UV radiation promotes flavonoid biosynthesis, while negatively affecting the biosynthesis and the de-epoxidation of xanthophylls: Consequence for photoprotection?

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1 **Abstract**

2 There is evidence that UV radiation may detrimentally affect the biosynthesis of carotenoids,

3 particularly de-epoxided xanthophylls, while strongly promoting phenylpropanoid,

4 particularly flavonoid biosynthesis in a range of taxa. Here we tested the hypothesis that

5 mesophyll flavonoids might protect chloroplasts from UV-induced photo-oxidative damage,

6 by partially compensating for the UV-induced depression of xanthophyll biosynthesis. To test

7 this hypothesis we grew two members of the Oleaceae family, *Ligustrum vulgare* L. and

8 *Phillyrea latifolia* L., under either partial shading or fully exposed to sunlight, in the presence

9 or in the absence of UV radiation. The examined species, which display very similar flavonoid

10 composition, largely differ in their ability to limit the transmission of UV and visible light

11 through the leaf and, hence, in the accumulation of flavonoids in mesophyll cells. We

12 conducted measurements of photosynthesis, chlorophyll *a* fluorescence kinetics, the

13 concentrations of individual carotenoids and phenylpropanoids at the level of whole-leaf, as

14 well as the content of epidermal flavonoids. We also performed multispectral fluorescence

15 micro-imaging to unveil the intra-cellular distribution of flavonoids in mesophyll cells. UV

16 radiation decreased the concentration of carotenoids, particularly of xanthophylls, while

17 greatly promoting the accumulation of flavonoids in palisade parenchyma cells. These effects

18 were much greater in *L. vulgare* than in *P. latifolia*. UV radiation significantly inhibited the

19 de-epoxidation of xanthophyll cycle pigments, while enhancing the concentration of luteolin,

20 and particularly of quercetin glycosides. Flavonoids accumulated in the vacuole and the

21 chloroplasts in palisade cells proximal to the adaxial epidermis. We hypothesize that

22 flavonoids might complement the photo-protective functions of xanthophylls in the

23 chloroplasts of mesophyll cells exposed to the greatest doses of UV radiation. However, UV

24 radiation might result in adaxial mesophyll cells being less effective in dissipating the excess

25 of radiant energy, e.g., by decreasing their capacity of thermal dissipation of excess visible

26 light in the chloroplast.

29

1 Introduction

The effects of UV, particularly UV-B radiation on plant physiology and biochemistry have received increasing interest from scientists over the last three decades, in view of the depletion of the stratospheric ozone layer, which is particularly severe in some regions of the Earth (for review articles, see Ballaré et al., 2011; Williamson et al., 2014; Bornman et al., 2015). High doses of UV radiation have the potential to damage Photosystem II (PSII) reaction centers (Vass, 2012) as well as DNA integrity (Frohnmeyer and Staiger, 2003; Biever and Gardner, 2016). Nonetheless, photosynthesis and biomass production decrease little in plants exposed to UV radiation under natural sunlight (Bassman et al., 2002; Wargent and Jordan, 2013; Kataria et al., 2014; Bornman et al., 2015; Siipola et al., 2015; Wargent et al., 2015). Blue light-activated photolyase, which repairs UV photoproducts in DNA (Biever and Gardner, 2016), effectively limits the damage driven by short-wave solar radiation (Aphalo et al., 2012; Hideg et al., 2013; Aphalo et al., 2015; Bornman et al., 2015; Klem et al., 2015).

During extended periods of exposure to UV and blue light radiation, the stimulation of phenylpropanoid biosynthesis (Agati and Tattini, 2010; Agati et al., 2013; Kaling et al., 2015; Siipola et al., 2015; Wargent et al., 2015; Huché-Thélier et al., 2016) offers further photoprotection to the photosynthetic apparatus, despite an initial decline in photosynthetic performance (Kolb et al., 2001; Tsormpatsidis et al., 2008). UV-absorbing hydroxycinnamates (HCA) and flavonoids serve a multiplicity of functions in photoprotection: they efficiently absorb short-wave solar radiation, thus decreasing the risk of photo-oxidative stress, as well as countering photo-oxidative damage by scavenging free radicals and reactive oxygen species, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (Agati et al., 2007, 2012). The potential of HCA and flavonoids to serve as antioxidants in photoprotection stems from the observation that these compounds accumulate in mesophyll, not only in epidermal cells, in response to high solar irradiance (Semerdejeva et al., 2003; Polster et al., 2006; Tattini et al., 2004, 2005; Ferreres et al., 2011). Flavonoids accumulate in the chloroplasts, other than in the vacuolar compartment in some species (Sanders and McClure, 1976), apparently associated to the chloroplast outer envelope membrane (Agati et al., 2007). High sunlight almost exclusively activates the biosynthesis of flavonoids with the greatest antioxidant capacity, in the presence or in the absence of UV-irradiance (Agati et al., 2009, 2011a; Siipola et al., 2015). This adds

further support to the idea that flavonoids may serve antioxidant functions in photoprotection (Ryan et al., 1998; Agati et al., 2007; Ferreres et al., 2011; Agati et al., 2012).

The effect of UV irradiance on carotenoid biosynthesis is less clear, possibly due to different experimental set-ups (UV supplementation vs. UV exclusion experiments), intensity of UV 'stress' (irradiance × time of exposure), plant species (woody vs herbaceous), and even genotype (Musil et al., 2002; Láposi et al., 2009; Newshman and Robinson, 2009; Li et al., 2010; Aphalo et al., 2012, 2015; Vodović et al., 2015). Nonetheless, the overall emerging picture describes a negative effect of UV radiation on the concentration of carotenoids (Hideg et al., 2006; Hui et al., 2015; Bernal et al., 2015), particularly in UV-exclusion experiments (Bischof et al., 2002; Liu et al., 2005; Newshman and Robinson, 2009; Albert et al., 2011), with few exceptions (Láposi et al., 2009; Klem et al., 2015). UV-B irradiance was additionally shown to partially inhibit the high light-induced down-regulation of xanthophyll epoxidation (Mewes and Richter, 2002; Moon et al., 2011), and the consequential nonphotochemical quenching (NPQ) of excess light in the chloroplast, by reducing the pH gradient across thylakoid membranes (Pfündel et al., 1992, Pfündel and Dilley, 1993).

This offers the intriguingly possibility that during UV acclimation plants might enhance their capacity to effectively counter the detrimental effects of the most energetic solar wavelengths, while partially decreasing their ability to cope with an excess of photosynthetic active radiation (PAR). This might have ecological significance, since an excess of visible light may translate into a severe stressful condition plants face on seasonal and daily basis (Li et al., 2009), further exacerbated by the concurrent impact of heat and drought stresses, particularly in a Mediterranean climate (Matesanz and Valladares, 2014; Tattini and Loreto, 2014).

In our study, we investigated the potential relationship between flavonoid and carotenoid biosynthesis in photoprotection mechanisms of plants growing in the presence or in the absence of UV radiation. We hypothesize that flavonoids might serve photoprotective functions of increasing significance in leaves growing in the presence of solar UV wavelengths, because of the decreased biosynthesis of carotenoids. To test this hypothesis we grew plants under either partial shading (40% of natural sunlight) or fully exposed to solar irradiance (100%) in the absence or in the presence of UV-radiation, in an UV-exclusion experiment. We analyzed the responses to different light treatments of two members of the Oleaceae family,

Ligustrum vulgare L. and *Phillyrea latifolia* L., which inhabit sunny or partially shaded areas, respectively, in the Mediterranean basin, and display a very similar flavonoid pool (Tattini et al., 2005; Fini et al., 2016). In *P. latifolia*, a constitutively higher frequency of secretory trichomes coupled with thicker cuticles and epidermises offer greater capacity in limiting the transmission of solar irradiance through the leaf, thus offering greater protection to the photosynthetic apparatus as compared to *L. vulgare* (Tattini et al., 2005). This hypothesis was consistent with the much higher accumulation of 'antioxidant' flavonoids in mesophyll cells of *L. vulgare* than of *P. latifolia* when plants grew in full sunlight. Therefore, in our study we tested the hypothesis that UV radiation, while promoting the biosynthesis of flavonoids might depress the biosynthesis of xanthophylls to greater extent in *L. vulgare* than in *P. latifolia*, with important consequences on photoprotection mechanisms.

