NaCl-induced physiological and biochemical adaptative mechanisms in the ornamental *Myrtus communis* L. plants

Running title: Salt stress and recovery in *Myrtus communis* L.

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Abstract

Physiological and biochemical changes in *Myrtus communis* L. plants after being subjected to different solutions of NaCl (44, and 88 mM) for up to 30 days (Phase I) and after recovery from the salinity period (Phase II) were studied. Myrtle plants showed salinity tolerance by displaying a series of adaptative mechanisms to cope with salt-stress, including controlled ion homeostasis, the increase in root/shoot ratio, the reduction of water potentials and stomatal conductance to limit water loss. In addition, they displayed different strategies to protect the photosynthetic machinery, including limiting toxic ion accumulation in leaves, increase in chlorophyll content, and changes in chlorophyll fluorescence parameters, leaf anatomy and increases in catalase activity. Anatomical modifications in leaves, including a decrease in spongy parenchyma and increased intercellular spaces, allow CO_2 diffusion in a situation of reduced stomatal aperture. In spite of all these changes, salinity produced oxidative stress in myrtle plants as monitored by increases in oxidative stress parameter values. The post-recovery period is perceived as a new stress situation, as observed through effects on plant growth and alterations in non-photochemical quenching parameters and lipid peroxidation values.

Keywords: ASC-GSH cycle, Gas exchange, Leaf anatomy, Oxidative stress, Recovery capacity, Water relations.

Abbreviations: APX: ascorbate peroxidase, ASC: Ascorbate reduced form; DHAR, dehydroascorbate reductase; GR, Glutathione reductase; GSH: glutathione reduced form; GSSG, glutathione oxidised form; MDHAR, monodehydroascorbate reductase; POX, peroxidase; SOD: superoxide dismutase.

Introduction

The presence of NaCl in the soil and the irrigation water is one of the main factors limiting plant growth. Salt-stress affects different physiological and biochemical processes, including photosynthesis, respiration, protein synthesis or lipid metabolism (Parida and Das, 2005; Tattini et al., 2006; Stepien and Johnson, 2009).

Salinity induced a water deficit as well as an ionic toxicity in the plants resulting in an alteration in the ionic homeostasis. In addition to the osmotic and toxic effects, salt stress is also manifested as an oxidative stress, with all these factors contributing to the deleterious effects of salinity in plants (Hernández et al., 2001; Barba-Espín et al., 2011; Acosta-Motos et al., 2014a; b).

The bibliography related to the effect of salt stress in ornamental shrubs is scarce. Few authors have studied the effect of salt-stress on plant growth and ion distribution in ornamental plants (Tattini et al., 2006; Cassaniti et al., 2009; Navarro et al., 2009; Álvarez et al., 2014; Acosta-Motos et al., 2014a; b). Saline water use can be a strategy for an efficient water management in landscaping projects, especially in arid zones characterized by limited water resources, such as the Mediterranean areas. For landscaping projects it is very important to select ornamental shrub species showing some degree of salt tolerance.

Myrtle (*Myrtus communis* L.) is a bushy evergreen sclerophyllous plant with significant ornamental interest used in re-vegetation projects in arid and degraded land and landscaping projects. Myrtle is a Mediterranean specie that is well adapted to abiotic stresses, although it may be affected by salinity and high light intensity. In general, *Myrtaceae* species such as *Eugenia myrtifolia* and *Leptospermum scoparium* are considered as salt-tolerant, showing less than 25% reduction in their relative growth rates

after 120 days of exposure to salinity levels up to 70 mMNaCl (7.4 dS m^{-1}) (Cassaniti et al., 2009).

In this work, the effect of different solutions of NaCl on plant growth, mineral nutrition, gas exchange parameters, water relations, chlorophyll fluorescence, leaf anatomy and antioxidative metabolism in *M. communis* plants was studied. The plant capacity of recovery following salinity relief has also been taken into account. In this way, there is scarce information about the response of plants to recovery from salinity and the physiological mechanisms involved in the recovery of plants subjected to salt stress are poorly understood (Chaves et al., 2011).

Material and Methods

Plant and experimental conditions

Native *Myrtus communis* L. plants from Viveros Muzalé S.L. (Cieza, Murcia, Spain) were cultivated in black polyethylene multi-alveolar trays under environmental conditions. The plants were immediately transplanted into 14 x 12 cm pots (1.2 l) and grown in a controlled growth chamber as described previously (Acosta-Motos et al., 2014a; b). The temperature in the chamber was 23ºC during the light phase (16 h photoperiod) and 18ºC during darkness. Relative humidity (RH) values ranged between 55-70%. A mean photosynthetic active radiation (PAR) of 350 µmol m^{-2} s^{-1} at canopy height was supplied during the light phase (08:00h-00:00h) by cold white fluorescent lamps.

Experimental design and treatments

Once myrtle plants (90 plants), were adapted to chamber conditions, they were exposed for up to 30 days (Phase I) to the following three irrigation treatments. Control plants were watered with a mixture of distilled water and tap water with an electrical conductivity (EC) = 0.3 dS/m. Saline treatments were designed as control treatment plus NaCl added specifically for each

treatment: S4 (4 dS/m) and S8 (8 dS/m), corresponding to 44 and 88 mM NaCl, respectively. The EC of different treatments was checked with a multirange Cryson-HI8734 electrical conductivity meter (Cryson Instrumnents, S.A., Barcelona, Spain) at the beginning and throughout the experimental period. Before starting the experimental period, the maximum water holding capacity of the soil was determined for each individual pot and considered as the weight at field capacity (WFC). Throughout the experiment, all pots were irrigated three times a week below the WFC in order to avoid drainage favouring an increase in soil salinity caused by time and severity of saline treatments. The maximum water holding capacity calculated for the substrate was 1226.16 ± 2.94 ml.

After the stress phase, all plants were exposed to a 23-days recovery period (Phase II) in which they were irrigated with the same solution used for the control plants. During the first three days of the recovery period, all plants were exposed to a further irrigation event with leaching with the same solution used for the control plants (a mixture of distilled water and tap water). The leaching fraction reached 10% (v/v) of the water applied in the control treatments, 27% of the water applied in S4 treatments, 50% of the water applied in S8 treatments.

