DE-FLIDAR: A NEW REMOTE SENSING INSTRUMENT FOR THE ESTIMATION OF EPIDERMAL UV ABSORPTION IN LEAVES AND CANOPIES

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ABSTRACT

A new FLIDAR is presented that can estimate the epidermal UV absorption of leaves and canopies from chlorophyll fluorescence (ChlF) measurements. The DE–FLIDAR (Dual-Excitation Fluorescence Light Detection And Ranging) performs a dual excitation of the chlorophyll present in leaves, in the UV (355 nm) and visible (532 nm) part of the spectrum, the latter being used as a reference excitation not absorbed by the epidermis. Therefore, the epidermal UV absorption of vegetation can be estimated from the ChlF excitation ratio "F(532)/F(355)". The DE–FLIDAR was used to analyse the epidermal UV absorption of the adaxial and abaxial side of tobacco leaves of different ages. The logarithm of the excitation ratio showed a good agreement with the absorbance of methanolic extracts obtained from the same leaves. We also analysed the presence of UV–absorbing compounds, at the canopy level, of different plant species grown indoors and outdoors. The ChlF excitation ratio (epidermal UV absorption) varied over a wide range for pea, barley and wheat canopies. The excitation ratio was much larger in outdoor grown plants, indicating an accumulation of UV-absorbing compounds, as expected. In addition, a dual ratio, red (RF) to far-red fluorescence (FRF) emission ratio, excited at 355 and 532 nm, [RF(355)/FRF(355)]/[RF(532)/FRF(532)], was shown to be linearly dependent to the chlorophyll content, and thus could be used as an accurate indicator of chlorophyll content, in plant leaves and canopies.

INTRODUCTION

UV-induced fluorescence can provide important information for plant ecophysiology and agronomy, like plant identification, monitoring of plant growth and development, mineral deficiency and presence of other stresses; for recent reviews see (1, 2). Under UV excitation, leaves emit blue (BF), green (GF), red (RF), and far-red (FRF) fluorescence, with maxima around 450, 530, 685, and 735 nm, respectively.

For outdoor vegetation fluorosensing, specific fluorescence LIDARs (Light Detection and Ranging) were developed, but, the main limitation of these FLIDARs is that they measure amplitude signals, which depend on atmospheric transmission and distance. This problem can be solved by performing simultaneous measurement of fluorescence emission, and using fluorescence ratios as signatures. On the other hand, fluorescence emission ratios (BF/RF or BF/FRF) present a limitation because they depend on two variables, which can vary independently, leading to ambiguous interpretation. Another alternative is to use fluorescence lifetime rather than amplitude. Presently, the major limitation for the use of lifetime as a signature is a more complex and more expensive instrumental development (3).

An increase in UVB (280-320 nm) radiation can lead to a reduction in growth rate and plant biomass (productivity), as well asto an inhibition of photosynthesis (4, 5). The exposure to UVB radiation also induces an accumulation of phenolic compounds, in the vacuoles and cell walls of leaf epidermis and in the cuticle (2, 4, 6). These compounds are mainly flavonoids and hydroxycinnamic acid derivatives that strongly absorb the UVB and UVA (320–400 nm) radiation and, then, can effectively protect the mesophyll against UV radiation. This screening effect of the epidermis on UV-induced chlorophyll fluorescence

(ChlF) has often been noted in the literature (1, 2), but UV-induced ChlF was only rarely used to actually estimate epidermal absorption, see for e.g. (7, 8), until Bilger and co-worker proposed the use of the comparison between UV-induced and blue-green-induced ChlF as a non-destructive method to estimate the epidermal UV absorption (9). The UV absorption by the leaf epidermis was initially demonstrated and analysed using epidermal peels (6). Later, fibre optic microprobes introduced into intact leaves were used to determine the amount of UV radiation reaching the mesophyll (10). Both of these established techniques for determining epidermal UV absorption showed that the adaxial epidermis of bifacial leaves is the major barrier for UV radiation, i.e. it is responsible for the major part of UV-absorption of the leaf.

