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Research article

The long-term resistance mechanisms, critical irrigation threshold and relief capacity shown by *Eugenia myrtifolia* plants in response to saline reclaimed water

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ABSTRACT

Salts present in irrigation water are serious problems for commercial horticulture, particularly in semi-arid regions. Reclaimed water (RW) typically contains, among others elements, high levels of salts, boron and heavy metal. Phytotoxic ion accumulation in the substrate has been linked to different electric conductivities of the treatments. Based on these premises, we studied the long-term effect of three reclaimed water treatments with different saline concentrations on *Eugenia myrtifolia* plants. We also looked at the ability of these plants to recover when no drainage was applied. The RW with the highest electric conductivity (RW3, EC = 6.96 dS m^{-1}) provoked a number of responses to salinity in these plants, including: 1) accumulation and extrusion of phytotoxic ions in roots; 2) a decrease in the shoot/root ratio, leaf area, number of leaves; 3) a decrease in root hydraulic conductivity, leaf water potential, the relative water content of leaves, leaf stomatal conductance, the leaf photosynthetic rate, water-use efficiency and accumulated evapotranspiration in order to limit water loss; and 4) changes in the antioxidant defence mechanisms. These different responses induced oxidative stress, which can explain the damage caused in the membranes, leading to the death of RW3 plants during the relief period. The behaviour observed in RW2 plants was slightly better compared with RW3 plants, although at the end of the experiment about 55% of the RW2 plants also died, however RW containing low salinity level (RW1, EC = 2.97 dS m^{-1}) can be effective for plant irrigation.

1. Introduction

The potential for sustainable agricultural activity in many arid and semi-arid regions is limited by the scarce fresh water resources for irrigation (Bezborodov et al., 2010; Cirillo et al., 2016). Reclaimed waters (RWs) used as a non-conventional water resource are of proven agronomic and environmental interest for irrigation of ornamental (Acosta-Motos et al., 2014, 2016; Gómez-Bellot et al., 2015a,b) and other crop plants (Pedrero et al., 2014, 2015; Dorta-Santos et al., 2016; Nicolás et al., 2016), especially in Mediterranean regions where water availability is a limiting factor (Yermiyahu et al., 2008). The use of RW has different benefits, including a reduction in the discharge of pollutants into natural water courses (Zekri and Koo, 1994), which can be particularly important when the treated water is used for landscaping (Dobrowolski et al., 2008). RWs are also characterised by their high

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate reduced form; CAT, catalase; DHA, ascorbate oxidized form; DHAR, dehydreascorbate reductase; DW, dry weight; EC, electrical conductivity; ETa, accumulated evapotranspiration; FW, fresh weight; GR, glutathione reductase; GSH, glutathione reduced form; GSSG, glutathione oxidized form; g_s, stomatal conductance; H_2O_2, hydrogen peroxide; J, absorption rate of ions by roots; LP, lipid peroxidation; L_p, root hydraulic conductivity; MDHAR, monodehydroascorbate reductase; NADH, nicotinamide adenine dinucleotide reduced form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; $O_2^{\bullet -}$, superoxide anion; $^{\bullet}OH$, hydroxyl radicals; PAR, photosynthetically active radiation; POX, peroxidase; P_n, net photosynthetic rate; RH, relative humidity; ROS, reactive oxygen species; RW, reclaimed water; RWC, relative water content; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive-substances; TW, turgid weight; WFC, weight at field capacity; WUE, water-use efficiency; WUE_i, intrinsic water-use efficiency; Ψ_l, leaf water potential.

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nutrient content, which can preclude the use of fertilizers, thus reducing the risk of environmental contamination (Khajanchi et al., 2015; Dorta-Santos et al., 2016). Despite these advantages, however, RW is of lower quality than fresh water. Furthermore, depending on the origin of the RW, the time of collection and the treatment applied, it may contain certain phytotoxic ions, heavy metals and fecal microorganisms. In such cases, this type of water could be used for landscaping and revegetation projects using ornamental plants where the impact is not as important (Gómez-Bellot et al., 2015b; Acosta-Motos et al., 2016) as it would be in other crops for human consumption (Pedrero et al., 2015; Nicolás et al., 2016).

Salinity is among the other harmful elements present in these waters and can result in a plant damage and reduced plant quality. Salinity in soils and irrigation water is one the main abiotic stresses affecting agriculture worldwide, limiting crop growth and production. In order to mitigate the negative effects of salinity, plants have developed different physiological and biochemical mechanisms including changes in biomass parameters, phytotoxic ion distribution, water relations, photosynthesis and the antioxidative metabolism response (Munns and Tester, 2008). The main negative effects produced by salinity include osmotic stress, related to a decrease in water potential in the roots, and ion toxicity, due to an excessive accumulation of phytotoxic ions in all plant organs, leading to nutritional imbalance resulting from a shortage of calcium, magnesium and potassium ions (Parida and Das, 2005). The response of plants to salinity is different depending on the plant species used. In salinity experiments it is important to select salt-resistant endemic plants adapted to Mediterranean areas, such as *Myrtus communis* (Miralles et al., 2010; Acosta-Motos et al., 2014, 2015a, 2016), or plants adapted to similar climates, such as *Eugenia myrtifolia* (Acosta-Motos et al., 2015b). Moreover, it is also important to set other experimental conditions such as the use of pots for growing of the plants and the drainage conditions applied (Bañón et al., 2012; Acosta-Motos et al., 2014, 2016).

In addition, salinity can limit CO_2 fixation in plants, producing oxidative stress which is mediated by an overproduction and accumulation of reactive oxygen species (ROS) such as superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) at the subcellular level (Corpas et al., 1993; Hernández et al., 1993, 1995). This response contributes to the appearance of symptoms such as a disruption in cellular metabolisms through membrane lipid peroxidation, enzyme inhibition and damage to nucleic acids (Parida and Das, 2005; Sabra et al., 2012). In order to cope with the ROS, plants have implemented a complex defence system that includes enzymatic and non-enzymatic antioxidant mechanisms (Noctor and Foyer, 1998). In general, salt-tolerant plants have a better response than other plants to oxidative stress, increasing the activity and/or the expression of antioxidant enzymes, as has been observed in different crops (Hernández et al., 2000, 2001; Demiral and Türkan, 2006; Moradi and Ismail, 2007; Duarte et al., 2013; Gil et al., 2014; Acosta-Motos et al., 2015a,b). This increase may also occur, however, in salt-sensitive species (Arbona et al., 2003; Lee et al., 2013). Other authors have correlated salt tolerance with higher constitutive levels of certain antioxidants (Hernández et al., 2003; López-Gómez et al., 2007). Overall, there is scarce and inconsistent information on the effect of RW with high salt levels on the antioxidative metabolism of ornamental crops.

In a previous work, under controlled environmental conditions, we studied the short-term effect of NaCl on *Eugenia myrtifolia* plants (an interesting plant useful for xeriscaping and landscaping projects in public areas) and their ability to recover (Acosta-Motos et al., 2015b). In the present work, the aim was to determine the long-term effect of the same responses when RWs containing different salt concentrations are used as an unconventional alternative water resource. This work thus evaluates the effect of salt accumulation due to the different RW treatments applied during a long period of time (23 weeks) and the plants' ability to recover following a salinity relief period (9 weeks) (with no drainage applied). To this effect, plant growth, ornamental quality parameters, water relations, gas exchange, mineral nutrition and antioxidative metabolism were evaluated. Furthermore, we established a set of guidelines to be considered by nurseries. These "lines of action" indicate how long irrigation should be applied and, on the one hand, which salt threshold levels are critical for optimum growth and even for improving the visual and ornamental qualities of the plants (positive approach), and, on the other hand, the levels that can cause irreversible damage and plant death (negative approach).

2. Materials and methods

2.1. Plant and experimental conditions

Single rooted cuttings (120) of native *Eugenia myrtifolia* plants were transplanted into 14 × 12 cm pots (1.2 l) filled with a mixture of coconut fibre, sphagnum peat and perlite (8:7:1) and amended with Osmocote plus (2 g l^{-1} substrate) (14:13:13 N, P, K + microelements) supplied by Agrosolmen S.L., Lorca (Murcia), Spain. The experiment was conducted in a controlled environment growth chamber set to simulate natural conditions. The temperature in the chamber was 23° C during the light phase (16 h photoperiod) and 18° C during darkness. Relative humidity (RH) values ranged between 55 and 70%. A mean photosynthetic active radiation (PAR) of 350 μmol m^{-2} s^{-1} at canopy height was supplied during the light phase (08:00 h-00:00 h) by cold white fluorescent lamps.

2.2. Water irrigation treatments, substrate analyses and experimental design

At the beginning of the experimental period three water samples from each irrigation water source were collected in glass bottles, transported in an ice chest to the laboratory and stored at 5 °C in order to determine the irrigation water quality. A chemical analysis of the waters used for each irrigation treatment was performed, and the results obtained are shown in Table 1.

