Short paper

Detection of photosynthetic activity and water stress by imaging the red chlorophyll fluorescence

Hartmut K. Lichtenthaler*, Fatbardha Babani

*a Botanisches Institut (Pflanzenphysiologie und Pflanzenbiochemie), University of Karlsruhe, Kaiserstr. 12, 76128 Karlsruhe, Germany

*b Institute of Biological Research, Academy of Sciences, Tirana, Albania

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Abstract – It is shown that with a new flash-lamp chlorophyll (Chl) fluorescence imaging system (FL-FIS), the photosynthetic activity of several thousand points of an intact attached leaf can be screened in a non-destructive way within a few seconds. The method allows the detection of the gradients in photosynthetic capacity over different parts of the leaf. The photosynthetic activity is sensed via imaging the Chl fluorescence at its maximum Fm and at steady state Chl fluorescence Fs of the induction kinetics and by the subsequent determination of the image of the fluorescence decrease ratio (RFd) which is known as the vitality index of the photosynthetic apparatus. Under water stress the photosynthetic activity decreases as seen in the images of the two fluorescence ratios RFd and Fm/Fs. Histogram and profile analysis of Chl fluorescence images and Chl fluorescence ratios allow the quantification of the differences between normal and stressed leaves with a high statistical significance. © 2000 Éditions scientifiques et médicales Elsevier SAS

chlorophyll fluorescence / flash light pulses / fluorescence decrease ratio / fluorescence imaging / Phaseolus vulgaris / photosynthetic activity / vitality index

Chl, chlorophyll / FL-FIS, flash-lamp fluorescence imaging system / Fd, fluorescence decrease from Fm to Fs / Fm, maximum chlorophyll fluorescence / Fs, steady state Chl fluorescence / Fm/Fs, ratio of maximum to steady state Chl fluorescence / PPFD, photosynthetic photon flux density / RFd, variable chlorophyll fluorescence decrease ratio

1. INTRODUCTION

Plants are exposed to a multitude of natural biotic and abiotic stressors [11]. Almost all stressors affect either directly or indirectly the photosynthetic performance of leaves and modify their optical and fluorescence properties. Early stress detection in plants, before visual damage symptoms are detectable, is required in order to reactivate the plant’s vitality by suitable countermeasures. In the last 20 years, chlorophyll fluorescence signatures of plants have been applied as an efficient tool to describe and investigate the photosynthetic light processes and quantum conversion at physiological conditions as well as to detect stress and senescence in the photosynthetic apparatus [3, 5, 7, 12, 13, 16]. The red Chl fluorescence provides ample information on the photosynthetic apparatus as first discovered by Kautsky [5, 6, 10]. The Chl fluorescence induction kinetics of pre-darkened leaves (known as the Kautsky effect) exhibits a fast fluorescence rise to a maximum (Fm) within ca. 200 ms and then a slow decline, parallel to the onset of photosynthesis, within 5 min to the much lower steady state fluorescence value Fs. The ratio of this fluorescence decrease Fd to the steady state fluorescence Fs (ratio Fd/Fs), also known as variable Chl fluorescence ratio RFd, has been established as an indicator of the potential photosynthetic capacity of leaves [1, 12, 13, 17].

So far only Chl fluorescence signatures of single leaf spots have been measured (e.g. [1, 3, 7, 13, 16]). The disadvantage of such punctuated Chl fluorescence measurements is the fact that these provide only
limited information on the state of health of plants and their photosynthetic apparatus, as a single leaf spot is often not representative of the whole leaf [8, 14]. Over the last 6 years, high resolution multi-colour fluorescence imaging techniques for whole leaves have been developed. These techniques offer the new possibility to study the distribution and irregularities of Chl fluorescence signatures over the whole leaf area [2, 4, 8]. The blue and green fluorescence signatures of the cell walls of leaves have been included as internal fluorescence standard [2, 8, 12, 14, 15]. Imaging of the red Chl fluorescence (F690) during the induction kinetics with the resulting R_fD-value images should provide quick information on the active photosynthetic performance of all leaf parts, and also allow the study of the successive loss of photosynthetic activity of leaves under stress conditions.

Our previous fluorescence imaging results were obtained using an expensive laser-equipped fluorescence imaging system (Laser-FIS) [8, 14]. For routine analysis, we recently constructed a new, compact and much cheaper flash-lamp induced fluorescence imaging system (FL-FIS). Using this Karlsruhe FL-FIS, we wanted to test if the photosynthetic activity of all parts of a green bean leaf could be screened by means of Chl fluorescence imaging and if the decline in photosynthetic activity during water stress affected all leaf parts in the same way.

