90 and 135 mM NaCl doses, fresh and dry weight of this rootstock decreased slowly. Root fresh weight of rough lemon was reduced sharply at 45 mM NaCl, and this value increased at 90 mM.

Leaf symptoms: Visible symptoms of leaf damage induced by NaCl stress were observed over a 2-month period. Leaf symptoms were generally chlorotic and brown-black necrotic spots. The first leaf symptoms were detected 20 days after treatment (DAT). Control and 45 mM NaCl-treated plants showed no symptoms throughout the experimental period (Table 3). Visible toxicity symptoms were not observed in Cleopatra and Carrizo at 90 mM NaCl. All rootstocks were affected with varying degree at 135 mM NaCl. The most affected rootstock was rough lemon, being similar to trifoliata orange and Volkamer lemon. Carrizo citrange exhibited the lowest visible leaf symptoms among the rootstocks.

Antioxidative enzyme activity: Few antioxidative enzyme activities were determined in leaves of control and NaCl-treated plants in order to assess responses of the six citrus rootstocks to NaCl-induced oxidative stress. For APX activity, a three-way interaction among rootstock, time and NaCl dose was detected ($F = 2.06$; $P < 0.001$). At 135 mM NaCl, leaves of salt treated plants showed significantly higher levels of APX than that in control plants (Fig. 1). APX activities of rough lemon, Volkamer lemon and Cleopatra mandarin increased at all doses (45, 90 and 135 mM) over the four time intervals (10, 20, 30 and 40 DAT). In sour orange, APX activity increased at 20 DAT. At 40 DAT, APX activity was lower than control in trifoliata orange at 45 and 90 mM treatments whereas it was not different from that of control at 135 mM NaCl.

For GP, a three-way interaction among rootstock, salt dose and sampling time was detected ($F = 2.78$; $P < 0.001$). At 40 DAT, GP level of trifoliata orange, rough lemon, Carrizo citrange and Volkamer lemon were higher than that of control at 135 mM salt concentration except sour orange and Cleopatra mandarin (Fig. 2). On the other hand, GP activity of sour orange was lower than that of control at all concentrations at 20 DAT. The enzyme activity showed little changes in Cleopatra mandarin at 30 and 40 DAT. Salt-induced decrease was observed in GP activity for Volkamer lemon during 20 days and then enzyme activity increased. The highest enzyme activity in relation to control was observed in salt-sensitive rough lemon at 135 mM salt stress (~2.5-fold higher than that in the control) at 40 DAT.

In general, change of SOD activity in leaves of rootstocks was different from the other enzymes studied. A three-way interaction among rootstock, time and salt dose was not detected for SOD ($F = 0.79$). However, two-way interactions were detected. At 40 DAT, SOD activities of all rootstocks were lower than the control (Fig. 3). In general, increase in salt dose lowered SOD activity in all rootstocks. The most drastic decrease in enzyme activity was determined in trifoliata orange compared to the control at 40 DAT. In rough lemon, enzyme level of salt treated plants was higher than the control at 30 DAT while treated and control plants showed similar level of SOD activity at 40 DAT.

Table 1. Shoot growth (cm) of rootstocks under different level of salinity.

Rootstocks	Control	45 mM[x]	90 mM	135 mM
Sour Orange	41.8[y] [abz]	31.7 [c]	23.1 [ab]	11.6 [b]
Trifoliata orange	10.9 [c]	11.2 [d]	6.1 [c]	5.1 [b]
Rough lemon	45.4 [a]	48.8 [ab]	30.1 [a]	12.7 [b]
Cleopatra	65.2 [a]	58.2 [a]	21.4 [ab]	27.7 [a]
Carrizo	18.8 [bc]	11.9 [d]	6.6 [c]	8.2 [b]
Volkamer lemon	56.8 [a]	43.9 [bc]	16.4 [bc]	13.4 [b]
P value	24.2	13.9	11.2	13.0

[x]Salt level treated [y]Mean of 3 replicates
[z]Mean seperation within columns and variable by Student's t multiple range test. $P < 0.05$.

Table 2 Fresh (FW) and dry root weight (DW) of rootstocks under different level of salinity.

