

Time-resolved blue-green fluorescence of sugar beet (*Beta vulgaris* L.) leaves. Spectroscopic evidence for the presence of ferulic acid as the main fluorophore of the epidermis

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Abstract

Sugar beet (*Beta vulgaris* L.) leaves emit blue-green fluorescence when excited with ultraviolet light. Comparative spectral analysis showed that the blue-green fluorescence of leaves, on both the adaxial and the abaxial side, was dominated by the blue-green fluorescence of the epidermis. A detailed investigation of the adaxial epidermis and of possible candidates for its blue-green fluorescence was carried out. The possible candidates were two hydroxycinnamic acid derivatives (ferulic acid and *p*-coumaric acid) and two flavonoids (kaempferol and quercetin). Each of these absorbed ultraviolet light, but ferulic acid was the only fluorophore that fluoresced similarly to epidermis. Decay-associated excitation and emission spectra were used to investigate further the blue-green fluorescence of the epidermis. In sugar beet epidermis, four kinetic components were resolved: the very fast (20–40 ps), the medium (0.8–0.9 ns), the slow (2.6–2.8 ns) and the very slow (7.1–7.6 ns) kinetic components. Several lines of evidence indicate the presence of ferulic acid as the main fluorophore of the epidermis. First, the very fast kinetic component, which contributes 70% to the overall blue-green fluorescence emitted from the epidermis, showed the spectral characteristics of ferulic acid. Second, the fluorescence lifetime of this kinetic component matched that of ferulic acid, which was 20–40 ps. Finally, the very fast kinetic component was also found in sugar beet leaves and in their phenolic extracts, and its thermal sensitivity (measured as the percentage of the blue-green fluorescence change per °C) matched that of ferulic acid. The results of this work lead us to conclude that ferulic acid is the fluorophore responsible for most of the blue-green fluorescence emitted from sugar beet epidermis.

Keywords: Fluorescence; Blue-green fluorescence; Decay-associated spectrum; Ferulic acid; (Leaf epidermis)

1. Introduction

Under UV excitation, leaves emit blue-green fluorescence (BGF) (maximum near 440 nm and shoulder near 520 nm) and red fluorescence (maxima in the 690 and 735 nm regions) [1–5]. Red fluorescence is emitted by chlorophyll *a*. The origin of the BGF is, however, not yet well understood. Recent investigations have suggested that the BGF is most probably a mixed signal composed of the fluorescence of various phenolic substances, emitting from vacuoles of the epidermis and of the mesophyll, and from cell walls [2–4,6–8].

Goulas et al. [3] and Lang et al. [4] have used two different approaches in a search for the phenolic substances responsible for the BGF. Goulas et al. [3] used synchrotron radiation and the time-correlated single-photon-counting technique to investigate the spectral and time-resolved characteristics of the BGF emitted from spinach leaves. They resolved four kinetic components. Excitation and emission spectra of these four kinetic components were also determined. They were compared with the excitation and emission spectra reported in the literature for a variety of phenolic substances. Goulas et al. concluded from these comparisons that two hydroxycinnamic acid derivatives (ferulic acid and *p*-coumaric acid), and one flavonoid (kaempferol) may contribute to the BGF of spinach leaves. On the other hand, Lang et al. [4] used two spectrofluorimeters, with a xenon lamp and a pulsed nitrogen laser as sources of excitation, to investigate emission spectra of various phenolic substances. They desig-

Abbreviations: BGF, blue-green fluorescence.

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nated, among other compounds, one of the hydroxycinnamic acid derivatives already suggested by Goulas et al. [3] (ferulic acid) and one flavonoid (quercetin) as possible candidates for the BGF.

The aim of this work was to identify the phenolic substances responsible for the BGF of sugar beet (*Beta vulgaris* L.) in relation to potential remote sensing by fluorescence of this important crop. In sugar beet, the presence of quercetin, *p*-coumaric acid, and especially kaempferol and ferulic acid is well documented [9,10]. In the present study, we further used the combined kinetic spectrofluorimetric approach of Goulas et al. [3] to investigate the BGF of sugar beet. We investigated not only the emission spectra of the phenolic substances candidates for the BGF of sugar beet, but also their excitation spectra and fluorescence lifetimes. Evidence is presented for the presence of ferulic acid as the main fluorophore of the epidermis of sugar beet leaves.

2. Material and methods

2.1. Plant material

Sugar beet (*Beta vulgaris* L. cv Monohil) was grown in a growth chamber in half-Hoagland nutrient solution. Plants were grown with $350 \mu\text{E m}^{-2} \text{s}^{-1}$ PAR, measured at canopy level. Since sugar beet leaves grow almost vertically, the PAR measured at leaf level was characteristically $110 \mu\text{E m}^{-2} \text{s}^{-1}$. Light was provided by a combination of halogen and sodium vapour lamps (Claude, Puteaux, France). They emit almost exclusively visible light (Fig. 1). Their UV emission was estimated to be approximately 2.5% of that emitted by most commercial UV lamps. Light was filtered through a plastic sheet (polycarbonate), effectively cutting off radiation below 386 nm (Fig. 1). Plants

grew at a temperature of 20°C (day) and 15°C (night), 80% relative humidity and a photo-period of 16 h light/8 h dark.

Young, rapidly expanding leaves (length of 13 to 14 cm) were used for all measurements. Leaf pieces and epidermis (adaxial or abaxial side), mesophyll and major vein were used for BGF measurements. To obtain the abaxial epidermis and the mesophyll, we used leaf pieces from which the epidermis was stripped away. The adaxial epidermis was obtained by removing the rest of the plant tissue with a scalpel. When we isolated the epidermis, care was taken to avoid mesophyll contamination.

For excitation and emission fluorescence spectra, samples of approximately 1 cm² of surface were used. For decay-associated excitation and emission spectra of epidermis, samples of approximately 2 cm² of surface were used. Samples, other than epidermises, were enclosed in commercial (106-QS, Hellma, Paris, France) quartz cells (1 mm optical path). Epidermises were placed between two commercial (106-QS, Hellma) quartz windows with HPLC grade water (see below). Samples were placed on a home-made holder, at controlled room temperature (20°C). For some experiments, the temperature was changed and kept at $\pm 0.25^\circ\text{C}$ by a bath, which allowed sample temperature to be varied between 3 and 33°C. Our procedure minimizes, or avoids completely, the water loss during the experiments, which might affect the optical properties of the plant material. Thus, we did not detect changes either in spectra or in time-resolved fluorescence measurements during the experiments, and samples were all turgid at the end of the experiments (not shown).

2.2. Chemicals

Fig. 2 shows the structures of ferulic acid, *p*-coumaric acid, kaempferol and quercetin. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (100% purity), *p*-coumaric acid (4-hydroxycinnamic acid) (100% purity), kaempferol (3,4',5,7-tetrahydroxyflavone) (purity > 96%) and quercetin (3,3',4',5,7-pentahydroxyflavone) (98.7% purity) were purchased from Sigma (St. Louis, MO, USA). Water (HPLC grade; pH 6.5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (Uvasol, for fluorimetry) was purchased from Merck (Darmstadt, Germany). All other chemicals used were of proanalysis grade.

