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Original Article

Growth, Physiological and Biochemical Responses of Two Greek Cotton Cultivars to Salt Stress and their Impact as Selection Indices for Salt Tolerance

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Abstract

Soil salinity is a major constrain of crop productivity. Upland cotton (*Gossypium hirsutum* L.) is an important fiber crop worldwide and a major agricultural product in Greece. Two commercial cotton cultivars ('Hersi' and 'ST 318') were studied to compare their response under non-saline and saline conditions in a greenhouse experiment. Salt stress on plants was imposed by two different approaches: a gradual and an initial acclimatization to a non-lethal NaCl concentration (150 mM). To explore salt stress responses, growth (height of plants, roots, shoots and leaves dry weight, reproductive shoots, Salinity Sensitivity Index), gas exchange (Photosynthetic rate, Stomatal conductance, Transpiration rate and Water Use Efficiency) and biochemical parameters (proline, H_2O_2 and MDA content), were examined as well as ion homeostasis. 'Hersi' had significantly higher dry weight of roots, shoots and leaves, lower salinity sensitivity index of roots compared to 'ST 318'. In this regard, it appears that 'Hersi' cultivar performed better than 'ST 318' to increased salinity conditions, due to better control of gas exchange parameters and K^+/Na^+ homeostasis as well as better membrane integrity. Furthermore, the gradual acclimatization to the 150 mM NaCl concentration had a milder effect on both cultivars compared to the initial acclimatization.

Keywords: *Gossypium hirsutum*; gas exchanges parameters; ion analyses; lipid peroxidation; salinity tolerance

Introduction

Soil salinity negatively affects 20% of the world's cultivated lands and more than half of all irrigated lands (FAO 2015). The problem is expanding due to intense agricultural practices and irrigation. High salt concentrations in the soil affect the yield of agricultural crops in various ways including delaying in plant growth and development, disturbance of enzymatic activities and malfunctions in photosynthetic activity (Parihar *et al*., 2015). In order to enhance productivity and utilize marginal areas, it is of great importance to improve salt tolerance of crop plants.

Soil salinity affects plant growth through two stages: the first stage is the osmotic stress which is then followed by ionic stress (Munns and Tester, 2008; Carillo *et al*., 2011) . During the first phase, water absorption of root decreases dramatically with a concomitant water loss from leaves due to osmotic stress of high salt concentration in soil and plants (Munns and Gilliham, 2015). Osmotic stress causes a sudden removal of water, resulting in a leaky plasma membrane. Thus, abnormal amounts of salt are allowed to enter within the root cell and move along the stem (Munns, 2002; Munns and Tester, 2008). The resulting low water potential prevents the process of photosynthesis and transpiration due to the reduction in stomatal conductance induced by stomatal closure (Brugnoli and Lauteri, 1991; Rahnama *et al*., 2010; Ozyigit *et al*., 2017). On the other

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hand, as far as ionic stress is concerned, elevated ion concentrations of Na^+ and Cl^- in the soil solution are antagonistic to other inorganic nutrients, resulting in an imbalance of cellular ionic homeostasis and toxic side effects at a metabolic level. Na^+ antagonizes K^+, which plays an important role in regulating the osmotic potential of plant cells and activates many enzymes involved in respiration and photosynthesis (Hasegawa *et al*., 2000).

Plants can survive under saline conditions and evolve to better tolerate saline conditions through alternations in morphological, physiological and metabolic level (Munns and Tester, 2008; Parihar *et al*., 2015). These special alterations include ion homeostasis, photosynthesis adjustment, antioxidant enzyme induction, osmotic adjustments, and combinations of these factors (Parida and Das, 2005; Ashraf and Foolad, 2007; Peng *et al.,* 2016; Wang *et al*., 2016; Rahneshan *et al.,* 2018). Furthermore, a crucial parameter for salinity tolerance is to maintain an optimal cytoplasmic K^+/Na^+ ratio. The K^+/Na^+ ratio has been used in many studies as a successful salinity resistance index for selecting resistant cotton varieties. Moreover the concentration of Ca+ and Mg+ in response to high salinity plays a critical role in osmoregulation and homeostasis of plant cells (Hadi and Karimi, 2012; Thu *et al.,* 2017)

