# Salinity stress constrains photosynthesis in *Fraxinus ornus* more when growing in partial shading than in full sunlight: consequences for the antioxidant defence system

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• **Background and Aims** A major challenge in plant ecophysiology is understanding the effects of multiple sub-optimal environmental conditions on plant performance. In most Mediterranean areas soil salinity builds up during the summer because of low availability of soil water coupled with hot temperatures. Although sunlight and soil salinity may strongly interact in determining a plant's performance, this has received relatively little attention.
• **Methods** Two-year-old seedlings of *Fraxinus ornus* were grown outdoors in pots during a Mediterranean summer in either 45 % (shaded plants) or 100 % (sun plants) sunlight irradiance and were supplied with either deionized water or deionized water plus 75 mM NaCl. Morpho-anatomical traits, water and ionic relations, gas exchange and photosystem II performance, concentrations of individual carotenoids, activity of antioxidant enzymes, concentrations of ascorbic acid and individual polyphenols were measured in leaves. Leaf oxidative stress and damage were estimated by *in vivo* analysis of stable free radicals and ultrastructural analyses.
• **Key Results** Leaf concentrations of potentially toxic ions did not markedly differ in shaded or sun plants in response to salinity. Leaves of sun plants displayed superior water use efficiency compared with leaves of shaded plants, irrespective of salinity treatment, and had both better stomatal control and higher $CO_2$ carboxylation efficiency than leaves of shaded plants. In the salt-treated groups, the adverse effects of excess midday irradiance were greater in shade than in sun plants. The activity of enzymes responsible for detoxifying hydrogen peroxide decreased in shaded plants and increased in sun plants as a result of salinity stress. In contrast, the activity of guaiacol peroxidase and the concentration of phenylpropanoids increased steeply in response to salinity in shaded plants but were unaffected in sun plants.
• **Conclusions** It is concluded that salinity may constrain the performance of plants growing under partial shading more severely than that of plants growing under full sun during summer. The results suggest co-ordination within the antioxidant defence network aimed at detoxifying salt-induced generation of reactive oxygen species.

**Key words:** Antioxidant enzymes, electron spin resonance (ESR), *Fraxinus ornus*, gas exchange performance, ionic relations, water relations, leaf morpho-anatomy, oxidative damage, photosynthesis, shade versus sun, salinity, transmission electron microscopy (TEM).

## INTRODUCTION

Root-zone salinity has long been hypothesized to deleteriously affect a plant's performance more in full sunlight than under partial shading (Munns and James, 2003; Tattini *et al.*, 2006; Munns and Tester, 2008). This is because 'sun' plants may have greater uptake and translocation rates of potentially toxic ions than their shaded counterparts (Munns and James, 2003). The matter is, however, more complex, and involves not only light-induced effects on ionic and water relations (García-Sánchez *et al.*, 2006; Melgar *et al.*, 2009; Cimato *et al.*, 2010), but also effects on the suite of morpho-anatomical and biochemical features of the leaf/plant that are inextricably linked to both uptake and transport of water and salt (Tattini *et al.*, 2006; López-Hoffman *et al.*, 2007).

First, the accumulation of potentially toxic ions in sensitive shoot organs largely depends on the irradiance at which shaded plants grow. In leaves of olive trees grown in full sunlight the leaf concentrations of $Na^+$ and $Cl^-$ were 73 % greater than those in leaves growing in 15 % sunlight, and were only in part countered by 20 % greater $K^+$ concentration (Melgar *et al.*, 2009). On the other hand, García-Sánchez *et al.* (2006) showed that leaf $Na^+$ concentration was higher in *Citrus* plants growing under moderate shade (50 % of full sunlight) compared with in full sun. These contrasting results may depend simply on stomatal conductance and transpiration rates of plants growing under deep or moderate shade (Barradas *et al.*, 2005; Nicolás *et al.*, 2008). Furthermore, interaction effects of sunlight irradiance and root-zone salinity on physiological and biochemical

traits depend upon a species-specific strategy to control the entry and allocation of potentially toxic ions to sensitive shoot organs and to manage properly the flux of sunlight irradiance reaching the leaf (broadly 'salt excluders' versus 'salt includers' and 'sun tolerant' versus 'sun sensitive'; Tattini *et al.*, 2006; Melgar *et al.*, 2009; Fusaro *et al.*, 2014).

Second, leaves growing in full sun usually display a suite of morpho-anatomical traits that may confer great capacity to counter salt-induced water/osmotic stress (Evans, 1999; Poorter *et al.*, 2009). There is clear evidence that drought stress may be more deleterious for leaves adapted to shade than to full sun (Valladares and Pearcy, 2002; Fini *et al.*, 2012). Salt-induced osmotic imbalance and leaf dehydration may more severely constrain plants growing at limited sunlight availability (García-Sánchez *et al.*, 2006; Cimato *et al.*, 2010), as sun plants may limit cuticular water loss more effectively and have superior control of stomatal aperture compared with shaded plants (Rhizopoulou *et al.*, 1991; Ponton *et al.*, 2002). This in turn greatly affects photosynthetic performance, particularly when the usage of radiant energy for $CO_2$ assimilation decreases as a consequence of stomatal and mesophyll limitations (for reviews see Flexas *et al.*, 2004; Chaves *et al.*, 2009). This is of great significance for plants inhabiting the Mediterranean regions, as excess soil salinity builds up concomitantly with hot temperatures, reduced soil water availability and high sunlight irradiance during the summer months (Tattini *et al.*, 2006; Fusaro *et al.*, 2014). In other words, Mediterranean plants in their natural habitats are challenged by severe excess light stress and hence by massive generation of reactive oxygen species (ROS) when suffering from an excess of soil salinity.

Finally, leaves of plants growing under partial shade or full sunlight also differ considerably in their capacity to avoid and counter stress-induced generation of ROS. In leaves acclimated to high sunlight, carotenoids, particularly violaxanthin-cycle pigments, may effectively dissipate excess radiant energy (Müller *et al.*, 2001; Demmig-Adams and Adams, 2006) while directly protecting chloroplast membranes against oxidative damage (e.g. by chemically quenching singlet oxygen; Ramel *et al.*, 2012). Furthermore, the activity of antioxidant enzymes and the concentrations of low molecular-weight antioxidants (such as ascorbate and phenylpropanoids) are usually greater in well-watered plants growing in full sun than in those growing in shade (Agati and Tattini, 2010; Agati *et al.*, 2012). Exploring the extent to which salt-induced limitation of photosynthesis actually translates into excess light stress under natural conditions is a central issue for the ecology of Mediterranean plants exposed to a wide array of abiotic stresses, including root-zone salinity. Indeed, stress-induced excess excitation energy received by the chloroplast (Zhu *et al.*, 2009; Fini *et al.*, 2012) may have great impact on the plant's suite of primary and secondary antioxidant defences. There is evidence that excess excitation energy depresses the activity of enzymes devoted to $H_2O_2$ removal, particularly ascorbate peroxidase (APX) (Mullineaux and Karpinski, 2002; Hatier and Gould, 2008; Fini *et al.*, 2011). Recently, phenylpropanoids in conjunction with vacuolar peroxidases (POXs) have been suggested to complement the depletion of primary antioxidant defences to scavenge excess $H_2O_2$ generated in the chloroplast and the peroxisome under severely excessive excitation energy (Ferreres *et al.*, 2011; Fini *et al.*, 2011; Agati *et al.*, 2012).

Here we investigated *Fraxinus ornus* (manna ash), a deciduous member of the Oleaceae family. This species is largely used for restoring vegetation in urban and rural areas in the Mediterranean basin. We are unaware of experiments exploring the strategies adopted by this species to cope with excess soil salinity. Woody deciduous species usually display greater fluxes of $Na^+$ and $Cl^-$ than woody evergreens, even at moderate soil salinities (Munns and Tester, 2008; Fusaro *et al.*, 2014), likely as a consequence of their relative inability to sustain both drastic reductions in transpiration rates and severe dehydration (Tattini *et al.*, 2006; Munns and Tester, 2008; Fusaro *et al.*, 2014). The extent to which this affects gas exchange performance and hence biochemical adjustments in response to root-zone salinity during summer is central for understanding the ecophysiology of the Mediterranean flora.

We hypothesized that in *F. ornus* (1) transpiration rates, and hence the rates of $Na^+$ and $Cl^-$ transport to the leaf, may not differ greatly between plants growing under moderate shade and those growing fully exposed to sunlight; but (2) sun plants are likely to display a suite of morpho-anatomical features conferring greater ability to counter salt-induced water stress than their shaded counterparts. To test these hypotheses, we grew plants at 45 or 100 % full solar irradiance and supplied them with deionized water (control, C) or this water with the addition of 75 mM NaCl (salt-treated, ST) during the Mediterranean summer. Measurements were performed of morpho-anatomy, water and ionic relations, gas exchange and photosystem (PS) II performance, the concentration of individual carotenoids, the activity of antioxidant enzymes and the concentrations of ascorbic acid and individual phenylpropanoids (mostly coumarins and quercetin 3-*O*-glycosides; Fini *et al.*, 2012). Leaf oxidative damage was investigated by *in vivo* analysis of stable free radical content using electron spin resonance (ESR) spectrometry)and ultrastructural analysis using electron transmission microscopy (TEM).

