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REVIEW

Technique of the modulated chlorophyll fluorescence: basic concepts, useful parameters, and some applications

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Abstract

The review summarizes basic information about slow and fast chlorophyll (Chl) *a* fluorescence induction kinetics (FIK) recorded using fluorimeters working on a principle of the pulse amplitude modulation (PAM) of a Chl fluorescence signal. It explains fundamental principles of the measuring technique, evaluates the terminology, symbols, and parameters used. Analysis of Chl FIK resulting in a set of Chl fluorescence parameters (FPs) provides qualitative and quantitative information about photosynthetic processes in chloroplasts. Using FPs, one can describe the

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Abbreviations (see also Tables 1 to 4): APs – pulses of actinic radiation; AR – continuous actinic radiation; Chl – chlorophyll; ChlF – chlorophyll fluorescence; DAS – dark-adapted state; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Φ_F – quantum yield of ChlF; FIK – fluorescence induction kinetics; FLT - fluoranthene-treated; FP – fluorescence parameter; FR – far-red radiation; FY – fluorescence yield; k_D – rate constant of thermal deactivation within LHCs of photosystem 2; k_F – rate constant of ChlF emission; k_P – rate constant of photochemical reactions at open PS2 reaction centres; k_T – rate constant of excitation energy transfer to nonfluorescent pigments; λ – wavelength; λ_A – wavelength of photon absorbed; λ_F – wavelength of photon emitted; LAS – light-adapted state; LED – light-emitting diode; LHC – light-harvesting chlorophyll-protein complex; MR – weak modulated measuring radiation; n_A – total number of photons absorbed; n_F – total number of photons emitted; NRD – non-radiative energy dissipation; OEC – oxygen-evolving complex; PAH – polycyclic aromatic hydrocarbon; PAM – pulse amplitude modulation; PFD – photon flux density; PQ – plastoquinone; PS – photosystem; Q_A, Q_B – primary quinone-type electron acceptors of PS2; RC – reaction centre; SP – saturation pulse of "white light"; SR - saturating radiation; τ_F – mean life-time of fluorescence emission; τ_D – mean life-time of thermal dissipation; t – time variable; v'', v' – vibronic numbers.
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functioning of the photosynthetic apparatus under different internal and external conditions. Brief comments on proper application of the fluorimetric method in photosynthesis research and some actual examples are also given.

Additional key words: chemicals; circadien cycle; dark-adapted state; fast kinetics; fluoranthene; light-adapted state; photosystem 2; slow kinetics; temperature.

Introduction

Measuring chlorophyll fluorescence (ChlF) has become a very useful technique in obtaining rapid qualitative and quantitative information on photosynthesis. Remarkable progress in the understanding and practical use of ChlF in plant science was induced by the urgent need of applied research for quantitative, non-invasive method capable to assess photosynthesis in intact leaves. Developments in instrumentation (Schreiber 1986, Schreiber *et al.* 1986, 1988) and methodology (*e.g.*, Genty *et al.* 1989, Krause and Weis 1991) have substantially increased these possibilities (van Kooten and Snel 1990). Also increased understanding of fundamental photosynthetic processes (*e.g.*, Krause and Weis 1991, van Grondelle *et al.* 1994, Govindjee 1995) has promoted the application of fluorimetric methods in the photosynthesis research. Over the last two decades, numerous types of fluorimeter have been developed and, recently, they are available worldwide for routine field and laboratory research. Among them, fluorimeter working on a principle of the *pulse amplitude modulation* (PAM) of the ChlF emission (Schreiber *et al.* 1986) is used in many fields of plant physiology. Application of those fluorimeters+ ranges from energy caption processes in LHCs and electron flow through a set of carriers in thylakoid membrane to physiological processes in single plant or canopy, depicted often in two-dimensional (2D) representation. Recent application of PAM-technique goes through cyanobacteria and almost all groups of plants from unicellular algae to vascular plants. In the field, it is used in a great variety of aquatic and terrestrial ecosystems.

The PAM-fluorescence technique allows to obtain both qualitative and quantitative information on organization and functioning of a plant photosynthetic apparatus by analysis of fast and slow Chl fluorescence induction kinetics (FIK). The information included in FIK can be decoded using a set of Chl fluorescence parameters (FPs) defined mostly within the last three decades (*e.g.*, Kitajima and Butler 1975, Bilger and Schreiber 1986, Lichtenthaler *et al.* 1986, Genty *et al.* 1989,

+At present, the German term 'fluorometry' is commonly used in the literature for the time-dependent measurements of ChlF recorded using the PAM-fluorescence technique. This therm has been asserted oneself all over the world due to papers of Ulrich Schreiber and his co-workers who developed the PAM-principle and due to wide distribution of the PAM-fluorometer (*H. Walz*, Germany). But in other languages, the term 'fluorimetry' is used for this kind of measurements and the Latin prefix 'fluoro-' is connected with the determination of an amount of the element fluorine in samples using quite different analytical methods. Hence the term 'fluorimetry' is preferentially used in this paper.

Bilger and Björkman 1990). Nevertheless, the extensive use of the technique requires an unification in terminology and interpretation of Chl FPs in order to minimize confusion in the literature. This paper reviews principles of ChlF, the basic concepts used in Chl fluorimetry, and evaluates Chl FPs used in the ChlF technique. In addition, some critical remarks and recommendations on the related terminology, and analysis of Chl FIK are given. Some applications of the PAM-fluorimetry in plant physiology and ecology are shown.

Basic concepts

Chlorophyll fluorescence: Photosynthetic processes in plants remain active only under a sufficiently long actinic irradiation and sufficient supply of water and CO_2 molecules. Once incident photons are absorbed by molecules of antenna pigments (Chls and carotenoids), the excitation energy is transferred *via* excitons to reaction centres (RCs) of the photosystems 2 (PS2) and 1 (PS1). Therein, the energy drives primary photochemical reactions that initiate the photosynthetic energy conversion (*e.g.*, Govindjee and Govindjee 1975). This *photochemical pathway*, involving a charge separation and electron transport *via* a set of carriers, is not the only way in which the excitation energy is consumed. Other two competitive pathways represent the *thermal dissipation*, *i.e.*, a non-radiative de-excitation of excited states of pigment molecules to heat, and the *chlorophyll fluorescence*, *i.e.*, the emission of photons by the radiative de-excitation of excited Chl molecules. The three energy pathways (Fig. 1) illustrate an application of the energy conservation law to a Chl *a* molecule located in PS2 RC.

