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Recycled Wastewater and Reverse Osmosis Brine Use for Halophytes Irrigation: Differences in Physiological, Nutritional and Hormonal Responses of *Crithmum maritimum* and *Atriplex halimus* Plants

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Abstract: Halophytes are capable of coping with excessive NaCl in their tissues, although some species may differ in their degree of salt tolerance. In addition, it is not clear whether they can tolerate other confounding factors and impurities associated with non-conventional waters. The experiment was performed in a greenhouse with *Crithmum maritimum* and *Atriplex halimus* plants, growing on soil and irrigated with two different water types: reclaimed wastewater (RWW) (EC: 0.8–1.2 dS m^{-1}) and reverse osmosis brine (ROB) (EC: 4.7–7.9 dS m^{-1}). Both species showed different physiological and nutritional responses, when they were irrigated with ROB. *Atriplex* plants reduced leaf water potential and maintained leaf turgor as consequence of an osmotic adjustment process. *Atriplex* showed higher intrinsic water use efficiency than *Crithmum*, regardless of the type of water used. In *Crithmum*, the water status and photosynthetic efficiency were similar in both treatments. *Crithmum* presented a higher leaf accumulation of B and Ca ions, while *Atriplex* a higher amount of K, Mg, Na and Zn. *Crithmum* plants irrigated with ROB presented higher concentrations of 1-aminocyclopropane-1-carboxylic acid and trans-zeatin-glucoside, whereas abscisic acid concentration was lower. *Atriplex* showed a lower concentration of trans-zeatin-riboside and scopoletin. The characteristics associated to water irrigation did not influence negatively the development of any of these species, which confirms the use of brine as an alternative to irrigate them with conventional waters.

Keywords: salinity; non-conventional irrigation; water status; photosynthetic efficiency; plant nutrition; phytohormones; growth

1. Introduction

The Mediterranean region is characterized by a climate with prolonged drought periods where isolated and torrential rains are frequent. In addition, soils usually present some extent of salinization, as well as a poor structure and a scarce vegetation cover, which increases water erosion [1]. Aridity might increase in this area due to climate change [2]. In this sense, water scarcity is becoming more frequent due to the overexploitation of aquifers as a result of an increasing demand [3]. This prevents the recovery of these sources of supply during recharge periods and it leads to the depletion of water resources. Agriculture is the largest user of water supplies, consuming over 70% of the abstracted freshwater globally [4]. In recent years, wastewater reclamation, recycling and reuse has gained attention in many countries, to ensure water security and to develop effective strategies for sustainable utilization of water resources in agricultural and landscape irrigation [5–8]. This kind of water is recovered from domestic, municipal, and industrial wastewater treatment plants and it may have specific treatments depending of its purpose [9]. Its use implies benefits of different nature, such as the pressure alleviation on other water resources [10] and the reduction of fertilizer cost due to its high nutrient content [11], producing high-value crops and crop commodities [12]. However, depending on its source and type of treatment, there is a wide range of chemical contaminants persisting in reclaimed wastewater, such as inorganic compounds, heavy metals, pathogens and many other complex compounds [13], that may affect negatively crop yield or be unsafe to human consumption [14,15]. In the case of plants with gardening and reforestation purposes, in addition to the problems related to the high salt concentrations and heavy metals which may affect growth, stabilization and quality of plants [16], the presence of persistent organic pollutants such as organochlorine pesticides can pose threats to ecosystems due to their biological accumulation through the trophic chain.

Some of these mentioned water treatments include membrane processes such as reverse osmosis (RO), whose main application is water desalination. Desalination technologies, particularly the reverse osmosis process, have been increasingly adopted to produce freshwater from alternative sources [17]. The water residue or RO brine resulting from this process is usually removed without using it, due to its high quantity of salts, causing environmental problems and high economic costs [18]. This has led some authors to consider that the reuse of agricultural and industrial brines for crop production can be beneficial in preventing discharge of brines into natural environmental [19]. Either way, there is a need to consider this water as a new non-potable water source [20] and to improve brine management strategies [21]. Numerous projects have been developed for the utilization of saline water on conventional crops and forages [22–24]. However, the low salinity tolerance of most crops limits the amount of saline water that can be applied for conventional crop production. In this sense, the selection and adoption of suitable plant species and genotypes are key factors to improve agricultural and green areas quality, as well as to decrease management costs. Halophytes plants are the native flora of saline soils, which survive completing their whole life cycle in such environments [25]. Halophytes are not only used in landscaping or as ornamental plants, they are also used to treat saline effluence, or cultivated with industrial purposes. Last but not least, they are being used for forage/fodder, human food and even gourmet vegetables [26]. The viability of plants in saline habitats depends on their ability to cope with several major constraints as (i) water deficit, (ii) restriction of CO_2 uptake, (iii) ion toxicity and (iv) nutrient imbalance [27]. Therefore, halophytic species may differ in their degree of salt tolerance [28]. To achieve this tolerance and be able to deal with salt stress, a wide range of morphological, physiological and biochemical mechanisms take place on the whole plant, at tissue and at cellular/molecular levels [29,30].

The main salt tolerance target is keeping the ionic excess away from the metabolic active tissues to preserve leaf photosynthesis and meristematic activity [31]. This aim is obtained generally thanks to processes such as accumulation of osmotic adjustment substances, ion-selective absorption and compartmentalization, morphological changes in root and leaf tissues, antioxidant and hormone regulation [32–34]. Taking into account this last process, phytohormones emerge as cellular signal molecules with key functions in the regulation of plant responses to abiotic stresses. Recently, a considerable amount of evidence has shown that phytohormones are signals connecting root and shoot, triggering responses to external stress [35]. To avoid water losses due to evapotranspiration, plants regulate cell biophysics promoting cellular turgor decrease and leaf stomatal closure. Consequently, a reduction of stomatal conductance limits CO_2 uptake and photosynthesis ultimately [36]. The reduction in photosystem II efficiency by excess salinity is associated with decreasing total chlorophyll content. In salt tolerant plants, PSII photochemistry is reported to be more resilient to salt stress than CO_2 fixation processes, with a balance between the light-harvesting processes and effective energy dissipating mechanisms [37,38]. Nevertheless, while halophytes are clearly capable of coping with excessive amounts of NaCl in their tissues [39,40], it is not clear whether they can tolerate other confounding factors and impurities associated with wastewater irrigation.

