Turkish Journal of Agricultural and Natural Sciences
Special Issue: 2, 2014

TÜRK
TARIM ve DOĞA BİLİMLERİ
DERGİSİ

TURKISH
JOURNAL of AGRICULTURAL
and NATURAL SCIENCES

www.turkjans.com

Effect of Calcium and Boron on the Ion Status, Carbohydrate and Proline Content, Gas Exchange Parameters and Growth Performance of Pomegranate cv. Wonderful Plants Grown Under NaCl Stress

Eleana SARAFI[a*], Christos CHATZISSAVVIDIS[b], Ioannis THERIOS[a]
[a] Laboratory of Pomology, School of Agriculture, Aristotle University, 541 24 Thessaloniki, Greece
[b] Laboratory of Pomology, Department of Agricultural Development, Democritus University of Thrace, 68200 Orestiada, Greece
* Corresponding author: eleanasarafi26@hotmail.com

Abstract

A greenhouse experiment was conducted to study the effects of sodium chloride (NaCl) on growth, nutrient status, carbohydrate and proline content and gas exchange parameters of pomegranate plants (*Punica granatum* L.) cv. Wonderful. One-year-old own-rooted pomegranate plants were grown for 58 days in a 1:1 sand–perlite medium. They were irrigated with nutrient solutions containing two concentrations of B (25 or 100 µM) in combination with 0, 40 or 80 mM NaCl and 1.0 or 10 mM $CaCl_2$, respectively. At the end of the experiment, the greatest height was observed in plants treated with 1.0 or 10 mM $CaCl_2$ or 100 µM B, whereas it was significantly reduced by the inclusion of NaCl into the nutrient solution. Similarly, a decline of fresh and dry matter weight was recorded in the treatment with 80 mM NaCl. The concentration of chlorophyll and carbohydrates in leaves was unaffected by the inclusion of NaCl into the nutrient solution, whereas in roots, the respective concentrations of carbohydrates were reduced by 50% compared to control. Moreover, as a result of salinity (mainly 80 mM NaCl), a decrease of photosynthetic parameters (photosynthetic rate and stomatal conductance) was recorded, while proline concentration of leaves increased and that of roots was reduced. Finally, the inclusion of NaCl in the nutrient solution led to increased Na and Cl, reduced P and Mg in leaves, and P, K and Zn in roots. However, B, Fe and Mn concentrations were not affected by NaCl treatments.

Keywords: Boron, Chlorophyll content, Photosynthetic parameters, Nutrient concentration, Pomegranate, Salinity

Introduction

Over one and a half billion hectares of land are salt-affected and soil salinity is a worldwide problem reducing the cultivated land and affecting the chain of food supply. Pomegranate is considered as moderately tolerant to salinity (Allen et al., 1988; Maas, 1993). Pomegranate (*Punica granatum L.*) is growing extensively in arid and semi-arid regions wordwide (Sarkhosh et al., 2006) and is one of the oldest edible fruits (Blumenfeld et al., 2006). In Greece, the last 10 years the plantations of pomegranate (mainly cv. Wonderful) has been significantly expanded.

Salinity can decrease plant growth (Munns, 1993; Navarro et al., 2007), assimilation rate of fundamental elements, photosynthetic rate (Marschner, 1995; Debez et al., 2008), impair nutrition and cause various other metabolic disturbances. A strategy to cope with salinity is to use salt tolerant cultivars. There was reported that some pomegranate cultivars present higher tolerance to salinity than others by maintaining their vegetative growth and lower chloride transport to the shoot (Karimi and Hasanpour, 2014). Interestingly, hydroponic experiments with pomegranates indicated that some cultivars may tolerate up to 40 mM NaCl (Naeini et al., 2006). Another technique to alleviate salinity stress is to supplement calcium (Ca) in the growth medium (Kaya et al., 2002; Chatzissavvidis et al., 2008; Nedjimi et al., 2009). Calcium contributes to the maintenance of membrane integrity and remediation of the adverse effects of salinity (Sotiropoulos, 2007).

Moreover, the behavior of plants to salt stress may be modified by the level of boron (B) concentration in the nutrient solution, due to salinity x B interactive effects (Alpaslan and Gunnes, 2001; Bastias et al., 2010).

Since there is not much data in the literature about the response of the commercial pomegranate cv. Wonderful to salinity, the aim of this paper is to examine the interactive effects of salinity, Ca and B on growth performance, nutrient composition, gas exchange parameters, chlorophyll, carbohydrates and proline content of that cultivar.

Materials and Methods

Plant material and treatments

One-year-old, uniform, own-rooted pomegranate plants of the commercial pomegranate cultivar Wonderful (*Punica granatum* L.) were planted in 3 L plastic bags containing inert sand:perlite (1:1) medium and then were randomized in the different treatments based on their initial fresh weight. The plants were placed into a glasshouse and irrigated every 2 days with 250 ml per plant of a 25% Hoagland No 2 nutrient solution (Hoagland and Arnon, 1950) modified with B (25 and 100 μM), NaCl (0, 40 and 80 mM) and $CaCl_2$ (1 and 10 mM). The experimental design was completely randomized with 10 treatments and six replicates per treatment. Every 15 days, 400 ml of distilled water was supplied to each plant in order to leach out any accumulated salts. This quantity is enough to allow some leaching in order to prevent build-up of salts. The experiment lasted 58 days and held in a glass greenhouse of the laboratory of Pomology at the Aristotle University of Thessaloniki (Northern Greece).

Plant growth and analysis of nutrient content

At harvest, plants were separated into leaves, stems and roots and their dry and fresh matter weights were measured. All samples were washed twice with distilled water, dried at 68°C for 48 h and then ground to a fine powder to pass through a 30-mesh screen. Dry samples were stored in a cool room (12±2°C) until analyzed in one batch in order to avoid batch-to-batch variation in analysis. Tissue B concentration determinations were made by dry ashing 0.5 g of dry plant material in a muffle furnace at 500°C for 6 h. The ash was dissolved in 0.1N HCl and B was determined colorimetrically (420 nm) by the Azomethine-H method (Wolf, 1974).

