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Research article

Epidermal UVA screening capacity measured *in situ* as an indicator of light acclimation state of leaves of a very plastic alpine plant *Soldanella alpina* L.

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ABSTRACT

Soldanella alpina differing in leaf epidermal UV-A absorbance (DEA_{375}), as measured with the Dualex, was investigated as a model alpine plant for the flavonoid (Flav) composition and concentration and for anatomical and pigment characteristics. In sun leaves, twenty-three flavones were characterised by their mass formula, their maximum absorption, their glycosylation, their methylation and dehydroxylation pattern. The flavones belonged to four subfamilies (tetra-hydroxy-flavones, penta-hydroxy-flavones, penta-hydroxy-methyl-flavones and tri-hydroxy-di-methoxy-flavones), abundant in sun and shade leaves. Their concentration was estimated by their absorption at 350 nm after HPLC separation. Sun leaves contained relatively higher contents of penta-hydroxy-methyl-flavones and shade leaves higher contents of tetra-hydroxy-flavones. The flavones were present mainly in vacuoles, all over the leaf. After shade-sun transfer, the content of most flavones increased, irrespective of the presence or absence of UV radiation. Highly significant correlations with the log-transformed DEA_{375} suggest that DEA_{375} can be readily applied to predict the flavone content of *S. alpina* leaves. Shade-sun transfer of leaves decreased the hydroxycinnamic acid (HCA) content, the mass-based chlorophyll (Chl) a + b content and the Chl/Carotenoid (Car) ratio but increased DEA_{375}, and the Car content. Together with previously reported anatomical characteristics all these parameters correlated significantly with the DEA_{375}. The Flav content is therefore correlated to most of the structural characteristics of leaf acclimation to light and this can be probed *in situ* by DEA_{375}.

1. Introduction

Because climate change threatens alpine plants, it becomes urgent to develop the knowledge of their plasticity in response to rapid environmental changes. Since about ten years ago, experiments are conducted each summer on *Soldanella alpina* L. at the Joseph Fournier Alpine Station to better understand its acclimation potential. *Soldanella alpina* plants growing in the alpine life zone belong to the family of the *Primulaceae* from which a rich composition of different flavonoids (Flav) have been described (Harborne, 1968). Total leaf Flav content of plants increases with altitude (Rozema et al., 1997; Filella and Penuelas, 1999). Flav synthesis is induced by UV-B radiation and their accumulation in cell walls, vacuoles and trichomes of epidermal cells was shown to attenuate the penetration of UV radiation (Winkel-Shirley, 2002; Agati et al., 2013). However, also visible light and different stress factors induce Flav synthesis and they were shown to be present in vacuoles and chloroplasts of mesophyll cells. This, as well as the known antioxidant activity of Flav, suggests that Flav accumulation also contribute to antioxidant protection (Agati et al., 2013). Hence, under high sunlight in the mountains, flavonoids could protect photosynthetic cells from UV-induced damage by reducing the UV radiation arriving in the chloroplast and by scavenging of reactive oxygen species

Abbreviations: Car, carotenoids; Chl, chlorophyll; Chl-index, chlorophyll optically measured; DEA_{322}, DEA_{375}, epidermal UV absorbance optically measured with the Dualex at 322 nm or 375 nm; Flav, flavonoids; HCA, hydroxycinnamic acids; LMA, leaf mass per area; m/z, mass to charge ratio; UV, ultraviolet

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formed under such conditions (Agati et al., 2013).

In recent years, portable fluorescence detectors, like the Dualex, allow to estimate epidermal leaf Flav contents *in situ* (Goulas et al., 2004). In *S. alpina*, the Dualex measurements of epidermal UV-A absorbance at 375 nm (DEA_{375}) suggest very high contents of epidermal flavonoids, which contribute to protection of photosystem II from photoinhibition under natural sunlight (Laureau et al., 2015). The UV filtering capacity of *S. alpina* leaves is very high as compared to most other investigated species (Meyer et al., 2006; Louis et al., 2009; Latouche et al., 2013; Bidel et al., 2015; Klem et al., 2015; Lefebvre et al., 2016) and as compared to several other alpine plant leaves growing at the same site (unpublished results).

However, *S. alpina* leaves with relatively low contents of UV absorbing compounds were also present at shaded growing sites. Similar differences in epidermal UV-A absorbance between shade and sun leaves have been published for various plant species (Krause et al., 2003; Barnes et al., 2013). In *S. alpina* leaves, high DEA_{375} in comparison to low DEA_{375} correlated with other characteristics indicating light acclimation as: 1) higher chlorophyll Chl a/b ratio 2) lower Chl/Car ratio 3) higher ascorbate peroxidase activity, 4) higher leaf mass per area (LMA) and leaf thickness, 5) higher stomatal, trichome and adaxial epidermal cell densities (Laureau et al., 2015). At least for *S. alpina* leaves, DEA_{375} measurements may be suitable to estimate rapidly and non-destructively the relative state of light acclimation of leaves *in situ*.

While UV absorbance and Flav contents vary in several plant species due to daily changing light conditions (Veit et al., 1996; Barnes et al., 2016), it was stable in *S. alpina* leaves (Laureau et al., 2015). DEA_{375} of *S. alpina* leaves did not decline after prolonged transfer of plants to low light intensity in a growth chamber neither during senescence of leaves (Laureau et al., 2015), confirming observations in *Populus tremuloides* and *Vicia faba* (Barnes et al., 2013) and several tree species (Mattila et al., 2018). In contrast, high epidermal UV absorbance was induced in shade leaves exposed to sunlight, even in the absence of UV radiation (Barnes et al., 2013; Laureau et al., 2015). Such a lowlight – highlight transfer occurs also in the natural environment in particular in autumn if the vegetation shading *S. alpina* leaves gets senescent and during grazing by animals. In spring, after snowmelt, all leaves from the previous year have a high DEA_{375}, while newly formed leaves of the present year can develop under shade conditions with low DEA_{375} (Laureau et al., 2015).

The Flav composition and their tissue distribution of *S. alpina* are unknown, as well as changes induced by shade-sun transfer. After sun exposure, the accumulation of Flav is generally accompanied by a decrease in the mass allocated to Chl within the leaf (Meyer et al., 2006). By optically measuring both Chl and epidermal Flav contents, using the Chl meter SPAD or the Dualex 4 Scientific (Cerovic et al., 2015), it is possible to probe this process *in situ* by assessing the Chl to Flav ratio of leaves. In woody plants, like beech, this ratio decreases from shade to sun leaves through the canopy (Meyer et al., 2006; Louis et al., 2012). However, it is unknown to which extent it could probe the short-term plasticity of shade leaves exposed to sun in the field.

S. alpina is an interesting model for this investigation, since it grows at various altitudes, it is already enzymatically and photosynthetically characterised (Laureau et al., 2011, 2015) and because the same leaf can experience very low and very high solar radiation during its life cycle in the Alps. Our specific objectives were (i) to investigate the Flav composition of *S. alpina* leaves and their distribution inside the leaf, (ii) to analyse changes of the Flav composition induced by sunlight in the presence and absence of UV and their relation to optically assessed epidermal Flav content and (iii) to investigate to which extent optical sensor measurements are correlated to Chl, Car, and to anatomical traits in different light acclimation states of *S. alpina* leaves.

2. Material and methods

2.1. Plant material and experimental designs

S. alpina plants growing at 2100 m (45° 02′ 12.3″ N 6° 24′ 068′ E) in the area of the Lautaret and at 2400 m altitude (45° 03′ 10.5″ N 6° 24′ 20.2″ E) between the Lautaret and the Galibier pass in the French Alps were investigated (Laureau et al., 2015). Plants at 2100 m altitude were used either from shaded sites covered by the surrounding vegetation or from more sun exposed sites in an area of approximately 30 m^2. At this altitude plants had fully developed leaves of the present year at the beginning of the experiments. Plants at 2400 m altitude were all sun exposed and investigations started directly after snowmelt in leaves from the previous year. As shown previously (Talhouët et al., 2020), only mature leaves, which did not change their leaf area during the experiment, were used.