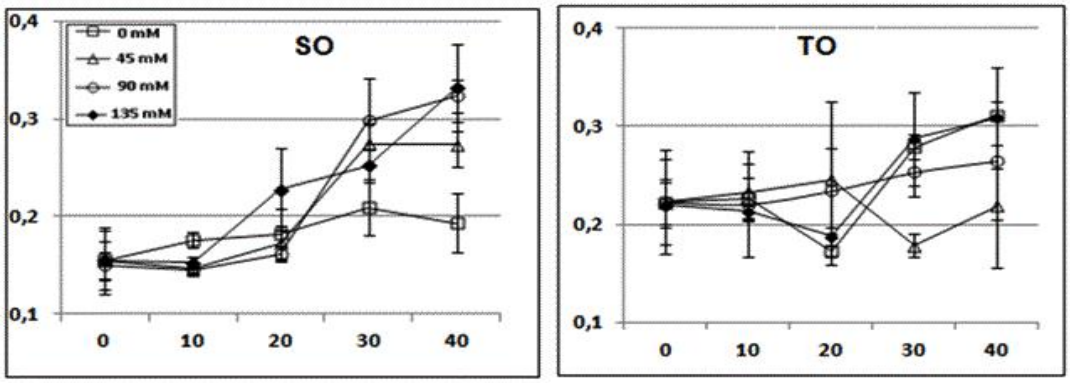
	Control		45 mM[x]		90 mM		135 mM	
Rootstocks	FW (g)	DW (g)	FW (g)	DW (g)	FW (g)	DW (g)	FW (g)	DW (g)
Sour Orange	9.41[y] [abz]	3.58 [b]	5.89 [bc]	2.86 [b]	6.86 [b]	2.21 [bc]	6.70 [bc]	2.54 [a]
Trifoliata orange	4.33 [b]	1.48 [c]	2.49 [de]	1.35 [d]	2.72 [b]	0.88 [d]	2.73 [c]	0.90 [b]
Rough lemon	18.35 [a]	5.79 [a]	16.11 [a]	3.71 [a]	15.46 [a]	4.31 [a]	11.60 [a]	3.45 [a]
Cleopatra	3.00 [b]	1.17 [c]	2.14 [e]	1.13 [d]	3.84 [b]	1.25 [cd]	3.37 [c]	1.10 [b]
Carrizo	3.93 [b]	1.55 [c]	5.22 [cd]	2.16 [c]	4.02 [b]	1.63 [bcd]	3.53 [c]	1.26 [b]
Volkamer lemon	9.55 [ab]	2.93 [bc]	8.16 [b]	4.01 [a]	7.03 [b]	2.28 [b]	7.92 [ab]	2.82 [a]
P value	9.01	2.00	2.83	0.65	7.62	1.01	4.12	1.11

[x]Salt level treated [y]Mean of 3 replicates
[z]Mean seperation within columns and variable by Student's t multiple range test. $P < 0.05$.

Table 3 Visible leaf injury of rootstocks occurred by different level of salinity.

Rootstocks	90 mM[x]	135 mM
Sour Orange	0.67[y] [bcz]	1.00 [ab]
Trifoliata orange	1.33 [ab]	2.33 [a]
Rough lemon	2.00 [a]	2.67 [a]
Cleopatra	0.00 [c]	1.67 [ab]
Carrizo	0.00 [c]	0.33 [b]
Volkamer lemon	1.00 [abc]	2.33 [a]
P value	1.18	1.87

All rootstocks showed no symptoms for control and 45 mM salt treatment.
[x]Salt level treated
[y]Mean of 3 replicates. Each value was generated according to 0 to 5 scale.
[z]Mean seperation within columns and variable by Student's t multiple range test. $P < 0.05$.



