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The F685/F730 Chlorophyll Fluorescence Ratio as a Tool in Plant Physiology: Response to Physiological and Environmental Factors*

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Summary

The effect of chlorophyll concentration, light intensity and leaf temperature on the chlorophyll fluorescence ratio F685/F730 of intact leaves was evaluated. Fluorescence reabsorption that affects mainly the F685 band increases with chlorophyll concentration. This phenomenon was studied on an aurea mutant of tomato and its wild type, with very different chlorophyll content. Fluorescence spectra of the two genotypes were corrected for reabsorption using their transmittance and reflectance properties. The correction removes most of the differences in the two fluorescence spectra. The F685/F730 decreases during the declining phase of the fluorescence induction kinetics. We demonstrated that when red light is used to induce the fluorescence kinetics the variation of F685/F730 is not due to a change in the leaf absorption, as proved by the simultaneous measurement of leaf transmittance. This evidence suggests that the F685/F730 ratio is sensitive to changes in the photosynthetic activity of the leaf. Under natural conditions, the F685/F730 ratio markedly decreases as light intensity and leaf temperature increase during a daily cycle. This behaviour can be due to photoinhibitory and heat stresses. In controlled laboratory conditions, the F685/F730 ratio was seen to decrease under high light intensity ($>1000\,\mu mol\,m^{-2}\,s^{-1}$) at constant leaf temperature. It decreases also when leaf temperature was decreased from 25 °C to 14 °C at low light intensity ($150\,\mu mol\,m^{-2}\,s^{-1}$). A possible interpretation of these experimental data relies on a non-negligible contribution of PSI to the total fluorescence at physiological temperatures with respect to PSII fluorescence. Changes in the photosynthetic activity of the two photosystems may induce variation in the F685/F730 ratio. Our results indicate that light intensity and leaf temperature are important parameters to take into account when the F685/F730 ratio is used as stress indicator.

Key words: Chlorophyll fluorescence; stress detection in plants; temperature; light intensity; continuous monitoring; environmental factors; Lycopersicon esculentum Mill.; Epipremnum aureum; Juglans regia L.; Fagus sylvatica L.; Ficus benjamini.

Abbreviations: Chl = chlorophyll; ChlFR = ratio between the red and near-ir bands of the chlorophyll fluorescence spectrum; F690/F735 = ratio of the chlorophyll fluorescence intensity at 690 nm and 735 nm; PPFD = photosynthetic photon flux density; ΔT = difference between leaf and air temperatures; LEAF = Laser Excited Automatic Fluorometer.

* Dedicated to Prof. Hartmut Lichtenthaler on the occasion of his 60th birthday.

Introduction

The study of the *in vivo* chlorophyll (Chl) fluorescence emitted by higher plants is of great interest because of its application to monitor the physiological state of plants. Measuring the Chl fluorescence induction kinetics of intact leaves has been proposed as a helpful method to evaluate the effect of different environmental stress factors on the plant photosynthetic apparatus (Krause and Weis, 1984; Schreiber, 1984; Renger and Schreiber, 1986). More recently, the extensive research of Lichtenthaler and co-workers suggested that useful information on the condition of the plant can be obtained by the shape of the chlorophyll fluorescence spectrum (Rinderle and Lichtenthaler, 1988). They showed that the ratio between the red and near-ir chlorophyll fluorescence bands, F690/F735, at steady state and room temperature can be used as a stress indicator of plants (Lichtenthaler and Rinderle, 1988). This method is particularly attractive since it does not require pre-darkening of the leaf and, therefore, can be used in remote sensing measurements (Cecchi et al., 1994; Valentini et al., 1994). However, the physiological meaning of the changes in F690/F735 under different environmental conditions of the plants is not completely known. It has been reported that F690/F735 is strongly dependent on the leaf chlorophyll concentration (Lichtenthaler et al., 1990) since the fluorescence band at shorter wavelengths undergoes partial reabsorption by the chlorophyll itself (Agati et al., 1993 a). Consequently, any stress factor that changes the leaf chlorophyll concentration can be detected by F690/F735. The Chl fluorescence ratio is also sensitive to the photosynthetic activity of the leaf. In plants treated with diuron (DCMU) to block the photosynthetic electron transport from PSII to PSI the F690/F735 ratio increases with respect to controls (Lichtenthaler and Rinderle, 1988). Furthermore, changes in F690/F735 were observed during the fluorescence induction kinetics (Kautsky effect) (Szabó et al., 1992).

Many different effects can influence the Chl fluorescence ratio; therefore, the correct use of F685/F730 in physiological studies and as plant stress indicator requires the knowledge of its dependence on all of the environmental factors. For this reason we started to analyse the changes of Chl fluorescence ratio in plants under different controlled conditions and also under natural environments. This study is far from being conclusive, however, it addresses the main factors affecting F685/F730 and proposes possible interpretation of the physiological meaning of the Chl fluorescence ratio.

Materials and Methods

Since the two maxima of the Chl fluorescence spectrum shift slightly in the range of 5–10 nm depending on the species, it is convenient to use the term ChlFR to indicate the ratio between the fluorescence intensities of the red (685 ± 5 nm) and near-ir (735 ± 5 nm) bands.

The ChlFR was measured by a portable Laser Excited Automatic Fluorometer, LEAF®, built in our laboratory. The instrument characteristics and the experimental procedures used for fluorescence measurements using LEAF are described elsewhere (Mazzinghi, to be published). The instrument was mainly used for field application even though it was also found useful in laboratory experiments.

Complete fluorescence spectra induced by laser light were detected in the laboratory by the apparatus described in detail by Agati et al. (1993 a) and reported in Fig. 1, for convenience. Briefly, the laser excitation beam was coupled to an optical fibre that was put in contact to the upper side of the leaf. The same fibre was used to collect the Chl fluorescence signal and deliver it to the entrance port of a monochromator (Jobin Yvon mod. HRP). Fluorescence spectra were recorded by an intensified diode array detector and analyzer (EG&G PAR OMA III mod 1421 and 1460). The system can acquire a complete spectrum in about 10 ms, making the measurement of the fluorescence induction kinetics possible at all wavelengths simultaneously. The high sensitivity of the system also allows for recording of spectra with a very low excitation power.

Total reflectance (R(λ)) and transmittance (T(λ)) spectra of leaves were measured using an integrating spheroid (PU7908/24, Pye Unicam, Cambridge, England) fitted inside a double beam spectrophotometer (PU8800, Pye Unicam, Cambridge, England). For details of the method see Agati et al. (1993 a).