## MATERIALS AND METHODS

### Plant material and growth conditions

Two-year-old *Fraxinus ornus* seedlings (whole-plant dry weight $16.7 \pm 1.4$ g, mean $\pm$ s.d., $n = 10$) were grown outdoors in 5.0-L pots in a peat/pumice substrate (50:50, v:v) fertilized with 2 kg m$^{-3}$ of a controlled release fertilizer (Osmocote Exact, 15:9:12, 5–6-month formulation, Everris International, Geldermalsen, Netherlands). Plants grew under 60 % sunlight irradiance ($\sim$1100 $\mu$mol m$^{-2}$ s$^{-1}$ at midday, corresponding to the light saturation point in healthy *F. ornus* leaves; see Table 3) over a 4-week period in Florence (43°46′ N, 11°15′ E) from 1 to 28 July. Plants were then transferred to screen houses (3 × 2 × 2 m, length × width × height) constructed with a roof and walls using plastic foils with specific transmittances, to receive 45 or 100 % solar irradiance over a 4-week experimental period. Plants grew under a 100-$\mu$m ethylene tetrafluoroethylene fluoropolymer transparent film (Nowoflon® ET-6235, Nowofol® Kunststoffprodukte, Siegsdorf, Germany) with the addition or not of a proper black polyethylene net. Photosynthetic photon flux density (PPFD) at midday inside the screen houses was measured with a calibrated Li-190 quantum sensor (Li-Cor, Lincoln, NE, USA), and was on average 1917 and 885 $\mu$mol m$^{-2}$ s$^{-1}$ at the

sun and shaded sites, respectively, on a clear day. Final light treatments were achieved by the end of an 8-d period during which plants were exposed to final light treatments for 2, 4, 8 and 12 h, every 2 d, when the experiment started. Temperature maxima/minima were measured daily with Tinytag Ultra2 data loggers (Gemini Dataloggers, UK) and averaged 31·4/18·7 °C and 32·6/17·5 °C at the shade and sun sites, respectively. Plants were supplied at 2-d intervals with deionized water (treatment C) or this water with the addition of 75 mM NaCl (treatment ST) until complete leaching of the substrate.

*Plant growth, leaf morphology and anatomy and water and ionic relations*

Plant growth was estimated in terms of relative shoot dry weight increments, i.e. relative growth rate of the shoot ($RGR_{shoot}$), using the equation:

$$RGR_{shoot} = (\ln W_1 - \ln W_0)/(t_1 - t_0) \tag{1}$$

where $W$ is shoot dry weight, $t$ is time and subscripts 0 and 1 denote initial and final harvest. Leaf lamina size and leaf mass per area (LMA) were determined as reported previously (Tattini *et al.*, 2000). Whole-leaf thickness and the thicknesses of adaxial and abaxial cuticular layers were estimated in 1-μm transverse sections that were fixed and embedded following standard methodology (Tattini *et al.*, 2005), using an Axiophot microscope (Carl Zeiss, Jena, Germany) equipped with a high-resolution TK 870E video camera (JVC, Yokoama, Japan). Cuticular waxes were quantified on six leaves immersed in chloroform (2 × 100 mL) for 30 s, following the gravimetric protocol of Hauke and Schreiber (1998).

Predawn water ($\psi_w$) and osmotic ($\psi_\pi$) potentials were measured for fully developed leaves (35 d old) using a standard method (Tattini *et al.*, 2002). Leaf osmotic potential was measured on expressed sap of frozen and thawed leaves using a boiling-point Vapro 5520 osmometer (Wescor, South Logan, UT, USA). Leaf osmotic potential at full turgor ($\psi_{\pi FT}$) was then calculated as:

$$\psi_{\pi FT} = \psi_\pi[(RWC - AWF)/(100 - AWF)] \tag{2}$$

where RWC is relative water content and AWF is apoplastic water fraction. RWC was measured as previously reported (Tattini *et al.*, 2002) and AWF was estimated from the analysis of pressure/volume isotherms at 6 %. The contribution of dehydration (D) to osmotic adjustment was calculated as:

$$D = \Delta\psi_\pi - \Delta\psi_{\pi FT} \tag{3}$$

where $\Delta\psi$ is the difference in leaf osmotic potential between salt-treated and control plants. The osmotic contributions of $Na^+$, $Cl^-$ and $K^+$ to $\psi_{\pi FT}$ were calculated by the Van't Hoff equation:

$$\psi_{\pi,s} = 0.002479 \times RDW \times C \tag{4}$$

where $\psi_{\pi,s}$ is the contribution ($-MPa$) of individual solutes ($s$) to $\psi_{\pi FT}$, RDW is relative dry weight at saturation ($kg\ m^{-3}$), $C$ is the molar concentration of solutes ($mol\ kg^{-1}$ DW) and $0.002479\ m^3\ MPa^{-1}\ mol^{-1}$ is the RT value at 25 °C.

Cation analysis was performed with a PerkinElmer 1100 emission-absorption spectrophotometer (PerkinElmer, Norwalk, CT, USA) and chloride was quantified with a Quanta 4000E Ion Capillary Electrophoresis Unit (Waters, Milford, MA, USA), based on the protocols previously reported by Tattini and Gucci (1999). Soluble carbohydrates were identified and quantified by high-performance liquid chromatography with refractive index detection (HPLC-RI) as reported previously (Tattini *et al.*, 1996).

*Leaf gas exchange, mesophyll conductance and PSII performance*

Net photosynthesis ($A$), stomatal conductance ($g_s$), transpiration rate ($E$) and intercellular $CO_2$ concentration ($C_i$) were measured on the medial leaflet of fully expanded leaves, under laboratory conditions (leaf temperature 28 °C), using a portable infrared gas analyser (Ciras-2; PP-systems, Amesbury, MA, USA) operating at 380 μL $L^{-1}$ ambient $CO_2$. Leaves were enclosed in a 2·5 $cm^2$ Parkinson cuvette and provided with saturating light irradiance using actinic light (CFM; PP-systems). The CFM also provides measurement of modulated chlorophyll fluorescence, allowing simultaneous measurements of gas exchange and fluorescence parameters. Mesophyll conductance to $CO_2$ diffusion ($g_m$) was estimated using the variable $J$ method (Harley *et al.*, 1992):

$$g_m = A/\{C_i - [\Gamma^* \cdot J_f + 8 \cdot (A + R_{day})]/[J_f - 4 \cdot (A + R_{day})]\} \tag{5}$$

where $A$ is $CO_2$ assimilation rate, $\Gamma^*$ is the chloroplastic $CO_2$ compensation point, $J_f$ is the rate of linear electron transport estimated from chlorophyll fluorescence and $(A + R_{day})$ is the gross photosynthetic rate (including non-photorespiratory respiration). $\Gamma^*$ was calculated using the Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase)-specific factor estimated for deciduous species (Galmés *et al.*, 2005) and $J_f$ was calculated as described by Genty *et al.* (1989):

$$J_f = \Phi_{PSII} \times \alpha \times 0.5\ PPFD \tag{6}$$

where $\Phi_{PSII}$ is the quantum yield of PSII in the light and $\alpha$ is leaf absorptance.

Leaf absorptance was measured with a spectroradiometer equipped with an integrating sphere (model 1800-12S; LI-Cor, Lincoln, NE, USA); the factor 0·5 was chosen assuming an equal distribution of absorbed photons between PSI and PSII (Loreto *et al.*, 1992). Before measurements, we verified that $J_f$ measurements corresponded to the quantification of electron transport rate estimated by gas exchange in non-photorespiratory conditions (i.e. lowering $O_2$ concentration to 1 %; Laisk and Loreto, 1996).

The $CO_2$ concentration in the chloroplasts ($C_c$) was then calculated as:

$$C_c = C_i - A/g_m \tag{7}$$

Responses of $A$ to changes in intercellular $CO_2$ concentration ($A/C_i$ curves) were analysed by decreasing stepwise external $CO_2$ concentration ($C_a$) from 380 to 30 μL $L^{-1}$, then restoring $C_a$ to 380 μL $L^{-1}$ and finally increasing $C_a$ stepwise to 1800 μL $L^{-1}$. The apparent maximum rate of carboxylation by Rubisco (referred to as carboxylation efficiency throughout

this paper) was calculated as described by Long and Bernacchi (2003). In addition, the activity of Rubisco *in vitro* was measured following the protocol of Ouerghi *et al*. (2000).

A quantitative analysis of stomatal (SL), mesophyll conductance (ML) and biochemical (BL) limitations on *A* was carried out as described by Grassi and Magnani (2005). Responses of *A* to changes in photosynthetic photon flux density (*A*/PPFD curves) were measured in the range $0-1800$ $\mu$mol quanta m$^{-2}$ s$^{-1}$. Daily changes in *A* and stomatal conductance (*g*$_s$) were also measured *in situ*, from 06:00 to 21:00 h. *In situ* measurements were conducted at 380 $\mu$L L$^{-1}$ CO$_2$, ambient temperature and natural irradiance, as described by Valladares and Pearcy (2002). Net daily carbon gain (CO$_{2,daily}$) was then calculated by the integration procedures reported by Tattini *et al*. (2004).

A modulated chlorophyll *a* fluorescence analysis was conducted on dark-adapted (over a 40-min period) leaves using a PAM-2000 fluorometer (Walz, Effeltrich, Germany) connected to a Walz 2030-B leaf-clip holder through a Walz 2010-F trifurcated fibre optic, under laboratory conditions. The maximum efficiency of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_0)/F_m$, where $F_v$ is the variable fluorescence and $F_m$ is the maximum fluorescence of dark-adapted (over a 40-min period) leaves. The minimal fluorescence, $F_0$, was measured using a modulated light pulse $<1$ $\mu$mol m$^{-2}$ s$^{-1}$, to avoid appreciable variable fluorescence. Values of $F_m$ and $F_m'$ were determined at 20 kHz using a 0·8-s saturating light pulse of white light at 8000 $\mu$mol m$^{-2}$ s$^{-1}$. We then estimated $\Phi_{PSII}$ and non-photochemical quenching [NPQ $= (F_m/F_m') - 1$] as described by Genty *et al*. (1989). The excitation pressure on PSII, (1 − *q*P), *q*P being the coefficient of photochemical quenching, was calculated as described by Schreiber *et al*. (1995).

