PHOTOSYNTHETICA 43 (3): 355-369, 2005

Chlorophyll fluorescence imaging of photosynthetic activity with the flash-lamp fluorescence imaging system

H.K. LICHTENTHALER*, G. LANGSDORF, S. LENK, and C. BUSCHMANN

Botanical Institute (Molecular Biology and Biochemistry of Plants), University of Karlsruhe, Kaiserstrasse 12, D-76128 Karlsruhe, Germany

Abstract

A flash-lamp chlorophyll (Chl) fluorescence imaging system (FL-FIS) is described that allows to screen and image the photosynthetic activity of several thousand leaf points (pixels) of intact leaves in a non-destructive way within a few seconds. This includes also the registration of several thousand leaf point images of the four natural fluorescence bands of plants in the blue (440 nm) and green (520 nm) regions as well as the red (near 690 nm) and far-red (near 740 nm) Chl fluorescence. The latest components of this Karlsruhe FL-FIS are presented as well as its advantage as compared to the classical single leaf point measurements where only the fluorescence information of one leaf point is sensed per each measurement. Moreover, using the conventional He-Ne-laser induced two-wavelengths Chl fluorometer *LITWaF*, we demonstrated that the photosynthetic activity of leaves can be determined measuring the Chl fluorescence decrease ratio, R_{Fd} (defined as Chl fluorescence decrease F_d from maximum to steady state fluorescence F_s: F_d/F_s), that is determined by the Chl fluorescence induction kinetics (Kautsky effect). The height of the values of the Chl fluorescence decrease ratio R_{Fd} is linearly correlated to the net photosynthetic CO_2 fixation rate P_N as is indicated here for sun and shade leaves of various trees that considerably differ in their P_N. Imaging the R_{Fd}-ratio of intact leaves permitted the detection of considerable gradients in photosynthetic capacity across the leaf area as well as the spatial heterogeneity and patchiness of photosynthetic quantum conversion within the control leaf and the stressed plants. The higher photosynthetic capacity of sun *versus* shade leaves was screened by Chl fluorescence imaging. Profile analysis of fluoresence signals (along a line across the leaf area) and histograms (the signal frequency distribution of the fluorescence information of all measured leaf pixels) of Chl fluorescence yield and Chl fluorescence ratios allow, with a high statistical significance, the quantification of the differences in photosynthetic activity between various areas of the leaf as well as between control leaves and water stressed leaves. The progressive uptake and transfer of the herbicide diuron *via* the petiole into the leaf of an intact plant and the concomitant loss of photosynthetic quantum conversion was followed with high precision by imaging the increase of the red Chl fluorescence F_{690}. Differences in the availability and absorption of soil nitrogen of crop plants can be documented *via* this flash-lamp fluorescence imaging technique by imaging the blue/red ratio image F_{440}/F_{690}, whereas differences in Chl content are detected by collecting images of the fluorescence ratio red/far-red, F_{690}/F_{740}.

Additional key words: blue-green fluorescence; chlorophyll fluorescence decrease ratio; diuron absorption; flash light pulses; nitrogen supply; R_{Fd}-ratio; sun and shade leaves, water stress.

Introduction

Plants are exposed to high or low irradiance and to a variety of biotic and abiotic stressors affecting photosynthesis, growth, various physiological functions, and crop yield (Lichtenthaler 1996). Well known in nature are the

Received 25 November 2004, *accepted* 26 April 2005.
*Author for correspondence; fax +49 721 6084874, e-mail: hartmut.lichtenthaler@bio.uka.de
Abbreviations: Chl – chlorophyll; F_d – fluorescence decrease from F_p to F_s; F_m – maximum Chl fluorescence at a saturating light pulse; F_s – steady state Chl fluorescence; F_d/F_s – ratio of Chl fluorescence decrease F_d to steady state Chl fluorescence F_s; FIS – fluorescence imaging system; FL – flash-lamp; P_N – net photosynthetic rate; PAR – photosynthetically active radiation; PPFD – photosynthetic photon flux density; PS2 – photosystem 2; R_{Fd} – variable Chl fluorescence decrease ratio.
Acknowledgements: This research was sponsored in part by EU grants within the INTERREG II research project, and the Inco-Copernicus project QAAFFI, as well as the programs CLOSYS and STRESSIMAGING which are gratefully acknowledged. We thank Mrs. Gabrielle Johnson, Rastatt, Germany for English language assistance and Mr. Martin Knapp for support during the preparation of the manuscript.

irradiance adaptation responses of chloroplasts and leaves, *i.e*. the formation of sun and shade leaves as well as sun and shade chloroplasts (Boardman 1977, Lichtenthaler *et al.* 1981, 1982, Meier and Lichtenthaler 1981). Sun leaves with sun chloroplasts possess a much higher photosynthetic capacity on a leaf area and a chlorophyll (Chl) content basis, higher values for the ratio Chl *a/b*, higher saturation irradiance of photosynthetic CO_2 fixation, a much lower content of light-harvesting Chl proteins LHC2 as well as a larger number of exposed, unappressed thylakoid membranes compared to shade leaves with low-irradiance chloroplasts (Lichtenthaler *et al.* 1981, Lichtenthaler and Burkart 1999). All environmental factors, such as excessive irradiance, heat, or water stress either directly or indirectly affect the photosynthetic function of leaves, the pigment composition, and the ultra-structure of the photosynthetic apparatus. In addition, they often modify the optical and fluorescence properties of leaves, as can be seen in the reflectance and fluorescence emission spectra. Early stress detection in plants, before visual damage symptoms are noticeable, is required in order to reactivate the plant's vitality by suitable countermeasures. Today the non-destructive fluorescence methods, in the future these also in combination with reflectance signals, are the most promising techniques for early stress detection in plants.

With respect to fast screening of the photosynthetic activity, the Chl fluorescence signatures of leaves have been applied in the last 25 years as an efficient tool to check the differences of the photosynthetic quantum conversion and to detect stress and senescence in the photosynthetic apparatus. Various papers dealing with these aspects are found in the following reviews: Lichtenthaler and Rinderle (1988), Krause and Weis (1991), Govindjee (1995, 2005), as well as in the books "Applications of Chlorophyll Fluorescence" (Lichtenthaler 1988) and "Chlorophyll *a* Fluorescence" (Papageorgiou and Govindjee 2004). Details on the different Chl fluorescence parameters and ratios, which were collected by single point measurements of leaves, are given by Schreiber *et al.* (1986), Lichtenthaler and Rinderle (1988), Genty *et al.* (1989), Šantrůček *et al.* (1992), Bolhàr-Nordenkampf and Öquist (1993), Šiffel *et al.* (1993), Schindler and Lichtenthaler (1996), Strasser *et al.* (1998), and Lichtenthaler and Burkart (1999).

Kautsky and Hirsch (1931) discovered first that Chl fluorescence provides ample information on the function of the photosynthetic apparatus. Chl fluorescence induction kinetics of pre-darkened leaves (known as Kautsky effect) exhibit a fast rise within *ca*. 200 ms to a maximum (denoted as F_m or F_p, depending on the excitation radiation being saturating or not) and then a slow decline to the much lower steady state fluorescence value F_s. The ratio of this fluorescence decrease F_d (= $F_m - F_s$) to the steady state fluorescence F_s, also known as the variable Chl fluorescence ratio R_{Fd} (ratio F_d/F_s), has been established as an indicator of the potential photosynthetic capacity of leaves (Lichtenthaler and Rinderle 1988, Tuba *et al*. 1994, Babani and Lichtenthaler 1996).

So far most published papers deal with Chl fluorescence signatures measured on single leaf spots (*e.g*. Schreiber *et al.* 1986, Lichtenthaler and Rinderle 1988, Krause and Weis 1991, D'Ambrosio *et al*. 1992, Šiffel *et al.* 1993, Babani and Lichtenthaler 1996, Schindler and Lichtenthaler 1996). This means that per each measurement the Chl fluorescence of only one rather small leaf point (leaf spot) is excited and sensed. However, such single leaf point measurements provide only limited information on the photosynthetic apparatus, since single leaf spots often are not representative of the photosynthetic activity of the whole leaf. For this reason high-resolution multi-colour fluorescence imaging techniques have been developed over the last ten years (Lang *et al*. 1994, Edner *et al*. 1995, Lichtenthaler *et al.* 1996, 1997, Lichtenthaler and Miehé 1997, Buschmann and Lichtenthaler 1998). They allow imaging of the natural blue (F_{440}) and green (F_{520}) fluorescences of leaves as well as the red (F_{690}) and far-red (F_{740}) Chl fluorescences (Fig. 1). The laser version (Laser-FIS) has also been applied in near-field fluorescence imaging (Sowinska *et al.* 1999). These techniques allow to consecutively screen the four fluorescence images of leaves with more than 100 000 pixels per leaf and thus they provide information of high statistical significance. Imaging of the red Chl fluorescence (F_{690}) during the induction kinetics at F_m and F_s, with determination of the R_{Fd} ratio images, provides quick information on the photosynthetic performance of the leaf and its different regions. In order to prove the reliability of the R_{Fd} values as indicators of the net photosynthesis rates (P_N) of intact leaves, one objective of this investigation was to determine and correlate the R_{Fd} values (with a conventional Chl fluorometer) and P_N of the same leaves.

Our early fluorescence imaging results were obtained with an expensive laser-equipped fluorescence imaging

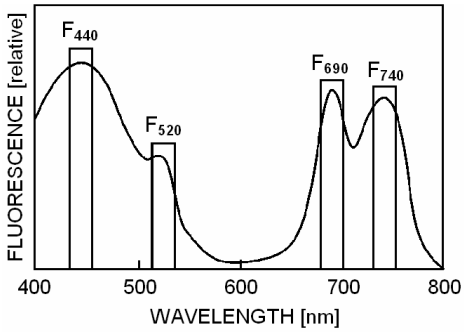

Fig. 1. Fluorescence emission spectrum of a green leaf showing the blue (F_{440}) and green (F_{520}) fluorescence bands (derived from the cell walls) as well as the red (F_{690}) and far-red (F_{740}) Chl fluorescence bands emitted from the chloroplasts in the mesophyll cells (excitation: UV 360 nm, *Perkin Elmer LS50* spectrofluorometer). The four fluorescence bands used for multi-colour fluorescence imaging by means of appropriate transmission filters are indicated.

system (Laser-FIS) (Lang *et al.* 1994, Lichtenthaler *et al.* 1996). The laser was later replaced by a much cheaper xenon flash lamp that gave rise to the flash-lamp induced fluorescence imaging system (FL-FIS) (Buschmann *et al.* 2000, Lichtenthaler and Babani 2000). For routine analysis of leaves, we modified and improved this Karlsruhe FL-FIS and describe here its new components and its application to determine differences in the photosynthetic activity of green leaves and to detect stress effects on the photosynthetic apparatus.

