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Leaf Water Relations and Net Gas Exchange Responses of Salinized Carrizo Citrange Seedlings during Drought Stress and Recovery

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• *Background and Aims* Since salinity and drought stress can occur together, an assessment was made of their interacting effects on leaf water relations, osmotic adjustment and net gas exchange in seedlings of the relatively chloride-sensitive Carrizo citrange, *Citrus sinensis* × *Poncirus trifoliata*.
• *Methods* Plants were fertilized with nutrient solution with or without additional 100 mM NaCl (salt and no-salt treatments). After 7 d, half of the plants were drought stressed by withholding irrigation water for 10 d. Thus, there were four treatments: salinized and non-salinized plants under drought-stress or well-watered conditions. After the drought period, plants from all stressed treatments were re-watered with nutrient solution without salt for 8 d to study recovery. Leaf water relations, gas exchange parameters, chlorophyll fluorescence, proline, quaternary ammonium compounds and leaf and root concentrations of Cl^- and Na^+ were measured.
• *Key Results* Salinity increased leaf Cl^- and Na^+ concentrations and decreased osmotic potential (Ψ_π) such that leaf relative water content (RWC) was maintained during drought stress. However, in non-salinized drought-stressed plants, osmotic adjustment did not occur and RWC decreased. The salinity-induced osmotic adjustment was not related to any accumulation of proline, quaternary ammonium compounds or soluble sugars. Net CO_2 assimilation rate (A_{CO2}) was reduced in leaves from all stressed treatments but the mechanisms were different. In non-salinized drought-stressed plants, lower A_{CO2} was related to low RWC, whereas in salinized plants decreased A_{CO2} was related to high levels of leaf Cl^- and Na^+. A_{CO2} recovered after irrigation in all the treatments except in previously salinized drought-stressed leaves which had lower RWC and less chlorophyll but maintained high levels of Cl^-, Na^+ and quaternary ammonium compounds after recovery. High leaf levels of Cl^- and Na^+ after recovery apparently came from the roots.
• *Conclusions* Plants preconditioned by salinity stress maintained a better leaf water status during drought stress due to osmotic adjustment and the accumulation of Cl^- and Na^+. However, high levels of salt ions impeded recovery of leaf water status and photosynthesis after re-irrigation with non-saline water.

Key words: Carrizo citrange, citrus, Cl^-, Na^+, salt stress, drought stress, proline, carbohydrates.

INTRODUCTION

In the Mediterranean area, low rainfall and high temperatures in summer along with high salinity of irrigation water often result in agricultural crops suffering simultaneous drought and salinity stress (Paranychianakis and Chartzoulakis, 2005). Drought stress in citrus reduces growth and metabolism, leading to a reduction in fruit yield and quality (Gómez-Cadenas *et al.*, 1998; Arbona *et al.*, 2005) and to increased costs of juice extraction (IEA, 2000). Drought also reduces peel thickness making citrus fruit more vulnerable to damage during handling and shipping (Agustí, 1999). Reductions in net assimilation of CO_2 (A_{CO2}) in leaves, stomatal conductance (g_s) and transpiration (E) are often used as indicators of drought stress (Sinclair and Allen, 1982; Shalhevet and Levy, 1990).

In addition to sensitivity to drought, *Citrus* species have been classified as relatively salt-sensitive (Maas, 1990; Shalhevet and Levy, 1990). The accumulation of solutes may allow plants to maintain a positive pressure potential that is required to keep stomata open and to sustain gas exchange and growth (White *et al.*, 2000). Several studies of salinized citrus have demonstrated that leaf turgor potential can be maintained at similar or even higher levels than in non-salinized control plants by the accumulation of Na^+ and Cl^- which contribute to the osmotic adjustment process (Bañuls and Primo-Millo, 1992; García-Sánchez and Syvertsen, 2006). During salt stress, the accumulation of compatible solutes in crop leaves can include the amino acids proline and betaines such as glycine betaine, proline betaine or β-alanine betaine (McNeil *et al.*, 1999), which can be similar to those that accumulate at low water potentials during dehydration stress (Verslues *et al.*, 2006), high temperature, freezing, UV radiation and heavy metal toxicity (Delauney and Verma, 1993; Siripornadulsil *et al.*, 2002). Salt-stressed citrus leaves do not accumulate proline (Syvertsen and Yelenosky, 1988) but drought-stressed (DS) citrus leaves can accumulate proline (Syvertsen and Smith, 1983) and other types of betaines but probably not glycine betaine (Nolte *et al.*, 1997).

In salt-stressed citrus leaves, reductions in gas exchange parameters have been associated with the specific toxicity of Cl^- and/or Na^+ rather than with osmotic stress (Bañuls and Primo-Millo, 1992; Levy and Syvertsen, 2004). These reductions in A_{CO2} have been attributed to a direct biochemical inhibition of photosynthetic capacity

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(Lloyd *et al.*, 1987; García-Sánchez and Syvertsen, 2006), which can be more important than low g_s in limiting A_{CO2}. The use of calculated C_i to describe non-stomatal limitations on A_{CO2} should be interpreted with caution due to changes in mesophyll conductance that can affect CO_2 diffusion and its concentration at the chloroplasts (Syvertsen *et al.*, 1995; Flexas *et al.*, 2004).

Recovery of citrus plants previously suffering drought stress is generally characterized by a rapid recovery of leaf water potential (within 2 d) followed by a later recovery of g_s which allows the plants to limit water losses via transpiration and regain full turgor after rewatering (Ruiz-Sánchez *et al.*, 1997). Physiological mechanisms involved in the recovery of salinized citrus is poorly understood but Cámara-Zapata *et al.* (2004) observed an increase in leaf Cl^- concentration during a 6-week recovery period in previously salinized sour orange but not in Cleopatra mandarin plants. It is possible that this apparent translocation of Cl^- from roots to leaves could impair the recovery of leaf water status and net gas exchange.

Although there have been many studies on physiological responses of citrus to high salinity (Levy and Syvertsen, 2004) and to drought (Kriedemann and Barrs, 1981; Arbona *et al.*, 2005), few investigations have studied interactions between drought and salinity stress at the same time (Syvertsen *et al.*, 1988) or plant recovery after these stresses have been relieved. It is difficult to quantify the combined effects of salinity and drought because salinity effects become more intense during dehydration. To gain insights into mechanisms of stress tolerance, the objectives of this study were to compare water relations, net gas exchange, organic solutes and osmotic adjustment responses to short-term drought stress and recovery, in leaves of salinized and non-salinized Carrizo citrange rootstock seedlings. We hypothesized that high solute concentrations in salinized plants could facilitate osmotic adjustment during drought and recovery after re-irrigation with good quality water.

