



Changes in growth, physiological parameters and the hormonal status of *Myrtus communis* L. plants irrigated with water with different chemical compositions



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ABSTRACT

Myrtus communis, an important Mediterranean ornamental shrub, was used to study the effect of irrigation water with different chemical compositions in the plant response. A treatment with NaCl was used to establish the plant resistance to high salinity at long term. Plants were subjected to four irrigation treatments with drainage for three months: Control (0.8 dS m^{-1}); two treatments using reclaimed water (RWs): RW1 (2.0 dS m^{-1}) and RW2 (5.0 dS m^{-1}); and NaCl (10.0 dS m^{-1}). High levels of electric conductivity of RWs not affected plant growth, while NaCl decreased leaf dry weight. Coinciding with the accumulation of Na^+ and Cl^- in the roots, soil water potential decreased, which hinders the mobilization of water to the leaves, decreasing leaf water potential. The osmotic adjustment in the NaCl treatment was due to Na^+ and Cl^- ions, although the proline could contribute as an Osmo compatible solute, increasing the turgor plants. Also changes in cell walls rigidity minimize the negative effects on the water balance; however, a higher lipid peroxidation was observed in these plants. Stomatal closure was associated with a decrease in K^+ and an increase in abscisic acid. NaCl produced an increase in salicylic acid and did not affect jasmonic acid contents at the end of the experiment. Similar behavior in soil and leaf water potentials, although less pronounced than in NaCl, was shown in RW2 plants. The abscisic acid increased in the RW2 with respect to the control and a decrease in stomatal conductance was observed at the end of the experiment. Plants irrigated with RW1 behaved similarly to the control.

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Abbreviations: ABA, abscisic acid; DW, dry weight; EC, electrical conductivity; FW, fresh weight; g_s , stomatal conductance; HPLC, high performance liquid chromatography; IAA, indoleacetic acid; J, absorption rate of ions by roots; JA, jasmonic acid; LP, lipid peroxidation; MDA, malondialdehyde; NETD, noise equivalent temperature difference; PAR, photosynthetically active radiation; P_n , net photosynthetic rate; RH, relative humidity; RW, reclaimed water; RWC_{tp} , relative water content at the turgor loss point; SA, salicylic acid; T, leaf temperature; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive-substances; TCA, trichloroacetic acid; WFC, weight at field capacity; Ψ_r , soil water potential at the root surface; Ψ_1 , leaf water potential; Ψ_s , leaf osmotic potential; Ψ_t , leaf turgor potential; Ψ_{100s} , leaf osmotic potential at full turgor; Ψ_{tp} , leaf water potential at the turgor loss point; ϵ , bulk modulus of elasticity.

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1. Introduction

Regions with a Mediterranean climate suffer a permanent scarcity of conventional water resources. This situation poses a double challenge, i.e., whether to use less freshwater for irrigation and urban needs or to use unconventional alternative water resources in order to preserve natural fresh water (Fereres and Connor, 2004; Pedrero et al., 2012). In this respect, reclaimed water (RW) are an example of an effective alternative to increasing exploitation of natural resources (Yermiyahu et al., 2008). Reclaimed water (RW), characterized by a high nutrient load (Ca^{2+} , K^+ , Mg^{2+} , P and S) and a high concentration of phytotoxic ions (Na^+ , Cl^- and B^{3+}), have been used in several recent studies (Acosta-Motos et al., 2014a,b; Gómez-Bellot et al., 2014, 2015a,b). However, this type of water requires fundamental disinfection processes before it can be used, including tertiary treatments to eliminate the presence of certain pathogenic microorganisms. These treatments are considered

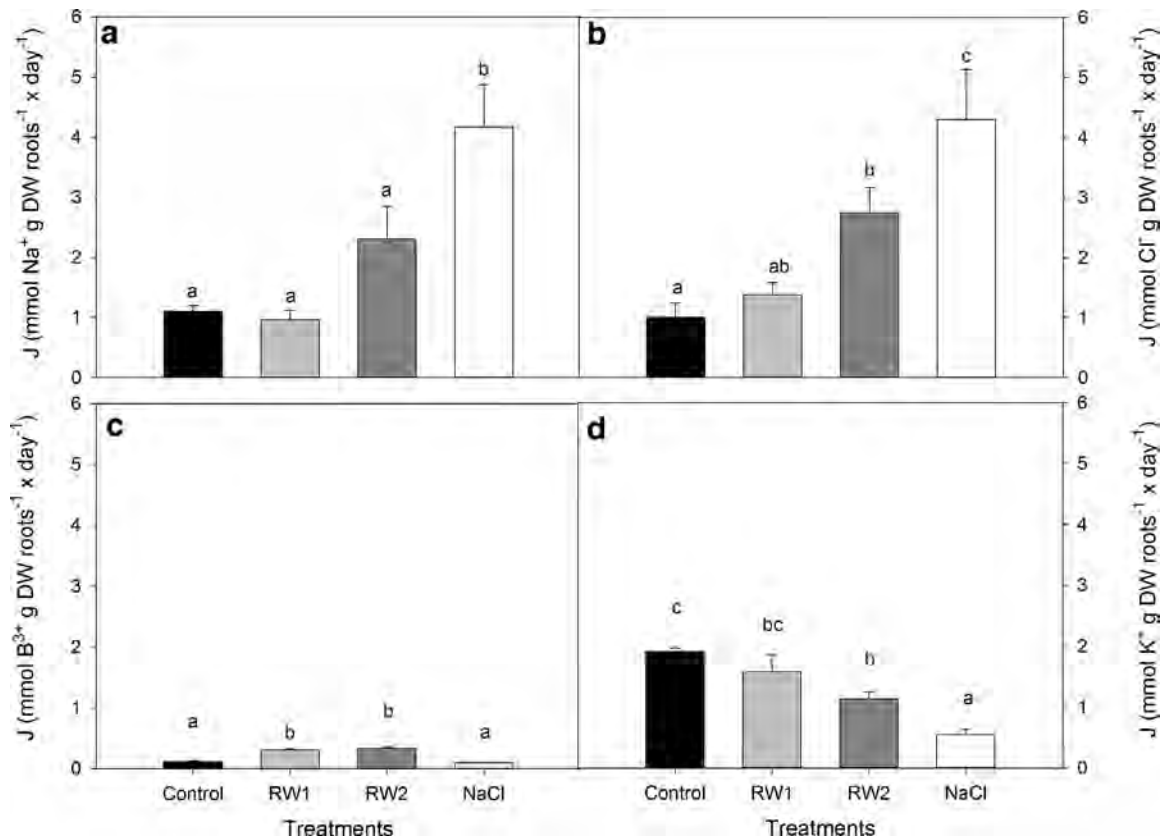


Fig. 1. Effect of the different irrigation treatments on the absorption rates of Na⁺ (a), Cl⁻ (b), B³⁺ (c) and K⁺ (d) ions in *M. communis* plants at the end of the experimental period. Data are means of 6 calculations \pm standard error (SE). Different letters in each column denote significant differences at $P < 0.05$ according to Duncan's Multiple Range Test.

