



## Article

# Changes in Morpho-Anatomical and Eco-Physiological Responses of *Viburnum tinus* L. var *lucidum* as Modulated by Sodium Chloride and Calcium Chloride Salinization

Veronica De Micco <sup>1</sup>, Carmen Arena <sup>2,\*</sup>, Chiara Amitrano <sup>1</sup>, Youssef Rouphael <sup>1</sup>, Stefania De Pascale <sup>1</sup> and Chiara Cirillo <sup>1,\*</sup>

<sup>1</sup> Department of Agricultural Sciences, University of Naples Federico II, 80055 Portici, Italy; demicco@unina.it (V.D.M.); chiara.amitrano@unina.it (C.A.); youssef.rouphael@unina.it (Y.R.); depascal@unina.it (S.D.P.)

<sup>2</sup> Department of Biology, University of Naples Federico II, 80126 Napoli, Italy

\* Correspondence: c.arena@unina.it (C.A.); chiara.cirillo@unina.it (C.C.); Tel.: +39-081-679173 (C.A.); +39-081-2539381 (C.C.)

**Abstract:** Salinity in water and soil is among the major constraints to the cultivation of ornamental crops since it can affect their growth and aesthetic value. A greenhouse experiment was carried out to assess whether the application of two different salts (80 mM NaCl or 53.3 mM CaCl<sub>2</sub>, with a final ionic concentration of 160 mM) could differently modulate the anatomical and physiological acclimation of an important ornamental species such as *Viburnum tinus* L. var. *lucidum*. Eco-physiological analyses (e.g., leaf gas exchange and chlorophyll *a* fluorescence emission) were performed and leaves were subjected to light microscopy analysis to quantify functional anatomical traits through digital image analysis. Results showed that the two iso-osmotic solutions induced different structure-mediated physiological alterations in *V. tinus* plants. Photosynthesis was lowered by CaCl<sub>2</sub> treatments (−58%) more than by NaCl (−37%), also due to the occurrence of photodamage apart from stomatal limitations. Neither Na<sup>+</sup> nor Cl<sup>−</sup> exhibited toxic effects in leaf lamina structure which was reflected in the limited reduction in dry matter accumulation. Overall data were interpreted focusing on the coordination among leaf structural and functional traits suggesting that the fine control of functional anatomical traits contributes to physiological acclimation to both stressful conditions.

**Keywords:** iso-osmotic salts; leaf anatomical traits; photochemistry; photosynthesis; toxic ions; phenolics



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## 1. Introduction

The genus *Viburnum*, belonging to the Adoxaceae family, comprises nearly 200 species widely used as ornamentals in urban landscapes. Among the most utilized species, *Viburnum tinus* L. var. *lucidum* (Mill.) Aiton [1] is an ornamental shrub known for being tolerant to drought and salinity stress, attributes making it suitable for xeriscaping [2,3].

In the last decades, the Mediterranean area has suffered serious limitations to plant growth, development, and production due to climate change consequences, such as enhanced frequency, severity, and duration of drought periods, also triggering the rising of the salinity levels in soil and water. Understanding the mechanisms of plants' responses to abiotic stresses due to climate changes in arid and semi-arid areas is increasing not only for food crops but also for ornamental species [4]. Furthermore, water scarcity and low water quality have recently induced municipalities to consider the use of reclaimed water or industrial wastewater (with high salt concentration) for landscape irrigation [5]. Salinity stress, due to the poor quality of irrigation water, can contribute to plants experiencing osmotic stress even under adequate levels of water availability in the soil. Furthermore, specific ionic effects due to unbalanced uptake and ionic toxicity can occur, causing in ornamental plants a visual quality depletion related to foliage damage and necrosis, growth

and flowering reduction [6]. In this context, the study of the effect of salinity on ornamental species becomes crucial to evaluate the most tolerant plants that can be used for landscaping especially in arid and semi-arid regions [7].

Plants living in conditions of water scarcity or high salinity levels have developed a series of morphological traits to adapt their metabolism to the limiting environment [8]. These changes are often considered to be alterations to enhance the ability of the plant to resist the stress imposed by the high salinity levels; however, they can easily be mistaken to be signs of damage and disruption of the plants' activity [9].

For example, one of the first reactions of plants subjected to saline stress is a conspicuous reduction in leaf area [4]. This is considered a mechanism to reduce the water loss by better regulating stomatal closure, and to enhance the efficiency of water use, in the same way it happens when plants are subjected to osmotic stress [10]. The limitation in transpiration, either due to the reduction in canopy in favor of root development or to physiological adjustment, is a means to favor the retention of toxic ions at the root level while reducing the translocation towards the aerial organs [10,11]. Generally, leaf area reductions when plants are subjected to different kind of stresses are also known to reduce "construction costs" and raise yields under global atmospheric changes [12,13].

The response to salinity and drought stress also involves anatomical changes of the leaf lamina. A salinity-induced increase in the leaf lamina thickness is often reported and ascribed to the increase in either palisade or spongy parenchyma thickness. In the first case, the response is considered a way to maximize the photosynthesis potential under the stressful saline condition due to the major content of chloroplasts in the palisade parenchyma [14,15]. Changes in spongy thickness instead are often accompanied by the increase in intercellular spaces, a phenomenon also happening under conditions of drought stress [14,16,17]. Indeed, the increase in intercellular spaces in the spongy parenchyma is interpreted as a way to facilitate CO<sub>2</sub> diffusion through the leaves, especially in a situation of reduced stomatal aperture. On the other hand, a larger incidence of intercellular spaces would represent a strategy for water saving by reducing transpiration, indicating a larger resistance to water flow due to reduced connections between cells [18].

Other anatomical and cytological changes induced by salinity include, apart from salt glands, alterations in the number and size of stomata, number and size of chloroplasts, ultrastructure of chloroplasts, as well as frequency of trichomes, all features related to the control of photosynthesis and transpiration [19,20]. Experiments based on the application of iso-osmotic solutions of different salts allow distinguishing between osmotic stress and ion toxicity, also helping to unravel the different strategies adopted by plants when subjected to different salt types. De Micco et al. [21] recently found that *C. citrinus* plants subjected to CaCl<sub>2</sub> solutions developed leaf morpho-anatomical traits (larger spongy cells, lowest percentage of phenolic compounds in leaves, reduced stomatal frequency) that did not allow plants to maintain a high photosynthetic performance compared to NaCl iso-osmotic solution, highlighting the key role of anatomical traits in maintaining the photosynthetic efficiency under stressful conditions.

