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RESEARCH ARTICLE

Response of pomegranate cv. wonderful plants to salinity

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Abstract

Three salts (NaCl; KCl; Na_2SO_4) were supplied to pomegranate cv. Wonderful plants, in order to investigate their effects on growth, nutrient status, chlorophyll, total carbohydrate content and antioxidant defense system. In general, high salt supply led to a significant decline in total N and K content of plants. Also, all salt treatments decreased Ca and Mg concentration of leaves. Both NaCl and KCl treatments increased leaf Cl concentration by up to 418%. Salt excess resulted in a significant decline of chlorophyll and carbohydrate concentration of leaves and/or roots. Finally, concerning antioxidants, diamine oxidase activity increased in the treatment of 120 mM Na_2SO_4. In conclusion, salinity impaired mineral nutrition of pomegranate cv. Wonderful. On the other hand, that cultivar presented mechanisms that alleviated the detrimental effects of salinity. Therefore, the studied plants, even under high saline treatments, managed to maintain water content, chlorophyll fluorescence and enzyme activity in normal levels. These results suggest that 'Wonderful' may be cultivated under saline conditions provided that a suitable fertilization program is used.

Keywords: Antioxidant, nutrient status, alternative crops, *Punica granatum* L., salinity, toxicity

1. Introduction

Soil salinity is one of the most serious environmental stresses limiting growth and yield of horticultural plants, worldwide. Almost 20% of the world's cultivated land and half of the world's irrigated land are affected by salinity and more severely, salinization is still expanding, posing a greater threat to sustainable development of agriculture. Factors contributing to soil salinization include the excessive application of chemical fertilizers, the use of saline irrigation water, the high water table and the exposure of plants to salt spray near the sea.

Pomegranate (*Punica granatum* L.) is considered to be moderately tolerant to salinity (Maas and Hoffmann, 1976). Although pomegranate is one of the oldest known edible fruits, its culture has always been restricted and generally is considered as a minor crop. In recent years, pomegranates are increasingly recognized as attractive fruit trees that provide highly valued health beneficial ingredients and a wide range of usages of the fruit and its products. Thus, it is of utmost importance to exploit its potential against salinity.

Salinity reduces plant growth due to osmotic and ionic effects on soil solution. The most damaging effects of salinity on plants include ion toxicity, water deficit and nutrient imbalance (Marschner, 1995). Additionally, components of photosynthetic electron transport in the chloroplasts as well as the enzyme responsible for carbon assimilation are highly sensitive to Na^+ and Cl^- concentrations (Nayidu *et al.*, 2013). The salt-related decline in the photosynthetic activity might trigger PSII photoinhibition and/or photodamage (Lu and Vonshak, 2002). Therefore, measurements of chlorophyll a fluorescence is a rapid and noninvasive tool used to screen varieties for salinity tolerance. Furthermore, plants subjected to salinity experience oxidative damages through an increase of reactive oxygen species (ROS) (Arora *et al*., 2008). These ROS cause oxidative stress by initiating lipid peroxidation that leads to a loss of membrane integrity (Dionisio-Sese and Tobita, 1998). To scavenge the ROS, plants possess protective enzymes like peroxidase [POD (EC 1.11.1.7)] and diamine oxidase [DAO (EC 1.4.3.6)] that protect plants from ROS damage (Xing *et al*., 2007; Wu *et al*., 2012).

In most cases, salinity problems are linked to an excess of NaCl in the irrigation water, but sometimes other salts like Na_2SO_4 and KCl are present. Many reports studied the effects of salinity induced by an excess of NaCl, but few works have been carried out on the effects of KCl and Na_2SO_4. The present study was undertaken to evaluate the effects of NaCl, KCl and Na_2SO_4 on growth, tissue ion, chlorophyll and sugar contents, and antioxidant response of *P. granatum.*

2. Materials and Methods

2.1. Plant material and treatments

Six-month-old pomegranate (cv. Wonderful) plants, uniform in height and girth, were transplanted to 3L plastic pots containing a 1:1 sand: perlite mixture. The experimental plants were maintained outdoors in the Agricultural Research Station of Orestiada city (Greece), under natural light conditions and a 3-meter high lath house covered with plastic on the top to avoid rain. The mean minimum, average and mean maximum temperatures were 10.3, 22.6 and 36.6 °C, respectively. Plants were initially irrigated with good quality tap water (0.80 dS m^{-1}) for 15 days. After 15 days, the plants were irrigated every two days with 250 ml of modified half-strength Hoagland nutrient solution containing NaCl, KCl, or Na_2SO_4 (0, 40, 80 and 120 mM). The electrical conductivities were

equal to 1.36 dS m^{-1} for control, 4.1, 8.5 and 11.8 for the treatments 40, 80 and 120 mM NaCl, respectively, 5.5, 9.5 and 12.9 for the treatments 40, 80 and 120 mM KCl, respectively, and 5.9, 12.7 and 12.6 for the treatments 40, 80 and 120 mM Na_2SO_4, respectively. The treatment with no salt addition was considered as control. Every 10 days, 450 ml of distilled water was supplied to each plant in order to leach out any accumulated salts.

After 60 days the experiment was terminated, when typical visual symptoms of salt toxicity appeared.