Growth and plant water measurements

At the end of Phase I and Phase II, the substrate was gently washed from the roots of six plants per treatment and plants were divided into shoots (leaves and stem) and roots, and oven-dried at 80ºC until they reached a constant weight to measure their respective dry weights (DW).

Leaf water potential (Ψ_l), leaf osmotic potential, leaf turgor potential (Ψt), leaf osmotic potential at full turgor (Ψ_{100S}) was estimated in six plants per treatment during the central hours of illumination at the middle and at the end of Phase I (15 d and 30d) and Phase II as described previously (Álvarez et al., 2012). Leaf proline was analysed in

six plants per treatment at the middle and at the end of Phase I (15 d and 30d) and Phase II according to Bates et al., (1973).

For the experiment of high light intensity (HL), a batch of plants, not used for the salt-stress study, were grown in the same conditions describes above for two months, and during the last four days of the experiment they were exposed to 100% solar radiation (sunlight), whereas another batch of plants were grown in a room chamber in the same conditions but in presence of low light intensity (LL) (100 PAR) for two months.

Determination of inorganic solutes

At the beginning, at the end of Phase I (30 d) and Phase II, six 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 concentration of Cl^{-}, Na^{+}, K^{+}, and Ca^{2+} ions were determined as described in Acosta-Motos et al. (2014 a; b).

The absorption rates of Na^{+}, Cl^{-}, K^{+}, and Ca^{2+} ions (J) by the root system at the end of Phase I and Phase II was calculated considering the total salt content of six plants per treatment at harvest, expressed as mmol Na^{+}, Cl^{-}, K^{+} and $Ca^{2+}$the mean root weight, using the formula described by Pitman (1975):

$$J = (M2-M1)/ (WR*t)$$

where M1 and M2 correspond to a concentration in mmol of ion studied in the total plant at the beginning and at the end of Phase I and Phase II, respectively; t corresponds to the time in days and WR is the logarithmic mean root biomass, calculated as (WR2-WR1)/Ln (WR2/WR1), with WR1 and WR2, being the dry weight of roots at the beginning and at the end of Phase I and Phase II, respectively.

Gas Exchange and chlorophyll determination

Leaf stomatal conductance (gs) and net photosynthesis rate (P_N) were determined in attached leaves in six plants per treatment during the central hours of illumination at middle and the end of Phase I (15 d and 30 d) and Phase II using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA). Intrinsic water efficiency P_N/gs was calculated. Total chlorophyll was analysed in leaf samples in six plants per treatment at middle of Phase I (15 d), at the end of Phase I (30 d) and Phase II as described by Inskeep and Bloom (1985).

Measurement of chlorophyll fluorescence

The fluorescence of chlorophyll was measured at middle and the end of Phase I (15 d, 30 d) and Phase II with a chlorophyll fluorometer (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany) in detached leaves from controls and salt-treated myrtle plants. After dark-incubation of plants (20 min), the minimum and the maximal fluorescence yields were monitored. Kinetic analyses were carried out with actinic light (81 µmol quanta m^{-2} s^{-1} PAR) and repeated pulses of saturating light at 2700 µmol quanta m^{-2} s^{-1} PAR for 0.8 s at intervals of 20 s. The effective PSII quantum yield (Y(II)), the quantum yield of regulated energy dissipation (Y(NPQ)), the non-photochemical quenching (NPQ), the maximal PSII quantum yield (Fv/Fm), the coefficients of non-photochemical quenching (qN) and the photochemical quenching (qP) were analysed (Maxwell and Johnson, 2000).

ETR (apparent rate of photosynthetic electron transport) parameter was performed at the end of Phase I (30 d) and Phase II as well as in the experiment with HL using a light curve analysis with increasing PAR light pulses (0-700 PAR).

Antioxidative metabolism

Enzyme extraction and analysis

All operations were performed at 4ºC. For the enzymatic determinations plants were sampled at middle and the end of Phase I (15 d, 30 d) and Phase II. Leaf samples (1 g) were homogenized and pre-purified as described previously (Acosta-Motos et al., 2014b). For the APX activity, 20 mM sodium ascorbate was added to the extraction buffer. The activities of the ASC-GSH cycle enzymes, POX, CAT, and SOD were assayed as described in Barba-Espín et al. (2011).

Oxidative stress parameters

The rate of passive electrolyte leakage from stress-sensitive plant tissue can be used as a measure of alterations of membrane permeability. Ion leakage was estimated at 15 and 30 days in Phase I and at the end of Phase II, according to the method described by Lafuente et al. (1991). The extent of lipid peroxidation was estimated by determining the concentration of thiobarbituric acid-reactive substances (TBARS) as previously described (Hernández and Almansa, 2002).

Light microscopy and morphometrical analysis

Leaves sections (1×1 mm from the most recent fully expanded leaves) from the central region of myrtle leaves, avoiding the main vein, were used for light microscopy. These samples were fixed and postfixed according to Agulló-Antón et al. (2013). Semi-thin sections (0.5-0.7 µm thick were cut with a Leica EM UC6 ultramicrotome. The sections were stained with 0.5% toluidine blue, mounted in DPX and observed with a Leica DMR light microscope.

For morphometric analysis, 10 different sections from each treatment (3 plants of each treatment), were studied at the end of Phase I (30 d). The percentages of area occupied by palisade parenchyma (PP), spongy parenchyma (SP) and intercellular spaces (IS) in leaves from myrtle plants were measured and expressed as the % of total area using Adobe Photoshop CS4 Extended software.

Statistical analyses of data

In the experiment plants were randomly attributed to each treatment. Statistical analysis of the data were performed using the SPSS 19.0 software (SPSS Inc., 2002) Data were subjected to analysis of variance (ANOVA) and the mean values were compared by a Duncan's Multiple Range Test at $p \leq 0.05$ to assess significant differences between C, S4 and S8 treatments.

Results

Effect of NaCl on plant growth

At the end of Phase I, 4 or 8 dS m^{-1} NaCl did not negatively affect the growth of myrtle plants. In addition, S4 plants showed a significant increase in root growth and in leaf and shoots DW, whereas S8 plants significantly increased the root DW by about 62% (Table 1), leading to a 50% increase in the DW root/DW shoot ratio (Table 1). After the recovery period (Phase II), a decrease in leaf and stem DW was produced in S4 and S8 plants.