necessary but are not so important when reclaimed water is used for the irrigation of ornamental plants whose visual appearance is the most important criterion to be considered (Gori et al., 2000; Álvarez et al., 2013).

Furthermore, it is known that about a third of cultivated lands are affected by salinity. In coastal areas or semiarid environments, this problem is accentuated by the high demand for high quality water for urban uses (Cassaniti et al., 2009). Therefore, in regions without adequate infrastructure to produce regenerated water, the use of marginal water still containing salts could be a solution for ornamental plant irrigation (Chartzoulakis et al., 2002; Acosta-Motos et al., 2015a,b). Moreover, using salt tolerant plants in landscaping and xeriscaping projects and in public areas would reduce the problem even further (Navarro et al., 2007). Among the species that can be used, myrtle plants are of particular interest because they show tolerance to different abiotic stresses, as described in Acosta-Motos et al. (2014a,b, 2015a), Navarro et al. (2009).

The responses to salinity in several ornamental plants studied vary, as has been observed with reclaimed water (RW) (Acosta-Motos et al., 2014a,b; Gómez-Bellot et al., 2014, 2015a,b) or saline water (NaCl) (Álvarez and Sánchez-Blanco, 2014; Acosta-Motos et al., 2015a,b). Salt stress is known to produce nutritional disorders, which affect the availability of nutrients through absorption and transport within the plant, changing the quality of vegetative organs (Grattan and Grieve, 1994, 1999a,b). High salt concentrations produce alterations in water relations and gas exchange, and mineral distribution (Cassaniti et al., 2009). Depending on the composition of the salt solution, ionic toxicity or nutritional deficiencies may occur due to competition between cations and anions (Shannon and Grieve, 1999). In reclaimed water used for irriga-

tion, the presence of nutrients can offset or reduce the incidence of damage caused by salt (Pedrero et al., 2012; Acosta-Motos et al., 2014a,b; Gómez-Bellot et al., 2014, 2015a,b). In addition, the use of drainage, leaching the accumulated salts, may minimize the negative effects of salinity. As is known, another factor to consider is the time of exposure to salinity. Early responses to salt stress on several ornamental species showed that it appeared to be tolerant to high salt concentrations. While, for long periods, salinity could delay their recovery and even cause permanent damage (Álvarez and Sánchez-Blanco, 2014). In the case of myrtle plants, previous research results indicated that these plants subjected to solutions of NaCl (4 and 8 dS m^{-1}) for up to 30 days accumulated toxic ions in roots in order to avoid leaf toxicity, and keep their water status and stomata regulation in order to limit water loss (Acosta-Motos et al., 2015a). Nevertheless, the response to NaCl solutions with high electrical conductivity has not yet been studied in this plant at long-term.

Moreover, in response to situations of high salinity, changes in gene expression may be mediated by plant hormones such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and indole acetic acid (IAA). Assays on the ecophysiological response and induction of phytohormones in plant species under different conditions of abiotic stress have been described by several authors (Albacete et al., 2008; Arbona and Gómez-Cadenas, 2008; Ghanem et al., 2008; De Ollas et al., 2015; Gómez-Cadenas et al., 2015; Puértolas et al., 2015).

In this study, the response of myrtle plants to watering with two reclaimed water was evaluated under controlled environmental conditions to check if under such conditions the components of these water could be of benefit to these plants, despite the high salinity. In addition, the physiological response to salinity was

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

Parameter	Irrigation water			
	Control	RW1	RW2	NaCl
EC (dS m ⁻¹)	0.80	2	5	10
pH (-log [H ⁺])	7.75	8.02	8.11	7.62
Na ⁺ (mmol L ⁻¹)	2.03	7.46	23.11	83.25
Cl ⁻ (mmol L ⁻¹)	1.88	6.62	22.55	97.58
Ca ²⁺ (mmol L ⁻¹)	1.72	3.55	5.37	0.80
B ³⁺ (mmol L ⁻¹)	0.01	0.03	0.10	0.01
K ⁺ (mmol L ⁻¹)	0.45	0.38	1.36	0.29
Mg ²⁺ (mmol L ⁻¹)	1.45	2.73	5.61	0.72
P (mmol L ⁻¹)	<0.003	0.01	0.03	<0.003
S (mmol L ⁻¹)	2.74	5.06	13.22	2.20

studied under the same conditions when myrtle plants were watered with a high NaCl concentration. For all the irrigation treatments applied, changes in plant growth, ion uptake and mineral distribution, water relations, gas exchange, leaf temperature and lipid peroxidation were analyzed. In addition, a series of phytohormones were also measured to obtain a hormonal profile at the end of the experimental period.

2. Materials and methods

2.1. Plant material and experimental conditions

Myrtle plants (120) were grown in 14 × 12 cm pots (1.2 L) filled with a composite of coconut fibre, sphagnum peat and perlite (8:7:1) and improved with Osmocote plus (2 g L⁻¹ substrate) (14:13:13 N, P, K + microelements). The experiment was conducted in a controlled growth chamber, where the temperature was set at 23 °C during the light period and 18 °C during darkness. Relative humidity (RH) values oscillated between 55 and 70%. A mean photosynthetic active radiation (PAR) of 350 μmol m⁻² s⁻¹ at canopy height was provided during the light period (07:00–23:00 h).

2.2. Water irrigation treatments

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 characterize the irrigation water quality. A chemical analysis for each irrigation water 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, Spain). The concentrations of B³⁺, Ca²⁺, K⁺, Mg²⁺, Na⁺, P 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 analyzed by ion chromatography (Metrohm, Herisau, Switzerland).