In this paper, we present a new FLIDAR instrument for the estimation of this epidermal UV absorption of leaves and canopies, based on dual excitation of Chl present in leaves. The DE–FLIDAR (Dual-Excitation FLIDAR) uses a dual excitation in the UV (355 nm) and visible (532 nm), the latter being used as a reference excitation. Therefore, the epidermal UV absorption of vegetation is estimated from the ChlF excitation ratio (FER), F(532)/F(355), where F(532) and F(355) are ChlF yields excited at 532 and 355 nm, respectively. Using the DE-FLIDAR, the presence of UV–absorbing compounds was investigated in individual leaves and canopies of different plant species, at different stages of development, grown indoors and outdoors. In addition, a new RF to FRF dual emission ratio, excited at 355 and 532 nm, [RF(355)/FRF(355)]/[RF(532)/FRF(532)] was proposed as an accurate indicator for the Chl content in plants.

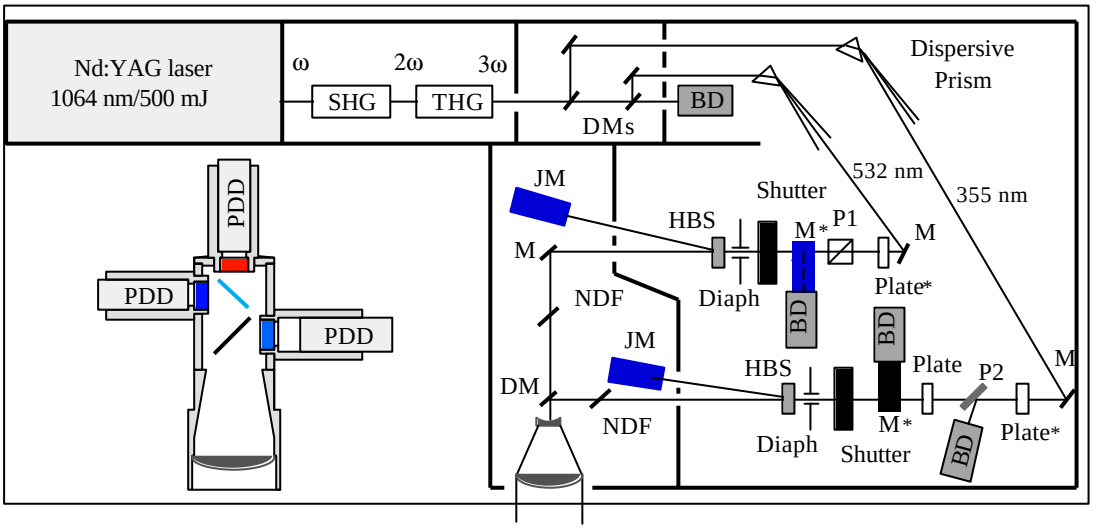

Figure 1: Schematic diagram of the DE–FLIDAR. See text for details. SHG and THG: Second and Third Harmonic Generation, respectively; (*) rotated by a step motor.

METHODS

Description of the DE-FLIDAR

The DE-FLIDAR is built around a Q-switched Nd:YAG laser (CGR-170, Spectra Physics) operating at 50 Hz and frequency doubled and tripled to give 532 and 355 nm radiation (Figure 1). The green and UV beams are separated with two pairs of dichroic mirrors (DMs) (Spectra-Physics). The residual 1064 nm light is trapped in a beam dump (BD) (BD-5, Spectra-Physics). The resulting excitation beams have a maximum energy per pulse of 90 mJ, with a duration of 4 to 7 ns (FWHM). For greatest spectral purity we used, for each beam, an isocel dispersive prism (J. Fichou). High reflectivity mirrors (M) (16MFB133 and 16MFB153, Melles Griot) are used to re-direct the beams (Figure 1). As the epidermal absorption of leaves can vary substantially, it is advantageous to adjust the energy of the beams independently, and to maintain the same intensity of the ChlF signal. In the DE-FLIDAR, the energy of each beam can be adjusted outside the laser cavity, by taking advantage of their linear polarisation. The insertion of a half-wave