Experiments were performed in four consecutive years. In 2015, selected leaves with low or high DEA_{375} values were marked and investigated regularly during 30 days. Leaves covered by the surrounding vegetation were either left under this condition (shade leaves) or the vegetation was cut (shade-sun transferred leaves) and leaves were exposed to sunlight either in the presence or absence of a UV cut-off filter (1 m^2; Plexiglas PVCG; Air and Eau Systèmes, Ludres, France; cutoff filter at 400 nm) which absorbs all radiation lower than 400 nm (Laureau et al., 2015). Shade-sun transferred leaves which were protected by the UV filter were used for the investigation of the individual induced Flav. Since the use of the UV filter had no major effect on Flav induction in 2015, all other experiments were performed in the absence of the UV filter.

2.2. Radiation and temperature measurements

The leaf temperatures were measured with a copper-constantan thermocouple attached to the lower surface of at least 2 independent leaves under each condition and the PFD with a quantum flux sensor (JYP 1000, SDEC France) which was placed in the same orientation and height of 2 independent representative leaves. The data were recorded with a Campbell data logger (CR23x, Logan, Utah) during the whole investigation period in the reference year 2015 at 2100 m and 2018 at 2400 m. Data were accumulated every 30 s. Hourly mean and maximum photon flux density values are shown in Table 1. UV-A and UV-B radiation were measured with a UV radiometer RM22 (Dr. Göbel, Ettlingen, Germany) for at least 1 h during three sunny cloudless days under the different conditions. Actual maximum values are presented as W cm^{-2} and accumulated values as J h^{-1} (Table 1).

Table 1
Mean and maximum values of Photon Flux Density (PFD) during the day (7–18h), leaf day and night (18-7h) temperature during the investigation period in the year 2015 at 2100 m altitude and during the investigation period in the year 2018 at 2400 m altitude of *S. alpina* leaves. UV-A and UV-B radiation was measured for 60 min at three independent cloudless days. Actual UV radiation was measured as mW cm^{-2}, total UV radiation was calculated as J h^{-1}. Leaves at 2100 m altitude were investigated under shaded conditions under vegetation and exposed conditions in sunlight or sunlight protected by a cut-off UV filter.

		Shade	Sun	Sun + UV Filter	Sun 2400 m
PFD µmol $m^{-2}s^{-1}$	mean	300	800	700	800
	max	2000	2500	2000	2700
T °C	day 7–18h	17	19.5	20	19.9
T °C	night 18-7h	11	9.5	10.5	9.1
UV-A	mW cm^{-2}	0.193	2.56	0.132	3.3
	J h^{-1}	1.1	10.22	0.51	11.8
UV-B	mW cm^{-2}	0.011	0.095	0.0085	0.14
	mJ h^{-1}	43	429	39	541

Table 2
Single ion record of Flav of *S. alpina* sun leaves after HPLC separation. In total 32 peaks with a typical Flav signature were measured and 23 major Flav were characterised. They are presented in the order of the estimated Flav subfamily and the elution of the peak (P), which was numbered subsequently. The retention time (RT) is shown. The table include the monoisotopic mass to charge ratio (M/C) (m/z), the major and important MS_2 ions (m/z), the proposed Flav formula, the wavelength of the maximum peak absorption in the UV-C, a shoulder in the UV-B and the maximum in the UV-A region in nm and the probable glycosylation.

P	RT	M/C ratio (m/z)	MS_2 ions (m/z)	Flavonoid formula	UV-C	UV-B	UV-A	Glycosylation
Subfamily: Tetra-Hydroxy-Flavone								
1	28.96	903.2770	741, 757, 611, 595, 449, 287	$C_{39}H_{50}O_{24}$	263	305	345.2	di-rhamnose-di-hexose
2	32.38	903.2770	741, 757, 611, 595, 449, 287	$C_{39}H_{50}O_{24}$	262	306.5	343	di-rhamnose-di-hexose
3	39.50	903.2770	741, 757, 611, 595, 449, 287	$C_{39}H_{50}O_{24}$	262	306	345	di-rhamnose-di-hexose
7	51.26	741.2242	595, 449, 287	$C_{33}H_{40}O_{19}$	263	302	344.4	di-rhamnose-hexose
8	52.16	595.1663	449, 287	$C_{27}H_{30}O_{15}$	263	305	345	rhamnose-hexose
9	52.97	741.2242	595, 449, 287	$C_{33}H_{40}O_{19}$	263	305	344	rhamnose-hexose
11	55.12	595.1663	449, 287	$C_{27}H_{30}O_{15}$	263	306.2	345.2	rhamnose-hexose
12	55.70	741.2242	595, 449, 287	$C_{33}H_{40}O_{19}$	253	306	348.1	di-rhamnose-hexose
Subfamily: Penta-Hydroxy-Flavone								
4	47.37	611.1612	465, 303	$C_{27}H_{30}O_{16}$	253	310	350	rhamnose-hexose
5	49.83	757.2191	627, 611, 465, 303	$C_{33}H_{40}O_{20}$	254	309	350	di-rhamnose-hexose
6	50.92	611.1612	465, 303	$C_{27}H_{30}O_{16}$	255	304.4	349	rhamnose-hexose
Subfamily: Penta-Hydroxy-Methyl-Flavone								
13	55.89	771.2348	625, 479, 317, 302, 285, 153	$C_{34}H_{42}O_{20}$	252	309	350.4	di-rhamnose-hexose
14	56.17	771.2348	625, 479, 317, 302, 285, 153	$C_{34}H_{42}O_{20}$	251.4	306.5	351.4	di-rhamnose-hexose
15	56.52	479.119	317	$C_{22}H_{22}O_{12}$	249.2	307	347	hexose
16	56.90	625.1769	479, 317, 302, 285, 153	$C_{28}H_{32}O_{16}$	263	306.2	347	rhamnose-hexose
Subfamily:Tri-Hydroxy-Di-Methoxy-Flavone								
10	54.14	683.146	331	$C_{29}H_{30}O_{19}$	263	304	348	di-glucuronide
17	57.79	507.1139	331	$C_{23}H_{22}O_{13}$	265.1	304.2	349.2	glucuronide
18	59.27	493.1346	331	$C_{23}H_{24}O_{12}$	265.2	304.2	333.2	hexose
19	59.51	859.178	829, 507, 331	$C_{35}H_{38}O_{25}$	266.1	304	326.1	tri-glucoronide
20	60.2	859.1780	829, 507, 331	$C_{35}H_{38}O_{25}$	265	304.2	341.8	tri-glucoronide
21	60.3	493.1346	331	$C_{23}H_{24}O_{12}$	269	306.2	333.6	hexose
22	60.3	859.1780	829, 507, 331	$C_{35}H_{38}O_{25}$	268.8	304	348.6	tri-glucoronide
23	62.44	493.1346	331	$C_{23}H_{24}O_{12}$	266.1	303.5	335.8	hexose

2.3. Optical measurements of epidermal UV absorbance and leaf Chl

The optical measurements of epidermal UV absorbance and leaf Chl followed Laureau et al. (2015). Epidermal UV absorbance was measured from both leaf sides of attached leaves with a leaf-clip device (Dualex, Force-A, Orsay, France) as described in Goulas et al. (2004). A Flav-Dualex (Dualex® FLAV 3.3) was used to measure absorbance at 375 nm by comparing Chl fluorescence excited at 650 and at 375 nm. The excitation at 375 nm lies in the absorption range of Flav (Goulas et al., 2004). Epidermal absorbance at 322 nm was measured with a HCA-Dualex from the same leaf location as measured with the UV-A Dualex. The HCA-Dualex compares fluorescence yield excited at 650 and at 322 nm and allows estimation of the epidermal hydroxycinnamic acid (HCA) content (Latouche et al., 2013). Since Flav contribute to DEA_{322}, HCA-Dualex measurements were corrected by the estimated Flav content according to Latouche et al. (2013). Ten readings from each Dualex were taken, including five from each leaf side. Results were used to estimate the total Flav and the total HCA content of the leaf epidermis (Goulas et al., 2004).