The electrical conductivity (EC) was measured with a multirange Cryson-HI8734 electrical conductivity meter (Cryson Instruments, S.A., Barcelona, Spain). The pH was calculated with a Cryson-507 pH-meter (Cryson Instruments, S.A., Barcelona,

Table 1
Chemical analyses of the water used in different treatments. Data are values collected at the beginning of the experimental period.

Parameters	Irrigation water			
	Control	RW1	RW2	RW3
EC (dS m^{-1})	0.88	2.97	4.38	6.96
pH (-log[H^+])	7.72	8.07	8.25	7.85
SDT (mg L^{-1})	–	754.02	1679.17	5340.00
OD (mg L^{-1})	–	5.10	9.05	6.20
SS (mg L^{-1})	–	2.56	8.65	5.46
Turbidity	–	7.00	3.22	1.65
Na^+ (mmol L^{-1})	2.26	11.31	15.78	64.90
Cl^- (mmol L^{-1})	1.96	20.68	24.28	43.86
Ca^{2+} (mmol L^{-1})	2.35	1.72	4.14	5.05
B^{3+} (mmol L^{-1})	0.01	0.02	0.05	0.12
K^+ (mmol L^{-1})	0.09	0.85	0.96	3.00
Mg^{2+} (mmol L^{-1})	1.72	1.67	4.08	8.50
S (mmol L^{-1})	2.67	1.17	6.38	8.79

Spain). The concentrations of B^{3+}, Ca^{2+}, K^{+}, Mg^{2+}, Na^{+} and S ions were determined by an inductively coupled plasma optical emission spectrometer (ICP-OES, IRIS intrepid II XDL, Thermo Fisher Scientific Inc., Loughborough, UK). Chloride (Cl^{-}) ion was analysed by ion chromatography (Metrohm, Herisau, Switzerland).

Five substrate samples per treatment (corresponding with the same plants used for biomass parameters) were collected at the end of the salinity period and sent to an external laboratory (Antonio Abellan Caravaca S.L. [Fitosoil]) for analysis. The substrate was dried at room temperature for a week. Na^{+}, Ca^{2+} and Mg^{2+} were determined by inductively coupled plasma ICP-AES in a saturated soil extract, and Cl^{-} was determined by ion chromatography. EC was determined on saturated soil paste (Table 3).

Once the *E. myrtifolia* plants (30 per treatment) were adapted to the chamber conditions, they were exposed for up to 23 weeks (stress period) to the following four irrigation treatments: a control, where plants were watered with tap water with an electrical conductivity (EC) of 0.88 dS m^{-1}, and three reclaimed water treatments (RW). The reclaimed water came from three sewage treatment plants located in the province of Murcia (Spain), namely: RW1 (EC = 2.97 dS m^{-1}) from Jumilla; RW2 (EC = 4.38 dS m^{-1}) from Campotejar; and RW3 (EC = 6.96 dS m^{-1}) from Mazarrón. FAO classifications indicated severe restrictions on the use of the last two types of water. All three waste water treatment plants applied a conventional activated-sludge process followed by ultraviolet radiation as the tertiary treatment. Before the experimental period began, the maximum water-holding capacity of the soil was determined for each individual pot, and was considered as the weight at field capacity (WFC). To determine the maximum water-holding capacity of the substrate, all the pots were uniformly mixed and packed to a bulk density of 0.165 g cm^{-3}. The substrate surfaces were covered with aluminium foil to prevent water evaporation, and the lower parts were submerged to half of the pot's height in a water bath and then left to equilibrate overnight. The next day, the pots were removed and left to drain freely until drainage was negligible. The fresh weight was then recorded and calculated for each individual pot and considered as the weight at field capacity (WFC). At the end of the experiment, the substrate was dried in an oven at 105° C until constant weight to obtain the dry weight and calculate the volumetric water content. Later, the difference between the fresh weight and oven-dry weight was measured and the volumetric water content was calculated (58 ± 1%), which was considered as the substrate's field capacity. Throughout the experiment, all pots were irrigated three times a week below the WFC in order to avoid drainage, favouring an increase in soil salinity due to the time and the severity of the saline treatments. After the salinity period, all plants were exposed to a 9 weeks (relief period) in which they were irrigated with the same water used for control plants.

2.3. Growth measurements

At the beginning of the experiment, before applying the different treatments, five plants per treatment were selected and all the substrate was gently washed from their roots. Each plant was then divided separately into shoots (leaves and stems) and roots. Then, once again, at the end of both experimental periods (salinity and relief), all the substrate was gently washed from the roots of five plants per treatment and each plant was divided separately into shoots (leaves and stems) and roots. These fresh materials were then oven-dried at 80° C, until they reached a constant weight, to measure their respective dry weights (DW). The total number of leaves and leaf area (cm^2) were determined from the same five plants per treatment using a leaf area meter (Delta-T Devices Ltd., Cambridge, UK).

2.4. Ionic balances and nutritional changes

At the beginning and at the end of both experimental periods (salinity and relief) the same plants used for the biomass parameters calculation were also used to determine the inorganic solute concentration. Plant material, which had been previously oven-dried at 80° C until it reached a constant weight, was ground to obtain dry vegetable powder. The level of Cl^{-} ions was analysed using a chloride analyser (Model 926; Sherwood Scientific Ltd., Cambridge, UK) in an aqueous extract obtained by mixing 100 mg dry vegetable powder with 40 ml of water followed by shaking for 30 min and filtering. The amounts of B^{3+}, Ca^{2+}, K^{+}, Mg^{2+}, Na^{+} and S ions were determined in a digestion extract of 100 mg of tissue powder with 50 ml of a mix of HNO_3:$HClO_4$ (2:1, v/v) using an inductively coupled plasma optical emission spectrometer (ICP-OES, IRIS intrepid II XDL, Thermo Fisher Scientific Inc., Loughborough, UK).

The absorption rate by the root system (J) of Na^{+}, Cl^{-}, B^{3+}, K^{+}, Ca^{2+}, Mg^{2+} and S ions was calculated considering the total ion content of five plants per treatment, expressed as the mmol of each ion and the mean root weight, using the following formula described by Pitman (1975):

Table 2
Influence of four irrigation treatments at the end of stress period and after recovery period on the growth of *E. myrtifolia*. Leaf DW, stem DW, shoot DW, root DW and total DW are given in (g $plant^{-1}$) and leaf area is given in (cm^2). RW3 treatment was not analysed at the end of the recovery period because these plants died during the relief period.

Growth parameters	Stress period					Recovery period			
	Control	RW1	RW2	RW3	F_{We}	Control	RW1	RW2	F_{We}
Leaf DW	4.05 ± 0.46b	2.61 ± 0.35 ab	2.24 ± 0.41 ab	1.25 ± 0.35a	6.71[a]	6.79 ± 0.75b	6.10 ± 0.28b	2.20 ± 0.84a	9.21[a]
Stem DW	1.70 ± 0.15b	1.37 ± 0.17 ab	1.52 ± 0.29 ab	1.15 ± 0.15a	3.52[a]	2.79 ± 0.29b	2.02 ± 0.18 ab	1.41 ± 0.21a	6.77[a]
Shoot DW	5.75 ± 0.61b	3.98 ± 0.45 ab	3.76 ± 0.68 ab	2.40 ± 0.41a	6.06[a]	9.58 ± 1.04b	8.12 ± 0.44b	3.62 ± 1.05a	8.52[a]
Root DW	1.88 ± 0.18a	2.11 ± 0.23a	1.65 ± 0.14a	1.51 ± 0.21a	1.28 ns	1.95 ± 0.24a	2.38 ± 0.46a	1.60 ± 0.58	0.53 ns
Total DW	7.63 ± 0.74b	6.09 ± 0.64 ab	5.41 ± 0.74 ab	3.91 ± 0.62a	4.33[a]	11.53 ± 1.21b	10.50 ± 0.70 ab	5.22 ± 1.62a	4.80[a]
Shoot/Root	3.09 ± 0.25b	1.90 ± 0.14 ab	2.29 ± 0.36 ab	1.58 ± 0.10a	9.59[b]	5.05 ± 0.64b	3.81 ± 0.79 ab	3.14 ± 0.53a	7.35[a]
Leaf area	1031.3 ± 169.1b	644.8 ± 80.9b	465.4 ± 31.2 ab	204.8 ± 50.4a	11.18[c]	1516.1 ± 205.3b	1291.7 ± 84.7b	358.9 ± 120.3a	20.63[b]
Number of leaves	293 ± 31b	250 ± 24b	215 ± 19 ab	113 ± 24a	7.15[b]	457 ± 60b	440 ± 11b	178 ± 59a	8.29[a]

Data are means of 5 calculations ± standard error (SE). Different letters in the same row denote significant difference according to Lincon's Multiple Comparisons Robust Test (p = 0.05). FWe: statistical values (welch approximation) from one-way Robust ANOVA for the different parameters analysed.
Non-significant differences are indicated by "ns".
[a] F_{We} values were significant at 95% levels of probability.
[b] F_{We} values were significant at 99% levels of probability.
[c] F_{We} values were significant at 99.9% levels of probability.

