



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

## FLORE

# Repository istituzionale dell'Università degli Studi di Firenze

### **Flavonoids in glandular trichomes of *Phillyrea latifolia* L. (Oleaceae) play a key role in the mechanisms of acclimation to excess of solar**

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Flavonoids in glandular trichomes of *Phillyrea latifolia* L. (Oleaceae) play a key role in the mechanisms of acclimation to excess of solar radiation / M. TATTINI; E. GRAVANO; P. PINELLI; N. MULINACCI; A. ROMANI. - In: NEW PHYTOLOGIST. - ISSN 0028-646X. - STAMPA. - 148(2000), pp. 69-77.

*Availability:*

This version is available at: 2158/219262 since:

*Terms of use:*

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

*Publisher copyright claim:*

(Article begins on next page)

# Flavonoids accumulate in leaves and glandular trichomes of *Phillyrea latifolia* exposed to excess solar radiation

M. TATTINI<sup>1\*</sup>, E. GRAVANO<sup>2</sup>, P. PINELLI<sup>3</sup>, N. MULINACCI<sup>3</sup>  
AND A. ROMANI<sup>3</sup>

<sup>1</sup>*Istituto sulla Propagazione delle Specie Legnose – Consiglio Nazionale delle Ricerche, Via Ponte di Formicola 76, I-50018 Scandicci, Florence, Italy*

<sup>2</sup>*Dipartimento di Biologia Vegetale, Università di Firenze, Piazzale delle Cascine 28, I-50144 Florence, Italy*

<sup>3</sup>*Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via G. Capponi 9, I-50121 Florence, Italy*

Received 5 July 1999; accepted 26 May 2000

## SUMMARY

Experiments were conducted on *Phillyrea latifolia* plants grown under a dense overstorey of *Pinus pinea* (shade plants) or on seashore dunes (sun plants) in a coastal area of Tuscany (42° 46' N, 10° 53' E). Total integrated photon flux densities averaged 1.67 and 61.4 m mol m<sup>-2</sup> d<sup>-1</sup> for shade and sun sites, respectively. A leaf morphological–structural analysis, a qualitative and quantitative analysis of phenylpropanoids of leaf tissue and leaf surface, and a histochemical localization of flavonoids were conducted. The area of sun leaves reached 57% of that of shade leaves, whereas leaf angle ( $\beta$ ), sclerophylly index (ratio of leaf d. wt:leaf area), and trichome frequency (trichome number mm<sup>-2</sup>) were markedly greater in leaves exposed to full solar radiation than in leaves acclimated to shade. The total thickness of sun leaves was 78% higher than that of shade leaves, mostly owing to a greater development of both palisade parenchyma and spongy mesophyll. The concentration, but not the composition, of leaf tissue phenylpropanoids varied significantly between sun and shade leaves, with a marked increase in flavonoid glycosides in sun leaves. Flavonoids occurred almost exclusively in the upper epidermal cells of shade leaves. By contrast, flavonoids largely accumulated in the upper and lower epidermis, as well as in the mesophyll tissue of leaves that were acclimated to full sunlight. Flavonoid glycosides were found exclusively in the secretory products of glandular trichomes of *P. latifolia* leaves exposed to high levels of light; luteolin 7-*O*-glucoside and quercetin 3-*O*-rutinoside were the major constituents. By contrast, verbascoside and an unidentified caffeic acid derivative constituted 72% of total phenylpropanoids secreted by glandular trichomes of shade leaves, whereas they were not detected in glandular trichomes of sun leaves. These findings suggest that the light-induced synthesis of flavonoids in glandular trichomes of *P. latifolia* probably occurs *in situ* and concomitantly inactivates other branch pathways of the general phenylpropanoid metabolism. This is the first report of the key role of glandular trichomes and of flavonoid glycosides in the integrated mechanisms of acclimation of *P. latifolia* to excess light.

Key words: flavonol glycosides, fluorescence microscopy, glandular trichomes, *Oleaceae*, phenylpropanoids.

## INTRODUCTION

*Phillyrea latifolia* is an evergreen sclerophyllous shrub of the *Oleaceae*, occurring widely in the Mediterranean coastal areas (Pignatti, 1982; Gellini *et al.*, 1983). *Phillyrea* is usually faced with intense periods of water deficit during the summer season as

well as with prolonged exposure to high solar irradiance during both the summer and winter (Harley *et al.*, 1987). *P. latifolia* is also distributed on seashore dunes, where excess soil salinity and salt spray are additional stressful agents. *P. latifolia* is highly tolerant to salinity stress, and excretion of toxic ions by 'salt glands' has previously been suggested as largely contributing to the salinity tolerance of the species (Gucci *et al.*, 1997). However, recent experiments have shown that glandular

\*Author for correspondence (fax +39 55 755 121; e-mail tattini@ipsl.fi.cnr.it).

trichomes of *P. latifolia* should be considered facultative 'salt glands', because their actual contribution to the toxic ion balance within the whole plant and the leaf is negligible (Gravano *et al.* 1998; Tattini & Gucci, 1999).

We therefore hypothesized that the functional role of the glandular trichomes of *P. latifolia* might be related to the species evolution in environments where excess solar irradiance, in combination with water deficit, is one of the major environmental constraints. It has previously been reported that in several species found in arid environments a dense covering of wax and trichomes might help in decreasing water loss by lowering the amount of light absorbed by the leaf and the accompanying heat load (Ehleringer *et al.*, 1976; Vogelmann, 1993). Flavonoids, especially flavonoid aglycones (Wollenweber, 1993), accumulate on the leaf surface of species from arid or semi-arid habitats and can act as filters for UV radiation (Wollenweber & Dietz, 1981; Fahn, 1988). In *Olea europaea*, another member of the Oleaceae that is widely distributed in the Mediterranean basin, the deposition of polyphenols in leaf hairs has been suggested to protect leaf cells from damage by UV radiation (Karabourniotis *et al.*, 1998). The ability of flavonoids to screen out UV wavelengths while maintaining the normal flux of PAR (400–700 nm) also decreases the photoinhibition of photosynthesis in plants suffering from excess light in combination with other environmental constraints (Björkman & Demmig-Adams, 1995).