In intact leaves of higher plants, the ChlF emission originates nearly exclusively from Chl *a* because the energy of excited states of Chl *b* is transferred with a high efficiency to the Chl *a* molecules (Govindjee and Govindjee 1975, Papageorgiou 1975). Moreover, at room temperature, ChlF is emitted predominantly (about 90 %) from Chl complexes of PS2 (Govindjee 1995) and represents only about 3–5 % of the absorbed energy (Govindjee and Govindjee 1975, Walker 1987). To describe the fluorescence emission process, the quantum yield of ChlF (Φ_F) has been introduced. It is defined as the total number of photons emitted (n_F) divided by the total number of photons absorbed (n_A). Another way of Φ_F expression is to use the rate constants of de-excitation processes (Krause and Weis 1991, Govindjee 1995):

$$\Phi_F = \frac{n_F}{n_A} = \frac{k_F}{k_F + k_D + k_T + k_P} \tag{1}$$

Here k_F is the rate constant of the Chl *a* fluorescence, k_D the thermal deactivation, k_T the excitation energy transfer to non-fluorescent pigments, and k_P the photochemistry. Only the relative quantum yield of ChlF can be reported in view of the homogeneous ChlF emission (Govindjee 1995). In the literature, the term of *fluorescence yield* (FY) is preferentially used (Papageorgiou 1975, Genty *et al.* 1989). Its application presupposes that the absorbed light flux is not affected

significantly in the course of experiment by some physiological and physical factors, *e.g.*, by inner structural changes in a plant tissue under light, non-homogeneous ChlF emission, changes in absorption cross-sections of fluorescent pigments, reabsorption processes, variations in sample geometry. Then the changes of fluorescence intensity reflect changes of relative Φ_F. Thus, symbol $F(t)$ means Chl FY (not only the ChlF intensity) at any time t of the light induction. All PFs described below are valid just under these conditions.

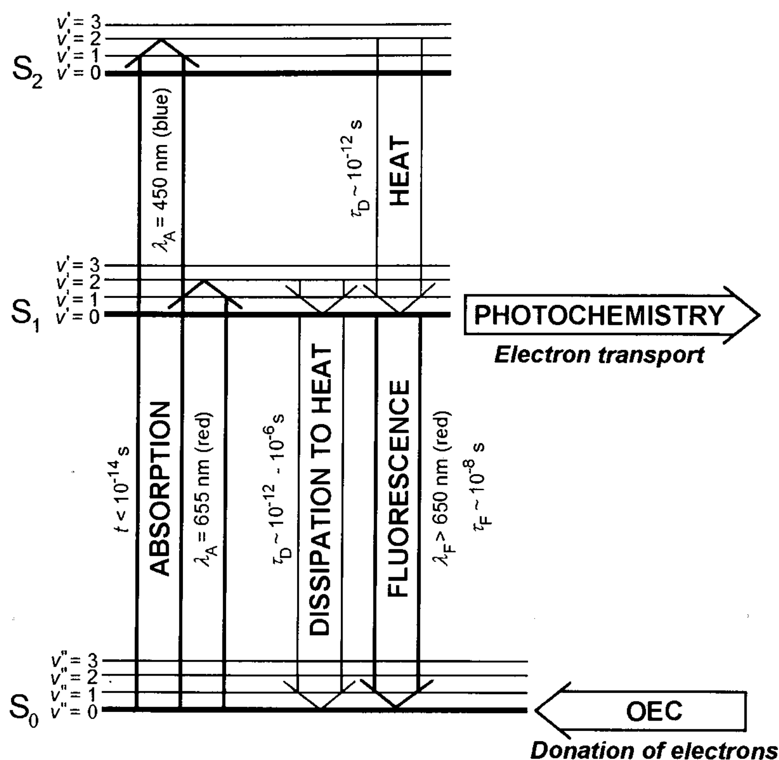

Fig. 1. Simplified three-level-scheme of the redistribution of excitation energy in a chlorophyll (Chl) molecule of the PS2 reaction centre. Energy diagram shows the ground singlet state (S_0) and excited singlet states (S_1, S_2) of a respective molecule with closed electron shells. Vibronic states of individual energy levels with different vibronic numbers (v" for the ground state, v' for the excited ones) are indicated by *solid horizontal lines*. *Solid vertical bars with arrows* represent following quantum processes: (*1*) the *absorption* of a radiation quantum with the distinct wavelength λ_A resulting in an electron transition from the ground to the excited singlet states within time $t \leq 10^{-15}$ s, (*2*) the *thermal dissipation* of excitation energy as a nonradiative internal conversion to heat, and (*3*) the *fluorescence*, *i.e.*, the radiative deexcitation in wavelengths $\lambda_F > 650$ nm. Parameter τ means the approximate life-time of electrons in the individual quantum states (Prosser *et al.* 1989). *Horizontal bars* indicate the *photochemical pathway*, *i.e.*, the origin of an electron transport starting with a charge separation in PS2 RC, and completed by the neutralization of a Chl ion by electrons donated from an oxygen-evolving complex (OEC); compiled after Govindjee and Govindjee (1975) and Owens (1996).

Although the emission of ChlF represents the minor competing process of deactivation of excited pigments, Chl FY varying in time gives a possibility to investigate the mechanisms by which thylakoids regulate the utilization of radiant energy absorbed by PS2 complexes. As follows from Fig. 1, the increase of one leads to the decrease of at least one of the other two components in which the excitation energy is redistributed. Thus, an antiparallel relation between photochemical and non-photochemical (heat dissipation, ChlF) processes can be supposed. Increased consumption of the excitation energy in the photochemical pathway or its increased dissipation to heat leads to the *quenching* of Chl FY. If the time changes of FY after irradiation, *Kautsky effect* (Kautsky and Hirsch 1934), are continuously measured, the Chl *fluorescence induction kinetics* (FIK) is recorded. In the photosynthesis research, photosynthetic activities of plants are derived from the shape of these curves (Bradbury and Baker 1981, 1984, Schreiber *et al.* 1986, 1995).