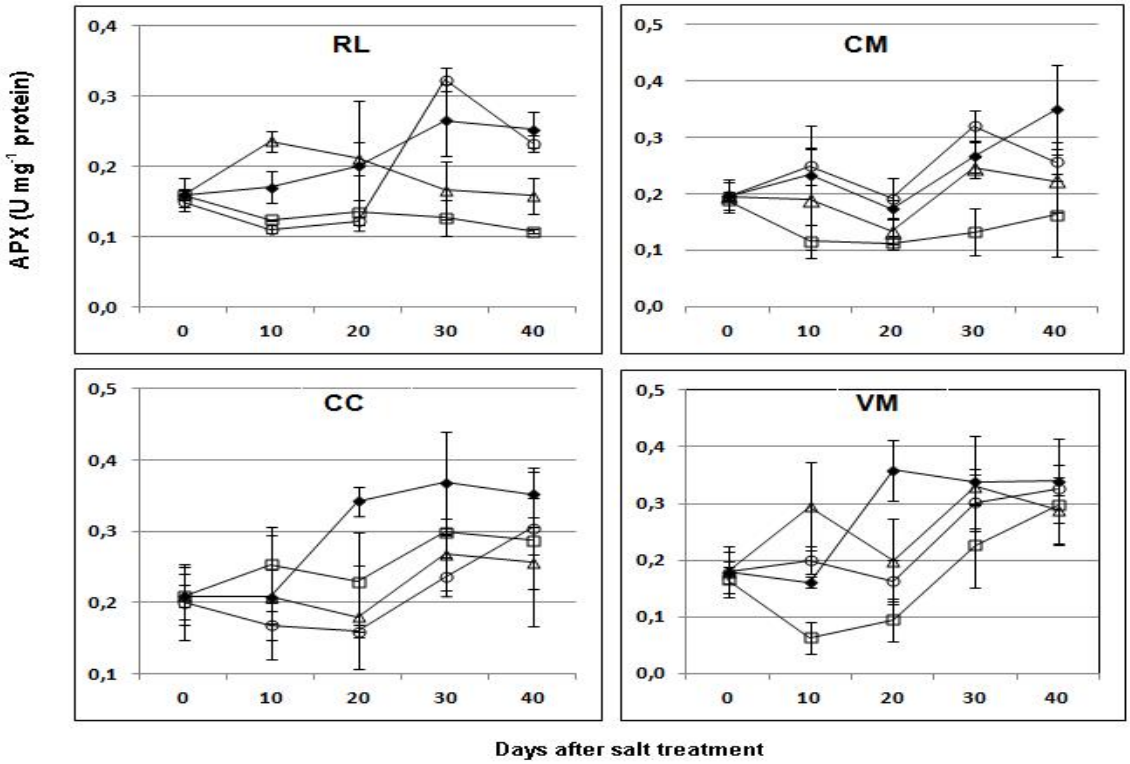
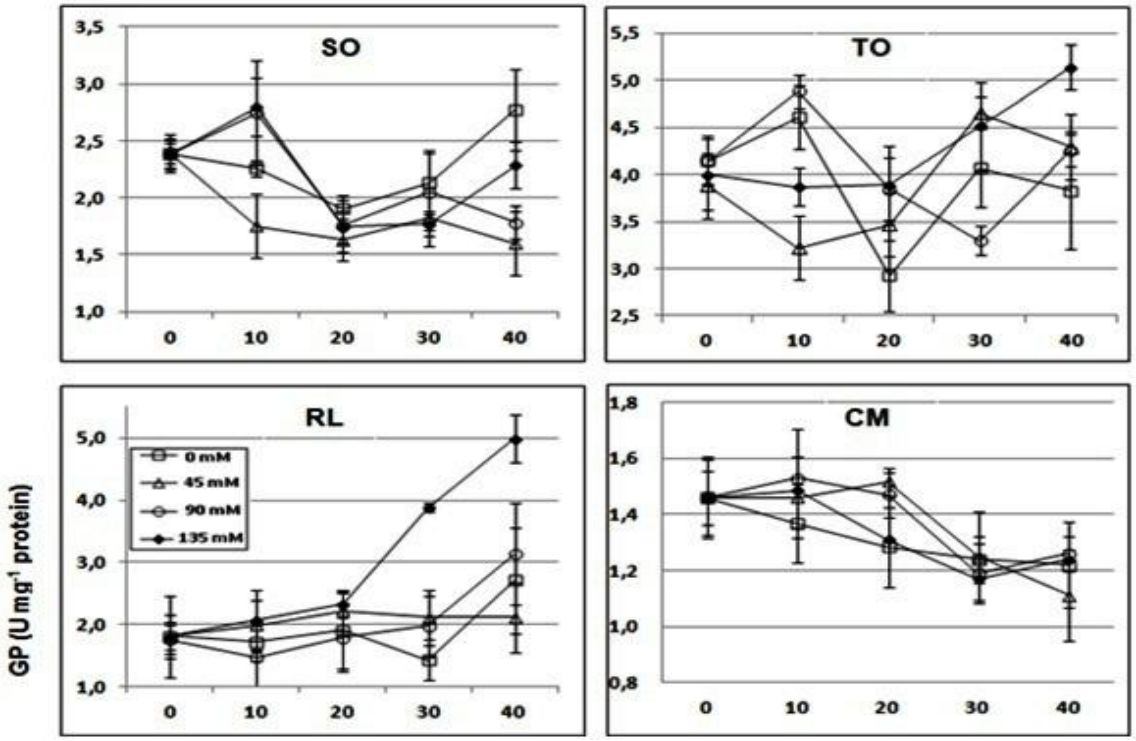


