



Special issue in honor of Prof. H.K. Lichtenthaler

REVIEW

## Multi-colour fluorescence imaging of photosynthetic activity and plant stress

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### Abstract

Imaging the four fluorescence bands of leaves, the red ( $F_{690}$ ) and far-red ( $F_{740}$ ) chlorophyll (Chl) fluorescence as well as the blue ( $F_{440}$ ) and green ( $F_{520}$ ) fluorescence of leaves and the corresponding fluorescence ratios is a fast and excellent nondestructive technique to detect the photosynthetic activity and capacity of leaves, of gradients over the leaf area as well as the effect of various strain and stress parameters on plants. This review primarily deals with the first and pioneering multi-colour fluorescence imaging results obtained since the mid-1990s in a cooperation with French colleagues in Strasbourg and in my laboratory in Karlsruhe. Together we introduced not only the joint imaging of the red and far-red Chl fluorescence but also of the blue and green fluorescence of leaves. The two instrumental setups composed for this purpose were (1) the Karlsruhe–Strasbourg UV-Laser Fluorescence Imaging System (Laser-FIS) and (2) the Karlsruhe Flash-Light Fluorescence Imaging System (FL-FIS). Essential results obtained with these instruments are summarized as well as the basic principles and characteristics of multi-colour fluorescence imaging. The great advantage of fluorescence imaging is that the fluorescence yield in the four fluorescence bands is sensed of several thousand up to 200,000 pixels per leaf area in one image. The multi-colour FIS technique allows to sense many physiological parameters and stress effects in plants at an early stage before a damage of leaves is visually detectable. Various examples of plant stress detection by the multi-colour FIS technique are given. *Via* imaging the Chl fluorescence ratio  $F_{690}/F_{740}$  it is even possible to determine the Chl content of leaves. The FIS technique also allows to follow the successive uptake of diuron and loss of photosynthetic function and to screen the ripening of apples during storage. Particularly meaningful and of high statistical relevance are the fluorescence ratio images red/far-red ( $F_{690}/F_{740}$ ), blue/red ( $F_{440}/F_{690}$ ), and blue/green ( $F_{440}/F_{520}$ ) as well as images of the fluorescence decrease ratio  $R_{Fd}$ , which is an indicator of the net  $CO_2$  assimilation rates of leaves.

**Keywords:** chlorophyll fluorescence decrease ratio; chlorophyll fluorescence induction kinetics; fluorescence ratio images; Kautsky effect.

### Highlights

- Introduction to the basic principles of imaging the fluorescence signatures of plants
- Determination of  $P_N$  rates and chlorophyll content by imaging the fluorescence ratios  $R_{Fd}$  and  $F_{690}/F_{740}$
- Early plant stress detection by imaging the fluorescence ratios blue/red and blue/far-red

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**Abbreviations:** Chl – chlorophyll;  $F_0$  – ground chlorophyll fluorescence;  $F_{440}$  and  $F_{520}$  – blue and green fluorescence;  $F_{690}$  and  $F_{740}$  – red and far-red Chl fluorescence;  $F_d$  – Chl fluorescence decrease from maximum to the steady state;  $F_m$  – maximum Chl fluorescence;  $F_s$  – steady-state Chl fluorescence;  $F_v$  – variable Chl fluorescence; FIS – fluorescence imaging system; LIDAR – laser-induced detection and ranging;  $P_N$  – photosynthetic net  $CO_2$  assimilation rate;  $R_{Fd}$  – Chl fluorescence decrease ratio  $F_d/F_s$ .

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## Introduction

Green leaves of plants exhibit a distinct genuine red to far-red Chl fluorescence which can be excited by UV radiation and also by blue light. Upon illumination of pre-darkened leaves, this Chl fluorescence goes through an induction kinetic (fast rise and slow decrease), known as Kautsky effect (*see reviews Lichtenthaler 1992, Govindjee 1995*). The slow Chl fluorescence decrease proceeds within 4 to 5 min, however, only when the process of photosynthetic quantum conversion is undisturbed. Many stress factors, such as high irradiance, heat, and water stress or application of herbicides (*e.g.* diuron), attack by mites or viruses affect the Chl fluorescence induction kinetics. For this reason, the study of special aspects of the Chl fluorescence emission proved to be a valuable method to detect plant stress at an early stage so that countermeasures can be taken (*see e.g.* the review Lichtenthaler and Rinderle 1988a and the two books: Lichtenthaler 1988a, Papageorgiou and Govindjee 2004). Usually, such investigations have been and are still performed by measuring the Chl fluorescence at single leaf spots. However, measuring the Chl fluorescence information of only a few spots per leaf is not sufficient, because these spots may not be representative of the physiological situation of the whole leaf, especially, when considerable gradients exist over the leaf area, which is often the case, even under nonstress conditions (*see e.g.* Lichtenthaler *et al.* 2013).

In addition to Chl fluorescence, plants also possess a genuine blue and green fluorescence emission that predominantly come from the cell walls (Fig. 1) and can be excited by UV-A radiation. A typical fluorescence emission spectrum of green leaves shows four fluorescence peaks or shoulders (Fig. 2). The origin and characteristics of the blue and green fluorescence had been investigated in my group already in the years before the technique of fluorescence imaging was developed (Stober and Lichtenthaler 1993a,b; Stober *et al.* 1994). By single-point measurements of leaves we also demonstrated that the fluorescence ratios blue/red, blue/far-red as well blue/green are valid indicators of plant stress.

A more powerful, reliable, and advanced investigation technique, which opened up in the 1990s, is the 'multi-colour fluorescence imaging' *i.e.* imaging of leaves in the two Chl *a* fluorescence emission bands red and far-red as well as in the blue and green fluorescence bands. This allows to image a larger leaf area or whole leaves providing the fluorescence information of several ten or hundred thousand of leaf pixels. Such multi-colour fluorescence imaging systems enable a much more accurate estimation of the extent of stress. Fluorescence images also precisely permit the evaluation of smaller or larger damage gradients over the leaf area as well as changes in *e.g.* Chl fluorescence yield showing up only at the leaf rim, which, quite often, are the first signs of plant stress.

Multi-colour fluorescence imaging is based on the excitation and registration of very short fluorescence pulses in the microsecond or picosecond range which are enhanced by a gated image intensifier and saved by a CCD camera. Pulsed light sources, such as UV-lasers or

pulsed UV-radiation *e.g.* from a flash-light xenon lamp, function as an excitation source. Applying a UV-laser or pulsed UV-radiation, one can record fluorescence images in all four fluorescence emission bands *i.e.* in the red and far-red Chl fluorescence emission bands as well as in the blue and green fluorescence bands.

Already back in July 1993, we remotely sensed in Karlsruhe – in cooperation with Swedish physicists using their LIDAR-based fluorescence imaging system – the red and far-red Chl fluorescence and simultaneously, by selecting appropriate filters, also the blue and green fluorescence of plants from a distance of 50 to 80 m. To my best knowledge, this was the very first time that multi-colour fluorescence images of plant leaves (single ones and/or a group of leaves) were obtained (Lichtenthaler 1994, Edner *et al.* 1995).

Based on these promising and pioneering first multi-colour fluorescence imaging results of 1993, it was my goal to develop a multi-colour fluorescence imaging system (FIS) for imaging the whole leaves in a near distance. This was realized in the mid-1990s in close cooperation

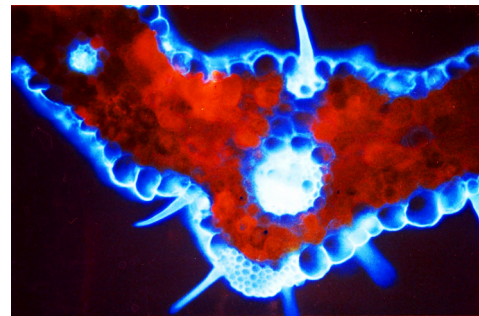


Fig. 1. Origin of the blue and green fluorescence (cell walls of epidermis, vascular bundles, and leaf hairs) and the red + far-red Chl fluorescence (from chloroplasts of mesophyll cells) shown here on a leaf cross-section of a 7-d-old green primary leaf of wheat as viewed in a fluorescence microscope (UV-excitation: 365 nm). (Based on Stober and Lichtenthaler 1993b)

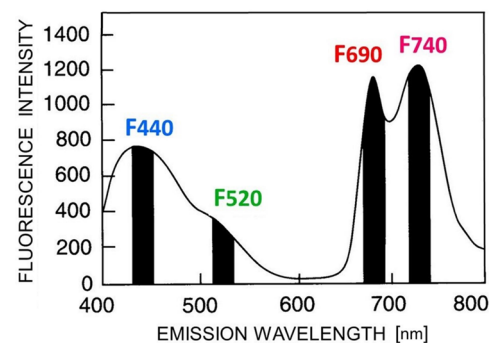


Fig. 2. Fluorescence emission spectrum of an aurea-green tobacco leaf (upper leaf side) excited by UV-A radiation ( $\lambda = 355$  nm) in a *Perkin-Elmer* spectrofluorometer. The four fluorescence bands in the blue (F<sub>440</sub>), green (F<sub>520</sub>), red (F<sub>690</sub>), and far-red (F<sub>740</sub>) spectral region are marked here as black bars.

with French physicists in Strasbourg who had the different technical parts in their laboratory which were required to establish a UV-laser-based multi-colour fluorescence imaging system (Lang *et al.* 1994a, Lichtenthaler *et al.* 1996, Lichtenthaler and Miehe 1997). This was followed by another, easier to handle flash-lamp FIS apparatus in my laboratory in Karlsruhe (Lichtenthaler and Babani 2000, Lichtenthaler *et al.* 2000, Buschmann *et al.* 2008). Early fluorescence imaging systems – some only dealing with imaging the red Chl fluorescence, others also including blue-green fluorescence – have been developed also by a few other groups *e.g.* Genty and Meyer (1995), Ciscato and Valcke (1998), Nedbal *et al.* (2000), Barbagallo *et al.* (2003). Presently, various groups are applying multi-colour fluorescence imaging systems in their research. The general application of these in the detection of plant stress and infections of plants by viruses or fungi has been summarized by Roland Valcke in an excellent overview here in this special issue on fluorescence imaging (Valcke 2021).