Phenolic acids, the secoiridoids oleuropein and oleuropein aglycone, verbascoside and the flavonol glycosides (i.e. glycosides of luteolin, apigenin, and quercetin) have previously been detected as the major phenylpropanoids in the leaf mesophyll of *Phillyrea* spp. (Romani *et al.*, 1996). Some of the phenylpropanoids already mentioned have interesting antioxidant properties (Takahama, 1989; Yamasaki *et al.*, 1997) in addition to their ability to screen off excess UV radiation (Caldwell *et al.*, 1983; Shirley, 1996), and might protect cell metabolism from the detrimental effects of reactive oxygen species that occur under severe stress (Husain *et al.*, 1987).

The objective of this study, which was conducted on *P. latifolia* plants that had developed in the shade or in full sunlight, was to look for the possible role of glandular trichomes and their secretory products in the plants' integrated mechanisms of acclimation to high solar radiation. We performed morphological and structural analyses of the leaves, histochemical localization of flavonoids, and identification and quantification of phenylpropanoids in leaf tissue and on the surface. Our results suggest strongly that flavonoid secretion by glandular trichomes is a central part in the acclimation mechanisms to excess light that operate in *P. latifolia*.

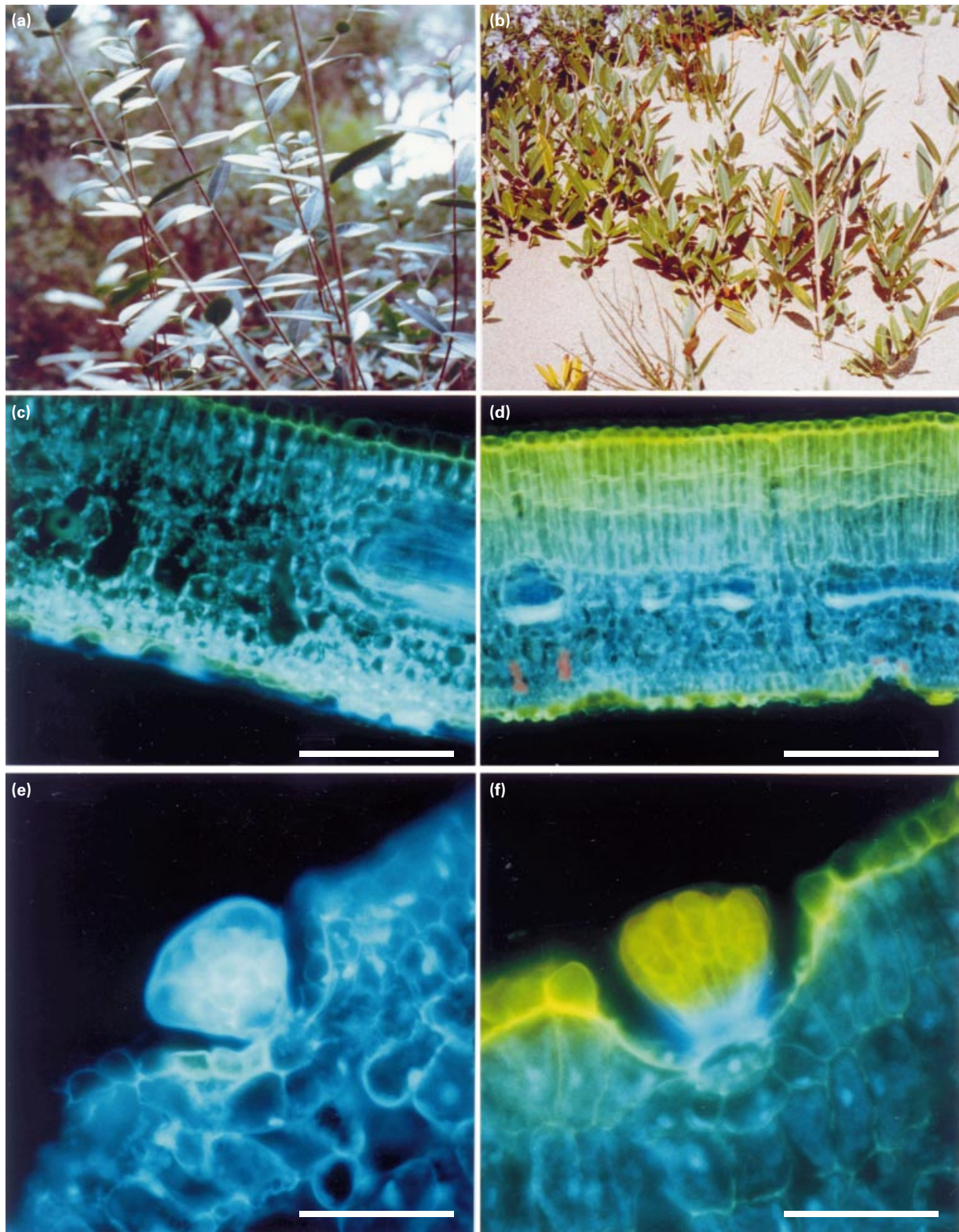
## MATERIALS AND METHODS

### *Plant material and growing conditions*

Leaves were collected from *Phillyrea latifolia* L. plants growing in the south of Tuscany at Castiglione della Pescaia (lat 42° 46' N, long 10° 53' E) at the end of September 1998. Plants were grown either under a dense overstorey of *Pinus pinea* L. (shade plants) or on seashore dunes approx. 2 m above sea level (sun plants). Plants acclimated to shade were small trees, whereas sun plants had several shoots (15–20 cm long) that emerged slightly from the sand (Fig. 1a,b). Total integrated photon flux densities were monitored on several clear days before and after the sampling date with a 1800 LI-COR spectroradiometer (LI-COR, Lincoln, NE, USA) and averaged 1.67 and 61.4 mol m<sup>-2</sup> d<sup>-1</sup> for shade and sun sites, respectively. Midday PAR values averaged 54 and 2037 µmol m<sup>-2</sup> s<sup>-1</sup> for shade and sun sites, respectively. Predawn leaf water potentials were measured as reported by Tattini *et al.* (1995) and averaged -0.6 and -1.8 MPa for shade and sun leaves, respectively.

