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ORIGINAL ARTICLE

Physiological and biochemical mechanisms of the ornamental *Eugenia myrtifolia* L. plants for coping with NaCl stress and recovery

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Abstract
Main Conclusion **We studied the response of *Eugenia myrtifolia* L. plants, an ornamental shrub native to tropical and subtropical areas, to salt stress in order to facilitate the use of these plants in Mediterranean areas for landscaping. *E. myrtifolia* plants implement a series of adaptations to acclimate to salinity, including morphological, physiological and biochemical changes. Furthermore, the post-recovery period seems to be detected by Eugenia plants as a new stress situation.**

Different physiological and biochemical changes in *Eugenia myrtifolia* L. plants after being subjected to NaCl stress for up to 30 days (Phase I) and after recovery from salinity (Phase II) were studied. Eugenia plants proved to be tolerant to NaCl concentrations between 44 and 88 mM, displaying a series of adaptative mechanisms to cope with salt-stress, including the accumulation of toxic ions in roots. Plants increased their root/shoot ratio and decreased their leaf area, leaf water potential and stomatal conductance in order to limit water loss. In addition, they displayed different strategies to protect the photosynthetic machinery, including the limited accumulation of toxic ions in leaves, increase in chlorophyll content, changes in chlorophyll fluorescence parameters, leaf anatomy and antioxidant defence mechanisms. Anatomical modifications in leaves, including an increase in palisade parenchyma and intercellular spaces and decrease in spongy parenchyma, served to facilitate CO_2 diffusion in a situation of reduced stomatal aperture. Salinity produced oxidative stress in Eugenia plants as evidenced by oxidative stress parameters values and a reduction in APX and ASC levels. Nevertheless, SOD and GSH contents increased. The post-recovery period is detected as a new stress situation, as observed through effects on plant growth and alterations in chlorophyll fluorescence and oxidative stress parameters.

Keywords ASC-GSH cycle · Gas exchange · Leaf anatomy · Oxidative stress · Recovery capacity · Water relations

Abbreviations

APX	Ascorbate peroxidase
ASC	Ascorbate reduced form
DHAR	Dehydroascorbate reductase
GR	Glutathione reductase
GSH	Glutathione reduced form
GSSG	Glutathione oxidised form
MDHAR	Monodehydroascorbate reductase
POX	Peroxidase
SOD	Superoxide dismutase

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Introduction

Mediterranean areas are characterised by limited water availability. Therefore, the use of non-conventional water resources is a common strategy for efficient water management. Saline waters can be an option in irrigation strategies particularly for ornamental shrubs in landscaping (Cassaniti et al. 2009).

Under saline conditions, plants have to activate different physiological and biochemical mechanisms in order to cope with the resulting stress. Such mechanisms include changes in water relations, photosynthesis, respiratory metabolism, the hormonal profile, toxic ion distribution and the antioxidative metabolism response (Hernández et al. 2001; Parida and Das 2005; Álvarez et al. 2012, 2014; Ashraf and Harris 2013). Physiological constraints imposed by salt stress include osmotic stress and ion toxicity, leading to a nutrient imbalance as well as a disruption of the plant's metabolism (Marschner 1995). Furthermore, and as previously reported, salt stress is also manifested as an oxidative stress at the subcellular level (Corpas et al. 1993; Hernández et al. 1995). These three factors mentioned above can all contribute to the negative effects produced by salinity in plants.

Salt-induced reductions in plant growth are associated with decreases in the net photosynthesis rate. It is known that salinity affects the photosynthetic process due to stomatal and non-stomatal limitations, including stomatal closure, a reduction in chlorophyll content, the inhibition of Calvin–Benson cycle enzymes and the degradation of membrane-associated proteins in the photosynthetic apparatus (Parida and Das 2005; Mittal et al. 2012; Shu et al. 2013). Many authors have reported the decrease in net photosynthesis and stomatal conductance resulting from short-term and long-term exposure to salinity. However, the reductions in these parameters have been found to be less marked in salt-tolerant than in salt-sensitive plants (Moradi and Ismail 2007; Duarte et al. 2013). Moreover, salt stress has been shown to produce a decrease in the photochemical quenching parameters in different plant species, suggesting inhibition of PSII electron transport (Moradi and Ismail 2007; Mehta et al. 2010). In addition, salt stress has been observed to produce either increases or reductions in the non-photochemical parameters, depending on the plant species studied (Moradi and Ismail 2007; Ikbal et al. 2014).

A correlation between salt stress tolerance and an improved oxidative stress response has been observed by different authors (Hernández et al. 2001; Moradi and Ismail 2007; Duarte et al. 2013; Gil et al. 2014), although increases in antioxidative enzymatic activities have also been described in some salt-sensitive species (Arbona et al. 2003; Lee et al. 2013). Different authors have reported that NaCl-tolerant plants either induce or show higher constitutive levels of antioxidant defences (Gueta-Dahan et al. 1997; Hernández et al. 2000, 2003; Mittova et al. 2003). In fact, it has been observed that halophytes present a higher antioxidant capacity than glycophytes, suggesting that this may be one of the reasons why halophytes tolerate high salinity levels (Ozgur et al. 2013; Bose et al. 2014; Gil et al. 2014).

The effect of salt stress on crop plants has been extensively studied. However, few authors have focused their attention on the effect of salinity on ornamental shrubs. Saline waters can be an option in irrigation strategies for ornamental shrubs in landscaping and is of particular interest in Mediterranean areas. Yet salinity may affect the aesthetic value of plants, which is a very important aspect when working with ornamental plants (Acosta-Motos et al. 2014a, b).

In this work, we used *E. myrtifolia* plants, an ornamental shrub native to tropical areas in Asia and Oceania and subtropical areas in South America. One of our goals was to study the response of this plant species to NaCl stress with the hypothesis that it would be a good candidate for use in Mediterranean environments for landscaping. The effect of moderate NaCl levels on plant growth and toxic ion distribution in different ornamental plants, including *E. myrtifolia*, has been studied in a previous work (Cassaniti et al. 2009) but no further analyses have been performed.

Based on the working hypothesis, the effect of different NaCl treatments at 15 and 30 days on plant growth, gas exchange, water relation, mineral nutrition, chlorophyll fluorescence, leaf anatomy and antioxidative metabolism in *E. myrtifolia* plants was studied. Furthermore, the relevance of studying the plants' capacity for recovery following salinity relief was also taken into account. Current information regarding the response of plants to recovery from salt stress is scarce, and the physiological mechanisms involved in this recovery process remain poorly understood (Chaves et al. 2011). We have also investigated a possible relationship between Na^+ and Cl^- uptake and partitioning among organs in order to evaluate if the plant response might be related to the retention of these ions in the roots.

Materials and methods

Plant and experimental conditions

Single rooted cuttings (120) of native *Eugenia myrtifolia* L. 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) (Agrosolmen S.L., Lorca (Murcia), Spain). The experiment was conducted in a controlled environment growth chamber set to simulate natural conditions as described in Acosta-Motos et al. (2014b). 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 (08.00–00.00 hours) by cold white fluorescent lamps.

Experimental design and treatments

Once *E. myrtifolia* plants were adapted to chamber conditions, they were exposed for up to 30 days (Phase I) to the following four irrigation treatments. Control plants were watered with a mixture of distilled water and tap water with an electrical conductivity (EC) = 0.3 dS/m. Saline treatments were designed as control treatment plus NaCl added specifically for each treatment: S4 (4 dS/m), S8 (8 dS/m) and S12 (12 dS/m), corresponding to 44, 88 and 132 mM NaCl, respectively. The EC of the different treatments was evaluated with a multirange Cryson-HI8734 electrical conductivity meter (Cryson Instrumnents, S.A., Barcelona, Spain) at the beginning of and throughout the experimental period. Before starting the experimental period, the maximum water holding capacity of the soil was determined for each individual pot and was considered as the weight at field capacity (WFC). 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 time and the severity of the saline treatments. After the stress phase (Phase I), all plants were exposed to a 16-day recovery period (Phase II) in which they were irrigated with the same solution used for the control plants. During the first three days of the recovery period, all plants were exposed to a further irrigation event with leaching with the same solution used for the control plants (a mixture of distilled water and tap water). The leaching fraction reached 10 % (v/v) of the water applied in the control treatments, 27 % of the water applied in S4 treatments, 50 % of the water applied in S8 treatments, and 72 % of the water applied in S12 treatments, respectively.

Growth, inorganic solutes and ionic absorption rate determinations

At the beginning and end of the salinity period (Phase I) and during the recovery period (Phase II), the soil was gently washed from the roots of six plants per treatment and each plant harvested was divided into shoots (leaves and stem) and roots, and the different organs were washed with distilled water. The leaf fresh weights (FW) and leaf relative water content were measured. Then, leaves, stems and roots were oven-dried at 80 °C until they reached a constant weight in order to measure their respective dry weights (DW). Leaf areas (cm^2) were determined for the same plants before drying using a leaf area meter (AM 200; ADC BioScientific Ltd., Hoddesdon, UK).