Although salinity tolerance changes severely among plant species and cultivars, research-based information on *Viburnum* salt tolerance is still limited [6], especially when subjected to iso-osmotic salt solutions. Based on the above reasoning, here we grew *Viburnum tinus* var. *lucidum* plants using the same agricultural practices but subjecting them to an irrigation treatment performed by using two different iso-osmotic salt solutions (CaCl<sub>2</sub>\_T, CaCl<sub>2</sub> and NaCl\_T, NaCl). We monitored growth, morpho-anatomical, and physiological traits of salt stressed plants comparing them to non-treated control plants, with a specific focus on the structure–function relations which dictate possible ion-specific acclimation strategies to salinity.

Since *Viburnum* is one of the most sold ornamental species in the European market as ornamental potted shrubs for the brilliant color of its foliage [22] and considering the climate change context we are experiencing in the Mediterranean area, the study of the

effect of salinity on ornamental species becomes crucial to evaluate the most tolerant plants that can be used for landscaping, especially in arid and semi-arid regions [23].

## 2. Materials and Methods

### 2.1. Plant Material, Cultivation Conditions, and Experimental Design

The experiment was conducted in an unheated greenhouse at the Azienda Agraria Torre Lama, an experimental station of the University of Naples Federico II, Southern Italy (43° 31' N, 14° 58' E; 60 m a.s.l.). Two-years-old rooted cuttings of *Viburnum tinus* L. var. *lucidum* (provided by the specialized nursery Vivaio Torsanlorenzo, Ardea, Italy) were transplanted in 1.5 L pots filled with peat moss, placed at a density of 2.4 per square meter, and cultivated for 4 months. Daily temperature ranged between 17 and 34°C, while relative humidity was maintained at 58/75% (day/night). Average Photosynthetic Photon Flux Density (PPFD) was 550  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , thanks to a 50% black shading net, and was measured by a light sensor operating in the FluorPen FP100max fluorometer (Photon System Instruments, Brno, Czech Republic).

Plants (n. 72) were fertigated with three nutrient solutions, 15 days after the transplant (DAT) in a randomized complete-block design with four replicates, per treatment, to obtain 3 salinity conditions: a  $\text{CaCl}_2$  salinity treatment ( $\text{CaCl}_2\text{-T}$ ), a NaCl salinity treatment ( $\text{NaCl-T}$ ), and a non-salt control (C). More specifically, the C plants were fertigated with a basic nutrient solution with an electrical conductivity (EC) of 2.0  $\text{dS m}^{-1}$  and containing: 13.6 mM N- $\text{NO}_3$ , 2.0 mM S, 1.4 mM P, 6.0 mM K, 4.5 mM Ca, 2.0 mM Mg, 20  $\mu\text{M}$  Fe, 9  $\mu\text{M}$  Mn, 1.5  $\mu\text{M}$  Cu, 3  $\mu\text{M}$  Zn, 20  $\mu\text{M}$  B, and 0.3  $\mu\text{M}$  Mo [24].  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$  plants contained the basic nutrient solution with the addition of 53.3 mM  $\text{CaCl}_2$  and 80 mM NaCl, respectively, leading to a total ion concentration of 160 mM in both saline solutions. The two salt solutions were characterized by iso-osmotic concentration, with EC values of 11.6 and 11.1  $\text{dS m}^{-1}$  for the  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$ , respectively. The pH in the three nutrient solutions was  $6.0 \pm 0.2$ .

### 2.2. Biometry, Morphological, and Anatomical Leaf Traits

At the end of the salinity treatment (127 DAT), 10 plants per treatment were sampled to quantify canopy volume (CV), total leaf area (TLA) per plant height (PH), leaf dry weight (LDW) and stem dry weight (SDW) per plant, area of the single leaf (ASL), and specific leaf weight (SLW).

After the measurement of plant height, canopy volume was estimated by approximating the canopy to the volume of a sphere and the leaves were collected to measure leaf area by means of an electronic area meter (Li-Cor3000, Li-182 Cor, Lincoln, NE, USA). The dry biomass was determined by drying the above ground biomass (leaves and stems) in a forced-air oven at 75 °C until constant weight. SLW was measured in 5 leaves per treatment as the ratio between leaf dry mass and leaf area [25] on six fully expanded leaves.

At 127 DAT, 9 fully expanded leaves were collected from three plants per treatment and fixed in the FAA chemical solution (40% formaldehyde/glacial acetic acid/50% ethanol-5/5/90 by volume). The median portion of each leaf was dissected under a dissection microscope (SZX16, Olympus, Hamburg, Germany) to obtain subsamples of 5 mm  $\times$  5 mm leaf lamina to be devoted to the preparation for thin sectioning, while the remaining part of the lamina was used to quantify epidermal traits (i.e., stomata and trichome features). For thin sectioning, the subsamples were dehydrated in an ethanol series up to 95%, infiltrated and embedded in the acrylic resin JB4<sup>®</sup> (Polysciences, Warrington, PA, USA). The 5  $\mu\text{m}$  thick cross sections of the leaf lamina were cut through a rotative microtome. Sections were stained with 0.5% Toluidine blue in water [26], mounted with mineral oil for microscopy, and observed under a transmitted light microscope (BX60, Olympus, Hamburg, Germany). Unstained sections were mounted with mineral oil for fluorescence microscopy and observed under the epi-fluorescence microscope equipped with the settings to detect the autofluorescence of phenolic compounds (Mercury lamp, band-pass filter 330–385 nm, dichromatic mirror > 400 nm, and barrier filter > 420 nm) [27,28].

For the quantification of stomata and trichome traits on the abaxial epidermis, 3 portions of the leaf lamina per chemically fixed leaf were flattened on microscope slides and mounted with distilled water. The epidermis was observed under the epi-fluorescence microscope with the same settings reported above which allowed highlighting the inner thick wall of stomata guard cells and trichomes with suberized walls, thus easing their identification and counting.

Images of the leaf sections and of the lamina abaxial surface were taken with a digital camera (CAMEDIA C4040, Olympus, Hamburg, Germany), avoiding main veins. The digital images were analyzed with the image analysis software program AnalySIS® 3.2 (Olympus). On the leaf cross sections the following parameters were quantified: thickness of palisade and spongy parenchyma (in 5 transects of the lamina), area of single parenchyma cells (in 15 cells per section), density of spongy parenchyma (as percentage of area occupied by cells over a given surface, in 5 regions of the section), area occupied by phenolics in the spongy parenchyma (as percentage of spongy tissue occupied by the auto-fluorescent phenolic compounds over a given surface, in 5 regions of the section), as reported in De Micco et al. [29]. On the leaf abaxial epidermis, the following parameters were quantified: frequency of stomata and trichomes (number of objects per surface unit), stomatal size (e.g., area of the single stomata calculating by measuring length and width of stomata guard cells and approximating the stomata to an ellipse, in 15 stomata per leaf), and stomatal area (by multiplying the stomatal size by stomata frequency and expressing the value as percentage over the given surface).