2.3. Optical microscopy

Observations were performed on adaxial epidermises, mounted in water, with an optical microscope (Wild, Heerbrugg, Switzerland).

2.4. Absorption spectroscopy

Absorption spectra were measured between 240 and 600 nm with a Beckman DU-40 spectrophotometer (Beck-

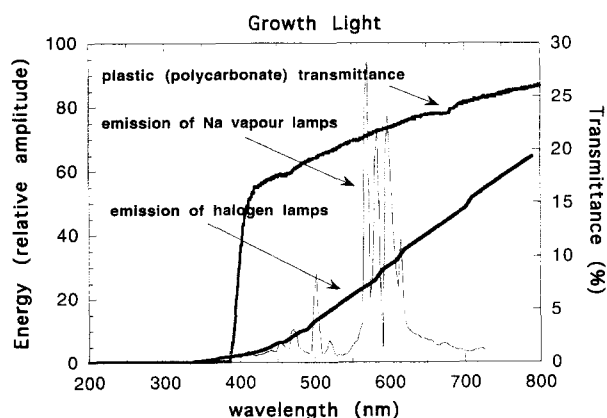


Fig. 1. Transmittance of the plastic sheet (polycarbonate) and the spectra of the radiation (relative energy) provided by a combination of halogen and sodium vapour lamps (Claude, Puteaux, France). The transmittance of the plastic sheet was measured with a Beckman DU-40 spectrophotometer (Beckman Instruments, CA, USA).

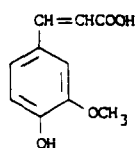
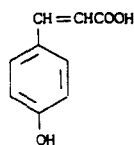
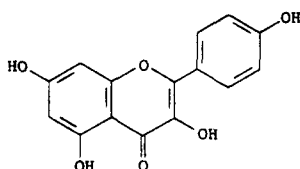
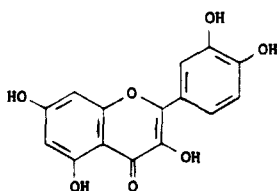
**Ferulic Acid****p-Coumaric Acid****Kaempferol****Quercetin**

Fig. 2. Structures of hydroxycinnamic acid derivatives (ferulic acid and *p*-coumaric acid), and flavonoids (kaempferol and quercetin).

man Instruments, CA, USA). For absorption studies of isolated epidermis, samples were placed against the photo-detector by a home-made holder. Epidermal pigments (approximately 1 cm² of epidermis was used) were extracted with 2 ml of methanol/water (4:1, v/v). Extracts were filtered through a 0.2 μm filter (Acrodisc 32, Gelman Sciences, MI, USA) before analysis. They were analyzed spectrophotometrically immediately after the extraction. For comparative purposes, ferulic acid and *p*-coumaric acid (both in water), kaempferol and quercetin (both in methanol) were also analyzed. Their concentrations were 10⁻⁵ M. All measurements were made in a

quartz cuvette of 1 cm optical path (QS, Hellma) at room temperature (20°C).

2.5. Time-correlated single-photon-counting measurements and decay-associated excitation and emission spectra

Time-correlated single-photon-counting measurements of fluorescence decays were performed on the SA4 line of the SUPERACO synchrotron in Orsay (France) as described previously [11,12], with some modifications. For the excitation part of the apparatus, a double-grating monochromator (6 nm band pass, H10 D UV, Jobin-Yvon, Longjumeau, France) was used, instead of a single-grating monochromator. For measurements of excitation spectra, a KV418 (Schott) anti-UV filter was placed before the emission monochromator. For measurements of emission spectra, it was replaced by a KV389 (Schott) anti-UV filter. Decay-histograms were acquired until 32 000 counts were accumulated at the maximum. 42 ns were covered by 512 channels at 0.083 ns per channel. Iterative convolution of fluorescence decays was performed as previously described [11] using a home-made program based on Marquardt search algorithm for non-linear parameters [13]. Decay-associated excitation spectra were obtained by varying the excitation wavelength at which the fluorescence decays were recorded with fixed emission wavelength. Decay-associated emission spectra were obtained as previously described [11]. Individual analysis was performed for each decay and then a global fit was performed to all decays in a spectrum simultaneously.

Fluorescence excitation spectra from 240 to 400 nm were recorded on the same apparatus by 'scrolling' the excitation monochromator and integrating the number of counts during one second at each new wavelength spaced by 2 nm. Fluorescence emission spectra from 400 to 600 nm were recorded in the same way by 'scrolling' the emission monochromator. As a special case, the fluorescence excitation and emission spectra of *p*-coumaric acid were measured between 240 and 380 nm and between 380 and 580 nm, respectively. For most cases, three forward and three backward scans were averaged. Excitation and emission spectra were corrected for instrumental response using rhodamine B as a quantum meter and quinine sulfate as a standard, respectively [14]. All measurements were made at controlled room temperature (20°C).

2.6. Thin-layer chromatography analysis

Epidermal pigments (approximately 5 cm² of epidermis were used) were extracted with 180 μl of methanol after grinding the sample in liquid N₂. After methanol evaporation, 8 μl of methanol were used to resuspend pigments. Extracts were filtered with a 0.45 μm filter (Shandon HPLC, Corgy Pontois, France). Ferulic acid, *p*-coumaric acid, kaempferol, quercetin (10 nmol of each in 1 μl of methanol), and a mixture of them (2.5 nmol of each in 1

μl of methanol) were run, together with the epidermis extract. Methanol was also run as a control.

Qualitative thin-layer chromatography was performed using silica gel 60 on plastic plates (Merck, Darmstadt, Germany). Toluene, ethyl formate and formic acid (5:4:1, by volume) were used as solvents. Plates were examined under UV excitation, using synchrotron radiation.

3. Results

3.1. Excitation and emission fluorescence spectra of different tissues of a sugar beet leaf

Fig. 3 shows the excitation and emission fluorescence spectra of different tissues of the same sugar beet leaf. The highest BGF within the leaf was found in the major vein, in line with data recently reported [15]. It had an emission maximum at 450 nm and two excitation maxima at 340 and 250 nm. The BGF emission of the mesophyll was approximately 10% that of the major vein. It showed

emission maxima at 450 and 510 nm, with a shoulder at 430 nm. Their excitation maxima were at 320 and 245 nm.

Leaf pieces showed an emission maximum at 420 nm, with a shoulder at 520 nm. Their excitation maxima were at 330 and 240 nm. Adaxial and abaxial sides of leaf pieces did not give significant differences. Isolated epidermis retained most of the BGF emitted from the mother leaf pieces. The fluorescence spectra were similar and they showed the same maxima. These data clearly confirm earlier work [4,7,8,11,16–18] suggesting that most of the BGF emitted from the leaf comes from the epidermis. A detailed investigation of the adaxial epidermis of sugar beet leaves was therefore carried out.