Crithmum maritimum (Apiaceae), or sea fennel, is a food halophyte found on rocky shores of Mediterranean Sea and Atlantic Ocean [41]. Several uses of *C. maritimum* are known for culinary purposes and its leaves have been used for aromatic and medicinal purposes as a tonic and diuretic [42]. *C. maritimum* is moderately tolerant to NaCl, known as a facultative halophyte, since it does not require salt for maximal growth. On the other hand, *Atriplex halimus*, or Mediterranean saltbush, is a xerohalophytic perennial shrub native to the Mediterranean. It is considered desirable due to its high fodder quality [43] and due to its potential for use in ecological restoration programs [44]. *A. halimus* is well adapted to salinity by tolerating salts internally and/or by its excretition [45] through its trichomes [46].

Based on the above considerations, in this study we evaluate if the irrigation with reclaimed wastewater and brine from a reverse-osmosis water treatment of two halophytes species (*Crithmum maritimum* and *Atriplex halimus*) growing on soil, is suitable for revegetation purposes. The objectives of the study were (i) to test the use of saline effluent such as brine from a reverse osmosis (RO) desalination treatment to irrigate *Crithmum maritimum* and *Atriplex halimus*, (ii) to study the growth of two halophyte forage species to factors associated with wastewater and (iii) to compare physiological traits, nutritional and hormonal status of two halophytes. Comparative data relating plant physiological and agronomic processes may prove beneficial information on the tolerance of plants to abiotic stresses.

2. Materials and Methods

2.1. Plant Material and Experiment Conditions

The experiment was performed in a greenhouse located in the municipal wastewater treatment plant (WWTP) of Balsicas (Murcia, Spain) (latitude 37°47′48″ N, longitude 0°57′36″ W), from April 2018 to January 2019. Two halophyte species typical from Mediterranean areas, *Crithmum maritimum* (CM) and *Atriplex halimus* (AH) were grown. Seedlings of both species (n = 72) were transplanted on 24 April 2018 into the greenhouse, which has a silty clay loam soil. The average bulk density was 1.46 g cm^{-3} and the volumetric soil water content at field capacity and permanent wilting point were 36.3% and 19.8%, respectively. The experimental plot consisted of 6 rows, with a total length of approximately 6 meters and 12 plants per row, following a planting pattern of 0.5 $\times$ 1 m^2. The microclimatic conditions showed that during the experimental period, the average values of the air temperature, relative humidity and radiation were around 21 °C, 63% and 500 Wm^{-2}, respectively. Irrigation and agronomic management were established by the farmer. Plants were irrigated by a drip irrigation system with one lateral pipe per plant row and one emitter (3 L h^{-1}). The volume of water applied depended on the season, the climatic conditions and the plant development. Irrigation was applied to bring soil moisture to the field capacity (up to a depth of 30 cm as the depth of root expansion). Soil moisture was measured using capacitive probes (ECHO-5, Decagon Devices Inc., Pullman, WA, USA) connected to a datalogger (CR1000, Campbell Scientific Inc., Logan, UT, USA). Irrigation was scheduled twice a week, activating it until soil moisture reached field capacity.

2.2. Irrigation Water Treatments and Experimental Design

Before starting with the different irrigation treatments, plants were irrigated with water from the Irrigation Community of Campo de Cartagena (< 0.9 dS m^{-1}). Irrigation treatments began on 23 May 2018, four weeks after transplanting. During the thirty-five following weeks, two irrigation treatments were applied at 100% field capacity: (1) Reclaimed wastewater (RWW) as control (EC: 0.8–1.2 dS m^{-1}), obtained by feeding wastewater to several tertiary treatments in the WWTP, such as ultrafiltration, granular activated carbon filter and four reverse osmosis membrane elements, and (2) reverse osmosis brine (ROB) (EC: 4.7–7.9 dS m^{-1}) which was a water residue result of the above mentioned process. The salinity level in the brine was medium, avoiding excessive salinization and soil degradation. Water quality of both treatments was similar during the experiment, it just varied in a narrow range, depending on the characteristics of the input wastewater treated at the WWTP (Table 1). In this sense, and in general terms, the concentration of the reverse osmosis brine components was much higher than those found in reclaimed wastewater. The concentration of Na, Cl and SO_4 was around six times higher in brine than in reclaimed wastewater, while the concentrations of anions such as F, NO_3 and PO_4 was around three times higher in brine than in reclaimed wastewater. Elements such as B, Ni, Cu and Zn showed a similar concentration in both waters (Table 1).

Table 1. Physicochemical analyses of the irrigation treatments. Data is presented as average values of the water samples collected during the experiment.

	RWW	ROB
EC (dS m^{-1})	0.994	5.403
pH	7.199	7.124
SS (mg L^{-1})	1.276	4.269
Turbidity (NTU)	0.570	0.686
E. coli (UFC 100 mL^{-1})	0.00	0.00
Fe (ppm)	0.04	0.07
K (ppm)	17.54	98.14
Mg (ppm)	8.45	56.12
Mn (ppm)	0.05	0.27
Na (ppm)	160.16	1003.29
Cl (ppm)	210.01	1208.92
P (ppm)	2.68	10.04
S (ppm)	1.88	9.11
B (ppm)	0.821	0.877
Ni (ppm)	0.008	0.008
Cu (ppm)	0.009	0.009
Zn (ppm)	0.054	0.044
F (ppm)	0.07	0.24
NO_2 (ppm)	0.10	0.10
NO_3 (ppm)	5.64	15.70
PO_4 (ppm)	8.21	30.72
SO_4 (ppm)	129.6	877.58

EC, electrical conductivity; SS, suspended solids; *E. coli*, *Escherichia coli* bacteria.

