Light-induced accumulation of ortho-dihydroxylated flavonoids as non-destructively monitored by chlorophyll fluorescence excitation techniques

Giovanni Agati a,*, Zoran G. Cerovic b, Patrizia Pinelli c, Massimiliano Tattini d

a Istituto di Fisica Applicata ‘Carrara’, IFAC, Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, I-50019, Sesto Fiorentino, Firenze, Italy
b Equipe de Biospectroscopie Végétale, Laboratoire d’Ecologie Systématique et Evolution, CNRS, UMR 8079, Bât. 362, Université Paris-Sud, 91405 Orsay Cedex, France
c Istituto per la Valorizzazione del Legno e delle Specie Arboree, IVALS, Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, I-50019, Sesto Fiorentino, Firenze, Italy
d Istituto di Fisica Applicata ‘Carrara’, IFAC, Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, I-50019, Sesto Fiorentino, Firenze, Italy

Abstract

Chlorophyll fluorescence excitation techniques have been used in plant science for more than one decade to non-destructively estimate phenolic compounds in the epidermal cell layer. These techniques have been used here to evaluate the effect of different light intensities and spectral quality on the accumulation of ortho-dihydroxylated flavonoids in Phyllirea latifolia L., Myrtus communis L. and Ligustrum vulgare L. In a first experiment, chlorophyll fluorescence excitation spectra were measured (with a double arm optical fiber bundle connected to a spectrofluorimeter) on the adaxial and abaxial leaf surfaces of container-grown P. latifolia and M. communis exposed to 20% or 100% full sunlight. Differences in epidermal absorption spectra (referred to as epidermal absorption spectra throughout the paper) were then calculated from the relative chlorophyll fluorescence excitation spectra. This allowed comparing the content of UV-absorbing compounds between differentially irradiated leaves as well as between adaxial and abaxial epidermal layers. The absorption spectra were characterized by a band centered at 360–380 nm, which was greater in sun than in shade leaves and in the adaxial than in the abaxial surfaces, irrespective of species. Based upon HPLC-DAD and HPLC–MS analyses of leaf extracts and UV-spectral features of individual flavonoids, we conclude that quercetin, luteolin and myricetin derivatives were responsible for the observed light-induced changes in the spectral features of examined tissues.

In a second experiment, we grew L. vulgare potted plants at 30% or 85% full sunlight in the presence or in the absence of UV radiation. We measured the absorbance characteristics at 370 nm of three leaf-pairs located in the apical portion of each shoot, using a portable fluorimetric sensor, the Multiplex®. This allowed estimating, non-destructively, an index closely related to the concentration of epidermal flavonols and flavones having an ortho-dihydroxyl substitution in the B-ring. This index was greater in leaves growing at 85% than at 30% sunlight, irrespective of UV irradiance. When plants acclimated for 3 weeks to 30% sunlight, in the presence or in the absence of UV irradiance, were transferred to 85% sunlight, the flavonoid index exponentially increased, reaching a maximum within 10 days.

On the whole, our experiments conclusively show that light-responsive flavonoids are mostly the dihydroxy B-ring-substituted structures. These flavonoids are the most effective, among the wide array of flavonoid structures, in preventing the generation and scavenging reactive oxygen species. As a consequence, we suggest that flavonoids do not merely serve as UV-screening agents, but behave as ROS-detoxifying agents in the mechanisms of photoprotection.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The chlorophyll fluorescence excitation technique has been developed, during the last decade, to non-destructively assess the content of UV-absorbing compounds on the leaf epidermal tissues (Bilger et al., 1997; Bidel et al., 2007). Basically, the method compares the Chl fluorescence signal measured under different excitation wavelengths. Because of the sub-epidermal localization of Chl, the excitation-light reaching the chloroplasts is attenuated by compounds located in the epidermal cell compartments and, hence, there is an inverse relationship between the concentration of UV-absorbing compounds and the intensity of the Chl fluorescence signal (Burchard et al., 2000; Bilger et al., 2001). In vivo absorption spectra of leaf epidermis have been previously measured using spectrofluorimeters equipped with optical fibers (Cerovic et al., 2002). Portable optical sensors (Goulas et al., 2004; Cerovic et
al., 2008) newly developed for in-field measurements (Kolb et al., 2005; Agati et al., 2008) are of particular significance in ecophysiology, permitting time-course measurements to assess the content of epidermal UV-absorbers on the very same leaf. Recent experiments have used the Chl fluorescence excitation technique for in vivo estimating the light-induced changes in leaf epidermal phenolics in woody (Kolb et al., 2001; Lenk and Buschmann, 2006; Bidel et al., 2007) and herbaceous species (Mazza et al., 2000; Barnes et al., 2008), as well as in phytoplankton (Mohovic et al., 2006).