retardation plate and a polarising beamsplitter (P1 or P2) (03PGL303/A and 16PPQ025, Melles Griot) in the beam path permits to adjust continuously the output beam irradiance by suitably rotating the half-wave retarder (Plate). The retardation plates are rotated by two step motors (82 971 002, Crouzet), and the residual reflected beam is trapped into beam dumps. The 532 and 355 nm-beams have orthogonal polarisation. Hence, to avoid any potential differences in interaction of the two beams with the surface of leaves, a half-wave retardation plate is inserted to rotate the UV beam to a vertical polarisation (the polarisation of the green beam). Although the laser operates continuously at 50 Hz, the effective excitation rate of the present DE-FLIDAR can be set between 0.2 Hz and 2 Hz, by using a combination of a moving mirror (M), mounted on a lever arm of a step motor (650 R726, Sonceboz), and a rapid electro-mechanical shutter (VS25S 2 ZM 0, Vincent Associates). The moving mirror and shutter, of each beam, are synchronised with the Q-switch synchro-output of the laser, and controlled, by a laboratory-built electronic circuit. A beam dump is used to trap the unused reflected pulses. Thus, the DE-FLIDAR generates an alternated excitation at 532 and 355 nm with 500 ms delay time between beams, at a variable repetition rate (1 Hz for the present study). Thanks to the alternated excitation of the DE-FLIDAR with only 500 ms delay time, it was possible to measure the FER independently from variable ChlF and leaf movement (variation of the angle of excitation).

To obtain an identical laser spot on the target for both beams we used an iris diaphragm (Diaph). It was followed by a Holographic Beam Sampler (HBS) (HBS-532-100-1C-10, Gentec). We used the transmitted first order diffraction, from the HBS, to sample the pulse energy. The diffracted radiation, which is proportional to energy of the excitation beam pulse, is measured by a pyroelectric Joulemeter (JM) (ED 200, Gentec). Additional Neutral density filters (NDF) are used when necessary (for leaves of high epidermal transmittance). The two monochromatic light beams are finally combined in front of the output beam-expander, by a dichroic mirror (DM) (16HSB105, Melles Griot). The beam-expander consists of a plano-concave lens (01LQS003, Melles Griot) and a plano-convex lens (01LQF116, Melles Griot). For the present study, the DE-FLIDAR was adjusted to monitor the sample at 3.5 m with a beam irradiating 190 cm^2 of the target area. Light from the target is received by a f/2.6 plano-convex lens with 77 mm entrance aperture (01LPX279, Melles Griot), which limits the field of view of the DE-FLIDAR detection to the area excited by the laser-beams. The DE-FLIDAR can perform three simultaneous measurements. The incoming light is split into different wavelength bands by two dichroic mirrors: first an UV mirror, at 45° (Balzers), followed by a blue mirror, at 45° (DC blue, Balzers). Furthermore, by changing the dichroic mirrors, simultaneous measurement of red, far-red and blue fluorescence is possible. For ChlF measurement, we used a long-pass filter (KV550, Schott) in addition to a 3 mm thick glass filter (RGN9, Schott) (for FRF), and an interference filter (682DF22EM, Omega) (for RF). The three-photodiode detectors (PDD) are identical. They consist of a fast PIN photodiode (S3590-01, Hamamatsu) followed by a differential amplifier, which make them insensitive to continuous light. The differential amplifier is built on the same feedback principle as described in (7). Stabilised signals from the photodiode detectors and the Joulemeters are sampled by a data acquisition card (410 series, Transera) on board of a personal computer. Signals from the photodiode detectors are divided by the energy level, obtained from the Joulemeters, in order to correct for the laser energy fluctuations. A proprietary program allows an on-line control of the experiment and display of measured signals. For quantitative measurement of the epidermal UV absorption, we used Rhodamine B on solid support, as a photon counter, in order to correct for optical losses downstream of the Joulemeter.

Plant material and extracts

Plants were grown either in a growth cabinet or outdoors in July. Tobacco (*Nicotiana tabacum*, cv Burley) was grown in soil, and all other species, peas (*Pisum sativum* L., var. Petit Provençal), barley (*Hordeum vulgare*, cv Nevada) and wheat (*Triticum aestivum*, cv Lloyd) in pure wet vermiculite. Thylakoids

were obtained from intact pea chloroplast isolated from young pea shoots (10 days old) and layered on glass-fibre filter supports as described in (11). Tobacco leaves were sampled for pigment extraction using a cork borer with 2 cm^2 aperture (two samples per leaf). Leaf samples were frozen, and then pigments extracted by heating for 30 minutes at 70 °C in 12 ml of methanol. Absorbance of cooled extracts was measured on a HP 8453 diode-array spectrophotometer. The distribution of total Chl content on tobacco leaves was also estimated using a portable Chl meter (SPAD-502, Minolta).

