On the role of flavonoids in the integrated mechanisms of response of *Ligustrum vulgare* and *Phillyrea latifolia* to high solar radiation

Massimiliano Tattini[1], Lucia Guidi[2], Laura Morassi-Bonzi[3,6], Patrizia Pinelli[4], Damiano Remorini[5], Elena Degl'Innocenti[2], Cristiana Giordano[6], Rossano Massai[5] and Giovanni Agati[7]

[1]Istituto per la Valorizzazione del Legno e delle Specie Arboree, IVALSA, Consiglio Nazionale delle Ricerche, Via Madonna del Piano, I-50019, Sesto F.no, Firenze; [2]Dipartimento di Chimica e Biotecnologie Agrarie, Università di Pisa, Via S. Michele degli Scalzi 2, I-56124, Pisa; [3]Dipartimento di Biologia Vegetale, Università di Firenze, Via U. La Pira 4, I- 50121, Firenze; [4]Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via Ugo Schiff 6, I-50019, Sesto F.no, Firenze; [5]Dipartimento di Coltivazioni e Difesa delle Specie Legnose 'G. Scaramuzzi', Università di Pisa, Via del Borghetto 80, I-56124, Pisa; [6]Centro di Microscopie Elettroniche, Ce.M.E., Consiglio Nazionale delle Ricerche, Via Madonna del Piano, I-50019, Sesto F.no, Firenze; [7]Istituto di Fisica Applicata 'Nello Carrara', IFAC, Consiglio Nazionale delle Ricerche, Via Madonna del Piano, I-50019, Sesto F.no, Firenze, Italy

Author for correspondence:
Massimiliano Tattini
Tel: +39 0555225 692
Fax: +39 0555225 656
Email: m.tattini@ivalsa.cnr.it

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Summary

• The role of flavonoids in mechanisms of acclimation to high solar radiation was analysed in *Ligustrum vulgare* and *Phillyrea latifolia*, two Mediterranean shrubs that have the same flavonoid composition but differ strikingly in their leaf morpho-anatomical traits.

• In plants exposed to 12 or 100% solar radiation, measurements were made for surface morphology and leaf anatomy; optical properties, photosynthetic pigments, and photosystem II efficiency; antioxidant enzymes, lipid peroxidation and phenylalanine ammonia lyase; synthesis of hydroxycinnamates and flavonoids; and the tissue-specific distribution of flavonoid aglycones and ortho-dihydroxylated B-ring flavonoid glycosides.

• A denser indumentum of glandular trichomes, coupled with both a thicker cuticle and a larger amount of cuticular flavonoids, allowed *P. latifolia* to prevent highly damaging solar wavelengths from reaching sensitive targets to a greater degree than *L. vulgare*. Antioxidant enzymes in *P. latifolia* were also more effective in countering light-induced oxidative load than those in *L. vulgare*. Consistently, light-induced accumulation of flavonoids in *L. vulgare*, particularly ortho-dihydroxylated flavonoids in the leaf mesophyll, greatly exceeded that in *P. latifolia*.

• We conclude that the accumulation of flavonoid glycosides associated with high solar radiation-induced oxidative stress and, hence, biosynthesis of flavonoids appear to be unrelated to 'tolerance' to high solar radiation in the species examined.

Key words: antioxidant enzymes, chlorophyll fluorescence, cuticular flavonoid aglycones, glandular trichomes, high solar radiation, microspectrofluorometry, ortho-dihydroxylated flavonoid glycosides, phenylalanine ammonia lyase.

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Introduction

Plants exposed to high solar radiation undergo morpho-anatomical, physiological and biochemical adjustments (Caldwell *et al.*, 1995; Rozema *et al.*, 1997; Lambers *et al.*, 1998) devoted, for the most part, to countering perturbations (mainly of oxidative origin) of cellular homeostasis (Mittler, 2002; Foyer & Noctor, 2003; Apel & Hirt, 2004). Of the

biochemical traits that may vary markedly as a result of UV-B stress and high solar radiation, considerable attention has been focused, over the past two decades, on the biosynthesis of phenylpropanoids, particularly flavonoids (for reviews, see Caldwell *et al.*, 1995; Dixon & Paiva, 1995; Rozema *et al.*, 1997; Jordan, 2002; Bassman, 2004). In fact, flavonoids potentially have several protective functions against the detrimental effects of short solar wavelengths (Bornman, 1999; Gould *et al.*, 2000; Jansen, 2002; Jordan, 2002; Tattini *et al.*, 2004). The location of flavonoids in trichomes (Karabourniotis *et al.*, 1998; Tattini *et al.*, 2000), on the cuticular wax layer and in epidermal cells (Wollenweber, 1993; Hutzler *et al.*, 1998; Kolb *et al.*, 2001), particularly those bound to the epidermal cell wall (Strack *et al.*, 1988; DeLucia *et al.*, 1992; Day, 1993), may to a great extent prevent short solar wavelengths from reaching sensitive targets within the leaf. However, other functional roles have been recently proposed for flavonoids, as flavonoids with ortho-dihydroxylated B-rings are preferentially synthesized, as compared with monohydroxy-substituted counterparts, in leaves exposed to both UV-B and high solar radiation (Ryan *et al.*, 1998; Agati *et al.*, 2002; Hofmann *et al.*, 2003; Tattini *et al.*, 2004). In fact, ortho-dihydroxylated B-ring flavonoids may efficiently dissipate excess energy through tautomeric interconversions (Smith & Markham, 1998) and scavenge reactive oxygen species (Yamasaki *et al.*, 1997; Neill & Gould, 2003), through a quenching mechanism as well as by 'inhibiting' the formation of free radicals (Rice-Evans *et al.*, 1997). The fundamental role assigned to flavonoids in the biochemical strategies adopted by plants to cope with light stress probably depends on the multiplicity of their potential functions. Biosynthesis of flavonoids has also been shown to be favoured relative to biosynthesis of hydroxycinnamates, which are as effective as flavonoids in providing effective UV screening in foliage (Landry *et al.*, 1995; Sheahan, 1996) and in tissue exposed to high doses of UV radiation as well as to wounding or pathogen attack (Christensen *et al.*, 1998; Burchard *et al.*, 2000; Schoch *et al.*, 2001; Tattini *et al.*, 2004).

Nevertheless, it is still a matter of controversy whether the ability to accumulate flavonoids, particularly flavonoids with ortho-dihydroxylated B-rings, and 'tolerance' to UV-radiation stress are correlated (Olsson *et al.*, 1998; Hofmann *et al.*, 2000; Smith *et al.*, 2000; Dixon *et al.*, 2001; Musil *et al.*, 2002; Tanaka *et al.*, 2002; Hofmann *et al.*, 2003). We note (i) that 'tolerance' is a merely qualitative (rather than quantitative), multicomponent descriptor of plant–environment interactions (Levitt, 1980; Mittler, 2002), and (ii) that mutants lacking or possessing the ability to synthesize flavonoids (Li *et al.*, 1993; Bieza & Lois, 2001) may be oversimplified plant model systems for quantifying the UV-tolerance/flavonoid-biosynthesis relationship (Casati & Walbot, 2003), because flavonoids are ubiquitously found in terrestrial plants (Rozema *et al.*, 1997; Jansen, 2002). In contrast, less attention has been devoted to analysing the tissue-specific locations of individual flavonoid classes (instead of merely quantifying flavonoid concentrations at the whole-leaf level), which may clarify the complex issue of their functional roles in mechanisms of acclimation to high UV irradiance (Day, 1993; Bornman, 1999; Olsson *et al.*, 1999; Agati *et al.*, 2002; Tattini *et al.*, 2004). A recent study conducted by Semerdjieva *et al.* (2003b) on *Vaccinium* spp. showed an inverse relation between cuticle thickness and UV-B-induced accumulation of mesophyll flavonoids. Although identification of flavonoids was not carried out, both the far blue-light excitation wavelength (λ_{exc} = 488 nm) and the staining reagent Naturstoff (Hutzler *et al.*, 1998) used for visualizing mesophyll flavonoids through confocal laser scanning microscopy were consistent with the accumulation of ortho-dihydroxylated B-ring flavonoids in response to UV radiation (Agati *et al.*, 2002; Tattini *et al.*, 2004).