2.2. Plant growth and determination of inorganic ions

At initiation of the experiment the mean plant height (and standard error) was 26.4 (3.06) cm and the mean trunk diameter (and standard error) was 1.6 (0.13) mm. At the end of the experiment, the plants were harvested and separated into leaves (basal and apical), stems and roots. The number of leaves, the plant height, the number and length of lateral stems, as well as their fresh weight (FW) and dry weight (DW) was measured. The DW% was equal to the ratio (DW/FW) × 100.

Determination of the N was carried out using the Kjeldahl method and Cl was extracted from the dried tissue with distilled water and measured by titration with 0.0141N $AgNO_3$. For K, Ca, Mg and Na analyses, 0.5 g of dried sample was dry ashed for 6 h at 550 °C, dissolved in 3 ml 6 N HCl and diluted to 50 ml with deionized water. The concentrations of K, Ca, Mg and Na were determined using atomic absorption spectroscopy (Perkin-Elmer model 2380; Perkin-Elmer, Salem, MA, USA). Nutrient use efficiency (NUE) which is defined as the amount of dry biomass produced (in g) per unit (mg) of nutrient was also calculated.

2.3. Determination of chlorophyll fluorescence and content

Chlorophyll fluorescence was measured using a portable fluorometer PAM-2100 (H. Walz, Effeltrich, Germany). Before measuring chlorophyll fluorescence parameters, leaves were put in dark-adapted state for 30 min using light exclusion clips. The following chlorophyll fluorescence yields were measured: minimum chlorophyll fluorescence yield in the dark-adapted state (F_0), and maximum chlorophyll fluorescence yield in the dark-adapted state (F_m). Using the above parameters, the F_v/F_m ratio (maximum quantum yield of PSII photochemistry, $F_v = F_m - F_0$: variable fluorescence) and F_v/F_0 ratio (maximum primary yield of PSII photochemistry) were calculated.

The chlorophyll concentration of the same leaves of the treatments of 0 (control), 40 and 120 mM of salts was determined. Leaf discs (0.95 cm^2) were collected at noon under full sun and placed in test tubes containing 15 ml of 96% ethanol (78 °C), until their total discoloration. The absorbance of the extract was measured with a spectrophotometer at 665 and 649 nm and the total chlorophyll ($a + b$) concentration was calculated according to Papadakis *et al*. (2007). The previous leaves were also used for SPAD (Soil Plant Analysis Development) measurements with a portable CCM-200 chlorophyll content meter (Opti-sciences Inc., USA).

2.4. Determination of carbohydrate and antioxidant activity

Carbohydrate concentrations in leaves and roots were assayed following the anthrone method (Khan et al., 2000). For POD and DAO assays, fresh leaf and root samples (0.25 g) were homogenized in a cold mortar with a pestle using 1.5 ml of 100 mM potassium

phosphate buffer (pH 7.0). The homogenate was centrifuged (12,000 g for 30 min at 4 °C) and the clear supernatant was used for the antioxidant enzyme assays. POD activity was measured by a modified method with guaiacol. Ten µl of the clear supernatant was added in 1.55 ml reaction mixture that contained 100 mM potassium phosphate buffer (pH 7.0) and 35 mM guaiacol. The reaction was started by injecting 0.2 ml H_2O_2 (100 mM), as a substrate. DAO activity was determined by using a coupled reaction with horseradish peroxidase and guaiacol. Fifteen µl of the supernatant was added in 1.55 ml reaction mixture that contained 100 mM potassium phosphate buffer (pH 7.0), 35 mM guaiacol, and horseradish peroxidase (0.1 g l^{-1}). The reaction was started by injecting 50 µl of putrescine as a substrate. For both enzymes, their activity was detected spectrophotometrically and the absorbance was continuously recorded at 436 nm for a period of 1 min.

A change in absorbance of 0.01 was regarded as a unit of the enzyme activity. All the above spectrophotometric analyses were conducted in a Shimadzu spectrophotometer (Kyoto, Japan) at room temperature (25 ± 2 °C).

2.5. Statistical data analysis

The experimental layout included three salt concentrations, three salts and six replicates (two plants per replicate) per treatment. Data were subjected to analysis of variance (ANOVA). For comparison of the means the Duncan's multiple range test was used ($P < 0.05$) using the SPSS 18.0 statistical package (SPSS, Inc., Chicago, USA).

3. Results

3.1. Growth parameters and toxicity symptoms

The number of leaves per plant decreased significantly in all salt treatments, except for 40 mM KCl (Table 1). The fewest leaves per plant (38.7) were measured in the plants treated with 120 mM KCl. Similarly, salinity negatively affected the number of lateral shoots, mainly in the highest salt concentrations, whereas neither the length of lateral shoots nor the plant height was affected by the inclusion of salts in the nutrient solution. The stem diameter at the end of the experiment was affected negatively only in the treatment 120 mM KCl (2.24 mm), compared to control (2.69 mm).

Concerning the percentage of dry weight of leaves it was significantly declined only in top leaves of Na_2SO_4-treated plants. Moreover, the total plant dry weight was reduced in the treatments 120 mM KCl and Na_2SO_4 compared to control. At the end of the experiment, DW% ranged between 16.41 and 40.17% in leaves, 31.31 and 47.03% in stems and 17.52 and 32.50% in roots (data not shown). Moreover, the ratio of DW of aerial parts to DW of root was reduced in the treatments 80 and 120 mM NaCl, KCl and Na_2SO_4, compared to control (data not shown). This reduction of shoot-to-root mass ratio is a common response of stressed plants in order to minimise water losses and thereby to maintain a favourable water status for growth.