In this article, I reviewed our basic and pioneering multi-colour fluorescence imaging results obtained with (1) the Karlsruhe–Strasbourg UV-laser fluorescence imaging system (Laser-FIS) and (2) with the Karlsruhe flash-lamp fluorescence imaging system (FL-FIS). Both are powerful instruments to sense changes and differences in the photosynthetic activity of leaves as well as to detect stress and disease events in plants as is outlined below.

### Origin and characteristics of the red and far-red Chl fluorescence of plant leaves

**Detection of Chl fluorescence:** In 1931, the chemist and former painter Hans Kautsky (1891–1966), then in Heidelberg, detected with bare eyes in a darkened room the red Chl fluorescence of green leaves. He also described the Chl fluorescence induction kinetics and correctly characterized the fast fluorescence rise, followed by a slow decrease to a very low steady-state fluorescence in all the details (Kautsky and Hirsch 1931). In addition, he also detected the inverse relationship between photosynthesis performance and Chl fluorescence emission in stating ‘*the larger the proportion of absorbed radiation converted into chemical energy, the lower the Chl fluorescence intensity in the steady state*’. Moreover, in 1943, Kautsky presented the first evidence on the existence of two photosynthetic light reactions in photosynthesis stating that ‘*two light reactions succeed one another almost immediately*’ (Kautsky and Franck 1943, *see also the review of Lichtenthaler 1992*). At room temperature, the Chl fluorescence and its variable part (the induction kinetic) primarily come from ‘Photosystem II’ of the photosynthetic apparatus, whereas ‘Photosystem I’ contributes relatively little.

**The Chl fluorescence decrease ratio  $R_{Fd}$ :** Since about 60 years, the Chl fluorescence induction kinetics are applied as fast and nondestructive method to study and judge the primary photosynthetic processes and to detect stress conditions in plants (*see review Lichtenthaler and*

*Rinderle 1988a, Lichtenthaler 1988b*). Yet such kinetics could only be measured at single leaf spots. Most authors focused on PSII dealing with details of the light-induced Chl fluorescence rise kinetics (O-J-I-P rise) and their variation and modification by various external and internal factors (*see e.g. Lichtenthaler et al. 1998, Stirbet and Govindjee 2011*). In contrast, my research concentrated on the slow Chl fluorescence decrease,  $F_d$ , from the maximum  $F_m$  to the steady-state fluorescence,  $F_s$ . In fact, the ratio of the Chl fluorescence decrease  $F_d$  to the steady-state fluorescence  $F_s$ , *i.e.* the  $R_{Fd}$ -ratio ( $F_d/F_s$ ), determined at light saturation of leaf photosynthesis, proved to be directly correlated to the photosynthetic net  $CO_2$  assimilation rates of leaves  $P_N$  (Fig. 3). We routinely measured  $R_{Fd}$  values with our laser-induced two-wavelengths Chl fluorometer *LITWaF* (Haitz and Lichtenthaler 1988). When  $F_s$  is determined at light saturation of leaf photosynthesis, the determination of  $R_{Fd}$  values is possible with most Chl fluorometers and also with the pulse-amplitude modulated PAM fluorometer (Lichtenthaler *et al.* 2005a). An example of the correlation of  $R_{Fd}$  values with the photosynthetic rates is given for sun and shade leaves in Fig. 4 (Lichtenthaler and Rinderle 1988a, Lichtenthaler and Babani 2004, Lichtenthaler *et al.* 2005b). The  $P_N$  of shade leaves were in the range of 0.1 to 3.8 and those of sun leaves in the range of 4.8 to 12  $\mu mol(CO_2) m^{-2} s^{-1}$ . In fact, the  $R_{Fd}$  ratio proved to be an excellent vitality index and indicator of stress conditions in plants.

### Shape of the Chl fluorescence emission spectrum:

The red to far-red Chl fluorescence emission spectrum of light-green leaves, measured at room temperature and excited by UV-A radiation or blue light, is characterized by a high maximum in the red (near 690 nm) and the second lower maximum or shoulder in the far-red spectral region between 735 to 745 nm as shown in Fig. 2. The fluorescence bands of these two maxima have been termed  $F_{690}$  and  $F_{740}$ . With the increasing Chl content of leaves, the

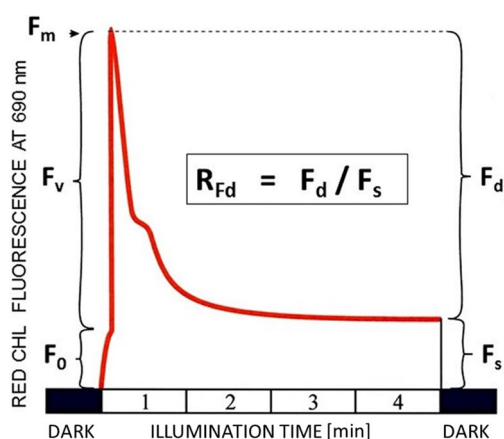


Fig. 3. Light-induced Chl fluorescence induction kinetics (Kautsky effect) in a pre-darkened leaf induced by saturating actinic light. The Chl fluorescence decrease ratio  $R_{Fd}$  is an indicator of the photosynthetic activity (Lichtenthaler and Rinderle 1988a, Lichtenthaler and Babani 2004)

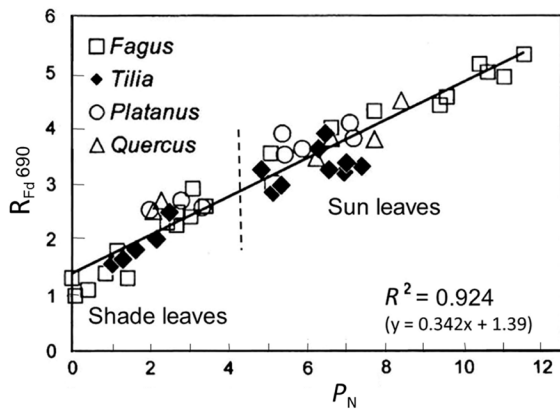


Fig. 4. Linear correlation of the values of the Chl fluorescence decrease ratio  $R_{Fd}$  with the photosynthetic net  $CO_2$  assimilation rates  $P_N$  of leaves, shown here for sun and shade leaves of different trees.  $P_N$  is expressed in  $\mu mol(CO_2) m^{-2} s^{-1}$ . (Based on Lichtenthaler and Rinderle 1988a, Lichtenthaler and Babani 2004)

$F_{690}$  emission maximum decreases due to overlapping of the  $F_{690}$  emission band with the *in vivo* absorption bands of the different Chl-proteins in the thylakoid membranes (Lichtenthaler and Rinderle 1988a, Lichtenthaler 1990, Gitelson *et al.* 1998). As a result, the  $F_{690}$  band decreases by reabsorption to a lower maximum or a shoulder, whereas the  $F_{740}$  band is little affected or slightly increased. As a consequence, the Chl fluorescence ratio  $F_{690}/F_{740}$  declines with increasing Chl content (it is a curvilinear relationship) and can be applied as a noninvasive inverse indicator of the Chl content of leaves (Lichtenthaler and Rinderle 1988a, Hák *et al.* 1990, Lichtenthaler *et al.* 1990). When measuring at slightly shifted neighbouring fluorescence emission bands (at 700 and 735 nm) and forming the corresponding Chl fluorescence ratio  $F_{700}/F_{735}$ , one obtains a direct linear relationship to the Chl content of leaves (Gitelson *et al.* 1999).

**Comparison of blue and red excitation light:** The shape of the Chl fluorescence emission spectrum depends on the excitation wavelength. Using UV-A radiation, the Chl fluorescence yield is relatively low. Since UV-A does not much penetrate into the leaf, the main Chl fluorescence emission peak  $F_{690}$  is well visible, because reabsorption processes of the  $F_{690}$  band play a minor role. The highest Chl fluorescence yield is obtained at blue light excitation (*e.g.* 470 nm), as blue light is absorbed by carotenoids (which transfer their excited states to chlorophylls) as well as by Chl, and this already in the upper leaf layers below the epidermis. Due to this fact, the blue light-induced Chl fluorescence is much less affected by reabsorption of the red emission band  $F_{690}$  as compared to red excitation light, which penetrates much deeper into the leaf layers than blue light. As a consequence, red-light-induced Chl fluorescence emission spectra of green and dark-green leaves usually exhibit only a shoulder in the  $F_{690}$  band, whereas the  $F_{740}$  band is characterized by a clear peak. These relationships have been described for the leaves of various plants (*e.g.* Lichtenthaler and Rinderle 1988a,b).

### Origin and characteristics of the blue and green fluorescence of plant leaves

In addition to the red and far-red Chl fluorescence, green plant leaves also possess a blue-green fluorescence emission, which – as compared to the red and far-red Chl fluorescence – is generally much lower. Also this rather low blue-green fluorescence was detected by Kautsky (Kautsky and Hirsch 1931). This blue-green fluorescence of green leaves is primarily emitted from the cell walls of the Chl-free epidermis cells as well as from those of the Chl-free cells of the leaf veins as is shown in the leaf cross-section together with the Chl fluorescence (Fig. 1). The major source of the blue-green fluorescence is the blue-green fluorophore, ferulic acid, covalently bound to all plant cell walls as has been directly shown *via* a detailed chemical analysis of hydrolyzed cell walls (Lichtenthaler and Schweiger 1998). This genuine blue-green fluorescence is characterized by a high blue fluorescence peak band ranging from 430 to 445 nm (termed  $F_{440}$ ) and a much lower shoulder in the green wavelength region near 520 to 530 nm (termed  $F_{520}$ ) as shown in Fig. 2. It is a characteristic of all cell walls in green, etiolated or white plant tissues (*e.g.* Stober and Lichtenthaler 1993b, Stober *et al.* 1994). Another genuine feature of the blue-green fluorescence of leaves is the fact that it is in general a stable constant signature which – in contrast to the Chl fluorescence induction kinetics (Kautsky effect) – does not change during illumination of green leaves with white light (Stober and Lichtenthaler 1993c). Thus, it can serve as an internal fluorescence standard in relation to the variable red and far-red Chl fluorescence.

Already in the late 1980s and beginning 1990s, we had intensively studied in my group in Karlsruhe the blue-green fluorescence as well as the red and far-red Chl fluorescence *via* fluorescence emission spectra as well as kinetic measurements at single leaf points (Lang *et al.* 1991, Stober and Lichtenthaler 1993b,c; Stober *et al.* 1994). At excitation wavelengths < 340 nm, the blue-green fluorescence yield is high. At longer UV excitation wavelengths, the blue-green fluorescence yield decreases because the absorption of wavelengths > 355 nm by the responsible fluorophores strongly declines. With respect to multi-colour fluorescence imaging of plant leaves, the use of a UV-laser of  $\lambda = 355$  nm is possible, because this wavelength still excites the blue-green fluorescence fairly well but also the red Chl fluorescence.