Materials and methods

Plant growth: Fully developed sun and shade leaves of different trees were taken from 30–70-year-old solitary trees at the university campus. In trees with a dense leaf crown, such as beech, the shade leaves at the inner tree crown received on sunny days *ca*. 80 µmol(photon) m^{-2} s^{-1}, whereas the sun leaves were exposed to a maximum photosynthetic photon flux density (PPFD) of 1 700–2 000 µmol(photon) m^{-2} s^{-1}. Bean plants (*Phaseolus vulgaris* L.) were grown in the botanical garden and sugar beet plants (*Beta vulgaris* L.) on an experimental research field in Durlach without supply (0 kg) and with additional nitrogen fertilizer (150 kg per hectare).

Determination of pigments: The content of Chl (*a*+*b*) of leaves was determined in the same 100 % acetone extract solution using the extinction coefficients re-evaluated by Lichtenthaler (1987). The values are expressed in mg(*a*+*b*) m^{-2}(leaf area).

Measurement of Chl fluorescence induction kinetics: The Chl fluorescence induction kinetics (Kautsky effect) of pre-darkened leaves (20 min) were measured at the red fluorescence band F_{690} using the laser-induced two-wavelengths fluorometer *LITWaF* as previously described (Lichtenthaler and Rinderle 1988, Babani and Lichtenthaler 1996). The excitation was performed with a 10 mW He-Ne laser [λ 632.8 nm, irradiance *ca*. 700 µmol(photon) m^{-2} s^{-1} at the leaf sample]. The Chl fluorescence decrease ratio R_{Fd} was determined from the readings at F_m and in the steady state F_s 5 min after the onset of irradiation (cf. Fig. 5 in Results). The obtained R_{Fd}-values were confirmed by parallel measurements with the portable two-wavelengths *BUKA* Chl fluorometer (for this home-made instrument see Langsdorf *et al.* 2000) and by control measurements with the *CCD-OMA* Chl spectrofluorometer [He-Ne laser 10 mW, irradiance at the leaf sample *ca*. 2 500 µmol(photon) m^{-2} s^{-1}] as described in Szabó *et al.* (1992). This charge coupled device–optical multichannel analyzer (*CCD-OMA*), developed by us, not only senses the Chl fluorescence induction kinetics, but in addition also the complete Chl fluorescence emission spectra at each time point of the kinetic.

Porometer P_N measurements were done in pre-darkened (20 min) leaves using a CO_2/H_2O-porometer system (*Walz*, Effeltrich, Germany). The irradiance of the "white light" applied was 1 100 µmol(photon) m^{-2} s^{-1} on the leaves and saturating with respect to the P_N. The latter ranges between *ca*. 300 (shade leaves) and *ca*. 900 (sun leaves) µmol(photon) m^{-2} s^{-1}. Stable maximum P_N rates were reached in sun leaves between 24–30 min after onset of irradiation and in shade leaves between 15 to 20 min.

The Karlsruhe fluorescence imaging system (FL-FIS)

Excitation: A new compact flash-lamp fluorescence imaging system (FL-FIS) (Fig. 2) has been established based on the early Karlsruhe/Strasbourg laser-induced fluorescence imaging system (Laser-FIS) for plants (Lang *et al.* 1994, Lichtenthaler *et al.* 1996, Lichtenthaler and Miehé 1997). In order to replace the expensive Nd-YAG laser, a xenon flash lamp with appropriate filters was successfully applied as excitation source (Buschmann *et al.* 2000, Lichtenthaler and Babani 2000). The latter has now been replaced by a new xenon lamp (*FX-4400, Perkin Elmer Optoelectronics*, Cambridge, UK) allowing measurements with shorter gating times and a higher pulse frequency. The flash duration time has been decreased to less than 20 µs operating at 50 Hz. The 20 µs flash consists of a 2.5 µs main peak, and a 16 µs low afterglow. Although the lamp is capable of an input energy greater than 1.0 J per flash, we have only been applying input energy of 0.5 J in order to guarantee a longer life span of the xenon lamp. The lamp and the camera are well synchronized and allow a 20 µs gating time at the intensifier. The plants' blue (F_{440}) and green (F_{520}) fluorescence as well as the red (F_{690}) and far-red (F_{740}) Chl fluorescence are excited simultaneously using a UV-A transmission filter (*DUG 11*, *Schott*, Mainz, Germany, central wavelength 340 nm, half width 75 nm, maximum spectral transmittance 79 %) in front of the xenon lamp. This filter is applied in stress and strain investigations when images of the four fluorescence bands are necessary. When only information on the photosynthetic performance of leaves is needed, then blue excitation radiation is applied to excite the red and far-red Chl fluorescence, because this yields a much higher Chl fluorescence than UV excitation. Then a blue filter (*BG-12*, *Schott*, Mainz, Germany, central wavelength 407 nm, half width 104 nm, maximum spectral transmittance 59 %) combined with a UV absorbing film (less than 1 % transmission below 390 nm) is applied (Fig. 3) to solely excite the red and far-red Chl fluorescence. The UV film is necessary to protect the blue filter from the UV radiation of the xenon lamp. The combined blue filter and UV-film has a central wavelength of 435 nm, a half width of 57 nm, and 40 % maximum spectral transmittance.

A higher Chl fluorescence yield is obtained by blue radiation excitation than by UV-A excitation. On the one hand this is due to the fact that blue radiation is better absorbed by Chl as well as carotenoids and is transferred to Chl *a*. On the other hand the exciting blue radiation passes unabsorbed through the epidermis cells, penetrates the green leaf mesophyll to a higher extent, and thus excites more Chl molecules than UV-A radiation. The latter, in turn, is partially absorbed by the flavonols and cinnamic acids in the colour-less leaf epidermis (Buschmann and Lichtenthaler 1998, Lichtenthaler and Schweiger 1998). This is the major reason that the Chl absorption bands may not fully overlap with the UV-A-excitation bands playing only a minor role. Thus, when screening the photosynthetic activity *via* Chl fluorescence induction kinetics it is advantageous to apply blue radiation excitation. In contrast, when the screening aims at the detection of early stress and strain events and damage, UV-A excitation is recommended since the fluorescence ratios blue to red (F_{440}/F_{690}) and blue to far-red (F_{440}/F_{740}) are very early stress indicators (Lichtenthaler and Miehé 1997, Buschmann and Lichtenthaler 1998).

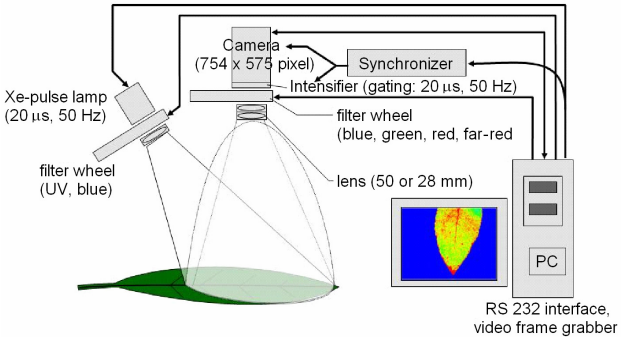

Fig. 2. The flash-lamp fluorescence imaging system (FL-FIS) for acquiring blue (F_{440}), green (F_{520}), red (F_{690}), and far-red (F_{740}) fluorescence images of green leaves. A pulsed xenon lamp with UV-transmission filter is used as an excitation source when images of the four fluorescence bands are collected. A CCD video camera with an intensifier collects, in each fluorescence band, the emitted fluorescence of several hundred thousand pixels per leaf. The images are processed by the image processing system of a PC. If only fluorescence images of the red and far-red Chl fluorescence are needed, a blue filter is applied to the filter wheel in front of the xenon lamp. For the spectral range of both filters see Fig. 3.

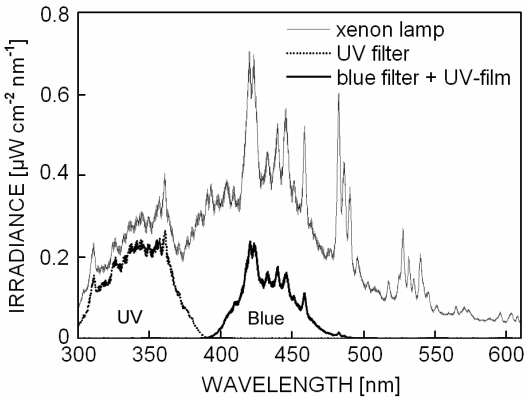

Fig. 3. The spectral irradiance of the xenon lamp without filters, with UV-A transmission filter (UV-filter), and with blue transmission filter + UV absorbing film. The UV-filter is applied for the excitation of all four fluorescence bands of leaves and the blue filter for excitation of the red and far-red Chl fluorescence only.

Detection: The fluorescence signals are detected using a gated intensified video camera with a CCD array of 565×754 elements (CCIR video output, *Optronics*, Kehl, Germany, 8 bit resolution on frame grabber; *Oculus F64*, *Coreo*, Québec, Canada, objective *Nikon* 50 mm). The image intensifier tube (2nd gen, *S25* photocathode, *P43* screen, gatable up to 50 ns, coupling by fibre reducing taper; *Optronics*, Kehl, Germany) is gated synchronously with the flash lamp. The decreased gating time of 20 µs reduces the background effects and increases the image quality. The flashes excite the leaves with a frequency of 50 Hz, but the frame grabber only reads a 'half image' at a time, so the effective image frequency is 25 Hz. 'Half image' means that the frame grabber reads the odd rows from the first image and the even rows from the second image. After the measurements, the background is measured using the same setting except the flashing of the lamp. The measurements are automatically corrected with the background. By applying appropriate interference filters (*Oriel*, France; 10 nm half-band width), installed into a filter wheel in front of the CCD camera, the fluorescence images of leaves can consecutively be sensed in the four fluorescence bands (440, 520, 690, and 740 nm). The changes in F_{690} of green leaves (*e.g.* induced by irradiance adaptation of the photosynthetic apparatus or by environmental stress) possess much higher amplitude during the fluorescence induction kinetics than the far-red Chl fluorescence band F_{740} (range *ca.* 725–750 nm). For this reason it is recommended to measure such changes, in particular R_{Fd}-values, at the F_{690} band. This yields clearer signals and earlier detection of stress events and damage.

