Quantitative study of fluorescence excitation and emission spectra of bean leaves

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Abstract

A quantitative and comprehensive knowledge of leaf fluorescence is required for the interpretation of fluorescence signals at the canopy level and also for the modelling of leaf and canopy fluorescence. In this work we present full range fluorescence excitation and emission spectra of intact leaves, expressed in units of apparent spectral fluorescence yield, from both the adaxial and the abaxial sides of the leaves, and for both front-side and back-side geometries. Emission spectra were measured for incident radiations in the blue and the green spectral range. The red/far-red fluorescence ratio depended on the measurement geometry and on the excitation wavelength. Excitation spectra were measured for emissions at 687 and 760 nm. When the abaxial side was illuminated, the measured spectra always had a larger intensity compared to adaxial side that is explained by the higher scattering of the spongy tissues. At 760 nm, the spectra had the same shape for front-side and back-side geometry, indicating that scattering predominated. At 687 nm, the shape of the spectra was very different for front-side and back-side geometry due to re-absorption of red fluorescence within the leaf. The comparison of excitation spectra measured from the adaxial or the abaxial side revealed differences in carotenoid absorption.

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1. Introduction

Chlorophyll-a fluorescence (ChlF) represents an intrinsic signal emitted by plants that can be used to monitor their physiological state. The most widely used ChlF parameters are related to the efficiency of light utilization by photosynthesis and are routinely measured according to the Saturation-Pulse method [1] at the leaf level, in the laboratory and in the field [2]. Spectral analysis of ChlF can provide further information concerning plant physiological state. The ratio of the two ChlF peaks in the red (near 685 nm) and far-red (near 740 nm) regions is a valuable indicator of leaf chlorophyll content [3–5]. Indeed, red fluorescence (RF) is re-absorbed by chlorophylls, while far-red fluorescence (FRF) is not, resulting in a negative curvilinear relation between the RF/FRF ratio and the leaf Chl content [5]. Also, the ratio of UV- and blue-green-induced ChlF can estimate the epidermal transmittance to UV [6]. Interestingly, spectral analysis of ChlF can be performed at leaf as well as canopy levels.

Recently, instruments have been developed to measure, at the canopy level, intensities of solar-induced RF and FRF using the Fraunhofer Line Principle applied to the oxygen absorption bands at 687 and 760 nm [7]. However, fluorescence signals measured by passive remote sensing at the canopy level remain difficult to interpret since they can be markedly influenced not only by leaves properties but also by the canopy structure. Fluorescence signals emitted at 687 and 760 nm have particularly different properties [7,8]. Leaf fluorescence depends on pigment contents,
chlorophylls, carotenoids and polyphenols (for a review see Cerovic et al. [9]), and on the scattering properties of the epidermal and mesophyll tissues [10]. These different factors determine the absorption profile of the incident radiation and the propagation of the fluorescence fluxes within the leaves, and consequently, the shape and the intensity of leaf fluorescence spectra. At the canopy level, fluorescence emitted by both sides of the leaves contributes to the detected signals and the adaxial or the abaxial side of the leaves can be illuminated. Moreover, in the case of passive remote sensing, fluorescence signals are induced by white light. Thus, they depend on leaves fluorescence excitation spectra and on the spectral composition of solar-light.

The interpretation and the modelling of canopy fluorescence require a quantitative and comprehensive study of leaf fluorescence. However, despite the importance of the above-mentioned factors on the absolute and relative intensities of RF and FRF, their impacts have not, to our best knowledge, been yet comprehensively quantified, and a complete interpretation of both fluorescence excitation and emission spectra on the same set of leaves has not been done previously. In this work we present for the first time full range fluorescence excitation and emission spectra of intact bean leaves, expressed in units of apparent spectral fluorescence yield (ASFY), from both the adaxial and the abaxial side of the leaves, and measured in front-side (epifluorescence) or back-side (transfluorescence) configurations. Excitation spectra were measured for an emission at 687 and 760 nm, and we present the full spectral dependency of the RF/FRF ratio.

2. Materials and methods

2.1. Plant material

Green beans (Phaseolus vulgaris L. var. Contender) were grown outdoors, in 460 x 345 x 750 mm trays filled with commercial potting compost, during summer 2003, in Orsay (France). They were watered daily and fertilized weekly. Leaves were collected from the top of the canopy and kept in water until the end of the measurements. The chlorophyll content was measured with the Minolta SPAD-502 and was around 24 μg cm⁻². Carotenoids content was determined spectrophotometrically according to [11] and was around 6 μg cm⁻².