2. Material and Methods

2.1. Plant material and growth conditions

Self-rooted *Ligustrum vulgare* L. and *Phillyrea latifolia* L. potted plants were grown in screen houses (2 m × 2 m × 2 m, length × width × height) constructed with roof and walls using plastic foils with specific transmittances, over a six-week experimental period. Plants were exposed to 40% or 100% solar irradiance in the absence (referred as PAR plants/leaves throughout the paper) or in the presence of UV irradiance (referred as to UV plants/leaves). Solar UV radiation was excluded by LEE #226 UV foils (LEE Filters, Andover, UK), which fully excluded solar wavelengths in the range 280–380 nm, and transmitted just 3% of radiation in the 380–390 nm range. Plants grew under a 100-µm ETFE fluoropolymer transparent film (NOWOFLON® ET-6235, NOWOFOL® Kunststoffprodukte GmbH & Co. KG, Siegsdorf, Germany) in the UV treatment. Attenuation of solar irradiance was achieved by adding a proper black polyethylene frame to the LEE #226 or NOWOFOL ET-6325 foils. UV irradiance (280–400 nm) and photosynthetic active radiation (PAR, over the 400 -700 nm spectral region) inside the screen houses were measured by a SR9910-PC double-monochromator spectroradiometer (Macam Photometric Ltd., Livingstone, UK), and a calibrated Li-190 quantum sensor (Li-Cor Inc., Lincoln, NE, USA), respectively. UV-A was 798 or 314, and UV-B 43.1 or 17.3 kJ m^{-2} d^{-1} in the UV treatment under 100 or 40% solar irradiance, respectively, on a clear day. Biologically effective UV-B radiation, UV-B$_{BE}$ (as weighed by the generalized plant action

spectrum proposed by Caldwell (1971)), was 3.54 or 1.39 kJ m^{-2} d^{-1}, at 100% or 40% solar

irradiance. UV-A irradiance was 33.2 or 13.9 kJ m^{-2} d^{-1} in plants at 100 or 40% solar irradiance

in the absence of UV radiation, respectively, on a clear day. Temperature maxima/minima were

measured daily with Tinytag Ultra2 data loggers (Gemini Dataloggers, UK) and averaged

30.8/17.7 °C or 32.6/16.9 °C in plants growing at 40% or 100% sunlight, over the whole

experimental period. We sampled six-week-old leaves, i.e., newly developed under the

different light treatments, for measurements at midday hours (from 12:00 to 14:00 hrs), when

photosynthetic and non-photosynthetic pigments play major photoprotective functions.

2.2 Photosynthesis and chlorophyll a fluorescence

Measurements of net CO_2 assimilation rate (P_n) were performed using a LI-6400

portable photosynthesis system (Li-Cor, Lincoln, NE, USA), at PPFD of 1000 µmol photons

m^{-2} s^{-1}, a CO_2 concentration of 400 µmol mol^{-1}, and a leaf temperature of 30 °C. 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 fiber optic. 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 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} in dark or light conditions,

respectively. PSII quantum yield in the light (Φ_{PSII}) and nonphotochemical quenching (NPQ =

$(F_m/F_m') - 1$) were then estimated as previously reported (Guidi et al., 2008).

1 *2.3 Identification and quantification of carotenoids and phenylpropanoids*

2   Individual carotenoids were identified and quantified as reported in Tattini et al. (2015).

3 Fresh leaf material (300 mg) was extracted with 2×5 mL acetone (added with 0.5 g L^{-1} CaCO$_3$)

4 and injected (15 µL) in a Perkin Elmer Flexar liquid chromatograph equipped with a quaternary

5 200Q/410 pump and a LC 200 diode array detector (DAD) (all from Perkin Elmer, Bradford,

6 CT, USA). Photosynthetic pigments were separated in a 250×4.6 mm Agilent Zorbax SB-

7 C18 (5 µm) column operating at 30°C, eluted for 18 min with a linear gradient solvent system,

8 at a flow rate of 1 mL min^{-1}, from 100% CH3CN/MeOH (95/5 with 0.05% triethylamine) to

9 100% MeOH/ethyl acetate (6.8/3.2). Xanthophyll cycle pigments (violaxanthin,

10 antheraxanthin, zeaxanthin, collectively named VAZ), neoxanthin, lutein, and β-carotene, were

11 identified using visible spectral characteristics and retention times. Individual carotenoids and

12 chlorophylls were calibrated using authentic standards from Extrasynthese (Lyon-Nord,

13 Genay, France) and from Sigma Aldrich (Milan, Italy), respectively, as previously reported

14 (Tattini et al.*,* 2014).

15   The analysis of individual phenylpropanoids, which was limited to hydroxycinnamic

16 acid and flavonoid derivatives, was conducted following the protocol of Tattini et al. (2015).

17 Leaf tissues was extracted with 3×5 mL 75% EtOH/H$_2$O adjusted to pH 2.5 with formic acid.

18 The supernatant was partitioned with 4×5 mL of *n*-hexane, reduced to dryness, and finally

19 rinsed with 2 mL of CH$_3$OH/H$_2$O (8/2). Aliquots of 10 μL were injected into the Perkin Elmer

20 liquid chromatography unit reported above. Phenylpropanoids were analyzed through a $150 \times$

21 4.6 mm Waters (Waters Italia, Milan, Italy) Sun Fire column (5 μm) operating at 30 °C at a

22 flow rate of 1 mL min^{-1}. The mobile phase consisted of (A) H$_2$O (adjusted to pH 2.5 with

23 H$_3$PO$_4$)/CH$_3$CN (90/10, v/v) and (B) H$_2$O (adjusted to pH 2.5 with H$_3$PO$_4$)/CH$_3$CN (10/90).

24 Metabolites were separated using a linear gradient elution from A to B over a 60 min run, and

25 identified using retention times and UV spectral characteristics of authentic standards

26 (Extrasynthese, Lyon-Nord, Genay, France), as well as by mass spectrometric data. HPLC-MS

27 analysis was performed with an Agilent LC 1200 chromatograph coupled with an Agilent 6410

28 triple-quadrupole MS-detector equipped with an ESI source (all from Agilent Technologies,

29 Santa Clara, CA, USA). Quantification of caffeic acid derivatives (HCA throughout the paper,

30 mostly verbascoside and echinacoside, Tattini et al., 2004, 2005), glycosides of apigenin (API,

31 mostly apigenin 7-*O*-rutinoside and glucoside), quercetin (QUE, the pool consisting of

1   quercetin 3-*O*-glucoside, 3-*O*-rhamnoside, and 3-*O*-rutinoside) and luteolin (LUT, luteolin 7-

2   *O*-glucoside and rhamnoside) was performed using calibration curves of verbascoside,

3   apigenin 7-*O*-rutinoside, quercetin 3-*O*-rutinoside, and luteolin 7-*O*-glucoside, respectively.

4

5   *2.4 Epidermal flavonoids and sub-cellular distribution of flavonoids in mesophyll cells*

6   Flavonoids located on the surface and epidermal cells of leaves (referred as to

7   'epidermal' flavonoids throughout the paper) were optically estimated *in vivo* using the

8   Multiplex® 2 (FORCE-A, Orsay, France) portable fluorimetric sensor, as detailed in Agati et

9   al. (2011b). The Chl fluorescence signals under red light excitation (λ_{exc} = 625 nm, FRF_R) and

10   UV-excitation (λ_{exc} = 375 nm, FRF_{UV}) were used to calculate the flavonoid index (FLAV),

11   FLAV = FRF_R/FRF_{UV}. This excitation set-up mostly estimates the epidermal content of

12   dihydroxy B-ring-substituted flavonoids (such as QUE and LUT derivatives), as both HCA

13   and mono-hydroxy flavones (such as API derivatives) have much smaller molar extinction

14   coefficients as compared to QUE and LUT derivatives at 375 nm (Agati et al., 2011; 2013).

