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Research

Morpho-anatomical, physiological and biochemical adjustments in response to root zone salinity stress and high solar radiation in two Mediterranean evergreen shrubs, *Myrtus communis* and *Pistacia lentiscus*

Massimiliano Tattini[1], Damiano Remorini[2], Patrizia Pinelli[3], Giovanni Agati[4], Erica Saracini[1], Maria Laura Traversi[1] and Rossano Massai[2]

[1]Istituto per la Valorizzazione del Legno e delle Specie Arboree, IVALSA, Consiglio Nazionale delle Ricerche, Via Madonna del Piano, Sesto F.no, I–50019 Firenze, Italy; [2]Dipartimento di Coltivazione e Difesa delle Specie Legnose 'G. Scaramuzzi', Università di Pisa, Via del Borghetto 80, I–56124, Pisa, Italy; [3]Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via Ugo Schiff 6, Sesto F.no, I–50019 Firenze, Italy; [4]Istituto di Fisica Applicata 'Nello Carrara', IFAC, Consiglio Nazionale delle Ricerche, Via Madonna del Piano, Sesto F.no, I–50019 Firenze, Italy

Author for correspondence:
Massimiliano Tattini
Tel: +39 0555225692
Fax: +39 0555225656
Email: tattini@ivalsa.cnr.it

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Summary

• Salt- and light-induced changes in morpho-anatomical, physiological and biochemical traits were analysed in *Myrtus communis* and *Pistacia lentiscus* with a view to explaining their ecological distribution in the Mediterranean basin.

• In plants exposed to 20 or 100% solar radiation and supplied with 0 or 200 mM NaCl, measurements were conducted for ionic and water relations and photosynthetic performance, leaf morpho-anatomical and optical properties and tissue-specific accumulation of tannins and flavonoids.

• Net carbon gain and photosystem II (PSII) efficiency decreased less in *P. lentiscus* than in *M. communis* when exposed to salinity stress, the former having a superior ability to use Na^+ and Cl^- for osmotic adjustment. Morpho-anatomical traits also allowed *P. lentiscus* to protect sensitive targets in the leaf from the combined action of salinity stress and high solar radiation to a greater degree than *M. communis*. Salt and light-induced increases in carbon allocated to polyphenols, particularly to flavonoids, were greater in *M. communis* than in *P. lentiscus*, and appeared to be related to leaf oxidative damage.

• Our data may conclusively explain the negligible distribution of *M. communis* in open Mediterranean areas suffering from salinity stress, and suggest a key antioxidant function of flavonoids in response to different stressful conditions.

Key words: flavonoid glycosides, fluorescence spectroscopy, gas exchange and photosynthetic performance, hydrolysable tannins, ionic and water relations, leaf morpho-anatomy and leaf optics, lipid peroxidation.

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Introduction

Response mechanisms of plants to either excess soil salinity (for recent reviews see Hasegawa *et al.*, 2000; Munns, 2002, 2005) or high solar radiation (particularly UV-radiation, Rozema *et al.*, 1997; Jansen *et al.*, 1998; Caldwell *et al.*, 2003) have been investigated in great details over the past three decades. To date, less is known about how plants perform when exposed to the concomitant action of root-zone salinity stress and high solar irradiance (Lu *et al.*, 2003; Munns & James, 2003) although world's land area afflicted by soil salinity increases because of clearing (Munns, 2005). The

knowledge of response mechanisms of both cultivated and wild species, particularly those distributed in Mediterranean-type ecosystems (Margaris, 1981), to the concerted action of excess soil salinity and high solar radiation is therefore of increasing significance from both a horticultural and an ecological point of view.

Basically, salt-induced decreases in leaf net CO_2 assimilation rate because of stomatal and mesophyll limitation to CO_2 diffusion (Evans & von Caemmerer, 1996), and because of reduced carboxylation efficiency (Allakhverdiev *et al.*, 2000; Tattini *et al.*, 2003), may induce severe conditions of 'excess-light' stress (Demmig-Adams & Adams, 1992) in plants growing at clearing sites, as the leaf's potential to use sunlight in photosynthetic processes is drastically reduced (Lu & Zhang, 2000; Wang *et al.*, 2001). Furthermore, the problem of allocating potentially toxic ions out of highly sensitive cellular compartments (Niu *et al.*, 1995; Hasegawa *et al.*, 2000) may be aggravated in plants – particularly evergreen species (Munns, 2002) – growing at sunny sites, as transpiration and growth rates and, hence, the transport of salt in the shoot may increase because of high solar irradiance (Munns & James, 2003). As a consequence, species exposed to salty and sunny environments have to highly integrate mechanisms primarily intended to control (1) both the penetration and distribution of solar wavelengths in the leaf (Evans, 1999; Tattini *et al.*, 2005) and (2) the allocation of 'toxic ions' at whole-plant and organismal level (Cheeseman, 1988; Colmer *et al.*, 1995). The relative efficiency and the integration (Lambers *et al.*, 1998; Tattini *et al.*, 2005) of these suites of morpho-anatomical and physiological traits will be largely responsible for interspecific and intraspecific 'tolerance' to salinity and excess light stress under field conditions, as it may reduce both the generation of reactive oxygen species (ROS) (Hasegawa *et al.*, 2000; Mittler, 2002) and damage to the photosynthetic apparatus (Allakhverdiev *et al.*, 2000; Lu & Zhang, 2000; Tattini *et al.*, 2005).

The increase in ROS content coupled with changes in net carbon gain because of light- and salt-stress may also strongly affect the biosynthesis of carbon-based secondary compounds, particularly leaf polyphenols (Mackerness *et al.*, 2001; Babu *et al.*, 2003; Tattini *et al.*, 2004), which serve key functions in the 'biochemical' (Semerdjieva *et al.*, 2003) strategies of most species to cope with harsh environments (Aerts, 1995; Tattini *et al.*, 2005). Slow-growing, long-lived evergreen sclerophylls, such as those typically distributed in the Mediterranean basin, make a great investment in leaf phenolics (Margaris, 1981; Romani *et al.*, 2002) to effectively counter damages from both pathogens and predators (Aerts, 1995; Dixon & Paiva, 1995), and at the same time, protect photosynthetic organs from the detrimental effects of highly energetic solar wavelengths (Tattini *et al.*, 2000; Semerdjieva *et al.*, 2003; Tattini *et al.*, 2005).

Here we report data of an experiment conducted on *Myrtus communis* and *Pistacia lentiscus*, two sclerophyll evergreen shrubs (Brosse, 1979; Gucci *et al.*, 1998; Mendes *et al.*, 2001) widely used in herbal medicine in Mediterranean countries, and both recently determined to contain very high amounts of hydrolysable tannins and flavonoid glycosides (Baratto *et al.*, 2003; Romani *et al.*, 2004). Both species largely occur in Mediterranean coastal areas suffering from both drought stress and high solar radiation (Gucci *et al.*, 1998; Mendes *et al.*, 2001), but *M. communis* is sparsely distributed on seashore dunes, which are also frequently exposed to excess soil salinity, over the long summer season. To date, experiments aimed at elucidating response mechanisms of *M. communis* and *P. lentiscus* to either root-zone salinity stress or high solar radiation are surprisingly scarce (Mendes *et al.*, 2001). To draw a comprehensive picture of the integrated mechanisms of response to the combined action of salinity stress and high solar radiation we estimated several traits related to morpho-anatomy, physiology and biochemistry, in plants exposed to 20% (referred as to shade plants throughout the paper) or 100% (sun plants) solar radiation, and supplied with 0 or 200 mM NaCl. In detail, we analysed: (1) leaf water and ionic relations; (2) gas exchange features estimated at both saturating light and on a daily basis *in situ*, and whole-plant growth rate; (3) basic leaf morphology (leaf size, leaf angle and leaf mass per area) and anatomy (whole-leaf and tissue layer thickness) together with leaf optical features; (4) the content and composition of photosynthetic pigments, the photochemical reflectance index (PRI) to estimate the radiation use efficiency, and the maximum potential efficiency of photosystem II (PSII) photochemistry (F_v/F_m); and (5) the leaf lipid peroxidation, the whole-leaf concentration and the leaf CO_2-based accumulation of hydrolysable tannins and flavonoids, together with their tissue-specific distribution.

Materials and Methods

Plant material and growing conditions

One-year-old potted plants of *M. communis* L. and *P. lentiscus* L. grown under glasshouse conditions (30°C/14°C day/night cycle; 480 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR, over 400–700 nm) at midday), were headed back at the beginning of May at Pisa, Italy (43°43′ N, 10°23′ E), and then supplied with one-third strength Hoagland's solution (Hoagland & Arnon, 1950) until complete leaching of the substrate, three times a week, over a 6-wk period. At that time, *M. communis* and *P. lentiscus* had produced nine pairs and four even-pinnate, fully expanded leaves, respectively. Then plants were placed outside under proper polyethylene nets to receive 20% or 100% solar irradiance and supplied, at 2-d intervals, with one-third strength Hoagland's solution with the addition of 0 and 200 mM NaCl. Final light and salinity treatments were reached by the end of a 4-d period, over which daily sunlight irradiance increased by 20% and root zone salinity concentration by 50 mM. At that time, each plant was either

placed into 1 m^3 box (constructed with both roof and walls) to receive 20% or be fully exposed (100%) to sunlight radiation. Plants were exposed to light and salt treatments over an 8-wk experimental period, with their positions changed at 1-wk interval. Sunlight shading was estimated over the 300–1100 nm waveband, using a Li-1800 spectroradiometer (Li-Cor Inc., Lincoln, NE, USA) equipped with a remote cosine receptor. The daily dose of UV-B irradiance was measured by an SUV100 scanning spectroradiometer (Biospherical Instruments, San Diego, CA, USA) on a total of 25 d, both clear and cloudy, over the whole experimental period. Plants at the sun site received a daily dose of 11.7, 0.99 MJ m^{-2} and 19.1 KJ m^{-2} in the PAR, UV-A and UV-B wavebands, respectively. These data were in excellent agreement with daily courses of irradiance obtained from the ELDONET dosimeter located at the Pisa station (Marangoni *et al.*, 2000). Mean daily doses of 2.23, 0.19 MJ m^{-2} and 3.3 KJ m^{-2} in the PAR, UV-A and UV-B wavebands, respectively, were recorded at the shade site.