All plants maintained their leaves during the experiment. Toxicity symptoms due to salinity were developed on the older leaves, in the form of tip chlorosis followed by marginal chlorosis and necrosis, firstly in the plants treated with 120 mM KCl followed by the Na_2SO_4-treated ones.

Table 1. Effects of salinity in the nutrient solution on growth parameters of the pomegranate cv. Wonderful. Data are the mean of six replicates. Different letters in each column indicated that there was a significant difference at the level of 0.05. The *P*-values of the two-ANOVA refer to the variables salt (S), salt concentration (C) and salt × salt concentration (S × C)

Treatments		Leaf number	Shoot length	Number of shoots	Plant height	Dry weight (%)	
(mM)			(cm)		(cm)	Basal leaves	Top leaves
Control		124.8a	16.4ab	4.4a	53.6ab	29.4bcd	31.3ef
NaCl	40	83.8bc	12.8ab	3.9ab	56.4a	31.3ab	32.3de
	80	71.0cd	20.0ab	2.2bcd	56.8a	29.3bcd	31.8def
	120	58.6cde	18.4ab	1.7cd	51.0ab	25.9d	29.6f
KCl	40	107.5ab	20.7a	2.9a-d*	57.8a	28.8bcd	31.2ef
	80	53.0de	12.5b	2.7a-d	45.7ab	29.9bc	34.2cd
	120	38.7e	8.4b	1.8cd	41.2b	26.9cd	31.4ef
Na_2SO_4	40	87.0bc	14.0b	2.9abc	55.6a	32.4ab	35.5bc
	80	77.0cd	16.3ab	2.0cd	53.8ab	33.9a	37.6ab
	120	54.3cde	11.4b	1.5d	42.0b	32.1ab	38.4a
P-values							
S		0.648	0.277	0.687	0.166	<0.001	<0.001
C		<0.001	0.090	0.005	0.004	0.050	0.201
S × C		0.083	0.052	0.628	0.480	0.013	<0.001

* "a-d" is the abbreviation of "abcd"

3.2. Absorption of inorganic ions

Final N concentration in plants ranged between 18.3 and 29.1 g kg^{-1} in leaves (Table 2a), 8.3 and 10.5 g kg^{-1} in stems and 9.4 and 11.8 g kg^{-1} in roots, (Table 2b). Also, the N concentration in top leaves was significantly reduced in all treatments compared to control. High salt supply led to a significant decline of total N content, whereas N use efficiency was significantly enhanced in all salt treatments (Table 3). At the end of the experiment, K concentration ranged between 11.1 and 49.8 g kg^{-1} in leaves (Table 2a), 8.5 and 25.8 g kg^{-1} in stems and 6.5 and 24.6 g kg^{-1} in roots (Table 2b). The inclusion of KCl in the nutrient solution led to an increase in K concentration of leaves and stems. Generally, NaCl-treated plants presented lower K concentration in basal leaves, stems and roots than the control. High Na_2SO_4 supply led to a decline in K concentration in leaves and roots. Also, high NaCl or Na_2SO_4 supply decreased total K content of plants. Concerning K use efficiency (Table 3), this was significantly increased in NaCl and Na_2SO_4 treatments but decreased in KCl treatments.

Table 2a. Effects of salinity in the nutrient solution on nutrient concentrations in basal and top leaves of pomegranate cv. Wonderful. Data are the mean of six replicates. Different letters in each column indicated that there was a significant difference at the level of 0.05.

Treatments		N	K	Ca	Mg	Cl	Na
(mM)				(g kg^{-1} dry weight)			
			Basal	leaves			
Control		28.0a	19.7c	20.9a	2.1a	2.2c	2.0e
NaCl	40	25.8a	16.5cd	18.5a	1.6ab	4.5c	5.3d
	80	25.6a	14.3cd	18.1a	1.6ab	8.7b	10.2c
	120	27.7a	11.1d	13.8b	1.1bc	11.4a	14.8a
KCl	40	25.8a	43.9a	9.7b	0.7c	9.1ab	1.0e
	80	18.3a	35.3b	9.7b	0.6c	7.1b	1.2e
	120	25.0a	40.9ab	10.3b	0.8c	3.0c	1.1e
Na_2SO_4	40	25.0a	15.4cd	12.1b	1.1bc	3.3c	8.5c
	80	25.6a	16.1cd	13.2b	1.2bc	1.6c	11.0bc
	120	25.3a	11.1d	9.3b	0.9c	2.3c	13.3ab
			Top	leaves			
Control		29.1a	20.4d	12.2a	1.7a	4.2e	1.5e
NaCl	40	26.3bc	20.2d	8.7b	1.4b	6.7d	3.5d
	80	27.2b	16.3def	7.2c	1.1c	8.0c	6.1c
	120	25.5bcd	15.7def	6.7c	0.8c	10.3b	10.9ab
KCl	40	25.2a-d	39.8b	3.2de	0.4ef	11.0b	0.7e
	80	24.9bcd	49.8a	2.2e	0.3f	11.2b	0.5e
	120	23.5cd	34.5c	3.6de	0.4ef	14.6a	0.8e
Na_2SO_4	40	24.9cde	19.4de	6.1c	1.0c	3.4ef	6.5c
	80	23.3e	15.5ef	4.3d	0.7d	2.9f	10.5b
	120	23.4e	13.5f	3.6de	0.5de	2.5f	12.4a