In the present experiments the Chl fluorescence excitation technique, both in the lab using spectrofluorimetric methods and in the field through fluorimetric measurements, has been used to evaluate the light-induced accumulation of ortho-dihydroxylated flavonoids in some Mediterranean species. We studied leaves of *Phyllirea latifolia* and *Myrtus communis* acclimated to 20% or 100% natural sunlight, and leaves of *Ligustrum vulgare* acclimated to 30% or 85% natural sunlight in the presence or in the absence of the UV radiation. These species have been previously reported to synthesize a wide array of phenylpropanoids (particularly the antioxidant dihydroxy B-ring-substituted flavonoid structures) in response to high-light, water and salinity stress (Tattini et al., 2000, 2004, 2005, 2006).

Since both the whole polyphenol composition and the leaf tissue-specific distribution of various polyphenol classes for these species have been previously reported (Agati et al., 2002; Tattini et al., 2004, 2005, 2006), they represent suitable models to test the potentiality of the new fluorimetric techniques.

2. Materials and methods

2.1. Experiment 1

One-year-old *P. latifolia* L. and *M. communis* L. potted plants (irrigated three times a week until complete leaching of the substrate) were grown outdoors under 20% (shade) or 100% natural sunlight (sun) over a 6-week period. Plants at the full-sun site received a daily dose of 12.1 MJ m$^{-2}$, 1.05 MJ m$^{-2}$ and 19.4 KJ m$^{-2}$ in the PAR (photosynthetic active radiation over 400–700 nm), UV-A and UV-B wavebands, respectively. Mean daily doses of 0.19 MJ m$^{-2}$ and 3.6 KJ m$^{-2}$ in the UV-A and UV-B wavebands, respectively, were detected at the shade site. Leaves sampled for measurements were newly developed under different light regimes.

Chlorophyll fluorescence excitation spectra of attached leaves were acquired by a spectrofluorimeter (Cary Eclipse, Varian, Les Ulis, France) through a double arm optical fiber bundle (C Technologies, Cedar Knolls, NJ, USA) and a fiber optic coupler (Varian, part n° FA-VAR00-AP15). The common end of the fiber bundle was maintained at fixed both distance (5 mm) and angle (30°) from the leaves, using a proprietary clip. The excitation spectra were recorded from 225 nm to 545 nm, setting the emission band at 685 nm, i.e., the shorter wavelength peak of chlorophyll fluorescence, and corrected for the spectrofluorimeter excitation efficiency.

The theoretical basis for determining in vivo pigment absorbance spectra in leaf epidermis from Chl fluorescence measurements have previously been described (Cerovic et al., 2002). Briefly, the Chl fluorescence excitation spectrum can be expressed as:

$$\text{ChlF}_{685}^{\lambda_s}(\lambda_{ex}) = I(\lambda_{ex}) \times T(\lambda_{ex}) \times A_{Chl}(\lambda_{ex}) \times \Phi_{Chl}(\lambda_{ex})$$

(1)

where $I$ is the incident irradiance, $T$ is the transmittance of the epidermal layers, $A_{Chl}$ is the Chl absorption efficiency and $\Phi_{Chl}$ is the Chl fluorescence apparent quantum yield. Comparing two samples with different epidermal pigment concentrations, $s_1$ and $s_2$, under identical irradiation and detection conditions, the Fluorescence Excitation Ratio (FER) between the relative Chl fluorescence excitation spectra will be:

$$\frac{\text{ChlF}_{685}^{s_1}(\lambda_{ex})}{\text{ChlF}_{685}^{s_2}(\lambda_{ex})} = \frac{I(\lambda_{ex}) \times T_s(\lambda_{ex}) \times A_{Chl}(\lambda_{ex}) \times \Phi_{Chl}(\lambda_{ex})}{I(\lambda_{ex}) \times T_s(\lambda_{ex}) \times A_{Chl}(\lambda_{ex}) \times \Phi_{Chl}(\lambda_{ex})} = \frac{T_s(\lambda_{ex})}{T_s(\lambda_{ex})}$$