Figure 1 APX activity of rootstocks leaves under different salinity levels. Enzyme activity was expressed as units (mg protein)$^{-1}$. Each point is the average value of three independent measurements. SO: sour orange, TO: trifoliata orange, RL: Rough lemon; CM: Cleopatra mandarin, CC: Carrizo citrange, VM: Volkamer lemon



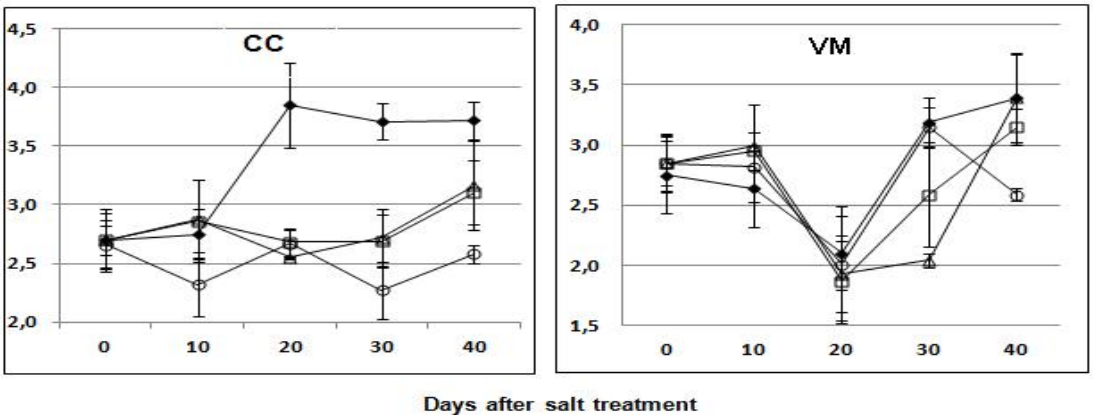


Figure 2 GP activity of rootstocks leaves under different salinity levels. Enzyme activity was expressed as units (mg protein)$^{-1}$. Each point is the average value of three independent measurements. SO: sour orange, TO: trifoliata orange, RL: Rough lemon; CM: Cleopatra mandarin, CC: Carrizo citrange, VM: Volkamer lemon