At the beginning and end of Phase I and during Phase II the same plants used for growth measurements were also used to determine the inorganic solutes and ionic absorption rate. Plant material that had been previously oven-dried at 80 °C until it reached a constant weight, was ground to obtain dry vegetable powder. The concentrations of Cl^- were analysed by a chloride analyser (Chloride Analyser Model 926, Sherwood Scientific Ltd.) in the aqueous extracts obtained by mixing 100 mg of dry vegetable powder with 40 mL of water before shaking for 30 min and filtering. The concentrations of Na^+, K^+, and Ca^{2+} ions were determined in a digestion extract with HNO_3:$HClO_4$ (2:1, v/v) by inductively coupled plasma optical emission spectrometer (ICPOES IRIS INTREPID II XDL). The absorption rate of Cl^-, Na^+, K^+, and Ca^{2+} (J) by the root system was calculated considering the total salt content, expressed as mmol of Cl^-, Na^+, K^+, and Ca^{2+} and the mean root weight, using the formula described by Pitman (1975):

$$J = (M2 - M1)/(\mathrm{WR} \times t)$$

where $M1$ and $M2$ correspond to a concentration of Cl^-, Na^+, K^+, and Ca^{2+} in mmol in the total plant at the beginning, at the end of the salinity (Phase I) and at the end of the recovery periods (Phase II), respectively. In this formula, t corresponds to the time in days and WR is the logarithmic mean root biomass, calculated as (WR2−WR1)/Ln (WR2/WR1), with WR1 and WR2 representing the dry weight of roots at the beginning and at the end of Phase I or at the end of Phase II, respectively.

Plant water measurements and gas exchange

The soil water potential at the root surface (Ψ_r), leaf water potential (Ψ_l), leaf osmotic potential (Ψ_s), leaf turgor potential (Ψ_t), and leaf osmotic potential at full turgor (Ψ_{100s}) were estimated in six plants per treatment during the central hours of illumination at middle (15 days) and end of Phase I and once Phase II was finished.

The soil water potential was estimated using the method described by Jones (1983), which assumes that $\Psi_r = 0$ for control plants. To calculate Ψ_r for NaCl treatments we used the following equation:

$$\Psi_r = \Psi_{NaCl} - (\Psi_C \times gs_{NaCl})/gs_C$$

where Ψ_C and Ψ_{NaCl} correspond to the mean value of leaf water potential in the control and NaCl treatments, respectively, while gs_C and gs_{NaCl} correspond to the mean value of stomatal conductance in the respective treatments. Leaf water potential was estimated using a pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA) in which leaves were placed in the chamber within 20 s of collection and pressurised at a rate of 0.02 MPa s^{-1}. Leaves from the Ψ_l measurements were frozen in liquid nitrogen (−196 °C) and stored at −30 °C. After thawing, the osmotic potential (Ψ_s) was measured in the extracted sap using a WESCOR 5520 vapour pressure osmometer (Wescor Inc., Logan, UT, USA). Ψ_t was estimated as the difference between leaf water potential (Ψ_l) and leaf osmotic potential (Ψ_s). Leaf osmotic potential at full turgor (Ψ_{100s}) was estimated as indicated above for Ψ_s, using excised leaves with their petioles placed in distilled water overnight to reach full saturation.

The contribution of ions to total $\Psi_{100scalculated}$ was calculated according to Munns and Weir (1981). From the relative dry weight (RDW, kg m^{-3}) (dry weight/leaf water content), the solute concentration on a dry-weight basis (C, g kg^{-1}), the molecular weight of each solute (M, g mol^{-1}) and the vańt Hoff relation (using a RT value for 25 °C of 0.002479 m^3 MPa mol^{-1}, Nobel 1983) for six plants per treatment. It is assumed that ions behaved as ideal osmotic:

$$\Psi_{100scalculated} = -0.002479 \times RDW \times C \times 1/M$$

The proline in leaf samples was analysed at middle (15 days) and end of Phase I and once Phase II was finished as described in Pérez-Clemente et al. (2012). Briefly, 0.1 g of frozen plant tissue (leaves) was homogenized with 5 mL of 3 % sulfosalicylic acid using a tissue homogenizer (Ultra-Turrax). After extraction, homogenates were centrifuged to pellet cell debris at 4 °C at 12,000*g* for 10 min and 1 mL aliquot of the supernatant was combined with an equal volume of glacial acetic acid and ninhydrin reagent. This mixture was boiled in a water bath for 1 h and then cooled in an ice bath (at least 5 min). The solution was partitioned against 2 mL of toluene and absorbance at 520 nm measured in this organic layer. A calibration curve was performed using commercial proline as a standard.

Evapotranspiration (ET) was measured gravimetrically during Phase I in 30 plants per treatment, based on the difference in weights (weight after irrigation and weight before irrigation again), using a balance (Analytical Sartorius, Model 5201; capacity 5.2 kg and accuracy of 0.01 g).

Leaf stomatal conductance (gs) and leaf photosynthetic rate (P_N) in attached leaves in six plants per treatment during the central hours of illumination were determined at middle (15 days) and end of Phase I and once Phase II was finished using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA). Intrinsic water-use efficiency (WUE) was calculated based on the P_N/gs balance registered. For leaf chlorophyll determination, 30 mg of fresh leaves from the central region, avoiding the main vein, were used. Leaf samples were incubated in 3 mL of *N*,*N*-dimethylformamide in darkness at least for 72 h. The absorbance was read at 645 nm and 664.5 nm with a Thermo Spectronic (model Helios alpha, UVA No. 092009) and used to calculate chlorophyll content (mg g^{-1} FW) according to Romero-Trigueros et al. (2014).

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured in detached leaves from control and salt-treated Eugenia plants with a chlorophyll fluorimeter (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany). After a dark incubation period (20 min), the minimum and the maximal fluorescence yields of the plants were monitored. Kinetic analyses were carried out with actinic light (81 µmol quanta m^{-2} s^{-1} PAR) and repeated pulses of saturating light at 2700 µmol quanta m^{-2} s^{-1} PAR for 0.8 s at intervals of 20 s. The following parameters were also analysed: effective PSII quantum yield [*Y*(II)]; the quantum yield of regulated energy dissipation [*Y*(NPQ)]; the non-photochemical quenching (NPQ); the maximal PSII quantum yield (F_v/F_m); the coefficients of non-photochemical quenching (qN); and the photochemical quenching (qP) (Maxwell and Johnson 2000).

Enzyme extraction and analysis

All operations were performed at 4 °C. For the enzymatic determinations, plants were sampled at 15 and 30 days of stress and after 16 days of recovery.

Leaf samples (1 g) were homogenized 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. 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 used for the enzymatic determinations. For the APX activity, 2 mM of sodium ascorbate was added to the equilibration buffer. APX (EC 1.11.1.11) was determined at 290 nm following the ASC oxidation by H_2O_2 (Hossain and Asada 1984). MDHAR (EC 1.6.5.4) was assayed by the decrease at 340 nm due to the NADH oxidation (Arrigoni et al. 1981).

Monodehydroascorbate was generated by the ascorbate/ascorbate oxidase system (Arrigoni et al. 1981). To determine the MDHAR activity, the rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the initial monodehydroascorbate-dependent NADH oxidation rate (with ascorbate and ascorbate oxidase). DHAR (EC 1.8.5.1) was determined by following the increase at 265 nm due to ascorbate formation (Dalton et al. 1993). The reaction rate was corrected for the nonenzymatic reduction of DHA by GSH. GR (EC 1.6.4.2) was assayed by the decrease at 340 nm to the NADPH oxidation, as described by Edwards et al. (1990). The reaction rate was corrected for the small, nonenzymatic oxidation of NADPH by GSSH. SOD (EC 1.15.1.1) was assayed by the ferricytochrome c method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich 1969). CAT (EC 1.11.1.6) was measured following the decrease of absorbance of H_2O_2 at 240 nm (Aebi 1984). POX activity (EC. 1.11.1.7) was analysed following the oxidation of 4-methoxy-α-naphtol at 593 nm according to Ros-Barceló (1998).

Oxidative stress parameters

The rate of passive electrolyte leakage from stress-sensitive plant tissue can be used as a measure of alterations in membrane permeability. Ion leakage was estimated at 15 and 30 days in Phase I and at the end of Phase II. Leaf discs (2 mm diameter) were incubated in 10 mL of 0.3 M mannitol in 50-mL plastic centrifuge tubes and the conductivity of the solutions was measured after 24 h with a conductivity-meter (Crison Mod. 524). Tubes containing the mannitol solution and the tissue were weighed and heated to boiling for 5 min. After cooling to room temperature with shaking, deionized water was added to make their initial weight, and the total conductivity was measured after an additional 0.5 h of shaking. Ratios of ion leakage are expressed as percentage of the total conductivity per hour (Acosta-Motos et al. 2014b).

The extent of lipid peroxidation was estimated by determining the concentration of thiobarbituric acid-reactive substances (TBARS). Briefly, leaf material (400 mg) was homogenized 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. Then, the samples were 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).