### *Leaf morphological-structural analysis and histochemical localization of flavonoids*

Leaf area was measured with a LI-COR LI-3100 area meter, and d. wt was measured after drying the leaves at 65°C to constant weight. The sclerophylly index (g d. wt m<sup>-2</sup>) was then calculated as the ratio between leaf d. wt and leaf area. Leaf angle ( $\beta$ ) was measured with respect to the horizontal plane, as proposed by Ehleringer & Comstock (1987), with an inclinometer. Shoot portions were immersed in distilled water in Erlenmeyer flasks, sealed with plastic bags and maintained at 4°C for not more than 12 h until the structural or histochemical analyses were started. Transverse sections of fresh leaf tissue were cut with a Reichert-Jung 2800 Frigo Cut (Cambridge Instruments, Nussloch, Germany). The total leaf thickness and the thickness of upper and lower cuticular, upper and lower epidermal, palisade parenchyma and spongy mesophyll layers were quantified by means of a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) equipped with a high-resolution TK 870 E JVC video camera (JVC, Yokohama, Japan). Trichome frequency was estimated by counting the number of glandular trichomes per unit leaf area at a magnification of  $\times 50$  (Gravano *et al.*, 1998). Histochemical localization of flavonoids was performed by modifying the protocol proposed by Schnitzler *et al.* (1996). Transverse sections (30 µm) of fresh leaf tissue were stained for 1.5 min in 1% diphenylboric acid 2-amino-ethyl ester (Sigma) in ethanol (Naturstoff reagent). Section thickness and staining time were optimized in preliminary experiments in



**Fig. 1.** Morphological features of *Phillyrea latifolia* plants acclimated to shade (a) or to full solar irradiance (b). Histochemical visualization of flavonoids in leaf tissue (c,d) and in glandular trichomes (e,f) of shade (c,e) or sun (d,f) plants. Fluorescence was observed in conventional microscopy (excitation at 365 nm) with transverse sections (30  $\mu\text{m}$ ) of fresh leaf material stained for 1.5 min with Naturstoff reagent. The yellow fluorescence indicates the localization of flavonoid metabolites. Bars: 200  $\mu\text{m}$ , (c,d); 50  $\mu\text{m}$ , (e,f).

which the thickness ranged between 10 and 50  $\mu\text{m}$  and the staining time between 30 s and 5 min. Fluorescence micrographs were taken with a Zeiss

Axioplan fluorescence microscope with a 365 nm exciter filter, and a FT-395 chromatic beam splitter. A barrier filter operated at 420 nm. The yellow

fluorescence of the stained material was consistent with the occurrence of flavonoids, as reported previously (Schnitzler *et al.*, 1996).

#### *Identification and quantification of phenylpropanoids in leaf surface and leaf tissue*

Leaves were weighed fresh and rinsed with 70 ml of 95% (v/v) ethanol in 250 ml Erlenmeyer flasks for 45 s; the ethanol washing solution was maintained under ice in the dark before analysis. Leaves were quickly blotted dry on absorbing paper, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The qualitative and quantitative analysis of phenylpropanoids in leaf tissue was performed by following a protocol similar to that of Romani *et al.* (1996).

Freeze-dried leaf tissue (after the removal of midribs), ground under liquid nitrogen in a mortar with a pestle, was extracted four times with 100 ml of ethanol in water (adjusted to pH 2.5 with formic acid; 80:20, v/v). The ethanolic solution was concentrated under vacuum (Rotavapor 144 R; Büchi, Switzerland) and 50 ml was extracted four times with 30 ml of n-hexane. The ethanol phase was dried under reduced pressure, rinsed with 15 ml of water, pH 2.5, and deposited on an Extrelut<sup>®</sup> 20 prepacked cartridge (Merck, Darmstadt, Germany). Elution was performed with (1) 200 ml of n-hexane to collect a colourless eluate, (2) 250 ml of ethyl acetate, and (3) 250 ml of methanol adjusted to pH 2 with formic acid. Finally, the ethyl acetate fraction was reduced to dryness under reduced pressure, diluted with 1 ml of pH 2 water:methanol:acetonitrile (20:60:20, v/v/v) and analysed by HPLC. Phenylpropanoids secreted by glandular trichomes were analysed in the rinsing ethanol solution, which was reduced to dryness under vacuum and rinsed with 1 ml of 95% (v/v) ethanol adjusted to pH 2.5 with formic acid.

Phenylpropanoids were separated by using an HP 1090L liquid chromatograph equipped with a diode array detector (DAD) and managed by a HP 9000 workstation (Hewlett Packard, Palo Alto, CA, USA). The column was a 4.6 mm  $\times$  250 mm LiChrosorb<sup>®</sup> RP18 (5  $\mu\text{m}$ ) (Merck) maintained at  $26^{\circ}\text{C}$  and equipped with a 4 mm  $\times$  10 mm LiChrosorb<sup>®</sup> RP18 precolumn. The eluent was a four-step linear gradient of acetonitrile in water (adjusted to pH 3.2 with phosphoric acid), from 100% water to 100% acetonitrile over a 106-min period, at a flow rate of  $1.0\text{ ml min}^{-1}$ , as reported previously (Romani *et al.* 1999b). UV-visible spectra were recorded in the range 190–450 nm and chromatograms were acquired at 254, 280, 330 and 350 nm. The identification of individual polyphenols was performed by comparison of their retention times and UV-visible spectra with those of authentic standards (Extrasynthese S. A., Lyon Nord, Genay, France) or isolated compounds. Elenolic acid and verbascoside

were extracted and isolated from *O. europaea* tissues as reported in Romani *et al.* (1999a). When necessary, the identification of phenylpropanoids was made by interfacing the HPLC–DAD with an HP1100 MSDI API–electrospray mass spectrometer (Hewlett Packard) operating in the negative-ion ionization mode as reported previously (Romani *et al.*, 1999b). Quantification of individual compounds was performed with four-point calibration curves ( $r^2 \geq 0.99$ ) in the range 0–40  $\mu\text{g}$ . In detail, phenolic acids, tyrosol and hydroxytyrosol were quantified at 280 nm with syringic acid and tyrosol as references, respectively. Oleuropein and the elenolic acid derivative were calibrated and quantified at 254 nm with oleuropein as standard; verbascoside and an unknown caffeic acid derivative were calibrated and quantified at 330 nm. The actual concentrations of phenylpropanoids were determined after applying corrections for changes in molecular mass. The flavonoids were quantified at 350 nm with the use of authentic standards.