15   The sub-cellular distribution of flavonoids in mesophyll cells was visualized in 100-

16   µm-thick cross-sections of fresh leaf material stained with 0.1% (w/v) diphenylborinic acid 2-

17   amino-ethylester (Naturstoff reagent (NR) as reported previously (Agati et al., 2007).

18   Fluorescence microscopy analysis was performed using a Leica SP8 confocal laser-scanning

19   microscope (Leica Microsystems CMS, Wetzlar, Germany) under the following excitation-

20   emission set-up: (1) λ_{exc} = 488 nm and λ_{em} over the 562-646 nm waveband for the detection of

21   dihydroxy B-ring-substituted flavonoids (Agati *et al*., 2009) (2) λ_{exc} = 488 nm and λ_{em} over the

22   687-7576 nm waveband for chlorophyll detection.

23   *2.5 Experimental design, data analysis and statistics*

24   The experiment was performed using a completely randomized block design, with four

25   blocks (screen houses), each consisting of three plants per species, for each light treatment, on

26   a total of 96 plants. Chl *a* fluorescence measurements were conducted on four replicate plants

27   per treatment (one plant per screen house) on two consecutive days. Metabolite analyses were

28   conducted on four replicate plants per treatment, each replicate consisting of three leaves

29   sampled from individual plants in the screen house. Epidermal flavonoids were estimated on

30   12 leaves per species and light treatment. Data were checked for homogeneity of variance using

==Levene's test.== Then data were analyzed using both three-way ANOVA with species (SP), solar irradiance (referred as to visible light, VIS, throughout the paper), and UV radiation (UV) as fixed factors (with their interaction factors) and two-way ANOVA with visible light (VIS) and UV (UV) as fixed factors (with their interaction factors), for each individual species. Significant differences among means were estimated at the 5% ($P < 0.05$) level, using Tukey's test (Statgraphics Centurion XVI, Stat Point Technologies Inc., Warrenton, VA, USA).

The extent to which physiological and biochemical traits (X) varied in response to visible (by comparing plants growing at 40% and 100% sunlight, irrespective of UV treatment) and UV light (by comparing UV- and PAR-treated plants, irrespective of visible light) was also estimated by the normalized index of variation (NIV) using the equations proposed by Tattini et al. (2006):

$$NIV^{VIS} = (X_{100\%} - X_{40\%})\,(X_{100\%} + X_{40\%})^{-1} \tag{1}$$

$$NIV^{UV} = (X_{UV} - X_{PAR})\,(X_{UV} + X_{PAR})^{-1} \tag{2}$$

3. Results

3.1 Overall effects of visible and UV radiation on physiological and biochemical traits

Visible light affected the suite of physiological and biochemical traits examined in our study to greater degree than UV radiation did. NIV^{VIS} and NIV^{UV}, calculated using absolute NIVs, averaged 0.23 and 0.12, respectively (Table 1; see Appendix Table A1). Visible light greatly affected the biosynthesis of phenylpropanoids (NIV = 0.36) and, to a lesser extent, the biosynthesis of photosynthetic pigments (NIV = 0.18) and the photosynthetic performance (NIV = 0.15). UV radiation had little impact on photosynthetic performance (NIV = 0.03), while it substantially affected the concentration of photosynthetic (NIV = 0.17) and non-photosynthetic pigments (NIV = 0.11). In detail, the pool of xanthophyll cycle pigments (VAZ) as well as the VAZ de-epoxidation state (DES) were significantly higher in sun than in shaded leaves. In contrast, UV radiation markedly depressed both VAZ and DES. Visible light mostly increased the biosynthesis of QUE and LUT derivatives, while its effect was minor on the biosynthesis of API derivatives. UV radiation had an effect similar to that of visible light on

1 the biosynthesis of individual phenylpropanoids (with the exception of API derivatives),

2 though at a substantially smaller degree. The flavonoid concentration at the level of the whole-

3 leaf varied more (NIV = 0.36) than 'epidermal' flavonoid concentration (NIV = 0.19) in

4 response to visible light and UV radiation.

5

Table 1. The normalized index of variation (NIV) for the effects of visible (NIV^{VIS}) and UV treatment (NIV^{UV}) on physiological and biochemical-related features of *L. vulgare* and *P. latifolia* leaves.

Trait	NIV^{VIS} (100% – 40%)		NIV^{UV} (UV – PAR)	
	L. vulgare	*P. latifolia*	*L. vulgare*	*P. latifolia*
P_n	−0.43	+0.02	−0.06	−0.02
F_v/F_m	−0.06	−0.03	−0.02	−0.01
Φ_{PSII}	−0.28	−0.16	+0.03	+0.02
NPQ	+0.15	+0.11	−0.05	−0.04
Total chlorophyll (Chl_{tot})	−0.14	−0.03	−0.06	−0.05
Total carotenoids (Car_{tot})	+0.06	+0.01	−0.23	−0.17
$Car_{tot}\ Chl_{tot}^{-1}$	+0.16	+0.04	−0.17	−0.11
Lutein Chl_{tot}^{-1}	+0.03	−0.03	−0.10	−0.09
β-carotene Chl_{tot}^{-1}	+0.02	−0.02	−0.17	−0.09
Zeaxanthin (Z) Chl_{tot}^{-1}	+0.70	+0.38	−0.46	−0.27
Antheraxanthin (A) Chl_{tot}^{-1}	+0.52	+0.43	−0.31	−0.23
Violaxanthin (V) Chl_{tot}^{-1}	−0.05	−0.05	+0.09	+0.03
VAZ (V+ A + Z)	+0.46	+0.18	−0.19	−0.05
DES $[(0.5A + Z) (V + A + Z)^{-1}]$	+0.36	+0.24	−0.24	−0.15
Hydroxycinnamates	+0.30	+0.34	+0.08	+0.04
Apigenin glycosides	+0.13	+0.12	+0.02	+0.01
Quercetin glycosides	+0.63	+0.44	+0.26	+0.18
Luteolin glycosides	+0.58	+0.40	+0.19	+0.18
'Epidermal' flavonoids	+0.33	+0.24	+0.10	+0.09

Net photosynthesis (P_n, µmol m^{-2} s^{-1}), the concentrations of chlorophyll (µmol g^{-1} FW), and carotenoids (µmol g^{-1} FW), the concentration of individual carotenoids relative to Chl_{tot}, the whole-leaf concentrations (µmol g^{-1} FW) of individual phenylpropanoids were measured on four replicate six-week-old leaves, newly developed under different light treatments, sampled between 12:00 and 14:00 hrs. 'Epidermal' flavonoids were estimated on 12 leaves per species and light treatment. Summary of three-way ANOVA of the effects of species (SP), visible light (VIS) and UV radiation (UV) as fixed factors with their interaction factors on the suite of physiological and biochemical traits is reported in Table A1 in the Appendix.

3.2. Visible and UV irradiance affect photosynthesis and photosynthetic pigments more in L. vulgare than in P. latifolia

L. vulgare displayed greater changes in response to light treatments examined in our study as compared to *P. latifolia* (Table 1; see Appendix Tables A1-A3). Photosynthesis was either unaffected in *P. latifolia* or strongly depressed in *L. vulgare* because of sunlight, irrespective of UV radiation (Fig. 1A). Similarly, declines in both maximal (F_v/F_m, Fig. 1B) and actual (Φ_{PSII}, Fig. 1C) efficiency of PSII photochemistry were greater in *L. vulgare* than in *P. latifolia* in response to visible light, as also observed for the light-induced increase in

nonphotochemical quenching (NPQ, Fig. 1D). UV radiation had a relatively minor, still significant effect on F_v/F_m, irrespective of species (Fig. 1A; see Appendix Tables A1-A3). Leaves growing at ambient UV radiation had slightly higher Φ_{PSII} than plants growing in the absence of UV (Table 1), particularly under shaded conditions (Fig. 1B). This is consistent with the observation that NPQ was also lightly lower (-8%, Table 1 and Fig. 1D) in leaves receiving ambient UV radiation than in leaves exposed to visible light only.

