Contribution of Chlorophyll Fluorescence to the Reflectance of Leaves in Stressed Plants as Determined with the VIRAF-Spectrometer

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Reflectance spectra allow the early detection of stressors causing differences in pigment content as well as changes of leaf tissue structure and photosynthetic activity. The reflectance decreased with increasing Chl content in greening bean leaves. In stressed leaves, in turn, the reflectance increased with decreasing Chl content. This also caused a shift of the red reflection rise ("red edge") to shorter wavelengths ("blue shift") associated with a blue shift of the inflection point of the red edge. The contribution of the red and far-red Chl fluorescence to the reflectance signal at the red edge of the spectrum and the shift of the wavelength position of the inflection point are demonstrated and discussed.

Introduction

At their natural habitat plants are exposed to various kinds of natural and anthropogenic stressors which can have consequences on plants ranging from reduced photosynthesis and growth to the death of plants when the stressors exceed these stress-coping mechanisms as reviewed by Lichtenthaler (1996 and 1998). Optimum stress detection should be non-destructive and fast. In case of crop plants and in forestry, stress should be realized as early as possible in order to be able to take countermeasures for the protection of plants. Examination of leaves is most promising since the leaves represent the largest surface of the plant. Good parameters for stress detection in leaves are: (i) leaf pigment content (Chls and carotenoids) and pigment ratios, such as *a/b* and $(a+b)/(x+c)$, (ii) leaf surface, (iii) leaf tissue structure and (iiii) photosynthetic activity. Stress to the photosynthetic apparatus decreases the pigment content and the physiological activity of plants which is reflected in a change of the optical leaf properties. By means of spectroscopic methods, such as measurements of reflectance, absorption and Chl fluorescence the four parameters (i) to (iiii) mentioned above can be detected. Some parameters can even be sensed remotely from aircrafts and satellites (for a review see Buschmann, 1993; Lichtenthaler *et al.*, 1998).

Many studies on the optical characteristics of leaves have been carried out using one type of spectroscopy (for a review see e.g. Gausman and Quisenberry, 1990). Here we present the VIRAF-spectrometer that allows measuring in parallel *in vivo* spectra of reflectance, absorption and Chl fluorescence with one leaf sample. The spectra are measured by illuminating the leaf sample with strong white light as is done in remote sensing of plants when detecting the reflected sunlight and this also provides a sufficiently high Chl fluorescence yield. In this study we compare the reflectance and Chl fluorescence spectra of green, greening and yellowish-green leaves with different Chl content and examine the influence of the Chl content on the optical spectra of leaves. In particular we investigated the possible contribution of the red Chl fluorescence to the reflectance spectra of leaves.

Abbreviations: Chl, chlorophyll; NIR, near infra-red (here: 700 to 900 nm); VIRAF, Visible Infra-red Reflectance Absorption Fluorescence, x+c, xanthophylls and carotenes.

Materials and Methods

Plant material

Leaves of the following plants were used for detection of reflectance, absorption and Chl fluores-

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cence: a) 20-year old cherry-laurel plant (*Prunus laurocerasus* L.) leaves of green plants and plants with a mineral deficiency growing on stony, sandy soil in a garden in Karlsruhe, b) 6-week old tobacco plants (*Nicotiana tabacum* L.) grown in pots in a greenhouse of the Botanical Garden of the University of Karlsruhe, c) 8-day old etiolated bean plants (*Phaseolus vulgaris* L.) grown in pots in a growth chamber (23° C, 60% relative humidity).

