Salts and nutrients present in regenerated waters induce changes in water relations, antioxidative metabolism, ion accumulation and restricted ion uptake in *Myrtus communis* L. plants.

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Abstract

The use of reclaimed water (RW) constitutes a valuable strategy for the efficient management of water and nutrients in landscaping. However, RW may contain levels of toxic ions, affecting plant production or quality, a very important aspect for ornamental plants. The present paper evaluates the effect of different quality RWs on physiological and biochemical parameters and the recovery capacity in *Myrtus communis* L. plants. *M. communis* plants were submitted to 3 irrigation treatments with RW from different sources (22 weeks): RW1 (1.7 dS m^{-1}), RW2 (4.0 dS m^{-1}) and RW3 (8.0 dS m^{-1}) and one control (C, 0.8 dS m^{-1}). During a recovery period of 11 weeks, all plants were irrigated with the control water. The RW treatments did not negatively affect plant growth, while RW2 even led to an increase in biomass. After recovery, only plants irrigated with RW3 showed some negative effects on growth, which was related to a decrease in the net photosynthesis rate, higher Na accumulation and a reduction in K levels. An increase in salinity was accompanied by decreases in leaf water potential, relative water content and gas exchange parameters, and increases in Na and Cl uptake. Plants accumulated Na in roots and restricted its translocation to the aerial part. The highest salinity levels produced oxidative stress, as seen from the rise in electrolyte leakage and lipid peroxidation. The use of regenerated water together with carefully managed drainage practices, which avoid the accumulation of salt by the substrate, will provide economic and environmental benefits.

Keywords: Reclaimed water; Ion transport; Oxidative stress; Recovery capacity; Gas exchange

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; DW, dry weight; EC, electrical conductivity; EL, electrolyte leakage; ET, evapotranspiration; GR, glutathione reductase; GPX, glutathione peroxidase; g_s, stomatal conductance; J, absorption rate by roots; MDHAR, monodehydroascorbate reductase; PAR, photosynthetic active radiation; POX, peroxidase; P_n, net photosynthesis rate; ROS, reactive oxygen species; RW, reclaimed water; RWC, relative water content; SOD, superoxide dismutase; WFC, weight at field capacity; Ψ_l, leaf water potential.

1. Introduction

Mediterranean areas are characterized by semiarid climatic conditions, with an average rainfall of 300 mm or less, where limited water availability is already a severe constraint to development. Therefore, the use of non-conventional water resources, such as reclaimed water (RW), is a common strategy for efficient water management (Yermiyahu et al. 2008). Moreover, economic benefits attributed primarily to the nutrient content of the RW have been suggested (Pedrero et al. 2010, Goméz-Bellot et al. 2013). Nevertheless, RW used for ornamental plant production has some peculiarities compared with the same practice applied in other agricultural fields (Lubello et al. 2004).

Disinfection is an important part of tertiary treatment, although the potential biological problems (microbial contaminants) associated with effluents applied to vegetable or fruits crops are not so important in the case of landscaping, where the most important aspect is visual appearance and ecological soundness (Gori et al, 2000). However, the high concentration of toxic elements present in these waters might cause damage and so decrease the quality of plants (Johnson and Parnell 1998).

Most landscape projects include a variety of species with different levels of tolerance to salinity (Franco et al. 2011, Sánchez-Blanco et al. 1991), while the response of species commonly as ornamental plants to irrigation with reclaimed wastewater varies (Fitzpatrick et al. 1986, Gori et al. 2000). For example, it was found that plant growth after three months of irrigation with wastewater was strongly dependent on the species used. It is therefore important to select among plants to be used, endemic salt-resistant species, including ornamental shrubs, as *Myrtus communis* L., which has special interest for landscaping projects and public areas (Navarro et al. 2009). However, the salinity tolerance of plants depends on the amount of water applied, especially when plants are grown in small commercial containers. In this respects, by controlling the leaching fraction it is possible to control salinity in the root zone (Bañón et al. 2011).

Salt stress is known to produce malfunctions in many physiological and metabolic processes with a resulting reduction in plant growth and productivity (Greenway and Munns 1980). However, irrigation management strategies, such as increased leaching, can partly minimise the negative effects of salinity (Bañón et al. 2011). The two main negative effects induced by salinity and which influence plant growth and development are osmotic stress (due to the decrease in the water potential of the root medium) and ion toxicity (associated with an excessive Cl, Na and B uptake and/or transport to aerial parts of the plant) which leads to Ca and K deficiency and other nutrient imbalances (Marschner 1995).

In addition to these stress effects, oxidative stress, which is mediated by an over-generation and accumulation of reactive oxygen species (ROS) at subcellular level, may also occur (Hernández et al. 1993, 1995, Corpas et al. 1993). These three factors contribute to the salt-induced symptoms and metabolic imbalances that can finally lead to membrane malfunction and cellular death (Hernandez et al. 2001, Parida et al. 2004). In order to cope with ROS, plants have developed a complex arsenal of defenses that include carotenoids, ascorbate, glutathione, tocopherols, anthocyanins and enzymes such as superoxide dismutase (SOD, EC1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidase (POX, EC. 1.11.1.7), as well as enzymes involved in the ascorbate-glutathione cycle (ASC-GSHcycle): ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC1.8.5.1), monodehydroascorbate reductase (MDHAR, EC1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2) (Noctor and Foyer 2008).

Little information is available on the effect of abiotic stress conditions on antioxidative metabolism in ornamental plants (Alguacil et al. 2003, Caravaca et al. 2005, Roldán et al. 2008, de Olivieira Jucoski et al. 2013) and no information is available on ornamental plants subjected to salt stress. It has been reported that the presence of high levels of antioxidants, constitutive or induced, can

induce greater resistance to oxidative damage in plants (Hernández et al. 2000). The activities of some antioxidative enzymes in plants increase under salt stress and a correlation between these enzymes levels and salt tolerance has been reported (Mittova et al. 2003, Parida et al. 2004, López-Gómez et al. 2007).