A Chl content index (Chl-index) was obtained using either the Dualex 4 Scientific (Force-A, Orsay, France) for the measurements between 2016 and 2018 or the SPAD-502 (Konica-Minolta, Tokyo, Japan) for the measurements in 2015 according to Cerovic et al. (2015). Five readings from the adaxial side were used to estimate the mean Chl content of the leaf. The measured Chl-index values with the Dualex 4 correlated to surface based total Chl content as measured in acetone extracts (Laureau et al., 2011) (Fig. S1) and correlated to SPAD (Chl-index = 0.61*SPAD + 4.98, R^2 = 0.771, n = 85). All measurements were done at the same leaf spots, with five spots per leaf. The ratio between Chl-index and DEA_{375} was calculated from the mean of Chl-index and the total Flav contents of each leaf.

The LMA, the Chl and the Car contents after extraction of leaves in 80% acetone and subsequent centrifugation, the Chl a/b ratio and the ratio of Chl a + b/Car were determined at the end of the experimental period as described in Laureau et al. (2011, 2015).

Biochemical analysis and imaging were done the following days after the experimental period, at the INRA (AGAP, Montpellier) and at an imaging facility (ImagoSeine, institute Jacques Monod, Paris), respectively. Harvested leaves were stored overnight under humidified atmosphere and low temperature conditions before imaging.

2.4. Sample extraction and mass spectrometry characterization of leaf flavonoids

The extraction protocol was derived from Bidel et al. (2015). The extraction solvent was composed of methanol/water (70/30, v/v) acidified with 0.1% HCl. The *5-O*-methoxyflavone, a compound that is very similar to *Soldanella* flavones but not synthesised by this plant species, was added at a concentration of 500 μM to the extraction solvent, as an internal standard. Results were corrected for the loss of analytical Flav during sample preparation or sample inlet by correcting for the internal standard. For individual leaf lamina analysis, leaves were harvested in their natural environment after Dualex measurements and freezed-dried. Quantification was done on both, a surface basis unit and a dry weight basis.

Leaf metabolites were characterized according to Bidel et al. (2015) using Synapt G2-S high resolution mass spectrometry system (Waters Corp., Milford, MA). Chromatographic separation was performed on C18 column (XTerra MS, 3.5 μm particle size, 2.1 × 100 mm) kept at 40 °C. A binary mobile phase gradient was delivered at a total flow rate of 0.21 mL min^{-1} using a binary HPLC pump (Waters 1525l, Waters, Manchester, UK). De-ionized water (solvent A) and acetonitrile (solvent B) were both acidified with 0.1% (v/v) formic acid to minimize the ionization of phenolics (around pH 3.0). The elution gradient started at 95:5 (v/v) of A:B, followed by a linear gradient for 20 min to 75:25 of (A:B), and second one for 20 min to 60:40 of (A:B). Including the washing and reconditioning of the column, the total running time was 60 min. They were quantified using a micromass ZQ ESCi multimode ionization mass spectrometer (Micromass Ltd, Manchester, UK) as described by Bidel et al. (2015).

Identification of metabolites was based on their retention times, UV absorbance spectra, m/z ratio value and MS fragmentation pattern. When it was possible, they were compared with commercial standards eluted with the same gradient. The absorbance peak of each Flav was measured with a DAD 996 detector (Waters) after HPLC separation and integrated at 350 nm, a wavelength close to their maximum absorbance peak indicated in Table 2.

2.5. UV screening compounds of adaxial and abaxial epidermis and mesophyll

Since *S. alpina* has a thick epidermis covered by a thick cuticle, it is resistant enough for manual removal. The epidermis was sampled under a binocular microscope using a razor blade incision located along the main nervure and scraped off with a thin tweezer. We verified the absence of chlorophyll and carotenoids of the epidermis samples using LC-MS in a positive electrospray mode, indicating that no mesophyll cells were removed with epidermis.

About 25 mm^2 of abaxial epidermis of fresh individual leaf was removed and immediately extracted in 100 µL of methanol/water (70/30, v/v) acidified with 0.1% HCl. Interveinal mesophyll located immediately beneath the epidermis was then harvested and similarly extracted. Finally, adaxial epidermis adjacent to this zone was extracted. The surface areas of collected tissues were estimated from images taken with a dissecting microscope. To improve disintegration of cell walls, samples were frozen in liquid nitrogen and warmed up three times in the dark, then sonicated at 0 °C for 15 min and stored at −20 °C. After thawing and clarification by centrifugation at 18 000 g for 15 min, 20 µL of the supernatant solution was injected into the Rheodyn® loop of the chromatographic system.

For the determination of epicuticular Flav, 5 g of intact freezed-dried leaves were immerged repeatedly three times in 5 mL chloroform by agitation in a glass bottle during 3 min to remove epicuticular waxes of the epidermis, according to the protocols of Lütz and Gülz (1985) and Fukuda et al. (2008). The extraction was repeated three times and the chloroform extracts were combined and evaporated under reduced pressure to obtain a crude wax fraction, which was taken up in methanol and analyzed by high resolution mass spectroscopy. Similar extraction was also carried out using hexane and acetone.

2.6. Reagents and chemicals

The *5-O*-metoxyflavone was provided by Sigma-Aldrich (Saint-Quentin Fallavier, France).

Methanol and acetonitrile from Carlo Erba (Val de Reuil, France) were HPLC-grade quality. Formic acid from Merck (Darmstadt, Germany) was of analytical grade quality. De-ionized water was produced using a reverse osmosis Milli-Q system from Millipore (Molsheim, France).

2.7. Fluorescence microscopy

Images of leaf surfaces were taken using an inverted epifluorescence microscope (AxioObserver.Z1, Zeiss) equipped with a monochrome-cooled sCMOS camera (ORCA-Flash4.0 LT, Hamamatsu), a colour-cooled CCD camera (Axiocam HRc, Zeiss) and a led (pE-300 white, CoolLED) for fluorescence excitation. Leaf visible fluorescence was imaged using a 330WB80 excitation filter (Omega, Battleboro, USA), a long pass dichroic beam splitter (DCLP400, Omega) and a LP400 emission filter (Omega). Leaf blue fluorescence was imaged using a 330WB80 excitation filter, a long pass dichroic beam splitter (390DRLP02, Omega) and a 450WB80 emission filter (Omega). Cells densities were determined by counting the cells on UV-excited blue and visible fluorescence images obtained by microscopy at 10× magnification (Plan-Neofluar x10 quartz objectives, Zeiss). Trichomes were counted on UV excited visible fluorescence 42-bit colour images, while stomata and ground epidermal cells were counted on UV excited blue fluorescence 12-bit monochrome images (Laureau et al., 2015). The spatial distribution of UV-A penetration through the adaxial epidermis was imaged using UV excited blue fluorescence at different depths of focus from the cuticle to the surface of the mesophyll, at x40 magnification (Plan-Apochomatic x40 quartz objectives, Zeiss). Experiments were automated using Zen software (Zen 2 blue edition, Zeiss) and images were processed using Image J 1.45s (National Institutes of Health, USA).

2.8. Data analysis

Statistical differences were analyzed with the softwares R studio (R studio 1.1.419, Inc., USA) and Sigma Plot (Systat Software, Inc., USA). Differences between means were assessed using a one-way analysis of variance (Anova) according to the GLM procedure. When the Anova identified significant differences between means, Tuckey's test was applied to identify which means are different from which. Significant differences were indicated by different letters or indicated in supplementary tables by the respective p-value. In the cases where normality of the residuals and homeodasticity were violated, the data were log-transformed. If despite the transformation, Anova hypotheses remained unverified, the difference among the medians was tested using a Kruskal-Wallis test and treatements were compared in a pairwise fashion using a Wilcoxon test. The relationships between optical and biochemical and anatomical parameters were tested by a regression analysis.

3. Results

3.1. Climatic conditions

At 2100 m elevation, during the investigation period, *S. alpina* leaves exposed to sunlight receive, on an average, more than 2.5 times more PFD during the day than shade leaves (Table 1). Plants grown under the UV filter receive 100 µmol m^{-2} s^{-1} mean PFD less than plants without filter. The mean day temperature was the lowest in shade conditions and slightly higher under the UV filter compared to sun conditions. The mean night temperature was lower in sun condition than in shade and under UV filter, hence sun plants were exposed to higher daily thermal amplitude than shade plants and plants under UV filter. Maximum PFD was similar under shade and UV filter conditions but markedly higher in sun plants. UV-A and UV-B radiation maximum and the UV dose were 10 times higher in sun than in shade plants. Plants grown under the UV filter receive also low UV radiation, which was lower than in shade plants. At 2400 m elevation, PFD and UV radiation were the highest and temperatures were similar to sun leaves at 2100 m elevation (Table 1).