Water and ionic relations, and osmotic adjustment

Water (ψ_w) and osmotic (ψ_π) potentials were measured at predawn on medial leaves (i.e. located in the medial portion of the newly developing shoot), which were 25- to 35-d-old, depending on treatment, using a standard methodology (Tattini *et al.*, 2003). Since *P. lentiscus* consists of even-pinnate leaves (Brosse, 1979), the medial pinna (referred to as leaf throughout the paper) was sampled for measurements. Leaf osmotic potential (ψ_π) was measured on expressed sap of frozen and thawed leaves using a freezing-point Osmomat 030 osmometer (Gonotec, Berlin, Germany) equipped with a 15-µl measuring cell. Leaf turgor potential (ψ_p) was calculated as the difference between ψ_w and ψ_π.

Leaf osmotic potential at full turgor was then calculated as:

$$\psi_{\pi FT} = \psi_\pi((RWC - AWF)/(100 - AWF)) \quad \text{Eqn 1}$$

(RWC is the relative water content; AWF is the apoplastic water fraction). The RWC was measured as previously reported (Tattini *et al.*, 2003) and AWF was estimated from the analysis of pressure/volume isotherms at 5% and 6% in *M. communis* and *P. lentiscus*, respectively. The contribution of dehydration (D) to osmotic adjustment was calculated as:

$$D = \Delta\psi_\pi - \Delta\psi_{\pi FT} \quad \text{Eqn 2}$$

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

$$\psi_\pi^i = -0.002479(RDW)C \quad \text{Eqn 3}$$

(ψ_π^i indicates the contribution (in MPa) of individual solutes to $\psi_{\pi FT}$; RDW is relative dry weight at saturation (kg m^{-3}); C is the molar concentration of solutes (mol kg^{-1}); 0.002479 m^3 MPa^{-1} mol^{-1} is the RT value at 25°C). Solutes were assumed to have an ideal behaviour (Gucci *et al.*, 1997).

Cation analysis was performed with a Perkin-Elmer 1100 emission-absorption spectrophotometer (Perkin Elmer, 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 & Gucci (1999). Analyses were conducted in leaves sampled for determination of ψ_π, in roots and in both leaf and stem tissues located in different portions of the current-year shoot. The amounts of Na^+ in different plant organs (Na^{organ} throughout the paper) were calculated by multiplying the tissue Na^+ concentration (Na_{organ}) by the dry weight of the relative organ at the end of the experiment. Net Na^+ fluxes (J_{Na}) were then calculated using the following equation (Tattini & Gucci, 1999):

$$J_{Na} = [(Na^+_1 - Na^+_0/t_1 - t_0) \times \ln(WR_1/WR_0)/(WR_1 - WR_0)] \quad \text{Eqn 4}$$

(Na^+ is the sodium content of the whole plant ($J_{Na,plant}$); WR is the root dry weight; $t_1 - t_0$ is the time interval). Rates of Na^+ transported the leaf ($J_{Na,leaf}$) were calculated using the Na^+ content in fully expanded leaves in Eqn 4.

Gas exchange features and relative growth rate

Gas exchange features at saturating light (namely at 600 and > 900 µmol $m^{-2}s^{-1}$ over the PAR waveband for shade and sun leaves, respectively) were measured on medial leaves under laboratory conditions, using a portable Li-Cor 6400 (Li-Cor Inc.) infrared gas analyser operating at 34 ± 0.5 Pa ambient CO_2. Maximum efficiency of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_0)/F_m$, in 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, as reported in Tattini *et al.* (2005). Total assimilated CO_2, over the whole experimental period (CO_{2tot}), was calculated by daily measurements of net assimilation rate of leaves labelled at the beginning of the experiment, the area of which accounted for approx. the 20% of the leaf lamina size at full development. Measurements were conducted *in situ* at 3-h intervals during the day, from 06:00 h to 20:00 h, while night-time respiration rates were determined at midnight and 04:00 h. Net daily carbon gain was then calculated by the integration procedures reported previously by Valladares and Pearcy (1997) and by Tattini *et al.* (2004).

Relative growth rate was calculated by the increase of whole plant dry weight (W), over the $t_1 - t_0$ period, using the equation:

$$RGR = (\ln W_1 - \ln W_0)/t_1 - t_0 \quad \text{Eqn 5}$$

Whole-plant dry weight at t_0 (i.e. W_0) was determined on a total of 12 replicate plants, and averaged 11.7 ± 1.9 or 16.6 ± 2.1 g in *M. communis* and *P. lentiscus*, respectively.

Morpho-anatomy, optical properties and photosynthetic pigments

Leaf size, leaf mass per area (LMA) and leaf angle (with respect to the horizontal plane) were measured as reported previously (Tattini *et al.*, 2000), on leaves exposed to salt and light treatments for 8 wk. Whole-leaf thickness and the thickness of cuticular (average of adaxial and abaxial cuticle thickness), palisade and spongy parenchymal layers were estimated in 1-μm thick transverse sections, fixed and embedded following a standard methodology (Semerdjieva *et al.*, 2003), using a Zeiss AxioPhot microscope (Carl Zeiss, Jena, Germany) equipped with a high-resolution TK 870E JVC video camera (JVC, Yokohama, Japan). Optical features were measured using a Li-1800 spectroradiometer equipped with a Li-1800–125 integrating sphere (Li-Cor Inc.). Absorption efficiency over the 400–700 nm waveband (%A_{Vis} mg^{-1} DW) and scattering efficiency (%$(R/T)_{850}$ mg^{-1} DW; i.e. the ratio of reflectance (R) to transmittance (T) at 850 nm) were calculated as reported in Knapp and Carter (1998). Photochemical reflectance index (PRI) was determined as follows:

$$PRI = (R_{531} - R_{570})/(R_{531} + R_{570}) \quad \text{Eqn 6}$$

where R_{570} is a reference wavelength (nm) and R_{531} depends on the content of xanthophyll cycle pigments (Gamon & Surfus, 1999). The PRI has been previously shown to be closely related with the photosynthetic radiation use efficiency in a number of species (Guo & Trotter, 2004). The contents of Chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b) and carotenoids (Car) were measured as in Lichtenthaler (1987) on leaves previously sampled for optical trait measurement.

Lipid peroxidation, hydrolysable tannins and flavonoid glycosides

Leaf lipid peroxidation was estimated by measuring the amount of malondialdehyde (MDA), using the protocol of Hodges *et al.* (1999), which takes into account the possible influence of interfering compounds in the assay for 2-thiobarbituric acid (TBA)-reactive substances.

Hydrolysable tannins and flavonoids were quantified following the protocols reported in Romani *et al.* (1996) and Romani *et al.* (2002). Briefly, 80–100 mg of freeze-dried leaf tissue were extracted with 3 × 10 ml of 70% ethanol adjusted to pH 3.2 using formic acid. The ethanol solution was then evaporated to dryness under vacuum at room T (Rotavapor 144R, Büchi, Flawil, Switzerland), and dissolved in H_2O (pH 2.5 by formic acid) to a final volume of 10 ml. The aqueous solution was then partitioned with 3 × 7 ml *n*-hexane, evaporated to dryness, and finally redissolved in 1 ml of pH 2.5 water–methanol–acetonitrile (20 : 60 : 20) and used for the separation, identification and quantification of polyphenols, which were performed using an HP 1100 liquid chromatograph equipped with a diode array detector (DAD) (all from Hewlett & Packard, Palo Alto, CA, USA). The column was a 4.6 × 250 mm (5 μm) LiChrosorb RP_{18} (Merck, Darmstad, Germany) kept at 26 ± 1°C, and eluted with H_2O (pH 3.2 by H_3PO_4)–acetonitrile using a four-step linear solvent gradient system at 1.0 ml min^{-1}, over a 106-min run, as suggested by Romani *et al.* (1996). Identification of individual polyphenols was performed by comparison of their retention times and UV-Vis spectra with those of authentic standards (Extrasynthese, Lyon, Nord-Genay, France) or isolated compounds. Hydrolysable tannins, mostly galloyl glucoses and ellagitannins in *M. communis* (Romani *et al.*, 2004), and galloyl quinic derivatives in *P. lentiscus* (Romani *et al.*, 2002), respectively, were quantified at 280 nm, using a calibration curve of gallic acid. In both species, flavonoid glycosides were myricetin 3-*O*-glycosides (myricetin 3-*O*-glucuronide was also detected in *P. lentiscus*, Romani *et al.*, 2002) and quercetin 3-*O*-glycosides (accounting for not more than 5% of the flavonoid pool, Romani *et al.*, 1996, 2002). Quantification of flavonoid glycosides was performed at 360 nm using calibration curves of myricetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside for myricetin and quercetin derivatives, respectively. Since neither change in root zone salinity concentration nor in solar irradiance substantially altered the composition of tannin (Tan) and flavonoid (Flav) pools, the whole classes of polyphenols have been reported thereafter.

Calculation of CO_2-based accumulation of hydrolysable tannins (Tan^{CO_2}) or flavonoids ($Flav^{CO_2}$) was as in Tattini *et al.* (2004):

$$Tan^{CO_2} = (\text{mol Tan m}^{-2})_{t_1-t_0} \times (\text{mol CO}_2\ \text{m}^{-2})^{-1}{}_{t_0-t_1} \quad \text{Eqn 7}$$

$$Flav^{CO_2} = (\text{mol Flav m}^{-2})_{t_1-t_0} \times (\text{mol CO}_2\ \text{m}^{-2})^{-1}{}_{t_0-t_1} \quad \text{Eqn 8}$$

($(\text{mol Tan m}^{-2})_{t_1-t_0}$ and $(\text{mol Flav m}^{-2})_{t_1-t_0}$, are, respectively, the increases in tannin and flavonoid concentrations on a leaf area basis; $(\text{mol CO}_2\ \text{m}^{-2})_{t_0-t_1}$ is the net assimilated CO_2 over the $t_0 - t_1$ period). The polyphenol concentration was measured of very young leaves, the area of which did not exceed 20% of the leaf area size at full development, at t_0. The CO_2-based accumulation of polyphenols, taking into account salt- and light-induced changes in net carbon gain, has been recently proposed, instead of the polyphenol content at whole-leaf level, to estimate newly assimilated carbon actually allocated to secondary metabolites (Tattini *et al.*, 2004; Tattini *et al.*, 2005).