The intensity and / or duration of stresses affect both the velocity and the extent of recovery after stress relief (Chaves et al. 2009). In general, when a severe stress is imposed, recovery can be partial and the maximum photosynthetic rates are sometimes never reached. It is possible that salinity irreversibly affects the photosynthetic capacity and accelerates leaf senescence (Chaves et al. 2011). Incomplete photosynthesis recovery has been linked to sustained oxidative damage (Galmés et al. 2007) and it is closely related to plant ability to avoid or to repair membrane damage when stress intensifies (Chaves and Oliveira 2004).

In this study, the effect of long-term treatment by RW with different levels of salinity, on gas exchange, water relations, mineral uptake and nutrition and antioxidative metabolism in *Myrtus communis* L. plants grown under controlled environmental conditions was studied. The relevance of studying the plant capacity to recover following salinity relief has also been taken into account. We investigate possible relations between Na and Cl uptake and partitioning between organs in order to evaluate whether the response of plants might be related to the retention of these ions in the roots.

2. Material and Methods

2.1. Plant and experimental conditions

Single rooted cuttings (120) of native myrtle (*Myrtus communis* L) were transplanted into 14 x 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). The experiment was conducted in a controlled growth chamber, where the environmental conditions were selected to simulate natural conditions. The temperature in the canopy was 23ºC during the light phase and 18ºC during darkness. Relative humidity 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) and the daily light integral was 20.16 mol m^{-2} d^{-1}.

2.2. Experimental design and treatments

M. communis plants were exposed to four irrigation treatments, using water from different sources for 22 weeks (Phase I). The irrigation treatments consisted of a control, where the electrical conductivity (EC) of the water was 0.8 dS m^{-1} (indicating no use restrictions or slight restrictions according to FAO classifications; FAO, 2003) and three reclaimed water treatments. In this case, the water came from three sewage treatment plants located in the Province of Murcia (Spain), namely: RW1 (EC 1.7 dS m^{-1}) from Jumilla; RW2 (4.0 dS m^{-1}) from Campotejar and RW3 (8.0 dS m^{-1}) from Mazarrón. FAO classifications indicated severe restrictions on the use of the last two types of water. All three waste

water treatment plants applied a conventional activated-sludge process, followed by ultraviolet radiation as the tertiary treatment. At the start of the experimental period, the pH and concentrations of Na^+, Cl^- K, Ca and B^{3+} ions, in each irrigation water were analysed. The results are shown in Table 1. After 22 weeks (Phase I), all plants were exposed to an 11-week recovery period (Phase II), when the plants were irrigated with the same water as used for the control plants. Throughout the 33 weeks of the experiment, all plants were irrigated twice a week to above container capacity. At the start of the experimental period the maximum water holding capacity of the substrate was determined for each individual pot and considered as the weight at field capacity (WFC). The volume of irrigation water applied was determined in each treatment as the point when the leaching fraction reached 10% (v/v) of applied water in the control treatment, 25% in RW1, 40% in RW2, or 55% of the applied water in RW3. Each plant (n = 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 treatment) was calculated and added to each plant. Average values of water added to each pot during the whole experimental period was 15.1 l for the control and 13.7, 16.6 and 13.9 l for RW1, RW2 and RW3 plants, respectively.

2.3. Growth and plant water measurements

At the end of Phase I and Phase II, the substrate was gently washed from the roots of eight plants per treatment and each plant was divided into shoots (leaves and stem) and roots. These were oven-dried at 80ºC until they reached a constant weight to measure their respective dry weights (DW). Leaf areas were determined for the same plants before drying, using a leaf area meter (AM 200; ADC BioScientific Ltd., Hoddesdon, UK). Plant height was periodically measured in 20 plants per treatment throughout the experimental period. To assess the compactness of the plants, the ratio of leaf area to plant height was calculated in 8 plants per treatment at the end of Phase I and Phase II by dividing leaf area by the respective plant heights.

Evapotranspiration (ET) was measured gravimetrically throughout the experimental period in 20 plants per treatment, based on the difference in weights (weight after irrigation and weight before irrigating again), using a balance (Analytical Sartorius, Model 5201; capacity 5.2 kg and accuracy of 0.01 g).

Seasonal changes in leaf water potential (ψ_l), relative water content (RWC), stomatal conductance (g_s) and net photosynthesis rate (P_n) were determined in six plants per treatment during the central hours of illumination. ψ_l was estimated 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} (Turner, 1988). The RWC of leaves was measured according to Barrs (1968). g_s and P_n were determined in attached leaves using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA).

2.4. Determination of inorganic solutes

The inorganic solute concentrations and EC values of each irrigation water were measured at the start of the experiment. At the end of the salinity and recovery periods (Phase I and Phase II), eight plants per treatment were separated into leaves, stem and roots, washed with distilled water, dried at 70ºC, and stored at room temperature for inorganic solute analyses. The concentrations of Cl^-, Na^+, B^{3+}, K^+, and Ca^{2+} ions were assayed as described in Álvarez and Sánchez-Blanco (2014). The absorption rate of Na^+ and Cl^- ions by the root system at the end of Phase I (J) was calculated by considering the total salt content of eight plants per treatment at harvest, expressed as mmol Na^+ and Cl^- ,and the mean root weight, using the formula described by Pitman (1975).

2.5. Enzyme extraction and analysis

All operations were performed at 4ºC. 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% PVPP (w/v) and 0.2% (v/v) Triton X-100. For the APX activity, 20 mM sodium ascorbate was added to the extraction buffer. The extracts were filtered through two layers of nylon cloth and centrifuged at 10000 g for 15 min. The supernatant fraction was filtered on Sephadex G-25 NAP columns equilibrated with the same buffer used for homogenization and used for the enzymatic determinations. For the APX activity, 2 mM sodium ascorbate was added to the equilibration buffer. The activities of the ASC-GSH cycle enzymes, POX, CAT, and SOD were assayed as described in Barba-Espín et al. (2011). The extent of lipid peroxidation in leaves was analysed only after the stress period and was estimated by determining the concentration of substances reacting with thiobarbituric acid (TBARS) (López-Gómez et al. 2007). The rate of passive electrolyte leakage from stress-sensitive plant tissue can be used as a measure of alterations of membrane permeability. In our case, ion leakage was estimated at the end of Phase I and Phase II, according to the method described by Lafuente et al. (1991).