3.2. Soluble Flav in S. alpina leaves

The total Flav profile of *S. alpina* leaves was first investigated in sun leaves collected randomly in the area of the Galibier pass at 2400 m elevation. Hydro-methanolic extracts of the lamina of full sun leaves are extremely rich in Flav. Thirty-two compounds with typical Flav absorbance spectra have been separated using a 70 min chromatographic gradient from which the 23 most abundant Flav were characterized. Peaks were numbered using their elution order (Table 2) and are separated by the estimated Flav subfamily. All Flav were glycosylated with various combinations of hexoses, rhamnoses and glucuronides (Table 2). Eight different tetra-hydroxy-flavones, three penta-hydroxy-flavones, four penta-hydroxy- methyl-flavones and eight tri-hydroxy-dimethoxy-flavones were identified (Table 2). The monoisotopic mass-to-charge ratio, the major and important MS_2 ions as well as the proposed formula and their glycosylation are shown in Table 2.

The maximum absorbance wavelength of the most abundant peaks

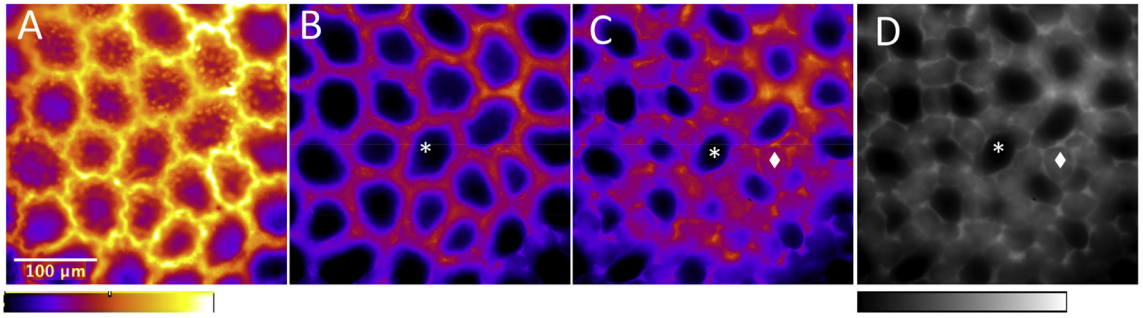

Fig. 1. Fluorescence images of the adaxial surface of a *S. alpina* sun leaves grown at 2100 m altitude with different increasing depth of focus from A through C. The focus was on the top of the epidermis and of the mesophyll in A and C, respectively. Images were obtained with a 3330WB80 excitation filter and a 450WB80 emission filter in order to show the cell outlines, and were taken with a similar exposure time. Image D is the grey image corresponding to C and shows the mesophyll cell delimitation under the epidermis. The three-bits false colour scale (A–C) and the eight-bits grey scales (D) correspond to the relative blue fluorescence intensity from 0 (black) to maximal value (white). The asterisk and the diamond indicate the vacuole of an epidermal cell and a mesophyll cell, respectively. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in the UV-A ranged between 347 and 355 nm (Fig. S2). All peaks show an absorption shoulder in the UV-B and another maximum in the UV-C region of the spectrum (Table 2, Fig. S2). The absorbance peak of each Flav was integrated at 350 nm, a wavelength close to their maximum absorbance peak indicated in Table 2 between 20 and 70 min elution. It should be mentioned, that additional eleven compounds with a retention time lower than 28 min contributed to the 350 nm absorption but to less than 1% and were therefore not further analyzed.

3.3. Flav distribution in the leaf

Individual leaves from intact plants were tested for their Flav distribution in the leaf (Fig. S3). Therefore, the adaxial and abaxial epidermis and the mesophyll were extracted separately and analyzed by HPLC. All Flav were identified in the abaxial and adaxial epidermis as well as in the mesophyll (Fig. S3) but no Flav were found in cuticular waxes (not shown). To examine to which extent the adaxial epidermis filtered the UV radiation, blue fluorescence images of the epidermis of sun leaves with different focus were recorded (Fig. 1). Blue fluorescence is not emitted by Flav but mainly by HCA and some various other metabolites like phenolamides, several coumarins or tryptophane derivatives (Cerovic et al., 1999).

Each epidermal cell was delimited by a bright fluorescent winding cell wall and the rough cuticle visible on the surface of some cells emitted also a blue fluorescence possibly from HCA (Fig. 1A). The peripheral cytoplasm emitted about 50% less blue fluorescence than the cell wall (Fig. 1A and B) and the central vacuole did not fluoresce (Fig. 1B and C, black area). The mesophyll blue fluorescence could be detected only at the level of anticlinal epidermal cell walls, not at the level of epidermal vacuoles (Fig. 1C and D) and similar pattern were observed using visible fluorescence (not shown), confirming that vacuoles contained non fluorescent UV absorbing compounds like Flav that screened the UV excitation of the mesophyll. Hence, from the spatial distribution of non fluorescent areas, we deduced that the UV screening was not uniform, depending on the epidermal cell distribution and the vacuoles size. Vacuoles and peripheral cytoplasm covered about 50 and 10% of the imaged area, respectively (Fig. 1B). UVA radiation reached the mesophyll, where blue fluorescence was visible under the epidermal anticlinal cell wall (Fig. 1C and D).

3.4. Changes of individual Flav after shade-sun transfer

The 23 major Flav were present in sun and shade leaves and analyzed on the same leaf area basis corrected by an internal standard (Fig. 2). The two tetra-hydroxy-flavones with the peak number P8 and P9 and a retention time of 52.1 and 53 min were the most abundant Flav. Their relative content of total 350 nm absorption was 38% in sun and 45% in shade leaves (Fig. 2). Both Flav shared a parent ion at m/z 741 $[M+H]^+$ with a fragment ion at m/z 287 $[M+H]^+$ corresponding to tetra-hydroxy-flavone and a fragmentation patter, which suggests two isomers containing hexose-di-rhamnose (Table 2). A second group of three penta-hydroxy-flavones peaking between 47 and 51 min (P4, P5 and P6) had a relative content of 350 nm absorption of 26% in sun and 33% in shade leaves. The relative content of a third group of two penta-hydroxy-methyl-flavones (P14 + P15 at 56 min retention time) was 12% in sun leaves but only to 6% in shade leaves. All other Flav shared the remaining 24% in sun and 16% in shade leaves. The relative content of tetra-hydroxy-flavones to the total 350 nm absorption was higher in shade than in sun leaves, while the relative content of penta-hydroxy-methyl-flavones was higher in sun than in shade leaves (Fig. 2). The other two Flav subfamilies were similarly distributed in shade and in sun leaves. The total peak area at 350 nm of sun leaves was four times higher than in shade leaves. The peak area of most individual peaks (Fig. 2) was significantly (at least $p = 0.05$) higher in sun than in shade leaves (Table S1). No significant difference was observed in the three tetra-hydroxy-flavones peaks P1,P3 and P7, in two penta-hydroxy-methyl-flavones (P16, P22) and one tri-hydroxy-di-methoxy-flavone (P18) (Table S1).

The 350 nm peak areas of all 23 Flav increased strongly after shade-sun transfer to values similar as in sun leaves (Fig. 2). Some of the shade-sun induced Flav had even higher peak areas compared to sun leaves. The flavonoids with the peak number P1 and P3 were significantly induced in sunlight in the presence of UV-radiation (Fig. 2 Table S1). Leaves transferred to sunlight in the absence of UV radiation had significantly higher peak areas of P18 and P19 than sun leaves, but not than leaves induced in the presence of UV radiation (Fig. 2 Table S1).

Independent of the treatment, tetra-hydroxy-flavones had the highest total peak area at 350 nm, followed by penta-hydroxy-flavones and penta-hydroxy-methyl-flavones (Fig. 3A). The glycosylation pattern of Flav by rhamnose-hexose and di-rhamnose-hexose were by far prevailing (Fig. 3B). All Flav subfamilies were similarly induced in shade-sun transferred leaves, independent of the presence of UV radiation. However, the peak area of tri-hydroxy-di-methoxy-flavones at 350 nm was higher than in sun leaves but the difference was not significant (Fig. 3A, Table S2).