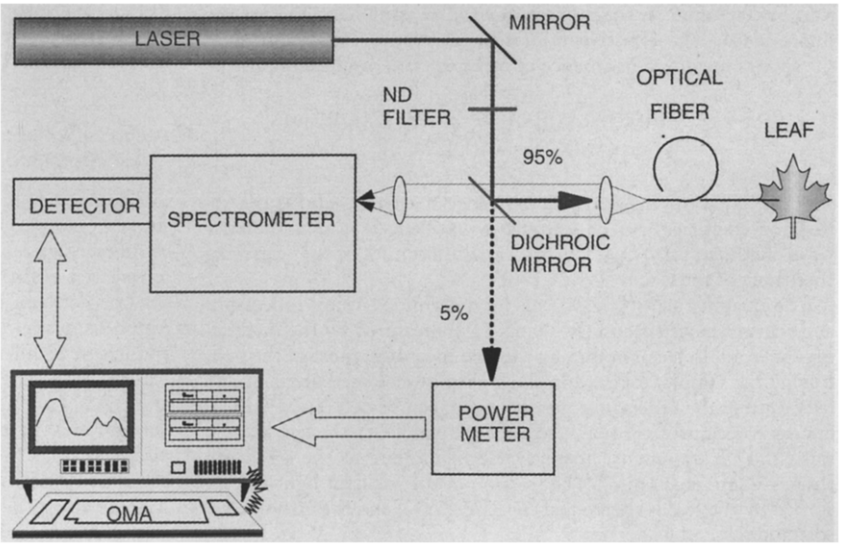

Fig. 1: Experimental set-up for laboratory measurements of laser-induced chlorophyll fluorescence spectra of intact leaves.

All measurements in the laboratory were performed at a room temperature of 22 ± 2 °C (if not otherwise specified) on plants grown in pots, without detaching the leaves.

Effect of chlorophyll concentration on the chlorophyll fluorescence ratio

The effect of Chl concentration on the leaf fluorescence spectrum was evaluated on the wild type cv. UC 105 of tomato (*Lycopersicon esculentum* Mill.) and its aurea mutant, isolated from tissue cultures by Lipucci Di Paola et al. (1988). The two genotypes had a marked difference in the pigment content as tested by acetone extraction and spectrophotometric determination (Lichtenthaler, 1987). The total Chl and carotenoids in the aurea mutant were 3.6 and 3 times less, respectively, than those of the wild type. The Chl *a* to Chl *b* ratio was higher in the mutant than in the wild type (3.7 vs. 2.5). Other phenotypic characteristics of the aurea mutant were described previously (Lipucci Di Paola et al., 1988; Lercari and Lipucci Di Paola, 1991).

The plants were grown in a greenhouse under natural light conditions (22/17 °C day/night mean air temperature). All of the spectral measurements were carried out on fully expanded leaves of apical and basal shoots in 2 months old plants.

Fluorescence measurements were performed on leaves placed in a leaf holder made by a metallic clip covered with soft neoprene rubber, to avoid any damage. The whole plant was dark adapted for at least 20 min and then excited at a laser photon fluence rate of less than 20 $\mu mol\, m^{-2} s^{-1}$, which avoids induction of fluorescence kinetics. This was checked measuring the total fluorescence intensity for 5 min after turning on the laser light. In these conditions, fluorescence is very close to the ground (initial) fluorescence F_0, when all the electron acceptors are open. Excitation was provided by the 632.8 nm line of a 5 mW HeNe laser (Uniphase mod. 105-1, Spectra Physics, Mt. View, CA) or by the 457.9 nm line of a 100 mW Argon-ion laser (NEC Model GLG3100, Japan).

Because of the partial overlapping of the transmission and fluorescence spectra, the measured fluorescence spectrum of the leaf is affected by fluorescence reabsorption. The spectral distortion induced by the reabsorption can be estimated by considering the absorption of the excitation and of the emitted light through the leaf. We have recently suggested a simple model for this purpose, reported in detail by Agati et al. (1993 a), which can be summarized as follows. The contribution of scattering to the light attenuation was taken into account by defining an effective average optical pathlength $Z(\lambda)$ into the leaf, due to diffuse propagation, and supposing it to be the same, on the average, for the exciting light and for the fluorescence. The effective fluorescence spectra, $I_F^e(\lambda)$, defined as the spectra measured in absence of reabsorption, can be computed:

$$I_F^e(\lambda) = I_F^m(\lambda)\,(\alpha(\lambda_L)+\alpha(\lambda)f(\lambda))\,[1-\exp(-\alpha(\lambda_L)Z(\lambda_L))]/\{\alpha(\lambda_L)\,[1-\exp(-(\alpha(\lambda_L)+\alpha(\lambda)f(\lambda))Z(\lambda_L))]\}, \quad (1)$$

where $I_F^m(\lambda)$ is the measured fluorescence spectrum, $\alpha(\lambda)$ is the absorption coefficient of the leaf at the wavelength λ, λ_L is the laser wavelength, and $f(\lambda)$ contains the variation with λ of the scattering coefficient of the leaf, with ($f(\lambda_L) = 1$).

The quantity $\alpha(\lambda)f(\lambda)Z(\lambda)$ can be determined using reflectance and transmittance spectra ($R(\lambda)$ and $T(\lambda)$) measured by the integrating spheroid. In fact, combining the two measures, most of the light leaving the sample, forward in the transmittance measurement and backward in the reflectance measurement, can be detected. Only radiation transmitted through the leaf edge parallel to the leaf plane is missed. This amount is, however, negligible due to the small leaf thickness (about 0.2 mm). The fraction of the incident light not absorbed by the leaf is then equal to $(R(\lambda)+T(\lambda))$ and the effective absorption is:

$$\alpha(\lambda)Z(\lambda) \approx \ln[1/(R(\lambda)+T(\lambda))]. \quad (2)$$

The use of these values in Eq. (1) makes the spectral correction of the laser induced fluorescence possible.

Chlorophyll fluorescence spectra during the Kautsky kinetics

The study was performed using a well-watered ornamental plant (*Epipremnum aureum* (Marbel Queen)).

The fluorescence induction kinetics was caused and monitored by the LEAF instrument on a dark adapted plant. Fluorescence was excited by a diode laser emitting at 635 nm through a 400 µm optical fiber, delivering at the leaf surface a PPFD of about 250 $\mu mol\, m^{-2} s^{-1}$. The LEAF system acquired fluorescence values in auto-repeat mode at time intervals of 1 sec. The leaf absorption was monitored during the Kautsky kinetics by measuring the total leaf transmittance at various irradiation times. The leaf was irradiated at 632.8 nm by a HeNe laser at the entrance port of the integrating spheroid inside the spectrophotometer. The low intensity of the measuring beam (PPFD less than 0.01 $\mu mol\, m^{-2} s^{-1}$) was insufficient to excite any fluorescence (Agati et al., 1993 a). The laser beam was expanded and deflected towards the leaf by a prism and gave an excitation PPFD of about 230 $\mu mol\, m^{-2} s^{-1}$. The intensity was chosen to be comparable with that of the LEAF instrument. The irradiation beam was shut off while T was recorded at various irradiation times.