3.2. Microscopic observations

The adaxial epidermis of sugar beet leaves consisted of a multilayer of cells, including stomates (not shown). Cells remained intact and alive, as demonstrated by the activity of the stomates (active movement of chloroplasts within the guard cells). The absence of mesophyll cells was confirmed by these observations (not shown).

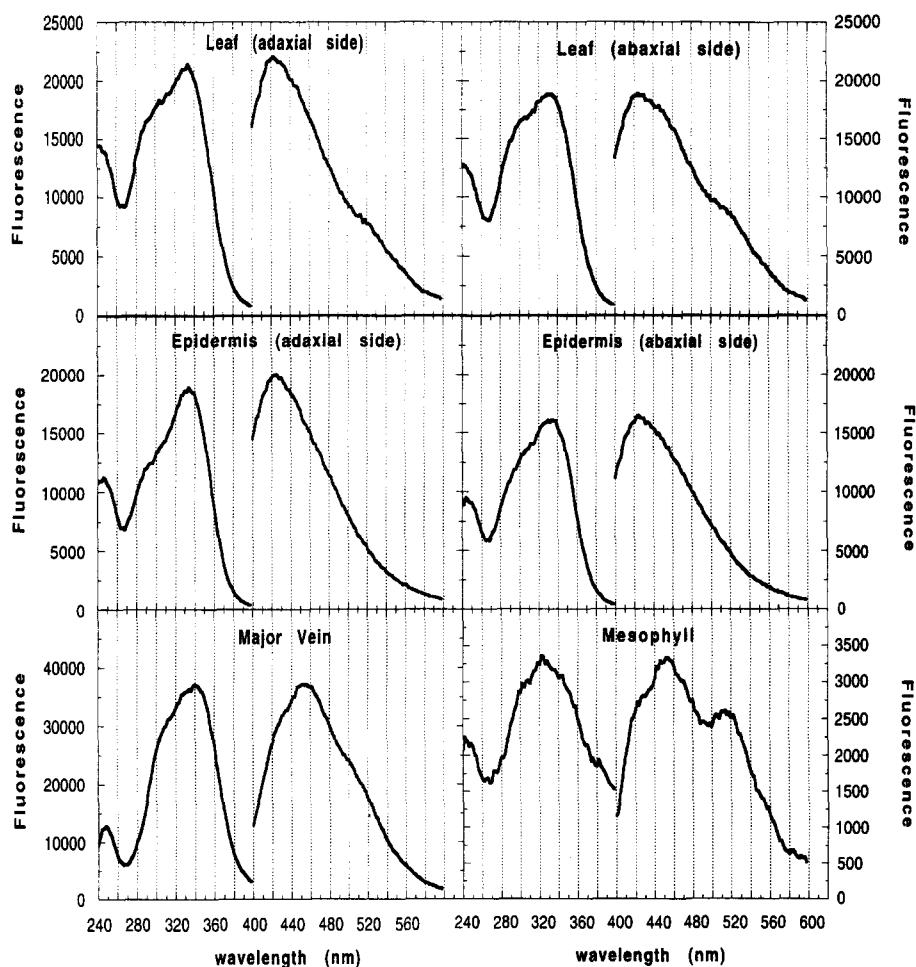


Fig. 3. Excitation (from 240 to 400 nm) and emission (from 400 to 600 nm) fluorescence spectra of sugar beet: leaf (uppermost panels) (left, adaxial side; right, abaxial side), epidermis (middle panels) (left, adaxial side; right, abaxial side), major vein (lower panel, left), and mesophyll (lower panel, right). Excitation spectra were recorded at emission maxima. Emission spectra were recorded at excitation maxima.

3.3. Absorption spectroscopy of epidermis, methanol/water extracts of epidermis, and standards

As can be seen from the absorption spectra in Fig. 4, the isolated epidermis (uppermost panel, left) strongly absorbed UV light, with a maximum at 290 nm and a shoulder at 325 nm. Although the intensity of absorption varies among samples (probably due to natural heterogeneities within the epidermis or caused by our isolation procedure), the form of the spectra remained identical. To avoid the absorption of proteins and nucleic acids and to avoid the optical dispersion by the intact tissues of the epidermis, we extracted the pigments of the epidermis (uppermost panel, right). Methanol/water extracts of adaxial epidermis gave two maxima at 260 and 325 nm, with a shoulder at 350 nm.

For comparative purposes, we measured the absorption of the hydroxycinnamic acid derivatives (ferulic acid and *p*-coumaric acid) and flavonoids (quercetin and kaempferol) that have been shown to be present in sugar beet [9,10]. Ferulic acid absorbed UV-B (280–320 nm), with maxima

at 285 and 310 nm (middle panel, left; [19]). It can be also seen that *p*-coumaric acid absorbed UV-B, with a maximum at 285 nm (middle panel, right). Kaempferol absorbed UV-C (250–280 nm) and UV-A (320–400 nm), with maxima at 265 and 365 nm (lower panel, left). Quercetin, like kaempferol, absorbed UV-C and UV-A, with maxima at 255 and 370 nm (lower panel, right). Similar maxima have been reported previously [20].

The contribution of fluorescent compounds to total absorbance of the epidermis seems to be minor. This can be concluded if we compare the absorption spectra of the methanol/water extracts of the epidermis (uppermost panel, right) with those of the hydroxycinnamic acid derivatives and flavonoids (middle panels and lower panels). In addition, other substances can screen the absorption of fluorophores blurring the spectroscopic identification. Still, excitation and emission spectra of fluorescence would be far more specific (there are far less fluorophores than chromophores in the epidermis). We therefore compared the fluorescence spectra of potential pure fluorophores of sugar beet to the fluorescence of the epidermis.