Upland cotton (*Gossypium hirsutum* L.) is one of the world's primary fiber crop which is widely cultivated throughout the world (Constable *et al*., 2015). Furthermore, is a major agricultural product in Greece, which is the biggest cotton producer in EU (Cotton Incorporated, 2017/18). In addition to fiber production, cottonseeds are an important source of oil for human consumption. Thirdly, cotton pie (a byproduct derived from the oil production process) is used in animal husbandry, making it an excellent feed for ruminants, due to its high protein level (Dogan *et al*., 2012). Moreover, cottonseed flour could be a great source of protein for the nutrition of the ever-growing population of the planet. It is, therefore, a considerable source of income for Greek farmers and an important factor in the development of agriculture (Papakosta-Tasopoulou, 2013). Cotton is one of the most salt tolerant crops (Ashraf and Wu, 1994; Ashraf, 2002; Basal, 2010). However, considerable variation exists between varieties and in several cases salinity stress causes a series of negative effects on cotton growth, yield, and fiber quality (Razzouk and Whittington, 1991; Ashraf, 2002; Zhang *et al*., 2014). Identification of salt-tolerance in cotton germplasm is an important goal for further improvement in cotton production. Previous studies conducted on this issue revealed great genotypic variability for salinity tolerance in cotton (Akhtar *et al.,* 2010; Zhang *et al*., 2014; Ozyigit *et al*., 2017; Wang *et al*., 2017ab;). Therefore, the present research was conducted in order to: a) determine salinity-induced changes in growth, gas exchange parameters, mineral concentrations, osmotic adjustment and H_2O_2 accumulation in two commercially cultivated Greek cotton cultivars and b) identify the parameters that are most crucial for rendering cultivars tolerant to salinity at early growth stages in upland cotton. Moreover, in the experimental procedure the plants are usually stressed by initial acclimatization of high concertation of NaCl. However, this is not the case in nature, where the NaCl concentration rises gradually. Thus in the present study plant responses were tested under both initial and gradual application of NaCl.

As it has been pointed out, cotton is the most important fiber culture and one of the most profitable crops in the world. Therefore, the information that was gathered from this study and the presenting results may provide useful criteria in further research into the understanding and improvement of the complex mechanisms of cotton plant resistance in order to utilize it in marginal environments.

Materials and Methods

Biological material and growth conditions

Two Upland cotton cultivars, 'Hersi' and 'ST 318', supplied by Greenco and Pioneer-Greece respectively, which are early maturing cultivars with adaptability to multiple environments, were evaluated for their salt tolerance. Three seeds of each cultivar were planted into every pot, of 24 cm diameter filled with Gramoflor's Gartner Substrate. After plant emergence, the most vigorous seedling remained in every pot. We used five pots per each treatment (number of total plants 40). The pots were placed in greenhouse the 1rst week of May and were exposed to normal sunlight and photoperiod. The NaCl treatments initiated 11 days after seedling emergence as follows: a) control, no salt; b) initial acclimatization of 50 mM NaCl; c) initial acclimatization of 150 mM NaCl and d) gradual acclimatization to 50-100-150 mM final NaCl concentrations. The experiment was arranged in a completely randomize block with five replications. The salt concentration in the gradual acclimatization was increased from 0 to 50, 100, and 150 mM NaCl every 10 days. Plants were watered every 3 days after the initiation of treatments. The experiment lasted 43 days. Then, at the stage of eight leaves, the above- and underground parts of plants were harvested. After harvesting soil was removed from the roots and plant parts (roots, shoots and leaves) were dried at 65 °C for 48 hours. Furthermore, leaves, roots and shoots of each plant were sampled, immediately frozen in liquid nitrogen and stored at -80 °C for the biochemical analyses.

Measurements of growth and gas exchange parameters

The net weight of roots, shoots and leaves, the aboveground plant length and the number of reproductive shoots were measured on the harvesting day. Moreover, the dry weight of roots, shoots and leaves was determined the day after harvesting. The salinity sensitivity index (IS) based on dry weight of roots, shoots and leaves was estimated according to (Hamrouni *et al*., 2008) as IS = (Ws-Wc)/Wc × 100, in which Ws and Wc were the values of weight of the salt-stressed and control plants, respectively.

The net photosynthesis (A), stomatal conductance (gs), transpiration rate (E), internal CO_2 concentration (Ci) were measured by using a portable photosynthetic system (LICOR-6400, Lincoln, NE, USA). The measurements were made on the uppermost fully expanded leaf, between 12:00 and 14:00 h, under a controlled atmosphere (atmospheric CO_2 380 μmol·mol^{-1} and air flow rate 500 μmol·s^{-1}). Photosynthetically active radiation (PAR) was set at 1700 μmol m^{-2} s^{-1} by a red/blue external light source. The instantaneous water use efficiency (WUE) was calculated from the ratio between CO_2 assimilation and leaf transpiration (A/E).

Determination of ion concentrations

Nutrient analyses were carried out on dried plant tissues. Samples were ground into a mill and after dry ashing in a muffle furnace at 500 °C for 8 hours and dissolution of ash with dilute aqua regia, the content of K, Na, Ca and Mg were determined (Jones, 2001). The concentration of potassium (K) and sodium (Na) was carried out using a flame photometer (Corning 410, Sherwood Scientific Ltd) and the determination of calcium (Ca) and magnesium (Mg) using a flame atomic absorption spectrophotometer (Varian AA-20). For the determination of Cl content, plant samples were extracted in distilled water by shaking for 3 h. The Cl content in the extracts was analyzed by potentiometric titration using an automatic titrator (Titrino 702 Methohm).