2.6. Statistical analyses of data

In the experiment 30 plants were randomly attributed to each treatment. The data were analysed 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. Statistical comparisons were considered significant at $P \leq 0.05$.

3. Results

3.1. Plant Growth, water status and gas exchange

The treatment of *Myrtus communis* L. plants up to 22 weeks (Phase I) with regenerated waste-water containing different salinity levels had no negative effects on biomass accumulation, and in the case of RW2 even resulted in a significant increase in the dry weight (DW) of shoots and roots compared with control plants (Fig. 1A). In addition, the treatment containing the highest salt concentration showed an increase in root DW compared to control plants (32%), although such differences were not significant. After 11 weeks of watering with control water (Phase II), RW2 plants again showed the highest root DW values (Fig. 1B), although such increases compared to control plants were lower than at the end of the Phase I and the differences in shoot DW between RW2 and control disappeared after Phase II. The growth of plants previously treated with the highest salinity level was affected after the recovery Phase since the shoot growth was reduced by 28% compared to control plants (Fig. 1B).

Another parameter used to analyze the effect of irrigation with reclaimed water in plant growth was the evolution in height during the experiment (Fig. 2A). According to this parameter, in general, all plants irrigated with RW were shorter than control plants from the beginning of the experiment, which remained so during the two phases of the experiment, an effect that was more pronounced in RW3 plants. RW2 produced the highest leaf area/plant height ratios at the end of both phases and RW3 plants had lower values than the controls after Phase II (Fig. 2B).

In all RW treatments ψ_l was lower than in the control treatment, especially in the RW3 treatment (Fig. 3A), which showed the most negative values from the first weeks after the beginning of the experiment, while RW1 and RW2 plants showed similar values (about -0.9 MPa). However, as the experiment progressed, differences in the ψ_l values widened until the end of Phase I, depending on the salinity levels. During the recovery period (Phase II), ψ_l showed a trend to recover in all plants previously irrigated with RW, although they did not reach the control values, and RW3 plants still presented the most negative values (Fig. 3A).

As regards the evolution of RWC, the decrease in leaf water potential caused by salinity was accompanied by leaf dehydration. In RW3 plants RWC displayed a similar behavior to ψ_l, these plants presenting the lowest RWC values in both phases of the experiment (Fig. 3B). However, the RWC values for RW1 plants were lower than those of RW2 plants, unlike in the case of ψ_l, whose values in RW1 were less negatives than in RW2. At the end of Phase II, only RW3 plants showed significant differences in RWC in relation to control plants (Fig. 3 B).

The gas exchange parameters were affected in plants irrigated with RW (Fig. 4). A reduction in g_s and P_n compared with the control was observed in all plants irrigated with RW from the beginning of the Phase I, which was maintained until the end of this phase. RW1 and RW3 plants were the most affected in this respects (Fig. 4A, B). During the recovery phase the reduction in these parameters was maintained in RW1 and RW3 plants, while in RW2 plants, P_n had similar values to control plants despite their lower g_s values than the control (Fig. 4A, B). Evapotranspiration (ET) was higher in control plants throughout the experiment and values fell proportionally to the imposed salt level in the RW treatments (Fig. 4C).

3.2. Uptake Rates and Mineral Content Distribution

The rates of both Na^+ and Cl^- absorption by roots (J) during Phase I increased with the Na and Cl concentrations of the irrigation waters (Fig. 5A). The ability of *M. communis* to restrict the entry of Na or Cl through thee roots was investigated by calculating the slope of the linear regression between the increasing Na and Cl concentration in the water and their relative absorption rate by the root system during Phase I (Fig. 5B). The absorption rates of Na showed a higher slope than Cl, which means that myrtle plants are able to restrict the Cl-uptake by roots to a greater extent than Na.

The Na and Cl concentrations measures in leaves, stems and roots at the end of Phase I increased with their relative concentrations increased in the irrigation water (Fig. 6A, B). The tendency of *M. communis* to accumulate Na and Cl preferentially in a given part of the plant (leaves, stem or roots) was investigated by calculating the slope of the linear regression between the Na and Cl concentration in plant tissue and their relative concentrations in the irrigation water (Table 2). The accumulation of Na in the root system showed a higher slope compared with the leaves and stem, while in the case of Cl accumulation no significant differences between slopes were found. This means that the transport of Na from the roots to the stems and leaves was restricted and that the distribution of each toxic ion differed.

At the end of Phase I, the concentration of B in the leaves and stem of the RW2 and RW3 plants was higher than in control plants (Fig. 6C). Surprisingly, the concentration of K increased in the leaves of RW2 plants (up to 24%), whereas a 25% of decrease was observed in leaves from plants treated with the most saline RW (Fig. 6D). As regards Ca, its concentration increased by nearly 50% in leaves from plants irrigated with the moderate and high salinity treatments (Fig. 6E).

After the recovery period, sodium concentrations were still statistically higher in the leaves and roots of RW1 plants as well as in all parts of the RW3 plants. However, no statistical differences were observed in RW2 plants compared with controls (Fig. 6F). Cl levels were similar in all treatments with no differences in any part of the plants (Fig. 6G). Plants submitted to the three reclaimed water irrigation treatments had higher B concentrations in all plant tissues than the control plants, RW1 and RW3 plants containing the highest concentrations (Fig. 6H). A general decrease in K concentrations occurred in the plants of all three RW treatments in relation to control plants, except in RW2, which showed similar leaf K values to the control plants (Fig. 6I). Finally, Ca levels increased in the roots from RW1 plants and in the leaves from RW3 plants but decreased in stems from RW2 plants, all in relation with the controls (Fig. 6J).

3.3. Antioxidative metabolism

The RW with the highest salt content induced an oxidative damage in leaves, as monitored by the increase in some oxidative stress parameters, such as electrolyte leakage (EL) and lipid peroxidation (Fig. 7). At the end of Phase I, plants treated with RW1 and RW3 showed increased EL values, being the rises of about 40% and 2-fold, respectively, in relation to control plants (Fig. 7). At the end of the

recovery period, despite the reduction in substrate EC, plants previously treated with the highest salt concentration showed a significant increase in EL, reaching values up to 1.9 times those of control plants. At the end of Phase I, the data of EL correlated with lipid peroxidation values, but in this case, the differences in lipid peroxidation were less pronounced than that in EL, and only RW3 plants had higher lipid peroxidation values than the controls.