The hypothesis tested here was that the biosynthesis of flavonoids, particularly 'internal flavonoid glycosides' (Gould *et al.*, 2000), may be largely controlled by constitutive morpho-anatomical and biochemical features (Gutschick, 1999), primarily intended both to prevent light penetration (Day, 1993; Vogelmann, 1993; Krauss *et al.*, 1997) and to remove the consequent oxidative damage (Asada, 1999; Mittler, 2002). An experiment was therefore designed in which two members of the Oleaceae family, namely *Ligustrum vulgare* and *Phillyrea latifolia*, were compared. These species have nearly identical flavonoid compositions (Agati *et al.*, 2002; Tattini *et al.*, 2004) but differ greatly in their morpho-anatomical characteristics. *P. latifolia* and *L. vulgare* are adapted to sunny and partially shaded areas, respectively, at Mediterranean latitudes (Brosse, 1979; Tattini *et al.*, 2000; Tattini *et al.*, 2004). In leaves developing at 12% (shade) or 100% (sun) solar irradiance, investigations were conducted to analyse (i) morpho-anatomical and optical features, and photosynthetic pigment content and composition; (ii) chlorophyll *a* (Chl *a*) fluorescence kinetics, the activity of antioxidant enzymes and lipid peroxidation; (iii) the activity of phenylalanine ammonia lyase (the enzyme involved in the first committed step of phenylpropanoid metabolism) and the accumulation of flavonoid glycosides and hydroxycinnamates, and finally (iv) the tissue-specific distribution of flavonoids, particularly cuticular aglycones and mesophyll flavonoids with ortho-dihydroxy-substituted B-rings.

Materials and Methods

Plant material and growing conditions

One-year-old self-rooted *Ligustrum vulgare* L. and *Phillyrea latifolia* L. plants were headed back to five or six shoots at the end of June 2003 at Pisa, Italy (43°43′ N, 10°23′ E). Plants were grown in 3.0-l pots with a pumice : sphagnum peat substrate [50 : 50, volume/volume (v/v)] outdoors under 20% sunlight radiation and supplied with a 1/3-strength Hoagland's

solution (three times a week) over an 8-wk period. At the end of this period, *P. latifolia* and *L. vulgare* plants had produced three and eight leaf pairs per shoot, respectively. Plants were then placed in 100-m^2 boxes constructed with black polyethylene nets to receive 12% (shade) or 100% (sun) sunlight irradiance. Before the start of the experiment, plants in the full-sunlight treatment were exposed to increasing sunlight for 6 d (starting from 40% solar radiation, and increasing the irradiance by 20% every 2 d). Light treatments were imposed for an additional 4 wk. 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 sensor. The daily doses of UV-B irradiance were measured with an SUV 100 scanning spectroradiatiometer (Biospherical Instruments, San Diego, CA, USA) on a total of 15 d, both clear and cloudy, over the whole experimental period, as previously reported (Tattini *et al.*, 2004). Plants at the full-sun site received mean daily doses of 10.9 MJ m^{-2}, 0.94 MJ m^{-2} and 18.7 kJ m^{-2} in the photosynthetically active radiation (PAR; over 400–700 nm), UV-A and UV-B wavebands, respectively. These data were in good agreement with daily courses of irradiance obtained from the European Light Dosimeter Network (ELDONET) dosimeter located at the Pisa station (Marangoni *et al.*, 2000). Mean daily doses of 0.09 MJ m^{-2} and 1.7 kJ m^{-2} in the UV-A and UV-B wavebands, respectively, were recorded at the shade site.

All measurements, except those for environmental scanning electron microscopy (ESEM), were carried out on newly developing leaves, the area of which was approx. 20–25% of the leaf lamina area of fully developed leaves at the beginning of the experiment.

Leaf morphology and anatomy

Leaf mass per area (LMA) and leaf angle (with respect to the horizontal plane) were determined as reported previously (Tattini *et al.*, 2000). Whole-leaf thickness and the thicknesses of adaxial and abaxial cuticular, adaxial and abaxial epidermal, and palisade and spongy parenchymal layers were estimated in 1-μm-thick transverse sections, fixed and embedded following standard methodology (Semerdjieva *et al.*, 2003a), using a Zeiss AxioPhot microscope (Carl Zeiss, Jena, Germany) equipped with a high-resolution TK 870E JVC video camera (JVC, Yokohama, Japan). Leaf surface morphology was analysed in fresh material using the Fei Quanta 200 Environmental Scanning Electron Microscope (ESEM; Fei Corporation, Eindhoven, the Netherlands) operating in low-vacuum mode (the chamber pressure was kept at 1 Torr). In contrast with other measurements, ESEM analysis was carried out on the newest leaves, the area of which did not exceed 1.2 mm^2. These leaves were chosen to enable monitoring of early events during leaf development, as the programming of epidermal cell differentiation for the development of glandular trichomes (Glover, 2000) may greatly affect the ability of the leaf to prevent light-induced damage.

Leaf optics, photosynthetic pigment content, and net CO_2 assimilation rate

Leaf optical characteristics were determined by recording reflectance (R) and transmittance (T) spectra in the 380–1100 nm waveband using a Li-Cor 1800 spectroradiometer equipped with a Li-Cor 1800-125 integrating sphere (Li-Cor Inc.). The percentage absorptance ($A\% = 100 - R - T$) over the 400–700 nm waveband ($A_{400-700}$) and the scattering index [$(R/T)_{850}$] were determined after Lee & Graham (1986). Both the 'absorptance efficiency' and the 'scattering efficiency' (Knapp & Carter, 1998) were then calculated by normalizing the corresponding indexes on the basis of the dry weight of 1.65 cm^2, i.e. the area over which reflectance and transmittance spectra were recorded. Normalization of optical features on a dry mass, rather than area basis, has been successfully used to compare the optical features of leaves differing markedly in their whole-leaf thickness and LMA (Knapp & Carter, 1998), as was also the case in our experiment (Table 1).

The contents of chlorophyll (Chl_{tot}) and carotenoids (Car) were determined after Lichtenthaler (1987). The leaf net CO_2 assimilation rate was measured using a Li-Cor 6400 portable infrared gas analyser operating at 34 ± 0.5 Pa ambient CO_2. Mean daily net CO_2 assimilation was calculated by subtracting dark respiration from diurnal net photosynthetic rate. The diurnal net CO_2 assimilation rate was measured at 3-h intervals from 06:30 to 20:30 hours, and dark respiration was measured at midnight and 04:00 hours, on two consecutive days every week. The integration procedures previously reported by Valladares & Pearcy (1997) and Tattini *et al.* (2004) were then used to calculate both daily assimilated CO_2 and total assimilated CO_2 over the 4-wk experimental period.

Chlorophyll fluorescence analysis

A modulated Chl *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 photosystem II (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 μmol m^{-2} s^{-1}, to avoid appreciable variable fluorescence. F_m and F'_m were determined at 20 kHz using a 0.8-s saturating light pulse of white light at 8000 μmol m^{-2} s^{-1}. F'_m, i.e. the maximum fluorescence in light conditions, was determined at 400 or 900 μmol m^{-2} s^{-1} of photons over the PAR waveband, i.e. at light intensities at which saturation of photosynthesis occurred for shade and

Table 1 Morphological and anatomical features of *Ligustrum vulgare* and *Phillyrea latifolia* leaves exposed to 12 or 100% solar radiation over a 4-wk period