The treatments followed a randomized design, with three replications per treatment (6 plants per replication, 18 plants per treatment and species). Three rows were irrigated with RWW from the tertiary effluent and the other three with ROB in the plot.

2.3. Plant Water Relations

Leaf water relations were measured throughout the experiment in nine plants per treatment (three plants per replication). Leaf water potential (Ψ_{leaf}) was measured at midday, collecting a mature leaf according to Scholander et al. [47] using a pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA). Leaves were placed in the chamber within 20 s of collection and pressurized at a rate of 0.02 MPa s^{-1} [48]. Adjacent leaves were also collected, frozen immediately in liquid nitrogen (−196 °C) and subsequently stored at −30 °C. After thawing, the leaf osmotic potential (Ψ_{os}) was measured in the extracted sap using a WESCOR 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA), according to Gucci et al. [49]. The leaf osmotic potential at full turgor (Ψ_{100s}) was estimated as indicated above for Ψ_{os}, after placed in distilled water overnight to reach full saturation. The leaf turgor potential (Ψ_t) was estimated as the difference between leaf water potential (Ψ_{leaf}) and leaf osmotic potential (Ψ_{os}).

2.4. Gas Exchange and Chlorophyll Fluorescence Parameters

Leaf gas exchange and chlorophyll fluorescence were measured simultaneously at midday throughout the experiment using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA), fitted with an infrared gas analyzer attached to a leaf chamber fluorometer (LCF) (6400-40B, 2 cm^2 leaf area, Licor Bioscience, Inc., Lincoln, NE, USA). The reference CO_2, photosynthetically active radiation (PAR) and speed of the circulating air flow inside the system were set at 400 ppm, at 2000 µmol m^{-2} s^{-1} and at 500 µmol s^{-1}, respectively. The leaf photosynthetic rate (P_n), stomatal conductance (g_s), internal CO_2 concentration (C_i), the excitation capture efficiency of open centers (F_v'/F_m'), the effective quantum efficiency of photosystem II (Phi PSII), photochemical quenching coefficient (qP) and the electron transport rate (ETR) were measured [50]. The intrinsic water use efficiency (WUE_i) was determined as the P_n/g_s ratio, and the photosynthetic efficiency was expressed as the relationship between the degree of stomatal opening necessary to reach a certain level of photosynthesis.

2.5. Qualitative and Quantitative Analysis of Phytohormones and Chlorophyll Content in Leaves

Analytical standards of the phytohormones 1-aminocyclopropane-1-carboxylic acid, giberellic-5 acid, trans-Zeatin glucoside, abscisic acid, salicylic acid and scopoletin were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). trans-Zeatin, trans-Zeatin riboside and [2H5]-trans-Zeatin were obtained from Olchemlm (Olomouc, Czech Republic). Ethanol, Water LC-MS quality, dimethyl sulfoxide, formic acid and methanol were bought from Panreac (Barcelona, Spain). Acetonitrile was from J.T. Baker (Thermo Fisher Scientific Inc., Waltham, MA, USA).

For the identification and quantification of hormones, 0.1g of fresh leaves from 6 samples per treatment were crushed in a mortar with liquid nitrogen and stored at −80 °C. Then, they were vortexed with 0.5 mL 80% methanol/water (*v*/*v*) and incubated at 4 °C during 30 min and finally centrifuged at 15,000 rpm (20,627× *g*), at 4 °C for 15 min. The supernatant was kept in ice and then it was further extracted with 0.5 mL 80% methanol/water (*v*/*v*) after being incubated and centrifuged under the same conditions described above. Finally, both supernatants from the two previous extractions were passed through Chromafix C_{18} solid phase extraction cartridge (Macherey Nagel, Düren, Germany) (previously activated with 3 mL 80% methanol/water (*v*/*v*). The eluted sample was concentrated to dryness by the use of a rotary vacuum evaporator during approximately 3 h (Speedvac, Thermo, Waltham, MA, USA). Then, the dry residue was resuspended with 200 µL de 20% metanol/water (*v*/*v*), sonicated for 8 min and filtrated through 0.45 µm polyethersulfone filter (Millipore) and finally injected in a ultra-high-performance liquid chromatography (UHPLC) coupled triple quadrupole mass spectrometry (UHPLC-ESI-QqQ-MS/MS) for qualitative and quantitative analysis [51].

Chromatographic separation of phytohormones and the phytoalexin scopoletine was performed by a method previously described by Albacete et al., 2008 with slight modifications. Briefly, we used a UHPLC coupled to a 6460 UHPLC-ESI-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), using a BEH C_{18} analytical column (2.1 × 100 mm, 1.7 µm) (Waters, Milford, MA, USA). Mobile phases A (H_2O) contained 0.01% formic acid (v/v) and B acetonitrile. The flow rate was 0.2 mL/min using a linear gradient scheme: (t; %B): (0.0; 19.00), (2.5; 90.00), (4.5; 90.00), (6.00; 19.00), (8.00; 19.00). The injection volume was 10 µL. The column temperatures were 40 °C. The operating conditions for the ionization source were as follows: Gas flow: 8 L/min, Nebulizer: 45 psi, Capillary Voltage: 4000 V (positive mode) and 2750 V (negative mode), Nozzle Voltage: 1000 V (positive mode) and 1500 V (negative mode), Gas Temperature: 300 °C, Sheath Gas Temperature: 375 °C and Jetstream Gas Flow: 11 L/min. The ion optics and fragmentation conditions are detailed in Table 2. Data acquisition and processing were performed using Mass Hunter software version B.08.00 (Agilent Technologies). The quantification of the phytohormones and scopoletin detected in the samples was performed according to standard curves freshly prepared each day of analysis.