Nutritional Changes

Salt stress increased the absorption rates of Na^+ and Cl^- by myrtle roots. The increase observed in Na^+ uptake was quite similar in S4 and S8 plants, reaching about 2 times the values reported for their respective control plants (Fig. 1A). In contrast, the

changes showed in Cl^- uptake were dependent on the NaCl level used in each treatment. Accordingly, Cl^- uptake rates increased up to 38% and 52% for S4 and S8 plants, respectively (Fig. 1C). The uptake rate of K^+ and $Ca^{2+}$was not affected in plants subjected to S4 and S8 treatments (Fig. 1E and G).

After Phase II, the absorption rates for Na^+ and Cl^- were still much higher in plants previously subjected to NaCl stress than in the controls (Fig 1B and D). The uptake rate for K^+ did not change in S4 and S8 plants (Fig. 1F), whereas Ca^{2+} uptake rate values were a 45% higher in S4 plants and a 56% higher in S8 plants in relation to the values presented by control plants (Fig.1H).

Ion Distribution

Concerning the distribution of the different ions, Na^+ accumulated mainly in roots, where its content increased up to 1.86-times in S4 plants and about 2-times in S8 plants (Fig. 2A). The Na^+ ions also accumulated in the aerial part but in a lower extent than in roots (Fig. 2A). Thereby, Na^+ content increased by 50% in leaves subjected to NaCl stress, whereas the Na^+ levels in stems increased up to 70% in S4 plants and up to 42% in S8 plants (Fig. 1A).

In contrast, Cl^- distribution was more uniform than that of Na^+, although Cl^- accumulated more in S8 than in S4 plants. In this case, Cl^- accumulated in the aerial part from S4 plants but not in roots, whereas in S8 plants Cl^- accumulated in all plant organs, especially in stems (52% increase) and roots (45% increase) (Fig. 2C).

No significant effects of NaCl were recorded for K^+ distribution (Fig 2E) whereas Ca^{2+} levels significantly increased in leaves from S4 plants (15%) and in stem from S8 plants (27%) (Fig. 2G).

After Phase II, again Na^+ accumulated in roots, being the increase up to 51% and 57% in S4 and S8 plants, respectively (Fig 2B). Na^+ also accumulated in the aerial part

of the plants, although data were lower to that showed by roots (Fig. 2B). Regarding Cl^- distribution, this toxic ion showed a 38% increase in roots from plants previously treated with 4 dS m^{-1}, whereas in S8 plants Cl^- levels rose up to 20% in stems and 72% in roots in relation to control plants (Fig 2D). K^+ contents declined in leaves and stems from S4 plants and in roots from S8 plants (Fig 2F). The increase in Ca^{2+} uptake observed after Phase II was parallel with a rise in the Ca^{2+}contents in roots in S4 (23%) and S8 (51%) plants (Fig. 2H).

Plant Water Relations

Leaf water potential (Ψ_l) showed a progressive decline with the severity and the duration of the NaCl treatments applied. Salt-treated plants showed more negative Ψ_l values than control plants. In this way, S4 plants presented Ψ_l values of -0.69 and -0.84 MPa at 15 and 30 days of stress, respectively (Table 2). The decline in Ψ_l was more noticeable in S8 plants, with values, of -0.78 and -0.96 MPa at 15 and 30 days of stress, respectively. At the end of Phase II, Ψ_l values increased in S4 (-0.72 MPa) and S8 (-0.75 MPa) plants but did not reach control values (Table 2).

Concerning leaf turgor potential (Ψt), at 15 days of salinity only S8 plants experienced a decline in leaf Ψt (0.47 MPa) (Table 2). However, at 30 days both S4 and S8 plants showed a decline in Ψt (0.82 and 0.82 MPa, respectively). At the end of Phase II, a significant increase in Ψt was observed in S4 and S8 plants, reaching values of 0.93 and 0.97 MPa, respectively (Table 2).

The osmotic potential at maximum saturation (Ψ_{100s}) values decreased in both saline treatments during the phase I, pointing to a slight osmotic adjustment in these treatments, while no differences in this parameter were found at the end of Phase II Table 2).

In parallel to water relation parameters, we analysed the leaf proline content during the experiment. No significant differences were observed in proline levels due to the effect of NaCl treatments, and only at the end of Phase II proline increased in plants previously treated with 8 dS m^{-1} NaCl (Table 2).

Gas exchange and chlorophyll fluorescence parameters

Myrtle plants showed unchanged (S4) or increased (S8) leaf chlorophyll levels at the end of Phase I (Table 3). At the end of Phase II, S8 plants presented significant higher chlorophyll contents than control plants (Table 3).

After 15 days of NaCl treatments, all plants showed similar gs values, although S4 and S8 plants showed lower P_N values than the control, resulting in a decrease of about 50% in the intrinsic water efficiency (P_N/gs) in both treatments (Table 3). At the end of Phase I, salt-treated plants appeared to have developed an ability to acclimate to the stress conditions. S4 showed similar P_N and gs values to control plants and S8 had similar intrinsic water use efficiency (P_N/gs), as P_N and gs were proportionally reduced compared with control. Finally, at the end of Phase II, S8 plants still showed lower P_N and gs values than control and S4 plants, although no significant differences in P_N/gs among treatments were found (Table 3).

After 15 days of NaCl-stress, myrtle plants irrigated with 4 and 8 dS m^{-1} showed increased photochemical quenching parameters [qP, Y(II), and Fv/Fm] values (Table 4, Fig S1). In addition, S8 plants increased non-photochemical quenching parameters [qN, NPQ and Y(NPQ)] (Table 4; Fig S1). At 30 days of NaCl stress, S4 plants only showed a decline in qP, whereas in S8 plants a reduction in qP and Y(II), and an increase in the non-photochemical quenching parameters was recorded (Table 4, Fig S1). After Phase II, the response was quite similar to that observed at 15 days of stress. In this case, plants

previously subjected to S4 treatment increased qP and Y(II), but reduced the non-photochemical quenching parameters. In contrast, S8 plants increased qP and decreased Y(II), whereas no change in qN and NPQ was observed (Table 4, Fig S1).

