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Principles and Characteristics of Multi-Colour Fluorescence Imaging of Plants[1,2]

CLAUS BUSCHMANN and HARTMUT K. LICHTENTHALER
Botanical Institute II, University of Karlsruhe, D-76128 Karlsruhe, Germany

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Summary

Green plants illuminated with UV-radiation emit a blue and green fluorescence as well as a red and far-red chlorophyll fluorescence with maxima near 440, 520, 690 and 740 nm, respectively. In contrast to point data measurements, the establishment of a high spatial resolution multi-colour fluorescence imaging system, the Karlsruhe/Strasbourg Fluorescence Imaging System (FIS), permits to simultaneously screen several hundred picture elements (pixels) of the leaf in the four fluorescence bands of plants, and opens new possibilities for physiology measurements and early stress detection. This review describes the principles and particular characteristics a) of the plants' fluorescence signatures, b) of fluorescence imaging in general and c) of the Karlsruhe/Strasbourg high resolution multi-colour fluorescence imaging system FIS. The origin of the fluorescence signals in the blue and green (cinnamic acids and other plant phenolics), the red and far-red (chlorophyll *a*) and the factors influencing these fluorescence signatures are explained. By means of an intensified video camera, fluorescence images with several hundred picture elements are taken, not only of the red and far-red Chl fluorescence, but also of the blue and green plant fluorescence, as multi-colour images. Via computer-aided data processing one obtains false colour images not only of the fluorescence intensity, but also of the fluorescence ratios of whole leaves. The fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F740) proved to be very sensitive early stress and strain indicators of plants. The fluorescence ratio F690/F740 is an indicator of the *in situ* Chl content. By fluorescence imaging of the variable Chl fluorescence (Rfd-values) one can also simultaneously measure the potential photosynthetic activity of all points of a leaf. At short-term stress, the fluorescence ratio blue/green (F440/F520) is relatively stable, but changes at long-term stress, often by an increase of the green fluorescence emission. Due to the high statistic confidence (large pixel numbers) and the excellent possibilities to detect specific distribution gradients and local irregularities in fluorescence emission and fluorescence ratios over the leaf area, fluorescence imaging is a superior means for early stress and damage detection in plants.

Key words: Chlorophyll fluorescence, blue-green fluorescence, fluorescence images, fluorescence ratios, stress detection.

Abbreviations: a+b = chlorophyll *a*+*b;* CCD = charge coupled device; Chl = chlorophyll; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethyl urea = diuron; FIS = fluorescence imaging system; F440 = blue fluorescence near 440 nm; F520 = green fluorescence near 520 nm; F690 = red chlorophyll fluorescence near 690 nm; F740 = far-red chlorophyll fluorescence near 740 nm; F440/F520 = fluorescence ratio blue/green; F440/F690 = fluorescence ratio blue/red; F440/F740 = fluorescence ratio blue/far-red; F690/F740 = Chl fluorescence ratio red/far-red; UV-A = ultraviolet radiation (315–390 nm); x+c = total leaf carotenoids (xanthophylls + carotenes).

[1] Dedicated to Prof. Dr. Martin Bopp, Heidelberg on the occasion of his 75th birthday.
[2] This review was presented at the International Congress «Stress of Life», July 1–5, 1997 in Budapest in the Session «Plant Stress Detection by Reflectance and Fluorescence».

1. Introduction

The growing demand for environmental control of vegetation requires the development of fast test and screening techniques which can be applied on a large scale. Spectroscopic detection of electromagnetic radiation has been successfully used as a non-contact and non-destructive method in remote sensing. Today remote sensing is almost exclusively carried out by measuring the reflectance signals and images via aircraft and satellite (e.g. Nilsson, 1995). In recent years, the screening of plants' fluorescence signatures has been developing as a specific tool, which can be applied to detect the functioning and state of health of a plant. Fluorescence represents emission of visible light by organic molecules that had been excited by the absorption of ultraviolet (UV) radiation or short wavelength visible light. Photos of the red Chl fluorescence and blue-green fluorescence of leaf sections using microscopes have already been taken for a longer time as reviewed in the books by Slavik (1996) and by Wang and Herman (1996). The red chlorophyll (Chl) fluorescence had been sensed in the past 30 years in order to obtain information on the functioning of the photosynthetic apparatus (Kautsky and Hirsch, 1931; Lichtenthaler and Rinderle, 1988; Karukstis, 1991; Krause and Weis, 1991; Govindjee, 1995). The blue-green fluorescence as a new genuine plant signature was introduced to plant stress detection in the past 12 years only (Chappelle et al., 1985; Lichtenthaler et al., 1991, 1992; Stober and Lichtenthaler, 1991–1994). The multi-colour fluorescence signatures blue, green, red and far-red have so far been sensed only by point data measurements which come from one single leaf spot and exclusively give information on this leaf spot.

Fluorescence imaging on a larger scale offers fast and remote measurement of fluorescence signals with a high spatial resolution from a leaf to a canopy. In contrast to reflectance signals, the different bands sensed in fluorescence imaging have the advantage of being plant-specific signatures. Since the intensity of a passive reflectance signal (reflectance of sunlight) is at least ten times higher than that of the actively induced fluorescence (UV or blue laser excitation), fluorescence images of plants can only be taken from smaller distances than reflectance images in order to have a good spectral resolution. Furthermore, the instrumentation for fluorescence measurements has to be more sophisticated than that for reflectance measurements, i.e. it needs a strong source of radiation for exciting the fluorescence, and in most cases a synchronized amplification for discriminating the low-intensity fluorescence from the background of high-intensity daylight reflectance. Fluorescence signals of leaves can also be taken by conventional fluorometers, but then only the fluorescence information of a small leaf spot is given (point data measurements) providing just one piece of information per leaf which is rarely representative for the whole leaf area. In contrast, fluorescence images have the advantage to show the variation of the fluorescence signal over the whole leaf area with possibly many hundreds of picture elements (pixels) of fluorescence information per leaf. The pattern and texture of the fluorescence signals do not only replace hundreds of measurements at a time, but also provide a good localization of signals, statistic confidence and the supplementary information of specific signal distribution and ratios which can be used for a better interpretation of the fluorescence signals.

Imaging of delayed Chl fluorescence

Delayed Chl fluorescence is recorded in the dark after a preceding illumination. The recorded delayed fluorescence spectra are identical to those of the prompt Chl *a* fluorescence. The first fluorescence images were taken by measuring the delayed Chl fluorescence at a near distance of a few centimeters to the leaf (Sundbom and Björn, 1977; Björn and Forsberg, 1979; Ellenson and Amundson, 1982; Ellenson and Raba, 1983; Ellenson, 1985). In these cases the samples were illuminated by blue light pulses, and the measurement of the red+far-red Chl fluorescence was carried out during the dark periods between the blue light pulses. A further system to study stress or herbicide induced changes in delayed Chl fluorescence emission of leaves via delayed fluorescence images was described by Blaich et al. (1982).

Imaging of the prompt Chl fluorescence

Later on, images of the prompt Chl fluorescence were taken (again at near distance). Prompt Chl fluorescence represents the red fluorescence emitted within nanoseconds after the onset of illumination of the sample (i.e. in principal simultaneously to the illumination). A sequence of fluorescence images during several minutes of illumination with continuous blue light allowed to follow the photosynthetic induction kinetics (Kautsky effect) of leaves (Omasa et al., 1987). Images of the Chl fluorescence were also taken by exciting with short blue light pulses illuminating the sample at a high repetition rate (Daley et al., 1989; Osmond et al., 1990; Esfeld et al., 1995; Meyer and Genty, 1995; Siebke and Weis, 1995 a–c; Bro et al., 1996). Using the technique of a PAM-fluorometer (pulse amplitude modulation) and applying short light pulses saturating photosynthesis the distribution and the height of photochemical and non-photochemical quenching was determined and presented as an image.

Multi-colour fluorescence imaging

The introduction of the blue and green fluorescences as genuine and additional fluorescence signatures in the stress detection of plants (Chappelle, 1985; Lichtenthaler and Stober, 1990; Lang et al., 1991; Stober and Lichtenthaler, 1992, 1993; Stober et al., 1994) opened new possibilities for fluorescence imaging of plants concentrating on the four main fluorescence bands of plants: blue (F440), green (F520) as well as red (F690) and far-red (F740) Chl fluorescence. Some participants of the EUREKA research programme LASFLEUR on remote sensing of vegetation by laser-induced chlorophyll fluorescence (see Briantais et al., 1993; Lichtenthaler, 1993; Lichtenthaler and Lang, 1995) also made the first approaches for multi-colour fluorescence imaging of plants (Lang et al., 1994 a and b, 1995 a and b; Edner et al., 1994 and 1995). The Karlsruhe/Strasbourg fluorescence imaging system (FIS) using a tripled frequency Nd:YAG laser (λ exc. = 355 nm) proved to be a very suitable system for simultaneous excitation of blue and green fluorescence as well as red and far-red

Chl fluorescence and was developed to an efficient imaging tool for detection of plant stress (Heisel et al., 1996; Lang et al., 1996; Lichtenthaler et al., 1996; Lichtenthaler and Miehé, 1997; Lichtenthaler et al., 1997a). This fluorescence imaging system also has the potential for far-distance remote sensing of the laser-induced fluorescence images.

In this report we are describing the new plants' fluorescence signatures and the technique of the high resolution multi-colour fluorescence imaging system. FIS has been developed in the last five years and offers the possibility to study not only the chlorophyll fluorescence (chlorophyll content and photosynthetic activity), but also the blue-green fluorescence signature as an indicator of bound (cell wall) and soluble cinnamic acids and flavonoids accumulating in leaf tissues (vacuoles) of plants. By forming fluorescence ratios, the multi-colour FIS opens many new possibilities for early stress and strain detection in plants, including the non-invasive simultaneous determination of the potential photosynthetic activity of all points of a leaf. This is not possible with any other ecophysiological method.

2. Characteristics of blue-green fluorescence and chlorophyll fluorescence

When excited by UV-radiation, plants exhibit a fluorescence emission spectrum showing a strong emission in the blue-green region (400–550 nm), and the Chl fluorescence in the red to far-red region (650–800 nm) as shown in figure 1.