VIRAF-spectrometer

For measuring in parallel the spectra of *in vivo* reflectance, absorption and Chl fluorescence the VIRAF-spectrometer, which has recently been developed (Buschmann *et al.* 1994), was used. A scheme of this instrument is shown in Fig. 1. A 100 W quartz halogen lamp (Oriel 66172, LOT, Darmstadt/Germany) with collimator optics (L1) is used as light source. For reflectance measurements the light beam is reflected by mirror M1 towards mirror M3 and via the lens L3 and the diaphragm D2 to the beam splitter BS (Spindler & Hoyer, Göttingen/Germany) that reflects 50% of the light, leading it to the sample. For Chl fluorescence measurements the diaphragm D2 behind lens L3 is exchanged for a blue filter F1 (Corning Nr. 9782, Schröder, Ellerau/Germany). For measuring transmittance mirror M1 is turned by 90 degrees and the light beam is then directed via mirror M2, lens L2, diaphragm D1, mirror M4 rectangularly on to the sample. The irradiance at the sample for all types of measurements amounts to 2000 μmol quanta · m^{-2} · s^{-1}. The illumination of the sample, both in reflectance and fluorescence mode, starts after opening an electro-mechanical shutter in front of the sample (Sh, 1 ms opening time) triggered by the controller via a signal from the computer interface. Light coming from the leaf sample crosses the beam splitter BS (reduced by 50%), is reflected by mirror M5 and finally imaged by lens L4 on the entrance slit of the spectrograph (Oriel 77400 Multispec, LOT, Darmstadt/Germany). The different spectral parts of the light are detected by a self-scanned photodiode array with 1012 detector elements (S2304–1024Q, Hamamatsu, Herrsching/Germany). Integration times can be chosen between 20 ms and 1 s for the 510 data points of one spectrum. The signals are transferred to a personal computer that controls all operations of the instrument and assists with the mathematical operations (e.g. calculation of means, differences and derivatives).

Measurement of spectra with a conventional spectrometer

In vivo spectra of reflectance and transmittance were also measured with a conventional spectrometer (Shimadzu UV 2101 PC, Shimadzu, Kyoto/Japan) using the integrating sphere attachment. The spectrum of absorbance A was calculated from the spectrum of transmittance T [A = – log (T/100)]. The spectrum of the absorption in percent (A%) was calculated by subtracting the reflectance R and the transmittance T (A% = 100 – R – T).

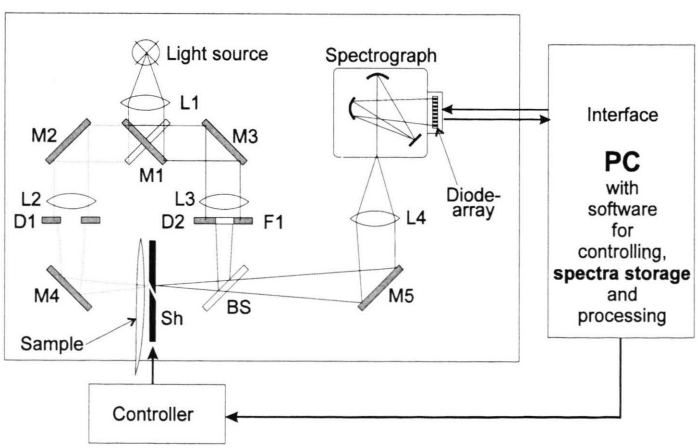

Fig. 1. Scheme of the VIRAF-spectrometer developed for parallel measurements of reflectance, absorption and Chl fluorescence in the visible and the near infra-red between 400 and 910 nm (L = lens, M = mirror, F = filter, D = diaphragm, Sh = shutter, BS = beam splitter). Reflectance and Chl fluorescence are measured with mirror M1 reflecting light towards mirror M3, whereas absorption is measured with mirror M1 reflecting light towards mirror M2 (dotted line). For reflectance measurements the diaphragm D2 is placed between the lens L3 and the beam splitter BS. In case of Chl fluorescence measurements filter F2 replaces the diaphragm D2. Further explanation see text under Materials and Methods.

Determination of the Chl content

The Chl content of leaves was determined from leaf pigment extracts with 100% acetone using the redetermined equations of Lichtenthaler (1987).