### Analysis of photosynthetic pigments and phenylpropanoids

Individual carotenoids in leaves sampled at predawn and midday were identified and quantified as reported by Beckett *et al*. (2012). Leaves were immediately frozen in liquid nitrogen and kept at −80 °C until analysis. Fresh leaf material ($120-150$ mg) was extracted with $2 \times 4$ ml acetone (containing 0·5 g l$^{-1}$ CaCO$_3$) and 15 $\mu$L aliquots were injected into a PerkinElmer Flexar chromatograph equipped with a quaternary 200Q/410 pump and LC 200 diode array detector (all from PerkinElmer, Bradford, CT, USA). Photosynthetic pigments were separated using a $250 \times 4$·6 mm Waters Spherisorb ODS1 (5 $\mu$m) column operating at 30 °C, eluted with a linear gradient solvent system, at a flow rate of 1·2 mL min$^{-1}$, consisting of CH$_3$CN/MeOH/H$_2$O (8·4/0·8/0·7, A) and MeOH/ethyl acetate (6·8/3·2, B) during an 18-min run: $0-12$ min from 100 to 0 % A; $12-18$ min at 0 % A. Carotenoids were tentatively identified using visible spectral characteristics and retention times. Violaxanthin and antheraxanthin were quantified using the calibration curve of an authentic lutein standard (Extrasynthese, Lyon-Nord, Genay, France); for zeaxanthin we used the calibration curve of an authentic zeaxanthin standard (Extrasynthese). Chlorophylls *a* and *b* were quantified using calibration curves of chlorophyll authentic standards (Sigma-Aldrich, Milan, Italy).

Phenylpropanoids, mostly coumarins and flavonols, were extracted, identified and quantified following the protocol of Fini *et al*. (2012). Freeze-dried leaf tissue ($30-40$ mg) was extracted with $3 \times 5$ mL of 75 % ethanol (adjusted to pH 3·0 with formic acid), and the supernatant was partitioned with $3 \times 8$ mL of *n*-hexane. The ethanol fraction was reduced to dryness under vacuum at room temperature with a Büchi P12 Multivapor unit equipped with a Büchi V-855 vacuum controller (Büchi, Flawil, Switzerland). The residue was dissolved in 1 mL of MeOH/H$_2$O (9:1, v:v) and 10 $\mu$L aliquots were injected into the Perkin-Elmer LC equipment described above. Phenylpropanoids were separated using a 250 mm × 4·6 mm Hypersil BDS C18 column (5 $\mu$m) operating at 30 °C and eluted with a linear gradient solvent system, at a flow rate of 1·0 mL min$^{-1}$, consisting of CH$_3$CN (A) and H$_2$O (pH 3·2 by H$_3$PO$_4$, B), during a 60-min run; $0-15$ min from 5 to 15 % A; $15-35$ min from 15 to 30 % A; $35-40$ min from 30 to 40 % A: $40-50$ min from 40 to 60 % A; $50-55$ min from 60 to 90 % A; $55-60$ min from 90 to 100 % A. Individual coumarins and flavonoids were identified using retention times and UV spectral characteristics of authentic standards (all from Extrasynthese) and by MS data. HPLC−mass spectrometry (MS) analysis was performed with an Agilent LC1200 chromatograph coupled with an Agilent 6410 triple quadrupole MS detector equipped with an electrospray ion source (all from Agilent Technologies, Santa Clara, CA, USA). Coumarins were quantified at 350 nm for esculetin and at 334 nm for both esculin (esculetin 6-*O*-glucoside) and chicorin (esculetin glycosides were pooled and are referred as to esculin throughout this article, as the chicorin concentration never exceeded 10 % of the esculin concentration) using calibration curves for authentic standards. Quercetin derivatives (quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside and quercetin 3-*O*-rutinoside) were quantified at 350 nm using calibration curves for the individual compounds.

### Antioxidant enzymes, oxidative stress and oxidative damage

Antioxidant enzyme activities and ascorbate concentration were measured in fresh leaf material as described by Guidi *et al*. (2008). Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured photometrically at 560 nm, based on the inhibition by SOD of nitroblue tetrazolium reduction. One unit of SOD was defined as the amount needed to bring about 50 % inhibition of the nitroblue tetrazolium reduction state. Catalase (CAT, EC 1.11.1.6) activity was measured photometrically at 270 nm by determining the rate of conversion of H$_2$O$_2$ to O$_2$. Total APX (EC 1.1.11.1) activity was measured as the decrease in absorbance at 290 nm resulting from ascorbate oxidation. Polyphenol oxidase (PPO, EC 1.14.18.1) activity was determined according to Espín *et al*. (1997) with 3,4-dihydroxyphenylpropionic acid as substrate, measuring the formation of *o*-quinones. Guaiacol POX (EC 1.11.1.7) was assayed as described by Bestwick *et al*. (1998), using 10 mM guaiacol (in 50 mM potassium phosphate buffer) as substrate and, following the addition of 0·25 % H$_2$O$_2$, measuring the concentration of oxidized guaiacol. The concentration of total ascorbate (ASA) was determined spectrophotometrically as described by Guidi *et al*. (2008).

Oxidative stress was estimated by determining the content of stable free radicals using ESR spectroscopy. Intact leaves were placed in a wide-bore (8·0 mm i.d.) quartz tube, and analyses were performed in continuous wave (CW) X-band using a Brüker Elexsys E500 series spectrometer equipped with a Brüker ER4122 SHQE cavity at 100 K (using an ER411VT Brüker variable temperature unit) as reported by Melgar *et al*.

(2009). Spectra were recorded under the following experimental conditions: modulation frequency 100 kHz, microwave power 20 mW, modulation amplitude 0·3 mT and frequency 9·39 GHz. The signal area was calculated using a double integration of the experimental spectrum. Our interest was in a signal centred at $g \sim 2{\cdot}003$ (peak-to-peak line width 1 mT), detectable on the fourth peak of the Mn(II) sextet component, using a scan width of 7 mT.

Cell ultrastructure and $H_2O_2$ accumulation were examined using TEM. Accumulation of $H_2O_2$ was determined cytochemically by detection of cerium perhydroxide formation upon the reaction of cerium chloride ($CeCl_3$) with endogenous $H_2O_2$, following the protocols of Guidi *et al.* (2010). Leaf pieces (area $\sim 0{\cdot}15$ mm$^2$) were vacuum-infiltrated in 5 mM $CeCl_3$ in 50 mM 3-(N-morpholino)-propanesulphonic acid (pH 7·2). Samples treated with $CeCl_3$ were then fixed in 10 % glutaraldehyde, 10 % acrolein and 6 % paraformaldehyde in 0·2 m phosphate buffer (pH 7·2) for 1 h, and washed twice with the same buffer before post-fixing with 2 % osmium tetroxide in phosphate buffer (pH 7·2). Specimens were dehydrated in a graded ethanol series (25, 50, 75, 90 and 100 %), and gradually embedded in Epon–Araldite. Ultrathin sections ($\sim 80$–90 nm) were obtained with an LKB IV ultramicrotome (LKB Produkter, New York, USA), mounted on Formvar-coated copper grids, stained with uranyl acetate and lead citrate and examined using a Philips CM12 transmission electron microscope (Philips, Eindhoven, Netherlands) operating at 80 kV.

### Statistics

The experimental set up was a split-plot design with eight replicates in which light intensity was the main plot and salinity the subplot. Measurements were made on fully developed leaves aged 5–6 weeks. Data collected under laboratory conditions, with the exception of $\psi_{\pi,s}$ (the contribution of individual solutes to osmotic potential at full turgor), were analysed using two-way ANOVA (light and salinity as fixed factors and their interaction factor). Daily trends in $A$ and $g_s$ *in situ* were analysed using repeated measures ANOVA. Statistical analysis was performed using the SPSS statistical package (IBM, New Orchard Road, Armonk, NY, USA). The extent to which a plant trait $X$ varied in response to sunlight irradiance (light) or root-zone salinity (salt) was quantified in terms of the normalized index of variation (NIV) as follows (Tattini *et al.*, 2006):

$$\mathrm{NIV}_{\mathrm{light}} = (X_{\mathrm{sun}} - X_{\mathrm{shade}})/(X_{\mathrm{sun}} + X_{\mathrm{shade}}) \qquad (8)$$

$$\mathrm{NIV}_{\mathrm{salt}} = (X_{\mathrm{salt}} - X_{\mathrm{control}})/(X_{\mathrm{salt}} + X_{\mathrm{control}}) \qquad (9)$$

## RESULTS

### Morpho-anatomy and water and ionic relations

Salinity stress did not markedly affect the whole set of leaf morpho-anatomical features examined in our study, which instead were greatly altered by sunlight irradiance (Table 1 and Supplementary Data Table S1). There were major light-induced alterations in cuticle thickness and cuticular wax content (average +58 %), but whole-leaf thickness, LMA and the proportion of palisade parenchyma to spongy parenchyma tissue were also significantly greater in leaves of sun plants than on those of shaded plants (average +34 %). Interestingly, leaf lamina size, which was on average 40 % greater in shaded than in full sun plants, decreased because of salinity stress only in leaves of shaded plants. The RGR$_{\mathrm{shoot}}$, which was slightly higher in control plants growing in full sun than in partial shade) declined more in shaded (−58 %) than in sun (−44 %) plants because of root-zone salinity (Table 1).