2. RESULTS AND DISCUSSION

2.1. Pigment content and pigment ratios

The fluorescence imaging was performed with fully developed green bean leaves. One leaf was submitted to water stress for 2 h by detaching the leaf from the plant. Other leaves came from a bean plant that had not been watered for 10 d. The chlorophyll and carotenoid contents of both leaves are shown in Table I. The pigment content of both leaves, which came from different plants, were not identical but were in the same range typical of fully grown leaves of bean plants. The pigment ratios also exhibited the values typically found in green leaf tissue [1, 13]. The Chl and carotenoid contents were relatively evenly distributed over the whole leaf area, as was shown by multiple pigment determinations of different leaf parts which exhibited a low pigment variation of < 3 %. The 2-h water stress of the detached leaf caused a 31 % loss in the relative water content, a breakdown of chlorophylls or carotenoids, however, did not occur. In addition, in the water-stressed bean plants, no decline in chlorophyll or carotenoid levels were detected. Due to a certain shrinkage of the leaf under water stress, the pigment level measured on a leaf area basis was slightly increased by about 5 %.

2.2. Chlorophyll fluorescence images

The photosynthetic activity of green, 20-min pre-darkened bean leaves was studied by imaging the red Chl fluorescence induction kinetics (F690) of the upper adaxial leaf side during the Chl fluorescence induction kinetics (Kautsky effect). By applying the newly developed Karlsruhe flash-lamp fluorescence imaging system (FL-FIS; cf. figure 1), the red Chl fluorescence F690, as excited here by blue light, was imaged at maximum fluorescence Fm (between 0.2 to 1 s after onset of illumination) and at steady state Chl fluorescence Fs after 5 min of white light exposure. At Fm, the Chl fluorescence intensity was more or less evenly distributed over the whole leaf area except for the leaf rim, the middle vein and the petiole where the F690 intensity was significantly lower (figure 2A). The lower F690 yield of the leaf petiole is due to a much lower Chl content of the petiole as compared to the green leaf tissue. With the onset of photosynthetic quantum conversion, the Chl fluorescence continuously declined within 5 min to the steady fluorescence Fs as seen by a change of the red false colour (highest Chl fluorescence intensity) to a light to dark blue false colour (low Chl fluorescence intensity). At Fs, the 690 intensity was not evenly distributed over the leaf area, as indicated by the light blue and dark blue leaf regions (figure 2B).

From the Chl fluorescence images at Fm and Fs, the computer can process the image of the Chl fluorescence ratio, known as R_fD-values. The latter are defined as the ratio of Chl fluorescence decrease Fd

<table>
<thead>
<tr>
<th>Leaf-type</th>
<th>Pigment content (mg·m⁻² leaf area)</th>
<th>Pigment ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a+b</td>
<td>x+c</td>
</tr>
<tr>
<td>Green bean leaf</td>
<td>271</td>
<td>41.3</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>295</td>
<td>46.5</td>
</tr>
</tbody>
</table>

Table I. Chlorophyll (a+b) and total carotenoid content (x+c) as well as pigment ratios Chl a/b and chlorophylls/carotenoids (a+b)/(x+c) in a fully functional green bean leaf and in a leaf of a water-stressed bean plant. Mean of six determinations from two leaves with a standard deviation of maximal ± 3 % (pigment levels) and less than ± 1 % (pigment ratios). The small differences in the pigment levels of the two leaves are not due to the water stress, as they are in the range of variation between different bean plants.
Figure 1. Set-up of the Karlsruhe flash-lamp fluorescence imaging system (FL-FIS) to measure the blue, green, red and far-red fluorescence signatures of whole leaves. The red chlorophyll fluorescence (F690) is excited by a pulsed Xenon lamp using a blue transmission filter. The emitted red Chl fluorescence band near 690 nm, shown here for a single leaf pixel, is simultaneously collected from several thousand pixels of the leaf using a CCD camera and stored by the image processing system.