The inorganic analyses of the rest elements were conducted by dry ashing of plant material as above. Afterwards the ash was dissolved in 3 ml 6N HCl and each solution was diluted (16-fold) with deionised water. Phosphorus (P) concentration was determined by the vanado-molybdo-phosphate yellow color method and potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn) and zinc (Zn) concentrations by atomic absorption spectroscopy (Perkin-Elmer 2380, Norwalk, CT, USA), using standard methods.

Carbohydrate and proline concentration

For the determination of carbohydrates and proline concentration the methods of Khan et al. (2000) were followed. In both parameters, three replications per treatment were used.

Chlorophyll content and measurement of photosynthetic parameters

The chlorophyll concentration in leaves was determined in fully developed leaves (6^{th} to 8^{th} leaf from the apex of the plant), according to Wintermans and De Mots (1965).

Moreover, at the termination of the experiment, photosynthetic rate and stomatal conductance were measured using the ADC BioScientific LC pro system Serial No.31613. For these measurements, young fully expanded leaves (2^{nd} and 3^{rd} leaves counting from the shoot apex) were used. Measurements were carried out in the noon (12.00–14.00 p.m.) at steady light intensity (>900 mol m^{-2} s^{-1}), and leaf temperature between 24 and 27°C.

Statistical analysis

The experimental layout which consisted of 10 treatments included one cultivar, two B concentrations (25 and 100 μM), three NaCl concentrations (0, 40, 80 mM), two $CaCl_2$ concentrations (1 and 10 mM) and six replicates (plants) per treatment. Data were subjected to analysis of variance (ANOVA). For comparison of means the Duncan's multiple range test was used ($P \leq 0.05$) using the SPSS 15.0 statistical package (SPSS, Inc., Chicago, USA).

Results

Fresh and dry weight

Our data indicated that salinity (NaCl 40 and 80 mM) reduced total fresh weight per plant and that of leaves (Table 1). This decrease is

about 60%. However, fresh matter weight (%) of stems and roots was not significantly different compared to control. Concerning dry matter weight, no difference was recorded of all treatments compared to control. However, the treatment NaCl 80 mM + $CaCl_2$ 10 mM + B 100 µM resulted in a decrease by 57% of dry matter weight compared to control.

Visible symptoms of NaCl toxicity were observed mostly with 80 mM NaCl without additional $CaCl_2$ or B. The toxic effect of NaCl was obvious both in basal and apical leaves (data are not presented). Initially, NaCl toxicity symptoms appeared as tip chlorosis and were extended to the base of leaf blade. Subsequently, chlorosis covered the whole leaf blade surface.

Chemical composition

B

Increasing NaCl supply in the nutrient solution led to a decline in B concentration (Table 1) in all plant parts but it did not differed significantly compared to control. Statistically significant increase of B was observed in the presence of 25 µM B or 100 µM B plus 1 mM $CaCl_2$. This B concentration was 2-3 times greater compared to control values.

P

The final P concentration ranged between 0.06 and 0.14% in leaves and between 0.06 and 0.18% in roots (Table 2). Maximum P concentration in the previous plant tissues was observed in the plants treated with B 100 µM. Addition of NaCl (40 and 80 mM) in combination with or without $CaCl_2$, reduced significantly the P concentration in leaves, whereas in roots the treatment NaCl 80 mM + $CaCl_2$ 10 mM + B 100 µM reduced by about 50% the concentration of P, in comparison to the control.

Na

At the termination of the experiment, Na concentration ranged from 0.06 to 2.13% in leaves and from 0.26 to 1.11% in roots. Na concentration in leaves of 'Wonderful' was higher than that in roots. Addition of NaCl (40 mM) increased significantly Na concentration both in leaves and roots. Increasing NaCl from 40 to 80 mM in combination with B 100 µM and with or without $CaCl_2$ doubled the concentration of Na in leaves which differed significantly in comparison to the control. On the contrary, Na concentration in roots was reduced by 20% in the plants treated with 80 mM NaCl+10 mM $CaCl_2$+100 µM B.

K

Leaves of 'Wonderful' plants showed higher concentration of K in comparison to roots. Maximum leaf K concentration was observed in the control plants. Addition of 40 mM NaCl with or without $CaCl_2$ and additional concentration of B 100 µM, reduced the K concentration but not differed significantly compared to the control. The highest decrease of K concentration (1.13%) was observed in the treatment NaCl 80 mM + $CaCl_2$ 1 mM + B 100 µM. In contrast, K concentration in roots increased significantly in the plants treated with B 100 µM. Inclusion of NaCl (40 and 80 mM) in the nutrient solution led to a significant reduction in K that ranged between 0.58 and 0.65%.

Ca

At the termination of the experiment, Ca concentration ranged between 0.93 and 4.00% in leaves and between 0.83 and 2.08% in roots. The maximum concentration of Ca in leaves and roots was recorded in the plants treated with 10 and 1 mM $CaCl_2$ combined with B, respectively. Addition of NaCl (40 and 80 mM) plus B 100 µM reduced significantly Ca concentration in leaves to 0.98-0.93%. In contrast, saline treatments affected negatively but not significantly Ca concentration in roots. Addition of 1 and 10 mM $CaCl_2$ increased by 23 and 97% Ca levels compared to 40 mM NaCl, respectively. In the case of NaCl 80 mM, the addition of 10 mM $CaCl_2$ increased its levels by 93%.

Cl

The final leaf Cl concentration increased from 0.50% in the control plants to 2.19% .The addition of NaCl with or without $CaCl_2$ and B 100 µM increased significantly the Cl concentration both in leaves and roots. Maximum Cl concentration in leaves was observed in the plants treated with 80 mM NaCl+10 mM $CaCl_2$+ B 100 µM, whereas in roots it was recorded in the respective treatment with $CaCl_2$ 1 mM. Furthermore, significant increase of Cl concentration in roots was observed in presence of 10 mM $CaCl_2$ and 100 µM B.

Mg

At the end of the experiment, Mg concentration ranged between 0.36 and 0.54%

in leaves (Table 2) and between 0.20 and 0.32 % in roots. Maximum Mg concentration in leaves was observed in the control plants. The addition of NaCl (40 and 80 mM) reduced significantly the Mg concentration both in leaves and roots in comparison to the control. Mg concentration in leaves decreased by 50% in comparison to control.