Sunlight irradiance (NIV = 0·08) had a relatively minor effect compared with root-zone salinity (NIV = 0·31) on water and ionic relations (Table 2 and Supplementary Data Table S1). Nonetheless, osmotic potential decreased more in leaves of sun plants than in shaded plant leaves in response to salinity. Furthermore, dehydration contributed more in shaded ($D = -0{\cdot}27$ MPa, 26 % of $\Delta\psi_\pi$) than in sun plants (−0·25 MPa, 16 % of $\Delta\psi_\pi$) to the salt-induced decline in $\psi_\pi$. The greater capacity for active osmotic adjustment in response to salinity stress displayed by leaves of sun plants than those of shaded plants (Table 2) was mostly due to higher concentrations of $Na^+$ (122 mM in shaded versus 167 mM in sun plants), $Cl^-$ (142 mM in shaded versus 195 mM in sun plants) and $K^+$ (240 mM in shaded versus 293 mM in sun plants). On the contrary, soluble carbohydrates (which contributed more to $\psi_{\pi FT}$ in sun than in shaded plants in the control) made a greater contribution to salt-induced osmotic adjustment in shaded than in sun plants

TABLE 1. *Shoot dry weight increments (RGR$_{shoot}$) and leaf morpho-anatomical features in control (C) and salt-treated (ST)* F. ornus *grown at 45 (shade) or 100 % (sun) solar irradiance*

| Trait | Shade | | Sun | | NIV$_{light}$ | NIV$_{salt}$ |
|---|---|---|---|---|---|---|
| | C | ST | C | ST | | |
| RGR$_{shoot}$ (d$^{-1}$ × 10$^3$) | 23·2 ± 2·8 | 9·8 ± 2·2 | 29·1 ± 3·6 | 15·6 ± 3·2 | +0·15 | −0·35 |
| Leaf area (cm$^2$) | 15·4 ± 1·8 | 11·5 ± 1·3 | 10·3 ± 1·1 | 8·9 ± 1·0 | −0·17 | −0·11 |
| Leaf mass per area (g DW m$^{-2}$) | 68·2 ± 4·6 | 70·5 ± 5·4 | 98·4 ± 7·3 | 95·3 ± 5·0 | +0·17 | +0·00 |
| Cuticle thickness (μm) | 3·2 ± 0·3 | 3·1 ± 0·2 | 4·7 ± 0·5 | 4·8 ± 0·3 | +0·21 | +0·01 |
| Cuticular wax (μg cm$^{-2}$) | 65·7 ± 7·7 | 74·2 ± 8·1 | 109·1 ± 8·8 | 116·0 ± 9·9 | +0·29 | +0·05 |
| Leaf thickness (μm) | 187 ± 21 | 194 ± 19 | 244 ± 19 | 252 ± 17 | +0·13 | +0·01 |
| Palisade to spongy ratio | 0·42 ± 0·4 | 0·40 ± 0·3 | 0·53 ± 0·3 | 0·54 ± 0·4 | +0·13 | −0·00 |

Data are mean ± s.d. ($n = 6$).
Leaves sampled for measurement were fully developed (5–6 weeks old).

TABLE 2. *Water and ionic relations in control (C) salt-treated (ST)* F. ornus *grown at 45 (shade) or 100 % (sun) solar irradiance*

|  | Shade | | Sun | | | |
|---|---|---|---|---|---|---|
|  | C | ST | C | ST | $NIV_{light}$ | $NIV_{salt}$ |
| RWC (%) | $93.4 \pm 2.8$ | $86.0 \pm 2.0$ | $92.5 \pm 1.8$ | $89.1 \pm 1.5$ | +0.00 | −0.03 |
| $\psi_w$ (−MPa) | $0.44 \pm 0.3$ | $0.83 \pm 0.5$ | $0.48 \pm 0.3$ | $0.86 \pm 0.4$ | +0.03 | +0.29 |
| $\psi_\pi$ (−MPa) | $1.88 \pm 0.09$ | $2.95 \pm 0.15$ | $1.94 \pm 0.17$ | $3.51 \pm 0.22$ | +0.06 | +0.32 |
| $\psi_{\pi FT}$ (−MPa) | $1.75 \pm 0.13$ | $2.51 \pm 0.16$ | $1.78 \pm 0.15$ | $3.10 \pm 0.18$ | +0.07 | +0.30 |
| $\psi_{\pi,FT,Na^+}$ (−MPa) | $0.05 \pm 0.01$ | $0.25 \pm 0.03$ | $0.07 \pm 0.01$ | $0.34 \pm 0.03$ | +0.15 | +0.66 |
| $\psi_{\pi FT,Cl^-}$ (−MPa) | $0.06 \pm 0.01$ | $0.29 \pm 0.04$ | $0.09 \pm 0.02$ | $0.40 \pm 0.02$ | +0.17 | +0.64 |
| $\psi_{\pi FT,K^+}$ (−MPa) | $0.35 \pm 0.03$ | $0.49 \pm 0.05$ | $0.38 \pm 0.02$ | $0.60 \pm 0.04$ | +0.08 | +0.22 |
| $\psi_{\pi FT,mannitol}$ (−MPa) | $0.21 \pm 0.02$ | $0.42 \pm 0.01$ | $0.23 \pm 0.02$ | $0.36 \pm 0.03$ | −0.03 | +0.28 |
| $\psi_{\pi FT,sugars}$ (−MPa) | $0.55 \pm 0.03$ | $0.50 \pm 0.04$ | $0.66 \pm 0.04$ | $0.65 \pm 0.02$ | +0.11 | −0.03 |

Data are mean $\pm$ s.d. ($n = 6$).
Leaves sampled for measurement were fully developed (5–6 weeks old).
Sugars include sucrose, glucose, galactose and fructose.
$\psi_w$, water potential; $\psi_\pi$ osmotic potential; $\psi_{\pi FT}$, osmotic potential at full turgor; $\psi_{\pi FT,s}$, contribution of solutes to $\psi_{\pi FT}$.

TABLE 3. *Gas exchange and PSII photochemistry in control (C) and salt-treated (ST)* F. ornus *grown at 45 (shade) or 100 % (sun) solar irradiance*

|  | Shade | | Sun | | | |
|---|---|---|---|---|---|---|
|  | C | ST | C | ST | $NIV_{light}$ | $NIV_{salt}$ |
| $A$ ($\mu$mol m$^{-2}$ s$^{-1}$) | $13.4 \pm 1.2$ | $4.7 \pm 0.8$ | $14.1 \pm 1.4$ | $7.1 \pm 0.7$ | +0.08 | −0.40 |
| $g_s$ (mmol m$^{-2}$ s$^{-1}$) | $245 \pm 21$ | $74 \pm 12$ | $267 \pm 29$ | $112 \pm 10$ | +0.08 | −0.47 |
| $g_m$ (mmol m$^{-2}$ s$^{-1}$) | $138 \pm 19$ | $69 \pm 10$ | $129 \pm 10$ | $65 \pm 9$ | −0.04 | −0.33 |
| $V_{c,max}$ ($\mu$mol m$^{-2}$ s$^{-1}$) | $75 \pm 8$ | $19 \pm 4$ | $106 \pm 9$ | $33 \pm 5$ | +0.19 | −0.56 |
| $J_{max}$ ($\mu$mol m$^{-2}$ s$^{-1}$) | $120 \pm 11$ | $33 \pm 5$ | $96 \pm 7$ | $46 \pm 6$ | −0.04 | −0.46 |
| $C_i$ ($\mu$mol mol$^{-1}$) | $217 \pm 21$ | $276 \pm 16$ | $210 \pm 11$ | $239 \pm 14$ | −0.05 | +0.09 |
| $C_c$ ($\mu$mol mol$^{-1}$) | $141 \pm 13$ | $248 \pm 22$ | $128 \pm 8$ | $198 \pm 14$ | −0.09 | +0.25 |
| $PPFD_{sat}$ ($\mu$mol quanta m$^{-2}$ s$^{-1}$) | $965 \pm 80$ | $356 \pm 58$ | $1056 \pm 93$ | $871 \pm 85$ | +0.19 | −0.24 |
| $F_v/F_m$ | $0.78 \pm 0.01$ | $0.77 \pm 0.01$ | $0.77 \pm 0.02$ | $0.76 \pm 0.01$ | −0.01 | −0.01 |
| $\Phi_{PSII}$ | $0.56 \pm 0.02$ | $0.36 \pm 0.04$ | $0.49 \pm 0.03$ | $0.38 \pm 0.03$ | −0.02 | −0.17 |
| NPQ | $0.95 \pm 0.10$ | $1.91 \pm 0.09$ | $1.30 \pm 0.12$ | $1.97 \pm 0.13$ | +0.07 | +0.29 |
| $1 - q$P | $0.14 \pm 0.03$ | $0.29 \pm 0.03$ | $0.18 \pm 0.02$ | $0.23 \pm 0.02$ | −0.02 | +0.24 |

Data are mean $\pm$ s.d. ($n = 6$).
Leaves sampled for measurements were fully developed (5–6 weeks old).
Measurements were conducted under laboratory conditions.
$A$, net $CO_2$ assimilation rate; $g_s$, stomatal conductance; $g_m$, mesophyll conductance to $CO_2$; $V_{c,max}$, apparent rate of maximum carboxylation by Rubisco; $C_i$, internal $CO_2$ concentration; $C_c$, chloroplast $CO_2$ concentration; $F_v/F_m$, maximum efficiency of PSII photochemistry; $\Phi_{PSII}$, actual efficiency of PSII photochemistry; $1 - q$P, reduction state of primary acceptors.