**Further compounds influencing the blue-green fluorescence emission:** In addition to ferulic acid bound to the cell walls, several other endogenous organic plant substances localized in other cell compartments *e.g.* in the cell vacuole, can also show a rather faint blue or green fluorescence, which can slightly increase and modify the major blue-green fluorescence emitted by the cell walls. These are often secondary phenolic plant products, such as chlorogenic acid, caffeic acid, sinapic acid, or in certain plants, the coumarins aesculetin or scopoletin, or the more widespread catechin as well as some flavonoids in the vacuoles of epidermis cells (*e.g.* the flavonol quercetin).

An overview on the fluorescence emission spectra of such compounds is given by Lang *et al.* (1991). Also reduced NADP, a functional compound in chloroplasts can slightly contribute to the overall blue-green fluorescence emission of leaf tissues. Some of the secondary plant products mentioned above can be accumulated under stress conditions or aging of plant tissues and several of these can increase the green fluorescence band near 520 nm. Tobacco leaves, however, respond to an infection with the *Tobacco mosaic virus* by formation of the blue fluorescent scopoletin (Lenk *et al.* 2007).

**Differences in fluorescence emission spectra between upper and lower leaf side:** The shape of the fluorescence emission spectrum of plant leaves with respect to the relative intensities of the blue-green and the red and far-red Chl fluorescence is quite different for the upper and the lower leaf side. This is mainly due to the fact that in bifacial leaves, the leaf veins, which possess a high blue-green fluorescence yield, stick out from the lower leaf surface. Thus, fluorescence emission spectra excited and sensed on the lower leaf side usually exhibit a higher blue-green fluorescence yield as compared to the red and far-red Chl fluorescence. In contrast, the blue-green fluorescence emission at the upper leaf-side is fairly low as compared to the red and far-red Chl fluorescence. These interrelationships have been investigated in detail by various fluorescence emission spectra of leaf spots with large and small leaf veins as well as of vein-free leaf spots (see Fig. 2 in Lang *et al.* 1994a).

### The Karlsruhe–Strasbourg UV-Laser Fluorescence Imaging System (Laser-FIS)

In cooperation with physicists of the Group d'Optic Appliquée of the Centre des Recherches Nucléaires in Strasbourg, we started in 1993 to establish the first fluorescence imaging system that allows to image and detect the blue and green fluorescence as well as the red and far-red Chl fluorescence emission of green leaves over the whole leaf area (Lang *et al.* 1994a,b, 1996; Lichtenthaler *et al.* 1995, 1996, see also the review of Lichtenthaler and Miché 1997). In this high resolution Laser-FIS, the fluorescence is excited by a pulsed Nd:YAG-laser at 355 nm with a pulse duration of 100 ps and a repetition frequency of 1 kHz. In order to illuminate the whole leaf surface, the excitation beam was broadened. Additional white light from a separate light source was applied before and during the measurements in order to measure in the steady state ( $F_s$ ) of the Chl fluorescence induction kinetics. The fluorescence images of leaves were sensed at a distance of 0.5 m, yet this is also possible from a distance of several meters. The fluorescence images were focused *via* a lens on the image intensifier. The latter was gated to reduce the influence of ambient light. The gate width was settled to a duration of 20 ns, which is sufficient to collect the fluorescence emitted even by one laser pulse. The UV-A excitation wavelength of the laser of 355 nm allows to simultaneously excite the blue and green fluorescence as well as the red and far-red Chl fluorescence which then

is consecutively imaged in the four fluorescence emission bands blue ( $F_{440}$ ), green ( $F_{520}$ ), red ( $F_{690}$ ), and far-red ( $F_{740}$ ) spectral region using four appropriate band-pass filters (half-band width of 10 nm). Depending on the leaf size and the selected leaf area, the individual fluorescence emission of several thousand leaf pixels are simultaneously collected by a CCD (charge-coupled device) camera and then stored by the image-processing system as shown in the scheme in Fig. 5.

This multi-colour fluorescence imaging system has a very high resolution and represents a new and efficient way of stress detection in plants. *Via* computer-aided data processing, the differential fluorescence intensity of the individual leaf pixels is indicated by false colours (false colour images) from blue (zero fluorescence yield) *via* light blue (low fluorescence yield) and green and yellow to red as the highest fluorescence yield. Moreover, the processing software of this Laser-FIS permits to directly present fluorescence ratio images. In fact, we recognized and established the fluorescence ratios of blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ), which contain supplementary information to the hitherto applied Chl fluorescence ratio  $F_{690}/F_{740}$ , as new and efficient additional parameters to detect early stress events in plants.

The fluorescence images are routinely measured using the Karlsruhe–Strasbourg Laser-FIS which excites the fluorescence of leaves by very short pulses of 355 nm. In order to sense fluorescence images under photosynthetic conditions of leaves *i.e.* in the steady state  $F_s$  of the Chl fluorescence induction kinetics, the leaves are illuminated by saturating white light coming from a separate light source. However, by firstly measuring pre-darkened leaves followed by fluorescence images taken during illumination of the leaf with continuous white light of a separate light source, one can also image the changes in Chl fluorescence emission during the Kautsky Chl fluorescence induction kinetics. The differential fluorescence intensity of individual leaf pixels in different parts of the leaf area is then shown by false colours (false colour images) from blue

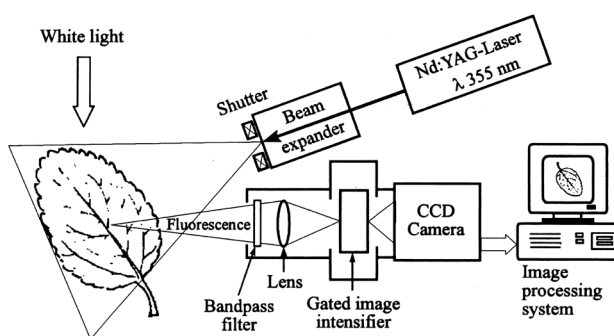


Fig. 5. Setup of the Karlsruhe–Strasbourg multi-colour Fluorescence Imaging System (Laser-FIS) for the simultaneous imaging of blue and green fluorescence as well as red and far-red Chl fluorescence of leaves. The fluorescence emitted, as shown here for one leaf pixel, is simultaneously collected from several ten thousand leaf pixels over the leaf area by a CCD camera and stored by the image processing system. (Based on Lang *et al.* 1994a, Lichtenthaler *et al.* 1996, Lichtenthaler and Miché 1997)

(zero fluorescence yield) *via* light blue and green and yellow to red as the highest fluorescence yield. With this first multi-colour FIS, being jointly composed in the Strasbourg laboratory, we obtained excellent insights into the behavior of the blue-green fluorescence and the red and far-red Chl fluorescence emission of leaves, into gradients over the leaf area, and into the changes induced by different plants stress events. These basic multi-colour fluorescence imaging results were later confirmed using other instruments.

### Multi-colour fluorescence imaging of leaves with the Laser-FIS

#### Fluorescence imaging of the lower leaf side of tobacco leaves:

One of the first examples of imaging the blue, green, red, and far-red fluorescence over the leaf area is shown in Fig. 6 for the lower leaf side of a light-green tobacco *aurea* mutant Su/su (Lang *et al.* 1994a). We detected considerable gradients in the blue-green and also in the red and far-red Chl fluorescence emission over the leaf area. The blue and green fluorescence come primarily from the leaf veins, and are particularly high in the major, the median leaf vein. This is due to the fact that at the lower leaf side of bifacial leaves, the leaf veins, in particular the median leaf vein, stick out from the leaf surface. In contrast, the red and far-red Chl fluorescence predominantly emanate from the vein-free leaf areas *i.e.* the intercostal fields, and come from the green mesophyll cells with their

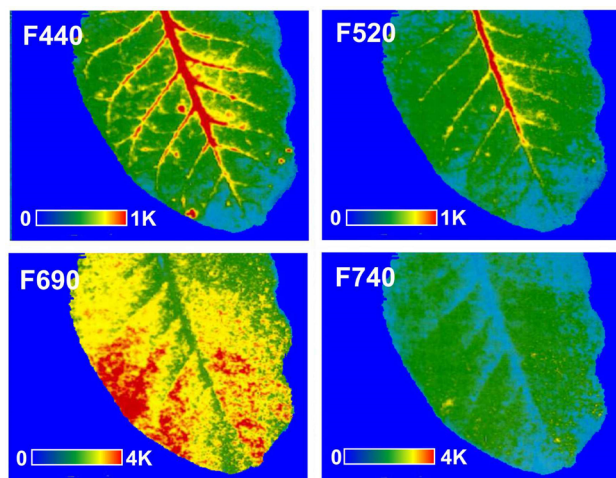


Fig. 6. Fluorescence images in the blue ( $F_{440}$ ) and green ( $F_{520}$ ) as well as red ( $F_{690}$ ) and far-red ( $F_{740}$ ) fluorescence bands of a light-green tobacco leaf (lower leaf-side). The highest blue and green fluorescence emanate from the leaf veins, whereas the highest chlorophyll fluorescence comes from the vein-free leaf regions. Imaging was performed with the Karlsruhe–Strasbourg Laser-FIS (see Fig. 5). The fluorescence yield is presented in false colours which increases from dark blue (no fluorescence) *via* green and yellow to red as the highest fluorescence. K in the scales means kilo (= 1,000) counts. Note that the scales for blue and green fluorescence are different from those of the red and far-red Chl fluorescence. (Based on Lang *et al.* 1994a, Lichtenthaler *et al.* 1996, Lichtenthaler and Miché 1997)

many chloroplasts. Although the cell walls of the green mesophyll cells exhibit a clear blue-green fluorescence (as shown for wheat in Fig. 1), their fluorescence does not or only very little contribute to the blue-green fluorescence sensed at the surface of the leaves, because this blue-green fluorescence is reabsorbed by the carotenoids and Chls in the chloroplasts of the green mesophyll cells. In fact, in this way, the blue-green fluorescence emitted in the mesophyll cell walls contributes to the red and far-red Chl fluorescence emission of leaves. The results, described here for a light-green tobacco leaf, were later found to be the same with bifacial green leaves of other plants, crops, and trees.