Ascorbate and glutathione analyses

Leaf samples (four replicates per treatment) were snap-frozen in liquid nitrogen and then ground to a fine powder and extracted in 1 mL of 1 M $HClO_4$. Homogenates were centrifuged at 12,000*g* for 10 min. The supernatant was neutralized with 5 M K_2CO_3 to pH 5.5–6. The homogenate was centrifuged at 12,000*g* for 1 min to remove $KClO_4$. The supernatant obtained was used to determine ascorbate and glutathione content (Diaz-Vivancos et al. 2010). Reduced ascorbate was measured by the change in absorption at 265 nm, where ascorbate was determined via oxidation to DHA in the presence of ascorbate oxidase (Pellny et al. 2009). Glutathione (GSH, GSSG) were analysed using dithio-bis-2- nitrobenzoic acid and glutathione reductase in the presence of NADPH (Pellny et al. 2009).

Light microscopy and morphometrical analysis

Leaves sections (1 × 1 mm from the most recent fully expanded leaves) from the central region of Eugenia leaves, avoiding the main vein, were used for light microscopy. These samples were fixed and postfixed according to Fernández-García et al. (2014). Semi-thin sections (0.5–0.7 µm thick were cut with a Leica EM UC6 ultramicrotome. The sections were stained with 0.5 % toluidine blue, mounted in DPX and observed with a Leica DMR light microscope. For morphometric analysis, 10 different sections from each treatment (3 plants of each treatment), were studied. The percentages of area occupied by palisade parenchyma (PP), spongy parenchyma (SP) and intercellular spaces (IS) in leaves from *E. myrtifolia* plants were measured and expressed as the % of total area using Adobe Photoshop CS4 Extended software.

Statistical analyses of data

In the experiment, 30 plants were randomly attributed to each treatment. The data were analysed by one-way ANOVA using the SPSS 20.0 software (SPSS Inc., 2002) software. Treatment means were separated with Duncan's Multiple Range Test ($P \leq 0.05$).

Results

Effect of NaCl on plant growth

At the end of Phase I, 4 dS/m NaCl (S4) stimulated the foliar area in Eugenia plants, whereas 8 dS/m NaCl (S8)

Table 1 Effect of NaCl on different growth parameters in *E. myrtifolia* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II). Leaf FW, Leaf DW, Leaf Water content, Stem DW and Root DW are given in (g $plant^{-1}$)

Growth parameters	Treatments				F^a
	Control	S4	S8	S12	
Phase I					
Total leaf area (cm^2)	925 ± 44b	1105 ± 63c	775 ± 10b	549 ± 55a	27.14***
Leaf FW	23.28 ± 3.06b	22.27 ± 2.44b	10.94 ± 1.74a	10.23 ± 0.96a	10.32**
Leaf DW	5.05 ± 0.54bc	6.07 ± 0.30c	4.28 ± 0.17b	2.87 ± 0.22a	15.89***
Leaf water content	18.23 ± 2.64b	16.20 ± 2.16b	8.45 ± 1.22a	7.36 ± 10.80a	8.67**
Stem DW	1.61 ± 0.32b	1.70 ± 0.14b	1.33 ± 0.05ab	0.90 ± 0.05a	4.18*
Root DW	3.17 ± 0.56	2.59 ± 0.10	2.34 ± 0.18	2.22 ± 0.09	3.48ns
Root DW/shoot DW	0.47 ± 0.03b	0.33 ± 0.01a	0.48 ± 0.02b	0.59 ± 0.04c	17.41***
Phase II					
Total leaf area (cm^2)	826 ± 67c	1102 ± 39d	637 ± 29b	480 ± 62a	35.00***
Leaf FW	22.99 ± 0.74b	27.67 ± 0.68c	14.59 ± 1.19a	11.73 ± 1.54a	45.22***
Leaf DW	6.39 ± 0.59b	8.98 ± 0.45c	5.38 ± 0.06b	3.46 ± 0.66a	21.30***
Leaf water content	16.59 ± 0.20b	18.69 ± 0.23c	10.22 ± 0.75a	8.27 ± 0.91a	67.37***
Stem DW	2.12 ± 0.14b	2.97 ± 0.23c	1.84 ± 0.10ab	1.27 ± 0.28a	12.42**
Root DW	3.64 ± 0.34b	3.73 ± 0.34b	3.53 ± 0.26b	2.41 ± 0.37a	4.48*
Root DW/shoot DW	0.43 ± 0.05ab	0.31 ± 0.01a	0.51 ± 0.03b	0.52 ± 0.06b	4.86*

Data represent the mean ± SE from six plants. Different letters in the same row indicate significant differences according to Duncan's test ($P \leq 0.05$)

ns non-significant values

F^a values from one-way ANOVA for the different plant growth parameters analysed. *F* values were significant at 99.9 % (***), 99 % (**) or 95 % (*) levels of probability

did not affect the studied growth parameters. In addition, control, S8 and S12 plants lost leaf area between Phases I and II. In general, the highest NaCl levels (S12) induced a significant decrease in biomass production as could be observed by the 40 % reduction in leaf and stem DW (Table 1). Although salt stress produced no statistically significant changes in the root DW, a concentration-dependent decrease in this parameter was observed, leading to an increase in the DW root/DW shoot ratio in plants treated with the highest NaCl level (Table 1).

After the recovery phase (Phase II), plants previously subjected to the S4 treatment displayed the best performance, showing higher values in foliar area as well as in the leaf and stem DW than control plants (Table 1). However, in plants previously irrigated with 8 dS/m NaCl, a reduction in foliar area was observed after the recovery period. The plants subjected to the S12 treatment did not show any signs of recovery, and a decrease of about 40 % was recorded in the growth parameters of these plants in relation to the control (Table 1).

Nutritional changes

Salt stress increased the uptake rate for Cl^- in a concentration-dependent manner. At the end of Phase I, these values increased 2-, 5.3- and 7-fold in S4, S8 and S12 plants, respectively, in relation to control plants (Fig. 1a). The absorption rate for Na^+ did not show statistically significant changes in S4 plants, whereas similar increases were produced in S8 and S12 plants. In contrast, the uptake rate of K^+ by roots significantly decreased in all NaCl-treated plants, whereas an increase in the Ca^{2+} uptake rate was observed in plants irrigated with 8 and 12 dS/m NaCl (Fig. 1a–c).

After Phase II, the uptake rate of Cl^- decreased in Eugenia plants, mainly in plants previously subjected to S8 and S12 treatments, although the values were still much higher than those observed for control plants. No statistically significant changes were observed for the Na^+ absorption rate, whereas the behaviour of K^+ uptake was similar to that observed in Phase I. Finally, similar to Cl^- absorption, Ca^{2+} uptake values decreased in all cases, but the data were higher in plants subjected to salt stress than in control plants (Fig. 1).

Concerning the distribution of the different ions, at the end of Phase I, Cl^- accumulated mainly in roots from S8 to S12 plants, and the Cl^- concentration was more limited in the aerial part of the plants (Fig. 2a). Similarly, Na^+ also accumulated in roots from plants subjected to the S8 and S12 treatments. No important changes were observed in leaves, whereas Na^+ only accumulated in the stems of S12 plants (Fig. 2b). After Phase II, Cl^- and Na^+ levels were

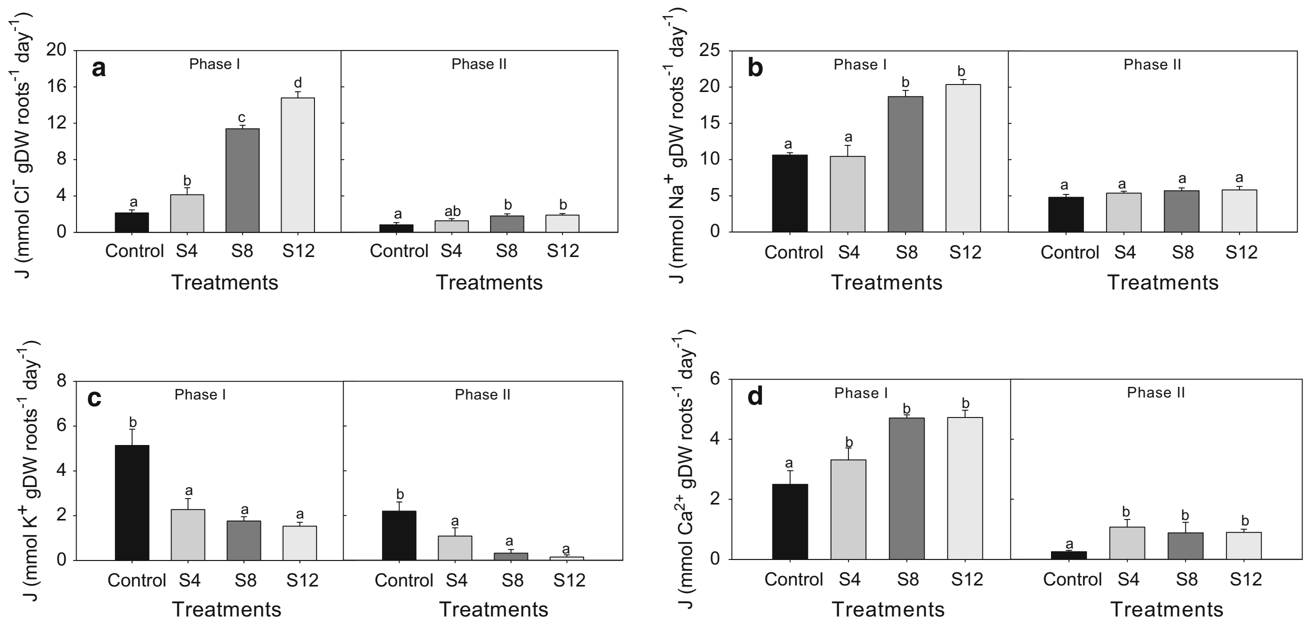

Fig. 1 Effect of increased concentrations of NaCl on the uptake rates of Cl^- **(a)**, Na^+ **(b)**, K^+ **(c)** and Ca^{2+} **(d)** ions in *E. myrtifolia* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II). Data represent the mean ± SE from six plants. Different letters in the same experimental period indicate significant differences according to Duncan's test ($P \leq 0.05$)