Results and Discussion

The *in vivo* reflectance, transmittance and absorption spectra of a green leaf taken with a conventional spectrometer with an integrating sphere attachment showed a high absorption in the visible spectral range (between 400 and 700 nm, Fig. 2A). A lower absorption and thus a higher reflectance and transmittance were visible in the near infrared (700 to 800 nm) and somewhat less pronounced in the green spectral range around 550 nm (for other leaves see: Woolley, 1971). The *in vivo* reflectance and transmittance spectrum, taken at the same leaf position, exhibited the same but inverse characteristics, both spectra showed minima in the spectral range of the absorption maxima and *vice versa*. The *in vivo* absorption bands are broad due to the fact that Chl is integrated into different Chl-carotenoid-protein complexes (French *et al.*, 1972; Lichtenthaler *et al.*, 1981) and that light is internally reflected, scattered and refracted (Butler, 1974; Vogelmann, 1993). The spectrum of absorbance A (logarithmic scale) is characterized by more pronounced differences between the blue to red wavelength range than the spectrum of percent absorption A% (Fig. 2B).

Changes during greening of bean leaves: The *in vivo* reflectance spectrum of a leaf decreased with increasing Chl content, especially in the range between 500 and 700 nm. This was shown for etiolated bean leaves which greened upon illumination with white light (Fig. 3). With increasing Chl content the reflectance decreased, first in the red Chl absorption band near 680 nm and later also in the range between 500 and 680 nm. It has been shown before that the decrease of reflectance is not linearly correlated to the increase of the Chl content (Buschmann and Nagel, 1993; Gitelson and Merzlyak, 1996). The red Chl absorption maximum near 680 nm remained at the same wavelength position with increasing illumination time and Chl content from leaf 1 (yellowish-green) to leaf 4 (dark-green). The inflection point's wavelength position of the "red edge" (i.e. the steep reflectance rise between 690 and 730 nm) shifted, however, from 686 nm (leaf 1) via 692 nm (leaf 2) and 697 nm (leaf 3) to 712 nm (leaf 4). In addition,

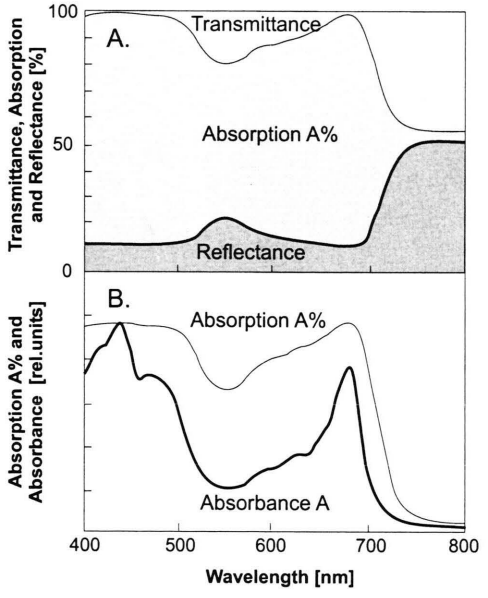

Fig. 2. *In vivo* spectra of a fully green tobacco leaf (*Nicotiana tabacum* L.) taken by means of a conventional Shimadzu spectrometer with an integrating sphere. A: *In vivo* spectrum of transmittance, absorption and reflectance in percent of the incident light (= 100%). B: Comparison of the *in vivo* spectrum of absorption (A% = 100 – T – R) and of absorbance (A = – log(T/100)). A% = absorption [%], T = transmittance [%], R = reflectance [%].

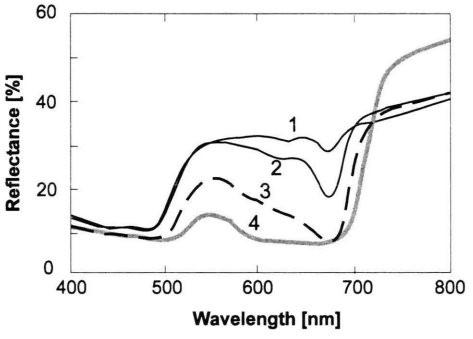

Fig. 3. *In vivo* reflectance spectra of primary leaves of an 8-day old etiolated bean plant (*Phaseolus vulgaris* L.) during greening in the light. The spectra 1 to 3 were taken from leaves with only few hours of light (0.1 h, 2 h and 4 h, respectively), whereas spectrum 4 is from a dark-green, fully developed leaf after 2 days of light. The Chl content was: 1 μg (leaf 1), 4 μg (leaf 2), 12 μg (leaf 3), and 42 μg cm^{-2} leaf area (leaf 4).

we observed a strong increase in reflectance in the near infra-red (NIR) between 730 and 800 nm from the young, developing bean leaves 1 to 3 to the adult, fully differentiated dark-green bean leaf 4, which had reached its full leaf size and thickness (Fig. 3). This increase in the NIR-region was associated with an increase in the size and length of the mesophyll cells and the size of the aerial interspaces in the spongy parenchyma causing this rise in the NIR-reflectance.

