Australian Journal of Crop Science AJCS
AJCS 7(10):1436-1441 (2013) ISSN:1835-2707

Comparative studies between diploid and tetraploid Dez Orange [*Citrus sinensis* (L.) Osb.] under salinity stress

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Abstract

Due to salt-sensitivity nature of citrus, it is imperative to provide rootstocks and cultivars that are tolerant to saline conditions. In this study, responses of tetraploid (4X) and its corresponding diploid (2X) Dez orange (*Citrus sinensis* (L.) Osb.) cultivar to different concentrations of NaCl (0, 20, 40 and 60 mM) were investigated using a randomized complete block design with four replications. Plant mineral concentrations, proline accumulation, malondialdehyde (MDA) and H_2O_2 contents were measured. Results indicated that increasing NaCl concentration significantly reduced leaf N and P contents in both diploid and tetraploid plants but reduction of these nutrients were lower in tetraploids (2.15 to 1.51%, 2.35 to 1.78% in diploid and tetraploid plants respectively, at $P \leq 0.05$). Results also showed that the accumulation of Na and Cl was lower in tetraploids. Reduction of K in tetraploid was lower as opposed to diploid plants (1.18 and 0.98% in leaves and, 0.50 and 0.48 in roots at 60 mM NaCl, respectively). Free proline content in the leaves of both plants increased with increasing NaCl level with a more marked increase was observed in tetraploid plants than those in diploids (62.3 and 54.8 µmol/g FW at 60 mM NaCl, respectively). Tetraploid plants contained lower concentration of MDA (37.1 and 44.3 nmol/g FW at 60 mM NaCl, respectively) and H_2O_2 (58.8 and 62.5 µmol/g FW at 60 mM NaCl, respectively). Results obtained in this study suggested that tetraploid Dez orange exhibited a better adaptation to salinity stress than its corresponding diploid in its seedling stage.

Keywords: Citrus, diploid, tetraploid, salt stress.
Abbreviations: MDA-malondialdehyde; H_2O_2-hydrogen peroxide; ROS-reactive oxygen species; TBA-thiobarbituric acid; TCA-trichloroacetic acid; KI-potassium iodide; DMRT-Duncan's Multiple Range Tests.

Introduction

Soil salinity is one of the major important abiotic stresses and plants expose to the stress may experience a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Gurmani et al., 2011). High NaCl salinity influence plant growth and development by affecting plant water status, causing nutrient imbalance and inducing specific ion toxicity which is related with excessive Na and Cl uptake (Marschner, 1995). For example, salinity was reported to affect germination rate and growth of seedlings through its effect on roots and shoots, chlorophyll content, malondialdehyde (MDA) accumulation and H_2O_2, concentration of mineral contents and their partitioning, and on activities of antioxidant enzymes (Meng et al., 2011). Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are a product of normal cellular metabolism, but under salinity stress, the balance between the production and the elimination of this substance is disturbed in cellular components of plants. Malondialdehyde (MDA), which is the first product formed during the lipid peroxidation reaction could be elevated under stress condition may indicate the decomposition of polyunsaturated fatty acids in the membranes. Thus, measurement of MDA production is often performed to show the level of oxidative damage in cells for the purpose of determining membrane function (Sheokand et al., 2008). In managing osmotic stress occurred during salt stress, plant tends to accumulate higher concentration of osmoticum. This is clearly seen as the ability of plant to increase the concentration of proline in tissues that would play important roles in the adjustment of its cellular water, detoxification of ROS, protection of membrane integrity, and stabilization of proteins and enzymes, thus help the plants in adapting to various stress conditions, particularly salinity. To overcome abiotic stress, plants tend to develop a series of complex nutritional, molecular and biochemical regulation mechanisms (Sardella et al., 2004). The role played by K, Na and Cl in salt tolerance species have been extensively reported (Amtmann et al., 2004; Rodriguez-Navarro and Rubio, 2006). According to Gurmani et al. (2011), the issue of salt tolerance plant is not only necessitated by adaptation to Na toxicity but rather by the presence of abundant K whose uptake by plant cell is considerably affected by high external Na concentration. Bayat et al. (2011) hypothesized that improved salinity tolerance in plants can likely be attained by devising mechanism that allows a plant to maintain desirable K/Na ratio in cytosol. It has been previously reported that all species in the genus *Citrus* are

Table 1. The ANOVA of mean square (MS) values of N, P, K, Na, Cl, K/Na, Proline, MDA and H_2O_2 content.