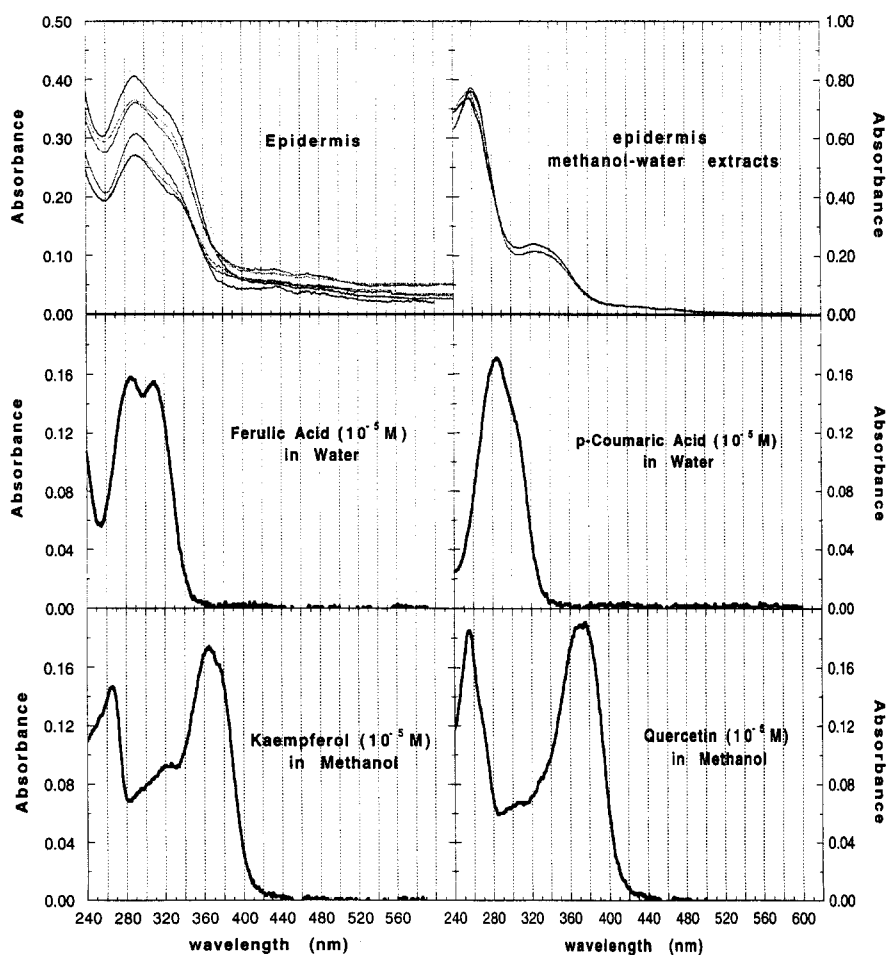


Fig. 4. Absorption spectra (from 240 to 600 nm) of sugar beet adaxial epidermis (uppermost panel, left), methanol/water extracts of sugar beet adaxial epidermis (uppermost panel, right), ferulic acid (10^{-5} M) in water (middle panel, left), *p*-coumaric acid (10^{-5} M) in water (middle panel, right), kaempferol (10^{-5} M) in methanol (lower panel, left), and quercetin (10^{-5} M) in methanol (lower panel, right).

3.4. Excitation and emission fluorescence spectra of standards

We used 310 nm for excitation, wavelength that is absorbed by all of the substances tested (see Fig. 4) and that efficiently induces BGF in the epidermis (see Fig. 3). The results are shown in Fig. 5A. Ferulic acid was strongly fluorescent, followed by *p*-coumaric acid. The flavonoids, kaempferol and quercetin, were weakly fluorescent under

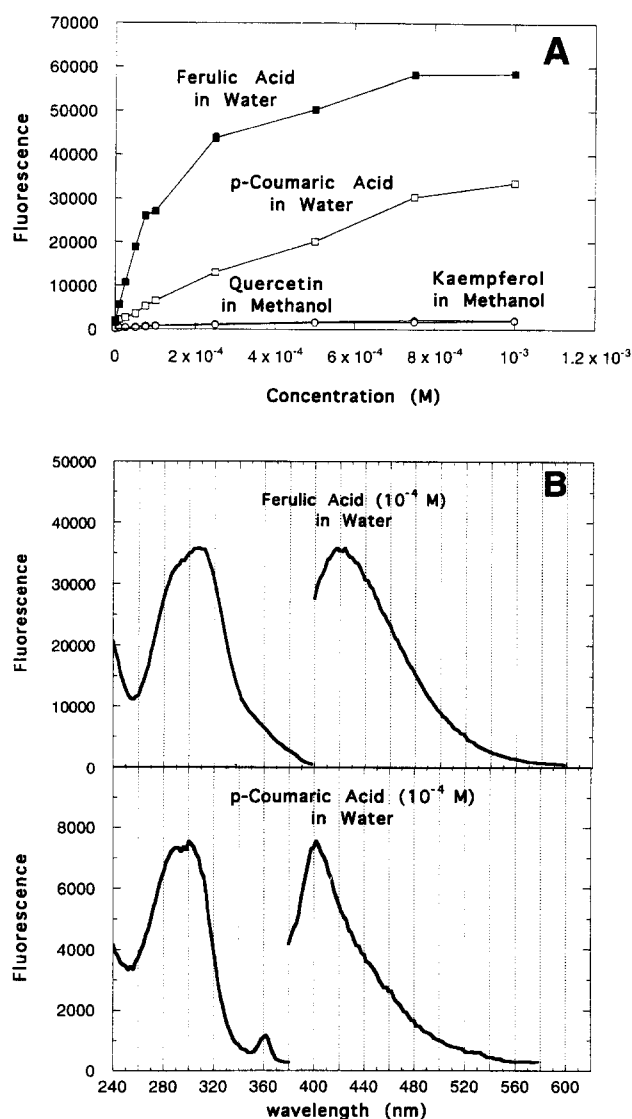


Fig. 5. (A) Relationship between fluorescence and concentration for ferulic acid in water, *p*-coumaric acid in water, kaempferol in methanol, and quercetin in methanol. Excitation was at 310 nm. Emission was at their maxima (see text). (B) Excitation and emission fluorescence spectra of ferulic acid (upper panel), and *p*-coumaric acid (lower panel). The concentration of the fluorophores was 10^{-4} M in water. Excitation spectra were recorded at emission maxima, and it was from 240 to 400 nm for ferulic acid and from 240 to 380 nm for *p*-coumaric acid. Emission spectra were recorded at excitation maxima, and it was from 400 to 600 nm for ferulic acid and from 380 to 580 nm for *p*-coumaric acid.

such excitation. These data clearly rule out flavonoids as the main fluorophores of the epidermis. When fluorescence was plotted versus concentration, all of them gave a linear increase in fluorescence until 10^{-4} M. This concentration was used to determine their fluorescence characteristics.

To obtain complete spectral information about the BGF emitted by these substances, we measured their excitation and emission fluorescence spectra. Ferulic acid showed an excitation maximum at 310 nm with an emission maximum at 420 nm (Fig. 5B; [21]). This is similar to the data obtained by Lang et al. [4] for ferulic acid in methanol (10^{-5} M), where the emission maximum was at 430 nm. In Fig. 5B it can be also seen that *p*-coumaric acid has an excitation maximum at 300 nm (the little peak at 360 nm is the Raman scattering of water), with an emission maximum at 400 nm. As has been already cited, kaempferol did not fluoresce under 310 nm excitation (Fig. 5A). Under UV-C (270 nm) excitation, kaempferol becomes slightly fluorescent. It showed an emission maximum at 500 nm (not shown). Similar characteristics were found for quercetin (not shown). These data agree with those reported previously for flavonoids [4,22].