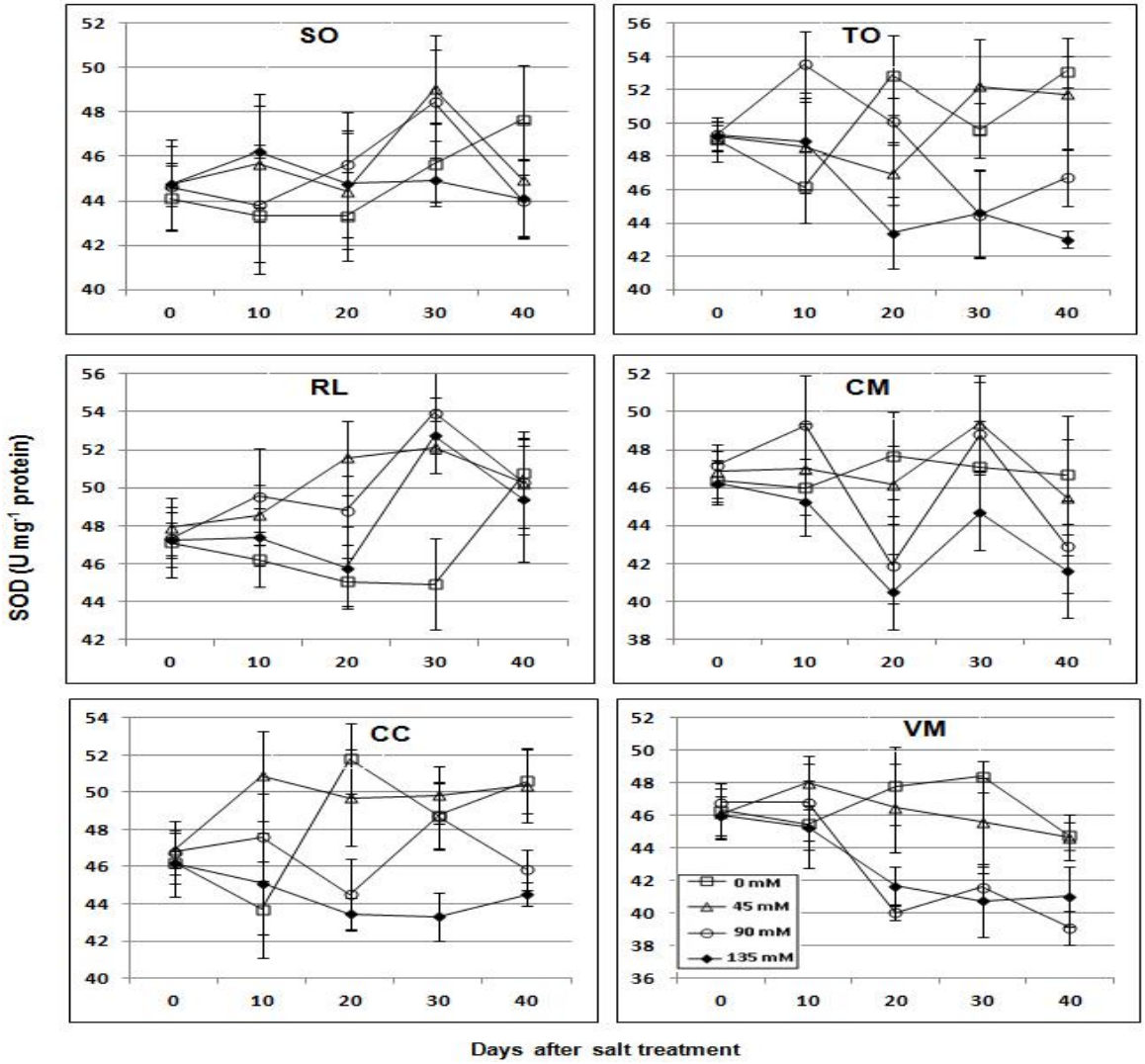


Figure 3 SOD activity of rootstock leaves under different salinity levels. Enzyme activity was expressed as units (mg protein)$^{-1}$. Each point is the average value of three independent measurements. SO: sour orange, TO: trifoliata orange, RL: Rough lemon; CM: Cleopatra mandarin, CC: Carrizo citrange, VM: Volkamer lemon

DISCUSSION

Salinity adversely affected plant growth in all rootstocks, but degree of this effect varied among the rootstocks (Table 1). It was reported plant adaptations to salinity are of three distinct types: osmotic stress tolerance, Na^+ or Cl^- exclusion, and the tolerance of tissue to accumulated Na^+ or Cl^-. (Munns and Tester 2008). In our study, the most growth reduction was determined in Volkamer lemon (~76% compared to the control) followed by sour orange and rough lemon. These species were previously reported as moderate in salt tolerance (Anjum *et al.,* 2000), however, present investigation proved them as sensitive to salt tolerance Rough lemon was found to be salt sensitive in our study. Trifoliata orange and Carrizo citrange produced the lowest plant growth at all salt concentrations. This might be caused by trifoliata that has relatively slow growth rate in normal growth conditions. Reduction in plant growth of the citrus rootstocks caused by salinity was previously reported. Ratio of plant growth reduction in our study was higher than that in Anjum *et al.* (2000). This might be caused by different growing conditions used, as in present study hydroponic culture was while other used commercial nursery soil. These results were also supported by Perez-Tornero *et al*. (2009). Salinity effect also varied in other crops such as among the wheat cultivars in previous study (Din *et al*. 2008).