MATERIALS AND METHODS

Plant material and growing conditions

This study was conduced at the University of Florida's Citrus Research and Education Center (Lake Alfred, FL, 28·09°N, 81·73°W). One-year-old seedlings of Carrizo citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* L.] were grown in 1·5-L containers filled with autoclaved native Candler fine sandy soil. In the field, the water content in this sandy soil ranges from about 10% at field capacity to about 2% at wilting point. Plants were grown during the summer in an evaporatively cooled greenhouse with maximum photosynthetically active radiation (PAR) (LI-170; LICOR, Inc., Lincoln, NE, USA) at plant level of 1500 $\mu mol\ m^{-2}\ s^{-1}$ and natural photoperiods. Average day/night temperature was 36/21°C and relative humidity varied diurnally from 40% to 100%.

At the beginning of the experiment, plants were watered three times per week with 100 mL of 0 mM or 100 mM NaCl (Salt) added to half-strength Hoagland's solution (Hoagland and Arnon, 1938). This was a sufficient volume to leach from the bottom of all pots. To avoid an osmotic shock, salinity was increased in increments of 25 mM NaCl per day until 100 mM NaCl was achieved. After 7 d of 100 mM NaCl treatment, half of the plants from each salt level were allowed to become drought stressed by withholding irrigation for 10 d while the other half were maintained well irrigated with the nutrient solution with or without the salt treatment as before. Thus, the experimental design was a 2 × 2 factorial of two salt treatments (0 mM NaCl or 100 mM NaCl) and two irrigation treatments (well irrigated or drought stressed) with 12 replicate plants in each treatment. The well-irrigated, no-salt treatment was the non-stressed, control treatment. After the period of drought stress, half of the plants in each treatment ($n = 6$) were harvested and the other half were re-irrigated with the nutrient solution without the salt treatment to study the recovery process during the following 8 d.

Leaf and soil water content

All leaf tissue evaluations were done using uniform fully expanded mature leaves from the mid-stem area of each of the six replicate plants per treatment. At the end of the period of drought stress (day 18) and again after the recovery period (day 26), individual petioles from sampled leaves were cut and leaves were immediately weighed to obtain a leaf fresh weight (f. wt). Leaf petioles were placed in a beaker of water overnight in the dark so that leaves could become fully hydrated. Leaves were reweighed to obtain turgid weight (t. wt) and dried at 80 °C for 24 h to obtain dry weight (d. wt). The relative water content (RWC) of the leaves was calculated as $RWC = [(f.\ wt - d.\ wt) \times (t.\ wt - d.\ wt)^{-1}] \times 100$ according to Morgan (1984).

Gravimetric soil water (W_g) content was also determined at the end of the drought period (day 18) using six pots per treatment. After the plants were removed, soil samples from each pot were weighed (W_w), dried at 65°C to a constant weight and reweighed again (W_d; Kasischke *et al.*, 2003). W_g was calculated as $W_g = (W_w - W_d) \times 100/W_d$ and expressed in g H_2O (100 g soil)$^{-1}$.

Water relations

Covered leaf stem water potential (Ψ_S) was periodically measured at midday (1100–1200 h) with a Scholander-type pressure chamber (PMS instrument, Corvallis, OR, USA; Scholander *et al.*, 1965) using mature leaves that were previously enclosed in aluminium foil-covered plastic envelopes at least 2 h before measurement (McCutchan and Shackel, 1992). After Ψ_S measurement, leaves were tightly enclosed in aluminium foil, frozen by immersing in liquid nitrogen and stored in a freezer at −18 °C. After thawing, leaf osmotic potential (Ψ_π) was measured in expressed cell sap collected from a syringe at 25 ± 1 °C and placed in an osmometer (Digital Osmometer, Wescor, Logan, UT, USA). Osmotic potential at full turgor (Ψ_π^{100}) was measured at the end of the stress and recovery period on one similar leaf per plant after rehydration to full turgor as above before freezing in liquid

nitrogen. Osmotic adjustment was calculated as the difference between Ψ_π^{100} of control and stressed plants.

Gas exchange and chlorophyll fluorescence

Net assimilation of CO_2 (A_{CO2}), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and photosynthetic water use efficiency ($WUE = A_{CO2}\ E^{-1}$) were measured periodically during the experiment using single mid-stem leaves in a portable photosynthesis system (LI-6200; LI-COR Inc.) with a 0·25-L cuvette. The cuvette was equipped with an external light source (model QB1205LI-670; Quantum Devices Inc., Barneveld, WI, USA) to maintain a constant PAR of $\geq 800\ \mu mol\ m^{-2}\ s^{-1}$ during measurements which is sufficient to saturate citrus leaves grown in full sun with light ($PAR \approx 2000\ \mu mol\ m^{-2}\ s^{-1}$; Syvertsen, 1984). All measurements were made in the morning from 0800 to 1000 h to avoid high afternoon temperatures and low humidity. During all measurements, the leaf temperature was $32 \pm 2\ ^\circ C$ and leaf-to-air vapour pressure difference was $2{\cdot}4 \pm 0{\cdot}4$ kPa within the cuvette.

At the end of both the stress (day 18) and recovery periods (day 26), chlorophyll *a* fluorescence (F) was measured with a pulse-modulated fluorometer (model OS1-Fl; Opti-Sciences, Tyngsboro, MA, USA) on leaves similar to those used for gas exchange and other measurements. Fluorescence measurements were made between 0900 and 1000 h under ambient light and also after 20 min of acclimation to dark under leaf clips (FL-DC; Opti-Sciences). The maximum quantum efficiency (F_v/F_m) of photosystem II was calculated as $F_v/F_m = (F_m - F_o)/F_m$; where F_m and F_o were maximal and minimal fluorescence of dark-adapted leaves, respectively (Maxwell and Johnson, 2000; Jifon and Syvertsen, 2003). Quantum yield (Y) was measured as $Y = (F'_M - F')/F'_M$ where F'_M and F' were the maximal and steady-state fluorescence yield in the light, respectively. This parameter measures the proportion of the light absorbed by chlorophyll associated with the photochemistry in photosystem II.