Table 2. Ultra-high-performance liquid chromatography (UHPLC) coupled triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS) parameters for the identification, identity confirmation (second MRM transition) and quantification of the phytohormones.

Hormone ^{Z}	Retention Time (min)	Ionization Mode	Parent Ion (m/z)	Ion Fragments (m/z)	Fragmentor (V)	Collision Energy (V)
ACC	1.312	Positive	102.1	56.0 ^{Y}	80	15
				28.0 ^{X}	80	15
tZ	1.724	Positive	220.2	202.0	80	15
				136.0	80	15
tZdeuter	1.744	Positive	225.2	136.3	80	15
tZ-GLC	1.742	Positive	382.4	220.0	80	15
				202.0	80	15
tZ-Rib	1.743	Positive	352.4	219.7	80	15
				136.0	80	15
SC	2.802	Positive	193.2	132.5	80	20
				149.1	80	20
GA5	3.095	Negative	329.4	145.0	80	39
				285.0	80	18
ABA	3.130	Negative	263.3	152.9	80	14
				204.1	80	18
SA	3.219	Negative	137.1	93.2	80	15
				65.4	80	15

^{Z} ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; GA5, giberellic-5 acid; SA, salicylic acid; SC, scopoletin; tZ, trans-zeatin; Tz-Glc, trans-zeatin glucoside; Tz-Rib, trans-zeatin riboside. ^{Y} MRM transition for quantification. ^{X} MRM transition for confirmation.

At the end of the experiment, the chlorophyll content was assayed according to Inskeep and Bloom [52] in the leaves of four plants per treatment. The extraction was made from 50 mg of fresh material in 5 mL of 80% acetone in the dark at 4 °C. The extract was read at 647 nm for chlorophyll-a, and 664 nm for chlorophyll-b in an Uvikon 940 spectrophotometer (Kontron Instruments AG, Zürich, Switzerland).

2.6. Determination of Mineral Content in Leaves and Plant Canopy

The inorganic mineral content of dry leaves was determined at the end of the experiment in three plants per treatment (one sample per replication) by means of emission spectrophotometry. The leaves were oven dried at 80 °C, ground, and sieved through a 2-mm nylon mesh before analysis. A chemical analysis of water irrigation treatments was performed. The nutrient concentrations were determined in an extract digested with HNO_3:$HClO_4$ (2:1, v/v) using an inductively coupled plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL). At the end of the experiment, the plant canopy was determined in both species by measuring height and width from the top, selecting nine representative plants per treatment (three plants per replication).

2.7. Statistics

In the experiment, all plants (n = 72) were randomly assigned to each treatment, with three replications for each treatment. The data were analyzed by one-way ANOVA and two-way ANOVA using IBM SPSS Statistics 25. The independent variables were irrigation water and species. Treatment means were separated with Duncan's multiple range test ($p \leq 0.05$).

3. Results

3.1. Plant Water Relations

Throughout the experiment, the highest values of leaf water potential and osmotic water potential were found in *Crithmum* plants, with no differences in the osmotic water potential by the type of water. The lowest values of these parameters were found in Atriplex plants, especially in those irrigated with ROB (Figure 1A,B). Atriplex plants showed also the lowest values of osmotic potential at full turgor during the experiment (Figure 1C). In general, leaf turgor potential was higher in Atriplex plants than in *Crithmum*, while there were differences only by the irrigation type at the beginning of the experiment, ROB treatment showing higher values than RWW treatment (Figure 1D).

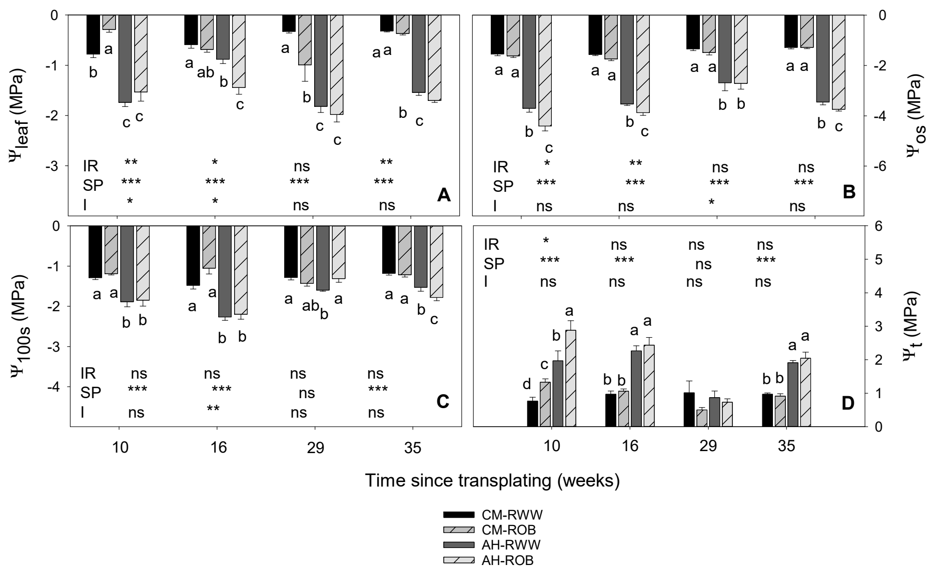

Figure 1. Leaf water potential (Ψ_{leaf}) (**A**), osmotic water potential (Ψ_{os}) (**B**), osmotic water potential at full turgor (Ψ_{100s}) (**C**) and leaf turgor potential (Ψ_t) (**D**) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one way and two-way ANOVA tests in these parameters, for independent variables (irrigation, IR, and species, SP) and their interaction (I). Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

3.2. Gas Exchange and Chlorophyll Fluorescence Parameters

In both species, the irrigation with ROB induced a decrease in g_s compared with the RWW treatment during the first half of the experiment, showing *Atriplex* irrigated with ROB the lowest values (Table 3). In fact, regardless the type of irrigation, the lowest gs values were found in *Atriplex*. Nevertheless, there were no differences in P_n by irrigation type, except at the beginning of the experiment (week 10), when irrigation with ROB decreased P_n in both species, being this decrease only significant for *Atriplex* plants (Table 3). Regardless the type of water used, *Atriplex* showed the highest values of P_n at week 12 and 16, while *Crithmum* showed the highest ones at the end of the experiment. The intrinsic water use efficiency (WUEi) barely experienced variations resulting from the type of irrigation water. Despite these unclear variations, in general terms, *Atriplex* plants showed a higher WUEi than *Crithmum* (Table 3).