RESULTS

Effect of Chl content on the fluorescence excitation ratio

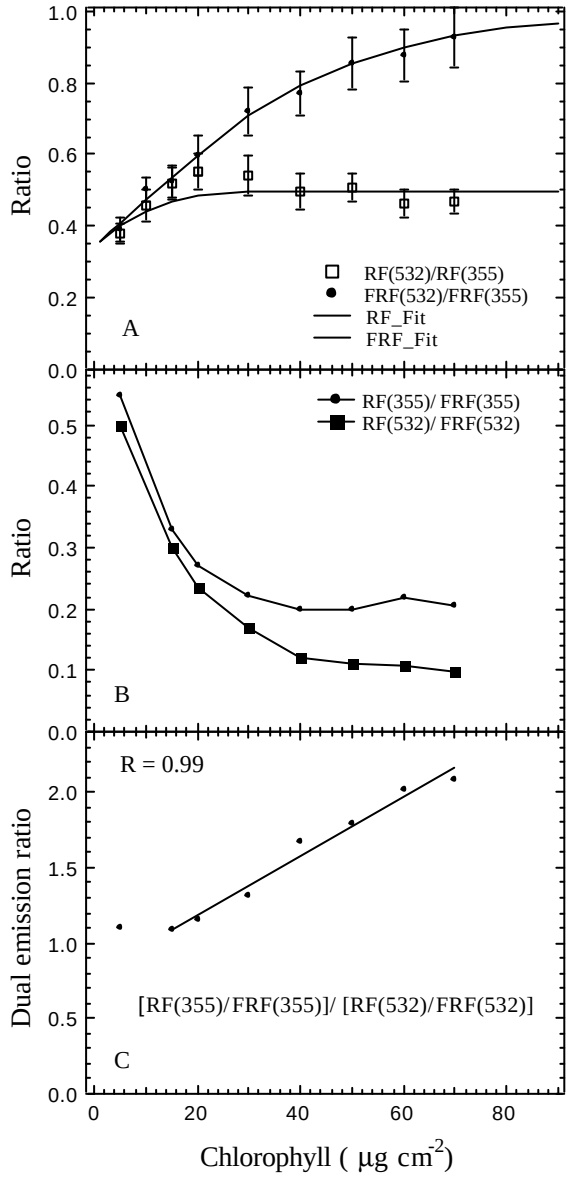

Figure 2: Dependency of the FER on the Chl content, and estimation of the Chl content by the dual emission ratio. See text for details. The error bars denote the standard deviation (N=170).

The FER was measured using the DE-FLIDAR on pea thylakoids layered on solid supports, at different Chl contents. Thanks to the absence of any UV screening, the measured excitation ratio directly reflects the effect of Chl content (Figure 2A). The excitation ratio was measured above 730 nm (FRF), and at 682 nm (RF). The experimental results were also fitted by the distortion function (see equation 2 in appendix). The FRF excitation ratio presents a significant increase with a tendency to saturation above 70 µg cm^{-2}. On the other hand, the RF excitation ratio showed a weaker dependency on Chl content. Indeed, above 20 µg cm^{-2}, the FER remains relatively constant with a value of about 0.5 (Figure 2A). This relatively weak dependency on Chl content can be explained by the reabsorption of the ChlF. The 355 nm-beam is absorbed within the first thylakoid layers, due to the high absorbance of Chl at this wavelength. On the other hand, the 532 nm-beam penetrates deeper in the sample, and consequently the green-induced ChlF is more reabsorbed. Hence, when ChlF, excited at 355 and 532 nm, is measured in a spectral region where fluorescence and absorption spectra overlap, this reabsorption leads to a saturation of RF at lower Chl content.