Differences between stressed and green cherry-laurel leaves: When the leaves from green cherry-laurel plants were compared to the yellowish-green leaves from plants grown and exposed to mineral stress and N-deficiency, the reflectance in the NIR-region above 750 nm was the same in all cases and independent of the Chl content (Fig. 4). In contrast, in the visible spectral range (here: 500 to 700 nm) of the reflectance spectrum considerable changes were found, similar to those of greening bean leaves. With increasing Chl content the wavelength position of the reflectance minimum in the red Chl absorption band near 680 nm remained constant. The reflectance signal decreased first in the red Chl absorption band and then in the spectral range between 500 and 680 nm. The inflection point of the reflectance rise at the red edge shifted from 692 nm of leaf 1 (with low Chl content) of the stressed plant to 720 nm for the intact green leaf 4 of the healthy plant (Table I). Parallel to the shift of the inflection point position a decrease of the reflectance at 700 nm occurred with increasing Chl content of the leaves (Table I). When one takes the reflectance spectrum of the healthy plant's green leaves as a standard, the leaves of the stressed plant exhibit a rise of the reflectance and a blue shift of the red edge as well as of the wavelength position of the inflection point with decreasing Chl content. As the Chl a/b ratio was in the same range (2.9–3.0) for all four leaves, this blue shift is due to the lower Chl content only, but not to changes in the Chl a/b ratio or different amounts of the light harvesting Chl-proteins LHC-II (cf. Lichtenthaler *et al.*, 1982).

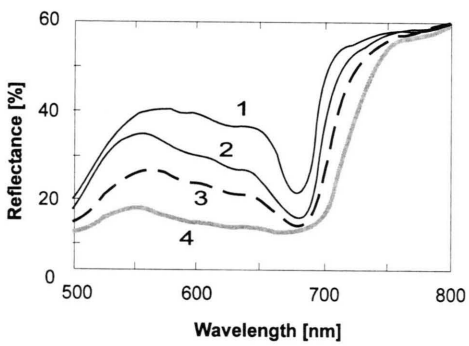

Fig. 4. *In vivo* reflectance spectra of cherry-laurel leaves (*Prunus laurocerasus* L.) from different plants taken by means of a conventional spectrometer with integrating sphere. The differences between leaf 4 (dark-green healthy plant) and leaves 1 to 3 (yellow to yellowish green plant with N- and mineral deficiency) were caused by mineral stress. The Chl content was: 11 μg (leaf 1), 29 μg (leaf 2), 31 μg (leaf 3), and 52 μg cm^{-2} leaf area (leaf 4).

Leaf reflectance and Chl fluorescence

In order to investigate the relationship between leaf reflectance and Chl fluorescence we compared reflectance and Chl fluorescence emission spectra of the cherry-laurel leaves with different Chl content. The *in vivo* spectrum of the Chl fluorescence overlapped with the *in vivo* spectrum of reflectance (Fig. 5). This was true for the green leaf with a high Chl content and for the yellowish-green leaf with a lower Chl content. Leaf 2 with

Table I. Reflectance at 700 nm and wavelength position of the inflection point of the red rise of the reflectance signal ("red edge") as determined for fully developed cherry-laurel leaves (*Prunus laurocerasus* L.) from plants with different Chl content. The yellowish-green to light-green leaves 1 to 3 with low Chl content came from a plant that grew on a stony, sandy stand (with mineral stress and N-deficiency), the dark-green leaf 4 was from a fully green plant growing on humus-rich soil.