**Imaging of the upper leaf side:** When the fluorescence is excited and sensed at the upper leaf side of the tobacco leaf mentioned above and of bifacial leaves of other plants, the situation is somewhat different. At the upper leaf side, the leaf veins (with their Chl-poor leaf bundle cells) are scarcely to be seen (as they do not stick out from the leaf surface as is the case on the lower leaf side). There, the blue-green fluorescence is much lower and more homogeneously distributed over the leaf area. Also the red and far-red Chl fluorescence are fairly evenly distributed over the leaf area when excited and sensed at the upper leaf side. However, differences and gradients in the fluorescence yield are still to be seen. Thus, even under full physiological photosynthesis conditions, the Chl fluorescence can show a slightly higher intensity at the leaf tip and leaf base as compared to the green leaf parts situated more in the center of the leaf. With respect to plant stress detection, it is noteworthy that *e.g.* an early stage of water and heat stress is indicated by a significant increase of the Chl fluorescence yield (both  $F_{690}$  and  $F_{740}$ ) specifically only at the leaf rim as compared to controls (see Fig. 4 in Lichtenthaler and Miché 1997). Similar leaf rim effects are also induced by salt stress to plants (unpublished results). Again, these effects, firstly observed using a tobacco leaf, were later confirmed for leaves of various other plants.

**Fluorescence ratio images:** The fluorescence images of Fig. 6 clearly show that the blue fluorescence yield ( $F_{440}$ ) is considerably higher than that of the green fluorescence ( $F_{520}$ ). In addition, also the intensity of the red fluorescence  $F_{690}$  (in the main maximum of Chl fluorescence emission) is clearly higher than that of the far-red fluorescence (range of 735–745 nm), whereas Chl fluorescence emission spectra (excited here by UV radiation) exhibit a lower peak or only a shoulder. The image processing system of the Laser-FIS permits to form fluorescence ratio images by a pixel-to-pixel division of the fluorescence yield. In this way, one can form fluorescence ratio images of the fluorescence ratios blue/green ( $F_{440}/F_{520}$ ), red/far-red ( $F_{690}/F_{740}$ ) as well as of the ratios blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ). These fluorescence ratio images, made from the fluorescence images of Fig. 6 were analyzed in detail and the results presented in a subsequent paper (Lichtenthaler *et al.* 1996). In fact, due to the gradients of the individual fluorescence bands over the leaf area, the

differences in fluorescence yield at the four fluorescence bands are much better and more reliably recognized in the fluorescence ratio images (see Figs. 3 and 6 in Lichtenthaler *et al.* 1996). This is due to the fact that, in general, the fluorescence ratio values are more evenly distributed over the leaf area and exhibit less gradients as compared to the direct fluorescence values which can show large gradients. This point is highly essential with respect to fluorescence imaging of plant stress. According to our experience, one should always rely on the higher validity of fluorescence ratio images as compared to pure fluorescence images. In addition, we also checked the possible values of images of the fluorescence ratios green/red ( $F_{520}/F_{690}$ ) and green/far-red ( $F_{520}/F_{740}$ ). These fluorescence ratios may exhibit slight differences to the ratio images blue/red and blue/far-red, yet for a routine investigation of plant stress, it is sufficient to analyze the images of the fluorescence ratios blue/red and blue/far-red as well as red/far-red and blue/green.

**Application of the herbicide diuron:** Applying diuron to one leaf half of a tobacco leaf (*via* the lower leaf side) resulted in an inhibition of photosynthetic quantum conversion with a concomitant strong increase of mainly the red ( $F_{690}$ ) and to a lower degree also the far-red Chl fluorescence ( $F_{740}$ ) as compared to the untreated leaf-half (control) (Lichtenthaler *et al.* 1996). The blue and green fluorescence emission remained practically constant. This was clearly visible in a significant decrease of the fluorescence ratios blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ), and an increase of the fluorescence ratio red/far-red ( $F_{690}/F_{740}$ ), whereas the ratio blue/green ( $F_{440}/F_{520}$ ) did not change as shown in a diagram in Fig. 7 (upper part). In a further experiment, we applied diuron ( $10^{-5}$  M) *via* the roots of a young foxglove plant (*Digitalis purpurea* L.). Several hours after diuron application, images of the Chl fluorescence ( $F_{690}$ ) were taken from a green attached leaves of a control plant and a diuron-treated plant during the Kautsky induction kinetics. Chl fluorescence images were taken at maximum fluorescence ( $F_m$ ) and 6 min after onset of illumination with white light in the steady state ( $F_s$ ). In the control leaf the Chl fluorescence strongly decreased from  $F_m$  to  $F_s$  as shown in Fig. 8. In contrast, in the diuron-treated plant, where diuron was successively transported from the roots *via* the bundle sheet cells of the stem to the leaf veins, maximum fluorescence  $F_m$  decreased to  $F_s$  only on those leaf parts, where diuron was not yet present (Lichtenthaler and Miehe 1997).

**Horizontal profiles of fluorescence intensities:** With the aim to test the Laser-FIS, we took fluorescence images in the four fluorescence bands of a variegated green-white leaf of *Campelia zanonina* L. with alternating green and white longitudinal stripes. As expected, the blue-green fluorescence was high in the white leaf stripes and the red and far-red fluorescence were high in the green leaf stripes. In fact, we found an inverse distribution of the  $F_{440}$  and green fluorescence ( $F_{520}$ ) as compared to the red ( $F_{690}$ ) and far-red ( $F_{740}$ ) Chl fluorescence (Lichtenthaler *et al.* 1996). This is visualized from fluorescence images of the leaf by forming horizontal profiles of the four fluorescence bands

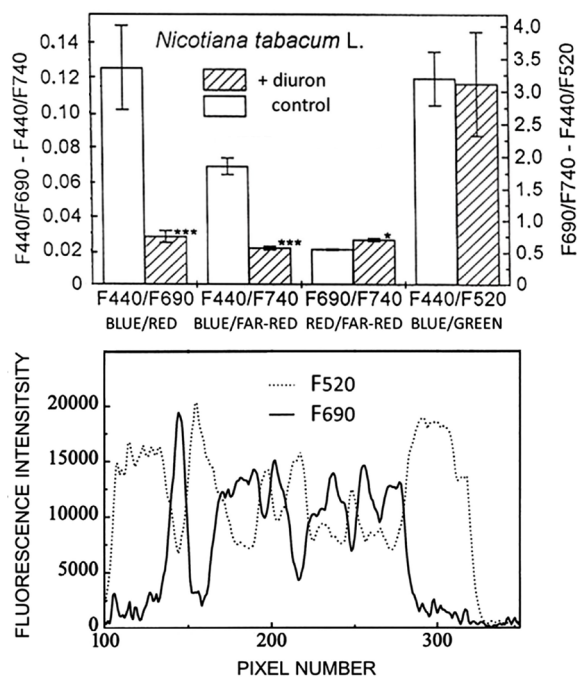


Fig. 7. Upper part: Significant differences in the four fluorescence ratios blue/red, blue/far-red, red/far-red, and blue/green of a tobacco leaf, where one leaf-half was treated with the herbicide diuron (high increase in red and far-red Chl fluorescence) and the other half remained untreated (control). The ratios marked by asterisks are significantly different from controls: \*\*\* highly significant ( $p < 0.001$ ) and \* significant ( $p < 0.05$ ). (Based on Lichtenthaler *et al.* 1996). Lower part: Horizontal profile of the yield of green ( $F_{520}$ ) fluorescence and red ( $F_{690}$ ) Chl fluorescence across the transverse axis of a variegated *Campelia* leaf. Green and red fluorescence show an inverse intensity distribution. (Based on Lichtenthaler *et al.* 1996)

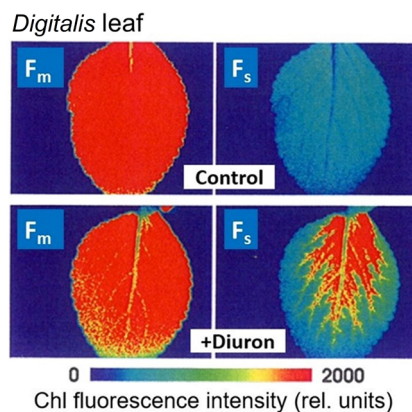


Fig. 8. Red chlorophyll fluorescence images ( $F_{690}$ ) (false colour images) of a green foxglove leaf (*Digitalis purpurea* L.) taken at maximum ( $F_m$ ) and steady-state fluorescence ( $F_s$ ) of the Chl fluorescence induction kinetics. Upper part: Leaf of a control plant. Lower part: Leaf from a plant 48 h after application of the herbicide diuron ( $10^{-5}$  M) *via* the roots. In those leaf parts, where diuron has already been taken up, the Chl fluorescence remains high and does no longer decrease to the steady-state fluorescence  $F_s$ . (Based on Lichtenthaler and Miehe 1997, modified)

across the transverse leaf axis. This inverse partition of the fluorescence intensities is shown for the  $F_{520}$  and  $F_{690}$  band in Fig. 7 (lower part).

**The Chl fluorescence ratio  $F_{690}/F_{740}$ :** Since the intensity of the red Chl fluorescence band  $F_{690}$  decreases with increasing Chl content of the leaf, whereas that of the far-red Chl fluorescence band  $F_{740}$  is little affected, the values of the ratio  $F_{690}/F_{740}$  decrease with increasing Chl content of the leaf as had been reported before (see review of Rinderle and Lichtenthaler 1988, Hák *et al.* 1990, Lichtenthaler *et al.* 1990). In this way, by forming red/far-red fluorescence ratio images of plants, one can determine *e.g.* the increase in Chl content during greening and development, a decrease in the Chl content during the autumnal Chl decline or at stress conditions. Due to the high statistical significance of several thousand pixel values per each leaf, such changes in the  $F_{690}/F_{740}$  ratio images and the Chl content can be well detected at an early stage. This also applies to the three other fluorescence ratios. In fact, changes in the four fluorescence ratios, as sensed *via* fluorescence imaging, can effectively be used in an early detection of various types of plant stresses because these fluorescence ratios change independently of each other and this depending on the type of stress applied. These applications possibilities of fluorescence imaging are mentioned in a later chapter of this review.