	Sunlight irradiance						
	12%		100%				
Parameter	*L. vulgare*	*P. latifolia*	*L. vulgare*	*P. latifolia*	F_S	F_L	$F_{S\times L}$
Leaf angle (°)	6.2 ± 1.9	7.2 ± 1.5	52.3 ± 8.8	10.7 ± 2.9	213.2**	317.6**	237.1**
LMA (mg d. wt cm^{-2})	3.8 ± 0.1	7.1 ± 0.2	7.1 ± 0.1	14.1 ± 0.4	4590.2**	4632.4**	617.4**
Adaxial cuticle (μm)	4.2 ± 0.6	6.7 ± 0.5	4.5 ± 0.5	7.7 ± 0.7	135.2**	7.7*	2.8 ns
Adaxial epidermis (μm)	13.3 ± 0.9	15.2 ± 0.9	14.6 ± 1.2	17.5 ± 1.0	33.5**	14.4*	0.9 ns
Palisade parenchyma (μm)	54.5 ± 6.1	76.1 ± 9.4	87.7 ± 10.7	117.0 ± 6.8	78.9**	166.0**	2.3 ns
Spongy parenchyma (μm)	90.2 ± 9.5	120.8 ± 12.2	121.3 ± 16.3	155.7 ± 10.5	51.4**	53.0**	0.3 ns
Abaxial epidermis (μm)	11.2 ± 1.5	10.4 ± 1.3	13.0 ± 1.5	11.1 ± 1.4	3.8 ns	3.4*	0.3 ns
Abaxial cuticle (μm)	2.9 ± 0.4	5.5 ± 0.7	3.8 ± 0.6	6.8 ± 0.9	97.6**	11.7*	0.6 ns
Whole-leaf thickness (μm)	180.5 ± 12.5	239.3 ± 17.5	248.5 ± 24.9	317.6 ± 15.4	117.4**	143.0**	0.7 ns

Leaf angle and leaf mass per area (LMA) were determined on 12 leaves or 12 leaf discs per species and light treatment. Whole-leaf thickness and the thicknesses of different tissue layers were measured on six transverse sections taken from three replicate leaves. The result of a two-way analysis of variance (ANOVA), for variation in morpho-anatomical parameters [total error degrees of freedom (df) = 47 for leaf angle and LMA, and df = 23 for other parameters] with species (S) and light (L) as fixed factors, with their interaction (S × L), is shown.
**, $P < 0.001$; *, $0.05 > P > 0.001$; ns, not significant. Data are mean ± standard deviation; $n = 12$ for leaf angle and LMA; $n = 6$ for tissue layer thickness.

sun leaves, respectively (data not shown). Photosystem II quantum yield in the light (Φ_{PSII}) and nonphotochemical quenching [$q_{NP} = (F_m/F'_m) - 1$] were estimated using the saturation pulse method described in Schreiber *et al.* (1986), and calculated according to Genty *et al.* (1989) and Bilger & Björkman (1990), respectively. The excitation pressure on PSII, $(1 - q_P)$, q_P being the coefficient of photochemical quenching, was calculated after Schreiber *et al.* (1995).

Antioxidant enzymes, phenylalanine ammonia lyase, and lipid peroxidation

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was measured photometrically at 560 nm, according to the inhibition by SOD of nitroblue tetrazolium (NBT) reduction. One unit of SOD was defined as the amount needed for 50% inhibition of the NBT reduction state (Beyer & Fridovich, 1987). The activity of ascorbate peroxidase (ASCPx; EC 1.11.1.11) was measured as a decrease in absorbance at 290 nm, resulting from ascorbate (ASC) oxidation (Nakano & Asada, 1981). Catalase (CAT; EC 1.11.1.6) activity was measured photometrically at 270 nm (Cakmak & Marschner, 1992) by determining the rate of H_2O_2 conversion to O_2. Finally, the activity of phenylalanine ammonia lyase (PAL; EC 4.3.1.5) was determined photometrically at 290 nm by measuring the conversion of phenylalanine to cinnamic acid, as recently reported by Guidi *et al.* (2005). Lipid peroxidation was estimated by measuring the amount of malondialdehyde (MDA), as described by Hodges *et al.* (1999), which takes into account the possible influence of interfering compounds in the assay for 2-thiobarbituric acid (TBA)-reactive substances.

'Cuticular' flavonoid aglycones and 'internal' soluble flavonoid glycosides

Analysis of cuticular flavonoid aglycones was performed after rinsing the leaves with diethyl ether for 2 h in the dark at room temperature, as reported by Gould *et al.* (2000). The diethyl ether fraction was then reduced to dryness, diluted with 0.5 ml of H_2O/MeOH/CH_3CN (20 : 60 : 20, v/v/v) and analysed using high-performance liquid chromatography with a diode array detector (HPLC-DAD). The analysis of internal flavonoid glycosides, that were extracted with soluble EtOH/H_2O (75 : 25, v/v), referred to as internal flavonoid glycosides throughout the paper, was carried out on diethyl ether-treated leaves, following the protocol of Tattini *et al.* (2004). Separation and identification of metabolites were achieved using an HP1100 liquid chromatograph equipped with a DAD, and managed using an HP workstation (all from Hewlett & Packard, Palo Alto, CA, USA). Column, eluent and operating conditions were as reported previously (Tattini *et al.*, 2004). Flavonoids, namely quercetin 3-O-rutinoside (que 3-O-rut), luteolin 7-O-glc (lut 7-O-glc), and both apigenin 7-O-glucoside (api 7-O-glc) and apigenin 7-O-rutinoside (api 7-O-rut), were identified by comparison of their retention times and UV spectra with those of authentic standards (Extrasynthese, Lyon-Nord, Genay, France), and quantified at 350 nm using individual calibration curves operating in the range 0–40 μg. Quantification was also performed for hydroxycinnamates, namely *p*-coumaric acid and echinacoside in *L. vulgare*, and verbascoside, plus an unidentified caffeic acid derivative, in *P. latifolia* (Agati *et al.*, 2002; Tattini *et al.*, 2004).

CO_2-based accumulation of 'internal' soluble phenylpropanoids

CO_2-based phenylpropanoid accumulation (phenylCO_2) was calculated by normalizing the increase in the internal soluble phenylpropanoid concentration on a leaf area basis (mol phenyl m^{-2}) to assimilated CO_2 (mol m^{-2}) during the experimental period (t_0 to t_1) using the equation recently reported by Tattini *et al.* (2004):

$$\text{phenyl}CO_2 = \text{mol phenyl mol}^{-1}\ CO_2 = (A - B)/C \quad \text{Eqn 1}$$

[A, mol phenyl m^{-2} at t_1; B, mol phenyl m^{-2} at t_0; C, assimilated CO_2 (diurnal carbon gain minus night-time respiration) over the whole experimental period t_0 to t_1.] The internal phenylpropanoid concentrations of *L. vulgare* were 125 ± 17 and 174 ± 15 μmol m^{-2} at the shade and sun sites, respectively, at t_0. Corresponding phenylpropanoid concentrations were 148 ± 19 and 212 ± 20 μmol m^{-2} in *P. latifolia*, at t_0. The CO_2-based accumulation of phenylpropanoids, taking into account the daytime assimilated carbon, which may vary depending on species and solar irradiance, has been proposed (instead of the phenylpropanoid content at whole-leaf level) to estimate newly assimilated carbon devoted to the synthesis of secondary metabolites (Tattini *et al.*, 2004).

Tissue-specific localization of flavonoids

The tissue-specific localization of flavonoids was estimated on transverse sections (50-μm-thick) of fresh leaf tissue stained with 0.1% (weight/volume) 2-amino ethyl diphenyl boric acid (Naturstoff reagent, NR) in phosphate buffer (pH 6.8) as reported by Tattini *et al.* (2004). Fluorescence microspectroscopy and fluorescence microimaging were performed using a standard inverted epi-fluorescence microscope (Diaphot, Nikon, Japan) coupled to both a charge-coupled device (CCD) camera and a multichannel spectral analyser (PMA 11-C5966; Hamamatsu, Photonics Italia, Arese, Italy) as described by Agati *et al.* (2002). The excitation wavelength was selected by a 10-nm bandwidth interference filter centred at 488 nm (488FS10-25; Andover Corporation, Salem, NH, USA) coupled to a ND510 (Nikon) dichroic mirror. It has been previously shown that only flavonoids contribute to fluorescence signals of NR-treated tissues excited at 488 nm (Hutzler *et al.*, 1998; Tattini *et al.*, 2004). Fluorescence spectra of adaxial and abaxial epidermal and both palisade and spongy parenchymal tissues were recorded using a ×40 Plan Fluor (Nikon) objective, which integrated (over a 5-s period) the fluorescence signal on a 500-μm^2 area. Residual excitation light was removed by a GG515 long-pass filter (Schott Glas, Mainz, Germany), and fluorescence spectra were finally corrected for the transmission spectra of optics and filters. Fluorescence images at 580 nm of blue light-excited (λ_{exc} = 488) cross-sections were recorded using a 10-nm bandwidth interference filter (580FS10-25; Andover Corporation). The image spatial calibration, using a ×10 Plan Fluor (Nikon) objective, was 0.79 μm $pixel^{-1}$. The images were subsequently digitized with a 14-bit dynamics and processed for background subtraction, flat-field correction for spatial nonuniformity of the excitation beam, and sharpen filtering (Tattini *et al.*, 2004), before being converted to a TIF format. Elaboration and pseudo-colour representations of fluorescence images were obtained using Image-Pro Plus version 4.0 software (Media Cybernetics, Silver Spring, MD, USA), as previously reported (Agati *et al.*, 2002).