The software *Camille 1.05* provided with the camera (*Optronics*, Kehl, Germany) with modifications allows a centralized controlling of all FL-FIS components *via* the PC. It is important to pick up the images in their best quality, therefore some general precautious and measuring adjustments need to be taken into account. In order to obtain a strong signal several hundred images are accumulated with that software. We chose the highest possible gain voltage (600 V) to increase the image intensity and maximise the contrast between the analyzed plant tissue and the background. We applied a uniformity correction to eliminate the effect of the inhomogeneous radiation distribution of the xenon lamp. For the uniformity correction the UV-A excited fluorescence at 440 nm of a white sheet of paper was measured, and the software corrected

the plants' fluorescence measurements by means of this uniformity image. This is the correction algorithm:

$$C^*(x,y) = \frac{C(x,y)}{U(x,y)}\,\mathrm{mean}(U)$$

U(x,y) is the intensity at the coordinates x and y of the uniformity image, C(x,y) is the intensity at the coordinates x and y of the current image, $C^*(x,y)$ is the intensity at the coordinates x and y of the resulting image. Mean(U) is the mean intensity of the uniformity image.

Image pre-processing: The fluorescence images of plants are separated from the background in the following way: First the image with the highest contrast between the plants' fluorescence and the background is selected. Then the histogram, *i.e.* the frequency distribution of fluorescence intensities of all (more than 100 000) leaf pixels of this image, is calculated. The resulting histogram has two peaks. The first maximum at the lower intensities represents the background; the second maximum at the upper intensities represents the plants' fluorescence. As intensity threshold we took the minimum of the frequency distribution between the two peaks. With the latter a 1-bit mask is calculated, in which the pixels with a lower intensity than the threshold value are black (= 0), and the pixels with a higher intensity than the threshold value are white (= 1). This mask is used for all images of the same leaf or plant tissue and with the same position. The resulting masked images, that do no longer contain any background pixels, are obtained by multiplication of the original image with the mask.

Applying computer-aided data-processing, pseudo colour (false colour) images of the measured fluorescence intensity and the Chl fluorescence ratio (R_{Fd}-values) are obtained by a pixel to pixel division process. The images are expressed in pseudo colours, whereby blue equals zero fluorescence and red is the highest fluorescence as indicated on the image scale (blue, green, yellow, red) shown for each figure. The relative height of the fluorescence ratio R_{Fd} is indicated in the same pseudocolours, from blue (zero value) to red (highest value).

Imaging procedures: Green leaves that had been predarkened for 20 min, were irradiated with "white light" (PPFD 1 500 μmol m^{-2} s^{-1}) for 1 s in order to determine the maximum Chl fluorescence level F_m. F_{690} was excited and sensed at the upper (adaxial) leaf side. After imaging the F_m value the leaves were continuously irradiated. Additional F_{690} images were taken 5 min after the onset of irradiation. One hundred image accumulations were chosen as a suitable number of successive readout images to obtain a good signal to noise ratio. After their measurements, the same amount of background images was acquired and subtracted automatically. The whole procedure of accumulation of F_{690} images and subtraction of the background images took 8 s. The Chl fluorescence decline F_d from F_m to the steady-state fluorescence F_s, reached after 5 min, was taken to determine the fluorescence ratio $R_{Fd} = F_d/F_s$ which is equivalent to $(F_m - F_s)/F_s$. The F_m and F_s values were obtained by dividing the sum of the fluorescence counts of all pixels by the total number of the leaf pixels.

After the collection of fluorescence images, the latter can be processed (*a*) to yield a histogram with the frequency distribution of the fluorescence intensity of all leaf pixels, (*b*) to give a profile analysis across a line of the leaf area, and (*c*) to create fluorescence ratio images, such as red/far-red or blue/red or R_{Fd} ratio images.

Results and discussion

Imaging possibilities: The flash-lamp fluorescence imaging system FL-FIS allows screening of the images of F_{440} and F_{520} of leaves, which primarily emanate from covalently bound cinnamic acids (mainly ferulic acid) in the cell walls of all leaf cells (Lang *et al.* 1992, Lichtenthaler and Schweiger 1998). The FL-FIS is also able to sense images of F_{690} and F_{740} Chl fluorescence with high precision. In contrast to the Chl fluorescence, which exhibits a photon-induced transient (Kautsky effect), the blue and green fluorescence represents a stable signature that does not show any induction kinetics (Stober and Lichtenthaler 1993, Stober *et al.* 1994). Once the fluorescence images have been collected and saved to the PC, the data processing software allows establishing of a frequency distribution histogram of the fluorescence yield either of all leaf pixels or of those of selected leaf areas. In addition, a profile analysis of the fluorescence yield of the pixels along a narrow or broader line across the leaf area is possible. An example of a profile analysis is shown for a variegated leaf of *Campelia zonania* L. with green, dark-green, and white longitudinal stripes (Fig. 4). The profile of F_{690} exhibits high signals in the Chl containing green leaf stripes, whereas F_{440} and F_{520} exhibit maximums only in the white leaf stripes. Thus, blue and red fluorescences show a negative contrast to each other. The green leaf stripes also possess a blue fluorescence; however, this is re-absorbed by Chls and carotenoids.

Histograms and profiles of fluorescence images allow the analysis of differences between control and stressed plants, thus permitting very early stress detection at times when countermeasures against the stressors can still be taken. Moreover, from two images measured at different fluorescence bands, fluorescence ratio images can be calculated: F_{440}/F_{690} as an early stress and strain indicator (Lichtenthaler *et al.* 1996, Lichtenthaler and Miehé 1997, Buschmann and Lichtenthaler 1998), F_{690}/F_{740} as an indicator of the Chl content of leaves (curvilinear inverse relationship) (Lichtenthaler and Rinderle 1988, Hák *et al.* 1990, D'Ambrosio *et al.* 1992, Gitelson *et al.* 1998, Lichtenthaler and Babani 2004), or the R_{Fd} ratio images

that indicate P_N (Babani and Lichtenthaler 1996, Tuba *et al.* 1994) as is demonstrated below in detail.

Correlation of R_{Fd} and P_N: The irradiation-triggered Chl fluorescence induction kinetics of 20-min pre-darkened leaves are well known (for basic principles see Fig. 5). After the fast rise *via* F_0 to maximum F_m (at a saturating flash) or to F_p (at a non-saturating flash), the Chl fluorescence slowly decreases parallel to the onset of photosynthetic electron flow and beginning CO_2 fixation. After 4 to 5 min irradiation the leaf is in the steady state fluorescence F_s. When the induction kinetics are measured at high PPFD of *ca.* 2 000 µmol(photon) m^2 s^{-1} of "white light" or at red radiation (He-Ne laser, λ 632.8 nm, 10 mW) of *ca.* 700 µmol(photon) m^{-2} s^{-1} (which are above the saturation irradiance of P_N), the measured Chl fluorescence is representative of all leaf chloroplasts, those at the upper and lower leaf halves. The upper and lower leaf halves give differential Chl fluorescence signals when measured at the non-saturating irradiance ["actinic light" of 80–300 µmol(photon) m^{-2} s^{-1}] of a PAM fluorometer (Lichtenthaler *et al.* 2005), this does, however, not apply to the R_{Fd} ratio measured at saturating irradiance. This especially applies to red He-Ne laser radiation that penetrates much deeper into the green leaf mesophyll than non-saturating "white light" that is readily absorbed by Chls and carotenoids in the upper leaf half. When measured at a high PPFD, the ratio of the Chl fluorescence decrease F_d (from F_m to F_s) to the steady state fluorescence F_s (R_{Fd} = F_d/F_s) reflects P_N as has been demonstrated before (Tuba *et al.* 1994, Babani and Lichtenthaler 1996) and is demonstrated below. Under environmental stress, *e.g.* at a prolonged high irradiation under water stress the Chl fluorescence induction curve first decreases from F_m but then increases to much higher steady level (Fig. 5). The corresponding Chl fluorescence-decrease ratios R_{Fd}' and R_{Fd}'' exhibit much lower values and are early indicators of stress to the photosynthetic apparatus. The normal range of R_{Fd} (measured in the red 690 nm band of Chl fluorescence) in fully functional green leaves is 1.3–2.7 for leaves of shade plants and shade leaves of trees, and of 3.0–5.5 or sometimes even higher in sun exposed plants and sun leaves of trees.

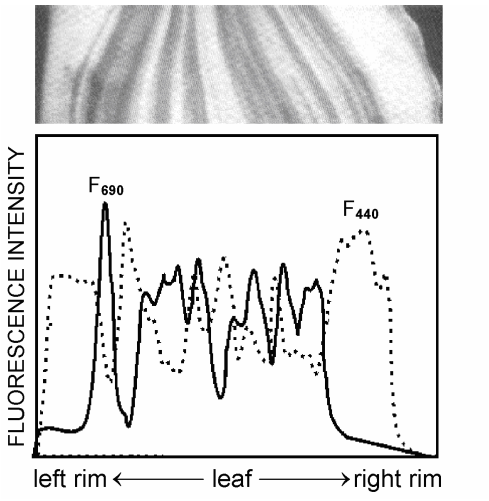

Fig. 4. Black and white photo of a white-green variegated leaf of *Campelia zonania* L. with a profile analysis of the red (F_{690}, *solid line*) and the blue (F_{440}, *broken line*) fluorescence measured across the leaf area from the left to the right leaf side of red and blue fluorescence images, respectively. The profile analysis is based on the fluorescence of more than 5 000 pixels in each case.

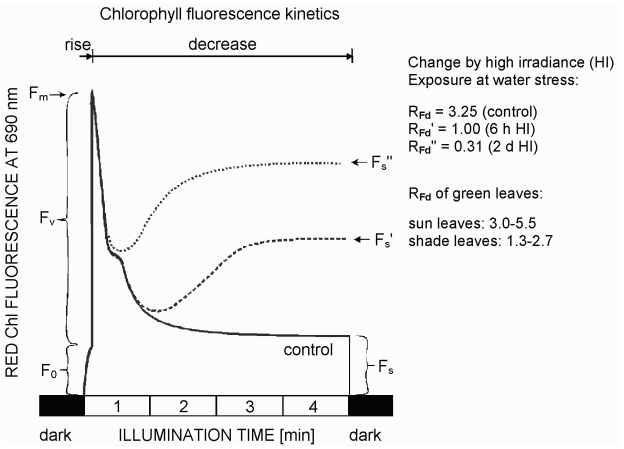

Fig. 5. Changes in the light-induced chlorophyll fluorescence kinetics (Kautsky effect) in green leaves with increasing high irradiance (HI) exposure at water stress. The decrease of the induction curve from F_m to the steady state F_s is slowed down *via* F_s' to F_s'' with increasing exposure time. As a consequence, the values of Chl fluorescence decrease ratio R_{Fd} (= F_d/F_s) decline from 3.25 (control) to 0.31 after a 2-d exposure.