2.2. Fluorescence spectra

Fluorescence excitation and emission spectra were measured with a commercial fluorimeter (Cary eclipse, Varian, Les Ulis, France), equipped with two optical fibres, one for the excitation and one for the detection. The source was a xenon lamp and the measuring beam was weak enough to avoid induction of variable fluorescence. The leaf was illuminated at an angle of about 30° to the leaf normal. The detection was normal to the leaf. Measurements were performed at four different configurations (Fig. 1). The two optical fibres could be on the same side of the leaf or on opposite sides, and the adaxial or the abaxial side of the leaf could be illuminated.

Fluorescence excitation spectra were measured at two emission wavelengths, 687 and 760 nm. The fluorescence emission spectra were measured for two excitation wavelengths, 440 (maximum excitation efficiency in the blue spectral range) and 535 nm (minimum excitation efficiency in the green spectral range). Both the excitation and emission bandpass were 5 nm. The spectra were fully corrected and expressed in ASFY units (nm⁻¹), number of photons emitted in a bandpass of one nanometer centred at a given emission wavelength for one photon received at a given excitation wavelength. The unit chosen was the ASFY, based on the incident number of photons, because it is difficult to assess precisely the number of absorbed photons and because the incident number of photons is the input variable needed for radiative transfer models in remote sensing.

2.3. Correction of the spectra

Both excitation and emission spectra had to be corrected for the spectrum of the exciting source, and for the detection sensitivity, at each wavelength combination. The light at the leaf level provided by the spectrophluorimeter excitation source, $S(λ)$, was measured with a calibrated photodiode (S1337-1010BQ, Hamamatsu). The detection sensitivity was assessed by measuring the output of a standard lamp (LI-COR 1800-02) that has a known spectrum, $B(λ)$, at the emission side of the spectrophluorimeter, yielding the spectrum, $B'(λ)$. The spectra of $S(λ)$ and $B(λ)$ were chosen to be in photon units in order that the corrected fluorescence spectrum $F(λ_{ex}, λ_{em})$ has photon units:

$$F(λ_{ex}, λ_{em}) = F_{exp}(λ_{ex}, λ_{em})S(λ_{ex})^{-1}B(λ_{em})B'(λ_{em})^{-1}$$  (1)
with \( F_{\text{exp}}(\lambda_{\text{ex}}, \lambda_{\text{em}}) \) the measured spectrum, \( \lambda_{\text{ex}} \) and \( \lambda_{\text{em}} \) the excitation and emission wavelength, respectively. At this stage, spectra have a correct shape but further calibration is needed in order to express \( F(\lambda_{\text{ex}}, \lambda_{\text{em}}) \) in units of ASFY (nm\(^{-1}\)), which allows to calculate the emitted fluorescence when the incident light is known.

### 2.4. Measurement of the apparent spectral fluorescence yield

The ASFY was measured for one wavelength combination. The fluorescence emitted by a leaf and the incident light diffused by a Lambertian reference (Spectralon\textsuperscript{TM}) were measured under the same experimental conditions (Fig. 2). However, under these conditions, the fluorescence signal is contaminated by reflected light, which is almost unavoidable in front-face configuration measurements. Thus, complementary measurements were performed to correct the fluorescence signal, as explained below.

Fluorescence was induced by a He–Ne laser and measured through an interference filter centred at 758 nm. The leaf was preadapted to the measuring beam for 15 min. The signal \( S_1 \) was obtained:

\[
S_1 = I \Omega \phi T_{\text{IF}}(758) B_{\text{IF}}(754-762) D(758)
+ I \Omega R(632.8) D(632.8) T_{\text{IF}}(632.8)
\]

(2)

with \( I \), quantum flux of the incident light (number of photons), \( \Omega \), the solid angle of detection (sr\(^{-1}\)), \( \phi \), the apparent spectral fluorescence yield (nm\(^{-1}\)), \( T_{\text{IF}} \), the interference filter transmission, \( B_{\text{IF}} \), the interference filter bandpass (nm) (the product \( T_{\text{IF}}(758) B_{\text{IF}}(754-762) \) was calculated as the integral of the filter transmission between 754 and 762 nm), \( D \), the photodiode sensitivity (\( V \) number of photons\(^{-1}\)), \( R(632.8) \), the leaf reflectance at 632.8 nm. The first term of the equation corresponds to the emitted fluorescence. The second term corresponds to the signal reflected by the leaf and unwillingly transmitted by the interference filter. In this term, the filter bandpass is not required as the source is a laser, with bandpass smaller than 1 nm.