Also the glycosylation pattern of the Flav was different in sun-induced and sun leaves. Both, the tri-gluconoride and the di-gluconoride flavones accumulated significantly more in shade-sun transferred leaves in the presence and the absence of UV radiation compared to shade leaves. Their content was also higher as compared to sun leaves but the difference was not significant. Furthermore, shade-sun transferred leaves in the presence of UV radiation accumulated significantly the highest content of di-rhamnose-di-hexose glycosides (Fig. 3B Table S3).

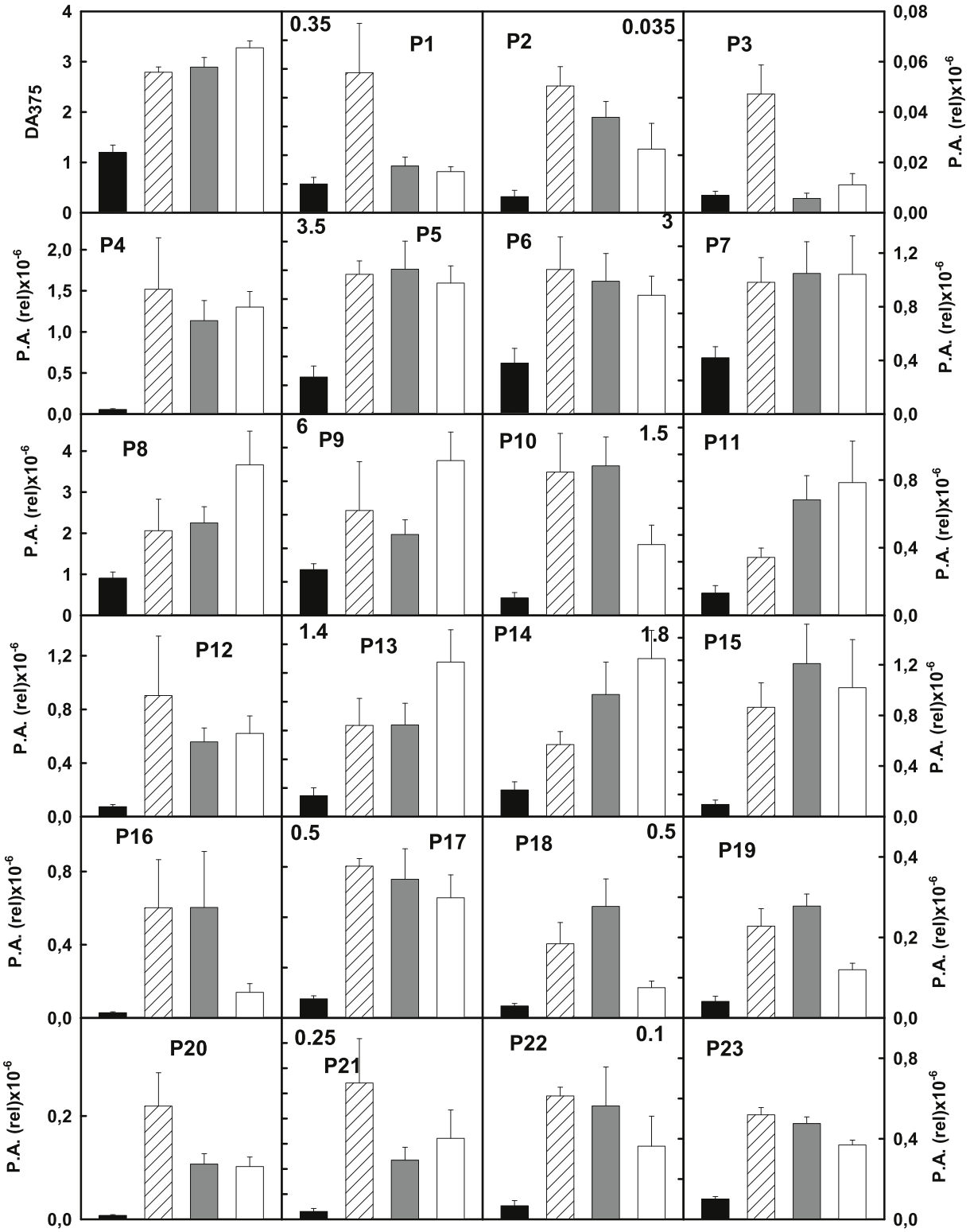

Fig. 2. Values of the DEA_{375} and of the 23 individual identified Flav (Table 2) in shade (black), sun (white) and shade-sun transferred leaves in the presence of UV radiation (white stripes) and in the absence of UV radiation blocked with an UV filter (grey). The peaks are numbered corresponding to their retention time and are characterized in Table 2. The relative peak area (P.A.) x 10^{-6} is shown. The maximum scale is indicated by numbers corresponding to their relative concentration. The statistic difference (*p*-value) is shown in Table S1.

3.5. Changes of epidermal UV absorbance, Chl and Car after shade-sun transfer

Shade-sun transfer also affected other pigments involved in photosynthesis and protection against UV radiation and oxidative stress. In leaf extracts, Chl a + b contents declined non-significantly on a fresh weight and surface basis to values similar as in sun leaves. The Car content increased significantly on a fresh weight and a surface basis (Fig. 4A, B, C, D). Interestingly, Chla/b ratios remained stable in shade-sun transferred leaves while the ratio was higher in sun than in shade

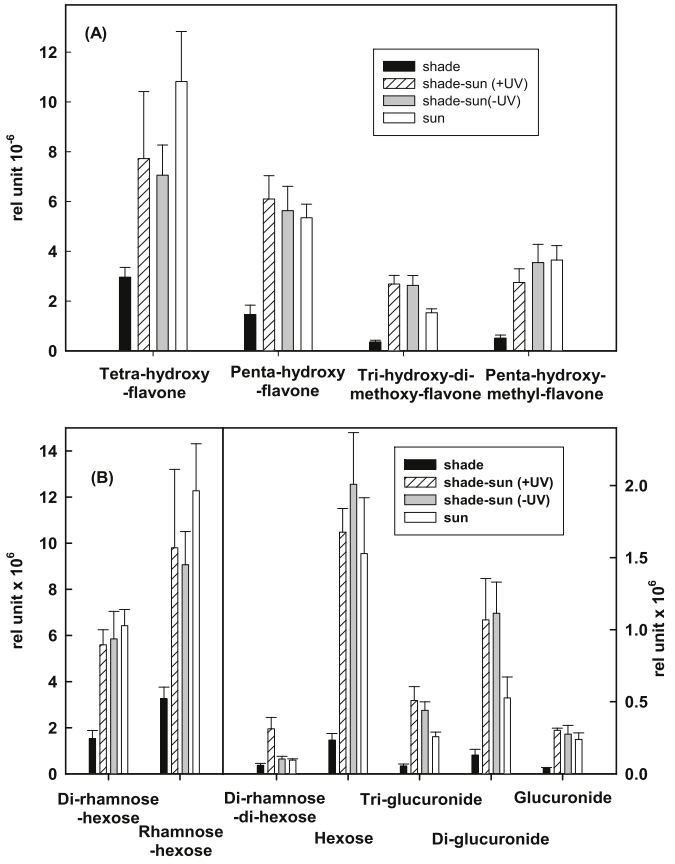

Fig. 3. A: Relative peak area at 350 nm of the four subfamilies of flavonoids grouped together in *S. alpina* shade leaves (black) shade-sun transferred leaves (white stripes) shade-sun transferred leaves protected by a UV filter (grey) and sun leaves (white). The statistic difference (*p* value) is shown in Table S2B: Relative peak area of the glycosylation pattern of the flavonoids in *S. alpina* shade leaves (black) shade-sun transferred leaves (white stripes) shade-sun transferred leaves protected by a UV filter (grey) and sun leaves (white). The statistic difference (*p* value) is shown in Table S3.

