

Allocation pattern, photosynthetic performance and sugar metabolism in hydroponically grown seedlings of loquat (*Eriobotrya japonica* Lindl.) subjected to salinity

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Abstract

Hydroponically grown loquat seedlings were subjected to increasing NaCl concentrations (0, 12, 24, and 48 mM) for 42 d. At the whole plant level, Na⁺ and Cl⁻ accumulated proportionally to the salt treatments; however, Na⁺ accumulated prevalently in leaves whilst Cl⁻ accumulated prevalently in stems. Reduction of leaf biomass was only observed in seedlings grown with 48 mM NaCl, whereas net photosynthesis declined along with increasing NaCl concentration and salinity led to general accumulation of glucose, reduction of mannitol and sorbitol, and changes in pigment composition in loquat leaves. Increasing level of salinity induced accumulations of both proline and H₂O₂ but not of malondialdehyde byproducts. Dramatic alteration of sugar patterns in the leaves poses serious concerns about possible effects of salinity to fruit production. Further research is necessary to investigate the concentration at which NaCl significantly compromises the yield of this economically important fruit species.

Additional key words: carotenoids; chlorophyll; gas exchange; NaCl; proline; salt stress.

Introduction

Loquat (*Eriobotrya japonica* Lindl.) is an evergreen fruit tree species belonging to the Rosaceae family (Lin *et al.* 2007) which is grown commercially in subtropical to mild temperate climates, including the Mediterranean area (García-Legaz *et al.* 2005, 2008). Salinity is one of the major abiotic stresses that affects plant growth and productivity in many arid and semiarid environments all over the world, including the major sites where loquat is cultivated, such as the Mediterranean basin (Chaves *et al.* 2009, Landi and Benelli 2016, Guidi *et al.* 2017). To date, there is insufficient data about the effect of salinity on the physiology/biochemistry of this species, though it has been observed that salinity can dramatically reduce photosynthetic performance (García-Legaz *et al.* 2005), alter antioxidant systems, and can seriously increase the level of oxidative stress (López-Gómez *et al.* 2007). In

addition, there is a lack of knowledge about the sugar (and polyalcohol) metabolism and the pigment composition of salt-stressed loquat trees.

There are two main negative effects induced by a hypersaline environment on plant growth and development: water deficit and ion toxicity associated with excessive Cl⁻ and Na⁺ (Chaves *et al.* 2009, Shabala and Munns 2012). Impairment of photosynthetic performance under salt stress is firstly a consequence of stomatal limitations induced by the osmotic stress perceived by the leaf when plants are grown in a hypersaline environment (Shabala and Munns 2012). Under these circumstances, there is a limited CO₂ availability, as well as the accumulation of ATP and NADH, which leads to an excess of excitation energy (Takahashi and Badger 2011). As a consequence, the deregulation of the cellular metabolism can cause an increase in rates of the reactive oxygen species (ROS) production. Indeed, ROS accumulation is one of the most

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Abbreviations: Car – carotenoids; Chl – chlorophyll; C_i – intercellular CO₂ concentration; E – transpiration; g_s – stomatal conductance; MDA – malondialdehyde; P_N – net photosynthesis; ROS – reactive oxygen species; TBA – 2-thiobarbituric acid; TCA – trichloroacetic acid; WUE – water-use efficiency.

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frequently observed reactions in salt-treated plants (Parihar *et al.* 2015), including loquat seedlings (Hernández *et al.* 2003), which can trigger oxidative damage to the chloroplasts (Triantaphyllides and Havaux 2009). Metabolic limitations of photosynthesis in plants affected by salinity can also be a result of harmful concentrations of Na^+ and Cl^- in the leaf tissue (Munns *et al.* 2006). In some species, Cl^- accumulation significantly impairs the photosynthetic process (Tavakkoli *et al.* 2010), whereas ion excess (both Na^+ and Cl^-) induces a reduction of leaf expansion as a consequence of reduced leaf transpiration rate. In turn, this results into a surplus of photosynthates in actively growing tissues (Munns *et al.* 2000). Excess of photosynthates induces feedback signals which down-regulate the photosynthesis to compensate the lower sugar demand due to the temporary inhibition of plant growth, even if in some cases sugar accumulation can occur before feed-back regulatory mechanisms take place (Lemoine *et al.* 2013). Furthermore, the accumulation of Na^+ and Cl^- at cytosolic or chloroplast level inhibits the activity of photosynthesis-related enzymes, thereby further reducing CO_2 photoassimilation (Sudhir and Murthy 2004).

Responses of plants to high salinity encompass a plethora of mechanisms at both biochemical and physiological levels, such as the control/exclusion of ion uptake by roots and transport into leaves, the compartmentalization of ions at the cellular and organ levels, the change in photosynthetic pathway, the alteration in membrane structure, the induction of antioxidant systems, stimulation of plant hormones, and the synthesis of compatible solutes including proline and sugars (*sensu lato* including polyols) (Sudhir and Murthy 2004, Parida and Das 2005). In plants grown under high salinity conditions, these metabolites can indeed act as osmoprotectants (Gupta and Huang 2014), whereas proline can also play a key role in the stabilization of proteins and protein complexes in the chloroplast and cytosol, and in the protection of the photosynthetic apparatus (as shown in the salt-hypersensitive *p5cs1 Arabidopsis* mutant) (Szabados and Savaure 2010).