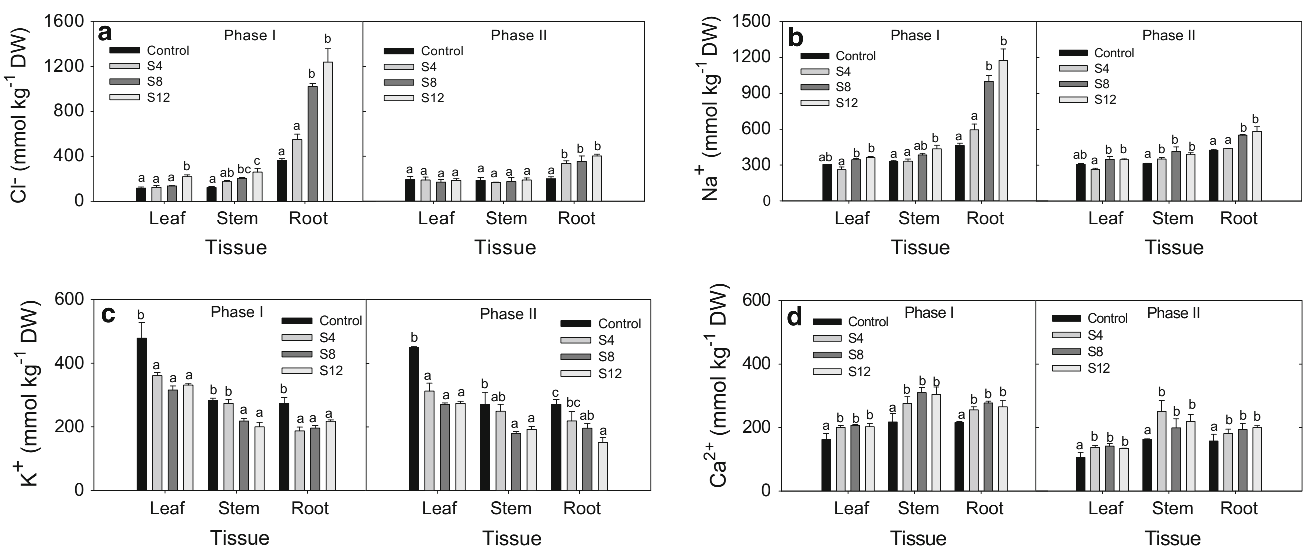

Fig. 2 Concentrations of Cl^- **(a)**, Na^+ **(b)**, K^+ **(c)** and Ca^{2+} **(d)** in different organs of *E. myrtifolia* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II). Data represent the mean ± SE from six plants. Different letters in the same experimental period indicate significant differences according to Duncan's test ($P \leq 0.05$)

much lower than those observed in Phase I. During the recovery period, even though the drainage conditions applied reduced Na^+ and Cl^- uptake, both ions still accumulated in roots. Na^+ concentration also increased in leaves and stems (Fig. 2a, b). At the end of Phase I, K^+ concentration dropped in all parts of NaCl-treated plants (Fig. 2c). After the recovery period, K^+ levels decreased in leaves from S4 plants as well as in all organs from plants previously irrigated with 8 and 12 dS/m (Fig. 2c). A significant increase in Ca^{2+} concentrations was produced in all parts of the plants in both phases of the experiment (Fig. 2d). NaCl had a similar effect on the absolute Na^+ and Cl^- contents as it had on Na^+ and Cl^- concentrations. S8 and S12 plants thus presented both a higher root content and concentration of Na^+ and Cl^- in Phase I (Fig. 2, Suppl. Fig. S1). However, in Phase II, due to drainage, a decrease in Na^+ and Cl^- contents also occurred in roots. In addition, a mobilisation of both toxic ions occurred in the canopy (Suppl. Fig. S1).

Plant water relations

Table 2 shows the effect of NaCl on plant-water relations. During Phase I the soil water potential at the root surface (Ψ_r) decreased in parallel with the severity of the saline treatments (Table 2). However, at the end of the recovery period, these values increased in relation to the data observed in Phase I (Table 2). Leaf water potential (Ψ_l) experienced a progressive decline with the severity of the NaCl treatments. At 15 and 30 days of salt-treatment Eugenia plants presented significantly more negative Ψ_l values than control plants, especially those treated with 8 and 12 dS/m NaCl (Table 2). At the end of Phase II, the Ψ_l values of stressed plants increased but did not reach the control values (Table 2).

Regarding leaf turgor potential (Ψ_t), only at the end of Phase I were significant differences observed among control and S8 and S12 plants (Table 2). Nevertheless, no differences in Ψ_t values were observed at the end of Phase II (Table 2).

During Phase I, the osmotic potential at maximum saturation (Ψ_{100s}) values were more negative in S8 plants, indicating an osmotic adjustment process (Table 2). However, the values were more negative at the end of the recovery period than after Phase I, and all previously stressed plants displayed lower Ψ_{100s} values than control plants (Table 2). At the end of Phase I the contribution of the ions to the level of osmotic adjustment differed with the saline treatment. The importance of Na^+, Cl^- and Ca^{2+} increased with increases in the NaCl level, whereas the importance of K^+ decreased (Supp. Table S1). At the end of Phase II, the contribution of Na^+ and Cl^- to osmotic adjustment was more important than at the end of the saline period (see data in bracket in Suppl. Table S1, see also Ψ_{100s} in Table 2).

In parallel to the water relation parameters, we studied the effect on NaCl on the proline levels during the experiment (Table 2). In general, during Phase I, proline contents were always higher in plants treated with the most severe NaCl treatments At the end of this period, only plants previously subjected to the 8 and 12 dS/m treatments showed higher levels of proline than control plants, as occurred also after Phase II (Table 2).

Gas exchange and chlorophyll fluorescence parameters

Eugenia plants showed unchanged or increased levels of total chlorophyll under saline conditions (Table 3), the effect being more evident in S12 plants at 15 days of stress and in S8 and S12 plants at the end of the recovery phase (Table 3).

Evapotranspiration (ET) was higher in control plants throughout the experimental period, and values fell

Table 2 Effect of increased NaCl levels on soil water potential at the root surface (Ψ_r as MPa), leaf water potential (Ψ_l as MPa), leaf turgor potential (Ψ_t as MPa); leaf osmotic potential at full turgor (Ψ_{100s} as MPa) and proline levels (μmol/g FW) after 15 and 30 days of salt treatment (Phase I) and after the recovery period (Phase II) in *E. myrtifolia* plants

	Ψ_r	Ψ_l	Ψ_t	Ψ_{100s}	Proline
15 days (Phase I)					
Control	0d	−0.58 ± 0.03d	0.64 ± 0.04	−1.27 ± 0.03c	8.27 ± 0.31a
S4	−0.41 ± 0.06c	−0.73 ± 0.02c	0.66 ± 0.09	−1.39 ± 0.01b	9.17 ± 0.40ab
S8	−0.58 ± 0.05b	−0.85 ± 0.05b	0.60 ± 0.11	−1.60 ± 0.03a	9.83 ± 0.30b
S12	−0.79 ± 0.04a	−1.00 ± 0.04a	0.55 ± 0.09	−1.44 ± 0.06b	9.77 ± 0.10b
F^a	52.58***	27.05***	1.37ns	15.41***	5.81*
30 days (Phase I)					
Control	0c	−0.63 ± 0.02d	0.54 ± 0.02c	−1.03 ± 0.05b	7.32 ± 0.24a
S4	−0.22 ± 0.03b	−0.85 ± 0.01c	0.52 ± 0.03c	−1.02 ± 0.03b	7.67 ± 0.28a
S8	−0.62 ± 0.07a	−0.96 ± 0.02b	0.40 ± 0.02b	−1.23 ± 0.07a	9.55 ± 1.02b
S12	−0.70 ± 0.10a	−1.12 ± 0.02a	0.26 ± 0.03a	−1.13 ± 0.07ab	9.95 ± 0.33b
F^a	27.83***	150.91***	29.87***	3.43*	5.42*
Recovery period (Phase II)					
Control	0b	−0.63 ± 0.02b	0.32 ± 0.03	−1.18 ± 0.11b	6.76 ± 0.52a
S4	0.16 ± 0.05b	−0.81 ± 0.02a	0.32 ± 0.02	−1.39 ± 0.01a	7.46 ± 0.55ab
S8	−0.42 ± 0.04a	−0.78 ± 0.03a	0.40 ± 0.01	−1.55 ± 0.01a	8.91 ± 0.24c
S12	−0.43 ± 0.01a	−0.75 ± 0.04a	0.29 ± 0.06	−1.43 ± 0.01a	8.38 ± 0.15bc
F^a	18.75**	7.11**	2.35ns	8.06**	5.48