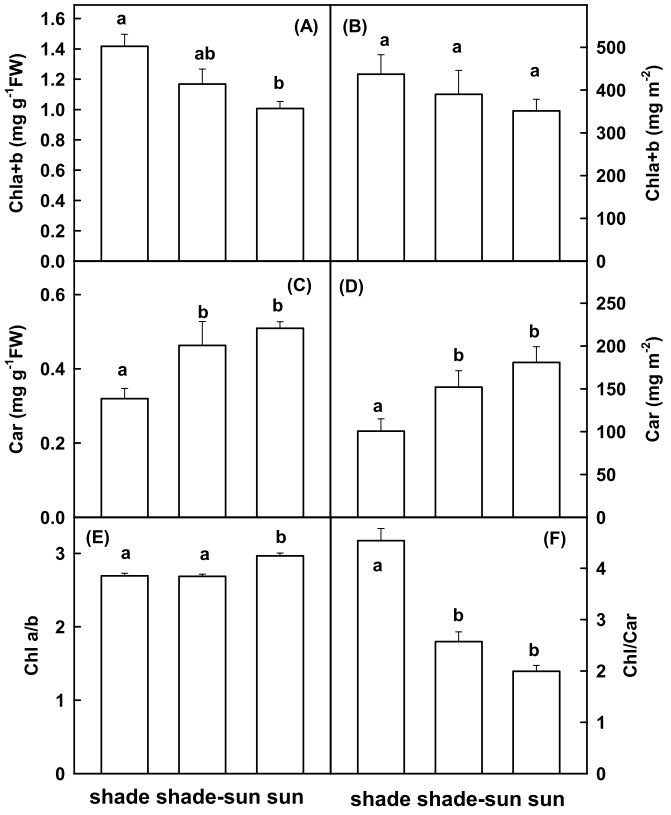

Fig. 4. Changes of Chl and Car content and Chl a/b and Chl/Car ratios of sun leaves shade leaves and leaves transferred from the shade to the sun after an observation period of 30 days under the respective conditions. Chl and Car contents were measured in leaf extracts and are expressed on a fresh weight and a surface basis. Significant differences at the $P \leq 0.05$ level is indicated by different letters. Statistic details are shown in Table S4.

leaves (Fig. 4E). In contrast, the Chl/Car ratio significantly differed between shade and sun leaves and declined significantly in shade-sun transferred leaves (Fig. 4F).

In Fig. 2, changes in DEA_{375} were documented for the same samples used for Flav extraction. However, optical measurements allowed the extension of Flav, HCA and Chl measurements to a much higher sample number and to higher altitude growing sites. Comparing Fig. 5A with Table 1 shows that DEA_{375} strongly depended on the mean PFDs received by the leaves but not on the maximum PFD and saturated at a mean PFD of 800 μmol $m^{-2}s^{-1}$. Contrary to DEA_{375}, DEA_{322} decreased from shade to sun conditions (Fig. 5C). A similar trend was measured for the Chl-index (Talhouët et al., 2020), leading to a strong decrease of the Chl-index/DEA_{375} ratio from shade to sun leaves at 2100 m elevation. The higher Chl-index/DEA_{375} ratio at 2400 m compared to 2100 m elevation was due to a higher Chl-index at higher altitude (Fig. 5B) and the higher Chl-index/DEA_{375} ratio in shade-sun transferred leaves in the presence compared to the absence of the UV filter was mainly due to a lower DEA_{375}.

3.6. Correlation between optical, biochemical and anatomical measurements related to leaf acclimation to light

The strong dependence of optical DEA_{375} and DEA_{322} on mean PFD (Fig. 5) allows investigating to which extent optical measurements correlate to individual Flav, photosynthetic pigments and to anatomical traits and are representative for light acclimation. Not surprisingly most of the log transformed peak area of the 23 individual Flav correlated highly significantly to DEA_{375} (Table 3). Therefore, UV-A absorbance was ascribed to a mixture of different Flav. No significant correlation was observed for P3, which were exclusively induced in shade-sun transferred leaves in the presence of UV radiation and for P7, which was not significantly different in shade and sun leaves (Table S1).

Although total Chl a + b contents on a fresh weight basis as measured in leaf extracts was lower in sun and shade-sun transferred leaves compared to shade leaves, no significant correlation between DEA_{375} and either the Chl a + b content or the Chl a/b ratio was found. DEA_{375} positively and significantly correlated with Car contents on a surface and mass basis as well as with a decrease of the Chl/Car ratio and with the increase of LMA (Table 4, Fig. S4).

Highly significant correlations were also observed between the abaxial DEA_{375} and the stomatal and abaxial trichome densities. Consequently, abaxial trichome density correlated also to stomatal density, as well as adaxial epidermal ground cell density. However, no clear difference between shade, shade-sun transferred and sun leaves were observed (not shown). In contrast, the stomatal density was negatively correlated to the abaxial DEA_{322}.

The Chl-index did not linearly correlate to LMA nor to the stomatal density. The increase of LMA as a function of light received by the leaf was greatly larger than that of the Chl-index because the mass-based Chl content, expressed as Chl-index/LMA, decreased according to a curvilinear relationship. After a logarithmic transformation, Chl-index/LMA and LMA were linearly and negatively correlated while the logarithmic transferred ratio of DEA_{375}/LMA against LMA correlated positively. Consequently, the Chl-index/DEA_{375} ratio highly and negatively correlated to LMA (Table 4, Fig. S4). In shade leaves, this ratio sharply

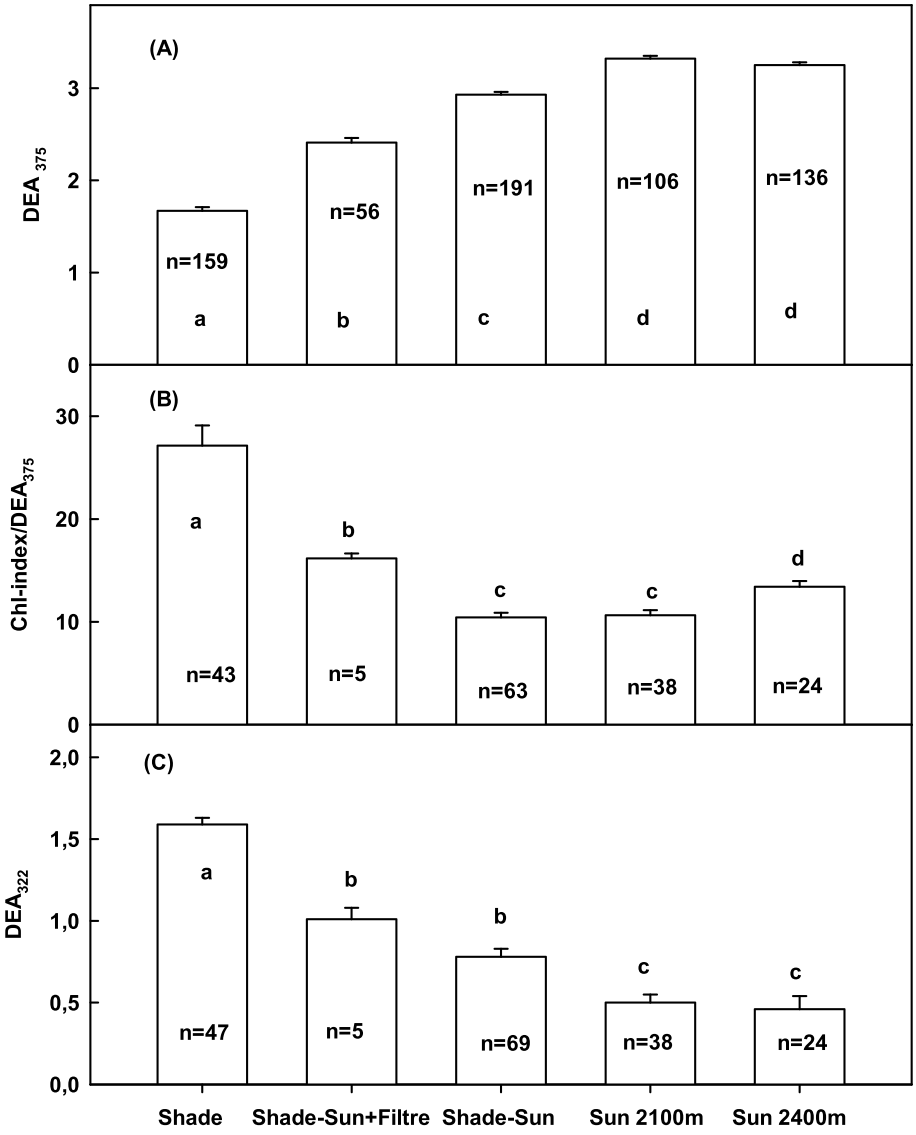

Fig. 5. Epidermal UV absorption at 375 nm (DEA_{375}), ratio of Chl-index to DEA_{375} and DEA_{322} in *S. alpina* leaves collected at 2100 m from the shade, transferred from shade to sun in the presence and absence of a UV filter, from the sun and from the sun at 2400 m elevation. DEA_{322} has been corrected according to Material and methods. Measurements on individual plants were performed at the end of the experimental period (end July or the beginning of August). Significant differences at the $p < 0.05$ level is indicated by different letters. Detailed statistics are shown in Table S 5.