By comparing the excitation and emission fluorescence spectra of the adaxial epidermis (see Fig. 3) with the excitation and emission fluorescence spectra of ferulic acid (see Fig. 5B) one can conclude that the BGF emitted from the epidermis contains fluorescence of ferulic acid. Epidermis and ferulic acid had identical emission spectra, with maxima at 420 nm. The form of the excitation spectrum of the epidermis looked like that of ferulic acid, but the maximum of the epidermis was 20 nm shifted to longer wavelengths (maximum at 330 nm) when compared with that of ferulic acid (maximum at 310 nm). This shift may be due to the esterification of ferulic acid (see below). Further corroboration for the presence of ferulic acid in the epidermis of sugar beet leaves comes from time-resolved fluorescence measurements.

3.5. Time-resolved measurements and decay-associated excitation and emission spectra of the epidermis

In Fig. 6 the instrumental function (excitation flash) and a fluorescence decay of epidermis are presented. Data were analyzed by applying a convolution model, as previously described [11]. The correctness of the convolution was judged from the examination of the χ^2 value, distribution of the weighted residuals between the calculated and the experimental function, and the autocorrelation function of the residue [23]. In sugar beet epidermis, four kinetic components were resolved: the very fast (20–40 ps), the medium (0.82–0.90 ns), the slow (2.60–2.77 ns) and the very slow (7.09–7.62 ns) kinetic components.

For comparative purposes we made time-resolved measurements of ferulic acid (10^{-4} M in water) in the same apparatus and temperature (20°C) as for epidermis. Ferulic acid showed a monoexponential decay with a fluorescence

lifetime of 21 ps, indicating that it fluoresces in the time domain of the very fast component found in epidermises.

Decay-associated excitation spectra of epidermis were recorded with the emission wavelength fixed at the maximum (Fig. 7, left panels). First, an individual analysis was performed for each decay. Second, we analyzed all the decays globally by fitting the best common lifetimes for a 4-component model to all decays. Then, average lifetimes for each wavelength were calculated. Finally, the absolute contribution to the excitation of the individual component was calculated by multiplying the fractional intensity by the excitation spectra at the corresponding wavelength recorded separately. Decay-associated emission spectra of epidermis were recorded with the excitation wavelength fixed at the maximum (Fig. 7, right panels). Decay-associated emission spectra of epidermis were calculated following the same procedure as for the decay-associated excitation spectra.

From the data presented in Fig. 7, it can be seen that in the epidermis the average lifetimes depend on the excitation or emission wavelength. It indicates that the BGF of the epidermis is the result of a superposition of several

emissions. As has already been pointed out, four kinetic components were resolved. At the excitation and emission maxima, the very fast kinetic component contributes 70% to the BGF. It has identical emission spectrum to ferulic acid (maxima at 420 nm), the form of its excitation spectrum resembles that of ferulic acid, and ferulic acid fluoresces in its time domain. We can therefore conclude that the very fast kinetic component contains fluorescence of ferulic acid. The medium, slow and very slow kinetic components contribute, at the excitation and emission maxima, with 16%, 11% and 3% to the BGF, respectively. They have excitation maxima in the UV-B (280–320 nm), but these are progressively shifted to shorter wavelengths. Their emission maxima are progressively shifted to longer wavelengths. The origin of this heterogeneity is not clear at present and deserves further investigation.

3.6. Temperature-induced changes in blue-green fluorescence: leaf, leaf phenolic extracts and ferulic acid

Further confirmation for the involvement of ferulic acid fluorescence in the very fast kinetic component came from

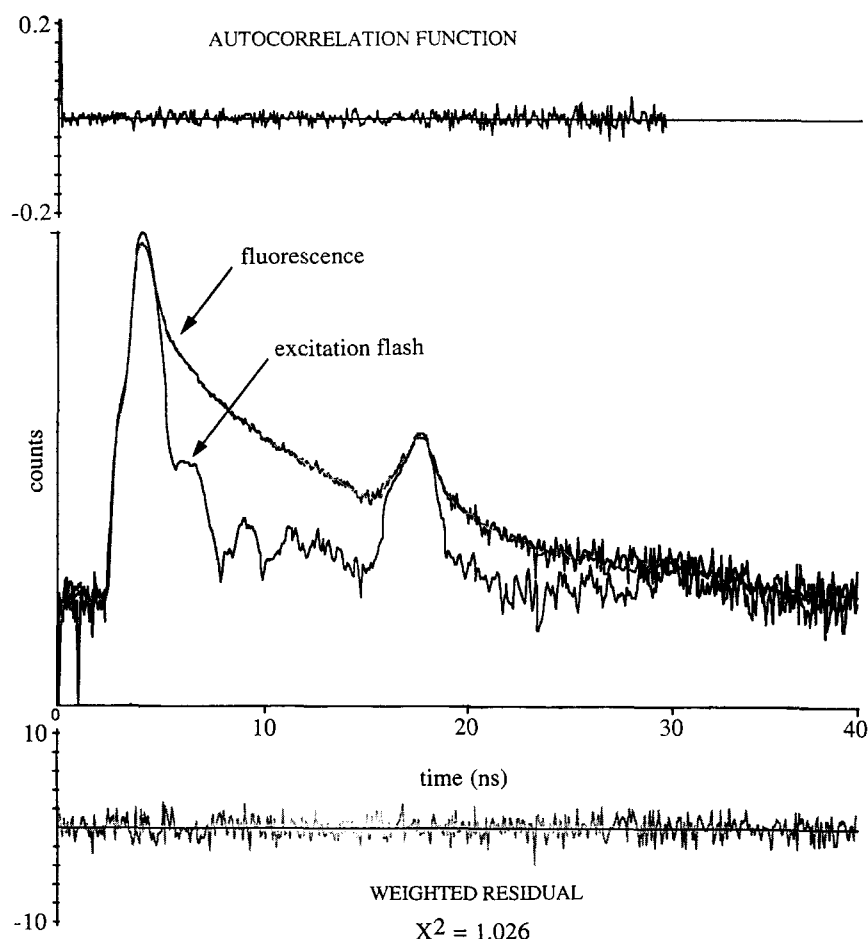


Fig. 6. Fluorescence decay of sugar beet adaxial epidermis. This is a typical decay from the series of measurements performed on epidermis to calculate the excitation and emission decay-associated spectra. The excitation wavelength was 370 nm, and emission wavelength was 430 nm. The residual and the corresponding autocorrelation function are also shown.