Experimental design and statistics

The experiment was a complete random design, with at least 20 plants per species and light treatment. Therefore, approx. 100–120 leaf pairs were available for sampling. Leaf angle and LMA were determined on 12 replicate leaves and leaf discs, respectively. Whole-leaf thickness and the thicknesses of different tissue layers were measured on six cross-sections taken from three replicate leaves, at the end of the experiment. Leaf surface morphology was analysed on three to four newly developing leaves of both species at the sun site. Optical variables and the concentration of photosynthetic pigments were determined on four replicate leaf samples at the end of the experiment. Diurnal net CO_2 assimilation and night-time respiration rates were measured on four replicate leaves, during two consecutive days, at weekly intervals. The same leaves were sampled, at the end of the experiment, to quantify soluble hydroxycinnamates and both cuticular and internal flavonoid glycosides. CO_2-based phenylpropanoid accumulation was then calculated by normalizing the increase in the leaf concentration of both hydroxycinnamates and individual flavonoid glycosides on the basis of assimilated CO_2, over the whole experimental period. Chlorophyll fluorescence analysis was conducted on three leaves per treatment, at 10-d intervals over the experimental period. Activities of SOD, ASCPx, CAT and PAL, and the content of malondialdehyde were estimated on three replicate samples after 10 and 25 d of light treatment. Fluorescence microspectroscopy and fluorescence microimaging were performed on six cross-sections taken from three replicate leaves at the end of the experiment. A total of 90 spectra were recorded for each individual tissue layer, namely adaxial and abaxial epidermal layers (cuticle plus epidermis), and both palisade and spongy tissue layers. All data, except those for surface morphology and CO_2-based phenylpropanoid accumulation and fluorescence spectroscopy, were subjected to a two-way analysis of variance (ANOVA), where species (S) and light (L) were modelled as fixed factors, with their interaction (S × L).

Results

Ligustrum vulgare and *Phillyrea latifolia* differed markedly in the morpho-anatomical traits of their leaves, irrespective of

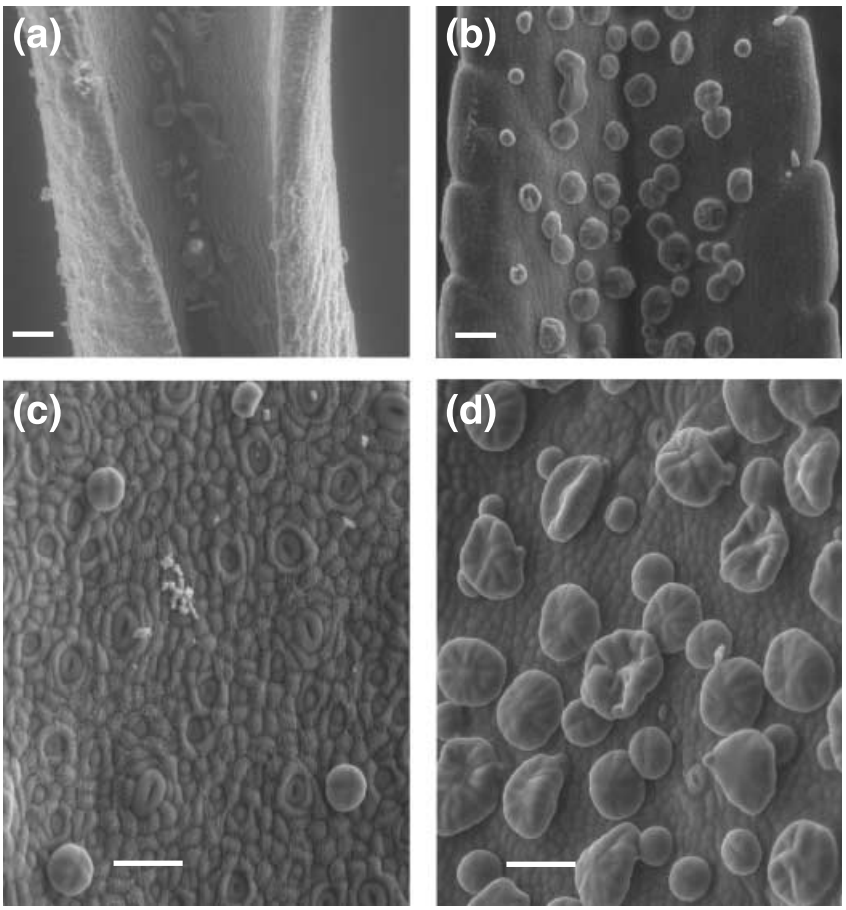


Fig. 1 Adaxial (a,b) and abaxial (c,d) surface morphology of *Ligustrum vulgare* (a,c) and *Phillyrea latifolia* (b,d) leaves, showing glandular trichome distribution. Environmental scanning electron microscopy (ESEM) analyses were performed in low-vacuum mode (chamber pressure kept at 1 Torr) on the youngest leaves (the area of which was approx. 1.2 mm^2) developing at the sun site. Bar, 100 μm (a,b) or 50 μm (c,d).

sunlight irradiance (Table 1; Fig. 1). LMA and the thicknesses of various leaf tissues, particularly the thicknesses of adaxial and abaxial cuticle layers, were much greater in *P. latifolia* than in *L. vulgare* (Table 1). Light-induced changes in leaf anatomy, mainly changes in the thicknesses of mesophyll tissues, did not differ significantly between the examined species. In contrast, leaf angle varied to a much greater extent in *L. vulgare* than in *P. latifolia* under high solar irradiance. In general, *L. vulgare* and *P. latifolia* mainly differed in their leaf surface morphology (Fig. 1), as few glandular trichomes (mostly distributed along the main vein and scarce on the remaining leaf blade) were detected on the adaxial surface of *L. vulgare* (Fig. 1a), while they densely covered that of *P. latifolia* (Fig. 1b). Furthermore, the frequency of trichomes (320 ± 24 vs 28 ± 4 trichomes mm^{-2}; mean ± standard error of the mean) on the abaxial surface, as well as trichome size (57 ± 4 vs 25 ± 3 μm), was dramatically greater in *P. latifolia* than in *L. vulgare* (Fig. 1c and d). Finally, differentiation of abaxial epidermal cells in emerging leaves of *P. latifolia* mainly produced trichomes rather than stomata.

The efficiencies of both absorbing photons over the PAR waveband ($A_{400–700}$) and scattering light (R/T_{850}; an estimate of the pathlength of photons within the leaf; Vogelmann, 1993) were significantly greater in *L. vulgare* than in *P. latifolia*, irrespective of sunlight (Fig. 2a and b). Chl_{tot}, expressed on a dry weight basis, was dramatically higher in *L. vulgare* than in *P. latifolia* at the shade site, and decreased to a much greater extent in the former (−65%) than in the latter (−41%) under high solar radiation (Fig. 2c). Moreover, Chl_{tot}, on a leaf area basis, was much lower in sun-exposed leaves than in shade leaves in *L. vulgare* (41.7 and 26.9 μg cm^{-2}, respectively), but was much higher in sun-exposed leaves than in shade leaves in *P. latifolia* (35.1 and 44.3 μg cm^{-2}, respectively). Finally, the Car : Chl_{tot} ratio also varied to a much greater extent in *L. vulgare* (+70%) than in *P. latifolia* (+25%) under high solar irradiance (Fig. 2d). Net carbon gain (as estimated from daily measurements of both diurnal net CO_2 assimilation and night-time respiration rates) did not differ between *L. vulgare* (3.2 mol CO_2 m^{-2}) and *P. latifolia* (3.0 mol CO_2 m^{-2}) at the shade site, but was substantially greater in *P. latifolia* (7.4 mol CO_2 m^{-2}) than in *L. vulgare* leaves (5.6 mol CO_2 m^{-2}) at the sun site.