The incident light was assessed by replacing the leaf by a white Lambertian reference (Spectralon\textsuperscript{TM}), with reflectance close to 100%. The reflected signal had to be attenuated by a neutral density filter, whose transmittance at 632.8 nm is \( T_{\text{NF}}(632.8) \). The signal \( S_2 \) was obtained:

\[
S_2 = I \Omega T_{\text{NF}}(632.8) D(632.8)
\]

(3)

The second term of the signal \( S_1 \) has to be determined to obtain the ASFY, \( \phi \). The reflectance of the leaf at 632.8 nm (\( R(632.8) \)) and the transmittance of the interference filter at 632.8 nm (\( T_{\text{IF}}(632.8) \)) were assessed using the following two configurations:

(a) with the leaf and the neutral density filter, giving \( S_3 \):

\[
S_3 = I \Omega R(632.8) D(632.8) T_{\text{IF}}(632.8)
\]

(4)

(b) with the Lambertian reference and the interference filter, giving \( S_4 \):

\[
S_4 = I \Omega T_{\text{IF}}(632.8) D(632.8)
\]

(5)

The combination of Eqs. (2)–(5) gave the ASFY:

\[
\phi = \left( \frac{S_1 - S_3}{S_2} \right) \frac{T_{\text{NF}}(632.8) D(632.8)}{T_{\text{IF}}(758) B_{\text{IF}}(754-762) D(758)}
\]

(6)

The contamination was very low under this configuration, about 1%. Measurements of the ASFY for the wavelength pair (632.8 and 758) and of a spectrum containing this wavelength pair, on the same leaf, enabled us to calibrate the intensity of all the spectra, that therefore are expressed in nm\(^{-1}\).

### 3. Results and discussion

#### 3.1. Fluorescence emission spectra

Figs. 3A and B show fluorescence emission spectra of a bean leaf for two excitation wavelengths, 440 and 535 nm. They are all expressed in ASFY units (nm\(^{-1}\)), and were measured for the four configurations previously described (Fig. 1). The spectra presented in Fig. 3A were measured for front-side geometry, the spectra in Fig. 3B for back-side geometry. The integral of these spectra corresponds to the apparent fluorescence yield (AFY), for a given excitation wavelength. AFYs were calculated for front-side and
back-side configurations and summed up. This yielded AFYs of 0.3% when the adaxial side was illuminated, and 0.9% when the abaxial side was illuminated, for an excitation at 440 nm.

The spectra presented in Figs. 3A and B enabled us to study the influence of the configuration on the global intensity of the fluorescence and on the RF/FRF ratio, as well as the influence of the excitation wavelength on the RF/FRF ratio. The ASFY was much larger for front-side measurements compared to back-side measurements, as absorption and diffusion effects are stronger in the second case. It was also larger when the abaxial side was illuminated, compared to the adaxial side. Beans are dicots so their leaves are dorso-ventral asymmetric. The fluorescence intensity is defined by the absorption profile of the incident radiation and by the propagation properties of the fluorescence fluxes that both depend partly on tissues scattering. The diffusion of light inside a leaf depends on the number of intercellular spaces [12] and also on the number and distribution of chloroplasts in cells [13,14]. The abaxial part of the mesophyll (spongy tissues), which contains more intercellular spaces than the adaxial part (palisade tissues), is more diffusive. Consequently, the path of light is lengthened by multiple reflection, and the apparent absorption of chlorophyll is higher [15]. Until recently, there was no straightforward way to measure absorption profiles within leaves. However, several approaches have been developed nowadays to predict them. Fibre optic microsensors were used to measure light gradient within leaves, which were shown to be relatively steep and wavelength dependent [16]. The depth of penetration of light was larger for radiations weakly absorbed by chlorophylls. An experimental–theoretical procedure based on internal measurements using microprobes and on the four-flux approximation of the radiative transfer theory was proposed by Richter and Fukshansky [17–19] to calculate gradients of light absorption, as affected by leaf optics and morphology. More recently, an experimental technique was described in which profiles of chlorophyll fluorescence within leaves were directly measured [20–22]. These profiles were bell-shaped and it was shown for spinach leaves, that when the abaxial side was illuminated, the fluorescence profiles within the leaves were narrower and closer to the irradiated surface compared to the adaxial side, due to the diffusive nature of the spongy tissues [21]. This may explain the increase of the fluorescence intensity when the abaxial side is illuminated.