Measuring principle: A fluorimeter for detection of modulated ChlF with high sensitivity and dynamic capability was developed in the 80's. It allowed to monitor photochemical processes in PS2 and, indirectly, in PS1 (Schreiber 1986, Schreiber *et al.* 1986, 1988). The fluorimeter typically consists of four radiation sources. The sources produce quantitatively and qualitatively different radiation incident on a photosynthetic object causing ChlF: (*1*) *measuring* (analytical) *modulated radiation,* (*2*) *actinic radiation*, (*3*) *saturation pulses*, and (*4*) *far-red radiation* (see Fig. 2).

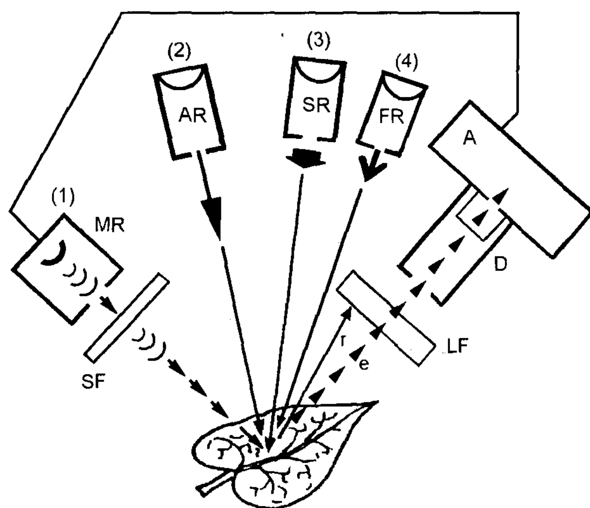

Fig. 2. Block scheme of the fluorimeter for detection of modulated ChlF. A – amplifier; AR – actinic radiation source; D – detector; e – emission of modulated ChlF; FR – far-red radiation source; LF – long-pass filter; MR – measuring radiation source; r – reflected radiation; SF – short-pass filter; SR – saturating radiation source. The radiation sources numbering (*1*) to (*4*) is explained in the text.

The modulated measuring radiation (MR) is provided by a light-emitting diode (LED) as short pulses of red radiation with very low integral photon flux density (PFD < 0.2 μmol m^{-2} s^{-1}). The pulses are 3 μs (1 μs) long and repeated at the frequency of 600 Hz or 20 kHz in a *PAM-2000* fluorometer and 1.6 kHz or 100 kHz in a *PAM101-103* fluorometer, respectively (Schreiber 1986, Anonymous 1993). The red radiation having a peak wavelength λ = 650 nm passes through a short-pass filter (λ<670 nm). After absorption by a sample, part of the radiant energy is emitted as

ChlF and then detected in a PIN-diode photodetector. The photodetector is protected by a long-pass filter (λ>700 nm) and a heat absorbing filter (Fig. 2). FY detected by the modulated measuring beam is denoted as the basic or background fluorescence (F_0) and represents a minimum Chl FY level.

The second radiation source (often an array of LEDs) provides the continuous red actinic radiation (AR) with the peak λ of 665 nm and PFD of up to hundreds of μmol m^{-2} s^{-1}. If a blue AR (λ = 450 nm) is used, Chl molecules are excited into the 2nd excited singlet state (see Fig. 1). Multifarious sources of AR (red, blue, green, white) are used to induce ChlF. Changes in ChlF are then detected as the amplitude modulation of the pulsing signal induced by MR. A highly selective amplification system ignores all signals except the fluorescence excited during the 3 μs (1 μs) measuring pulses. It allows to tolerate ratios of up to 1 : 10^7 between MR and AR by the measuring system (Schreiber 1986). A part of the exciting radiation is reflected and transmitted by a sample. The reflected part is cut off by the long-pass filter. As the radiation source of saturation pulses (SPs) 0.4 to 1 s long, mostly a halogen lamp is used with "white" radiation of very high PFD (up to 10 000 μmol m^{-2} s^{-1}) which temporarily converts all RCs of PS2 to fully reduced (closed) state. In this state, the photochemical processes in PS2 are saturated by the saturating radiation (SR), it means k_P = 0 in Eq. (1). Then the absorbed energy is fully converted to ChlF and heat, therefore the maximum FY (F_M) is reached. During SP, the frequency of MR is usually increased in order to achieve a better signal-to-noise ratio as well as time resolution. The source of far-red radiation (FR) with λ of 735 nm is used to promote activity of PS1 resulting in a rapid reoxidation of the plastoquinone (PQ) pool.

Saturation pulse method: To quantify contributions of the PS2 photochemistry and non-radiative energy dissipation (NRD) processes to the total Chl FY quenching, the *saturation pulse method* was developed, originally introduced as a "light-doubling" (Bradbury and Baker 1981, 1984, Schreiber *et al.* 1986). In this method, an equilibrium is assumed between photochemical and non-photochemical pathways at any time of the radiation induction. The application of a short pulse of SR during an induction phase causes a complete saturation of the photochemical processes leading to an over-reduction of the acceptor side of PS2. If actual, $F(t)$, and maximum, $F_M'(t)$, FYs are compared to the reference F_M level, quantification of the photochemical and non-photochemical ChlF quenching is possible (see Fig. 4). In fluorescence experiments, two defined states of the photosynthetic apparatus should always be induced: (*a*) the *dark-adapted state* (DAS), photochemically inactive state, and (*b*) the *light-adapted state* (LAS), steady-state of photosynthesis. The former occurs if all electron transport processes in thylakoid membranes are stopped, a transthylakoidal pH-gradient is minimized as well as the concentrations of NADPH and ATP which are necessary for CO_2 fixation. The latter is connected with fully balanced photosynthetic processes being active in chloroplasts after the sufficiently long irradiation by AR, resulting in a synthesis of NADPH and ATP, and in concurrent CO_2 fixation.