The aim of the present research was thus to: (1) characterize the allometric responses of loquat organs to prolonged salinity exposure, as well as the allocation pattern of ions mainly perturbed by salinity stress (Na^+ , Cl^- , and K^+), and (2) determine the photosynthetic responses and changes in pigment and carbon metabolism of loquat saplings subject to increasing levels of salinity.

Materials and methods

Plant material and growth conditions: Six-month-old seedlings of *E. japonica*, uniform in stem diameter, leaf area, and height, were initially grown in black plastic pots containing a mixture of peat:perlite (2:1, v/v) at a glasshouse located at the arboretum of the Agricultural University of Athens (latitude 37.981907, longitude 23.705639).

Afterward, the plants were individually transplanted into black plastic bags containing 2 l of a mixture of silica sand:perlite (1:2, v/v) for 21 d (acclimation period). During transplanting, the seedling roots were thoroughly washed firstly with tap water and then with distilled water in order to remove residuals of the initial mixture. During the acclimation period, loquat seedlings were irrigated three times per week with tap water. Afterwards, the plants were irrigated, thrice per week, with a full-strength Hoagland's nutrient solution, differing in NaCl concentration (0, 12, 24, and 48 mM). In total, 20 plants were used (five replicates per each NaCl treatment). During the growing period, maximum and minimum average temperatures were $28.8 \pm 4.7^\circ\text{C}$ and $19.9 \pm 2.7^\circ\text{C}$, respectively. The irradiance averaged $580 \mu\text{mol}(\text{quantum}) \text{m}^{-2} \text{s}^{-1}$.

One week after the appearance of the initial mild visible symptoms of salinity stress (in the form of yellow-brown necrosis on the leaf margin; see Fig. 1S – *supplement*) in basal leaves of seedlings grown under 48 mM NaCl, *i.e.*, 41 d after NaCl imposition, gas-exchange parameters were measured (details below). The next day, the third and the fourth fully-expanded mature leaf from the top to the base of the shoot (not symptomatic; completely developed under the salt treatment), were sampled for biochemical determinations. After the measurement of the number of leaves and their total area per plant, including the leaves sampled for the aforementioned determinations, each plant part was weighted (FM), washed initially with tap water and afterwards twice with distilled water. Finally, all plant parts were oven-dried at 75°C until they become a constant mass (DM).

Na^+ , Cl^- and K^+ concentration in plant organs: Dried plant parts were milled to a fine powder. The concentrations of Na^+ , Cl^- , and K^+ were determined in all plant parts. For measurements of K^+ and Na^+ , dried powder (0.5 g) of each sample was ashed for 5.5 h at 550°C , dissolved in 3 ml of 6 N HCl and diluted with deionized water up to 50 ml. Then, K^+ and Na^+ concentrations were measured using a flame spectrophotometer (PGI 2000, PG Instruments Ltd., Alma Park-Leicestershire, UK) standard methods. For Cl^- analysis, dried powder 0.2 g of each plant part was weighed and put in falcon tubes containing 25 ml of deionized water and were continuously shaken for 24 h. Then a centrifugation ($4,400 \times g$ for 15 min at room temperature) was carried out, and afterwards the supernatant was further filtered by using lab filter paper. Cl^- concentration was determined after titration with 0.0141 N AgNO_3 using the 702 SM Titrimetric potentiometric automatic titrator (Metrohm AG, Herisau, Switzerland).

Chlorophyll (Chl) and carotenoid (Car) content: Fresh leaf samples (75 mg) were ground in a mortar with 10 ml of cold acetone (80%), transferred in falcon tubes, kept in the dark for 1 h, and vortexed at 15-min intervals. A

centrifugation at $4,400 \times g$ for 5 min at 4°C was then performed. Chl and Car concentrations were determined spectrophotometrically at absorbance of 470, 647, and 663 nm and using the equation described by Lichtenthaler and Buschmann (2001):

$$[\text{Chl } a] = 12.25A_{663} - 2.79A_{647}$$

$$[\text{Chl } b] = 21.5A_{647} - 5.1A_{663}$$

$$[\text{Car}] = \{1000A_{470} - 1.82 [\text{Chl } a] - 85.02 [\text{Chl } b]\}/198$$

Sugar, sorbitol, mannitol, and starch: For soluble carbohydrates determination, 30 mg of freeze-dried leaf tissue were added to 2 ml of HPLC grade water (*Carlo Erba Reagents S.A.S.*, Val-de-Reuil, France) and vortexed for 20 s. Then extraction of the water-soluble carbohydrates was performed in a microwave oven for 2 min at 400 W. After centrifugation ($4,400 \times g$ for 10 min at 4°C), the supernatant was removed and this process was repeated. The two supernatants were pooled together and filtered using syringe filters (0.2 μm pore size). HPLC analyses were conducted using a HPLC pump (*model 510 Waters*, Milford, MA, USA) equipped with an HP refractive index-RI (*HP 1047A*, HP, Palo Alto, CA, USA). The mobile phase consisted of HPLC grade water. Twenty μl of extract was injected into an *Agilent HI-PLEX Ca²⁺* column (7.7×300 mm, 8 μm , mobile phase: 100% DI H_2O , flow rate of 0.6 ml min^{-1} , temperature of 80°C , detector: refractive index) (*Agilent*, Santa Clara, CA, USA). The processing of the chromatograms was conducted by means of *PeakSimple* chromatography data system consisted of a hardware (*Model 302*, *SRI Instruments*, Bad Honnef, Germany) and the *PeakSimple 4.51* chromatography acquisition and integration software for *Windows* (*SRI Instruments*, Bad Honnef, Germany). Sucrose, glucose, fructose, sorbitol, and mannitol quantitative determination was based on a specific reference curve with HPLC pure grade standards. The concentration of starch in loquat leaves was measured according to the procedure described by Vemmos (1999).