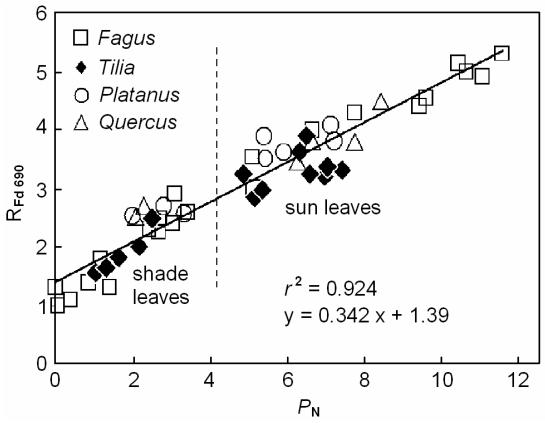

Fig. 6. Linear correlation of the Chl fluorescence decrease ratio R_{Fd} (measured with the *LITWaF* at the F_{690} band) with the photosynthetic net CO_2 fixation rates (P_N) of sun and shade leaves of trees (beech, linden, platane, oak). P_N [µmol(CO_2) m^{-2} s^{-1}] was determined with a CO_2/H_2O porometer at an irradiance of 1 100 µmol(photon) m^{-2} s^{-1} (sun leaves) and at 500 µmol(photon) m^{-2} s^{-1} (shade leaves) that is well above the photosynthetic saturation irradiance of *ca.* 900 and 300 µmol(photon) m^{-2} s^{-1}, respectively.

In order to prove the reliability of RFd-values, measured as F690, as indicators of the leaf P_N, we determined in various trees the R_{Fd}-values of sun and shade leaves by means of single leaf point measurements (using the *LITWaF*). We compared them with P_N of the same leaves measured at saturation irradiance with a CO_2/H_2O porometer. The leaf spots where the Chl fluorescence kinetics are determined are much smaller (*ca*. 0.64 cm^2) than the leaf area (*ca*. 30 cm^2) that has been placed into the porometer. Hence, in order to obtain a mean R_{Fd}-value per leaf that is representative of the investigated leaf and can be correlated with P_N we measured at least 5–6 R_{Fd}-values for each leaf. We found a very good correlation between R_{Fd} and P_N of sun and shade leaves (Fig. 6). This demonstrates that the height of R_{Fd} indicates P_N of leaves. When a clear P_N could no longer be measured (P_N = 0 to >0.5), the R_{Fd} was still 1.0–1.4. This is an indication that even with closed stomata the photosynthetic apparatus is active in photosynthetic quantum conversion, *e.g.* using the internal respiration CO_2 for photosynthesis.

Imaging of the Chl fluorescence decrease ratio R_{Fd} of leaves

Basic principle of the measurement: By imaging F_{690} of 20 min pre-darkened leaves at the Chl fluorescence maximum $F_{m,}$ and after 5 min at the steady state level F_s one can calculate the R_{Fd} for each leaf pixel (see Fig. 7). These images show that not all the leaf parts possess the same Chl fluorescence yield during continuous irradiation and that the R_{Fd} values are not the same for the different leaf areas. This heterogeneity of photosynthetic activity across the leaf area is documented by the R_{Fd} image demonstrating that certain pixels (marked in red) have high R_{Fd}-values of *ca*. 4.0 and high P_N, whereas others (yellow and green pixels) possess much lower R_{Fd}. Thus, the photosynthetic activity is unevenly distributed across the leaf area and shows a spatial heterogeneity.

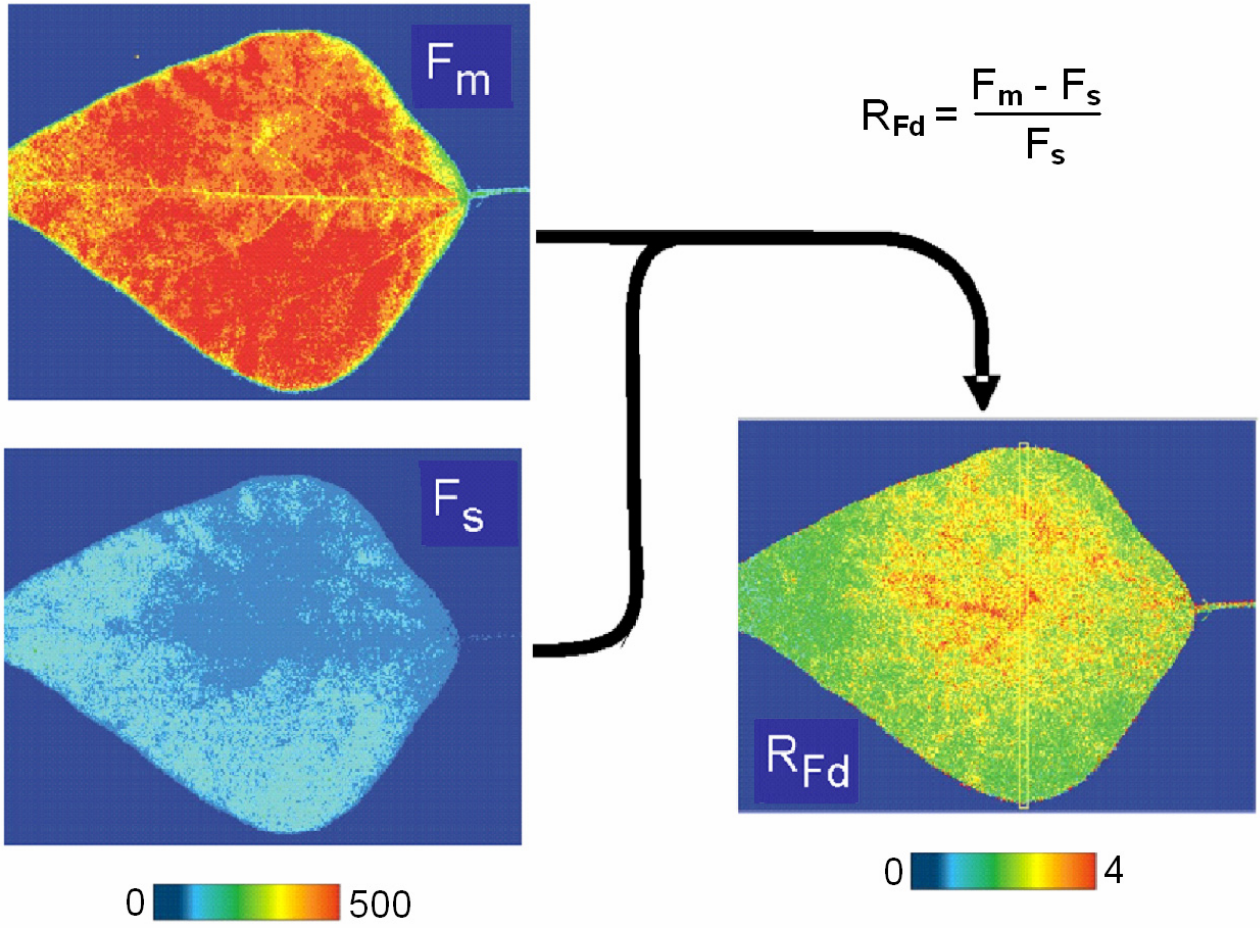

Fig. 7. Images of the red Chl fluorescence F_{690} of a bean leaf (*Phaseolus vulgaris* L.) measured at the maximum fluorescence level, F_m and the steady state level F_s (5 min after onset of saturating "white" irradiation). From both images the R_{Fd} ratio image was determined by a pixel to pixel calculation. The fluorescences at F_m and F_s of each leaf pixel are given in false colours from zero (blue) to high fluorescence (red). In the case of the R_{Fd} ratio image the false colours state the absolute values of the ratio. (Excitation with pulsed blue radiation of the xenon flash lamp.)

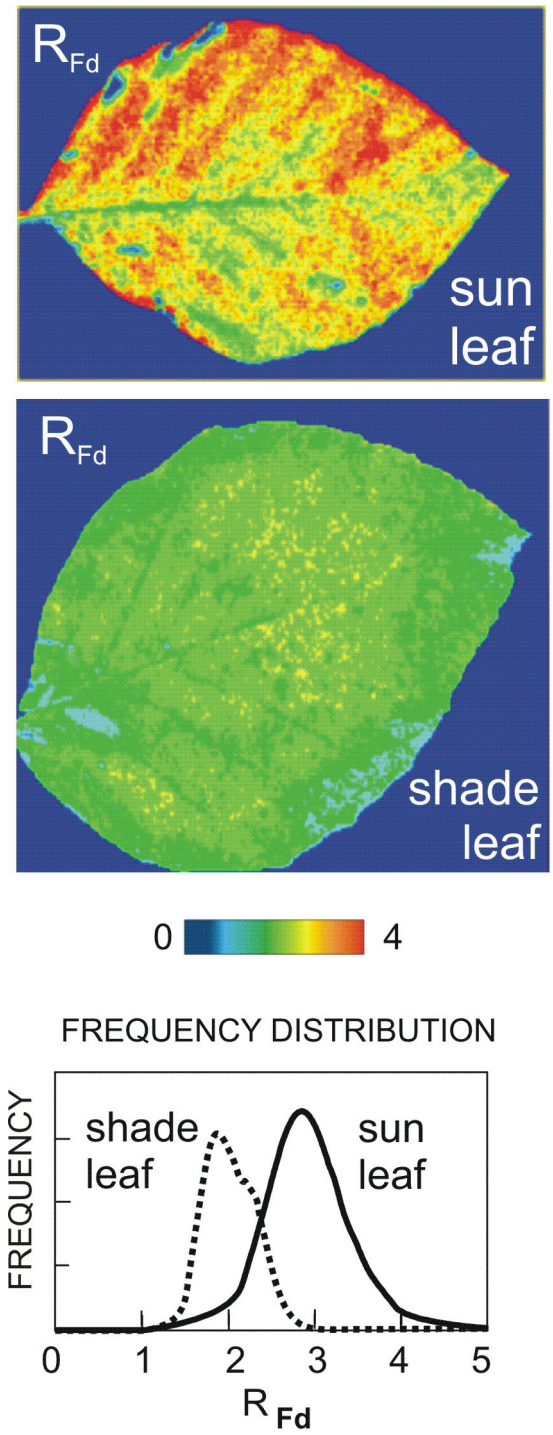

Fig. 8. Images of the R_{Fd}-values (Chl fluorescence decrease ratio) of sun and shade leaves of beech (*Fagus sylvatica* L.). The histogram (*lower part*) of the frequency distribution of R_{Fd} values of all leaf pixels shows the large difference in photosynthetic quantum conversion of sun and shade leaves. Similar differences as in R_{Fd} values were also found in net photosynthetic rate (see Fig. 6). Excitation: pulsed blue radiation of the xenon lamp. (Based on Lichtenthaler *et. al.* 2000.)