At a known flux of incident photosynthetically active radiation (PAR) the relative rate of photosynthetic electron transport (ETR) was determined in *M. communis* L. leaves. This parameter was analysed at the end of both experimental phases, and showed that myrtle plants presented low ETR data. At the end of Phase I, the maximum ETR values were recorded at low light intensities (56-111 PAR), and then data decreased with the increase of PAR. In this phase, S4 plants displayed the highest ETR values (Fig 3A). At the end of Phase II, ETR values were much higher to those observed in Phase I, being the maximum ETR data reached at higher PAR values (111-186 PAR). At 111 PAR, plants recovered from NaCl-stress displayed higher ETR values than control plants. Again, ETR data progressively decreased with the increase of PAR, with the values close to zero at 300 PAR (Fig 3B). The ETR/P_N ratio observed in non-stressed plants was close to 4.5, both at the end of Phase I and Phase II. This ratio increased with the NaCl levels used, reaching a 23% increase in S4 plants and a 2-fold in S8 plants (Fig 3C). Furthermore, the recovered plants, previously subjected to NaCl-stress, showed higher ETR/P_N values than those observed at the end of the salinization phase. In this case, the rise in ETR/P_N was up to 84% and 5.3-fold in S4 and S8 plants, respectively (Fig 3C).

ETR data reflected that myrtle plants appear to be adapted to low light intensity (LL). In this sense, we studied the effect of high light intensity (HL) in the chlorophyll fluorescence parameters. The data, obtained for apical and basal leaves (3rd-4th branch), were different depending on the light conditions. For example, plants subjected to HL showed decreased Fv/Fm values compared to plants subjected to LL, especially the apical leaves, which were more exposed to sunlight (Table 5). In addition, these leaves presented

decreased non-photochemical quenching parameters, reflecting a reduced capacity for a safe dissipation of excess light energy (Table 5). However, basal leaves, more protected from HL, showed increased qP values and non-photochemical quenching parameters in relation to control basal leaves as well as than apical leaves from HL stressed plants. In addition, these leaves from HL-stressed plants also showed the highest ETR values (Fig S2).

Leaf anatomy

Salt stress induced some changes in the leaf anatomy from *M. communis* plants. At the end of Phase I, plants treated with 8 dS m^{-1} NaCl presented a decrease in the percentage of spongy parenchyma and increased percentage of intercellular spaces. However, no significant changes were recorded in S4 plants (Table 6, Fig S3).

Antioxidative metabolism

NaCl treatment induced an oxidative stress in myrtle plants after 30 days of treatment only in S8 plants, as evidenced by electrolyte leakage (EL) and lipid peroxidation (LP) in leaves, indicative of membrane damage (Table 7). The NaCl-induced oxidative stress was also evidenced by the H_2O_2 accumulation observed in leaves from plants treated with 8 dS m^{-1} NaCl (Fig S4). After Phase II, both S4 and S8 plants showed increased EL values but only S8 plants still presented increased LP values (Table 7), suggesting some damage to cell membranes.

The effect of salt stress on the activity of some antioxidant enzymes was studied in plants treated with 4 and 8 dS m^{-1} NaCl. Under our experimental conditions, DHAR and POX activities as well as ascorbate and glutathione levels were not detected in myrtle leaves. At 15 days of NaCl stress, a decrease in CAT activity and an increase in GR activity were produced in myrtle plants (Table 8). In addition, plants subjected to S8

treatment showed decreased APX and SOD activities (Table 8). At the end of Phase I, a 2-fold increase in CAT activity occurred in S4 plants, whereas an increase in MDHAR was observed only in S8 plants (Table 8). At 30 days of stress, APX activity values were lower than those observed at 15 days, and a 60% decrease in APX occurred in S4 and S8 plants. In addition, a 30% drop in SOD was observed in S8 plants (Table 8). At the end of Phase II, the antioxidants activities analysed were lower than those observed in Phase I except for CAT activity. Moreover, we were not able to detect GR activity in recovered plants. In this period, plants previously treated with NaCl displayed a significant increase in CAT activity, especially S4 plants that showed a 3-fold increase. However, APX activity showed no sign of recovery, and even decreases of 35% in S4 plants and 58% in S8 plants were recorded (Table 8).

Discussion

Growth and ion accumulation

Myrtus communis L. plants are considered to be moderately tolerant to NaCl levels of 25 mM (Cassaniti et al. 2012), whereas other *Myrtaceae* species such as *E. myrtifolia* and *L. scoparium* are tolerant to salinity levels up to 70 mM NaCl (7.4 dS m^{-1}) (Casanitti et al., 2009). In the present work, we used higher levels of NaCl, and under our experimental conditions, 4 and 8 dS m^{-1} NaCl levels (equivalents to 44 and 88 mM NaCl, respectively) did not negatively affect the plant growth, and S4 treatment even produced stimulation in plant biomass production. S8 plants showed increase root biomass, and as a consequence a rise in DW root/ DW shoots ratio took place. DW roots is a parameter considered to be important in the response to salt stress, because the higher root growth the higher water and nutrient uptake can take place, favouring the accumulation of toxic ions in roots, especially Na^+, and thus minimizing its negative effects in the shoot growth (Marchner, 1995). In Eugenia plants, reduced toxic ions accumulation seems to be due to

a tight control of its uptake rates and its translocation to the aerial part (Acosta-Motos et al., 2014b). However, myrtle plants seemed to control Na^+ accumulation in the aerial part by reducing its translocation from the roots and/or by its accumulation in roots. At the end of Phase II plant growth seemed to be retarded by the new applied conditions in previously stressed plants. This response was observed also in other ornamental plants (Cassaniti et al., 2009; Acosta-Motos et al., 2014b; 2015?).

A correlation between K^+ and /or Ca^{2+} contents and plant growth effect was observed in myrtle plants. The treatments with 4 and 8 dS m^{-1} NaCl did not affect either the Ca^{2+} and K^+ uptake rates nor the Ca^{2+} and K^+ contents in roots and shoots. It is known that Ca^{2+} and K^+ can play an important role in plant growth and development as well as in the maintenance of osmotic adjustment and cell turgor (Marchner, 1995). Moreover, the addition of Ca^{2+} in the irrigation water can reduce the Na^+ and Cl^- levels in loquat and anger plants, but was not able to improve plant growth (Hernández et al., 2003), probably due to the displacement of Ca^{2+} from cell membranes affecting the membrane functions (Lynch et al., 1987). It is known that Ca^{2+} plays a role maintaining the structure and functionality of membranes as well as the improvement of the K^+/Na^+ selectivity (Cramer et al., 1987; Lynch et al., 1987).