Table 3
Effect of four irrigation treatments on different substrate parameters at the end of the stress period in *E. myrtifolia* plants.

Parameters	Control	RW1	RW2	RW3	F_{We}
EC (dS $m^{-1)}$	10.06 ± 0.57a	12.48 ± 0.54a	21.54 ± 1.66b	27.45 ± 0.79b	99.14[a]
Na^+ (mmol kg^{-1} DW)	34.57 ± 1.16a	60.73 ± 2.34b	119.34 ± 8.56c	163.34 ± 3.58d	343..85[a]
Cl^- (mmol kg^{-1} DW)	56.53 ± 2.98a	85.50 ± 4.43b	159.81 ± 14.03c	239.07 ± 6.68d	178.04[a]
Ca^{2+} (mmol kg^{-1} DW)	12.63 ± 0.99a	9.15 ± 0.64a	18.65 ± 1.71b	19.47 ± 1.33b	18.27[a]
Mg^{2+}(mmol kg^{-1} DW)	10.26 ± 0.87a	9.42 ± 0.63a	23.61 ± 1.71b	22.86 ± 0.98b	50.19[a]

Data are means of 5 calculations ± standard error (SE). Different letters in the same row denote significant difference according to Lincon's Multiple Comparisons Robust Test ($p = 0.05$). F_{We}: statistical values (welch approximation) from one-way Robust ANOVA for the different parameters analysed.
[a] F_{We} values were significant at 99.9% levels of probability.

Table 4
Effect of different irrigation treatments at the end of stress period, on TBARS and some antioxidant enzymes on *E. myrtifolia* leaves.

Antioxidative metabolism	Control	RW1	RW2	RW3	F_{We}
LP-TBARS (nmol min^{-1} g^{-1} FW)	3.63 ± 0.15a	4.42 ± 0.40a	4.38 ± 0.51a	7.60 ± 0.79b	16.35[c]
CAT (μmol min^{-1} g^{-1} FW)	22,2 ± 1,9a	31,3 ± 2,2 ab	33,3 ± 4,8 ab	36,6 ± 2,1b	8.37[b]
APX (nmol min^{-1} g^{-1} FW)	111,9 ± 11,8b	119,9 ± 7,3b	76,9 ± 9,4a	72,5 ± 8,4a	19.32[c]
MDHAR (nmol min^{-1} g^{-1} FW)	11,5 ± 0,6d	9,4 ± 0,4c	5,4 ± 0,3b	3,7 ± 0,2a	20.05[c]
GR (nmol min^{-1} g^{-1} FW)	3,9 ± 0,3c	3,7 ± 0,2c	1,4 ± 0,1b	0,4 ± 0,1a	23.82[c]
SOD (U g^{-1} FW)	9,7 ± 1,8a	10,3 ± 2,1 ab	13,1 ± 2,5 ab	13,8 ± 2,76b	5.88[a]
POX (μmol min^{-1} g^{-1} FW)	23,01 ± 2,3a	18,4 ± 3,4a	43,7 ± 8,6b	196,2 ± 15,5c	25.57[c]

Data are means of 5 calculations ± standard error (SE). Different letters in the same row denote significant difference according to Lincon's Multiple Comparisons Robust Test ($p = 0.05$). F_{We}: statistical values (welch approximation) from one-way Robust ANOVA for the different parameters analysed.
[a] F_{We} values were significant at 95% levels of probability.
[b] F_{We} values were significant at 99% levels of probability.
[c] F_{We} values were significant at 99.9% levels of probability.

$$J = (M2 - M1)/(WR \times t),$$

where M1 and M2 correspond to a concentration of the different ions expressed as mmol per the total plant at the beginning and at the end of experimental periods (salinity and relief), respectively.

In this formula, t corresponds to the time in days and WR is calculated as (WR2−WR1)/ln (WR2/WR1), with WR1 and WR2 representing the dry weight of the roots at the beginning and at the end of the experimental period, respectively.

2.5. Water status

Root hydraulic conductivity (L_p) was determined at the end of both experimental periods (salinity and relief) in five plants per treatment, according to Ramos and Kauffman (1979). Plants were de-topped and the substrate was carefully washed from the roots, which were submerged in a container of water and placed in a pressure chamber with the cut stump exposed. The air pressure in the chamber was increased at an approximate rate of 0.4 MPa min^{-1} up to a final pressure of 0.8 MPa min^{-1}. A small piece of plastic tubing was fitted to the stump, and the exudate was collected every 5 min and its volume measured. After exudation measurements, the root systems were placed in an oven at 80° C until they reached a constant dry weight. Root hydraulic conductivity was calculated using the following formula:

$$L_p = \frac{J}{P \times W}$$

where L_p is expressed in mg s^{-1} g^{-1} MPa^{-1}, P is the applied hydrostatic pressure (MPa), W is the dry weight of the root system (g) and J is the water flow rate through the entire root system (mg s^{-1}).

Leaf water potential (Ψ_l) was estimated according to Acosta-Motos et al. (2014), using a pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA) in which the leaves were placed within 20 s of collection and pressurised at a rate of 0.02 MPa s^{-1}.

The relative water content of leaves (RWC) was measured according to Barrs (1968). The relative water content (RWC) of a plant tissue is expressed by RWC (%) = [(FW − DW)/(TW − DW)] * 100, where, FW, DW, and TW are the fresh, dry and turgid weight, respectively, of the leaf. The fresh leaf weight corresponds to the weight at the time of sampling; the turgid weight is the weight of the leaves after saturation in distilled water at 4 °C in the dark to avoid weight loss by transpiration and biomass synthesis. Dry weight is the weight of the leaves after placing them in the oven at 80 °C to constant weight.

2.6. Gas exchange

Leaf stomatal conductance (g_s) and leaf photosynthetic rate (P_n) were determined throughout the experiment in the attached leaves of five plants per treatment during the central hours of illumination, using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA). Water-use efficiency (WUE) was calculated based on total DW (g)/ET_a (Kg), and intrinsic water use efficiency (WUE_i) was estimated according to the P_n/g_s ratio. Evapotranspiration (ET) was measured gravimetrically in 30 plants per treatment, based on the difference in weights (weight after irrigation and weight before irrigation), using a balance (Analytical Sartorius, Model 5201; 5.2 kg capacity and 0.01 g accuracy). Accumulated evapotranspiration (ET_a) was obtained throughout the trial.

2.7. Enzyme extraction and catalytic activities

For the enzymatic determinations, plants were sampled at the end of the salinity period. Leaf samples (1 g) were homogenised with an extraction medium (1/3, w/v) containing 50 mM Tris−acetate buffer (pH 6.0); 0.1 mM EDTA; 2 mM cysteine; 1% (w/v) PVP; 1% (w/v) PVPP; and 0.2% (v/v) Triton X-100. All operations were performed at 4° C. For the APX activity, 20 mM of sodium ascorbate was added to the extraction buffer. The extracts were filtered through two layers of nylon cloth and centrifuged at

10 000 g for 15 min. The supernatant fraction was filtered on Sephadex NAP-10 columns (GE Healthcare) equilibrated with the same buffer used for homogenisation and the enzymatic determinations. For the APX activity, 2 mM of sodium ascorbate was added to the equilibration buffer. APX (EC 1.11.1.11), MDHAR (EC 1.6.5.4), GR (EC 1.6.4.2), SOD (EC 1.15.1.1), CAT (EC 1.11.1.6) and POX (EC. 1.11.1.7) were analysed following protocols described in our laboratory (Hernández et al., 2000; Barba-Espín et al., 2011; Faize et al., 2011).

2.8. Oxidative stress parameter

The extent of lipid peroxidation (LP) was estimated at the end of the salinity period by determining the concentration of thiobarbituric acid-reactive substances (TBARS). Briefly, leaf material (400 mg) was homogenised in 1 M perchloric acid solution. The homogenate was centrifuged at 15 000 g for 10 min, and 0.5 ml of the supernatant obtained was added to 1.5 ml 0.5% TBA in 1 M perchloric acid. The mixture was incubated at 90° C in a shaking water bath for 20 min, and the reaction was stopped by placing the reaction tubes in an ice water bath. The samples were then centrifuged at 10 000 g for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of TBARS (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1} (Hernández and Almansa, 2002).

2.9. Statistical analyses of data

In the trial, 30 plants were randomly assigned to each treatment. The data were analysed by one-way ANOVA (specifically was used the Welch's Test Robust Generalization). Treatment means were separated with Lincoln's Multiple Comparisons Robust Test. All statistical analyses were carried out with R software [R version 3.2.2 (2015-08-14)].