Tissue-specific localization of tannins and flavonoids

The tissue-specific localization of hydrolysable tannins and flavonoids was estimated on transverse sections (50-μm thick) of fresh leaf tissue stained with 0.1% (w : v) 2-aminoethyl diphenyl boric acid (Naturstoff reagent, NR) in phosphate buffer (pH 6.8) as reported in Tattini *et al.* (2004). Multispectral fluorescence microimaging was performed using a standard inverted epifluorescence microscope (Diaphot; Nikon, Tokyo, Japan) coupled with a charge-coupled device (CCD) camera as described in Agati *et al.* (2002). The excitation wavelength was selected using a 10-nm bandwidth interference filter centred at 365 nm, and fluorescence images at 440 nm (F_{440}) and 580 nm (F_{580}) were recorded using 10 nm bandwidth interference filters, 440FS10-25 and 580FS10-25, respectively (Andover Corporation, Salem, NH, USA). The fluorescence intensity was corrected for the CCD camera spectral response. The NR-stained myricetin 3-*O*- and quercetin 3-*O*-derivatives have a maximum fluorescence emission at around 580 nm, and do not emit below 460 nm (Agati *et al.*, 2002; Tattini *et al.*, 2004). Therefore, fluorescence in the blue waveband of NR-stained cross-sections was exclusively caused by soluble tannins, which showed fluorescence maxima at around 440 nm (data not shown), and wall-bound hydroxycinnamates, which have a peak of maximum fluorescence at around 470 nm when stained with NR (Agati *et al.*, 2002). The contribution of wall-bound hydroxycinnamates to F_{440} accounted for approx. 20% that from hydrolysable tannins, irrespective of species and light treatment. As a consequence, the tissue localization of hydrolysable tannins and flavonoids throughout the leaf depth was estimated by analysing profiles of F_{440} and F_{580} from adaxial to abaxial epidermal tissues, respectively, following the protocol reported in Tattini *et al.* (2004).

Experimental design, data analysis and statistics

The experiment was a complete random design, with each pot representing a replicate plant. A minimum of 25 plants per species, for a total of approx. 120 and 60 shoots for *M. communis* and *P. lentiscus*, respectively, were available for sampling in both the salinity and the light treatment. Pre-dawn leaf ψ_w and ψ_π, and RWC were estimated at 2-wk intervals over the whole experimental period on five replicate plants. The concentrations of Na^+, Cl^- and K^+ in the leaf and stem tissues located in the apical, medial and basal portion of the current-year shoot were measured in five replicate plants, both at 2-wk intervals (in medial leaves) and at the end of the experiment. The whole-plant dry weight and the dry weights of different plant organs were determined in 10 replicate plants at the end of the experiment, and elemental analyses in different plant organs were carried out on five replicate tissue samples, constituted by two plants each. Gas exchange features and the F_v/F_m ratio were measured in five replicate plants (on 3- to 5-wk-old leaves), at 2-wk intervals, over the whole experimental period. Daily net carbon gain (CO_{2tot}) and daily transpiration rate (E_{tot}) were estimated in four replicate plants, on leaves labelled at the beginning of the experiment, by measuring diurnal assimilation and night-time respiration rates at 2-wk intervals. Leaf size and leaf angle were measured in 12 replicate plants at the end of the experiment. The LMA was measured in both 1 wk and 8 wk-old leaves taken from 12 replicate plants, at the beginning and at the end of the experiment, respectively. The thickness of cuticular and of both palisade and spongy parenchymal layers were determined of six cross-sections taken from three 8-wk-old leaves (one per plant), at the end of the experiment. Leaf optical features and photosynthetic pigment content were measured in four replicate plants, after 4 wk and 8 wk of treatment. The concentration of hydrolysable tannins and flavonoids was estimated in both 7-d-old (four replicate samples each constituted by four to five leaves per plant) and 8-wk-old leaves (four replicate samples each comprising two leaves per plant, namely those used for diurnal measurements of CO_2 assimilation plus the opposite leaf). Fluorescence images were recorded of leaves from four replicate 'control' plants growing at 100% solar irradiance. Data were subjected, when appropriate, to a three-way ANOVA, with species, light and salinity as fixed factors, with their interaction factors. Data resulting from time-course experiments were pooled together before statistical analysis.

The extent to which morpho-anatomical, physiological and biochemical traits varied in response to changes in root zone salinity and sunlight irradiance was estimated by the 'normalized index of variation' (NIV) using the following equations:

$$NIV^{salinity} = (X_{200mM} - X_{control})/(X_{200mM} + X_{control}) \quad \text{Eqn 9}$$

$$NIV^{sunlight} = (X_{100\%} - X_{20\%})/(X_{100\%} + X_{20\%}) \quad \text{Eqn 10}$$

(X is the parameter to be examined). The normalized index of variation does not markedly differ from the well-known plasticity index (PI = (max value − min value)/maximum value; Valladares *et al.*, 2000), but NIV allows one to visualize the actual treatment effect on the examined trait, because it may assume both positive and negative values. Furthermore, the 'normalization factor' includes traits of both 'treated' and 'control' plants (i.e. shade leaves or plants supplied with good quality water in the present experiment), in a way that a greater 'weight' is also given to species-specific 'constitutive' features.

Results

Myrtus communis and *P. lentiscus* significantly differed in 84% of traits examined (see the Appendix, Table A1), except for

root Na^+ concentration (Na_{root}, on a tissue water molar basis), RWC, and both P_n and total chlorophyll on a leaf area basis (Chl_{tot} cm^{-2}). We note, however, that both P_n and Chl_{tot} cm^{-2} underwent significant species–salt (as also observed for RWC) and species–light interactions. Changes in root zone salinity concentration significantly affected 69% of the examined traits, mostly ionic and water relations, and, consistently, gas exchange features. Salinity did not significantly alter morpho-anatomical and optical features of leaves, except for PRI. Finally, the total chlorophyll content (Chl_{tot}), both the lipid peroxidation (estimated on the basis of leaf MDA content) and the content of hydrolysable tannins varied significantly because of salinity stress (see the Appendix, Table A1). Overall, a highly significant effect of changes in solar irradiance was seen in 75% of the traits examined, particularly leaf morpho-anatomy and leaf optics, and both the content and composition of photosynthetic pigments (see the Appendix, Table A1). Noticeably, light treatment also dramatically affected ionic relations: both the Na^+ concentration (on a tissue water molar basis) in different plant organs and the leaf K^+ : Na^+ ratio (K/Na_{leaf}). Overall, changes in solar irradiance did not significantly affected gas exchange performances estimated at saturating light, as a result of significant salt–light and species–light interactions on most examined features. The light treatment affected the concentration of leaf flavonoids to a significantly greater degree than that observed for hydrolysable tannins (see the Appendix, Table A1). Finally, significant species–salt–light interactive effects were seen in: (1) both the leaf Na^+ concentration (Na_{leaf}, $F = 225.1$, df = 39) and the amount of Na^+ allocated to the leaf tissue (Na^{leaf}, $F = 165.7$, df = 39), which varied at a substantially greater degree in *P. lentiscus* than in *M. communis* (Figs 1–3); (2) gas exchange features ($F = 10.4$, 27.6 and 8.4 for P_n, g_s and E, respectively, df = 159), PRI ($F = 454.2$, df = 63) and both the content (Chl_{tot} g^{-1} DW, $F = 16.8$ for df = 63) and the composition of photosynthetic pigments (Car : Chl, $F = 37.5$ for df = 63), which, in contrast, underwent larger changes in *M. communis* than in *P. lentiscus* (Fig. 1; see Table 2).

Species-specific responses to changes in root zone salinity concentration

The species-specific response to changes in root zone salinity stress has been reported on the basis of the normalized index of variation ($NIV^{salinity}$) for a total of 26 parameters (Fig. 1a). The amount of Na^+ allocated to the leaf (moles per organ, Na^{leaf}) in salt-treated *P. lentiscus* (NIV = 0.69) largely exceeded that in salt-treated *M. communis* (NIV = 0.35), whereas the amounts of Na^+ in stem (Na^{stem}) and roots (Na^{root}) varied similarly in both species due to changes in root zone salinity. In agreement with this, we observed that $J_{Na,plant}$ in salt-treated *P. lentiscus* exceeded by only 30% that in salt-treated *M. communis* (particularly in plants at the sun site, Fig. 2a), whereas the rate of Na^+ transport to the leaf (i.e. $J_{Na,leaf}$) was 3.5 times greater in the former than in the latter (Fig. 2b). These results were mostly due to markedly different leaf Na^+ concentration (Na_{leaf}, on a tissue water molar basis) in the species examined (Fig. 3), as the root to shoot ratio varied from 0.32 to 0.44 and from 0.37 to 0.49 in *M. communis* and *P. lentiscus*, respectively, because of salinity stress (data not shown). Indeed, Na_{leaf} in salt-treated *M. communis* (Fig. 3a) accounted for just 42% Na_{leaf} in salt-treated *P. lentiscus*, irrespective of solar irradiance (Fig. 3b). Furthermore, in salt-treated *M. communis* Na^+ stem concentration (Na_{stem}) was greater (+28%) than Na_{leaf}, and the latter slightly superior (+22%) in basal than in apical leaves. By contrast, Na_{leaf} did not vary because of leaf age in salt-treated *P. lentiscus*, and dramatically exceeded Na_{stem} (Fig. 3). No species-specific response to salinity stress was detected for leaf the K^+ : Na^+ ratio, which decreased sharply (NIV = −0.69 for both species) from controls to salt-treated plants (Fig. 1a).