Physiological adjustments to counter high solar irradiance-induced perturbations on PSII were also significantly greater in *L. vulgare* than in *P. latifolia*, particularly during the first 3 wk of exposure at the sun site (Table 2). *L. vulgare* leaves suffered from a greater excitation pressure on PSII (estimated from measurements of the reduction state of primary acceptors;

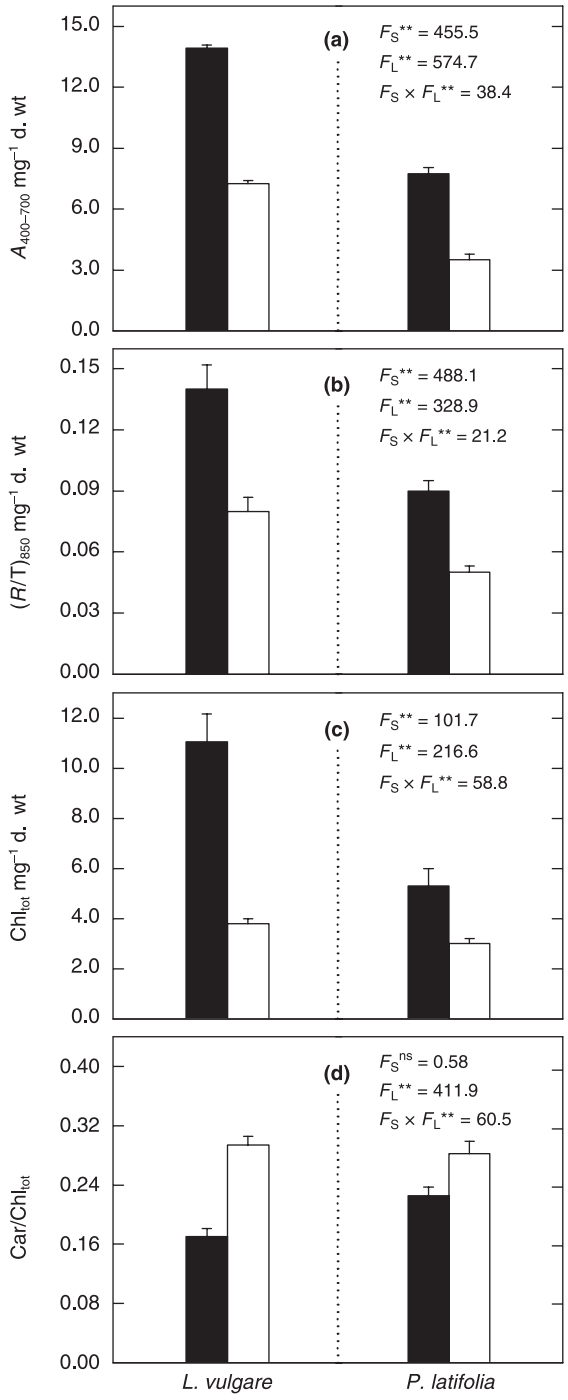


Fig. 2 Absorptance efficiency over the 400–700 nm waveband (a), scattering efficiency (b), total chlorophyll content (c) and the ratio of the content of carotenoids to that of chlorophyll (Car : Chl_{tot} ratio) (d) in *Ligustrum vulgare* and *Phillyrea latifolia* leaves exposed to 12% (closed bars) or 100% (open bars) solar radiation over a 4-wk period. The result of a two-way analysis of variance (ANOVA) [total error degrees of freedom (df) = 15] for variation in both optical features (data were arcsine-transformed before statistical treatment) and photosynthetic pigments, with species (S) and light (L) as fixed factors, with their interaction (S × L), is shown. **, $P < 0.001$; *, $0.05 > P > 0.001$; ns, not significant. Data are mean ± standard deviation; $n = 4$.

$1 - q_p$) and removed excess energy through nonphotochemical quenching mechanisms (q_{NP}) to a greater extent than *P. latifolia* leaves, at the sun site. Consistently, the actual potential efficiency of PSII (Φ_{PSII}) of *P. latifolia* leaves significantly exceeded that of *L. vulgare* leaves at the sun site. In contrast, species-specific responses to high solar irradiance were not detected in terms of the maximum potential efficiency (F_v/F_m) of PSII (Table 2).

The antioxidant enzyme system in *P. latifolia* appeared to be 'constitutively' more efficient than that in *L. vulgare* in removing reactive oxygen species. The activities of SOD and CAT in *P. latifolia* were much higher than those in *L. vulgare* at the shade site (Fig. 3a and c). Light-induced changes in SOD activity were similar in *L. vulgare* and *P. latifolia*, while the activity of CAT rose to a much greater extent in the former (still remaining lower than in *P. latifolia*) under high solar radiation. However, the examined species had similar ASCPx activity at the shade site (Fig. 3b), but only in *P. latifolia* did ASCPx activity increase steeply (+270%) under high solar radiation. Consistently, the content of MDA, here taken as an indicator of membrane lipid peroxidation, increased to a greater extent in *L. vulgare* than in *P. latifolia* under high solar radiation (Fig. 3d). Finally, the examined species did not differ (Fig. 3e) in their PAL activity (the enzyme involved in the first committed step of the phenylpropanoid biosynthetic pathway) at the shade site, but the light-induced increase in PAL activity was much greater in *L. vulgare* (+300%) than in *P. latifolia* (+68%).

The amount of daily assimilated carbon devoted to the synthesis of 'internal' soluble phenylpropanoids was consistently greater in *L. vulgare* (+22%) than in *P. latifolia* leaves at the sun site, although phenylpropanoid accumulation, on the basis of assimilated CO_2, was lower in the former at the shade site (Table 3). On the whole, the CO_2-based accumulation of 'internal' flavonoid glycosides was most strongly affected by high solar radiation in both species, as the hydroxycinnamate to flavonoid ratio was much lower in sun-exposed (0.50) than in shade (0.97) leaves. However, the usage of newly assimilated carbon for the synthesis of flavonoid glycosides with ortho-dihydroxylated B-rings, relative to that of mono-hydroxy-substituted apigenin glycosides, was substantially greater in *L. vulgare* than in *P. latifolia*, at the sun site. Moreover, the que 3-O-rut : lut 7-O-glc ratio increased sharply in *L. vulgare*, but not in *P. latifolia*, in response to high solar irradiance (Table 3). Finally, the concentration of cuticular flavonoids (aglycones of quercetin, luteolin and apigenin) was constitutively higher (+230%) in *P. latifolia* than in *L. vulgare* (Table 4), and varied similarly with sunlight in both species.

The tissue-specific distribution of flavonoids, as revealed by both fluorescence spectroscopy and fluorescence microimaging (Figs 4 and 5), was closely related to the contents of both 'cuticular' flavonoid aglycones and 'internal' flavonoid glycosides. The fluorescence intensity (maximum peak at around 565–570 nm) of epidermal layers, which included both

Table 2 Time-course of maximum efficiency of PSII photochemistry (F_v/F_m), PSII quantum yield in light conditions (Φ_{PSII}), reduction state of PSII reaction centres ($1 - q_P$), and nonphotochemical quenching (q_{NP}) of *Ligustrum vulgare* and *Phillyrea latifolia* leaves exposed to 12 or 100% solar radiation over a 4-wk period