#### Further results obtained with the Laser Fluorescence Imaging System (Laser-FIS)

**Imaging some stress events:** The negative contrast between the leaf veins and the Chl-rich intercostal fields in the blue-green ( $F_{440}$ ,  $F_{520}$ ) and red fluorescence images ( $F_{690}$ ,  $F_{740}$ ) was confirmed by fluorescence imaging the upper leaf side of a croton leaf (Lang *et al.* 1995), where the leaf veins can well be seen on the upper leaf-side, since they are little masked by green cells. In this investigation, the differences in fluorescence yield of the croton leaf were presented in grey scales with black (as zero fluorescence) *via* grey tones to white as the highest fluorescence yield. In the same paper, we also described early water stress effects in tobacco leaves which were only detectable *via* fluorescence imaging and visually not yet seen. Thus, the lateral leaf parts on the leaf rim of a green water-stressed tobacco leaf showed a strong increase in red and far-red Chl fluorescence, whereas the other leaf parts showed no change as compared to controls. Despite the higher Chl yield on the leaf rim, the blue fluorescence remained nearly constant. Hence, the water-stressed lateral leaf parts were characterized by a considerable decrease in the ratio of blue/red ( $F_{440}/F_{690}$ ), whereas the ratio of red/far-red ( $F_{690}/F_{740}$ ) remained almost unchanged. In addition, UV-A treatment of green tobacco plants caused a considerable increase in red and far-red Chl fluorescence with the consequence of extremely decreased fluorescence ratios of blue/red and blue/far-red. Moreover, a mite attack in green bean leaves could be detected by fluorescence imaging which was not yet visually detectable. Already very tiny leaf spots, which were visually undetectable and

had resulted from a mite attack, showed an increase in the blue-green fluorescence and a decrease of the red and far-red Chl fluorescence (Lang *et al.* 1994b, 1995). Hence, the affected very small leaf spots exhibited much higher values of the fluorescence ratios of blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ).

**Nitrogen fertilizing:** The application of the high-resolution imaging technique, *i.e. via* using the Karlsruhe–Strasbourg Laser-FIS, also allows to differentiate between N-deficiency as well as medium or high N-supply. This has been proven with leaves of winter wheat grown in several fields in the Alsace with different nitrogen treatments (Heisel *et al.* 1997). The measurements were carried out on leaves of two storeys of wheat plants in May and June 1996. The red and far-red Chl fluorescence were enhanced by the N-fertilization, whereas blue and green fluorescence remained much the same. As a consequence, we observed a significant decrease of the fluorescence ratios blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ) with an increasing nitrogen concentration. The fluorescence imaging results were in excellent correlation with the crop yields at a higher N-supply. In contrast, N-deficiency is characterized by high values for the fluorescence ratios blue/red and blue/far-red (Table 1). These results demonstrated that nitrogen fertilization can well be monitored by imaging the two fluorescence ratios blue/red and blue/far-red.

**The imaged ratios of blue/red and blue/far-red in early stress detection in plants:** Plants are exposed to various kinds of natural and anthropogenic, biotic and abiotic stress constraints (Lichtenthaler 1996). The Chl fluorescence ratio red/far-red ( $F_{690}/F_{740}$ ) had been proven before to be an excellent nondestructive indicator of stress-induced changes in Chl content by measuring fluorescence emission spectra and kinetics at several leaf spots (*e.g.* Lichtenthaler and Rinderle 1988a, Stober and Lichtenthaler 1993a,c). By imaging the four fluorescence ratios, in combination with fluorescence emission spectra, we found that the fluorescence ratios blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ) are very sensitive indicators of ongoing stress events as well (Lichtenthaler *et al.* 1997a). In fact, both ratios are even more sensitive to changes in the environment and to stress constraints as compared the Chl fluorescence ratio of red/far-red ( $F_{690}/F_{740}$ ). Based on all our results obtained with the Karlsruhe–Strasbourg UV-laser fluorescence imaging system (Laser-FIS), these findings were summarized in a review on the principles and characteristics of multi-colour fluorescence imaging of plants (Buschmann and Lichtenthaler 1998). This review also includes original findings of various other fluorescence imaging investigations obtained using the UV-laser fluorescence imaging system *e.g.* changes induced by high irradiance stress of white light to single spots of the same leaf or by UV-radiation exposure of plants, or by heat and water stress.

The results of all these strain and stress effects – obtained *via* fluorescence imaging of plant leaves – on changes of the four fluorescence ratios of blue/red, blue/far-red, red/far-red, and blue/green are summarized in



Table 1. Differences in the fluorescence ratios of blue/red ( $F_{440}/F_{690}$ ), blue/far-red ( $F_{440}/F_{740}$ ), red/far-red ( $F_{690}/F_{740}$ ), and blue/green ( $F_{440}/F_{520}$ ) between different types of leaves, developmental stages as well as strain and stress conditions including partial inhibition of photosynthetic activity. The table is based on the multiple results obtained by fluorescence imaging leaves and plants using either the Karlsruhe–Strasbourg fluorescence imaging system (Laser-FIS) or the Karlsruhe flash-lamp-FIS described here. The individual data listed here are based on several thousand up to hundred thousand or more leaf pixels per fluorescence image taken from various papers (as listed in Buschmann and Lichtenthaler 1998; see also Buschmann *et al.* 2000, Lichtenthaler *et al.* 1996, 1997a,b). ++: strong rise; +: rise; - -: strong decrease; -: decrease; 0: no significant change of the fluorescence ratio.

Conditions	$F_{440}/F_{690}$ blue/red	$F_{440}/F_{740}$ blue/far-red	$F_{690}/F_{740}$ red/far-red	$F_{440}/F_{520}$ blue/green
Different development of leaf parts				
Variigated/green leaf	++	++	++	0
Lower/upper leaf-side	++	++	+	0
Yellow-green/green leaf	+	++	++	+
2 <sup>nd</sup> flush/1 <sup>st</sup> flush	- -	- -	++	-
Strain and stress				
Water deficiency	++	++	0	0
N-deficiency	++	++	+	0
Sun exposure	++	++	+	- -
Mite attack	++	++	0	+
Inhibition of photosynthetic activity				
Heat treatment	- -	- -	0	-
UV-A treatment	- -	- -	0	-
Diuron inhibition	- -	- -	+	0
Photoinhibition	++	++	- -	0

Table 1. These data are based on the fluorescence imaging information of the whole leaves, yet they are fully supported by the fluorescence ratios formed from fluorescence emission spectra of single leaf spots. Under many conditions and environmental stress constraints, the fluorescence ratios of blue/red and blue/far-red proved to be very valuable and very sensitive indicators of ongoing changes or damage by stress. Both fluorescence ratios showed either a considerable and significant increase or decrease to changing environment and plant stress. For this reason, images of both ratios are the best tool to detect early plant stress. The Chl fluorescence ratio of red/far-red ( $F_{690}/F_{740}$ ) can increase or decrease, too, however, not to that degree as the ratios of blue/red and blue/far-red. The blue and green fluorescence, in turn, are less sensitive to changes in environment. Hence, the fluorescence ratio of blue/green ( $F_{440}/F_{520}$ ) is less variable, and therefore less suited for stress detection in plants.

### The Karlsruhe multi-colour Flash-Lamp Fluorescence Imaging System (FL-FIS)

The first fluorescence imaging system, the Laser-FIS described above, is a relatively expensive setup which provides excellent results. Yet it also has the disadvantage that the red and far-red Chl fluorescence of plant leaves could only be excited with an excitation wavelength of 355 nm of the applied laser. In addition, for each imaging measurement, we had to take the plants, cultivated in our greenhouse or field station in Karlsruhe, to the laboratory

of our French colleagues to Strasbourg, a distance of 110 km. To overcome this gap, we developed in 1999 in our Karlsruhe laboratory a cheaper and easier to handle fluorescence imaging system, the Karlsruhe multi-colour flash-lamp FIS. This FL-FIS allows to excite all four fluorescence bands by pulsed UV-radiation or optionally to concentrate solely on the excitation of the red and far-red Chl fluorescence by pulsed blue light excitation. The latter delivers a much higher yield of the red and far-red Chl fluorescence as compared to UV-excitation. Blue light excitation is wanted *e.g.* when one only deals with imaging of particular photosynthetic parameters, such as  $R_{Fd}$  values.

The expensive Nd:YAG laser was replaced by a xenon flash lamp as an excitation source with a pulse energy of 0.7 J, a flash duration of 20  $\mu$ s, operated at 16.6 Hz. For the simultaneous excitation of blue-green as well as red and far-red Chl fluorescence, a broad UV-A transmission filter (*DUG II*, range 280 to 400 nm) is applied. The instrumentation also permits to excite exclusively the red and far-red Chl fluorescence by pulsed blue light. For this purpose, a blue transmission filter is applied (*Corning No. 9782*,  $\lambda_{max} = 465$  nm) in front of the xenon flash lamp. In both cases, fluorescence detection is performed using a gated intensified CCD-digital camera with an array of  $565 \times 754$  elements. The image intensifier is gated synchronously with the flash lamp in order to collect the very short fluorescence pulse emitted by each very short excitation pulse. The fluorescence images in the four fluorescence bands of blue ( $F_{440}$ ), green ( $F_{520}$ ), red ( $F_{690}$ ), and far-red ( $F_{740}$ ) are sensed using appropriate

interference filters (half-band width of 10 nm) built into a filter wheel in front of the CCD camera. The setup of this Karlsruhe multi-colour flash-lamp FIS is shown in Fig. 9. A special software (*Camille 1.05*) allows a centralized control of all FL-FIS components. Usually, one hundred image accumulations are chosen as a suitable number of successive readout images including accumulation and subtraction of background images. This is achieved in a few seconds.