Plant water relations

The decrease in leaf water potential (Ψ_l) in salt-treated plants reflects an osmotic effect and as consequence difficulty for water uptake during the Phase I, probably as a result of salts accumulation in the substrate, especially in S8 plants. Even at the end of Phase II these plants showed reduced Ψ_l, despite the availability of water in the substrate (Hardikar and Pandey, 2008). This behaviour has been described in other ornamental species irrigated under saline conditions (Sánchez-Blanco et al, 1998; Navarro et al.,

2007; Acosta-Motos et al., 2014b). These results are supported by the decrease in Ψt at the end of phase I, indicating lower leaf turgor.

Moreover, myrtle plants showed an osmotic adjustment at the end of Phase I as observed by the reduction in Ψ_{100S}, especially in S8 plants, that could be due to the ion compartmentation inside the vacuoles (Koyro et al., 2006). However, no effect of proline in the osmotic adjustment was observed during Phase I, and only a limited contribution can be observed at the end of Phase II only in S8 plants.

Gas exchange and chlorophyll fluorescence parameters

NaCl levels of 8 dS m^{-1} did not negatively affect plant biomass although a clear reduction in P_N took place. Similarly, Tattini et al. (2006) previously reported the negative effect of NaCl on photosynthesis in myrtle plants. As a compensatory mechanism to protect the photosynthetic process, *M. communis* plants showed unchanged (S4) or increased (S8) chlorophyll contents at the end of Phase I. It is known that salt-tolerant species show increased or unchanged chlorophyll content under salinity conditions whereas chlorophyll levels decrease in salt-sensitive species, suggesting this parameter as a biochemical marker of salt tolerance in plants (Ashraf and Harris, 2013; Stepien and Johnson, 2009). Thus, the loss of chlorophyll can lead to photo-damage in the photosynthetic apparatus (Havaux and Tardy, 1999).

Myrtle plants showed low P_N data and this result was associated with lower gs values, which can also influence the response of myrtle plants to salt stress through a fine stomatal control regulation. Decreased gs values is a common response of NaCl-adapted plant species, and this response allows plants to maintain a level of toxic ions lower than expected through a limited transpiration, as described previously (Alarcón et al., 2006; Fernández-García et al., 2014).

At the end of Phase II, S8 plants significantly increased chlorophyll contents, but no recovery of P_N was produced, that were linked again to the decrease in gs. This lack of photosynthesis recovery correlated with a decrease in plant biomass and an osmotic effect in P_N decline cannot be ruled out. In spite of the drainage conditions applied at the beginning of Phase II, Na^+ and Cl^- accumulated in the substrate. A similar lack of P_N recovery was found in olive trees after salinity relief (Tattini et al., 1995). These authors attributed the decline in P_N, after NaCl recovery, to an osmotic effect outside the roots rather than a specific effect of toxic ions on leaf photosynthesis.

After Phase II, and although a partial recovery in P_N occurred in S4 plants, a general reduction in plant growth was observed. Probably, in this case, myrtle plants have to invest most of the produced photosynthates in different mechanisms to cope with the new imposed growth conditions, for example, i) to form roots in order to retain toxic ions and to increase water uptake, and ii) to produce more energy (ATP) for proton pumps to compartmentalize toxic ions and/or for ion exclusion. That would mean that both chloroplast and mitochondrial electron transport chains could be working at full capacity, with the risk of ROS overproduction.

No correlation between P_N and fluorescence parameters was observed at 15 days of salt stress. At 30 days of stress the drop in P_N parallel with a decline in qP and Y(II) and increases in non-photochemical parameters. The long-term NaCl treatment (75-150 mM) also decreased Y(II) in *Lawsonia inermis* L. plants along with a decrease in Y(NPQ) (Fernández-Garcia et al., 2014). At the end of Phase II, the partial recovery in P_N observed in S4 plants correlated with increases in qP and Y(II) whereas in S8 plants, both qN and Y(NPQ) dropped, reflecting a decrease in the safe dissipation of excess energy that could induce photo-oxidative damage in the photosynthetic apparatus (Maxwell and Johnson, 2000).

Myrtle leaves showed low Fv/Fm data that could be related with the low electron transport rates observed. Different authors described optimal Fv/Fm values around 0.8 in most non-succulent leaves (Krall and Edwards 1992; Karpinski et al., 1997; Maxwell and Johnson 2000; Hernández 2004, 2006a), and values lower than 0.8 can be observed after the exposition of plants to stress, indicating a phenomenon of photoinhibition. However, we have recorded lower Fv/Fm data in some woody species such as apricot (about 0.76-0.78; Hernández et al., 2006b) or in Eugenia plants (0.68-0.76; Acosta-Motos et al., 2015 Planta). For this reason, other authors suggested that plants can be considered as stressed with Fv/Fm values lower than 0.7 (Colom and Vazzana, 2003, Percival et al., 2006, Bacelar et al., 2007).

The ETR/P_N ratio reflects a balance between the PSII activity and the CO_2 fixation (Krall and Edwards 1992). In C3 plants, both CO_2 fixation and photorespiration are the major sinks of electrons from PSII (4 electrons are required for CO_2 or O_2 reacting with Rubisco), whereas in C4 plants there is a linear relationship between PSII activity and CO_2 fixation (Krall and Edwards 1992). In different C4 species, ETR/P_N values ranging 4.6-6.1 have been recorded. According to these data, both control plants as S4 plants behave as C4 plants. However, S8 plants and especially recovered plants showed increased ETR/P_N ratios, emphasizing that there was an important electron transport to other acceptors different to CO_2. The increased ETR/P_N values were correlated with an increase in NPQ and qN in S8 plants, reflecting a dissipation of excess energy as heat to avoid PSII damage (Maxwell and Johnson 2000). The involvement of photorespiration as a sink for electrons from PSII was more evident in the recovered plants that showed increased CAT activity values.