Estimation of the Chl content by fluorescence emission ratios

The reabsorption phenomenon, described above, has already been exploited to estimate the Chl content of a leaf; for recent reviews see (1). The RF/FRF emission ratio can be measured by the DE-FLIDAR using an excitation at 355 nm, and specifically at 532 nm (Figure 2B). As expected, when increasing the Chl content, due to the reabsorption of the RF emission, both RF(355)/FRF(355) and RF(532)/FRF(532) ratios show a decline reaching a steady state, which corresponds to a saturation of the Chl absorption. Thanks to a smaller absorbance of Chl in the green spectral region, the 532 nm excitation beam penetrates deeper in the leaf, and consequently, the green-induced ChlF emission RF(532) is more attenuated than the UV-induced one. The RF(532)/FRF(532) ratio shows then saturation at much larger Chl contents. Because both RF(355)/FRF(355) and RF(532)/FRF(532) ratios are governed by the same propagation laws, the dual emission ratio, defined as [RF(355)/FRF(355)]/[RF(532)/FRF(532)], is linearly dependent on the Chl above 15 µg cm^{-2}, as can be seen in Figure 2C.

Epidermal UV absorption of tobacco leaves

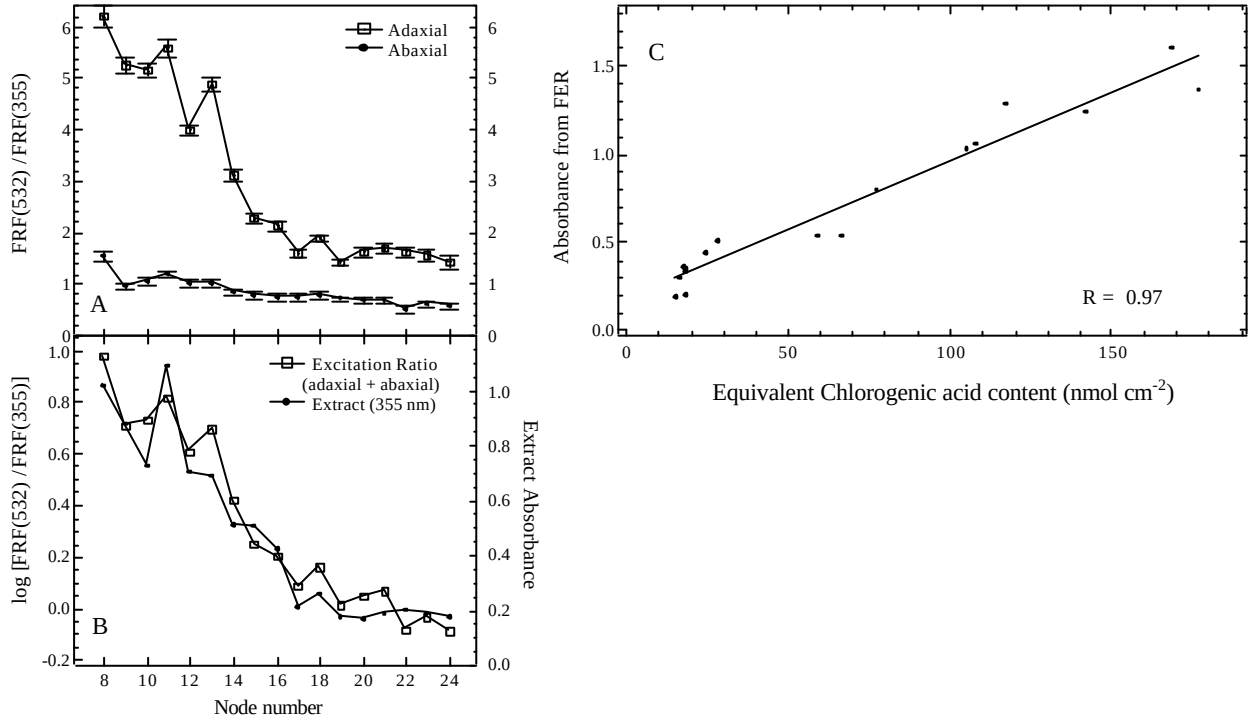

Figure 3: (A) FER of adaxial and abaxial sides of tobacco leaves at different nodes on the stem. The error bars denote the standard deviation. (B) Comparison of the estimated epidermal UV absorbance using the DE-FLIDAR and the UV absorbance of the corresponding leaf extracts, at 355 nm. (C) Comparison of the content of leaf phenolic compounds computed from the FER and from the maximum of absorbance of the extracts (326 nm).