Calcium concentration of plants ranged between 2.2 and 20.9 g kg^{-1} in leaves (Table 2a), 5.7 and 10.5 g kg^{-1} in stems and 4.8 and 9.0 g kg^{-1} in roots (Table 2b). In general, all salt treatments led to a significant decrease of Ca concentration in leaves, as well as in total plant Ca content. Moreover, KCl-treated plants had lower Ca concentration in stems, compared to control. The Ca use efficiency of plants was positively affected by salt inclusion in the nutrient solution (Table 3).

The final Mg concentration in plants ranged between 0.3 and 2.1 g kg^{-1} in leaves (Table 2a), 0.4 and 2.1 g kg^{-1} in stems and 0.5 and 2.1 g kg^{-1} in roots (Table 2b). All salt treatments decreased Mg concentration of leaves. Also, NaCl and Na_2SO_4 treatments decreased Mg concentration in roots, whereas Na_2SO_4 supply led to an increase in Mg concentration of stems. Generally, the total plant Mg content was depressed, whereas Mg use efficiency of plants treated with NaCl or KCl was significantly increased, compared to control (Table 3). At the end of the experiment, Na concentration ranged between 0.5 and 14.8 g kg^{-1} in leaves (Table 2a), 1.7 and 9.5 g kg^{-1} in stems and 1.5 and 9.1 g kg^{-1} in roots (Table 2b). Total Na content and Na concentration in all parts of the plants treated with

NaCl or Na_2SO_4 were significantly increased, whereas Na use efficiency was decreased compared to control (Table 3). The order of magnitude of Na concentration among plant parts was: basal leaves > stems > apical leaves = roots for NaCl treatments, and basal leaves > apical leaves > roots > stems for Na_2SO_4 treatments.

Table 2b. Effects of salinity in the nutrient solution on nutrient concentrations in stems and roots of pomegranate cv. Wonderful. Data are the mean of six replicates. Different letters in each column indicated that there was a significant difference at the level of 0.05. The *P*-values of the two-way ANOVA refer to the variables salt (S), salt concentration (C) and salt × salt concentration (S × C) for all plant parts, as well as their total plant content.

Treatments (mM)		N	K	Ca	Mg	Na
			(g kg^{-1} dry			
			Stems			
Control		9.9a	13.3bc	0.89ab	0.6c	3.7de
NaCl	40	8.7a	9.2e	0.73bc	0.5c	5.9c
	80	9.2a	9.8de	1.05a	0.6c	7.4b
	120	8.7a	8.5e	0.93ab	0.7c	9.5a
KCl	40	9.2a	23.1a	0.63c	0.6c	1.7f
	80	10.4a	24.9a	0.57c	0.4c	2.1f
	120	9.9a	25.8a	0.58c	0.4c	2.4ef
Na_2SO_4	40	8.3a	12.5cd	0.88ab	2.1a	8.5ab
	80	9.2a	10.4cde	0.77bc	1.6b	8.2ab
	120	10.5a	16.0b	0.73bc	1.7b	8.8ab
			Roots			
Control		10.4a	24.6a	0.71abc	1.7ab	1.5d
NaCl	40	9.9a	15.9b	0.71abc	0.9cde	3.7c
	80	10.8a	7.9c	0.80ab	0.5e	7.5ab
	120	10.5a	6.5c	0.67bcd	0.6e	9.1a
KCl	40	10.3a	7.6c	0.65bcd	1.3bcd	7.1ab
	80	11.8a	10.6c	0.90a	2.1a	5.8b
	120	9.4a	23.2a	0.54cd	1.4bcd	1.8cd
Na_2SO_4	40	11.1a	9.6c	0.68bcd	1.5abc	8.9a
	80	9.5a	8.2c	0.56cd	1.0cde	8.4a
	120	10.8a	7.2c	0.4.8d	0.8de	9.0a
			Total plant	content		
Control		206.6a	206.4a	129.5a	15.6a	26.8c
NaCl	40	186.4a	173.8bc	109.3a	12.0ab	52.6b
	80	173.9ab	122.3cd	102.5ab	9.30bcd	76.1a
	120	121.9bcd	79.0d	65.3cd	6.03de	76.9a
KCl	40	174.5ab	299.9a	64.3cd	7.80b-e	23.8cd
	80	110.0cd	194.8bc	39.1de	5.14de	15.2cd
	120	69.3d	139.2bcd	28.9e	3.48e	8.14d
Na_2SO_4	40	151.4abc	134.0bcd	76.0bc	14.3a	74.1a
	80	149.0abc	119.0cd	67.0cd	11.4abc	89.0a
	120	103.3cd	84.9d	38.7de	7.17cde	57.1b
P-values						
S		0.727	<0.001	<0.001	<0.001	<0.001
C		0.795	0.821	<0.001	<0.001	<0.001
S × C		0.996	<0.001	<0.001	<0.001	<0.001