According to the ETR data recorded, myrtle plants seem to be adapted to LL and the exposure to HL strongly decreased Fv/Fm values, indicating that photoinhibition of

photosynthesis occurred (Karpinski et al., 1997; Hernández et al., 2006). In addition, apical leaves, more exposed to solar irradiation, showed reduced non-photochemical quenching parameters reflecting a reduced capacity for a safe dissipation of excess light energy. The decline observed in Fv/Fm in myrtle plants, after exposure to 100% solar irradiation, was much lower than that described in pea or Arabidopsis plants subjected to HL stress (Karpinski et al., 1997; Hernández et al., 2006).

Anatomical changes

It is known that prolonged salt stress may cause changes in leaf anatomy (Garrido et al., 2014; Fernández-García et al., 2014). The observed morphological changes at 30 days of stress (increased root/canopy ratio) in S8 plants were accompanied by leaf anatomical changes. These NaCl-induced anatomical changes might facilitate the CO_2 to reach the chloroplast in a more efficient manner in a situation of reduced stomatal aperture. These changes seemed to be an adaptative response to protect the photosynthetic process. However, in spite of these anatomical changes, S8 plants strongly reduced P_N in response to NaCl stress.

Effects on the antioxidative metabolism

At 15 days of NaCl treatment no oxidative stress was observed. However, at longer term, S8 plants showed increased oxidative stress parameters such as H_2O_2 accumulation, EL and LP, indicating membrane damage. The salt-induced oxidative stress has been described in other plant species, including herbaceous, woody plant or even *in vitro* plants (Hernández et al., 2001, 2003; Diaz-Vivancos et al., 2013; Ikbal et al., 2014). The membrane damage was also evident in salt recovered pea plants (Hernández and Almansa, 2002). These authors suggested that the new growth conditions

could be perceived by plants as a new stress conditions, at least during the period of recovery studied.

Under NaCl stress, S8 plants undergo a decline of H_2O_2-scavenging enzymes as well as SOD activity. On the other hand, myrtle plants suffered a sharp decline in the APX activity during the experimental period, contrary to that happen in CAT activity. APX and CAT appear to compensate each other at least to a certain degree, as reported in knock-out cytAPX Arabidopsis plants in response to light stress that showed elevated CAT expression (Pnueli et al., 2003). At the end of the recovery period, APX, although showing low activity data, is partially recovered in S4 in relation to the values observed in control plants, but not in S8 plants. The effect of NaCl in reducing APX activity has been described in other plant species, including *in vitro* grapevine plantlets (Ikbal et al., 2014), anger plants (Hernández et al., 2003) or in pea leaves (Hernández et al., 2001). Thus, APX activity seems to be crucial for plant stress tolerance as well as for growth and development (Shigeoka and Maruta, 2014). In a wheat mutant line, showing a 40% decrease in thylakoidal APX, a decline in P_N as well as in growth and seed production was reported (Danna et al., 2003). This lack of APX recovery can be related with the decline observed in plant growth in recovered plants after Phase II.

The decrease in CAT activity observed at 15 days of salinity was also described in pea leaf peroxisomes after 15 days of growth with 70 mM NaCl (Corpas et al., 1993). The increase in CAT activity after Phase I (S4 plants) and after recovery from salt stress (S4 and S8 plants) may suggest an involvement of photorespiration in the response of myrtle plants to long-term NaCl stress and recovery. A correlation between CAT activity and photosynthesis has been described since the increase in CAT reduces the photorespiratory loss of CO_2 (Brisson et al., 1998).

Conclusion

This work integrates morphological, anatomical, physiological and biochemical responses of myrtle plants to NaCl stress. As a general conclusion, myrtle plants are able to grow in the presence of high NaCl levels. To perform this tolerant response, myrtle plants implement different adaptative mechanisms to cope with salt stress (See Figure 6). In the case of S4 plants, at the end of Phase I, they maintained non-photochemical parameters values and increased CAT activity, whereas S8 plants increased the mechanisms for safe dissipation of excess energy [(qN, NPQ and Y(NPQ)]. In addition, S8 plants increased the root/canopy ratio and the chlorophyll content in addition to changes in the leaf anatomy to favour the photosynthesis process. Moreover, in both treatments plants 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. Finally, myrtle plants cope with the established oxidative stress by maintaining and/or activating certain defence mechanisms. Nevertheless, irrigation with the same water used on the controls for 23 days (Phase II) seems to be perceived by myrtle plants as a new challenge, as previously described in other plant species. Finally, we would point out that the present work was carried out under controlled environmental conditions. However, myrtle is a Mediterranean plant, and under field conditions more than one abiotic stress condition occurred. Stress combination can have deleterious effect on plant productivity, and prolonged exposure of plants to abiotic stresses, such as drought, extreme temperature, light stress, or salinity, resulted in the weakening of plant defenses and enhanced susceptibility to biotic stresses (Suzuki et al., 2014). For this reason, under filed conditions, myrtle plants could respond differently to salinity than those observed in this experiment where plants were grown under controlled conditions.

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Table 1.- Effect of NaCl on different growth parameters in *M. communis* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II).

	Treatments			
Growth parameters	**Control**	**S4**	**S8**	**F**
(Phase I)				
DW Leaf (g plant^{-1})	1.61±0.17a	2.94±0.34b	1.80±0.02a	13.02**
DW Stem (g plant^{-1})	1.78±0.15a	2.38±0.24b	1.85±0.08ab	3.88*
DW Root (g plant^{-1})	3.61±0.30a	5.51±0.78b	5.85±0.26b	4.26*
DW Root / DW Shoot	1.06±0.12a	1.04±0.09a	1.60±0.08b	8.29**
(Phase II)				
DW Leaf (g plant^{-1})	4.08±0.27b	2.41±0.32a	2.63±0.34	7.07a*
DW Stem (g plant^{-1})	4.46±0.34b	2.47±0.42a	2.75±0.36a	8.32**
DW Root (g plant^{-1})	5.08±0.49	5.06±0.46	3.83±0.28	3.01 ns
DW Root/ D.W. Shoot	0.60±0.06	1.04±0.08	0.71±0.08	1.12 ns

Data represent the mean ± SE from 6 plants. Different letters in the same row indicate significant differences according Multiple Range Duncan´s Test ($p \leq 0.05$). F values from one-way ANOVA for the different plant growth parameters analysed. F values were significant at 99.9% (***), 99% (**) or 95% (*) levels of probability. Non-significant values are indicated by “ns”.