Determination of lipid peroxidation and H_2O_2 : Leaf tissue (0.5 g FM) was homogenized in 10 ml 0.1% trichloroacetic acid (TCA) at 4°C . After centrifugation at $4,400 \times g$ for 15 min at 4°C , the supernatant was used for the determination of both lipid peroxidation level and H_2O_2 concentration. Lipid peroxidation was measured as malondialdehyde (MDA) byproduct content determined by reaction with 0.5% 2-thiobarbituric acid in 20% TCA (w/v). The concentration of MDA was calculated from the difference of the absorbance at 532 and 600 nm using the extinction coefficient of $155 \text{ mmol}^{-1} \text{ cm}^{-1}$ (Heath and Packer 1968). Hydrogen peroxide was also measured spectrophotometrically after a reaction with potassium iodide (KI). The reaction mixture consisted of 0.5 mL of 0.1% TCA, leaf extract supernatant, 0.5 ml of 0.1 M potassium-phosphate buffer (pH 7), and 1 ml of 1 M KI (w/v). The reaction color was developed within 45 min

in darkness and absorbance was measured at 390 nm (*Spectronic Helios Gamma UV-VIS*, *Thermo Fisher Scientific*, UK). The amount of hydrogen peroxide was calculated using a standard curve prepared with eight known concentrations of H_2O_2 .

Proline estimation: Fresh leaves (0.1 g) were cut into small pieces and ground with a mortar with 10 ml of 80% (v/v) ethanol. The homogenate was placed in falcon tubes and then centrifuged at $4,400 \times g$ for 5 min at 4°C . One milliliter of the supernatant was transferred into test tubes containing 2 ml of acid-ninhydrin. After mixing, test tubes were maintained at 95°C for 25 min in a water bath and then transferred to an ice bath and allowed to cool at room temperature. After centrifugation (5 min for $4,400 \times g$ at 4°C), the absorption of the supernatant was recorded at 520 nm (*Spectronic Helios Gamma UV-VIS*, *Thermo Fisher Scientific*, UK) and proline was quantified as described by Bates *et al.* (1973).

Gas-exchange parameters were measured using a portable *Li-Cor 6400* (*Li-Cor*, Lincoln, NE, USA) infrared gas analyzer. Net photosynthetic (assimilation) rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were measured twice per plant and per NaCl treatment, using both the 3rd and 4th fully developed leaves from the shoot apex. To avoid the effects of fluctuating environments on gas-exchange measurements, all plants were measured between 9:50 and 11:15 h (GMT), under steady-state light intensity [$1,200 \mu\text{mol}(\text{quantum}) \text{ m}^{-2} \text{ s}^{-1}$] and CO_2 concentration (390 mg l^{-1}), while the leaf temperature ranged between 28.1 and 29.6°C . Intrinsic water-use efficiency (WUE_i) was calculated as P_N/g_s , whereas instantaneous WUE was calculated as P_N/E . Carbon dioxide-use efficiency was estimated as P_N/C_i .

Statistical analysis: Homogeneity of variance was assessed by *Bartlett's* test and then the data were subjected to one-way analysis of variance (*ANOVA*) using the '*PASW Statistics 18*' statistical package (*SPSS INC.*, Chicago, IL, USA). Percentage values were arcsine transformed prior to analyses. For mean separation, the *Tukey's* multiple range post-hoc test was applied ($P \leq 0.05$). Linear correlations were calculated using the *Pearson's* correlation test using *GraphPad* (La Jolla, CA, USA). The experiments were arranged following a completely randomized experimental design.

Results

Dry mass partitioning and biometric parameters: After 42 d of NaCl treatment, the salt-treated plants maintained similar stem biomass production, independently of the NaCl concentration imposed (Table 1). Increased root DM was observed in salt-treated plants, even though the

Table 1. Biometric parameters of *Eriobotrya japonica* seedlings grown under control (0 mM) and excess of NaCl (12, 24, 48 mM) for 42 days. Means ($n = 5 \pm \text{SE}$) with *different letters* are different after one-way *ANOVA* followed by multiple range *Tukey's* post-hoc test ($P \leq 0.05$).

Parameter	NaCl [mM]			
	0	12	24	48
Leaves				
[g(DM) per plant]	6.18 ± 0.32^b	7.13 ± 0.38^b	6.91 ± 0.41^b	5.37 ± 0.45^a
Stem [g(DM) per plant]	1.68 ± 0.13^a	1.44 ± 0.14^a	1.77 ± 0.14^a	1.60 ± 0.17^a
Root [g(DM) per plant]	1.64 ± 0.13^a	1.86 ± 0.14^a	1.93 ± 0.15^a	1.74 ± 0.15^a
Total [g(DM) per plant]	9.43 ± 0.55^a	10.43 ± 0.67^a	10.60 ± 0.66^a	8.71 ± 0.66^a
Shoot/root	4.81 ± 0.19^a	5.55 ± 0.34^a	6.00 ± 0.47^a	5.17 ± 0.48^a
Leaf area [cm ² per leaf]	72.95 ± 6.20^a	67.10 ± 2.77^a	73.47 ± 9.80^a	76.48 ± 12.98^a
Leaf number	11.25 ± 0.63^b	12.00 ± 0.41^b	12.00 ± 0.41^b	9.50 ± 0.65^a
Plant leaf area [cm ²]	817.6 ± 69.7^b	796.3 ± 118.4^b	872.9 ± 100.2^b	704.5 ± 72.1^a

Table 2. Na⁺, Cl⁻, and K⁺ concentration [% (DM)] as well as K⁺/Na⁺ ratio in leaves, stem and roots of *Eriobotrya japonica* seedlings grown under control (0 mM) and excess of NaCl (12, 24, 48 mM) for 42 days. Means ($n = 5 \pm \text{SE}$) with *different letters* are different after one-way *ANOVA* followed by multiple range *Tukey's* post-hoc test ($P \leq 0.05$).

