REVIEW

Imaging of the blue, green, and red fluorescence emission of plants: An overview

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Abstract

An overview is given on the fluorescence imaging of plants. Emphasis is laid upon multispectral fluorescence imaging in the maxima of the fluorescence emission bands of leaves, *i.e.*, in the blue (440 nm), green (520 nm), red (690 nm), and far-red (740 nm) spectral regions. Details on the origin of these four fluorescence bands are presented including emitting substances and emitting sites within a leaf tissue. Blue-green fluorescence derives from ferulic acids covalently bound to cell walls, and the red and far-red fluorescence comes from chlorophyll (Chl) *a* in the chloroplasts of green mesophyll cells. The fluorescence intensities are influenced (*1*) by changes in the concentration of the emitting substances, (*2*) by the internal optics of leaves determining the penetration of excitation radiation and partial re-absorption of the emitted fluorescence, and (*3*) by the energy distribution between photosynthesis, heat production, and emission of Chl fluorescence. The set-up of the Karlsruhe multispectral fluorescence imaging system (FIS) is described from excitation with UV-pulses to the detection with an intensified CCD-camera. The possibilities of image processing (*e.g.*, formation of fluorescence ratio images) are presented, and the ways of extraction of physiological and stress information from the ratio images are outlined. Examples for the interpretation of fluorescence images are given by demonstrating the information available for the detection of different developmental stages of plant material, of strain and stress of plants, and of herbicide treatment. This novel technique can be applied for near-distance screening or remote sensing.

Additional key words: photosynthetic activity; quality control; remote sensing; strain; stress detection.

Introduction

Fluorescence represents radiation emitted during the de-excitation of pigments that have been excited by absorption of visible radiation (PAR) or UV-radiation. The intensity of the blue-green fluorescence of leaves is constant (Chappelle *et al.* 1985, Stober and Lichtenthaler 1993a), whereas the Chl fluorescence of intact leaves varies with time being inversely related to the photosynthetic activity (Chl fluorescence induction kinetics or "Kautsky-effect", see, *e.g.*, Krause and Weis 1991, Lichtenthaler 1992). The fluorescence measurements performed in the last decades concentrated on the measurements of single leaf points (point data measurements). A new dimension of fluorescence measurements is the two-dimensional resolution of the emitted leaf fluorescence by fluorescence imaging. This provides ample fluorescence information of many ten thousand pixels (= picture elements) over the whole leaf area. Thus it allows detection of local disturbances and gradients in fluorescence emission (*e.g.*, Sundbom and Björn 1977, Omasa *et al.* 1987, Daley *et al.* 1989, Genty and Meyer 1994, Lang *et al.* 1994, Siebke and Weis 1995a,b, Lichtenthaler *et al.* 1996, Buschmann and Lichtenthaler 1998).

Fluorescence imaging has already had a long tradition on the microscopic scale (fluorescence microscopy, summarising literature: Slavik 1996, Wang and Herman 1996, Gilroy 1997). Macroscopic fluorescence imaging of whole leaves and whole plants is becoming an essential

technique with the prospect of non-destructive monitoring of plant diseases and stress from near distance (quality control by automated inspection and sorting) and far-distance (remote sensing) (see Lang *et al.* 1996 and the reviews of Lichtenthaler and Miehé 1997, Buschmann and Lichtenthaler 1998, Cerovic *et al.* 1999; for field application see Sowinska *et al.* 1999). Single spot or point data fluorescence measurements, which are still widely used today, usually have the advantage of low costs and the possibility for relatively high spectral resolution but the disadvantage of the fluorescence information from one leaf spot only which is seldom representative for the whole leaf. Laser or flash light induced fluorescence imaging, in turn, expands the vision of research, reveals spatial differences of a leaf sample, and provides the distribution and localisation of the fluorescence signals across the leaf area. This allows better interpretation, higher statistical accuracy, and the screening of gradients or local disturbances permitting early stress detection.