Fe

At the termination of the experiment, Fe concentration ranged between 51 and 386 μg g^{-1} in leaves (Table 2) and between 66 and 341 μg g^{-1} in roots. The maximum Fe concentration both in leaves (47 times greater than control) and roots was observed in the plants treated with 100 μM B and 10 mM $CaCl_2$. The presence of 80 mM NaCl with or without $CaCl_2$ plus 100 μM B reduced significantly the Fe in the above plant parts compared to control.

Mn

The final Mn concentration ranged between 11 and 28 μg g^{-1} in leaves and between 9 and 20 μg g^{-1} in roots. Minimum Mn concentrations in leaves were observed in control plants or in those treated with B 100 μM, while maximum concentration of Mn was observed with further inclusion of 10 mM $CaCl_2$ and this concentration differed significantly from the control. Mn concentration was lower in the roots than in the leaves of Wonderful. Addition of 40 mM NaCl and 100 μM B decreased by 30% the Mn concentration in roots. However, further NaCl supply (80 mM) with or without $CaCl_2$ increased significantly the concentration of Mn in comparison to the control.

Zn

At the end of the experiment, Zn concentration ranged between 11 and 18 μg g^{-1} in leaves and between 13 and 18 μg g^{-1} in roots. Inclusion of 100 μM B plus 1 or 10 mM $CaCl_2$ in the nutrient solution significantly increased the concentration of Zn compared to control. Salinity (mainly 80 mM NaCl) combined with B 100 μM reduced the concentration of Zn whereas, the further addition of 10 mM $CaCl_2$ caused a significant increase by 18.35% compared to control.

Photosynthetic parameters

Photosynthetic rate (P_n) values ranged between 2.31 to 6.32 μmol CO_2 m^{-2} s^{-1}. 'Wonderful' plants treated with additional B 100 μM presented the maximum photosynthetic rate (6.32 μmol CO_2 m^{-2} s^{-1}). Addition of 40 mM NaCl with or without $CaCl_2$ plus B 100 μM decreased significantly the photosynthetic rate in comparison to the control. On the contrary, stomatal conductance was not affected significantly by any of the treatments applied in the cv. Wonderful. The lowest P_n value (2.31 μmol m^{-2} s^{-1}) was recorded in the treatment with 40 mM NaCl plus 100 μM B.

Chlorophyll concentration

Chl *α* concentration values ranged between 3.1 and 6.2 mg g^{-1}. On the other hand, Chl *b* concentration was about half the value of Chl *α* and ranged between 1.5 to 2.8 mg g^{-1}. Chl *α*, as well as Chl *b* and total chlorophyll (Chl *a* + Chl *b*) concentration in leaves of 'Wonderful' increased significantly in the treatment with 10 mM $CaCl_2$ and 100 μM. Inclusion of NaCl (40 and 80 mM) in combination with or without $CaCl_2$ did not affect the Chl *α* and *Chl b* values, whereas total chlorophyll (Chl *a* + Chl *b*) in the treatment NaCl 80 mM + $CaCl_2$ 1 mM + B 100 μM decreased significantly in comparison to the control.

Table 1. Effect of B, NaCl and $CaCl_2$ in the nutrient solution on B concentration and dry matter weight of pomegranate cv. Wonderful plants.

Treatments	Boron (µg g^{-1})			Fresh matter weight (g)					Dry matter weight (g)		
	Leaves	Stems	Roots	Total	Leaves	Stems	Roots	Total	Leaves	Stems	Roots
Control	28 b	46 b	71 cd	20.36 a	5.58 a	5.51 a	9.27 abc	7.43 a	2.30 a	2.87 a	2.27 a
B 100	57 a	130 a	244 a	20.41 a	5.87 a	5.41 a	9.13 abc	7.67 a	2.47 a	2.78 a	2.42 a
$CaCl_2$ 1 + B 100	50 a	133 a	204 b	20.16 a	5.54 a	5.33 a	9.29 abc	6.77 a	2.16 ab	2.58 a	2.04 a
$CaCl_2$ 10 + B 100	14 c	45 b	81 c	16.85 ab	4.00 b	5.15 a	7.71 abc	5.82 a	1.53 abc	2.76 a	1.53 a
NaCl 40 + B 100	19 bc	32 b	46 cd	17.54 ab	3.64 b	4.00 a	9.90 ab	5.77 a	1.40 abc	2.05 a	2.32 a
NaCl 40 + $CaCl_2$ 1 + B 100	28 b	46 b	65 cd	18.67 ab	3.63 b	4.34 a	10.70 a	5.58 a	1.43 abc	2.20 a	1.95 a
NaCl 40 + $CaCl_2$ 10 + B 100	23 bc	35 b	46 cd	15.53 b	3.71 b	4.00 a	7.83 abc	7.22 a	1.33 abc	2.17 a	3.72 a
NaCl 80 + B 100	20 bc	30 b	43 cd	12.86 b	3.25 b	3.28 a	6.32 bc	4.53 a	1.11 bc	1.71 a	1.72 a
NaCl 80 + $CaCl_2$ 1 + B 100	24 bc	31 b	35 d	12.21 b	2.82 b	2.90 a	6.50 bc	5.04 a	2.00 abc	1.56 a	1.48 a
NaCl 80 + $CaCl_2$ 10 + B 100	28 b	35 b	41 cd	11.67 b	3.03 b	3.13 a	5.52 c	4.46 a	1.00 c	1.65 a	1.83 a
P-values (3-way ANOVA)											
B	***	***	***	0.916 ns	0.867 ns	0.897 ns	0.741 ns	0.862 ns	0.909 ns	0.996 ns	0.808 ns
NaCl	***	***	***	0.001 **	***	0.004 **	0.004 **	0.112 ns	0.034 *	0.016 *	0.238 ns
$CaCl_2$	***	***	***	0.349 ns	0.155 ns	0.961 ns	0.176 ns	0.975 ns	0.130 ns	0.967 ns	0.646 ns
NaCl x $CaCl_2$	***	***	***	0.960 ns	0.113 ns	0.990 ns	0.959 ns	0.678 ns	0.372 ns	0.994 ns	0.480 ns

Means followed by the same letters within each column are not significantly different at $P \leq 0.05$ (Duncan's multiple range test, n=6).

ns: Not significantly different at the *P* B 0.05 probability level

***/**/*: Significantly different at the *P* B 0.001/0.01/0.05 probability level, respectively.

Table 2. Effect of B, NaCl and $CaCl_2$ in the nutrient solution on nutrient concentrations in leaves and roots of pomegranate cv. Wonderful plants.