(2)

According to the Beer–Lambert’s law ($A = -\log T$), the logarithm of the FER (logFER) equals the difference in the epidermal absorbance between $s_2$ and $s_1$:

$$\text{logFER} = \log \frac{\text{ChlF}_{685}^{s_1}(\lambda_{ex})}{\text{ChlF}_{685}^{s_2}(\lambda_{ex})} = A_{s_2} - A_{s_1}$$

(3)

Before logFER calculations, Chl fluorescence excitation spectra were normalised to the value at 540 nm in order to take into account differences among the leaves in Chl content and tissue scattering properties. The Chl fluorescence excitation spectra were recorded of the adaxial/abaxial surfaces of the same aged leaves, of *P. latifolia* and *M. communis*.

Hydroxycinnamic acid derivatives, gallotannins and flavonoids were extracted, identified and quantified using HPLC-DAD and HPLC-MS analyses, as reported in Romani et al. (1999) and in Tattini et al. (2004). In brief, 40 mg of freeze-dried tissue were extracted with 3 × 8 ml of EtOH/H$_2$O (75/25, v/v) adjusted to pH 2.5 with formic acid. The supernatant was partitioned with 3 × 8 ml of n-hexane to remove lipophylic compounds and reduced to dryness under reduced pressure at room T. The residue was dissolved in 1 ml of MeOH and 10–20 ml aliquots injected in a HP1100 liquid chromatograph equipped with a diode array detectors (DAD) and managed by a HP work station (all from Agilent Technologies, Palo Alto, CA, USA). Column, eluent and analytical conditions were as reported previously (Romani et al., 1999; Tattini et al., 2004). All compounds were identified by comparison of their retention times and UV-spectra with those of authentic standards (Extrasynthese, Lyon–Nord, Genay, France) or isolated compounds. Hydroxycinnamates were quantified at 330 nm using a calibration curve of caffeic acid, and hydroxytyrosol derivatives and oleanuropein at 280 nm. Gallotannins were quantified at 260 nm, using a calibration curve of gallic acid. Flavonoids were quantified at 350 nm, using calibration curves of quercetin 3-O-rutinoside, luteolin 7-O-glucoside, apigenin 7-O-glucoside, and myricetin 3-O-rhamnoside for quercetin, luteolin, apigenin, and myricetin derivatives, respectively.

2.2. Experiment 2

*L. vulgare* plants were grown in screen houses constructed with roof and walls using plastic foils with specific transmittances (Supplemental Fig. S1), over a 3-week experimental period. The screen houses were N-S exposed with small shielded opening below the front roof and at the NE and NW corners to permit air circulation. Ultraviolet radiation was excluded by LEE 226 UV foil (Supplemental Fig. S1B). Total transmittance of the shading net and foils, as measured using a spectroradiometer, was 13.5% in the PAR, 65% in the UV-A and UV-B wavebands, respectively, and 90% in the UV-C wavebands. The UV-screen houses were N-S exposed with small shielded opening below the front roof and at the NE and NW corners to permit air circulation. Ultraviolet radiation was excluded by LEE 226 UV foil (Supplemental Fig. S1B). Total transmittance of the shading net and foils, as measured using a spectroradiometer, was 13.5% in the PAR, 65% in the UV-A and UV-B wavebands, respectively, and 90% in the UV-C wavebands.