The activity of the antioxidant enzymes, catalase, SOD, POX and the ASC-GSH cycle enzymes are shown in Table 3. Of ASC-GSH cycle enzymes, DHAR and MDHAR activities, which are involved in ascorbate recycling, were not detected. We only observed an effect in the enzyme activities in plants treated with the lowest and the highest salinity levels, but not in plants subjected to the intermediate salt level. Also in plants subjected to the RW1 or RW3 treatment, significant increases in SOD and POX occurred. The increase in SOD was about 20%, and, also worthy of mention is the pronounced increase in POX activity observed in RW1 plants (up to 1,9-fold) and especially in RW3 plants (up to 3.2-fold) (Table 3). Finally, APX showed a significant decrease of about 28%, but only in RW1 plants (Table 3).

4. Discussion

Alternative water sources, such as reclaimed water, contain different nutrients that may be beneficial for plant growth, providing a possibility to reduce the fertilization, making their use an environmentally safe water management strategy requirement (Gori et al. 2000). Indeed, RW constitutes a significant plant nutrient source for soils of low fertility. It can contribute to increasing P, K, Fe and S concentrations and also the accumulation of organic matter (Kalavrouziotis et al. 2005, Rattan et al. 2005). In our experiment, the analysis of regenerated waste water revealed high levels of B, K and Ca (Gómez-Bellot et al. 2013), and as a result their concentrations in plants could increase.

The different RW used in the present study have different salt contents depending on their origin, and may include toxic ions such as Na, Cl or B. However, the accumulated levels of these elements in plants during the application of different treatments in Phase I were not sufficient to affect the growth parameters of the plants. In this sense, after 22 weeks of irrigation with RW of different salinity levels, were no effects of note on total dry weight, while the plants irrigated with RW2 even exhibited significantly higher growth than those irrigated with control water, as previously reported for other woody species (Davies et al. 2005, Zekri and Koo 1990). However, salinity may affect the aesthetic value of plants, which is a very important trait for ornamental plants (Álvarez et al. 2012). In our conditions, RW2 improved the relationship between leaf area and plant height (compactness), whereas, RW3 reduced this parameter. This means that the level of salinity is important and irrigation with reclaimed water has the potential to improve crop quality in woody ornamentals by reducing excessive vigour and promoting a more compact habit (Franco et al. 2006). These results suggest a specific response of plants to the different regenerated waters used. In this response to salinity, the presence of other nutrients in the water may also be involved. For example, the regenerated waste water may contain cations and anions, such as K, Ca, B, HPO_4^{2-}, NO_3^-, that can interfere with Na and Cl uptake by plants.

In addition, visual symptoms related to B accumulation were not detected in plants irrigated with RW, although decreases in g_s, P_n and plant dry weight were observed in the plants showing the highest accumulation of B (RW3).

The effect of salinity on different ornamental plants was studied by Cassaniti et al. (2009), who classified ornamental plants as sensitive, moderately sensitive, moderately salt-tolerant, and tolerant depending on the percentage that the relative growth rate was reduced in the presence of NaCl in the range 10 to 70 mM. In salt sensitive species, the reduction in plant growth and injury symptoms correlated with increased Cl and/or Na accumulation as well as a reduction in k in leaves. However, some salt-tolerant species accumulate high ion levels in leaves, whereas in other cases the salt tolerance is related to a higher ion concentration in roots in relation to leaves, which is indicative of limited transport to the shoots (Cassaniti et al. 2012). Myrtle plants accumulate Na in roots and limite its transport to the aerial part of the plant, a trait which, along with the retention of Cl in roots, has been proposed as being related to salt tolerance in plants. The ability of plants to control the salt concentration of the aerial part by its accumulation in roots or by a reduced root uptake is an important mechanism to allow plant survival and growth under salt stress conditions (Colmer et al. 2005). However, such mechanisms did not avoid an over-accumulation of Na and Cl in leaves of the RW3 plants, at least during the time that the experiment lasted.

Although the application of drainage according to the electrical conductivity of the different irrigation waters seems to minimize the negative effects of salts in the growth of *Myrtus communis* L. plants (Bañón et al. 2011), in our experimental conditions the salt leaching could have been insufficient, especially in RW3 plants, since even when plants were irrigated with control water, these plants still displayed a decrease in biomass and plant height, which correlated with decreased P_n, higher Na accumulation and a reduced K level. In relation to the distribution of biomass, several authors have pointed out that in some ornamental species the growth of stems is more sensitive to salinity than roots (Álvarez and Sánchez-Blanco 2014), as occurred in the *M. communis* L. plants studied. According to Munns (2002), the behavior of roots to saline stress treatments is surprisingly robust in terms of tolerance, when compared with other plant tissues.

Higher K and Ca concentrations were observed in the leaves of plants irrigated with RW2, which could have partially prevented leaf tissue dehydration (Slama et al. 2008). In addition, RW plants maintained relatively high K/Na and Ca/Na ratios in leaves, shoots and roots (data not shown), which correlated with their response to salinity. In this sense, K and Ca play an important role in plant growth and development, but are also key players in the maintenance of osmotic adjustment and cell turgor (Osakabe et al. 2014).

The decrease in water potential and RWC in RW3 plants reflects the greater difficulty for water uptake during the first weeks of treatments as a result of the greater accumulation of salts in the substrate (Álvarez et al. 2012). This became clear in the RW1 and RW2 treatment as the experiment progressed. Despite the availability of water in the substrate, salts can promote an osmotic effect near the rooting

zone, limiting water uptake (Hardikar and Pandey 2008). This behavior has been observed in other ornamental species grown under the same conditions (Navarro et al. 2007, Miralles et al. 2011). As a response to this osmotic effect, a reduction in evapotranspiration and stomatal conductance occurred during the first weeks of the treatments acting, as a mechanism to avoid excessive loss of water (Munns and Tester 2008), particularly in the plants subjected to the highest saline concentration.