Fast Chl fluorescence induction kinetics: Upon irradiation of the sample in DAS, a rise of Chl FY is induced from an initial minimum value F_0 (point O) to a maximum level F_P (point P) as shown in Fig. 3. The time to reach F_P (usually 0.3–1.0 s) depends on structural and functional properties of PS2 complexes. Absolute level of F_P depends on the irradiance used to induce the kinetics. It reaches its maximum F_M when the saturating PFD is used that converts all RCs of PS2 to closed (fully reduced) state. Between O and P, several intermediate states can be distinguished and characterized as points K, J, I, D (K under high temperature only). The points O to P are reached steadily in a time-course of the ChlF induction. Due to low time resolution of *PAM*-fluorimeters (sampling rate of 30 μs), only I and D points can be usually identified (see Fig. 3). To record and distinguish the points K

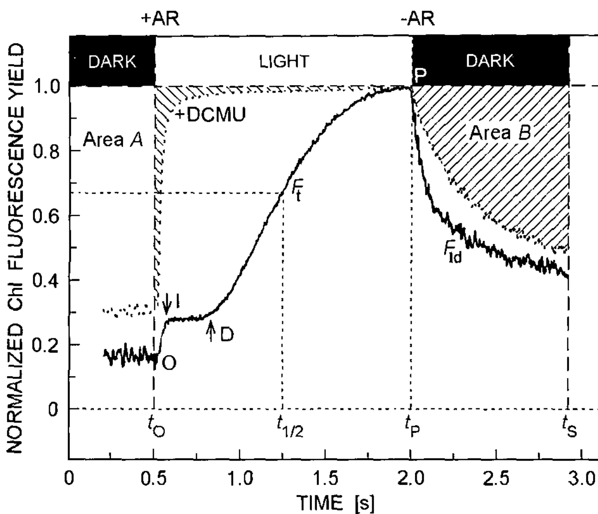

Fig. 3. Fast chlorophyll (Chl) FIKs recorded on dark-adapted leaves of *Pisum sativum* (cv. Laura) using a *PAM-2000* fluorometer. Position of the O, I, D, P points is indicated by the respective letters (O, P) and arrows (I, D). Both area *A* over the rising part of FIK (in light) and area *B* over the relaxing part of FIK (in dark) are indicated only for the leaf incubated with DCMU. Used symbols and abbreviations are explained in Tables 1 and 3. Both records were normalized to the F_M values.

and J, devices having faster sampling rate (at least 1 μs) must be used. The point K can be recognized when the sample is exposed to strong heat stress. Under these conditions, the capacity of an oxygen-evolving complex (OEC) decreases due to the blockage of PS2 electron transport between OEC and tyrosine which results in a FY increase to the step K (Strasser and Tsimilli-Michael 1999). Usually, the K step is seen at about 300 μs after beginning of the ChlF rise. The point J occurs at about 2 ms and is related to a partial closure of RCs of PS2 (Strasser and Strasser 1999). The summary and glossary of the O, I, D, and P points which can be measured by the technique of modulated ChlF is given in Table 1.

Commonly speaking, the time-course of fast FIK represents the increasing number of closed RCs of PS2. During the ChlF rise, the primary quinone-type electron acceptor Q_A is steadily reduced (reaching fully reduced state at point P) because the rate of concurrent Q_A reoxidation by PQ is lower than the rate of Q_A reduction. The points I to P thus represent different phases of the redox state of Q_A. Point I is at that part of the curve (in the 10 to 50 ms range) where the initial ChlF rise starts to bend

Table 1. Definitions of the basic ChlF terms related to fast Chl FIK measured by a PAM-fluorimeter on a dark-adapted sample.

Symbol	Explanation
O, F_0	Point O: *Minimum* Chl FY measured on a dark-adapted sample with *open* PS2 RCs and all non-photochemical processes in a thylakoid membrane minimized
I, F_I	Point I: Chl FY at the time in the ms-range at which FIK starts to bend or form a plateau. Reduced Q_A starts to be reoxidised by the plastoquinone
D, F_D	Point D: Chl FY at the time at which induction kinetics starts to rise from a plateau. The rate of Q_A reduction starts to overreach the rate of Q_A reoxidation
P, F_P	Point P: *Maximum* Chl FY reached at the induction curve. If a saturating flash is applied, then $F_P = F_M$ (*i.e.*, all PS2 RCs are *closed*)
t_O	Time at which F_0 is reached (time at initial point of the fast FIK rise)
t_P	Time at which F_P is reached
$t_{1/2}$	Half-time of Chl FY rise from F_0 to F_P
t_S	Time at which a dark relaxation is stopped
F_t	Actual Chl FY at any time of a light induction period
F_{td}	Actual Chl FY at any time of a dark relaxation period
$dF(t)/dt$	Rate of Chl FY rise/decay at any time t of fast FIK

or form a shoulder. Decrease of ChlF or plateau between I and D points is caused by oxidation of Q_A and electron transport from Q_A to Q_B. If the plateau is observed, then the rates of reduction and reoxidation are equal. Starting from I, Q_B is steadily reduced and electrons are transferred to the PQ pool (Bolhàr-Nordenkampf and Öquist 1993). The following ChlF rise from D to P is due to an increasing reduction of the PQ pool and it reaches the maximum in the P-point when the PQ pool is maximally reduced (full reduction occurs if the saturating PFD is used). For more detailed overview see Papageorgiou (1975). The rise of modulated ChlF, reflecting all photochemical processes discussed above, is presented in Fig. 3.

Slow Chl fluorescence induction kinetics: If the dark-adapted sample is irradiated by AR, the detected ChlF signal rises very fast (in *ca.* 1 s) to the P-point and then declines (in terms of minutes) to the steady-state level T (*e.g.*, Papageorgiou 1975, Walker 1987). This O-P-S-M-T curve is demonstrated in a middle part of Fig. 4 which shows the fast ChlF rise followed by slow Chl FIK with some characteristic points described below. The Chl FY levels presented in Fig. 4 as well as important abbreviations and terms are summarized in Table 2.

The record of slow Chl FIK (Fig. 4) can be divided into three main parts. The first part corresponds to DAS that is characterized by minimum (F_0) and maximum (F_M) Chl FY levels. DAS is reached after a short-termed darkness, usually of 10–20 min, which is sufficient for reoxidation of electron carriers of the PS2 acceptor side and

Table 2. Terminology valid for slow Chl FIK; q_P – the photochemical quenching of F_V ($0 \le q_P \le 1$), q_N – the non-photochemical quenching of F_V ($0 \le q_N < 1$); compiled after Genty *et al.* (1989) and van Kooten and Snel (1990).