The intercellular CO_2 (C_i) in both species was hardly affected by the type of water used during the experiment (Figure 2A).

Regardless the irrigation type, *Crithmum* showed higher C_i values than *Atriplex*. No statistical differences in $F_{v'}/F_{m'}$ were observed by the type of water used during the experiment, while irrespective of the irrigation type, *Crithmum* showed the highest values throughout almost the whole experiment (Figure 2B). Regardless the type of water used, *Atriplex* showed increased PhiPSII, qP and ETR values most of the weeks (Figure 2C–E). The photosynthetic efficiency showed a similar trend line for both irrigation treatments in *Crithmum*, since for the same photosynthesis value, the stomatal conductance was similar, although from 12 μmol m^{-2} s^{-1} of P_n approximately, plants irrigated with ROB showed a g_s slightly higher (Figure 3A). In *Atriplex*, plants irrigated with ROB had a lower photosynthetic efficiency than those irrigated with the RWW, since from 13 μmol m^{-2} s^{-1} of P_n approximately, for the same P_n value, the stomatal conductance of plants irrigated with ROB was higher (Figure 3B).

Table 3. Stomatal conductance (g_s), net photosynthetic rate (P_n), and intrinsic water use efficiency (WUE_i) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (Irrigation, IR, and species, SP) and their interaction (I). Values are means of nine samples.

g_s (mmol m^{-2} s^{-1})					
Week	**10**	**12**	**14**	**16**	**19**
CM-RWW	268.4 a	210.9 a	199.2 b	110.5	333.3 a
CM-ROB	211.9 ab	177.3 ab	299.1 a	122.8	285.4 ab
AH-RWW	152.8 bc	123.6 bc	120.4 bc	94.1	204.4 b
AH-ROB	105.0 c	58.4 c	95.5 c	92.4	196.7 b
Sig.	**	**	***	ns	*
IR	*	*	ns	ns	ns
SP	**	**	***	ns	**
I	ns	ns	*	ns	ns
P_n (μmol m^{-2} s^{-1})					
Week	**10**	**12**	**14**	**16**	**19**
CM-RWW	16.93 a	10.47 b	10.76	6.587 b	26.69 a
CM-ROB	12.90 ab	9.56 b	14.10	7.104 b	24.18 ab
AH-RWW	18.82 a	16.89 a	14.52	14.68 a	18.21 b
AH-ROB	10.17 b	15.60 a	11.64	14.42 a	17.57 b
Sig.	*	**	ns	***	*
IR	***	ns	ns	ns	ns
SP	ns	***	ns	***	**
I	*	ns	ns	ns	ns
WUEi (P_n/g_s)					
Week	**10**	**12**	**14**	**16**	**19**
CM-RWW	69.0 b	51.4 b	54.8 b	62.2 b	80.9
CM-ROB	65.3 b	105.8 b	50.2 b	60.2 b	87.6
AH-RWW	119.1 a	157.1 b	113.6 a	157.6 a	92.2
AH-ROB	91.0 b	369.6 a	120.0 a	155.9 a	93.5
Sig.	***	**	***	***	ns
IR	*	*	ns	ns	ns
SP	***	**	***	***	ns
I	*	ns	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