Plant part	Treatments	P (%)	Na	K	Ca	Cl ($\mu g\ g^{-1}$)	Mg	Fe	Mn	Zn
Leaves	Control	0.14 a	0.12 c	1.87 a	2.04 c	0.50 f	0.54 a	143 b	11 b	13 cd
	B 100	0.14 a	0.13 c	1.57 a	1.95 c	0.46 f	0.52 a	95 b	14 b	14 bc
	$CaCl_2$ 1 + B 100	0.14 a	0.07 c	1.67 a	2.84 b	0.88 ef	0.47 abc	76 b	17 ab	16 ab
	$CaCl_2$ 10 + B 100	0.08 bc	0.07 c	1.61 a	4.00 a	1.43 cd	0.50 ab	386 a	28 a	18 a
	NaCl 40 + B 100	0.08 bc	0.90 b	1.58 a	0.98 d	1.20 de	0.38 d	50 b	18 ab	16 ab
	NaCl 40 + $CaCl_2$ 1 + B 100	0.09 bc	0.84 b	1.55 a	1.21 d	1.27 de	0.39 cd	51 b	18 ab	16 ab
	NaCl 40 + $CaCl_2$ 10 + B 100	0.06 c	0.90 b	1.65 a	1.94 c	1.50 bcd	0.40 cd	55 b	23 ab	13 cd
	NaCl 80 + B 100	0.08 bc	1.76 a	1.57 a	0.93 d	1.97 ab	0.37 d	63 b	20 ab	11 d
	NaCl 80 + $CaCl_2$ 1 + B 100	0.10 b	2.13 a	1.13 b	0.95 d	1.89 abc	0.36 d	54 b	16 ab	12 cd
	NaCl 80 + $CaCl_2$ 10 + B 100	0.06 c	1.65 a	1.69 a	1.80 c	2.19 a	0.43 bcd	59 b	14 b	18 a
	P-values (3-way ANOVA)									
	B	1.000 ns	0.939 ns	0.078 ns	0.748 ns	0.819 ns	0.832 ns	0.749 ns	0.520 ns	0.331 ns
	NaCl	***	***	0.320 ns	***	***	***	0.037 *	0.553 ns	0.003 **
	$CaCl_2$	***	0.721 ns	0.176 ns	***	0.002 **	0.255 ns	0.133 ns	0.247 ns	0.003 **
	NaCl x $CaCl_2$	0.131 *	0.689 ns	0.115 ns	0.006 **	0.163 ns	0.510 ns	0.163 ns	0.135 ns	***
Roots	Control	0.18 a	0.51 de	0.93 ab	1.25 bcd	0.11 d	0.32 ab	177 ab	13 ab	25 a
	B 100	0.18 a	0.46 def	1.07 a	1.48 bc	0.09 d	0.33 a	222 ab	16 ab	22 a
	$CaCl_2$ 1 + B 100	0.14 b	0.30 ef	0.95 ab	1.58 b	0.45 c	0.23 cd	137 b	13 ab	23 a
	$CaCl_2$ 10 + B 100	0.06 d	0.26 f	0.77 bc	2.08 a	0.57 c	0.29 abc	341 a	16ab	15 b
	NaCl 40 + B 100	0.09 c	0.81 bc	0.65 c	0.83 d	0.84 b	0.26 bcd	133 b	9 b	14 b
	NaCl 40 + $CaCl_2$ 1 + B 100	0.11 c	0.91 ab	0.66 c	1.26 bcd	0.75 b	0.31 ab	240 ab	15 ab	14 b
	NaCl 40 + $CaCl_2$ 10 + B 100	0.06 d	0.63 cd	0.62 c	1.56 bc	0.81 b	0.21 d	73 b	11 b	13 b
	NaCl 80 + B 100	0.10 c	1.06 a	0.60 c	0.89 d	1.12 a	0.21 d	66 b	15 ab	14 b
	NaCl 80 + $CaCl_2$ 1 + B 100	0.10 c	1.11 a	0.73 c	1.07 cd	1.15 a	0.30 abc	90 b	20 a	14 b
	NaCl 80 + $CaCl_2$ 10 + B 100	0.09 c	0.83 b	0.56 c	1.46 bc	1.05 a	0.19 d	67 b	16 ab	14 b
	P-values (3-way ANOVA)									
	B	0.364 ns	0.904 ns	0.062 ns	0.270 ns	0.631 ns	0.640 ns	0.459 ns	0.520 ns	0.331 ns
	NaCl	***	***	***	***	***	0.021 *	0.004 **	0.553 ns	0.003 **
	$CaCl_2$	***	***	0.022*	***	0.003 *	0.018 *	0.938 ns	0.247 ns	0.003 **
	NaCl x $CaCl_2$	***	0.213 ns	0.084 ns	0.847 ns	***	***	0.040 *	0.135 ns	***

Means followed by the same letters within each column are not significantly different at $P \leq 0.05$ (Duncan's multiple range test, n=6).
ns: Not significantly different at the *P* B 0.05 probability level; ***/**/*: Significantly different at the *P* B 0.001/0.01/0.05 probability level, respectively.