Leaf chlorophyll concentration was also analysed at the end of both the drought-stress and recovery periods. Chlorophyll was eluted from two 1-cm-diameter leaf discs per leaf by submerging discs in 2 mL of *N,N*-dimethylformamide in the dark for at least 72 h. Absorbance of extract solutions was read at 647 nm and 664 nm with a UV-vis spectrophotometer (model UV2401PC; Shimadzu, Riverwood Drive, Columbia, MD, USA) and used to calculate leaf chlorophyll concentrations using equations in Inskeep and Bloom (1985).

Concentration of Cl^- and Na^+

When plants were harvested at the end of the drought and recovery periods, the Cl^- and Na^+ concentrations were determined in both leaves and roots. Mature leaves at mid-stem and fibrous roots of each plant were briefly rinsed with deionized water and oven-dried at 60 °C for at least 48 h. Dried tissues were ground to a powder and Cl^- concentration in $mmol\ kg^{-1}$ d. wt was measured using a silver ion titration chloridometer (Haake Buchler, Sandle Brook, NJ, USA) after the tissue had been extracted with 0·1 N solution of nitric acid and 10% acetic acid. Titrations were calibrated against known chloride standards bracketing the range of Cl^- in tissues. Sodium concentration in the tissues was determined with an inductively coupled plasma atomic emission spectrometer after the tissue samples had been dry-ashed overnight at 500 °C and suspended in 1 M HCl. Chloride concentration was also determined in the same leaf cell sap extracted from previously frozen leaves for Ψ_π evaluation using a silver ion titration chloridometer and expressed in units of $mmol\ L^{-1}$.

Proline, quaternary ammonium compounds (QAC) and soluble sugar concentration

Leaf proline, QAC and soluble sugar concentrations were also analysed at the end of the drought stress (day 18) and recovery period (day 26). Proline was extracted from fresh leaf tissue with sulfosalicilic acid (3%) and quantified according to the protocol described by Bates *et al.* (1973). QAC were also extracted from fresh leaf tissues with 2 N H_2SO_4 and quantified using glycine betaine as the standard (Grieve and Grattan, 1983). Proline and QAC concentrations were expressed as $mmol\ kg^{-1}$ d. wt. Soluble sugars were extracted from dry plant tissues with 80% ethanol, and starch was extracted from the pellet with MES solution with amyloglucosidase; carbohydrates were quantified using glucose as standard (Hodge and Hofreites, 1962). Glycine betaine, proline and glucose standards were from Sigma-Aldrich Corp. (Fluka, St Louis, MO, USA).

Statistical analysis

Data were subjected to analysis using a two-way variance (ANOVA; SPSS statistical package; SPSS, Chicago, IL, USA) with two salt treatments × two irrigation levels and six replicate plants per treatment. When there was a significant ($P < 0{\cdot}05$) salt × irrigation treatment interaction, means were separated using Duncan's multiple range test (Little and Hills, 1987). Pearson's correlation coefficients (r) were tested among selected leaf water relation variables and leaf tissue constituents using combined data from all four treatments ($n = 24$). Correlations were tested separately on leaves sampled at the end of the drought-stress and recovery periods.

RESULTS

Soil water content

Soil water content (W_g) in pots of well-watered treatments was $14{\cdot}5 \pm 0{\cdot}23\%$. At the end of the drought stress treatment, W_g was reduced to $2{\cdot}0 \pm 0{\cdot}7\%$, so was near the wilting point in the soil of both the saline and non-saline treatments.

Water relations and osmotic adjustment

Saline irrigation prior to the beginning of the drought period (day 7) did not affect Ψ_S or leaf Ψ_π (Fig. 1). At the end of the drought period (day 18), the largest reduction