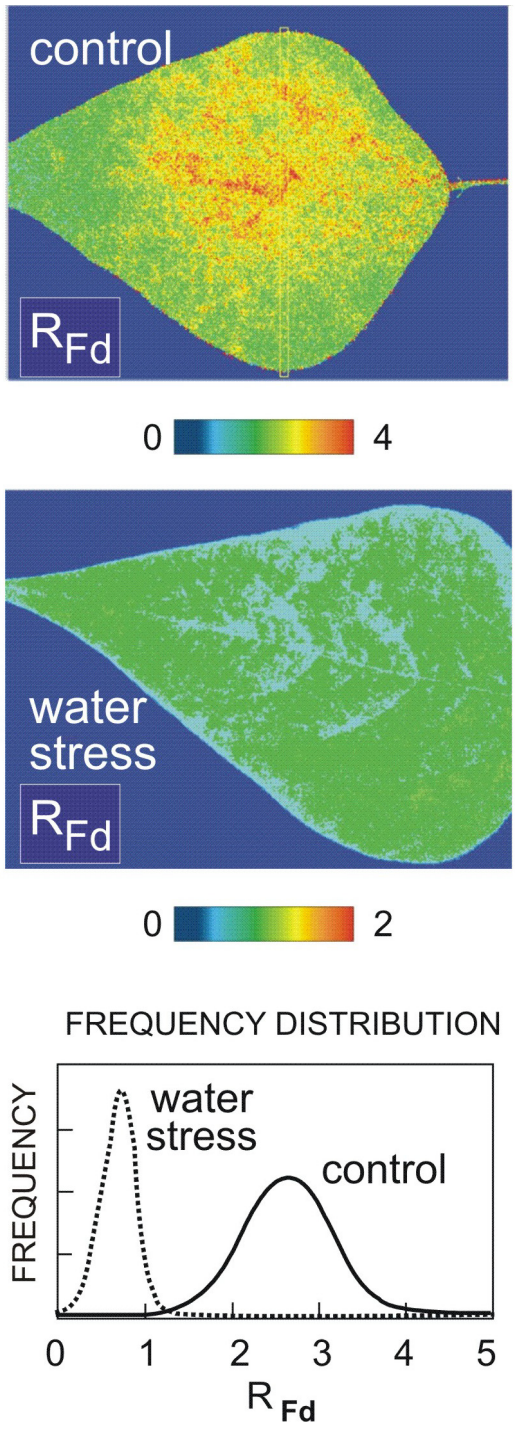

Fig. 9. Images of the R_{Fd} values as indicators of photosynthetic activity in bean leaves of control and water stressed plants. The scales are different in both cases. The histogram of the R_{Fd} values of all leaf pixels shows the high decline of R_{Fd} values at water stress. The stressed bean plants had not been watered for 10 d, and their leaves exhibited a water content of 69 % as compared to 87 % in the watered controls. Excitation was performed with pulsed blue radiation of the xenon lamp. (Based on Lichtenthaler and Babani 2000.)

Differences in the R_{Fd} ratio between sun and shade leaves: The large differences in P_N of sun and shade leaves (Fig. 6) can easily be sensed by Chl fluorescence imaging of R_{Fd}. The latter possess much higher values in sun leaves than in shade leaves, as is clearly visible when viewing the R_{Fd} images of Fig. 8. As already found in the bean leaf (Fig. 7), the R_{Fd} even within one sun or shade leaf possesses a large spatial heterogeneity. There are considerable differences in R_{Fd} and hence P_N across the leaf area; and these are particularly large in the sun leaves of beech with red pixels (high P_N) down to some local leaf spots with blue pixels (*i.e.* almost no photosynthetic quantum conversion). By creating the histogram of the R_{Fd}-values of all leaf pixels the differences in P_N (as judged from R_{Fd}) are quantified (Fig. 8). Similar differences in the R_{Fd} are found in other sun and shade leaves of beech as well as in those of other trees mentioned in Fig. 6.

Decline of R_{Fd}-values at water stress is also verified by images of the R_{Fd}. The latter declines in leaves of water stressed bean plants as compared to those of well watered control plants (Fig. 9). The histogram with the frequency distribution of the R_{Fd}-values of all leaf pixels shows two separate distribution curves for the control leaves and the water stressed leaves.

Uptake of herbicide diuron (DCMU): Diuron inhibits the photosynthetic quantum conversion by blocking the photosynthetic electron flow at photosystem 2 (PS2). Under full diuron influence the Chl fluorescence induction kinetics are suppressed, the Chl fluorescence rises to F_m and remains at that high level. The progressive diuron uptake by the leaves, after diuron application to the plant roots, can precisely be followed by Chl fluorescence imaging (Fig. 10). About 30 min after the diuron application practically no change in Chl fluorescence yield is detectable as compared to the control leaf at zero time. A low Chl fluorescence yield is found in the irradiated bean leaf indicated by mostly green and some yellow pixel areas. At about 2.5 h after the application, the diuron absorption is clearly detectable by a strong increase of Chl fluorescence (here shown as false colour red pixels) at the transition of the leaf petiole (not shown) to the central leaf vein. With increasing time diuron penetrates deeper into the leaf *via* the different leaf veins and by absorption into the Chl containing mesophyll cells. Apparently two pools of diuron exist, the larger part of it is transported slowly and is detectable by the strong increase of the false red colour in Fig. 10. However, a small pool of diuron is transported more rapidly within the leaf and the leaf veins as shown by the increase of the dominantly green leaf pixels to a higher proportion of yellow pixels. The latter indicate a partial absorption and a partial block of photosynthetic quantum conversion. This partial increase of Chl fluorescence, as seen by means of the yellow pixels, always precedes the full block of photosynthetic quantum conversion as is evident in all Chl fluorescence images in Fig. 10.

Differentiation of plants with low and high N-supply: Crop plants growing on soil poor in nitrogen (N_0 plants, no fertilizer), develop in a different way than plants cultivated with a full supply of nitrogen fertilizer (*e.g.* with 150 kg per hectare, N_{150} plants). In sugar beet, the leaves of N_0 plants possessed much lower amounts of Chl *a*+*b* [271 mg m^{-2}(leaf area)] than the leaves of N_{150} plants which contained 585 mg(Chl *a*+*b*) m^{-2}(leaf area). The light-green leaves of the N_0 plants possessed an approximately 2.5–3.0 times higher amounts of flavonol and soluble cinnamic acids in their leaf epidermis cells than the dark-green leaves of the N_{150} plants. As a consequence the UV induced F_{440} was considerably lower in the N_0 leaf than in the N_{150} leaf (Fig. 11). This is due to the fact that the exciting UV radiation in N_0 leaves is absorbed by the soluble flavonols and soluble cinnamic acids of the epidermis vacuole that do not show any blue fluorescence. These plant phenolics form a protective screen of the green mesophyll cells against damaging UV-A radiation. In other words, the leaves of N_0 plants are protected much better against UV-A radiation and damage than the fully green leaves of N_{150} plants.

For the same reasons F_{690} was significantly lower in the leaves of N_0 plants than N_{150} plants (Fig. 11). Due to the amounts of high soluble flavonol and cinnamic acids in the epidermis cells of N_0 leaves, the exciting UV-A radiation was absorbed to a large degree in the epidermis cells and less UV-A reached the green mesophyll cells. The consequence was a lower amount of Chl fluorescence being excited than in leaves of N_{150} plants.

The corresponding images of F_{440}/F_{690} indicated higher values in the N_0 leaves (with low Chl fluorescence) and, as expected, lower values in the N_{150} sugar beet leaves (which exhibited a very high Chl fluorescence yield) as seen in the ratio images (Fig. 11). Again there existed large differences and gradients in F_{440}/F_{690} in the different leaf parts across the leaf area as had been described also for the R_{Fd}-ratio in several other leaves. The histogram of the frequency distribution of F_{440}/F_{690} of all leaf pixels clearly showed these differences between leaves of N_{150} and N_0 plants.

The Chl fluorescence ratio red/far-red is a curvilinear inverse indicator of the Chl *a*+*b* content of leaves (Lichtenthaler and Rinderle 1988, Babani *et al.* 1996). In leaves of many different plants, F_{690}/F_{740} decreased with increasing Chl content of the leaf. This was due to the fact that F_{690} overlaps with the absorption bands of the *in vivo* Chl *a* bands in the pigment protein complexes of thylakoids of the leaf cell chloroplasts (see also Gitelson *et al.* 1998).

F_{740}, in turn, was not greatly affected by this re-absorption. Hence, F_{690} decreased by re-absorption with increasing Chl content, whereas F_{740}, at low Chl content