Effect of natural factors on the chlorophyll fluorescence ratio

The ChlFR was measured during a complete diurnal cycle with the LEAF fluorometer in continuous mode on leaves of a well watered walnut tree (*Juglans regia* L.) located in an open area, and exposed to direct sun light during the entire day. The experimental set-up used is sketched in Fig. 2. Data were collected by LEAF, using a clip-on probe, at time intervals of 4 sec, averaged every 15 sec, for a resulting sampling time of 1 min. Data were then averaged again on a PC every 10 min, which was the sampling time of all other micro-meteorological parameters. At the same time the PPFD was also monitored by the fluorometer using a cosine corrected Quantum Sensor (LICOR), placed parallel and close to the leaf surface. Leaf temperature was monitored continuously by a portable infrared thermometer (Land Cyclops Compac 3, Minolta) connected to the same PC used for downloading fluorescence data. The environmental parameters (air temperature, relative humidity, PPFD, total solar irradiance, wind speed an direction) were detected by a meteorological station, located in an open area close (10 m) to the plant, and were acquired by a data logger (DELTA-T).

Effect of temperature and light intensity on the chlorophyll fluorescence ratio

The effect of high light intensity and temperature on ChlFR was studied on 3 year-old seedlings of *Fagus sylvatica* L. grown in a forest nursery in central Italy. Before bud burst seedlings were transferred to a greenhouse and kept well watered but covered with a shading network to have a PPFD as low as 50 $\mu mol\, m^{-2} s^{-1}$. After 3 months, plants were moved to a controlled environment chamber and left to acclimate for two weeks in the same shaded condition as before. Then the plant under investigation was exposed to full light and the F685/F730 ratio and the leaf temperature were monitored continuously by the same experimental set-up used in the field experiment (Fig. 2). The PPFD of the laser excitation at 635 nm was about 150 $\mu mol\, m^{-2} s^{-1}$. The growth chamber was programmed to have a daily cycle with a 14 h light period, a temperature of 25 °C and a relative humidity of 60 %. Light was provided by 12 metal

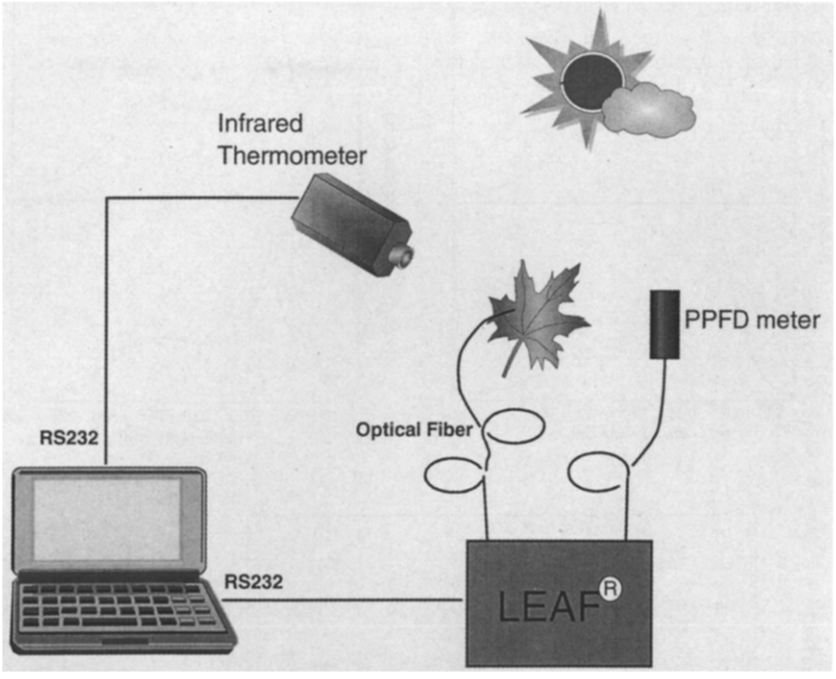

Fig. 2: Experimental set-up for continuous monitoring of the chlorophyll fluorescence ratio, leaf temperature and photosynthetic photon flux density. This set-up was used for measurements in the field and in the growth chamber.

iodur vapour lamps, which resulted in a PPFD of about 1000 $\mu mol\, m^{-2} s^{-1}$ at the plant top. Four complete daily cycles were monitored continuously.

To examine the dependence of F690/F735 by the temperature alone the chamber was programmed to reduce the temperature to 20 °C during the dark period over a 1 h period. The data were acquired and averaged with the same procedure used for the experiment on plants in natural conditions.

Laser excited fluorescence spectra under controlled conditions of light and temperature were recorded in a laboratory experiment on leaves of *Ficus benjamini*. The plant was adapted to low light with a PPFD of 150 $\mu mol\, m^{-2} s^{-1}$ (Schott KL1500 halogen lamp illuminator). The leaf attached to the plant was placed in a home-made holder thermoregulated by circulating water to maintain the leaf temperature at the desired value. Leaf temperature was monitored continuously by a portable infrared thermometer (Land Cyclops Compac 3, Minolta). Fluorescence was excited by a HeNe laser through an optical fiber irradiating the upper side of the leaf and collected by the same fiber as described above. The optical fiber was placed at 45 degrees with respect to the leaf surface to avoid covering the measuring area. The laser intensity was controlled by neutral density optical filters. Two different experiments were carried out. In the first, a laser intensity was kept at about 200 $\mu mol\, m^{-2} s^{-1}$ (30 μW on a spot of 1 mm diameter) and the ChlFR was measured continuously changing the leaf temperature at a rate of 0.5 °C/min. In the second experiment, the leaf was kept at constant temperature (25.5 ± 0.5 °C) and irradiated continuously with the laser light at a PPFD of about 2000 $\mu mol\, m^{-2} s^{-1}$.