Myrtus communis plants (30 per treatment) were exposed to four irrigation treatments for three months, using water from different sources. The treatments consisted of a control irrigated with tap water reaching an EC of 0.8 dS m⁻¹. The reclaimed water came from two sewage treatment plants located in the Province of Murcia (Spain), namely RW1 (EC 2.0 dS m⁻¹) from Jumilla and RW2 (EC 5.0 dS m⁻¹) from Campotejar and a saline treatment (NaCl) with an EC of 10.0 dS m⁻¹. All plants were watered three times a week to above container capacity. At the beginning of the assay, the maximum water field capacity (WFC) for each substrate was calculated. Each plant (30 plants per treatment) was weighed before each

irrigation event, and the volume of irrigation water required to refill the pot to its threshold level (i.e., its WFC plus its pre-determined level of leaching, depending on the treatment) was calculated and added to each plant. Specifically, the volume of irrigation applied was determined in each treatment as the point at which the leaching fraction reached 15% (Control), 30% (RW1), 45% (RW2) and 60% (NaCl) (v/v) of applied water. Thus, electrical conductivity of the drainage water was about ± 10% EC of the irrigation water supplied for each treatment.

2.3. Biomass parameters

At the beginning and at the end of the experiment period, the substrate was gently washed from the roots of six plants per treatment. Each harvested plant was divided into shoots (leaves and stem) and roots, and the different organs were washed with distilled water to measure fresh weights (FW). Then, leaves stems and roots were oven-dried at 80 °C until they reached a constant weight to measure their respective dry weights (DW). Succulence was calculated by dividing the total fresh weight by the total dry weight of the plant.

2.4. Inorganic solutes concentration

At the beginning and at the end of the experiment the same plants used for biomass parameters were also used to determine the inorganic solute concentration. 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 level of Cl⁻ ions was analysed using a chloride analyzer (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 B³⁺, Ca²⁺, K⁺, Mg²⁺, Na⁺, P and S ions, were determined in a digestion extract of 100 mg of tissue powder with 50 mL of a mix of HNO₃:HClO₄ (2:1, v/v) by 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⁻, B³⁺, K⁺, Ca²⁺, Mg²⁺, P and S ions was calculated considering the total ion content of six plants per treatment, expressed as the mmol of each ion and the mean root weight, using the following formula described by Pitman (1975):

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

where M1 and M2 correspond to a concentration of different ions in mmol in the total plant at the beginning and at the end of experimental period, 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 relations and gas exchange

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 determined in six plants per treatment during the central hours of illumination along the experiment.

Leaf water potential was measured according to Scholander et al. (1965), using a pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA) in which each leaf was 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 Ψ_s was measured in the extracted sap using a WESCOR

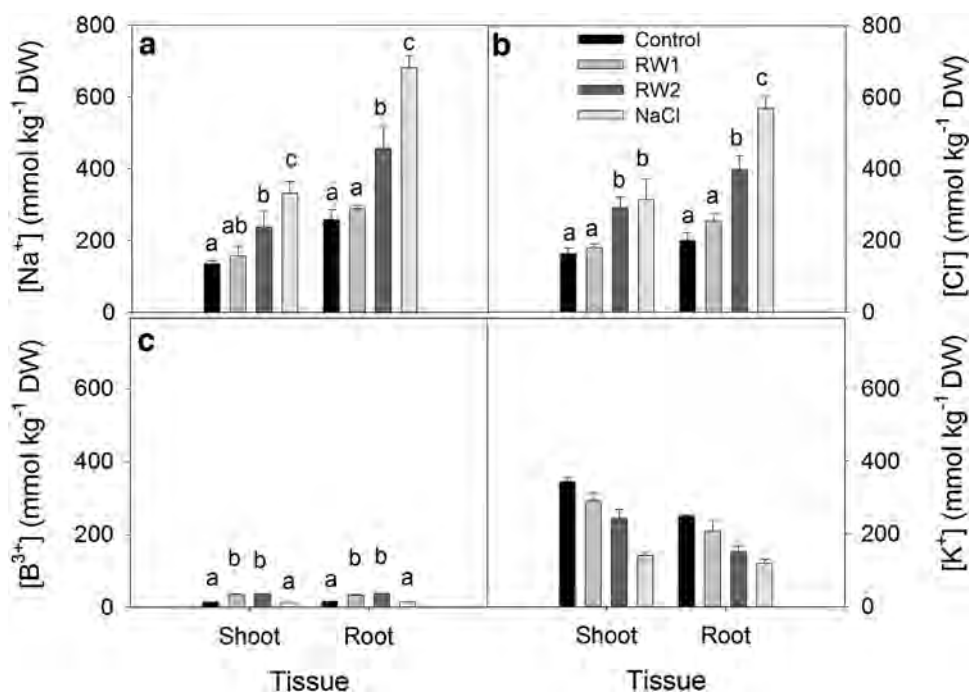


Fig. 2. Concentrations of Na⁺ (a), Cl⁻ (b), B³⁺ (c) and K⁺ (d) in different organs of *M. communis* plants at the end of the experimental period. Data are means of 6 calculations ± standard error (SE). Different letters in each tissue denote significant differences at $P < 0.05$ according to Duncan's Multiple Range Test.

5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA). Leaf turgor potential was estimated as the difference between Ψ_1 and Ψ_s . Finally, 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 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 RW and NaCl treatments we used the following equation:

$$\Psi_r = \Psi_{RW/NaCl} - (\Psi_C \times g_s RW/NaCl) / g_s C$$

where Ψ_C and $\Psi_{RW/NaCl}$ correspond to the mean value of leaf water potential in the control and RW and NaCl treatments, respectively. The $g_s C$ and $g_s RW/NaCl$ correspond to the mean value of stomatal conductance in the respective treatments. Leaf stomatal conductance (g_s) and net photosynthesis rate (P_n) were determined in attached leaves in six plants per treatment during the central hours of illumination along the experimental period using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA).