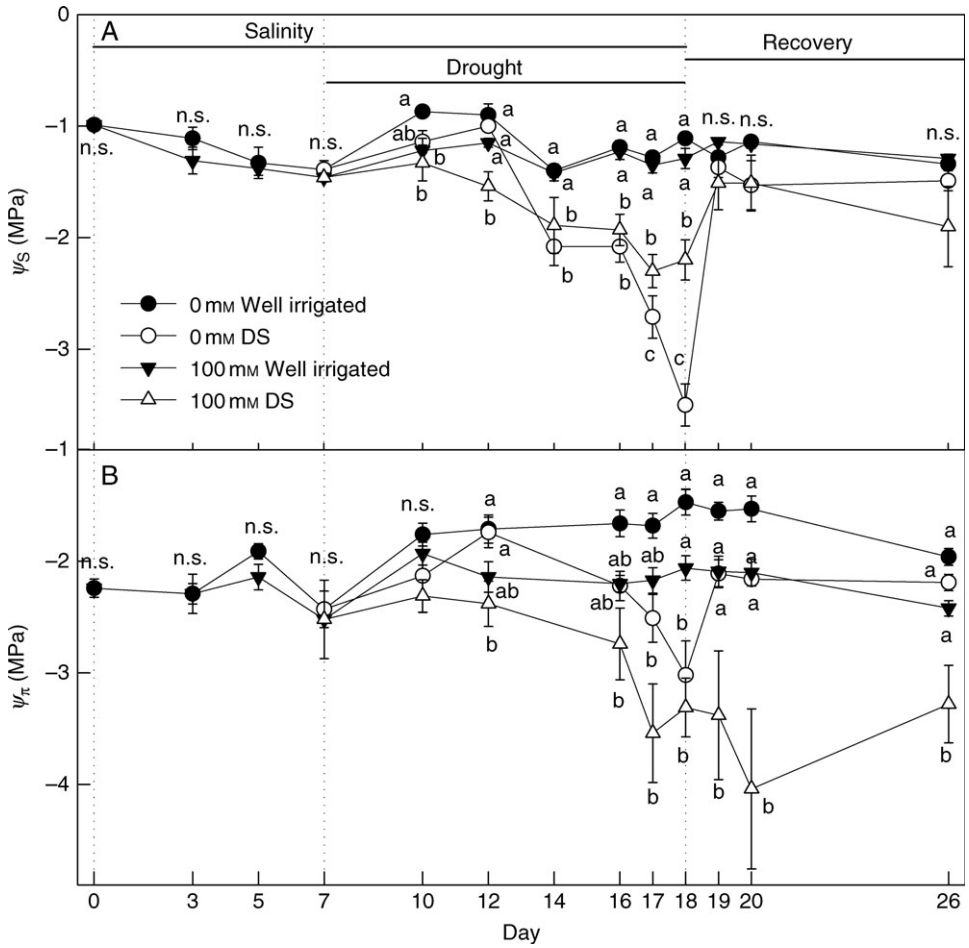

FIG. 1. Effects of soil salinity (0 or 100 mM NaCl) and irrigation (well-irrigated or drought stress, DS) during the 10-d drought stress period and the 8-d recovery period on (A) midday stem water potential (Ψ_S) and (B) osmotic potential (Ψ_π) of fully expanded leaves on Carrizo citrange seedlings. Vertical bars indicate the s.e.m. ($n = 6$). Within each day, means followed by the different letters are significantly different at $P < 0{\cdot}05$; n.s., non-significant differences between means.

in Ψ_S occurred in leaves from the non-salinized drought-stress treatment followed by salinized DS plants. Ψ_π declined in leaves from drought-stress treatments regardless of salinity treatment (Fig. 1B). The small decline in Ψ_π of leaves from the well-irrigated saline treatment was not significantly different from the non-stressed control treatment by day 18. When the previously salinized well-irrigated plants were watered with non-salinized water during the recovery period (days 19–26), there was little change in Ψ_S and Ψ_π (Fig. 1). Previously non-salinized drought-stress treatment began recovery after the first 12 h of rewatering (day 19) such that Ψ_S and Ψ_π were similar to the control. In plants from the previously salinized drought-stress treatment, Ψ_S also recovered completely in the first 12 h but Ψ_π remained lower than the control treatment on day 26.

At the end of the drought-stress period, leaf RWC in previously non-salinized plants was significantly lowered by drought stress but there was no additional decrease in RWC from drought stress in salinized plants (Table 1). Ψ_π^{100} was significantly lower and osmotic adjustment was significantly greater in salt-stressed than non-saline leaves regardless of irrigation treatment. By the end of the recovery period, RWC was lowest in previously salinized DS plants but RWC of the previously DS and well-watered salinized plants fully recovered. Ψ_π^{100} remained lower and osmotic adjustment was greater in the salinity and drought-stress treatments than in non-stressed control plants but there was no significant interaction between the stress treatments.

Gas exchange and fluorescence parameters

Salinity had no effect on the rate of A_{CO2}, WUE, g_s and C_i prior to the beginning of the drought period (day 7; Fig. 2). During the drought-stress period (days 8–18), both drought-stress treatments (non-saline and salinized plants) progressively decreased A_{CO2}, WUE and g_s such that C_i increased compared with well-irrigated treatments. Salinity also reduced A_{CO2} but was reduced less by salinity in well-irrigated than in DS plants. Stomatal conductance was lower in all stressed treatment than in the control

TABLE 1. *Effects of soil NaCl (0 or 100 mM) and irrigation (well irrigated or drought stressed) after the 10-d drought-stress period and at the end of the 8-d recovery period on mean (n = 6) relative water content (RWC), osmotic potential at full turgor (Ψ_π^{100}) and osmotic adjustment (OA = Ψ_π^{100} of control – Ψ_π^{100} of stressed plants) of fully expanded leaves on Carrizo citrange seedlings*

Salt treatment	Irrigation treatment	Leaf RWC (%)	Ψ_π^{100} (MPa)	OA
Stress period				
0 mM	Well-irrigated	92·2[a†]	−1·7	–
	Drought stressed	57·2[b]	−1·77	0·07
100 mM	Well irrigated	91·1[a]	−2·14	0·44
	Drought stressed	88·6[a]	−2·63	0·93
		ANOVA		
Salt		***	***	**
Irrigation		***	n.s.	n.s.
Salt × irrigation		***	n.s.	n.s.
Recovery period				
0 mM	Well irrigated	93·5[a]	−1·53	–
	Drought stressed	92·2[a]	−1·96	0·42
100 mM	Well irrigated	92·8[a]	−2·07	0·53
	Drought stressed	86·7[b]	−2·77	1·23
		ANOVA		
Salt		*	**	***
Irrigation		**	**	**
Salt × irrigation		*	n.s.	n.s.

† Within each column, different letters indicate significant differences at $P \leq 0{\cdot}05$ (Duncan's test). n.s., *, ** and *** indicate non-significant or significant differences at $P < 0{\cdot}05$, 0·01 or 0·001, respectively.

treatment but no significant differences were found between stressed treatments (Fig. 2C). Thus, at the end of the drought period (day 18), DS plants had the lowest A_{CO2} and WUE, and the highest C_i regardless of the salt treatment.

During the recovery period (days 19–26), previously non-salinized DS plants and salinized well-irrigated plants began to increase A_{CO2}, g_s and WUE along with decreases in C_i during the first 48 h after rewatering such that they reached similar values to those of non-stressed control plants by the end of the recovery period (Fig. 2). The response to irrigation during recovery was different to previously salinized drought stress, however, as A_{CO2} and WUE did not recover. Stomatal conductance of salinized DS plants recovered from days 20–26 to values comparable to those of the non-stressed treatment such that C_i remained higher than in the other treatments.

At the end of the drought-stress period (day 18), leaf chlorophyll concentration was not affected by the relatively short-term salinity or drought-stress treatments (Table 2). Salinity did not affect F_o but the other fluorescence parameters F_m, F_v/F_m and Y were decreased by salinity in both irrigation treatments. Drought stress increased F_o and decreased F_m, F_v/F_m and Y regardless of salt treatment. At the end of the recovery period (day 26), the previous drought stress reduced leaf chlorophyll concentration in the previously salinized plants but chlorophyll was not affected in non-salinized plants. Even after recovery, F_o of the previous DS plants remained high while F_v/F_m and Y remained lower than in the well-irrigated treatments, regardless of previous salinity treatment.