Determination of proline, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA)

Proline accumulation in leaves was determined spectrophotometrically (Perkin-Elmer LAMBDA™ 1A UV/Vis Spectophotometer) according to the ninhydrin method described by (Bates *et al*., 1973). The method was implemented with some modifications.

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) (Leidi and Saiz, 1997) leaf contents were measured spectrophotometrically according to the methods described by (Heath and Packer, 1968) and (Alexieva *et al*., 2001), which were implemented with some modifications.

Statistical analysis

The effect of treatments and cultivars on growth, gas exchange and biochemical parameters was detected by a two-way ANOVA using the Statgraphics Centurion XVI statistical analysis package. The means were compared by Tukey test at 0.05 probability level. The biochemical parameters (proline, H_2O_2, MDA) were estimated as the ratio of the biochemical parameters of plants under salt stress in relation to the values of the controls.

Results

Plant growth and gas exchange parameters

The salt treatments significantly ($P < 0.05$) affected the growth parameters (height, dry weight, number of reproductive shoots), salinity sensitivity index and gas exchange parameters (A, gs, Ci, E, WUE) (Table 1, 2, 3). On the other hand, the cultivars had a significant effect only on dry weight, salinity sensitivity index, A, gs and Ci (Tables 1, 2, 3). In addition, the interaction between salt treatments and cultivars was not statistically significant for any of the growth and gas exchange parameters (Tables 1, 2, 3) indicating that the salt treatments effect on the cultivars was consistent.

The growth parameters were not significantly affected by 50 mM treatment with only exception the height and shoot dry weight, which were significantly reduced, compared to control (Table 1). The lowest values for all the growth parameters were recorded under initial acclimatization of 150 mM NaCl (Table 1). However, the root dry weight and the number of reproductive shoots at gradual acclimatization and initial acclimatization of 150 mM NaCl did not significantly differ (Table 1). The highest salinity sensitivity index (absolute number) was recorded at the initial acclimatization of 150 mM NaCl, while the salinity sensitivity index at 50 mM NaCl and gradual acclimatization for shoot and leaf dry weight did not significantly differ (Table 2).

Gas-exchange parameters of gs and Ci were the only that was significantly reduced at 50 mM NaCl treatment compared to the control (Table 3). Moreover, the gradual acclimatization and initial acclimatization of 150 mM NaCl had significantly lower gas-exchange parameters compared to the control and 50 mM NaCl treatment, while no significant differences were detected between them (Table 3).

'Hersi' had significantly higher dry weight of roots, shoots and leaves (Table 1) and lower salinity sensitivity index of roots (Table 2) compared to 'ST 318', while the cultivars did not significantly differ in height, number of reproductive shoots and salinity sensitivity index of shoots and leaves. The gas exchange parameters of A, gs and Ci were significantly higher in 'Hersi' than in 'ST 318' but E and WUE were similar (Table 3).

Ionic homeostasis

NaCl treatments affected significantly all studied parameters of plant's ionic homeostasis (leaves' and roots' ion content in K^+, Na^+ and Cl^-, K^+/Na^+ ratio) (Table 4 and 5). Contents of Na and Cl were increased in the presence of NaCl in both leaves and roots while the concentration of K^+ was reduced in leaves (Table 4) but increased in roots (Table 5). For micronutrient Mg^{2+}, an increase was observed comparing with the controls and no reduction of accumulation of Ca^{2+} was found in both cultivars (Fig. 1). The $Mg^{2+}$and $Ca^{2+}$was not affected for both cultivars in the roots (data not shown).

In contrast, leaves' Na^+ content and K^+/Na^+ ratio were cultivar-dependent. Moreover, significant interactions in Na^+, K^+ content and K^+/Na^+ ratio were observed. That means that the two cultivars behaved differently under stressed conditions for Na^+ and K^+ accumulation. In particular, 'Hersi' tended to accumulate less Na^+ in leaves, with no reduction of K^+ content, maintaining higher values of K^+/Na^+ ratio compared to 'ST 318' under salt stress (Fig. 1). Leaves' Cl^- content in salt stressed plants was much higher than Na^+ content, in both cultivars, indicating that they can restrict the influx of Na^+ ions, comparing to Cl^- ions. This mechanism seems to be more efficient for 'Hersi' cultivar than for 'ST 318'. Under gradual acclimatization both cultivars showed better ion homeostasis compared to initial acclimatization i.e. higher K^+, lower Na^+ and Cl^- and a higher value of the K^+/Na^+ ratio.

Biochemical parameters

The ratio of proline content was gradually increased, while the corresponding ratio of H_2O_2 and MDA content was decreased by increasing the salt concentration from 50 mM NaCl to 150 (both by gradual and initial acclimatization) (Table 6) in relation to the control. Regarding the tested cultivars, 'Hersi' obtained higher ratio of proline content compared to 'ST 318' at 50 mM NaCl and at gradual acclimatization (50-100-150 mM), whereas lower at the initial acclimatization of 150 mM NaCl (Fig.