Figure 2. Images of the red chlorophyll fluorescence of a bean leaf at maximum fluorescence Fm (A) and at steady state fluorescence Fs (B) of the Chl fluorescence induction kinetics (Kautsky effect). C, Image of the Chl fluorescence decrease ratio (RFd = Fd/Fs); D, Chl fluorescence ratio Fm/Fs, both of which are indicators of the photosynthetic quantum conversion. The scale in A and B indicates the intensity of the Chl fluorescence. In C and D, it indicates the values of the two fluorescence ratios. The black lines show the leaf area where profile measurements were performed (cf. figure 4).
from Fm to Fs over the steady state fluorescence Fs ($R_{Fd} = Fd/Fs$). $R_{Fd}$-values are direct indicators of the photosynthetic activity of leaves as has been shown before by parallel photosynthetic CO$_2$ measurement [1, 17]. The $R_{Fd}$-image of the bean leaf is also given in false colour, whereby red means a high $R_{Fd}$-value of 4 and the green colour values of about 2. The photosynthetic activity was very high (red colour) only at some leaf parts, and it was fairly low (mainly green colour) in others, such as at the leaf tip (figure 2C). It is of interest in this respect that the leaf petiole also exhibited good $R_{Fd}$-values with red to green false colours indicating that, despite the lower Chl content, the Chl present is functionally organized and is involved in photosynthetic quantum conversion and CO$_2$-fixation.

Instead of the $R_{Fd}$-values, one can also form the variable Chl fluorescence Fm/Fs, which is a similar ratio as the $R_{Fd}$-values, and possesses somewhat higher values. Also, the Fm/Fs image indicated that the intensity of photosynthetic quantum conversion of the bean leaf was not evenly distributed over the whole leaf area (figure 2D).

### 2.3. Histograms and profiles

The Chl fluorescence imaging technique allows the screening of histograms of the frequency of the Chl fluorescence distribution of Fm and Fs for all leaf pixels measured (figure 3A). This is also possible for the values of the fluorescence ratios $R_{Fd}$ and Fm/Fs (figure 3B). Such histograms also show the differences in absolute values between both Chl fluorescence ratios, $R_{Fd}$ and Fm/Fs.

Another option of the Chl fluorescence imaging technique is the formation of profiles of the Chl fluorescence parameters, such as the fluorescence intensity of Fm or values of the fluorescence ratios Fm/Fs of $R_{Fd}$ over a vertical or horizontal leaf section (figure 4A, B). These profiles are an excellent means of detecting differences and gradients in Chl fluorescence parameters and photosynthetic activity within the leaf area.

### 2.4. Water stress detection

By Chl fluorescence imaging, it is also possible to screen the decline in photosynthetic activity under stress conditions. Thus, in a bean leaf detached for 2 h, water stress effects were detected through a large...
decrease in the values of the Chl fluorescence ratios $R_{F_{d}}$ and $F_{m}/F_{s}$ (compare figure 5A with figure 3B). This was due to the fact that the total Chl fluorescence increased and the decrease from $F_{m}$ to $F_{s}$ was very low (figure 5B) as compared to the control leaf (figure 5A). In fact, the Chl fluorescence $F_{s}$ was almost twice as high in the water-stressed leaf compared to the control. A similar decrease in values of $R_{F_{d}}$ and $F_{m}/F_{s}$ was obtained in leaves from a bean plant that had not been watered for 10 d.

2.5. Measurements of the Kautsky effect

We also followed the changes in $R_{F_{d}}$-values under water stress by performing kinetic measurements during the Chl fluorescence induction kinetics. For this purpose, Chl fluorescence images were taken at $F_{m}$ and after 1, 5 and 8 min after onset of illumination of the bean leaf which had been pre-darkened for 20 min. During the induction phase, the leaf of the control plant developed to normal $R_{F_{d}}$-values of ca. 2.6, whereas those of the water-stressed bean plant remained very low near 0.8 (figure 6A). In addition, the much lower decline in the Chl fluorescence $F$ from $F_{m}$ to the steady state $F_{s}$ in the leaf of the water-stressed bean plant, expressed here as a standardized ratio $F/F_{m}$, demonstrated that the photosynthetic quantum conversion was disturbed under water stress (figure 6B).

3. CONCLUSION

The newly constructed compact Karlsruhe fluorescence imaging system, FL-FIS, allows quick screening of the red Chl fluorescence (F690) emission over the whole leaf area and the detection of differences and gradients in photosynthetic quantum conversion. Kinetic Chl fluorescence measurements are possible, since the screening process is completed within a few seconds. Thus, the FL-FIS technique allows the taking of Chl fluorescence images during the light induced Chl fluorescence induction kinetics, known as the Kautsky effect [5, 6, 10]. From the images taken at maximum...
and at steady state Chl fluorescence, \( Fm \) and \( Fs \), one can process the images of the fluorescence ratios, such as \( Fm/Fs \) and \( R_{Fd} \)-values, which are known to reflect the photosynthetic quantum conversion and CO\(_2\) fixation capacity of leaves [1, 13, 17]. The ratio images, expressed in false colours, allow a) the quick detection of gradients in photosynthetic quantum conversion between different parts of the leaf area and b) the detection of a decline in photosynthetic activity under stress conditions, such as with water stress as demonstrated here. Thus, the FL-FIS allows a very early stress detection, which leaves time to overcome stress conditions by taking suitable countermeasures. In addition, this investigation showed that fluorescence imaging does not require a laser, but instead a much cheaper Xenon flash-lamp, with appropriate filters to select the region of the excitation light, yields the same results.