(Table 2). This resulted from a much greater (+61 %) salt-induced increase in the molar concentration of mannitol in shaded than in sun plants.

### Gas exchange and PSII photochemistry

Salinity stress markedly (NIV = 0.33) affected leaf gas exchange performance under laboratory conditions, whereas the overall effect of sunlight irradiance (NIV = 0.10) was of relatively minor significance (Table 3 and Supplementary Data Table S1). Sunlight irradiance did not significantly alter net photosynthesis and stomatal conductance measured at saturating light under laboratory conditions in control plants, whereas salt-induced declines in gas exchange were more pronounced in shaded (on average −67.5 %) than in full sun plants (average −55 %). Sun plants had much greater carboxylation efficiency ($V_{c,max}$) than leaves of shaded plants (+47 %), as estimated by $A/C_i$ curves,

particularly when exposed to salinity stress (+68 %). The apparent maximum electron transport rate contributing to Rubisco regeneration ($J_{max}$) was higher (+25 %) in control plants growing in the shade than in full sun plants, but salt-induced decreases in $J_{max}$ were again greater in leaves of shaded plants (−72 %) than in leaves of sun plants (−52 %) (Table 3). The photosynthetically active radiation at which photosynthesis saturated ($PPFD_{sat}$), which did not differ significantly between leaves of sun and shaded control plants, decreased unexpectedly much more in shaded (−61 %) than in full sun plants (−17 %) in response to salinity stress. At midday PPFD exceeded $PPFD_{sat}$ by 255 % in shaded plants and by 210 % in sun plants (Table 3).

Mesophyll conductance to $CO_2$ did not vary significantly with sunlight, but decreased in response to salinity in both sun and shaded plants (Table 3). In leaves of sun plants and especially in shaded plants, root-zone salinity increased the intercellular $CO_2$ concentration compared with control leaves (Table 3),
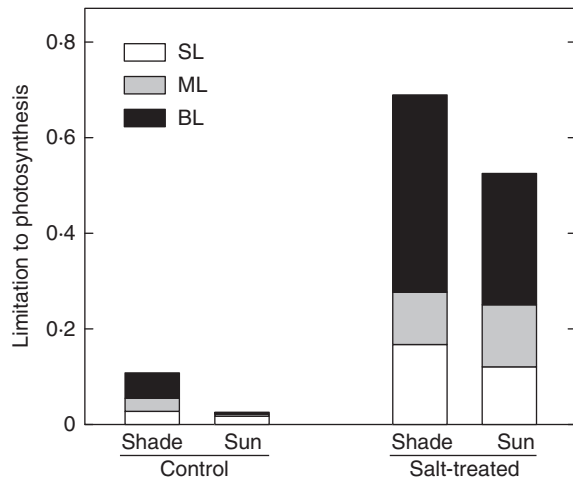
F₁G. 1. Stomatal (SL), mesophyll (ML) and biochemical (BL) limitations on photosynthesis in leaves (5–6 weeks old) of *F. ornus* grown at 45 (shade) or 100 % sunlight irradiance (sun) and supplied with 0 (control) or 75 mM NaCl (salt-treated). Data are means for four replicate leaves.

indicating that, though stomatal limitations increased because of root-zone salinity, non-stomatal limitations played the major role in constraining *A* (Fig. 1). Diffusive limitations (SL + ML) accounted for 40 and 50 % of the total limitation on *A* in shaded and sun salt-treated plants, respectively (Fig. 1). Thus, biochemical limitations (BL) constrained *A* more in shaded than in sun plants when suffering from salinity stress. Consistently, though salinity depressed $g_m$, the $CO_2$ concentration in the chloroplast ($C_c$) increased greatly in response to salinity, particularly in shaded plants (Table 3).

Shaded and sun plants displayed more evident differences in gas exchange performance under field conditions irrespective of salt treatment, as revealed by the daily courses of net photosynthesis and stomatal conductance estimated *in situ* (Fig. 2). Briefly, in control plants in full sun, stomatal conductance (Fig. 2B) decreased more than in corresponding shaded plants during the central hours of the day, without a concomitant depression in net $CO_2$ assimilation rate (Fig. 2A). In salt-treated leaves, photosynthesis was much higher in the sun than in the shade (+33 %), though sun plants had slightly higher stomatal conductance (+9 %) compared with shaded plants. Overall, on a daily basis, instantaneous water use efficiency (WUE, inset in Fig. 2A) was greater in sun (+16 %) than in shaded plants, especially during the hottest hours. This occurred despite sun plants having to cope with a higher vapour pressure deficit (VPD; +23 %) than shaded plants (inset in Fig. 2B).

PSII photochemistry in the dark-adapted state, i.e. $F_v/F_m$, did not vary in response to either sunlight or salt treatment (Table 3 and Supplementary Data Table S1). This is consistent with light- and salt-induced declines in PSII quantum yield ($\Phi_{PSII}$) and increases in NPQ, the extent of these changes being greater in leaves of shaded plants (on average 44 %) than in those of sun (28 %) plants. The reduction state of primary acceptors, $1 - qP$, here taken as a measure of excess excitation pressure on PSII (Maxwell and Johnson, 2000), increased much more in the shade (+107 %) than in full sun (+28 %) in response to salinity stress. Overall, leaves of salt-treated plants had greater excess excitation energy when grown under partial shading than in full sun (Table 3).
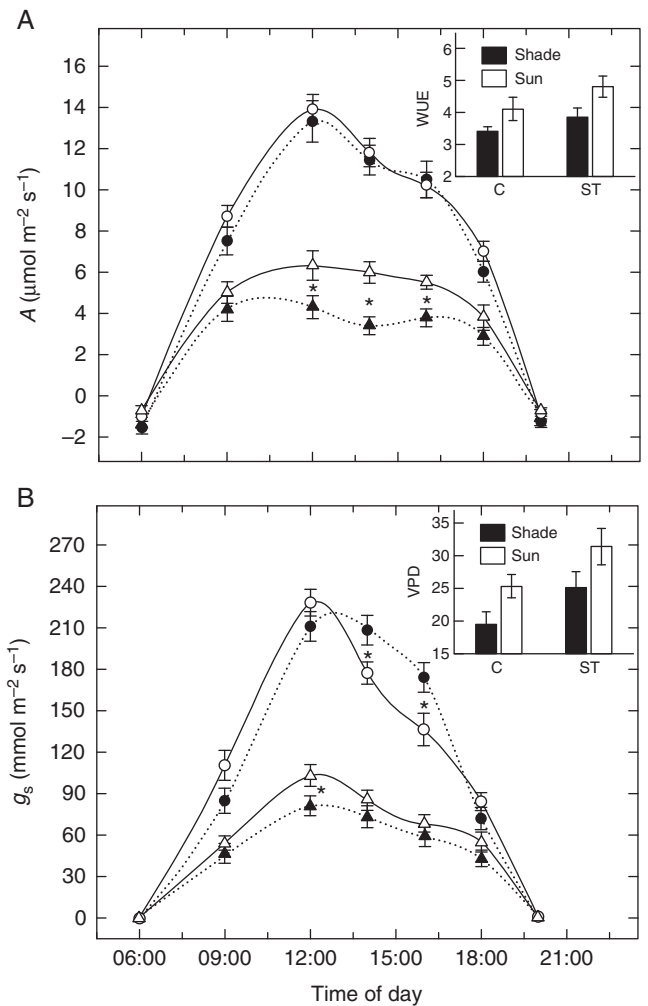


F₁G. 2. (A) Daily changes in net photosynthesis (A) and (B) stomatal conductance ($g_s$) in leaves of *F. ornus* grown at 45 (black symbols) or 100 % sunlight irradiance (open symbols) and supplied with 0 (circles) or 75 mM NaCl (triangles). Insets in (A) and (B) denote water use efficiency (*A/E*) and VPD, respectively, for control (C) and salt treatment (ST). Measurements were conducted after 2 (4-week-old leaves) and 4 (6-week-old leaves) weeks of treatment on four replicate plants, and data were pooled prior to statistical analysis. Data are means ± s.d. ($n = 8$). *$P < 0.05$ (LSD test).