*Hydroxycinnamic acid derivatives, gallotannins and flavonoids were extracted, identified and quantified using HPLC-DAD and HPLC-MS analyses, as reported in Romani et al. (1999) and in Tattini et al. (2004). In brief, 40 mg of freeze-dried tissue were extracted with 3 × 8 ml of EtOH/H$_2$O (75/25, v/v) adjusted to pH 2.5 with formic acid. The supernatant was partitioned with 3 × 8 ml of n-hexane to remove lipophylic compounds and reduced to dryness under reduced pressure at room T. The residue was dissolved in 1 ml of MeOH and 10–20 ml aliquots injected in a HP1100 liquid chromatograph equipped with a diode array detectors (DAD) and managed by a HP work station (all from Agilent Technologies, Palo Alto, CA, USA). Column, eluent and analytical conditions were as reported previously (Romani et al., 1999; Tattini et al., 2004). All compounds were identified by comparison of their retention times and UV-spectra with those of authentic standards (Extrasynthese, Lyon–Nord, Genay, France) or isolated compounds. Hydroxycinnamates were quantified at 330 nm using a calibration curve of caffeic acid, and hydroxytyrosol derivatives and oleanuropein at 280 nm. Gallotannins were quantified at 260 nm, using a calibration curve of gallic acid. Flavonoids were quantified at 350 nm, using calibration curves of quercetin 3-O-rutinoside, luteolin 7-O-glucoside, apigenin 7-O-glucoside, and myricetin 3-O-rhamnoside for quercetin, luteolin, apigenin, and myricetin derivatives, respectively.

L. vulgare plants were grown in screen houses constructed with roof and walls using plastic foils with specific transmittances (Supplemental Fig. S1), over a 3-week experimental period. The screen houses were N-S exposed with small shielded opening below the front roof and at the NE and NW corners to permit air circulation. Ultraviolet radiation was excluded by LEE 226 UV foils (LEE Filters, Andover, UK), whereas plants exposed to full-sun radiation were grown under a 100 μm ETTE fluoropolymer film (NOWOFILON® ET-6235, NOWOFILON® Kunststoffprodukte GmbH & Co. KG, Siegsdorf, Germany) to maintain similar conditions of temperature and humidity. Control plants were grown under 30% sun irradiance (30% sun), adding a proper black polyethylene net to the LEE 226 UV foil (Supplemental Fig. S1B).
UV irradiance (280–400 nm) and PAR inside the greenhouses were measured using a SR9910-PC double-monochromator spectroradiometer (Macam Photometric Ltd, Livingston, Scotland) and a calibrated Li-190 quantum sensor (Li-COR, Lincoln, NE, USA), respectively. Total UV irradiance was 24.5 W m⁻² and 1.1 W m⁻² in the 85% sun and 85% sun-UV treatments, respectively, at midday on a clear day (PAR was 1450 ± 46 μmol m⁻² s⁻¹ at the two sites).

Epidermal flavonoids were optically estimated in situ using the Multiplex® 2 (Mx) (FORCE-A, Orsay, France) portable fluorimetric device, after 4 weeks of treatment with different light regimes. This hand-held battery-operated optical sensor was a new version of that previously used to assess winegrape phenolic maturity (Cerovic et al., 2008). It consisted of 4 excitation Light Emitting Diode (LED) sources in the UV-A (370 nm), blue (460 nm), green (515 nm) and red (637 nm) and 3 detection channels in the blugreen, red and far-red spectral regions. These two last detection bands at 680–690 nm and 730–780 nm, respectively, corresponded to the two emission peak of chlorophyll. The LED sources being pulsed and synchronized to detection, the sensor is insensitive to ambient light. Acquisition time for a standard acquisition sequence of all 4 excitation bands and averaging 500 shots per measurement was of about 1 s, permitting high sampling numbers. The collected data were visible on a real-time display and stored on a Secure Digital (SD) card for further analysis.

Different combinations of the blue-green (BGF), red (RF) and far-red (FRF) fluorescence signals at the various excitation bands could be used as indices of different compounds, such as flavonols and flavones, anthocyanins and chlorophyll. Here, the flavonoids, FLAV, index was calculated as

\[
\text{FLAV Index} = \log \left( \frac{\text{FRF}_R}{\text{FRF}_UV} \right) = \log \left[ \frac{I_F^{\text{TP}} (1 - 10^{-A_{\text{Chl}}})}{I_U^{\text{TP}} (1 - 10^{-A_{\text{Chl}}})} \right] \quad (4)
\]

where FRF, I, T⁵⁻⁴ and A⁷⁻⁴ were the far-red Chi fluorescence, LED intensity, epidermal transmittance and Chl absorbance, respectively. The R and UV subscripts referred to red and UV-A light, respectively. Once the index was corrected for the difference in \( I \) between the UV and red LED sources and for the difference in \( A_{\text{Chl}} \) at the two excitation bands, and it was assumed that the T⁵⁻⁴ in the red was equal to 1, the FLAV Index given from Eq. (1) corresponded to the epidermal UV-A absorbance, as previously described (Goulas et al., 2004). The large area of detection of the sensor, 8-cm diameter, permitted to acquire the signal from the whole apical part, three leaf-pairs for each shoot. For each light regime 33–39 measurements (2–4 shoots per plant, 10–12 plants per treatment) on the adaxial sides were collected and the average values for the FLAV Index were calculated.