Fluorescence signals measured in the blue, green, red,

and far-red spectral regions are specific to plants. The red and far-red fluorescence is only emitted by the Chls, whereas the blue and green fluorescence is emitted by cinnamic acids (with ferulic acid as major substance) and other plant phenolics covalently bound to cell walls (Lichtenthaler and Schweiger 1998) (for details see below). Because of this high specification, fluorescence signals may be more suitable for remote sensing of vegetation than today's widely used reflectance signals, although fluorescence signatures are usually much less intense than reflectance signatures. However, with the advanced, highly sensitive new sensors and with pulsed excitation it is possible to measure fluorescence images with good distinction from the high background of light environment (*e.g.*, sun light).

In this review emphasis is put on multispectral fluorescence imaging that adds the spectral dimensions to the two-dimensional image. This, and in particular the images of fluorescence ratios, provide more extensive information that is beneficial for the interpretation of the fluorescence results.

Fluorescence bands and their origin

The fluorescence emission spectrum of a green leaf, as induced by UV radiation, is characterised by four bands with maxima or shoulders near 440 nm (blue), 520 nm (green), 690 nm (red), and 740 nm (far-red) (Lichtenthaler *et al.* 1992a) (Fig. 1). The intensity of the bands may vary within a plant (Lang *et al.* 1991) and between species (Johnson *et al.* 2000). There is a large

variety of fluorescing substances in plant material (Goodwin 1953, Fry 1979, 1982, Lang *et al.* 1991, Rost 1995). The blue and green fluorescence emission of leaves, first measured by Chappelle *et al.* (1985), is emitted from cinnamic acids (mainly ferulic acid) covalently bound to the cell walls (Hartley 1973, Harris
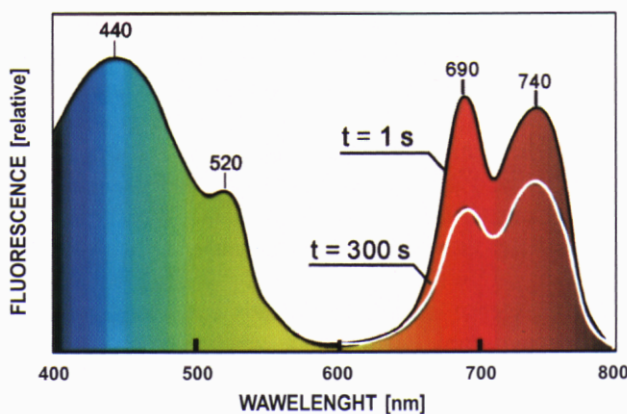

Fig. 1. Fluorescence emission spectrum of a 20 min pre-darkened green tobacco leaf taken *via* a fluorescence spectrometer with excitation in UV-A (340 nm) directly after onset of irradiation (t = 1 s) and after 300 s of irradiation with "white light" saturating photosynthesis (t = 300 s). During the light-triggered induction kinetics (Kautsky effect) the Chl fluorescence near 690 nm decreases to a stronger degree than the far-red Chl fluorescence near 740 nm. As a consequence the Chl fluorescence ratio F_{690}/F_{740} decreases from 1.10 (1 s) to 0.85 after 300 s of irradiation.

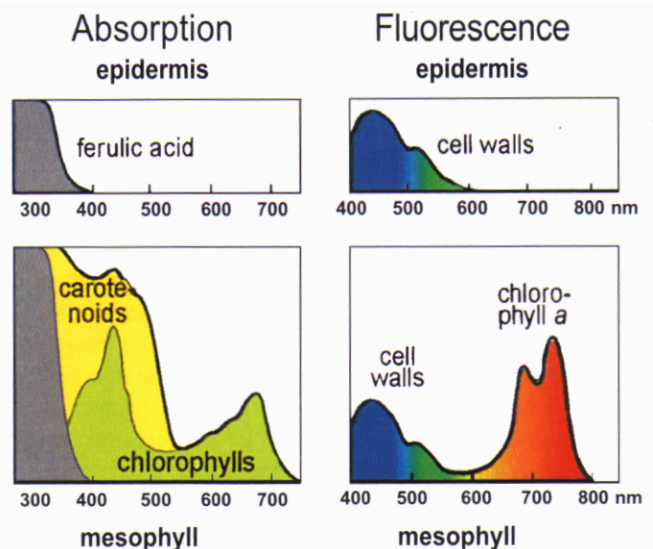Fig. 2. Absorption spectra of leaf substances (*left*) and the corresponding fluorescence emission spectra (*right*) of the upper leaf epidermis and of the green mesophyll cells beneath the epidermis. The blue-green fluorescence intensity of the mesophyll cells is less strong than in the pigment-free epidermis, since part of it is re-absorbed by the blue-green absorption bands of the chlorophylls and carotenoids.