3. Results

3.1. Plant growth

Growth parameters measured at the end of the salinity period were not significantly different between the plants irrigated with RW1 and RW2 treatments and the control plants (Table 2). In contrast, when the plants were irrigated with RW3 treatment, almost all the growth parameters studied were negatively affected in the same period. Root DW was the only growth parameter that did not show significant differences among the four treatments studied. At the end of the relief period, no significant differences were observed between RW1 and control plants. However, the RW2 plants showed a significant decrease in biomass production in relation to control plants, and the RW3 plants did not show recovery because they died during the relief period (Table 2).

3.2. Ionic balances and nutritional changes

Regarding the substrate analyses, the RW2 and RW3 treatments showed the highest values in electrical conductivity (EC) compared with the control and RW1 treatments at the end of the salinity period (Table 3). These results correlated with the Na^+ (F_{We} = 343.85***) and Cl^- (F_{We} = 178.04***) concentrations observed among treatments. Finally, in the same period, the RW2 and RW3 treatments produced a significantly greater increase in Ca^{2+} and Mg^{2+} ion concentrations in the substrate than the control and RW1 treatments (Table 3).

RW treatments increased the absorption rate for Na^+ in a concentration-dependent manner. At the end of the salinity period, the Na^+ absorption values increased 1.5-, 2.4- and 2.5-fold in the RW1, RW2 and RW3 treatments, respectively, in relation to the control plants. However, the absorption rate for Na^+ was not significantly different in RW1 plants compared with the control (Fig. 1A). The absorption rate for Cl^- did not display significant differences in RW1 and RW2 plants, whereas a significant increase in RW3 (3.3-fold) was observed (Fig. 1B). Regarding the uptake rate for B^{3+}, a significant increase was produced in RW2 (67%) and RW3 (83%) plants with respect to the control treatment (Fig. 1C). An opposite effect was observed with K^+ ions, whose uptake rate was lower in all RW treatments than in the control plants (Fig. 1D). After the relief period, the absorption rate values for Na^+ were very similar in plants previously irrigated with RW1 and RW2 treatments (Fig. 1E). The response was different, however, in the uptake rate for Cl^-, because no statistical changes were observed between the different treatments (Fig. 1F). Similar tendencies in the same treatments (RW1 and RW2) in B^{3+} and K^+ uptake were too observed at the end of relief period (Fig. 1G and H). The absorption rate for Ca^{2+}, Mg^{2+} and S during the salinity period ("see Appendix S1 in Supporting Information") was higher in RW2 and RW3 plants than in control and RW1 plants. After the relief period ("see Appendix S1 in Supporting Information") the responses were different in RW2 in Ca^{2+} and Mg^{2+} uptakes. A progressive decrease in Ca^{2+} uptake values was observed, whereas no significant difference was noted in Mg^{2+} uptake. Only the S uptake had similar responses in both periods in RW2 treatment.

Concerning the accumulation of the different nutrients in each tissue, at the end of the salinity period, Na^+ had mainly accumulated in leaves, stems and roots of RW2 and RW3 plants (Fig. 2A). The Cl^- concentration was higher in roots from RW2 and RW3 plants than in the other treatments, whereas in leaves and stems, Cl^- only accumulated in RW3 plants (Fig. 2B). After the relief period, Na^+ displayed a significant increase in stems and roots from RW1 and RW2 plants. In leaves, however, Na^+ concentrations only increased significantly in RW2 plants (Fig. 2E). The Cl^- distribution did not show significant changes in leaves and stems after relief period (Fig. 2F). On the other hand, the RW2-treated plants accumulated more Cl^- in the roots than RW1 plants (Fig. 2F). Regarding B^{3+} distribution, a higher mobilisation of this nutrient toward leaves was appreciated in all treatments studied (Fig. 2C). In general, a greater increase in the B^{3+} ion concentration in plants previously subjected to RW3 (during the salinity period) and RW2 (during the relief period) was observed compared with the control plants (Fig. 2C and G). At the end of the salinity period, the K^+ concentration dropped in all tissues from RW-treated plants (Fig. 2D). After the relief period, K^+ levels decreased in the leaves and roots of RW1 plants as well as in the stems of RW2 plants (Fig. 2H). Regarding the Ca^{2+}, Mg^{2+} and S concentrations ("see Appendix S2 in Supporting Information"), a greater accumulation of these nutrients occurred in the RW2 and RW3 treatments at the end of the salinity period in practically all tissues. After the relief period ("see Appendix S2 in Supporting Information"), higher levels of Mg^{2+} and S were observed in roots from the RW2 treatment. However, the Ca^{2+} concentrations decreased in the RW2-treated plants.

3.3. Water relations

The RW applied produced decreases in root hydraulic conductivity (L_p) with absolute values of 0.50, 0.26, 021 and 0.18 mg s^{-1} MPa^{-1} g^{-1} obtained for the control, RW1, RW2 and RW3 treatments, respectively (Fig. 3A). The same tendencies were remarked at the end of the relief period, and an even greater separation was observed between RW treatments (RW1 and RW2

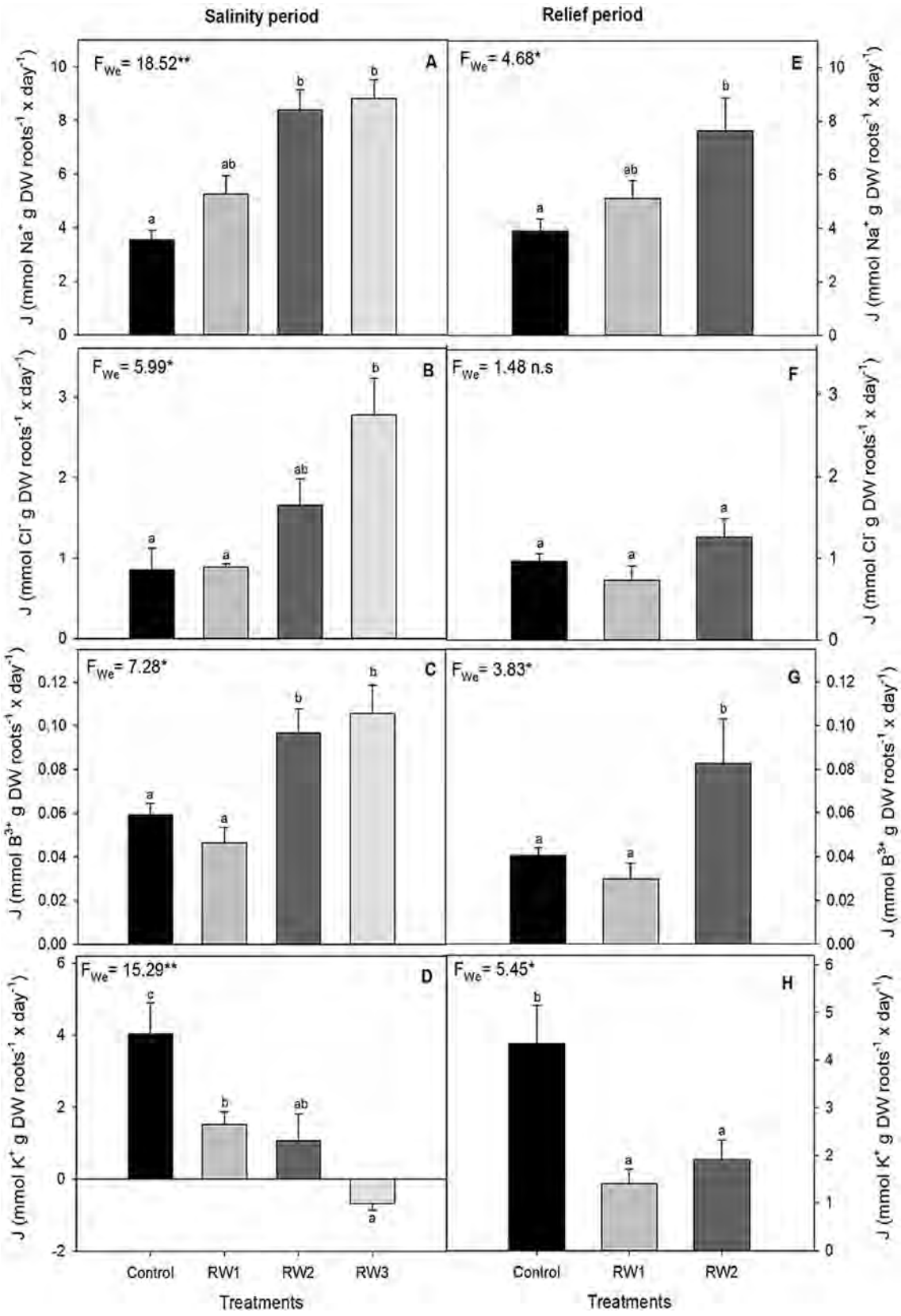

Fig. 1. Effect of the different irrigation treatments on the absorption rates of Na^+ (A and E), Cl^- (B and F), B^{3+} (C and G) and K^+ (D and H) ions in *E. myrtifolia* plants at the end of the salinity period and relief period. Data are the means of 5 calculations ± standard error (SE). Different letters in the same row denote significant difference according to Lincoln's Multiple Comparisons Robust Test ($p = 0.05$). F_{We} values from one-way ANOVA for the different treatments analysed.
*FWe values were significant at 95% levels of probability.
**FWe values were significant at 99% levels of probability.
***FWe values were significant at 99.9% levels of probability.