Parameter	NaCl [mM]			
	0	12	24	48
Na ⁺ leaves [% DM]	$0.09 \pm <0.01^a$	$0.23 \pm <0.01^b$	0.51 ± 0.03^c	0.73 ± 0.09^d
Na ⁺ stem [% DM]	$0.08 \pm <0.01^a$	0.13 ± 0.02^b	0.14 ± 0.02^b	0.20 ± 0.01^c
Na ⁺ roots [% DM]	0.11 ± 0.02^a	0.21 ± 0.01^b	0.30 ± 0.04^c	0.57 ± 0.09^d
Cl ⁻ leaves [% DM]	$0.03 \pm <0.01^a$	$0.04 \pm <0.01^a$	0.12 ± 0.02^b	0.24 ± 0.03^c
Cl ⁻ stem [% DM]	$0.09 \pm <0.01^a$	0.11 ± 0.01^a	0.32 ± 0.03^b	0.45 ± 0.03^c
Cl ⁻ roots [%DM]	0.11 ± 0.01^a	0.61 ± 0.1^b	0.63 ± 0.01^b	0.81 ± 0.07^c
K ⁺ leaves [% DM]	2.55 ± 0.15^a	2.46 ± 0.10^a	2.27 ± 0.18^a	2.36 ± 0.62^a
K ⁺ stem [% DM]	2.74 ± 0.11^a	2.14 ± 0.75^a	2.21 ± 0.13^a	2.30 ± 0.22^a
K ⁺ roots [% DM]	2.27 ± 0.21^a	2.10 ± 0.20^a	2.66 ± 0.14^a	2.54 ± 0.10^a
K ⁺ /Na ⁺ leaves	28.0 ± 1.2^d	11.0 ± 0.6^c	4.5 ± 0.3^b	3.5 ± 0.5^a
K ⁺ /Na ⁺ stem	35.9 ± 1.7^c	18.4 ± 2.8^b	16.2 ± 1.6^b	11.9 ± 0.9^a
K ⁺ /Na ⁺ roots	22.2 ± 3.3^c	10.3 ± 1.2^b	9.5 ± 1.2^b	4.8 ± 0.6^a

difference was not significant (Table 1). Of note, the root apparatus was the only organ in which a significant increase of moisture content was recorded ($\sim +3\%$ on average than controls; data not shown). Conversely, we observed a reduction of leaves DM only at the highest NaCl concentration (6.2 vs. 5.4 g per plant in controls and plants treated with 48 mM of NaCl, respectively). This reduction was attributable to the reduced number of leaves of the 48 mM-salt-treated plants, which correlates with a reduced leaf area per plant (-14% with respect to controls) (Table 1). No significant changes were observed between controls and salt-treated plants in terms of shoot/root ratio (Table 1).

Partitioning of Na⁺, Cl⁻ and K⁺ between plant organs:

Na⁺ and Cl⁻ accumulation paralleled the severity of salt treatment (Table 2) and significant linear correlations were found between NaCl supply against Na⁺/Cl⁻ accumulated in different plant organs (Fig. 1A,B). Even though Na⁺ and Cl⁻ accumulated with a similar extent at the whole plant

level, at each salt treatment (e.g., at 48 mM, Na⁺ and Cl⁻ averaged 0.50 and 0.42% DM, respectively), Na⁺ and Cl⁻ had different patterns of distribution to different plant organs. Na⁺ accumulated principally in leaves, then in roots and stems (slope of linear correlation was 0.013, 0.0095, and 0.0024, respectively), whereas Cl⁻ accumulation occurred preferentially in roots followed by stems and leaves (slope of linear correlation was 0.013, 0.008, and 0.004, respectively). Of note, linear correlation between NaCl supply and Cl⁻ accumulation in roots (Fig. 1B) only had a r^2 of 0.74 due to the high variability observed in Cl⁻ accumulation at 12 mM NaCl. Roots accumulated proportionally more Cl⁻ than Na⁺ at 48 mM NaCl, whereas the accumulation of both Na⁺ and Cl⁻ in leaves was similar under the same treatment (~ 8 -fold higher than controls for both the elements). No differences were found regarding K⁺ partitioning, even though the ratio of K⁺/Na⁺ decreased severely along with NaCl treatments, with the highest decrement observed in leaves, followed by roots and stems (Table 2).