Dependent variables	Sources of variation					
	NaCl		Cultivar		NaCl × Cultivar	
	Leaf	Root	Leaf	Root	Leaf	Root
N	6.41 **	0.263**	7.26 **	0.056**	2.69 **	0.009**
P	1.45 **	0.001**	4.82 **	0.001**	0.23 **	0.0**
K	0.12 **	0.096*	0.117 **	0.107**	0.098 **	0.009**
Na	0.170 *	0.125*	0.017 *	0.125**	0.069 **	0.060**
Cl	21.528 **	1.903*	4.626 **	1.051**	0.95 **	0.048*
K/Na	0.195 *	0.221**	1.54 **	0.049**	0.185 *	0.01**
Proline content	0.002 **	-	0.0004 **	-	0.0005 **	-
MDA content	0.171 **	-	0.053 *	-	0.046 *	-
H_2O_2 content	107.52 **	-	14.839 *	-	44.532 **	-

*, ** significant difference at P≤0.05 and 0.01

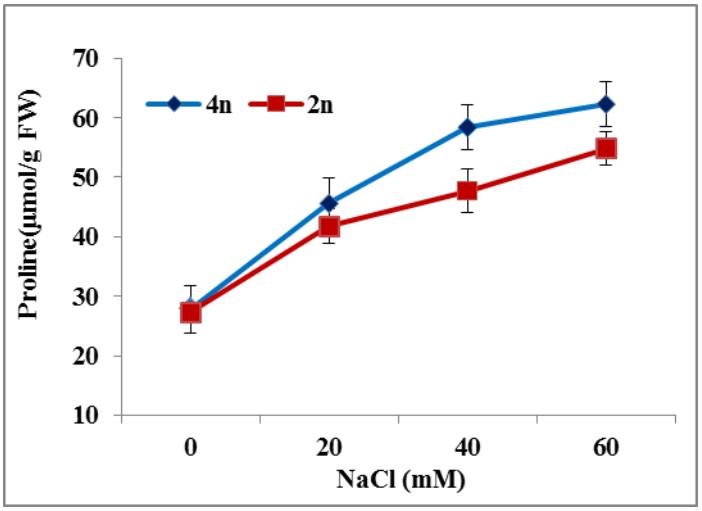

Fig 1. Comparison between the tetrapolyploid (4n) and diploid (2n) Dez sweet orange in term of leaf proline content.