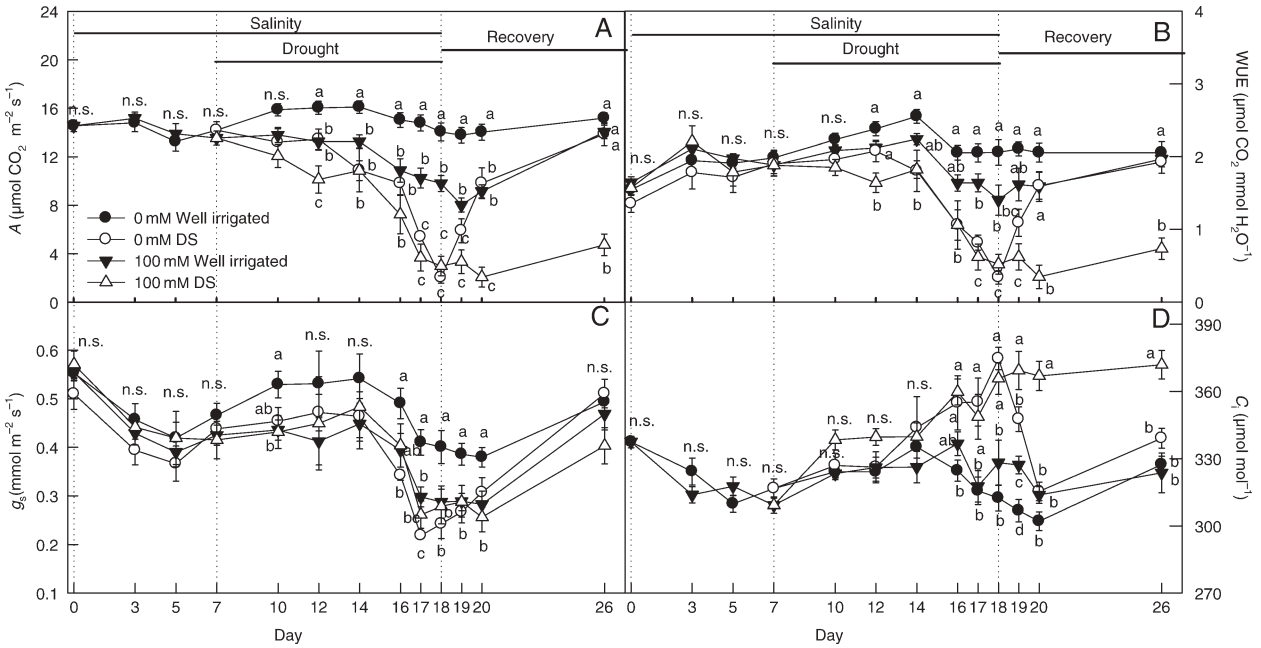

FIG. 2. Effects of soil salinity (0 or 100 mM NaCl) and irrigation (well-irrigated or drought stress, DS) during the 10-d drought stress period and the 8-d recovery period on (A) net CO_2 assimilation rate (A_{CO2}, μmol CO_2 m^{-2} s^{-1}), (B) leaf water use efficiency (WUE, μmol CO_2 $mmol^{-1}$ H_2O), (C) stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) and (D) intercellular CO_2 concentration (C_i; μmol mol^{-1}) of fully expanded leaves on Carrizo citrange seedlings. Vertical bars indicate the s.e.m. ($n = 6$). Within each day, means followed by the different letters are significantly different at $P < 0{\cdot}05$; n.s., non-significant differences between means.

TABLE 2. *Effects of soil NaCl (0 or 100 mM) and irrigation (well irrigated or drought stressed) after the 10-d drought-stress period and at the end of the 8-d recovery period on mean (n = 6) leaf total chlorophyll (Chl) maximal (F_m, relative units) and minimal fluorescence of dark-adapted leaves (F_o, relative units), maximum quantum yield of dark-acclimated leaves (F_v/F_m, relative units) and quantum yield (Y, relative units) of fully expanded leaves on Carrizo citrange seedlings*

Salt treatment	Irrigation treatment	Total Chl (mg dm^{-2})	F_o	F_m	F_v/F_m	Y
Stress period						
0 mM	Well irrigated	5·18	94·5	447·7	0·79	0·61
	Drought stressed	4·78	101·5	417·7	0·75	0·45
100 mM	Well irrigated	5·27	94·2	423·8	0·78	0·54
	Drought stressed	4·99	109·7	375·5	0·7	0·36
		ANOVA				
Salt		n.s.	n.s.	*	*	*
Irrigation		n.s.	**	*	**	***
Salt × irrigation		n.s.	n.s.	n.s.	n.s.	n.s.
Recovery period						
0 mM	Well irrigated	5·30[a†]	76	380·7	0·8	0·61
	Drought stressed	5·10[a]	91·3	333·8	0·73	0·41
100 mM	Well irrigated	5·34[a]	80·7	361·7	0·78	0·59
	Drought stressed	3·87[b]	97·7	356·2	0·71	0·46
		ANOVA				
Salt		*	n.s.	n.s.	n.s.	n.s.
Irrigation		**	*	n.s.	*	**
Salt × irrigation		*	n.s.	n.s.	n.s.	n.s.

[†] Within each column, different letters indicate significant differences at $P \leq 0{\cdot}05$ (Duncan's test). n.s., *, ** and *** indicate non-significant or significant differences at $P < 0{\cdot}05$, 0·01 or 0·001, respectively.

Concentration of Cl^- and Na^+ in leaves and roots and Cl^- concentration in leaf cell sap

At the end of the period of drought stress, leaf and root Cl^- and Na^+ concentrations were, of course, increased by salinity but drought stress did not significantly increase the relatively low leaf and root Cl^- and Na^+ levels in the non-salinized plants (Table 3). Cl^- concentration in the leaf cell sap was significantly increased by both salt and drought treatments but there were no significant interactions between the stress treatments. At the end of the recovery period, leaf Cl^- concentration remained higher in the previously salinized DS plants than in salinized well-irrigated plants (Table 3). However, root Cl^- concentration was higher for salinized DS plants than for salinized well-irrigated plants. Leaf and root Na^+ concentrations were increased by the previous salt treatment but drought-stress treatment had no effect on tissue Na^+. At the end of the recovery period, salinized DS plants tended to have higher leaf Cl^- (though not significantly so) and significantly lower root Cl^- concentration than at the end of the stress period. Similarly, leaf Na^+ concentration also was higher and root Na^+ concentration was lower at the end of the recovery period than at the end of the stress period. The previous salt and drought-stress treatment maintained high Cl^- concentrations in leaf cell sap during the recovery period but there were no significant interactions between the stress treatments.