		Sunlight irradiance						
		12%		100%				
Days of exposure	Parameter	*L. vulgare*	*P. latifolia*	*L. vulgare*	*P. latifolia*	F_S	F_L	$F_{S\times L}$
10	F_v/F_m	0.83 ± 0.007	0.80 ± 0.026	0.75 ± 0.011	0.71 ± 0.025	11.1*	72.5**	0.4 ns
	Φ_{PSII}	0.70 ± 0.011	0.69 ± 0.017	0.35 ± 0.019	0.46 ± 0.031	17.9*	562.4**	23.6*
	$1 - q_P$	0.08 ± 0.004	0.07 ± 0.015	0.33 ± 0.027	0.24 ± 0.028	21.4*	292.7**	11.6*
	q_{NP}	0.28 ± 0.023	0.25 ± 0.052	0.81 ± 0.041	0.58 ± 0.102	13.1*	147.9**	7.7*
20	F_v/F_m	0.83 ± 0.015	0.81 ± 0.014	0.79 ± 0.018	0.75 ± 0.013	11.8*	20.6*	0.2 ns
	Φ_{PSII}	0.74 ± 0.007	0.72 ± 0.017	0.49 ± 0.022	0.59 ± 0.011	18.3*	472.8**	42.3**
	$1 - q_P$	0.03 ± 0.008	0.03 ± 0.004	0.20 ± 0.022	0.13 ± 0.007	21.9*	299.9**	19.5*
	q_{NP}	0.24 ± 0.019	0.20 ± 0.011	0.65 ± 0.044	0.39 ± 0.018	93.8**	407.8**	1.3 ns
30	F_v/F_m	0.83 ± 0.008	0.84 ± 0.014	0.83 ± 0.006	0.84 ± 0.012	1.9 ns	0.4 ns	0.6 ns
	Φ_{PSII}	0.74 ± 0.011	0.74 ± 0.032	0.61 ± 0.014	0.67 ± 0.015	5.4*	79.0**	7.03*
	$1 - q_P$	0.04 ± 0.005	0.04 ± 0.016	0.15 ± 0.015	0.09 ± 0.009	16.9*	123.3**	16.5*
	q_{NP}	0.25 ± 0.011	0.26 ± 0.012	0.47 ± 0.063	0.44 ± 0.021	0.6 ns	103.3**	1.3 ns

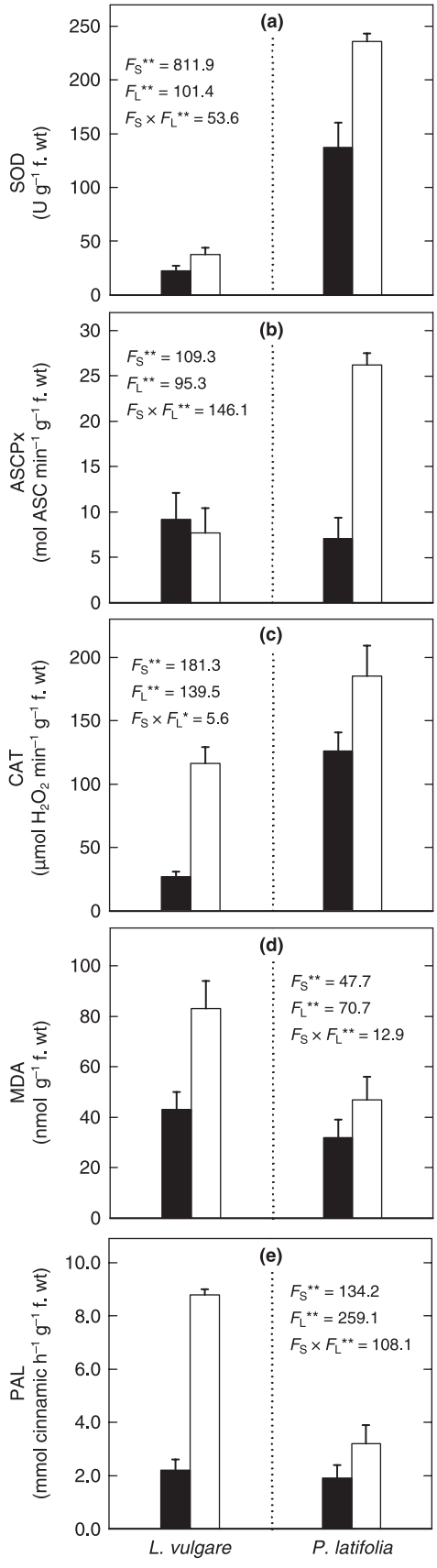
Analyses were performed on three replicate leaves after 10, 20 and 30 d of exposure to contrasting solar radiation. The result of a two-way analysis of variance (ANOVA) [degrees of freedom (df) = 11] for variation in chlorophyll fluorescence-derived parameters, with species (S) and light (L) as fixed factors, with their interaction (S×L), is shown at each sampling date. Statistical treatment of data as reported in Table 1. **, $P < 0.001$; *, $0.05 > P > 0.001$; ns, not significant. Data are mean ± standard deviation; $n = 3$.

Table 3 The CO_2-based accumulation (μmol mol^{-1} CO_2) of internal flavonoid glycosides and hydroxycinnamates in the leaf tissue of *Ligustrum vulgare* and *Phillyrea latifolia* exposed to 12 or 100% solar radiation over a 4-wk period

	Sunlight irradiance			
	12%		100%	
Pigment	*L. vulgare*	*P. latifolia*	*L. vulgare*	*P. latifolia*
Que 3-O-rut	24.5 ± 4.2	35.4 ± 8.5	192.4 ± 22.8	69.8 ± 12.0
Lut 7-O-glc	59.4 ± 9.7	64.8 ± 9.6	211.5 ± 32.1	167.7 ± 23.2
Api 7-O-gly	41.6 ± 4.9	65.8 ± 9.8	34.6 ± 5.8	59.7 ± 9.2
Hydroxycinnamates	122.5 ± 13.7	160.1 ± 24.3	152.0 ± 22.7	192.8 ± 22.0

Analyses were performed on leaves previously treated with diethyl ether to remove cuticular flavonoids (Gould *et al*., 2000). Flavonoid glycosides and hydroxycinnamates were extracted with EtOH/H_2O (75 : 25, volume/volume), and quantified by high-performance liquid chromatography with a diode array detector (HPLC-DAD). Api 7-O-gly includes both apigenin 7-O-glucoside (api 7-O-glc) and apigenin 7-O-rutinoside (api 7-O-rut). The CO_2-based accumulation was calculated by normalizing the light-induced changes in the tissue concentration of individual phenylpropanoids on the basis of assimilated CO_2 over the 4-wk period (Eqn 1). Data are mean ± standard deviation; $n = 4$.
Que 3-O-rut, quercetin 3-O-rutinoside; Lut 7-O-glc, luteolin 7-O-glc; Api 7-O-gly, apigenin 7-O-glycosides.

cuticle and epidermis, was approx. 3.5 times greater in *P. latifolia* than in *L. vulgare* (Fig. 4a and c). By contrast, que 3-O-rut and lut 7-O-glc (the contribution of apigenin derivatives to the fluorescence signal at the 570–600 nm waveband has been shown to be negligible under excitation at 488 nm; Agati *et al*., 2002) in the leaf mesophyll accumulated to a much greater extent in *L. vulgare* than in *P. latifolia* in response to high solar radiation (Fig. 4b and d). Specifically, quercetin and luteolin glycosides appreciably accumulated in the adaxial portion of palisade tissue in *P. latifolia* (Fig. 5d) or throughout the whole palisade parenchyma in *L. vulgare* (Fig. 5c), respectively, at the sun site. Finally, the cuticles appeared to be the tissues with the highest flavonoid concentration (Fig. 5a and b), although our false-colour representations did not actually separate the contribution of 'pure cuticular' from that of 'epidermal' flavonoids (Fig. 5c and d). The greatest concentration of flavonoids in the 'cuticular' layers merely depended on the small 'volume' in which cuticular flavonoids were actually



'dissolved', as the concentration of internal flavonoid glycosides (201.6 nmol cm^{-2}) was much greater than that of cuticular flavonoid aglycones (37.8 nmol cm^{-2}).

Discussion

The data obtained in our experiment highlight the role played by flavonoids in the biochemical strategies adopted by *L. vulgare* and *P. latifolia* to cope with high solar radiation (Caldwell *et al.*, 1995; Gould *et al.*, 2000; Tattini *et al.*, 2000; Tattini *et al.*, 2004). In particular, we found that 'constitutive' morphological and anatomical features strictly determined the accumulation of 'mesophyll' ortho-dihydroxylated B-ring flavonoid glycosides (Semerdjieva *et al.*, 2003b), probably controlling the degree to which oxidative load was generated in response to high solar radiation (Babu *et al.*, 2003; Tattini *et al.*, 2004).