2). Additionally, 'Hersi' had lower ratio of H_2O_2 content than 'ST 318' at gradual and initial acclimatization of 150 mM NaCl, but higher at 50 mM NaCl (Fig. 2). Finally, 'Hersi' kept lower ratio of MDA content in comparison to 'ST 318' at 50 mM NaCl, while higher at 150 mM NaCl initial acclimatization (Fig. 2).

Table 1. The effect of salt treatment and cultivar on plants' growth parameters (mean ± SE)

Salt Treatment (mM NaCl)	Height (cm)	Dry Weight (g)			Reproductive Shoots
		Root	Shoot	Leaf	
Control	69±1.9a	2.93±0.2a	9.5±0.4a	17±1.5a	7.1±0.4a
50	60±2.5b	2.61±0.2ab	6.9±0.7b	13±1.4ab	6.6±0.6ab
50-100-150	56±1.3b	1.93±0.2bc	5.5±0.2b	11±0.4b	5.3±0.2bc
150	44±0.91c	1.19±0.1c	3.5±0.3c	7±0.5c	4.5±0.3c
Cultivar					
'Hersi'	58±2.7	2.6±0.2a	6.7±0.6a	14±1.2a	6.0±0.4
'ST 318'	56±2.0	1.68±0.2b	5.8±0.5b	11±0.9b	5.8±0.4
Source of variation					
Treatment (A)	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05
Cultivar (B)	Ns**	P<0.05	P<0.05	P<0.05	ns
AXB (Interaction)	ns	ns	ns	ns	ns

Note: Means followed by the same letter in the column did not significantly differ (Tukey test, p > 0.05). ns: not significant at 0.05 level.

Table 2. The effect of salt treatment and cultivar on Salinity Sensitivity Index for dry weight (mean ± SE)

Salt Treatment (mM NaCl)	Salinity Sensitivity Index for Dry Weight		
	Root	Shoot	Leaf
50	-11±4.4a	-27±7.1a	-20±7.3a
50-100-150	-36±4.9b	-42±2.0a	-30±2.6a
150	-60±2.1c	-63±2.5b	-60±2.6b
Cultivar			
'Hersi'	-27±6.2a	-41±6.1	-32±6.4
'ST 318'	-43±5.9b	-46±4.8	-38±5.3
Source of variation			
Treatment (A)	P<0.05	P<0.05	P<0.05
Cultivar (B)	P<0.05	ns	ns
AXB (Interaction)	ns	ns	ns

Note: Means followed by the same letter in the column did not significantly differ (Tukey test, p > 0.05). ns: not significant at 0.05 level.

Table 3. The effect of salt treatment and cultivar on plants' gas exchange parameters (A: Photosynthetic rate; gs: stomatal conductance; Ci: Intercellular CO_2 concentration; E: Transpiration rate; WUE: Water Use Efficiency) (mean ± SE)

Salt Treatment (mM NaCl)	A (μmol CO_2 $m^{-2}s^{-1}$)	gs (mol H_2O $m^{-2}s^{-1}$)	Ci (μmol CO_2 mol air^{-1})	E (mmol H_2O $m^{-2}s^{-1}$)	WUE (μmol CO_2 $mmol^{-1}$ H_2O)
Control	21.1±0.60a*	0.32±0.011a	218±7a	10.3±0.12a	2.05±0.57a
50	19.3±0.80a	0.26±0.012b	189±6b	9.5±0.33a	2.10±0.11a
50-100-150	16.1±0.48b	0.15±0.008c	156±6c	6.6±0.21b	2.46±0.69b
150	14.0±0.49b	0.14±0.011c	134±7c	6.04±0.24b	2.41±0.11b
Cultivar					
'Hersi'	18.2±0.79a	0.23±0.021a	183±9a	8.1±0.50	2.32±0.87
'ST 318'	16.8±0.93b	0.20±0.022b	164±10b	8.1±0.54	2.18±0.69
Source of variation					
Treatment (A)	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05
Cultivar (B)	P<0.05	P<0.05	P<0.05	ns**	ns
AXB (Interaction)	ns	ns	ns	ns	ns

Note: Means followed by the same letter in the column did not significantly differ (Tukey test, p > 0.05). ns: not significant at 0.05 level.

Table 4. The effect of salt treatment and cultivar on plants' ion contents in leaves (mean ± SE)

Salt Treatment (mM NaCl)	K^+ Content ($mg\ g^{-1}$)	Na^+ Content ($mg\ g^{-1}$)	K^+/Na^+ Ratio	Cl^- Content ($mg\ g^{-1}$)
Control	26.95 ±1.43a	0.69 ±0.06a	40.82 ±4.54a	7.74±1.61c
50-100-150	25.20 ±1.04ab	8.42 ±0.50b	3.04 ±0.24b	47.38 ±1.68b
150	24.38 ±1.16b	15.41 ±2.42c	1.80 ±0.34b	60.95 ±2.64a
Cultivar				
'Hersi'	24.78 ±1.09a	6.57 ±2.10a	12.94 ±6.95a	37.77 ±9.96a
'ST 318'	26.23 ±1.40a	9.78 ±3.73b	17.50 ±10.17b	39.61±11.26a
Source of variation				
Treatment (A)	P<0.05	P<0.05	P<0.05	P<0.05
Cultivar (B)	ns	P<0.05	P<0.05	ns
AXB (Interaction)	P<0.05	P<0.05	P<0.05	ns

Note: Means followed by the same letter in the column did not significantly differ (Tukey test, p > 0.05). ns: not significant at 0.05 level.