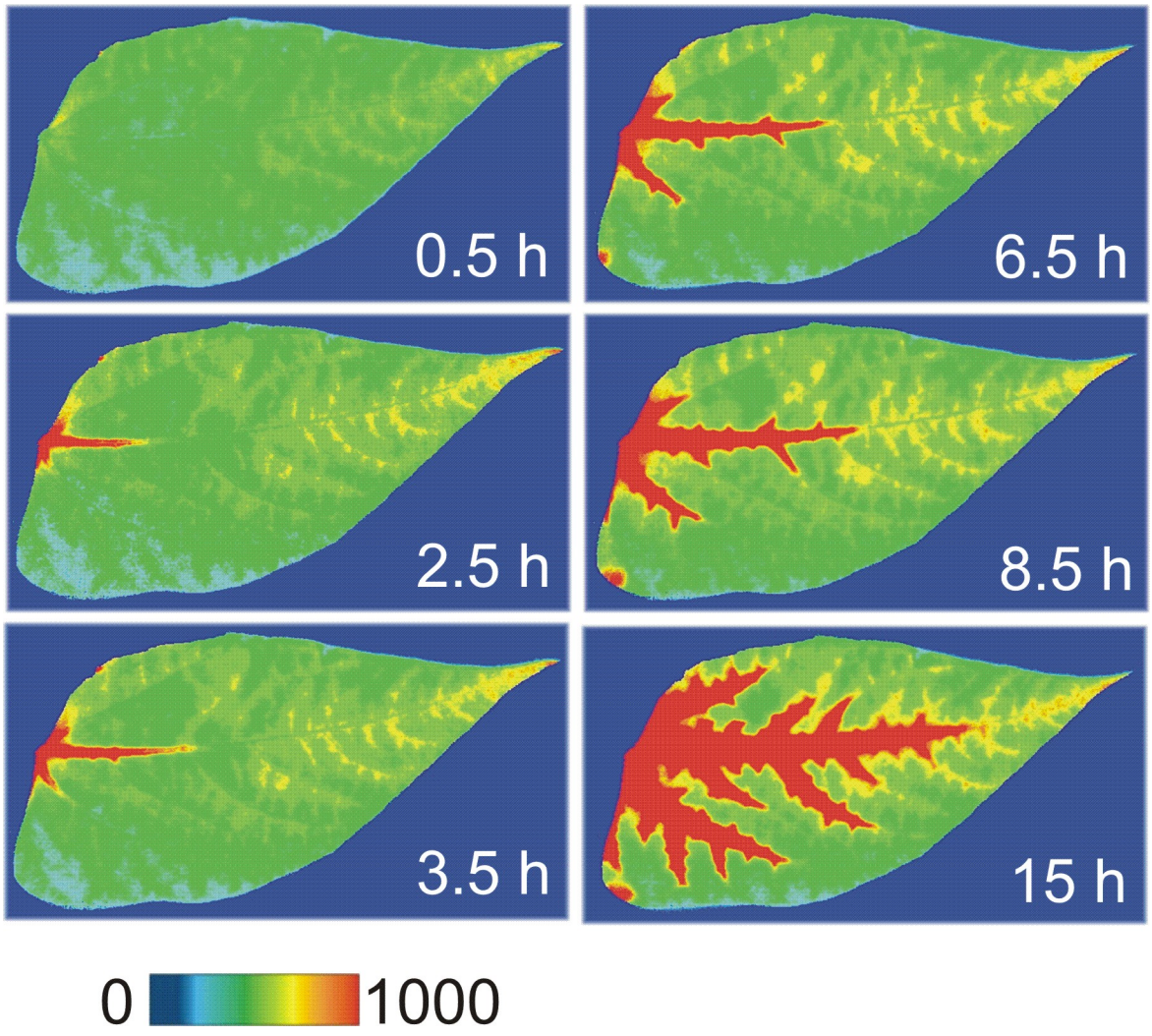

Fig. 10. Successive absorption of the herbicide diuron (DCMU) into a bean leaf as visualized by the strong rise in red Chl fluorescence (screened here at the F_{690} band). The increase in the red Chl fluorescence band is shown in false colours with green as a low and red as the highest fluorescence yield. Diuron, which blocks photosynthetic quantum conversion at photosystem 2 by binding to the Q_B-protein, was applied (10^{-4} M) *via* the root.

only present as a shoulder, developed into new fluorescence maximum at high Chl *a*+*b* contents of the leaf. The calculation of F_{690}/F_{740} for sugar beet leaves showed a more even distribution of these ratio values across the leaf area (Fig. 12) compared to the other fluorescence ratios mentioned above. This indicates that Chl was homogeneously distributed across the leaf area. Due to the low Chl contents in the leaves of the N_0 plants, the mean values for F_{690}/F_{740} were significantly higher than in the leaves of the N_{150} plants (see histogram in Fig. 12). These results demonstrated that the *in vivo* Chl content of leaves can be measured in a non-destructive way by imaging the red/far-red Chl fluorescence ratio.

Conclusions: The results presented in this overview demonstrate that Chl fluorescence imaging (based on several hundred-thousand leaf pixels per measurement and leaf) is much superior to the hitherto applied single leaf point measurements with the classical Chl fluorometers supplying only one piece of fluorescence information per one leaf spot and leaf per measurement. Thus, fluorescence imaging of a few typical leaves of a plant is a superior technique for ecophysiological plant research. One could argument that the "classical" single-leaf-point Chl fluorescence information is based on many thousands of chloroplasts of the measured leaf point. However, it is only the average of that particular leaf point but not the average of the whole leaf with all its different leaf parts that usually have different information. For this reason a single-leaf-point measurement is not representative for the whole leaf. In contrast, Chl fluorescence imaging simultaneously provides fluorescence information on all leaf parts and shows differences in Chl fluorescence yield, *e.g.* on the rim, the basis, top, or middle parts of

leaves as well as the differences between leaf veins and the inter-costal fields. The advantages of fluorescence imaging of whole leaves in comparison to single-leaf-point measurements are shown in Fig. 13. Certain parts of

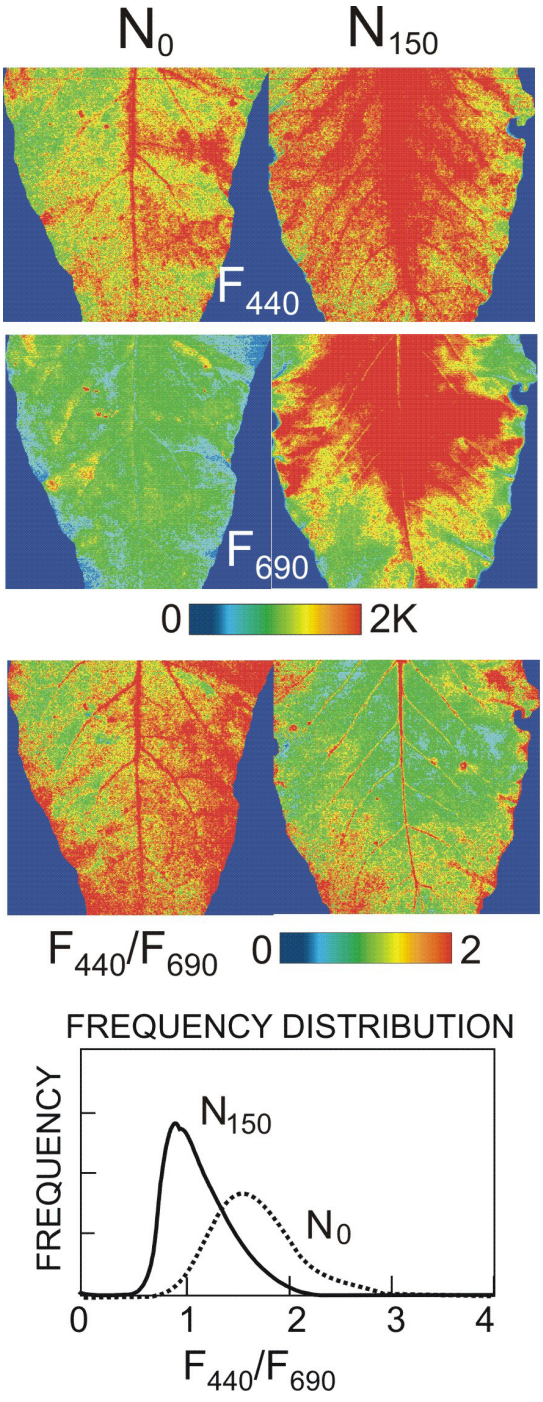

Fig. 11. Blue (F_{440}) and red (F_{690}) fluorescence images of sugar beet leaves from plants grown either on soil with no extra N supply (N_0) or with the addition of nitrogen fertilizer (N_{150}, with 150 kg N per hectare). The histogram of the fluorescence ratio F_{440}/F_{690} allows to differentiate both plant types. (Based on Langsdorf *et al.* 2000.)

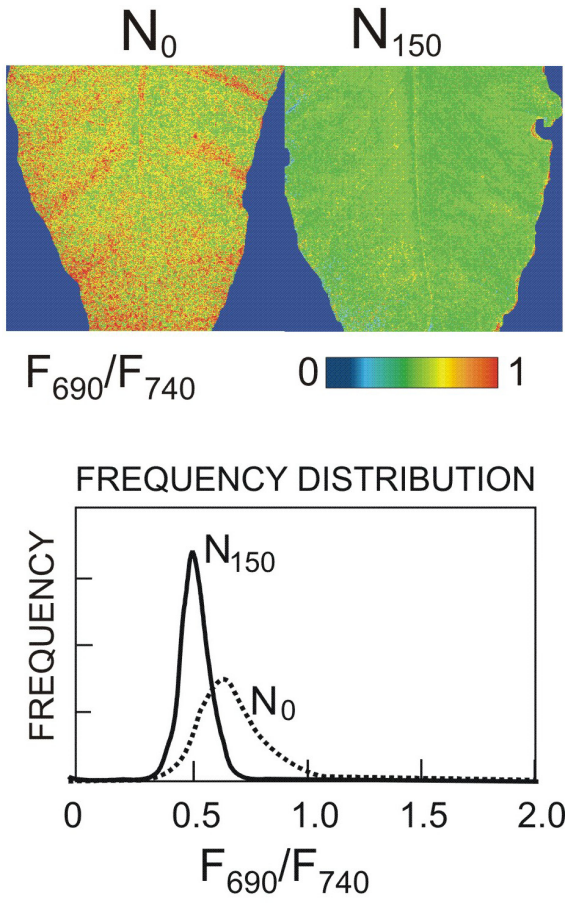

Fig. 12. Images of the Chl fluorescence ratio red/far-red (F_{690}/F_{740}) and a histogram with the frequency distribution of the ratio values in leaves of sugar beet plants grown on soil either without (N_0 plants) or with 150 kg per hectare N-fertilizer (N_{150} plants).

such a leaf can have a high, others a medium, and again other parts a very low Chl fluorescence yield resulting in high, medium, or low R_{Fd}. Imaging of R_{Fd} unanimously proved that the photosynthetic activity is not evenly distributed across the leaf area. Instead a certain patchiness and spatial heterogeneity exist in leaves of control and stressed plants as shown in our investigations (*e.g.* Figs. 8 and 9). This can only be detected by fluorescence imaging but not by measuring P_N *e.g.* of a whole leaf or a major leaf part or *via* the R_{Fd} determined of a few individual leaf spots using the classical fluorometers. Fig. 13 shows that measuring Chl fluorescence on two or three leaf spots would result in a wrong evaluation of the leaf's photosynthetic characteristics, since one never knows whether one takes fluorescence information from a leaf spot with high, medium, or low activity. Only imaging provides the overall information on all leaf parts and in more than 100 000 pixels per leaf; thus it supplies a high statistical confidence which permits also a very early stress detection.