In Figure 3A, the FER, FRF(532)/FRF(355), is shown for tobacco leaves grown in a growth cabinet. The FER was measured on adaxial and abaxial leaf sides, at different stages of development, from the older leaf (node N° 8) to the youngest one (node N° 24), on the same plant. As reported for other plant species (9), the adaxial side had a higher FER (i.e. higher UV absorption) than the abaxial sides, in all leaves. This difference is attributed to the light exposure of plants, which is much higher for the adaxial leaf side. The weak value of the FER of abaxial side reflects the weaker epidermal UV absorption. Furthermore, the FER de-

creases from aged leaves to younger ones, for both leaf sides, with some variations from leaf to leaf around a mean decreasing value. The FER of the abaxial side of the youngest leaves (node 16 to 24) remains low and almost constant. The absolute value of the ratio is smaller than 1 because of the smaller efficiency of ChlF excitation at 532 nm than at 355 nm.

Esters of caffeic acid like chlorogenic acid and its isomers, have been repeatedly reported as being the major UV-absorbing compounds in tobacco. In addition, Takahama showed that chlorogenic acid was accumulated during leaf ageing (12). Absorption spectra of methanolic extract of old tobacco leaves (not shown), indicated that esters of caffeic acid, could be the main component responsible for the epidermal UV absorption. Other esters of cinnamic acids cannot be excluded at this point, because of the similarity of their absorption spectra. Still, it is reasonable to say that the increased FER in aged leaves, corresponds to an accumulation of chlorogenic acid.

The logarithm of the FER, which after correction should be equal to the epidermal UV absorbance at 355 nm (see appendix), was compared to absorbances of extracts obtained from leaves at different nodes along the tobacco stem (Figure 3B). As we used total extracts of the leaf, containing the phenolic compounds of both the adaxial and abaxial epidermis (and even mesophyll), we compared the extracts absorbances to the sum of the logarithm of the adaxial and abaxial FER. It can be seen that qualitatively equivalent estimation of UV-absorbing compounds was obtained by both methods. The negative values of the logarithmic FER were mainly influenced by the abaxial leaf side. Indeed, on this side, the UV epidermis absorption is weak. However, a relative comparison of epidermal UV absorbances using FER is possible. To be able to correct for the effect of Chl content on FER, we measured the Chl content for each tobacco leaf (not shown) and then the distortion function was calculated for each leaf. The FER corrected for the chlorophyll effect using the distortion function has a quantitative meaning, and can be directly compared to extract absorbances. In Figure 3C the corrected FER was plotted against the content of UV-absorbing compounds calculated using the maximal absorbency of the extracts (326 nm), and expressed as concentration of chlorogenic acid. This shows that after proper calibration, the FER can be used to measure the content of UV-absorbing compounds in the leaf epidermis. The higher value for absorbance (offset) obtained by FER can be explained by the differences in total absorbance seen by this parameter compared to absorbance depending only on methanol extractable compounds (soluble compounds).

Changes in epidermal UV absorption during the development of pea canopies

The analysis of the epidermal UV absorption at the canopy level was performed on pea, from the 4th day after imbibition to the 14th day of development. Pea canopies were grown indoors, in a growth cabinet, and outdoors, in July. Figure 4A shows the apparent FRF yield, excited at 355 and 532 nm, on indoor and outdoor grown pea canopies. Both canopies show an increasing green-induced FRF(532) up to the 10th day, which corresponds to the full covering of the vermiculite trays (closed canopies). After the 10th day, the FRF(532) remains constant. On the other hand, the UV-induced FRF(355) increases over the whole growing period, for both indoor and outdoor grown canopies. Furthermore, both the UV and green-induced FRF had lower levels when peas were grown outdoors under natural illumination containing an UV component, than those grown indoors. Accordingly, the FER, FRF(532)/FRF(355), was always higher in outdoor grown canopies (Figure 4B). Furthermore, the FER is constantly changing during the development of both types of canopies, reaching a maximum value on the 7th and 8th day, for indoor and outdoor grown canopies, respectively. The FER decreases thereafter. Because the FER is inversely proportional to the epidermal UV transmittance (see equation 1 in appendix), the differences in FER presented in Figure 4B can be interpreted as a consequence of a higher accumulation of UV-absorbing compounds in leaves of the canopy exposed to UV radiation. Indeed, Day *et al.* reported that the concentration of UVB-absorbing compounds were over 80% higher in pea leaves of UVB-treated plants (13). Furthermore, UVB radiation induced significant increases in UV-absorbing compounds on buds and expanded pea leaves (5). The in-