Table 5. The effect of salt treatment and cultivar on plants' ion contents in roots (mean ± SE)

Salt Treatment (mM NaCl)	K^+ Content ($mg\ g^{-1}$)	Na^+ Content (mg/g)	K^+/Na^+ Ratio	Cl^- Content (mg/g)
Control	14.09 ±1.13a*	0.41 ±0.10a	37.45±3.32a	2.57±0.65c
50-100-150	19.08 ±0.94b	6.69 ±0.29b	2.99 ±0.34b	14.09 ±1.98b
150	18.05 ±1.04b	10.50 ±1.53c	1.81 ±0.24c	22.64 ±2.02a
Cultivar				
'Hersi'	16.86 ±1.12a	5.34 ±1.26a	15.55 ±7.89a	12.93 ±3.83a
'ST 318'	18.06 ±1.30a	6.40 ±1.82a	12.62 ±6.58a	13.27 ±2.23a
Source of variation				
Treatment (A)	P<0.05	P<0.05	P<0.05	P<0.05
Cultivar (B)	ns**	ns	ns	ns
AXB (Interaction)	ns	ns	ns	ns

Note: Means followed by the same letter in the column did not significantly differ (Tukey test, p > 0.05). ns: not significant at 0.05 level.

Table 6. The ratio of the biochemical activity of plants under salt treatments in relation to plants under control (mean ± SE)

Salt Treatment	Proline	Hydrogen peroxide	Malondialdehyde
(mM NaCl)	($mg\ 100g^{-1}$)	($\mu mol\ 25g^{-1}$)	($\mu mol\ g^{-1}$)
50	1.9±0.01c*	1.2±0.04a	1.0±0.06a
50-100-150	3.5±0.11b	1.1±0.02b	0.8±0.05b
150	8.0±0.24a	0.6±0.01c	0.4±0.03c
Cultivar			
'Hersi'	4.3±0.59b	0.95±0.08	0.7±0.04b
'ST318'	4.7±0.79a	0.94±0.06	0.8±0.10a
Source of variation			
Treatment (A)	P<0.05	P<0.05	P<0.05
Cultivar (B)	P<0.05	ns**	P<0.05
AXB (Interaction)	P<0.05	P<0.05	P<0.05

Note: Means followed by the same letter in the column did not significantly differ (Tukey test, p > 0.05). ns: not significant at 0.05 level.