The RF/FRF ratio decreases when the chlorophyll content increases, due to the re-absorption of red fluorescence [3] but it also depends on the measurement configuration.

**Fig. 3.** Leaf fluorescence emission spectra and RF/FRF ratio. Adaxial or abaxial indicates the illuminated side. (A) Fluorescence emission spectra measured with a front-side configuration. (B) Fluorescence emission spectra measured with a back-side configuration. (C,D) Variations of the RF/FRF ratio with the wavelength. The curves were obtained by dividing the excitation spectrum for an emission at 687 nm by that for an emission at 760 nm. (C) Front-side configuration. (D) Back-side configuration.
The curves presented in Figs. 3C and D were obtained by dividing the excitation spectrum for an emission at 687 nm by that for an emission at 760 nm. They show the variations of the RF/FRF ratio with the excitation wavelength for different geometries. We observed that it was higher when the abaxial side was illuminated. This ratio was smaller for an excitation wavelength in the green spectral range compared to excitations in the blue and red spectral ranges in the case of front-side geometry, and slightly higher compared to excitations in the blue and red spectral range for back-side geometry. The RF/FRF ratio depends on the pathlength followed by fluorescence inside the leaf. When the abaxial side is irradiated, the fluorescence profile is narrower and closer to the surface. Thus, the re-absorption of red fluorescence is weaker with this configuration and the RF/FRF ratio increases. The green incident light, weakly absorbed by chlorophyll, penetrates further inside the leaf than blue and red radiations (Fig. 4) [16]. Thus, the proportion of fluorescence emitted from inside the leaf is greater than for these two excitations. As a consequence, in the case of front-side geometry, the re-absorption of RF is stronger for a green excitation, and the RF/FRF ratio is smaller. These results are in accordance to the results obtained by Agati [23] who showed a decrease of the RF/FRF ratio between 440 and 635 nm. After 650 nm, this ratio increases, due to the second absorption peak of Chls, in accordance to [16]. In the case of back-side geometry, the re-absorption of RF is stronger for blue and red excitations and the RF/FRF ratio is smaller than for green excitation.

3.2. Fluorescence excitation spectra

Fluorescence excitation spectra were measured from 300 to 800 nm (Fig. 5), for two emission wavelengths, 687 and 760 nm, and for the four configurations previously described (all presented spectra are available as additional data, in an excel file). The in vivo leaf absorption, and

![Fluorescence excitation spectra](image)
therefore excitation spectrum, extending much further than the first emission peak (685 nm), we measured fluorescence excitation beyond this emission wavelength. For a fluorescence emission at 687 nm, the apparent energy uphill is possible thanks to a transfer of thermal energy between the environment and the fluorescing molecules. The ASFY and the shape of the spectra depend on absorption and diffusion effects. Chlorophyll molar absorptivity coefficient is weak in the green spectral area. However, leaf absorption is high in the green. This is due to the high chlorophyll content of leaves, and also to a larger effect of multiple scattering in spectral area with weak absorption [24]. We also observed that the ASFY was much higher when the abaxial side was illuminated, for front-side or back-side geometry, and was weaker for back-side measurements compared to front-side measurements.

At 687 nm (Figs. 5A and B), the shape of the spectrum was very different for front-side and back-side measurements, due to absorption effects. The fluorescence intensity in the 400–500 nm spectral range was weaker compared to the 500–750 nm spectral range when measured with back-side geometry. This is explained again by the pathlength of the fluorescence inside the leaf as blue radiation is absorbed close to the illuminated surface (Fig. 4). The fluorescence intensity was weaker when the adaxial side was illuminated, at any excitation wavelength, as already seen with fluorescence emission spectra. In the UV region, this was due to an absorption by polyphenols located predominantly in the adaxial epidermis [25].

Figs. 5C and D show the fluorescence emitted at 760 nm for each measurement configuration. For both detection configurations, the shape of the spectra corresponding to a given illuminated side remained about the same. This indicated that at 760 nm, diffusion effects are predominant.