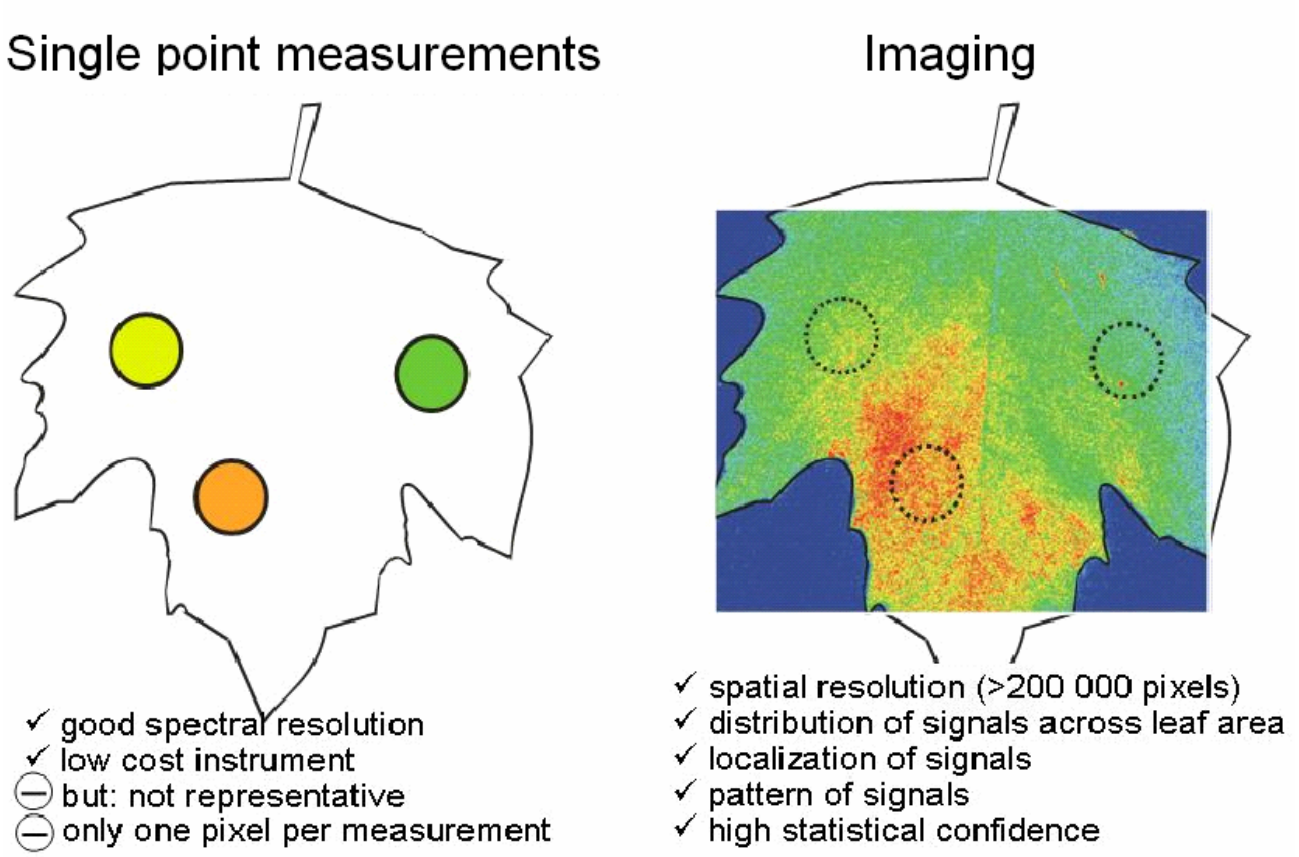

Fig. 13. The advantages and superiority of leaf fluorescence imaging compared to the fluorescence measurement at single leaf spots here shown for a maple leaf. The spatial heterogeneity of the Chl fluorescence signals and ratios shows up *via* imaging, but not at all or not necessarily *via* single leaf point measurements, even if several points of a leaf are measured.

Imaging of R_{Fd} and quantifying the values by histograms (the frequency distribution of the Chl fluorescence yield of all leaf pixels) is an excellent tool to detect differences in photosynthetic quantum conversion, *e.g*. between sun and shade leaves or between control and stressed plants. In view of the fluorescence results of the very high number of leaf pixels screened per each measurement such differences are screened with a very high statistical significance. The flash lamp fluorescence imaging system FL-FIS further demonstrates that by imaging the red as well as the far-red Chl fluorescence one obtains, *via* F_{690}/F_{740}, also valuable information on the Chl content of leaves. Such non-destructive measurements can be made before and during stress influence, resulting in the detection of a possible decrease of Chl amounts. Also, the regeneration of the Chl amounts of stressed leaves can be observed when the stressor is removed. Moreover, differences in the Chl content between plants grown in different environments as well as mineral supply can be visualised *via* the F_{690}/F_{740} ratio images.

In addition, the described FL-FIS with its simultaneous screening of the images of the genuine blue and green fluorescence of leaves, besides the red and far-red Chl fluorescence, allows determining stress and strain to plants by imaging the fluorescence ratio blue/red (F_{440}/F_{690}) or blue/far-red (F_{440}/F_{740}). Both are very early stress indicators long before a drop or damage of the photosynthetic performance of leaves or a visual loss of pigments can be detected. The Karlsruhe multicolour flash-lamp FL-FIS is unique, not only in its ability to image the red Chl fluorescence together with the far-red and the blue and green fluorescence of leaves, but additionally in its ability to image the R_{Fd}-values which are the indicators of P_N of leaves. We demonstrated here that R_{Fd} values, determined at or very close to saturation irradiance of the Chl fluorescence induction kinetics at F_m and at F_s, reflect the photochemical activity and photosynthetic performance and the Chl fluorescence signals of all leaf chloroplasts. Thus, they are representative for the whole leaf.

In contrast to the flash-lamp FL-FIS, the Chl fluorescence induction kinetics, usually screened with the PAM fluorometer, are not measured at saturation irradiance of the kinetics but at a much lower irradiance which is an essential requirement of the PAM technique. Hence, the measurements of the Chl fluorescence ratio F_v/F_m, F_v/F_0, or $\Delta F/F_o'$, $\Delta F/F_m'$ *etc*. in the PAM fluorometer primarily reflects the photochemical activity of PS2. Moreover, at

the non-saturating low irradiances used to induce the Chl fluorescence induction kinetics, the Chl fluorescence results of the PAM-measurements in green and dark-green leaves only reflect the PS2 quantum conversion characteristics of the chloroplast portion at the upper part of the palisade mesophyll cells at that leaf side where the Chl fluorescence kinetics has been induced by photons and where the fluorescence has been sensed (Lichtenthaler *et al.* 2005). Since the chloroplasts at the lower leaf half can not be sensed in green and dark-green leaves with the PAM measuring technique, the PAM information is, except for light-green leaves, in most cases not really representative for the whole leaf. In fact, maple leaves (*Acer platanoides* L.), that, *via* the strong decline of the PAM measured F_v/F_m, $\Delta F/F_m'$, and F_v'/F_m' as well as the photochemical quenching coefficient q_P, should have been fully photoinhibited, still exhibited *ca*. 70 % of their maximum P_N (Schindler and Lichtenthaler 1996). This happened since the chloroplasts in the lower leaf parts, which were protected against photoinhibition by the photoinhibited upper outer chloroplast layer, apparently were still fully photosynthetically active. However, the great advantage of the PAM instrument is that, once a decline in P_N and quantum conversion has been detected, one can determine the cause and mechanism of this decline, *e.g*. a photoinhibition of the photosynthetic apparatus by determination of q_N and its components such as the quenching coefficient q_I.

Since the first development of a laser equipped fluorescence imaging system (Laser-FIS) sensing the blue, green, red, and far-red fluorescence of leaves (Lang *et al.* 1994, Lichtenthaler and Miehé 1997), several other fluorescence imaging techniques have been established by various other work groups. These techniques, however, only image the Chl fluorescence, *i.e*. one band in one wavelength region (*e.g*. Bro *et al.* 1995, Osmond *et al.* 1999, Holub *et al.* 2000, Nedbal *et al.* 2000, Baker *et al.* 2001, Chaerle and van der Straeten 2001, Barbagallo *et al.* 2003, Hill *et al.* 2004, Nedbal and Whitmarsh 2004, Oxborough 2004). Most of these instruments are based on PAM-type Chl fluorescence measurements and thus these PAM-type imaging results may be, in various cases, only of limited worth and significance, since they exclusively reflect the photochemical activity of PS2. For this reason such PAM fluorometer imaging results may, especially in fully green and dark-green leaves, not be representative for all leaf chloroplasts but only for those of the upper chloroplast layer of the leaves' palisade cells. In contrast, the flash-lamp FL-FIS, which images the red and far-red Chl fluorescence as well as the blue and green fluorescence, exhibits much broader application possibilities in photosynthetic and eco-physiological research and in the stress detection of plants.

References

Babani, F., Lichtenthaler, H.K.: Light-induced and age-dependent development of chloroplasts in etiolated barley leaves as visualized by determination of photosynthetic pigments, CO_2 assimilation rates and different kinds of chlorophyll fluorescence ratios. – J. Plant Physiol. **148**: 555-566, 1996.

Baker, N.R., Oxborough, K., Lawson, T., Morison, J.I.L.: High resolution imaging of photosynthetic activities of tissues, cells and chloroplasts in leaves. – J. exp. Bot. **52**: 615-621, 2001.

Barbagallo, R.P., Oxborough, K., Pallett, K.E., Baker, N.R.: Rapid, non-invasive screening for perturbation of metabolism and plant growth using chlorophyll fluorescence imaging. – Plant Physiol. **132**: 485-493, 2003.

Boardman, N.K.: Comparative photosynthesis of sun and shade plants. – Annu. Rev. Plant Physiol. **28**: 355-377, 1977.

Bolhàr-Nordenkampf, H.R., Öquist, G.: Chlorophyll fluorescence as a tool in photosynthesis research. – In: Hall, D.O., Scurlock, J.M.O., Bolhàr-Nordenkampf, H.R., Leegood, R.C., Long, S.P. (ed.): Photosynthesis and Production in a Changing Environment: A Field and Laboratory Manual. Pp. 193-206. Chapman & Hall, London – Glasgow – New York – Melbourne – Madras 1993.

Bro, E., Meyer, S., Genty, B.: Heterogeneity of leaf CO_2 assimilation during photosynthetic induction. – In: Mathis, P. (ed.): Photosynthesis: from Light to Biosphere. Vol. V. Pp. 607-610. Kluwer Academic Publ., Dordrecht – Boston – London 1995.

Buschmann, C., Langsdorf, G., Lichtenthaler, H.K.: Imaging of the blue, green and red fluorescence emission of plants. – Photosynthetica **38**: 483-491, 2000.

Buschmann, C., Lichtenthaler, H.K.: Principles and characteristics of multi-colour fluorescence imaging of plants. – J. Plant Physiol. **152**: 297-314, 1998.

Chaerle, L., van der Straeten, D.: Seeing is believing: imaging techniques to monitor plant health. – Biochim. biophys. Acta **1519**: 153-166, 2001.

D'Ambrosio, N., Szabó, K., Lichtenthaler, H.K.: Increase of the chlorophyll fluorescence ration F690/F735 during the autumnal chlorophyll breakdown. – Radiat. environ. Biophys. **31**: 51-62, 1992.