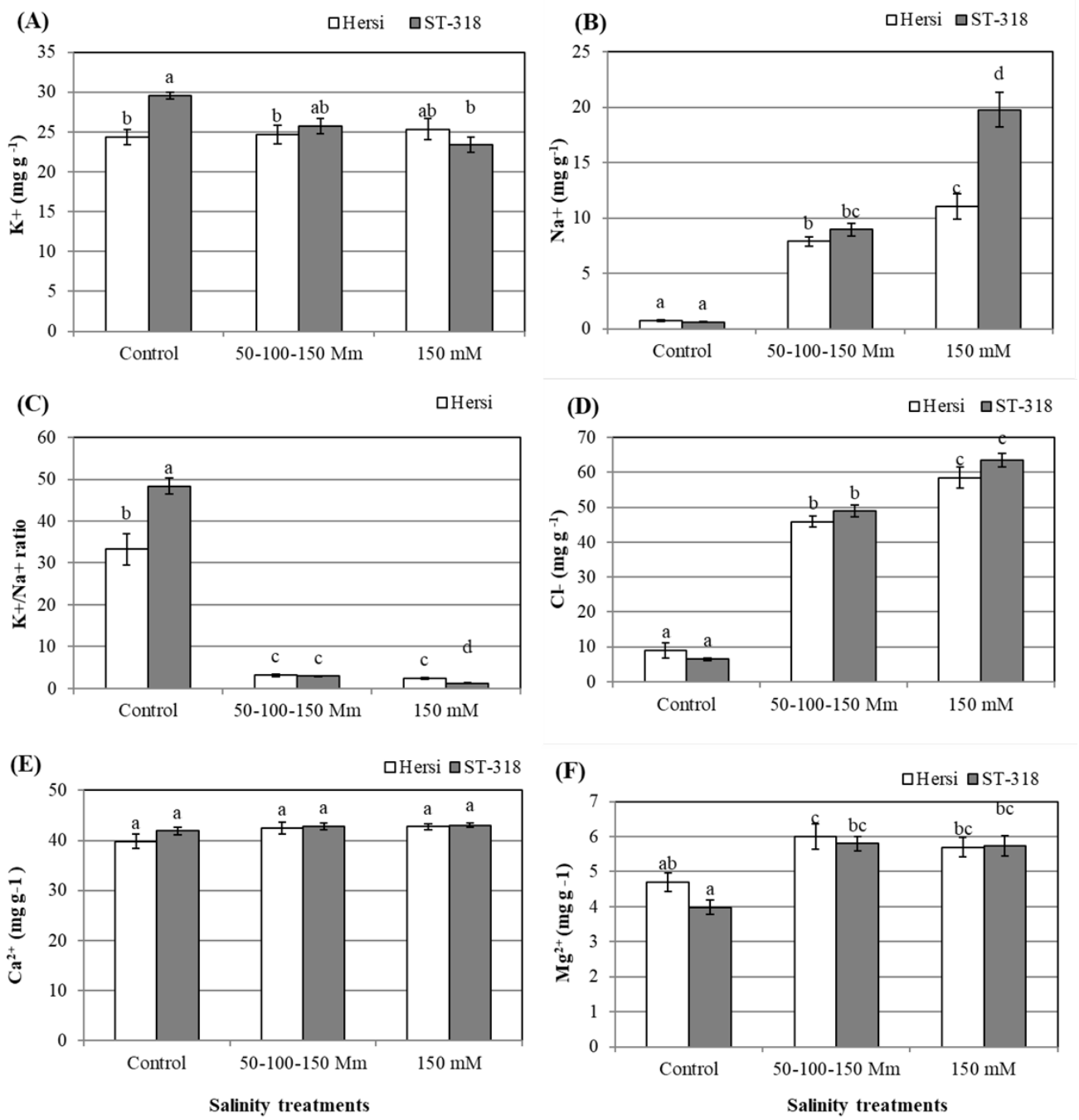

Fig. 1. The content of (A) Na^+, (B) K^+, (C) the ratio of K^+/Na^+, (D) the content of Cl^-, (E) the content of Ca^{2+} and (F) the content of Mg^{2+} for cultivars 'Hersi' and 'ST- 318' under salinity treatments in shoots. The vertical bars indicate the mean's SE. The different letters refer to the significant differences at $p < 0.05$ (Tukey test)

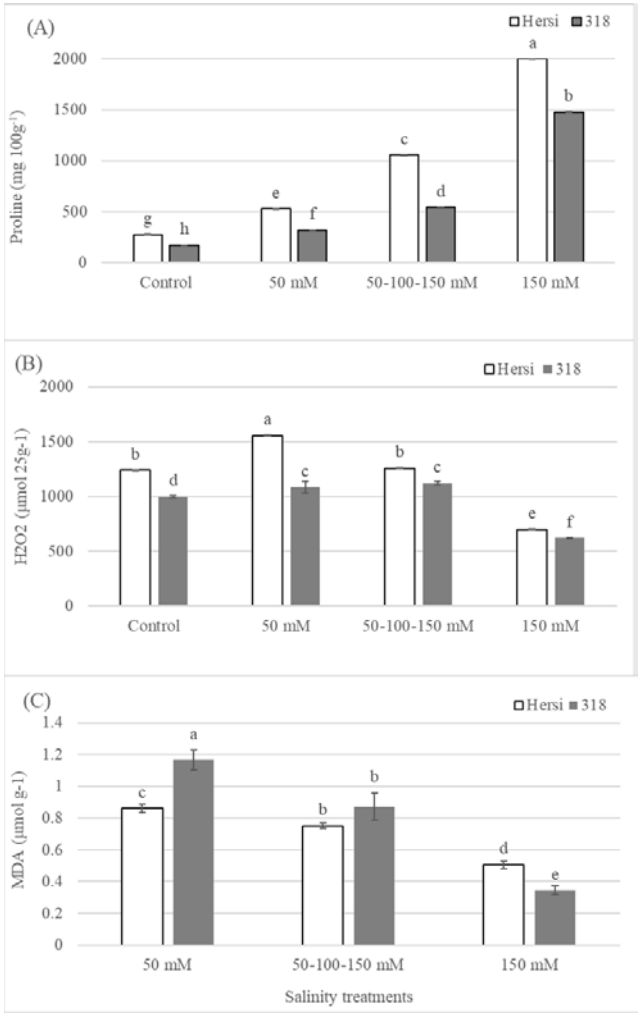

Fig. 2. The ratio in relation to control of (A) Proline (mg 100 g^{-1}) content, (B) Hydrogen peroxide (μmol $25g^{-1}$) content and (C) Malondialdehyde (μmol g^{-1}) content for 'Hersi' and 'ST 318' cultivars under salinity treatments. The vertical bars indicate the mean's SE. The different letters refer to the significant differences at $p < 0.05$ (Tukey test)