Visible and ambient UV radiation had opposite effects on the concentration and composition of carotenoids (Table 1 and Fig. 2). While visible light slightly increased, UV radiation greatly depressed the leaf total carotenoid concentration, expressed on both tissue fresh weight and Chl_{tot} basis (Table 1, Fig. 2B,C), with major effects observed in *L. vulgare* (Fig. 2; see Appendix, Tables A1-A3). As expected, leaves growing in full sunlight displayed a larger pool of VAZ and higher DES as compared to leaves that grew under shaded conditions. The VAZ pool as well as DES also increased much more in *L. vulgare* (+178% for VAZ and +110% for DES) than in *P. latifolia* (+46% for VAZ and +63% for DES) because of visible light. Similarly, decreases in both VAZ (-35% vs -15%) and DES (-32% vs. -24%) because of the UV treatment were more pronounced in *L. vulgare* than in *P. latifolia* (Table 1, Fig. 2). It is finally noted that the VAZ pool was high relative to the Chl pool in our study, ranging from 76 in shaded to 155 mmol mol^{-1} Chl_{tot} in full sun exposed leaves. The VAZ to Chl_{tot} ratio was particularly high in plants growing in full sunlight in the absence of UV radiation, ranging from 258 in *L. vulgare* to 120 mmol Chl_{tot}^{-1} in *P. latifolia*, respectively.

Figure 1. Photosynthesis (P_n, A), maximum (F_v/F_m, B) and actual (Φ_{PSII}, C) efficiency of PSII photochemistry, and nonphotochemical quenching (NPQ, D) in *L. vulgare* and *P. latifolia* leaves grown under partial shading (40% full sunlight) or fully exposed to sunlight (100%) in the presence (UV) or in the absence (PAR) of UV radiation. Measurements were conducted on four replicate six-week-old leaves, newly developed under different light treatments, between 12:00 and 14:00 hours. Data (means ± SD, *n* = 4) were analyzed using both three-way ANOVA with species (SP), solar irradiance (VIS), and UV radiation (UV) as fixed factors (with their interaction factors) and two-way ANOVA with VIS and UV as fixed factors (with their interaction factors), for each individual species. Summary of three-way and two-way ANOVA is in Tables A1-A3 in APPENDIX.
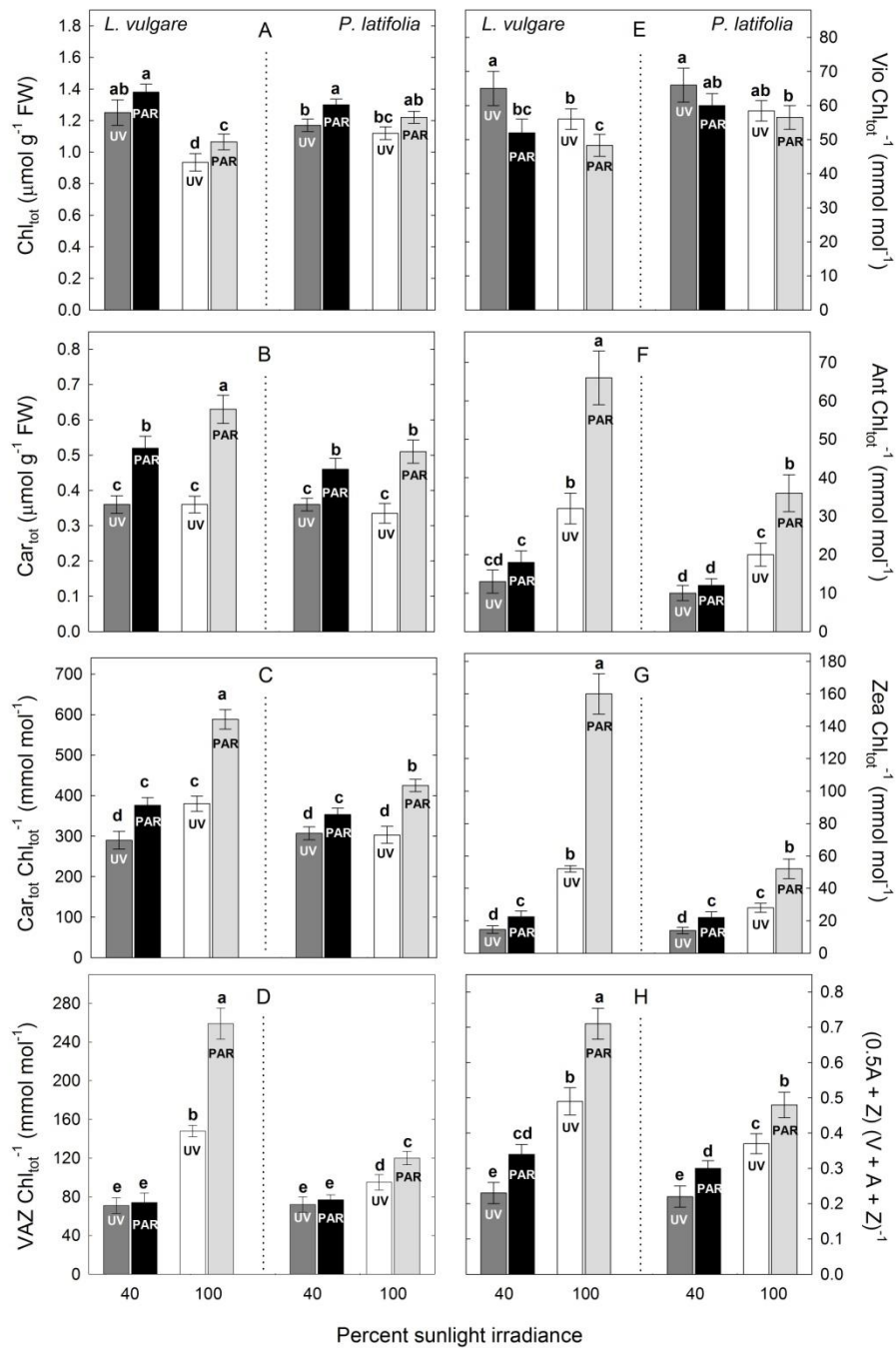
Figure 2. The concentrations of total chlorophyll (Chl_{tot}) and carotenoids (Car_{tot}, B), the relative (to Chl_{tot}) concentration of carotenoids (C), xanthophyll cycle pigments, (D-G), the de-epoxidation state of VAZ (H) in *L. vulgare* and *P. latifolia* leaves grown under partial shading (40% full sunlight) or fully exposed to sunlight (100%) in the presence (UV) or in the absence (PAR) of UV radiation. Data are means ± SD, *n* = 4. Statistical treatment of data as reported in Fig. 1.

1   *3.3. Visible and UV radiation affect the biosynthesis of phenylpropanoids more in L. vulgare*

2   *than in P. latifolia*

3   An increase in visible light was the main driver for the biosynthesis of

4   phenylpropanoids, irrespective of species (Table 1; Fig. 3; see Appendix Table A1). The

5   investment of fresh assimilated carbon to phenylpropanoid biosynthesis, calculated by

6   normalizing the whole-leaf phenylpropanoid concentration to total assimilated carbon over the

7   six-week-experimental period, was much higher in *L. vulgare* (3.45 mmol mol^{-1} CO_2) than in

8   *P. latifolia* (1.15 mmol mol^{-1} CO_2) growing in full sunlight (Fig. 3E). UV radiation also

9   promoted the biosynthesis of phenylpropanoids (with the exception of API glycosides), with a

10   greater increase in *L. vulgare* (+49%) than in *P. latifolia* (+33%), in both shaded and full sun

11   leaves (Fig. 3). Both visible and UV radiation mostly affected the concentration of dihydroxy

12   B-ring-substituted flavonoids, especially QUE derivatives, particularly in *L. vulgare* (Fig. 3C).

13   The ratio of QUE to other phenylpropanoids (PHENYL) varied from 0.25 to 0.51 in *L. vulgare*

14   or from 0.20 to 0.32 in *P. latifolia* because of visible light (data not shown, but see Fig. 3A-

15   D). The QUE to PHENYL ratio further increased because of UV radiation, by 60% in *L.*

16   *vulgare* and by 37% in *P. latifolia*.

17   'Epidermal' flavonoids, mostly QUE and LUT derivatives in our study, increased

18   considerably because of visible light, but varied much less in response to UV-B radiation (Fig.