Blue-green fluorescence

The blue-green fluorescence of green leaves is primarily emitted from the epidermis cell walls and the leaf veins (Figs. 2A, B and C) by ferulic acid which is covalently bound to the cell wall carbohydrates (Fry, 1982; Hartley, 1973; Harris and Hartley, 1976; Morales et al., 1996; Lichtenthaler and Schweiger, 1998). The blue-green fluorescence is characterized by a maximum in the blue region (440–460 nm) and referred to as F440 or F450, and a shoulder or maximum in the green region (520–530 nm) is termed F520 or F530 (see Table 1). The chemical analysis of ferulic acid content and intensity of blue-green fluorescence demonstrated that the members of the *Poaceae* possess a higher blue fluorescence emission than dicotyledonous plants due to their higher con-

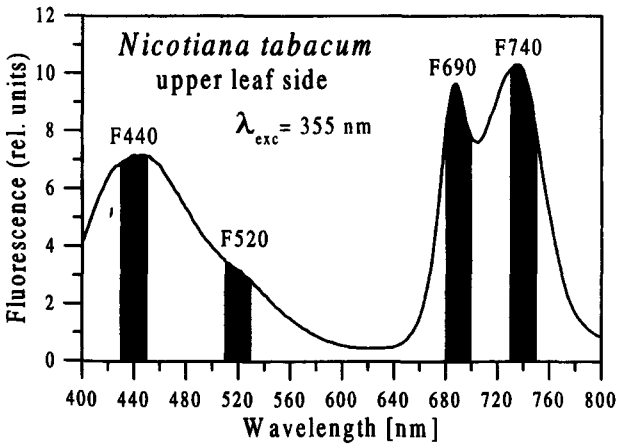
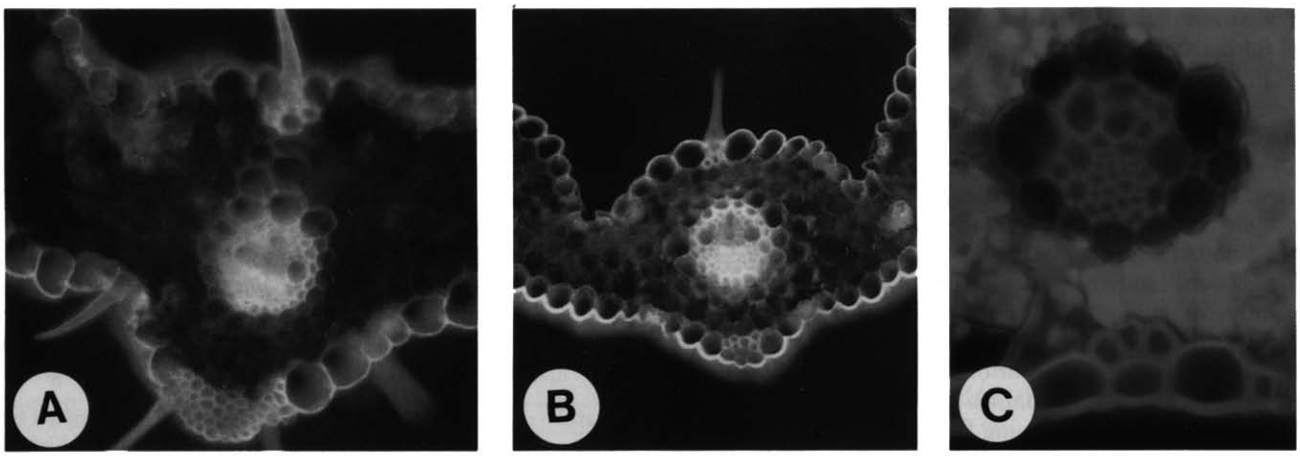
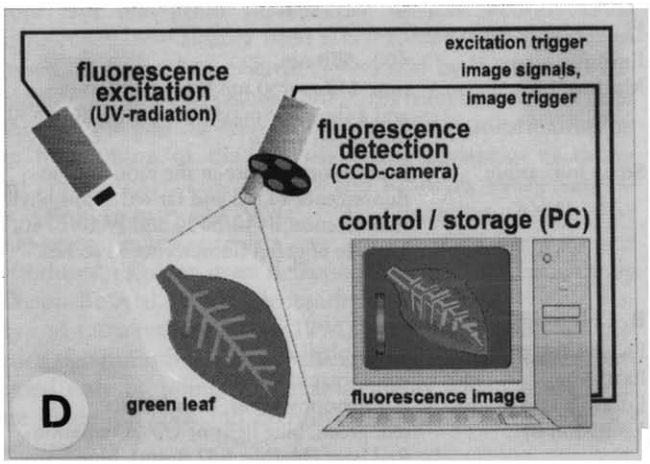


Fig. 1: UV-radiation induced fluorescence emission spectrum of a green tobacco leaf (λ exc. = 355 nm). The fluorescence bands in the blue (F440), green (F520), red (F690) and far-red (F740) region, used for fluorescence imaging of the physiological state of health of plants, are marked by black bars.

Table 1: Characteristics of the blue-green fluorescence and the red and far-red chlorophyll fluorescence emitted by plants.

A. Blue-green fluorescence	
Fluorescing pigments:	mainly ferulic acid covalently bound to cell walls, modulation by cinnamic acids and flavonoids in vacuoles
Extraction:	a) cell wall bound ferulic acid: after alkaline hydrolysis b) soluble cinnamic acids and flavonoids: with aqueous methanol
Location and origin:	a) cell walls (signal coming mainly from leaf epidermis), b) vacuoles (interaction with soluble phenols)
Excitation:	UV radiation (N_2 laser: 337 nm, tripled frequency Nd:YAG laser: 355 nm), pulsed flash lamp (with filters)
Fluorescence characteristics:	
Emission range:	400–570 nm
Maxima:	near 440 to 450 nm (blue F440) and a shoulder/maximum near 520 to 530 nm (green F530)
Stress indication:	increase or decrease in the ratio of blue fluorescence to red and far-red chlorophyll fluorescence: F440/F690 and F440/F740, increase of green fluorescence in some plants
B. Chlorophyll fluorescence	
Fluorescing pigment:	chlorophyll *a*
Extraction:	with organic solvents
Location and origin:	leaf mesophyll cells: chloroplasts
Excitation by:	red, green, blue light or UV-A radiation Red laser (He/Ne: 632.8 nm), blue laser (dye laser) UV-laser (e.g. Nd: YAG laser: 355 nm) Green laser (e.g. doubled frequency Nd: YAG laser: 532.5 nm) Pulsed flash lamp (with appropriate filters)
Fluorescence characteristics:	
Emission range:	650–800 nm
Maxima:	near 690 nm (red F690) and near 730 to 740 nm (far-red F740)
Stress indication:	
short-term stress:	changes in fluorescence induction kinetics at inhibition of photosynthesis, increase of the ratio F690/F740 by 30%.
long-term stress:	decline of chlorophyll content, large increase of the ratio F690/F740; the ratio F690/F740 is an inverse indicator of the *in situ* leaf chlorophyll content


A
B
C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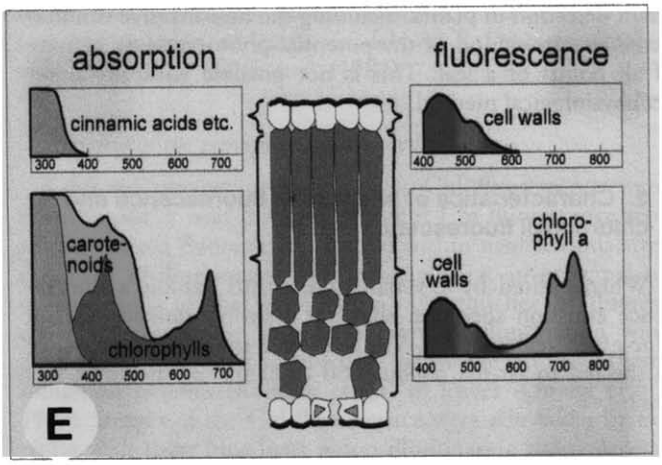
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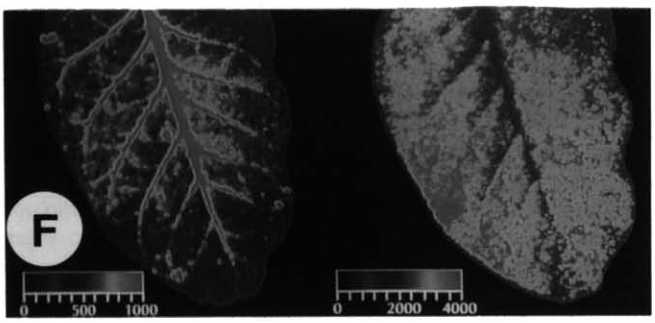
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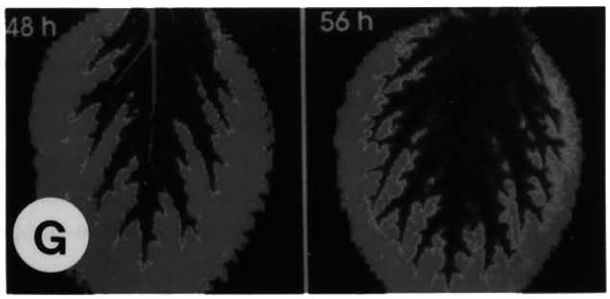
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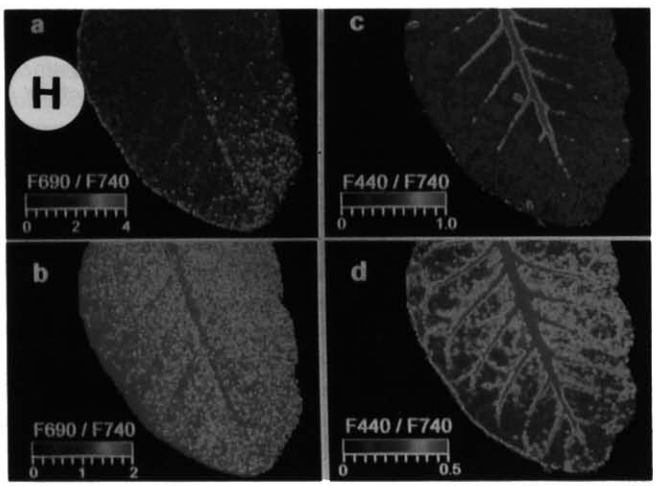
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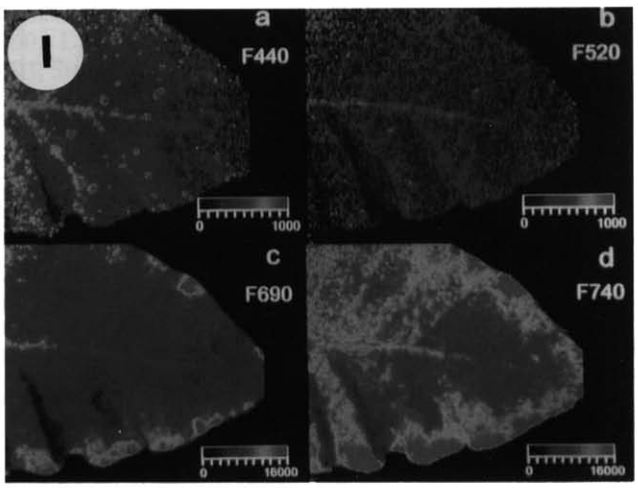
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tent of covalently bound ferulic acid in their cell walls (Lichtenthaler and Schweiger, 1998). The soluble plant phenolics (flavonoids, cinnamic acids etc.), being present in the vacuole of plant cells in particular in epidermis cells, seem to contribute very little to the overall blue-green fluorescence emission of plants (Lichtenthaler and Schweiger, 1998). The blue-green fluorescence is a genuine property of all plant cell walls and can also be seen in the mesophyll cell walls of etiolated leaves (Fig. 2 B), of white chlorophyll-free leaves (treatment with bleaching herbicides) (Stober and Lichtenthaler, 1993 b), of white leaves where pigments had been extracted by repeated shaking in acetone (Stober et al., 1994) and of white leaf parts of variegated plants (Lichtenthaler et al., 1996). With increasing content of chlorophylls and carotenoids in the chloroplasts during greening, the blue-green fluorescence emission decreases due to its partial re-absorption by the chlorophylls and carotenoids in the chlorophyll-carotenoid-protein complexes of the mesophyll chloroplasts (Stober and Lichtenthaler, 1992; Stober et al., 1994). The absorption and fluorescence characteristics of the chlorophyll-free epidermis cells and the green mesophyll cells with their numerous chloroplasts are summarized in a scheme in figure 2 E.