Plant growth, measured as biomass production, is a measure of net photosynthesis. The long-term effects of salinity on photosynthesis and the reduction in carbon assimilation are due to the accumulation of salt in leaves (Termatt and Munns 1986). However, low salt concentrations can stimulate these parameters (Rajesh et al. 1998) or have hardly any effect, as occurred in *M. communis* L. plants. In plants irrigated with reclaimed water the P_n was barely affected during Phase I and the highest P_n rates during Phase II corresponded to the control and RW2 treated plants, correlating with their greater biomass production. The effects of salinity on P_n and g_s seem to be dependent on the plant species, the level of salinity and the duration of any imposed stress (Tattini et al. 2002). In RW3 plants, the reduction of photosynthesis observed at the end of the experiment was reflected in the reduction of dry matter production, as we have already mentioned, which could be related to the greater accumulation of Na and Cl in leaves compared with the other treatments (Álvarez et al. 2012), although, more probably, with the increased levels of Na an B since RW2 plants did not show statistical differences in Na accumulation, being the least affected plants. In addition, Cl levels were statistically similar in all treatments.

There is little information available about the effect of abiotic stress conditions on the antioxidative metabolism of ornamental plants in this respect (Alguacil et al. 2003, Caravaca et al. 2005, Roldán et al. 2008, de Olivieira Jucoski et al. 2013) and no information for ornamental plants subjected to salt stress. In our experiment, we observed that the highest salinity treatment induced an oxidative stress in *M. communis* L. plants, which suffered leaf membrane damage, as monitored by the increases in EL and lipid peroxidation. Both parameters are used as markers of membrane damage under both biotic and abiotic challenges (Diaz-Vivancos et al. 2006, Faize et al. 2011). An increase in antioxidant enzymes was observed in plants irrigated with the RW containing the lowest and the highest salt contents, but no changes was produced in RW2 plants, confirming that this treatment did not produce strong stress conditions in myrtle plants, as observed from the plant growth parameters. It is well known that salt stress induces an oxidative stress in sensitive plants mediated by O_2^- and H_2O_2 at the subcellular level contributing in the symptoms caused by salinity (Hernández et al. 1993, 1995, 2001). In this sense, the activity of some of antioxidant enzymes in myrtle increased in response to salt stress in order to cope with the ROS that may be over-accumulated under such stress conditions. It has been suggested that the capacity of induction is one of the mechanisms of tolerance in plants to salinity (Hernández et al. 2000), but the cellular compartment where the increase and/or induction of antioxidant enzymes take place also seen to be important (Hernández et al. 2000). It has been claimed that greater tolerance to NaCl requires the induction of specific antioxidant enzymes in specific cell organelles (Hernández et al. 1993, 1995,

2000, Gómez et al. 1999, Mittova et al. 2003). Increased POX and/or SOD activity has also been described in different ornamental plants, including *Juniperus oxycedrus* L., *M. communis* L. and *Phyllirea angustifolia* L., subjected to drought stress (Caravaca et al. 2005; Roldán et al. 2008). However, in plants treated with the highest salinity levels, in spite of the increase in SOD and POX activities, membrane damage occurred, suggesting the induction of such antioxidant defenses may not have been sufficient to cope with the oxidative stress induced by long-term salt stress. In addition, POX activity not only functions as a H_2O_2-scavenger but also catalyzes the formation of H_2O_2 (Riedle-Bauer 2000). In this sense, plants treated with the highest salt level showed a huge increase in POX activity may also have been involved in H_2O_2 generation, contributing to the observed oxidative stress in leaves.

Taken together, the data show that the presence of salts in the RW did not negatively affect long-term plant growth, and mild salinity levels even stimulated biomass production. After recovery, the plants previously treated with the highest salinity levels manifested the negative effects of such treatment, which correlated with decreased P_n, greater Na accumulation and a reduction in K levels, especially in stems. These responses reflect the establishment of oxidative stress, reducing the membrane functionality.

5. Contributions

J.R.A. performed the experiment and carried out statistical analysis. G.B.E performed the antioxidative metabolism experiment. S.A. performed the experiment and was involved in data interpretation and manuscript writing. J.A.H. performed the antioxidative metabolism experiments and was involved in data interpretation and manuscript writing. M.J.S. designed and instructed the research work, coordinated the study, provided study material and facilities for the experiments and was involved in data interpretation and manuscript writing.

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FIGURE LEGENDS

Fig. 1. Influence of the different irrigation treatments on biomass accumulation in *M communis*. plants at the end of Phase I (A) and Phase II (B). Data represent the mean ± SE from at least 6 plants. Different letters indicate significant differences ($P<0.05$) according to Duncan's test ($p<0.05$).

Fig. 2. Influence of the different irrigation treatments on plant height evolution throughout the experiment (A) and on leaf area to plant height ratio at the end of Phase I and Phase II (B) in *M communis* plants. Vertical line indicates irrigation change between the end of the Phase II and the beginning of Phase II. Data represent the mean ± SE from at least 6 plants. Different letters indicate significant differences ($P<0.05$) according to Duncan's test ($p<0.05$).

Fig. 3. Influence of the different irrigation treatments on leaf water potential (ψ_l; A) and leaf relative water content (RWC; B) in *M. communis* plants during the experiment. Vertical lines indicate irrigation change between the end of the Phase I and the beginning of Phase II.

Fig. 4. Influence of the different irrigation treatments on stomatal conductance (g_s; A), net photosynthetic rate (P_n; B) and accumulated evapotranspiration (ET; C) in *M. communis* plants during the experiment. Vertical lines indicate irrigation change between the end of the Phase II and the beginning of Phase II.

Fig. 5. (A) Absorption rate by roots (J) in *M. communis* plants at the end of Phase I. (B) The slopes of linear regressions between Na or Cl concentration in the irrigation water and absorption rate by roots (J) in *M. communis* plants at the end of Phase I are shown in the bottom part of the figure.

Fig. 6. Concentrations of Na^+, Cl^-, B^{3+}, K^+ and Ca^{2+} in *M. communis* plants at the end of Phase I (A-E) and Phase II (F-J). Data represent the mean ± SE from at least 6 plants. Different letters indicate significant differences ($P<0.05$) according to Duncan's test ($p<0.05$).