### 2.3. Leaf Gas-Exchanges and Chlorophyll *a* Fluorescence Emission

Eco-physiological behavior of plants was monitored by performing measurements of leaf gas exchanges and chlorophyll *a* fluorescence emission at 106 and 121 DAT. The measurements were performed within 2 h across solar noon, on fully expanded leaves using nine replicates per each treatment.

With a portable gas-exchange analyzer (LCA 4, ADC BioScientific Ltd., Hoddesdon, UK), equipped with a broad-leaf chamber, the following parameters were measured: net CO<sub>2</sub> assimilation rate ( $P_N$ ), sub-stomatal CO<sub>2</sub> concentration ( $C_i$ ), stomatal conductance ( $g_s$ ), transpiration rate ( $E$ ), and intrinsic water use efficiency (iWUE).

On the same day and using a portable FluorPen FP100max fluorometer, equipped with a light sensor (Photon System Instruments, Brno, Czech Republic), the chlorophyll *a* fluorescence emission measurements were performed following Lichtenthaler and Babani [30]. The ground fluorescence ( $F_0$ ) was induced by an internal LED blue light ( $1\text{--}2\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ) on 30 min dark-adapted leaves. The maximal fluorescence in the dark ( $F_m$ ) was induced by a saturating pulse of  $3000\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ . Maximum quantum yield of PSII photochemistry ( $F_v/F_0$ ) was determined as the ratio of variable to ground fluorescence. For the fluorescence measurements in the light, the quantum yield of PSII electron transport ( $\Phi_{\text{PSII}}$ ) was determined following Genty et al. (1989) [31], while the photochemical ( $q_P$ ) and non-photochemical quenching ( $q_N$ ) were calculated as reported in [32].

### 2.4. Mineral Composition and Organic Acids

The anions, cations, and organic acids were separated and quantified by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA). More specifically, at 127 DAT, the leaf and root dried tissues were ground separately in a Wiley mill (IKA, MF10.1, Staufen, Germany) with a 0.5 mm sieve, and 1 g of leaf and root dry powder per plant was used for chemical analyses. For the analysis of ions ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Cl}^-$ ) and organic acids (quinic, acetic, formic, malic, tartaric, oxalic, and citric acids), 250 mg of leaf and root dried matter were extracted in 50 mL of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany) using a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80 °C for 10 min following Roupheal et al. [20]. Nutrient accumulation was

calculated by multiplying the dry weight of each plant part by the concentration of a given nutrient.

### 2.5. Data Elaboration

All experimental data were subjected to one-way ANOVA using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Eco-physiological data were analyzed with a two-way ANOVA with the salt treatment and DAT used as independent factors, also showing their interactions. Multiple comparison tests were performed with the Student–Newman–Keuls coefficient using  $p < 0.05$  as the level of probability. Percent data were transformed through arcsine function before statistical analysis. Shapiro–Wilk and Kolmogorov–Smirnov tests were performed to check for normality [21].

## 3. Results

### 3.1. Growth and Morpho-Anatomical Traits

Both salinity treatments significantly reduced all plant growth parameters whereas an opposite trend was recorded for the specific leaf weight (SLW) (Table 1). Canopy volume (CV), leaf and stem dry weight per plant (LDW, SDW) showed similar values in  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$  plants which were significantly lower than control (C) ones. As regards total leaf area per plant height (TLA/PH) and the area of single leaf (ASL), both parameters were significantly lower in  $\text{NaCl-T}$  than in  $\text{CaCl}_2\text{-T}$  plants which in turn showed significantly lower values than the non-saline (C) treatment. Finally, specific leaf weight was similar in  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$  plants and significantly higher than the non-saline control treatment.

**Table 1.** Effects of salt treatment in the nutrient solution on canopy volume (CV), total leaf area (TLA) per plant height (PH), leaf dry weight (LDW), and stem dry weight (SDW) per plant, area of the single leaf (ASL), and specific leaf weight (SLW) of *Viburnum tinus* L. var. *lucidum* plants. Mean values and standard errors are shown. Different letters correspond to significantly different values ( $p < 0.05$ ).

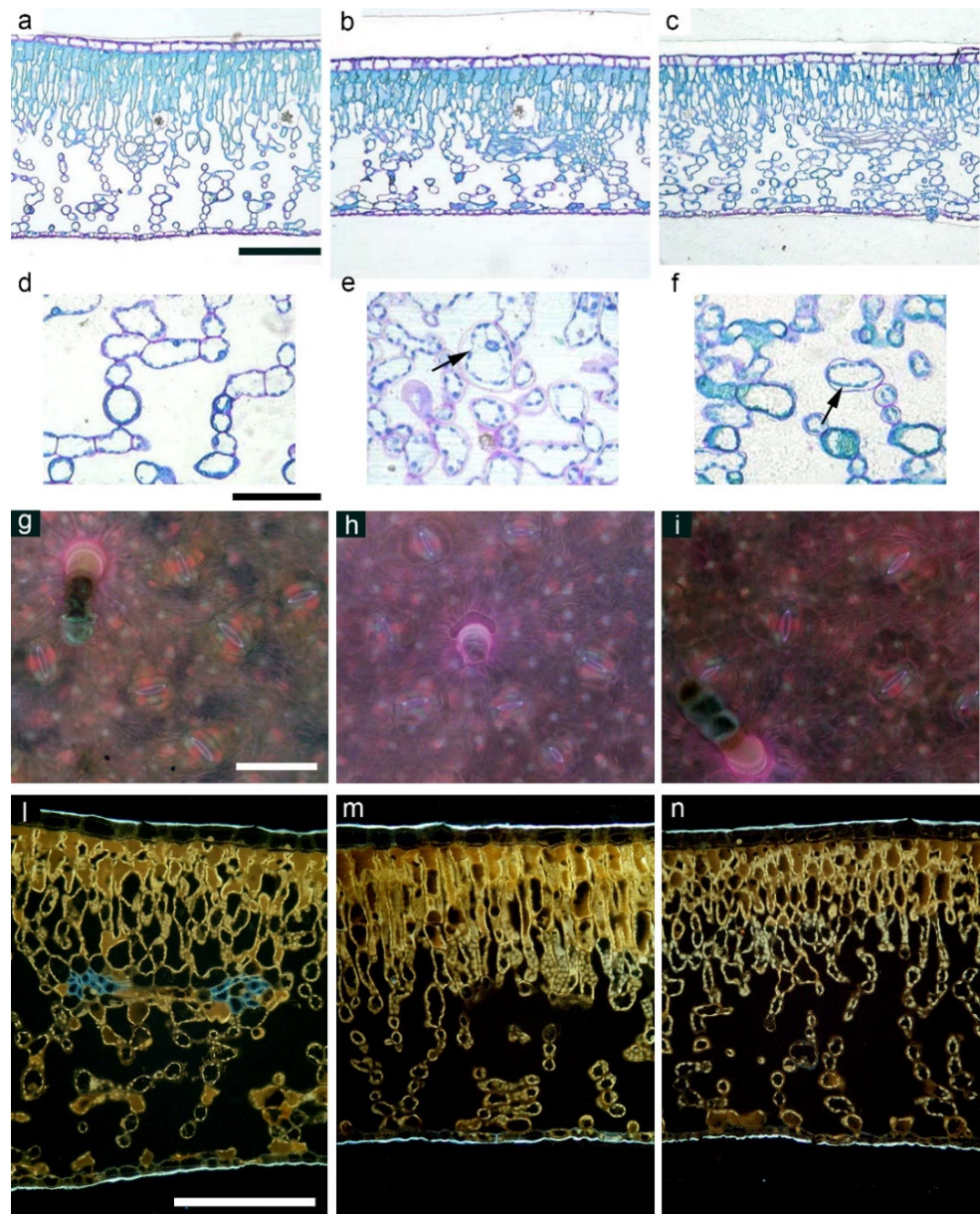
	CV ( $\text{m}^3 \text{ Plant}^{-1}$ )	TLA/PH ( $\text{m}^2 \text{ m}^{-1}$ )	LDW ( $\text{g Plant}^{-1}$ )	SDW ( $\text{g Plant}^{-1}$ )	ASL ( $\text{cm}^2$ )	SLW ( $\text{g DW cm}^{-2}$ )
Salt treatment						
C	$1.61 \pm 0.09$ a	$2.41 \pm 0.08$ a	$133.18 \pm 6.18$ a	$80.93 \pm 3.12$ a	$28.22 \pm 0.87$ a	$81.83 \pm 1.51$ b
$\text{CaCl}_2\text{-T}$	$1.02 \pm 0.15$ b	$1.88 \pm 0.10$ b	$92.50 \pm 6.03$ b	$69.64 \pm 3.16$ b	$25.10 \pm 0.81$ b	$98.73 \pm 1.53$ a
$\text{NaCl-T}$	$0.93 \pm 0.06$ b	$1.54 \pm 0.08$ c	$81.06 \pm 4.32$ b	$64.85 \pm 2.98$ b	$21.45 \pm 1.00$ c	$101.03 \pm 2.07$ a
Significance	***	***	***	**	***	***