Chlorophyll fluorescence

The red and far-red Chl fluorescence with maxima near 690 and 740 nm (F690 and F740) is emitted by the chlorophyll *a* molecules in the antenna and reaction centre of the photosynthetic photosystem II of the chloroplasts of the mesophyll cells. Some characteristics of the Chl fluorescence are summarized in Table 1 B, details are found in various review papers (Lichtenthaler et al., 1986; Lichtenthaler and Rinderle, 1988; Govindjee, 1995; Lichtenthaler and Miehé, 1997). At room temperature the measurable leaf Chl fluorescence seems to emanate solely from photosystem II, whereas photosystem I apparently contributes only very little (e.g. Lichtenthaler and Rinderle, 1988 a; Govindjee, 1993; Govindjee, 1995; Gitelson et al., 1998).

By point data measurements of leaves it had been proved that the Chl fluorescence ratio red/far-red (F690/F740) is in-

Figs. 2 A–I: Fluorescence of leaf cross sections, schemes of fluorescence imaging and fluorescence emission of leaves as well as multi-colour fluorescence images of differently treated leaves.

Figs. 2 A and B: Fluorescence of the cross section of a 7-day-old green primary leaf of wheat, and a 7-day-old etiolated primary leaf of wheat (*Triticum aestivum* L.) as viewed in a fluorescence microscope (excitation: 365 nm) (Stober et al., 1993 b).

Fig. 2 C: Fluorescence of the cross section of a green leaf of the C_4-plant maize (*Zea mays* L.) as viewed in a fluorescence microscope (excitation: 365 nm). The blue fluorescence is seen in the cell walls of the leaf vein cells and the cell walls of the lower epidermis. The outer cell wall of the epidermis shows a more greenish fluorescence. The red chlorophyll fluorescence is mainly emanated from the mesophyll cells, whereas the bundle sheet cells have only a very faint Chl fluorescence (photo taken by H. R. Bolhàr-Nordenkampf, Vienna, 1997).

Fig. 2 D: Scheme of a multi-colour fluorescence imaging system. The leaf sample is illuminated by a radiation source for fluorescence excitation (in most cases a pulsed UV-laser with a beam expanding optic). The fluorescence excited is detected by a video camera (usually with an intensified Charge-Coupled-Device (CCD) detector). A tele-lense focuses the emitted fluorescence to the CCD-camera. In front of the camera is a filter wheel with the filters transmitting in the blue, green, red and far-red fluorescence emission maximum. The intensified image of the camera is transferred into a personal computer (PC) where the image is stored and processed. The blue, green, red and far-red fluorescence images of the leaf shown on the screen consist of several hundreds of picture elements (pixels), whereby the relative fluorescence intensity is indicated by false colours. The fluorescence intensity increases from the blue (lowest intensity) via green and yellow to red (highest intensity). Here the screen shows an image of the blue fluorescence which is higher in the leaf veins than in the vein-free leaf parts.

Fig. 2 E: Example of absorption and UV-induced fluorescence emission in the chlorophyll-free epidermis cells and the green mesophyll cells of a leaf. Cinnamic acids, covalently bound to the cell walls, absorb in the UV, the carotenoids located in the chloroplasts absorb in the blue, and chlorophylls in the blue and red spectral region. The fluorescence emission spectra of leaves showing a maximum in the blue (440 to 450 nm), a shoulder in the green (520 nm) as well as maxima in the red (690 nm) and the far-red (735 nm) spectral region are composed of the blue green fluorescence of epidermis cells, and the red and far-red chlorophyll fluorescence of the green mesophyll cells.

Fig. 2 F: False colour fluorescence image of a green tobacco leaf (*Nicotiana tabacum* L.). Left: Sum of the blue and green fluorescence, right: sum of the red and far-red chlorophyll fluorescence. Blue-green and chlorophyll fluorescence show a negative contrast (Lang et al., 1994).

Fig. 2 G: False colour images of the variable chlorophyll fluorescence ratio Rfd of a green attached *Digitalis* leaf indicating the progressing loss of photosynthetic activity by the uptake of the herbicide diuron (DCMU) applied via the roots. Left: 48 h, and right: 56 h after herbicide application. The Rfd-values (indicators of photosynthetic activity; see Lichtenthaler and Rinderle, 1988 a) decline from red (highest values at normal photosynthesis) via yellow and green to dark-blue (fully inhibited photosynthesis) (photo M. Lang and M. Sowinska, 1995).

Fig. 2 H: False colour images of the fluorescence ratios a) red/far-red (F690/F740) and c) blue/far-red (F440/F740) of a green tobacco leaf (*Nicotiana tabacum* L.). By choosing a smaller scale (b and d) more details of the gradients in fluorescence ratios can be seen (Lichtenthaler et al., 1996).

Fig. 2 I: False colour images of the blue (F449), green (F520), red (F690) and far-red fluorescence (F740) of a green tobacco leaf (*Nicotiana tabacum* L.) submitted to water stress (2 weeks) and a short-term heat stress of 6 h. Note: the high chlorophyll fluorescence yield (in c and d) at the leaf rim where photosynthesis was impaired by the heat treatment. (For further details see Lang et al., 1996).

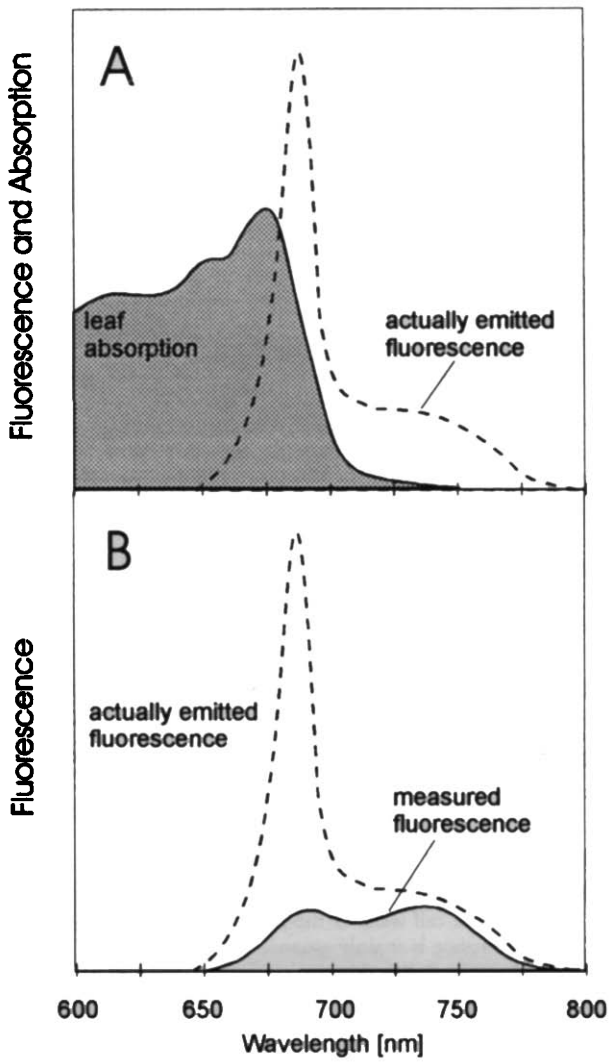


Fig. 3: Effect of partial re-absorption of the actually emitted fluorescence on the measurable chlorophyll fluorescence emission spectrum of a leaf. A. Absorption spectrum of the leaf (chlorophylls) and spectrum of the actually emitted fluorescence, B: Comparison of measured fluorescence emission spectrum with the actually emitted spectrum (see also Fig. 4A).

versely correlated (curvilinear correlation) with the *in situ* Chl content of leaves (Hák et al., 1990; Lichtenthaler and Rinderle, 1988 a; Lichtenthaler et al., 1990; D'Ambrosio et al., 1992; Babani and Lichtenthaler, 1996). This is due to a partial re-absorption of the emitted red Chl fluorescence F690 by the absorption bands of the leaf chlorophyll as indicated in figure 3, whereas the far-red Chl fluorescence F740 is much less affected by re-absorption (see also Gitelson et al., 1998).

Blue and red light excitation: The shape of the Chl fluorescence emission spectrum of leaves also depends on the wavelength of the excitation light. Incident blue light is readily absorbed by carotenoids and by the chlorophylls of the chloroplasts already at the upper part of the palisade cells of the leaf mesophyll. Hence, the major part of the actually emitted Chl fluorescence (excited by blue light) has to cover a short distance for leaving the leaf at the epidermis of the upper leaf side, and the Chl fluorescence is only slightly re-absorbed by *in situ* chlorophyll. In the case of red light (e.g. He-Ne laser; 632.8 nm), however, which is only absorbed by chlorophylls but not by carotenoids, a substantial part of the excitation light penetrates deeper into the leaf mesophyll. As a consequence, the actually emitted average Chl fluorescence excited by red light has to pass more layers of chloroplasts of the leaf mesophyll cells than the blue light emitted fluorescence before it finally leaves the leaf at the upper epidermis. This means, there is more re-absorption of the red light induced Chl fluorescence F690 than in the case of blue light excited Chl fluorescence. Hence, the shape of the Chl fluorescence emission spectrum measured at red light excitation is quite different from that of the blue light excited Chl fluorescence as indicated in figure 4A. As a consequence, the Chl fluorescence ratio red/far-red (F690/F740) exhibits much higher values after blue than after red light excitation (Lichtenthaler and Rinderle, 1988 b).

Green excitation light: Green light (e.g. 532.5 nm; a Nd-YAG laser with doubled frequency) is much less absorbed by the chlorophylls, and therefore some of it penetrates even further into the leaf and to the lower leaf region before it is absorbed and transferred into red Chl fluorescence as compared to red light. The re-absorption of the short wavelength Chl fluorescence form F690 at green light excitation is thus considerably higher, and the Chl fluorescence ratio F690/F740 correspondingly lower than at blue or red light excitation (Fig. 4A).