Edner, H., Johansson, J., Svanberg, S., Lichtenthaler, H.K., Lang, M., Stober, F., Schindler, C., Björn, L.-O.: Remote multi-colour fluorescence imaging of selected broad-leaf plants. – EARSel Adv. remote Sensing **3**: 2-14, 1995.

Genty, B., Briantais, J.-M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim. biophys. Acta **990**: 87-92, 1989.

Gitelson, A.A., Buschmann, C., Lichtenthaler, H.K.: Leaf chlorophyll fluorescence corrected for re-absorption by means of absorption and reflectance measurements. – J. Plant Physiol. **152**: 283-296, 1998.

Govindjee: Sixty three years since Kautsky: Chlorophyll *a* fluorescence. – Aust. J. Plant Physiol. **22**: 131-160, 1995.

Govindjee: Chlorophyll *a* fluorescence, a bit of basics and history. – In: Papageorgiou, G.C., Govindjee (ed.): Chlorophyll Fluorescence: a Signature of Photosynthesis. Pp. 1-42. Springer, Dordrecht 2004.

Hák, R., Lichtenthaler, H.K., Rinderle, U.: Decrease of the chlorophyll fluorescence ratio F690/F730 during greening and development of leaves. – Radiat. environ. Biophys. **28**:

329-336, 1990.
Hill, R., Schreiber, U., Gademann, R., Larkum, A.W.D., Kühl, M., Ralph, P.J.: Spatial heterogeneity of photosynthesis and the effect of temperature-induced bleaching conditions in three species of corals. – Mar. Biol. **144**: 633-640, 2004.
Holub, O., Seufferheld, M.J., Gohlke, C., Govindjee, Clegg. R.M.: Fluorescence lifetime imaging (FLI) in real-time – a new technique in photosynthesis research. – Photosynthetica **38**: 581-599, 2000.
Kautsky, H., Hirsch, A.: Neue Versuche zur Kohlenstoffassimilation. – Naturwissenschaften **10**: 1964, 1931.
Krause, G.H., Weis, E.: Chlorophyll fluorescence and photosynthesis: the basics. – Annu. Rev. Plant Physiol. Plant mol. Biol. **42**: 313-349, 1991.
Lang, M., Lichtenthaler, H.K., Sowinska, M., Summ, P., Heisel, F.: Blue, green and red fluorescence signatures and images of tobacco leaves. – Bot. Acta **107**: 230-236, 1994.
Lang, M., Šiffel, P., Braunová, Z., Lichtenthaler, H.K.: Investigations of the blue-green fluorescence emission of plant leaves. – Bot. Acta **105**: 435-440, 1992.
Langsdorf, G., Buschmann, C., Sowinska, M., Babani, F., Mokry, M., Timmermann, F., Lichtenthaler, H.K.: Multicolour fluorescence imaging of sugar beet leaves with different nitrogen status by flash lamp UV-excitation. – Photosynthetica **38**: 539-551, 2000.
Lichtenthaler, H.K.: Chlorophylls and carotenoids – pigments of photosynthetic biomembranes. – In: Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. **148**. Pp. 350-382. Academic Press, San Diego – New York – Berkeley – Boston – London – Sydney – Tokyo – Toronto 1987.
Lichtenthaler, H.K. (ed.): Applications of Chlorophyll Fluorescence. – Kluwer Acad. Publ., Dordrecht – Boston – London 1988.
Lichtenthaler, H.K.: The Kautsky effect: 60 years of chlorophyll fluorescence induction kinetics. – Photosynthetica **27**: 45-55, 1992.
Lichtenthaler, H.K.: Vegetation stress: an introduction to the stress concept in plants. – J. Plant Physiol. **148**: 4-14, 1996.
Lichtenthaler, H.K., Babani, F.: Detection of photosynthetic activity and water stress by imaging the red chlorophyll fluorescence. – Plant Physiol. Biochem. **38**: 889-895, 2000.
Lichtenthaler, H.K., Babani, F.: Light adaptation and senescence of the photosynthetic apparatus. Changes in pigment composition, chlorophyll fluorescence parameters and photosynthetic activity. – In: Papageorgiou, G.C., Govindjee (ed.): Chlorophyll Fluorescence: a Signature of Photosynthesis. Pp. 713-736. Springer, Dordrecht 2004.
Lichtenthaler, H.K., Babani, F., Langsdorf, G., Buschmann, C.: Measurement of differences in red chlorophyll fluorescence and photosynthetic activity between sun and shade leaves by fluorescence imaging. – Photosynthetica **38**: 521-529, 2000.
Lichtenthaler, H.K., Burkart, S.: Photosynthesis and high light stress. – Bulg. J. Plant Physiol. **25**: 3-16, 1999.
Lichtenthaler, H.K., Buschmann, C., Döll, M., Fietz, H.-J., Bach, T., Kozel, U., Meier, D., Rahmsdorf, U.: Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. – Photosynth. Res. **2**: 115-141, 1981.
Lichtenthaler, H.K., Buschmann, C., Knapp, M.: How to correctly determine chlorophyll fluorescence parameters and the chlorophyll fluorescence decrease ratio R_{Fd} of leaves with the PAM fluorometer. – Photosynthetica **43**: 379-393, 2005.
Lichtenthaler, H.K., Kuhn, G., Prenzel, U., Meier, D.: Chlorophyll-protein levels and stacking degree of thylakoid stacking in radish chloroplasts from high-light, low-light and bentazon-treated plants. – Physiol. Plant. **56**: 183-188, 1982.
Lichtenthaler, H.K., Lang, M., Sowinska, M., Heisel, F., Miehé, J.A.: Detection of vegetation stress via a new high resolution fluorescence imaging system. – J. Plant Physiol. **148**: 599-612, 1996.
Lichtenthaler, H.K., Lang, M., Sowinska, M., Summ, P., Heisel, F., Miehé, J.A.: Uptake of the herbicide diuron (DCMU) as visualized by the fluorescence imaging technique. – Bot. Acta **110**: 158-163, 1997.
Lichtenthaler, H.K., Miehé, J.A.: Fluorescence imaging as a diagnostic tool for plant stress. – Trends Plant Sci. **2**: 316-320, 1997.
Lichtenthaler, H.K., Rinderle U.: The role of chlorophyll fluorescence in the detection of stress conditions in plants. – CRC crit. Rev. anal. Chem. **19**: S29-S85, 1988.
Lichtenthaler, H.K., Schweiger, J.: Cell wall bound ferulic acid, the major substance of the blue-green fluorescence emission of plants. – J. Plant Physiol. **152**: 272-282, 1998.
Meier, D., Lichtenthaler, H.K.: Ultrastructural development of chloroplasts in radish seedlings grown at high and low light conditions and in the presence of the herbicide bentazon. – Protoplasma **107**: 195-207, 1981.
Nedbal, L., Soukupová, J., Kaftan, D., Whitmarsh, J., Trtílek, M.: Kinetic imaging of chlorophyll fluorescence using modulated light. – Photosynth. Res. **66**: 3-12, 2000.
Nedbal, L., Whitmarsh, J.: Chlorophyll fluorescence imaging of leaves and fruits. – In: Papageorgiou, G., Govindjee (ed.): Chlorophyll Fluorescence, a Signature of Photosynthesis. Pp. 389-407. Springer, Dordrecht 2004.
Osmond, C.B., Kramer, D., Lüttge, U.: Reversible, water stress-induced non-uniform chlorophyll fluorescence quenching in wilting leaves of *Potentilla reptans* may not be due to patchy stomatal responses. – Plant Biol. **1**: 618-624, 1999.
Oxborough, K.: Imaging of chlorophyll *a* fluorescence: theoretical and practical aspects of an emerging technique for the monitoring of photosynthetic performance. – J. exp. Bot. **55**: 1195-1205, 2004.
Papageorgiou, G.C., Govindjee (ed.): Chlorophyll *a* Fluorescence, a Signature of Photosynthesis. – Springer, Dordrecht 2004.
Šantrůček, J., Šiffel, P., Lang, M., Lichtenthaler, H.K., Schindler, C., Synková, H., Konečná, V., Szabó, K.: Photosynthetic activity and chlorophyll fluorescence parameters in *aurea* and green forms of *Nicotiana tabacum*. – Photosynthetica **27**: 529-543, 1992.
Schindler, C., Lichtenthaler, H.K.: Photosynthetic CO_2 assimilation, chlorophyll fluorescence and zeaxanthin accumulation in field grown maple trees in the course of a sunny and a cloudy day. – J. Plant Physiol. **148**: 399-412, 1996.
Schreiber, U., Schliwa, U., Bilger, W.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorimeter. – Photosynth. Res. **10**: 51-62, 1986.
Šiffel, P., Šantrůček, J., Lang, M., Braunová, Z., Šimková, M., Synková, H., Lichtenthaler, H.K.: Age dependence of photosynthetic activity, chlorophyll fluorescence parameters and chloroplast ultrastructure in *aurea* and green forms of *Nicotiana tabacum Su/su* mutant. – Photosynthetica **29**: 81-94, 1993.
Sowinska, M., Cunin, B., Deruyver, A., Heisel, F., Miehé, J.-A., Langsdorf, G., Lichtenthaler, H.K: Near-field measurements

of vegetation by laser-induced fluorescence imaging. – SPIE **3868**: 120-131, 1999.

Stober, F., Lang, M., Lichtenthaler, H.K.: Blue, green and red fluorescence emission signatures of green, etiolated, and white leaves. – Remote Sens. Environ. **47**: 65-71, 1994.

Stober, F., Lichtenthaler, H.K.: Studies on the constancy of the blue and green fluorescence yield during the chlorophyll fluorescence induction kinetics (Kautsky effect). – Radiat. environ. Biophys. **32**: 357-365, 1993.

Strasser, R.J., Srivastava, A., Tsimilli-Michael, M.: The fluorescence transient as a tool to characterize and screen photosynthetic samples. – In: Yunus, M., Pathre, U., Mohanty, P., (ed.): Probing Photosynthesis: Mechanism, Regulation and Adaptation. Pp. 445-483. Taylor & Francis, London 1998.

Szabó, K., Lichtenthaler, H.K., Kocsányi, L., Richter, P.: A CCD-OMA device for the measurement of complete chlorophyll fluorescence emission spectra of leaves during the fluorescence induction kinetics. – Radiat. environ. Biophys. **31**: 153-160, 1992.

Tuba, Z., Lichtenthaler, H.K., Csintalan, Z., Nagy, Z., Szente, K.: Reconstitution of chlorophylls and photosynthetic CO_2 assimilation upon rehydration of the desiccated poikilochlorophyllous plant *Xerophyta scabrida* (Pax) Th. Dur. et Schinz. – Planta **192**: 414-420, 1994.