diploid, with 2n= 2x=18 chromosomes (Rose et al., 1998) and they are easily crossed amongst themselves producing fertile hybrids (Barrett, 1992). Several studies have revealed that chromosome doubling in citrus, may possibly arise during seed formation and tetraploid seedlings arise spontaneously in nuclear cells (Cameron and Soost, 1968). Zhang et al. (2010) stated that polyploidy seems to have the potential to enhance accumulation of secondary metabolites and may increase tolerance of plants to a number of abiotic stresses. The occurrence of tetraploidy in some citrus species has led to smaller, more desirable trees with larger fruits and possibly superior drought tolerance in contrast to the original diploids (Barrets, 1992). In general, polyploidy in many of species is often associated with several changes in plant physiological parameters such as increase in mesophyll cell volume, thickness of leaves, increase in the concentration of photosynthetic enzymes and pigments in the cell in contrast to diploids. . The superiority of polyploids over their diploids counterparts in terms of genetic adaptability and tolerance to environmental stresses is believed to have a significant improvement in enhancing agricultural output. Li et al. (2009) showed that hexaploid wheat have survived severe drought conditions and therefore maintained yield stability, while the growth and yield of diploids were negatively affected. Recent report by Meng et al. (2011) stated that the tetraploid turnip exhibited an enhanced adaptation to salinity stress. Similar results were also reported for ligneous plants, where tetraploid, pentaploid and hexaploid plants exhibited a higher tolerance to water deficiency than their corresponding diploid (Pustovoitova et al., 1996; Li et al., 1996). In citrus, Saleh et al. (2008) attempted to compare between three tetraploid rootstock genotypes (*Poncirus trifoliata*, Carrizo citrange, Cleopatra mandarin) to their respective diploid rootstocks and their results showed that the tetraploids plants were more tolerant to salinity, and these plants were found to accumulate less toxic ions in leaves. The mechanisms related to chromosome doubling in citrus and performances of tetraploid citrus under salinity are not adequately investigated. Due to these concerns, there is growing interest in tetraploid citrus to tap their potentials as parents for seedless triploid breeding (Ollitrault et al., 2008) in view of developing plant that tolerant to many environmental stresses including salinity. Dez orange [*Citrus sinensis* (L.) Osb.] is one of the orange varieties that are grown extensively in south west of Iran. It appears that there are no reports that compare the response of diploid and tetraploid levels of this cultivar under salinity stress. This study was carried out to determine the nutritional and biochemical responses of tetraploid Dez orange in comparison to its diploid progenitor in relations to their adaptation to NaCl-saline condition.

Results

Effects of salinity on mineral composition of Dez orange cultivars

The results on the effects of NaCl salinity on N contents in the leaves and roots of both diploid (2n) and tetraploid (4n) Dez orange are presented in Tables 1 and 2. Salinity stress

Table 2. Mineral composition of leaf and root tissues (dry weight basis) of diploid (2n) and tetraploid (4n) Dez orange under different NaCl levels.

Treatments	Variables		N (%)		P (%)		K (%)		Na (%)		Cl (%)		K/Na (%)	
			Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
NaCl × Cultivar	0 mM	2n	2.15a	2.30a	0.491a	0.038a	1.54a	0.81a	0.26c	0.41c	0.93d	1.82d	5.92a	1.97a
	0 mM	4n	2.35a	2.23a	0.489a	0.044a	1.50a	0.78a	0.29c	0.38d	0.98c	1.76c	5.17a	2.05a
	20 mM	2n	2.12a	2.21b	0.473b	0.029b	1.43ab	0.68b	0.32c	0.49c	1.34c	2.14c	4.47b	1.39b
	20 mM	4n	2.18b	2.10b	0.461b	0.028b	1.48a	0.68b	0.34c	0.46c	1.28bc	1.86c	4.36b	1.48b
	40 mM	2n	1.75b	1.87c	0.423c	0.018c	1.25b	0.54c	0.53b	0.58b	2.11b	2.45b	2.36c	0.93c
	40 mM	4n	1.98c	1.85d	0.465b	0.020c	1.36b	0.58c	0.50b	0.52b	1.78b	2.15b	2.72c	1.11c
	60 mM	2n	1.51c	1.73d	0.401d	0.016c	0.98c	0.48d	0.76a	0.72a	2.79a	2.91a	1.29d	0.66d
	60 mM	4n	1.78d	1.99c	0.451c	0.019d	1.18c	0.50d	0.64a	0.69a	2.12a	2.43a	1.84d	0.72d

Means in each column with the different letters indicate significant differences at P≤5 % level according to DMRT.