Carbohydrate and proline concentration

At the termination of the experiment carbohydrate concentration in leaves of Wonderful plants was found not to be significantly altered by the salinity as well as by the addition of $CaCl_2$ (Table 4). On the contrary, addition of NaCl (40 and 80 mM) in the nutrient solution with or without $CaCl_2$ and B 100 µM decreased significantly the carbohydrate concentration of roots in comparison to the control. Regarding the concentration of proline, the treatment with 10 mM or without $CaCl_2$ and 100 µM B decreased proline concentration which differed significantly only in roots. Addition of 40 mM NaCl plus 10 mM $CaCl_2$ and 100 µM B increased significantly the concentration of proline in leaves as well as in roots. Further addition of NaCl (80 mM) with or without $CaCl_2$ and B 100 µM increased proline concentration in leaves, whereas decreased it in roots, in comparison to the control.

Table 3. Effect of B, NaCl and $CaCl_2$ in the nutrient solution on photosynthetic rate and stomatal conductance of pomegranate cv. Wonderful plants.

Treatments	Photosynthetic rate (P_n) ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	Stomatal conductance (g_s) ($mmol\ CO_2\ m^{-2}\ s^{-1}$)
Control	4.90 ab	0.05 ab
B 100	6.32 a	0.07 a
$CaCl_2$ 1 + B 100	5.13 ab	0.05 ab
$CaCl_2$ 10 + B 100	3.45 bc	0.04 b
NaCl 40 + B 100	2.31 c	0.03 b
NaCl 40 + $CaCl_2$ 1 + B 100	3.55 bc	0.03 b
NaCl 40 + $CaCl_2$ 10 + B 100	2.39 c	0.03 b
NaCl 80 + B 100	4.10 abc	0.03 b
NaCl 80 + $CaCl_2$ 1 + B 100	3.60 bc	0.04 b
NaCl 80 + $CaCl_2$ 10 + B 100	3.11 bc	0.04 b
P-values (3-way ANOVA)		
B	0.180 ns	0.191 ns
NaCl	0.005 **	0.043 *
$CaCl_2$	0.155 ns	0.667 ns
NaCl x $CaCl_2$	0.359 ns	0.371 ns

Means followed by the same letters within each column are not significantly different at $P \leq 0.05$ (Duncan's multiple range test, n=6).

ns: Not significantly different at the *P* B 0.05 probability level.

***/**/*: Significantly different at the *P* B 0.001/0.01/0.05 probability level, respectively.

Table 4. Effect of B, NaCl and $CaCl_2$ in the nutrient solution on leaf chlorophyll, proline and carbohydrate concentrations of pomegranate cv. Wonderful plants.

Treatments	Chlorophyll (mg g^{-1} dry matter wt)			Carbohydrates (mmol g^{-1})		Proline (mmol g^{-1})	
	Chl α	Chl *b*	Chl α+*b*	Leaves	Roots	Leaves	Roots
Control	6.216 a	2.766 a	8.98 a	545 a	0.61 a	7.09 cd	3.63 a
B 100	5.593 a	2.515 a	8.11 a	609 a	0.56 ab	6.72 cd	2.53 bc
$CaCl_2$ 1 + B 100	6.236 a	2.761 a	8.99 a	619 a	0.56 ab	7.00 cd	3.03 ab
$CaCl_2$ 10 + B 100	3.196 b	1.517 b	4.71 b	514 a	0.48 abc	5.64 d	1.68 cd
NaCl 40 + B 100	6.240 a	2.836 a	9.07 a	460 a	0.33 cd	8.00 bc	1.42 d
NaCl 40 + $CaCl_2$ 1 + B 100	6.116 a	2.727 a	8.85 a	543 a	o.37 bcd	8.64 bc	1.78 cd
NaCl 40 + $CaCl_2$ 10 + B 100	5.416 a	2.281 ab	7.70 a	518 a	0.39 bcd	11.50 a	1.42 d
NaCl 80 + B 100	5.670 a	2.610 a	8.28 a	509 a	0.29 cd	10.40 ab	1.38 d
NaCl 80 + $CaCl_2$ 1 + B 100	4.436 ab	1.954 ab	6.39 ab	493 a	0.24 d	11.30 a	1.73 cd
NaCl 80 + $CaCl_2$ 10 + B 100	5.646 a	2.466 a	8.11 a	457 a	0.40 bcd	11.04 a	2.18 bcd
P-values (3-way ANOVA)							
B	0.454 ns	0.523 ns	0.475 ns	0.370 ns	0.530 ns	0.765 ns	0.025 *
NaCl	0.181 ns	0.298 ns	0.212 ns	0.031 *	***	***	0.014 *
$CaCl_2$	0.029 *	0.026 *	0.028 *	0.524 ns	0.557 ns	0.572 ns	0.240 ns
NaCl x $CaCl_2$	0.024 *	0.057 ns	0.031 *	0.852 ns	0.670 ns	0.262 ns	0.121 ns

Means followed by the same letters within each column are not significantly different at $P \leq 0.05$ (Duncan's multiple range test, n=6).
ns: Not significantly different at the *P* B 0.05 probability level
***/**/*: Significantly different at the *P* B 0.001/0.01/0.05 probability level, respectively.

Discussion

Salinity exerted a significant effect on growth; hence, salinity reduced the total fresh and dry weight of leaves. Leaves and roots accumulate the toxic ions of Na and Cl. Similar data are reported for several pomegranate cultivars (Doring and Ludders, 1987; Naeini et al., 2006).

The depression of plant growth by NaCl stress is not merely due to osmotic stress but ion toxicity also is participated (Greenway and Munns, 1980; Marschner, 1995; Martin and Korbner 1995). Excess Na is frequently believed to be mainly responsible for the stress, although high levels of Cl are found in salinized pomegranate plant tissues (Patil and Waghmare, 1982). The decrease of fresh weight of the pomegranate plants is largely attributable to reduced leaf area and therefore to light interception, which leads to a decrease of carbon assimilation. A smaller part of this decrease is due to stomatal closure by decreasing stomatal conductance as a consequence of salinity.

According to our data the harmful effects of salinity (visual toxicity symptoms) was recorded only in leaves with 80 mM NaCl. This indicates that the general statement in the literature that pomegranate is a tolerant species to salinity is not accurate. The cv. Wonderful seems not to tolerate saline treatments above 80 mM NaCl. Except for the reduced leaf expansion and photosynthetic rate, a similar decrease in gas exchange (Zhao et al, 2007) could explain the observed growth reduction at high salinity. However, other minor factors cannot be excluded (Greenway and Munns, 1980) suggesting that salinity exerts its effects directly on cell extension or cell division. This is in line with our data that the main effect of salinity in pomegranate cv. Wonderful was a reduction in leaf area.