the investigation of the effect of temperature on leaf BGF. Leaf BGF intensity has been shown to be temperature-dependent, increasing when leaf temperature decreased [7,8]. Thermal sensitivities (measured as % variation $(^{\circ}\text{C})^{-1}$) of the overall leaf BGF ranged from 0.75 to 2.75 depending on plant species [7,8]. We tested sugar beet leaves, their phenolic extracts (prepared as previously described [24]) and ferulic acid for thermal sensitivity. All of them increased their overall BGF by decreasing temperature (Fig. 8, insets). Time-resolved BGF measurements were carried out to investigate the thermal sensitivity of each kinetic

component in these three samples. Thus, we fixed the excitation and emission wavelengths to the fluorescence maxima and we measured BGF decays at low and high temperature. In sugar beet leaves, five kinetic components were resolved: the very fast (30–40 ps), the fast (0.26–0.27 ns), the medium (0.84–0.93 ns), the slow (2.39–3.14 ns), and the very slow (7.47–7.91 ns) kinetic components (Table 1). The same five kinetic components were also resolved for the phenolic extracts of sugar beet leaves (Table 2). Their fluorescence lifetimes and the fluorescence lifetime of ferulic acid were temperature-indepen-

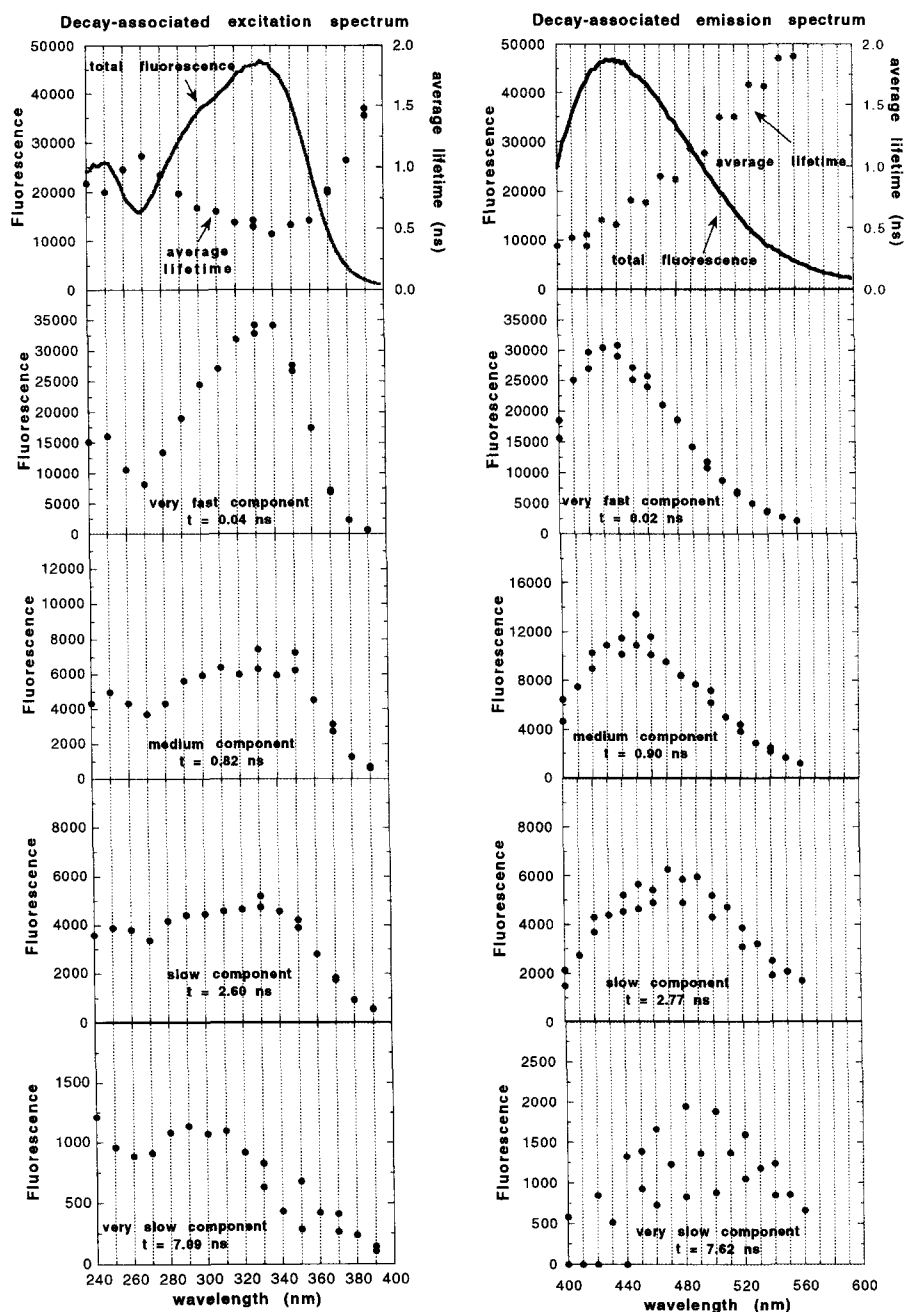


Fig. 7. Decay-associated excitation spectrum (left panels) and decay-associated emission spectrum (right panels) of sugar beet adaxial epidermis. For details see text.

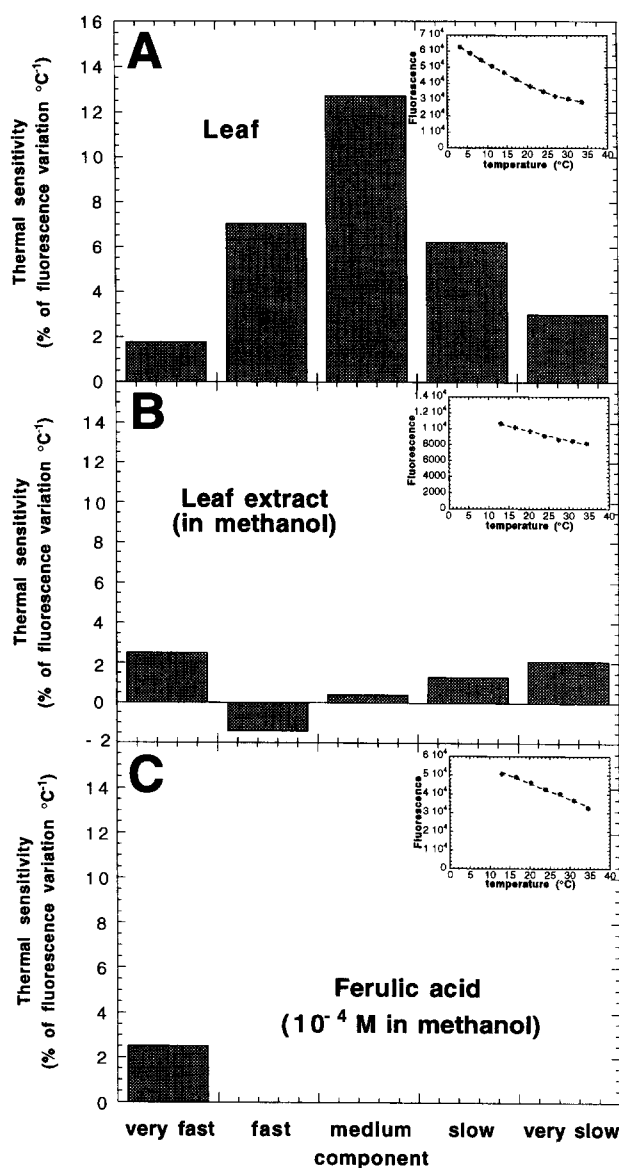


Fig. 8. Thermal sensitivities of the kinetic components of the blue-green fluorescence. Inserts, changes induced by temperature in the intensity of the blue-green fluorescence. (A) Sugar beet leaves; (B) phenolic extracts of sugar beet leaves and (C) ferulic acid. Although there are two minor components in the fluorescence of ferulic acid, they only contribute 6% to the overall fluorescence and they were not included for thermal sensitivity calculations.