Fig. 7. Influence of the different irrigation treatments on oxidative stress parameters in leaves from *Myrtus communis* plants. Electrolyte leakage (EL) was analyzed at the end of Phase I (A) and Phase II (B) of the experiment, whereas lipid peroxidation (TBARS) was analyzed only after the stress period (C). Data represent the mean ± SE from at least 6 plants. Different letters indicate significant differences ($P<0.05$) according to Duncan's test ($p<0.05$).

598 **Table 1.** Chemical analyses of the reclaimed waters used in the different treatments. Data are values
599 from samples collected at the beginning of the experimental period.

	Irrigation water			
Parameter	Control	RW1	RW2	RW3
Na^+ (mmol l^{-1})	1.826	11.304	15.652	69.130
Cl^- (mmol l^{-1})	1.944	6.901	14.873	29.887
Ca^{2+} (mmol l^{-1})	0.250	1.725	4.125	5.425
B^{3+} (mmol l^{-1})	0.012	0.018	0.055	0.133
K^+ (mmol l^{-1})	0.087	0.854	0.963	3.078
pH	7.52	8.07	8.25	7.74

600

601 **Table 2**. Slopes of the linear regression between Na^+ or Cl^- concentration in the irrigation water and
602 their relative amounts in the plant tissues in *M. communis* plants at the end of Phase I.
603

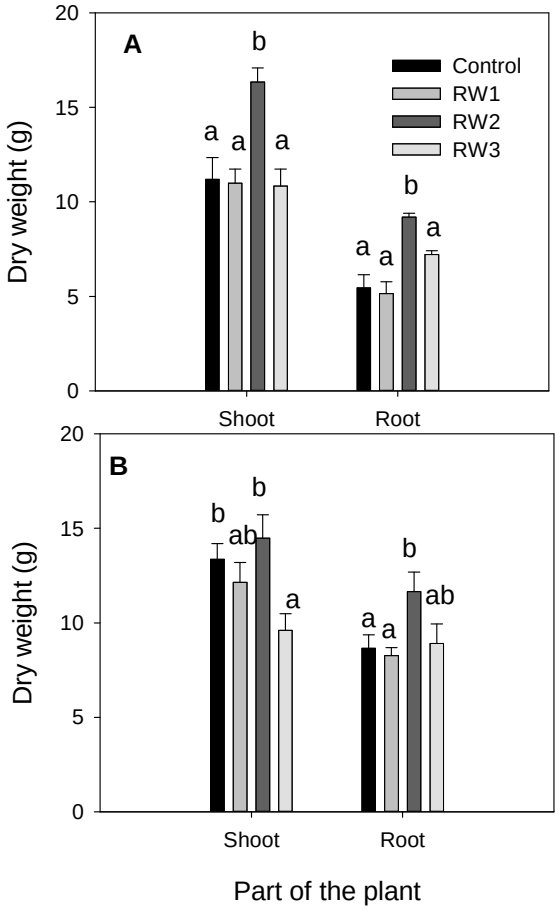
	Ion	
Tissue	Na^+	Cl^-
Leaves	1.281±0.090a	1.520±0.164
Stem	1.399±0.185a	1.408±0.265
Root	3.021±0.164b	1.912±0.442
P	***(0.000)	ns (0.504)

604 Data represent the mean ± SE from at least 6 plants. Different letters indicate significant differences
605 ($P<0.05$) according to Duncan's test ($p<0.05$). Asterisks indicate the level of probability: * ($P<0.05$). **
606 ($P<0.01$). *** ($P<0.001$). Non-significant values are indicated by "ns".

607 **Table 3.** Effects of the different irrigation treatments with different salinity levels on some antioxidant
608 enzymes in *M. communis* leaves at the end of Phase I.
609

	Control	RW1	RW2	RW3	P
CAT (µmol min^{-1} g^{-1} FW)	8.18 a	13.92 a	8.48 a	11.77 a	ns
APX (nmol min^{-1} g^{-1} FW)	119.64 a	86.43 b	105.98 ab	96.82 ab	**
GR (nmol min^{-1} g^{-1} FW)	4.22 ab	4.08 ab	5.04 a	3.74 b	**
SOD (U g^{-1} FW)	35.75 b	42.39 a	35.05 b	43.62 a	**
POX (µmol min^{-1} g^{-1} FW)	49.97 c	93.79 b	47.55 c	160.02 a	***

610 Data represent the mean ± SE from at least 6 plants. Different letters indicate significant differences
611 ($P<0.05$) according to Duncan's test ($p<0.05$). Asterisks indicate the level of probability: * ($P<0.05$),
612 ** ($P<0.01$), *** ($P<0.001$). Non-significant values are indicated by "ns".



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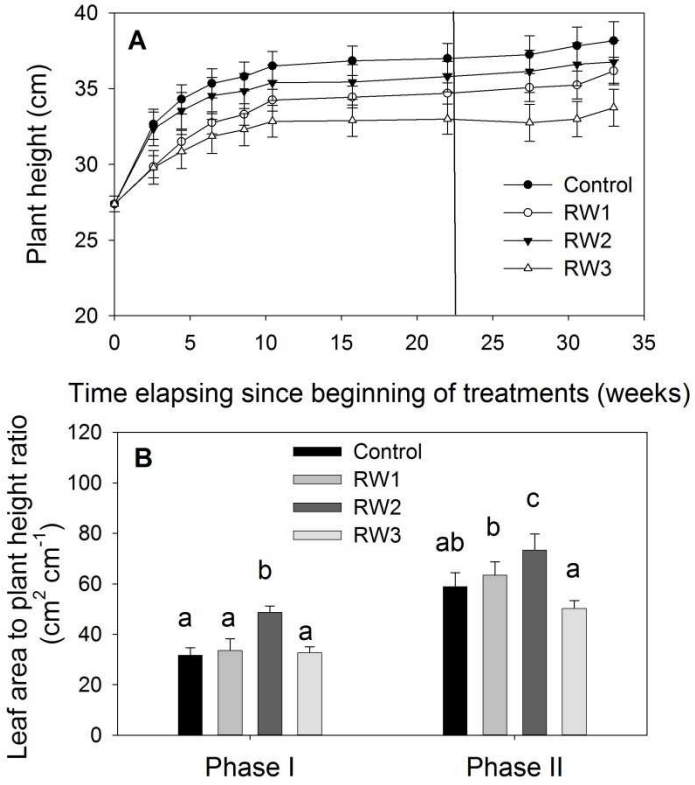