decreased as LMA increased due to a decrease in Chl-index and an increase in DEA_{375} values (Fig. S5A). Therefore shade leaves were in a heterogeneous state of acclimation to light climate, suggesting more or less deep shade conditions in the field. By contrast, in sun acclimated leaves, from all the experiments (Fig. S5A), as from a representative plants (Fig. S5B), the leaf acclimation state to light expressed by the Chl-index/DEA_{375} ratio was more uniform, with higher and more steady mass allocation to Flav than in shade leaves.

4. Discussion

4.1. Flav composition in S. alpina leaves

Soldanella alpina is a case study of an alpine species for investigating the biochemical and anatomical leaf plasticity in response to varying solar radiation in the field. Plants growing in the sun received on average 2.6 times higher PFD but approximately 10 times higher UV-A and UV-B radiation than shade plants. In this and previous studies, it was shown that *S. alpina* leaves increase the epidermal UV absorbance in response to the light condition and this increase was explained with the accumulation of Flav (Laureau et al., 2015; Talhouët et al., 2020) as described from various other plant species (Kolb et al., 2001; Bidel et al., 2007; Siipola et al., 2015; Klem et al., 2015; Barnes et al., 2016; Zivcak et al., 2017). Mass spectrometry and absorbance analysis of *S. alpina* sun leaves after HPLC separation indicated the presence of up to 32 different Flav and several minor compounds absorbing in the 350 nm range. The identification of these Flav was out of the scope of the present study and requires the availability of a huge number of leaves for further NMR analysis. However, we could divide them into four flavone subfamilies depending on their hydroxylation degree, their methoxylations and esterifications to glycosides from which Harborne (1968) postulated tri-hydroxy-flavones to be present in the *Primulaceae* family. Two penta-hydroxy-flavones absorb up to 45% of the radiation at 350 nm and were present in sun and shade leaves. In conclusion, the extremely high epidermal UV-A absorbance of *S. alpina* leaves results from a mixture of different Flav.

All these Flav were found across the leaf, in epidermal and mesophyll cells but not in the cuticular waxes (Fig. S3). Such a Flav distribution is typical for sun acclimated plant leaves (Agati et al., 2013) and epicuticular Flav are not found in several different genera

Table 3
Correlation coefficients and significance of the relationships between the total DEA_{375} and the area based Flav content from HPLC analysis of *S. alpina* leaves from different growth conditions at 2100 m and 2400 m altitude and from different light and UV exposures. Data (of peak area or/and of DEA_{375}) were log transformed. THF, Tetra-hydroxy-flavone; PHF, Penta-hydroxy-flavone; THDMF, Tri-hydroxy-di-méthoxy-flavone; PHMF, Penta-hydroxy-methoxy-flavones. The number of leaves (n) used for the correlation is indicated. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., non significant.

Flav	Flav skeleton	Glycosylation	r	n
P1	THF	di-rhamnose-di-hexose	0.750***	22
P2	THF	di-rhamnose-di-hexose	0.637*	20
P3	THF	di-rhamnose-di-hexose	0.028ns	21
P4	PHF	hexose-rhamnose	0.884***	22
P5	PHF	hexose-di-rhamnose	0.893***	22
P6	PHF	hexose-rhamnose	0.818***	22
P7	THF	hexose-di-rhamnose	0.339ns	22
P8	THF	rhamnose-hexose	0.719***	22
P9	THF	hexose-di-rhamnose	0.612*	22
P10	THDMF	di-glucuronide	0.750***	22
P11	THF	rhamnose-hexose	0.806***	22
P12	THF	hexose-di-rhamnose	0.910***	22
P13	PHMF	hexose-di-rhamnose	0.804***	22
P14	PHMF	hexose-di-rhamnose	0.900***	22
P15	PHMF	hexose	0.736***	22
P16	PHMF	hexose-rhamnose	0.497ns	22
P17	THDMF	glucuronide	0.736***	22
P18	THDMF	hexose	0.625*	22
P19	THDMF	tri-glucuronide	0.731**	22
P20	THDMF	tri-glucuronide	0.819***	22
P21	THDMF	hexose	0.655***	22
P22	THDMF	tri-glucuronide	0.602*	22
P23	THDMF	hexose	0.910***	22

Table 4
Correlation coefficients (r^2) and significance levels between optical, biochemical and anatomical measurements. The regression equation is shown as y = a*x + b and correlation graphs are shown in Fig. S4. The relationships between optical measurements of total DEA_{375} was correlated to biochemical measurements of Chl a + b (mg g^{-1} FW) and Car (mg g^{-1} FW and mg m^{-2}) and the Chla + b/Car ratio and to LMA (g m^{-2}). Abaxial or adaxial DEA_{375} and DEA_{322} were correlated to the anatomical determined stomatal and trichome densities. Anatomical determinations of the adaxial trichome density and the abaxial ground cell density were correlated to the stomatal density. Chl-index was correlated to LMA and the mass-based Chl-index (Chl index/LMA) to the stomatal density. After logarithmic transformation, the mass based Chl-index (Chl index/LMA), the mass-based DEA_{375} measurement (DEA_{375}/LMA) and the Chl-index to the DEA_{375} ratio (Chl-index/DEA_{375}) were correlated to LMA. The number of leaves used (n) is indicated. DEA_{322} was corrected according to material and methods. $^{***}P < 0.001$; $^{**}P < 0.01$; n.s., non significant.

Relationship	r^2	a	b	n
DEA_{375} vs Chl a + b (mg g^{-1}FW)	0.043^{ns}	−0.095	1.34	36
DEA_{375} vs Car (mg g^{-1}FW)	0.427***	0.097	0.08	36
DEA_{375} vs Car (mg m^{-2})	0.509***	33.5	10.3	36
DEA_{375} vs Chl a/b	0.113^{ns}	0.136	2.29	36
DEA_{375} vs Chl/Car	−0.425***	1.11	6.40	36
DEA_{375} vs LMA	0.599***	5.49	−0.14	103
Abaxial DEA_{375} vs stomatal density	0.325 ***	0.067	−0.071	53
Abaxial DEA_{322} vs stomatal density	0.126**	−0.0024	0.95	53
Abaxial DEA_{375} vs abaxial trichome density	0.189**	0.053	0.62	53
Abaxial trichome density vs stomatal density	0.412***	0.069	−3.51	53
Adaxial epidermal ground cell density vs stomatal density	0.404***	1.26	86.8	53
Chl-index vs LMA	0.021^{ns}	−0.139	43.8	103
Chl-index/LMA vs stomatal density	0.034^{ns}	−0.0014	1.15	53
log(Chl-index/LMA) vs log(LMA)	0.576***	−1.13	1.77	103
log(DEA_{375}/LMA) vs log(LMA)	0.127***	0.321	−1.68	103
Chl-index/DEA_{375} vs log(LMA)	0.367***	−61.3	112.6	103

(Barberan and Wollenweber, 1990). None of the flavones were specific for the abaxial or adaxial leaf side or the mesophyll. Epidermal glycosylated Flav absorb most of the UV-A radiation. In *S. alpina*, they were mainly localised in the vacuole confirming the pattern known in mountain herbaceous dicots species (Day, 1993). However, UV-A was not completely blocked at the anticlinal cell walls of the epidermis and thus penetrate to the upper mesophyll surface where it could be absorbed by the mesophyll Flav (Fig. 1).