Leaf proline, QAC and sugar concentration

At the end of the period of drought stress, leaf proline concentrations were increased by drought-stress treatments, and levels were highest in non-salinized DS plants (Table 4). Leaf proline was not affected by salinity under well-watered conditions. Concentrations of QAC were not affected by salinity or drought stress. Concentrations of soluble sugars in leaves were significantly reduced by drought stress in non-salinized plants but not in the already lower values in salt-stressed plants. Leaf starch was significantly decreased by both salt and drought-stress treatments.

At the end of the recovery period, the previous drought-stress treatments maintained higher leaf proline and soluble sugars than the previously well-irrigated treatments regardless of the previous salinity treatment (Table 4). Concentrations of QAC were higher in previously DS than in well-watered leaves, and values were highest in previously salinized DS plants. Leaves of the previous salt and drought-stress treatments had higher soluble sugar concentrations than previously non-salinized and well-watered treatments. Leaf starch increased after stress relief as there were no significant treatment effects on leaf starch concentrations after the recovery period.

Pearson's correlation coefficients

When data at the end of the drought-stress period were pooled across treatments, A_{CO2} was positively correlated with leaf RWC and starch but negatively correlated with proline (Table 5). A_{CO2} was negatively correlated to leaf Cl^- but not related to leaf Na^+. There were significant negative correlations between Ψ_π^{100} and leaf tissue Cl^- and Na^+ but there was a positive correlation between Ψ_π^{100} and leaf starch. Leaf RWC was not correlated to Ψ_π^{100} but RWC was negatively correlated to leaf proline and increased with leaf Na^+ and soluble sugars. Leaf

TABLE 3. *Effects of soil NaCl (0 or 100 mM) and irrigation (well irrigated or drought stress) after the 10-d drought-stress period and at the end of the 8-d recovery period on mean (n = 6) concentrations of leaf and root tissue chloride (Cl^-), sodium (Na^+) and leaf cell sap Cl^- of Carrizo citrange seedlings*

Salt treatment	Irrigation treatment	Cl^- (mmol kg^{-1} d. wt)		Na^+ (mmol kg^{-1} d. wt)		Cl^- (mmol L^{-1})
		Leaf	Root	Leaf	Root	Cell sap
Stress period						
0 mM	Well irrigated	141[c†]	153[c]	38[bc]	128[c]	35
	Drought stressed	173[c]	112[c]	14[c]	104[c]	119
100 mM	Well irrigated	319[b]	319[b]	73[b]	462[b]	179
	Drought stressed	535[a]	416[a]	129[a]	637[a]	354
		ANOVA				
Salt		***	***	***	***	***
Irrigation		***	n.s.	n.s.	n.s.	***
Salt × irrigation		**	*	***	*	n.s.
Recovery period						
0 mM	Well irrigated	122[c]	159[b]	24	133	98
	Drought stressed	159[bc]	192[b]	14	99	186
100 mM	Well irrigated	333[b]	286[a]	177	438	219
	Drought stressed	637[a]	197[b]	323	362	367
		ANOVA				
Salt		***	**	***	***	***
Irrigation		**	n.s.	n.s.	n.s.	***
Salt × irrigation		*	**	n.s.	n.s.	n.s.

[†] Within each column, different letters indicate significant differences at $P \leq 0{\cdot}05$ (Duncan's test). n.s., *, ** and *** indicate non-significant or significant differences at $P < 0{\cdot}05$, 0·01 or 0·001, respectively.

starch was negatively related to leaf Cl^- and Na^+. Leaf proline was negatively related to soluble sugars and starch but QAC were not related to any water relation characteristics or to other leaf constituents.

At the end of the recovery period, A_{CO2} was positively related to Ψ_π^{100} and RWC, but negatively related to the other leaf constituents measured except for leaf starch (Table 5). Ψ_π^{100} increased with RWC but was negatively correlated with leaf Cl^-, Na^+, soluble sugars and proline concentrations. At this time, there was no correlation between Ψ_π^{100} and leaf starch. In addition, RWC was negatively correlated with leaf Cl^-, Na^+, soluble sugars,

TABLE 4. *Effects of soil NaCl (0 mM or 100 mM) and irrigation (well irrigated or drought stress) after the 10-d drought-stress period and at the end of the 8-d recovery period on mean (n = 6) concentrations of proline, quaternary ammonium compounds (QAC), soluble sugars and starch in fully expanded leaves of Carrizo citrange seedlings*

Salt treatment	Irrigation treatment	Proline (mmol kg^{-1} d. wt)	QAC (mmol kg^{-1} d. wt)	Soluble sugars (mmol kg^{-1} d. wt)	Starch (mmol kg^{-1} d. wt)
Stress period					
0 mM	Well irrigated	41·6[c†]	38·6	555[a]	133
	Drought stressed	154·1[a]	44·5	370[b]	93
100 mM	Well irrigated	55·2[c]	43·1	458[ab]	106
	Drought stressed	107·3[b]	45	538[a]	67
		ANOVA			
Salt		n.s.	n.s.	n.s.	**
Irrigation		***	n.s.	n.s.	***
Salt × irrigation		*	n.s.	*	n.s.
Recovery period					
0 mM	Well irrigated	53·1	34·0[c]	305	166
	Drought stressed	89·6	43·9[b]	366	175
100 mM	Well irrigated	41·8	34·7[c]	339	161
	Drought stressed	92·9	54·8[a]	394	130
		ANOVA			
Salt		n.s.	n.s.	*	n.s.
Irrigation		***	***	**	n.s.
Salt × irrigation		n.s.	*	n.s.	n.s.

[†] Within each column, different letters indicate significant differences at $P \leq 0{\cdot}05$ (Duncan's test). n.s., *, ** and *** indicate non-significant or significant differences at $P < 0{\cdot}05$, 0·01 or 0·001, respectively.

TABLE 5. *Pearson's correlation coefficients between A_{CO2}, osmotic potential at full turgor (Ψ_π^{100}), relative water content (RWC), Cl^-, Na^+, soluble sugar, starch, proline and quaternary ammonium compounds (QAC) of fully expanded leaves on Carrizo citrange seedlings at the end of the 10-d irrigation treatment period and after the 8-d recovery period (*n = 24).