Myrtus communis and *P. lentiscus* also largely differed in their ability to counter salt-induced osmotic unbalance, as the salt-induced decrease in leaf ψ_w was substantially greater in *M. communis* (NIV = 0.50) than in *P. lentiscus* (NIV = 0.35), but changes in ψ_π caused by salinity stress were similar in both species over the whole experimental period (Fig. 1a). Consequently, the loss of leaf turgor (ψ_p) was severe passing from controls (ψ_p = 1.15 MPa) to salt-treated *M. communis* (ψ_p = 0.61 MPa), whereas leaf ψ_p varied to a much lesser degree in *P. lentiscus*, from 1.23 MPa in controls to 1.02 MPa in salt-treated plants (Fig. 1a). Furthermore, salt-induced decreases in ψ_π (Table 1) were mostly due to dehydration in *M. communis* (D accounted for 57% to salt-induced changes in ψ_π) or net solute accumulation in *P. lentiscus* (D = 38%), since nonosmotic volume change (Gucci *et al.*, 1997) contributed little (less than 5% in both species) to salt-induced decreases in ψ_π. Moreover, the per cent contributions of Na^+ ($\psi_{\pi FT}^{Na^+}$) and Cl^- ($\psi_{\pi FT}^{Cl^-}$) to $\psi_{\pi FT}$ in salt-treated P. lentiscus (43%) largely exceeded those in salt-treated M. communis (27%), whereas the contribution of K^+ to $\psi_{\pi FT}$ of salt-treated leaves was similar in both species (Table 1).

The effect of salinity stress on gas exchange features measured at saturating light (i.e. at 600 and > 900 μmol $m^{-2}s^{-1}$ PAR for shade and sun leaves, respectively) was clearly species-specific (see the Appendix, Table A1). In detail, net assimilation rate (P_n), and both stomatal conductance (g_s) and transpiration rate (E) decreased to a much greater extent in *M. communis* (on average NIV = –0.52) than in *P. lentiscus* (on average NIV = –0.28) in response to salinity stress (Fig. 1a, Table 2). Similarly, both net carbon gain (CO_{2tot}) and transpiration rates (E_{tot}), estimated on a daily basis, were also more severely depressed in *M. communis* ($NVI_{CO_{2tot}} = -0.51$, $NVI_{E_{tot}} = -0.54$) than in *P. lentiscus* ($NVI_{CO_{2tot}} = -0.31$ and $NIV_{E_{tot}} = -0.24$) by salinity stress (Fig. 1a, Table 2). Salt-induced inhibition in the efficiency of PSII photochemistry (here estimated on the basis of the maximum

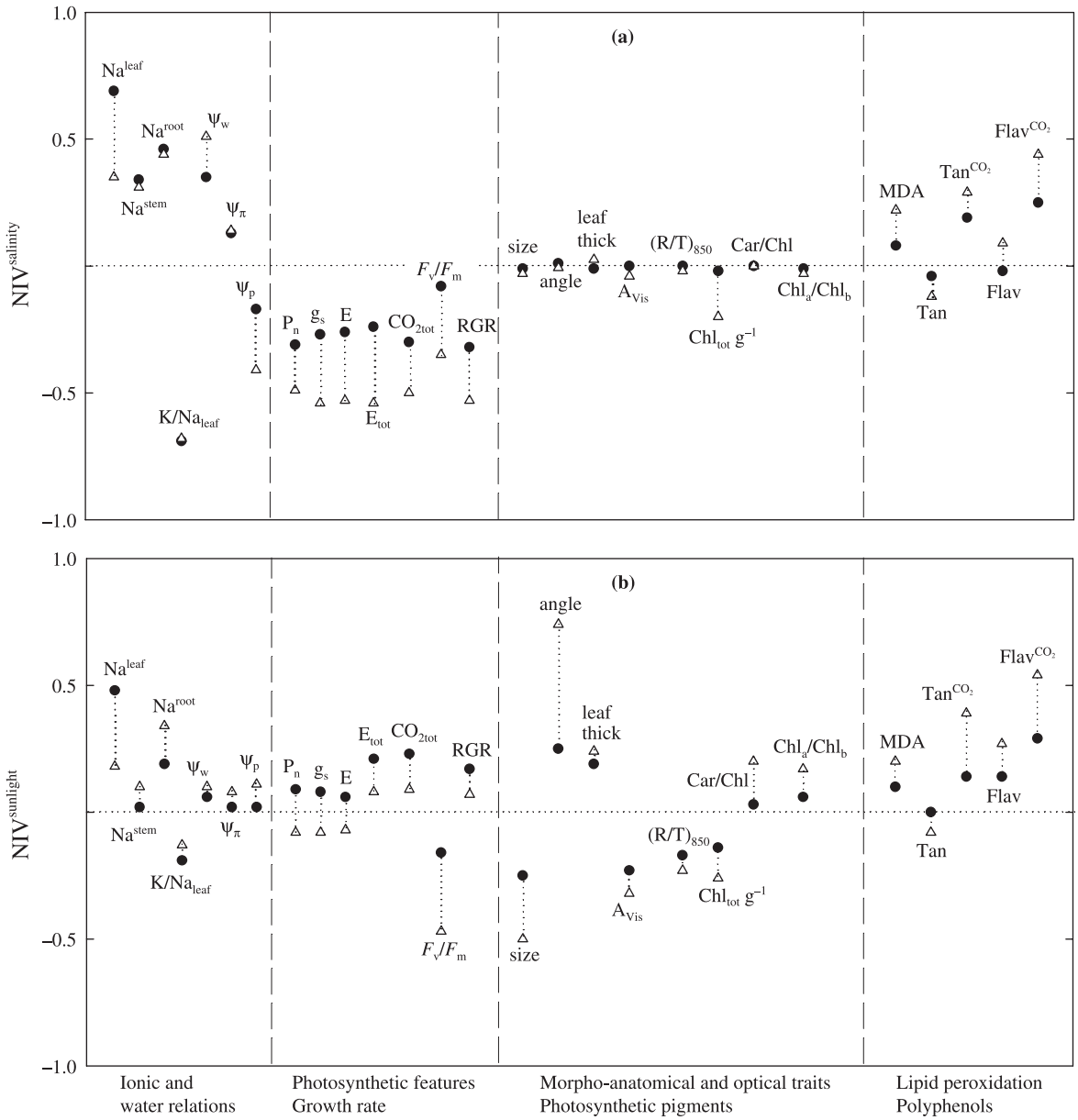

Fig. 1 Normalized index of variation to changes in root zone salinity concentration (a, $NIV^{salinity}$) or solar irradiance (b, $NIV^{sunlight}$) for 26 parameters (see Table A1 and text) related to (1) ionic and water relations; (2) photosynthetic features and growth rate; (3) both morpho-anatomical and optical traits, and photosynthetic pigments; (4) lipid peroxidation and polyphenol metabolism in *Myrtus communis* (open triangles) and *Pistacia lentiscus* (closed circles). Na^{organ} represents the amounts of Na^+ in different plant organs (i.e. moles per organ), whereas K/Na_{leaf} has been calculated on a tissue water cation molar basis. NIVs × 10 have been reported for F_v/F_m for illustrative purposes only.

potential efficiency of PSII in dark-adapted leaves, F_v/F_m) was appreciable only in *M. communis*, and F_v/F_m of salt-treated *M. communis* was 88% of that of *P. lentiscus* at the sunny site (Table 2). Finally, relative growth rate decreased more in *M. communis* (NVI_{RGR} = –0.53) than in *P. lentiscus* (NVI_{RGR} = –0.32) because of salinity stress, irrespective of solar irradiance (Fig. 1a, Table 2).

Salinity stress poorly affected morpho-anatomical traits (see the Appendix, Table A1), as well as the absorption efficiency over the visible waveband (A_{Vis}) and the efficiency ($(R/T)_{850}$) to scatter light (Fig. 1a; Table 3). Conversely, the photochemical reflectance index (which is negatively related to the content of xanthophyll cycle pigments, and positively related to the photosynthetic radiation use efficiency, Guo & Trotter, 2004) decreased to a much greater extent in *M. communis* than in *P. lentiscus* due to salinity stress, as also occurred for the net carbon gain (CO_{2tot}, Tables 2 and 3). Moreover, both species had similar PRI when supplied with good-quality water at the shade site, but PRI was dramatically smaller in *M. communis* (−0.079) than in *P. lentiscus* (−0.016),

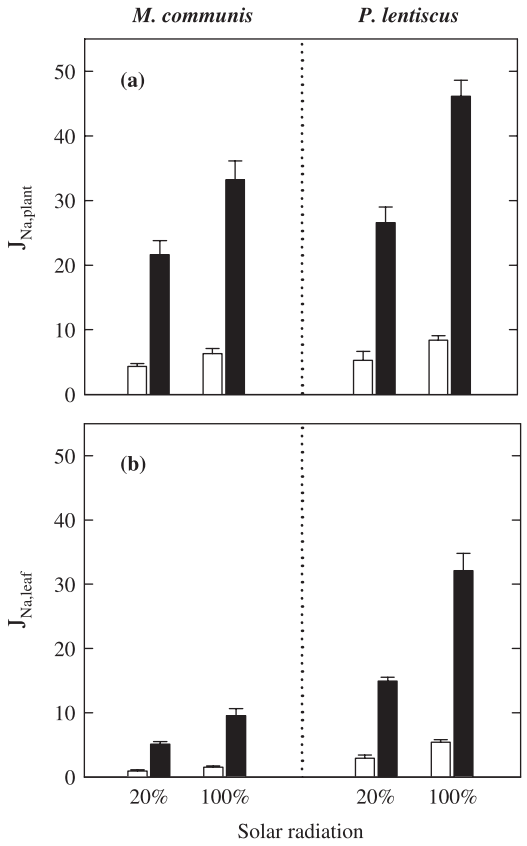

Fig. 2 Na^+ fluxes (J_{Na}, μmol g^{-1} root DW d^{-1}) at the level of whole-plant (a) or leaf tissue (b) in *Myrtus communis* and *Pistacia lentiscus* exposed to 20% or 100% solar irradiance and supplied with 0 (open bars) or 200 mM NaCl (closed bars), over an 8-wk experimental period. Data are means ± SD, $n = 5$.