UV-excitation of Chl fluorescence: UV-radiation, when it is not absorbed in the epidermis layer of leaves, will excite red and far-red Chl fluorescence as well as blue-green fluorescence in the top chloroplast layers of the palisade parenchyma cells just below the epidermis. Laboratory or greenhouse plants, usually grown at a much lower irradiance than outdoor plants, possess a strong UV-A radiation-induced Chl fluorescence. However, the latter is progressively reduced with increasing irradiation under which the plants are grown (Stober and Lichtenthaler, 1993 a) as seen in fluorescence excitation spectra (Schweiger et al., 1996). In outdoor plants exposed to full sunlight and to natural UV-A and UV-B, the level of soluble and extractable flavonoids (flavones, flavonols or chalkons) and cinnamic acid derivatives, in the vacuole including chlorogenic acid is increased several fold in many plants as compared to greenhouse plants (Lichtenthaler and Schweiger, 1998). Most of the incident UV-radiation is then absorbed by the epidermis cells with the consequence that only little UV-radiation penetrates into the green mesophyll cells to excite Chl fluorescence. Hence, the UV-radiation induced measurable red and far-red Chl fluorescence of leaves of many outdoor plants is extremely low.

In such cases it is recommended to switch the tripled frequency Nd:YAG laser (λ exc. = 355 nm), used for exciting the blue-green and red/far-red fluorescence, to the doubled frequency position (λ exc. = 532.5 nm) and to solely excite Chl fluorescence by green light as well. This yields a strong Chl fluorescence signal which can be used to determine induction kinetics and photosynthesis measurements and the *in situ* leaf Chl content via the red/far-red fluorescence ratio F690/F740.

The fluorescence ratios blue/red and blue/far-red

Due to the strong decline of Chl fluorescence in outdoor plants, the strain imposed to the plants by high irradiance and UV-exposure is easily detectable via strongly increasing values of the fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F740) (Stober and Lichtenthaler, 1993 a; Schweiger et al., 1996; Lang et al., 1996). This strain or stress response (Lichtenthaler, 1996) of increasing levels of UV-absorbing plant phenolics in the vacuoles in outdoor plants (Lichtenthaler and Schweiger, 1998) proceeds as part of the UV-protection response and takes place long before a damage of the photosynthetic apparatus can be seen. Damage of the photosynthetic apparatus in the chloroplasts of green mesophyll cells only occurs when the stress-tolerance capacity of the leaves is overcharged by a too high stress dose over a too long time period (for the stress concept of plants see Lichtenthaler, 1996). Thus strain, a stress response of plants that occurs before a damage, is easily detectable via the changes in the values of the fluorescence ratios blue/red and blue/far-red. In fact, both ratios proved to be very sensitive and early indicators of stress and strain exposure of plants and will change as soon as the growth conditions of plants are modified.

The fluorescence ratio blue/green

The ratio of the blue to the green fluorescence F440/F520 is much less influenced by variation in the growth conditions than the fluorescence ratios blue/red and blue/far-red. This may be explained by the fact that under physiological growth conditions both signatures come from one substance, i.e. the cell wall bound ferulic acid. The level of the latter does not seem to rapidly change at stress exposure (Lichtenthaler and Schweiger, 1998). In fact, blue and green fluorescence can serve as an internal fluorescence standard with respect to the more rapidly changing chlorophyll fluorescence. Thus, a short-term inactivation of the photosynthetic quantum conversion by heat stress or after a diuron treatment of leaves, which increases the red and far-red Chl fluorescence and changes the fluorescence ratios blue/red and blue/far-red, does neither affect the blue and green fluorescence emission nor the fluorescence ratio blue/green (Lichtenthaler et al., 1997 a). Under various long-term stress conditions, however, the green fluorescence band F520 can considerably increase, apparently by accumulation of particular green fluorescing organic compounds. This is seen in an increased green fluorescence (sometimes with a green fluorescence emission maximum) and in considerably lower values of the fluorescence ratio blue/green (Lang et al., 1996; Lichtenthaler et al., 1997 b).

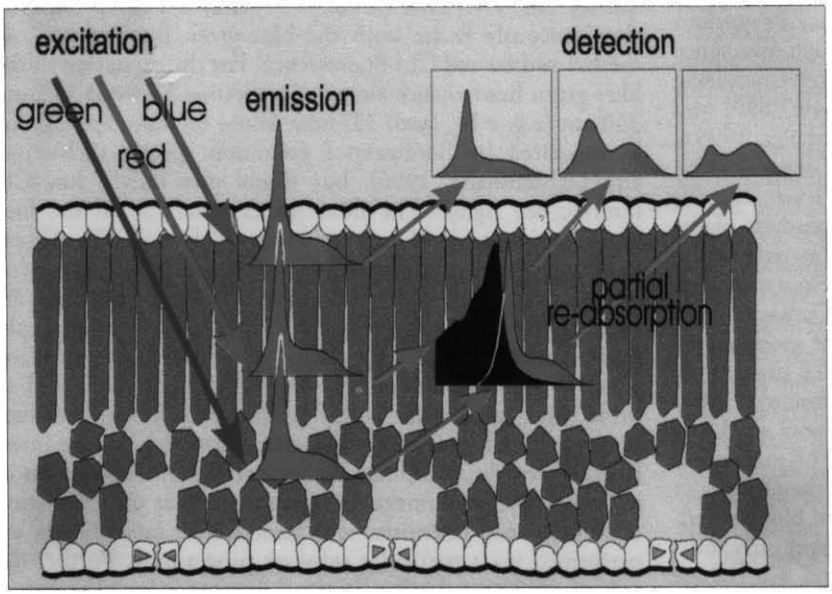
3. Multi-colour fluorescence imaging

3.1 Principles

The basic principles of the set-up of fluorescence imaging of plant leaves as applied in the Karlsruhe/Strasbourg fluorescence imaging system FIS is shown in figure 2 D. The leaf is excited by pulsed UV-radiation (UV-lasers or a pulsed flash light lamp with appropriate filters). The applied tripled frequency Nd:YAG laser (λ exc. = 355 nm) is a compromise to simultaneously excite both the blue-green fluorescence and the red and far-red Chl fluorescence. For the excitation of the blue-green fluorescence alone, an excitation between 330 and 340 nm (e.g. a N_2-laser, 337 nm) would be more appropriate as suggested by fluorescence excitation spectra (Schweiger and Lichtenthaler, 1996), but would give only a low Chl fluorescence signal. The fluorescence detection in the four fluorescence bands blue, green, red and far-red (Fig. 1), excited at 355 nm, is performed via a CCD video camera using appropriate interference filters. The fluorescence images are focused by a lens to the image intensifier tube, gating (amplification of the signal only during the fluorescence emission time) is required to optimize the signal/noise ratio and to avoid interaction of the ambient light with the emitted fluorescence. The fluorescence signals are transferred to the interface card of the computer. Image correction, subtraction of leaf reflectance and image processing as well as the calculation of fluorescence intensities and fluorescence ratio images are performed via a particular software programme. Further details of the Karlsruhe/Strasbourg fluorescence imaging system have been described (Lang et al., 1994; Lichtenthaler et al., 1996; Heisel et al., 1996; Lichtenthaler and Miehé, 1997). The fluorescence intensities in the four fluorescence bands blue, green, red and far-red are expressed in false colours with a colour scale from dark blue (zero fluorescence) via green and yellow to red as highest fluorescence intensity.

The different approaches of the multi-colour fluorescence imaging technique in the past four years are summarized in Table 2. The multi-colour fluorescence imaging system FIS of plants, based on all four fluorescence bands, was established in a joint cooperation of the plant physiologists of Lichtenthaler's group in Karlsruhe and the physicists of Miehé's group in Strasbourg. The aim was to develop a system for near distance and remote sensing of the state of health of plants. The Karlsruhe group also cooperated with the group of Svanberg (Lund, Sweden) who used a tripled frequency, Raman shifted Nd:YAG laser (λ exc. = 397 nm) to excite the red and far-red Chl fluorescence and to obtain remote images and ratio images of the Chl fluorescence (Edner et al., 1995). For the latter, an excitation at 397 nm is very suitable. The blue and green fluorescence intensities, however, when excited at 397 nm, are too low to be used as fluorescence signatures. This has been confirmed by extensive studies using fluorescence excitation spectra (Schweiger and Lichtenthaler, 1996). This fact excluded the Raman shifted Nd:YAG laser as an excitation source for a multi-colour fluorescence imaging, including blue and green fluorescence. The excitation by the frequency tripled Nd:YAG laser (λ exc. = 355 nm) proved, however, to be a good compromise to simultaneously excite blue-green as well as red and far-red Chl fluorescence. A nitrogen laser (λ exc. = 337 nm) is very suitable for blue and green fluorescence excitation, yet it gives only a very low Chl fluorescence intensity since most of the exciting 337 nm UV-radiation is already absorbed in the chlorophyll-free epidermis.

The fluorescence imaging technique with several hundreds of pixels per leaf is an advantage that allows screening of the whole leaf area. Therefore, gradients and irregularities in the fluorescence emission over the leaf area are easily recognized.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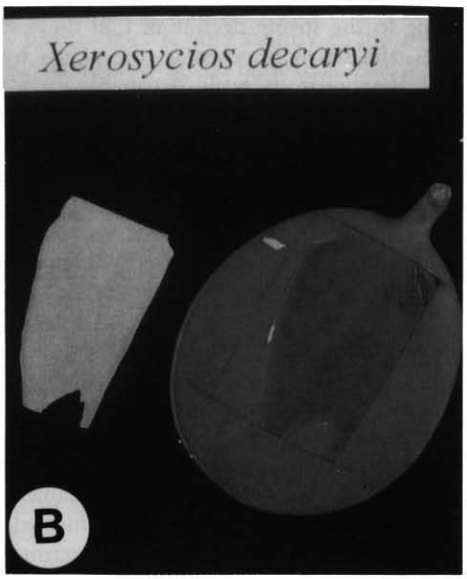
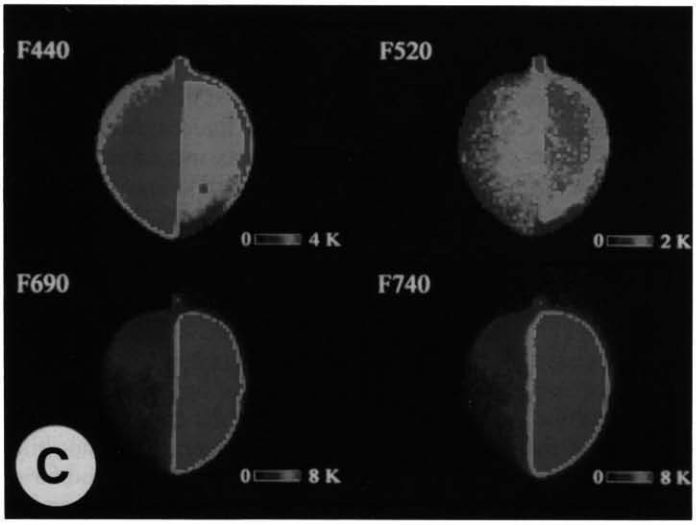