By applying other transmission filters in front of the xenon flash lamp, an excitation of Chl fluorescence by orange (e.g. 570 nm) or red light (e.g. 620 to 650 nm) is also possible. With this Karlsruhe multi-colour FL-FIS one can simultaneously sense the fluorescence emission of 40,000 to more than 200,000 pixels per leaf depending on the size of leaves. Due to these high pixel numbers one can detect even small differences between control and stressed plants at a very early stage of plant stress and this with a high statistical significance. Fluorescence ratio images are particularly good indicators of stress to plants. By forming images of the Chl fluorescence decrease ratio  $R_{Fd}$  (ratio  $F_d/F_s$ ), a direct linear indicator of the photosynthetic  $CO_2$  assimilation rates  $P_N$  (see Fig. 4), one can also measure the differences in photosynthetic rates e.g. between sun and shade leaves or follow the successive onset of photosynthesis during greening of leaves or the decline of photosynthetic function due to uptake of the herbicide diuron. The software also allows to form histograms of the frequency distribution of the fluorescence intensity or of the  $R_{Fd}$  values of a leaf. In addition, also profiles of the values of the Chl intensity or the Chl fluorescence ratios  $R_{Fd}$  or  $F_m/F_s$  along a crossline of the leaf can be made. The different fluorescence intensity of the individual leaf pixels is, also in the FL-FIS, indicated by false colours from blue (zero fluorescence yield) *via* light blue (low fluorescence yield) and green and yellow to red as the

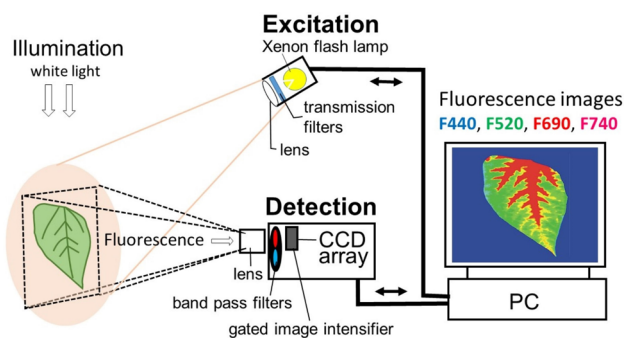


Fig. 9. Setup of the Karlsruhe multi-colour Flash-Lamp Imaging System (FL-FIS) to image the blue and green fluorescence as well as of the red and far-red Chl fluorescence. Fluorescence is excited by a pulsed xenon lamp. For an excitation of all four fluorescence bands a UV-transmission filter is applied. Using a blue transmission filter allows to solely excite Chl fluorescence. The fluorescence emitted in the four spectral regions is collected simultaneously from 30,000 up to 200,000 pixels over the whole leaf area by a CCD camera (using special band pass filters in front of the camera) and stored by the image processing system. (Based on Lichtenthaler and Babani 2000)

highest fluorescence yield. Further details on the FL-FIS and examples of its application are given by Lichtenthaler and Babani (2000) as well as Lichtenthaler *et al.* (2000, 2005b). We also compared the Karlsruhe FL-FIS with the Karlsruhe–Strasbourg Laser-FIS using green leaves of different plants and found that both FIS instruments yielded practically the same results.

### Imaging photosynthetic activity *via* imaging the fluorescence ratio $R_{Fd}$ of leaves

**Differences in  $R_{Fd}$  values between control and water-stressed bean plants:** Applying the Karlsruhe FL-FIS (using blue light as the excitation source), we sensed images of the Chl fluorescence  $F_{690}$  at maximum  $F_m$  and steady-state fluorescence  $F_s$  of bean leaves from control and water-stressed bean plants (Fig. 10A,B) (Lichtenthaler and Babani 2000). The fluorescence images at  $F_s$  were sensed 5 min after onset of illumination of the pre-darkened leaves with saturating white light. Using the image processing program, we determined from these images *via* a pixel-to-pixel subtraction ( $F_d = F_m - F_s$ ) images of the Chl fluorescence  $F_d$ . From these  $F_d$  and  $F_s$  images, one obtains images of the  $R_{Fd}$  values by a pixel-to-pixel division ( $R_{Fd} = F_d/F_s$ ). In the control plants, we found a strong decrease in the red Chl fluorescence from  $F_m$  to  $F_s$  (Fig. 10, upper part) which was much lower in the leaf of the water-stressed bean plant. Hence, the corresponding  $R_{Fd}$  images (Fig. 10C,D) demonstrated that the  $R_{Fd}$  values at water stress are significantly lower as compared to the control bean leaf. This is well documented in the corresponding histogram of the  $R_{Fd}$  frequency distribution, which is based on more than 100,000 values per leaf (Fig. 11, lower part). These results clearly demonstrate that

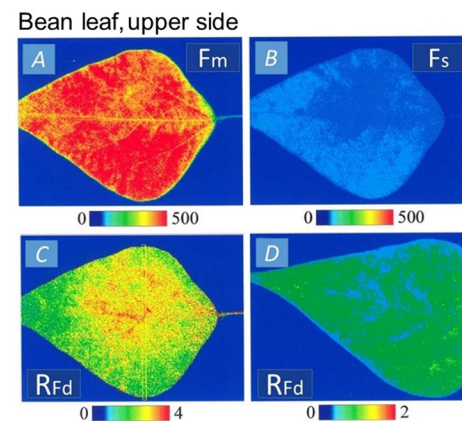


Fig. 10. Images of the red chlorophyll fluorescence of a bean leaf using the FL-FIS. (A) at  $F_m$  and (B) at steady-state fluorescence  $F_s$  of the Chl fluorescence induction kinetics (Kautsky effect). (C) Image of the Chl fluorescence decrease ratio values ( $R_{Fd} = F_d/F_s$ ) of the control plant. (D) Image of the Chl fluorescence decrease ratio values  $R_{Fd}$  of a bean leaf from a ten-day water-stressed plant. The scale in (A) and (B) indicates the intensity of the Chl fluorescence. In (C) and (D), it indicates the values of the fluorescence ratios  $R_{Fd}$ . (Based on Lichtenthaler and Babani 2000, Lichtenthaler *et al.* 2005b)

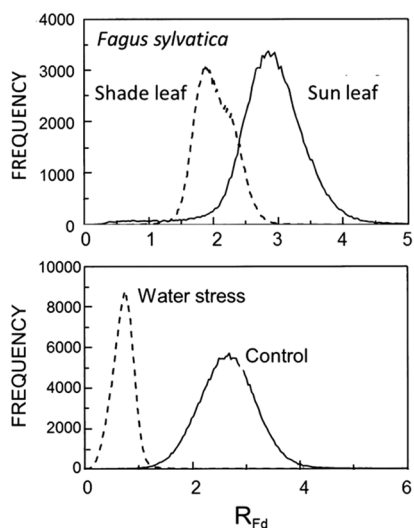


Fig. 11. Histogram showing the differences in the frequency distribution of  $R_{Fd}$  values. *Upper part*: Differences in sun and shade leaves of beech (*Fagus sylvatica* L.) based on ca. 100,000 leaf pixels of each leaf type. *Lower part*: Differences in the  $R_{Fd}$  values of bean leaves (*Phaseolus vulgaris* L.) from a control and a water-stressed plant based on ca. 200,000 leaf pixels. (Based on data from Lichtenthaler and Babani 2000 and 2004, Lichtenthaler et al. 2005b)

the large and highly significant decline in photosynthetic quantum conversion at water stress conditions can well be detected by the noninvasive technique of imaging the  $R_{Fd}$  values.

#### Differences in $R_{Fd}$ values between sun and shade leaves of beech:

Applying blue light as the excitation source, we also determined – using the Karlsruhe FL-FIS – the red Chl fluorescence ( $F_{690}$ ) of sun and shade leaves of beech (*Fagus sylvatica* L.) (Lichtenthaler et al. 2000). The fluorescence images were again taken at maximum fluorescence  $F_m$  and at steady fluorescence  $F_s$ . The latter was sensed 5 min after onset of illumination of the pre-darkened leaves with saturating white light. Using the image processing program, we determined from these images Chl fluorescence decrease ratio images ( $R_{Fd}$  images), both of sun and shade leaves of the beech (Fig. 12). These images revealed large gradients in  $R_{Fd}$  values (and correspondingly in photosynthetic rates) over different parts of the leaf area, both in sun and shade leaves of the beech. Such large gradients were not known or anticipated before. The histogram with the frequency distribution of more than 100,000 individual  $R_{Fd}$  value pixels per leaf clearly demonstrate with a high statistical significance ( $p < 0.001$ ) that sun leaves possess much higher  $R_{Fd}$  values and correspondingly much higher  $CO_2$  assimilation rates as compared to shade leaves (Fig. 11, upper part).

#### The $R_{Fd}$ values of sun and shade leaves of three other trees:

By using again the Karlsruhe FL-FIS, the same differences in  $CO_2$  assimilation rates  $P_N$ , as seen via  $R_{Fd}$  ratio images, were detected between sun and shade leaves

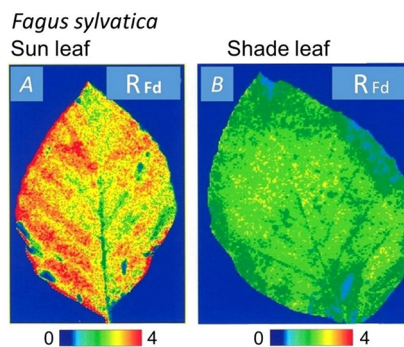


Fig. 12. Images of the Chl fluorescence decrease ratio values  $R_{Fd}$  of a sun (A) and a shade leaf (B) of beech (*Fagus sylvatica* L.). The  $R_{Fd}$  images were determined from the Chl fluorescence images taken first at  $F_m$  and then at  $F_s$  5 min after onset of continuous illumination of the pre-darkened leaves. The measurements were performed with the Karlsruhe flash-lamp imaging system (FL-FIS) at the red Chl fluorescence maximum near 690 nm. (Based on Lichtenthaler et al. 2000, Lichtenthaler and Babani 2004)

of three other single standing trees of the Karlsruhe University Campus: linden tree (*Tilia cordata* Mill.), platanus (*Platanus acerifolia* Wild.), and poplar (*Populus alba* L.). The frequency distribution of  $R_{Fd}$  values is based on more than 100,000 pixels per leaf. The differences between sun and shade leaves of the three trees were highly significant ( $p < 0.001$ ) (Lichtenthaler et al. 2007b). In this paper, also the stomatal conductance  $g_s$  of the sun and shade leaves was determined as well as pigment content (chlorophylls and carotenoids) and some other photosynthetic standard parameters. The  $g_s$  of sun leaves was also much higher in sun as compared to shade leaves and showed a similar relationship to  $P_N$  as did the  $R_{Fd}$  values determined via Chl fluorescence imaging.