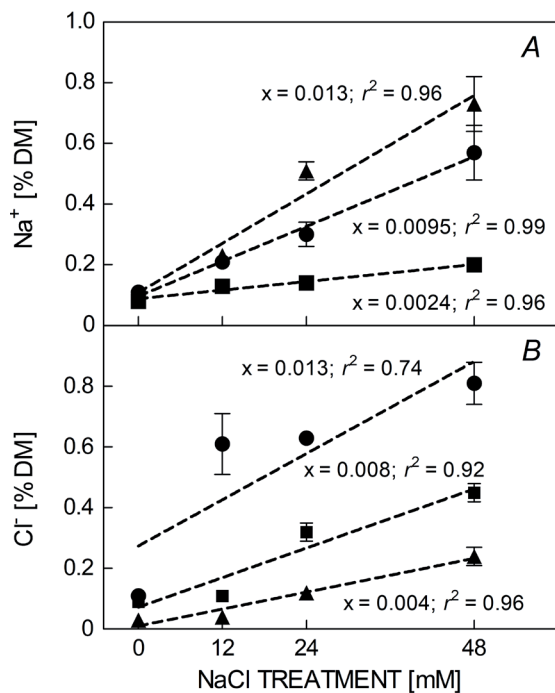


Fig. 1. Linear relation between increasing concentrations of NaCl in the nutrient solution (0, 12, 24, and 48 mM) vs. (A) Na^+ and (B) Cl^- concentration [% DM] in leaves (triangle), stem (circle), roots (square) of *Eriobotrya japonica*. Linear correlations were tested using Pearson's correlation test ($P \leq 0.05$). Coefficient of determination (r^2) and equation slope (x) were reported.

Sugar metabolism in leaves: At the end of the experimental period, we observed a strong accumulation of glucose, independent of the severity of salt treatment (from 0.24–0.44, 0.56, 0.52% in plants grown with 0, 12, 24, and 48 mM NaCl, respectively) (Table 3). These increments led to higher contents of nontranslocated sugars in the leaves, given that the concentration of fructose

was mostly constant regardless of the level of imposed salinity. Conversely, we observed a general decline of sucrose and other translocating polyols (mannitol and sorbitol, which are typical of loquat) but these decrements were not consistent with the severity of salinity. In fact, plants grown with 12 mM NaCl exhibited the lowest foliar concentration of total sugars, which is dependent on the strongest reduction of sorbitol, as evidenced by the lowest concentration of translocated sugars observed in plants belonging to this treatment. The ratio between translocated to non-translocated sugars was approximately 50% lower in salt-treated plants, consistently at each salt level. No significant changes were observed between controls and treated plants in terms of starch amount (Table 3).

Pigment composition: Plants grown under salinity showed a lower content of total Chl than that of controls, even though we did not observe a reduction of Chl *b* at any salt treatment, and only a significant decline of Chl *a* was observed in plants submitted to the most severe salt treatment (Fig. 2A). A consistent reduction of Chl *a/b* ratio was observed at all the NaCl concentrations (Fig. 2B), whereas no significant variations were observed in Car contents (Fig. 2C).

H_2O_2 , MDA and proline accumulation: Higher contents of H_2O_2 were observed in leaves of salt-treated plants, with the highest accumulation shown by plants grown at 48 mM NaCl [1.82 in controls vs. 1.95, 2.32, and 3.11 $\mu\text{mol g}^{-1}(\text{FM})$ at 12, 24 and 48 mM NaCl, respectively] (Fig. 3A). No differences were observed between plants grown at 12 and 24 mM NaCl concerning leaf H_2O_2 content. No significant differences in MDA byproduct contents were found between controls and salt-treated plants (Fig. 3B), whilst leaves of salt-treated plants had higher content of proline

Table 3. Concentrations of glucose (Glu), fructose (Fru), sucrose (Suc), mannitol, (Man), sorbitol, (Sorb), total sugars (Suc + Glu + Fru + Man + Sorb), translocated sugars (Suc + Man + Sorb), nontranslocated sugars (Fru + Glu), the ratios between translocated/nontranslocated, translocated/total, nontranslocated/total, and starch content in *Eriobotrya japonica* seedlings grown under control (0 mM) and excess of NaCl (12, 24, and 48 mM) for 42 days. Means ($n = 5 \pm \text{SE}$) with different letters are different after one-way ANOVA followed by multiple range Tukey's post-hoc test ($P \leq 0.05$).

Parameter	NaCl [mM]			
	0	12	24	48
Glucose [% DM]	0.24 \pm 0.01 ^a	0.44 \pm 0.02 ^b	0.56 \pm 0.02 ^c	0.52 \pm 0.02 ^c
Fructose [% DM]	0.20 \pm 0.01 ^a	0.20 \pm 0.02 ^a	0.22 \pm 0.01 ^a	0.21 \pm 0.03 ^a
Sucrose [% DM]	6.59 \pm 0.09 ^b	5.63 \pm 0.22 ^a	6.05 \pm 0.15 ^a	5.60 \pm 0.18 ^a
Mannitol [% DM]	1.59 \pm 0.16 ^b	0.94 \pm 0.15 ^a	0.96 \pm 0.06 ^a	1.18 \pm 0.03 ^a
Sorbitol [% DM]	6.31 \pm 0.22 ^c	4.91 \pm 0.31 ^a	5.63 \pm 0.32 ^b	5.64 \pm 0.27 ^b
Total sugars [% DM]	14.93 \pm 0.38 ^c	12.12 \pm 0.60 ^a	13.41 \pm 0.47 ^b	13.13 \pm 1.05 ^b
Translocated	14.49 \pm 0.38 ^c	11.48 \pm 0.61 ^a	12.64 \pm 0.45 ^b	12.42 \pm 1.03 ^b
Nontranslocated	0.44 \pm 0.01 ^a	0.64 \pm 0.02 ^b	0.78 \pm 0.02 ^c	0.73 \pm 0.02 ^c
Trans/nontrans	32.60 \pm 0.68 ^b	18.00 \pm 1.36 ^a	16.27 \pm 0.36 ^a	17.20 \pm 0.99 ^a
Trans/total	0.97 \pm <0.01 ^b	0.95 \pm <0.01 ^a	0.94 \pm <0.01 ^a	0.94 \pm 0.01 ^a
Nontrans/total	0.03 \pm <0.01 ^a	0.05 \pm <0.01 ^b	0.06 \pm <0.01 ^b	0.06 \pm <0.01 ^b
Starch [% DM]	0.14 \pm 0.01 ^a	0.15 \pm 0.01 ^a	0.14 \pm 0.01 ^a	0.14 \pm 0.01 ^a