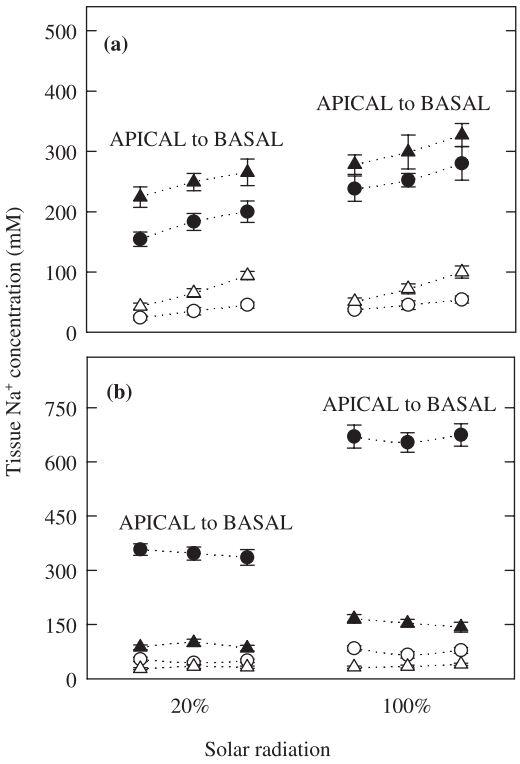

Fig. 3 The Na^+ concentration (on a tissue water molar basis) in leaf (circle) and stem (triangle) tissues of different age (apical to basal tissues) in *Myrtus communis* (a) and *Pistacia lentiscus* (b) exposed to 20% or 100% solar irradiance and supplied with 0 (open symbols) or 200 mM NaCl (closed symbols), over an 8-wk experimental period. Data are means ± SD, $n = 5$.

when plants were concomitantly exposed to salinity stress and high solar radiation. Finally, the total chlorophyll content (on a tissue dry weight basis, Chl_{tot} g^{-1}) did not appreciably vary in *P. lentiscus*, whereas substantially decreased in *M. communis* because of salinity stress (Fig. 1a).

Peroxidation of leaf membrane lipids because of salinity stress (Fig. 1a) was substantially greater in *M. communis* ($NVI_{MDA} = 0.22$) than in *P. lentiscus* ($NVI_{MDA} = 0.08$). The release of MDA because of salinity stress was also superior at the sun (+66%) than at the shade (+36%) in *M. communis*, whereas salt-induced lipid peroxidation did not vary in *P. lentiscus* because of light treatment (Fig. 4a). A relation emerged between the extent of leaf oxidative damage and the share of newly assimilated carbon allocated to tannins (Tan^{CO_2} and particularly to flavonoids ($Flav^{CO_2}$) as a result of salinity stress ($NIV_{Flav}^{CO_2} = 0.44$ and 0.24 in *M. communis* and *P. lentiscus*, respectively, Figs 1a and 4b,c). However, salinity stress had a negligible effect on the polyphenol content at the level of whole-leaf (NIV = –0.05, see also the Appendix, Table A1), mostly due to salt-induced reductions in the carbon actually available (CO_{2tot}) for their synthesis (Table 2).

Species-specific responses to changes in solar radiation

Overall, changes in solar irradiance affected the whole set of examined parameters to a much greater extent in *M. communis* (mean $NIV_{1,26} = 0.24$ using absolute NIVs) than in *P. lentiscus* (mean $NIV_{1,26} = 0.14$), with the remarkable exceptions of Na_{leaf} (mM), RGR, and both CO_{2tot} and E_{tot} (Fig. 1b). The light-induced increases in Na_{leaf} were closely related with $J_{Na,leaf}$, which varied from 10.4 to 20.1 μmol g^{-1} root DW d^{-1} in plants growing at the shade or sun site, respectively (Fig. 2a). As a consequence, the contribution of Na^+ and Cl^- to $\psi_{\pi FT}$ was greater in sun (43%) than in shade (34%) salt-treated plants (Table 3), particularly in *P. lentiscus* ('toxic ions' accounted for 50% and 38% of $\psi_{\pi FT}$ in sun and shaded *P. lentiscus* leaves, respectively, Table 1). Overall, water relations and gas exchange features at saturating light, underwent minor variations in both species because of high solar irradiance, as P_n, g_s and E were more severely depressed by salinity stress at the sun than at the shade site (see the Appendix, Table A1; Fig. 1b), particularly in *M. communis* (Table 2, and data not shown). Similarly, light-induced reductions in both F_v/F_m and photosynthetic radiation use efficiency (i.e. PRI) were greater in *M. communis* (NIV_{F_V/F_m}

Table 1 Water relations and osmotic adjustment in *Myrtus communis* and *Pistacia lentiscus* leaves in response to changes in root zone salinity stress and solar radiation

Species	NaCl (mM)	Sunlight (%)	RWC (%)	ψ_w (–MPa)	ψ_π (–MPa)	$\psi^{Na^+}_{\pi FT}$ (–MPa)	$\psi^{Cl^-}_{\pi FT}$ (–MPa)	$\psi^{k^+}_{\pi FT}$ (–MPa)
M. communis	0	20	97.8 ± 0.8	0.41 ± 0.04	1.51 ± 0.07	0.04 ± 0.01	0.04 ± 0.01	0.38 ± 0.03
	200		86.1 ± 2.6	1.39 ± 0.41	1.95 ± 0.31	0.21 ± 0.03	0.18 ± 0.03	0.43 ± 0.02
	0	100	96.5 ± 0.9	0.56 ± 0.08	1.75 ± 0.08	0.06 ± 0.01	0.06 ± 0.02	0.44 ± 0.02
	200		83.7 ± 2.4	1.64 ± 0.42	2.29 ± 0.33	0.30 ± 0.03	0.28 ± 0.02	0.36 ± 0.02
P. lentiscus	0	20	95.6 ± 0.4	0.76 ± 0.05	2.01 ± 0.07	0.07 ± 0.01	0.08 ± 0.01	0.59 ± 0.03
	200		88.9 ± 1.8	1.51 ± 0.39	2.56 ± 0.31	0.41 ± 0.03	0.40 ± 0.03	0.73 ± 0.04
	0	100	94.7 ± 0.6	0.84 ± 0.05	2.05 ± 0.06	0.11 ± 0.01	0.10 ± 0.02	0.66 ± 0.04
	200		86.7 ± 1.5	1.71 ± 0.39	2.71 ± 0.29	0.56 ± 0.04	0.59 ± 0.05	0.60 ± 0.02

RWC, relative water content; ψ_w, water potential; ψ_π, osmotic potential; $\psi_{\pi FT}$, leaf osmotic potential at full turgor.

Measurements were conducted on medial leaves (25- to 35-d-old) at predawn, at 2-wk intervals over an 8-wk experimental period. Data have been pooled together before statistical analysis and are means ± SD; $n = 20$. The contributions of cations ($\psi^{Na^+}_{\pi FT}$ and $\psi^{k^+}_{\pi FT}$) and chloride ($\psi^{Cl^-}_{\pi FT}$) to osmotic potential at full turgor were calculated using the Van't Hoff equation, assuming an ideal behaviour of the osmolites.

Table 2 Net CO_2 assimilation rate at saturating light (P_n), net carbon gain over the experimental period (CO_{2tot}), maximum efficiency of photosystem II (PSII) photochemistry (F_v/F_m), photochemical reflectance index (PRI) and relative growth rate (RGR) in *Myrtus communis* and *Pistacia lentiscus* in response to changes in root zone salinity concentration and solar radiation

Species	NaCl (mM)	Sunlight (%)	P_n (μmol m^{-2} s^{-1})	CO_{2tot} (mol m^{-2})	F_v/F_m	PRI	RGR ($d^{-1} \times 10^3$)
M. communis	0	20	15.8 ± 1.0	16.5 ± 1.3	0.818 ± 0.008	0.015 ± 0.003	23.6 ± 3.1
	200		6.1 ± 3.6	5.6 ± 1.1	0.783 ± 0.019	0.006 ± 0.002	8.5 ± 1.8
	0	100	14.6 ± 0.9	20.8 ± 1.6	0.764 ± 0.017	–0.041 ± 0.006	29.4 ± 1.9
	200		4.2 ± 1.8	6.3 ± 0.9	0.693 ± 0.048	–0.079 ± 0.010	7.8 ± 2.4
P. lentiscus	0	20	11.6 ± 0.9	14.3 ± 0.8	0.824 ± 0.011	0.014 ± 0.002	17.6 ± 2.4
	200		6.9 ± 1.2	8.1 ± 1.5	0.821 ± 0.009	0.010 ± 0.002	11.5 ± 2.6
	0	100	14.9 ± 1.1	25.0 ± 1.8	0.808 ± 0.015	–0.009 ± 0.002	28.5 ± 2.2
	200		7.0 ± 2.4	12.6 ± 2.0	0.785 ± 0.027	–0.016 ± 0.004	12.3 ± 1.5

P_n and F_v/F_m were measured at 2-wk intervals over an 8-wk experimental period, and data are means ± SD; $n = 20$. Net carbon gain was calculated by integrating curves of daily net CO_2 assimilation rates (conducted at 2-wk intervals over the experimental period) vs time and data are means ± SD; $n = 4$. PRI was estimated after 4 and 8 wk of treatment and data area means ± SD; $n = 8$. RGR was calculated by the increase in whole-plant dry weight over the whole experimental period and data are means ± SD; $n = 10$.

$= -0.047$) than in *P. lentiscus* $NIV_{F_v/F_m} = -0.016$, particularly in plants concomitantly exposed to root zone salinity stress (Fig. 1b, Table 2). Both CO_{2tot} and RGR (Fig. 1b; Table 2) consistently increased less in M communis ($NIV_{CO_2} = 0.09$, $NIV_{RGR} = 0.07$) than in P. lentiscus ($NIV_{CO_2} = 0.23$, $NIV_{RGR} = 0.17$), because of high solar radiation.