Finally, the fluorescence responses of leaf surface phenylpropanoids were compared with those of authentic standards or isolated compounds by high-performance thin-layer chromatography (HPTLC): 10  $\mu\text{l}$  of the concentrated (1 ml) rinsing ethanolic solution was deposited on 5 cm  $\times$  5 cm Silica Gel 60 F<sub>254</sub> HPTLC plates (Merck), and eluted with ethyl acetate:methanol:water : formic acid (77:13:10:4, v/v/v/v), in a Desaga horizontal separating chamber equipped with a tightly fitting glass lid (Carlo Erba, Milano, Italy). The plates were then dried at room temperature and sprayed with Naturstoff reagent and spots identified by their fluorescence characteristics at 365 nm.

#### *Experimental design and statistics*

The experimental design was a complete random, with six plants (replicates) at each site. Morphological measurements were performed on 10 leaves per plant randomly sampled from the medial part of the shoots. Leaf structural analysis was performed on six leaves per plant, by sampling two leaves from the medial part of three shoots per plant. Measurements were subjected to ANOVA and means were compared by least significant differences. The analysis of phenylpropanoids in both the leaf tissue and the leaf surface was performed on 20 leaves per plant, sampled as already described on four plants per site. Samples were collected three times during the day on 2 consecutive days. Results are reported as means  $\pm$  SD ( $n = 24$ ).

#### RESULTS

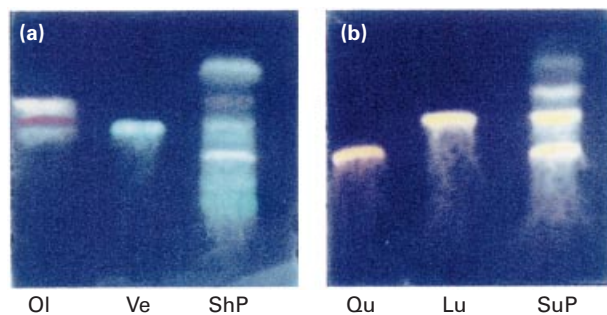
Leaves of plants acclimated to shade or full solar irradiance differ markedly in their morphological characters (Fig. 1a,b, Table 1). The leaf area of sun leaves reached only 57% of that of shade leaves,

**Table 1.** Morphological and structural features of *Phillyrea latifolia* leaves acclimated to shade or to full solar irradiance, collected at the end of September in a coastal area of Tuscany (lat 42° 46' N, long 10° 53' E)

Morphological-structural parameter	Site	
	Shade	Sun
Leaf area (cm <sup>2</sup> )	4.9 ± 0.3	2.8 ± 0.2**
Sclerophylly index (g d. wt m <sup>-2</sup> )	12.8 ± 1.7	24.2 ± 2.5**
Leaf angle (degrees)	8.4 ± 4.6	71.7 ± 9.8**
Trichome frequency (mm <sup>-2</sup> )	3.7 ± 0.5	7.1 ± 0.4**
Total leaf thickness (µm)	200.9 ± 19.5	357.8 ± 32.4**
Upper cuticle (µm)	7.1 ± 0.3	19.3 ± 0.6**
Upper epidermis (µm)	10.9 ± 0.9	16.1 ± 1.4*
Palisade parenchyma (µm)	80.4 ± 5.4	147.5 ± 14.7**
Spongy parenchyma (µm)	81.7 ± 8.8	150.4 ± 19.3**
Lower epidermis (µm)	8.7 ± 1.4	11.2 ± 2.3
Lower cuticle (µm)	7.1 ± 0.7	8.8 ± 1.1

Leaf area, leaf angle and sclerophylly index were measured on 60 leaves randomly sampled from the medial part of the shoot, from six plants.

The thickness of the whole leaf and leaf tissue layers were estimated on 36 leaf transverse sections from six plants as reported in Materials and Methods section. Results are means ± SD ( $n = 6$ ). Asterisks indicate least significant differences at the 5% (\*) or the 1% (\*\*) level.



**Fig. 2.** High-performance thin-layer chromatography (HPTLC) analysis of phenylpropanoids secreted by glandular trichomes of *Phillyrea latifolia* leaves acclimated to shade (a) or to full solar irradiance (b). Fluorescence responses of leaf surface phenylpropanoids were compared with those of authentic standards or isolated compounds, after spraying with Naturstoff reagent (details in the Materials and Methods section). Ol, oleuropein; Ve, verbascoside; ShP, shade phenylpropanoids; Qu, quercetin 3-*O*-rutinoside; Lu, luteolin 7-*O*-glucoside; SuP, sun phenylpropanoids.

whereas the sclerophylly index (g d. wt m<sup>-2</sup>) was 89% higher for sun leaves than for shade leaves. The frequency of glandular trichomes (number mm<sup>-2</sup>) was also considerably higher in sun leaves than in shade leaves (+92%). Leaves of *P. latifolia* plants acclimated to full solar radiation developed with a steep leaf angle (b), whereas shade leaves were positioned approximately parallel to the horizontal plane (Fig. 1a,b, Table 1). Total leaf thickness was 78% higher in sun leaves than in shade leaves, and was mostly accounted for by an increase in thickness of the palisade parenchyma and spongy mesophyll layers. The thicknesses of upper cuticle and epidermis were also significantly higher in sun leaves

than in shade leaves, whereas the development of both lower epidermal and cuticular layers did not significantly differ between the two types of leaf (Table 1). Marked changes in tissue compactness were also detected for leaves that had developed under different light conditions. In fact, the palisade parenchyma of sun leaves consisted of three or four layers of columnar cells with very reduced intercellular spaces, whereas the palisade parenchyma of shade leaves consisted of one or two layers of cells with larger intercellular spaces (Fig. 1c,d). Similarly, the spongy mesophyll layer of sun leaves seemed more compact than the corresponding tissue of shade leaves, as a result of a greater number of cells and smaller intercellular spaces (Fig. 1c,d). Glandular trichomes of shade and sun leaves did not differ in width and height (results not shown).