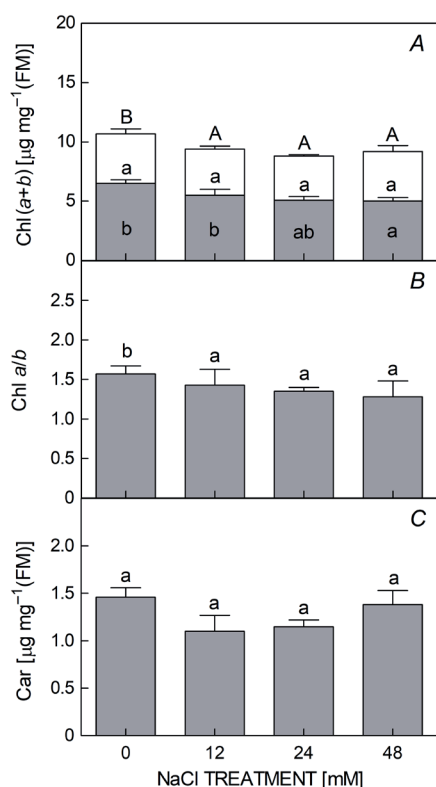


Fig. 2. Content of (A) total chlorophyll (Chl_{TOT}) as the sum of Chl *a* (grey bar) and Chl *b* (white bar); (B) Chl *a/b* ratio, and (C) total carotenoid (Car) in leaves of *Eriobotrya japonica* seedlings in relation to increasing concentrations of NaCl in the nutrient solution (0, 12, 24, and 48 mM). Means ($n = 5 \pm \text{SE}$) with similar lowercase letters are not statistically different according to multiple range Tukey's post-hoc test ($P \leq 0.05$). Means with different capital letters indicate that averages of Chl_{TOT} are statistically different according to multiple range Tukey's post-hoc test ($P \leq 0.05$).

than that of controls (+32.3, +54.4, and +36.7% at 12, 24, and 48 mM NaCl, respectively) (Fig. 3C).

Photosynthetic parameters: Photosynthetic rate was severely constrained by salinity and the most severe reduction of P_N was observed with the highest NaCl concentration (−63.1%). The decline of P_N was less severe and similar in plants grown with 12 and 24 mM NaCl (−36.7 and 44.3%, respectively) (Fig. 4A). Values of g_s declined similarly in salt-treated plants and independently to the NaCl concentration that were imposed (Fig. 4B). Intrinsic WUE (P_N/g_s ; Fig. 4C) as well instantaneous WUE (P_N/E ; data not shown) was similar in both controls and 12-mM-NaCl-treated plants whilst lower and similar values were observed in plants treated with 24 and 48 mM NaCl. Values of P_N/C_i declined under salinity, with the most severe reduction found in plants grown with 24 and 48 mM of NaCl in the nutrient solution (−55.6 and −66.6%, respectively) (Fig. 4D).

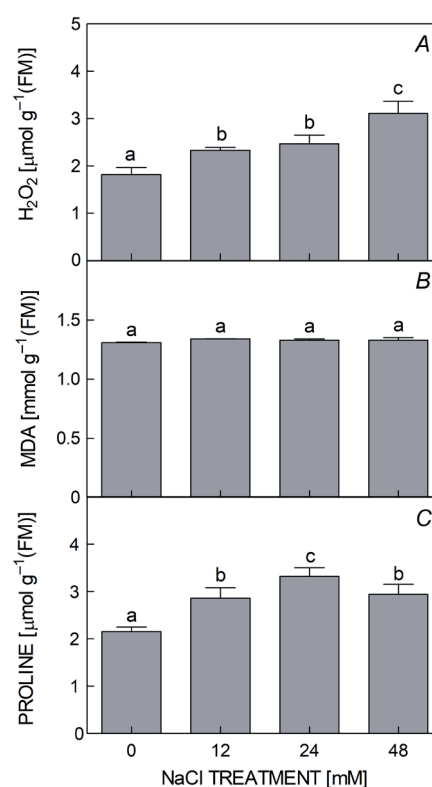


Fig. 3. Contents of (A) H_2O_2 , (B) malondialdehyde byproducts (MDA), and (C) proline in leaves of *Eriobotrya japonica* seedlings in relation to increasing concentrations of NaCl in the nutrient solution (0, 12, 24, and 48 mM). Means ($n = 5 \pm \text{SE}$) with the same letters are not statistically different according to multiple range Tukey's post-hoc test ($P \leq 0.05$).