Data represent the mean ± SE from five plants. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$). For more details, please see Table 1

Table 3 Effect of increased NaCl levels on total chlorophyll content (mg g^{-1} FW), net photosynthetic rate (P_N as μmol m^{-2} s^{-1}); stomatal conductance (gs as mmol m^{-2} s^{-1}); and water use efficiency (WUE as μmol CO_2 mol^{-1} H_2O) after 15 and 30 days of salt treatment (Phase I) and after the recovery period (Phase II) in *E. myrtifolia* plants

	Total chlorophyll	P_N	Gs	WUE
15 days (Phase I)				
Control	1.62 ± 0.07a	6.76 ± 0.75b	57.74 ± 7.07b	123 ± 14a
S4	1.79 ± 0.03ab	6.20 ± 0.77b	39.54 ± 8.30a	169 ± 11b
S8	1.81 ± 0.05ab	5.83 ± 0.21ab	31.33 ± 3.65a	197 ± 17c
S12	2.04 ± 0.10b	4.37 ± 0.22a	23.99 ± 2.62a	189 ± 12bc
F^a	4.19*	3.35*	6.07**	8.82**
30 days (Phase I)				
Control	1.74 ± 0.13	5.88 ± 0.43b	43.60 ± 2.50b	135 ± 6ab
S4	1.69 ± 0.12	5.58 ± 0.39ab	47.62 ± 4.38b	121 ± 12a
S8	2.20 ± 0.06	4.60 ± 0.44a	27.42 ± 2.79a	170 ± 11b
S12	2.07 ± 0.25	4.48 ± 0.15a	31.32 ± 2.81a	148 ± 15ab
F^a	1.87ns	3.66*	9.08**	3.22*
Recovery period (Phase II)				
Control	1.10 ± 0.03a	6.92 ± 0.42a	69.64 ± 11.64a	108 ± 13ab
S4	1.37 ± 0.01ab	9.56 ± 0.19b	120.16 ± 11.52b	82 ± 8a
S8	1.67 ± 0.04bc	6.06 ± 0.76a	45.70 ± 6.37a	139 ± 16b
S12	1.61 ± 0.10c	8.52 ± 0.35ab	61.66 ± 4.50a	142 ± 12b
F^a	14.32**	10.89***	12.57***	4.99*

Data represent the mean ± SE from six plants. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$). For more details, please see Table 1

proportionally with respect to increasing NaCl treatments (Suppl. Fig. S2). At 15 days of salt stress, a NaCl-dependent fall in gs occurred. In this case, the gs values decreased by about 32, 46 and 59 % in S4, S8 and S12 plants, respectively (Table 2). Regarding P_N values, a 35 % decrease occurred in S12 plants (Table 3). The gs decrease produced a rise in WUE values (Table 3). After 30 days of stress, S4 plants appear to have developed an ability to acclimate to the stress conditions, showing similar gs values to control plants (Table 3), whereas S8 and S12 plants showed decreased gs values (Table 3). At the end of Phase I, P_N values only decreased in S8 and S12 plants (Table 3).

At the end of Phase II, gs values slightly increased in all treatments with respect to the values observed after Phase I. For example, gs values increased by up to 70 % in S4 plants in relation to control plants, and, as a consequence, there was a significant increase in P_N as well (Table 3).

After 15 days of NaCl-stress, plants irrigated with 8 and 12 dS/m showed decreased photochemical quenching parameters [qP and *Y*(II)] and increased non-photochemical quenching parameters [qN, NPQ and *Y*(NPQ)]. However, at 30 days of salt-stress, an inverse response took place: the photochemical quenching parameters increased in salt-treated plants, whereas the non-photochemical quenching parameters decreased (Table 4, Suppl. Fig. S1). After Phase II, an alteration in the chlorophyll fluorescence parameters occurred, particularly in plants previously irrigated with 8 and 12 dS/m. In these plants, a decrease in qP as well as in qN and NPQ was recorded (Table 4 and Suppl. Fig. S3).

Anatomical changes

Salt stress induced changes in the leaf anatomy of Eugenia plants, and such changes were most evident in plants irrigated with 8 dS/m NaCl. Accordingly, at the end of Phase I, plants treated with 8 dS/m NaCl showed an increase in the percentage of palisade parenchyma and intercellular spaces but a decrease in spongy parenchyma (Table 5 and Suppl. Fig. S2). Changes produced in S4 and S12 plants were related to an increase in intercellular spaces (Table 5 and Suppl. Fig. S4).

After Phase II, anatomical modifications were observed for all treatments, especially in S4 plants. In these plants, an increase in palisade parenchyma and intercellular spaces as well as a decrease in spongy parenchyma could be observed. Plants previously treated with 8 dS/m maintained increases in the percentage of palisade parenchyma and decreases in spongy parenchyma, and similar changes occurred in S12 plants after the recovery period (Table 5 and Suppl. Fig. S4).

Antioxidative metabolism

The NaCl treatment induced oxidative stress in Eugenia plants as evidenced by electrolyte leakage (EL) and lipid peroxidation (LP), indicative of membrane damage. Such

Table 4 Effect of increased NaCl levels on fluorescence parameters after 15 and 30 days of salt treatment (Phase I) and after the recovery period (Phase II) in *E. myrtifolia* plants

	qP	Y(II)	F_v/F_m	qN	NPQ	Y(NPQ)
15 days (Phase I)						
Control	0.773c	0.473c	0.761b	0.620a	0.273a	0.273a
S4	0.765bc	0.463ab	0.751a	0.639a	0.271a	0.278a
S8	0.754ab	0.469ab	0.765c	0.663b	0.313b	0.302b
S12	0.745a	0.419a	0.758b	0.717c	0.368c	0.344c
F^a	5.54***	19.98***	28.69***	33.44***	36.84***	28.80***
30 days (Phase I)						
Control	0.754a	0.401a	0.744b	0.743c	0.396c	0.365c
S4	0.829d	0.453b	0.705a	0.644b	0.260a	0.276b
S8	0.769b	0.470c	0.764c	0.633b	0.272b	0.275b
S12	0.805c	0.480c	0.741b	0.606a	0.240a	0.252a
F^a	67.71***	56.59***	86.00***	57.34***	82.38***	69.41***
Recovery period (Phase II)						
Control	0.715b	0.295a	0.682b	0.832c	0.522d	0.476c
S4	0.735b	0.291a	0.647a	0.820c	0.461c	0.458c
S8	0.622a	0.299a	0.706c	0.765b	0.404b	0.431b
S12	0.648a	0.343b	0.730d	0.725a	0.367a	0.391a
F^a	15.32***	9.70***	49.87***	61.21***	43.55***	19.57***

Data represent the mean from 50 measurements. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$). For more details, please see Table 1

Table 5 Quantitative analysis for morphometric data in leaves from control and NaCl-treated *E. myrtifolia* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II)

	Treatments				F^a
	Control	S4	S8	S12	
30 days (Phase I)					
Palisade parenchyma (%)	36.92 ± 0.68a	36.16 ± 1.08a	45.71 ± 0.74b	36.31 ± 0.95a	14.64***
Spongy parenchyma (%)	46.57 ± 0.70b	40.93 ± 1.80b	31.41 ± 1.91a	42.92 ± 1.35b	9.83***
Intercellular space (%)	16.34 ± 0.83a	21.94 ± 0.97b	22.89 ± 1.55b	20.78 ± 0.75b	6.41**
Recovery period (Phase II)					
Palisade parenchyma (%)	33.85 ± 0.87a	44.94 ± 0.83c	43.04 ± 0.79c	39.27 ± 1.28b	22.94***
Spongy parenchyma (%)	49.60 ± 1.42d	30.67 ± 1.09a	39.79 ± 1.78b	43.88 ± 0.19c	40.51***
Intercellular space (%)	16.53 ± 0.65a	24.43 ± 0.93b	17.33 ± 1.49a	16.86 ± 0.17a	12.34***

Data represent the mean ± SE 10 different sections from each treatment (three plants of each treatment). Different letters in the same row indicate significant differences according to Duncan's test ($P \leq 0.05$). For more details, please see Table 1

effects were most noticeable in S12 plants. After Phase II, these plants still presented membrane damage as evidenced by an increase in the oxidative stress parameters (Table 6). In the case of S8 plants, although EL data returned to control values, there was nevertheless an increase in LP in relation to control plants (Table 6).