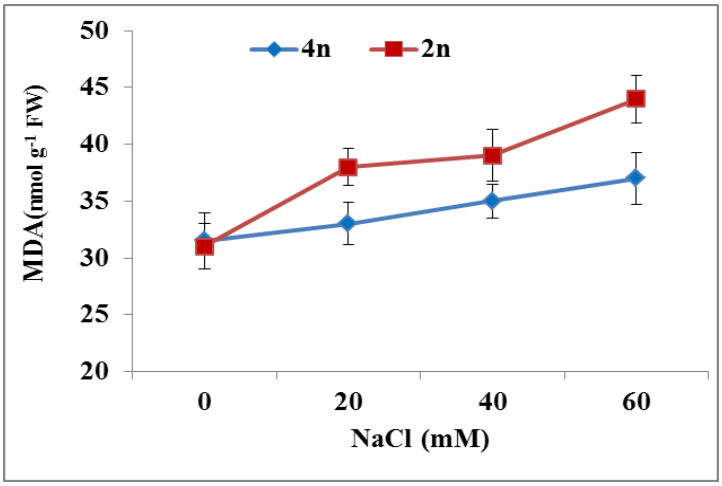

Fig 2. Comparison between the tetrapolyploid (4n) and diploid (2n) Dez sweet orange in term of MDA accumulation in leaves.

significantly decreased the N content of roots in both tetraploid and diploid plants at 20 mM of NaCl (P≤0.05). The leaves of both cultivars were also affected significantly by salinity at 40 mM of NaCl (P≤0.05) However, the highest effect of salinity on the N content of both the leaves and the roots of tetraploid and diploid plants was observed at 60 mM of NaCl (1.78 and 1.99% compared to 1.51 and 1.73% for the leaves and the roots of tetraploid and diploid plants at P≤0.05, respectively).Tetraploid plants had significantly higher N content in both their leaves and roots compared to their diploid counterparts starting at 40 mM of NaCl (Table 2, P≤0.05). Phosphorus content in leaves and roots of both cultivars was significantly affected by salinity levels (Tables 1 and 2). Salinity stress decreased the content of P in both leaves and roots of diploid and tetraploid plants at 20 mM of NaCl, significantly (P≤0.05). Salinity also significantly decreased the content of P in the leaves of diploid plants more than their tetraploid counterparts, starting at 40 mM of NaCl (0.423 compared to 0.465 %, respectively). The same was observed in the roots of plants at 60 mM of NaCl (0.016 compared to 0.019% for diploid and tetraploid plants, respectively). Potassium content in leaves and roots of both cultivars was significantly affected by salinity levels (Tables 1 and 2). Both cultivars showed similar responses to increasing levels of salinity in terms of the K content in both their leaves and roots. Salinity decreased the content of K in roots of both tetraploid and diploid plants starting at 20 mM of NaCl. The content of K in leaves of both cultivars was also affected by salinity stress at 40 mM of NaCl. Again, the highest effect of salinity was observed in leaves and roots of both cultivars at 60 mM of NaCl (Table 2). Sodium content in leaves and roots of both cultivars was significantly affected by salinity levels (Tables 1 and 2). Both cultivars showed similar response to the accumulation of Na in their leaves at different levels of salinity. The response of cultivars to the accumulation of K in their roots was also similar starting at 20 mM of NaCl. The content of Na in leaves of both cultivars started to increase at 40 mM of NaCl. Tetraploid plants had the lowest content of Na in their roots under no salinity stress conditions (0.38%). However, the content of Na in roots of diploid plants increased starting at 40mM of NaCl. The highest content of Na for the leaves and roots of both cultivars was also achieved at 60 mM of NaCl (Table 2). Chloride content in leaves and roots of both cultivars was significantly affected by salinity levels (Tables 1 and 2). Both cultivars showed similar responses to increasing of salinity levels in their leaves and roots, starting at 20 mM of NaCl. Under no salinity stress conditions, tetraploid plants had higher contents of Cl in their leaves compared to diploid ones while diploid plants had higher contents of Cl in their roots at similar conditions. Salinity increased the content of Cl in the leaves of diploid plants starting at 20 mM of NaCl. The same was observed in the leaves of tetraploid plants, starting at 40 mM of NaCl. Salinity increased the content of Cl in the roots of tetraploid plants starting at 20 mM of NaCl. The same was also observed in the roots of diploid plants, starting at 40 mM of NaCl. The highest Cl content was also observed at 60 mM of NaCl in leaves and roots of both cultivars (Table 2). Potassium and sodium ratio in leaves and roots of both cultivars was significantly affected by salinity levels (Tables 1 and 2). Both cultivars showed similar responses to increasing levels of salinity in terms of K/Na in both their leaves and roots. Salinity decreased the K/Na of leaves and roots of both cultivars, starting at 20 mM of NaCl. The

lowest K/Na was observed in leaves and roots of both cultivars at 60 mM of NaCl (Table 2).