Inclusion of extra B (100 µM) in the nutrient solution increased the growth rate of the main stem of plants, indicating that the studied cultivar requires higher B level for optimum growth compared to other fruit trees, without presenting problems of B toxicity. However, the results of other investigators indicated that the extra B did not completely restore growth reduction caused by salt stress (Ortiz et al., 1994).

Inclusion of Ca in the salinized nutrient solution exerted a significant effect in reducing the negative effects of salinity. In fact, Ca has a role in stabilizing membranes, in signal transduction through second messenger and controlling enzyme activity (White and Broadley, 2003), membrane integrity and ion transport (Martinez-Ballesta et al., 2006). Similar results for the role of Ca in remediating the adverse effects of salinity on plants were also reported for other plant species (Cabanero et al., 2004; Silva et al., 2007; Chatzissavvidis et al., 2008).

Chlorophyll concentration was affected by the various combinations of NaCl, $CaCl_2$ and B (100 µM) in solution. Hence, inclusion of $CaCl_2$ to nutrient solution containing 40 or 80 mM NaCl restored Chl *α* and Chl *b* concentrations to control levels. On the contrary, 80 mM NaCl plus 1 mM $CaCl_2$ decreased significantly chlorophyll concentration. Chlorophyll changes induced by salinity were reported also for other plants such as lentil (Tewari and Singh, 1991). This indicates that the high level of salinization induced a significant effect on the content of chlorophyll. This may be due to that total chlorophyll depends on the biological processes and stage of plant development. Various trends in chlorophyll concentration as affected by salinity were reported for other plant species. According to Misra et al. (1997) salinity stress increased the concentration of chlorophyll due to increased number of chloroplasts in the salt stressed plants. As a general rule, chlorophyll content is reduced due to salinity in salt sensitive plants (Hamada and El-Enany, 1991) and increased in plants tolerant to salinity (Singh et al., 1990).

The concentration of carbohydrates in leaves was unaffected by the inclusion of NaCl in the nutrient solution, whereas in roots the respective concentration was reduced by 50% compared to control. Many plant species which are NaCl-stressed accumulate soluble carbohydrates, a fact which indicates impairment of carbohydrate utilization. However, the constant level of carbohydrates in our plants under NaCl stress indicates no impairment of carbohydrate in the leaves. The reduced level of carbohydrates in roots (six times) may indicate that the transport of carbohydrates from leaves to roots was reduced. This reduced transport helps the leaves to maintain high carbohydrate level necessary for osmotic adjustment after NaCl stress (Zidan and Al-Zahrani, 1994). The constant carbohydrate level in leaves is consistent with no blockage in utilization of sugars in the growing leaves.

The photosynthetic process can be negatively affected by salinity as reported by Naumann et al. (2007). A decrease of Pn with increasing salinity takes place in spite of osmotic adjustment in *Punica granatum* L. The reduced Pn recorded in our experiment is partly associated with a toxic action of Na and Cl ions, inadequate compartmentation of ions in the vacuoles and very low synthesis of osmoregulators (Franco et al., 1999). The process of photosynthesis can be inhibited by NaCl effects on stomatal conductance leading to stomatal closure which limit CO_2 diffusion

to the chloroplasts (Centritto et al., 2003). In our experiment, stomatal conductance under salinity due to NaCl was reduced, maybe due to K-Na competition. Decrease of K in stomata results in their closure, which reduce internal (Ci) CO_2 concentration. The decrease of CO_2 assimilation may be ascribed to NaCl effect on stomatal and also to non stomatal factors. However, according to Meinzer et al (1994), photosynthesis was impaired by the biochemical capacity (mesophyll conductance) rather than stomatal conductance at high salinity. Our results for the cv. Wonderful are in agreement with several reports about other plant species (e.g. Zhao et al., 2007; Debez et al., 2008).

Proline is a compatible solute and evidence exists for its role as osmotic agent and protector of cellular structures. Accumulation of proline increases cellular osmotic pressure that can results in influx of water and creates turgor pressure which is required for cell expansion (Perez-Alfocea et al., 1993). Proline accumulation under NaCl stress was high in leaves and significantly lower in roots of pomegranate. The increase in proline concentration under NaCl in our experiments may be due to breakdown of proline – rich protein or its de novo synthesis (Tewari and Singh, 1991). It could also be due to prevention of feedback inhibition of the biosynthetic enzyme caused by sequestering proline away from its site of synthesis. Proline accumulation may be a general response to salinity stress. Many investigators found proline accumulation in various plants exposed to salt stress (Karimi et al., 2005; Chatzissavvidis et al., 2014).

The effect of NaCl stress in the presence or absence of 10 mM $CaCl_2$ affected differently the concentration of the various nutrient elements. Hence, P concentration was decreased. The greatest decrease in leaves and roots was recorded in the plants treated with 40 and 80 mM NaCl plus 10 mM $CaCl_2$. Phosphorus is an essential nutrient element important for ATP synthesis and thus plant growth. The greatest Na concentration was recorded with 80 mM NaCl. Salt stress (80 mM NaCl) also reduced K, Mg and Fe concentration and did not affect Zn and B concentration.

Nutrient imbalance may arise due to salt stress concerning nutrient availability, absorption and transport of nutrients and nutrient competition. Salinity increases energy consumption necessary for osmotic adjustment. This probably leads in the reduction of concentration of metabolic important ions like K^+ (Kwon et al., 1995). Numerous studies with many horticultural crops have shown that K^+, Mg^{2+} and P concentration in plant tissue declined as the NaCl salinity in the root media increased (Perez - Alfocea et al., 1996). Reduction of K^+ and Mg^{2+} is a competitive process and occurs regardless of whether the solution is dominated by Na^+ or Cl^-. At the same time the K^+ uptake is impaired by salinity whereas high K^+ concentrations in tissues are required for normal shoot growth. Moreover, the presence of adequate Ca^{2+} in the substrate influences the K^+/Na^+ ratio and enhances plant growth (e.g. in strawberry, Kaya et al., 2002), or leads to osmoregulation (e.g. in cowpea, Franco et al., 1999). Finally, high concentration of substrate Ca^{2+} often results in increased leaf Ca along with a marked reduction in leaf Mg (Ruiz et al., 1997).