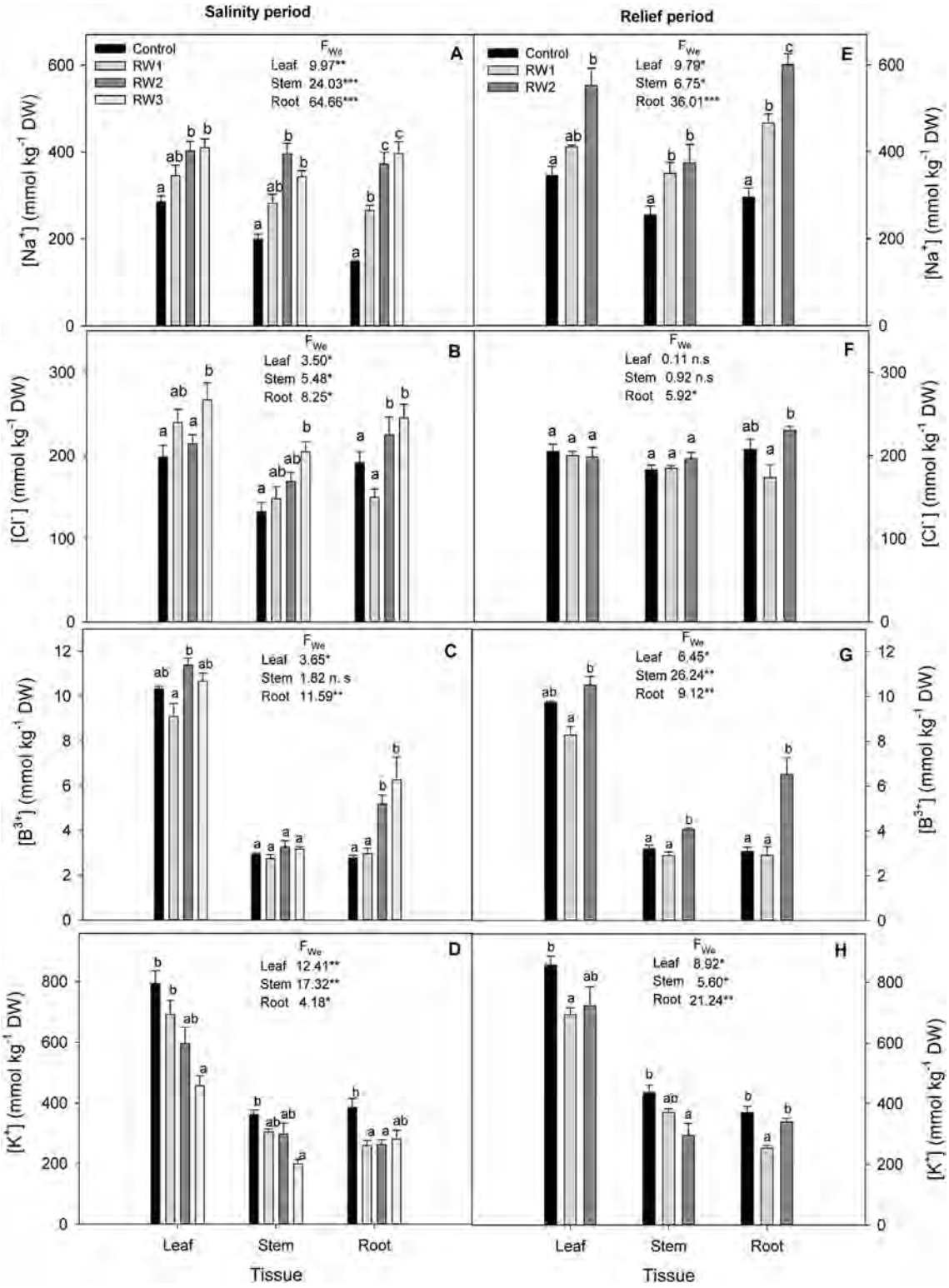

Fig. 2. Concentrations of Na^+ (A and E), Cl^- (B and F), B^{3+} (C and G) and K^+ (D and H) in different tissues of *E. myrtifolia* plants at the end of the salinity period and relief period. Data are the means of 5 calculations ± standard error (SE). Different letters in the same row denote significant difference according to Lincoln's Multiple Comparisons Robust Test ($p = 0.05$). F_We values from one-way ANOVA for the different treatments analysed.

treatments) and control plants (higher F_{We} value = 56.25***) during the relief period (Fig. 3B). WUE, expressed as total DW (g)/ET_a (Kg), responded similarly to the L_p. The differences were only significant, however, for the RW3 treatment (at the end of the salinity period) and the RW2 treatment (at the end of the relief period) compared with the other treatments (Fig. 3C and D).

Leaf water potential (Ψ_l) decreased in parallel with the increase in CE of the RW treatments throughout the salinity period. The Ψ_l

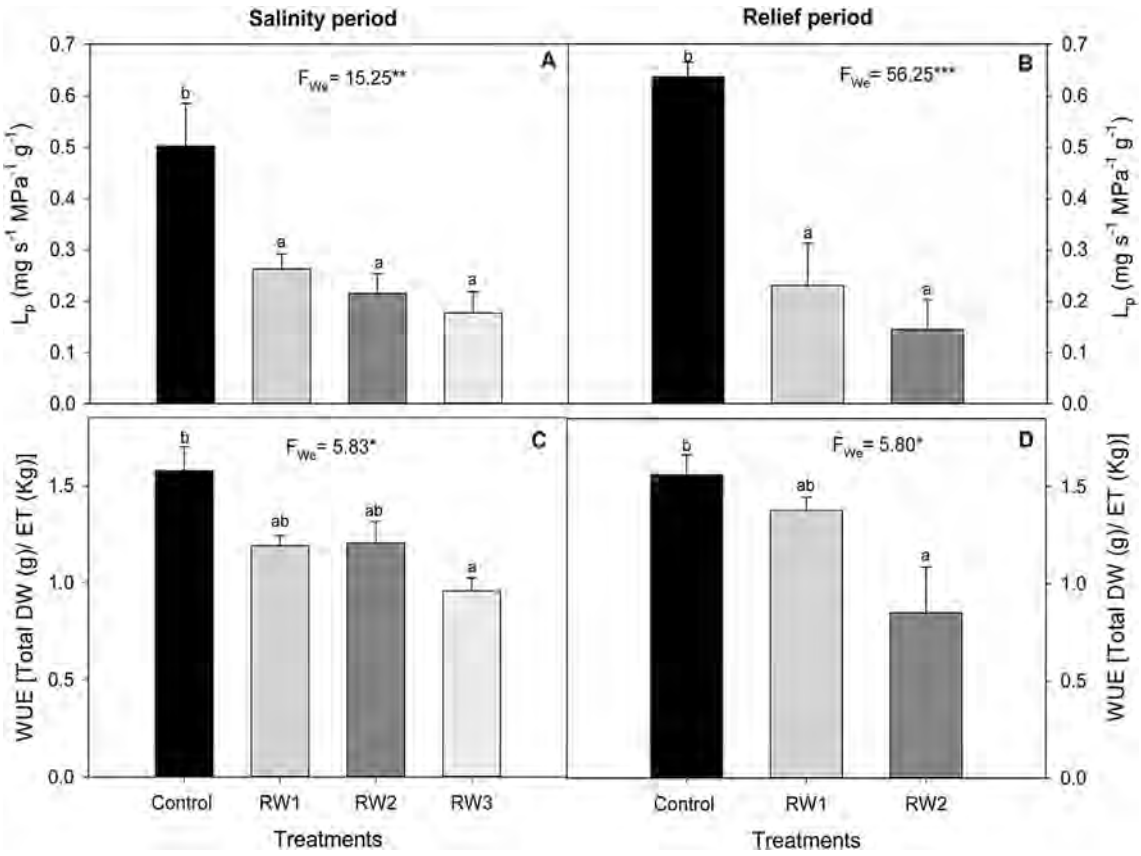

Fig. 3. Influence of different irrigation treatments on root hydraulic conductivity (L_p, A and B) and water-use efficiency based on Total DW/ET_a (WUE, C and D) in *E. myrtifolia* plants at the end of the salinity period and relief period. Data are the means of 5 calculations ± standard error (SE). Different letters in the same row denote significant difference according to Lincoln's Multiple Comparisons Robust Test (p = 0.05). F_{We} values from one-way ANOVA for the different treatments analysed.

values during the relief period were very similar to the Ψ_l values found at the end of the salinity period (Fig. 4B). The response of RW1 plants in this parameter was similar to and even better than control plants throughout the trial (Fig. 4A).