and Hartley 1976, Lichtenthaler and Schweiger 1998), and only to some degree from phenolics in the cell walls. The soluble phenolics and cinnamic acids do not contribute to the blue-green fluorescence emission (Lichtenthaler and Schweiger 1998). From kinetic measurements on the dependency of the blue-green fluorescence it had been assumed that ferulic acid could be the essential cinnamic acid (Morales *et al.* 1996). A detailed chemical analysis of soluble and covalently bound ferulic acid unequivocally proved that the covalently bound ferulic acid of cell walls is the major blue-green fluorescing substance (Lichtenthaler and Schweiger 1998). In addition, green fluorescence can emanate also from other secondary plant substances, such as quercitin and berberin, and to some extent also from riboflavin and reduced phylloquinone K_1 (H_2) (Lang *et al.* 1991). The red and far-red fluorescence, in turn, is exclusively emitted from Chl *a* of the antenna system of photosystem (PS) 2 in the thylakoids of the chloroplasts of the mesophyll cells. The far-red Chl fluorescence near 735 to 740 nm may contain at room temperature a small contribution of Chl *a* from PS1 (Holzwarth *et al.* 1987, Pfündel 1998). At low temperature (liquid nitrogen) this fluorescence band at 740 nm is, however, mainly from PS1 (Strasser and Butler 1977, Šiffel and Šesták 1988). Recent work on the de-convolution of the Chl fluorescence emission spectrum of green leaves using *in vivo* reflectance and absorption measurements demonstrated that more than 96 % of the shape of the Chl fluorescence emission spectrum at room temperature (including the 740 nm peak) can be explained by re-absorption processes that mainly affect the red Chl fluorescence of PS2 in the 690 nm region (Gitelson *et al.* 1998).

In a cross section of a leaf the fluorescence emitting substances are not homogeneously distributed. In the epidermis layer, blue and green fluorescence of the cell walls are very strong since Chls and carotenoids, which could re-absorb the emitted blue-green fluorescence, are not present in the epidermis cells except for the stomata. Inside the leaf mesophyll the green palisade cells and spongy parenchyma cells primarily emit the red and far-red Chl fluorescence. The blue-green fluorescence of those cell walls is very low, as most of the blue-green fluorescence is re-absorbed by the Chls and carotenoids of the numerous cell chloroplasts (Fig. 2) (Stober *et al.* 1994).

The shape of fluorescence emission spectrum may already undergo drastic changes during leaf development (Stober and Lichtenthaler 1992, 1993a,b, Šesták and Šiffel 1997). The intensity of fluorescence depends not only on the concentration of emitting substances but to a great extent also on the optical properties of the leaf which determine the penetration of the excitation radiation into the tissue as well as the penetration and partial re-absorption of the fluorescence on its way to the leaf surface where it is measured. On their way through the leaf tissue the excitation radiation and the emitted fluorescence are changed by absorption of the pigments, by internal reflection and infraction as well as by scattering. Thus, the increase of soluble UV-absorbing substances during growth of plants in the sunlight as compared to growth in a greenhouse (Lichtenthaler and Schweiger 1998) leads to a decrease of the UV-induced red Chl fluorescence since the penetration of UV-excitation into the leaf mesophyll is reduced (Stober and Lichtenthaler 1993b). With high amounts of Chls and carotenoids in leaves the emitted blue and green fluorescence is reduced by re-absorption on their way to the leaf surface. High amounts of Chls also reduce the short-wavelength red Chl fluorescence, since the 690-nm fluorescence band (F_{690}) overlaps with the red *in vivo* absorption bands of the Chls at 670 to 680 nm (Gitelson *et al.* 1998). The 740-nm Chl fluorescence maximum, in turn, is only slightly influenced by the Chl concentration. The re-absorption of the red Chl fluorescence by the Chls can be applied for deducing leaf Chl concentrations from the values of the Chl fluorescence ratio F_{690}/F_{740}. This ratio is inversely correlated to the Chl content (Hák *et al.* 1990, Lichtenthaler *et al.* 1990, D'Ambrosio *et al.* 1992, Babani and Lichtenthaler 1996, Gitelson *et al.* 1998).