Stress	A_{CO2}	Ψ_π^{100}	RWC	Cl^-	Na^+	Sol. sugar	Starch	Proline
Irrigation								
Ψ_π^{100}	n.s.†							
RWC	0·57**	n.s.						
Cl^-	−0·41*	−0·56**	n.s.					
Na^+	n.s.	−0·56**	0·51**	0·87***				
Sol. sugar	n.s.	n.s.	0·64***	n.s.	n.s.			
Starch	0·65***	0·52**	n.s.	−0·74***	−0·56**	n.s.		
Proline	−0·68***	n.s.	−0·58**	n.s.	n.s.	−0·34*	−0·44*	
QAC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Recovery								
Ψ_π^{100}	0·75***							
RWC	0·71***	0·63***						
Cl^-	−0·62***	−0·46*	−0·77***					
Na^+	−0·84***	−0·76***	−0·83***	0·83***				
Sol. sugar	−0·57**	−0·59**	−0·69***	0·59**	0·65***			
Starch	n.s.	n.s.	n.s.	−0·42*	n.s.	n.s.		
Proline	−0·51*	−0·41*	−0·52**	n.s.	n.s.	0·60**	n.s.	
QAC	−0·53**	n.s.	−0·76***	0·61**	0·62**	0·71***	n.s.	0·68***

Critical r of $n-2 = 22$ at $P < 0{\cdot}05 = \pm 0{\cdot}40$.
† 'n.s.', *, ** and *** indicate non-significant or significant differences at $P < 0{\cdot}05$, 0·01 or 0·001, respectively.

proline and QAC. Soluble sugars were negatively related to RWC and Ψ_π^{100} but positively correlated with leaf Cl^- and Na^+. Leaf proline was negatively related to Ψ_π^{100} and RWC but was positively related to soluble sugars. QAC were high when leaf RWC was low, whereas QAC were positively related to leaf Cl^-, Na^+, soluble sugars and proline.

DISCUSSION

Drought-stress period in salinized and non-salinized plants

Since RWC and Ψ_S were higher in salinized DS plants, the short-term preconditioning by salt stress prior to the drought-stress period improved the water relation responses to drought stress compared with non-salinized plants. Salinity can also enhance tolerance to freezing of citrus seedlings by modifying growth, water relations and mineral nutrition (Syvertsen and Yelenosky, 1988). In this short-term experiment, osmotic adjustment maintained RWC under drought stress in previously salinized plants. The negative correlation between Ψ_π^{100} and leaf Cl^- and Na^+ concentrations and the non-significant correlations between Ψ_π^{100} and the potential osmolytes proline, QAC or solubles sugars, indicated that this osmotic adjustment was mainly due to an increase in leaf Cl^- and Na^+ concentration (Table 5). Accumulation of leaf Cl^- can be a passive process which depends on transpirational water flow (Moya *et al.*, 1999; García-Sánchez *et al.*, 2006). DS plants apparently were not able to regulate the Ψ_π in order to compensate for the water deficit. Although leaf Cl^- and proline concentrations were increased by drought stress, this was solely a passive process resulting from the decreased water content since Ψ_π^{100} was similar in leaves from well-watered and DS plants (Table 1). In addition, there was no correlation between leaf proline concentration and Ψ_π^{100} at that time (Table 5). Thus, leaf proline accumulation was not sufficient to contribute to any decrease in Ψ_π^{100}. There was a significant negative correlation between leaf proline concentration and RWC at the end of the period of drought stress, however, supporting the idea that leaf dehydration increased proline concentration either passively (Bray, 1997) and/or by an increase in synthesis of proline (Lazcano-Ferrat and Lovatt, 1999). Despite no apparent role in osmotic adjustment to drought stress, proline could have played a role in drought tolerance since proline can be involved in protection of cellular structures against oxidative damage by scavenging free radicals during leaf dehydration (Tsugane *et al.*, 1999). This idea is supported by the elevated proline response to drought stress without any apparent changes in Ψ_π^{100} (Table 4). Similar to the results of Syvertsen and Yelenosky (1988), salinity alone did not affect leaf proline concentrations and there were no correlations between leaf proline and leaf Cl^- or Na^+ (Table 5). In other citrus salinity studies, an increase in leaf proline level was <2-fold compared with a non-salinized control treatment (Walker *et al.*, 1993). Thus, the regulation of proline in citrus leaves may be atypical of salinized glycophytes like potato plants (Fidalgo *et al.*, 2004) or other species (Verslues *et al.*, 2006) that accumulate high levels of proline in response to salinity stress.

Since both salinity and drought stress reduced A_{CO2}, decreases in leaf carbohydrates could be anticipated. A depletion of the leaf carbohydrate concentrations when citrus plants were grown under stress has been observed (Arbona *et al.*, 2005) but starch hydrolysis can partially buffer fluctuations in sugar concentrations when

photosynthesis is low (Praxedes *et al.*, 2006). In the present experiment, a large reduction in leaf starch concentration in salinized plants could have maintained high soluble sugar concentrations in leaves. However, soluble sugars were reduced by drought stress in non-salinized plants but not in salinized plants (Table 4). The significant negative correlation between leaf soluble sugar concentrations and leaf proline concentrations (Table 5), suggests that the reduction in leaf soluble sugar concentration in non-salinized DS plants could have been due to a diversion of sugars to increased proline synthesis (Ennajeh *et al.*, 2006; Knipp and Honermeier, 2006).

During the period of drought stress (days 8–18), A_{CO2} progressively decreased in plants from all the stressed treatments compared with well-irrigated treatment and despite concomitantly lowered g_s, calculated values of C_i were maintained in the stressed treatments until day 1. This implied that that stomatal closure was not the dominant limitation on A_{CO2} but rather increased mesophyll limitations were likely to be more important (Farquhar and Sharkey, 1982). The accumulation of leaf Cl^- undoubtedly contributed to this decrease as A_{CO2} was negatively related to leaf Cl^- concentrations. After day 14, as stress became more severe, C_i increased in both non-salinized and salinized DS leaves. This increase in C_i as A_{CO2} decreased under stress conditions also supports the idea that non-stomatal factors were more important than stomatal limitations on the reduced A_{CO2}. In addition, reductions in F_m, F_v/F_m and Y in stressed leaves support the notion that decreased biochemical factors in the mesophyll were responsible for the decline in photosynthesis. Lowered F_v/F_m can indicate photoinhibitory damage and decreased Y can lower electron transport to carbon fixation (Maxwell and Johnson, 2000).