Concerning the evolution of relative water content (RWC), the decrease observed in leaf water potential provoked by salinity was followed by leaf dehydration. This response was more pronounced in the RW2 and RW3 treatments (Fig. 4B). At the end of the relief period, a slight recovery was noted in RW2 plants, and similar values were observed in RW1 plants (Fig. 4B). A very good correlation was observed between leaf water potential and relative water content ($P < 0.001^{***}$; $r = 0.84$) (see Appendix S3 in Supporting Information).

3.4. Gas exchange

The gas exchange parameters: leaf stomatal conductance (g_s) and leaf photosynthetic rate (P_n) were plotted for the RW treatments in relation to control plants (Fig. 4C and D). Regarding g_s values, during the salinity period, RW1 plants, compared with control plants, showed increases of 45%, 22%, 13% and 3% after 7, 13, 18 and 20 weeks of treatment, respectively. Along the relief period, decreases of 11% and 5% were observed at 28 and 31 weeks, respectively (Fig. 4C). In RW2 plants in the salinity period, an increase of 14% was first appreciated in g_s values after 13 weeks of treatment, and then decreases of 33% and 53% were noted at 18 and 20 weeks, respectively (Fig. 4C). During the relief period, RW2 plants showed decreases of 56% and 44% in g_s values at 28 and 31 weeks, respectively. Finally, in RW3 plants, a progressive decrease was observed along the salinity period (8%, 44%, 63% and 78%) (Fig. 4C). Regarding P_n values, a salt concentration-dependent decrease was observed in this parameter at the end of the salinity period (11%, 27% and 36% in RW1, RW2 and RW3, respectively, relative to the control) (Fig. 4D). RW1 plants only showed a surprising increase at week 18. Along the relief period, at 28 weeks, the decline in P_n continued in RW1 (21%) and RW2 (53%) plants, but at the end of relief period, similarly to g_s, a slight improvement in P_n was noted for the same treatments (16% and 46%, respectively) (Fig. 4D). Intrinsic water-use efficiency (WUE_i) based on the P_n/g_s ratio showed important increases related with the severity of the RW treatments at the end of salinity period (Fig. 4E). A remarkable 3-fold increase in WUE_i was observed in RW3 plants. At the end of the relief period, no important changes in this parameter were observed for RW1 and RW2 plants in relation to control plants (Fig. 4E). Moreover, the accumulated evapotranspiration (ET_a), a parameter closely related to g_s, was recorded daily throughout the trial. A progressive decline in ET_a related with the severity of the RW treatments was observed, especially in RW3 and RW2 plants (Fig. 4F). These treatments showed different behaviour in this parameter than the control and RW1 treatments starting from weeks 8 (RW3 treatment) and 15 (RW2 treatment). The response of ET_a in the control and RW1 plants was very similar, although we detected higher absolute values in RW1 than in control plants (Fig. 4F).

A very good correlation was observed between leaf stomatal conductance and accumulated evapotranspiration ($P < 0.001^{***}$; $r = 0.90$) (see Appendix S3 in Supporting Information).

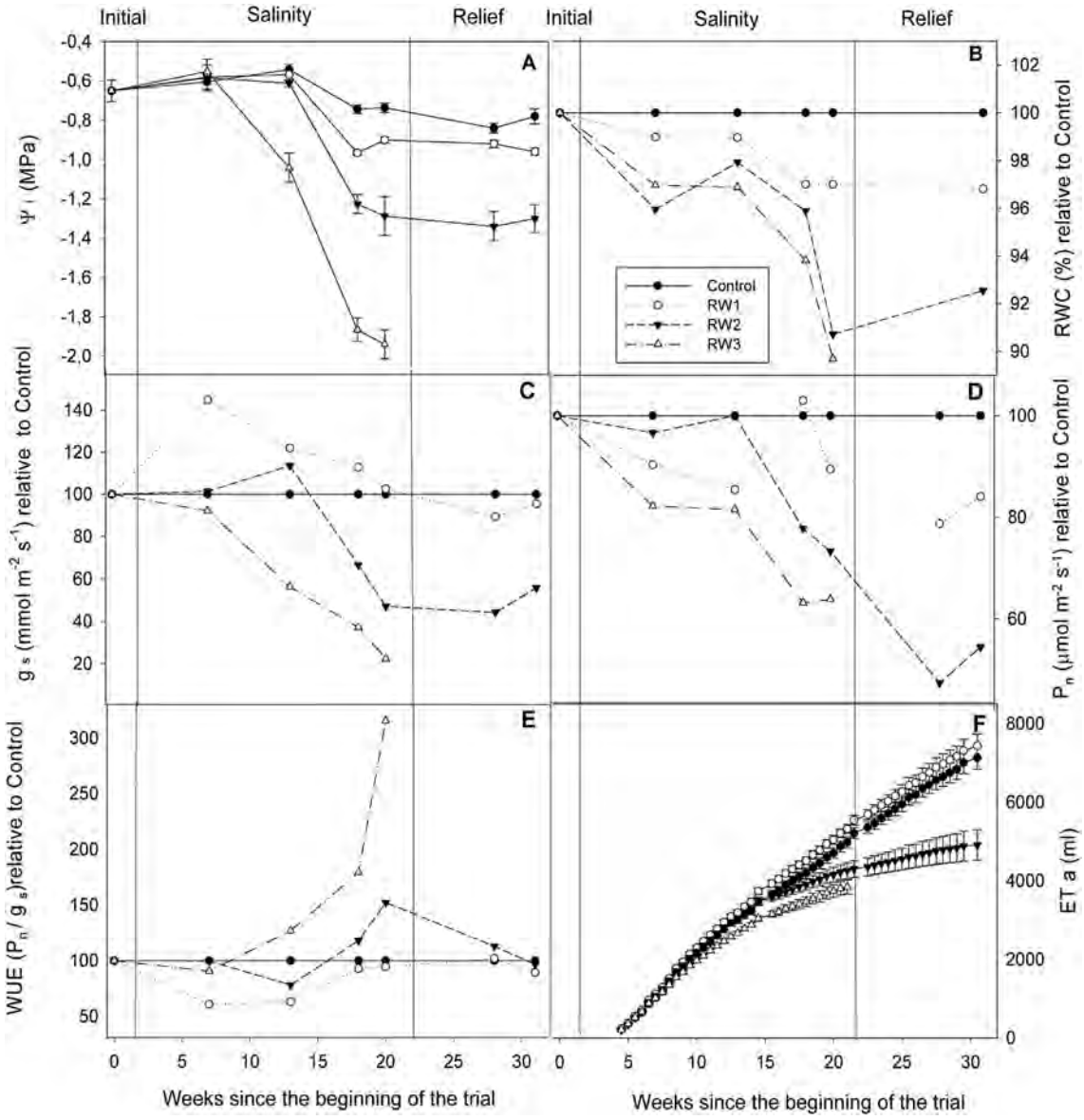

Fig. 4. Influence of different irrigation treatments on, leaf water potential Ψ_l, A), relative water content relative to control (RWC, B), leaf stomatal conductance (g_s, C), net photosynthetic rate relative to control (P_n, D), intrinsic water use efficiency relative to control based on P_n/g_s (WUE_i, E), accumulated evapotranspiration (ET_a; F)) in *E. myrtifolia* plants along of the experimental period. Data are the means of 5 calculations ± standard error (SE).

3.5. Antioxidative metabolism

In general, most of the changes observed in RW1 plants in the different enzyme activities studied were not statistically significant. Only a decrease of 20% was observed in MDHAR activity. The RW2 treatment induced a decrease in all the ASC-GSH cycle enzymatic activities. More specifically, the RW2 treatments induced a decrease in APX, MDHAR and GR activities by 30%, 53% and 3-fold, respectively, compared with control plants (Table 4). The RW3 treatment provoked a massive alteration in the antioxidative metabolism of *E. myrtifolia* leaves. Specifically, the RW3 treatment produced an increase in SOD (42%), CAT (68%) and POX (8.5-fold) activity compared with control plants. Similar to the RW2 treatment, the RW3 treatment induced greater decreases in APX, MDHAR and GR activities than control plants, of 35%, 3-fold and 10-fold, respectively (Table 4).

The RW3 treatment induced oxidative stress as evidenced by the lipid peroxidation (TBARS) values at the end of the salinity period, indicative of membrane damage. This treatment showed a significant 2-fold increase in TBARS compared with the control treatment. In the RW1 and RW2 treatments, however, the changes observed in this parameter were not statistically relevant with respect to the control (Table 4).