The blue and green fluorescences are constant with time as long as there are no changes in pigment concentration or in the optics of the leaf. The intensity of the red and far-red Chl fluorescence, however, is inversely related to the photosynthetic activity. The Chl fluorescence decreases with increasing photosynthetic activity of a leaf or a chloroplast that can be followed from the induction kinetics in the microsecond to minute range. The Chl fluorescence decrease is more pronounced at 690 nm than at the 740 nm band (Buschmann and Schrey 1981, Kocsányi *et al.* 1988). Another factor influencing the height of Chl fluorescence is the competition between radiative (fluorescence) and non-radiative (heat) de-excitation processes. Thus, in the case of photoinhibition (inhibition of photosynthesis by excessive irradiance) heat production is increased and Chl fluorescence decreased (Buschmann 1987). A decrease in variable Chl fluorescence under photoinhibitory irradiance and zeaxanthin formation has also been shown in various plants (Krause *et al.* 1990, Demmig-Adams and Adams 1992, Lichtenthaler *et al.* 1992b, Schindler and Lichtenthaler 1994).

Instrumentation for fluorescence imaging

A fluorescence imaging system (FIS) consists of a source for fluorescence excitation, a camera for acquiring the images, and a computer controlling these components as well as carrying out the image processing (Fig. 3). Various imaging systems have been used. Imaging of Chl fluorescence was applied to detect delayed fluorescence (Sundbom and Björn 1977, Björn and Forsberg 1979, Blaich *et al.* 1982, Ellenson and Amundson 1982), Chl fluorescence induction kinetics known as "Kautsky effect" (Omasa *et al.* 1987, Omasa 1990, Siebke and Weis 1995a,b), and non-photochemical quenching (Daley *et al.* 1989, Genty and Meyer 1994, Oxborough and Baker 1997a,b). Several multispectral fluorescence imaging systems have been described (Edner *et al.* 1994, 1995, Lang *et al.* 1994, Heisel *et al.* 1996, Lichtenthaler *et al.* 1996, Saito *et al.* 1997a,b, 1998, 1999, 2000, Sowinska *et al.* 1999, Lichtenthaler and Babani 2000). The laser-induced fluorescence imaging system (laser-FIS) developed in co-operation of Lichtenthaler's and Miehé's groups includes the blue (F_{440}), green (F_{520}), red (F_{690}), and far-red Chl fluorescence (F_{740}) and enables wide application possibilities (Lang *et al.* 1994, 1996, Lichtenthaler *et al.* 1996, Lichtenthaler and Miehé 1997).

Fluorescence excitation: For fluorescence excitation the sample must be irradiated with a source which is readily absorbed and the wavelength of which is shorter than that of the emitted fluorescence. Thus, blue and green fluorescence excitation can only be carried out *via* UV-radiation. In contrast, the red and far-red Chl fluorescence can be excited by either UV-radiation or "visible light" (blue, green, or red radiation up to about 620 nm). As UV-source lasers, *e.g.*, tripled Nd:YAG (355 nm) or tripled Nd:YAG Raman shifted (398 nm) can be applied. Lichtenthaler developed a flash lamp system with the appropriate filters as excitation source (*e.g.*, pulsed xenon lamp with UV-transmitting filter) as in the case of the Karlsruhe FL-FIS (Lichtenthaler and Babani 2000). For excitation in the visible field either light-emitting diodes (LED) or pulsed halogen or xenon lamps with appropriate filters can be used. The angle from which the sample is irradiated should be constant for different measurements and be as similar as possible to the angle of imaging. Since the geometry of the system influences the penetration of the excitation radiation into the sample and also the penetration and emission of the fluorescence measurable outside the leaf sample, the measurable fluorescence intensity changes with the angle of irradiation and detection. This may cause problems with respect to the interpretation of the fluorescence images, particularly when the sample surface is not flat (*e.g.*, fruits or wavy leaves).