Table 3. Effects of salinity in the nutrient solution on nutrient use efficiency (plant dry biomass produced (g) per mg of nutrient) of pomegranate cv. Wonderful. Data are the mean of six replicates. Different letters in each column indicated that there was a significant difference at the level of 0.05. The *P*-values of the two-way ANOVA refer to the variables salt (S), salt concentration (C) and salt × salt concentration (S × C)

Treatments (mM)		N	K	Ca	Mg	Na
Control		0.054d	0.054d	0.086e	0.723de	0.419b
NaCl	40	0.062bc	0.069c	0.107cd	0.981c	0.221c
	80	0.059c	0.085b	0.100de	1.108bc	0.136d
	120	0.061bc	0.098a	0.118cd	1.299ab	0.095d
KCl	40	0.060bc	0.036e	0.162ab	1.446a	0.444b
	80	0.060c	0.033e	0.167ab	1.291ab	0.438b
	120	0.071a	0.035e	0.174a	1.486a	0.575a
Na_2SO_4	40	0.062bc	0.070c	0.123c	0.656e	0.125d
	80	0.065b	0.083b	0.147b	0.870cde	0.108d
	120	0.063bc	0.080b	0.171a	0.932cd	0.114d
P-values						
S		<0.001	<0.001	<0.001	<0.001	<0.001
C		<0.001	0.253	<0.001	0.007	0.177
S × C		<0.001	<0.001	<0.001	<0.001	<0.001

Chloride concentration in leaves (Table 2a) ranged between 1.6 and 14.6 g kg^{-1}. NaCl and KCl treatments (except 40 mM NaCl and 120 mM KCl) increased Cl concentration in top and basal leaves. KCl-treated plants presented higher Cl concentration in top leaves than basal ones.

3.3. Chlorophyll content and fluorescence

The concentrations of chlorophyll in leaves (expressed as mass per unit area or as SPAD units) were affected by the salt concentration in the nutrient solution (Table 4). Excess of salts resulted in a significant decline of the leaf chlorophyll concentration of pomegranate plants, with 120 mM KCl treatment presenting the lowest values compared to control.

As regards chlorophyll fluorescence, the maximum quantum yield of PSII (F_v/F_m), F_v/F_0 ratio, F_0 and F_m values of the salt-treated pomegranate plants were not significantly different than the values of the control plants, irrespectively of the salt used (data not shown). The mean values of F_v/F_m, F_v/F_0, F_0 and F_m of the control plants were 0.65, 1.88, 6.25 and 1785, respectively.

3.4. Carbohydrate content and antioxidant activity

Carbohydrate concentrations recorded in leaves were 2.4 to 5.6 times higher than those in roots (Table 4).

Table 4. Effects of salinity in the nutrient solution on leaf chlorophyll concentration and SPAD units as well as on carbohydrates (leaves, roots) of pomegranate cv. Wonderful. Data are the mean of four replicates. Different letters in each column indicated that there was a significant difference at the level of 0.05. The *P*-values of the two-way ANOVA refer to the variables salt (S), salt concentration (C) and salt × salt concentration (S × C).

Treatments		Chlorophyll		Carbohydrates (mmol g^{-1} fresh weight)	
(mM)		(μg cm^{-2})	SPAD units	Leaves	Roots
Control		37.0a	31.4a	18.6ab	7.50a
NaCl	40	32.4ab	22.4b	20.8a	4.05b
	80	31.2ab	18.8bc	18.6ab	3.90b
	120	29.5b	19.9bc	17.0b	5.89ab
KCl	40	28.2b	18.4bc	14.4c	2.99b
	80	17.8c	15.9cd	19.4a	3.61b
	120	14.5c	12.7d	11.6d	4.78ab
Na_2SO_4	40	28.7b	19.8bc	14.5c	4.46b
	80	28.0b	19.8bc	14.8c	4.53b
	120	27.0b	17.8bc	13.6cd	4.39b
P-values					
S		<0.001	<0.001	<0.001	0.142
C		<0.001	<0.001	<0.001	0.134
S × C		<0.001	<0.001	<0.001	0.242

Almost all salt treatments led to a decrease in carbohydrate concentration in leaves and roots. However, plants treated with NaCl did not alter carbohydrate content in their leaves.

An increment (non significant) in the activity of POD in leaves was recorded in all treatments in comparison to control (Figure 1). However, Na_2SO_4 at 40 mM increased POD activity in roots in comparison to control. Similarly, DAO activity in leaves and roots was significantly increased in the treatments with 120 and 40 mM Na_2SO_4, respectively.