We note, first, that the suite of morphological and anatomical traits in the examined species (Lambers *et al.*, 1998), which reflects the evolutionary adaptations of *P. latifolia* and *L. vulgare* in harsh and shade areas of the Mediterranean basin, respectively (Brosse, 1979; Tattini *et al.*, 2000, 2004), largely controlled both the absorption and the distribution of sunlight radiation in the leaf (DeLucia *et al.*, 1992; Day, 1993; Vogelmann, 1993). The greater thickness of cuticle, coupled with the greater thickness and compactness (data not shown) of the palisade parenchyma tissue, probably gave *P. latifolia* a greater ability to both limit the absorption of light and decrease the pathlength of photons within the leaf (Fig. 2; Caldwell *et al.*, 1983; Knapp *et al.*, 1988; McClendon & Fukshansky, 1990; Gonzales *et al.*, 1996), and, at the same time, allowed a more equal distribution of highly collimated solar radiation in deeper tissues as compared with *L. vulgare* (Bornman & Vogelmann, 1988; Evans, 1999). The programming of epidermal cell differentiation to glandular trichomes in *P. latifolia* (Fig. 1b and d) and *L. vulgare* (Fig. 1a and c), which also reflects species-specific evolution in areas of contrasting light and water availability (Ehleringer *et al.*, 1976; Glover, 2000; Pérez-Estrada *et al.*, 2000), was of particular interest. Glandular trichomes, in fact, have been shown to efficiently protect sensitive targets in the leaf from highly

Fig. 3 The activity of (a) superoxide dismutase (SOD), (b) ascorbate peroxidase (ASCPx) and (c) catalase (CAT), (d) the content of malondialdehyde (MDA) and (e) the activity of phenylalanine ammonia lyase (PAL) in *Ligustrum vulgare* and *Phillyrea latifolia* leaves exposed to 12% (closed bars) or 100% (open bars) solar radiation over a 4-wk period. Analyses were conducted on three replicate leaves, after 10 and 25 d of exposure to light treatment. Data have been pooled together before statistical treatment of data. The result of a two-way analysis of variance (ANOVA) [degrees of freedom (df) = 23] for variation in the activity of antioxidant enzymes, and both MDA content and PAL activity, with species (S) and light (L) as fixed factors, with their interaction (S×L) is shown. **, $P < 0.001$; *, $0.05 > P > 0.001$. Data are mean ± standard deviation (SD); $n = 6$.

Table 4 The concentration (nmol cm^{-2}) of flavonoid aglycones in the cuticular layer of *Ligustrum vulgare* and *Phillyrea latifolia* leaves exposed to 12 or 100% sunlight irradiance over a 4-wk period

	Sunlight irradiance						
	12%		100%				
Flavonoid	*L. vulgare*	*P. latifolia*	*L. vulgare*	*P. latifolia*	F_S	F_L	$F_{S\times L}$
Quercetin	1.5 ± 0.4	3.6 ± 0.6	5.9 ± 0.9	15.6 ± 2.2	100.2**	155.7**	43.4**
Luteolin	3.3 ± 0.7	6.5 ± 1.0	10.1 ± 1.9	20.2 ± 2.7	52.3**	144.1**	11.3*
Apigenin	2.6 ± 0.6	5.4 ± 1.1	7.5 ± 1.3	16.5 ± 2.1	72.4**	139.4**	19.7**
Total	7.4 ± 1.3	15.7 ± 1.5	23.5 ± 4.1	52.4 ± 6.5	87.9**	177.5**	27.2**

Cuticular flavonoids were removed by rinsing leaves with diethyl ether for 2 h following the protocol of Gould *et al*. (2000). The diethyl ether fraction was then analysed by high-performance liquid chromatography with a diode array detector (HPLC-DAD) to identify and quantify flavonoids. The result of a two-way analysis of variance (ANOVA) [degrees of freedom (df) = 15] for variation in the concentration of flavonoids with species (S) and light (L) as fixed factors, with their interaction (S×L) is shown.
**, $P < 0.001$; *, $0.05 > P > 0.001$. Data are mean ± standard deviation; $n = 4$.

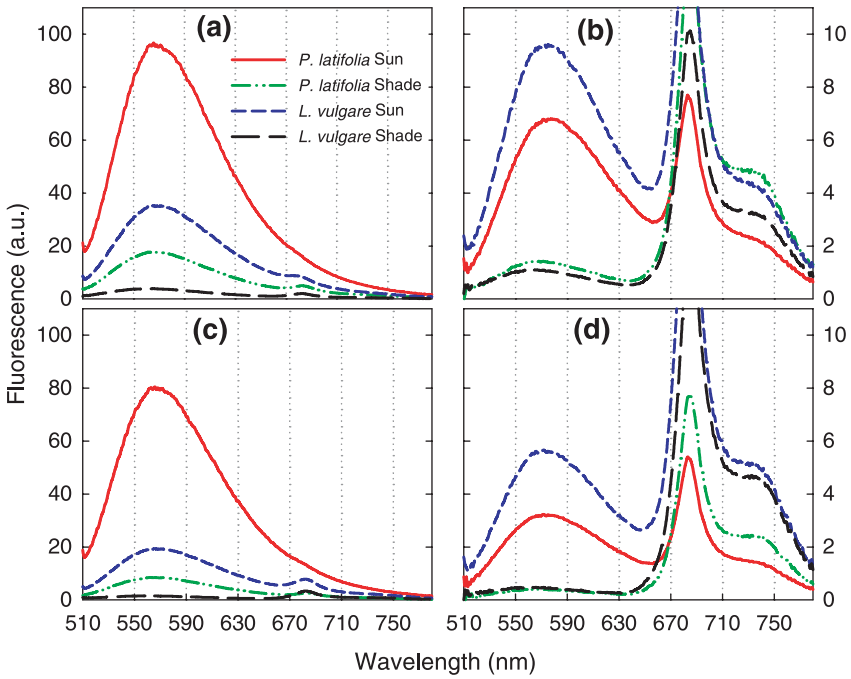


Fig. 4 Tissue fluorescence spectra of *Ligustrum vulgare* and *Phillyrea latifolia* leaves exposed to 12% or 100% solar radiation, showing the flavonoid distribution in adaxial (a) and abaxial (c) epidermal (cuticle plus epidermis), and both palisade (b) and spongy (d) parenchymal tissues. Cross-sections were stained with Naturstoff reagent and excited at 488 nm. Fluorescence intensities, over the 510–800 nm waveband, were recorded using an inverted epifluorescence microscope coupled to a multichannel spectral analyser (Agati *et al*., 2002) on a total of 90 spectra for each tissue layer.

damaging short solar wavelengths (see below; Karabourniotis *et al*., 1993; Tattini *et al*., 2000; Agati *et al*., 2002; Manetas, 2003), in addition to increasing reflectance over the visible waveband (Johnson, 1975; Pierce *et al*., 2001). From this standpoint, the much steeper angle at which *L. vulgare* leaves developed at the sun site (Table 1), together with leaf rolling (data not shown), might be explained simply by the greater need of *L. vulgare* to prevent light reaching the leaf surface, as compared with *P. latifolia* (Ehleringer & Comstock, 1987; Werner *et al*., 1999). We also suggest that the greater variation in leaf angle might have been partially responsible for the smaller net carbon gain detected in *L. vulgare* (5.6 mol CO_2 m^{-2}) than in *P. latifolia* leaves (7.4 mol CO_2 m^{-2}) at the sun site (Falster & Westboy, 2003).