4. Discussion

In a context of climate change, which is aggravated in Mediterranean climate regions, the aim of this work was to offer more efficient irrigation systems employing non-conventional water resources. Such systems are necessary today and will continue to be needed in the future for landscaping, revegetation and xeriscaping projects using ornamental plants as a source of plant material. Reclaimed water (RW) is a good example of an alternative water source that can be used when fresh water (FW) is scarce. Plants

cope with the resulting salt stress by implementing different growth, physiological and biochemical mechanisms (Munns and Tester, 2008; Stepien and Johnson, 2009; Acosta-Motos et al., 2015a,b; Rangani et al., 2016; Sazzad-Hossain and Dietz, 2016), which are discussed in the following paragraphs.

4.1. Plant growth

In agricultural crops, the damage associated with salt stress is usually measured as an effect on productivity. However, in ornamental crops used for xeriscape and landscaping projects, the impact of salinity on visual quality and plant ornamental value is more relevant. In a previous work carried out with *E. myrtifolia* plants (Acosta-Motos et al., 2015b), reductions in plant growth were considered as an adaptive avoidance mechanism under short-term salt stress. Nevertheless, in long-term salt stress conditions like those in the present work, decreases in leaf area and in the shoot/root ratio should be considered as a survival avoidance mechanism (Álvarez and Sánchez-Blanco, 2014). The progressive decline in leaf area and the number of leaves limited significant water loss by transpiration. Moreover, a similar decrease in the shoot/root ratio involved greater phytotoxic ion accumulation in roots, limiting the transport of these ions to the aerial parts of the plants (Munns and Tester, 2008; Cassaniti et al., 2009; Acosta Motos et al., 2015a,b). Yet although these responses may still serve as an adaptive mechanism for RW1 and RW2 plants, they did not prevent harmful ion accumulation in the leaves. In the case of the RW3 treatment, plants displayed this survival mechanism to delay death, which ultimately could not be avoided before the end of the relief period. During the relief period, the growth rate of RW2 plants was lower than that of the control and RW1 plants because the osmotic and toxic effects the RW2 plants suffered during the salinity period were not reversible. In general, when salt stress is applied during a long period of time, the improvement in plant growth after the post-salinity period is limited and is related with the recovery of the photosynthetic machinery. Salt accumulation during the post-salinity period could have affected the leaf photosynthetic rate, reducing the leaf area in RW2 plants and leading to leaf senescence. This behaviour has also been observed in others works, as described in Chaves et al. (2009, 2011).

4.2. Ionic balances and nutritional changes

The greater accumulation of Na^+ and especially Cl^- in the substrate related with the salinity treatments provoked an increase of both ions in the roots of RW-treated plants. A lower Cl^- concentration in the root was observed, however, indicating that *Eugenia* plants also developed an avoidance extrusion mechanism for Cl^- ions. If the exposure to salt stress is prolonged in time, the phytotoxic ions will reach the leaves by the end of the salinity period, as occurred in *Eugenia* plants irrigated with RW2 and, especially, RW3 treatments. Plant resistance to salinity can be defined as the ability to either avoid, by means of salt regulation, excessive amounts of salt inside the protoplasm, or, alternatively, to tolerate the toxic and osmotic effects associated with the increased ion concentrations (Larcher, 2003).

High B^{3+} concentration is another problem associated with the use of reclaimed water. Higher B^{3+} levels observed in leaves and roots, particularly in RW3 plants, could also explain the irreversible damage to these plants at the end of the salinity period. Nevertheless, typical damage related to boron was not observed. It is probable that high Na^+ levels interfere with B^{3+} absorption (Lopez-Gómez et al., 2007). Furthermore, the symptoms associated with Na^+ and Cl^- accumulation may mask and/or mitigate typical damage related to B^{3+} excess (Bañón et al., 2012).

On the other hand, it has also been reported that reductions in plant growth are related to a decrease in Ca^{2+} and K^+ concentrations in plant tissues (Valdez-Aguilar et al., 2009). In our experiment, irrigation with the RW2 and RW3 treatments produced a progressive decrease in K^+ in all plant tissues, and a major accumulation in Ca^{2+} was observed at the end of the salinity period. At the end of the relief period, limited Cl^- transport to the aerial part was maintained in RW2 plants. The Na^+, B^{3+} and K^+ ions showed very similar responses in the relief and salinity periods. An opposite response, however, was noted for the Ca^{2+} ion uptake rate and distribution. These responses could partly explain why the observed damage was partially irreversible in RW2 plants after the post-salinity period.

With good irrigation management, the potential benefits provided by other nutrients in the RWs could counteract some of the negative effects caused by the high salinity of these waters. For example, RW2 and RW3 treatments increased the concentrations of Ca^{2+} Mg^{2+}, S and P (data not shown) in the substrate at the end of salinity period. Moreover, a similar accumulation of the same ions (Ca^{2+}, Mg^{2+}, S and P) was observed in leaves and roots of RW2 plants during both experimental periods, which could help mitigate the negative effects of phytotoxic ions. As for RW3 plants, despite the greater accumulation of the same nutrients in all plant tissues at the end of the salinity period, the plants could not avoid the damage provoked by phytotoxic ions and ultimately died.

4.3. Water relations

The changes observed in root hydraulic conductivity (L_P) occurred as a result of the decrease in soil matric potential associated with progressive ion accumulation (toxic or beneficial) in the substrate. The study of this parameter (L_p) allows us to conclude that all plants irrigated with RW had difficulty in water uptake from the substrate, as could be observed at the end of both experimental periods. This response was aggravated by the high salt levels reached in the treatments, especially in RW3. A decrease in the conductivity of the water flow from the substrate to plants subjected to different abiotic stresses has been described in different papers (Navarro et al., 2007; Álvarez and Sánchez-Blanco, 2014, 2015). Nonetheless, the RW1 plants were able to improve their water use efficiency (WUE) by the end of both experimental periods, whereas in RW2 plants, an increase in WUE was only observed at the end of the salinity period. If the water flow in the soil-plant-atmosphere system is affected, the plants must display a plethora of mechanisms to ensure adequate leaf hydration for optimum plant growth and development. In this regard, RWC is a useful parameter, acting as a sensor of leaf water status and providing information about the leaf dehydration level. In response to the observed changes in RWC, leaves can also modify their leaf water potential (Ψ_l). Regarding the evolution of RWC, a progressive decline in Ψ_l was observed along the salinity period, and a similar response was also noted at the end of the relief period. The values observed in Ψ_l can also be used as markers of stress (Taiz et al., 2015). Such low Ψ_l values (−1.94 MPa) were reached in RW3 plants, indicating that this parameter could also be a good sensor for stress severity leading up to plant death (which occurred in RW3 plants after the stress period).

The decrease in Ψ_l and RWC, mainly in RW3 plants, reflected greater difficulties in terms of water mobilisation as a consequence of salt accumulation in the substrate. Despite the availability of water in the soil, salts can favour an osmotic effect near the rooting zone, reducing water mobilisation. This response has been described in other ornamental crops subjected to the same conditions (Slama et al., 2008; Álvarez and Sánchez-Blanco, 2015).

4.4. Gas exchange

In relation to the gas exchange parameters studied, the lowest values in leaf stomatal conductance (g_s) were observed in RW3 plants at the end of salinity period. Such values are related with salinity. Koyro (2006) and Álvarez and Sánchez-Blanco (2014, 2015) suggested that decreases in g_s represent adaptive or resistance mechanisms (according to the severity and/or the time of the stress) to cope with excessive salt levels, reducing the salt load in the leaves and helping increase longevity by maintaining salts at subtoxic levels for longer periods, which would not occur if transpiration rates were not diminished. In addition, changes in the transpiration by stomatal opening can be used by the leaves as a mechanism for cooling (Acosta-Motos et al., 2016; Chaves et al., 2016). Along the experimental period a similar response related with evapotranspiration (ET_a) was observed in RW1 plants, and an inverse response was seen in RW2 and RW3 plants. All these parameters (RWC, Ψ_l, gs and ET_a) act sequentially in an attempt to mobilise water to the leaves in order to reduce water loss by transpiration.

The same responses were also produced in the leaf photosynthetic rate (P_n), negatively affecting plant growth in RW2 and especially in RW3 plants by the end of the salinity period. In the experiment a decrease in P_n could be related with 1) stomatal closure and mesophyll CO_2 conductance (Flexas et al., 2004); 2) concurrent non-stomatal factors; 3) a decline in photosynthetic pigments; and 4) changes in ion accumulations (high concentration of Na^+ and Cl^- ions in roots and leaves, see ionic balances and nutritional changes). Nevertheless, photosynthesis activity remained high in spite of the reduced g_s values. Some anatomical changes have also been observed in mesophilic parenchyma in *Myrtus* and *Eugenia* leaves (Acosta-Motos et al., 2015a,b), improving internal CO_2 conductance. This indicates an increase in intrinsic water use efficiency ($WUE_i = P_n/g_s$ ratio) related with high salinity levels, as has been described in De Pascale et al. (2011), Fernández-García et al. (2014), Acosta-Motos et al. (2015a,b) and Lu et al. (2016). A similar response in WUE_i was observed in our experiment at the end of the salinity period (especially in RW3 plants).