19   3F). Flavonoids were detected in higher concentrations in the epidermal layers of *P. latifolia*

20   leaves as compared to corresponding tissues of *L. vulgare*, irrespective of light treatments.

21   Therefore, the greater concentrations of QUE and LUT, at the level of the whole-leaf, observed

22   *L. vulgare* than in *P. latifolia*, when plants grew at full sunlight (irrespective of the UV-

23   treatment) were attributable to mesophyll flavonoids.

24

1
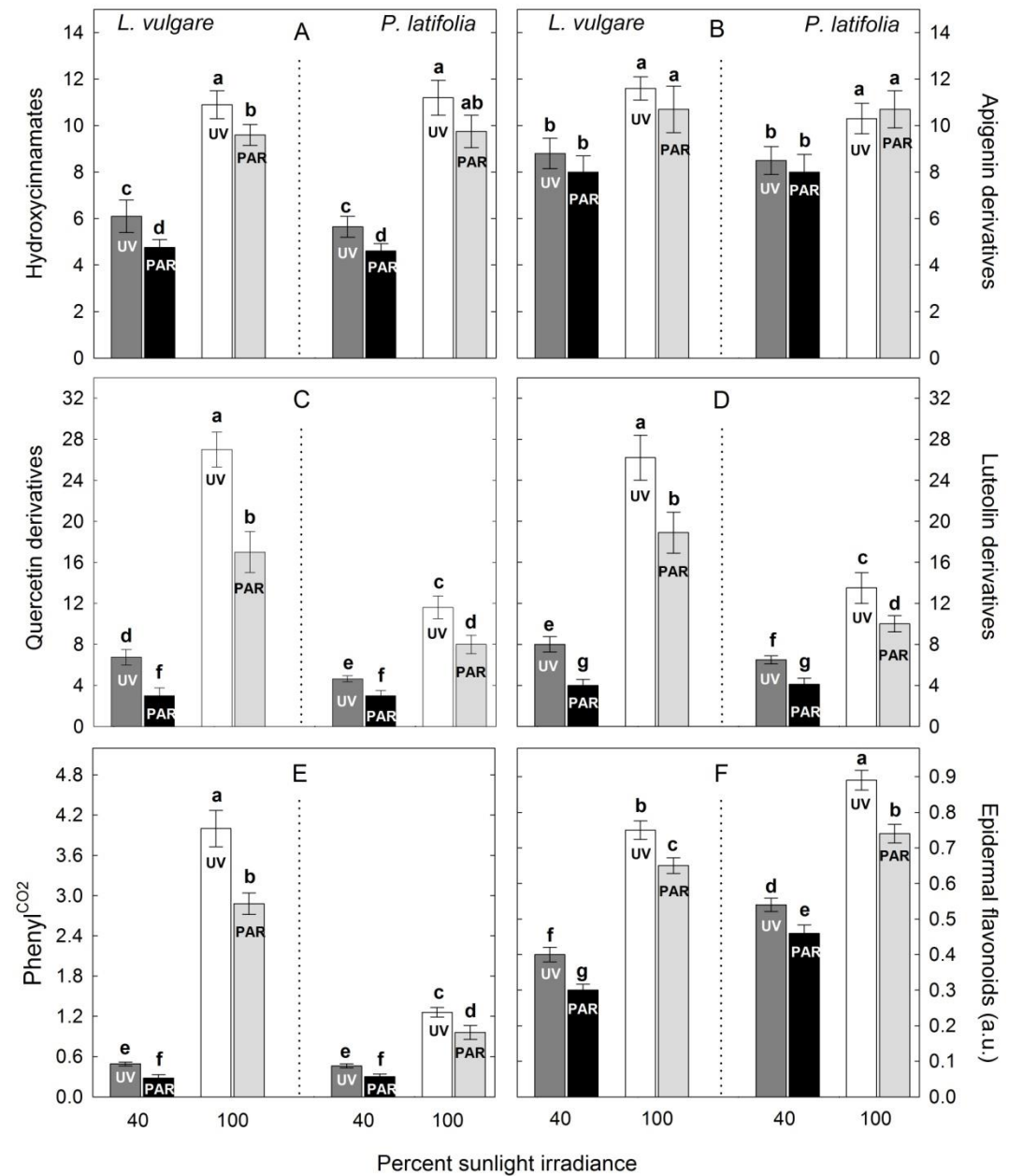
Figure 3. The whole-leaf concentration (μmol g^{-1} FW) of individual phenylpropanoids (A-D), the concentration of total phenylpropanoids normalized to assimilated CO_2 (Phenyl^{CO2}, E), the content of epidermal flavonoids (F) in *L. vulgare* and *P. latifolia* leaves grown under partial shading (40% full sunlight) or fully exposed to sunlight (100%) in the presence (UV) or in the absence (PAR) of UV radiation. Data are means ± SD, $n = 4$. Statistical treatment of data as reported in Fig. 1.

1   Finally, the three-dimensional fluorescence micro-imaging of *L. vulgare* leaves

2 exposed to full sunlight in the presence of UV radiation offers clear evidence that QUE and

3 LUT derivatives occur in the vacuole and chloroplasts of palisade parenchyma cells (Fig. 4A).

4 The overlap between Chl and flavonoid fluorescence (Fig. 4B) in intact cells is interesting, and

5 suggests that QUE and LUT might be located not only in the chloroplast outer envelope

6 membrane, as previously hypothesized by Agati et al. (2007), but possibly also in thylakoid

7 membranes.

8
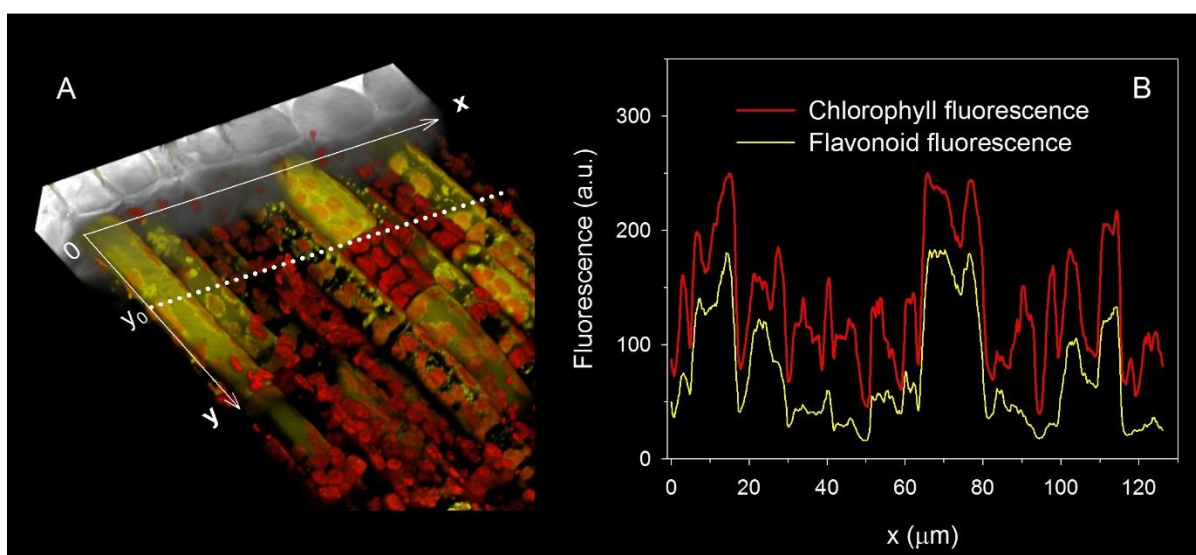
9

10 **Figure 4**. (A) Three-dimensional view of a Naturstoff-stained cross section of a *L. vulgare* leaf exposed
11 to full sunlight in the presence of UV radiation. Sixty fluorescence images were recorded (at 0.3-µm-
12 steps) along the *z*-axis in a Confocal Laser Scanning Microscope. Excitation-emission set-up: λ_{exc} =
13 488 nm and λ_{em} over the 562-646 nm waveband for the detection of QUE and LUT derivatives (yellow
14 channel); λ_{exc} = 488 nm and λ_{em} over the 687-7576 nm waveband for chlorophyll detection (red
15 channel). (B) Profiles of Chlorophyll and Flavonoid fluorescence obtained by plotting the mean
16 fluorescence intensity of each longitudinal row of pixels (x^0 to x^1) over the y_0 to y_1 leaf depth (see white
17 arrows in A).