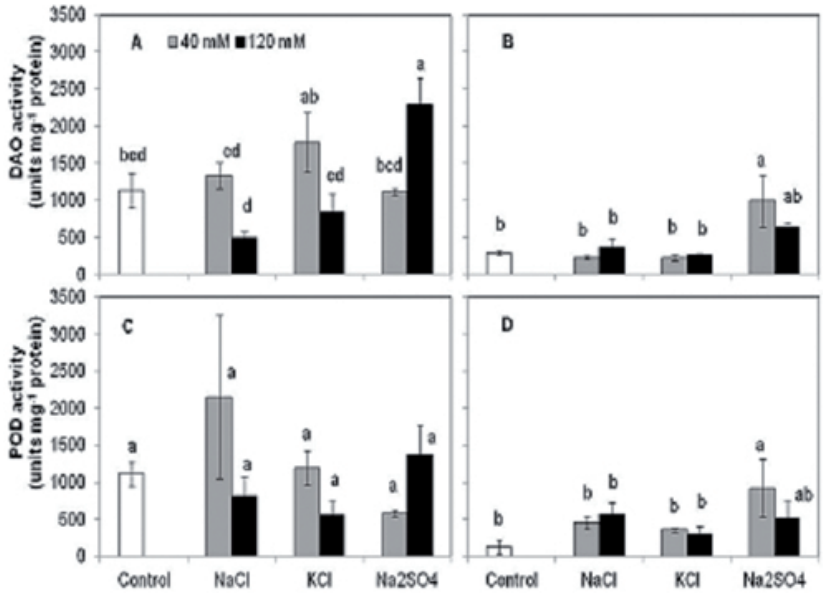

Figure 1. Effects of salinity in the nutrient solution on diamine oxidase (DAO) in leaves (A) and roots (B), and peroxidase (POD) activity in leaves (C) and roots (D) of pomegranate cv. Wonderful. Data are the mean of four replicates. Different letters in each plate showed that there was a significant difference at the level of 0.05. Vertical bars represent standard error (±SE). Only the low and high dose of salts was measured.

4. Discussion

The general pattern of plant response to salinity is growth suppression depending on salt concentration and composition, physiological stage of plant and plant species. Toxicity symptoms were developed on the older leaves, especially, in their tips and margins, since NaCl is carried in the transpiration stream and it is accumulated where that stream ends.

Because older leaves had more time to accumulate NaCl, their symptoms appear first and are more severe (Benton Jones, 2002; Chatzissavvidis *et al*., 2008). In the present study, salinity affected negatively, mainly the number of leaves, number of lateral shoots and dry weight (%) of leaves. In line, leaf number of citrus rootstocks decreased with the increase in salinity (NaCl) level in the irrigation water (Anjum, 2008) and the number of lateral shoots of Cornus plants decreased in 50 and 100 mM of NaCl or Na_2SO_4 (Renault *et al*., 2001). Munns and Tester (2008) suggested that moderate salinity inhibits lateral shoot development that becomes apparent over weeks and it is a response to the osmotic effect of NaCl. Contrary to the suggestion of Tattini *et al*. (1995) that shoot elongation is more sensitive to salinity than the number of leaves, we did not found any effect on shoot length or plant height. However, works have reported that plant height, number of leaves, and stem diameter of pomegranate plants decreased significantly with increasing soil salinity (Khayyat *et al*., 2014).

Regarding dry weight (%) of leaves, a decline has been also reported for other NaCl or Na_2SO_4-treated pomegranate or other woody species (Doring and Ludders, 1986a; Papadakis *et al.*, 2007; Melgar *et al*., 2008). The observed consequences of salinity on plant growth may be the end result of reduced photosynthetic rate, or a complex sum of osmotic effects, ion toxicities and mineral perturbations in plants (Naeini *et al*., 2004). Especially, the leaf growth rate decreases when soil salinity is elevated, primarily due to the osmotic effect of the salt accumulation around the roots (Munns and Tester, 2008; Khayyat *et al*., 2014). As a consequence, a significant decline in water content has been also observed for salt-treated pomegranate plants (Doring and Ludders, 1986a). It is well known that the water availability for the plants grown under saline conditions is quite low, because of the increased osmotic potentials existing in the root environment. Reduced water uptake results in reduced turgor of leaves and subsequently closure of stomata, leading to a reduction in transpiration and photosynthesis. A decrease in plant water potential due to salt stress has been usually correlated with stomatal closure, leading to various physiological disturbances.

As previously mentioned, salinity affects plants in different ways such as osmotic effects, ion toxicity and/or nutritional disorders. Nutrient imbalances may result from the effect of salinity on nutrients availability, on competition of nutrients during uptake, and on nutrients transport and/or partitioning within the plant (Marschner, 1995). Therefore, nutrient uptake by plants can be reduced by excessive salts in the soil solution, either by direct competition between ions or by decreased osmotic potential of the solution reducing the mass flow of mineral nutrients to the root surface (Grattan and Grieve, 1992). Our results indicated that salinity affected negatively N concentration in top leaves and total N content. Similarly, Naeini *et al*. (2004) and Kulkarni *et al*. (2007) working on pomegranate found that N concentration in leaves decreased with an increase in salinity. Also, N concentrations of leaves of sour orange decreased with increasing NaCl in the nutrient solution (Chatzissavvidis *et al*., 2008). In line, Banuls and Primo-Millo (1995) found that N accumulation in orange plants was negatively correlated with Cl accumulation during salinity stress, speculating that this was due to some form of competition between nitrate and Cl ions. According to Grattan and Grieve (1992) that competition between Cl- and NO_3- uptake can occur in plants grown under saline stress. In detail, high concentration of Cl in nutrient solution may inhibit the low affinity nitrate transport system. Therefore, the reduction in N concentration of leaves and roots is closely related to the accumulation of Cl in tissues. On the other hand, there has been reported that there is a stronger relationship between reduced water use efficiency and N uptake than between N uptake and Cl content under salt stress. Accordingly, Chatzissavvidis *et al*. (2008) noted that leaf N of salt-stressed sour orange was reduced because of water stress. Finally, salinity might interfere with N metabolism in a number of ways starting with the uptake of nitrate and ammonium N. It is likely that changes occur at the site of nitrate reduction from the leaves to roots, altering nitrate transport to the shoots.