Secondly, we suggest that the suites of morpho-anatomical traits in *L. vulgare* and *P. latifolia* leaves produced strikingly different frameworks within which physiological and biochemical features underwent adjustments under high solar radiation (Mittler, 2002; Pastori & Foyer, 2002). Consistently, loss of chlorophyll, which may effectively help to reduce light-induced perturbations on PSII merely by reducing centres of light absorption (Kyparissis *et al*., 1995; Havaux & Tardy, 1999), only played a role in the acclimation mechanisms of *L. vulgare* to high solar radiation (Fig. 2c). Nevertheless, excitation pressure on PSII was greater and nonphotochemical quenching mechanisms were activated to a substantially greater extent (which, in turn, was responsible for differential light-induced variation in Φ_{PSII}) in *L. vulgare* than in *P. latifolia* (Table 2) under high solar

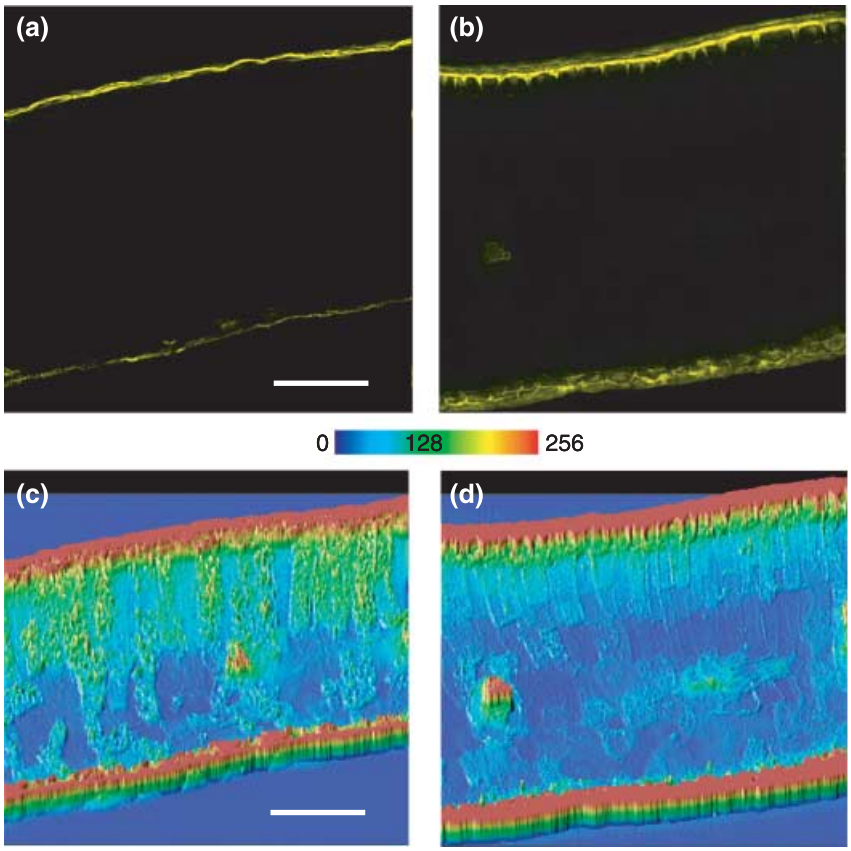


Fig. 5 Fluorescence images (a,b) recorded at 580 nm (F_{580}) and false-colour representations of flavonoid distribution throughout the leaf (c,d) in *Ligustrum vulgare* (a,c) and *Phillyrea latifolia* (b,d) at the sun site. The F_{580} signal is shown in yellow for illustrative purposes only. False-colour representations of flavonoid fluorescence (c,d) are shown on an expanded scale (×3 compared with F_{580}). F_{580} intensity increases from blue to red. Bar, 100 μm.

radiation (Björkman & Demmig-Adams, 1995; Demmig-Adams & Adams III, 1996; Müller *et al.*, 2001). Conversely, the retention of chlorophyll in *P. latifolia* at the sun site (which reflects the ability of the species to screen out UV radiation and should be taken as an indicator of lower sensitivity to high sunlight as compared with *L. vulgare*; Greenberg *et al.*, 1997; Kirchgessner *et al.*, 2003), coupled with the relatively low ability of the species to quench excess energy through non-photochemical mechanisms (Table 2; Munné-Bosch & Peñuelas, 2003), should have increased considerably the generation of reactive oxygen, unless antioxidant enzymes operated efficiently (Alscher *et al.*, 1997; Asada, 1999). However, both the 'constitutive' (in leaves at the shade site) and light-inducible systems of antioxidant enzymes (Fig. 3a and b) should have preserved chloroplasts from an excessive load of reactive oxygen to a greater extent in *P. latifolia* than in *L. vulgare* (Foyer *et al.*, 1994; Asada, 1999). This idea is further supported here by the extent of oxidative stress, which was estimated on the basis of bulk lipid peroxidation (Fig. 3d), actually suffered by *L. vulgare* and *P. latifolia* leaves when exposed to high solar radiation (Britt, 1996).

Finally, we note that the denser indumentum of glandular trichomes coupled with the 'constitutively' higher concentration of cuticular flavonoids in *P. latifolia* (Table 4) probably preserved the mesophyll cells from UV-induced perturbations to a greater extent in *P. latifolia* than in *L. vulgare* (Krauss *et al.*, 1997; Stephanou & Manetas, 1997; Krause *et al.*, 2003; Semerdjieva *et al.*, 2003b). We hypothesize that these perturbations were mainly of oxidative origin, and strictly controlled the light-induced increases in both the activity of PAL (Fig. 3e) and the accumulation of mesophyll flavonoids (Liu & McClure, 1995; Kalbin *et al.*, 2001; Mackerness *et al.*, 2001; Babu *et al.*, 2003), particularly that of ortho-dihydroxylated B-ring metabolites (Table 3), in the examined species (Figs 4 and 5). In fact, high solar radiation did not affect the CO_2-based accumulation of apigenin derivatives and slightly increased that of hydroxycinnamates (Table 3), which are as efficient as (or even more efficient than; Sheahan, 1996; Gould *et al.*, 2000) flavonoids with ortho-dihydroxylated B-rings in absorbing wavelengths in the 280–390 nm waveband (Tattini *et al.*, 2004). Therefore, the finding of a greater accumulation of mesophyll ortho-dihydroxylated flavonoids, coupled with the higher flavonoid to hydroxycinnamate and que 3-O-rut to lut 7-O-glc ratios in *L. vulgare* than in *P. latifolia* at the sun site (Table 3), is hard to explain simply by the greater need of the former to absorb deep-penetrating UV-A wavelengths in the leaf interior (as a result of the lower UV-screening effectiveness offered by cuticular flavonoid aglycones; Figs 4 and 5). Rather, ortho-dihydroxylated flavonoids, particularly que 3-O-rut, probably played key roles as both scavengers of reactive oxygen and excess energy dissipaters in highly disturbed mesophyll cells (Yamasaki

et al., 1997; Markham *et al.*, 1998; Smith & Markham, 1998). As a consequence, the greater shift in both the general phenylpropanoid (Christensen *et al.*, 1998; Schoch *et al.*, 2001) and the flavonoid-branch biosynthetic pathways (Ryan *et al.*, 1998; Olsson *et al.*, 1999; Agati *et al.*, 2002; Tattini *et al.*, 2004), i.e. metabolic plasticity as defined by Logemann *et al.* (2000), detected in *L. vulgare* than in *P. latifolia* (Table 3) can probably be interpreted as reflecting a greater need of the former to counter oxidative damage (Beggs *et al.*, 1985; Olsson *et al.*, 1998; Babu *et al.*, 2003) caused by high solar radiation. This idea is supported here by the observation that the same flavonoids accumulated both on the cuticular layer (Table 4) and in the leaf interior (Table 3), although cuticular and internal flavonoids should serve or have to serve very different functions in the response to high solar irradiance (Stephanou & Manetas, 1997; Gould *et al.*, 2000; Krause *et al.*, 2003). We hypothesize that highly specialized (Gravano *et al.*, 1998; M. Tattini, unpublished) glandular trichomes (which are autonomous in phenylpropanoid metabolism; Fahn, 1986, 1988; Tattini *et al.*, 2000) and mesophyll tissues experienced similar oxidative stress caused by high solar radiation in the examined species. In support of this hypothesis was the almost complete deactivation of the hydroxycinnamate-branch pathways in favour of flavonoid biosynthesis previously detected in glandular trichomes of *P. latifolia* leaves, when exposed to an acute UV-radiation stress (Tattini *et al.*, 2000; Agati *et al.*, 2002).

We conclude that flavonoids with ortho-dihydroxylated B-rings probably accumulated as a consequence of high solar radiation-induced oxidative damage in the mesophyll of *L. vulgare* and *P. latifolia* leaves. Therefore, accumulation of flavonoids in response to high solar radiation appeared to be unrelated to tolerance to high solar radiation in the examined species. Further experiments to estimate the tissue-specific location of flavonoids, a prerequisite for elucidating their actual functions in the response mechanisms to high solar radiation, are urgently needed to address the questions raised in the present work.

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