The flavonoid index was additionally estimated on plants which were exposed for 3 weeks to 30% sunlight in the presence or absence of UV irradiance and then transferred to 85% sunlight irradiance, during a 12-day-period. The same apical parts of each plant, adaxial leaf sides, were monitored daily by the Mx sensor up to 12 days of treatment. Hydroxycinnamates and flavonoids were extracted, identified and quantified using HPLC-DAD and HPLC–MS analyses, as reported above.

### 3. Results

#### 3.1. In situ chlorophyll fluorescence excitation spectroscopy – *Phyllirea latifolia*

The average Chl fluorescence excitation spectra of attached leaves of *Phyllirea latifolia*, by setting the fluorescence emission band at 685 nm, are reported in Fig. 1A. The spectra did not appreciably differ beyond 430 nm, as the epidermal layers are supposed to be transparent over this spectral region. By contrast, the fluorescence signal in the UV-A (320–390 nm) region and up to 425 nm, was markedly greater in shade than sun leaves, particularly in the adaxial-side of shade leaves. These differences in epidermal absorbances are evidenced by the logFER spectra reported in Fig. 1B and C. The small apparent absorbance difference in the 425–545 nm waveband is due to the difference in efficiency of carotenoid energy transfer to chlorophylls (Bidel et al., 2007; Pfundel et al., 2007).

Sun leaves had a larger epidermal absorbance in the 360–380 nm band than shade leaves, irrespective of the leaf side (Fig. 1B). Similar changes in absorbance were observed by comparing the logFER spectra of adaxial vs. abaxial surface in the...
same leaf (Fig. 1C). It is noted here that the apparent peak centered at 385 nm, was likely the result of a very low intensity of the fluorescence signal in the adaxial surface of sun leaves, due to very high concentrations of epidermal absorbers. As a consequence, the logFER spectral shapes obtained when the adaxial surface of sun leaves was included in the calculation were probably distorted.

logFER spectra of sun vs. shade leaves as well as of adaxial vs. abaxial surfaces in shade leaves were in agreement with the leaf flavonoid composition of *P. latifolia*, which consists of quercetin 3-O-rutinoside (que 3-O-rut), luteolin 7-O-glucoside (lut 7-O-glu) and apigenin 7-O-glycosides (api 7-O-gly) (Table 1). The absorbance spectrum of que 3-O-rut, with \( \varepsilon_{\text{max}} \) at 354 nm (Tattini et al., 2004), is actually similar to the logFER of (i) sun vs. shade abaxial surfaces and (ii) adaxial vs. abaxial epidermis in shade leaves. Lut 7-O-glu, which shows \( \varepsilon_{\text{max}} \) at 344 nm with a substantial tail to longer wavelengths, also contributed to the logFER spectra reported in Fig. 1. On the contrary, the UV-absorption features of api 7-O-gly, with an absorption peak centered at 335 nm, gave a negligible contribution to the logFER spectra reported in Fig. 1. These findings are consistent with the light-induced enhancements in the concentration of flavonoids, which was limited to the glycosides of quercetin and luteolin (Table 1).

3.2. *In situ* chlorophyll fluorescence excitation spectroscopy – *M. communis*

The Chl fluorescence excitation spectral analysis for *M. communis* is shown in Fig. 2. The highest UV-transmittance of the abaxial epidermis in shade leaves (Fig. 2A) resembles closely that detected in *P. latifolia* (Fig. 1A). The logFER spectrum of the adaxial surface of sun vs. shade leaves had a wide absorption band centered at 360 nm (Fig. 2B). By contrast, the spectral analysis in the UV-B region was difficult due to the low intensity of the fluorescence signal originated from the adaxial side of sun leaves. The sun vs. shade logFER spectrum of the abaxial epidermis showed an absorption band centered at 325 nm, in addition to the classical “flavonoid” band centered at 360 nm, indicating a larger presence of hydroxycinnamic acids in the abaxial epidermis, like seen before in *Morus nigra* (Bidel et al., 2007). The logFER spectrum of adaxial vs. abaxial epidermis in sun leaves (Fig. 2C) was similar to the logFER of adaxial surfaces of sun vs. shade leaves. These logFER data conform to the leaf flavonoid composition of *M. communis* (Table 2). Sunlight irradiance steeply increased the concentration of both myricetin 3-O-glycosides (88% of the total flavonoid pool) and quercetin 3-O-glycosides.