Symbol	Explanation
F_0	*Minimum* Chl FY measured on a dark-adapted sample with *open* PS2 RCs ($q_P = 1$) and all non-photochemical processes in a thylakoid membrane minimized ($q_N = 0$)
F_M	*Maximum* Chl FY measured on a dark-adapted sample with *closed* PS2 RCs ($q_P = 0$) and all non-photochemical processes in a thylakoid membrane minimized ($q_N = 0$)
F_S	*Steady-state* Chl FY remaining constant during induction by an actinic radiation as far as external circumstances do not change ($q_P > 0$, $q_N > 0$)
F_M'	*Maximum* Chl FY measured on a light-adapted sample with *closed* PS2 RCs ($q_P = 0$) and all active non-photochemical processes in a thylakoid membrane optimized ($q_N > 0$)
F_0'	*Minimum* Chl FY measured on a light-adapted sample with *open* PS2 RCs ($q_P = 1$) and all active non-photochemical processes in a thylakoid membrane optimized ($q_N > 0$)
$F(t)$	Actual Chl FY at any time t of induction by an actinic radiation
F_V	Maximum *variable* Chl FY measured on a dark-adapted sample $F_V = F_M - F_0$ (2)
F_P	Maximum of Chl FY measured on a dark-adapted sample after switching on of an actinic radiation (Kautsky peak, P-point)
F_V'	Maximum *variable* Chl FY measured on a light-adapted sample $F_V' = F_M' - F_0'$ (3)
$F_0''(t)$	*Minimum* Chl FY at any time of dark relaxation if all PS2 RCs are *open* ($q_P = 1$) and active non-photochemical processes decrease to their minimum ($q_N \ge 0$)
$F_M''(t)$	*Maximum* Chl FY at any time of dark relaxation if all PS2 RCs are *closed* ($q_P = 0$) and active non-photochemical processes decrease to their minimum ($q_N \ge 0$)
$F_V''(t)$	Maximum *variable* Chl FY at any time of dark relaxation $F_V''(t) = F_M''(t) - F_0''(t)$ (4)

relaxation of fast components of the non-photochemical quenching (Horton and Hague 1988). The middle part reflects changes of actual Chl FY during the radiation induction phase. If AR is switched on, the observed pronounced changes of $F(t)$ are linked to a simultaneous co-action of photochemical processes (charge separation in PS2 RCs, variations in efficiency of electron transport chain), and non-photochemical processes (*e.g.*, restoration of pH-gradient, NRD within a thylakoid membrane, LHC2 phosphorylation, photoinhibition of PS2 RCs). After a sufficiently long irradiation (5 min as minimum), the steady-state FY level (F_S) is reached when electron transport processes and coupled biochemical reactions in the carbon reduction cycle are equilibrated. If a pulse of SR is applied at this moment, maximum Chl FY (F_M') is measured in LAS. The third part of the record represents a back relaxation of minimum (F_0'') and maximum (F_M'') Chl FYs in darkness leading to a renewal of DAS. When AR is switched off, the initial minimum FY value (F_0') is measured after a short pulse (2–5 s) of a weak FR which is used to accelerate a reoxidation of the PS2 acceptor side.

The radiation phase of slow Chl FIK exhibits a few typical traits. The initial F_P peak (Fig. 4) covers the activation of both photochemical and non-photochemical processes in PS2. Its magnitude is determined by PFD of AR and depends on the

actual redox-state of the PS2 acceptor side. Following decrease of Chl FY is caused by continuous electron transport *via* electron carriers in the thylakoid membrane towards PS1. This phase of a linear electron transport creates the transthylakoidal

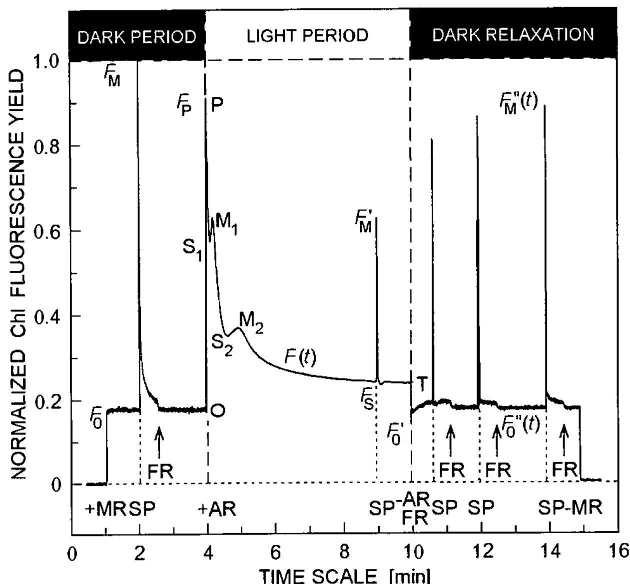

Fig. 4. Slow chlorophyll (Chl) *a* FIK recorded using a portable *PAM-2000* fluorometer (*H. Walz*, FRG) in the saturation pulse regime on a fully developed leaf of 10-week-old potted tobacco plant (*Nicotiana tabacum* cv. White Burley). The experiment was carried out *in vivo* using a small pre-darkening chamber at the following instrumental settings: Measuring radiation (MR) PFD < 0.2 μmol m^{-2} s^{-1} (λ = 650 nm), *PAM*-frequency of 600 Hz, switched on/off (+MR/-MR); actinic radiation (AR) PFD ≅ 290 μmol m^{-2} s^{-1} (λ = 655 nm), *PAM*-frequency of 20 kHz, switched on/off (+AR/-AR); saturation pulse (SP) PFD ≈5000 μmol m^{-2} s^{-1} (halogen lamp), pulse duration of 0.8 s, *PAM*-frequency of 20 kHz; far-red radiation (FR) PFD ≈10 μmol m^{-2} s^{-1} (λ = 735 nm), pulse duration of 3 s; time of the dark adaptation 10 min until the F_M determination; temperature of the leaf surface during a record 22.3 ± 0.1 °C. PFDs were measured at the leaf surface in the distance of 0.5 mm from the end of a fiber optics. For explanation of symbols and abbreviations see text and Table 2. Normalization to F_M was applied.

pH-gradient, a prerequisite of the ATP-synthesis and reduction of $NADP^+$ to NADPH (Mitchell 1966, Krause and Weis 1991). The first local peak on FIK (a sharp shoulder M_1) has its origin in the time-dependent changes of minimum Chl FY, $F_0'(t)$, in the first minute of irradiation by AR (see Fig. 5).