Flexas *et al.* (2004) made a convincing argument that g_s and internal mesophyll conductance can be regulated together during salt and drought stress and mesophyll conductance can influence the extent to which leaves can recover photosynthetic capacity after stress. Changes in internal leaf conductance to CO_2, however, can reduce CO_2 concentration at the chloroplast (Syvertsen *et al.*, 1995) and invalidate the estimation of C_i (Flexas *et al.*, 2004). Thus, even in species like *Citrus* where patchy stomatal closure is not considered an important issue (Syvertsen and Lloyd, 1994), the use of calculated C_i to describe non-stomatal limitations on A_{CO2} should be interpreted with caution as changes in mesophyll conductance can affect CO_2 diffusion and its concentration at the chloroplasts (Flexas *et al.*, 2004).

It is clear that reductions of A_{CO2} in leaves from drought-stress treatment in salinized and non-salinized plants were due to different mechanisms. In salinized DS plants, the decrease in A_{CO2} was apparently due to high Cl^- but not Na^+ toxicity (Table 5) since RWC was maintained (Table 1). High leaf Cl^- concentrations could have reduced A_{CO2} by a loss of cell turgor due to salt accumulation in the apoplast or by ion toxicity (Marschner, 1995). In non-salinized DS plants, the reduction in A_{CO2} was probably related to osmotic stress since RWC was reduced. Therefore, despite the positive effects of previously applied NaCl in improving leaf water relations after plants were subjected to drought stress, high Cl^- concentrations had a negative impact on A_{CO2} and g_s in both drought-stress and salinized drought-stress treatments (Fig. 2).

Recovery after drought stress

Recovery of plant water status parameters in previously non-salinized DS plants showed a typical recovery pattern similar to peach (*Prunus persica*; Girona *et al.*, 1993) or almond trees (*Prunus amygdalus*; Romero *et al.*, 2004) where rapid recovery of Ψ_S occurred within 24 h after re-irrigation. The recovery pattern in previously salinized plants was also typical for trees where Ψ_S recovered to non-stressed levels in 24 h while Ψ_π remained lower than in the non-salinized plants (Tattini *et al.*, 2002). Non-recovery of Ψ_π may have been due to the high levels of leaf Cl^- and/or Na^+ concentrations. On the other hand, the recovery of Ψ_π in salinized DS plants followed a similar pattern to that of the salinized well-irrigated plants even though Ψ_S and RWC remained lower than in non-stressed plants. This recovery pattern in salinized DS plants could have been related to the high Cl^- and Na^+ concentrations accumulated in roots during the stress period. Re-irrigation with non-salinized water apparently caused a translocation of previously accumulated Cl^- and Na^+ from roots to leaves. Similar increases in Cl^- and Na^+ concentrations in leaves and decreases in roots have been reported in sour orange but not in Cleopatra mandarin (Cámara-Zapata *et al.*, 2004). High leaf Cl^- concentration could have damaged the photosynthetic system as leaf chlorophyll concentration (García-Sánchez *et al.*, 2006), F_v/F_m and Y all decreased (Table 2). In addition, plants from the non-saline drought-stress treatment had a lower Y and F_v/F_m than plants from the control treatment. Thus, fluorescence parameters remained low while A_{CO2} recovered. High ion concentrations in roots could also have decreased hydraulic conductance (García-Sanchez *et al.*, 2000) and reduced Ψ_S and RWC in the previously salinized drought-stress treatment as a consequence of decreased water uptake after re-irrigation.

After recovery of DS plants, leaf proline concentration was decreased. Proline reductions may be due to their use for root growth, their transport to the shoots or to a dilution effect after regrowth during recovery (Lacerda *et al.*, 2005). The principal QAC in citrus is proline betaine, an osmolyte synthesized from proline with better osmoprotectant characteristics than proline (Nolte *et al.*, 1997). In the present experiment, the reduction in leaf proline after recovery from drought stress could have occurred along with a degradation of proline betaine since leaf QAC levels only increased in previously salinized DS plants. The accumulation of QAC apparently was not related to improved leaf water relations under drought stress, however, since QAC levels only increased after the recovery period. QAC are compatible solutes that can function as osmoprotectants and can be related to the function of other macromolecules during stress (Su *et al.*, 2006).

At the end of the recovery period, A_{CO2} was similar to pre-stress levels in all treatments except in previously salinized DS plants. Recovery of A_{CO2} in previously non-salinized DS plants occurred with the increase in RWC. High leaf proline concentration may have protected the photosynthetic system from permanent damage during drought (Lawlor, 2001) as recovery was complete after 8 d in non-salinized DS and salinized plants. A_{CO2} in salinized well-irrigated plants also recovered to non-stressed levels despite little change in leaf Cl^- concentrations. High leaf Cl^- concentration from foliar-applied Cl^- sprays had little effect on A_{CO2} even though leaf Cl^- concentrations were comparable to levels associated with reductions in A_{CO2} when salts were applied to the soil (Romero-Aranda and Syvertsen, 1996). In this experiment, therefore, whole plant water relations and Cl^- concentrations in the soil solution may have been more important than Cl^- levels in leaves for limiting A_{CO2}. Although salinized DS plants suffered less in terms of water relations than DS plants, salinized DS plants did not recover to the original values of A_{CO2}, WUE and C_i. The increase in leaf Cl^- concentration during the recovery period apparently inhibited this recovery.

In conclusion, water relation parameters of the relatively salt-sensitive Carrizo citrange were less affected by drought stress when seedlings were preconditioned by salinity stress. Plants from the salinized drought-stress treatment were able to maintain their RWC whereas RWC was reduced in plants under drought stress alone. Osmotic adjustment was related to the accumulation of Cl^- and not due to accumulation of proline, QAC or soluble sugars. Accumulation of Cl^- damaged leaves, however, when Cl^- concentrations became high enough. Salinized DS plants had high levels of leaf Cl^- and Na^+ that impeded recovery after re-irrigation with non-saline water, as translocation of Cl^- and/or Na^+ from roots to leaves continued in these plants. In contrast, DS plants were able to recover water relations and gas exchange parameters after 8 d of re-irrigation with non-saline water.

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