### Photosynthetic pigments

Consistent with gas exchange and leaf photochemistry, the concentrations and composition of photosynthetic pigments varied more in shaded than in sun plants in response to salinity (Table 4 and Supplementary Data Table S1). First, total chlorophyll ($Chl_{tot}$) concentration was higher in shaded than in sun plants under control conditions, but salt-induced declines in $Chl_{tot}$ were detected in shaded plants only. Second, sun plants had a higher pool of violaxanthin-cycle pigments relative to $Chl_{tot}$ compared with shaded plants, irrespective of salt treatment (149 versus 119 mmol mol⁻¹ $Chl_{tot}$). The de-epoxidation state (DES) of violaxanthin cycle pigments was also greater (+47 %) in sun than in shaded plants under control conditions. However, salt-induced increases in DES were much higher in shaded (+195 %) than in sun plants (+68 %). This did not result from higher concentrations of antheraxanthin and

TABLE 4. *Concentration of total chlorophyll (μmol g$^{-1}$ DW), concentrations of individual carotenoids relative to total chlorophyll concentration (mmol mol$^{-1}$) and de-epoxidation state (DES) of violaxanthin cycle pigments in leaves of control (C) and salt-treated (ST)* F. ornus *grown at 45 (shade) or 100 % (sun) solar irradiance*

| Pigment | Shade | | Sun | | NIV$_{light}$ | NIV$_{salt}$ |
| --- | --- | --- | --- | --- | --- | --- |
| | C | ST | C | ST | | |
| Total chlorophyll | 6·8 ± 0·5 | 4·9 ± 0·4 | 5·6 ± 0·3 | 5·0 ± 0·4 | −0·05 | −0·11 |
| β-carotene | 111 ± 14 | 114 ± 12 | 117 ± 9 | 83 ± 10 | −0·06 | −0·07 |
| Lutein | 130 ± 14 | 145 ± 8 | 122 ± 10 | 138 ± 12 | −0·03 | +0·06 |
| Violaxanthin (V) | 86 ± 7 | 58 ± 8 | 100 ± 6 | 87 ± 5 | +0·09 | −0·10 |
| Antheraxanthin (A) | 11 ± 2 | 35 ± 4 | 16 ± 2 | 36 ± 3 | +0·10 | +0·47 |
| Zeaxanthin (Z) | 9 ± 1 | 40 ± 3 | 20 ± 3 | 40 ± 4 | +0·10 | +0·47 |
| DES = (Z + A)/ (V + A + Z) | 0·19 ± 0·02 | 0·56 ± 0·04 | 0·28 ± 0·02 | 0·47 ± 0·03 | +0·00 | +0·37 |

Data are mean ± s.d. (*n* = 4).
Leaves (5–6 weeks old) were collected at 09:00 and 13:00 h and pooled before analysis.

TABLE 5. *Activity of antioxidant enzymes (normalized to mg of protein) and concentrations of ascorbate and phenylpropanoids in leaves of well-watered and salt-treated* F. ornus *grown at 45 (shade) or 100 % (sun) solar irradiance*

| Parameter | Shade | | Sun | | NIV$_{light}$ | NIV$_{salt}$ |
| --- | --- | --- | --- | --- | --- | --- |
| | C | ST | C | ST | | |
| SOD (U) | 0·26 ± 0·04 | 0·30 ± 0·04 | 0·57 ± 0·03 | 0·62 ± 0·06 | +0·35 | +0·04 |
| CAT (mmol H$_2$O$_2$ min$^{-1}$) | 10·4 ± 1·5 | 7·8 ± 0·9 | 15·3 ± 2·30 | 18·6 ± 1·24 | +0·30 | +0·00 |
| APX (mol ASA min$^{-1}$) | 0·17 ± 0·02 | 0·06 ± 0·02 | 0·15 ± 0·03 | 0·25 ± 0·01 | +0·27 | −0·01 |
| POX (μmol oxidized guaiacol) | 0·24 ± 0·04 | 0·53 ± 0·06 | 0·38 ± 0·02 | 0·34 ± 0·04 | −0·03 | +0·16 |
| PPO (μmol quinones) | 0·15 ± 0·03 | 0·63 ± 0·06 | 0·14 ± 0·02 | 0·36 ± 0·04 | −0·22 | +0·55 |
| ASA (μg g$^{-1}$ DW) | 48·0 ± 5·6 | 56·5 ± 4·4 | 74·7 ± 6·0 | 60·5 ± 5·8 | +0·05 | −0·02 |
| DHASA (μg g$^{-1}$ DW) | 60·2 ± 3·6 | 24·3 ± 5·1 | 84·1 ± 4·3 | 98·4 ± 8·0 | +0·38 | −0·08 |
| Esculetin (μmol g$^{-1}$ DW) | 45·1 ± 7·1 | 68·1 ± 5·5 | 45·9 ± 5·7 | 43·4 ± 4·1 | −0·12 | +0·12 |
| ESC (μmol g$^{-1}$ DW) | 54·1 ± 3·6 | 40·6 ± 5·0 | 58·2 ± 3·7 | 60·5 ± 7·2 | +0·11 | −0·04 |
| QUE (μmol g$^{-1}$ DW) | 4·3 ± 0·8 | 12·9 ± 1·8 | 8·9 ± 0·7 | 8·7 ± 1·1 | +0·01 | +0·24 |

Data are mean ± s.d. (*n* = 4).
Leaves sampled for measurement were fully developed (5–6 weeks old), collected at midday.
ESC, esculin (esculetin 6-*O* + esculetin 7-*O*-glucoside); QUE, quercetin 3-*O*-glycosides.

zeaxanthin in shaded than in sun plants, but depended on a salt-induced decline in violaxanthin concentration, which was indeed much greater in shaded (−33 %) than in sun plants (−13 %). On the contrary, the concentration of β-carotene decreased and remained constant in sun and shaded plant leaves, respectively, in response to salinity stress (Table 4).

*Antioxidant defences and oxidative damage*

Sun and shade plants mostly differed in the pool of antioxidant defences (NIV$_{light}$ = 0·19) compared with all other traits examined in our study, particularly when supplied with good-quality water (Table 5 and Supplementary Data Table S1). These differences mostly concerned SOD, CAT and APX, as well as total ASA and quercetin 3-*O*-glycosides, the activities and the concentrations of which were significantly higher (on average +76 %) in leaves of sun plants than in those of shaded plants. Enzymes and metabolites aimed at removing ROS varied either little in the sun (25 %) or markedly in the shade (118 %) in response to salinity. Notably, CAT and particularly APX decreased markedly in the shade or increased in the sun

because of salinity stress. Such decreases were paralleled by steep increases in the activities of POX and PPO in leaves of shaded plants (Table 5). Salinity stress decreased the concentration of total ASA in the shade but did not affect it in the sun. This was due to salt-induced reduction of the concentration of oxidized ascorbate (DHASA) in leaves of shaded plants, thus increasing the ASA/DHASA ratio from 0·80 (0·88 in sun) in the control to 2·32 (0·61 in sun) in leaves of salt-treated plants. The phenylpropanoid concentration and composition did not vary with salinity in sun plants, whereas major phenylpropanoids increased in concentration with salinity in leaves of shaded plants.

Overall, salt-treated *F. ornus* suffered from oxidative damage both in the shade and in full sun, as revealed by the TEM images in Fig. 3. In particular, damage was evident in palisade parenchyma cells in sun plants: aggregated chloroplasts occurred in the centre of the cell, were round in shape and displayed a disorganized lamellar system (Fig. 4A). No signs of damage were apparent in spongy cells (Fig. 4B). Palisade parenchyma cells suffered less from salinity stress in leaves of shaded plants: chloroplasts had swollen thylakoid membranes and an increased
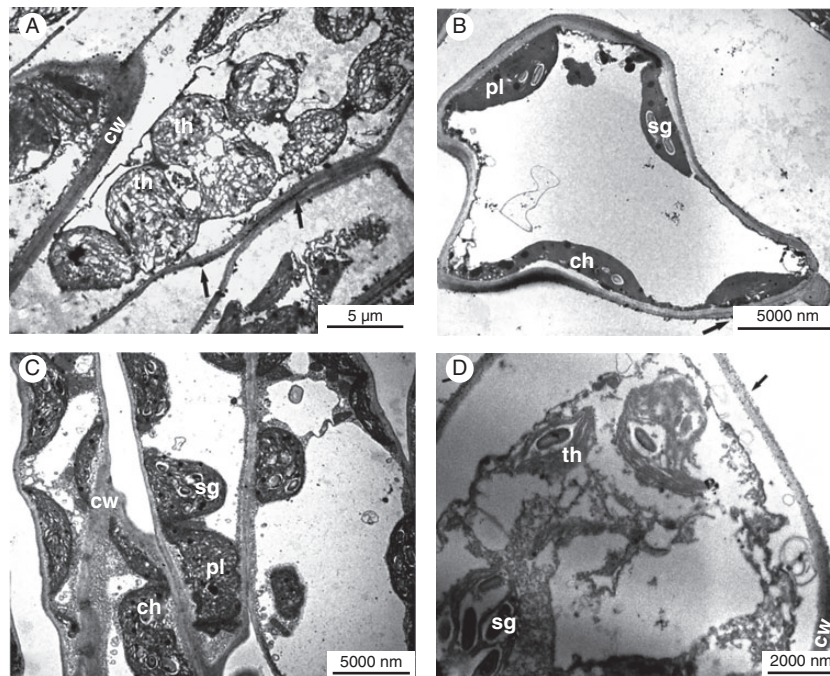
FIG. 3.  TEM of palisade (A, C) and spongy (B, D) mesophyll cells in salt-treated *F. ornus* leaves (5–6 weeks old) grown at 45 (C, D) or 100 % sunlight irradiance (A, B). Arrows denote $H_2O_2$ accumulation. Palisade parenchyma cells in sun plants (A) have round-shaped chloroplasts with disorganized lamellar system. Disruption of both the chloroplast envelope and the tonoplast membrane is evident in spongy cells of leaves of shaded plants (D). ch, chloroplast; cw, cell wall; th, thylakoids; pl, plastoglobules; sg, starch grains.
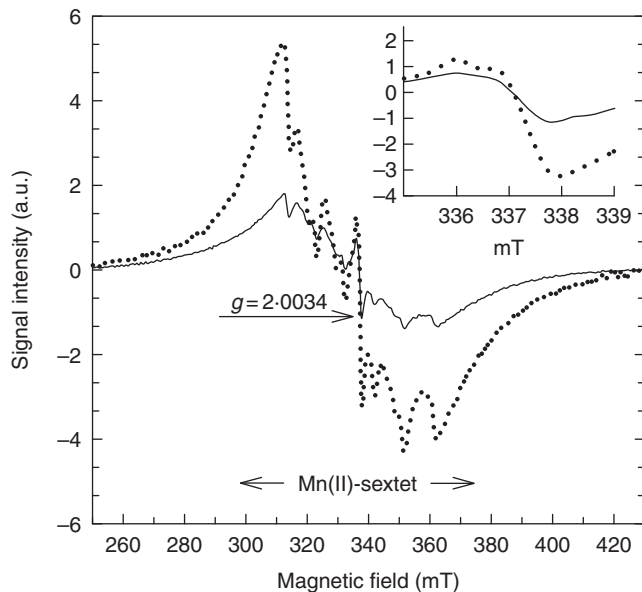