Xerosycios decaryi
B


F440
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0 4 K
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0 8 K
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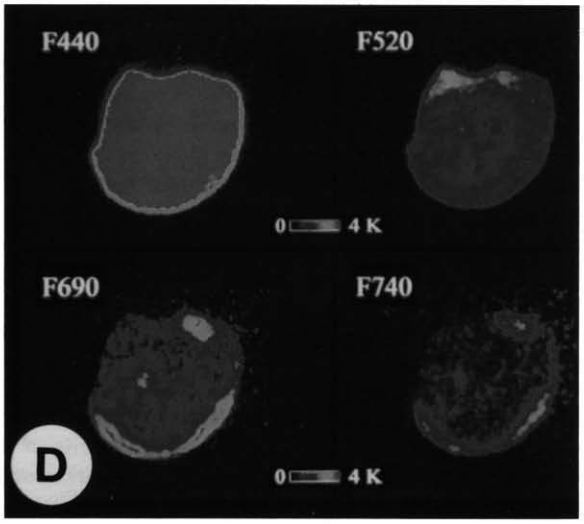
C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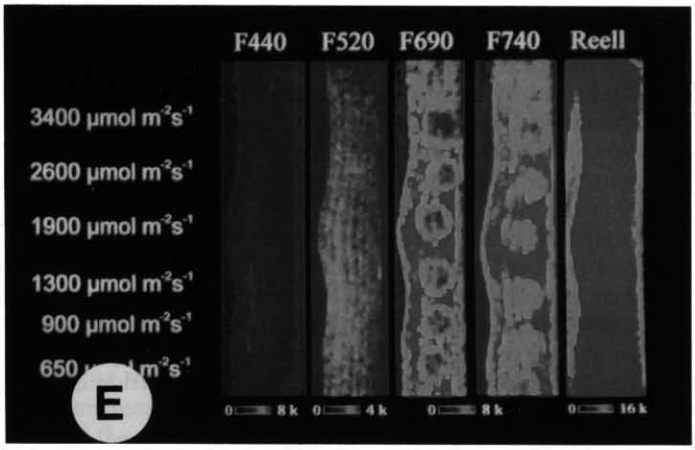
F440
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E

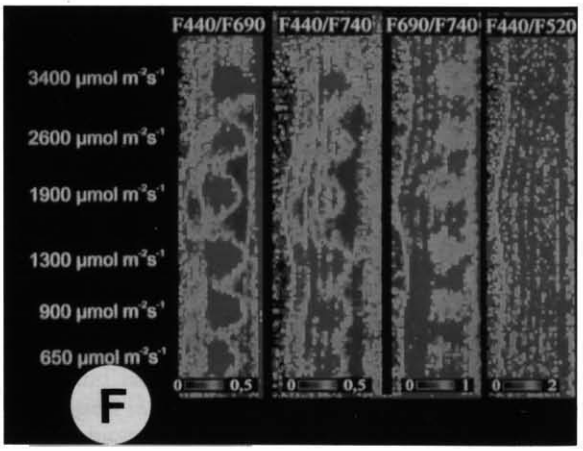
F440/F690
F440/F740
F690/F740
F440/F520
3400 µmol m-2s-1
2600 µmol m-2s-1
1900 µmol m-2s-1
1300 µmol m-2s-1
900 µmol m-2s-1
650 µmol m-2s-1
0 0,5
0 0,5
0 1
0 2
F

Table 2: Applications of multi-colour fluorescence imaging of plants.

Conditions, Effects of	Fluorescence images, Fluorescence type	Plants	Reference
1. Leaf veins/vein-free area, high and low chlorophyll content	blue, green, red, far-red	*Nicotiana tabacum*	Lang et al., 1994a Lang et al., 1994b
2. High and low chlorophyll concentrations, + UV-stress	blue, red, far-red and ratio images	*Acer, Quercus, Brassica, Zea mays*	Edner et al., 1994
3. Water stress, temperature, stress, high irradiance, DCMU, mites	blue, green, red, far-red, ratio images	*Nicotiana tabacum, Prunus laurocerasus*	Lang et al., 1995a Lang et al., 1995b, Lichtenthaler et al., 1995
4. High and low chlorophyll content, DCMU	red, far-red and ratio images	*Bergenia, Clivia, Prunus Iris, Nicotiana tabacum*	Edner et al., 1995
5. Deficiency of nitrogen, zinc, magnesium, iron	blue, red and ratio images	*Zea mays*	Heisel et al., 1996
6. Heat, water and light stress	blue, green, red, far-red and ratio images	*Nicotiana tabacum, Rhododendron* spec.	Lang et al., 1996
7. Leaf veins/vein-fress area, green/white parts of variegated plants	blue, green, red, far-red and ratio images, profiles through a leaf	*Nicotiana tabacum, Campelia zanonia*	Lichtenthaler et al., 1996
8. DCMU-treatment and uptake	blue, green, red, far-red and ratio images	*Nicotiana tabacum*	Lichtenthaler et al., 1997a
9. Drought, DCMU	red, far-red and ratio images of the photosynthetic activity	*Nicotiana tabacum, Digitalis purpurea*	Lichtenthaler and Miehé, 1997

Thus, only fluorescence images of the lower leaf side of a tobacco leaf show that the blue-green fluorescence emanates primarily from the main and lateral leaf veins, whereas the red and far-red Chl fluorescence predominantly come from the vein-free leaf parts (Fig. 2 F). Blue-green fluorescence on the one hand and red and far-red fluorescence on the other hand possess an inverse distribution.

The imaging technique not only allows to present the fluorescence intensities over the leaf area in false colours but also images of the fluorescence ratios as shown for the fluorescence ratios red/far-red and blue/far-red in figure 2H (upper part). Whereas the values of the fluorescence ratio red/far-red exhibit a more or less homogeneous distribution over the leaf area, the fluorescence ratio blue/far-red (F440/F740) posses-

Figs. 4 A–F: Scheme of fluorescence emission in leaves and fluorescence imaging of differently treated leaves and leaf parts.

Fig. 4 A: Scheme of the chlorophyll fluorescence emission of green leaves as induced by blue, red and green excitation light. The actually emitted chlorophyll fluorescence shows *in situ* a high band at 690 nm. Due to the overlap of the F690 band with the absorption spectrum of the chlorophylls inside the leaf a major part of the red fluorescence band is re-absorbed on its way to the leaf surface. The fluorescence measured outside the leaf is thus reduced by re-absorption of the 690 nm fluorescence. The re-absorption is stronger for red and green excitation light penetrating deeper into the leaf than blue light which is readily absorbed by chlorophylls and carotenoids in the first part of the palisade mesophyll cells below the epidermis cells (for further details see Gitelson et al., 1998).

Fig. 4 B: Photo of a leaf of *Xerosycios decaryi* L. where part of the epidermis (left) was removed.

Fig. 4 C: False colour fluorescence images of a leaf of *Xerosycios decaryi* L. with the epidermis removed from the right half of the leaf. Note the decrease in the blue fluorescence F440 and the increase in the green fluorescence F520, and red and far-red fluorescence (F690 and F740) after removal of the epidermis in the right leaf half (Lang, 1995).

Fig. 4 D: False colour fluorescence images of the epidermis peeled-off from a leaf of *Xerosycios decaryi* L.. Note the high fluorescence yield of the blue fluorescence F440 (Lang, 1995).

Fig. 4 E: False colour fluorescence images of a secondary maize leaf (*Zea mays* L.) irradiated by means of a light fiber (circular spots of 12 mm diameter) with white light of different irradiance (quanta fluence rates given on the left hand side). Note the decrease in the red and far-red chlorophyll fluorescence F690 and F740 induced by irradiation. Reel: False colour image of the intensity of white light reflected from the leaf (Lang, 1995).

Fig. 4 F: False colour images of the fluorescence ratios of a maize leaf (*Zea mays* L.) irradiated at circular spots with light of different irradiance (confer Fig. 4 E). The fluorescence ratios are blue/red (F440/F690), blue/far-red (F440/F740), red/far-red (F690/F740) and blue/green (F440/F520). (Image by M. Sowinska and M. Lang, see Lang, 1995).

ses higher values in the leaf veins. Reducing the intensity of the false colour scale by half, one can see further details of the fluorescence ratio distribution over the leaf area (Fig. 2 H, lower part) which might not have shown up when applying the full scale.

3.2 *Applications*

Chl fluorescence induction kinetics, Rfd-values and photosynthetic activity

When green leaves had been pre-darkened for 15 min, upon illumination they show the Chl fluorescence induction kinetics known as Kautsky effect (Kautsky and Hirsch, 1931, reviewed in Lichtenthaler, 1992). These Chl fluorescence transients had first been imaged by Omasa et al. (1987). The Chl fluorescence rises to a maximum (Fm) within 200 ms and then slowly declines to a much lower value within 4 or 5 min, the steady state Fs. The ratio of the fluorescence decrease Fd (from Fm to Fs) to the steady state fluorescence Fs (ratio Fd/Fs), also known as variable Chl fluorescence ratio Rfd (Lichtenthaler and Rinderle, 1988a; Lichtenthaler and Miehé, 1997), is an indicator of the potential photosynthetic capacity of leaves, as has been demonstrated by parallel determination of the net CO_2 assimilation rates (Tuba et al., 1994; Babani and Lichtenthaler, 1996).

Young, fully photosynthetically active leaves usually possess Rfd-values of 3 to 5 which decline with senescence and become zero when the photosynthetic electron transport is blocked by the herbicide diuron (DCMU). By taking red Chl fluorescence images of leaves pre-darkened for 15 min, first at the beginning of the illumination with white light (at Fm), and then again after 5 min (at Fs), one can directly obtain via image processing the false colour images of the Rfd-values of all parts of the leaf area (Lichtenthaler and Miehé, 1997). In other words, imaging of the red Chl fluorescence kinetics and Rfd-value images provide quick information on the actual photosynthetic capacity and performance of all parts of the whole leaf area. No other method exists that would provide such exact ample information on the photosynthetic performance of all leaf points of the leaf area. This opens new possibilities for a non-invasive and distant screening of the photosynthetic activity of leaves.

Using this Rfd-value imaging technique, one can also study the successive loss of photosynthetic activity of a leaf (via decline of the Rfd-values) during the uptake of the photosystem II herbicide diuron applied via the roots (Fig. 2 G). The increasing loss of photosynthetic function at 48 and 56 h after herbicide application is seen via the enlargement of the dark blue parts (Rfd-values and photosynthetic activity = 0) of the leaf. Only the lateral leaf parts (leaf rim) still possess a functioning photosynthetic apparatus with Rfd-values of about 4 (= red colour) (Fig. 2 G).