The effect of NaCl on the activity of some antioxidant enzymes was studied in plants subjected to 4 and 8 dS/m NaCl. At 15 days of salt stress, CAT activity increased in NaCl-treated plants, especially in S4 plants. This CAT increase was accompanied by a decrease in APX activity. In addition, a twofold increase in SOD as well as a strong decrease in POX activity was produced in S8 plants (Table 7). At 30 days of NaCl-stress, S4 plants showed an increase in GR and SOD and a drop in APX (Table 7). In S8 plants, we observed increases in MDHAR and SOD but significant decreases in APX and POX activities (Table 7).

At the end of Phase II, CAT activity increased and APX activity reached control values in stressed plants (Table 7). In contrast, MDHAR and GR decreased in S4 plants and SOD increased in both treatments. A general decrease in POX activity was produced in Phase II, but S8 plants displayed a significant increase in this enzymatic activity in relation to control plants (Table 7).

Table 6 Effect of increased NaCl levels on oxidative stress parameters in leaves from *E. myrtifolia* plants. Electrolyte leakage (EL) and lipid peroxidation (TBARS) were analysed at the end of the salinity period (Phase I) and after the recovery period (Phase II)

	EL (%)	TBARS (nmol/g FW)
15 days (Phase I)		
Control	32.70 ± 0.34a	2.87 ± 0.07 a
S4	33.52 ± 0.45a	3.29 ± 0.11ab
S8	38.75 ± 0.69b	3.82 ± 0.26b
S12	39.45 ± 0.44b	4.23 ± 0.38b
F^a	16.49***	6.33*
30 days (Phase I)		
Control	33.65 ± 0.64a	3.20 ± 0.22a
S4	34.33 ± 0.54a	3.52 ± 0.19a
S8	39.66 ± 0.99b	4.12 ± 0.26b
S12	42.11 ± 1.22b	4.51 ± 0.31b
F^a	21.35***	4.92*
Recovery period (Phase II)		
Control	34.07 ± 1.44a	4.87 ± 0.16a
S4	34.35 ± 0.32a	4.45 ± 0.14a
S8	35.26 ± 0.59a	5.78 ± 0.28b
S12	40.48 ± 1.07b	6.05 ± 0.35b
F^a	19.85***	10.85**

Data represent the mean ± SE from 10 plants. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$). For more details, please see Table 1

After 15 days of NaCl treatment, a strong increase in GSH was observed in Eugenia plants. Furthermore, this increase was much higher in S4 plants (fivefold) than in S8 (2.5-fold) plants with respect to the control, but no accumulation of GSSG occurred (Table 8). This response produced an increase in the redox state of GSH. At 30 days of NaCl stress, irrigated S8 plants maintained a significant increase in GSH. At the end of Phase I, an accumulation of GSSG was observed, producing a decrease in the redox state of glutathione in all cases (Table 8). After Phase II, control plants maintained GSH levels, whereas S4 plants displayed duplicate GSH values, and the data were three times higher in S8 plants (Table 8). In this period, GSSG values were much higher in control than in salt-stressed plants, which displayed a higher redox state of glutathione.

No oxidized ascorbate was detected in Eugenia plants under our experimental conditions. At 15 days of NaCl stress, reduced ascorbate (ASC) levels showed no significant differences among the treatments, although values were higher in NaCl-treated plants. However, at 30 days of NaCl irrigation, decreased ASC levels were observed in plants subjected to both saline treatments. After Phase II, ASC content increased dramatically in all treatments. Nevertheless, plants previously subjected to NaCl displayed lower ASC levels (threefold in S4 and fourfold in S8) than control plants (Table 8).

Discussion

Our data suggested that *E. myrtifolia* plants could be used for landscaping projects in Mediterranean areas. This plant species implements a series of adaptations to acclimate to salinity at the physiological level (plant growth, ion accumulation, water relations, gas exchange, chlorophyll fluorescence and anatomical changes), and at the biochemical level (antioxidative metabolism). Furthermore, the post-recovery period seems to be detected by Eugenia plants as a new stress situation, as observed through effects on plant growth and alterations in chlorophyll fluorescence and oxidative stress parameters.

Growth and ion accumulation

Tolerance to salt stress is a complex phenomenon that enables plants to adapt via different physiological and biochemical processes (Stepien and Johnson 2009). One of the most prominent effects of salt stress is the reduction in plant growth (Parida and Das 2005). However, the reduction in leaf area as well as the increase in the root DW/shoot DW ratio can be viewed as adaptive mechanisms to salt stress. The reduction in leaf area produces an indirect benefit, because plants can thus limit water loss by transpiration, which in turn can favour the retention of toxic ions in roots, limiting the accumulation of these ions in the aerial part of the plant (Munns and Tester 2008), as occurred in the most severe NaCl treatments. The ability of plants to control salt concentration in their aerial parts, either by salt accumulation in roots, by reduced salt uptake rates and/or by controlled translocation to leaves, can constitute an important mechanism of plant survival under saline conditions (Colmer et al. 2005; Cassaniti et al. 2009). This was the case of Eugenia plants, which accumulated high concentrations of Na^+ and Cl^- in roots. According to this response, Eugenia plants behaved as tolerant to NaCl concentrations up to 44 and 88 mM, especially if we consider that the saline irrigation treatments applied were carried out without any drainage. Our findings agree with a previous study performed by Cassaniti et al. 2009, who classified Eugenia plants as tolerant up to 70 mM NaCl after 2 months of treatment according to the relative growth rate parameter. After the recovery period, and although the analysed roots were not subjected to "free-space washing", the concentration of root Na^+ and especially Cl^- strongly decreased. Other authors also used the same methodology to study ion content and/or concentration in roots. In these cases, roots were washed to

Table 7 Effect of NaCl on the activity of some antioxidant enzymes in leaves from *E. myrtifolia* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II)

	CAT (µmol min^{-1}/g FW)	APX (nmol min^{-1}/g FW)	MDHAR (nmol min^{-1}/g FW)	GR (nmol min^{-1}/g FW)	SOD (U/g FW)	POX (µmol min^{-1}/g FW)
15 days (Phase I)						
Control	8.1 ± 0.6a	96.1 ± 0.1b	564.4 ± 29.2a	34.8 ± 2.0a	71.4 ± 3.1a	182.3 ± 24.1b
S4	19.6 ± 3.7b	54.2 ± 6.5a	560.1 ± 29.3a	37.5 ± 4.4a	85.1 ± 2.6a	152.9 ± 12.4b
S8	14.8 ± 0.3b	83.1 ± 4.9ab	646.5 ± 35.8a	39.5 ± 8.2a	146.9 ± 12.1b	56.4 ± 5.4a
F^{a}	9.0**	15.4**	2.4ns	0.1ns	13.0**	11.9**
30 days (Phase I)						
Control	13.2 ± 1.1ab	88.0 ± 7.2b	274.9 ± 15.9a	23.1 ± 2.4a	100.9 ± 2.8a	159.0 ± 15.9b
S4	17.1 ± 2.3b	47.1 ± 6.4a	283.0 ± 19.5a	39.6 ± 2.3b	119.0 ± 6.2b	153.1 ± 4.6b
S8	11.0 ± 0.4a	24.4 ± 4.6a	402.0 ± 20.5b	26.7 ± 1.3a	127.3 ± 8.3b	45.6 ± 4.6a
F^{a}	4.1*	20.3***	18.2***	16.2**	5.6*	30.9***
Recovery period (Phase II)						
Control	20.2 ± 1.0a	40.4 ± 3.1a	244.7 ± 21.2b	38.5 ± 6.3b	168.2 ± 7.8a	30.7 ± 1.2a
S4	26.3 ± 054b	42.2 ± 3.7a	188.1 ± 5.5a	20.6 ± 4.4a	232.1 ± 11.1b	31.4 ± 2.9ab
S8	27.8 ± 0.6b	44.7 ± 3.6a	233.6 ± 15.7b	32.0 ± 2.8b	295.8 ± 5.6c	37.9 ± 2.4b
F^{a}	22.1***	3.3*	4.6*	4.19*	41.26***	3.2*

Data represent the mean ± SE from six plants. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$). For more details, please see Table 1

Table 8 Effect of NaCl on the ascorbate and glutathione content in leaves from *E. myrtifolia* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II)

	GSH (nmol/g FW)	GSSG (nmol/g FW)	Redox state [GSH/(GSH + GSSG)]	Ascorbate (µmol/g FW)
15 days (Phase I)				
Control	1.07 ± 0.12a	1.62 ± 0.15a	0.40	6.20 ± 0.09ba
S4	5.35 ± 0.13c	1.55 ± 0.26a	0.79	8.91 ± 0.46b
S8	2.64 ± 0.35b	1.12 ± 0.07a	0.70	7.56 ± 0.40ab
F^{a}	41.89***	3.02ns		6.80*
30 days (Phase I)				
Control	0.83 ± 0.25a	3.58 ± 0.07b	0.20	9.61 ± 0.28b
S4	1.02 ± 0.20a	2.73 ± 0.18a	0.27	6.94 ± 0.20a
S8	2.29 ± 0.26b	2.26 ± 0.21a	0.50	7.50 ± 0.78a
F^{a}	10.31**	15.29**		9.32**
Recovery period (Phase II)				
Control	0.86 ± 0.19a	4.01 ± 0.16b	0.18	48.04 ± 8.31b
S4	2.09 ± 0.28b	2.66 ± 0.34a	0.44	15.83 ± 3.93a
S8	2.73 ± 0.33b	2.55 ± 0.23a	0.52	11.89 ± 1.88a
F^{a}	7.88**	8.00**		12.65**

Data represent the mean ± SE from four plants. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$). For more details, please see Table 1

remove surface ions (Cassaniti et al. 2009; Álvarez et al. 2012; Acosta-Motos et al. 2014b).