Discussion

One of the initial effects of salinity is the reduction of growth rate which can be observed either at whole-plant level or more evidently on specific plant organs (Parida and Das 2005, Parihar *et al.* 2015). The reduction of plant growth is mostly the result of the deleterious effects of root-zone salinity that induces low osmotic potential, nutritional imbalance, toxicity of Na^+ and/or Cl^- (and combination of these factors). In the attempt to contrast/counteract salt-promoted effects, multiple biochemical, physiological and molecular alterations occur *in planta* (Munns 2002, Krasensky and Jonak 2012). Among the biometric parameters measured in the present experiments, we only observed a significant reduction of leaf area per plant in plants grown with the highest NaCl concentration (48 mM), which was attributable to both fewer in number and smaller in size leaves per plant with respect to controls. In these plants, typical symptoms of NaCl toxicity (marginal leaf necrosis and necrotic patches close to the margin of the leaf, Fig. 1S, *supplement*) appeared in basal, older leaves (not contemplated for biochemical and physiological analyses). No symptoms were observed

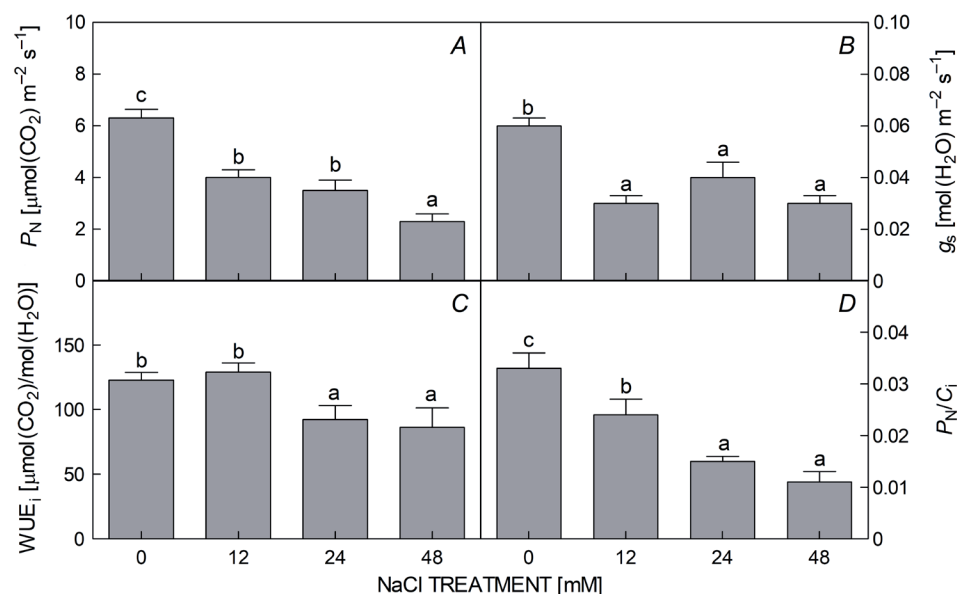


Fig. 4. (A) Net photosynthesis (P_N), (B) stomatal conductance (g_s), (C) intrinsic water-use efficiency (WUE_i), and (D) CO_2 -use efficiency (P_N/C_i) in leaves of *Eriobotrya japonica* seedlings in relation to increasing concentrations of NaCl in the nutrient solution (0, 12, 24, and 48 mM). Means ($n = 10 \pm \text{SE}$) with the same letters are not statistically different according to multiple range Tukey's post-hoc test ($P \leq 0.05$).

in leaves of plants grown with 12 and 24 mM NaCl. This suggests that, at least under the experimental conditions applied in current study, loquat seedlings were able to adjust their metabolism to successfully counteract the effect of NaCl when the concentrations were lower than 48 mM. Other authors have observed a more severe reduction of loquat biomass, imposing similar levels of salinity contrary to those tested in the present experiments (García-Legaz *et al.* 2005, López-Gómez *et al.* 2007). However, other differences related to the experimental protocol (*e.g.*, longer exposure, different age of plant material used and/or different cultivation methods) make the comparison very difficult.

The maintenance of ion balance is crucial for the maintenance of plant growth under salinity because one of the main effects of salinity is the disruption of ion homeostasis (Zhu 2003). Despite its toxicity, Cl^- is considered as the most detrimental factor when plants are grown in hypersaline environment. The perturbation of cation homeostasis (such as K^+) caused by excessive Na^+ uptake can severely perturb the plant metabolism as well (Tavakkoli *et al.* 2011). It is well known that K^+ is essential for osmoregulation and protein synthesis, maintaining cell turgor and stimulating photosynthesis (Flowers *et al.* 2015). In addition, both K^+ and Ca^{2+} are required for maintaining the integrity and functionality of cell membranes. In our experiment, we observed different allocation patterns of Na^+ and Cl^- : stoichiometrically, Na^+ accumulated prevalently in leaves whereas the roots appeared to provide a strong barrier against Cl^- , where the anion was strongly accumulated. García-Legaz *et al.* (2005) demonstrated that loquat grafted onto anger (*Cydonia oblonga*) resulted in

more tolerant to high salinity due to a rootstock ability to reduce the transport of Na^+ and Cl^- to the shoots (García-Legaz *et al.* 2005). However, in view of the considerable accumulation of Na^+ and Cl^- in the leaves (~8-fold higher than controls), compartmentalization mechanisms (such as vacuole storage) are likely to assist loquat leaves once both Na^+ and Cl^- are inexorably absorbed and translocated to the leaves (Zhu 2003).