Results and Discussion

Effect of chlorophyll concentration on the chlorophyll fluorescence ratio

Chlorophyll fluorescence spectra of the wild type and aurea mutant of tomato show the typical double peaked curve with maxima at about 685 and 735 nm (Fig. 3). The ratio of the two bands, F685/F735, which is dependent on the excitation wavelength, is higher in the aurea than in its wild type. This can be seen in Figs. 3 a and 3 b for excitation at 457.9 and 632.8 nm, respectively. Values of F685/F735 for the aurea mutant were 30 % and 60 % higher than those for the wild type at 457.9 and 632.8 nm, respectively. The relation between the ChlFR and the total Chl concentration (30.4 and 8.4 $\mu g\, cm^{-2}$ for the wild type and aurea, respectively) was in agreement with that found by the Lichtenthaler research group on different species during the autumnal Chl breakdown and the leaf greening development periods (D'Ambrosio et al., 1992; Hák et al., 1990). The well known process of fluorescence reabsorption explains the differences in ChlFR between aurea and wild type of tomato and the wavelength dependence of the effect. In fact, red light penetrates deeper into the leaf than blue light. The fluorescence emitted by deeper layers must go through a longer path length before exiting the leaf surface, and then undergoes a larger reabsorption.

The effect of the Chl concentration on the fluorescence spectrum of intact leaves has been quantified using a simple but sufficiently accurate model. The method uses the effective absorption spectra calculated by the leaf reflectance and transmittance spectra according to Eq. 2 and shown in Fig. 4. The absorption spectrum largely overlaps the 685 nm fluorescence band, which therefore undergoes reabsorption. In fact, in the Chl fluorescence spectra corrected for reabsorption according to Eq. 1, the intensity of the red band relative to the near-ir band was largely increased for both genotypes. The correction also makes the Chl fluorescence spectra of aurea and wild type much closer to each other (Fig. 3 c and d); values of F685/F735 for the aurea mutant are 9 % and 30 % higher than those for the wild type at 457.9 and 632.8 nm, respectively.

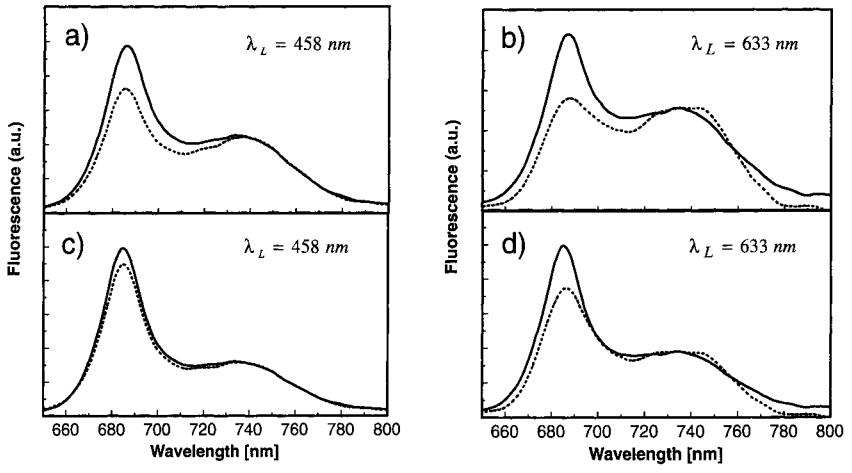

Fig. 3: Laser-induced chlorophyll fluorescence spectra of the aurea mutant (solid line) and wild type (dotted line) of tomato: a) measured spectra for excitation at 457.9; b) measured spectra for excitation at 632.8; c) spectra excited at 457.9 nm corrected for reabsorption; d) spectra excited at 632.8 nm corrected for reabsorption. Spectra of each figure are normalized at the 735 nm fluorescence peak.

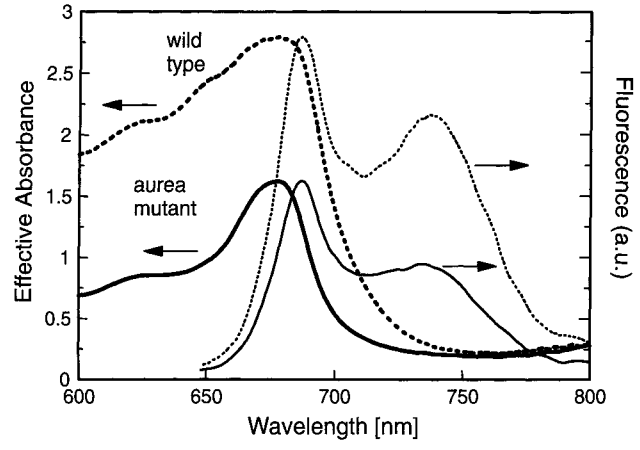

Fig. 4: Effective absorbance spectra calculated by Eq. (2) for the aurea mutant (solid line) and wild type (dashed line). The measured fluorescence spectra for the two genotypes (excitation at 457.9 nm), normalized at the relative absorption maximum, are also reported.

The correction for reabsorption eliminates almost completely the difference between the mutant and its wild type in the Chl fluorescence spectra excited at 457.9 nm. The fluorescence spectra of intact leaves became similar to that of isolated chloroplasts (Murata and Satoh, 1986). With 632.8 nm excitation some distinction still remains after correction (Fig. 3 d). This could be ascribed to the approximations introduced in the model. In fact, the assumption that the excitation light and the fluorescence travel the same average path length inside the leaf may not be completely valid for the 632.8 nm excitation when deeper leaf layers are involved.

Besides the fluorescence reabsorption dependent on the Chl concentration, other mechanisms can affect the emission bands of the two tomato genotypes to a diverse extent. Contributions to the difference in fluorescence spectra of the tomato aurea mutant and its wild type may also be due to their different aggregation of the thylakoid membranes. The wild type of tomato presents stacked thylakoid membranes, while in the mutant thylakoids are mostly isolated (Koornneef et al., 1985). The ultrastructure of chloroplasts affects the degree of energy transfer between PSII and PSI and the absorption by the two photosystems. A higher Chl *a/b* ratio indicates the presence of a larger amount of PSI relative to PSII (Anderson and Osmond, 1987). Since PSII is located mainly in the grana stacks and PSI mostly in the isolated lamellae, they are close to each other only in unstacked thylakoids, allowing for a good energy transfer between them (Briantais et al., 1986; Canaani, 1990). In granal chloroplasts, the absorption, associated mainly to the Chl of PSII, is reduced with respect to agranal chloroplast due to the sieve effect present in stacked thylakoids (Jennings and Zucchelli, 1985). To which degree the above factors contribute to the two Chl fluorescence bands at room temperature is still unknown.

Chlorophyll fluorescence spectra during the Kautsky kinetics

We observed that during the fluorescence induction kinetics the Chl fluorescence spectrum changes continuously. After the fluorescence reached its maximum, both red and near-ir bands decrease exponentially, but with different time constants. The behaviour of the total fluorescence (F685 + F730) and F685/F730 during the fluorescence decline from the maximum P to the terminal level T in *Epipremnum aureum* leaves is reported in Fig. 5 a. Similar results were observed in early studies on tomato plants (Agati et al., 1993 b).