Leaf number	Chl Content [μg Chl $a+b$ cm^{-2}]	% Reflectance at 700 nm	Inflection point of the "red edge" [nm]
Leaf 1	11	48	692
Leaf 2	29	38	697
Leaf 3	32	25	704
Leaf 4	52	17	720

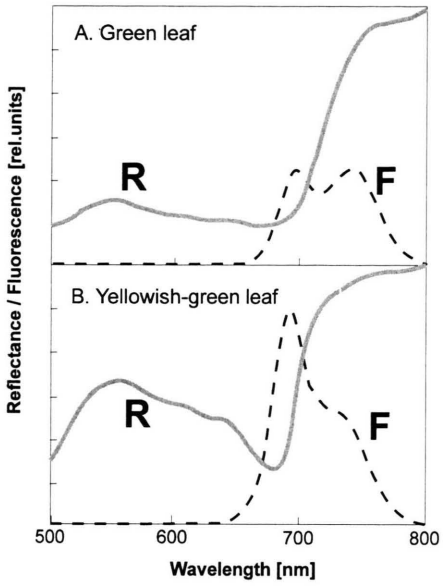

Fig. 5. *In vivo* spectra of A) a green and B) a yellowish-green cherry-laurel leaf (*Prunus laurocerasus* L.) showing the dependence of both spectra on the leaves' Chl content. The spectra of reflectance (R) and of Chl fluorescence (F) were taken here by means of a conventional spectrometer with integrating sphere (cf. Fig. 4). The Chl content of the leaves was: 52 μg (green leaf 4) and 29 μg cm^{-2} leaf area (yellowish-green leaf 2).

lower Chl content also showed a much higher Chl fluorescence with a particular, stronger increase of the red 690 nm band (F690) in comparison to the far-red 740 nm fluorescence band F740. This yellowish-green leaf also exhibited a much higher reflectance which paralleled the higher red maximum of the Chl fluorescence (near 690 nm) as compared to the green leaf 4. In addition, the inflection point of the rise of the reflectance at the red edge was shifted to shorter wavelengths from 723 to 696 nm (blue shift). The contribution of the Chl fluorescence to the blue shift of the red edge became clearly visible when modelling the reflectance and Chl fluorescence spectra measured separately by conventional spectrometers illuminating the sample with low-intensity monochromatic light (Fig. 5). The reflectance signal in the red edge region increased significantly due to the contribution of the Chl fluorescence. The inflection point of the red edge, however, was less affected by the Chl fluorescence, it shifted in the order of 1 nm (fully green leaf 4) and 3 nm (yellowish green leaf 2) towards shorter wavelengths when the Chl fluorescence of the two leaves was integrated into the reflectance spectrum.

In order to further investigate the relationship between reflectance and Chl fluorescence we measured the reflectance of the same leaf at high and low Chl fluorescence yield. Predarkened green leaves exhibit the Chl fluorescence induction kinetics (Kautsky effect) upon illumination with high Chl fluorescence yield after ca. 100 ms (Fp) and a much lower Chl fluorescence after onset of photosynthetic CO_2 fixation in the steady state Fs