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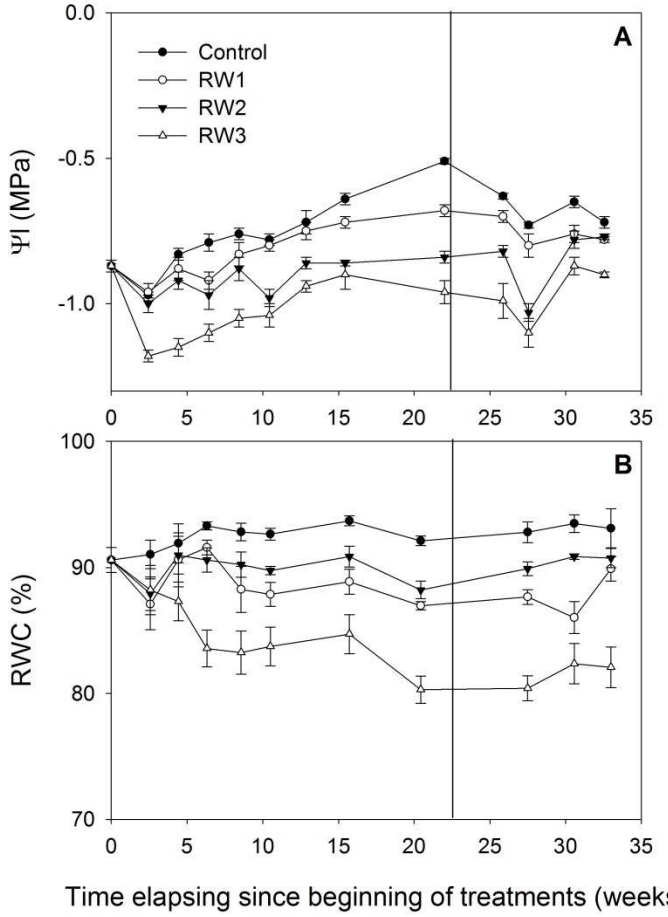

623

624 **Fig. 3.** Influence of the different irrigation treatments on leaf water potential (ψ_l; A) and leaf relative
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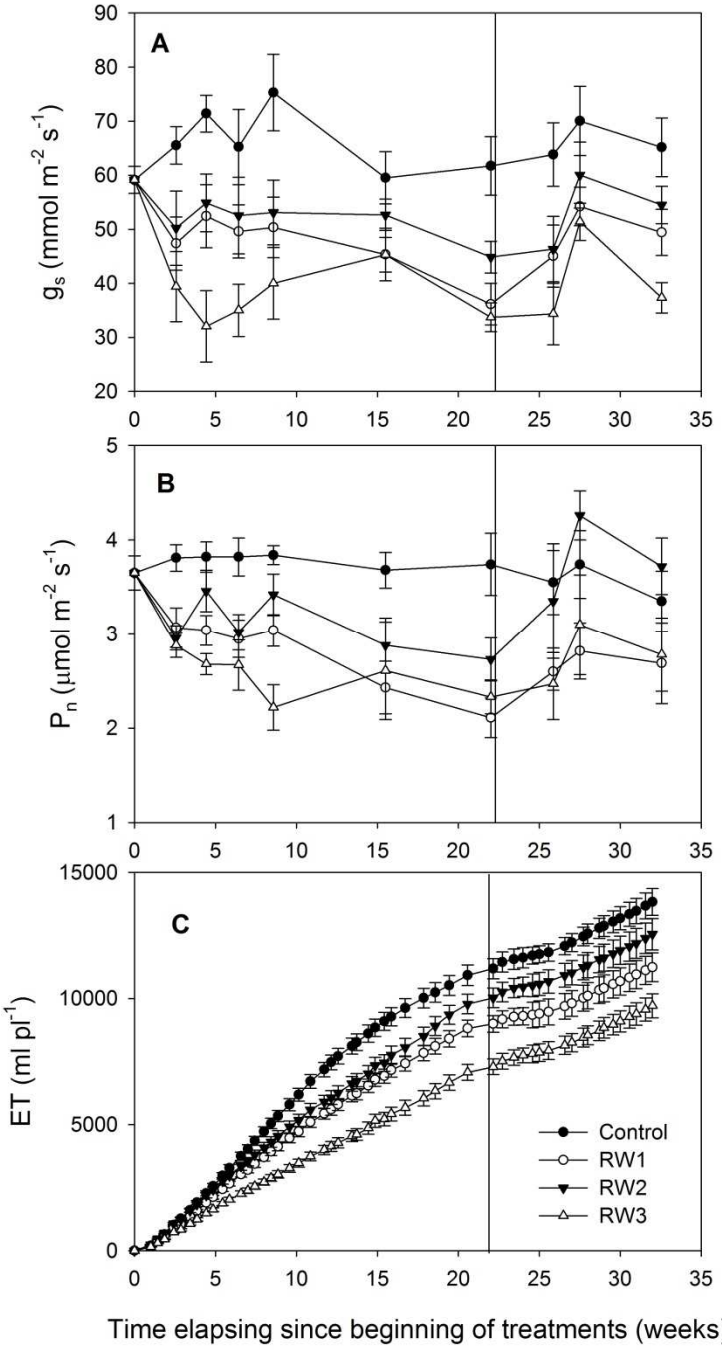

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629 photosynthetic rate (P_n; B) and accumulated evapotranspiration (ET; C) in *M. communis* plants during
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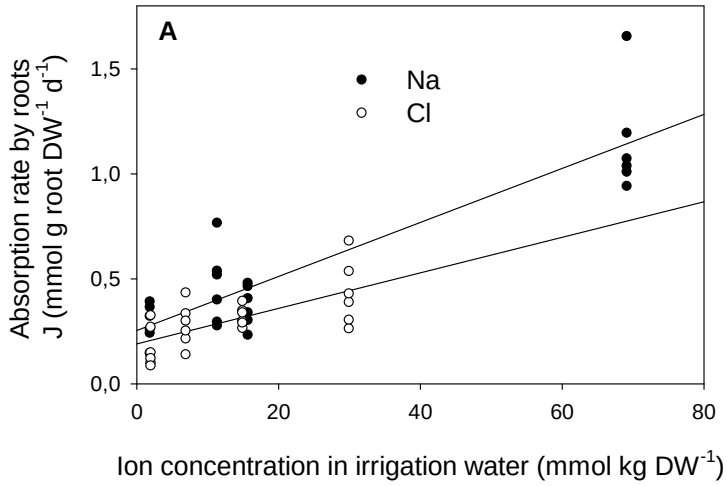
631 beginning of Phase II.