Acquisition of fluorescence images: Fluorescence images are acquired with a video camera equipped with a CCD (charged-coupled-device) area array sensor. The sensor array may contain about 500 lines with 700 pixels. In a distance of 0.5 m one pixel represents a size of less than 0.1 mm^2 of the leaf sample. An image intensifier with adjustable gain in front of the sensor panel drastically increases the sensitivity of the camera but does no longer allow a spectral resolution (*i.e.*, no colours but only the distribution of the intensity is sensed). In order to exclude reflected excitation radiation, a cut-off filter must be placed in front of the sensor system. For multispectral fluorescence imaging usually the images for the blue, green, red, and far-red fluorescence (the spectral range of the fluorescence maxima/shoulders) are acquired sequentially for the identical field of view, each with the appropriate filter. Interference filters with transmittance maximum at 420, 550, 690, or 740 nm were installed in a filter wheel in front of the image intensifier. This is the basis for the Karlsruhe-Strasbourg laser-FIS and the Karlsruhe FL-FIS. In order to exclude interfering background radiation (sunlight, light in the laboratory, or light from excitation sources) one should record the images in total darkness. This is, however, not a suitable method for routine measurements. For this reason another technique is applied in which the pulses of the excitation radiation are synchronised with the operation of the image intensifier. The latter procedure is called "gating", which means that the image intensifier of the camera is active only in a time window ("gate") when the fluorescence signal is expected to hit the sensor plate. Since the fluorescence signal of the four fluorescence bands of plants is several orders of magnitudes smaller than the reflectance signal of "white light", the acquisition of one image requires the accumulation of a few hundred images which can be achieved within a few seconds. When making less than 50 acquisitions the images would generally be too noisy.

Image processing: The accumulated images acquired by the camera are stored and further processed in a computer usually controlling the functions of both the excitation source and the camera. If necessary (recommended in most cases, especially when leaves are excited with a flash lamp), the raw image is corrected for non-uniform excitation. This is done by dividing each pixel with the pixel of an image of uniform fluorescence (*e.g.*, a blue fluorescence image of a white paper is acquired with a lower gain of the image intensifier). Images with a resolution of about 500 to 700 pixels are displayed in false colours with a scale analogue to the temperature, *i.e.*, ranging from a low fluorescence intensity with
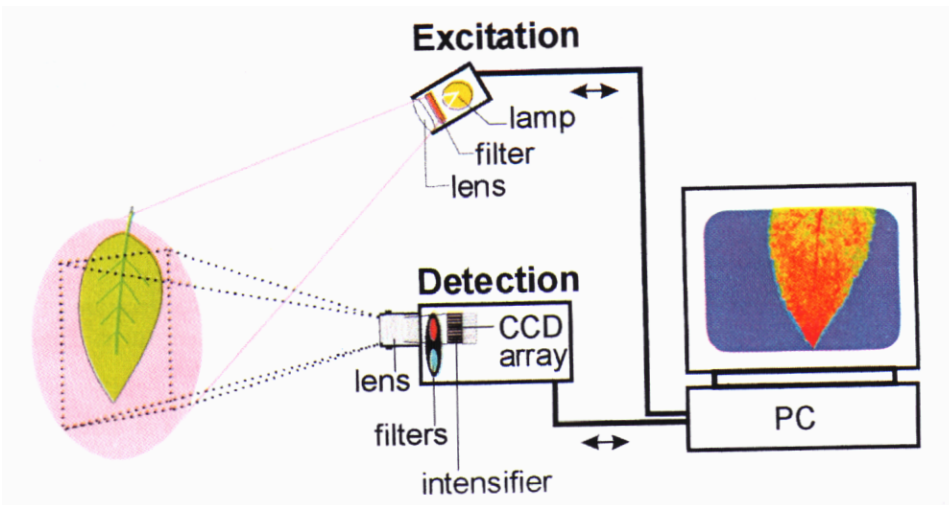
Fig. 3. Scheme of the Karlsruhe multispectral fluorescence imaging system (FIS) for measuring green plant material. Excitation is performed by irradiation with a pulsed xenon flash-lamp using a UV-transparent filter and a beam-expanding lens. For the detection the fluorescence image is acquired *via* a lens through one of the four filters of a filter wheel. The fluorescence signals in the blue, green, red, and far-red region are sensed using a CCD-array with amplification by a gated intensifier.
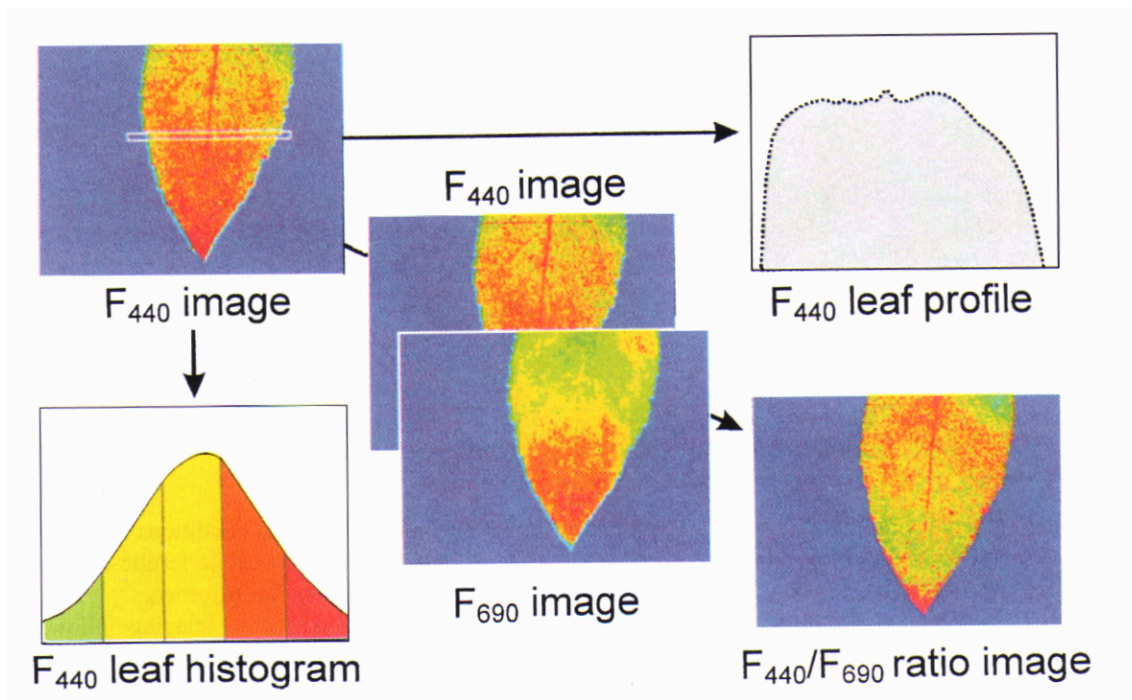