From the shape of the curves labeled *1* follows that $F_0'(t)$ exhibits pronounced changes almost exclusively in the initial phase of the irradiation. In this phase, the equilibrium between photochemical and non-photochemical processes dramatically varies, in connection with the pH-gradient creation and a feedback balanced synthesis of NADPH and ATP (Gerst *et al.* 1994). After switching AR on, $F_0'(t)$ rises by about 60 % in comparison to the initial F_0 level. After reaching a maximum, the likely

exponential decrease of $F_0'(t)$ to the steady-state value in approximately 2 min is observed. The initial F_0'-increase, followed by the exponential decline, influences as well the actual overall FY and the distinct shoulder (2) appears (cf. Fig. 4).

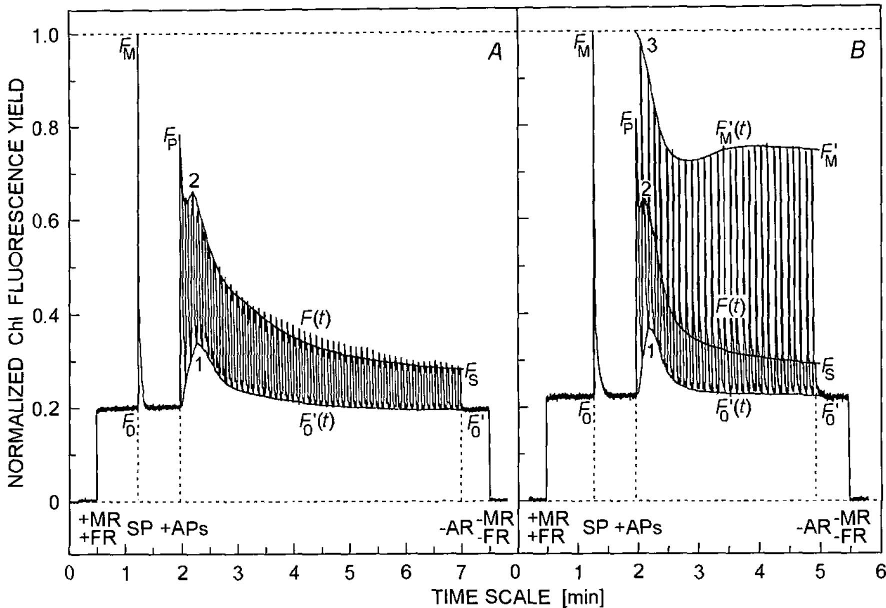

Fig. 5. Experimental records of the time-dependent changes of minimum, $F_0'(t)$, actual, $F(t)$, and (*B* only) maximum, $F_M'(t)$, chlorophyll (Chl) FYs. Both kinetic curves were measured *in vivo* using a *PAM-2000* fluorometer on leaves of 10-week-old potted tobacco plant (*A*) and 6-week-old hydroponically grown plant (*B*). The same experimental settings were used in both measurements: PFD of MR < 0.2 μmol m^{-2} s^{-1}, continuously switched-on FR with PFD ≈10 μmol m^{-2} s^{-1}, AR with PFD of 140 μmol m^{-2} s^{-1} triggered in 3-s-long pulses followed by 3 s period of a dark relaxation in the FR irradiation applied to the fast PQ pool reoxidation, 10 min dark adaptation of samples until the F_M determination, leaf surface temperature of 23.5 ± 0.5 °C. In *B*, the start of SPs with PFD of *ca.* 5000 μmol m^{-2} s^{-1} and duration of 0.8 s was synchronized with the triggered pulses of AR (APs). The solid curves *1* to *3* were spaced through experimental values by straight lines. Both records were normalized to the respective F_M values.

The second broader local maximum on FIK (M_2 in Fig. 4) can be associated with a start-up of the photosynthetic CO_2 fixation in carboxylation and reduction phases of the Calvin cycle. It corresponds to a feedback limitation of the linear electron transport due to an accumulation of NADPH and ATP in a close surrounding of the thylakoid membrane. Whenever the products are utilized photosynthetically (mostly by CO_2 fixation), FY declines and, when the rates of NADPH and ATP consumption become constant, Chl FY reaches its steady-state at the point T (Walker 1987). Moreover, conformational changes within the thylakoid membranes can be expected during the whole ChlF transition from F_P to F_S.

Table 3. List of ChlF parameters that can be calculated from fast Chl FIK measured on a dark-adapted sample.

Symbol	Explanation	
F_0/F_P	Ratio of minimum Chl FY (point O) to maximum Chl FY (point P)	
F_V/F_0	Ratio of variable Chl FY ($F_P - F_0$) to minimum Chl FY	
$(F_I - F_0)/F_V$	Ratio of $(F_I - F_0)$ to variable Chl FY	
A_{max}	Area over the part O – P of induction kinetics delimited by a vertical at point O and a horizontal at point P; N – normalization term $A_{max} = 1/N\,[F_P\,(t_P - t_0) - \int_{t0}^{tp} F_t\,dt\,];\ N \equiv F_V\,,\,F_0$	(6)
A_t	Area over FIK at any moment t within the time of Chl FY growth $A_t = 1/N\,[F_t\,(t - t_0) - \int_{t0}^{t} F_t\,dt\,];\ N \equiv F_V\,,\,F_0$	(7)
B	Area over the part P – end of induction kinetics (dark relaxation period) delimited by a horizontal at P and a vertical at the terminal point (t_S) of a record $B = 1/N\,[F_P\,(t_S - t_P) - \int_{tp}^{ts} F_{td}\,dt\,];\ N \equiv F_V\,,\,F_0$	(8)
β	Share of inactive RCs of PS2. Numerical value Z is found as an extrapolation of a linear part of the expression $\ln(A_{max} - A_t)$ for t going to 0 $\beta = e^Z \times 100$ [%]	(9)

Chl fluorescence parameters

Parameters related to fast Chl fluorescence induction kinetics: From fast FIK (Fig. 3), several Chl FPs can be derived (see Table 3). They can be divided into three groups: (*1*) those using extreme Chl FY values (F_0, F_P) as input, (*2*) those using F_t (*i.e.*, actual Chl FY located between F_0 and F_P), and (*3*) those related to areas over the kinetics. Mathematically, most FPs are ratios of FYs reached at two different times (Table 3). Ratios such as F_0/F_P, $(F_P - F_0)/F_P$, $(F_P - F_0)/F_0$ and analogously, when SR is applied, F_0/F_M, $(F_M - F_0)/F_M$, $(F_M - F_0)/F_0$ belong to the first group of parameters. Here, the *variable* Chl FY in DAS (F_V) is defined as

$$F_V = F_P - F_0 \qquad (5)$$

Before any calculations, values taken from fast Chl FIK must be normalized. Normalization is made by dividing the given Chl FY value by either F_0 or F_V.