NS, \*, \*\*, \*\*\*, not significant or significant at  $p < 0.05$ , 0.01, and 0.001, respectively.

Microscopy analysis of *V. tinus* leaves showed that salinity treatments did not cause severe alteration of the tissues in the dorsi-ventral leaf lamina, since no tissue rupture or thickened cell walls were observed. A few episodes of slight plasmolysis in some spongy parenchyma cells were evidenced (Figure 1a–f,l–n). Palisade parenchyma was made of up to three layers of cells independently from the treatment; the spongy parenchyma seemed to be denser in the salinity treated plants compared to controls. This was confirmed by the quantification of density of spongy parenchyma which was  $40 \pm 1.4\%$  (mean value  $\pm$  standard error) and  $39 \pm 1.2\%$  in  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$  plants, respectively, values significantly higher than the  $34 \pm 1.1\%$  of the control. Both palisade and spongy parenchyma were rich in phenolics, which appeared yellow-fluorescent and localized along the cell membranes especially at the chloroplast level (Figure 1l–n). The quantification of anatomical traits allowed to determine that salinity significantly reduced both palisade and spongy parenchyma thickness (Table 2). In detail, palisade parenchyma was significantly lower in  $\text{CaCl}_2\text{-T}$  than  $\text{NaCl-T}$  plants, which in turn showed significantly higher values than C plants. Spongy parenchyma instead was similar in  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$  plants but lower than control. The reduced spongy parenchyma thickness was likely due only to the salinity-induced lower incidence of intercellular spaces and lower number of cell layers given that the size of the single parenchyma cells was not significantly different in  $\text{CaCl}_2\text{-T}$



and NaCl\_T compared to C plants (Table 2). Stomatal size was significantly reduced in  $\text{CaCl}_2$ \_T and NaCl\_T plants compared to control, with not significant differences among the two salinity treatments (Table 2). The reduced stomatal size in the salinity treated plants was accompanied by a significantly reduced percent stomatal area which showed minimum values in  $\text{CaCl}_2$ \_T plants also suggesting a reduced stomata frequency. The area (%) of the palisade and spongy parenchyma occupied by phenolics was significantly lower in NaCl\_T and  $\text{CaCl}_2$ \_T leaves than controls (Table 2), notwithstanding the denser tissue.



**Figure 1.** Light (a–f) and epi-fluorescence (g–i,l–n) microscopy views of leaf lamina cross sections (a–f,l–n) and epidermal peels (g–i) of *Viburnum tinus* L. var. *lucidum* leaves of not-salt control (C) (a,d,g,l),  $\text{CaCl}_2$  salinity treatment ( $\text{CaCl}_2$ \_T) (b,e,h,m), and NaCl salinity treatment (NaCl\_T) (c,f,i,n). Arrows in e and f point to cells undergoing plasmolysis. Images in (a–i,l–n) are at the same magnification; scale bars = 100  $\mu\text{m}$  in (a) and (l), 50  $\mu\text{m}$  in (d) and (g).

**Table 2.** Effects of salt treatment in the nutrient solution on leaf anatomical functional traits (PP, palisade parenchyma; SP, spongy parenchyma) of *Viburnum tinus* L. var. *lucidum* plants. Mean values and standard errors are shown. Different letters correspond to significantly different values ( $p < 0.05$ ).

	PP Thickness ( $\mu\text{m}$ )	SP Thickness ( $\mu\text{m}$ )	Cell Area ( $\mu\text{m}^2$ )	Stomatal Size ( $\mu\text{m}^2$ )	Stomatal Area (%)	Trichome Frequency ( $\text{n mm}^{-1}$ )	Phenolics in PP (%)	Phenolics in PS (%)
Salt treatment								
C	85.9 $\pm$ 3.50 a	176 $\pm$ 3.79 a	188 $\pm$ 6.80	463 $\pm$ 7.35 a	4.33 $\pm$ 0.08 a	3.04 $\pm$ 0.39 b	57.9 $\pm$ 1.33 a	13.0 $\pm$ 1.13 a
CaCl <sub>2</sub> _T	74.3 $\pm$ 1.87 c	142 $\pm$ 3.92 b	209 $\pm$ 9.40	333 $\pm$ 6.97 b	3.13 $\pm$ 0.06 c	6.90 $\pm$ 0.63 a	47.6 $\pm$ 2.66 b	9.95 $\pm$ 1.24 b
NaCl_T	81.2 $\pm$ 2.87 b	143 $\pm$ 4.53 b	197 $\pm$ 7.21	339 $\pm$ 5.65 b	3.36 $\pm$ 0.06 b	5.73 $\pm$ 0.53 a	48.0 $\pm$ 22.7 b	10.1 $\pm$ 1.25 b
Significance	*	***	NS	***	***	***	*	**

NS, \*, \*\*, \*\*\*, not significant or significant at  $p < 0.05$ , 0.01, and 0.001, respectively.