18

1   **4. Discussion**

2   Data of our study offer a clear picture of the interaction effects of visible and UV

3   radiation on the concentration and composition of photosynthetic and non-photosynthetic

4   pigments, in two species that inhabit areas at largely different sunlight availability. Since the

5   biosynthesis of flavonoids represents a biochemical adjustment of much greater significance

6   in *L. vulgare* than in *P. latifolia* in response to high sunlight (Tattini et al., 2005), our study

7   may help understanding the relative significance of carotenoids and flavonoids in

8   photoprotection.

9   *4.1 Visible, not UV radiation affects photosynthetic performance in L. vulgare and P. latifolia*

10   In our study, UV radiation did not greatly affect photosynthetic performance in either

11   species, as maximal efficiency of PSII photochemistry (F_v/F_m) declined little, while quantum

12   yield of PSII photochemistry (Φ_{PSII}) was even slightly higher in UV-exposed than in PAR-

13   exposed leaves. Visible light greatly controlled photosynthetic performance, as F_v/F_m and Φ_{PSII}

14   decreased from shaded to full sun exposed leaves, particularly in *L. vulgare*. Data of our study

15   conforms to the general observation that in plants experiencing high solar irradiance, ambient

16   UV radiation may have a limited impact on photosynthesis (Bassman et al., 2002; Searles et

17   al. 2003; Newsham and Robinson, 2009; Klem et al., 2012; Hideg et al., 2013; Wargent et al.,

18   2015). This is exactly the case of plants grown under a Mediterranean climate. Cumulated daily

19   photon flux (over the visible portion of the solar spectrum) as well as high air temperatures,

20   may render UV radiation a 'primer of metabolic adjustment' (Hideg et al., 2013), rather than a

21   severe stress agent (Paoletti, 2006; Verdaguer et al., 2012; Bussotti et al., 2014; Bornman et

22   al., 2015; Klem et al., 2015; Wargent et al., 2015). The steep decline (-59%) in photosynthesis

23   because of high sunlight observed in *L. vulgare*, but not in *P. latifolia*, adds further

24   experimental validation to previous suggestions that *L. vulgare* is sensitive to high light (Tattini

25   et al., 2005). Light-induced depression of photosynthesis in *L. vulgare* has multiple reasons:

26   significant reductions in chlorophyll concentration (P_n when expressed on Chl_{tot} basis declined

27   by only 38% indeed, data not shown), in electron transport rate, and particularly in CO_2

28   mesophyll conductance, as recently observed by Fini et al. (2016). Mechanisms aimed

29   dissipating an excess of visible light, such as NPQ, operated indeed more in *L. vulgare* than in

30   *P. latifolia*, particularly when plants grew in the absence of UV radiation.

1 *4.2. UV radiation greatly reduces xanthophyll de-epoxidation, but slightly depresses NPQ*

2 Our study offers clear evidence that UV radiation negatively affected the biosynthesis

3 of carotenoids in both species, irrespective of sunlight irradiance. UV radiation significantly

4 reduced the pool of xanthophyll cycle pigments (VAZ) and the conversion of V to its de-

5 epoxided forms A and Z. Our data conform to those previously reported for plants that grew

6 under either ambient (Bischof et al., 2002; Li et al., 2010; Albert et al., 2011) or supplemental

7 UV radiation (Pfündel et al., 1992; Hideg et al., 2006; Yang et al., 2007; Moon et al., 2011;

8 Bernal et al., 2015). UV-induced decline in DES, likely resulted from alteration of the cyclic

9 electron flow, thus reducing the pH gradient across thylakoid membranes (Takahashi and

10 Badger, 2010; Murchie and Niyogi, 2011) and consequentially favoring epoxidation rather

11 than de-epoxidation of the VAZ pool, as compared to PAR exposed leaves (Bischof et al.,

12 2002). In our study, DES and linear electron transport rate (as estimated by Φ_{PSII}) were

13 unrelated indeed, as also observed in previous experiments (Yang et al., 2007; Bernal et al.,

14 2015).

15 It is worth noting that UV-induced marked decrease in DES (on average −30%) did not

16 result in corresponding declines in NPQ (−8%), particularly when plants grew in full sunlight.

17 This suggests that just a portion of VAZ, particularly Z, was likely involved in the thermal

18 dissipation of excess energy in the chloroplast (Peguero-Pina et al., 2013). This observation is

19 consistent with the high concentration of VAZ relative to Chl_{tot} detected in our experiment

20 (Demmig-Adams et al., 2012; Esteban et al., 2015a). Therefore, UV-induced decline in Z

21 concentration, possibly derived from a free pool of xanthophylls in thylakoid membranes rather

22 than VAZ bound to the light harvesting complex, and therefore not directly involved in

23 sustaining NPQ (Peguero-Pina et al., 2013; Havaux and García-Plazaola, 2014; Esteban et al.,

24 2015a,b). This suggests that zeaxanthin might have served an important antioxidant role in our

25 study (Peguero-Pina et al., 2013; Esteban et al., 2015a), the significance of which was greater

26 in plants that grew in the absence of UV radiation, especially in the sun sensitive *L. vulgare*.

27 Zeaxanthin behaves as a direct antioxidant, replacing the functions of tocopherol, and as a

28 membrane stabilizer ('indirect antioxidant') indeed, when the pool of xanthophyll cycle

29 pigments exceeds their potential binding sites in antenna proteins, as exactly occurs in leaves

1 challenged against a severe excess of sunlight irradiance (Havaux et al., 2007; Demming-
2 Adams et al., 2012; Esteban et al., 2015a).

3 *4.3 Visible and UV-induced accumulation of 'antioxidant' QUE and LUT is higher in*
4 *mesophyll cells of L. vulgare*

5 Our study offers further compelling evidence that UV radiation is not necessary for the
6 biosynthesis of both hydroxycinnamic acid and flavonoid derivatives, which have a strong
7 absorption in the UV region of the solar spectrum (Kolb et al., 2001; Agati et al., 2009, 2011;
8 Klem et al., 2012, 2015; Siipola et al., 2015). Data of our study conform to recent findings that
9 blue light may be even more effective than UV-B radiation in stimulating the biosynthesis of
10 flavonoids (Siipola et al., 2015). Nevertheless, UV radiation significantly promoted the
11 biosynthesis of QUE and LUT derivatives, irrespective of visible light, particularly in *L.*
12 *vulgare*. The great investment of carbon in the biosynthesis of flavonoids represents an
13 important component of the suite biochemical adjustments induced by high light (broadly
14 metabolic plasticity, Logemann et al., 2000; Di Martino et al., 2014) in *L. vulgare* (Tattini et
15 al., 2004, 2005). This may perhaps contribute to widespread distribution of this species, as also
16 observed for deciduous and semi-deciduous species with wide geographical distribution
17 (Tattini et al., 2015).

18 As already reported (Agati et al., 2013), QUE and LUT derivatives do not display
19 greater capacities as compared to API and HCA derivatives detected in our study to absorb
20 over the whole range of solar UV wavelengths. It is worth noting, that greater increases in the
21 whole-leaf concentration of QUE and LUT in *L. vulgare* than in *P. latifolia*, in response to
22 different light treatments, did not result in higher levels of epidermal flavonoids (as previously
23 observed, Tattini et al., 2005). These data, when taken together, support the idea that QUE and
24 LUT might have played a role in countering photo-oxidative stress generated by an excess of
25 visible and UV radiation, particularly in *L. vulgare*. Our multispectral fluorescence micro-
26 imaging analysis is consistent with putative antioxidant functions of flavonoids in
27 photoprotection, as QUE and LUT accumulated in the vacuole as well as in the chloroplasts of
28 palisade parenchyma cells proximal to the adaxial epidermis in sun leaves.

4.4 Could flavonoids protect against UV-induced inhibition of xanthophyll biosynthesis in countering photo-oxidative damage to chloroplasts?