All growth variables (shoot length, root number and length, shoot/root fresh/dry weight, root biomass) decreased significantly by NaCl treatments. Several reasons may cause these adverse effects. It was reported that plants have to spend more energy to extract water from saline solution, resulting in poor turgor pressure (Hassine and Lutts, 2010). Sattar *et al.* (2010) reported that survival percentage of cotton seedlings was significantly reduced by all the concentrations of the salt solution. Therefore, in addition to osmotic effects and inhibitory effects of high concentrations of Cl^- and Na^+, imbalance of essential nutrients may also contribute to reduction in plant growth under saline conditions (Anjum *et al.* 2000). Understanding these relationships would be important, but, unfortunately, this was beyond the aim of our study.

The first symptom of leaves was a progressive burn of leaf tips followed by leaf abscission in citrus (Banuls and Primo-Millo 1995). Further, exposure of plants to salt stress caused dieback in young shoots of the six rootstocks studied as also reported by Mer *et al.* (2000). In this study, some leaf injury was observed in salt-treated plants (Table 3). In general younger leaves rolled while elder leaves showed dried necrotic lesions when subjected to elevated and extended salt stress. No leaf abscission was observed during the experimental period, but chlorosis and necrotic spots on leaves were present.

Some discrepancies between the results of this and previous studies were detected. Cleopatra mandarin was reported to be more salt tolerant than sour orange (Arbona *et al.* 2003; Al-Yassin 2004). In contrast Cleopatra was found to be less tolerant to salinity than sour orange in another study (Hassan and Galal, 1989). Our study agreed with the second. Carrizo was the least affected rootstock from salinity for leaf injury in this study. The similar results were reported by (Zekri, 1993). In contrast Carrizo citrange were reported as salt sensitive (Al-Yassin, 2004). These were examples of inconsistencies in the ranking of rootstocks for salt tolerance. It was reported that age of citrus plants, experimental media (water, soil etc.) and salt types were critical factors in assessing citrus responses to salinity (Storey and Walker, 1999). Without direct comparisons of, for example, rootstocks of the same clone or growing conditions, it is impossible to determine whether experimental differences are responsible (Maas, 1993). Genome of these citrus rootstocks is made of several species and clonal variations in citrus are widespread (Uzun *et al.,* 2009; Gulsen and Roose, 2001a). Results of this study together with previous reports indicated that accordance of experimental procedures (i.e. growth media, salt source) and materials (plant age, genotypic variation) are crucial for avoiding inconsistencies among studies.

Antioxidative enzyme responses of the six citrus rootstocks subjected to varying levels of NaCl concentrations indicated complex mechanism. Two and three-way interactions among rootstocks, salt concentrations and time intervals were detected for APX, GP and SOD activity. Although a three-way interaction among rootstocks, salt concentrations, and sampling time was detected, the APX activity generally increased with elevated salinity level except trifoliata orange (Fig. 1). On the other hand, GP activity increased in most of the rootstocks (Fig. 2). The rootstocks showed different trends in antioxidative responses ranging from zigzagging to clear trends. Probably, each of cultivars has unique response to salinity stress.

SOD enzyme was localized in chloroplast, mitochondrion, cytoplasm and peroxisome acts as the first line of defence against ROS by dismutating O_2 to H_2O_2 (Liau *et al.,* 2007). In this study although there was no three-way interaction, two-way interactions were observed. This may indicate less complexity of SOD-based salt tolerance in citrus. SOD activity of the six rootstocks differed during the experimental period (Fig. 3). In rough lemon, SOD activity was higher or equal than the control, and sour orange showed zigzag, while the other rootstocks decreased SOD activities compared to the control. In previous studies the similar trends were also reported in lemon (Piqueras *et al*., 1996), Carrizo citrange (Arbona *et al.*, 2003), Valencia and Carvalhal (Ferreira and Lima-Costa, 2006). These findings agreed

with our results with non-linear increasing or decreasing of SOD activity in response to salt concentrations.

For antioxidative enzyme responses, two- or tree-way interactions among rootstocks, salt concentrations and sampling time indicated complexity of salt tolerance mechanism in the six citrus rootstocks, which were probably caused by their genetic make-up contributed by different genera and species as observed in Carrizo citrange (Gulsen and Roose, 2001b). Probably different plant species evolved independent defense mechanism against saline conditions. In addition these stress-related gene families located in cytoplasmic organelle (mitochondria, chloroplast) of cells were reported to evolve in different pace and mode of diversification compared to those located in nucleus, resulting in different defence mechanisms among the *Citrus* species (Clegg *et al.* 1984). For example manganese-dependent SOD encoding gene is localized in mitochondria of plants (Moller, 2001). This may partially explain variations in antioxidative enzyme responses of under similar stress factors. Molecular studies such as peroxidase specific markers and differential analysis of transcripts and proteins might be useful to understand salt tolerance mechanism and genetics (Gulsen *et al.* 2010b; Maserti *et al.* 2011).