dent (Tables 1–3). The fractional intensities of all kinetic components were, however, changed by temperature (Tables 1 and 2), indicating that their thermal sensitivity may be different. We therefore calculated the thermal sensitivity of each component by the following procedure. Since we know the overall fluorescence of the leaf, of the leaf extract and of ferulic acid at low and high temperature (see inserts of Fig. 8), and since we know the fractional intensities (in percentage of fluorescence) of each kinetic component at the same temperatures (see Tables 1–3), we can calculate the actual fluorescence change of each compo-

Table 1
Fluorescence lifetimes and fractional intensities of sugar beet leaves

Component	Temperature	
	33°C	3°C
Very fast		
lifetime	0.03 ± 0.01 ns	0.04 ± 0.01 ns
fractional intensity	45 ± 1%	28 ± 1%
Fast		
lifetime	0.26 ± 0.06 ns	0.27 ± 0.04 ns
fractional intensity	20 ± 2%	26 ± 2%
Medium		
lifetime	0.93 ± 0.25 ns	0.84 ± 0.08 ns
fractional intensity	12 ± 1%	24 ± 1%
Slow		
lifetime	3.14 ± 0.92 ns	2.39 ± 0.25 ns
fractional intensity	12 ± 1%	14 ± 1%
Very slow		
lifetime	7.91 ± 1.75 ns	7.47 ± 0.56 ns
fractional intensity	11 ± 1%	8 ± 1%
Mean lifetime	1.21 ± 0.09 ns	1.18 ± 0.04 ns

The wavelengths of excitation and emission were 337 and 450 nm, respectively. The temperature was fixed at 33 or 3°C. Data are mean ± S.D.

nent multiplying the overall fluorescence by each percentage of fluorescence at a given temperature. Then, we calculated the percentage of fluorescence variation of each component and we divided them by the change of temperature used. The results are shown in Fig. 8. The thermal sensitivity of ferulic acid (2.5% variation (°C)⁻¹, Fig. 8C) matched with that of the very fast kinetic component found in leaves (1.8% variation (°C)⁻¹, Fig. 8A) and with that of the leaf phenolic extracts (2.5% variation (°C)⁻¹, Fig. 8B).

Table 2
Fluorescence lifetimes and fractional intensities of sugar beet leaf phenolic extracts

Component	Temperature	
	33°C	14°C
Very fast		
lifetime	0.06 ± 0.01 ns	0.06 ± 0.01 ns
fractional intensity	22 ± 1%	26 ± 1%
Fast		
lifetime	0.39 ± 0.08 ns	0.25 ± 0.10 ns
fractional intensity	10 ± 1%	5 ± 1%
Medium		
lifetime	1.29 ± 0.16 ns	0.98 ± 0.06 ns
fractional intensity	21 ± 1%	19 ± 1%
Slow		
lifetime	3.76 ± 0.35 ns	3.19 ± 0.19 ns
fractional intensity	25 ± 1%	25 ± 1%
Very slow		
lifetime	9.44 ± 0.21 ns	9.23 ± 0.14 ns
fractional intensity	22 ± 1%	25 ± 1%
Mean lifetime	3.12 ± 0.02 ns	3.26 ± 0.03 ns

The wavelengths of excitation and emission were 320 and 420 nm, respectively. The temperature was fixed at 33 or 14°C. Data are mean ± S.D.

These data further support our contention that ferulic acid contributes to the BGF in the time domain of the very fast kinetic component.

3.7. Thin-layer chromatography analysis of methanolic extracts of epidermis and standards

Hydroxycinnamic acid derivatives and flavonoids occur commonly in plants as sugar-bound derivatives [25–29]. We therefore used thin-layer chromatography to investigate the possible presence of ferulic acid in the epidermis in a form bound to sugars. Free standards (ferulic acid, *p*-coumaric acid, kaempferol and quercetin), a mixture of them, and a methanolic extract of epidermis were run. Identification of the spots was made on the basis of their position, colour, and excitation and emission characteristics under UV excitation. Our solvent mixture separated the standards among them, with R_F values of 6.8 for ferulic acid, 6.6 for *p*-coumaric acid, 6.5 for kaempferol, and 6.0 for quercetin (Table 4). In the methanolic extract of the epidermis, two spots, one more diffuse and larger and the other more concentrated, remained at the bottom of the plate (R_F values of 0.6 and 0.15, respectively; Table 4), suggesting the presence of highly polar substances. They were sprayed with Mo/Ce (Molibden/Cerium) reagent, giving a positive test for sugars (not shown). The more diffuse spot showed the fluorescence characteristics of ferulic acid, fluorescing blue under UV light and with an excitation maximum in the UV-B. It was therefore attributed to a sugar-bound derivative of ferulic acid. The presence of ferulic acid in the sugar beet epidermis in a form bound to sugars agrees with data obtained from

Table 3
Fluorescence lifetimes and fractional intensities of ferulic acid (10^{-4} M in methanol)

Component	Temperature	
	33°C	14°C
Very fast		
lifetime	0.04 ± 0.01 ns	0.04 ± 0.01 ns
fractional intensity	$94 \pm 1\%$	$94 \pm 1\%$
Fast		
lifetime	–	–
fractional intensity	–	–
Medium		
lifetime	1.55 ± 0.08 ns	1.79 ± 0.10 ns
fractional intensity	$3 \pm 1\%$	$3 \pm 1\%$
Slow		
lifetime	–	–
fractional intensity	–	–
Very slow		
lifetime	8.70 ± 1.18 ns	10.72 ± 0.62 ns
fractional intensity	$3 \pm 1\%$	$3 \pm 1\%$
Mean lifetime	0.37 ± 0.05 ns	0.42 ± 0.03 ns

The wavelengths of excitation and emission were 325 and 420 nm, respectively. The temperature was fixed at 33 or 14°C. Data are mean \pm S.D.