Conclusion

High salt concentrations in the nutrient solution caused a significant decrease in the fresh weight of the leaves and total plants. The effect of 80 mM NaCl with or without $CaCl_2$ and with additional 100 µM B caused a significant increase in the concentration of proline in leaves, but no significant effect on the concentration of chlorophyll and carbohydrates. As regards photosynthetic rate and stomatal conductance, the maximum value observed in the treatment B 100 µM while a decrease was observed in the presence of NaCl with or without $CaCl_2$. The inclusion of NaCl in the nutrient solution led to increased Na and Cl, reduced P and Mg in leaves, and P, K and Zn in roots. However, B, Fe and Mn concentrations were not affected by NaCl treatments.

References

Allen, R.G., Pereira, L.S., Raes, D., Smith, M., 1998. Crop Evapotranspiration: Guidelines for Computing Crop Water Requirements. FAO Irrig. Drain. Paper 56. UN-FAO, Rome.Alpaslan, M., Gunes, A., 2001. Interactive effects of boron and salinity stress on the growth, membrane permeability and mineral composition of tomato and cucumber plants. Plant Soil. 236: 123-128.

Bastías, E., Alcaraz-López, C., Bonilla, I., Martínez-Ballesta M.C., Bolaños, L., Carvajal, M., 2010. Interactions between salinity and boron toxicity in tomato plants involve apoplastic calcium. J. Plant Physiol. 167: 54-60.

Blumenfeld, A., Shaya, F., Hillel, R., 2000. Cultivation of pomegranate. CIHEAM: Options Mediterraneans, http:// resources. Ciheam. Org/om/pdf/α42/ 00600264.

Cabanêro, F.J., Martinez, V., Carvajal, M., 2004. Does calcium determine water uptake under saline conditions in pepper plants, or is it water flux which determine calcium uptake? Plant Sci. 166: 443-50.

Centritto, M., Loreto, F., Chartzoulakis, K., 2003. The use of low [CO_2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. Plant Cell Environ. 26: 585-94.

Chatzissavvidis, C., Papadakis, I., Therios, I., 2008. Effect of calcium on the ion status and growth performance of a citrus rootstock grown under NaCl stress. Soil Sci. Plant Nutr. 54: 910-915.

Chatzissavvidis, C., Antonopoulou, C., Therios, I., Dimassi, K., 2014. Responses of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) to continuously and gradually increasing NaCl concentration. Acta Bot. Croatica 73 (1): 1-6.

Chen, T.H.H., Murata, N., 2002. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr. Opin. Plant Biol. 5: 250-257.

Cruz, V., Cuartero, J., Bolarin, M.C., Romero, M., 1990. Evaluation of characters for ascertaining salt stress responses in *Lycopersicon* species. J. Am. Soc. Hort. Sci. 115: 1000-1003.

Debez, A., Koyro, H.W., Grignon, C., Abdelly, C., Huczermayer, B., 2008. Relationship between the photosynthetic activity and the performance of *Cakile maritime* after long-term salt treatment. Physiol. Plant. 133: 373-85.

Doring, J., Ludders, P., 1987. Influence of sodium salts on Na, Cl and SO_4 content in leaves, shoots and roots of *Punica granatum*. Gartenbauwissenschaft. 52: 26–31.

Flowers, T.J., Yeo, A.R., 1995. Breeding for salinity resistance in crop plants-where next? Aust. J. Plant Physiol. 22: 875-884.

Franco, D.L., Filho, J.E., Prisco, J.T., Filho, E.G., 1999. Effects of $CaCl_2$ on growth and osmoregulator accumulation in NaCl stressed cowpea seedlings. Rev. Bras. Fisiol. Vegetal. 11 (3): 145-151.

Grattan, S.R., Grieve, C.M., 1999. Salinity-mineral nutrient relations in horticulture crops. Sci. Hortic.78: 127–157.

Greenway, H., Munns, R., 1980. Mechanisms of salt tolerance in nonhalophytes. Ann. Rev. Plant Physiol. 31: 149–190.

Hamada, A.M., El-Enany, A.E., 1994. Effect of NaCl salinity on growth, pigment and mineral element contents, and gas exchange of broad bean and pea plants. Biol. Plant. 36: 75-81.

Hasanpour, Z., Karimi, H.R., Mirdehghan, 2014. Effects of salinity and water stress on ecophysiological parameters and micronutrients concentration of pomegranate (*Punica granatum* L.). J. Plant Nutr. DOI: 10.1080/01904/67.2014.944711.

Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. California Agriculture Experiment Station Circular 347: 1-39.

Karimi, G., Ghorbanli, M., Heidari, H., Khavari Nejad, R.A., Assareh, M., 2005. The effects of NaCl on growth, water relations, osmolytes and ion content in *Kochia prostrata*. Biol. Plant. 49: 301-304.

Karimi, H.R., Hasanpour, Z, 2014. Effects of salinity and water stress on growth and macronutrients concentration of pomegranate (*Punica granatum* L.) J. Plant Nutr. 27 (12): 1937-1951.

Kaya, C., Kirnak, H., Higgs, D., Saltali, K, 2002. Supplementary calcium enhances plant growth and fruit yield in strawberry cultivars grown at high (NaCl) salinity. Sci. Hortic. 93: 65–74.

Khan, A., McNeilly, A.T., Collins, J.C., 2000. Accumulation of amino acids, proline and carbohydrates in response to aluminum and manganese stress in maize. J. Plant Nutr. 23: 1303–1314.

Kwon, T., Abe, T., Sasahara, T., 1995. Enhanced saline stress resistance in threonine and methionine overproducing mutant cell line from protoplast culture of rice (*Oryza sativa* L.). Plant Physiol. 145: 551-556.

Maas, E.V., Hoffmann, G.J., 1976. Crop salt tolerance: evaluation of existing data. In: Proc. Int. Conf. Texas Technical Univ. 187–197.