The occurrence of flavonoids in shade leaves, as evidenced by the weak yellow fluorescence of transverse sections stained with Naturstoff reagent, was minor and confined to the cells of the upper epidermis (Fig. 1c). By contrast, the intense yellow fluorescence of stained transverse sections of sun leaves clearly indicated a great accumulation of flavonoidic compounds. Flavonoids accumulated in cells of the upper and lower epidermis and also in those of palisade parenchyma, and were apparently bound to the cell wall (Fig. 1d). Flavonoids were also detected in guard cells of stomata of sun leaves. Fluorescence responses of glandular trichomes of leaves acclimated to shade or to full sunlight were also markedly different (Fig. 1e,f), and similar to those of the secretory products analysed by HPTLC. Surface phenylpropanoids of shade leaves gave a series of brightly blue spots, when sprayed with

**Table 2.** The concentration of individual phenylpropanoids in the leaf tissue and on the leaf surface of *Phillyrea latifolia* plants acclimated to shade or to full solar irradiance

Phenylpropanoid	Leaf tissue concentration ( $\mu\text{mol g}^{-1}$ d. wt)		Leaf surface concentration ( $\mu\text{mol g}^{-1}$ d. wt)	
	Shade	Sun	Shade	Sun
Syringe acid	1.01 $\pm$ 0.34	1.12 $\pm$ 0.17	15.3 $\pm$ 1.2	25.3 $\pm$ 6.8
Elenolic acid	0.91 $\pm$ 0.14	1.02 $\pm$ 0.25	7.6 $\pm$ 2.5	21.4 $\pm$ 4.3
Tyrosine	0.44 $\pm$ 0.14	1.26 $\pm$ 0.44	74.7 $\pm$ 21.5	34.5 $\pm$ 13.2
Hydroxytyrosine	0.38 $\pm$ 0.06	1.62 $\pm$ 0.32	58.2 $\pm$ 14.4	73.2 $\pm$ 17.4
Luteolin 7- <i>O</i> -glucoside	0.31 $\pm$ 0.07	3.64 $\pm$ 0.22	nd	94.6 $\pm$ 6.6
Luteolin derivative	0.09 $\pm$ 0.06	0.63 $\pm$ 0.09	nd	29.1 $\pm$ 3.4
Luteolin	0.08 $\pm$ 0.04	0.28 $\pm$ 0.03	nd	nd
Apigenin 7- <i>O</i> -rutinoside	1.12 $\pm$ 0.11	0.92 $\pm$ 0.32	nd	36.3 $\pm$ 1.5
Apigenin 7- <i>O</i> -glucoside	0.72 $\pm$ 0.21	3.37 $\pm$ 0.16	nd	35.2 $\pm$ 2.1
Quercetin 3- <i>O</i> -rutinoside	0.46 $\pm$ 0.08	5.81 $\pm$ 0.48	nd	108.1 $\pm$ 5.7
Oleuropein	0.49 $\pm$ 0.14	0.40 $\pm$ 0.03	234.0 $\pm$ 65.2	362.4 $\pm$ 26.8
Verbascoside	0.20 $\pm$ 0.06	0.14 $\pm$ 0.03	558.2 $\pm$ 34.9	nd
Caffeic acid derivative	nd	nd	446.3 $\pm$ 67.8	nd

Leaves were collected from four plants at the two sites, three times a day on two consecutive days, as shown in Table 1. Phenylpropanoids in the leaf tissue were identified and quantified after the leaves had been rinsed with 95% (v/v) ethanol for 45 s, by using HPLC equipped with a diode array detector, and by HPLC–MS analysis. The concentration of phenylpropanoids in the secretory products of glandular trichomes was estimated by analysing the rinsing ethanol solution. Results are means  $\pm$  SD ( $n = 24$ ). nd, not detected.

Naturstoff reagent, that were consistent with a large quantity of phenolic acids (Wagner & Bladt, 1996) and verbascoside. In addition, the white–yellow spot corresponded to the fluorescence response of an authentic standard of oleuropein (namely, the upper band of the three-band series of the commercial oleuropein standard) (Fig. 2a). By contrast, in the secretory products of sun leaves, the series of intense yellow–orange spots indicated a large quantity of flavonoids, which were identified as quercetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside in addition to oleuropein (Fig. 2b).

Detailed information about the phenylpropanoid composition and concentration of both leaf tissue and on the surface is given in Table 2, and is in complete agreement with the qualitative results of fluorescence microscopy and of HPTLC (Figs 1,2). Leaf tissue of plants acclimated to shade or sun had identical phenylpropanoid compositions, but phenylpropanoid concentration was 3.2-fold higher in sun leaves than in shade leaves. The increase was largely accounted for by the accumulation of flavonoids except for apigenin 7-*O*-rutinoside, which did not vary between shade and sun leaves (Table 2). Changes in the concentration of both tyrosol and hydroxytyrosol, which also increased in sun leaves, were of minor importance. The situation was very different when the composition and concentration of phenylpropanoids in the leaf surface were considered (Table 2). Secretory products of glandular trichomes of *P. latifolia* leaves acclimated to shade did not contain flavonoids; verbascoside and an unidentified caffeic acid derivative accounted for 72% of leaf surface phenylpropanoids, and 17% consisted of the secoiridoid oleuropein. By contrast, both the leaf

surface and the leaf tissue of sun plants had very similar flavonoidic compositions. Oleuropein was the main component (44%) of the total phenylpropanoids secreted by glandular trichomes of sun leaves, but the contribution of flavonoid glycosides was also relevant (37%). Esters of caffeic acid were not detected in the secretory products of glandular trichomes of sun leaves (Table 2).