#### Imaging $R_{Fd}$ values of sun and shade needles using the FluorCam imaging system:

In July 2005, I visited Czech colleagues in their experimental Ecological Research Station of the Czech Academy of Sciences, which is situated in a natural stand of trees in a forest in the Czech Beskydy Mountains (Bílý Kříž) at an altitude of 900 m. There I found the Czech imaging fluorometer (*FluorCam*, Photon System Instruments Ltd., Brno, Czech Republic) which regularly uses pulsed blue light (blue LEDs) as an excitation source. In modifying the *FluorCam* program, which normally measures only images during the Chl fluorescence rise kinetics, we took Chl fluorescence images at  $F_m$  and also at the steady-state  $F_s$ . To sense images at  $F_s$ , we applied continuous actinic saturating light [approximately  $2,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ ] for 5 min which was generated by a separate *Oriel 150 W* xenon lamp. In a joint measurement, we recorded images of the Chl fluorescence decrease ratio ( $R_{Fd}$  images) at the  $F_{690}$  emission band of sun and shade leaves of four tree species. The large differences in  $R_{Fd}$  values between sun and shade leaves of trees – described above for several trees of the Karlsruhe area – were confirmed there for beech (*Fagus*

*sylvatica* L.), linden tree (*Tilia cordata* Mill.), sycamore or mountain maple (*Acer pseudoplatanus* L.), and also for sun and shade needles of small shoots of the coniferous fir (*Abies alba* Mill.) (Lichtenthaler *et al.* 2007a). Like in sun and shade leaves, the chloroplasts of needles of fir branches growing under in full sun light exhibited a sun-type adaptation response with higher photosynthetic rates as compared to needles growing on branches in the shade which possess a shade-type adaptation response (confer the reviews Lichtenthaler 1983, Lichtenthaler and Babani 2004). Shade leaves and needles were taken from the inner tree crown, where on sunny days, they received approximately  $150 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ . The frequency distribution of the  $R_{Fd}$  values of fir sun and shade needles is shown in Fig. 13. The much higher  $R_{Fd}$  values significantly indicate the higher photosynthetic quantum conversion capacity of sun as compared to shade leaves or needles. Fluorescence imaging was performed of 20 min pre-darkened leaves and needles.

### Studying the successive transport and uptake of diuron into leaves

We also applied the Karlsruhe fluorescence imaging system (FL-FIS) to study the successive uptake of the herbicide diuron into bean leaves, when applied *via* the roots by dipping the roots of the bean plant into a nutrition solution containing  $10^{-5}$  M diuron (Lichtenthaler *et al.* 2013). Diuron is known to bind to the  $Q_B$ -protein and inhibits photosynthetic quantum conversion by blocking the electron flow of PSII. The progressive uptake of diuron, which is transported from roots *via* the bundle sheets of the shoot and leaf-petiole to the leaf basis and from there into the leaf can precisely be followed *via* imaging the Chl fluorescence at the  $F_{690}$  emission band. In presence of diuron, the maximum Chl fluorescence  $F_m$  does no longer decrease to the steady-state  $F_s$  as in controls. At about 2.5 h of application, the diuron uptake is detectable in the leaf veins and the neighboring mesophyll cells on the leaf basis. From there, the diuron uptake

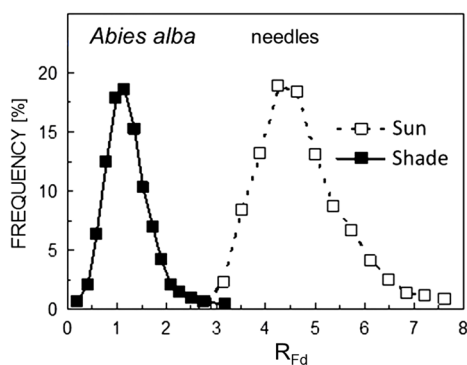


Fig. 13. Histogram showing the differences in the frequency distribution of  $R_{Fd}$  values of sun and shade needles of silver fir (*Abies alba* Mill.). The values are based on ca. 40,000 needle pixels of each needle type. Imaging was performed using the imaging fluorometer *FluorCam* (Based on Lichtenthaler *et al.* 2007a)

proceeds fairly slowly. This uptake is seen as red colour in the false colour images (Fig. 14). However, a small portion of diuron is rapidly transported to the central leaf tip and parts of the leaf rim, which is seen as a yellow pixels in the false colour images. This yellow colour in the false colour images signifies a partial increase in red Chl fluorescence indicating a partial inhibition of photosynthetic quantum conversion which always precedes the false red colour pixels indicating full inhibition. The results indicate that fluorescence imaging is an excellent technique to study time-dependent changes in the physiology of leaves.

### Differentiation of plants with low and high nitrogen supply

Crop plants *e.g.* sugar beets (*Beta vulgaris* L.) of an Agricultural Testing Station in Ladenburg, Germany, growing on soil poor in nitrogen ( $N_0$ -plants) can be well differentiated from plants which are cultivated with medium to full supply of nitrogen fertilizer [up to  $150 \text{ kg(N)}$  per hectare,  $N_{150}$ -plants] by fluorescence imaging. Using the Karlsruhe FL-FIS using UV-radiation for fluorescence excitation, we took fluorescence images in the four fluorescence bands  $F_{440}$ ,  $F_{520}$ ,  $F_{690}$ , and  $F_{740}$ . The best indicator to differentiate between low and high nitrogen supply are the fluorescence ratio of blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ) (Langsdorf *et al.* 2000, Lichtenthaler *et al.* 2005b). These fluorescence ratios proved to be significantly lower in nitrogen-fertilized sugar beet plants as compared to nonfertilized plants. With increasing N-supply, the intensity of all fluorescence bands increased, the red and far-red Chl fluorescence, however, to a higher degree than the blue-green fluorescence. The FL-FIS proved to be a valuable diagnostic tool for screening site-specific differences in N-availability which is required for precision farming.

### Multi-colour fluorescence imaging of bell pepper fruits and green apples during storage

With the FL-FIS, we measured the fluorescence images of freshly harvested green and also of orange bell pepper fruits as well as light green Braeburn apples in the four fluorescence bands blue, green, red, and far-red. An example of such fluorescence images is shown for green bell pepper in Fig. 15 together with the four fluorescence ratio images of blue/green ( $F_{440}/F_{520}$ ), blue/red ( $F_{440}/F_{690}$ ), blue/far-red ( $F_{440}/F_{740}$ ), and the Chl fluorescence ratio of red/far-red ( $F_{690}/F_{740}$ ) immediately after harvest (Buschmann *et al.* 2008, Lichtenthaler *et al.* 2012). The storage of fruits is usually paralleled by a successive water loss and a breakdown of Chl and changes of the fluorescence yield. In the case of Braeburn apples, we studied the changes in the four fluorescence bands and fluorescence ratios during storage and ripening of the apples during 2, 3, and 6 months at standard storage conditions of  $4^\circ\text{C}$  and 90% relative humidity. The blue and green fluorescence bands increased during storage and ripening, whereas the far-red band continuously declined with decreasing Chl content. In contrast, the intensity of

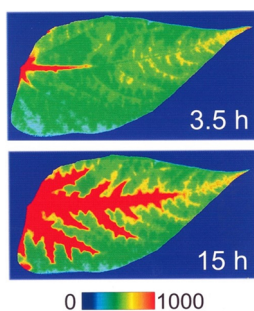


Fig. 14. Demonstration of the successive uptake of the herbicide diuron by an attached leaf of bean plants *via* imaging the red Chl fluorescence. Diuron uptake is visualized by a strong increase in Chl fluorescence shown here 3.5 and 15 h after diuron application ( $10^{-4}$  M) *via* the roots. The Chl fluorescence increase is given in false colours with light blue (low intensity), via green and yellow to red as the highest intensity. (Based on Lichtenthaler *et al.* 2005b and 2013, modified).

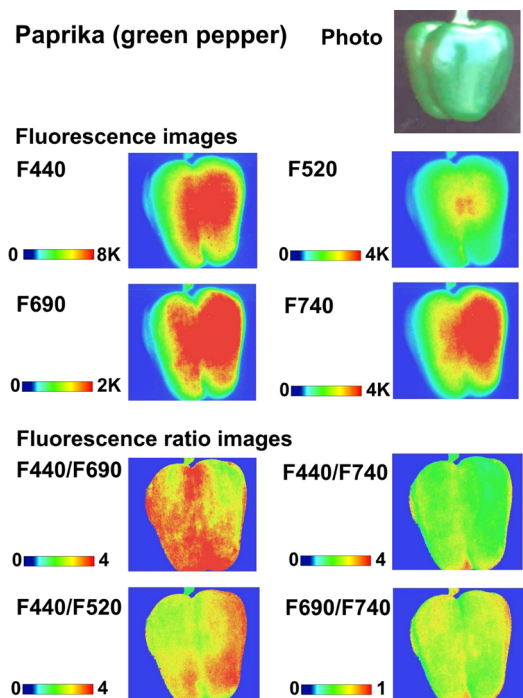


Fig. 15. Fluorescence images of green bell peppers (*Capsicum annuum* L.) after harvest. *Upper part*: Images in the blue ( $F_{440}$ ), green ( $F_{520}$ ), red ( $F_{690}$ ), and far-red ( $F_{740}$ ) fluorescence bands. *Lower part*: Images of the four fluorescence ratios blue/red, blue/far-red, blue/green, and red/far-red. The fluorescence intensities are presented in false colours: blue (no fluorescence) *via* green and yellow to red as the highest fluorescence. Note that the scales for the individual fluorescence signatures are different. K in the scales means kilo (1,000) counts. (Based on Buschmann *et al.* 2008, modified)

the red Chl fluorescence band  $F_{690}$  slightly increased up to three months of storage and then declined by 26% after six months of storage (Lichtenthaler *et al.* 2012).

This differential behavior of the four fluorescence bands is reflected in the fluorescence ratio images. The results demonstrate that the postharvest changes of apples and bell pepper fruits, the partial Chl loss and ripening can be well assessed by forming the four fluorescence ratio images. Multi-colour fluorescence imaging *via* the fluorescence ratio images has the great advantage of being more reliable and less sensitive to errors as compared to imaging only at one single fluorescence band *e.g.* red or blue.