Fig. 4. Fluorescence image of a green sugar beet leaf in the blue (F_{440}) and red (F_{690}) regions as well as various types of processing: histogram of fluorescence intensity (*lower left*), horizontal profile through the leaf (*upper right*), and fluorescence ratio image, *i.e.*, an image of the pixel-to-pixel ratio of the fluorescence yields measured at the two fluorescence bands blue (F_{440}) and red (F_{690}) (*lower right*).

dark blue *via* green and yellow to red as the highest fluorescence intensity. Depending on the software, various procedures of image processing are possible that enable or facilitate the interpretation of the images. Thus, the distribution of the fluorescence intensity can be displayed as a histogram, fluorescence profiles in hori-zontal or vertical lines can be indicated as a cross section of the leaf sample, and fluorescence ratio images can be achieved by calculating a fluorescence ratio for each pixel which must be identical in size for each of the four bands (Fig. 4).

Interpretation of fluorescence images

The Chl fluorescence has long been applied for stress detection (see Lichtenthaler et al. 1986, Lichtenthaler and Rinderle 1988 and references cited there). Fluorescence imaging has been used for the detection of various types of strain and stress (Heisel et al. 1996, 1997, Lang et al. 1996, Lichtenthaler et al. 1996, 1997, Saito et al. 1997a,b, 1998, Balota et al. 1999, Osmond et al. 1999a,b, Sowinska et al. 1999, Valcke et al. 1999) as well as for plant pathology (Osmond et al. 1990, 1998, Nilsson 1995, Ning et al. 1995, 1997). The images can be interpreted by comparing the fluorescence intensity distribution of a control sample (reference). Good referencing may sometimes only be possible for different pixels within one image. When applying multispectral fluorescence imaging, the ratios of the four fluorescence bands are usually more conclusive than an image acquired at a single wavelength only. In particular, the fluorescence ratio images blue/red (F_{440}/F_{690}) and blue/far-red (F_{440}/F_{740}) are very early stress and strain indicators. This is true even if rise of the ratio is caused by a decrease of the denominator or an increase of the numerator. The ratio also includes a kind of internal referencing.