#### DISCUSSION

*Phillyrea latifolia* seems an interesting case study for elucidating the integrated mechanisms of acclimation to high solar radiation operating at morphological–structural and biological levels in woody species of the Mediterranean basin. Morphological features of leaves exposed to full solar radiation, such as steep angle, reduced area, and high sclerophylly index (Table 1), might help *P. latifolia* plants in minimizing light absorption (Ehleringer 1988; Vogelmann, 1993) as usually occurs in species growing in excessive light under conditions of heat or drought stress (Ludlow & Björkman, 1984; Koller, 1986; Groom & Lamont, 1997). *P. latifolia* plants exposed to full sunlight actually suffered water stress, because predawn leaf water potential averaged  $-1.8$  MPa at the end of September. The frequency of glandular trichomes, which was higher in sun leaves than in shade leaves (Table 1), might help in decreasing air movement at the leaf surface and increasing leaf surface reflectance, and consequently might decrease water loss by *P. latifolia* leaves (Ehleringer *et al.*, 1976; Fahn, 1986). The superior thickness of sun leaves (Fig. 1b,c, Table 1), as detected in *P. latifolia*, has been also proposed as

a mechanism of acclimation to excess light operating in evergreen sclerophylls (Rhizopoulou *et al.*, 1991), resulting in better water use efficiency than thinner leaves (Yun & Taylor, 1986). It has been previously suggested that, in thicker leaves, cells of more structured palisade parenchyma allow the light to penetrate farther into the leaf and to have a more equal distribution of directional light to cell layers within the leaf that are specifically devoted to photosynthetic processes (Cui *et al.*, 1991; Vogelmann, 1993). From this standpoint, the changes in compactness of palisade parenchyma tissue between shade and sun leaves of *P. latifolia* (Fig. 1c,d, Table 1) is not surprising (Chazdon & Pearcy, 1991).

Changes in the UV optical properties of leaves acclimated to shade or to high solar radiation, in addition to morphological–structural changes, seem to have a key role in the acclimation mechanisms of *P. latifolia* to short-wave radiation (Caldwell *et al.*, 1983; Karabourniotis *et al.*, 1992). Leaf tissue of *P. latifolia* plants acclimated to shade or to full solar radiation differed greatly in flavonoid concentration but not in composition (Table 2), confirming a general response of plants exposed to excess UV and visible light wavelengths (Beggs *et al.*, 1987; Dixon & Paiva, 1995; Lavola, 1998). The accumulation of flavonoids in the epidermal cells of *P. latifolia* sun leaves (Fig. 1d) might attenuate the major proportion of the UV radiation reaching physiological targets (Caldwell *et al.*, 1983; Day *et al.*, 1984). The synthesis of flavonoids has been suggested to be confined to cells of epidermal layers (Schmelzer *et al.*, 1988) and the accumulation of flavonoids in the epidermal layers of sun leaves has been reported as constituting a primary adaptation of several species to the impact of short-wave radiation from the sun (Krauss *et al.*, 1997). However, the fact that flavonoids accumulated in the mesophyll tissue of *P. latifolia* leaves acclimated to full solar irradiance (i.e. palisade parenchyma cells; Fig. 1d), as occurs in analogous tissues of *Oenothera stricta* and *Avena sativa* (Robberecht & Caldwell, 1983; Knogge & Weissenböck, 1986), supports the idea that each tissue type is autonomous in flavonoid biosynthesis and accumulation (Ibrahim, 1992; Lois, 1994). It should also be taken into account that in the leaves of plants exposed to high levels of light, UV-A radiation penetrates farther than UV-B and can reach and damage target sites deep within the leaf (Bornman & Vogelmann, 1988). Flavonoid glycosides detected in *P. latifolia* leaves, such as quercetin 3-*O*-rutinoside and luteolin glycosides (Table 2), have relative maxima of absorbance in the UV-A region of the spectrum (320–350 nm) and might effectively protect cells specifically designated for photosynthesis from the formation of free radicals and other UV-A-induced damage (Takahama, 1989; Yamasaki *et al.*, 1997; Olsson *et al.*, 1998).

Although this experiment was not undertaken to elucidate the cellular distribution of phenylpropanoids in *P. latifolia* leaves, our fluorescence microscopy results suggest that flavonoids are probably bound to the epidermal and palisade parenchyma cell walls (Fig. 1c,d). The cellular distribution of flavonoids is still a matter of debate, because early reports on their unique vacuolar accumulation (Wagner, 1982) have been more recently confuted by both compartmental analysis and immunolocalization studies on flavonoid conjugates (Hrazidina, 1992; Ibrahim, 1992). Instead, our findings confirm previous results on the specific localization of hydrophilic flavonol glycosides, such as astragalin, quercetin 3-*O*-rutinoside and kaempferol 3-*O*-glucoside in several conifer species (Strack *et al.*, 1988; Schnitzler *et al.*, 1996; Fischbach *et al.*, 1999). The deposition of polyphenols in the cell wall has also been reported to occur in leaf hairs of *O. europaea* and *Quercus ilex* (Karabourniotis *et al.*, 1998).

Finally, the striking differences in phenylpropanoid composition between leaf tissue and secretory products of shade or sun leaves might help in elucidating the functional role of the glandular trichomes in the acclimation mechanisms to high levels of light that operate in *P. latifolia*. Differential conditions of light irradiance affected only the relative concentrations of constitutive metabolites of leaf tissue (Lois, 1994; Lavola *et al.*, 1997), whereas they deactivated branch pathways of the phenylpropanoid metabolism leading to the biosynthesis of caffeic acid esters or flavonoids in sun or shade trichomes, respectively (Table 2). These findings closely resemble the mutual deactivation induced by UV radiation and pathogens of furanocoumarin and flavonoid branch pathways in parsley cells (Schmelzer *et al.*, 1989; Douglas *et al.*, 1992) and suggest further that phenylpropanoids accumulated in and secreted by glandular trichomes are synthesized in the cells of the secretory organs (Fahn, 1988). In this regard, it should be pointed out that glandular trichomes occur almost exclusively on the lower surface of *P. latifolia* leaves (Gucci *et al.*, 1997 and results not shown). As a consequence, in leaves that had developed with a steep angle, glandular trichomes were irradiated for a long time during the day by direct or reflected sunlight from the sand, and biosynthesis of flavonoids was activated (Lois, 1994; Katz & Weiss, 1998). By contrast, under shade conditions both the low irradiance rates (total integrated photon flux density 1.64 mmol m<sup>-2</sup> d<sup>-1</sup>; PAR at midday 54 μmol m<sup>-2</sup> s<sup>-1</sup>) and the leaf angle did not allow light to reach glandular trichomes. Thus the signal that triggers the biosynthesis and the accumulation of flavonoids did not operate, whereas the syntheses of phenolic acids and of caffeic acid esters were unaffected (Lewis *et al.*, 1998). In conclusion, our results suggest that light-



induced changes in the morphological–structural features of leaves strongly affect the phenylpropanoid metabolism of glandular trichomes, thus clarifying their protective role in the integrated mechanisms of acclimation to excess light that operate in *P. latifolia*.