4. METHODS

4.1. Plant growth

Bean leaves (Phaseolus vulgaris L. var. nanus ‘Fori’) were grown in a greenhouse on a mineral peat (TKSII) at 23 \(^\circ\)C, at an average relative humidity of 60 %, and a maximal PPFD of 300 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \). Leaves from 4-week-old plants were used for Chl fluorescence imaging. The leaves of the well watered bean plants had an average water content of 86.8 \( \pm \) 2 % of fresh weight. They were water saturated and did not take up water when floating overnight on a water surface. The leaves of bean plants, grown under the same conditions but not watered for 10 d, had a significant lower water level of 69 % of fresh weight.

4.2. Determination of pigments

The level of the chlorophylls (\( a+b \)) and total carotenoids (\( x+c \)) of bean leaves was determined in the same acetone extract solution using the re-evaluated extinction coefficients of Lichtenthaler [9]. The chlorophyll and carotenoid levels within one leaf blade varied by less than 3 % and between fully grown leaves of different position on the plant by less than 8 %.

4.3. Fluorescence imaging system (FL-FIS)

The new compact flash-lamp fluorescence imaging system (FL-FIS) (figure 1) is based on the Karlsruhe/Strasbourg laser induced fluorescence imaging system (Laser-FIS) for plants [8, 12, 15]. Instead of an expensive Nd-YAG laser, a Xenon flash-lamp is used as an excitation source (FX 300, Cermax, ILC Technology Sunnyvale) with a pulse energy of 0.7 J, flash duration 20 \( \mu \)s operated at 16.6 Hz. For the simultaneous excitation of the blue, green, red and far-red leaf fluorescence, a broad UV-A transmission filter (DUG 11, Schott, Germany, range 280 to 400 nm, \( \lambda_{\text{max}} = 340 \) nm) is applied. To exclusively excite the red Chl fluorescence F690, as in this investigation, a blue filter was applied (Corning No. 9782, range 370–600 nm, \( \lambda_{\text{max}} = 465 \) nm). Blue light excitation provides a higher Chl fluorescence yield than UV-A excitation, because it penetrates deeper into the green leaf mesophyll and excites more Chl molecules than UV-A excitation which is partially absorbed by the flavonols in the leaf epidermis. Chl fluorescence detection was performed using a gated intensified CCD-digital camera with an array of 565 \( \times \) 754 elements (Photonetics, Kehl, Germany). The image intensifier tube has an adjustable gain with a factor of 3 000 and is gated synchronously with the flash lamp (gating time 75–100 \( \mu \)s). The images in the four fluorescence bands (440, 520, 690 and 740 nm) are sensed by using appropriate interference filters (Oriel, France; 10-nm half band width) that are built into a filter wheel in front of the CCD-camera. In this investigation, only the 690-nm Chl fluorescence band was measured which shows a higher amplitude during the induction kinetics than the 740-nm far-red Chl fluorescence band. Image correction was carried out taking into account non-uniform excitation of the leaf sample. The software ‘Camille 1.05’ (Photonetics, Kehl, Germany) allows a centralized control of all FL-FIS components via the PC. Via computer-aided data-processing, one obtains false colour images of the measured fluorescence intensity and the Chl fluorescence ratios (\( R_{Fd} \)-values and \( Fm/Fs \)) by a pixel to pixel division procedure, whereby blue is the lowest and red the highest fluorescence intensity and the lowest and highest values of the two fluorescence ratios \( R_{Fd} \) and \( Fm/Fs \).

4.4. Imaging procedures

Leaves of normal and water-stressed bean plants which had been pre-darkened for 20 min, were illuminated with white light (PPFD 1 000 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)). The red Chl fluorescence images (F690) were sensed at the upper adaxial leaf side at 0.2–1 s, 1 and 5 min after onset of illumination. One hundred image accumulations were chosen as a suitable number of successive readout images that took – including accumulation and subtraction of the background images – a
few seconds. The histograms of the frequency distribution of the Chl fluorescence intensity and the fluorescence ratios over the whole leaf area are based on all leaf pixels (i.e. 200,000 pixels). The profiles were obtained from a selected area line across the leaf area (black line in figure 2) and are based on ca. 5,000 pixels.

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