### 3.2. Leaf Gas-Exchanges and Chlorophyll *a* Fluorescence Emission

The measured gas-exchange parameters were not significantly influenced by the interaction between the two tested factors, namely salinity treatment (S) and the DAT (D). Net photosynthetic rate ( $P_N$ ) and stomatal conductance ( $g_s$ ) were significantly influenced only by S as main factors. In particular,  $P_N$  showed values significantly lower in both of the salinity treated plants compared to C plants, with CaCl<sub>2</sub>\_T even lower than NaCl\_T, whereas only CaCl<sub>2</sub>\_T plants showed  $g_s$  values significantly lower than control (Table 3). Transpiration rate (E) was significantly influenced by both S and D as main factors; more specifically, E was, similarly to  $P_N$ , significantly lower in both salt stressed plant treatments compared to C plants, and to in a greater extent in CaCl<sub>2</sub>\_T plants, and at 106 DAT compared to the second date. The lower value of E at 106 DAT corresponded to the significantly higher intrinsic water use efficiency (iWUE) compared to the other date. iWUE was not significantly influenced by either of the salinity treatments (Table 3). Sub-stomatal CO<sub>2</sub> concentration (Ci) was not significantly influenced by S or D as main factors.

**Table 3.** Effects of salt treatment in the nutrient solution net photosynthetic rate ( $P_n$ ), sub-stomatal CO<sub>2</sub> concentration (Ci), stomatal conductance ( $g_s$ ) transpiration rate (E), of *Viburnum tinus* L. var. *lucidum* plants. Mean values and standard errors are shown. Different letters correspond to significantly different values ( $p < 0.05$ ).

	$P_n$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	Ci ( $\mu\text{mol mol}^{-1}$ )	$g_s$ ( $\text{mmol m}^{-2} \text{ s}^{-1}$ )	E ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ )	iWUE ( $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ )
Salt treatment					
C	7.50 $\pm$ 0.34 a	191.28 $\pm$ 11.69 a	97.55 $\pm$ 14.20 a	2.11 $\pm$ 0.13 a	86.22 $\pm$ 12.19 a
CaCl <sub>2</sub> _T	3.15 $\pm$ 0.35 c	171.70 $\pm$ 11.97 a	31.64 $\pm$ 14.54 b	0.93 $\pm$ 0.13 c	122.57 $\pm$ 12.48 a
NaCl_T	4.73 $\pm$ 0.37 b	172.29 $\pm$ 12.67 a	64.61 $\pm$ 15.39 ab	1.36 $\pm$ 0.14 b	113.28 $\pm$ 13.21 a
DAT					
106	5.12 $\pm$ 0.32 a	173.33 $\pm$ 10.92 a	58.34 $\pm$ 13.27 a	1.22 $\pm$ 0.12 b	123.47 $\pm$ 11.39 a
121	5.13 $\pm$ 0.25 a	183.52 $\pm$ 8.74 a	70.86 $\pm$ 10.62 a	1.71 $\pm$ 0.10 a	91.25 $\pm$ 9.11 b
Significance					
S	***	NS	**	***	NS
D	NS	NS	NS	**	*
S $\times$ D	NS	NS	NS	NS	NS

NS, \*, \*\*, \*\*\*, not significant or significant at  $p < 0.05$ , 0.01, and 0.001, respectively.

Regarding the chlorophyll *a* fluorescence analysis (Table 4), all analyzed parameters were significantly influenced by S as the main factor; while D as the main factor had a significant influence only on photochemical (qP) and non-photochemical quenching (qN). The interaction S  $\times$  D was significant only in the case of qP. CaCl<sub>2</sub>\_T plants showed significantly lower values of quantum yield of PSII electron transport ( $\phi_{\text{PSII}}$ ) and maximum quantum yield of PSII photochemistry ( $F_v/F_0$ ) compared to NaCl\_T and C plants (Table 4). qP and qN were significantly lower in CaCl<sub>2</sub>\_T and NaCl\_T plants than control, with

minimum values always after the addition of  $\text{CaCl}_2$  in the nutrient solution. With the prolongation of treatments with both salts, both qP and qN showed a significant increase.

**Table 4.** Effects of salt treatment on quantum yield of PSII electron transport ( $\phi\text{PSII}$ ), photochemical quenching (qP), non-photochemical quenching (qN), and potential quantum efficiency of photosystem II ( $F_v/F_0$ ), of *Viburnum tinus* L. var. *lucidum* plants. Mean values and standard errors are shown. Different letters correspond to significantly different values ( $p < 0.05$ ).

	$\phi\text{PSII}$	qP	qN	$F_v/F_0$
Salt treatment				
C	0.641 ± 0.009 a	0.923 ± 0.009 a	0.509 ± 0.021 b	1.842 ± 0.071 a
$\text{CaCl}_2\text{-T}$	0.540 ± 0.018 b	0.874 ± 0.014 c	0.597 ± 0.038 a	1.221 ± 0.123 b
$\text{NaCl-T}$	0.615 ± 0.014 a	0.905 ± 0.012 b	0.565 ± 0.042 a	1.686 ± 0.140 a
DAT				
106	0.593 ± 0.019 a	0.873 ± 0.014 b	0.499 ± 0.032 b	1.667 ± 0.143 a
121	0.604 ± 0.019 a	0.928 ± 0.015 a	0.615 ± 0.035 a	1.499 ± 0.154 a
Significance				
S	***	***	**	***
D	NS	***	***	NS
S × D	NS	*	NS	NS

NS, \*, \*\*, \*\*\*, not significant or significant at  $p < 0.05$ , 0.01, and 0.001, respectively.

### 3.3. Mineral and Organic Acids

Chemical analyses showed that both cation and ion contents were significantly influenced by the two salinity treatments (Table 5), while among organic acids only quinic and oxalic acids were significantly influenced.

**Table 5.** Effects of salt treatment in the solution on leaf and root mineral accumulation of main ionic species in *Viburnum tinus* L. var. *lucidum* plants. Mean values and standard errors are shown. Different letters correspond to significantly different values ( $p < 0.05$ ).