Our data confirm previously reported spectral measurements during the fluorescence induction curve on maple leaves (Szabó et al., 1992). The decrease in the ChlFR during the dark-light transition could be due to a change in the leaf absorption as a result of a possible rearrangement of the chloroplasts (Inoue and Shibata, 1973; Brugnoli and Bjorkman, 1992). In order to investigate this hypothesis we repeated the experiment measuring also the total transmittance of the leaf during the Kautsky kinetics. Transmittance values at 685 and 730 for *Epipremnum aureum* excited with 632.8 nm light are plotted in Fig. 5 b. No changes in transmittance were observed over the time interval investigated (1 h), which is much longer than that of the Kautsky kinetics (7–12 min). Consequently, under this condition, the variation of the ChlFR during the Kautsky kinetics appears to be related to changes in the photosynthetic activity of the leaf rather then to leaf structural variations.

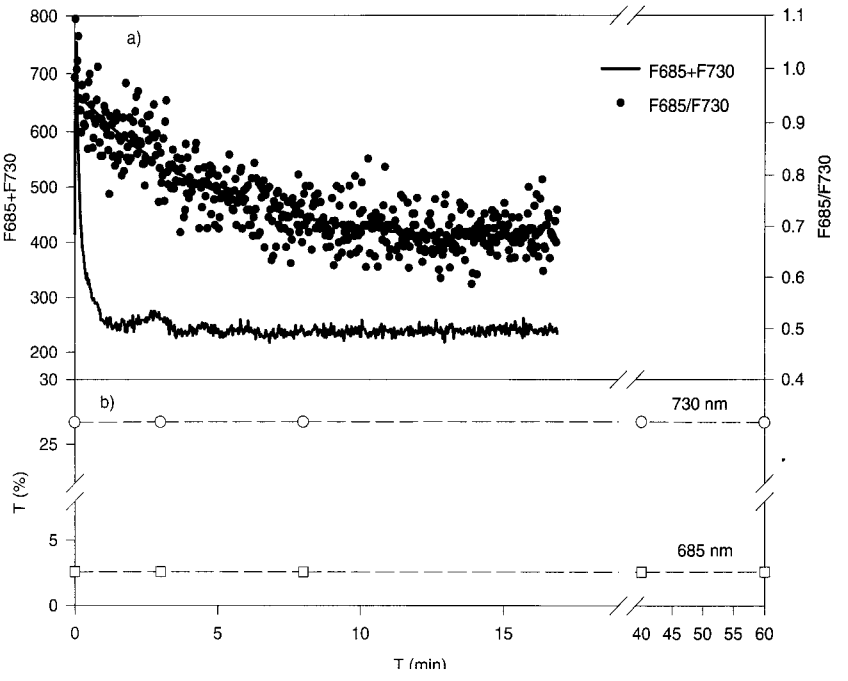

Fig. 5: Fluorescence (a) and transmittance kinetics (b) induced by red (632.8 nm) laser light in *Epipremnum aureum:* total chlorophyll fluorescence, F685+F730 (solid line); chlorophyll fluorescence ratio, F685/F730 (closed circles); leaf transmittance at 730 nm (open circles) and at 685 nm (open squares).

The physiological interpretation of these results is difficult because of the complex processes that occur in the intact leaf. At room temperature, most of the fluorescence in both spectral regions is emitted from PSII. Emission by PSI, which is higher at longer wavelengths than at 685 nm, is only considered a minor contribution (Krause and Weis, 1991). However, when the amount of excitation energy distributed on PSI is increased with respect to PSII, a corresponding increase in the longer wavelengths fluorescence peak relative to the shorter wavelength's peak is observed (Bradbury and Baker, 1981; Kyle et al., 1983; Murata and Satoh, 1986).

Our results on the change of the ChlFR during the declining phase of the Kautsky kinetics agrees with data reported by Bradbury and Baker (1981). They suggested that the reoxidation of the secondary PSII electron acceptors decreases the fluorescence at both emission maxima from the peak to the terminal state but with a larger effect on the 685 nm band. Other contributions to the ChlFR decrease can be provided by a change in the pH gradient across the thylakoid membranes (Bradbury and Baker, 1981) and by phosphorylation of thylakoid proteins (Kyle et al., 1983). Both mechanisms increase the excitation energy distribution to PSI, which results in higher values of F735 with respect to F685. Our and the above evidence indicate the potential use of the ChlFR as a probe of variations in the energy distribution between PSII and PSI at physiological temperature. This is also supported by the direct correlation found between the change in F710/F684 measured at room temperature and the changes in F735/F685 measured at 77 K for different degrees of thylakoid phosphorylation (Kyle et al., 1983). A potential relationship between the ChlFR at room temperature and the photosynthetic activity of PSII and PSI was also indicated by Lichtenthaler and Rinderle (1988). They reported that when the electron flow from PSII to PSI is blocked by DCMU, the total fluorescence increases but with a larger enhancement for the 690 nm band.

Effect of natural factors on the chlorophyll fluorescence ratio

Chlorophyll fluorescence was monitored continuously, along with leaf temperature and light intensity, during a diurnal cycle in the field on a *Juglans regia* L. tree. The whole set of data is shown in Fig. 6. Total fluorescence calculated as the sum of F685 and F730 decreased strongly when the fluorescence induction kinetics was started at the dark-light transition (Fig. 6a). Fig. 6a shows also the difference between the total sun irradiation and that effectively reaching the leaf surface (dashed line). The variation of the F685/F730 ratio was opposite to that of PPFD on the leaf (Fig. 6b). A similar behaviour was observed in *Zea mays* in the field with a complete different method of measure (Fig. 7) [the F685/F730 ratio was sampled manually all along the leaf, from the base to the vertex at different times of a sunny clear day]. As seen in Fig. 6 for the walnut tree, F685/F730 ranged from about 0.98 during the night to about 0.65 at the maximal sunlight intensity (PPFD = 1500 $\mu mol\, m^{-2} s^{-1}$). Air temperature changed from 17 °C to 31 °C (Fig. 6c). Leaf temperature remained close to air temperature until about 11 am, when PPFD was equal to about 100 $\mu mol\, m^{-2} s^{-1}$; then it increased much more than air temperature reaching a maximum of 47 °C. In the afternoon, leaf temperature decreased, again matching the air temperature at about 7 pm, when PPFD was reduced to about 100 $\mu mol\, m^{-2} s^{-1}$ (Fig. 6c) and F685/F730 values matched those at the beginning of the day.