In line with our results, a negative effect of salinity on K concentration has been found in pomegranate or other woody species (Renault *et al*., 2001; Kulkarni *et al*., 2007; Papadakis *et al*., 2007). An apparent antagonistic relationship between Na and K has been distinguished in salt-treated pomegranate plants (Naeini *et al*., 2004). This antagonism may be related to the direct competition between uptake of K and Na at the root absorption site. Accordingly, we observed an accumulation of Na accompanied by a decrease in K in the roots and this has been also reported by Renault *et al*. (2001). A high concentration of Na can interfere

with K uptake, resulting in K deficiency and stunted growth. It is well known that the two ions compete for uptake at the plasma membrane level (Marschner, 1995). What is more, reduction in K^+ uptake in plants by Na^+ is a competitive process and occurs regardless of whether the solution is dominated by Na^+ salts of Cl^- or SO_4^{2-}. The decline of K concentration in roots during salinity stress period may also provide a mechanism by which plants achieve ionic balance following uptake of high Na concentrations in roots (Marschner, 1995). Therefore, the active absorption of K^+ versus Na^+ has to be sufficient in order to cover the K^+ metabolic needs of plants, and for the survival of plants grown in saline medium. Salinity may increase the energy consumption required for osmotic regulation and competition of transported ions. This may subsequently lead to a reduction of metabolically important ions such as K^+ (Kwon *et al*., 1995).

Calcium is important in cell biology during salt stress, for example, in preserving membrane integrity, signaling in osmoregulation and influencing K/Na selectivity (Marschner, 1995). It plays a critical role in the growth of roots under salinity conditions; under such conditions, Ca content may be reduced (Kwon *et al*., 1995), as was also observed in the present experiment. Esechie and Rodriguez (1998) reported that root pressure, which is a mechanism for Ca transport to plant, was decreased at high salinity and consequently decreased plant transpiration, leading to Ca deficiency. In accordance to our results, it has been reported that increasing salinity in growth medium led to a decline in Ca content of pomegranate and cherry leaves (Kulkarni *et al*., 2007; Papadakis *et al*., 2007). Also, Naeini *et al*. (2004) experimenting with pomegranate, suggested that the reduction in Ca absorption and translocation was perhaps due to the elevated Na concentration, as Na and Ca are antagonistic in their absorption and translocation. Interestingly, in saline stressed citrus plants, the decline in leaf Ca concentration was attributed to inhibition of Ca uptake by the roots (Melgar *et al.*, 2008).

Similarly to our results, Naeini *et al*. (2004) working with pomegranate found that Mg concentration in roots presented a decreasing trend with an increase in salinity. In line, a negative correlation between salinity and Mg concentration in leaves of pomegranate was also recorded by Kulkarni *et al*. (2007). Karimi and Hasanpour (2014) reported that increases in salinity (NaCl + $CaCl_2$) reduced shoot and root Mg concentration in pomegranate. A connection between salinity and Mg deficiency has also been reported by other researchers, although no antagonism has been confirmed between Na or Cl and Mg in absorption and translocation (Doring and Ludders, 1986a).

Doring and Ludders (1987) reported that Na concentration increased in pomegranate leaves of NaCl-treated plants compared to control, as was also observed in our experiment for NaCl and Na_2SO_4 treatments. Moreover, the findings of Karimi and Hasanpour (2014) and Naeini *et al*. (2004) that Na in roots of pomegranate enhanced significantly up to 40 and 30 mM NaCl, respectively, whereas at higher levels of salinity the increase was not significant, were in accordance to our results. Roots accumulate Na up to a determinate concentration and since root capacity for Na accumulation is saturated, then Na concentration of leaves increased with increasing of Na in nutrient media (Esechie and Rodriguez, 1998). Concerning Na partitioning, studies on pomegranate and olive (Naeini *et al*., 2004) grown in saline growth media showed that Na concentration of basal leaves was higher than that of apical leaves. In the present study, likely, NaCl and/or Na_2SO_4 treated plants had higher Na in basal leaves than in apical ones. However, unlike the results of Naeini *et al*. (2004), we found that the average Na concentration of leaves was higher than that of roots.