	$\text{Na}^+$ (mg Plant <sup>-1</sup> )		$\text{K}^+$ (mg Plant <sup>-1</sup> )		$\text{Ca}^{2+}$ (mg Plant <sup>-1</sup> )		$\text{Mg}^{2+}$ (mg Plant <sup>-1</sup> )		$\text{Cl}^-$ (mg Plant <sup>-1</sup> )	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Salt treatment										
C	40.8 ± 5.02 b	209 ± 23.0 b	3744 ± 218.8 a	1216 ± 136.5 a	244 ± 34.3 b	136 ± 12.3 b	191 ± 13.9 a	167 ± 22.6 a	393 ± 25.1 b	236 ± 33.4 c
$\text{CaCl}_2\text{-T}$	53.3 ± 11.2 b	137 ± 15.7 b	1837 ± 162.5 b	788 ± 73.9 b	679 ± 95.5 a	227 ± 24.8 a	150 ± 16.1 b	123 ± 7.17 ab	1209 ± 127 a	832 ± 96.9 a
$\text{NaCl-T}$	335 ± 54.5 a	716 ± 42.7 a	1607 ± 243.9 b	418 ± 28.7 c	73.6 ± 13.12 b	72.4 ± 10.2 c	75.8 ± 8.77 c	98.3 ± 11.2 b	588 ± 106 b	593 ± 32.8 b
Significance	***	***	***	***	***	***	***	*	***	***

NS, \*, \*\*, \*\*\*, not significant or significant at  $p < 0.05$ , 0.01, and 0.001, respectively.

As regards ions,  $\text{NaCl-T}$  plants showed a significantly increased content of  $\text{Na}^+$  in both leaves and roots compared to the other two treatments ( $\text{CaCl}_2\text{-T}$  and C), while  $\text{CaCl}_2\text{-T}$  plants showed a significantly increased content of  $\text{Ca}^{2+}$  in both leaves and roots compared to the other two treatments ( $\text{NaCl-T}$  and C) (Table 5).

$\text{K}^+$  resulted reduced in  $\text{NaCl-T}$  and  $\text{CaCl}_2\text{-T}$  leaves and roots compared to C, with the lowest values found in  $\text{NaCl-T}$  roots. Similarly,  $\text{Mg}^{2+}$  resulted reduced in  $\text{NaCl-T}$  and  $\text{CaCl}_2\text{-T}$  leaves and roots compared to C, but with the lowest values found in  $\text{NaCl-T}$  leaves.  $\text{Cl}^-$  in leaves was significantly increased only in  $\text{CaCl}_2\text{-T}$  plants compared to both C and  $\text{NaCl-T}$  plants; in roots,  $\text{Cl}^-$  content was significantly lower in C plants compared to  $\text{NaCl-T}$  which showed, in turn, significantly lower values than  $\text{CaCl}_2\text{-T}$  ones.

Organic acids content in leaves was significantly influenced by the addition of  $\text{NaCl}$  and  $\text{CaCl}_2$  in the nutrient solution in the case of quinic, malic, and oxalic acids. Quinic acid content was significantly reduced in  $\text{CaCl}_2\text{-T}$  plants compared to the control, while  $\text{NaCl-T}$ s showed intermediate values. Malic acid content was reduced only in  $\text{NaCl-T}$  plants. The content of oxalic acid was significantly decreased and increased in  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$  plants, respectively, compared with the control (Table 6).



**Table 6.** Effects of salt treatment in the solution on main organic acids (quinic, acetic, formic, malic, oxalic, fumaric, citric, and isocitric acids) leaf content in *Viburnum tinus* L. var. *lucidum* plants. Mean values and standard errors are shown. Different letters correspond to significantly different values ( $p < 0.05$ ).

	Quinic ac. (g kg <sup>-1</sup> DW)	Acetic ac. (g kg <sup>-1</sup> DW)	Formic ac. (g kg <sup>-1</sup> DW)	Malic ac. (g kg <sup>-1</sup> DW)	Oxalic ac. (g kg <sup>-1</sup> DW)	Fumaric ac. (g kg <sup>-1</sup> DW.)	Citric ac. (g kg <sup>-1</sup> DW)	Isocitric ac. (g kg <sup>-1</sup> DW)
Salt treatment								
C	27.0 ± 2.28 a	3.04 ± 0.18	0.38 ± 0.03	25.8 ± 2.5 a	3.30 ± 0.39 b	1.36 ± 0.10	8.10 ± 1.48	0.21 ± 0.04
CaCl <sub>2</sub> _T	13.3 ± 2.29 b	2.39 ± 0.28	0.38 ± 0.03	26.3 ± 3.5 a	1.54 ± 0.19 c	1.27 ± 0.14	9.43 ± 1.64	0.27 ± 0.03
NaCl_T	19.6 ± 2.89 ab	3.01 ± 0.42	0.36 ± 0.08	14.0 ± 2.3 b	5.66 ± 0.84 a	1.30 ± 0.21	8.56 ± 1.43	0.28 ± 0.03
Significance	**	NS	NS	*	***	NS	NS	NS

NS, \*, \*\*, \*\*\*, not significant or significant at  $p < 0.05$ , 0.01, and 0.001, respectively.

#### 4. Discussion

The decrease in plant growth and eco-physiological parameters due to salinity stress is a well-known phenomenon reported in many crops including ornamental species [33–36]. Several species have shown different degrees of acclimation responses due to salinity stress; however, the coordination among morpho-functional traits to achieve the acclimation to osmotic or toxic stress has not been unraveled yet. This study shows how in *Viburnum tinus* L. var. *lucidum* morpho-anatomical and eco-physiological parameters are coordinated to achieve different acclimation responses when plants are subjected to iso-osmotic solutions of NaCl and CaCl<sub>2</sub>.

##### 4.1. The Effect of Salinity Treatment on Morpho-Anatomical Traits

When *V. tinus* var. *lucidum* plants were subject to NaCl and CaCl<sub>2</sub> salinity (NaCl\_T and CaCl<sub>2</sub>\_T plants), they showed a similar reduction in their canopy volume and dry weight compared to controls. However, CaCl<sub>2</sub>\_T plants presented a total leaf area (TLA) per plant height (PH) and the area of leaf lamina significantly higher than NaCl\_T plants (but still lower than control), indicating that salinity influences the compactness of the habitus depending on the salt. On the contrary, the salt type did not influence the specific leaf weight, which was increased under salinity compared to controls, together with increased mesophyll density. Leaf area is an important plant growth trait to consider in physiological and agronomic studies since it influences light interception, photosynthetic efficiency, evapotranspiration, and the plant response to salts and irrigation. The more compact habitus of CaCl<sub>2</sub>\_T plants compared to NaCl\_Ts would indicate a lower light interception and a different microclimate within the canopy, which could explain the reduced photosynthesis and evapotranspiration in these plants. The morphological modifications found in *V. tinus* var. *lucidum* plants were different from recent findings in *C. citrinus* plants subjected to the same iso-osmotic solutions, where salinity did not induce any changes in leaf lamina expansion, but canopy reduction was more severe in plants treated with CaCl<sub>2</sub> compared to NaCl [21]. Such different combinations of morphological traits indicate that, although there are some common morpho-anatomical responses linking salinity stress, the modality, and the intensity of them are strictly linked to the species, thus ultimately to the genotype [2,36]. Indeed, it is recognized that specific suites of structural features, and no single traits, determine the ability of a species to acclimate and cope with stressful conditions [37].