One of the risks of growing plants in small containers under salt stress conditions is the accumulation of Na^{+} and Cl^{-} ions in the substrate, which can bring about an excessive accumulation of toxic ions in all parts of the plant (Álvarez et al. 2012). In addition, salt stress produced an increase in Ca^{2+} in the different parts of the Eugenia plants. The increase in Ca^{2+} concentrations in response to salinity has been reported in other plant species such as *Vicia faba* L. and *Myrtus communis* L. (Gadallah 1999; Acosta-Motos et al. 2014b). Although Ca^{2+} concentrations increased in Eugenia roots by effect of saline stress, an increase in Na^{+}/Ca^{2+} ratio occurred that could induce an increase in membrane permeability, favouring passive Cl^- and Na^+ transport inside the roots (Greenway and Munns 1980). In contrast, and despite the fact that salt stress reduces K^+ concentrations in all parts of the plants, this decrease was about 30 %. The observed increase in Ca^{2+} along with the limited decline in K^+ can be considered important in the response of Eugenia plants to salinity conditions in view of the importance of both nutrients in plant growth and development. As well as, in the stomatal response, cellular turgor, cell wall and membrane stability, enzyme activation and cell signalling (Marschner 1995; Osakabe et al. 2014).

Plant water relations

The decrease in water potential in NaCl-treated plants can reflect an adaptation in water uptake during the beginning of the stress period as a result of the greater accumulation of salts in the substrate (Álvarez et al. 2012). Such accumulation was more evident in the S8 and S12 treatments. Despite the availability of water in the substrate, salts can promote an osmotic effect in the soil, limiting water uptake (Hardikar and Pandey 2008). This behaviour has been observed in other ornamental species grown under the same conditions (Koyro 2006; Acosta-Motos et al. 2014b). As a response to this osmotic effect, a reduction in evapotranspiration and stomatal conductance occurred during the stress period, acting as a mechanism to prevent excessive loss of water (Munns and Tester 2008), particularly in the plants subjected to the highest saline concentrations. Ψ_r data reflected the accumulation of toxic ions on the root surface and may have direct effects on the reduction of Ψ_l in order to guarantee water transport to the leaves.

The contribution of the ions to osmotic adjustment was different, but the contribution of Na^+ and Cl^- was the most important in NaCl-treated plants. This adjustment by toxic ion accumulation can be positive only if plants have the ability to compartmentalise the ions (Alarcón et al. 1999; Koyro 2006). This response has also been described in other ornamental plants subjected to salt stress (Sánchez-Blanco et al. 1998; Navarro et al. 2008).

However, a role for proline in osmotic adjustment, although limited, cannot be ruled out. It has been described that proline can act as an osmoprotectant as well as an antioxidant molecule, protecting different macromolecules during dehydration and reducing power storage (Ashraf and Foolad 2007; Planchet et al. 2014).

Gas exchange and chlorophyll fluorescence

As mentioned above, the aerial parts of the Eugenia plants studied were reduced, but chlorophyll levels on the other hand increased as a strategy to protect the photosynthetic machinery. It is known that salt-tolerant species show increased or unchanged chlorophyll content under saline conditions but that chlorophyll levels decrease in salt-sensitive species, suggesting this parameter as a biochemical marker of salt tolerance in plants (Stepien and Johnson 2009; Ashraf and Harris 2013).

At 15 days of stress, an increase in WUE was observed, mainly due to decreased gs values. However, at longer-term (30 days of stress) S4 plants appeared to adapt to the salinity conditions. Decreases in gs during the stress period can be also considered as an adaptative mechanism of salt tolerance (Flowers and Yeo 1981). After Phase II, the gas exchange parameters of plants seemed to stabilise, and P_N and gs even increased in plants previously treated with 4 dS/m NaCl.

Studies investigating the capacity for photosynthetic recovery after a salinity period are very scarce, yet this capacity can determine a plant's resilience to salt stress. Recovery depends on the intensity of photosynthesis decline during the stress period (Chaves et al. 2009). In our data, S12 plants did not show a significant decline in photosynthesis after the recovery period. This response likely allowed these plants to recover photosynthetic rates. However, S12 plants displayed a reduction in plant growth after Phase II, and a role for the accumulation of Na^+ and Cl^- in disturbing cell metabolism cannot be ruled out.

The response of Eugenia plants to NaCl was also reflected in the chlorophyll fluorescence parameter, data that were parallel with P_N and gs changes. In general, salt-sensitive plants show a drop in photochemical quenching parameters but an increase in non-photochemical quenching parameters (Moradi and Ismail 2007; Lee et al. 2013; Ikbal et al. 2014). However, and depending on the plant species and the severity of the stress, a decrease in photochemical and non-photochemical quenching parameters can take place. In Eugenia plants, after 15 days of salt treatments, plants subjected to 8 and 12 dS/m NaCl responded to the imposed stress with decreases in qP and *Y*(II) and a concomitant increase in the non-photochemical quenching parameters, a mechanism for safely dissipating excess light energy and minimising ROS generation (Maxwell and Johnson 2000). At 30 days of stress, the increase in qP and *Y*(II) and the decrease in the non-photochemical quenching parameters observed in salt-treated plants paralleled the response observed in gas exchange parameters, indicating an adaptative response to the imposed stress conditions. The recovery period was detected by plants as a new challenge, as evidenced by an alteration in the fluorescence parameters, especially in S8 and S12 plants. The observed decrease in qP as well as the drop in non-photochemical quenching parameters in this period suggested the generation of ROS in the chloroplasts as well as photooxidative damage (Foyer and Harbison 1994), a response similar to that in NaCl-sensitive plants.

Anatomical changes

It is known that prolonged water and salt stress may cause changes in leaf anatomy (Olmos et al. 2007; Fernández-García et al. 2014). In this study, the observed morphological changes at 30 days of stress (increased root/canopy ratio) were accompanied by leaf anatomical changes. For example, there was an increase in the percentage of intercellular spaces observed in all stressed plants, which allows for better CO_2 diffusion. In addition, S8 plants experienced an increase in palisade parenchyma, involving an increase in the number of chloroplasts and a reduction in spongy parenchyma, making it easier for CO_2 to reach the chloroplasts present in the palisade parenchyma. These changes were reflected in the P_N and gs values. After 30 days of stress, although gs decreased in plants treated with 8 and 12 dS/m NaCl, the anatomical changes made it possible for CO_2 to reach the chloroplast in a more efficient manner in a situation of reduced stomatal aperture. These alterations seem to be another strategy to protect the photosynthetic process. The same anatomical changes also took place in Phase II, especially in S4 plants. These changes correlated with the best P_N performance in the recovery period.

Information regarding the effect of salinity on the leaf anatomy of ornamental plants is very scarce. One study found that the leaf structure of *Rosmarinus officinalis* L. plants was modified in response to water stress, including a reduction in the intercellular spaces in the spongy mesophyll (Olmos et al. 2007). Salt stress also produced anatomical alterations in other shrub species. In *Lawsonia inermis* L plants, a 150 mM NaCl treatment produced a significant increase in leaf thickness due to a higher mesophyll cell area as an strategy to maximise photosynthesis potential (Fernández-García et al. 2014).

Antioxidative metabolism

In this study, salt stress was found to produce oxidative stress, as evidenced by damage in membranes, ROS accumulation and changes in antioxidative metabolism. Nevertheless, the response of S4 and S8 plants to salt stress was somewhat different. At 30 days of stress, the induction of an H_2O_2-generating enzyme (SOD) was observed in S8 plants in addition to a decrease in H_2O_2-scavenging enzymes (APX, POX and CAT), which would entail the accumulation of H_2O_2. However, DAB-staining did not show significant H_2O_2 accumulation in leaves. In fact, only S12 plants showed some H_2O_2 staining in leaves (data not shown). The S4 plants showed a more balanced ASC-GSH cycle than S8 plants with higher APX activity, unchanged MDHAR levels and an increase in GR activity. In addition, S4 plants presented similar SOD values to S8 plants, but higher CAT and POX activities, suggesting tightly controlled ROS generation. In general, salt-tolerant plants show increased levels of antioxidant mechanisms, including enzymatic and non-enzymatic defences, whereas salt-sensitive species display a decreased response in antioxidative defences (Hernández et al. 1995; Moradi and Ismail 2007; Diaz-Vivancos et al. 2013; Lee et al. 2013; Shu et al. 2013; Ikbal et al. 2014).