terpretation of changes in the content of UV-absorbing compounds during the development of a canopy, obtained from fluorescence measurements, is far more difficult. A canopy is a mixture of leaves of different ages with a structure constantly changing. For instance, Gonzalez *et al*. reported a difference in the UV absorbance between bud tissue and mature pea leaves, which were not exposed to UVB radiation. The UV absorbance of extracts from buds was 50% lower than that of mature leaves (5). At an early stage of canopy development, pea buds predominate, with a low concentration of UV-absorbing compounds. Until the 7th day of development (cf. Figure 4B), corresponding to the maximum value of the UV absorption, the buds were still closed and show the abaxial leaf side to the excitation beams. After the 7th day, the leaves start to open and the canopy showed a complex structure with presence of a mixture of adaxial sides of old leaves and young closed leaves (abaxial side). Since pea plants initiate leaves at the rate of about one leaf every 2 days, the decreasing level of UV absorption of the canopy could be attributed to the presence of such young leaves which cover up the older ones.

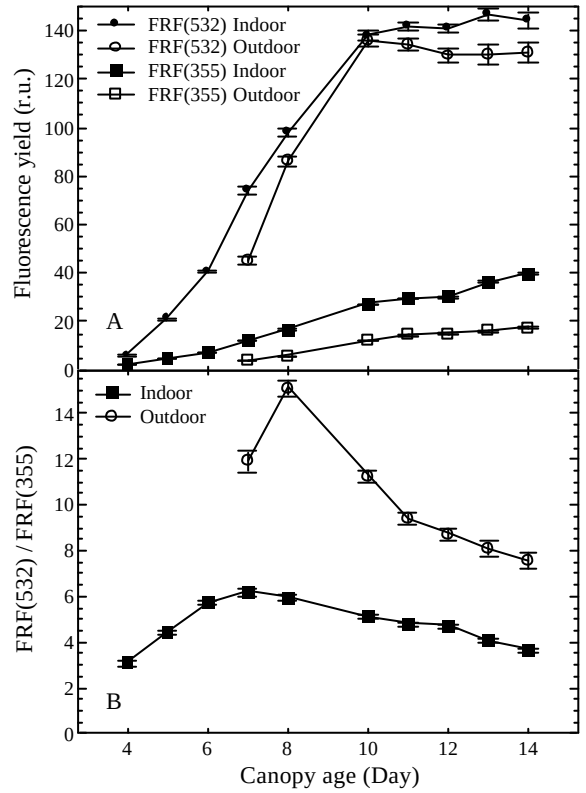

Figure 4: Changes of the FER during the development of pea canopies, in a growth cabinet and outdoor, in July. See text for details. (A) Apparent FRF yields, FRF(532) and FRF(355), excited at 532 and 355 nm. (B) Changes of the FER of pea canopies grown indoors and outdoors, with time of development. The error bars denote the standard deviation (N=170).