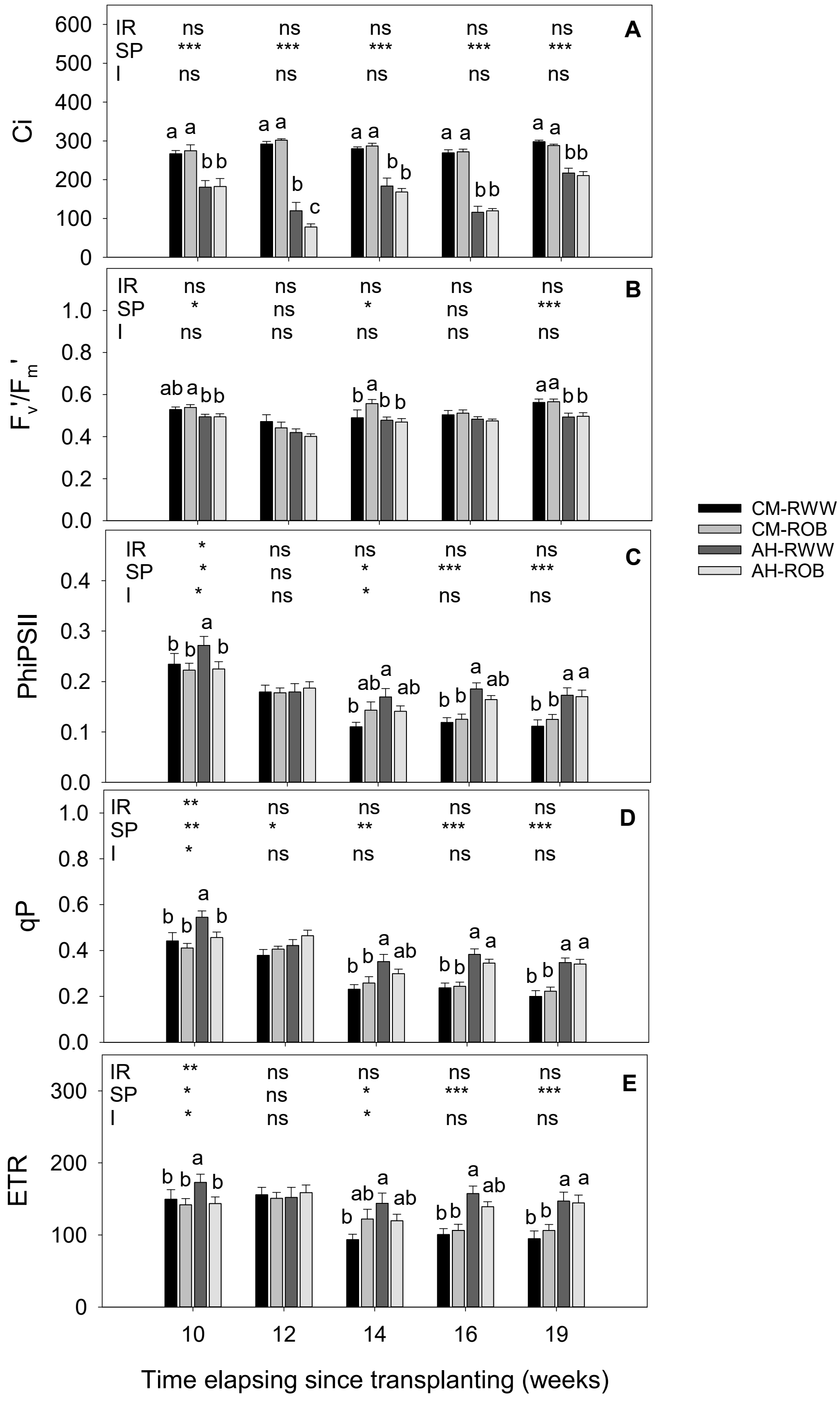

Figure 2. Internal CO_2 concentration (C_i) (**A**); The excitation capture efficiency of open centers (F_v'/F_m') (**B**); PSII effective quantum yield (PhiPSII) (**C**); Photochemical quenching coefficient (qP) (**D**); and the apparent electron transport rate (ETR) (**E**) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH) irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one way and two-way ANOVA tests in these parameters, for independent variables (irrigation, IR, and species, SP) and their interaction (I). Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

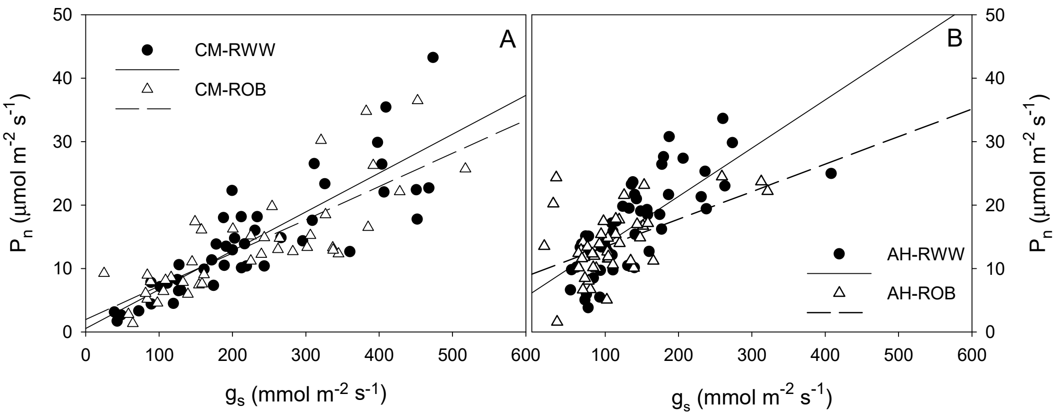

Figure 3. Photosynthetic efficiency as the relationship between photosynthesis and stomatal conductance in *Crithmum maritimum* (CM) (**A**) and *Atriplex halimus* (AH) (**B**) irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB).

3.3. Phytohormones and Chlorophyll Content in Leaves

The leaf chlorophyll content was not affected by the type of water although the highest values were found in *Atriplex* (Table 4).

Table 4. Leaf chlorophyll content (Chl A, Chl B and Chl T) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (Irrigation, IR, and species, SP) and their interaction (I). Values are means of four samples.

	Leaf Mineral Content (mg g^{-1})		
	Chl A	**Chl B**	**Chl T**
CM-RWW	0.539 b	0.171 b	0.710 b
CM-ROB	0.719 b	0.242 b	0.961 b
AH-RWW	2.048 a	0.549 a	2.597 a
AH-ROB	2.107 a	0.556 a	2.663 a
Sig.	***	**	***
IR	ns	ns	ns
SP	**	*	*
I	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

Several phytohormones were identified in leaves in both species: the precursor of Ethylene, 1-Aminocyclopropane-1-carboxylic acid (ACC), cytokinins (TZ, TZ-rib and TZ-glc), gibberellins (GA5), abscisic acid (ABA), salicylic acid (SA) and the phytoalexin scopoletin (SC).

Some of them (ACC, TZ-rib and TZ-glc) were modified by the type of irrigation water. However, they were affected differently depending on the species. A higher ACC and TZ-glc concentration and lower ABA concentration were observed in *Crithmum* plants irrigated with ROB compared with those irrigated with water from RWW (Figure 4). *Atriplex* plants irrigated with ROB showed lower concentration of TZ-rib and SC compared with those irrigated with water from RWW (Figure 4). Regarding only species, higher values of ACC, TZ-glc and SA were found in *Crithmum* than in *Atriplex* plants, while higher values of TZ, GA5, ABA and the phytoalexin SC were found in *Atriplex* than in *Crithmum* (Figure 4).