3.3. *In situ* estimation of a flavonoid index with a portable fluorimetric sensor

The effect of four different light regimes, examined in the Experiment 2, on the epidermal accumulation of flavonoids in *L. vulgare* leaves, as non-destructively evaluated by the fluorimetric Multiplex sensor, is shown in Fig. 3. The FLAV Index, corresponding to the epidermal absorbance at 370 nm, increased as sunlight

![Fig. 2.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Light</th>
<th>Tyrosol derivatives</th>
<th>Caffeoyl derivatives</th>
<th>Oleuropein</th>
<th>Quercetin 3-O-rutinoside</th>
<th>Luteolin 7-O-glucoside</th>
<th>Apigenin 7-O-glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>0.25 ± 0.07 b</td>
<td>0.38 ± 0.04 b</td>
<td>4.7 ± 0.76 b</td>
<td>0.14 ± 0.03 b</td>
<td>0.30 ± 0.05 b</td>
<td>1.62 ± 0.23 b</td>
</tr>
<tr>
<td>100%</td>
<td>0.95 ± 0.11 a</td>
<td>0.68 ± 0.08 a</td>
<td>12.5 ± 1.22 a</td>
<td>1.54 ± 0.18 a</td>
<td>1.74 ± 0.24 a</td>
<td>2.06 ± 0.16 a</td>
</tr>
</tbody>
</table>

Measurements were conducted on leaves newly developed under different sunlight irradiance. Identification and quantification of individual polyphenols were conducted using HPLC-DAD and HPLC–MS analyses. Data are means ± standard deviation (n = 4) and those in a column not accompanied by the same letter differ significantly for P<0.05, using a least significant difference (LSD) test.

Caffeoyl derivatives: echinacoside + verbascoside.
irradiance increased. The FLAV Index was 9%, 33% or 60% greater in 30% sun, 85% sun-UV and 85% sun treatments, respectively, than in the 30% sun-UV treatment. We note that the exclusion of UV radiation under 85% sunlight reduced the FLAV Index by only 20%. Instead, sunlight shading, as estimated by comparing 85% sun-UV and 30% sun-UV, decreased the FLAV Index to a considerably greater extent (−33%).

The leaf flavonoid pool in *L. vulgare* did not substantially differ from that previously reported for *P. latifolia*, as mostly constituted by quercetin 3-O-glycosides, luteolin 7-O-glycosides and apigenin 7-O-glycosides (Fig. 4). The time course of the FLAV Index, when plants acclimated to shade conditions (in the presence or in the absence of UV irradiance) were transferred to the 85% sunlight is reported in Fig. 5. The FLAV accumulation in the adaxial epidermis increased greatly during the first 4 days of treatment, and to a considerably smaller extent from day 4 to day 10. Interestingly, the FLAV Index at the end of 12 days of exposure to 85% sunlight (1.6) did not differ from that detected at the end of 3 weeks of acclimation to the same sunlight irradiance (which was estimated on many replicate plants, as shown in Fig. 3).

4. Discussion

Changes in the concentration and composition of leaf flavonoids, taken together with their spectral features, indicate that the main difference in epidermal absorbance (as non-destructively quantified by logFER) of (i) adaxial vs. abaxial tissues (Fig. 1C) and (ii) sun vs. shade (Fig. 1B) leaves in *P. latifolia* are attributable to the relative concentrations of the ortho-dihydroxy B-ring substituted quercetin and luteolin glycosides. The logFER analysis reports here conforms to microspectrofluorimetry and fluorescence microimaging analyses previously reported for shade and sun leaves of *P. latifolia* (Agati et al., 2002; Tattini et al., 2005). In *M. communis*, both myricetin and quercetin glycosides, which have similar UV-spectral features, likely contributed to the difference in epidermal absorbance at 360 nm. Both flavonoid classes were observed to change between the two leaf sides (Fig. 2C) as well as in response to sunlight irradiance (Fig. 2B), as conclusively monitored by the logFER analysis.