Discussion

Most crop plants that provide food for the world population are glycophytes and are very sensitive to high concentrations of salts in the soil, mainly NaCl. Thus, salinity is one of the major abiotic stresses harmfully affecting crop growth and development and consequently yield and quality (Bojórquez-Quintal *et al*., 2014). Although cotton is considered as a relatively salt-tolerant crop, a long-term exposure may cause adverse effects on crop growth and development (Higbie *et al*., 2010). To this end three salinity treatments were applied: a low dose (50mM of naCl) a gradual (step-wise) acclimatization and an initial acclimatization to a non-lethal NaCl concentration (150 mM) in two commercial Greek cultivars in order to determine the degree of the tolerance of these cultivars to salt stress and unravel their responses with respect to different salt treatments. The two cultivars did not differ significantly regarding their tolerance to low levels of salt stress (50 mM). On the contrary they showed differences when subjected to both initial and gradual acclimatization of 150 mM of NaCl as indicated by several growth, gas exchange and biochemical parameters.

Growth and physiological parameters

The inhibition of normal growth of the plant is the immediate visual result of salinity stress. As it was found, all salinity treatments resulted in impairment of plant biomass for the two cotton cultivars. The inhibition of the growth consists of the significant reduction of the net and dry weights of leaves, shoots and roots as it is demonstrated in several studies (Basal, 2010; Rahnama *et al*., 2010; Zhang *et al*., 2014). The reduction of plant height was similar in both cultivars and higher under the initial acclimatization of 150 mM NaCl compared to the gradual acclimatization. On the other hand, Hersi showed lower reduction rates in growth parameters such as shoot, root and stem dry weight indicating better tolerance ability. Similar conclusions were reported by (Zhang *et al*., 2014) when they estimated the salinity tolerance of two cotton cultivars by comparing their morphological responses. This reduction in growth parameters was again more profound when plants were subjected to initial salt stress instead of gradual acclimatization and may be due to a reduction in water potential and accumulation of toxic ions (Zhu, 2001; Munns, 2002). In addition, the highest salinity sensitivity index (Campanelli *et al*., 2013), regarding root dry weight was recorded for 'ST 318' indicating that 'Hersi' responded better than 'ST318' under salt stress in terms of growth parameters.

Application of NaCl caused a decrease in A, E, gs and Ci concentration. Interestingly, no profound differences were monitored in photosynthetic parameters between the initial and the gradual acclimatization. However, 'Hersi' was the one that demonstrated higher gas exchange parameters (i.e A, gs, Ci, E) compared to 'ST 318'. On the other hand the reduction of A in 'ST 318' as well as the reduction in Ci is mainly due to the closure of the stomata, as indicated in other reports as well (Brugnoli and Lauteri, 1991; Zhang *et al*., 2014). In spite of the higher stomatal conductance of 'Hersi', the two varieties did not differ significantly in terms of the utilization of available water. Interestingly, initial and gradual acclimatization on NaCl had no effect on WUE, and plants from both cultivars managed to maintain their WUE at similar levels compared to the controls.

Since 'Hersi' showed higher gs, but both cultivars displayed almost the same water use efficiency, it could be suggested that 'Hersi' was capable of maintaining the same E with increased gs and increased A so overall it demonstrated better adaptability to salt stress. The ability to maintain open stomata and to perform fully functional photosynthesis even under stressful conditions has been documented as an indication of tolerance in several other studies (Holá *et al*., 2017; Zhu *et al*., 2018). In this regard, the superiority of 'Hersi' in biomass production under salt stress probably could be attributed to this ability.

Ion homeostasis

In this study, although salinity treatment disrupted the K^+/Na^+ balance only Na^+ content was significantly increased in response to salt treatments. More specifically,

under salinity treatments and especially under initial acclimatization of 150 mM NaCl, the Na^+ contents of leaves and roots in both cultivars were profoundly increased. However, 'Hersi' seemed to control Na^+ accumulation in both leaves and roots better than 'ST 318'. In several studies, plant adaptation to salt stress is attributed to the ability of maintaining a low Na^+ content and better regulating Na^+ ion homeostasis (Munns and Tester, 2008; Dai *et al*., 2014; Kong *et al*., 2016; Wang *et al*., 2016).

Salinity decreased K^+ concentrations in leaves, but increased its concentration in roots, suggesting that salt stress prohibited K^+ transport from roots to leaves. Similar results were shown by Wang *et al*. (2017a). On the other hand, K^+ content maintained at the same level in leaves of the two cultivars (Table 4). Tolerant genotypes are also capable of preserving K^+ homeostasis during all salt stress stages (Hauser and Horie, 2010; Shabala, 2013; Almeida *et al*., 2017). Therefore, K^+ is considered as a key regulatory element in plant metabolic process by promoting Na^+ exclusion and regulating osmotic adjustment (Chakraborty *et al*., 2016).

Having a stable K^+/Na^+ ratio in tissues is critical for plant growth and metabolism under saline conditions (Wang *et al*., 2016). According to the results of the present study, K^+/Na^+ ratio was profoundly higher in leaves of 'Hersi' cultivar's plants subjected to initial acclimatization compared to 'ST 318's plants. However, this ratio was not differentiated between the two cultivars under gradual acclimatization. These results are in accordance to recent findings by (Wang *et al*., 2017a) were they demonstrated that the K^+/Na^+ ratio of the salt sensitive cultivar 'Z571' decreased 14 times whereas in tolerant cultivars ('CCRI44', 'CZ91') the K^+/Na^+ ratio was reduced 4-8 times between control and salt treated plants.