The second group of parameters uses FYs at some significant points on fast FIK. Basically, Chl FYs at I to P points (Fig. 3) and the time at which they are reached represent parameters characterizing, after the proper normalization, the physiological status of a plant, PS2 in particular. The half-time ($t_{1/2}$) of Chl FY rise from O to P represents the parameter which is considered to be indirectly proportional to the size of LHC2 (Öquist and Wass 1988). Another FP, the $(F_I - F_0)/F_V$ ratio is proposed to be a measure of the proportion of inactive (Q_B-non-reducing) PS2 centres

(Klinkovský and Nauš 1994). The shorter time to reach the points and the higher level of Chl FY at these points, the more stressed the plant material is. In the presence of DCMU, a blocker of electron transport from Q_A to Q_B, Chl FY rises steeply after irradiation because there is no decrease in FY due to linear electron transport (Fig. 3), and, consequently, I and D points are not distinguished.

At any point of Chl FIK, the rate of the fluorescence rise can be calculated within the time resolution of the instrument. This parameter is of particular importance at the initial part of the curve (usually the first 300 μs of irradiation), where it represents the maximum rate of accumulation of closed RCs. Area A over the rising part of fast FIK (see Fig. 3) is proportional to the total number of electrons transported through PS2 during the time of ChlF induction and considered to be an equivalent of the size of PQ pool. When A is evaluated over the kinetics recorded under DCMU, it reaches a much smaller value which is equivalent to the proportion of PS2 RCs capable to transfer electrons *via* surrounding molecules of pigments or proteins (Cao and Govindjee 1990). The rise of A expressed as $(A_{max} - A_t)$, where A_{max} means the overall area over the rising part of FIK and A_t represents the area at any moment within the time of Chl FY growth (see Table 3), may be plotted against time. When logarithmic expression $\ln(A_{max} - A_t)$ is used instead of $(A_{max} - A_t)$, the relationship *9* in Table 3 can be used for the estimation of a share of PS2 β centres, *i.e.*, inactive PS2 RCs (Q_B-non-reducing). Generally, more detailed analysis of A growth can distinguish up to four types of PS2 (Sinclair and Spence 1990).

Area B (see Fig. 3) is a measure of Q_A reoxidation in dark. The larger area B, the faster dark reoxidation. On the decay part of fast Chl FIK, several phases of Q_A reoxidation (represented by the particular areas) can be distinguished (Krause and Weis 1991): very fast (≈500 μs), fast (≈10 ms), and slow (≈2 s). The very fast phase is attributed to electron transport from Q_A to Q_B in those PS2 RCs that do have Q_B bound. The fast phase is considered to be associated with those PS2 RCs that lack Q_B before the sample is irradiated. The slow phase of FY relaxation may be related to those RCs which are unable to transfer electrons to the PQ pool. Those phases have different rates of ChlF decrease which can be distinguished by using a semilog-plot $\ln(F_{td} - F_{min})$ *versus* time, where F_{td} is Chl FY at any time of the dark relaxation phase and F_{min} is minimum Chl FY at the end of a record (Table 3).

Parameters related to slow Chl fluorescence induction kinetics: Important information about the photochemical, non-photochemical, protective, regulative, destructive, and other processes acting in PS2 and the whole photosynthetic apparatus can be gained from an analysis of slow Chl FIK. For this purpose, many Chl FPs have been introduced to photosynthesis research within the last 25 years (*e.g.*, Kitajima and Butler 1975, Lichtenthaler *et al.* 1984, Schreiber *et al.* 1986, Genty *et al.* 1989, Bilger and Björkman 1990, Havaux *et al.* 1991). They are connected with both DAS and LAS. For their definitions and calculations, five mutually independent Chl FY levels have to be measured: F_M, F_0 (in DAS), F_M', F_S, and F_0' (in LAS). The correct determination of the F_M and F_0 values is essential because all changes of actual Chl FY during the irradiation period reflecting ChlF quenching mechanisms are

compared to these reference levels. Chl FPs derived from slow Chl FIK (Fig. 4) using five above-mentioned FYs are summarized in Table 4. Herein, the commonly used symbols, terms, and definitions for respective parameters are presented.

First Chl FP, the *maximum quantum yield of PS2 photochemistry* (Φ_{P_0}), Eq. (10), is the most frequently used parameter related to DAS. It is also called the maximum yield of primary photochemistry (Kitajima and Butler 1975), potential yield of PS2 photochemical reactions (Krause and Weis 1991), potential maximum PS2 quantum yield (Schreiber *et al.* 1995), or briefly the F_V/F_M ratio (Kitajima and Butler 1975). Its maximum value is almost constant for many different plant species and ecotypes measured under non-stressed conditions and equals to 0.832 ± 0.004 (Björkman and Demmig 1987). For stressed or damaged plants, Φ_{P_0} is markedly reduced. Thus, it can serve as an indicator of photoinhibition or other kind of injury cased to the PS2 complexes.

Two basic FPs describe the quenching of maximum *variable* Chl FY (F_V) during the inducing irradiation period: the *photochemical quenching of variable Chl FY* (q_P), Eq. (11), and the *non-photochemical quenching of variable Chl FY* (q_N), Eq. (12). q_P quantifies the photochemical capacity of PS2 in LAS and corresponds to the steady-state fraction of *open* (oxidized) PS2 RCs (Schreiber *et al.* 1986). Its complement to 1 ($1 - q_P$) approximates the reduction state of Q_A (Bilger and Björkman 1990) and is called the degree of PS2 reaction centre closure (Björkman and Demmig-Adams 1995). q_N (Schreiber *et al.* 1986) reflects influences of the non-photochemical processes on the ChlF emission during a transition of the sample from DAS to LAS, *e.g.*, changes in the transthylakoidal pH-gradient, inactivation of PS2 RCs (photoinhibition), disconnection of mobile LHCs of PS2, zeaxanthin formation, *etc.* (Horton and Hague 1988, Bilger and Björkman 1990, Krause and Weis 1991, Björkman and Demmig-Adams 1995).