In all excitation spectra, two peaks were present in the blue spectral range, at 439 and 475 nm, that may correspond to the peaks of chlorophyll a and chlorophyll b, respectively. The disappearance of the 475-nm peak in leaves lacking chlorophyll b [26] argue in favour of this hypothesis. But, the valley between the two peaks can also be due to absorption by carotenoids (lutein, neoxanthin and violaxanthin) present in the LHCII. Indeed, carotenoids have two main functions. They increase the cross section of absorption in the blue-green spectral range and they are essential to photoprotection. They quench Chl triplet states very efficiently [27], thus preventing the formation of singlet oxygen, and violaxanthin and zeaxanthin are involved in non-photochemical quenching mechanisms [28], that protect photosystems from excess light. Recent studies showed that the overall transfer efficiency from carotenoids to Chls in the LHClII was about 80% [29,30]. These molecules have a major absorption maximum between 450 and 470 nm and an other one between 480 and 500 nm [31]. Due to their antagonist functions, absorption by carotenoids may either create valleys or additional peaks in fluorescence excitation spectra. For an average Chl a/Chl b ratio of 3, in the case of the first hypothesis, the intensity of the 439-nm peak should be higher than the 475-nm peak. But the second peak may be increased by carotenoids absorption, the latter corroborated by the appearance of an additional peak at 486 nm.

The comparison of fluorescence excitation spectra measured with front-side configuration, from the adaxial or the abaxial side of the leaf highlights the two previously defined spectral ranges, 400–500 nm and 500–750 nm. These spectra were drawn on different axis scale in order to compare their shape (Figs. 6A and B), which is equivalent to normalize them. They were superimposed in the green and red, where they have the same shape. For the measurements from the adaxial side, the relative efficiency in the 400–500-nm range was lower compared to the 500–700-nm range, and the additional peak at 486 nm was less pronounced on this side. This could be the consequence of higher carotenoids content on the adaxial side that decreases Chl a excitation. There may be either a greater pool of carotenoids on the adaxial side, or a greater conversion of violaxanthin to zeaxanthin, that dissipates more energy thermally, compared to other carotenoids [32]. Indeed, a greater pool size of violaxanthin and zeaxanthin in sun vs. shade leaves has been reported consistently for a number of plant species [33]. It is also known

![Fig. 6. Comparison of fluorescence excitation spectra of a bean leaf, measured from the adaxial or from the abaxial side, with a front-side configuration. The spectra are drawn on different axis scale. They were superimposed in the green and red spectral ranges. (A) Fluorescence emitted at 687 nm. (B) Fluorescence emitted at 760 nm.](image)
that sun and shade leaves have similar properties like the adaxial and the abaxial side of a sun leaf [34]. The ratio of the 439- to the 475-peak was higher for the spectra measured from the adaxial side, compared to abaxial side. This may be again due to higher carotenoids content transferring energy only partly to Chls on the adaxial side, or can be due to higher Chl b content on the abaxial side, as the Chl a/Chl b ratio is higher for sun leaves than for shade leaves [33].

4. Conclusion

This work presents for the first time full range fluorescence excitation and emission spectra of intact leaves, from both the adaxial and the abaxial side of the leaves, for front-side and back-side geometry, and expressed in units of apparent spectral fluorescence yield. This enables a quantitative work on fluorescence fluxes calculation and modelling, or the interpretation of these complex spectra influenced by pigment contents and leaf anatomy. The measurement configurations, and the different excitation and emission wavelengths used, highlight the relative influence of absorption and scattering on the spectra, and particularly on the RF/FRF emission ratio. Moreover, fluorescence excitation spectra are useful to show the presence of other pigments than chlorophyll, such as polyphenols and carotenoids. Further development of this work could include measures of directional and hemispherical fluorescence yields.

5. Abbreviations

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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ASFY</td>
<td>apparent spectral fluorescence yield</td>
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<tr>
<td>Chl</td>
<td>chlorophyll</td>
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<td>FRF</td>
<td>far-red fluorescence</td>
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<td>F</td>
<td>fluorescence</td>
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<td>LHCII</td>
<td>light harvesting complexes of photosystem II</td>
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<td>RF</td>
<td>red fluorescence</td>
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotobiol.2006.03.009.

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