The leaf proline content was analysed at the end of experimental period following the procedure described in Pérez-Clemente et al. (2012). Briefly, 0.05 g of frozen plant tissue (leaves) was homogenised with 5 mL of 3% sulphosalicylic acid (Panreac) using a tissue homogenizer (Ultra-Turrax). After extraction, the homogenates were centrifuged to pellet cell debris at 4 °C at $12\,000 \times g$ for 10 min, and a 1 mL aliquot of the supernatant was combined with an equal volume of glacial acetic acid (Panreac) and ninhydrin reagent (Panreac). 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 (ACS grade; Panreac) and the absorbance at 520 nm was measured in this organic layer. A calibration curve was performed using commercial proline as a standard (Sigma-Aldrich).

As regards the pressure volume curves, the relative water content at the turgor loss point (RWC_{tlp}), the leaf water potential at the turgor loss point (Ψ_{tlp}), and the bulk modulus of elasticity (ϵ) were obtained at the end of the experimental period, using five leaves per

plant and six plants per treatment. Pressure–volume analysis of the leaves was performed as outlined by Wilson et al. (1979). The bulk modulus of elasticity (ϵ) at 100% RWC was calculated using the formula $\epsilon = (RWC_{tlp} \times \Psi_{100s}) / (100 - RWC_{tlp})$, where ϵ is expressed in MPa, Ψ_{100s} is the osmotic potential at full turgor (MPa), and RWC_{tlp} is the relative water content at the turgor-loss point, expressed as a percentage. Leaves were excised in the dark, placed in plastic bags, and allowed to reach full turgor by dipping their petioles in distilled water overnight. Pressure–volume curves were obtained from periodic measurements of leaf weight and balance pressure as the leaves dried on the bench at a constant temperature of 20 °C. The leaf-drying period for each curve was approximately 4–5 h.

2.6. Lipid peroxidation

The extent of lipid peroxidation (LP) was estimated by determining the concentration of thiobarbituric acid-reactive-substances (TBARS-MDA) following the procedure described in Hodges et al. (1999). Plant material (leaves) was homogenized in 5 mL of 80% cold ethanol (Panreac, Barcelona, Spain) using a tissue homogenizer (Ultra-Turrax; IKA-Werke, Staufen, Germany). Homogenates were centrifuged at 4 °C to pellet debris and different aliquots of the supernatant were mixed either with 20% trichloroacetic acid (TCA) (Panreac) or a mixture of 20% TCA and 0.5% thiobarbituric acid (Sigma-Aldrich, Madrid, Spain). Both mixtures were allowed to react in a water bath at 90 °C for 1 h. After this time, samples were cooled in an ice bath and centrifuged at $12\,000 \times g$ for 10 min. In the case of mixture A, absorbance was measured at two wavelengths (532 and 600 nm) against a target of TCA. In the case of the mixture B, the absorbance was measured at three wavelengths (440, 532 and 600 nm) against a target of TCA + TBA. The MDA concentration in the extracts was calculated as in Arbona et al. (2008).

2.7. Leaf temperature

Thermal images of canopy were obtained with a thermal imager (ThermaCam FLIR-e50 System, Inc., UK), which consisted of a

240 × 180 pixel line scan imager operating in the 7.5–13 μm wavebands, with a noise equivalent temperature difference (NETD) of 0.05 °C at 30 °C and an accuracy of 2 °C or 2% of the reading. This camera supplies a combination of high resolution infrared and visible spectrum images, facilitating the identification of leaves in the thermograms. The background temperature required for calculating object temperatures was estimated as the radiative temperature of a crumpled sheet of aluminum foil in a similar position to the leaves of interest with the emissivity set at 1.0 (Jones et al., 2002). Emissivity for leaf measurements was set at 0.96 (Grant et al., 2006). Images were taken at a distance of 0.7 m from the canopy of interest. Thermal images were processed with ThermoCam Explorer software (FLIR Quick Report).

2.8. Hormonal determination

Phytohormones were analyzed by HPLC coupled to tandem mass spectrometry as described in Arbona and Gómez-Cadenas (2008). Briefly, frozen plant material (leaves) was ground to a fine powder with a prechilled mortar and pestle, and then 0.2 g of powdered tissue was extracted in ultrapure water using a tissue homogenizer (Ultra-Turrax). Before extraction, samples were spiked with 100 ng of [²H₆]-ABA, 100 ng of [²H₄]-SA, 100 ng of dihydrojasmonic acid and 5 ng of [²H₂]-IAA to assess recovery and matrix effects. The tissue was immediately homogenized in 5 mL of ultra-pure water. Authors have previously demonstrated the efficiency of water as a solvent for acidic hormones (Durgbanshi et al., 2005). Extracts were then centrifuged at 4500 rpm for 30 min to pellet debris. The pH of the supernatant was adjusted to 3.0 with 30% CH₃COOH and partitioned twice against an equal volume of diethyl ether. After discarding the aqueous phase, the organic fraction was evaporated in a centrifuge vacuum evaporator (Jouan, Saint-Herblain, France). The dry residue was thereafter resuspended in a water:methanol (9:1) solution which was filtered through a 0.22 μm cellulose acetate filter. A 20 mL aliquot of this solution was then directly injected into the HPLC system (Alliance 2695, water Corp., Milford, USA). Hormones were then separated in a reversed-phase Kromasil 100 C18 column (100 × 2.1 mm 5 μm particle size) using methanol and ultrapure water, both supplemented with glacial acetic acid to a concentration of 0.05%. The mass spectrometer, a triple quadrupole (Quattro LC, Micromass Ltd., Manchester, UK), was operated in negative ionization electrospray mode, and concentrations of each plant hormone were determined using calibration curves performed with known amounts of pure standard samples.

2.9. Statistical analysis

In the experiment 30 plants were randomly attributed to each treatment. The data were analyzed by one-way ANOVA using SPSS 17.0 software (SPSS Inc., 2002). Ratio data were subjected to an arcsine square-root transformation before statistical analysis to ensure homogeneity of variance. Treatment means were separated with Duncan's Multiple Range Test ($P \leq 0.05$).

3. Results

3.1. Plant growth

The plant growth parameters measured at the end of the experiment did not show significant differences when the plants were irrigated with RWs compared with the control treatment (Table 2). On the other hand, the highest level of salinity corresponding to the NaCl treatment had a negative effect on leaf DW. The root/shoot DW ratio only increased in the NaCl treatment, while root DW did not show significant differences between any of the treatments

(Table 2). The leaves in the NaCl treatment also had lower values of succulence compared with the rest of the treatments (Table 2).