Marschner, H., 1995. Mineral Nutrition of Higher Plants. Academic Press, London. 379-396.

Martin, P.K., Korbner, R.M.D., 1995. Sodium and chloride ions contribute synergistically to salt toxicity in wheat. Biol. Plant. 37 (2): 265-271.

Martinez- Ballesta, M.C., Martinez, V., Carvajal, M., 2000. Regulation of water channel activity in whole roots and in protoplasts from roots of melon plants grown under saline conditions. Aust. J. Plant Physiol. 27: 685-691.

Martinez-Ballesta, M.C., Silva, C., Lopez-Berenguer, C., Cabanero, F.J., Carvajal, M., 2006. Plant aquaporins: New perspectives on water and nutrient uptake in saline environment. Plant Biol. 8: 535–546.

Meinzer, F.C., Plaut, Z., Saliendra, N.Z., 1994. Cation isotope discrimination, gas exchange and growth of sugarcane cultivars under salinity. Plant Physiol. 104: 521-526.

Misra, A.N., Sahl, S.M., Misra, M., Singh, P., Meera, T., Das, N., Har, M., Sahu, P., 1997. Sodium chloride induced changes in leaf growth, and pigment and protein contents in two rice cultivars. Biol. Plant. 39: 257–262.

Munns, R., 1993. Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. Plant, Cell Environ. 16: 15-24.

Naumann, J.C., Young, D.R., Anderson, J.E., 2007. Linking leaf chlorophyll fluorescence properties to physiological responses for detection of salt and drought stress in coastal plant species. Physiol. Plant. 131: 422-33.

Navarro, A., Bañon, S., Olmos, E., Sánchez-Blanco, M.J., 2007. Effects of sodium chloride on water potential components, hydraulic conductivity, gas exchange and leaf ultrastructure of *Arbutus unedo* plants. Plant Sci. 172: 473-480.

Naeini, M.R., Khoshgoftarmanesh, A.H., Lessani, H., Fallahi, E., 2004. Effects of sodium chloride – induced salinity on mineral nutrients and soluble sugars in three commercial cultivars of pomegranate. J. Plant Nutr. 27: 1319-1326.

Naeini, M.R., Khoshgoftarmanesh, A.H., Fallahi, E., 2006. Partitioning of chlorine, sodium, and potassium and shoot growth of three pomegranate cultivars under different levels of salinity. J. Plant. Nutr. 29: 1835-1843.

Nedjimi, B., Daoud, Y., 2009. Effects of calcium chloride on growth, membrane permeability and root hydraulic conductivity in two Atriplex species grown at high (sodium chloride) salinity. J. Plant Nutr. 32: 1818-1830.

Ortiz, A., Martínez, V. Cerdà, A., 1994. Short-term effects of osmotic shock and calcium on growth and solute composition of *Phaseolus vulgaris* L. plants. Physiol. Plant. 911: 468-476.

Patil, V.K., Waghmare, P.R., 1982. Salinity tolerance of pomegranate. Journal Maharashtra Agricultural University. 7: 268–269.

Perez-Alfocea, F., Estañ, M.T., Caro, M., Bolarin, M.C., 1993. Response of tomato cultivars to salinity. Plant Soil. 150: 203-211.

Perez-Alfocea, F., Balibrea, M.E., Santa Cruz, A., Estañ, M.T., 1996. Agronomical and physiological characterization of salinity tolerance in a commercial tomato hybrid. Plant Soil. 180: 251-257.

Rengel, Z., 1992. The role of calcium in salt toxicity. Plant Cell Environ. 15: 625-632.

Ruiz, J.M., Belakbir, L., Ragala, J.M., Romero, L., 1997. Response of plant yield and leaf pigments to saline conditions: effectiveness of different rootstocks in melon plants (*Cucumis melo* L.). Soil Sci. Plant Nutr. 43: 855-862.

Sarkhosh, A., Zamani, Z., Fatahi, R., Ebadi, A., 2006. RAPD markers reveal polymorphism among some Iranian pomegranate (*Punica granatum* L) genotypes. Sci. Hortic. 111: 24–29.

Silva, C., Aranda, F.J., Ortiz, A., Carvajal, M., Martinez, V., Teruel, J.A., 2007. Root plasma membrane lipid changes in relation to water transport in pepper: a response to NaCl and $CaCl_2$ treatment. J. Plant Biol. 50: 650-7.

Singh, M.P., Pandey, S.K., Singh, M., Ram, P.C., Singh, B.B., 1990. Photosynthesis, transpiration, stomatal conductance and leaf chlorophyll content in mustard genotypes grown under sodic conditions. Photosynthetica. 24: 623-627.

Sotiropoulos, T.E., 2007. Effect of NaCl and $CaCl_2$ on growth and contents of minerals, chlorophyll, proline and sugars in the apple rootstock M4 cultured *in vitro* Biol. Plant. 51 (1): 177-180.

Termaat, A., Munns, R., 1986. Use of concentrated macronutrient solutions to separate osmotic from NaCl-specific effects on plant growth. Aust. J. Plant. Physiol. 13: 509-522.

Tewari, T.N., Singh, B.B., 1991. Stress studies in lentil (*Lensesculenta* Moench). II. Sodicity-induced changes in chlorophyll, nitrate, nitrite reductase, nucleic acids, proline, yield, and yield components in lentil. Plant Soil. 135: 225-250.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92: 487–511.

Wintermans, J.F., De Mots, A., 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Bioch. Biophys. Acta. 109: 448-453.

Wolf, B., 1974. Improvement in the azomethine-H method for the determination of boron. Commun. Soil Sci. Plant Anal. 5: 39-44.

Zhao, G.Q., Ma, B.L., Ren, C.Z., 2007. Growth, gas exchange, chlorophyll fluorescence, and ion content of naked oat in response to salinity. Crop Sci. 47: 123-31.

Zidan, M.A. Al-Zahrani, H.S., 1994. Effect of NaCl on the germination seedling and some metabolic changes in sweet basil (*Ocimum basilicum*). Pak. J. Sci. Ind. Res. 37: 541-543.