### Multi-colour fluorescence imaging by a Hungarian and a Spanish group

In the years 1997 to 2000, I initiated and coordinated the research program QAAFFI (Quality Assessment of Agro-food by Fluorescence Imaging), which was supported by the European Union. Within this program, the group of Zoltán Szigeti, Budapest, received a copy of the Karlsruhe multi-colour flash-light fluorescence imaging system FL-FIS. They successfully applied the FL-FIS to detect changes in photosynthetic function in cabbage leaves infected with the *Turnip yellow mosaic virus* (Szigeti *et al.* 2002), to characterize the physiological status of cultivated plants (Szigeti 2008) and to map a Cd-induced chlorosis in poplar leaves (Solti *et al.* 2008). The research group of Matilde Barón also applied this FL-FIS in Budapest and the joint research endeavors of both groups are summarized in the interesting article 'Multi-colour fluorescence imaging: a useful tool to visualize compatible viral infection in plants' (Pineda *et al.* 2008).

Later, the Spanish research group of Matilde Barón applied an open *FluorCam 800C* imaging system which was customized with *Photon System Instruments* to permit multi-colour fluorescence imaging of leaves. This group successfully presented images of pathogen infections in plants (Barón *et al.* 2016). In addition, they also showed the application of multi-colour fluorescence imaging for plant disease detection (Pérez-Bueno *et al.* 2016), for diagnosis of bacterial and fungal infections on zucchini plants (Pineda *et al.* 2017), for diagnosis of mildew disease (Polonio *et al.* 2019) or the detection of toxic stress biomarkers (Segečová *et al.* 2019). They also demonstrated the combined use of blue-green fluorescence and thermal imaging in the detection of sunflower infection by the root parasitic weed *Orobanche* (Ortiz-Bustos *et al.* 2017).

### Near-field remote measurements of vegetation by fluorescence imaging

Based on our positive experience with plant leaves in the laboratory, a mobile laser-induced fluorescence imaging system, implemented in an all-road car (*see* Figs. 16, 17), was established by the physicists of our Strasbourg cooperation partners for near-field remote measurements of crops (Sowinska *et al.* 1999). This fluorescence imaging system is based on the Karlsruhe–Strasbourg UV-A Laser-FIS described above with a frequency-tripled ND:YAG laser as excitation source. The spot-size for imaging plants in the field was made adjustable by a variable beam expander. Fluorescence images were



Fig. 16. Photo of the Strasbourg mobile UV-laser fluorescence imaging system for 'near-field remote sensing' of crop plants and trees. (Based on Sowinska *et al.* 1999)

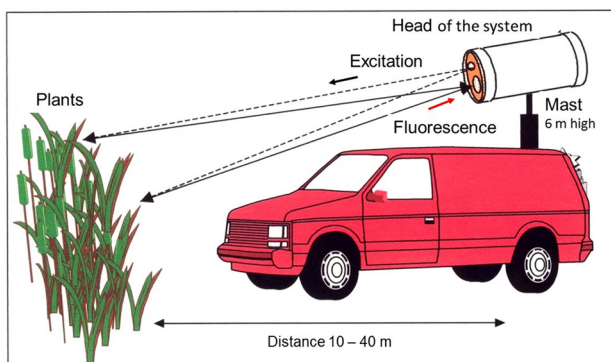


Fig. 17. Scheme of the experimental setup of the Strasbourg mobile UV-laser 'near-field fluorescence imaging system'. (Based on Sowinska *et al.* 1999)

recorded in the four fluorescence bands  $F_{440}$ ,  $F_{520}$ ,  $F_{690}$ , and  $F_{740}$ . The laser head and the CCD camera were placed on an azimuth platform on top of the car. The telescopic mast (with laser and camera) could be elevated up to 6 m, which allowed to sense laser-induced fluorescence images of plants at a distance of 10 to 40 m (Figs. 16, 17). In this way, we sensed the blue  $F_{440}$  and red  $F_{690}$  fluorescence bands and formed the fluorescence ratio images of blue/red ( $F_{440}/F_{690}$ ) of two younger birch trees, one grown in full sun and the second one grown in the shade. By imaging branches with approximately ten leaves in each case from a distance of 20 m we could well differentiate sun leaves from shade leaves. In fact, the differing blue/red ratio values of sun and shade leaves were practically identical to those obtained with single leaves sensed in very close distance to the CCD camera of a second, smaller, yet complete fluorescence imaging system inside the car.

In addition, in the same way, we remotely sensed sugar beet plants in a field of an experimental research station in Ladenburg, Germany, from a distance of 20 m (Sowinska *et al.* 1999). There were various parcels with sugar beet plants which had received different concentrations of nitrogen fertilizer. The average values of the fluorescence ratio of blue/red ( $F_{440}/F_{690}$ ) obtained from the fluorescence

images of unfertilized sugar beet parcels and those that had received 90 and 120 kg(N) per hectare were significantly different from each other and ranged from 1.05 *via* 0.92 to 0.75. They were slightly lower to those obtained of cut leaves measured in close distance to the CCD camera in the second fluorescence imaging system inside the car. As expected, the remotely sensed fluorescence images contained leaves from the upper and lower leaf side, from side angles, *etc.* As a consequence, the variability of the intensity of the individual fluorescence pixels of leaves in the field was considerably larger as compared to cut leaves that were measured in parallel in near distance, where the fluorescence was excited and sensed perpendicular to the leaf surface. However, the average values of usually more than 100,000 pixels of the blue/red fluorescence ratio of the canopy plants allowed a clear and significant differentiation. The Chl fluorescence ratio images of red/far-red ( $F_{690}/F_{740}$ ) of the field plants also showed differences between zero, medium or high nitrogen supply, the differences were, however, lower as compared the ratio of blue/red ( $F_{440}/F_{690}$ ) indicating that the Chl fluorescence ratio  $F_{690}/F_{740}$  is less suited to remotely monitor nitrogen supply to fields plants.

Some of these investigations were complemented by a direct measurement of leaves using the Karlsruhe FL-FIS. Also there, the best indicators to differentiate between low and high nitrogen supply were found to be the fluorescence ratios of blue/red ( $F_{440}/F_{690}$ ) and blue/far-red ( $F_{440}/F_{740}$ ) (Langsdorf *et al.* 2000, Lichtenthaler *et al.* 2005b). The results obtained unambiguously indicate the suitability of near-field remote sensing of crop plants by multi-colour fluorescence imaging in the four fluorescence bands blue, green, red, and far-red in order to monitor nitrogen fertilization rates or detect plant stress events. The 'near-field imaging system' described here is also suitable for monitoring the health conditions of forest and urban trees which are essential tasks in the present time of climate change.

#### Remote multi-colour fluorescence imaging of plants with a Swedish LIDAR system

A Swedish group around Sune Svanberg had established a mobile, truck-bound fluorescence LIDAR system which was mainly designed and applied for atmospheric pollution monitoring. I invited this group to test their fluorescence LIDAR system in the multi-colour fluorescence imaging of selected broad-leaf plants. In July 1993, we had a joint measuring campaign with plants of our experimental station in the Botanical Garden of the Karlsruhe University. With this Swedish LIDAR fluorosensor system, we sensed remotely plants in a distance of 50 to 80 m in the three fluorescence bands of blue ( $F_{440}$ ), red ( $F_{685}$ ), and far-red ( $F_{740}$ ). The excitation wavelength near 397 nm was generated by a frequency-tripled ND:YAG laser. The gate width of the CCD image intensifier was set at 20 ns to efficiently suppress the influence of daylight illumination of the target. Typically, the images were recorded during 100 laser shots. This technique enabled us to characterize vegetation in terms of the approximate

Chl content by sensing the Chl fluorescence ratio of  $F_{685}/F_{740}$ , to differentiate younger and older leaves *via* the fluorescence ratio of blue/red ( $F_{440}/F_{685}$ ) by measuring single or the whole group of leaves. Also the increase in the red and far-red Chl fluorescence induced by the herbicide diuron, whereby the blue-green fluorescence remained constant, could be sensed *via* decreasing values of the fluorescence ratio of blue/red ( $F_{450}/F_{685}$ ). This was the very first time that fluorescence ratio images (red/far-red and blue/red) of the whole leaves or leaf groups were recorded and this even by remote sensing. In fact, such fluorescence ratio images allow to detect plant stress in a much better way as compared to sensing of only the red Chl fluorescence. Some of these findings were found in the review of Lichtenthaler (1994), the major results are found in our joint paper (Edner *et al.* 1995). These pioneering results unambiguously demonstrated that multi-colour UV-laser induced fluorescence imaging *via* forming fluorescence ratio images is a valuable tool also in the remote assessment of the vegetation status in relation to environmental stress.

### Concluding remarks

In contrast to single point measurements of Chl fluorescence, multi-colour fluorescence imaging has the advantage to enable the determination of the overall distribution of the red and far-red Chl fluorescence and of the blue and green fluorescence of many thousand points over the whole leaf area. This is performed *via* pulsed UV-radiation excitation in already one single extremely short measurement event, but more precisely by about 20 up to 100 shots. Examples have been shown in this review for two instruments, the Karlsruhe–Strasbourg Laser-FIS and the Karlsruhe FL-FIS. The great advantage of multi-colour fluorescence imaging is the fact that it permits to form ‘fluorescence ratio images’ of the four ratios of blue/red, blue/far-red, red/far-red, and blue/green. In these images, the ratio value is given for each single point of a leaf as well as the average ratio value of hundred thousand or more leaf points over the whole leaf area. The image processing programs allow to make histograms with the frequency distribution of the fluorescence intensity and ratios between control leaves and stressed plants. Moreover, one can create profiles of the variability of individual fluorescence bands or ratios across the transverse leaf axis, and thus detect *e.g.* the negative contrast between the emission of the blue fluorescence  $F_{440}$  and the red Chl fluorescence  $F_{690}$ . By providing the individual fluorescence information of 40,000 up to more than 200,000 leaf pixels per measurement, the noninvasive high resolution multi-colour fluorescence imaging technique is a very powerful and advanced technique supplying reliable results with a high statistical confidence. It is an optimal and advanced modern technique of eco-physiological plant research which allows to detect early plant stress and also to sense *via*  $R_{Fd}$  images the actual photosynthetic activity of leaves. *Via* imaging the fluorescence ratio  $F_{690}/F_{740}$ , it also provides information on the chlorophyll content. In addition, it offers ample possibilities for the follow-up of changes of

agro-food during development, storage, and ripening. Moreover, our results also demonstrate that multi-colour fluorescence imaging can successfully be applied for remote sensing of crop plants, trees, and terrestrial vegetation. Multi-colour fluorescence imaging can be regarded as an ‘artificial eye’ that recognizes valuable fluorescence information of plants which remains hidden to our eyes.

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