Besides the compartmentation and accumulation of Na^+ and Cl^- in tissues/organs, where both are less harmful for plant metabolism, ion homeostasis can be maintained by the reduced stomatal conductance, which reduces transpiration flux, resulting in lower rates of Na^+ and Cl^- absorption and transport. Accordingly, we found a reduction of g_s in all the salt treatments, which was considered one of the main reason of the reduced CO_2 assimilation in salt-stressed plants (Chaves *et al.* 2009, Shabala and Munns 2012). It is plausible that the reduction of g_s was principally dependent on the severe reduction of K^+/Na^+ ratio in leaves, as observed in several other species (for a review see Chaves *et al.* 2009). Besides the observed stomatal limitations, other mechanisms can hamper the photosynthetic performance of loquat leaves, as demonstrated by the accumulation of intercellular CO_2 (decline of P_N/C_i). For example, the biochemical limitation imposed by impairment of Rubisco activity (Galmés *et al.* 2013) or, as observed in the current experiment, the reduction of Chl content (Amirjani 2011, Chutipaijit *et al.* 2011) which is probably attributable to the excessive accumulation of Cl^- in leaves (Tavakkoli *et al.* 2011), a common finding in salt-stressed plants. The higher decline of Chl *a* than that of Chl *b* is in agreement with other studies focusing on

salinity stress (Mane *et al.* 2010) and represents a mean to preserve the functionality of PSII, which is richer in Chl *a* and more prone to photoinhibition than PSI (Tikkanen *et al.* 2014). We observed that salinity depressed much more the processes leading to CO₂ fixation (biochemical limitations), than the influx of CO₂ *per se* (the decrease of P_N was greater than that of g_s), as already reported in other species (Rady 2011). This is the reason why we did not observe any WUE increment in our salt-treated seedlings, which is often a response to minimize water loss and Na⁺ and Cl⁻ accumulation, as demonstrated by other authors (Yang *et al.* 2008, Shabala and Munns 2012).

Severe accumulation of Na⁺ and Cl⁻ causes osmotic stress in plant cells and can lead to the accumulation of solute compatibles, including proline, sugar, and/or polyols (Gupta and Huang 2014, Penella *et al.* 2016, Guidi *et al.* 2017, Pompeiano *et al.* 2017). Enhancement of proline content caused by elevated salinity might further contribute to sustaining the electron flow between photosystems, stabilizing the redox balance, reducing photoinhibition and the level of damage to photosynthetic apparatus (Ashraf and Foolad 2007, Szabados and Savaure 2010); for example preserving Rubisco activity (Hamilton and Heckathorn 2001). Accumulation of glucose in loquat leaves is a common response of other species in reaction to salinity (Bohnert *et al.* 1995, Kerepesi and Galiba 2000) that can be attributed to the cleavage of sucrose in favor of simpler reducing sugars (Farrant *et al.* 1993). Under salt excess, glucose accumulation was likely to be dependent on both the reduction of phloem integrity as well as the sink strength of younger leaves, which negatively influences the sucrose biosynthesis by the mature (source) leaves (Lemoine *et al.* 2013). Similarly, and according to our results, it has been reported that salt excess has the potential to reduce the amounts of polyols load into the phloem due to the reduced sink strength of young leaves and/or the disruption of phloem integrity (Pommerrenig *et al.* 2007), which can also lead to hexose accumulation, at least until some other regulatory mechanisms take place to restore the balance. Apart from the role as osmoprotectant, glucose can also act as signaling molecule in plants under stress (Gupta and Kaur 2005), including salinity (Chaves *et al.* 2009), influencing the activation of stress-induced mechanisms (Lastdrager *et al.* 2014). It is well known that genes coding for enzymes involved in photosynthesis and other source activities (*e.g.*, export of photoassimilates and nutrient mobilization) are down-regulated under high sugar availability, whereas those involved in sink activities (*e.g.*, degradation of carbohydrates and the synthesis of storage polysaccharides, lipids, and proteins) are induced (Smith and Stitt 2007). In addition, strong accumulation of hexoses can promote a premature leaf senescence (Landi *et al.* 2015), and this partially explains the lower Chl *a/b* ratio and the lower content of total Chl found in salt-treated loquat leaves, as well as the incremented contents

of H₂O₂ (Molassiotis *et al.* 2006, Tanou *et al.* 2009, Miller *et al.* 2010). Salinity-induced ROS formation can lead to oxidative damage to various cellular components, such as proteins, lipids, and DNA, interrupting vital cellular functions of plants, and finally provoking cellular death and necrosis (reviewed by Gupta and Huang 2014). Accumulation of H₂O₂ did not parallel the incremented content of MDA in loquat leaves, independently to the salt concentration applied in the present experiment. This suggests that the propagation of ROS and occurrence of lipid peroxidation remained confined to the leaves of high-salt-treated plants, and thus the reduction of net photosynthesis can be efficiently used as a marker for the evolution of salt-triggered effects.

To conclude, we confirm that NaCl significantly impairs plant photosynthetic performances of loquat seedlings, principally due to stomatal limitations aimed at reducing the concentration of accumulated Na⁺ and Cl⁻ into the leaves. We also pointed out that salinity alters pigment composition and sugar patterns in the leaves. In particular, the incremented Chl *a/b* ratio, associated with accumulation of glucose and fructose into the leaves suggests that NaCl can reduce the leaf lifespan by promoting events of early senescence in fully developed leaves. Conversely, an impairment of translocating sugars (including polyols) can seriously compromise the development of sink organs, such as young leaves and fruits. Overall, these effects pose serious concerns about the possible effect of salinity to fruit production. Further research is necessary to investigate the interplay between Na⁺ and Cl⁻ accumulation, photosynthesis performance, and sugar metabolism in loquat leaves subjected to NaCl toxicity and to understand the level at which NaCl stress can significantly compromise the yield of this economically important fruit species.

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