To date this study was the most comprehensive because of higher number of treatments of rootstocks, antioxidative enzymes and time intervals. We investigated responses of the six common citrus rootstocks (trifoliata orange, sour orange, rough lemon, Volkamer lemon, Carrizo citrange, and Cleopatra mandarin) in relation to three major antioxidative enzymes (APX, GP, and SOD) and up to 40 days (10, 20, 30, and 40 days) under hydroponic growth conditions containing three different NaCl concentrations. This study provided valuable insights into mechanisms of salt tolerance of citrus.

REFERENCES

Al-Yassin, A. (2004). Influence of salinity on citrus. J. Central Eur. Agric. 5: 263-272.

Anjum, M.A., M. Abid and F. Naveed (2000). Effect of soil salinity on the performance of some citrus rootstocks at seedling stage. Pakistan J. Biol. Sci. 3:1998-2000.

Arbona, V., V. Flors, J. Jacas, P. Garcia-Agustin and A. Gomez-Cadenas (2003). Enzymatic and non-enzymatic antioxidant responses of Carrizo citrange, a salt-sensitive citrus rootstock, to different levels of salinity. Plant Cell Physiol. 4:388-394.

Arbona, V., Z. Hossain, M.F. Lopez-Climent, R.M. Perez-Clemente and A. Gomez-Cadenas (2008). Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. Physiol. Plant. 132:452-466.

Banuls, J. and E. Primo-Millo (1995). Effects of salinity on some Citrus scion-rootstocks combinations. Annals of Botany, 76:97-102.

Bar, Y., A. Apelbaum, U. Kafkafi and R. Goren (1997). Relationship between chloride and nitrate and its effect on growth and mineral composition of avocado and citrus plants. J. Plant Nut. 20: 715-731.

Cakmak, I. and H. Marschner (1992). Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. Plant Physiol. 98: 1222-1227.

Clegg, M.T., J.R.Y. Rawson and K. Thomas (1984). Chloroplast DNA variation in pearl millet and related species. Genetics, 106: 449–461.

Din, J., S.U. Khan and I. Ali (2008). Physiological response of wheat (*Triticum aesitivum* L.) varieties as influenced by salinity stres. The J. of Animal and Plant Sci. 18: 125-129.

FAO, (2010). FAOSTAT Online Database. Food and Agriculture Organization of the United Nations, Online available: http://faostat.fao.org. Accessed 23 Jan 2012.

Fernandez-Crespo, E., R. Gomez-Pastor., E. Matallana., L. Lapena., L. Scalschi., G. Camanes and P. Garcia-Agustin. (2012). Role of ammoium nutrition on salt-induced oxidative stres in 'Carrizo' citrange plants. XII. Int. Citrus Congress, Abstract Book, p: 122.

Ferreira, A.L. and M.E. Lima-Costa (2006). Metabolic responses to salt stress in cell suspension cultures of sensitive and resistant *Citrus.* J. Hort. Sci. Biotech. 81:983-988.

Garcia-Sanchez, F. and J.P. Syvertsen (2006). Salinity tolerance of Cleopatra mandarin and Carrizo citrange citrus rootstocks seedlings is affected by CO_2 enrichment during growth. J. Amer. Soc. Hort. Sci. 131:4-31.

Gueta-Dahan, Y., Z. Yaniv, B.A. Zilinskas and G. Ben-Hayyim (1997). Salt and oxidative stress: s imilar and speciec responses and their relation to salt tolerance in Citrus. Planta, 203:460-469.

Gulsen, O. and M.L. Roose (2001a). Lemons: diversity and relationships with selected Citrus genotypes as measured with nuclear genome markers. J. Amer. Soc. Hort. Sci. 126:309-317.

Gulsen, O. and M.L. Roose (2001b). Chloroplast and nuclear genome analysis of the parentage of lemons. J. Amer. Soc. Hort. Sci. 126:210-215.