The F685/F730 ratio was found to be correlated with both PPFD and leaf temperature. However, the best correlation was obtained for F685/F730 with the difference in temperature, ΔT, between leaf and air temperature (r^2 = 0.863). The correlation coefficient is good enough taking into ac-

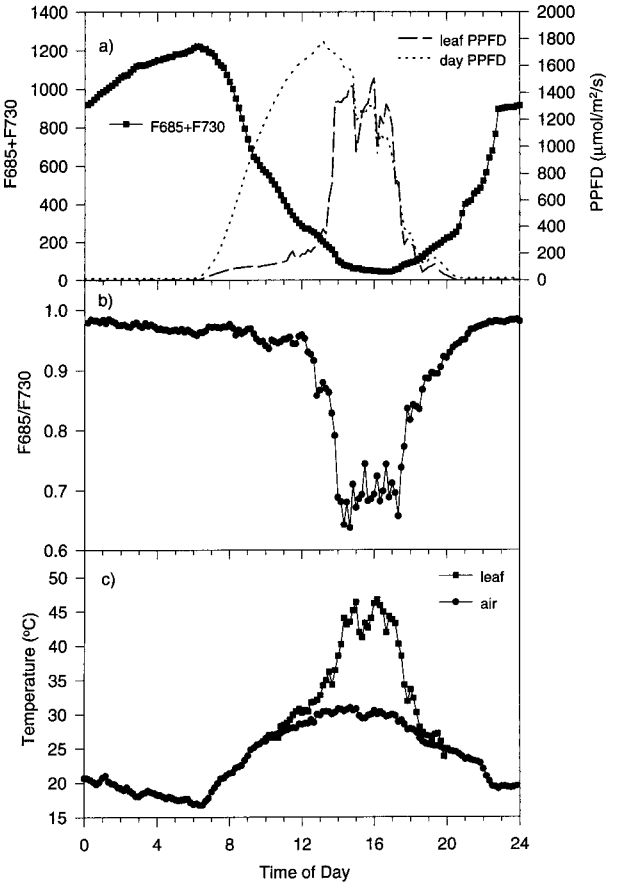

Fig. 6: Data from a continuous daily cycle monitoring on a *Juglans regia* tree under natural conditions: a) total chlorophyll fluorescence, F685+F730 (closed squares), sun PPFD (dotted line) and leaf PPFD (dashed line); b) chlorophyll fluorescence ratio, F685/F730; c) leaf temperature (squares) and air temperature (circles).

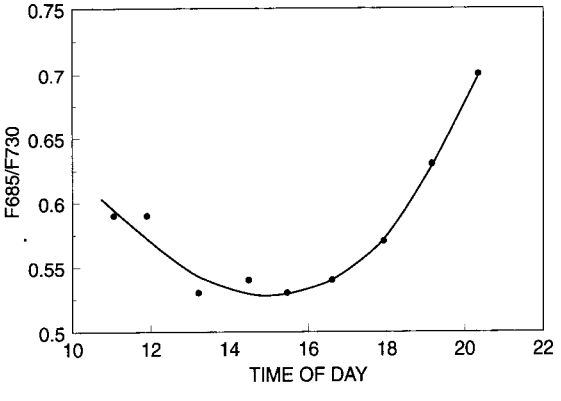

Fig. 7: Diurnal cycle of F685/F730 on a healthy young Maize plant (*Zea mays*). The points plotted are the average of about 100 measurements, scanning the leaf from base to vertex.

count the difficulty of measuring laser induced fluorescence spectra in full daylight, using a low exciting power in order to avoid any possible influence of the laser light on the photosynthetic status. Actually, in these conditions the signal/noise ratio was very poor, with a fluorescence signal of 40 counts of the A/D converter of the LEAF fluorometer superimposed to 4000 counts due to the scattered solar radiation. Only extensive averaging allowed signal extraction from background. As shown in Fig. 8, the opposite of F685/F735 and the ΔT have a very close behaviour during the daily cycle.

The variation of ChlFR under natural environmental conditions provides interesting insights on the physiological meaning of the Chl fluorescence ratio. It is well known that high light intensities and high temperatures can induce photoinhibition of photosynthesis (Ludlow, 1987). Photoinhibition can also occur under natural conditions, even at light intensities lower than full sunlight and at temperatures within the optimal range for photosynthesis (Ögren, 1988). Photoinhibition acts mainly on PSII by complex mechanisms that dissipate the excess of absorbed energy. The extent of the photoinhibitory effect can be estimated by the ratio (at 77 K) of the variable to maximum Chl fluorescence, Fv/Fm, at 690 nm, which has been shown to equal the photochemical quantum yield of PSII (Butler, 1978). The same fluorescence parameter measured at room temperature is, however, well correlated to the value at 77 K (Ögren, 1988) and hence it can also be used to measure the inhibition of PSII photochemistry. Under photoinhibition, Fv/Fm decreases drastically. PSI appears to be more resistant to photoinhibition than PSII (Havaux and Eyletters, 1991). PSI is also more tolerant to heat stress than PSII and its activity is even stimulated by high-temperatures (Havaux et al., 1991). Furthermore, by measurements at 77 K, the fluorescence quenching at 690 nm caused by photoinhibition, resulted also to occur at 735, but to a lower extent (Demmig and Bjorkman, 1987; Somersalo and Krause, 1989).

From the viewpoint of the above considerations, it is likely that the *Juglans regia* leaf under our investigation underwent photoinhibitory and heat stresses during the daily cycle. Both high sunlight and high temperature may have caused a greater inhibition and/or damage to PSII than to PSI. It is possible that under these particular conditions the contribution of PSI to the F735 fluorescence band is not negligible with respect to PSII fluorescence. Consequently, changes in the activity of the two photosystems may reflect on the relative intensity of the Chl fluorescence bands, since PSII contributes to both F685 and F735 while PSI contributes only to F735. Moreover, the energy distribution between PSII and PSI in spinach leaves was shown to be dependent on both temperature and light quality (Weis, 1985). In leaves that are in State 1, the energy distribution is unaffected by temperature. In State 2, however, above 20 °C the rate constant for the PSII → PSI energy transfer increases with temperature.