In addition, there was a profound increase in Cl^- content in the salt-treated plants in leaves and roots of both cultivars. The fact that high Cl^- content doesn't seem to influence the salt tolerance of the two cultivars could be a consequence of the restriction of entry of Cl^- ions into metabolically active areas of cells as a mechanism to maintain ionic equilibrium when ions are highly concentrated in the external environment (Niu *et al*., 1995).

Calcium plays a central role in membrane stability and K^+/Na^+ homeostasis (Grattan and Grieve, 1999). In addition, calcium is implicated in salt tolerance by enhancing ion uptake and ameliorating plant osmotic adjustment (Epstein, 1998). Moreover, it has been documented that salinity negatively affects Mg^{2+} uptake, and this decrease may influence the activity of some enzymes, which require Mg^{2+} for catalysis as well as chlorophyll synthesis (Khan *et al*., 2000). In the present study, both calcium and magnesium content remained almost unchanged in leaves of both cultivars in all salt treatments. The capacity of both cultivars in preserving the same ion uptake in control and salt stress conditions further supports the hypothesis that both cultivars are tolerant.

Lipid peroxidation and proline accumulation

Salt stress is known to result in extensive lipid peroxidation (measured by MDA content), as a consequence of accumulation of ROS. In both 'Hersi' and 'ST 318' an increase in both H_2O_2 (the most abundant ROS) and MDA was monitored in the plants watered with 50 mM NaCl compared to the controls, however in the plants subjected to higher concentrations of NaCl (either initial or gradual acclimatization) decreased levels of both H_2O_2 and MDA were observed. Although these observations are a bit unusual, similar results were obtained by (Ozturk *et al*., 2012) when they subjected pea varieties to different concentrations of NaCl and they also measured reduced levels of H_2O_2 and MDA in higher concentrations of NaCl. These results are also in agreement with studies of (Kennedy and De Filippis, 1999) in *Grevillea ilicifolia* and (Freitas *et al*., 2011) in cotton varieties.

Wang Y *et al*. (2016) measured the H_2O_2 content and MDA content on a salt tolerant cotton variety over a period of 24 h after imposition of NaCl (plants were transferred to a nutrient solution containing 200 mmol/ L NaCl). They found that both H_2O_2 and MDA content increased rapidly until 6 and 12 hours respectively after NaCl treatment and then they both dropped. These results probably suggest that in tolerant genotypes H_2O_2 accumulation up to a certain point my act as a signaling molecule mediating responses and triggering antioxidant activity but then it drops to lower levels in order not to cause oxidative stress. The reduction of MDA and H_2O_2 compared to controls in both cultivars suggest that they both possess an effective ROS scavenging mechanism, although the measurement of antioxidant enzymes activity is required.

Finally accumulation of osmolytes, especially that of proline, is one of the most common plant responses in order to adapt to adverse environmental conditions such as drought and salt stress. Proline has a multifunctional role besides being an osmolyte, and contributes to a plethora of biochemical pathways leading to scavenging ROS, stabilizing subcellular structures, modulating cell redox homeostasis etc (Verbruggen and Hermans, 2008; Bojórquez-Quintal *et al*., 2014; Gharsallah *et al*., 2016)). Proline is accumulated mainly in leaves in order to maintain chlorophyll level and cell water content and consequently to protect photosynthetic activity under salt stress (Silva-Ortega *et al*., 2008).

In the present study, the extent of proline accumulation varied in the two cotton cultivars, and proline accumulation in Hersi cultivar increased greatly (up to 8 times) in initial and gradual acclimatization of 150 mM NaCl compared to the controls. In 'ST 318', there was a proline accumulation in all salt treatments compared to control but not to the same extent as in 'Hersi'.

Conclusions

As an overall conclusion, both cultivars performed quite well in saline conditions compared to other cotton cultivars. The observed salt tolerance could be correlated with two characters (defense mechanisms) that they share: a) a rapid antioxidant capacity, and b) K^+/Na^+ homeostasis. The gradual acclimatization had a mild effect of all studied parameters apart from the gas exchange ones for both cultivars. However, 'Hersi' cultivar was found more tolerant to salinity as it performed better in terms of growth. This can be attributed to better gas exchange parameters, better control of K^+/Na^+ ratio and more efficient membrane homeostasis. It has to be noted that a special feature of

'Hersi' as indicated by the company that distributes it, is its deep rooting system. It would be very interesting to further explore the impact of it towards salt stress tolerance in cotton plants. Identification of salt tolerant cotton genotypes for salt affected areas can benefit the farming community through increased seed cotton yield and quality thus cotton cultivation could be expanded to these areas as well.

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