Effects of salinity on leaf proline content in Dez orange cultivars

Based on the obtained results from this investigation, the Proline content in the leaves of both diploid and tetraploid cultivars, was increased significantly ($p \leq 0.05$) due to increasing of NaCl rate (Fig. 1). The maximum amount of proline content in leaves was observed at the maximum salinity concentration (60 mM) for both diploid and tetraploid cultivars. Comparison of both plants indicated that increasing proline content at tetraploid plant leaves was more than that of the diploid one.

Effects of salinity on leaf MDA content in Dez orange cultivars

The results concerning MDA content in Dez orange in response to NaCl salinity are presented in Figure 2. There was a marked increase in MDA content when the concentration of NaCl increased from 0 to 60 mM NaCl. The MDA content in diploid plants was relatively higher than that in tetraploids. The highest MDA content (44.3 nmol/g FW) was observed in diploid Dez orange grown at the salinity level of 60 mM NaCl, while MDA contents in tetraploid plants under similar condition was 37.1 nmol/g FW.

Effects of salinity on leaf H_2O_2 content in Dez orange cultivars

Figure 3 shows that increasing NaCl salinity significantly elevated H_2O_2 content in the leaves of both diploid and tetraploid cultivars. However, the diploid plants generally contained a much higher concentration of H_2O_2 compared to the tetraploids. The highest H_2O_2 content (62.5 µmol g^{-1} FW) was observed in diploid plants at the salinity level of 60 mM NaCl.

Discussion

In this study, the N and P content in leaves and roots significantly decreased with increasing salinity in both diploid and tetraploid plants. The mineral contents in tetraploid Dez orange were higher than those in diploid plants. The findings of present investigation are consistent with the results of previous studies stating that mineral content of all the plant parts was higher in tetraploids than that of diploids (El-Morsy et al., 2009; Jones et al., 1995). Additionally, Na and Cl contents of leaves and root generally increase with increasing salt concentrations in the nutrient solution. It seems that tetraploids had a higher ability to sequester Na^+ into vacuole compared with diploid plants, thus making tetraploid Dez orange limits transport of Cl^- and Na^+ from root to shoot. According to several reports, lack of ion compartmentation leads to toxic effects of ions in susceptible plants (Greenway and Munns, 1980; Flowers et al., 1977). The nature of Na content in the tissue as reported in the present study seems to be paralleled with the result of Saleh et al. (2008) who reported that tetraploid citrus rootstocks had accumulated Na ion in their roots more than the diploid. Recently, Meng et al. (2011) found that NaCl-salinised tetraploid turnip had a higher concentration of Na in its roots, but a lower Na concentration in its shoots. Avoidance of high concentrations of toxic ions in leaves can result in a reduction in net uptake of Na and Cl, a high

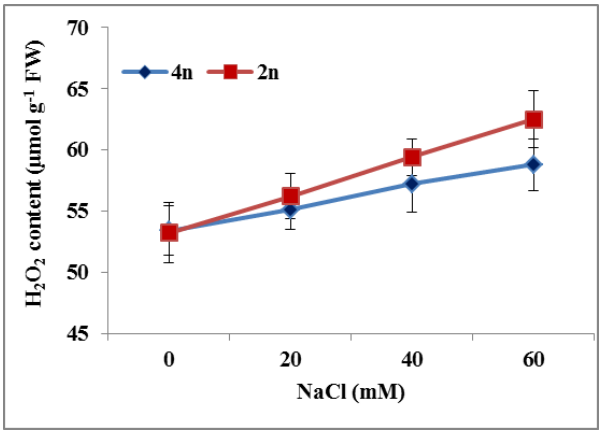

Fig 3. Hydrogen peroxide contents in leaves of the tetrapolyploid (4n) and diploid (2n) Dez sweet orange seedlings under NaCl stress.