3.2. Ion uptake and mineral distribution

The root absorption rate (J) for Na⁺ and Cl⁻ ions was significantly higher in plants treated with NaCl (Fig. 1a and b). The plants irrigated with RWs had higher J of B³⁺ levels than the plants of the other treatments (Fig. 1c). In contrast, the absorption rate for K⁺ decreased when the saline level increased (Fig. 1d). A higher value in the Ca²⁺ absorption rate was observed in plants irrigated with RW2 and NaCl treatments, and J for Mg²⁺ significantly increased in plants irrigated with RWs (Fig. S1). In general, the different treatments induced a significant increase in the absorption rate for S, and a decrease in P (Fig. S1).

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.jplph.2015.11.010>.

The ions distribution through the plant was different depending on the applied treatment. The concentrations of Na⁺ and Cl⁻ were higher in the aerial part than in the roots of plants submitted to the NaCl treatment than the rest of treatments (Fig. 2a and b). The B³⁺ ion concentration increased in all organs of the plants irrigated with reclaimed water (RW1 and RW2) (Fig. 2c). The K⁺ concentration decreased in the aerial part and roots of NaCl treated plants compared with the other treatments (Fig. 2d). The concentration of Ca²⁺ was higher in the all parts of the plant of RW2 and NaCl than in control plants (Fig. S2). The aerial part concentration of Mg²⁺ was highest in RWs plants than control and NaCl plants (Fig. S2). Finally, the concentration of P in the aerial part was lower in the RW2 and NaCl treatments compared to control plants (Fig. S2).

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.jplph.2015.11.010>.

3.3. Water relations and gas exchange

During the experiment, the soil water potential at the root surface (Ψ_r) fell in parallel with increasing salinity treatments (Fig. 3a). The leaf water potential (Ψ_l) decreased in the RW2 and NaCl plants from the beginning of the respective treatments, although this value fell more sharply in the most saline treatment. At the end of the experiment, Ψ_l decreased parallel to the salinity level applied to each treatment, with values of -0.75 MPa, -0.9 MPa, -1.1 MPa and -1.7 MPa for Control, RW1, RW2 and NaCl, respectively (Fig. 3b). The same behavior was observed for leaf osmotic potential (Ψ_s), resulting in a significantly higher leaf turgor potential (Ψ_t) value in NaCl plants compared with the rest of the treatments (Fig. 3c and d). In addition, a slight increase in Ψ_t was observed at some moments of the experiment in RW2 plants (Fig. 3d). Regarding gas exchange (g_s and P_n) a progressive decrease in both parameters was observed at the end of the experimental period, being related to the salinity applied (Fig. 3e and f).

The increase in plant leaf temperature coincided with the lowest values of g_s , with a difference of about one degree between plants subjected to NaCl and the rest of the treatments (Table 3).

The decrease in Ψ_s in the plants of both treatments, RW2 and NaCl, was due to solutes active accumulation (osmotic adjustment), which can be explained by the data from osmotic potential at full saturation (Ψ_{100s}) (Fig. 3g). Plants with higher salinity levels (RW2 and NaCl) exhibited osmotic adjustment, especially during the last phase of the experiment (Fig. 3g). At the end of the experiment, the proline content of leaves was similar in the control and RW1 plants, with values of around 20 μmol g⁻¹ FW. The plants irrigated with the NaCl solution showed the highest proline accumulation, while RW2 plants had an intermediate value between control and NaCl plants (Fig. 3h).