Table 4

Thin-layer chromatography of ferulic acid, *p*-coumaric acid, kaempferol, quercetin, a mixture of the four standards, and extracts of adaxial epidermis

	Number of spots	R_F values		
Ferulic acid	1	6.8		
<i>p</i> -Coumaric acid	1	6.6		
Kaempferol	1	6.5		
Quercetin	1	6.0		
Mixture of the four standards	3	6.0	6.55	6.8
Epidermis extract	2	0.15	0.6	
Methanol	0	–		

Methanol was also run as a control.

primary cell walls of suspension cultures of spinach in which free ferulic acid was not detected before hydrolysis [28]. In that work after hydrolysis, only 8% of ferulic acid was free, 25% was polysaccharide-bound, and 67% was esterified. Further work of the same group demonstrated that it was esterified with galactose and arabinose residues of polysaccharides [29]. In the case of wheat coleoptiles, ferulic acid was associated with a fraction of the cell walls rich in arabinose and xylose [27]. The more concentrated spot was yellow under visible light (like free kaempferol) and showed a yellow fluorescence under UV light, with an excitation maximum in the UV-A. It may be therefore attributed to kaempferol glycosides, form in which most of the flavonoids are found in nature [25,26].

4. Discussion

Sugar beet leaves emit BGF when excited with UV light. A comparative spectral analysis showed that the BGF of sugar beet leaves, on both adaxial and abaxial side, was dominated by the BGF of the epidermis. These data agree with those that suggested, for several plant species, that most of the BGF emitted from the leaf comes from the epidermis [4,7,8,11,16–18]. Our data do not show significant differences between the adaxial and the abaxial epidermis of sugar beet leaves. Other leaves show, however, a significantly lower BGF in the adaxial side when compared with the abaxial side [1,4,6], using plants grown in greenhouses or outdoors. Under our growth conditions, plants do not receive UV-B radiation at all, which is unrealistic for plants grown outdoors under a natural solar spectrum. In the presence of UV light, plants accumulate UV-absorbing pigments mainly in the adaxial epidermis of their leaves ([30] and references therein). They are mostly flavonoids and hydroxycinnamic acid derivatives [30], which protect leaves from UV light [31,32]. Moreover, it is thought that natural UV-protective mechanisms do not become fully developed in low levels of visible light [30], conditions in which our plants were grown. After the work of Stober and Lichtenthaler [33], the differences in BGF, found between the adaxial and abaxial side of the leaves,

may be clearly attributed to an accumulation of flavonoids in the adaxial epidermis of leaves growing in field conditions, or in growth chambers at high light intensities. In *Vicia faba*, flavonoids are exclusively found in the vacuoles of epidermal cells (59% in the adaxial epidermis, 7% in the abaxial epidermis, and 34% in the guard cells) [25]. The situation in pea and soybean seems to be similar [26]. Flavonoids absorb UV light and therefore attenuate the UV light that reaches the fluorophores fluorescing blue-green. This is referred to as the inner-filter effect due to excitation blocking (screening). The result is a significant reduction of BGF coming from the adaxial sides of leaves growing outdoors.

The intensity of the BGF was also shown to depend on plant species [1]. In their work, corn and soybean were chosen as representatives of monocotyledons and dicotyledons, respectively. The BGF of corn was much higher than that of soybean. The origin of the differences found in BGF among species is not yet known. Differences in inner-filter effect cannot be excluded, although one possible explanation is that different species had different fluorophores. Some works have suggested that the BGF is most probably a mixed signal composed of the fluorescence of various phenolic substances, including hydroxycinnamic acid derivatives and flavonoids [2–4,6–8]. Due to these differences among species and to this large number of possible candidates, case studies have to be undertaken to define the fluorophores responsible for the BGF in different plant species. We used sugar beet as a model system to study BGF ([11,12]; this work) and in view of using BGF for remote sensing of this important crop. In sugar beet, the presence of two hydroxycinnamic acid derivatives (ferulic acid and *p*-coumaric acid; the former in higher concentration) and two flavonoids (kaempferol and quercetin; the former in higher concentration) is well documented [9,10]. Flavonoids can be ruled out as being responsible for the BGF in sugar beet, since they do not fluoresce at the wavelengths that efficiently excite the epidermis. Between the two hydroxycinnamic acid derivatives, ferulic acid was the only one that fluoresced similarly to the epidermis.

Several lines of evidence indicate the presence of ferulic acid as the main fluorophore of the epidermis of sugar beet leaves: (i) the emission spectrum of ferulic acid matches that of the epidermis; (ii) ferulic acid fluoresces in the time domain of the very fast kinetic component (20–40 ps) found in the epidermis, which contributes with 70% to the total BGF, at the excitation and emission maxima, and whose emission spectrum matches that of ferulic acid; and (iii) the thermal sensitivity of the very fast kinetic component matches that of ferulic acid. On the other hand, the excitation spectrum of epidermis (and that of the very fast kinetic component) looks like that of ferulic acid, although a shift of 20 nm towards longer wavelengths is evident. The shift may be explained when taking into account that in different plant species most of ferulic acid was found to

be esterified to sugars of the cell wall polysaccharides [27,29]. Our thin-layer chromatography analysis also indicated that ferulic acid is present in sugar beet epidermis as glycosides and/or esters. Esters of ferulic acid have been shown to absorb at longer wavelengths (approximately 330 nm) [28,34] when compared with free ferulic acid. Our data indicate that free ferulic acid fluoresces similarly to its glycosides and/or esters isolated by thin-layer chromatography. As has been shown in Fig. 7, the BGF of the epidermis of sugar beet leaves is heterogeneous. Although 70% (i.e., the very fast kinetic component) of this BGF is due to ferulic acid (probably esterified to sugars), 30% (i.e., the medium, slow and very slow kinetic components) remains unexplained. Unfortunately, data on fluorescence lifetimes of most plant phenolics are missing from the literature, and the assignment of the medium, slow and very slow kinetic components should await time-resolved studies of a large variety of candidates.

The leaf BGF was shown to depend on stage of development [1,6,35] and on environmental factors experienced by plants [1,2,36]. These changes of BGF can be related to modifications of phenylpropanoid metabolism. Thus, changes in composition and accumulation of hydroxycinnamic acid and flavonoid derivatives have been shown to occur in plants under stress conditions [26,30,37]. In sugar beet, we tested a nutritional stress such as iron deficiency [12]. Iron deficiency induces a decrease of the blue-to-green fluorescence ratio, attributed to an accumulation of flavins (mostly riboflavin and/or flavin mononucleotide phosphate) fluorescing in the green. This BGF change may be used as a method to develop a technique for an early detection of iron deficiency in sugar beet plants. On the other hand, we have suggested the use of time-resolved BGF measurements to extract the information on nucleotide fluorescence from the overall leaf fluorescence, and to evaluate changes in mesophyll redox state [11]. To maximize the leaf nucleotide fluorescence (which comes mainly from the mesophyll) the excitation of the sugar beet epidermis should be avoided, using a band of excitation ranging from approximately 370 to 400 nm (see Fig. 3). Extensive characterization of the divers compounds contributing to the blue-green fluorescence (BGF) is necessary in order to develop the potential of BGF as a signature for remote sensing of plants.

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