capacity for accumulation of toxic ions in the root and stem or the maintenance of a high shoot/root ratio (Fernandez-Ballester et al., 2003). Differences in Na and Cl content of tetraploid and diploid plants recorded in this study could be associated with the higher shoot/root ratio or lower uptake rate in tetraploid than in diploid. The reduction of K content observed in salinized plants could be related to the increased K efflux into the growth medium, possibly due to disintegration of cell membrane that caused by Na toxicity which inhibits transport of K into root and up to the shoot, and the antagonism between K and Na cations, which increased considerably as the concentration of NaCl salinity in the root raised (Sakr and Arafa, 2009). The cellular roles of K^+ are to balance the charge in the cytoplasm, activate key enzymatic reactions and contribute to structural rigidity by maintaining the osmotic pressure of vacuole and hence, the cell turgor. Diploid plants tended to accumulate less Na in roots, thus more damage of salinity appeared as compared with leaves of tetraploid. Accumulation of solutes such as proline is a common observation under stress condition (Qasim et al., 2003). In the present study, a significant increase in proline content was found in citrus seedlings. The results showed that proline accumulation was associated with the concentration of NaCl in the root zone and proline content in leaves of tetraploid plant was higher than diploid citrus. Proline is an important osmolyte that acts as a mediator of osmotic adjustment under stress conditions, a stabilizer of sub-cellular structures, a sink for energy and even a stress-related signal. It is also involved in cell osmoregulation and protection of proteins during dehydration. Salt stress is also known to result in extensive lipid peroxidation, which has often been used as indicator of stress-induced damage at the cellular level (Tayebimeigooni et al., 2012). We observed that MDA content was affected by NaCl in leaves of both type of plants. Lower level of lipid peroxidation in tetraploid Dez orange suggests that salt tolerant plants are better protected from oxidative damage under salinity. The result gave an indication that diploid plants tend to suffer severe membrane damage as opposed to tetraploid plants under saline conditions. Similar results were recently described by Zhang et al. (2010). It is interesting to note that relatively low lipid peroxidation in salt-tolerant seedling implies that it may possess an improved protection against the damage. Salinity generally induced generation of H_2O_2 radical which is potentially harmful to plants including citrus (Kim et al., 2005; Gueta-Dahan et al., 1997). Fortunately, plants are able to deal with these ROS by

eliminating them with an effective scavenging system (Peroni et al., 2007). Detoxification of excess ROS produced during stress is an important intervention in reducing ROS-induced membrane lipid peroxidation, nucleic acid damage, and enzyme scavenging (Kim et al., 2005) which is often related to the enhanced tolerance to salt stress (Gueta-Dahan et al., 1997; Tayebimeigooni et al., 2012). Therefore lower concentration of H_2O_2 in the tissues of tetraploid Dez orange as observed in this study can be regarded as an evident of its increased resistance to salinity as compared to diploid ones.

Materials and methods

Plant materials and growth conditions

The plant materials used in the investigation were two Dez orange cultivars, specifically the Dez orange diploid (2n) and the Dez orange tetraploid (4n). These cultivars were provided by the Safiabad Agricultural Research Centre, Iran. The tetraploid Dez orange was developed from the diploid Dez orange and possesses the double number of complete chromosome sets of the diploid. The seedlings were grown in 25×30 cm black plastic bags filled with approximately 5.5 kg sandy-loam soil (pH 6.5, EC 0.6 dS/m and 0.64% organic matter) and raised in an experimental greenhouse in Universiti Putra Malaysia (02°N 59.476´ 101°E 2.867´, 51 m altitude). The average day/night temperatures were 32 and 27 °C, respectively under natural photoperiod conditions.