In fruit crops, sensitivity to salinity is closely correlated with Cl concentration in various plant tissues

and mainly in leaves. Leaves are the main sinks of Cl, since its concentration was found to be much higher in leaves than in stems and roots (Doring and Ludders, 1987; Papadakis *et al*., 2007). Naeini *et al*. (2004) experimenting with pomegranate reported that Cl accumulation in an increasing order was basal leaves > apical leaves > root, whereas we found higher Cl in apical than in basal leaves of KCl-treated plants. Since the transport of Cl^- ions occurs mainly in the transpiration stream, the above results were not unexpected. Chloride concentration in leaves increased with an increase in NaCl-induced salinity in three pomegranate cultivars (Naeini *et al*., 2004), as was found in the present work. We also observed that the increase in leaf Cl concentration was proportional to NaCl concentration in the nutrient solution. It is suggested that increasing salinity enhances Cl uptake and this is partly due to lower availability of Ca and as a result, enhanced permeability of root cell membranes (Marschner, 1995; Naeini *et al*., 2006). A study on NaCl-stressed citrus plants has shown that the toxic ion was Cl and leaf injury was associated with Cl (Bar *et al*., 1998). A particular threshold of leaf Cl concentration (around 15 g kg^{-1} DW) triggers leaf abscission through increased ethylene production (Raveh and Levy, 2005). Indeed, in this experiment, plants showing the first toxicity symptoms were those with the highest Cl (and not Na) concentration, in the treatment 120 mM KCl. In general, Cl concentration of 5 to 15 g kg^{-1} DW can be toxic depending on the type of plant, whereas resistant plants can tolerate up to 50 g kg^{-1} Cl without any noticeable damage (White and Broadley, 2001). The relatively low leaf Cl (≤14.6 g kg^{-1}) in our study could be linked to low leaf transpiration, high shoot to root ratio and/ or the ability of roots to retain a high Cl concentration. Specifically, it is established that the tolerant Citrus species are able to reduce the upward translocation of the Cl ions to the leaves (Melgar *et al*., 2008).

As pomegranate of the present experiment, many other species, e.g. citrus showed a decline in chlorophyll content in response to an increasing salt stress (Melgar *et al*., 2008). In general, chlorophyll concentration in plants has a strong negative correlation with salinity. Salinity may influence absorption of some ions, such as Mg^{2+} and Fe^{2+}, which are involved in chlorophyll formation (Sivstev *et al*., 1973), and in this study Mg concentration in plants was depressed by saline treatments. Also, it is well known that the chlorophyll degrading enzyme chlorophyllase is more active under salt stress. However, changes in chlorophyll during salinity vary depending on plant species, salt treatments and plant age. Specifically, NaCl-treated cherry plants showed significant reductions of chlorophyll concentration of leaves containing Na and Cl higher than 5 mg g^{-1} and 20 mg g^{-1}, respectively (Papadakis *et al*., 2007). In our experiment, the respective threshold values were 6.5 mg g^{-1} for Na (NaCl and Na_2SO_4 treatments) and 10 mg g^{-1} for Cl (NaCl and KCl treatments). Also, whereas Doring and Ludders (1986b) observed that the lowest chlorophyll contents of pomegranate leaves was observed at medium salt level (60 mM NaCl or Na_2SO_4), our results indicated the most pronounced decline in the chlorophyll concentration of plants treated with 120 mM NaCl or Na_2SO_4.

Furthermore, salts might built up in the chloroplast and exert a direct toxic effect on photosynthetic processes (Munns and Tester, 2008). However, Doring and Ludders (1986b) reported that the rate of photosynthesis in pomegranate was hardly influenced, despite the reduced chlorophyll concentration. Therefore, they concluded that an activity-increase of the existing amount of chlorophyll can be determined. This may also be the explanation for chlorophyll fluorescence parameters not having been affected by salinity in our experiment. Although chlorophyll fluorescence parameters were strongly affected by NaCl

stress in two Iranian pomegranate cultivars (Khayyat *et al*., 2014), other researchers reported that F_v/F_m ratio was not affected by salinity in citrus or olive plants (Melgar *et al*., 2008).

Similarly to our results for KCl and Na_2SO_4 treatments, many studies on pomegranates subjected to saline treatments indicated that carbohydrates in leaves decreased as salinity increased (Doring and Ludders, 1986a, 1986b; Naeini *et al*., 2004). Khayyat *et al*. (2014) suggested that with salinity (NaCl) increment, accumulation of total carbohydrates in two pomegranate cultivars was depressed significantly. As an explanation, Doring and Ludders (1986a) concluded that high osmotic pressure caused by salt accumulation may inhibit activity of hydrocarbons-synthesizing enzymes and, as a result, decreased soluble sugars concentration.

Interestingly, there was a similar pattern between the activities of the two studied enzymes (POD, DAO) demonstrating their close relationship, as was also found in chick-pea plants (Angelini *et al*., 1990).

In our case, the supplied salt form and concentration did not influence the activity of the above enzymes in four of the six saline treatments, but Na_2SO_4 treatments increased POD and DAO activity. In terms of antioxidant activity, a higher sensitivity to Na_2SO_4 than NaCl was also found by Tarchoune *et al*. (2010) experimenting with basil plants.

Many reports have referred the enhanced POD and DAO activity under salinity stress (e.g. Dionisio-Sese and Tobita, 1998). However, our results have shown that even at 120 mM NaCl or KCl antioxidant enzyme activities remained unchanged compared to control, indicating that these salt concentrations were not capable to produce oxidative damage. This antioxidant response may be indicative of the high tolerance of pomegranates to salinity stress.

5. Conclusions

Overall, the results show that pomegranate plants present mechanisms, such as that of shoot-to-root growth regulation, that alleviate the detrimental effects of salinity. Therefore, our plants, even under high saline treatments, managed to maintain water content, chlorophyll fluorescence and enzyme activity in normal levels. These results suggest that 'Wonderful' plants may be cultivated under saline conditions provided that a suitable fertilization program is used.

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