Our study shows that UV radiation, while increasing the mesophyll concentration of flavonoids, strongly inhibited the biosynthesis as well as the de-epoxidation of xanthophylls. The effect of UV radiation on the content and composition of photosynthetic and non-photosynthetic pigment was particularly evident in the sun-sensitive *L. vulgare*, which does not display an affective shield to protect mesophyll tissues against an excess of both visible and UV radiation (Tattini et al., 2005; Fini et al., 2016). This raises the question whether flavonoids may serve functions similar to those played by carotenoids in UV-exposed leaves, though flavonoids and carotenoids are known as serving distinct functions in photoprotection, based on relative physical-chemical features and intra-cellular distribution.

In our study, QUE and LUT derivatives had a clear chloroplast location, but the exact location of flavonoids in the chloroplast is not easily resolved issue with detection techniques currently available. There is still uncertainty whether flavonoids are located in thylakoids or instead associated to the chloroplast outer envelope membrane (Agati et al., 2007). The overlap between Chl and flavonoid fluorescence observed in our study is of interest. This observation conforms to previous findings that QUE derivatives may insert in hydrophilic and hydrophobic domains of thylakoid membranes (Pawlikoska-Pawlega et al., 2007), mostly at the stromal side of thylakoids at basic pHs, as occurs when chloroplasts suffer from a severe excess of light (Takahashi and Badger 2012; Dobrikova and Apostolova, 2015; Ruban, 2015). Therefore, in our study, QUE may have served multiple functions in protecting chloroplasts from photo-oxidative damage: by both absorbing UV radiation and protecting membrane lipids from peroxidation (Yoku *et al*., 1995; Pawlikoska-Pawlega et al., 2007) as well as through direct quenching of reactive oxygen species, such as $^1O_2$ (Agati et al. 2007).

The significance of flavonoids in the network of chloroplast antioxidants is an interesting issue, which deserves further investigation. Nonetheless, we note that flavonoids and carotenoids do have different inter-cellular, not only intra-cellular distribution in the leaf. While flavonoids accumulate mostly in adaxial (i.e. proximal to adaxial epidermis) mesophyll cells (this study, Tattini et al., 2004, 2005; Agati et al., 2007), carotenoids (and chlorophyll) are distributed in tissues located deep in the leaf (Nishio et al., 1993; Ålenius et al., 1995;

Gould et al., 2002; Vogelmann and Evans, 2002). The inverse inter-cellular gradient in non-photosynthetic and photosynthetic pigment distribution might be even more evident in sun leaves (Nishio et al., 1993; Agati et al., 2010). In high light-stressed leaves (as our leaves were, see F_v/F_m and Φ_{PSII} values), the degree of blue and red light-induced photoinhibition was shown to be markedly greater in adaxial than abaxial mesophyll cells (Oguchi *et al.*, 2011). Since F_v/F_m decreased more than Φ_{PSII} did (Oguchi *et al.*, 2011), it is possible that NPQ did not operate much in regulating PSII photochemistry in adaxial mesophyll cells (Meyers et al., 1997). Consequently, we put forward the idea that the antioxidant functions of chloroplast flavonoids might be of particular significance just in adaxial mesophyll cells, in which high doses of UV-radiation strongly inhibit xanthophyll de-epoxidation.

5. Conclusions

Our study, which extends previous suggestions of a potential functional relationship between carotenoids and flavonoids in leaves exposed to excess visible light (Havaux and Kloppstech, 2001) offers the hypothesis that flavonoids might complement the photo-protective functions of xanthophylls in the chloroplasts of mesophyll cells exposed to the greatest doses of UV radiation. However, UV radiation might result in adaxial mesophyll cells being less effective in dissipation of excess radiant energy, e.g., by decreasing their capacity of thermal dissipation of excess visible light in the chloroplast. This might be of particular significance, in view of future climate change, when the use of radiant energy to photosynthesis in high light grown plants will be severely constrained by concurrent environmental stressors, such as heat waves coupled with transient but severe drought stress events. The much higher depression in the biosynthesis and the de-epoxidation of xanthophylls in response to ambient UV radiation observed in *L. vulgare* than in *P. latifolia* may also help explain the infrequent distribution of *L. vulgare* facing harsh Mediterranean environments.

Acknowledgments

APPENDIX

Table A1. Summary of three-way ANOVA of the effects of species, solar irradiance and UV radiation as fixed factors with their interaction factors on photosynthesis (P_n), maximum (F_v/F_m) and actual (Φ_{PSII}) efficiency of PSII photochemistry, nonphotochemical quenching (NPQ), the concentrations (μmol g^{-1} FW) of total chlorophyll (Chl_{tot}), total carotenoids (Car_{tot}), and individual phenylpropanoids, as well as the concentrations of Car_{tot} and individual carotenoids relative to Chl_{tot} concentration in *L. vulgare* and *P. latifolia* leaves exposed to 40% or 100% sunlight in the absence or in the presence of UV radiation. Total error degrees of freedom (df) = 31, except for 'epidermal' flavonoids, for which df = 95.

$P^{***} < 0.0001$; $P^{**}, < 0.001$; $P^* < 0.05$; n.s., not significant.

Variable	$F_{species}$ (SP)	$F_{irradiance}$ (IR)	F_{UV} (UV)	$F_{SP \times IR}$	$F_{SP \times UV}$	$F_{IR \times UV}$	$F_{SP \times IR \times UV}$
P_n	21.2***	27.8***	0.2 n.s.	20.6	0.7 n.s.	0.5 n.s.	0.3 n.s.
F_v/F_m	35.5***	230.8***	69.2***	5.2*	1.0 n.s.	0.4 ns	0.6 n.s.
Φ_{PSII}	1.9 n.s.	793.5***	54.6***	29.6***	0.1 n.s.	6.9*	1.1 n.s.
NPQ	56.1***	138.3***	41.1***	1.9 n.s.	0.5 n.s.	0.5 ns	0.7 n.s.
Chl_{tot}	3.4 n.s.	73.7***	42.5***	4.1 n.s.	1.2 n.s.	0.5 n.s.	0.1 n.s.
Car_{tot}	5.7*	5.5*	202.2***	19.3***	7.0*	23.5***	5.3*
Car_{tot} Chl_{tot}^{-1}	8.3*	45.1***	124.1***	34.8***	3.8 n.s.	47.7***	3.9 n.s.
Lutein Chl_{tot}^{-1}	0.9 n.s.	3.4 n.s.	80.2***	9.9**	0.1 n.s.	19.9***	0.8 n.s.
β-carotene Chl_{tot}^{-1}	7.1*	0.1 n.s.	158.1***	12.9**	0.1 n.s.	17.3***	0.5 n.s.
Zeaxanthin (Z) Chl_{tot}^{-1}	28.3***	161.2***	101.7***	19.9***	9.6**	52.6***	7.8*
Antheraxanthin (A) Chl_{tot}^{-1}	33.4***	264.5***	91.4***	5.9*	5.6*	38.5***	0.8 n.s.
Violaxanthin (V) Chl_{tot}^{-1}	2.6 n.s.	6.1*	54.7***	4.1 n.s.	5.1*	0.7 n.s.	0.4 n.s.
VAZ (V + A + Z) Chl_{tot}^{-1}	35.4***	142.5***	50.7***	17.2***	9.8**	39.6***	5.1*
DES (0.5A + Z) (V + A + Z)$^{-1}$	17.3***	354.1***	220.6***	6.2*.	5.9*.	13.5**	0.1 n.s.
Hydroxycinnamic derivatives	0.3 n.s.	493.5***	55.4***	2.3 n.s.	0.1 n.s.	1.8 n.s.	0.7 n.s.
Quercetin derivatives.	126.7***	455.8***	110.4***	69.7***	31.8***	5.7*	8.2*
Luteolin derivatives	78.1***	239.8***	48.2***	14.5**	8.0*	2.7 n.s.	1.1 n.s.
Apigenin derivatives	2.9 n.s.	86.7***	1.5 n.s.	0.1 n.s.	1.2 n.s.	3.1 n.s.	1.2 n.s.
Flavonoid index	112.9***	810.1***	75.0***	6.6*	4.0 n.s.	1.2 n.s.	7.8*

1   **APPENDIX**

2   Table A2. Summary of two-way ANOVA of the effects solar irradiance and UV radiation as
3   fixed factors with their interaction factor on photosynthesis (P_n), maximum (F_v/F_m) and actual
4   (Φ_{PSII}) efficiency of PSII photochemistry, nonphotochemical quenching (NPQ), the
5   concentrations (μmol g^{-1} FW) of total chlorophyll (Chl$_{tot}$), total carotenoids (Car$_{tot}$), individual
6   phenylpropanoids, and the concentrations of Car$_{tot}$ and individual carotenoids relative to Chl$_{tot}$
7   concentration in *L. vulgare* leaves exposed to 40% or 100% sunlight in the absence or in the
8   presence of UV radiation. Total error degrees of freedom (df) = 15, except for epidermal
9   flavonoids, for which df = 47.