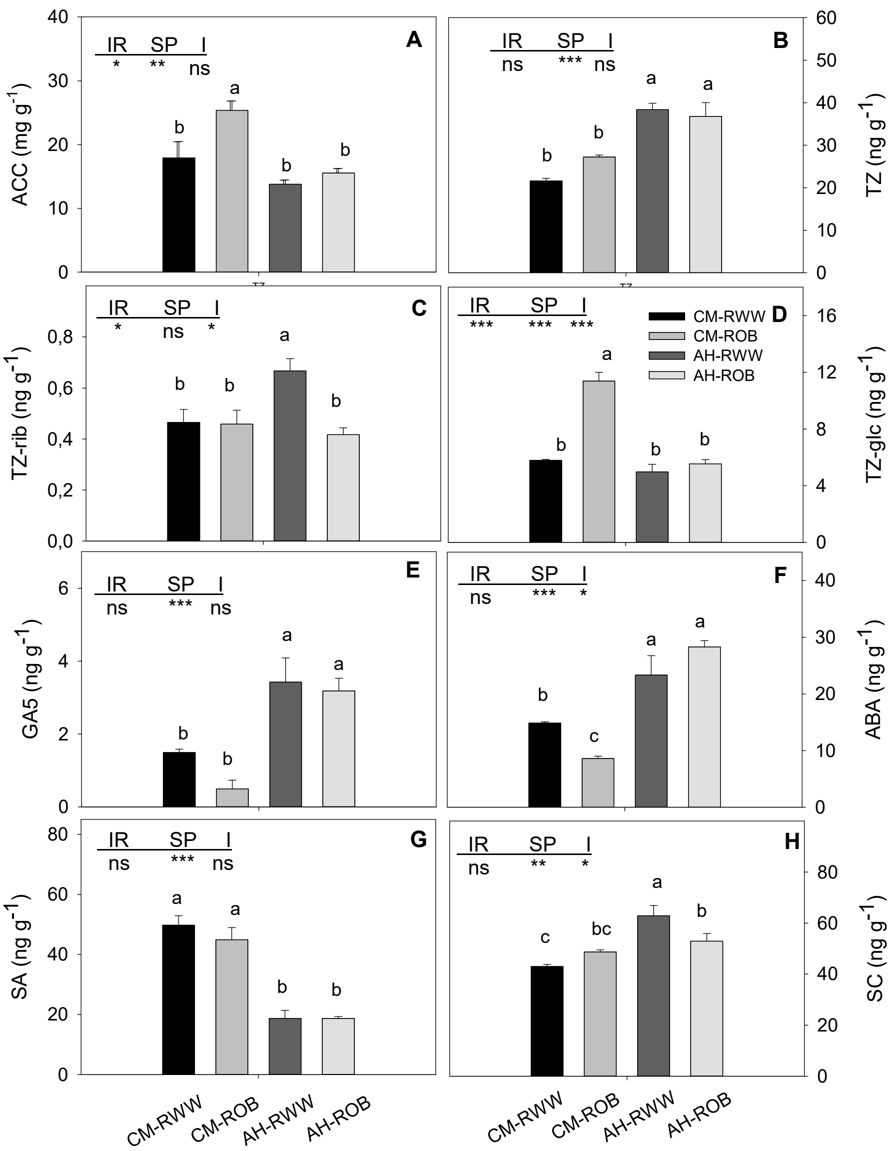

Figure 4. 1-Aminocyclopropane-1-carboxylic acid (ACC) (**A**), cytokinins (TZ) (**B**), (TZ-rib) (**C**), (TZ-glc) (**D**), gibberellins (GA5) (**E**), abscisic acid (ABA) (**F**), salicylic acid (SA) (**G**) and scopoletin (SC) (**H**), in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH) irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one way and two-way ANOVA tests in these parameters, for independent variables (irrigation, IR, and species, SP) and their interaction (I). Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

3.4. *Leaf Mineral Concentration and Plant Canopy Development*

Regardless the species, the concentrations of B, Ca, Fe, K, Mg, Na, P and Zn ions in leaves did not show any significant changes by the type of water used (Table 5). Only B concentration in *Crithmum* leaves was lower after irrigating with ROB. Regardless the type of water, a higher amount of B and Ca ions accumulated in *Crithmum* leaves than in *Atriplex*, and a higher amount of K, Mg, Na and Zn accumulated in *Atriplex* leaves than in *Crithmum* (Table 5).

Table 5. Leaf mineral concentration in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (irrigation, IR, and species, SP) and their interaction (I). Values are means of three samples.

ppm	B	Ca	Fe	K	Mg	Na	P	Zn
CM-RWW	329.0 a	45,143.3 a	126.5	34,673.3 b	3497.0 b	24,931.7 b	3313.2	31.1 b
CM-ROB	263.5 b	44,260.0 a	112.9	19,582.8 b	3343.0 b	17,809.2 b	3324.8	40.4 ab
AH-RWW	248.9 b	11,013.3 b	92.6	76,266.7 a	9628.3 a	56,661.7 a	3218.5	52.2 a
AH-ROB	248.0 b	9726.7 b	94.5	65,616.7 a	9315.0 a	71816.7 a	2879.2	50.1 a
Sig.	*	*	ns	**	***	**	ns	ns
IR	ns	ns	ns	ns	ns	ns	ns	ns
SP	*	**	ns	***	***	***	ns	*
I	ns	ns	ns	ns	ns	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

At the end of the experiment, the structure of the plant or its canopy was not statistically affected by the type of water used (Table 6). *Atriplex* showed a slight reduction of the canopy caused by the irrigation with brine but with no significant differences, while *Crithmum* reached numerically greater canopy by the irrigation with ROB, but not statistically. Regardless the type of water used, *Atriplex* plants developed a greater canopy than *Crithmum* (Table 6).

Table 6. Plant canopy determined by measuring height and width of plants in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (irrigation, IR, and species, SP) and their interaction (I). Values are means of three samples.