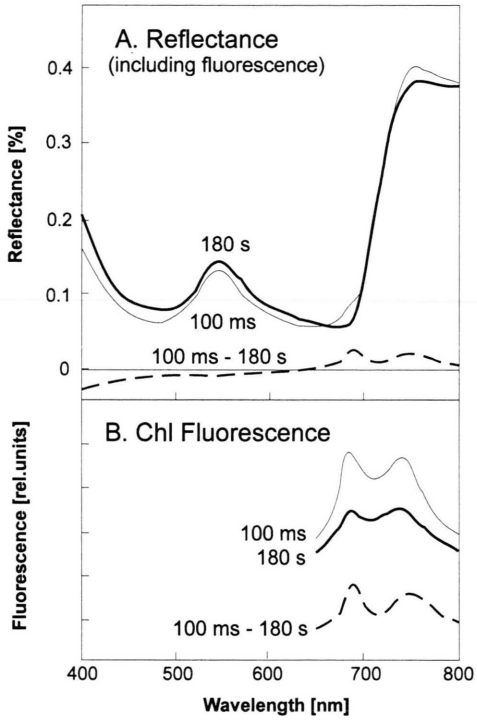

Fig. 6. *In vivo* spectra of a tobacco leaf (*Nicotiana tabacum* L.) taken using the VIRAF-spectrometer. A) Reflectance spectra measured by illumination with strong white light (2000 μmol m^{-2} s^{-1}) which also induces Chl fluorescence contributing to the reflectance spectrum. The spectra were taken 100 ms and 180 s after the onset of illumination in a predarkened (20 min) tobacco leaf. The difference spectrum (100 ms – 180 s) between the spectra at 100 ms (measured at Fm) and 180 s (measured at Fs) shows the decrease of the Chl fluorescence due to the "Kautsky effect". B) Spectra of the Chl fluorescence measured in parallel (excitation by strong blue light, 2000 μmol m^{-2} s^{-1}) at 100 ms and 180 s. The spectra of the Chl fluorescence show the typical maxima (near 690 and 740 nm) of the Chl fluorescence. The shape of the difference spectrum (100 ms – 180 s) in B resembles that of the difference spectrum from the reflectance spectra in A.

(e.g. Lichtenthaler *et al.*, 1986; Krause and Weis, 1991; Govindjee, 1995). When taking the *in vivo* reflectance spectra of an intact tobacco leaf (predarkened for 20 min) with the VIRAF-spectrometer at the beginning of the illumination (within 100 ms) and 180 s after the onset of illumination (Fig. 6A), there was a slight increase of the reflectance between 400 and 650 nm in the second spectrum and a decrease in reflectance between 650 and 800 nm. This can clearly be seen in the difference spectrum (100 ms – 180 s) (Fig. 6A). The latter showed a maximum (of the signal's decrease) at 690 and 740 nm and indicated that the differences in the two reflectance spectra measured at maximum (Fp) and steady state fluorescence (Fs) were caused by the Chl fluorescence, the emission of which had decreased from the first to the second reflectance spectrum due to the Kautsky effect.

The *in vivo* spectrum of the Chl fluorescence measured separately with the VIRAF at the same intact tobacco leaf point showed the same maxima (Fig. 6B). The Chl fluorescence also decreased there between 100 ms (Fp) and 180 s (Fs) after the onset of illumination (see difference spectrum: 100 ms – 180 s in Fig. 6B), due to the well-known Kautsky effect (Buschmann and Schrey, 1981; Lichtenthaler *et al.*, 1986). This Chl fluorescence decrease was stronger for the short- wavelength red maximum than for the long-wavelength far-red maximum of the Chl fluorescence. Therefore, the ratio between the two Chl fluorescence maxima (ratio F690/F740) decreased from a value of ca. 1.2 in the first fluorescence spectrum to a value of ca. 0.9 in the second spectrum. Similar differences in the reflectance spectra of the same tobacco leaf were obtained when the Chl fluorescence had been increased by treatment with the herbicide diuron (10^{-4} M) that blocks the photosynthetic quantum conversion and increases the Chl fluorescence yield (data not shown).

The Chl fluorescence contributing to the reflectance spectrum led to an increase of the reflectance signals between 650 and 800 nm and it slightly shifted the inflection point of the red edge by 1 nm to shorter wavelengths. This blue shift could not be easily seen in the VIRAF reflectance spectrum of the tobacco leaf (Fig. 6A) as its spectral resolution is limited due to the applied diode array detector.

Conclusion

Our data show that the *in vivo* reflectance spectra of leaves were strongly altered during greening and in stressed plants as induced by changes in the Chl content, the leaf tissue structure (cell size, aerial interspaces), and the loss of photosynthetic activity (Kautsky effect, diuron treatment). With the VIRAF-spectrometer, which is able to measure the reflectance, absorption and Chl fluorescence in parallel with one leaf sample, one can obtain information on the contribution of Chl fluorescence to the reflectance signal in the region of the red edge. The contribution of Chl fluorescence to the reflectance spectrum is higher in leaves with low Chl content exhibiting a higher Chl fluorescence.

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