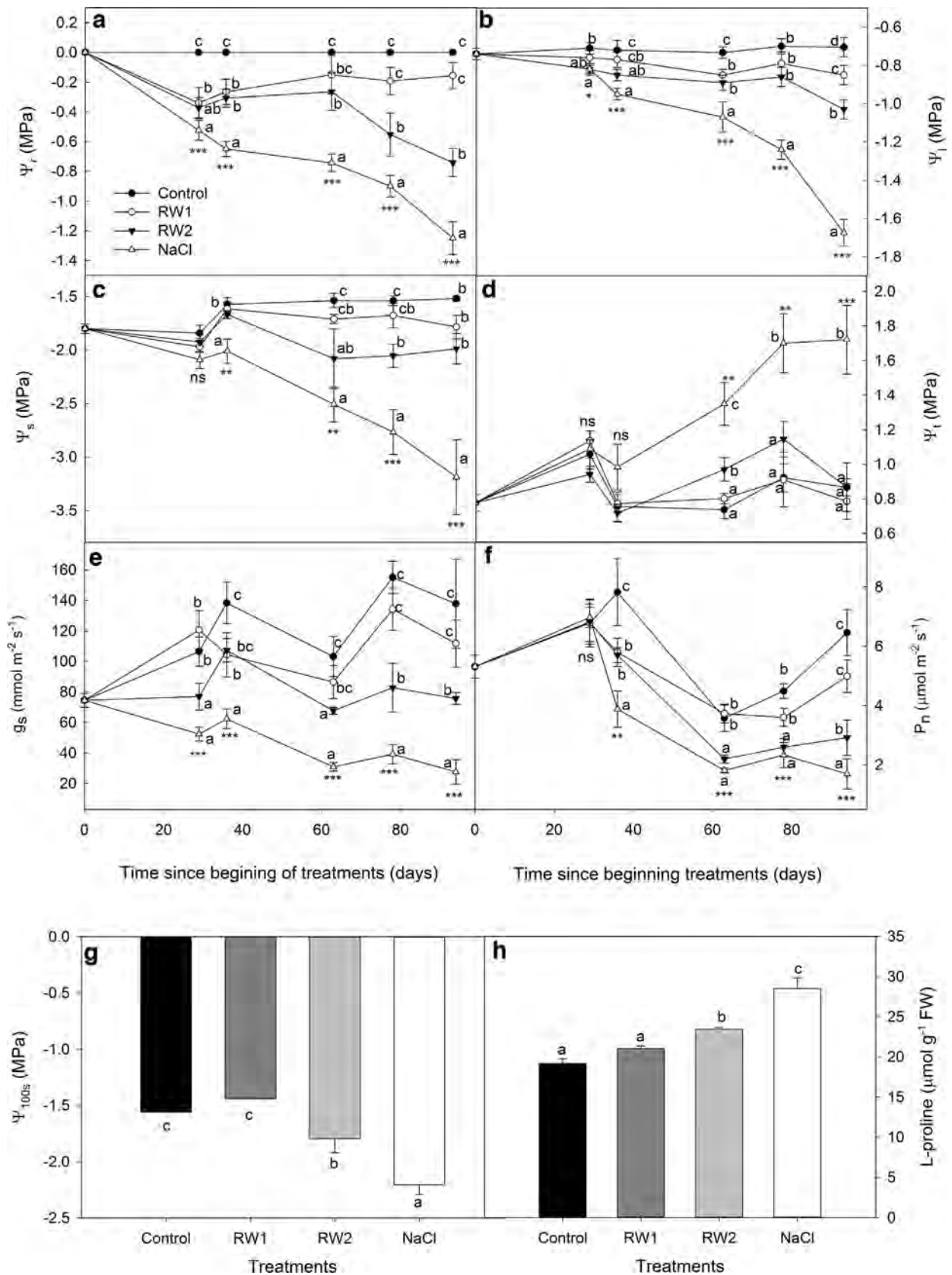


Fig. 3. Influence of different irrigation treatments on soil water potential at the root surface (Ψ_r , a), leaf water potential (Ψ_l , b), leaf osmotic potential (Ψ_s , c), leaf turgor potential (Ψ_t , d), leaf stomatal conductance (g_s , e), net photosynthetic rate (P_n , f), leaf osmotic potential at full turgor (Ψ_{100s} ; g) and proline (h) in leaves from *M. communis* plants along of the experimental period. Data are means of 6 calculations \pm standard error (SE). Symbols represent the different treatments: control (filled circles), RW1 (open circles), RW2 (filled triangles) and NaCl (open triangles). Asterisks indicate significant differences between treatments and different letters denote significant differences at $P < 0.05$ according to Duncan's Multiple Range Test.

As regards the pressure–volume (PV) analysis, the water potential at the turgor loss point (Ψ_{tlp}) significantly decreased in plants irrigated with the highest salinity level in relation to the control plants (Table 3). Meanwhile, the turgor loss point occurred at a higher value of relative water content (RWC_{tlp}), 81% versus 70% for the NaCl and control, respectively. The values of the elastic modulus (ϵ) increased significantly in plants treated with NaCl solution (Table 3).

3.4. 4Hormonal quantification and lipid peroxidation

At the end of the experimental period, the abscisic acid (ABA) content in leaves increased in the RW2 and NaCl treatments with respect to the control (Fig. 4a). The levels of salicylic acid (SA) also increased in proportion to the salinity applied (Fig. 4b). By contrast, decreases in indoleacetic acid (IAA) and jasmonic acid (JA) levels were observed as salinity increased (Fig. 4c and d).

Structural damage at the level of the membrane caused by oxidative stress was assessed through the lipid peroxidation (LP). The extent of LP was evidenced by the accumulation of MDA, which increased with the salinity treatments (Table 3).

4. Discussion

Salinity stress is a current environmental problem and an important factor limiting crop production. Plants respond to this abiotic stress through complex interactions among different morphological, physiological and biochemical processes (Munns and Tester, 2008). According to Cassaniti et al. (2009), the salinity can induce toxicity in plants due to the ions present (Na^+ and Cl^-) as well as nutritional deficiency due to increased competition among cations and anions (Shannon and Grieve, 1999). A severe reduction in growth has been attributed to increases in Na^+ and Cl^- ions, accompanied by a major reduction in Ca^{2+} and K^+ ion concentrations in plant tissues (Valdez-Aguilar et al., 2009). In our experiment, in spite of the high salinity in the RW2 ($\text{EC} = 5 \text{ dS m}^{-1}$), good results were obtained in both RWs plants, not reduction of biomass in all plant parts (leaves, stems and roots) and relatively high K^+ and Ca^{2+} ion concentrations were observed. K^+ and Ca^{2+} ions not only

play important roles in plant growth and development, but are also vital for osmotic adjustment and the maintenance of cell turgor (Osakabe et al., 2014). Moreover, it has been observed that adding nutrients to a saline medium improves crop quality (Grattan and Grieve, 1999a,b), as occurred when an excess of Na^+ was alleviated with added Ca^{2+} (García-Legaz et al. (2008)).

Similarly, the contribution of nitrates can reduce the incidence of damage caused by Cl^- (Iglesias et al., 2004). As regards the presence of boron, some studies suggest that the combined effect of salinity and excess B^{3+} are antagonistic, minimizing saline stress (Bañón et al., 2012). In our experimental conditions, the plants irrigated with reclaimed water (RW1 and RW2) had higher concentrations of B^{3+} , Ca^{2+} , Mg^{2+} and S. It is possible that the toxic effects produced by the highest concentrations of Na^+ in these plants might be offset by the effect of ions like Ca^{2+} , Mg^{2+} and S, which might help to improve plant growth. Therefore, nutritional components of reclaimed water, used as alternative water sources have a beneficial effect on plant growth and minimized the negative effects of the toxic ions present in the water. This has been observed in some ornamental species (Bañón et al., 2011; Miralles et al., 2011; Valdés et al., 2012; Cassaniti et al., 2013; Gómez-Bellot et al., 2015a,b) where higher nutrient contents promote better growth and ornamental quality of these plants (Lubello et al., 2004). All this justifies the beneficial effects of using RW for irrigation of ornamental plants in containers (Acosta-Motos et al., 2014a,b) and in the field with no obvious damage in the soil (Gómez-Bellot et al., 2015c).

In general, the osmotic stress caused by the salts present in the root environment induces a decrease in soil water potential at the root surface, and as consequence difficulty for water uptake by the plants (decreases in leaf osmotic potential and leaf water potential). These decreases became more evident as the salts are accumulated, which was observed as our experiment progressed, especially in the NaCl treatment. Similar observations are described in Álvarez et al. (2012) and Acosta-Motos et al. (2015b). At the end of the experimental period, an osmotic adjustment could be seen in the RW2 and NaCl treatments, which produced an increase in turgor, especially in the latter treatment. The main contributions to this osmotic adjustment would be related to the higher Na^+ and Cl^- contents observed in the aerial part, as well as to the leaf

Table 2
Influence of four irrigation treatments on the growth of *M. communis* plants at the end of the experimental period. Leaf DW, Stem DW, Root DW and Total DW are given in (g plant^{-1}). Data are means of 6 calculations \pm standard error (SE). Different letters denote significant differences at $P < 0.05$ according to Duncan's Multiple Range Test.