	CANOPY (cm)		
	Height (H)	**Width (W)**	**H × W**
CM-RWW	24.44 b	28.94 b	770.50 c
CM-ROB	28.72 b	33.94 b	1008.17 c
AH-RWW	65.94 a	104.56 a	6880.00 a
AH-ROB	57.72 a	97.72 a	5723.67 ab
	***	***	***
IR	ns	ns	ns
SP	***	***	***
I	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

4. Discussion

Crithmum maritimum and *Atriplex halimus* are naturally salt-tolerant plants, being great candidates to replace conventional edible sensitive plants in marginal and degraded lands [53]. *Atriplex* may even be useful for phytoremediation of former mining areas [54]. Although the salinity range in our experiment was moderately low, the use of reclaimed wastewater and brine has scarcely been proved in these plants. It is not clear whether they can tolerate other confounding factors and impurities associated with wastewater [55]. Regarding water relations, both species performed differently to the use of saline effluent from reverse osmosis (RO) brine. The reduction of leaf water potential by the irrigation of ROB was more evident in *Atriplex* plants, which reduced the osmotic leaf potential to maintain leaf turgor values similar to those plants irrigated with water from RWW. When water potential is suddenly reduced, osmotic adjustment occurs rapidly to allow partial turgor recovery and re-establishment of water potential gradient for water uptake, and the loosening ability of the cell wall increases [56]. Many plants accumulate organic osmolytes in their cytoplasm [57] to increase cellular water retention without affecting normal metabolic processes. However, *Crithmum* plants did not need to reduce the osmotic potential. ROB water seemed to decrease gas exchange, especially $g_{s,}$ in both plant species respect to the water from RWW during the first half of experiment, *Atriplex* in a greater extent. However, leaf chlorophyll content was not affected by the type of irrigation water, most halophyte plants are able to stabilize chloroplasts and thylakoids to protect photosynthesis mechanisms [58]. Little literature has been found about the role of the photosynthetic activity in these species [59,60]. Benzarti et al. [60] observed that g_s in *Atriplex portulacoides* was only reduced above 200 Mm ($\approx$18 dS m^{-1}) NaCl in the water. Although there were hardly any changes in the fluorescence parameters and WUEi by the type of water in these plants, the photosynthetic efficiency results indicated that *Atriplex* plants irrigated with RO brine needed to open more their stomas than those irrigated with RWW, to reach the same level of P_n. As a consequence, *Atriplex* plants had a greater loss of water and higher energy costs than *Crithmum* [61]. The greater concentration of mineral ions in brine water than in water from RWW, such as K, Mg, Na, S and P, did not lead to a higher leaf ion accumulation in both species. Nevertheless, each plant species had a different preference to accumulate ions in their leaves. *Crithmum* accumulated higher B and Ca content, the latter transported to the leaves by the transpiration process [62], suggesting a better flow of water to leaves, while *Atriplex* accumulated higher K, Mg, Na and Zn content. This indicated that each plant species had different mechanisms of ion uptake. In addition, both irrigation waters presented levels of heavy metals within the recommended concentration limits, although levels of salts such as Na, Cl and SO_4 were considered toxic for most crops [63–65].

Phytohormones have also important roles in salt stress tolerance [66,67]. The biosynthesis of ethylene is induced by many stresses, however, its role is controversial regarding salt stress [68]. Khan et al. [69] reported that increased ethylene biosynthesis in wheat was related to salt tolerance, while other authors claimed that its production might play a negative role in tomato growth, coinciding with an oxidative stress and leaf senescence [70,71]. In our experiment, the increase of ACC in *Crithmum* plants irrigated with ROB was not related to a negative response of plant physiology, since there was no evidence of oxidative stress or reduction of plant growth. A significant accumulation of ABA is essential to active plant protective mechanisms [72], which regulate leaf water potential and stomatal closing to avoid water losses [73]. It is well known that the increase of ABA synthesis and the decrease of TZ production is an effective defense mechanism of plants in response to salt stress [74]. However, in our experiment this behavior was not so clear in *Crithmum* plants, since RO brine did not induce the accumulation of ABA. *Atriplex* performed differently to *Crithmum*, since a lower leaf accumulation of TZ-rib and SC was observed in plants irrigated with brine compared to those irrigated with water from RWW. Scopoletin (phytoalexin) is a coumarin compound with antifungal properties and inhibitory effects on abiotic stresses [75]. Its accumulation has been correlated with resistance to stresses, such as dehydration and salt toxicity [75,76]. This fact might explain that the plants did not suffer salt stress or that salinity was not high enough to cause the accumulation of SC. Some studies showed that *Crithmum maritimum* is able to maintain growth at high salinity levels, even up to 340 mM NaCl [77,78]. However, Ben Hamed et al. [79] found that DW biomass of *Crithmum* begun to reduce even at 50 (4.5 dS m^{-1}) mM NaCl. In our case, the growth of both species was not affected by the different irrigation treatments, despite that the brine was 5.4 dS m^{-1} on average. In the *Atriplex* plants, irrigation with brine slightly reduced the canopy, surely due to lower photosynthetic efficiency showed in these plants. The physiological adaptations to salinity were more evident in *Atriplex* than in *Crithmum* plants to maintain growth, when they were irrigated with RO brine. Nedjimi and Daoud [80] and Boughalleb and Denden [81] showed an optimal plant growth in *Atriplex* at 50 mM Na_2SO_4, and at 100 mM NaCl, respectively, declining with a further increase in salinity.

5. Conclusions

In general, both species had a different physiological and nutritional response when they were irrigating with ROB. In the case of *Atriplex*, plants performed adaptations such as osmotic adjustment and stomatal regulation to maintain growth in comparison with *Crithmum*. In *Crithmum* plants irrigated with ROB, water status and photosynthetic activity performance, including photosynthetic efficiency, were similar to those irrigated with RWW. Nevertheless, the particular characteristics associated to this kind of waters did not seem to influence negatively the development of both species during the stabilization period. Therefore, reclaimed wastewater from tertiary effluence and reverse osmosis brine, such as the wastewater used in this experiment, could be an alternative to irrigation with conventional waters in both species for revegetation or soil preservation purposes.

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