Pistacia lentiscus and *M. communis* also strikingly differed in their 'constitutive' (i.e. in leaves at the shade site) morpho-anatomical traits (see the Appendix, Table A1; Table 3). The LMA, the whole-leaf thickness and the thickness of the cuticular layer (data reported in Table 3 are means of adaxial and abaxial cuticle thickness) in *M. communis* accounted for 53, 71 and 54%, respectively, of corresponding features in *P. lentiscus*. Generally, leaf morpho-anatomical features mostly varied in response to changes in solar irradiance (see the Appendix, Table A1; Fig. 1b). Leaf size decreased and leaf angle increased to a much greater extent in *M. communis* than in *P. lentiscus*, as also occurred for the increases in both whole-leaf and cuticular thickness (and in the ratio of palisade to spongy parenchyma thickness), in response to high sunlight (Fig. 1b, Table 3). The efficiency to both absorb visible wavelengths (A_{Vis}) and to scatter light [$(R/T)_{850}$] consistently decreased more in *M. communis* (on average NIV = –0.28) than in *P. lentiscus* (NIV = –0.20) because of high solar radiation, but still remained significantly higher in the former than in the latter (Fig. 1b, Table 3). Finally, both the content (Chl_{tot} g^{-1}) and the composition of photosynthetic pigments (i.e. Chl_a : Chl_b and Car : Chl_{tot} ratios) (1) were larger in *M. communis* than in *P. lentiscus* (Fig. 1b) and (2) underwent significant species–salt–light interactive effects. We found that Chl_{tot} g^{-1} ranged from 6.1 to 2.9 (Car : Chl_{tot} from 0.20 to 0.30) or from 3.1 to 2.2 mg g^{-1} DW (Car : Chl_{tot} from 0.23 to 0.24) from shade 'control' to sun 'salt-treated' plants of *M. communis* or *P. lentiscus*, respectively.

The increase in leaf lipid peroxidation – which was markedly greater in *M. communis* (+56%) than in *P. lentiscus* (+ 21%) – was accompanied by a parallel increase in the share of newly assimilated carbon allocated to secondary metabolites

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Table 3 Morpho-anatomical and optical features in *Myrtus communis* and *Pistacia lentiscus* leaves in response to changes in root zone salinity concentration and solar radiation

Species	NaCl (mM)	Sunlight (%)	LMA (g DW m^{-2})	Whole-leaf thickness (μm)	Cuticle thickness (μm)	Palisade to spongy ratio	A_{Vis} (% mg^{-1} DW)	$(R/T)_{850}$ (% mg^{-1} DW)
M. communis	0	20	58.3 ± 3.4	184.8 ± 8.2	4.1 ± 0.4	0.41 ± 0.03	9.98 ± 0.15	0.14 ± 0.02
	200		64.1 ± 2.6	200.6 ± 12.9	4.1 ± 0.3	0.44 ± 0.03	9.01 ± 0.25	0.13 ± 0.02
	0	100	112.3 ± 6.7	309.8 ± 10.4	5.2 ± 0.2	0.54 ± 0.05	4.92 ± 0.32	0.09 ± 0.02
	200		113.5 ± 4.3	314.5 ± 25.2	5.3 ± 0.5	0.56 ± 0.02	4.75 ± 0.27	0.08 ± 0.01
P. lentiscus	0	20	110.5 ± 2.9	258.2 ± 11.6	7.6 ± 0.6	0.56 ± 0.04	4.92 ± 0.32	0.07 ± 0.01
	200		111.2 ± 2.4	251.1 ± 10.7	7.4 ± 0.3	0.54 ± 0.02	4.85 ± 0.14	0.07 ± 0.01
	0	100	183.5 ± 8.6	380.4 ± 23.6	8.1 ± 0.4	0.67 ± 0.03	2.82 ± 0.28	0.05 ± 0.01
	200		182.2 ± 9.8	372.0 ± 27.1	8.3 ± 0.4	0.64 ± 0.03	2.88 ± 0.24	0.05 ± 0.01

LMA, leaf mass per area; A_{Vis}, absorptance efficiency over the visible waveband; R/T_{850}, scattering efficiency (i.e. the ratio of reflectance (R) to transmittance (T) at 850 nm)).
Measurements of LMA and tissue thickness were conducted on 8-wk-old leaves, whereas optical features were estimated in both 4- and 8-wk-old leaves (data have been pooled together before statistical treatment). The cuticle thickness has been reported by averaging the thickness of adaxial and abaxial cuticular layers. Data are means ± SD; n = 12 for LMA, n = 6 for anatomical features and n = 8 for optical traits.

in response to high solar radiation (Figs 1b and 4a,b), although the polyphenol content did not appreciably vary between sun and shade leaves (NIV = 0.02, Fig. 1b; see the Appendix, Table A1). Light-induced accumulation (on the basis of assimilated CO_2) was greater for flavonoids than for hydrolysable tannins (the $Flav^{CO_2}$: Tan^{CO_2} ratio varied from 0.05 in the shade to 0.09 in full-sun leaves, respectively), particularly in leaves of *M. communis* (Figs 1b and 4c). There was a close relation between the fluorescence profiles in both the blue (F_{440}) and yellow-red waveband (F_{580}), and light-induced increases of hydrolysable tannins and flavonoid glycosides (Fig. 4a,b). Indeed, the contribution of epidermal flavonoids to F_{580} was slightly greater in *P. lentiscus* than in *M. communis*, but in the latter, mesophyll flavonoids accumulated to a dramatically higher degree than in the former (Figs 5a,b). Profiles of F_{440} also showed that hydrolysable tannins accumulated to a greater extent throughout the whole depth in *P. lentiscus* (798 μmol g^{-1} DW as measured by high-pressure liquid chromatography (HPLC-DAD) than in M. *communis* leaves (572 μmol g^{-1} DW; see also the Appendix, Table A1, Fig. 4). However, we note that our fluorescence spectroscopy technique did not allow us compare the actual amounts of hydrolysable tannins and flavonoid glycosides, as the contribution of NR-stained hydrolysable tannins and NR-stained flavonoids at F_{440} and F_{580}, respectively, may have differed sharply owing to markedly different absorption coefficients at the excitation wavelength (λ_{exc} = 365 nm) as well as to large variations in fluorescence quantum yields of individual polyphenol classes.

Discussion

The data of our experiment may offer new insights in the mechanisms of response, which highly integrate morpho-anatomical, physiological and biochemical adjustments (Tattini *et al.*, 2005) of woody species to excess soil salinity and high solar irradiance, and may help to explain the ecological distribution of *M. communis* and *P. lentiscus* in Mediterranean-type ecosystems.

First, we highlight the strikingly different strategies adopted by the species examined to cope with excess root zone salinity concentration, which mostly concern the allocation of salt at organismal level (see the Appendix, Table A1; Fig. 1a) and strongly affect leaf water relations (Gucci *et al.*, 1997) and carbon acquisition (Cheeseman, 1988; Tattini *et al.*, 2003). *Pistacia lentiscus* and *M. communis* may be roughly classified (Munns, 2002) as salt-including or a salt-excluding species, respectively, based on the relative contributions of $J_{Na,leaf}$ (68% and 28% in *P. lentiscus* and *M. communis*, respectively) to $J_{Na,plant}$ (Fig. 2). We note, however, that (1) the actual Na^+ concentrations in the xylem sap (which may conclusively estimate the actual degree of 'Na^+ exclusion'; e.g. Munns, 2002) were not measured in the present experiment; and (2) the Na^+ flux at whole-plant level differed no more than 30% in the species examined (Fig. 2a). Nevertheless, a massive allocation of Na^+ to the leaf tissue taken together with (1) the lack of a gradient in Na^+ concentration between leaves of different age (Fig. 3b) and (2) a leaf Na^+ concentration (650 mM in apical leaves at the full-sun site) dramatically greater than that in both the stem tissue (on average 150 mM at the full-sun site; Fig. 3b) and in soil solution, led us to hypothesize that a very active secretion of Na^+ in the leaf vacuole effectively operated in *P. lentiscus* (Niu *et al.*, 1995; Hasegawa *et al.*, 2000). Indeed, neither *P. lentiscus* nor *M. communis* have specialized organs to excrete toxic ions out of the leaf (Brosse, 1979). Conversely, the efficiency of mechanisms aimed to sequester potentially toxic ions in 'less sensitive' organs, such as the stem and the old leaves in *M. communis*

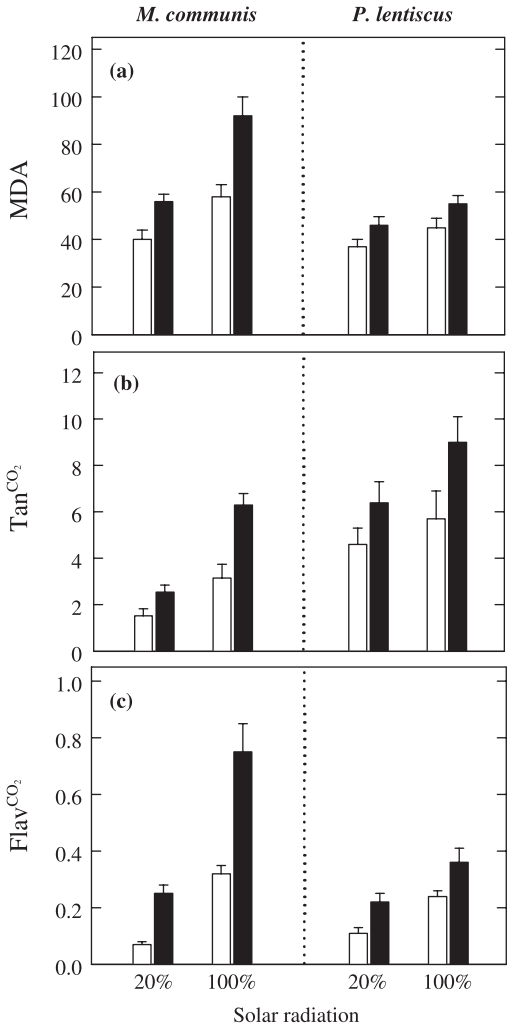

Fig. 4 The content of malondialdehyde (MDA, nmol g^{-1} DW) and the CO_2-based accumulation of hydrolysable tannins (Tan^{CO_2}, mmol mol^{-1} CO_2) and flavonoids ($Flav^{CO_2}$, mmol mol^{-1} CO_2) in leaves of *Myrtus communis* and *Pistacia lentiscus* supplied with 0 (open bars) or 200 mM NaCl (closed bars) and exposed to 20% or 100% solar irradiance, over an 8-wk experimental period. Data are means ± SD, $n = 4$.