632

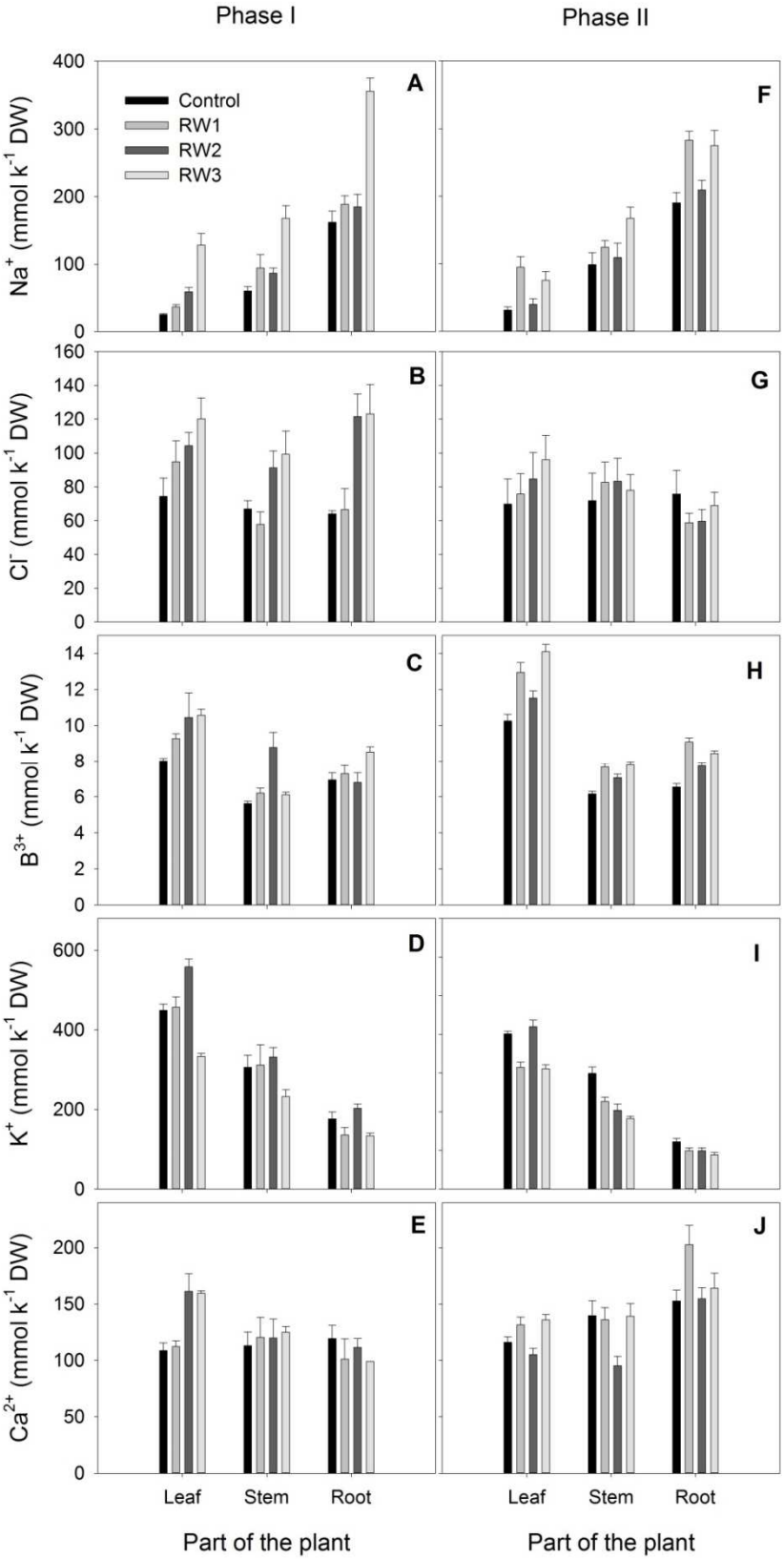


B

Na		Cl		P
12.86±1.12	b	8.47±0.81	a	***

633

634 **Fig. 5.** (A) Absorption rate by roots (J) in *M. communis* plants at the end of Phase I. (B) The slopes of
635 linear regressions between Na or Cl concentration in the irrigation water and absorption rate by roots
636 (J) in *M. communis* plants at the end of Phase I are shown in the bottom part of the figure.



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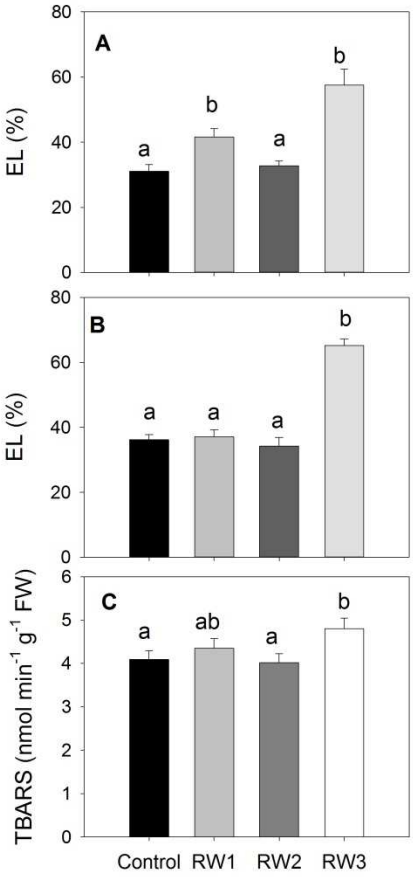

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