**Table 2**

<table>
<thead>
<tr>
<th>Light</th>
<th>Gallotannins</th>
<th>Myricetin 3-O- (6′-galloyl-gal)</th>
<th>Myricetin 3-O-galactoside</th>
<th>Myricetin 3-O-rhamnoside</th>
<th>Quercetin 3-O-galactoside</th>
<th>Quercetin 3-O-rhamnoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>61.2 ± 7.1 a</td>
<td>0.2 ± 0.05 b</td>
<td>0.04 ± 0.01 b</td>
<td>1.4 ± 0.26 b</td>
<td>0.07 ± 0.02 b</td>
<td>0.12 ± 0.03 b</td>
</tr>
<tr>
<td>100%</td>
<td>58.0 ± 8.7 a</td>
<td>1.2 ± 0.14 a</td>
<td>0.76 ± 0.06 a</td>
<td>3.9 ± 0.58 a</td>
<td>0.34 ± 0.05 a</td>
<td>0.53 ± 0.10 a</td>
</tr>
</tbody>
</table>

Measurements were conducted on leaves newly developed under different sunlight irradiance. Identification and quantification of individual polyphenols were conducted using HPLC-DAD and HPLC–MS analyses. Data are means ± standard deviation (*n* = 4) and those in a column not accompanied by the same letter differ significantly for *P* < 0.05, using a least significant difference (LSD) test. Myricetin 3-O-(6′-galloyl-gal) denotes myricetin 3-O-(6′-galloyl-galactoside).
The epidermal absorbance at 370 nm as measured using the Multiplex sensor in *L. vulgare* leaves is also attributable to the ortho-dihydroxylated B-ring of que 3-O- and lut 7-O-glycosides. The differences in the FLAV Index in differentially irradiated leaves of *L. vulgare* are in complete agreement with the distribution of flavonoids in the adaxial/abaxial epidermises as assessed previously using microspectrofluorimetry and fluorescence microimaging (Tattini et al., 2004, 2005). The non-destructive Mx measurements of FLAV were consistent with leaf FLAV as quantified by HPLC-DAD analysis, as changes in sunlight irradiance mostly affected the biosynthesis of the ortho-dihydroxylated flavonols (que 3-O-glycosides) and flavones (lut 7-O-glycosides). However, Mx and HPLC-DAD analyses may have differed because of (i) HPLC analysis quantifies whole-leaf soluble FLAV; (ii) flavonoids are distributed not only in the adaxial but also in the mesophyll tissues and abaxial epidermis (Agati et al., 2007, 2009); (iii) the Mx method estimates both soluble and wall-bound flavonoids in the epidermal cells; (iv) epidermal FLAV may be in part underestimated because of UV-transparency of anticalin cell-wall (i.e., the sieve effect) (Kolb and Pfundel, 2005). Nevertheless, good correlations have been observed between in vivo fluorimetrically determined epidermal UV-A absorbance, leaf FLAV concentration (Agati et al., 2008) and UV-A absorbance of leaf extracts (Kolb and Pfundel, 2005; Barthod et al., 2007). These findings suggest that the non-invasive FLAV Index is a reliable descriptor of whole-leaf flavonoids.

We conclude that non-invasive chlorophyll fluorescence techniques, performed using both spectrofluorimeters in the lab and optical portable sensors in the field, can be actually used to assess the epidermal accumulation of ortho-dihydroxy B-ring substituted flavonoids.

These flavonoids, which are synthesized in response to high sunlight in the absence or in the presence of the UV-wavelengths (Babu et al., 2003; Bilger et al., 2007; Kotilainen et al., 2008) have an effective ability to scavenge reactive oxygen forms (Agati and Tattini, 2010). We suggest that UV irradiance has to be considered as to an oxidative stress and sunscreen compounds are aimed to counter the UV-induced oxidative damage (Landry et al., 1995; Agati et al., 2007, 2009), in addition to reduce the risk of oxidative damage by attenuating the flux of UV radiation to sensitive leaf targets.