Diuron uptake by leaves

One can also follow the progressive uptake of the herbicide diuron by imaging the increase in red and far-red Chl fluorescence in places where the photosynthetic electron transport has been blocked by diuron (Lichtenthaler et al., 1997 a;

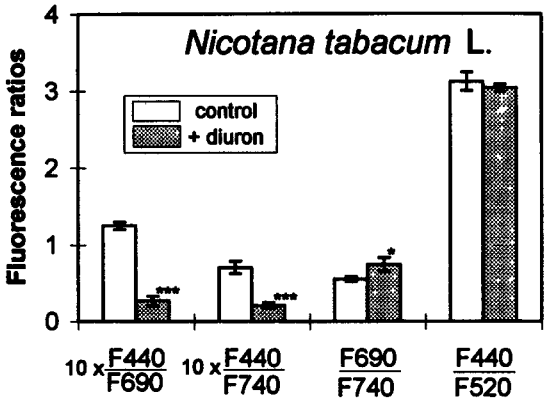

Fig. 5: Decline of the fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F740) in a green tobacco leaf treated with the herbicide diuron (10^{-5} M) via the lower leaf side. The chlorophyll fluorescence ratio red/far-red increased by ca. 30 % as compared to the control. Fluorescence imaging (n = 5) with 300 pixels each. Significance: *** $p < 0.001$ and * $p < 0.05$. (Based on Lang, 1995 and Lichtenthaler et al., 1996). Note: the fluorescence ratios blue/red and blue/far-red are shown at ten times higher value.

Lichtenthaler and Miehé, 1997). In these cases the Chl fluorescence of the induction kinetics remains at maximum fluorescence Fm, and the decline of F690 or F740 to the steady state Fs does not occur (Lichtenthaler and Rinderle, 1988 a). At a diuron inhibition of the photosynthetic quantum conversion, the blue-green fluorescence emission does not change and can be taken as internal standard. As a consequence, in diuron-treated leaves the fluorescence ratios blue/red and blue/far-red declined, the ratio red/far-red significantly increased by 30 % inhibition of photosynthetic electron transport and the ratio blue/green remained constant (Lichtenthaler et al., 1997 a) as shown in Fig. 5.

Effects of heat stress

When water-stressed tobacco leaves were treated with additional heat stress (6 h at 40 °C) the fluorescences F690 and F740 increased in the lateral leaf parts (leaf rim) due to the loss of photosynthetic function, whereas in the centre parts they did not change as compared to control plants (Fig. 2 I). Such gradients from outer to centre parts of the leaf representing early stress symptoms, permit an early stress diagnosis, which can only be observed by the fluorescence imaging technique. Since the blue and green fluorescence did not change during the heat treatment, they can be used as internal fluorescence standard. As a consequence, the fluorescence ratios blue/red and blue/far-red declined, the ratio red/far-red increased by about 25–30 %, whereas the fluorescence ratio blue/green was not changed (Lang et al., 1996).

Fluorescence images with and without epidermis

In order to check the contribution of the epidermis layer to the blue and green fluorescence signatures of leaves, we measured in leaves of *Xerosycios decanji* Humbert (Cucurbita-

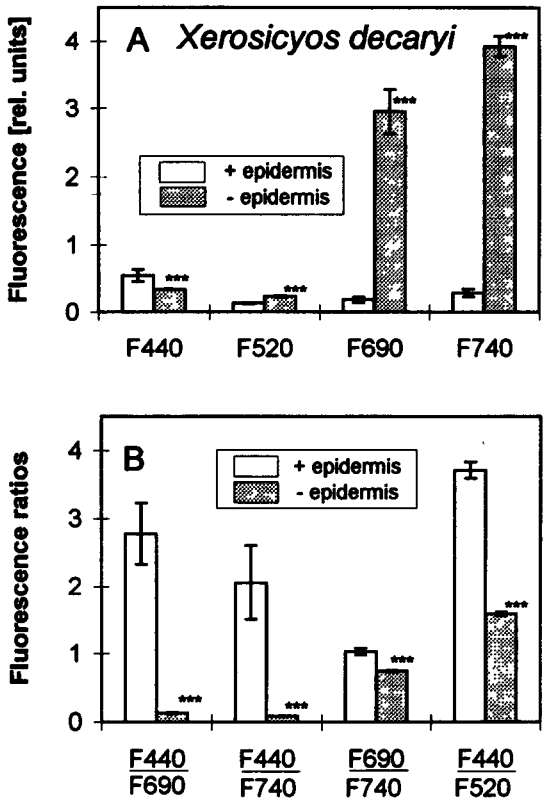

Fig. 6: Intensities of the blue, green, red and far-red fluorescence and differences in the values of fluorescence ratios of a leaf of *Xerosycios decaryi* L. with epidermis (left columns), and with the epidermis removed (right columns), (by Lang, 1995).

ceae) the multi-colour fluorescence emission with and without epidermis. In this plant, the epidermis can easily be peeled off (Fig. 4 B). Multi-colour fluorescence images were taken of *Xerosycios* leaves, where the epidermis had been peeled-off from the right leaf-half. After taking off the epidermis layer, the blue fluorescence F440 strongly declined, the green fluorescence F520 considerably increased, and the red and far-red fluorescences (F690 and F740) were enormously augmented at the right leaf-half (Fig. 4 C). This indicates that the green mesophyll cells can emit strong green, red and far-red fluorescence when the epidermis as barrier for the penetration of exciting UV-radiation into the leaf mesophyll is removed. The differences in fluorescence intensity and in fluorescence ratios with and without epidermis are shown in Fig. 6. When solely the peeled-off epidermis of *Xerosycios* was imaged, a very strong blue fluorescence emission F440 and a distinct green fluorescence F520 (Fig. 4 D) were seen. Some Chl fluorescence was detectable in such places of the epidermis where some chloroplasts of mesophyll cells stuck to it peeling it off. The strong blue fluorescence of the peeled-off epidermis underlines the fact that the major blue fluorescence emission of leaves comes from the epidermis cells as already suggested by the fluorescence photos of leaf sections (Figs. 2 A, B and C).

Photoinhibition treatment

When green maize leaves, grown at low light conditions, were treated by high irradiance of white light of different fluence rates administered via a glass fibre for 10 min, the red and far-red chlorophyll fluorescence emission declined in the illuminated spots (Fig. 4 E). This decline is apparently due to the photoinhibition of the photosystem II catalyzed electron transport in the illuminated spots. Photoinhibition, which is known to also decompose the D1-protein of photosystem II (Krause and Weis, 1991), is associated with a decrease in Chl fluorescence. The blue and green fluorescence emissions F440 and F520, in turn, were not affected by the photoinhibition of the photosynthetic apparatus (Fig. 4 E). As a consequence, the values of the fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F740) are increased, whereas the chlorophyll fluorescence ratio F690/F740 decreased, and the blue/green fluorescence ratio F440/F520 did not change (Fig. 4 F).

Black and white fluorescence images

The differences in fluorescence intensities of different leaf parts cannot only be expressed by means of false colours (increasing from dark-blue to red), but also as black and white images, whereby the fluorescence intensity is shown as an increase from black (no fluorescence) via grey tones to white (highest fluorescence). Two examples are given here.

a) Leaf affected by the white tobacco fly: Black and white images of the blue-green and the red and far-red fluorescence of a green tobacco leaf are shown in Fig. 7. The blue-green fluorescence emanates primarily from the main and lateral leaf veins, whereas the red and far-red Chl fluorescence is predominantly emitted by the vein-free leaf regions. The negative contrast of blue-green and red and far-red fluorescences are as evident in this black and white image (Fig. 7) as given by false colours shown in figure 2 F. When visually examined in white light the leaf looks homogeneously green, since our eyes cannot resolve the emitted fluorescences from the reflected light which exhibits a much higher intensity. The blue-green fluorescence image (Fig. 7, left part) shows, besides the white leaf veins, also various bright white spots distributed over the whole leaf area. These represent punctures of the white tobacco fly. As a phyto-response against the insects, the plant produced phenolic compounds or stilbens at the punctures (probably a phytoalexin response) and these are not visible but detectable by their strong blue-green fluorescence.

b) Leaf affected by a mite attack: Leaves of bean plants (*Phaseolus vulgaris* L.), when grown in the greenhouse, are often affected by mites which at the lower side suck the chloroplasts from the mesophyll cells and damage the leaves. A mite attack is detectable by a strong increase in the blue fluorescence, a slight increase of the green fluorescence, whereas the two chlorophyll fluorescences red and far-red are slightly decreased (Lang, 1995). As a consequence, the fluorescence ratio blue/red (F440/F690) is strongly increased (Fig. 8), whereas the Chl fluorescence ratio red/far-red (F690/F740) is only slightly increased.

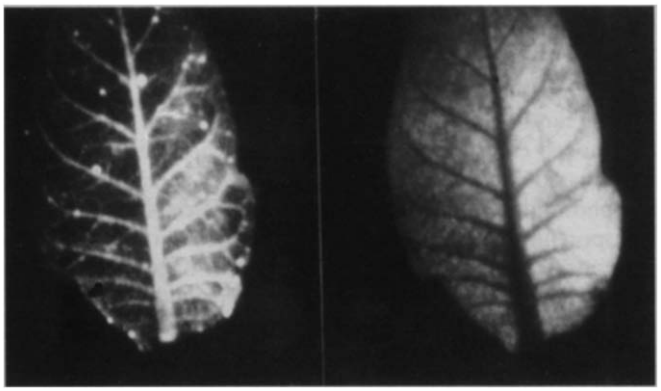

Fig. 7: Images of the blue (left) and the red fluorescence (right) of the lower leaf side of a tobacco leaf (24 cm length) affected by white tobacco flies (by Lang et al., 1994 b).

Changes induced by UV-A treatment

When tobacco plants were grown in the greenhouse for four weeks in white light at a fluence rate of 150 µmol $m^{-2} s^{-1}$ with and without additional UV-A radiation (UV-A fluorescent tubes, Philips TL80W/10R UVA), the fluorescence emission of leaves of controls and UV-A exposed plants was quite different. The Chl fluorescence emission F690 and F740 of the UV-A exposed leaves was considerably lower, and the blue fluorescence F440 higher than in control plants. As a consequence, the fluorescence ratios blue/red and blue/far-red decreased very much (Fig. 9). Since the green fluorescence increased to a higher extent than the blue fluorescence, a significant decrease was observed in the fluorescence ratio blue/green (F440/F520). These latter changes might have been caused by an accumulation of flavonoids and/or other secondary plant products in the vacuoles of the epidermis cells.

N-deficiency

Maize plants (*Zea mays* L.) were grown in the greenhouse: a) on a sand/peat mixture with 50 kg nitrogen per ha and b) without nitrogen addition causing N-deficiency symptoms in the long run. After three weeks, at an early stage of growth and before N-deficiency symptoms were visually detectable, the secondary leaf was assayed for its fluorescence signatures. The plants grown without nitrogen showed a higher blue-green fluorescence than the controls and considerably higher values of the fluorescence ratios blue/red and blue/far-red (Fig. 10). At this stage, differences in the chlorophyll content were not yet detectable, and consequently the Chl fluorescence ratio red/far-red was not yet increased. At later stages, however, also the Chl fluorescence ratio red/far-red increased which then was correlated by a lower Chl content as determined via extraction and photometric analysis. The fluorescence ratio blue/green initially showed little changes, but later a tendency for slightly higher values was observed. An increase in the Chl fluorescence ratio at N-deficiency had also been described by point data measurements in wheat (Hák et al., 1993). Similar changes in fluorescence ratios were also

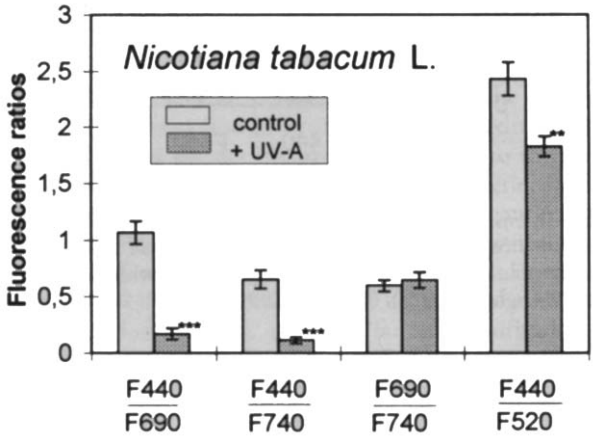

Fig. 9: Changes in the fluorescence ratios blue/red (F440/F690), blue/far-red (F440/F740) and blue/green (F440/F520) in leaves of tobacco plants (*Nicotiana tabacum* L.) grown in white light without and with irradiance by UV-A. Values based on 5 fluorescence emission spectra each. Significance: *** $p<0.001$; ** $p<0.01$.