Growth parameters	Treatment				P-value
	Control ($\text{EC}: 0.8 \text{ dS m}^{-1}$)	RW1 ($\text{EC}: 2 \text{ dS m}^{-1}$)	RW2 ($\text{EC}: 5 \text{ dS m}^{-1}$)	NaCl ($\text{EC}: 10 \text{ dS m}^{-1}$)	
Leaf DW	1.88 \pm 0.14b	1.96 \pm 0.23b	1.90 \pm 0.22b	1.14 \pm 0.22a	*
Stem DW	3.95 \pm 0.24ab	3.89 \pm 0.81ab	4.25 \pm 0.31b	2.70 \pm 0.30a	*
Root DW	5.83 \pm 0.30	5.30 \pm 0.64	6.68 \pm 0.78	5.21 \pm 0.42	ns
Total DW	11.36 \pm 0.42ab	11.16 \pm 1.42ab	12.83 \pm 1.15b	9.06 \pm 0.90a	*
Root DW/Shoot DW	1.07 \pm 0.08a	1.00 \pm 0.14a	1.08 \pm 0.08a	1.43 \pm 0.11b	*
Succulence	3.16 \pm 0.12b	2.85 \pm 0.14b	3.01 \pm 0.10b	2.20 \pm 0.29a	**

Asterisks indicate the level of probability: * $P < 0.05$ and ** $P < 0.01$. Non-significant values are indicated by "ns".

Table 3
Influence of different irrigation treatments on relative water content at the turgor loss point (RWC_{tlp} as%), leaf water potential at the turgor loss point (Ψ_{tlp} as MPa), bulk modulus of elasticity (ϵ as MPa), lipid peroxidation (LP as MDA equivalents [nmol l^{-1}]) and leaf temperature (T as $^{\circ}\text{C}$) at the end of the experimental period in *M. communis* plants. Data are means of 8 calculations \pm standard error (SE). Different letters denote significant differences at $P < 0.05$ according to Duncan's Multiple Range Test.

Parameters	Treatment				P-value
	Control ($\text{EC}: 0.8 \text{ dS m}^{-1}$)	RW1 ($\text{EC}: 2 \text{ dS m}^{-1}$)	RW2 ($\text{EC}: 5 \text{ dS m}^{-1}$)	NaCl ($\text{EC}: 10 \text{ dS m}^{-1}$)	
RWC_{tlp}	70.22 \pm 5.49a	71.58 \pm 7.24ab	71.85 \pm 10.54ab	81.30 \pm 1.79b	**
Ψ_{tlp}	-1.78 \pm 0.40a	-2.25 \pm 0.50ab	-2.31 \pm 0.14ab	-2.55 \pm 0.54b	**
ϵ	4.03 \pm 1.80a	4.37 \pm 0.60a	5.19 \pm 1.40ab	7.48 \pm 2.61b	**
LP	7.80 \pm 0.68a	30.85 \pm 2.34b	41.75 \pm 4.22bc	53.17 \pm 4.99c	***
T	25.55 \pm 0.17a	25.82 \pm 0.27	26.18 \pm 0.35	27.08 \pm 0.19	**

Asterisks indicate the level of probability: ** $P < 0.01$ and *** $P < 0.001$.

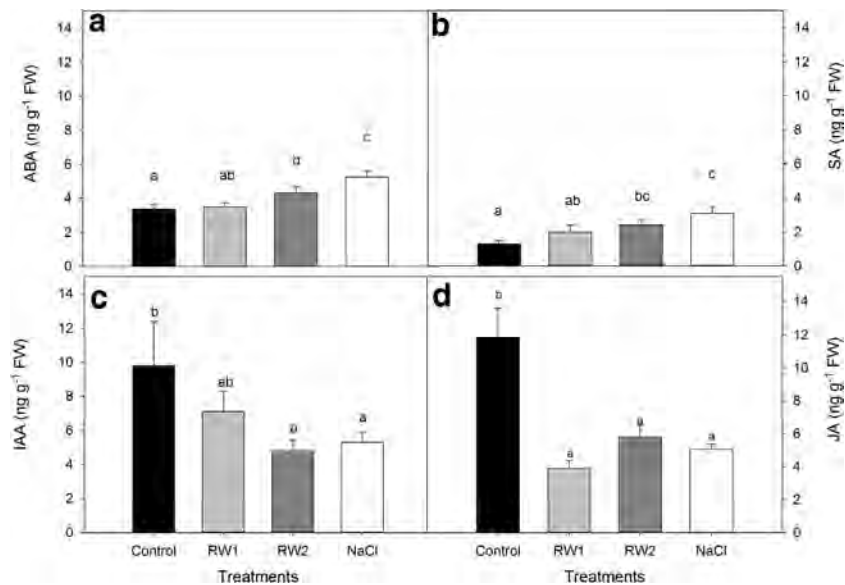


Fig. 4. Hormonal profile: Abscisic acid (ABA, a), salicylic acid (SA, b), indoleacetic acid (IAA, c) and jasmonic acid (JA, d) concentrations measured in leaves at the end of the experimental period in *M. communis* plants subjected to different irrigation treatments. Data are means of 6 calculations \pm standard error (SE). Different letters denote significant differences at $P < 0.05$ according to Duncan's Multiple Range Test.

proline content in the leaves. The plants may use these ions and proline to lower the osmotic potential and maintain turgor, which has also been described in some Mediterranean species (Álvarez et al., 2012; Acosta-Motos et al., 2015b). The ability of plants to perform osmotic adjustment in saline conditions allows them to reach a turgor loss point at more negative water potential values and maintain a relative level of water content. In addition, the presence of NaCl in the medium reduced cell wall elasticity, increasing the elasticity modulus (ϵ) (Navarro et al., 2007). Under salinity conditions, rigid cells may be advantageous as diminishing cell volume would confine existing solutes and ions to a level that could disrupt cellular processes (Touchette, 2007). This indicates that combining solute accumulation and changes in cell wall rigidity minimises the negative impacts of salinity on the water balance in myrtle plants. Similar results were obtained in *Alternanthera philoxeroides* (Bolaños and Longstreth, 1984) and in *Acacia nilotica* (Nabil and Coudret, 1995) species in response to salt stress. With higher values of bulk modulus elasticity, tissues became less elastic and the effect is to allow lower water potentials to be reached for a given change in water volume. An increase in ϵ concomitant with osmotic adjustment was an effective means of counteracting the negative effects of salinity on water balance of *A. unedo* plants (Navarro et al., 2007).