(which largely contribute to confer 'salt-tolerance' in 'salt-excluding' species; see Colmer *et al.*, 1995; Tattini & Gucci, 1999; Tattini *et al.*, 2003; Munns, 2005), was markedly smaller (particularly at the full-sun site, Fig. 3a) than that previously reported for 'salt-tolerant', salt-excluding, Mediterranean evergreens, such as *Olea europaea* and *Phillyrea latifolia* (Gucci & Tattini, 1997; Tattini *et al.*, 2003). Furthermore, nonstructural carbohydrates (mostly sucrose, glucose and fructose) (Lucchesini *et al.*, 2001) in *M. communis* contributed little to salt-induced decreases in $\psi_{\pi FT}$ (data not shown), in contrast with findings previously reported for salt-tolerant, mannitol-synthesizing Oleaceae (Tattini *et al.*, 1996; Tattini *et al.*, 2003).

Strikingly different abilities in using Na^+ and Cl^- to counter salt-induced osmotic unbalance (which, in turn, likely depended upon the relative abilities to sequester potentially toxic ions out of highly sensitive cellular compartments) (Niu *et al.*, 1995; Hasegawa *et al.*, 2000) were likely responsible for the markedly greater reductions in both photosynthetic performance and relative growth rates detected in *M. communis* than in *P. lentiscus* (Tattini *et al.*, 1997, 2003; James *et al.*, 2002). Indeed, although Na_{leaf} increased to a dramatically greater degree in *P. lentiscus* than in *M. communis* (Figs 1b and 3), both the efficiency of PSII photochemistry (Lu *et al.*, 2002; Lu *et al.*, 2003) and the photosynthetic radiation use efficiency (Wang *et al.*, 2001) were more severely depressed in the latter when both species suffered from concomitant conditions of salinity stress and high solar radiation (Table 2). We note that an increase in the mesophyll limitation to CO_2 diffusion (Evans & von Caemmerer, 1996) may have been also responsible for significant salt–light interactive effects on photosynthetic performance in both species (Table 2; see the Appendix, Table A1), as sun leaves were thicker, had a major proportion of palisade to spongy tissue (Table 3), and reduced intercellular spaces (data not shown) than the shade counterparts (Miyazawa & Terashima, 2001; Niinemets *et al.*, 2005).

Overall, reductions in net carbon gain and RGR taken together with (1) decreases in both the chlorophyll content (Greenberg *et al.*, 1997) and the PSII efficiency (Lu & Zhang, 2000; Lu *et al.*, 2002) and (2) increases in leaf oxidative damage (Shalata & Neumann, 2001) because of root zone salinity, allow us conclude that *M. communis* was actually 'less tolerant' than *P. lentiscus* to excess soil salinity (we note that individual parameters may fail in estimating 'salt-tolerance', which is both a multicomponent and a qualitative descriptor; see Cheeseman, 1988; Munns, 2002; Munns & James, 2003; Tattini *et al.*, 2003).

Second, data presented here may allow drawing a comprehensive picture of how the suite of 'constitutive' (i.e. in leaves at the shade site) leaf morpho-anatomical traits (see the Appendix, Table A1; Table 3) in the examined species (which probably reflects the evolutionary adaptations of *P. lentiscus* and *M. communis* in areas of contrasting light and water availability, Lambers *et al.*, 1998) may have actually determined the extent to which physiological and biochemical traits underwent adjustments in response to high solar irradiance (Gutschick, 1999; Semerdjieva *et al.*, 2003; Tattini *et al.*, 2005). In detail, the greater cuticle thickness, coupled with both a greater thickness and compactness (data not shown) of the palisade parenchyma tissue (Table 3), probably gave *P. lentiscus* a greater efficiency in both limiting the absorption of visible light – absorption over the visible waveband on a leaf area basis, i.e. A_{Vis} cm^{-2}, was also smaller in *P. lentiscus* (85.1%) than in *M. communis* (90.7%) – and decreasing the pathlength of photons within the leaf (Table 2, Knapp *et al.*, 1988). At the same time, mesophyll anatomy in *P. lentiscus* should have allowed a more equal distribution of highly collimated solar radiation in deeper tissues compared with

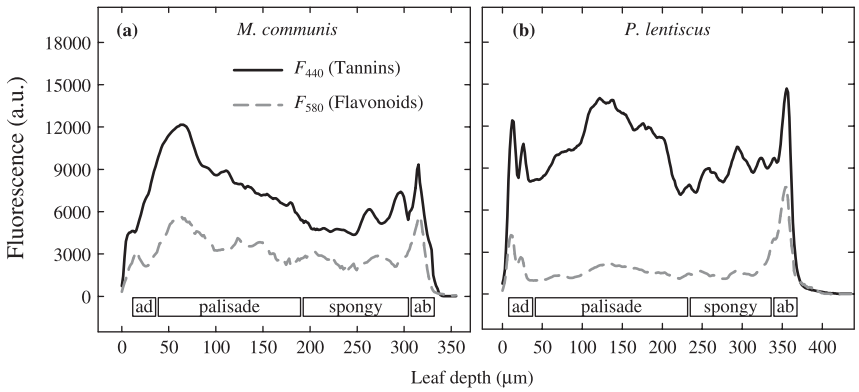

Fig. 5 The tissue-specific distribution of hydrolysable tannins (F_{440}) and flavonoids (F_{580}) in *Myrtus communis* and *Pistacia lentiscus* leaves exposed to 100% solar radiation, over an 8-wk experimental period. Measurements were conducted on 50-μm-thick cross-sections stained with Naturstoff reagent and excited at 365 nm. Profiles of fluorescence intensity (from adaxial through abaxial tissues) were recorded by a charge-coupled device (CCD) camera, at the maximum emission wavelengths of tannins (440 nm, F_{440}) and flavonoid glycosides (580 nm, F_{580}), respectively. Ad and ab indicate adaxial and abaxial epidermal (including cuticle) layers, respectively; palisade and spongy refer to palisade parenchyma and spongy parenchyma layers, respectively.

M. communis (Evans, 1999). As a consequence, morphological adjustments primarily intended to avoid light from reaching leaf surface, such as the decrease in leaf size and the increase in leaf angle (Ehleringer & Comstock, 1987; Werner *et al.*, 1999), operated to a much greater degree in *M. communis* than in *P. lentiscus* in response to high solar radiation (see the Appendix, Table A1; Fig. 1b). Similarly (1) loss of chlorophyll, which may also effectively help to reduce centres of light absorption and photo-damage to photosynthetic apparatus (Havaux & Tardy, 1999), (2) the increase in the Car : Chl_{tot} ratio (particularly xanthophyll cycle pigments; see PRI values in Table 3) aimed to dissipate thermally excess radiant energy (Demmig-Adams, 1998) and (3) the increase in the Chl*a* : Chl*b* ratio probably linked to the reduction of the light-harvesting complex II (Kirchgeβner *et al.*, 2003), served prominent roles in the acclimatization mechanisms of *M. communis* (McKinnon & Mitchell, 2003) to high solar radiation. The superior light-induced plasticity (Valladares *et al.*, 2000) in morpho-anatomical traits, and in the content and composition of photosynthetic pigments (which probably contributed to limit the usage of solar radiation in photosynthetic processes and new growth to a greater extent in *M. communis* than in *P. lentiscus* at the full-sun site (Fig. 1b, Table 2, Falster & Westboy, 2003), did not preserve *M. communis* from a more severe oxidative damage to leaf membrane lipids (i.e. MDA) and photosynthetic apparatus (i.e. F_v/F_m) compared with *P. lentiscus*, particularly when plants were also exposed to salinity stress (Fig. 4, Table 2). The lack of a direct relationship between light-induced plasticity in morpho-anatomical traits and adaptation to excess solar radiation has been widely discussed in the very recent past (for a review, see van Kleunen & Fischer, 2004) and previously reported in some species differing in light demand (Valladares *et al.*, 2000; Tattini *et al.*, 2005).

Overall, species-specific responses to light treatment may allow one conclude that *M. communis* is 'less tolerant' than *P. lentiscus* to high solar radiation. This finding, taken together with significant species–salt–light effects on most photosynthetic features may offer a conclusive explanation for the very sparse distribution of the former in seashore dunes of the Mediterranean basin, where excess soil salinity may frequently occur in concomitance with clear days, over the long summer season.

Our final discussion concerns the potential roles served by hydrolysable tannins and flavonoids in the biochemical strategies adopted by *M. communis* and *P. lentiscus* to cope with high solar radiation and salinity stress (Figs 4 and 5). First, we show that the share of newly assimilated carbon allocated to polyphenols markedly increased in response to both salinity stress and high solar radiation (Fig. 4), although neither stressors appreciably affected the whole-leaf polyphenol content (see the Appendix, Table A1; Tattini *et al.*, 2004). These findings fit with a widely reported plant behaviour to allocate carbon preferentially for defence than for new growth when exposed to environmental constraints of different origin (Lavola *et al.*, 2000; Kandil *et al.*, 2004). We suggest, therefore, that the whole-leaf polyphenol content seems inadequate to estimate environment-induced alterations in leaf biochemistry (Tattini *et al.*, 2004), although it is a key determinant of plant–environment interactions (Aerts, 1995; Kandil *et al.*, 2004).