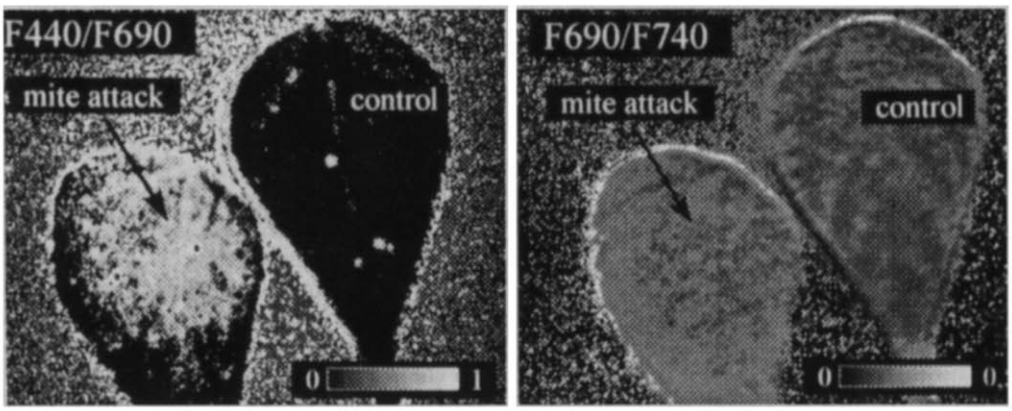

Fig. 8: Fluorescence ratios calculated from images of a bean leaf (*Phaseolus vulgaris* L.) without and with mite attack. The fluorescence ratio blue/red (F440/F690, scale 0–1) increased more than two-fold ($p<0.001$), whereas the chlorophyll fluorescence ratio red/far-red (F690/F740, scale 0–0.5) was increased by about 20% ($p<0.05$). The differences are based on 5 determinations with 180 pixels each (Lang et al., 1995 b).

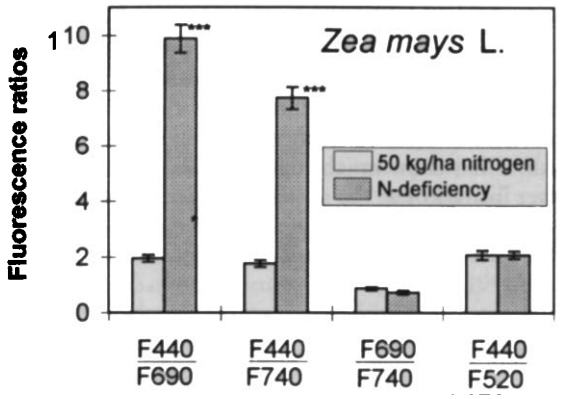

Fig. 10: Differences in the fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F740), red/far-red (F690/F740) and blue/green (F440/F520) in a green maize leaf (control 50 kg N ha^{-1}), and a leaf from an N-deficient maize plant grown on sand/peat. Values taken from 5 fluorescence emission spectra each. Significance: *** $p<0.001$.

seen in N-deficient maize leaves grown at a higher irradiance (Lichtenthaler et al., 1997b).

Sun exposure of leaves

Leaves of *Rhododendron* were exposed to full sunlight for several weeks, whereas other leaves of the same plant were kept in the shade. As compared to shade leaves, the sun-exposed leaves developed a significantly higher blue and green fluorescence, whereas the red and far-red chlorophyll fluorescence decreased drastically (Lang et al., 1996). This was associated with a 2.2 fold increase of the total leaf flavonol content in sun-exposed leaves with quercetin as main substance. The larger part of the leaf flavonols are bound to the vacuoles of the epidermis cells. The decrease in the Chl fluorescence intensity in sun-exposed leaves was thus due to the fact that a larger part of the exciting UV-A radiation (λ exc. = 355 nm) was absorbed in the epidermis, and much less UV-A penetrated into the leaf mesophyll cells to excite Chl fluorescence compared to shade leaves. This has also been observed in leaves of other sun-exposed outdoor plants (Schweiger and Lichtenthaler, 1996; Lichtenthaler and Schweiger, 1998). These changes resulted in a strong increase in the fluorescence ratios blue/red and blue/far-red, a smaller but significant increase of the Chl fluorescence ratio red/far-red (associated with a 25 % lower Chl content), and a strong decrease in the fluorescence ratio blue/green (Fig. 11). The latter was caused by an increase stronger in the green than in the blue fluorescence of un-exposed leaves. It may have been caused by quercetin which, in a solution, can exhibit a green fluorescence.

Green and aurea leaves (differences in Chl content)

The fluorescence emission of a green tobacco variety (su/su) and its dominant aurea mutant (Su/su) exhibiting a retarded greening and a lower Chl content (Schindler et al., 1994), was determined by multi-colour fluorescence imaging and fluorescence emission spectra. The green tobacco plants exhibited a very low blue-green fluorescence. The aurea plants, in turn, exhibited a much higher blue and green fluorescence and also a higher Chl fluorescence, which was different for the red and far-red fluorescence. All four fluorescence ratios blue/red, blue/far-red, red/far-red and blue/green were increased in leaves of aurea plants (Fig. 12). The strong increase in the Chl fluorescence ratio red/far-red (F690/F740) was caused by a chlorophyll content much lower in the aurea leaves. Also, the increase in the blue-green fluorescence is related to the lower content of chlorophylls and carotenoids in the aurea leaf which readily absorb part of the emitted blue-green fluorescence.

Upper and lower leaf sides (differences in Chl content)

The UV-A induced fluorescence signatures sensed from the upper and lower leaf sides of C_3-plants differ considerably. The upper leaf-half with its densely packed long palisade parenchyma cells containing high numbers of chloroplasts possess a much higher chlorophyll content than the lower leaf-half with its spongy parenchyma cells separated by large aerial

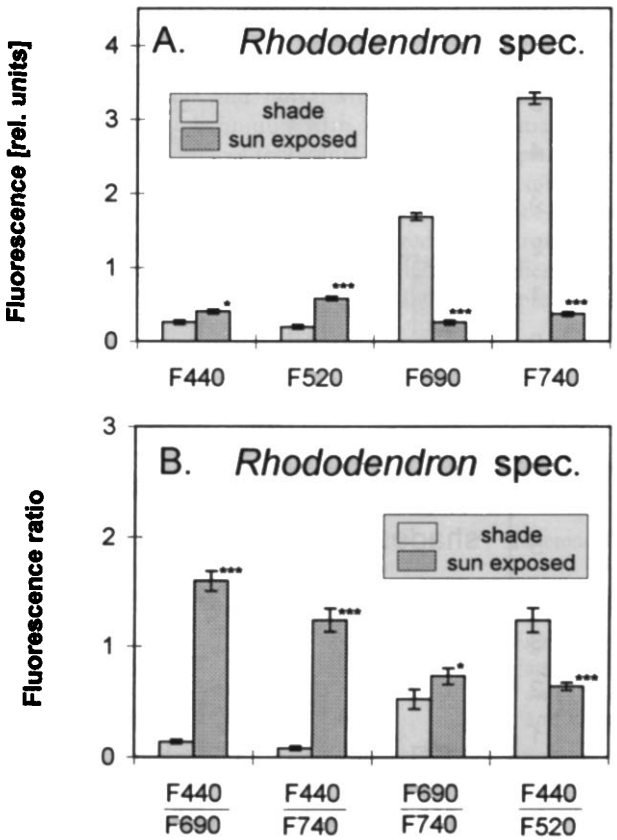

Fig. 11: Differences between shade and sun-exposed leaves of *Rhododendron spec.* (A) in blue (F440), green (F520), red (F690) and far-red (F740) fluorescence emission, and (B) in the fluorescence ratios blue/red, blue/far-red, red/far-red and blue/green as obtained from multi-colour fluorescence images. Mean values of 10 determinations with 200 pixels each. The differences are significant: *** $p<0.001$ and * $p<0.05$ (from Lang et al., 1996).

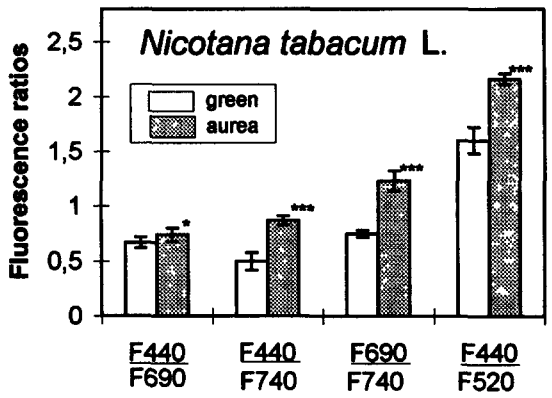

Fig. 12: Differences in the fluorescence ratios blue/red (F440/F690), blue/far-red (F440/F740), red/far-red (F690/F740), and blue/green (F440/F520) in a normal green tobacco variety (*Nicotiana tabacum* L., su/su), and its chlorophyll-poor aurea mutant (su/su). Chlorophyll content: 38 µg a+b /cm^2 leaf area (green leaf) and 18 µg a+b/cm^2 leaf area (aurea leaf). The mean values are based on 5 fluorescence emission spectra each (λ exc. = 355 nm) of different leaves. The differences are significant: *** $p < 0.001$ and * $p < 0.05$.