4.5. Antioxidative metabolism

It has been reported that salinity, in addition to its osmotic and toxic effects, also produces oxidative stress at the subcellular level mediated by reactive oxygen species (ROS) (Hernández et al., 1993, 1995, 2001). Salinity affects the antioxidant defences, giving rise to proportional responses to the salt concentrations present in each RW. This explains the major changes that occurred in plants treated with the highest salinity levels (RW2 and RW3). In both cases, we observed a reduction in stomatal conductance (gs) (see the previous section) as a consequence of the increase in salinity. The stomatal closure, in turn, affects the transpiration rate, reducing the salt concentration in leaves but also preventing excessive water loss. Although these effects are good for the plant, they can indirectly affect CO_2 diffusion inside the leaf. As a consequence, a decrease in the amount of CO_2 available for the Rubisco enzyme could produce an increase in photorespiration, draining C2 compounds (glycolate) into the peroxisome, producing H_2O_2 and inducing an increase in catalase (CAT) in this organelle. In addition, poor efficiency in CO_2 fixation could increase the reducing potential inside the chloroplast, producing superoxide radicals ($O_2^{\bullet -}$). The observed increase in superoxide dismutase (SOD) is necessary to dismutate $O_2^{\bullet -}$ to H_2O_2. An increase in SOD and CAT activities was only produced in plants subjected to the RW3 treatment. These data indicate that a significant production of ROS such as $O_2^{\bullet -}$, H_2O_2 and hydroxyl radicals ($^{\bullet}OH$) could be taking a place in these plants, because an induction in these activities may be mediated by an increase in ROS generation (Coego et al., 2005; Xing et al., 2008). Both photorespiration and oxidative stress together thus lead to a dangerous excess in H_2O_2, and new enzymes need to be recruited to detoxify it. The ASC-GSH cycle plays an important role in H_2O_2 removal and the recycling of reduced ascorbate (ASC) and reduced glutathione (GSH) forms (Noctor and Foyer, 1998). Nevertheless, a decrease in ASC-GSH cycle enzymes was observed, especially in RW3 plants. The sensitivity of ascorbate peroxidase (APX) (Class I-peroxidase) against H_2O_2 has been described as APX inactivation by H_2O_2 (Hiner et al., 2000). Consequently, a decline in these enzyme activities under stress conditions can cause an accumulation of H_2O_2 and the oxidised forms of ascorbate and glutathione (DHA and GSSG, respectively). In addition, dehydroascorbate reductase (DHAR) activity (one ASC-recycling enzyme) was not detected in *E. myrtifolia* leaves, which suggests that these plants recycled the ASC through monodehydroascorbate reductase (MDHAR) activity (via [NAD(P)H]). On the other hand, an increase in peroxidase (POX) activity took place in plants treated with RW2 and RW3 treatments. This increase could be the last defence line once H_2O_2 reaches the vacuole and/or cell walls where these enzymes (class III-peroxidases) are located.

Ultimately, however, the induction of SOD, POX and CAT activities in plants irrigated with the RW3 treatment did not appear to be enough to cope with the oxidative stress induced by long-term exposure to salinity. In addition, salt stress also produced permanent oxidative stress, which could be observed by damage to the plant membranes (lipid peroxidation), especially in RW3 plants. Plasma membrane alteration is one of the primary impairments caused by abiotic stresses such as saline or water stress, and such damage is mediated by an increase in ROS generation (Hernández et al., 1993, 1995; Faize et al., 2011). The same results have been described in similar assays under salt stress conditions in different plant species (López-Gómez et al., 2007; Díaz-Vivancos et al., 2013; Acosta-Motos et al., 2014). The perturbation produced in the antioxidant defence system of the plants treated with the highest salinity levels (RW3) resulted in an oxidative stress situation which can explain the damage caused in the membranes that ultimately lead to the death of these plants after the salinity period.

Taken together, *Eugenia* plants irrigated with reclaimed water with low salinity (RW1) showed higher biomass production than plants treated with the other RW treatments, which confirms the effectiveness of using RW1 for irrigation. In contrast, *Eugenia* plants irrigated with RW2 (EC = 4.38 dS m^{-1}) and, especially, with RW3 (EC = 6.96 dS m^{-1}) responded through different morphological, physiological and biochemical mechanisms to adapt to (RW2) or survive (RW3) a situation of salt stress that ultimately had irreversible negative effects on the plants, even after the plants were irrigated with good quality water for an appropriate period of time (Fig. 5).

The parameters shown in Fig. 4 allow us to establish a threshold for the use of the different RWs. All the studied parameters show that RW3 provoked significant stress after 18 weeks of treatments, leading to the death of Eugenia plants (Fig. 5). This effect can be observed with RW2 after 20 weeks of treatment, when some plant deaths also occurred. However, plants treated with RW1 showed similar responses to control plants in the different parameters. Given our results, we thus recommend that nurseries use reclaimed water with an EC no higher than 4 dS m^{-1} for the long-term irrigation of Eugenia plants without drainage (RW1, EC = 2.97 dS m^{-1}). When RWs with higher ECs are used (RW2 and RW3), the visual aspects of the plant and plant growth are irreversibly negatively affected, despite the application of a relief period.

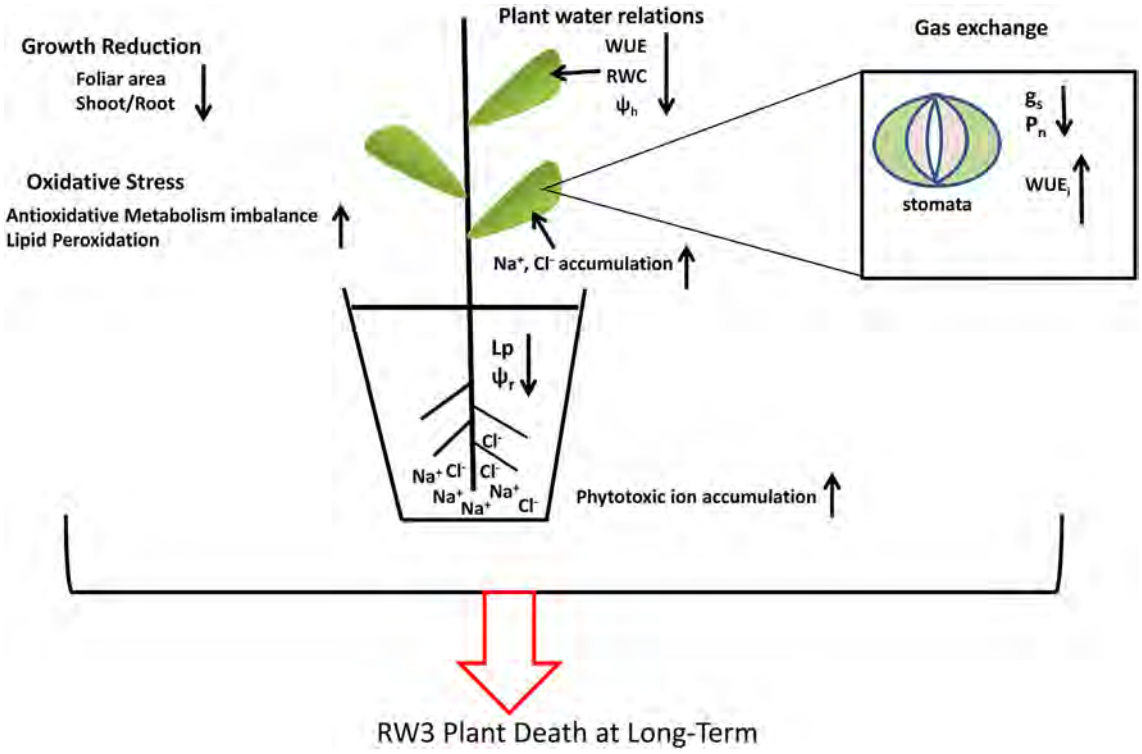

Fig. 5. Schema showing the effect of long-term salt stress (23 weeks) and the plethora of mechanisms developed by *Eugenia myrtifolia* plants irrigated with RW3 treatment in response to such stress. The RW3 plants died during the relief period (9 weeks).

Contributions

JR Acosta-Motos: performed the experiment, carried out statistical analysis and was involved in data interpretation and manuscript writing. JA Hernández: performed the experiment, carried out statistical analysis and was involved in data interpretation and manuscript writing. S. Álvarez: performed experiments related with plant water relations and gas exchange. G Barba-Espín: Involved on the antioxidative enzyme measurements. M.J. Sánchez-Blanco provided plant material and facilities for the experiments and was involved in data interpretation and manuscript writing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.12.003.

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