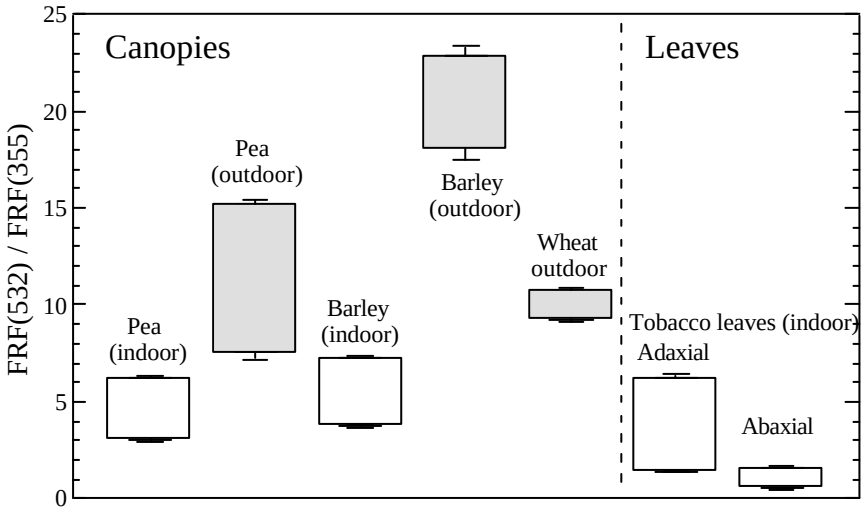

Figure 5: Summary of observed FER for different plant species, grown indoors and outdoors. FER measured on single leaves (right side) and in a canopy structure (left side). For each species and growth conditions, the bar encompasses all the values of FER recorded, which depended on the stage of development. Hatched bars indicate plants grown outdoors, in July (in presence of natural UV radiation). The error bars denote the standard deviation (N=170).

Comparison of epidermal UV absorption among species and conditions of growth

Pea and barley canopies exhibit a weak difference in FER, when grown indoors (Figure 5). But when grown under natural illumination, barley plants exhibit a much higher FER and consequently a higher epidermal UV absorption. For comparison with another monocotyledonous plant of agronomic importance, wheat canopies were also tested. They showed a lower UV absorption than barley, when both species were grown outdoors. Indoor grown tobacco leaves show a similar FER as pea and barley, when the adaxial leaf side is analysed. By contrast, abaxial leaf sides of tobacco show much smaller FER. So, by using the FER, it is easy to distinguish the adaxial from abaxial leaf side, and also to distinguish outdoor-grown from indoor-grown plants.

CONCLUSIONS

The DE-FLIDAR, presented in this report, is based on a dual-wavelength excitation and multi-wavelength emission. The main new parameter available with this LIDAR is the FER, F(532)/F(355), which logarithm corresponds to the UV absorbency, at 355 nm, of the leaf epidermis. Yet, one has to take into account the distortion effect due to variable Chl content. On the other hand, we also showed that quantitative measurements could be performed easier if ChlF is measured in the red part of the emission spectrum. Indeed, the distortion function, in this case, exhibits only a relatively constant offset of 0.5, for a Chl content above 20 µg cm^{-2}, which is generally the case for most leaves. In addition, using a reference wavelength close to the minimum absorption of Chl, at 532 nm, enables the estimation of the Chl content over a wide dynamic range. Indeed, a dual RF to FRF emission ratio, excited at 355 and 532 nm, [RF(355)/FRF(355)]/[RF(532)/FRF(532)] was shown to be linearly dependent on the Chl content, above 15 µg cm^{-2}, and thus could be used as an accurate indicator of Chl content, in plant leaves and canopies. The epidermal UV absorption, estimated using the DE–FLIDAR, was shown to vary over a wide range, for different plant species (tobacco, pea, and barley). The FER was much larger in outdoor grown plants, as expected, indicating an accumulation of UV-absorbing compounds. Indeed, the leaf epidermis is screening and protecting the mesophyll against UV radiation. And this effect is very much dependent on the irradiance under which the leaf has grown. Furthermore, with this LIDAR, epidermal UV absorption can be coupled to blue-green fluorescence and leaf Chl content to provide more complete information for plant identification and for the monitoring of plant growth and development, mineral deficiency or other stress conditions.

APPENDIX

The Fluorescence Excitation Ratio (FER) was shown to be inversely proportional to the epidermal UV transmittance,

$T(355)$, at 355 nm (14):
$$\frac{F(532)}{F(355)} = \frac{1}{T(355)} D(C_{Chl}, l_{em}) \qquad (1)$$

$D(C_{Chl}, l_{em})$ is the distortion function, depending on the specific absorbance $e(l)$ and the emission wavelength l_{em}.

$$D(C_{Chl}, l_{em}) = \frac{e(532)}{e(532)+e(l_{em})} \frac{e(355)+e(l_{em})}{e(355)} \left[\frac{1-10^{-[e(532)+e(l_{em})]C_{Chl}}}{1-10^{-[e(355)+e(l_{em})]C_{Chl}}}\right] \qquad (2)$$

In a logarithmic form, the FER, corresponds to the epidermal UV absorbance, at 355 nm, as seen below.

$$\log\left[\frac{F(532)}{F(355)}\right] = A(355) + \log[D(C_{Chl}, l_{em})] \qquad (3)$$

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