Gulsen, O., R.C. Shearman, T.M. Heng-Moss, N. Mutlu, D.J. Lee and G. Sarath (2007). Peroxidase gene polymorphism in buffalograss and other grasses. Crop Science, 47:767-772.

Gulsen, O., T. Eickhoff, T.M. Heng-Moss, R. Shearman, F. Baxendale, G. Sarath and D. Lee (2010a). Characterization of peroxidase changes in resistant and susceptible warm-season urfgrasses challenged by *Blissus occiduus.* Arthropod-Plant Interac. 4:45-55.

Gulsen, O., S. Kaymak, S. Ozongun and A. Uzun (2010b). Genetic analysis of Turkish apple germplasm using peroxidase gene-based markers. Sci. Hortic. 125:368-373.

Hassan, M.M. and M.A. Galal (1989) Salt tolerance among some citrus rootstocks. Agric. Sci. J. King. Saud Univ. 1:87-93.

Hassine, A.B. and S. Lutts (2010). Differential responses of saltbush *Atriplex halimus* L. Exposed to salinity and water stress in relation to senescing hormones abscisic acid and ethylene. J. Plant Physiol. 167:1448-1456.

Hoagland, D.R. and D.J. Arnon (1950). The water culture method for growing plants without soil. Circular No. 347. California Agricultural Experimental Station, Berkeley, USA.

Kusvuran, S., S. Ellialtioglu, K. Abak and F. Yaşar (2007). Bazı Kavun (*Cucumis* sp.) genotiplerinin tuz stresine tepkileri. Ankara Univ. Tarım Bil. Der. 13:395-404 (in Turkish).

Liau, Y., L. Wen, J. Shaw and C. Lin (2007). A highly stable cambialisticsuperoxide dismutase from *Antrodia camphorata*: expression in yeast and enzyme properties. J. Biotechnol. 131:84-91.

Maas, E.V. (1993). Salinity and citriculture. Tree Physiol. 12:195-216.

Maserti, B.E., R. Del Carratore, C.M. Della Croce, A. Podda, Q. Migheli, Y. Froelicher, F. Luro, R. Morillon , P. Ollitrault, M. Talon and M. Rossignol (2011). Comparative analysis of proteome changes induced by the two spotted spider mite *Tetranychus urticae* and methyl jasmonate in citrus leaves. J. Plant Physiol. 168:392-402.

Mer, R.K., P.K. Prajith, D.H. Pandya and A.N. Pandey (2000). Effect of salt on germination of seeds and growth young plants of *Hordeum vulgare*, *Triticum aestivum, Cicer arietinum and Brassica juncea*. J. of Agronomy and Crop Sci.185: 209-217.

Moller, I. (2001). Plant mitochondria and oxidative stress: Electron Transport, NADPH Turnover, and Metabolism of Reactive Oxygen Species. Ann. Rev. Plant. Physiol. Plant Mol. Biol. 52: 561–91.

Munns, R. and M. Tester (2008).Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59: 651-681.

Nakano, Y. and K. Asada (1981). Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-880.

Perez-Tornero, O., C.I. Tallon, I. Porras and J.M. Navarro (2009). Physiological and growth changes in micropropagated *Citrus macrophylla* explants due to salinity. J. Plant Physiol. 166: 1923-1933.

Piqueras, A., J.A. Hernandez, E. Olmos, E. Hellin and F. Sevilla (1996). Changes in antioxidant enzymes and organic solutes associated with adaptation of citrus cells to salt stress. Plant Cell Tissue Org. Cult. 5: 53-60.

Sattar, S., T. Husnain and A. Javaid (2010). Effect of NaCl salinity on cotton (*Gossypium arboreum* L.) grown on MS medium and in hydroponic cultures. The J. Anim. Plant Sci. 20: 87-89.

Storey, R., and R.R. Walker (1999). Citrus and salinity. Sci. Hort. 78: 39-81.

Uzun, A., T. Yesiloglu, Y. Aka-Kacar, O. Tuzcu and O. Gulsen (2009). Genetic diversity and relationships within Citrus and related genera based on sequence related amplified polymorphism markers (SRAPs). Sci. Hortic. 121: 306-312.

Zekri, M. (1993). Effects of salinity and calcium on seedling emergence, growth and sodium and chloride concentrations of citrus rootstocks. Proc. Flo. State Hort. Soc. 106: 18-21.