The large decrease of ChlFR with increasing sunlight and leaf temperature (Fig. 6) can be explained as due to a quenching of PSII fluorescence, while PSI fluorescence is reduced by a lower factor or remains unchanged or is even increased. The process appears to be completely reversible when light intensity and temperature decreased, proving that no alteration to the Chl concentration occurred. Other explanations such as changes in the leaf absorption due to chloroplast rearrangements (Inoue and Shibata, 1973; Brugnoli and Bjorkman, 1992) can not be ruled out. However, as shown

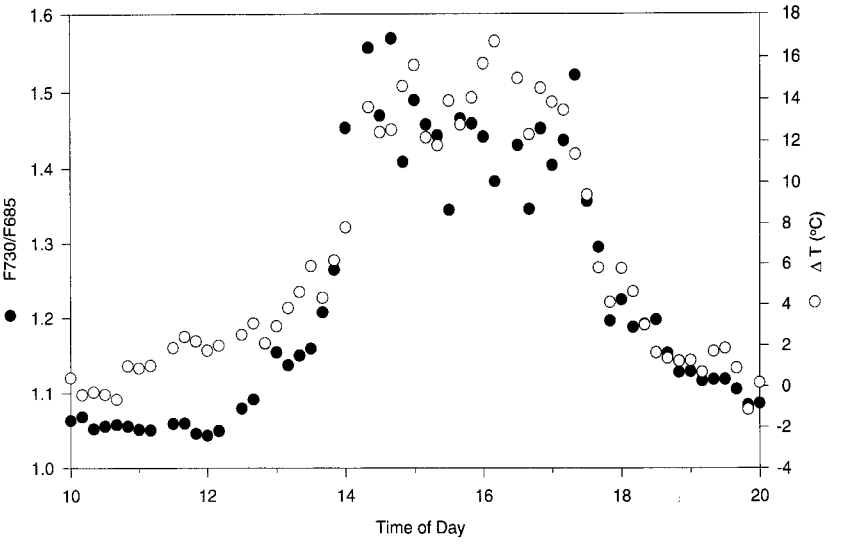

Fig. 8: Daily variation of the opposite, F730/F685, of the chlorophyll fluorescence ratio (closed circles) and the differential temperature, ΔT (open circles), as difference between the leaf and air temperatures.

below, a decrease of F690/F735 was also observed when plants were irradiated by intense red light (Fig. 10) which does not induce any leaf transmittance change (Inoue and Shibata, 1973; Agati et al., 1992).

The high light effect on F685/F730 can also be a consequence of a different contribution to fluorescence from the chloroplasts according to their distance from the upper leaf layer. Because of self reabsorption, F685 mainly originates from chloroplasts of the upper layers, while F730 receives contribution also from deeper layers. F685 and F730 can, therefore, be considered to represent different populations of chloroplasts which may be affected by stress factors to a diverse extent. The environmental light intensity at the upper layer chloroplasts is higher than inside the leaf. Consequently, F685 will be relatively more affected than F730 by all those fluorescence quenching processes occurring under high light such as photoinhibition and the zeaxanthin-dependent non-photochemical quenching.

Effect of temperature and light intensity on the chlorophyll fluorescence

The effect of light intensity and leaf temperature on ChlFR was investigated in a growth chamber on *Fagus sylvatica* seedlings and in a laboratory experiment on leaves of *Ficus benjamini*. In Fig. 9, a 24 h cycle of continuous monitoring of fluorescence, temperature and PPFD on an apical leaf of beech inside a growth chamber is shown. The leaf temperature and PPFD are given in Fig. 9 a. It is seen that the leaf temperature remains quite constant at about 28 °C during the light period except for the peak observed at 9 am corresponding to the dark-light transition. The large variation in the leaf temperature observed between midnight and 2 am was due to the fluctuation of the chamber thermostat system when the temperature was reduced to 20 °C by purpose. After that, leaf temperature is stabilized around 25 °C for the rest of the dark period. The leaf temperature was about 3 °C higher than the air temperature during the light period. Total Chl fluorescence (F685+F730) and F685/F730 show a peculiar behaviour (Fig. 9 b). Interestingly, the

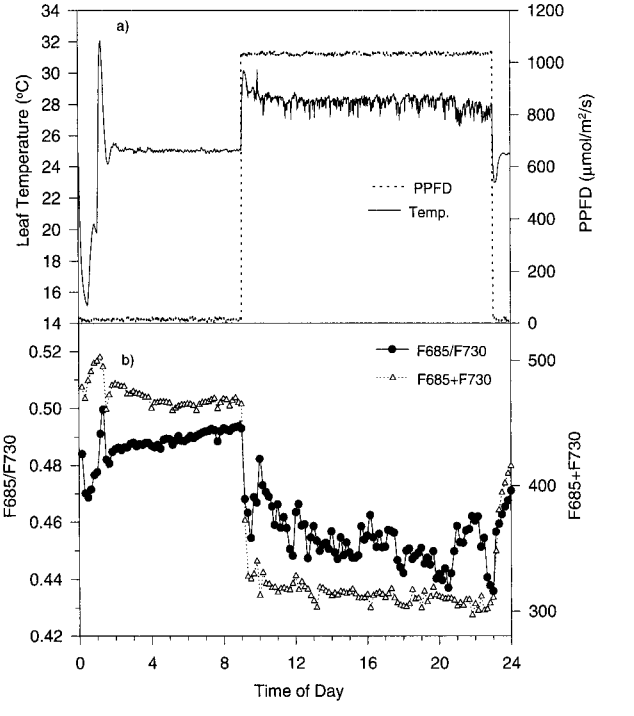

Fig. 9: Data from a continuous daily cycle monitoring on a *Fagus sylvatica* seedling in a growth chamber: a) leaf temperature (solid line) and PPFD (dotted line); b) total chlorophyll fluorescence, F685+F730 (open triangles) and chlorophyll fluorescence ratio, F685/F730 (closed circles).

ChlFR follows closely the variation of leaf temperature induced in the 0–2 h period. The total fluorescence seems instead to have an opposite response. When the light is switched on, both total fluorescence and F685/F730 decrease rapidly due to induction of the Kautsky kinetics. A slower decline in ChlFR remains after the steady state was reached.

Similar results were found when the high light intensity (PPFD of about 2000 µmol $m^{-2} s^{-1}$) was provided by the

632.8 nm radiation used to excite Chl fluorescence on *Ficus benjamini*. Values of F685/F730 as function of the irradiation time are given in Fig. 10. Curve fitting of experimental data results in a two-exponential decrement (r^2 = 0.986). The solid line in Fig. 10 is the combination of a rapid exponential decay with a time constant of about 2.5 min, followed by a second exponential curve with a 70-folds longer time constant. The leaf temperature remained constant at 25.5 ± 0.5 °C. In the laboratory and growth chamber experiments, the high light-induced decrease of ChlFR can result from both photoinhibition and the induction of the Kautsky kinetics as described above.