## ACKNOWLEDGEMENTS

We are grateful to Roberto Tafani and Maria Laura Traversi for their excellent technical assistance. M. T. was partly supported by the EU Environment and Climate Research and Development Programme within the Project 'Modelling Mediterranean Vegetation Dynamics' (ENV-CT97–0680).

## REFERENCES

- Beggs CJ, Kuhn K, Bocher R, Wellmann E. 1987. Phytochrome-induced flavonoid biosynthesis in mustard (*Sinapis alba* L.) cotyledons: enzymic control and differential regulation of anthocyanin and quercetin formation. *Planta* **172**: 121–126.
- Björkman O, Demmig-Adams B. 1995. Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. In: Schulze ED, Caldwell MM, eds. *Ecophysiology of photosynthesis*. Berlin, Germany: Springer-Verlag, 17–47.
- Bornman, JF, Vogelmann TC. 1988. Penetration of blue light and UV radiation measured by fibre optics in spur and fir needles. *Physiologia Plantarum* **72**: 699–705.
- Caldwell MM, Robberecht R, Flint SD. 1983. Internal filters: prospects for UV-acclimation in higher plants. *Physiologia Plantarum* **58**: 445–450.
- Chazdon RL, Percy RW. 1991. The importance of sunflecks for forest understorey plants. *Bioscience* **41**: 760–766.
- Cui M, Vogelmann TC, Smith WK. 1991. Chlorophyll and light gradients in sun and shade leaves of *Spinacia oleracea*. *Plant, Cell & Environment* **14**: 493–500.
- Day TA, Howells BW, Rice WJ. 1984. Ultraviolet absorption and epidermal-transmittance spectra in foliage. *Physiologia Plantarum* **92**: 207–218.
- Dixon R, Paiva NL. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**: 1085–1097.
- Douglas CJ, Ellard M, Haufler KD, Molitor E, Moniz de Sá M, Reinold S, Subramaniam R, Williams F. 1992. General phenylpropanoid metabolism: regulation by environmental and developmental signals. In: Stafford HA, Ibrahim RK, eds. *Phenolic metabolism in plants*. New York, USA: Plenum Press, 63–89.
- Ehleringer JR. 1988. Changes in leaf characteristics of species along elevational gradients in the Wasatch Front, Utah. *American Journal of Botany* **75**: 680–689.
- Ehleringer J, Björkman O, Mooney HA. 1976. Leaf pubescence effects on absorbance and photosynthesis in a desert shrub. *Science* **192**: 376–377.
- Ehleringer JR, Comstock J. 1987. Leaf absorbance and leaf angle: mechanisms of stress avoidance. In: Tenhunen JD, Catarino FM, Lange OL, Oechel WL, eds. *Plant response to stress*. Berlin, Germany: Springer-Verlag, 55–76.
- Fahn A. 1986. Structural and functional properties of trichomes of xeromorphic leaves. *Annals of Botany* **57**: 631–637.
- Fahn A. 1988. Secretory tissue in vascular plants. *New Phytologist* **108**: 229–257.
- Fischbach RJ, Kossmann B, Panten H, Steinbrecher R, Heller W, Seidlitz HK, Sandermann H, Hertkorn N, Schnitzler JP. 1999. Seasonal accumulation of ultraviolet-B screening pigments in needles of Norway spruce (*Picea abies* (L.) Karst.). *Plant, Cell & Environment* **22**: 27–37.
- Gellini R, Pantani F, Grossoni P, Bussotti F, Barbolani E, Rinallo C. 1983. Survey of the deterioration of the coastal vegetation in the park of San Rossore in central Italy. *European Journal of Forest Pathology* **13**: 296–304.
- Gravano E, Tani C, Bennici A, Gucci R. 1998. The ultrastructure of glandular trichomes of *Phillyrea latifolia* L. (*Oleaceae*) leaves. *Annals of Botany* **81**: 327–335.
- Groom PK, Lamont BB. 1997. Xerophytic implications of increased sclerophylly: interactions with water and light in *Hakea psilorrhyncha* seedlings. *New Phytologist* **136**: 231–237.
- Gucci R, Aronne G, Lombardini L, Tattini M. 1997. Salinity tolerance in *Phillyrea* species. *New Phytologist* **135**: 227–234.
- Harley PC, Tenhunen JD, Lange OL, Beyschlag W. 1987. Seasonal and diurnal patterns of *Phillyrea angustifolia* growing in Portugal. In: Tenhunen JD, Catarino FM, Lange OL, Oechel WL, eds. *Plant response to stress*. Berlin, Germany: Springer-Verlag, 329–337.
- Hrazidina G. 1992. Compartmentation in aromatic metabolism. In: Stafford HA, Ibrahim RK, eds. *Phenolic metabolism in plants*. New York, USA: Plenum Press, 1–24.
- Husain SR, Gillard J, Gillard P. 1987. Hydroxyl radical scavenging activity of flavonoids. *Phytochemistry* **29**: 2489–2491.
- Ibrahim RK. 1992. Immunolocalization of flavonoid conjugates and their enzymes. In: Stafford HA, Ibrahim RK, eds. *Phenolic metabolism in plants*. New York, USA: Plenum Press, 25–61.
- Karabourniotis G, Kofidis G, Fasseas C, Liakoura V, Drossopoulos I. 1998. Polyphenol deposition in leaf hairs of *Olea europaea* (*Oleaceae*) and *Quercus ilex* (*Fagaceae*). *American Journal of Botany* **85**: 1007–1012.
- Karabourniotis G, Papadopoulos K, Papamarkou M, Manetas Y. 1992. Ultraviolet-B radiation absorbing capacity of leaf hairs. *Physiologia Plantarum* **86**: 414–418.
- Katz A, Weiss D. 1998. Photocontrol of *chs* gene expression in petunia flowers. *Physiologia Plantarum* **102**: 210–216.
- Knogge W, Weissenböck G. 1986. Tissue-distribution of secondary phenolic biosynthesis in developing primary leaves of *Avena sativa* L. *Planta* **167**: 196–205.
- Koller D. 1986. The control of leaf orientation by light. *Photochemistry Photobiology* **44**: 819–826.
- Krauss P, Markstadter C, Riederer M. 1997. Attenuation of UV radiation by plant cuticles from woody species. *Plant, Cell & Environment* **20**: 1079–1085.
- Lavola A. 1998. Accumulation of flavonoids and related compounds in birch induced by UV-B irradiance. *Tree Physiology* **18**: 53–58.
- Lavola A, Julkunen-Tiitto R, Aphalo P, De La Rosa T, Lehto T. 1997. The effect of u.v.-B radiation on u.v. absorbing secondary metabolites in birch seedlings grown under simulated forest soil conditions. *New Phytologist* **137**: 617–621.
- Lewis CE, Walker JRL, Lancaster JE, Conner AJ. 1998. Light regulation of anthocyanin, flavonoid and phenolic acid biosynthesis in potato minitubers *in vitro*. *Australian Journal of Plant Physiology* **25**: 915–922.
- Lois R. 1994. Accumulation of UV-absorbing flavonoids induced by UV-B radiation in *Arabidopsis thaliana* L. *Planta* **194**: 498–503.
- Ludlow MM, Björkman O. 1984. Paraheliotropic leaf movement in *Siratro* as a protective mechanism against drought-induced damage to primary photosynthetic reactions: damage by excessive light and heat. *Planta* **161**: 505–518.
- Olsson LC, Veit M, Weissenböck G, Bornman JF. 1998. Differential flavonoid response to enhanced UV-B radiation in *Brassica napus*. *Phytochemistry* **49**: 1021–1028.
- Pignatti S. 1982. *Flora d'Italia*. Bologna, Italy: Edagricole.
- Rhizopoulou S, Meleti-Chrstou MS, Diamantoglou S. 1991. Water relations for sun and shade leaves of four mediterranean evergreen sclerophylls. *Annals of Botany* **42**: 627–635.
- Robberecht R, Caldwell MM. 1983. Protective mechanisms and acclimation to solar ultraviolet-B radiation in *Oenothera stricta*. *Plant, Cell & Environment* **6**: 477–485.
- Romani A, Baldi A, Mulinacci N, Vincieri FF, Tattini M. 1996. Extraction and identification procedures of polyphenolic compounds and carbohydrates in *Phillyrea* (*Phillyrea angustifolia* L.) leaves. *Chromatographia* **42**: 571–577.
- Romani A, Mulinacci N, Pinelli P, Vincieri FF, Cimato A. 1999a. Polyphenolic content in five tuscan cultivars of *Olea europaea* L. *Journal of Agricultural and Food Chemistry* **47**: 964–967.