4.2. Flav induction after shade-sun transfer

From the total Flav measured, 23 were abundant in shade and sun leaves and their induction after shade-sun transfer in the presence and absence of UV radiation was monitored semi quantitatively by their absorption at 350 nm after HPLC separation. As reported earlier, the increase of UV radiation compared to shade conditions was not required to increase DEA_{375} in *S. alpina* leaves although UV radiation amplifies the effect (Laureau et al., 2015). Similar results were described for other species by Barnes et al. (2013, 2016) and Klem et al. (2015). Siipola et al. (2015) presented evidence that blue visible light was even more effective in inducing Flav accumulation in pea leaves than UV radiation. In several other species, UV-B radiation was specific in inducing flavonoid dependent UV screening of leaves (Kolb et al., 2001; Latouche et al., 2013; Bidel et al., 2015; Vidovic et al., 2015). While most Flav accumulated equally in the presence and absence of UV radiation and remained localised in vacuoles (not shown), some distinct differences were observed. Remarkably, P1 and P3 accumulated only in shade-sun transferred leaves in the presence of UV radiation, but their contribution to UV-A absorption were negligible (Fig. 2). Furthermore, leaves accumulated more tri-hydroxy-di-methoxy-flavones after shade-sun transfer than sun leaves (Fig. 3A). This suggests that higher hydroxylation of Flav is either limited by enzymatic hydroxylation and requires prolonged sun exposure, or that higher hydroxylation is limited by antioxidant activity of the Flav leading to breakdown products. Increased hydroxylation is generally correlated to a higher antioxidant potential (Winkel-Shirley, 2002; D'Amelia et al., 2018). In accord, sun leaves with higher oxidative pressure had higher relative contents of penta-hydroxy-methyl-flavones while in shade leaves tetra-hydroxy-flavones were more abundant (Fig. 3A). In *Phillyrea latifolia,* sun and shade leaves differed in flavonoid concentration but not in composition (Tattini et al., 2000). Interestingly, shade-sun transferred leaves increased also their activity of the antioxidant enzyme ascorbate peroxidase (Talhouët et al., 2020), while the content of ascorbate remained similar in shade and sun leaves (Streb, unpublished results), suggesting that increased Flav contents contribute to antioxidative protection in *S. alpina*.

In addition, the glycosylation pattern of the Flav depended on the light condition in shade-sun transferred leaves. Flav glycosylated with di-rhamnose-di-hexose only accumulated in the presence of UV radiation (Fig. 3B). Shade-sun transferred leaves have a lower photosynthetic activity compared to shade and sun leaves, increased photoinhibition and show indications of oxidative stress (Laureau et al., 2015; Talhouët et al., 2020). Carbon assimilation limited the photosynthetic activity in shade-sun transferred leaves (Talhouët et al., 2020). If sinks for assimilated carbon are limiting, this can increase the leaf sugar content and reduce carbon assimilation (Paul and Foyer, 2001). In this context, Flav with di-rhamnose-di-hexose in shade-sun transferred leaves in the presence of UV radiation may act as a possible sink for accumulating leaf sugars. Furthermore, sugars can act as antioxidants (Keunen et al., 2013) leading to sugar oxidation and possibly glucuronide accumulation. Again, shade-sun transferred leaves accumulated more flavones with tri- and di-glucuronides compared to sun and shade leaves, supporting further the possibility that Flav synthesis may also act as a sink for excess sugar acids in the leaf.

4.3. Shade-sun induced changes of Chl, Car and of DA_{322}

Shade-sun transfer affected also photosynthetic pigments and the epidermal HCA content. The epidermal HCA content decreased after shade-sun transfer opposite to the Flav content and was lowest in *S. alpina* sun leaves (Fig. 5; Laureau et al., 2015). Although no specific HCA could be identified after HPLC separation, DEA_{322} and blue fluorescence of the cuticle, the cell wall and the trichomes suggest the presence of esterified or etherified HCA in *S. alpina* leaves (Bidel et al., 2015; Laureau et al., 2015). Furthermore, HCA are widespread metabolites in the *Primulaceae* family (Harborne, 1968). Flavones are synthesised from products of the phenylpropanoid and malonic acid pathway with the intermediate HCA (Winkel-Shirley, 2002). The negative correlation between epidermal HCA and Flav contents (Fig. 5) suggests that sun conditions stimulate preferentially the synthesis of flavones downstream of HCA than HCA synthesis itself as also observed by Bidel et al. (2007) in different woody species.

Shade-sun transfer of *S. alpina* leaves decrease their Chl a + b content as estimated from leaf extracts (Fig. 4) and by optical measurements (Talhouët et al., 2020). Similar results were described in high-light transferred shade leaves of rice, *Chenopodium album* and *Plectranthus coleoides* (Oguchi et al., 2003; Murchie et al., 2005; Vidovic et al., 2015). This together with Flav accumulation, decrease the Chl-index/DEA_{375} ratio in *S. alpina*, which was a typical signature of sun compared to shade leaves, indicating carbon allocation to Flav instead to Chl and holds true for various species (Meyer et al., 2006). While the Chl a/b ratio did not acclimate to higher light conditions in shade-sun transferred *S. alpina* leaves, similar to photosynthetic activity (Talhouët et al., 2020) the Car content increased markedly (Fig. 4). Similar observations were reported by Klem et al. (2015) in barley varieties. Since Car and Flav are involved in antioxidative protection (Young, 1991; Agati et al., 2013), their accumulation further indicates increased oxidative stress. In accord, shade-sun transferred leaves are more vulnerable to PSII photoinhibition and leaves have higher mortality than sun or shade leaves (Talhouët et al., 2020).

4.4. Epidermal UV absorbance as an indicator of light acclimation

With the exception of P3 and P7, all other Flav correlated highly significantly with the log-transferred total DEA_{375} (Table 4, Fig. S4), showing that not a single or some but nearly the whole Flav signature contribute to epidermal UV-A absorbance in *S. alpina* leaves and acclimate to a change in sunlight intensity.

S. alpina shade and sun leaves as well as shade-sun transferred leaves in the presence and absence of UV radiation represent different states of light acclimation, influenced by the mean PFD received by the leaf. This affects LMA, leaf thickness, stomatal density, Chl and Car mass based contents and the Chl/Car ratio (Laureau et al., 2015; Talhouët et al., 2020) as generally reported (Lambers et al., 1998). DEA_{375} is correlated to each of these variables and therefore provides a signature of the leaf acclimation to light. In addition, correlation between the Chl-index/DEA_{375} ratio and LMA was similar to those of tree leaves from various light acclimation states (Meyer et al., 2006). Sun leaves have a lower Chl-index/DEA_{375} ratio and a higher LMA than shade leaves. In *S. alpina*, each leaf of a given shade plant could acclimate to the sun and reach a similar low Chl-index/DEA_{375} ratio (Fig. S4). Therefore the acclimation of one leaf estimated by Chl-index/DEA_{375} is representative of that of others in *S. alpina*, which could simplify routine monitoring in the field. Consequently, on the whole, leaf Flav content assessed by DEA_{375} should be included as a general characteristic of the leaf light acclimation process.

In *S. alpina* leaves, the Chl a/b ratio did not increases in response to shade-sun transfer and therefore did not correlated to DEA_{375} (Fig. 4; Table 4). The ability to change the Chl a/b ratio after changing the light intensity seems to be species dependent. Increasing Chl a/b ratio were described in low-high light transferred leaves of rice and *Chenopodium album* (Oguchi et al., 2003; Murchie et al., 2005) but not in variegated *Plectranthus coleoides* (Vidovic et al., 2015).

In conclusion, rapid optical measurements *in situ* with DEA_{375} and the Chl-index can be used as indicators of structural and pigments components of light acclimation as well as of Flav contents. However, they cannot indicate the functional acclimation state of photosynthetic reactions (Talhouët et al., 2020) due to shade-light induced oxidative stress and damage. Optical assessments of Flav and Chl should then be associated to non-invasive gas exchange and Chl fluorescence measurements to provide a complete picture of the leaf light acclimation *in situ*.

Authors contributions

SM LB and PS designed the experiments and contribute to write the manuscript. All authors participate on measuring, data sampling and calculation of the results.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.02.045.

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