NaCl treatments

Three-month-old seedlings were treated with different levels of NaCl concentrations (0, 20, 40 and 60 mM) which were respectively added to a complete basic nutrition solution containing (mg L^{-1}) 232 N, 67 P, 239 K, 120 Ca, 30 Mg, 3 Fe, 0.62 Mn, 0.44 B, 0.02 Cu, 0.11 Zn and 0.048 Mo. To avoid osmotic shock, the concentrations of NaCl were raised gradually within two weeks. The treatments were given at every two days for 120 days in a sufficient volume until drainage.

Mineral ion contents

For determination of leaf ion contents, fully expanded leaves were harvested and washed with deionized water and dried at 70°C for 72 hrs before the leaves were ground and stored in dry plastic vials. The nutrient concentrations were measured with the method described by Awang et al. (2009). Briefly 0.25 g of the dry samples was transferred to 100 ml digestion flask and 5 ml concentrated H_2SO_4 was added to each digestion flask. Then the flasks were heated for 7 minutes at 450 °C and 10 ml 50% H_2O_2 was added. The flasks were removed from the digestion plate, cooled to room temperature and then made up to 100 mL with distilled water. N and P concentrations were analyzed using Auto analyzer (LACHART Instruments, Model Quickchem IC+FIA 8000 Series). Na and K were quantified using an atomic absorption spectrophotometer (Perkin Elmer, Model 3110, USA). Chloride was determined using silver ion titration method (Richards, 1954).

Proline content

Free proline content was determined from the leaf tissues according to the method described by Bates *et al.* (1973). Plant tissues were without delay frozen in liquid nitrogen. Briefly 0.5 g of frozen leaves was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and filtered through Whatman's No.2 filter paper. Two ml of the filtrate was mixed with 2 ml acid-ninhydrin and 2 ml glacial acetic acid in a test tube. The reaction mixture was extracted with 4 ml toluene and the chromophore containing toluene was aspirated and the absorbance was quantified spectro-photometrically at 520 nm. Proline content was computed using L-proline as standard.

MDA determination

To estimate lipid peroxidation, the concentration (MDA was assessed by the thiobarbituric acid (TBA) test according to the procedure of Wang et al. (2009). One gram of fresh leaf samples were homogenized in 5 mL 0.6% TBA in 10% trichloroacetic acid (TCA). The mixture was heated at 100°C for 15 min in a water bath. After cooling in ice, the mixtures were centrifuged at 5000 rpm for 10 min. The absorbance of supernatants were read at 450, 532 and 600 nm and MDA content was calculated on a fresh weight basis using the following formula: MDA (nmol g^{-1}FW)= 6.45 (OD_{532}-OD_{600}) -0.56 (OD_{450}) × 1000.

H_2O_2 determination

H_2O_2 concentration in the leaves was measured spectrophotometrically after reaction with potassium iodide (KI) as described by Velikova and Loreto (2005). Fresh leaf samples (1.0 g) were homogenized with 1 mL 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12000×g for 15 min, then 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL reagent (1 M KI in fresh distilled water) was prepared. The absorbance of the supernatant was measured at 390 nm. The blank prob consisted of 0.1% TCA in the absence of leaf extract.

Experimental design and statistical analysis

The study was conducted in a randomized complete block design with two cultivars and four salinity levels (8 treatments) in four replications including 32 pots. The data obtained were analyzed by ANOVA using SAS (Version 9, SAS Institute Inc. Cary, North Carolina, USA) and differences between treatment means were compared by using Duncan's Multiple Range Test (DMRT) at ≤ 0.05% level of probability.

Conclusion

In this study, we examined several nutritional and biochemical indicators for salt (NaCl salinity) tolerance in diploid and tetraploid Dez orange. Results on plant mineral status, proline, MDA and H_2O_2 composition suggested that tetraploid orange display a higher degree of tolerance to salt stress than the diploid. Thus tetraploidization in citrus might be responsible for the stronger capability to scavenge ROS and higher tolerance to the stress.

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