The relationship between ChlFR and leaf temperature was further investigated in the laboratory on leaves of *Ficus benjamini*. As the leaf temperature was decreased from about 25 °C to about 14 °C the F685/F730 ratio changed from 0.68 to 0.61 (Fig. 11). A good linear correlation (r^2 = 0.94) is present between the two parameters. In Fig. 11, the total Chl fluorescence (F685+F730) is also reported. As observed in the growth chamber experiment (Fig. 9), the trend of the total fluorescence is opposite to that of ChlFR increasing towards lower temperatures. Light intensity was low enough (150 μmol m^{-2} s^{-1}) to avoid any photoinhibition. We observed, therefore, that the ChlFR varies with temperature, at physiological temperatures, also in plants under controlled non-stressing conditions.

It is known that cooling intact leaf cells, isolated chloroplasts and thylakoids membranes causes their fluorescence spectra to change and the total fluorescence yields to increase. The most pronounced increase in Chl fluorescence upon cooling is between 275 and 100 K for the F735 band and between 100 and 4 K for the shorter wavelength band (Murata and Satoh, 1986). The reason for these different temperature profiles of the Chl fluorescence bands in not well understood.

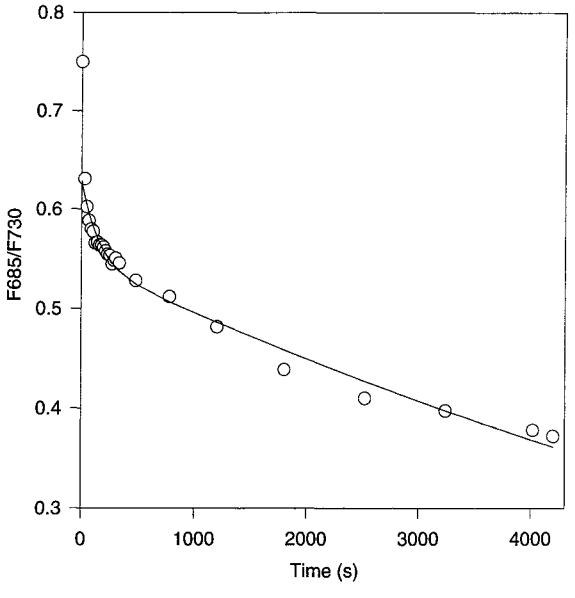

Fig. 10: Variation of the chlorophyll fluorescence ratio, F685/F730, under high 632.8 nm light intensity (2000 μmol m^{-2} s^{-1}) in *Ficus benjamini*. Solid line is the result of curve fitting analysis.

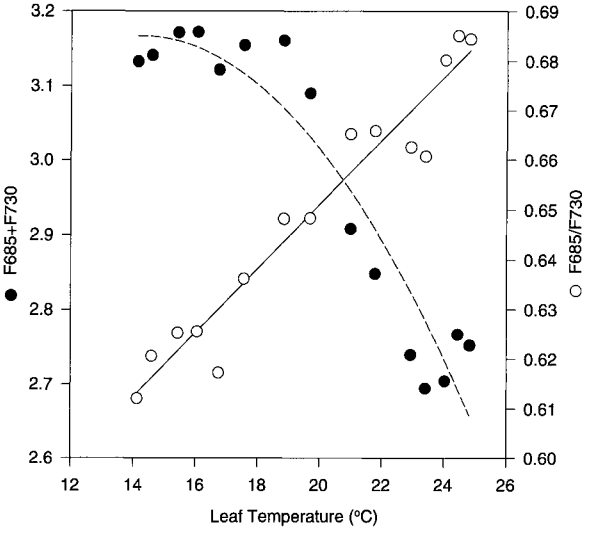

Fig. 11: Variation of the chlorophyll fluorescence ratio, F685/F730 (open circles) and total chlorophyll fluorescence, F685+F730 (closed circles) as function of the leaf temperature under moderate light intensity (150 μmol m^{-2} s^{-1}) in *Ficus benjamini*.

The increase in total fluorescence upon decreasing leaf temperature in the 5–30 °C range can be explained as due to a lowering of the fluidity of the thylakoid membranes, which inhibits the reoxidation of plastoquinones (Havaux and Gruszecki, 1993). Sundbom et al. (1982) reported an increase of *in vivo* Chl fluorescence (the detection wavelength was not specified) as the leaf temperature was reduced from 20 °C to −10 °C. Lipucci et al. (1992) found the F685/F730 ratio to decrease in *Phaseolus vulgaris* plants transferred from 20 °C to 4 °C. To our knowledge this is the only previous work about monitoring of spectral changes in leaf Chl fluorescence during a continuous temperature decrease. In our experiment, both fluorescence intensities at 685 and 730 nm increase as leaf temperature is reduced but the enhancement of F730 is larger than that of F685. At present, we are not able to explain this effect. However, it gives indirect evidence that the two Chl fluorescence bands at physiological temperatures contain contributions from two diverse systems. Although other experimental work is needed, our observation supports the hypothesis that the different contributions of PSII and PSI to the total fluorescence can also be discriminated at room temperature.

Conclusion

Our study showed that the Chl fluorescence ratio F685/F730 measured on intact leaves depends on many different factors. The well known effect of fluorescence reabsorption was compared in two tomato genotypes with marked different Chl concentration. The extent of the reabsorption was estimated by a simple method measuring the total reflectance and transmittance spectra of the leaf. This method can be used to discriminate the Chl concentration effect from other factors in comparing the ChlFR values from different species and under diverse growing conditions.

It has been proved that the ChlFR is sensitive to both light intensity and leaf temperature. These two parameters must therefore be taken into account in the use of F690/F735 as a stress indicator. In the laboratory, these two effects can be discriminated by the opposite trend of the total fluorescence F685+F730 and ChlFR. However, when this behaviour cannot be discriminated, as for example in remote sensing measurements, the simultaneous measurement of leaf temperature and irradiation is necessary to extract parameters of physiological interest from the ChlFR.

Results of this work provided evidence that the photosynthetic state of the leaf can be reflected on the ChlFR at physiological temperature. The usefulness of ChlFR in the study of plant ecophysiology then goes far beyond the simple detection of changes in chlorophyll concentration, and of plant stresses leading to changes in this parameter. We hope that our study will stimulate future experimental work in order to confirm the hypothesis that changes in ChlFR at room temperature are correlated to changes in the PSII versus PSI photosynthetic activity.

The F685/F730 ratio, or other parameters based on the fluorescence spectral shape, could then become useful tools to investigate plant physiology, as in the case for the fluorescence induction kinetics parameters, now routinely used for this purpose. Nevertheless, because of the influence of physical parameters on the ChlFR, a standardization of the measuring methods is needed to obtain reliable information.

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