Table 1. Changes of the fluorescence ratios blue/red, blue/far-red, red/far-red, and blue/green in leaves as indicators of different developmental stages, strain, and stress, as well as inhibition of photosynthetic activity of plants. The results obtained with the Karlsruhe-Strasbourg Laser-FIS are taken from several papers (as summarised in Buschmann and Lichtenthaler 1998). ++ = strong rise, + = rise, - - = strong decrease, - = decrease, 0 = no significant change of the fluorescence ratios given on top of each column.

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 or leaf parts				
Variegated/green leaf	++	++	++	0
Lower/upper leaf side	++	++	+	0
Yellow-green/green leaf	+	++	++	+
2nd flush/1st flush leaf	- -	- -	++	-
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 shows some examples of fluorescence imaging obtained with the Karlsruhe-Strasbourg laser-FIS. The fluorescence ratio images have been demonstrated for different developmental stages, several types of strain and stress, as well as different inhibitory treatments. The differences in fluorescence signatures must be interpreted by taking into account the causes for changes of fluorescence intensity mentioned above (concentration of the emitting substance, internal optics of the sample, photosynthetic activity, and energy loss by heat production). Thus, the reduction of the blue fluorescence with high temperature might be due to changes in optical properties of the leaf or in the chemical "micro-environment" of ferulic acid (as postulated by Morales et al. 1998 from measurements with beet leaves). One also has to consider the accumulation of UV-absorbing, non-fluorescing phenolic substances in the epidermis under heat and other stress conditions reducing the amount of incident UV that can excite ferulic acid in cell walls and thus reduce the blue fluorescence.

The non-destructive fluorescence imaging allows measurements for longer periods, i.e., one can follow changes of the fluorescence signature with time, e.g., the Kautsky effect: changes of the Chl fluorescence related to the onset of photosynthetic activity in pre-darkened leaves (Lichtenthaler and Babani 2000, Lichtenthaler et al. 2000). Furthermore, repetition of measurements is possible several days or weeks later provided one is able to fix the attached leaf sample in the same position (of course, taking in account changes induced by leaf growth and ageing – cf. Šesták and Šiffel 1997, Šesták 1999). Thus, stress development as well as regeneration of the leaf and the photosynthetic apparatus, after countermeasures have been taken, can be followed.

The non-homogeneous distribution of the fluorescence intensity or fluorescence ratios across the leaf area might help to interpret high Chl fluorescence on the peripheral border of a leaf as water or temperature stress (Lang *et al.* 1996), the dotted distribution of high fluorescence intensity indicating mite attack (Buschmann and Lichtenthaler

1998), or appearance of many spots with high blue fluorescence that indicate an attack of the white tobacco fly (Lang *et al.* 1994). Laser-FIS and FL-FIS can be applied also for the fluorescence analysis of fruits, vegetables, grains, wood, and their products.

Conclusion

This review shows the potential capacity of fluorescence imaging *via* pulsed laser or flashed (FL) fluorescence imaging systems (Laser-FIS or FL-FIS). Fluorescence imaging is a successfully developed research technique that is excellent for non-destructive screening of different developmental stages, growth conditions, senescence of leaves, and development and maturity of fruits. It also permits to detect strain, stress, and herbicide treatments affecting the physiological function and photosynthetic quantum conversion of plants as well as the maturity and

senescence of agro-food. The two existing functional systems for sensing all four fluorescence bands (blue, green, red, and far-red), the laser-FIS and the FL-FIS, are fully appropriate for routine analysis of plant materials. Further improvement of image sensors sensitivity for plant-specific fluorescence signals will make fluorescence imaging the major tool for quality control by automated inspection and sorting as well as remote sensing in agriculture, horticulture, forestry, and environmental analysis.

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