- Romani A, Pinelli P, Mulinacci N, Vincieri FF, Tattini M. 1999b.** Identification and quantitation of polyphenols in leaves of *Myrtus communis* L. *Chromatographia* **49**: 17–20.
- Schmelzer E, Jahnen W, Halbrock K. 1988.** *In situ* localization of light-induced chalcone synthase mRNA, chalcone synthase, and flavonoid end products in epidermal cells of parsley leaves. *Proceedings of the National Academy of Sciences, USA* **85**: 2989–2993.
- Schmelzer E, Kroeger-Lebus S, Halbrock K. 1989.** Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. *Plant Cell* **1**: 993–1001.
- Schnitzler JO, Jungblut TP, Heller W, Kofferlein K, Hutzler P, Heinmann U, Schmelzer E, Ernst D, Langebartels C, Sandermann H Jr. 1996.** Tissue localization of u.v.-B-screening pigments and chalcone synthase mRNA in needles of Scots pine seedlings. *New Phytologist* **132**: 247–258.
- Shirley BW. 1996.** Flavonoid biosynthesis: 'new functions' for an 'old' pathway. *Trends in Plant Science* **1**: 301–317.
- Strack P, Heilemann J, Momken M, Wray V. 1988.** Cell wall-conjugated phenolics from Coniferae leaves. *Phytochemistry* **27**: 3517–3521.
- Takahama U. 1989.** A role of hydrogenperoxide in the metabolism of phenolics in mesophyll cells of *Vicia fabia* L. *Plant Cell Physiology* **30**: 295–301.
- Tattini M, Gucci R. 1999.** Ionic relations of *Phillyrea latifolia* L. plants during NaCl stress and relief from stress. *Canadian Journal of Botany* **77**: 969–975.
- Tattini M, Gucci R, Coradeschi MA, Ponzio C, Everard JD. 1995.** Growth, gas exchange and ion content in *Olea europaea* plants during salinity stress and subsequent relief. *Physiologia Plantarum* **98**: 117–124.
- Vogelmann TC. 1993.** Plant tissue optics. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**: 231–251.
- Wagner GJ. 1982.** Compartmentation in plant cells: the role of the vacuole. *Recent Advances in Phytochemistry* **16**: 1–46.
- Wagner H, Bladt S. 1996.** *Plant drug analysis*. Berlin, Germany: Springer-Verlag.
- Wollenweber E. 1993.** Flavones and flavonols: In: Harborne JB, ed. *The flavonoids. Advances in research since 1986*. London, UK: Chapman & Hall, 259–335.
- Wollenweber E, Dietz VH. 1981.** Occurrence and distribution of free flavonoid aglycones in plants. *Phytochemistry* **20**: 869–932.
- Yamasaki H, Sakihama Y, Ikehama N. 1997.** Flavonoid-peroxidase reaction as a detoxification mechanism of plant cell against H<sub>2</sub>O<sub>2</sub>. *Plant Physiology* **115**: 1405–1412.
- Yun JI, Taylor SE. 1986.** Adaptative implication of leaf thickness for sun- and shade-grown *Abutilon theophrasti*. *Oecology* **67**: 1314–1318.