Here, *V. tinus* var. *lucidum* plants under salinity reduced growth and parameters such as leaf expansion, compared to control plants in agreement with main salinity-induced growth trends [38,39]. However, the higher leaf lamina expansion in CaCl<sub>2</sub>\_T plants compared to NaCl\_Ts, with the same dry biomass, can be interpreted in different ways. Firstly, it could be a way to compensate for the shading due to the more compact habitus to limit the reduction in photosynthetic performance. However, the lowest values of photosynthesis recorded in CaCl<sub>2</sub>\_T plants indicate that this strategy was not efficient, also because CaCl<sub>2</sub>\_T leaves were characterized by “less efficient” anatomical traits (e.g., reduced stomatal area and thickness of palisade parenchyma) than controls and NaCl\_Ts. Such features are linked with reduced CO<sub>2</sub> flow into the mesophyll and limited amount

of tissue deputed to the utilization of incident light radiation for photosynthesis [40]. On the other hand, the reduction in leaf area expansion in NaCl\_T plants might be a sign of a prompt adaptation to the stressful condition to reduce the “costs” linked to the formation of a greater leaf area and to allocate resources where they are most needed. Indeed, the reduction in leaf area and the specific leaf area (SLA) has often been related to increments in the water use efficiency of plants [41]. Here, no significant differences were detected between treatments concerning the efficiency of water use. However, an increasing trend was visible in both treated plants (CaCl<sub>2</sub>\_T and NaCl\_T). Finally, in several species of *Viburnum*, Chen et al. [42] found that the leaf Cl<sup>−</sup> concentration increases with increased electrical conductivity of saline solution containing NaCl and CaCl<sub>2</sub> (1.5:1) and that leaf chloride concentration correlates negatively with visual score, plant height, and leaf P<sub>n</sub> and g<sub>s</sub>, but not with the leaf area.

Moreover, osmotic stress reduces the photosynthetic activity in plants, by inducing stomatal closure [43] until provoking plant senescence and death under severe conditions [44]. Therefore, increments in stomatal area, along with reduction in leaf area in NaCl\_T plants, could be an added value to maintain good photosynthetic rates and a strategy to avoid plant senescence. In *V. tinus* var. *lucidum* plants treated with both salt solutions, the reduced stomatal size, a phenomenon often found as a response to stressful conditions, would indicate high ability to quickly control stomata opening/closure [45]. However, apart from stomata size, the regulation of stomata movements is strictly dependent on the availability of K<sup>+</sup> ions to regulate the guard cell turgor pressure [46,47]. The strong reduction in K<sup>+</sup> ion content in leaves of CaCl<sub>2</sub>\_T and NaCl\_T *Viburnum* plants, also accompanied by a similar decrease in Mg<sup>+</sup> ions, could be another reason for the reduced photosynthesis and stomatal conductance. In fact, low contents of K<sup>+</sup> have been reported as responsible for reduced g<sub>s</sub> [48,49]. Once again, this is a different strategy if compared to *C. citrinus* plants subjected to NaCl salinity in which the translocation of K<sup>+</sup> towards leaves likely compensated for the slow stomata reactivity due to their large size [21]. Vice versa, according to the same study on *C. citrinus* plants [21], the reduced growth in CaCl<sub>2</sub>\_T plants, more than NaCl\_Ts can be ascribed to less efficient leaf anatomical traits leading to less efficient gas-exchanges and photochemistry. It is noteworthy that even if both salinity treatments induced a decline in net-photosynthesis and stomatal conductance compared to control plants, the effects of CaCl<sub>2</sub> on photosynthetic apparatus were stronger in inducing stress conditions.

#### 4.2. The Effect of Salinity Treatment on Photosynthesis

The leaf gas exchange measurements indicated that, in both salt treatments (NaCl\_T and CaCl<sub>2</sub>\_T), the reduction in photosynthesis was due to stomatal closure, limiting the entrance of CO<sub>2</sub> and water vapor within the mesophyll. This is supported by the parallel reduction in P<sub>n</sub>, g<sub>s</sub>, and E despite no differences in sub-stomatal CO<sub>2</sub> concentration (C<sub>i</sub>). The stomatal behavior under these circumstances may represent an advantage for saline-stressed plants because it is an efficient strategy against water loss, especially in the condition of chronic saline stress [50–52]. Indeed, iWUE under both saline treatments was similar to the control. However, while in the case of NaCl\_T plants, only stomatal limitations seem to affect the gas exchanges, under CaCl<sub>2</sub> saline stress the situation appears different because PSII photochemistry was also negatively affected, indicating the occurrence of non-stomatal limitations. Therefore, it could be hypothesized that reducing PSII photochemistry in CaCl<sub>2</sub>\_T plants compared to control, together with P<sub>n</sub> decline, could be a safety strategy engaged by photosynthetic apparatus to reduce the high excitation pressure at PSII reaction centers under limiting carbon fixation. This strategy would limit the occurrence of photodamage, as demonstrated for other abiotic stresses, such as drought and high temperatures [53,54]. However, in these plants, the drop in photochemical processes was accomplished by the rise in non-photochemical quenching (qN), indicating that the excess of absorbed light was dissipated as heat. This is a very common photoprotective response and highlights the importance of thermal dissipation mechanisms in maintaining

the health status of photosynthetic apparatus in stressed plants [54]. In our case, the heat dissipation processes increase their importance in response to prolonged stress being more pronounced at the end of salt treatments.

Nevertheless, our data also demonstrated that such a compensatory mechanism is not adequate to avoid the photodamage in  $\text{CaCl}_2\text{-T}$  treated plants because a significant drop in  $F_v/F_0$  was found compared to  $\text{NaCl-T}$  and control, suggesting the occurrence of stress to photosystems [21]. Neither  $\text{CaCl}_2\text{-T}$  nor  $\text{NaCl-T}$  plants adopted the strategy to improve phenolics accumulation along cell membranes to dissipate the excess of light, thus avoiding photo-inhibitory processes [55,56]. It is likely that reduced photosynthesis did not allow the allocation of resources to implement this biochemical defense strategy as also found in other species subjected to salinity [20,57]. Conversely,  $\text{NaCl-T}$  plants exhibited a reduction in  $P_n$  and  $g_s$ , but the photochemical efficiency remained unchanged compared to control. In this case, it is likely to suppose that at the basis of reduced photosynthesis, there were different adaptation mechanisms more linked to differences in leaf anatomical traits, such as the reduced number and accessibility of carboxylation sites due to reduced palisade parenchyma thickness and reduced volume for  $\text{CO}_2$  flow towards the mesophyll.