FIG. 4.  ESR analysis of intact leaves (5–6 weeks old) of salt-treated *F. ornus* grown at 45 (dotted line) or 100 % sunlight irradiance (solid line). The signal centred on the fourth peak of the Mn(II) sextet, with $g = 2.0034$ (inset shows an enlarged view) is attributable to semiquinone radicals. a.u., arbitrary units.

stable free radical, centred on the fourth peak of the Mn(II) sextet, between salt-treated shaded and sun plants (Fig. 5). The area of this signal, with $g = 2.0034$ (consistent with the presence of semiquinone radicals) was indeed 88 % higher in leaves of salt-treated plants grown in the shade than in those of salt-treated sun plants.

## DISCUSSION

Our study draws a comprehensive picture of the effects of sunlight irradiance on the performance of *F. ornus*, a salt-sensitive species, under mild root-zone salinity. The results highlight that light-induced changes in morpho-anatomy, in addition to relevant biochemical characters, have great impact on the use of assimilated carbon in plants challenged by root zone salinity during summer, and profoundly affect the biochemical machinery aimed at countering salt-induced oxidative stress.

First, we suggest that the suite of morpho-anatomical features were responsible for greater net photosynthesis in leaves of sun plants than in those of shaded plants during the hottest hours of the day, irrespective of salinity treatment (Evans, 1999; Poorter *et al*., 2009). Light-induced increases in cuticle thickness and cuticular wax content may effectively limit cuticular transpiration, which is of particular significance in thin leaves, such as those of *F. ornus* (leaves acclimated for 5 weeks to full sunlight were 250 μm thick), particularly when stomatal conductance is depressed by salt-induced osmotic unbalance (Boyer *et al*., 1997; Ristic and Jenks, 2002). Light-induced reduction in leaf lamina size also confers on sun plants a great capacity for dissipating heat by conduction and convection (Nobel, 2005), a key feature for plants challenged by hot

number of plastoglobules (Fig. 4C). By contrast, spongy cells in leaves of shaded plants showed clear signs of degeneration, such as disruption of both the chloroplast envelope and the tonoplast membrane (Fig. 4D). Actually, our ESR analysis performed on intact leaves showed marked differences in the content of a

temperatures during summer. We calculate, using the method described by Nobel (2005), that sun plants dissipated 37 % more heat by conduction and convection during the warmest hours of the day (air temperature was on average 32·5 °C between 11:00 and 15:00 h). This may also help explain why stomatal conductance was higher in shaded than in sun plants during the hottest hours of the day. Sun plants regulated transpiration through stomata more effectively than shaded plants; stomata are indeed very sensitive to VPD in the sun (Nicolás *et al.*, 2008), whereas daily changes in light irradiance, rather than VPD, have been reported as the main factor controlling stomatal opening in leaves of shaded plants (Nicolás *et al.*, 2008). In our study, VPD was 14 % higher on a daily basis, and 33 % higher at midday, in full sun than in partial shading. Thus, enhanced stomatal control of water loss and heat dissipation by conduction/convection allowed control leaves in the sun to maintain transpiration rates similar to those in leaves of shaded plants during the warmest hours of the day. This is remarkable, as sun plants had to cope with both higher temperature (air temperature was ~1 °C higher in the sun than in the shade) and higher VPD than shaded plants. Salt-induced decrease in soil water potential was instead the main driver of stomatal responsiveness, as stomatal conductance did not differ markedly between leaves of sun and shaded plants supplied with root-zone NaCl, in agreement with the results of Perez-Martin *et al.* (2009).

Despite greater stomatal control, stomata did not limit net photosynthesis more in the sun than in the shade under either the control condition or with root-zone salinity. Salt-induced decreases in mesophyll conductance were indeed similar in leaves of sun and shaded plants (Loreto *et al.*, 2003). Therefore, diffusive limitations (SL + ML) were not the main determinants of the salt-induced declines in *A* observed in our study. In contrast, biochemical limitations on photosynthesis were markedly higher in shaded than in sun plants under control conditions, and these differences became even larger during salinity. This conforms to the values of both carboxylation efficiency ($V_{c,max}$) and mesophyll conductance ($g_m$) estimated in our study as well as in a number of previous experiments (Flexas *et al.*, 2004; Galmés *et al.*, 2013). Lower constitutive (i.e. in control leaves) coupled with steeper salt-induced reductions in $V_{c,max}$ may have been responsible for the larger increases in $C_c$ observed in shaded than in sun plants in response to salinity (Galmés *et al.*, 2011). An increase in $C_c$ mostly occurs under severe stress conditions, as a result of biochemical limitations largely prevailing over the diffusive ones (Flexas *et al.*, 2004; Galmés *et al.*, 2011), as was also the case in our study in leaves of shaded salt-treated plants. Therefore, greater photosynthetic rates – not lower transpiration rates – were responsible for higher WUE in leaves of sun plants than in those of shaded plants during the hottest hours of the day (Medrano *et al.*, 2012), particularly in salt-treated leaves. As mentioned above, morphoanatomical features indeed have a crucial role in leaf water balance under field conditions, a role that is not revealed in experiments conducted under laboratory (or controlled) conditions (Mielke and Schaffer, 2010; Gardiner and Krauss, 2011). WUE was indeed similar in leaves of sun and shaded plants measured under laboratory conditions at saturating light as well as in leaves sampled *in situ* at 09:00 and 18:00 h.

The relatively small differences in the accumulation of potentially toxic ions and K+ detected in our experiment relate to

similar daily $g_s$ (and instantaneous transpiration rate, not reported; García-Sánchez *et al.*, 2006) displayed by salt-treated plants growing under contrasting sunlight. Our study therefore suggests that the effect of sunlight irradiance on ionic transport to actively growing shoot organs may have relatively minor significance under field conditions (Reynolds, 2000; López-Hoffman *et al.*, 2007), particularly when soil salinity builds up in concomitance with hot temperatures. In turn, this leads us to conclude that in our study the performance of salt-treated shaded and sun plants depended mostly on the osmotic component of excess soil salinity rather than on the ionic component. In support of this, the K+/Na+ ratio was apparently high in salt-treated leaves (Greenway and Munns, 1980), ranging from 1·96 in shaded to 1·76 in sun plants. In contrast, the steeper salt-induced increase in mannitol concentration observed in shaded than in sun plants reveals that the leaves of shaded plants were less effective in active osmotic adjustment: dehydration indeed made a much greater contribution in shaded than in sun plants to salt-induced osmotic changes (Gucci *et al.*, 1997; Tattini *et al.*, 2002). Nonetheless, it is worth noting that sun plants made considerable use of 'cheap' osmolytes, i.e. Na+, Cl− and K+, for osmotic adjustment (Greenway and Munns, 1980; Gucci *et al.*, 1997) compared with shaded plants. This likely allowed sun plants to devote more fresh carbon to growth than to biosynthesis of compatible solutes (Cimato *et al.*, 2010) – a very costly process (Chen *et al.*, 2007) – compared with shade plants. This conforms to greater salt-induced decreases in $RGR_{shoot}$ observed in shaded than in sun plants in our study. Furthermore, in salt-treated sun plants photosynthetic performance might have benefited from higher concentrations of K+ compared with their shaded counterparts (Anschütz *et al.*, 2014; Shabala and Potosin, 2014). On the contrary, the steeper salt-induced increase in mannitol concentration possibly feedback-inhibited photosynthesis more in shaded than in sun plants (Paul and Pellny, 2003). However, the massive use of Na+ and Cl− for osmotic adjustment may raise concern about the performance of 'glycophytes' on a long-term basis (Gucci *et al.*, 1997; Tattini *et al.*, 2002). This issue merits further investigation (e.g. by performing field experiment), as *F. ornus* and other deciduous tree species are transiently challenged by excess soil salinity in Mediterranean areas.