At the end of the experiment, the myrtle plants suffered a functional loss in membrane permeability because of oxidative stress evaluated through the MDA content due to a higher accumulation of ions Na^+ and Cl^- in the leaves. This loss was particularly confirmed by the results obtained in the more saline treatment, indeed visual symptoms of toxicity were seen in these plants. Salt-induced oxidative stress has been described in other plant species (Ikbali et al., 2014; Acosta-Motos et al., 2015a).

Furthermore, stomatal conductance was more altered in plants treated with higher salt levels. The changes in stomatal conductance associated with salinity may be a consequence of reduced soil water potential at the root surface and a decrease in leaf water potential (Fig. 3). These parameters are strongly correlated with the absorption of water and its transport to the shoot. In general, plants show a tendency to reduce stomatal opening in response to salt stress (López-Climent et al., 2008; Álvarez and Sánchez-Blanco, 2014; Acosta-Motos et al., 2015a,b).

Moreover, a low photosynthetic rate was associated with lower stomatal opening in plants subjected to the saline treatment (Fig. 3), since photosynthesis can be affected by either a change in stomatal conductance or changes in the metabolic capacity of the mesophyll cells (Flexas et al., 2004). Furthermore, the presence of NaCl meant that myrtle aerial part accumulated significant amounts of Na^+ and Cl^- ions, resulting in an increased transport of these ions from roots to shoots, which may provoke a decrease in the photosynthetic rate and plant growth. However, in previous works in myrtle plants submitted to high salts concentration at shorter period of time (30 days) and without drainage, toxic ions, especially Na^+ , were accumulated in roots minimizing its negative effects in the shoot growth (Acosta-Motos et al., 2015a,b). In addition, variations in stomatal opening affect transpiration, which is a mechanism that leaves use for cooling. Accordingly, a decrease in stomatal conductance led to an increase in leaf temperature (Table 3) (Gómez-Bellot et al., 2015b).

Plant hormones play important roles in plant growth, regulate plant development and participate in signaling networks when plants are under abiotic stress. Abscisic acid (ABA) is considered as a marker in stomatal opening and closure induced by salt. It has been proposed that ABA may be a mediator in plant responses to stresses, acting as an internal signal for plants to survive in saline conditions (Keskin et al., 2010). In accordance with this function, myrtle plants exposed to high salinity showed increased ABA concentrations in leaves (Fig. 4). Similar results have been described in salinity-induced leaf senescence in tomato (Ghanem et al., 2008). An increase in ABA content thus led to a decrease in leaf stomatal conductance, when salinity increased. Moreover, in the same plants, an inverse relation between the decrease in K^+ levels in leaves and increased ABA content was observed.

Jasmonic acid (JA) has been described as part of a defence mechanism when plants are under biotic or abiotic stresses (Cheong and Choi, 2003). In two different tomato cultivars, JA leads changes in the presence of salt stress, increasing in a tolerant cultivar while decreasing in the salt-sensitive cultivar (Pedranzani et al., 2003). According to these authors, the highest levels of JA in tolerant plants could act as a protective mechanism against salinity. However, there is little information about how salinity affects endogenous JA levels in plants. Our results (Fig. 4) did not find significant changes

in JA levels for the different saline treatments, and the changes observed at the end of the experimental period could not be linked to the different effects produced by the treatments. This could be explained by the fact that JA acts very early. In this sense, the concentration of JA transiently increased after a few hours of stress but soon returned soon to control levels after the onset of the condition (De Ollas et al., 2015).

In contrast, salicylic acid (SA) levels rose proportionally with increasing salinity (Fig. 4). It seems that SA and JA have antagonistic interactions, and the SA/JA ratio has been suggested as a marker to indicate saline stress (Gupta et al., 2000). SA has an effect as protector against damage by salinity (Kaya et al., 2002) and as a compound that increases salt tolerance in certain species (Shakirova and Sahabudinova, 2003). SA acts by increasing the rate of photosynthesis, maintaining membrane stability and decreasing Cl^- and Na^+ accumulation in plants. In general, low concentrations of SA alleviate sensitivity to abiotic stresses, while high levels of SA can induce oxidative stress, leading to a decrease in tolerance to abiotic stresses (Miura and Tada, 2014). Some authors have described how SA application (0.1–0.5 mM) alleviated the damage caused by salinity in bean plants, decreasing photosynthesis and increasing foliar Na^+ and Cl^- levels (Nazar et al., 2011). However, high SA concentrations (1 mM) caused growth retardation (Nazar et al., 2011).

In contrast to the other stress-related hormones, indoleacetic acid (IAA) is a phytohormone that plays an important role in plant growth regulation. In response to salinity, rice plants reduced their IAA levels (Nilsen and Orcutt, 1996), and the same was observed in tomato roots (Dunlap and Binzel, 1996), which may cause delays in plants acquiring compounds for plant growth. The levels of IAA observed at the end of our experiment showed lower values as salinity increased (Fig. 4), which could be related to changes in growth parameters. These results are similar to those described by Albacete et al. (2008) and Ghanem et al. (2008) in studying hormonal changes in salinized tomato plants.

In conclusion, the results obtained in the present paper confirm the effectiveness of using reclaimed water for irrigation in spite of its high electrical conductivity, because the higher nutrient content present in such water may minimize the negative effects of Na^+ and Cl^- ions. On the other hand, myrtle plants are able to grow in the presence of high NaCl levels but a prolonged exposure of plants to salinity resulted in the weakening of plant defences and enhanced susceptibility of the negative effects on the leaf biomass by the accumulation of toxic ions in the aerial part. In spite of it the plants keep their water status by osmotic adjustment, changes in cell walls rigidity and stomata regulation, related with a decrease in K^+ and an increase in abscisic acid, in order to limit water loss.

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