The progression of salt stress did not affect the net photosynthesis and stomatal conductance but increased leaf transpiration with negative implications on the intrinsic water use efficiency (iWUE). However, the latter was not influenced by salinity likely thanks to the contribution of the increased frequency of trichomes which would have limited leaf transpiration. In fact, among the many roles played by trichomes, it is recognized that dense trichome layers limit water losses in two ways. Firstly, they may increase the thickness of the boundary layer and consequently the resistance to water vapor diffusion from the leaf lamina surface, and secondly, they can reduce the temperature of the leaf surface [58].

#### 4.3. The Effect of Salinity Treatment on Ion Accumulation

The use of low-quality irrigation water in agriculture results in salt accumulation in the root zone decreasing the osmotic potential and, as a consequence a decrease in the water available to the root zone [59,60]. Given the use of isosmotic solutions adopted in the present work, the osmotic effect induced in the plants has been the same [24]. Indeed, similar signs of slight loss of cell turgor have been evidenced in both  $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$  plants. Therefore, the differences observed between the growth of the plants irrigated with the two different saline solutions ( $\text{CaCl}_2\text{-T}$  and  $\text{NaCl-T}$ ) are likely due to the ionic effect rather than the osmotic one. Here, as expected, the uptake of  $\text{Na}^+$  was greater in both roots and leaves when plants were treated with  $\text{NaCl}$ , whereas uptake of  $\text{Ca}^{2+}$  was greater when the plants were treated with  $\text{CaCl}_2$ . Furthermore,  $\text{CaCl}_2$  salinity had no effect on uptake of  $\text{Na}$  in the plant tissues, but  $\text{NaCl}$  salinity reduced  $\text{Ca}$  uptake in both leaves and roots. Specifically,  $\text{NaCl}$  resulted in lower plant growth than  $\text{CaCl}_2$ , significantly in the average leaf size and the ratio between total leaf area and plant height. This phenomenon was expected, given the known deleterious effects of  $\text{Na}^+$  on uptake of cations and other nutrients [61]. The accumulation of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Cl}^-$  increased appreciably in the corresponding salinity treatments in comparison with the control, indicating that *Viburnum* does not rely on ion exclusion to counteract salinity. As stated by Munns and Gilliam [62], the majority of plants from moderately to highly salt tolerant act as salt includers characterized by efficient salt compartmentalization into the cells. In our experiment, compartmentalization in the mesophyll cells was likely due to increased mesophyll density found in both salinity treatments.

Under saline sodic conditions, high levels of external  $\text{Na}^+$  not only interfere with  $\text{K}^+$  acquisition by the roots, but also may alter the selectivity of root for  $\text{K}^+$  over  $\text{Na}^+$ . The  $\text{K}^+$  is a major inorganic nutrient cation in non-halophytes [63] and plays important roles in plant cell activities [64–66] and stress responses [67].

High  $\text{Ca}^{2+}$  concentrations are also known to inhibit stomatal regulation and lead to water stress in certain species, such as *Aster tripolium* L. [68], *Gerbera jamesonii* Adlam [69], and *Vaccinium sect. cyanococcus* [70]. This could explain the even more reduced  $P_n$  and  $g_s$  in

CaCl<sub>2</sub>\_T plants than in NaCl\_T plants. Furthermore, according to Bryla et al. [70] a stronger leaf injury incidence occurs in plants of blueberry irrigated with a saline solution of CaCl<sub>2</sub> compared to NaCl treated and control plants. The observed toxic effect was attributed to the high concentration of Ca<sup>2+</sup> rather than the chloride ions, by considering that calcifuge plants such as blueberry are highly efficient in Ca<sup>2+</sup> uptake and cannot regulate Ca<sup>2+</sup> influx, thus accumulating excessive amounts of this cation. A similar behavior might be related to low capacity for compartmentation or physiological inactivation of Ca<sup>2+</sup> (e.g., precipitation as calcium oxalate). In our case, the observed significant reduction in the leaf concentration of oxalic acid in CaCl<sub>2</sub>\_T and not in NaCl\_T plants may well be explained by possible compartmentation by precipitation of calcium oxalate in the first. As previously reviewed by Franceschi and Horner [71], oxalic acid plays a key role in the ion balance, mainly calcium, in plants. Besides sodium, Ca<sup>2+</sup> is also competitive with Mg<sup>2+</sup> and the binding sites on the root plasma membrane appear to have less affinity for the highly hydrated Mg<sup>2+</sup> than for Ca<sup>2+</sup>. Thus, high concentrations of substrate Ca<sup>2+</sup> often result in increased leaf-Ca along with a marked reduction in leaf-Mg [72].

Cl<sup>−</sup> is a plant micronutrient and regulates leaf osmotic potential, and turgor, and stimulates growth in plants [73,74]. Both the salts increased Cl<sup>−</sup> uptake at root level, particularly when CaCl<sub>2</sub> was applied, compared to control. Since the total leaf dry biomass was similar in CaCl<sub>2</sub>\_T and NaCl\_T plants, the observed highly increased accumulation at leaf level in CaCl<sub>2</sub>\_T plants is due to the higher concentrations of chloride in the CaCl<sub>2</sub> irrigation water. As reviewed by Wu and Li [75], the main traits related to controlling Cl<sup>−</sup> transport, its role in plant salt tolerance, and how root and shoot regulate Cl<sup>−</sup> exclusion may differ among species.

## 5. Conclusions

Results showed that plants of *V. tinus* var. *lucidum* reacted to salinity stress due to isosmotic solutions of NaCl and CaCl<sub>2</sub> with the adoption of different strategies of acclimation involving both structural and physiological defenses. We suggest that CaCl<sub>2</sub>\_T plants developed a less efficient leaf anatomical structure that contributed to the overall reduction in the plant photosynthetic performance. The occurrence of photodamage to photosystems in CaCl<sub>2</sub>\_T plants was also detected as well as a stronger nutritional unbalance and toxic effect compared to NaCl\_T plants. In conclusion, this study highlighted that the toxic effect due to specific salts can provoke damage and lower the yield and ornamental value of horticulture crops more than osmotic disfunction. Therefore, understanding the possible strategies adopted by plants by coordination structural and physiological acclimation to salinity induced by different salts is becoming more and more important for urban landscaping and restoration purposes.

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