By the end of the experiment, salt-treated leaves in the shade were subjected to a more severe excess of light during the central hours of the day compared with sun plants. Saturation of photosynthesis occurred at 33·2 and 42·2 % of the maximum sunlight irradiance at which shaded and sun plants grew, respectively. In the leaves of salt-treated shaded plants, *A* and $CO_{2,daily}$ accounted for 63 and 38 %, respectively, of the corresponding values in sun plants. This is also consistent with the salt-induced increase in the reduction state of primary acceptors, $1 - qP$, which was markedly higher in shaded than in sun plants. Consequently, the salt-induced activation of de-epoxidation processes within the violaxanthin cycle (VAZ) pool to sustain thermal dissipation of excess energy through NPQ was greater in the shade than in the sun. A correlation between DES and NPQ has been reported in many instances (Müller, 2001; Demmig-Adams and Adams, 2006). However, in our study, DES, but not NPQ, was significantly higher in shaded than in sun plants suffering from salinity stress. We note first that salt-treated leaves in the shade underwent greater decreases (−33 %)

in violaxanthin compared with sun plants (−13 %). In sun plants, therefore, the salt-induced increase in zeaxanthin may have been in part derived from β-carotene, the concentration of which decreased (−29 %) in response to salinity (Davison *et al.*, 2002; Du *et al.*, 2010). Second, zeaxanthin-dependent NPQ occurs at relatively low concentrations of VAZ relative to $Chl_{tot}$ (usually <50 mmol mol$^{-1}$ $Chl_{tot}$; Havaux and Niyogy, 1999; Niinemets *et al.*, 2003), and in our study the concentration of the VAZ pool greatly exceeded 100 mmol mol$^{-1}$ $Chl_{tot}$. This supports a role of zeaxanthin, located in other parts of the thylakoid membranes, which is not directly involved in NPQ, possibly aimed at directly preserving the chloroplast membrane from photo-oxidation (Havaux and Niyogy, 1999; Beckett *et al.*, 2012). Third, we conducted NPQ measurements under laboratory conditions, whereas we analysed carotenoids in leaves sampled in the field. Components of the VAZ cycle have key roles in preserving chloroplast membranes from heat (Havaux, 1998; Fernández-Marin *et al.*, 2011), and this may have been of particular value in leaves of salt-treated shaded plants, which underwent steep declines in *A*, particularly during the central hours of the day. In other words, leaves have to sustain greater NPQs in the field during the hottest hours of the day, which is not revealed by NPQ measurements under laboratory conditions. Our data do not support the notion that slow-relaxing components that are independent of xanthophylls play a prominent role in NPQ. Slow-relaxing components of NPQ become relevant when leaves are severely photo-inhibited (Müller *et al.*, 2001), which was not the case in our study.

The activity of antioxidant enzymes has been reported to be related to stress tolerance in some instances, though it is still uncertain whether a constitutive or stress-induced increase in antioxidant enzyme activity confers greater tolerance to abiotic stresses (for reviews see Smirnoff, 1998; Gill and Tuteja, 2010). The results of our study therefore support the idea that sun plants were more tolerant of root-zone salinity than shaded plants (Mittal *et al.*, 2012; Turkan *et al.*, 2013). Indeed, enzymes aimed at $H_2O_2$ removal were more active in control and particularly in salt-treated leaves growing in the sun than in those in partial shade, as recently observed in tomato fruits (Zushi *et al.*, 2014). We hypothesize that greater reduction in radiation use efficiency coupled with hot temperatures may have detrimentally affected the activities of enzymes aimed at detoxifying ROS in leaves of shaded plants under salinity stress (Peltzer and Polle, 2001; Hatier and Gould, 2008; Fini *et al.*, 2011, 2012). A salt-induced decrease in total ASA concentration in leaves of shaded plants, mostly due to reduction in DHASA concentration, is indeed consistent with the very low APX activity in leaves of salt-treated shaded plants. Interestingly, following salt-induced reduction of CAT and APX activities, the activity of guaiacol POX increased in leaves of shaded plants (Fini *et al.*, 2012; Maia *et al.*, 2013). This increase occurred in parallel with marked increases in the concentrations of effective antioxidant phenylpropanoids (e.g. the ratio of esculetin to esculin increased by as much as 102 % in leaves of shaded plants in response to salinity). It is therefore possible that POX and antioxidant phenylpropanoids will have complemented the action of primary antioxidant defences in countering salt-induced ROS generation in the shade (Ferreres *et al.*, 2011; Agati *et al.*, 2012).

Both shaded and sun plants underwent salt-induced damage at the sub-cellular level. Interestingly, in leaves of shaded plants

damage mostly occurred in spongy mesophyll cells, which might indicate that direct flux of solar energy was not the main driver of oxidative stress, which was likely the case in sun plants. It is apparent that, in sun plants, palisade cells preserved the underlying tissues from severe photo-oxidative stress, whereas in leaves of shaded plants the radiant energy that reached the spongy cells was likely much more that usable for photosynthesis, due to the very low ability to carboxylate $CO_2$. The signal centred on the fourth line of the $Mn(H_2O)_6^{2+}$ sextet at $g = 2.0034$ is consistent with the formation of semiquinone radicals (Hatano *et al.*, 1997; Gallez *et al.*, 2000). Semiquinones had possibly originated in the incomplete recycling of phenylpropanoids following their oxidation mediated by vacuolar POX (Hernández *et al.*, 2009; Agati and Tattini, 2010). However, it is likely that polyphenols flowed out from the vacuole of severely damaged cells and were then oxidized by PPO, the activity of which rapidly increased because of salinity stress, particularly in leaves of shaded plants.

## CONCLUSIONS

*Fraxinus ornus*, a glycophyte distributed mostly in the southern Mediterranean basin, may transiently face excess soil salinity during the summer months. Here we show that root-zone salinity may be more deleterious for plants growing in partial shade than for those growing in full sunlight, though the accumulation of potentially toxic ions is greater in sun than in shaded plants. This is because the suite of morpho-anatomical traits coupled with an inherently greater efficiency to carboxylate $CO_2$ allows greater photosynthetic rates in leaves of sun plants than in those of shaded plants, particularly when $CO_2$ diffusion in the leaf declines considerably because of salinity. Furthermore, potentially toxic ions and $K^+$ may contribute to active osmotic adjustment to a greater extent in leaves of sun plants than in those of shaded plants (which mostly use compatible solutes for osmotic adjustment), thus diverting less carbon and energy from growth while exerting a positive effect on photosynthetic performance. Following more severe reductions in photosynthesis, leaves of shaded plants are more likely to unexpectedly experience excess light stress compared with leaves growing in full sunlight. Drastic salt-induced reductions in radiation use efficiency in leaves of shaded plants may therefore result in severe depletion of primary antioxidant defences and photo-oxidative damage. We therefore conclude that, under mild root-zone salinity [relatively low soil salt concentration on a short-term (weeks) basis], deciduous tree species, such as *F. ornus*, are more resistant when inhabiting sunny than shaded habitats.

## SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford journals.org and consist of Table S1: summary of two-way ANOVA for the effects of irradiance and salinity treatments with their interactions on morpho-anatomical, physiological and biochemical features.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

$1 - q$P, reduction state of primary acceptors; $A$, net $CO_2$ assimilation rate (net photosynthesis); APX, ascorbate peroxidase; ASA, ascorbate; BL, biochemical limitations on net $CO_2$ assimilation rate; C, control (group); $C$, molar concentration of solutes (mol $kg^{-1}$ dry weight); $C_a$, external $CO_2$ concentration; CAT, catalase; $C_c$, $CO_2$ concentration in chloroplasts; Chl$_{tot}$, total chlorophyll; $C_i$, intercellular $CO_2$ concentration; $C_i$, internal $CO_2$ concentration; $CO_{2,daily}$, net daily carbon gain; $D$, dehydration; DES, de-epoxidation state; DHASA, oxidized ascorbate; DW, dry weight; $E$, transpiration rate; $F_0$, minimal fluorescence; $F_m$, maximum fluorescence of dark-adapted leaves (over a 40-min period); $F_v$, variable fluorescence of dark-adapted leaves (over a 40-min period); $F_v/F_m$, maximum efficiency of PSII photochemistry; $g_m$, mesophyll conductance to $CO_2$ diffusion; $g_s$, stomatal conductance; $J_f$, rate of linear electron transport estimated from chlorophyll fluorescence; $J_{max}$, maximum electron transport rate contributing to Rubisco regeneration; LMA, leaf mass per area; ML, mesophyll conductance limitations on net $CO_2$ assimilation rate; MS, mass spectrometry; NIV, normalized index of variation; NPQ, non-photochemical quenching; POX, peroxidase; PPFD, photosynthetic photon flux density; PPFD$_{sat}$ photosynthetic active radiation at which saturation of photosynthesis occurs; PPO, polyphenol oxidase; POX, peroxidase; PS photosystem; $q$P, coefficient of photochemical quenching; RDW, relative dry weight; RGR$_{shoot}$, relative growth rate of the shoot; Rubisco, activity of ribulose-1,5-bis-phosphate carboxylase/

oxygenase; ROS, reactive oxygen species; RWC, relative water content; SL, stomatal limitations on net $CO_2$ assimilation rate; SOD, superoxide dismutase; ST, salt-treated (group); TEM, transmission electron microscopy; VAZ, violaxanthin cycle; $V_{c,max}$, apparent rate of maximum carboxylation by Rubisco; $V_{c,max}$, carboxylation efficiency; VPD, vapour pressure deficit; $W$, shoot dry weight; WUE, water use efficiency; $\Phi_{PSII}$, quantum yield of photosystem II in the light; $\psi_\pi$, osmotic potential; $\psi_{\pi FT}$, osmotic potential at full turgor; $\psi_{\pi FT,s}$, contribution of solutes to $\psi_{\pi FT}$; $\psi_{\pi,s}$, contribution ($-MPa$) of individual solutes ($s$) to $\psi_{\pi FT}$; $\psi_w$, water potential; $\psi_\pi$, osmotic potential; $\psi_{\pi FT}$, osmotic potential at full turgor; $\Phi_{PSII}$, actual efficiency of PSII photochemistry; $\Delta\psi$, difference in leaf osmotic potential between salt-treated and control plants;