The salt- and light-induced greater increases in the allocation of carbon to flavonoids than to hydrolysable tannins (Figs 1 and 4) appear of particular interest. The light-induced preferential accumulation of flavonoids over that of hydrolysable tannins (Tegelberg *et al.*, 2004) may have depended simply upon relative UV-spectral features, as galloyl derivatives have absorption maxima in the low UV-B waveband (270–285 nm, Romani *et al.*, 2002) and offer a negligible protection against UV-A wavelengths (which mostly accounts for the UV-waveband of solar radiation), compared with that due to

both myricetin and quercetin glycosides, which effectively absorb wavelengths in the range 330–370 nm (Tattini *et al.*, 2004). We also hypothesize that, the greater concentration (and CO_2-based accumulation) of flavonoids in the mesophyll of *M. communis* than in *P. lentiscus* (Fig. 5) probably depended on a reduced ability of the former to limit the flux of UV-radiation in the leaf because of both a thinner cuticle and a smaller contribution from epidermal flavonoids (Day, 1993; Krauss *et al.*, 1997). However the fact that (1) flavonoid glycosides accumulated to a greater extent than did hydrolysable tannins because of salinity stress (Fig. 1a); and (2) there was a close relationship between leaf oxidative damage and preferential accumulation of flavonoids, irrespective of stressful conditions (Figs 1 and 4), supports the idea that myricetin and quercetin glycosides probably played antioxidant roles in *M. communis* and *P. lentiscus* exposed to salt and light stress (Tattini *et al.*, 2004; Tattini *et al.*, 2005). Flavonoids have the potential for serving antioxidant functions in tissues exposed to both high UV-radiation (Rice-Evans *et al.*, 1996; Ryan *et al.*, 1998) and oxidative damages of different origin (Schoch *et al.*, 2001; Babu *et al.*, 2003). However, flavonol 3-*O*-glycosides and galloyl derivatives do not actually differ in their *in vitro* abilities to scavenge free-radicals, such as the hydroxyl or the superoxide radicals (Rice-Evans *et al.*, 1996; Baratto *et al.*, 2003), and, hence, the high 'constitutive' content of hydrolysable tannins (we note that carbon allocated to hydrolysable tannins was at least one order of magnitude greater than that allocated to flavonoids irrespective of growing conditions; Fig. 4a,b) in the mesophyll cells should have been in large excess of that necessary to quench light- or salt-induced highly reactive oxygen species in both species. However, tannins (Bussotti *et al.*, 1998) and flavonoid glycosides (Yamasaki *et al.*, 1997; Takahama, 2004) have been shown to have a nearly exclusive vacuolar distribution (wall-bound flavonoid glycosides have been also reported in some species, Schnitzler *et al.*, 1996; Agati *et al.*, 2002), and therefore, both polyphenol classes have merely to reduce hydrogen peroxide, which may freely diffuse from cellular organelles to enter the vacuole under conditions of severe oxidative damage (Yamasaki *et al.*, 1997; Takahama, 2004). We therefore hypothesize that the preferential accumulation of flavonoids with respect to that of hydrolysable tannins might be linked to a superior ability of the former class to serve as substrates for class III peroxidases, which reduce hydrogen peroxide in the presence of phenolics to form more stable phenoxyl radicals in cell vacuoles (Yamasaki *et al.*, 1997; Takahama, 2004). The electron-donor ability of flavonol 3-*O*-glycosides, however, has been recently questioned by Pearse *et al.* (2005), as 3-*O*-glycosylation may both preserve flavonoids from autoxidation and increase their solubility in the vacuolar milieu, but, at the same time, reduces sharply their abilities to be oxidized via the hydrogen peroxide–peroxidase system compared with those of corresponding aglycones (Hodnick *et al.*, 1988; Yamasaki *et al.*, 1997). Future experiments should be aimed at addressing the complex issue of the functional roles of polyphenols in light of the chemical structure–electron-donor ability relationship for class III peroxidases.

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Appendix

Table A1 Summary of three-way anova of effects of species, salinity and light as fixed factors, with their interaction factors, on 32 parameters related with (1) ionic and water relations; (2) photosynthetic performance; (3) morpho-anatomical and optical traits; (4) the contents of photosynthetic pigments and polyphenols in *Myrtus communis* and *Pistacia lentiscus* plants

Parameter	df	$F_{species}$	F_{salt}	F_{light}	$F_{species \times salt}$	$F_{species \times light}$	$F_{salt \times light}$
Na^{leaf} (mol per organ)	39	152.1**	46.1**	94.8**	33.4**	60.1**	34.6**
Na^{stem} (mol per organ)	39	4.5*	249.6**	5.5*	11.1*	2.1 ns	1.7 ns
Na^{root} (mol per organ)	39	20.3**	158.4**	379.0**	2.1 ns	21.3**	27.3**
Na_{leaf} (mM)	39	184.1**	103.5**	788.2**	43.8**	139.6**	58.9**
Na_{stem} (mM)	39	542.3**	106.6**	1487.5**	0.5 ns	201.3**	53.7**
Na_{root} (mM)	39	0.1 ns	188.4**	1255.0**	3.1 ns	1.3 ns	269.7**
K/Na_{leaf}	39	3.2 ns	798.7**	365.5**	1.9 ns	7.6*	45.5**
ψ_w (MPa)[a]	159	18.4**	293.1**	8.1*	2.7 ns	0.1 ns	1.8 ns
ψ_π (MPa)[a]	159	117.8**	159.6**	19.6**	0.4 ns	5.1*	1.1 ns
$\psi_{\pi FT}$ (MPa)[a]	159	204.1**	98.6**	18.7**	5.3 ns	5.8*	0.2 ns
RWC (%)[a]	159	0.1 ns	423.4**	8.4*	31.9**	0.8 ns	1.9 ns
P_n (mmol m^{-2} s^{-1})[a]	159	0.5 ns	531.5**	0.2 ns	26.8**	13.2**	9.7**
g_s (mmol m^{-2} s^{-1})[a]	159	13.5**	707.4**	1.1 ns	43.4**	18.1**	12.7**
E (mmol m^{-2} s^{-1})[a]	159	39.8**	680.3**	0.1 ns	11.2**	0.5 ns	4.9*
F_v/F_m[a]	159	99.6**	25.5**	121.4**	11.3**	12.7**	1.7 ns
Leaf size (cm^2)	95	838.4**	4.1*	904.2**	2.5 ns	153.4**	3.3 ns
Leaf angle (°)	95	1352.6**	0.3 ns	2278.2**	1.1 ns	1444.7**	0.1 ns
LMA (g DW m^{-2})	95	812.7**	1.4 ns	880.7**	4.5*	4.7*	6.9*
Leaf thickness (μm)	47	276.9**	0.2 ns	757.5**	3.1 ns	11.4**	0.1 ns
Cuticle thickness (μm)	47	654.4**	0.1 ns	72.3**	0.3 ns	4.4*	0.1 ns
Palisade/spongy	47	304.9**	0.4 ns	280.9**	3.8 ns	0.3 ns	0.1 ns
A_{vis} (% mg^{-1} DW)[a]	63	885.8**	5.9*	905.2**	7.1*	168.4**	8.7*
R/T_{850} (% mg^{-1} DW)[a]	63	492.4**	1.5 ns	272.3**	4.5*	57.5**	0.2 ns
PRI[a]	63	3747.8**	751.7**	8133.9**	624.7**	3508.2**	154.7**
Chl_{tot} (mg g^{-1} DW)[a]	63	259.2**	52.9**	176.8**	30.9**	31.8**	12.4*
Chl_{tot} (μg cm^{-2})[a]	63	0.8**	96.4**	57.5**	21.8**	19.9**	7.8*
Chl_a : Chl_b[a]	63	227.1**	1.9 ns	80.4**	2.0 ns	7.2*	20.7**
Car : Chl[a]	63	33.8**	6.8*	235.9**	6.5*	75.3**	0.2 ns
MDA (nmol g^{-1} DW)[a]	63	108.7**	156.9**	99.6**	32.1**	6.2*	5.9*
Polyphenols (mol g^{-1} DW)	31	432.2**	2.2 ns	4.3 ns	3.2 ns	23.7**	2.1 ns
Tannins (mol g^{-1} DW)	31	691.5**	96.3**	14.8*	2.6 ns	19.7**	5.0 ns
Flavonoids (mol g^{-1} DW)	31	232.0**	3.2 ns	222.3**	11.5*	64.8**	0.5 ns

A_{vis}, absorptance efficiency over the visible waveband; Car, carotenoids; Chl_a, chlorophyll *a*; Chl_b, chlorophyll *b*; Chl_{tot}, total chlorophyll; E, transpiration rate; F_v/F_m, efficiency of photosystem II (PSII); g_s, stomatal conductance; K/Na_{leaf}, leaf K^+ : Na^+ ratio; LMA, leaf mass per area; MDA, malondialdehyde; Na^{leaf}, amount of Na^+ allocated to the leaf tissue; Na_{leaf}, leaf Na^+ concentration; Na^{root}, amount of Na^+ allocated to the root tissue Na_{root}, root Na^+ concentration; Na^{stem}, amount of Na^+ allocated to the stem tissue; Na_{stem}, stem Na^+ concentration; P_n, net assimilation rate; PRI, photochemical reflectance index; R/T_{850}, scattering efficiency (i.e. the ratio of reflectance (R) to transmittance (T) at 850 nm)); RGR, relative growth rate; RWC, relative water content; ψ_π, osmotic potential; $\psi_{\pi FT}$, leaf osmotic potential at full turgor; ψ_p, turgor potential; ψ_w, water potential.

The amounts of Na^+ in different plant organs (Na^{organ}) were calculated by multiplying the tissue Na^+ concentration (mol g^{-1} DW) by the dry weight of the relative organ.

[a]Data resulting from time-course measurements have been pooled together before statistical analysis.

*,**, $0.05 > P > 0.001$ and $P < 0.001$, respectively; ns, not significant.