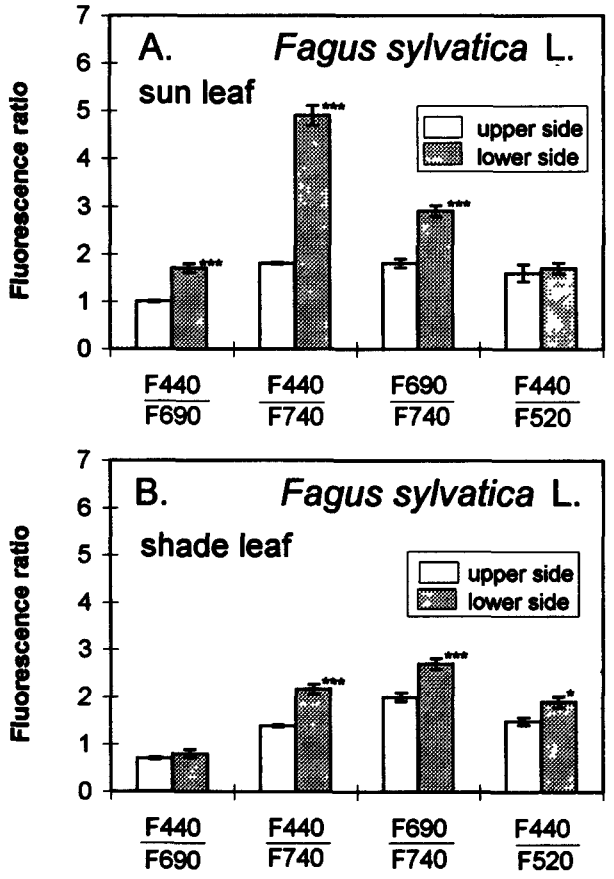

Fig. 13: Differences in the fluorescence intensity ratios blue/red, blue/far-red, red/far-red and blue/green from the upper and lower leaf sides of sun and shade leaves of beech (*Fagus sylvatica* L.). Mean values based on 6 fluorescence emission spectra each (λ exc. = 355 nm). The differences are significant: *** $p < 0.001$ and * $p < 0.05$.

interspaces (see also Fig. 4 A). As a consequence, the blue, green and red fluorescences and, to a lower extent, also the far-red fluorescence are re-absorbed to a higher degree when excited and leaving the upper instead of the lower epidermis. For this reason, the fluorescence ratios blue/red, blue/far-red and red/far-red show much higher values when measured at the lower instead of at the upper leaf side (Fig. 13). These results also indicate that the fluorescence signals are representative for that leaf-half where the fluorescence is excited and measured.

First flush and second flush leaves (high and low Chl content)

In the case of cherry laurel (*Prunus laurocerasus* L.) the leaves with different Chl content were compared using the Karlsruhe/Strasburg fluorescence imaging system in order to obtain further information on the influence of the Chl level on the fluorescence emission signals. In the light green 2nd flush leaf all four measured fluorescence signals were significantly higher than in the dark-green 1st flush leaf (the blue fluorescence 1.5×, the green 1.8×, the red 9.5× and the far-red chlorophyll fluorescence 4.4×) (Lang, 1995). As a consequence, the fluorescence ratios blue/red and blue/far-red were significantly lower in the 2nd flush leaf, the Chl fluorescence ratio red/far-red (F690/F740) much higher and the fluorescence ratio blue/green distinctively lower, as shown in Fig. 14. This demonstrates that a high proportion of the emitted blue and green fluorescence is re-absorbed by the the chlorophylls and carotenoids in the dark-green 1st flush leaf.

With decreasing Chl content of leaves (from 60 to about 12 µg a+b cm^{-2} leaf area), e.g. in senescing leaves, the red and far-red Chl fluorescence emissions first rise to much higher intensities, whereby the rise for the red fluorescence F690 is higher than for the far-red fluorescence F740. At Chl values <10 µg Chl a+b cm^{-2} leaf area, the red and far-red Chl

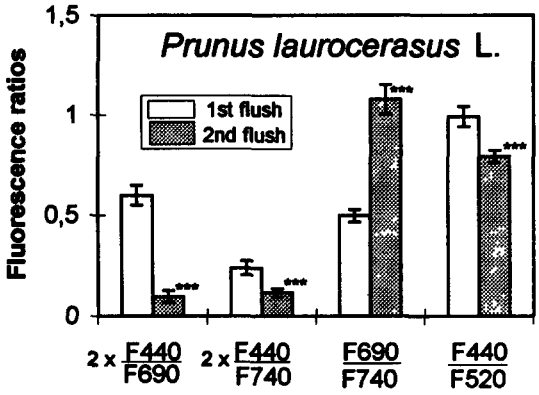

Fig. 14: Differences in the fluorescence intensity ratios blue/red, blue/far-red, red/far-red and blue/green of green cherry laurel leaves (*Prunus laurocerasus* L.). Comparison of a dark-green leaf of the 1st flush and a light green leaf (2nd flush in July) which did not turn fully green. The pigment content amounted to 55.6 µg a+b and 13.1 µg x+c cm^{-2} (green leaf, 1st flush), and 17.1 µg a+b and 6.9 µg x+c cm^{-2} leaf area (light green, 2nd flush leaf). The mean values are based on 10 images with 200 pixels each. The differences are highly significant: *** $p < 0.001$ (taken from Lang, 1995). Note: the fluorescence ratios blue/red and blue/far-red are shown as two fold values.

fluorescences F690 and F740 decline with decreasing Chl content. Thus, the light-green cherry laurel 2nd flush leaf with 17.1 µg Chl a+b cm^{-2} leaf area almost reached the maximum Chl fluorescence intensity a plant leaf can yield. This enormous increase in Chl fluorescence in the 2nd flush leaf with a much lower Chl content demonstrates that in dark-green leaves the largest portion of the originally *in situ* emitted Chl fluorescence in the leaf is re-absorbed by the leaf chlorophyll. In fact, by deconvolution of the measurable Chl fluorescence emission spectrum Gitelson et al. (1998) showed that the recalculated retrieved red Chl fluorescence F690, originally emitted in the leaf mesophyll, was eight to ten times higher than the measured Chl fluorescence.

White and green leaf parts of variegated leaves

When the red and far-red Chl fluorescence signals decline below 10 µg a+b cm^{-2} leaf area at a decreasing Chl content, e.g. during autumnal senescence, the blue and green fluorescences still rise. Then the fluorescence ratios blue/red and blue/far-red will again yield higher values which further increase with increasing loss of Chl content. As a consequence, white leaf parts of variegated plants possessing only trace amounts of Chl a+b are characterized by very much higher values for the fluorescence ratios blue/red and blue/far-red than the green leaf parts, as can be calculated from the imaging results in the variegated leaf *Campelia zanonia* L. (Lichtenthaler et al., 1996) indicated in Table 3.

Thus, when judging the changes of the fluorescence ratios blue/red and blue/far-red of leaves as induced by stress constraints, one always has to consider the Chl content of the leaves because the fluorescence ratios blue/red and blue/far-red can decrease (from high to low Chl content) or again increase from low to very low Chl content.

4. Fluorescence imaging and stress detection

The absolute emission signal of leaves can vary from sample to sample due to small differences a) in the excitation and sensing angles of the fluorescence, and b) the roughness and scattering properties of the leaf surface. Thus, the absolute fluorescences usually vary to a larger degree than the fluorescence ratios. Although one always needs to know if in a stressed plant the four fluorescence bands blue, green, red and far-red increase or decrease in the mean as compared to a control, one should never rely on the increase or decrease of the fluorescence intensities alone. The fluorescence ratios, in turn, exhibit a much lower variation from leaf to leaf, and represent reliable and reproducible means for the quantification of changes in the fluorescence characteristics of leaves and plants as induced by changes in the growth conditions (temperature, humidity, indoor and outdoor plants etc.), or by the various kinds of stress constraints and mineral deficiencies. It is known that during their lifetime plants can be exposed to various types of biotic and abiotic stresses as well as to natural and anthropogenic stressors (Lichtenthaler, 1996). In the long run all stressors can and will change one or several of the four fluorescence ratios presented here. The fluorescence ratios blue/red and blue/far-red proved to be very early stress and strain indicators, since they change with strain and stress begin and long before damage of the photosynthetic apparatus is detectable.

The non-invasive and non-destructive multi-colour fluorescence imaging technique with fluorescence images and fluorescence ratio images represents an excellent means to differentiate between certain groups of stress constraints. Although one cannot identify a stressor by fluorescence imaging alone, one can, however, considerably reduce the number of possible stress constraints to a few. The latter can then be further diminished to a single one by applying complementary

Table 3: Changes of fluorescence ratios blue/red, blue/far-red, red/ far-red and blue/green in leaves as indicators of strain, stress and damage situations in plants. The fluorescence ratios were determined by fluorescence imaging, and in some cases via fluorescence emission spectra.

Conditions	F440/F690 blue/red	F440/F740 blue/far-red	F690/F740 red/far-red	F440/F520 blue/green	References
Variegated/greeen tissue	++	++	++	0	[4] and text
Photoinhibition	++	++	−−	0	Figs. 4E+4F
Water deficiency	++	++	0	0	[1] and [3]
Sun exposure	++	++	+	−−	[3] and Fig. 11
Mite attack	++	++	0	+	[2] and Fig. 8
Heat treatment	−−	−−	0	−	[3] and Fig. 2I
UV-A treatment	−−	−−	0	+	Fig. 9
Diuron	−−	−−	+	0	[4,5]+Fig. 5
2nd flush/1st flush leaf	−−	−−	++	−	Fig. 14
Aurea/green leaf	+	++	++	+	Fig. 12
N-deficiency	++	++	+	0	Fig. 10
Lower/upper leaf side (sun leaf of beech)	++	++	+	0	Fig. 13

++ = strong rise, + = rise in fluorescence ratio; −− = strong decrease, − = decrease of fluorescence ratio, 0 = no significant change
References: [1] Lang, 1995; [2] Lang et al., 1995 b; [3] Lang et al., 1996; [4] Lichtenthaler et al., 1996; [5] Lichtenthaler et al., 1997

methods, e.g. a chemical analysis in cases of mineral deficiencies. This considerably reduces the time consuming search and efforts applied so far and also the costs of large scale chemical and other investigations.

Some stressors increase the fluorescence ratios blue/red and blue/far-red, whereas others can decrease them as summarized in Table 3. The fluorescence ratios red/far-red and blue/green may show the same tendency, increase or decrease, or change in opposite directions as the blue/red and blue/far-red fluorescence ratios. This is then a fast means for obtaining quick information on type of changes occurred and stressors involved. By multi-colour fluorescence imaging one can also obtain fast information on the photosynthetic capacity of leaves via determination of the Chl fluorescence images of 15-min pre-darkened plants immediately after illumination at Fm, and 5 min later in the steady state Fs of the Chl fluorescence induction kinetics. The consecutive processing of the Rfd-ratio images (Rfd = Fd/Fs) provides direct information on the actual photosynthetic activity of leaves (Lichtenthaler and Miehé, 1997).

Conclusion

The multi-colour fluorescence imaging technique described here is applicable to near and far distance up to 10 or 20 m. It can easily be extended for remote sensing of plants. One cannot only detect strain, stressors or damage to plants, and to the photosynthetic apparatus of leaves, but also follow the regeneration of the plants, when, after early stress detection, countermeasures are taken and the stressors are removed. Thus, laser-induced or flash-light induced high resolution fluorescence imaging is a superior technique for stress detection in plants. Its application in agriculture in the cultivation of crop plants from germination to harvest, and in the controlled application of nutrients, herbicides, fungicides and insecticides only at times when really necessary is possible. This topic is matter of the present cooperative research of the Karlsruhe and Strasbourg research groups within a joint EU Interreg II research programme.

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