Table 2.- Effect of increased NaCl levels on leaf water potential (ψl, as MPa), leaf turgor potential (ψt, as MPa); leaf osmotic potential at full turgor ($\psi 100s$, as MPa) and

Proline levels (µmol/g FW) in M. communis plants after 15 and at the end of salinity (Phase I) and after the recovery period (Phase II).

Data represent the mean from 5 plants. For more details, please see Table 1.

	Ψ_l	Ψ_t	Ψ_{100s}	**Proline**
15 Days(Phase I)				
Control	-0.61c	0.65 b	-1.33 b	7.56
S4	-0.69b	0.62 b	-1.47 a	6.99
S8	- 0.78a	0.47a	-1.44a	7.32
[a]F	12.38***	10.70***	20.14**	0.31n.s
30 Days(Phase I)				
Control	-0.68b	0.99b	-1.49 b	8.62
S4	-0.84a	0.86a	-1.59 a	8.22
S8	-0.96a	0.82a	-1.67 a	7.72
[a]F	10.73***	4.93*	13.78**	1.46n.s
Recovery period (Phase II)				
Control	-0.66b	0.76a	-1.33	7.91a
S4	-0.72ab	0.93b	-1.24	7.63a
S8	-0.75a	0.97b	-1.03	10.85b
[a]F	3.04*	8.58***	1.79n.s	3.88*

Table 3.- Effect of increased NaCl levels on total chlorophyll content (mg mg^{-1} FW), net photosynthetic rate (P_N, as µmoles m^{-2} s^{-1}); stomatal conductance (g_s, as mmoles m^{-2} s^{-1}); intrinsic water efficiency (P_N/g_s, as µmol CO_2 mol^{-1} H_2O) in *M. communis* plants after 15 and at the end of salinity period (Phase I) and after the recovery period (Phase II).

	Chlorophyll	**P_N**	**g_s**	**P_N/g_s**
15 Days(Phase I)				
Control	2.36 ±0.05a	1,09±0.08b	23.57±4.02a	51.35±9.96b
S4	2.19±0.08a	0.71±0.12a	24.71±3.54a	28.86±3.74a
S8	2.73±0.02b	0.63±0.09a	23.62±3.38a	26.71±1.50a
[a]F	16.50**	6.33*	0.03n.s	4.84*
30 Days(Phase I)				
Control	2.30 ±0.04a	1.79±0.33b	22.89±4.04b	78.20±2.54b
S4	2.10±0.07a	1.46±0.37ab	24.05±2.42b	60.70±1.78a
S8	2.43±0.01b	0.86±0.16a	10.43±1.30a	82.45±4.35b
[a]F	5.58*	4.90*	3.81*	3.53*
Revovery period (Phase II)				
Control	1.88±0.04a	2.70±0.75b	73.94±24.40 b	36.51±1.00
S4	1.93±0.03a	1.80±0.50ab	57.51±8.83b	31.30±2.04
S8	2.20±0.01b	0.63±0.32a	24.67±3.61a	25.53±8.90
[a]F	20.43**	4,09*	3,51*	0,94n.s

Data represent the mean ± SE from 6 plants. For more details, please see Table 1.

Table 4.- Effect of increased NaCl levels on fluorescence parameters in *M. communis* plants after 15 and at the end of salinity period (Phase I) and after the recovery period (Phase II).

	qP	**Y(II)**	**Fv/Fm**	**qN**	**NPQ**	**Y(NPQ)**
15 Days (Phase I)						
Control	0.667a	0.288a	0.670a	0.755b	0.370a	0.396a
S4	0.720b	0.382c	0.702b	0.700a	0.378a	0.411a
S8	0.697ab	0.352b	0.717b	0.796b	0.535b	0.555b
[a]F	3.15*	25.46***	9.16***	10.25***	8.82***	6.61**
30 Days (Phase I)						
Control	0.712b	0.372b	0.683a	0.649a	0.268a	0.318a
S4	0.636a	0.344b	0.706a	0.658a	0.282a	0.342a
S8	0.643a	0.279a	0.692a	0.815b	0.505b	0.472b
[a]F	4.69*	11.87***	0.97n.s	58.87***	69.47***	44.66***
Recovery period (Phase II)						
Control	0.588a	0.275a	0.670a	0.711b	0.450b	0.480c
S4	0.684b	0.355b	0.693a	0.670a	0.284a	0.338a
S8	0.666b	0.275a	0.666a	0.763b	0.380b	0.415b
[a]F	8.33***	12.10***	1.35n.s	12.10***	10.11***	12.81***

Data represent the mean from at least 50 measurements. For more details, please see Table 1.

Table 5.- Effect of lighting on fluorescence parameters in *M. communis* plants. A batch of plants were grown in the presence of 350 PAR for two months, and the last four days of the experiment, they were exposed to100% solar radiation (HL treatment), whereas other batch of plants were grown in a room chamber in the presence of low light intensity (LL) (100 PAR) for two months.

Lighting	Leaf type	qP	Y(II)	Fv/Fm	qN	NPQ	Y(NPQ)
LL	**Apical**	0.434a	0.240a	0.712c	0.762c	0.392c	0.392c
	Basal	0.444a	0.246ab	0.716c	0.659b	0.280b	0.280b
HL	**Apical**	0.465a	0.249b	0.587a	0.620a	0.193a	0.194a
	Basal	0.639b	0.274b	0.659b	0.780c	0.409c	0.409c
[a]F		32.46***	8.00***	41.13***	48.17***	71.03***	6.54***

Data represent the mean from at least 50 measurements. For more details, please see Table 1.

Table 6.- Quantitative analysis for morphometric data in leaves from control and NaCl-treated plants in *M. communis* plants at the end of the salinity period (Phase I).

	Treatments			
	Control	**S4**	**S8**	**[a]F**
(Phase I)				
Palisade parenchyma	22.51±0.83a	22.40±0.80a	24.36±0.48b	1.68n.s
Spongy parenchyma	49.57±1.38b	48.62±1.22b	42.35±1.29a	7.96**
Intercellular space	27.92±0.58a	28.99±0.89b	33.28±1.51b	7.06**

Data represent the mean ± SE 10 different sections from each treatment (3 plants of each treatment). For more details, please see Table 1.