Salt stress affects the ASC content, but an increase in reduced glutathione (GSH) occurred. Different authors (Hernández et al. 1999, 2000; Mittova et al. 2003; Diaz-Vivancos et al. 2013) have suggested a role for ASC in salt tolerance. In addition to playing a significant role in the protection and regulation of photosynthesis, ASC also plays an important role as a co-factor of many enzymes (Gest et al. 2013). At 30 days of stress, ASC decreased by up to 30 % in S4 plants. In S8 plants there was a nearly 21 % decrease in ASC, which correlated with an increase in the ASC-recycling enzyme MDHAR. In salt-tolerant plants, ASC levels can also suffer a decrease ranging from 30 to 35 % due to salinity, as observed in salt-tolerant pea plants or in salt-tolerant transgenic plum lines (Hernández et al. 2000; Diaz-Vivancos et al. 2013). Eugenia plants seemed to use GSH instead of ASC to tackle salt stress. Reduced glutathione can be used not only in H_2O_2 elimination but also to eliminate other peroxides (lipid peroxides or hydroperoxides) by GST and/or GPX enzymes (Noctor et al. 2012). It has been reported that glutathione-dependent enzymes, such as GST and GPX, play a crucial role in the limitation of oxidative processes under salt stress conditions (Roxas et al. 2000; Naliwajski and Skłodowska 2014). It is important to remark that in Eugenia plants the increase in GSH was not accompanied by changes in GR activity, suggesting that GSH biosynthesis could be enhanced. In contrast, in Phase II, Eugenia plants seemed to use both ASC and GSH to respond to the new imposed growth conditions. It is important to highlight the strong increase in ASC levels as well as the restoration of APX activity in recovered plants in relation to Phase I. Recovered plants could use both ASC-dependent and GSH-dependent mechanisms to control ROS metabolism.

Surprisingly, after stress release (Phase II), plants previously treated with 8 or 12 dS/m NaCl behaved as salt-sensitive according to the foliar area, root DW and the lipid peroxidation data. It is likely that the new irrigation conditions produce hypoosmotic stress, leading to an oxidative burst inducing cell damage (Cazalé et al. 1998). This response may be due the fact that plants, once adapted to NaCl stress, can detect new growth conditions as a new challenge. However, literature regarding the removal of salt stress is scarce. This response has also been described in pea leaves in response to short-term salt stress and after 8 h of the post-stress period, suggesting that plants can perceive the removal of NaCl as another stress situation (Hernández and Almansa 2002).

In response to the new conditions, previously stressed plants exhibited the highest values for CAT and SOD activity and recovered APX activity values. In pea plants recovered from drought or salt stress, an increase in APX, SOD and GR has also been described (Mittler and Zilinskas 1994; Hernández and Almansa 2002). Increased CAT and SOD values were a common response in salt-stressed Eugenia plants, especially in recovered plants. The response of CAT activity suggested that the photo-respiratory pathway can be induced under salinity conditions, whereas SOD is considered to act as the 'first line of defence' against oxidative stress in plants (Alscher et al. 2002). Photorespiration can supply electron acceptors to PSI and CO_2 for the chloroplast from the decarboxylation of glycine in the mitochondria (Halliwell and Gutteridge 2000). In addition, a close correlation between CAT activity and the photosynthetic rate has been described. Increased CAT activity has been found to reduce the photorespiratory loss of CO_2 by limiting the H_2O_2-dependent decarboxylation of the keto-acids glyoxylate and hydroxypyruvate in the peroxisome (Brisson et al. 1998).

Conclussions

Globally, the results of this study showed that Eugenia plants are able to withstand salt stress and can be considered for landscaping project in Mediterranean areas characterized by semiarid climatic conditions. Eugenia plants react to avoid leaf ion toxicity, to keep their water status in order to limit water loss and protect the photosynthesis process. Other responses implemented by Eugenia plants to adapt to salt stress include increases in the root/canopy ratio and in the chlorophyll content in addition to changes in the leaf anatomy. Finally, Eugenia plants cope with the established oxidative stress by activating certain defence mechanisms (Fig. 3). Nevertheless, irrigation with the

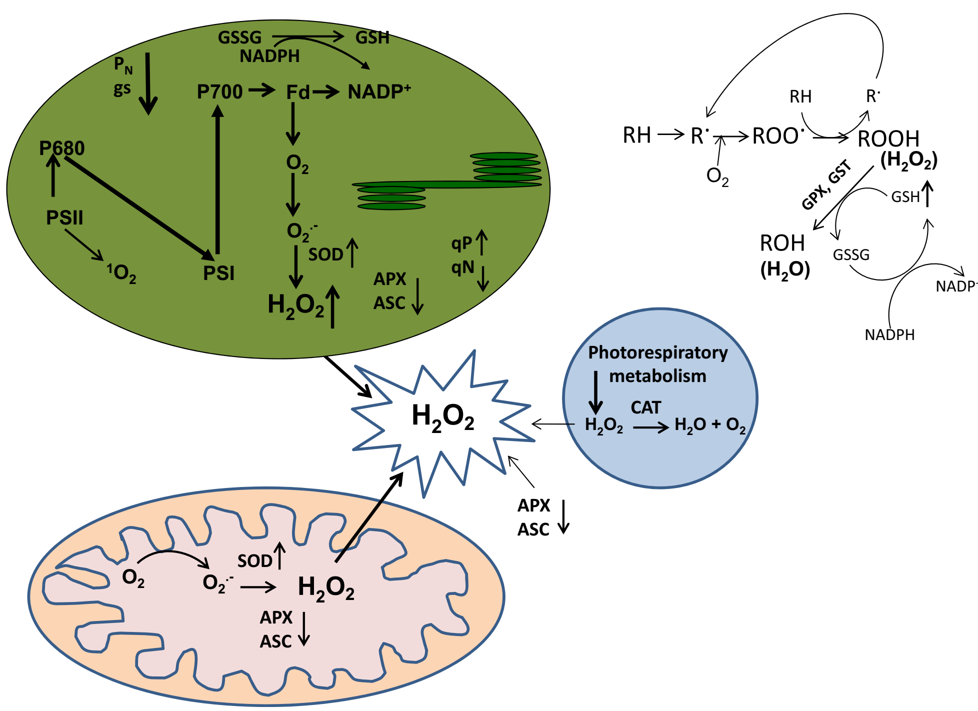

Fig. 3 Schema showing the effect of long-term salt stress (30 days) on the antioxidative metabolism of Eugenia leaves. Under salinity conditions, a decrease in P_N and gs took place, with an increase in qP and the electron transport rate and a decrease in qN. Under these conditions, increases in $^1O_2$ in PSII and $O_2^{\cdot-}$ in PSI could occur. The recycling of GSH can supply $NADP^+$, which could be considered as an additional response to protect the photosynthetic process in order to minimise ROS generation during the stress period. The increase in SOD activity and the drop in APX activity and ASC content can favour the accumulation of H_2O_2 in different cell compartments as described in other plant species (Corpas et al. 1993; Hernández et al. 1995, 2001; Gómez et al. 1999). In addition, photorespiratory metabolism can be increased and an overproduction of H_2O_2 can occur (Corpas et al. 1993). The H_2O_2 accumulated in chloroplasts, mitochondria and peroxisomes can leak into the cytosol, inducing an oxidative stress. The observed increase in GSH can induce GSH-dependent mechanisms [(Glutathione peroxidase (GPX), Glutathione-S-Transferase (GST)] to control H_2O_2 as well as hydroperoxides. However, these mechanisms cannot prevent damage to membranes after 30 days of stress

same water used on the controls for 16 days (Phase II) seems to be detected by Eugenia plants as a new stress situation. This can be due to the fact that Eugenia plants implement a plethora of mechanisms that have to be reversed once the saline treatment is finished. In other words, the plants have to retrace their steps to behave as control plants, but it appears that they would need more than 16 days to be able to perform once again as control plants.

Author contribution J.R.A.M. performed the experiment, carried out statistical analysis and was involved in data interpretation and manuscript writing and corrections. P.D.V. performed the antioxidative metabolism experiments and was involved in data interpretation and manuscript writing and corrections. S.A. performed the experiments of plant water relations and was involved in manuscript writing and corrections. N.F.G. performed the experiment of anatomical changes and was involved in manuscript writing. M.J.S.B. provided plant material and facilities for the experiments and was involved in data interpretation and manuscript writing and corrections. J.A.H. performed all the antioxidative metabolism experiments, chlorophyll fluorescence analysis, provided facilities and was involved in data interpretation and manuscript writing and corrections.

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