10   P***< 0.0001; P** < 0.001; P* < 0.05; n.s., not significant

Variable	F_{IR}	F_{UV}	$F_{IR \times UV}$
P_n	170.7***	1.6 n.s.	0.4 n.s
F_v/F_m	117.0***	31.5 ***	0.4 ns
Φ_{PSII}	929.8***	37.2***	4.7 n.s.
NPQ	140.6***	34.2***	0.1 n.s.
Chl$_{tot}$	81.7***	28.7***	0.5 n.s.
Car$_{tot}$	5.4*	135.2***	21.6**
Car$_{tot}$ Chl$_{tot}^{-1}$	163.2***	177.5***	79.9***
Lutein Chl$_{tot}^{-1}$	8.5*	28.9***	10.1**
β-carotene Chl$_{tot}^{-1}$	6.7*	95.6***	11.6**
Zeaxanthin (Z) Chl$_{tot}^{-1}$	506.5***	287.8***	169.4***
Antheraxanthin (A) Chl$_{tot}^{-1}$	248.3***	99.6***	33.4***
Violaxanthin (V) Chl$_{tot}^{-1}$	0.2 n.s.	48.2***	0.1 n.s.
VAZ (V + A + Z) Chl$_{tot}^{-1}$	497.7***	167.0***	136.5***
DES [(0.5A + Z) (V + A + Z)$^{-1}$]	229.5***	148.3***	7.2*
Hydroxycinnamic derivatives	237.1**	34.7***	0.4 n.s.
Quercetin derivatives	362.4***	117.1***	31.5***
Luteolin derivatives	340.9***	78.6***	4.9 n.s.
Apigenin derivatives	31.9***	0.9 n.s.	3.0 n.s.
'Epidermal' flavonoids	926.5***	51.2***	0.9 n.s.

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1   **APPENDIX**

2   Table A3. Summary of two-way ANOVA of the effects solar irradiance and UV radiation as

3   fixed factors with their interaction factor on photosynthesis (P_n), maximum (F_v/F_m) and actual

4   (Φ_{PSII}) efficiency of PSII photochemistry, nonphotochemical quenching (NPQ), the

5   concentrations ($\mu mol \ g^{-1}$ FW) of total chlorophyll (Chl_{tot}), total carotenoids (Car_{tot}), individual

6   phenylpropanoids, and the concentrations of Car_{tot} and individual carotenoids relative to Chl_{tot}

7   concentration in *P. latifolia* leaves exposed to 40% or 100% sunlight in the absence or in the

8   presence of UV radiation. Total error degrees of freedom (df) = 15, except for epidermal

9   flavonoids, for which df = 47.

10   P*** < 0.0001; P**, < 0.001; P* < 0.05; n.s., not significant

Variable	F_{IR}	F_{UV}	$F_{IR \times UV}$
P_n	0.2 n.s.	0.6 n.s.	0.0 n.s
F_v/F_m	97.9***	26.1***	0.1 n.s.
Φ_{PSII}	728.2***	51.1***	7.7*
NPQ	115.4***	23.5***	0.3 n.s.
Chl_{tot}	10.8*	18.9**	0.1 n.s.
Car_{tot}	4.4 n.s.	70.2***	2.4 n.s.
$Car_{tot} \ Chl_{tot}^{-1}$	0.2 n.s.	98.3***	21.5***
Lutein Chl_{tot}^{-1}	0.7 n.s.	47.3***	7.6*
β-carotene Chl_{tot}^{-1}	3.6 n.s.	19.3**	0.2 n.s.
Zeaxanthin (Z) Chl_{tot}^{-1}	289.9***	202.7***	56.8***
Antheraxanthin (A) Chl_{tot}^{-1}	170.0***	38.5***	18.9**
Violaxanthin (V) Chl_{tot}^{-1}	10.4**	14.2**	2.6 n.s.
VAZ (V + A + Z) Chl_{tot}^{-1}	119.2***	32.9***	20.8***
DES [(0.5A + Z) (V + A + Z)$^{-1}$]	197.8***	113.1***	5.8*
Hydroxycinnamic derivatives	276.8***	23.7***	1.6 n.s.
Quercetin derivatives	240.2***	53.0***	6.5*
Luteolin derivatives	170.3***	57.3***	3.3 n.s.
Apigenin derivatives	42.1***	1.1 n.s.	0.3 n.s.
'Epidermal' flavonoids	520.3***	65.3***	5.4*

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1 **Legends for Figures**

2 **Figure 1.** Photosynthesis (P_n, A), maximum (F_v/F_m, B) and actual (Φ_{PSII}, C) efficiency of PSII
3 photochemistry, and nonphotochemical quenching (NPQ, D) in *L. vulgare* and *P. latifolia* leaves
4 grown under partial shading (40% full sunlight) or fully exposed to sunlight (100%) in the
5 presence (UV) or in the absence (PAR) of UV radiation. Measurements were conducted on four
6 replicate six-week-old leaves, newly developed under different light treatments, between 12:00
7 and 14:00 hours. Data (means ± SD, $n = 4$) were analyzed using both three-way ANOVA with
8 species (SP), solar irradiance (VIS), and UV radiation (UV) as fixed factors (with their interaction
9 factors) and two-way ANOVA with VIS and UV as fixed factors (with their interaction factors),
10 for each individual species. Summary of three-way and two-way ANOVA is in Tables A1-A3 in
11 APPENDIX.

12 **Figure 2.** The concentrations of total chlorophyll (Chl_{tot}) and carotenoids (Car_{tot}, B), the relative
13 (to Chl_{tot}) concentration of carotenoids (C), xanthophyll cycle pigments, (D-G), the de-
14 epoxidation state of VAZ (H) in *L. vulgare* and *P. latifolia* leaves grown under partial shading
15 (40% full sunlight) or fully exposed to sunlight (100%) in the presence (UV) or in the absence
16 (PAR) of UV radiation. Data are means ± SD, $n = 4$. Statistical treatment of data as reported in
17 Fig. 1.

18 **Figure 3.** The whole-leaf concentration (μmol g^{-1} FW) of individual phenylpropanoids (A-D),
19 the concentration of total phenylpropanoids normalized to assimilated CO_2 ($Phenyl^{CO2}$, E), the
20 content of epidermal flavonoids (F) in *L. vulgare* and *P. latifolia* leaves grown under partial
21 shading (40% full sunlight) or fully exposed to sunlight (100%) in the presence (UV) or in the
22 absence (PAR) of UV radiation. Data are means ± SD, $n = 4$. Statistical treatment of data as
23 reported in Fig. 1.

24 **Figure 4**. (A) Three-dimensional view of a Naturstoff-stained cross section of a *L. vulgare* leaf
25 exposed to full sunlight in the presence of UV radiation. Sixty fluorescence images were recorded
26 (at 0.3-μm-steps) along the *z*-axis in a Confocal Laser Scanning Microscope. Excitation-emission
27 set-up: $\lambda_{exc} = 488$ nm and λ_{em} over the 562-646 nm waveband for the detection of QUE and LUT
28 derivatives (yellow channel); $\lambda_{exc} = 488$ nm and λ_{em} over the 687-7576 nm waveband for
29 chlorophyll detection (red channel). (B) Profiles of Chlorophyll and Flavonoid fluorescence
30 obtained by plotting the mean fluorescence intensity of each longitudinal row of pixels (x^0 to x^1)
31 over the y_0 to y_1 leaf depth (see white arrows in A).

32