Table 7.- Effect of increased NaCl levels on oxidative stress parameters in leaves from *M. communis* plants. Electrolyte leakage (EL) and lipid peroxidation (TBARS) were analysed at the end of the salinity period (Phase I) and after the recovery period (Phase II).

	EL (%)	**TBARS (nmoles/g FW)**
15 Days (Phase I)		
Control	29.70±1.25	5.60±0.21
S4	30.62±0.85	6.20±0.43
S8	30.47±0.77	5.82±0.28
[a]F	1.53 ns	0.93n.s
30 Days (Phase I)		
Control	34.43±1.20a	5.97±0.14a
S4	36.27±0.54a	5.95±0.20a
S8	39.73±0.97b	7.51±0.55b
[a]F	8.07***	6.55*
Revovery period (Phase II)		
Control	28.03±1.70a	7.14±0.45a
S4	37.10±1.22a	7.67±0.49a
S8	41.54±2.20a	8.65±0.15b
[a]F	15.39***	6.63*

Data represent the mean ± SE from 10 plants. For more details, please see Table 1.

Table 8.- Effect of NaCl on the activity of some antioxidant enzymes in leaves from *M. communis* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II).

	CAT	**APX**	**MDHAR**	**GR**	**SOD**
	µmol/g FW	**nmol/gFW**	**nmol/g FW**	**nmol/g FW**	**U/g FW**
15 Days (Phase I)					
Control	462,2±6,8b	90,0±2,7b	180,0±18,7a	28,4±1,7a	35,5±1,2b
S4	363,6±10,5a	88,0±4,3b	140,1±5,7a	43,8±3,1c	36,4±4,6b
S8	370,3±16,8a	76,1±1,9a	134,0±13,1a	36,2±2,5b	26,1±1,6a
[a]F	31,49***	8,97*	2,96n.s	10,78**	3,88*
30 Days (Phase I)					
Control	306,4±10,5a	29,8±2,4b	171,2±22,0a	20,7±3,4a	25,5±1,8b
S4	628,6±14,5b	11,4±0,7a	186,9±9,3a	29,0±6,1a	25,3±2,2b
S8	321,2±24,5a	11,3±1,4a	239,9±6,2b	31,9±3,6a	17,9±1,0a
[a]F	81,01***	36,24***	7,22**	1,65n.s	6,53*
Revovery period (Phase II)					
Control	402,2±91,2a	12,9±0,9c	8,6±0,5b	nd	12,6±2,9a
S4	1247,5±63,7c	8,4±0,6b	7,5±0,6a	nd	10,5±0,4a
S8	654,1±54,1b	5,9±0,6a	7,8±0,8ab	nd	13,8±0,8a
[a]F	36,96***	23,28***	3,18*		1,04n.s

Data represent the mean ± SE from at least 6 plants. For more details, please see Table 1; nd: non detected

Legend to Figures

Fig 1.- Effect of increased concentrations of NaCl on the uptake rates of Na^+ (A-D), Cl^- (E-H), K^+ (I-L) and Ca^{2+} (M-P) ions in *M. communis* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II). Data represent the mean ± SE from 6 plants. Different letters in the same experimental period indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$) in C, S4 and S8 treatments. Different letters in the same experimental period indicate significant differences according to t test ($p \leq 0.05$) in C and S12 treatments.

Fig 2.- Distribution of Na^+ (A-D), Cl^- (E-H), K^+ (I-L) and Ca^{2+} (M-P) in different organs of *M. communis* plants at the end of the salinity period (Phase I) and after the recovery period (Phase II). For more details, please see legend to Figure 1.

Fig 3.-Light response curves of the relative photosynthetic ETR in leaf of *M. communis* plants at the end of the salinity period (A, Phase I) and after the recovery period (B, Phase II). (C) ETR/P_N ratio at the end of salinity period (Phase I) and after the recovery period (Phase II). The maximum ETR values in each period were used to obtain the ETR/P_N ratios.

Fig 4.- Scheme showing the main adaptative responses implemented by *M. communis* plants after 30 days of NaCl exposure. Myrtle plants avoid ionic stress in leaves, maintaining low Na^+ and Cl^- contents and unchanged K^+ and Ca^{2+} levels. Myrtle plants protect the photosynthetic process by maintaining chlorophyll levels (S4 and S8 plants), but also by increasing (S8) or maintaining (S4) the non-photochemical quenching parameters. In addition, S8 plants displayed a best stomatal control and experimented anatomical leaf changes aimed to facilitate the CO_2 diffusion in a situation of reduced stomatal aperture. S4 plants increased CAT activity, suggesting increased photorespiratory activity. Myrtle plants decreased the root DW/ shoot DW ratio by

increasing root biomass, a mechanism to favour higher water and nutrient uptake as well as toxic ions accumulation (Marchner 1995). Finally, roots from myrtle plants showed increased Ca^{2+} rate uptake and unchanged K^{+} rate uptake, allowing the maintenance of Ca^{2+} and K^{+} contents in roots.

Fig S1.- Chlorophyll fluorescence parameters in leaves of *M. communis* at 15 and 30 days of NaCl stress (Phase I) and after the recovery period (Phase II). Images of the coefficient of photochemical quenching (qP), the effective PSII quantum yield [Y(II)] and the maximal PSII quantum yield (Fv/Fm), the non-photochemical quenching coefficient (qN), non-photochemical quenching (NPQ) and the quantum yield of regulated energy dissipation [Y(NPQ)].

Fig S2.- Light response curve of the relative photosynthetic ETR in leaf of *M. communis* plants exposed to low light (LL) or to high light (HL).

Fig S3.- Light microscopy images showing the effect of NaCl on the percentage of area occupied by palisade parenchyma (PP), spongy parenchyma (SP), intercellular spaces (IS) and idioblaste (ID) in leaves from *M. communis* plants at the end of the salinity period (Phase I; A, control; B, S4; C, S8).

Fig S4.- Effect of NaCl (12 dS m^{-1}) on ROS accumulation in leaves from *M. communis* plants at the end of the salinity period. Hydrogen peroxide was detected by staining with 3,3-diaminobenzidine (DAB). A, control leaves; B, S4 leaves; C, S8 leaves; D, detail from C showing DAB-staining zones.