Relative change of minimum Chl FY (q_0), Eq. (13), is FP describing changes of the initial F_0 level during an adaptation to irradiation of the sample exposed to AR (Bilger and Schreiber 1986). The parameter is associated with the electron flow regulating mechanisms (triggered by pH-gradient), inactivation of PS2 RCs, and conformation changes within pigment complexes in the thylakoid membrane (Bilger and Björkman 1990, Horton and Ruban 1992). Because the negative values of q_0 resulting from $F_0' > F_0$ can be measured in some cases (see Figs. 5 and 7) q_0 is not denominated as the 'quenching' of F_0 (cf. Bilger and Schreiber 1986).

For a monitoring of the efficiency of photochemical processes in PS2 in LAS, the *effective quantum yield of PS2 photochemistry* (Φ_P), Eq. (14), and the *effective quantum yield of photochemical energy conversion in PS2* (Φ_2), Eq. (15), are used. The former FP quantifies the extent to which the photochemistry of PS2 is limited by a competition with thermal decay processes (Oxborough and Baker 1997). Originally, Φ_P was called the efficiency of excitation energy capture by open PS2 RCs (Genty *et al.* 1989). For the parameter Φ_2 (Paillotin 1976) following terms exist in the literature: the quantum yield of PS2 electron transport (Genty *et al.* 1989), efficiency of PS2 electron transport per quantum absorbed by PS2 complexes (ibidem), overall quantum yield of the photochemical energy conversion in PS2

(Anonymous 1993), actual efficiency of energy conversion in PS2 (Björkman and Demmig-Adams 1995), fraction of light absorbed in PS2 antennae which are utilized in PS2 photochemistry (Demmig-Adams *et al.* 1996), and effective photochemical quantum yield of PS2 (Buffoni *et al.* 1998). Nevertheless, Eq. (15) shows that Φ_2 can be interpreted as the effective quantum yield of PS2 photochemistry (Φ_P) related to the fraction of photochemically active (open) PS2 RCs (q_P) in LAS. Φ_2 is often used for field investigations because it does not require the previous dark adaptation of samples as well as knowledge of the F_0' level.

Processes leading to a depression of F_M during the period of adaptation to irradiation quantify the following three FPs, definitions of which do not also require determination of F_0': the *non-photochemical ChlF quenching* (NPQ), Eq. (16), the *complete non-photochemical quenching of Chl FY* (q_{CN}), Eq. (17), and the *total quenching of Chl FY* (q_{TQ}), Eq. (18). The parameter NPQ (Bilger and Björkman 1990) was derived from the Stern-Volmer equation based on a matrix model of the antenna organization (Knox 1975, Schreiber *et al.* 1995). Björkman and Demmig-Adams (1995) report that NPQ is linearly related to the excess radiation over a wide range of incident PFDs and the extent of NPQ development in leaves is correlated with zeaxanthin formation. NPQ is often used as an indicator of the excess-radiant energy dissipation to heat in PS2 antenna complexes in LAS (Demmig-Adams *et al.* 1996, Gilmore 1997). FP q_{CN}, also termed the non-photochemical quenching quantum yield (Buffoni *et al.* 1998), has been introduced by analogy with q_N. It quantifies energy dissipating processes connected with both the non-photochemical quenching of F_V (q_N) and the relative change of F_0 (q_0). Hence, q_{CN} can be used as a measure of NRD within thylakoid membranes in LAS. The parameter q_{TQ}, also called the total quenching yield (Buffoni *et al.* 1998), integrates co-action of all quenching mechanisms (q_P, q_N, q_0) and can serve as a measure of the overall excitation energy consumption by photochemical and non-photochemical processes in LAS.

The *effective photosynthetic electron transport rate* (ETR), Eq. (19), and the *ratio of ChlF decrease* (Rfd), Eq. (20), are FPs frequently used in ecophysiological studies. ETR (Weis and Berry 1987, Anonymous 1993) is quantified in µmol(electron) m^{-2} s^{-1}. It takes into account actual PFD, Φ_2, and two semi-empirical factors (f_1 reflects that two photons must be absorbed by PS2 and PS1 *per* one electron transported, f_2 represents an absorption coefficient of a given plant material; see Table 4). The Rfd parameter (Brown 1967), also termed the "vitality index", has been often used as an indicator of the "vitality" of plants and evolution of stress (Lichtenthaler *et al.* 1984, 1986). From its values, conclusions about the potential photosynthetic activity and capacity of leaves, intactness and functionality of whole plants were made (Lichtenthaler 1988).

For the simplicity and better understanding, all parameters in Table 4 are presented in their time-independent forms. It assumes that the defined DAS or steady-state of photosynthesis are reached after the sufficiently long darkness or irradiation of the sample. Of course, FPs can be determined throughout the entire light induction period, not only in LAS. Therefore, the actual values of $F_M'(t)$, $F_V'(t)$, $F(t)$, and $F_0'(t)$

Table 4. List of ChlF parameters that can be calculated from slow Chl FIK measured on a sample in the dark/light-adapted state (DAS/LAS).

Symbol State	Explanation Definition relationship	
Φ_{P0} DAS	Maximum quantum yield of PS2 photochemistry; the F_V/F_M ratio: $\Phi_{P0} = \frac{F_V}{F_M} = 1 - \frac{F_0}{F_M}$; $0 < \Phi_{P0} < 1$	(10)
q_P LAS	Photochemical quenching of *variable* Chl FY: $q_P = \frac{F'_M - F_S}{F'_V} = \frac{\Delta F}{F'_V} = 1 - \frac{F_S - F'_0}{F'_M - F'_0}$; $0 \leq q_P < 1$	(11)
q_N LAS	Non-photochemical quenching of *variable* Chl FY: $q_N = \frac{F_V - F'_V}{F_V} = 1 - \frac{F'_V}{F_V} = 1 - \frac{F'_M - F'_0}{F_M - F_0}$; $0 \leq q_N \leq 1$	(12)
q_0 LAS	Relative change of minimum Chl FY: $q_0 = \frac{F_0 - F'_0}{F_0} = 1 - \frac{F'_0}{F_0}$; $-0.2 < q_0 < 1$	(13)
Φ_P LAS	Effective quantum yield of PS2 photochemistry: $\Phi_P = \frac{F'_V}{F'_M} = 1 - \frac{F'_0}{F'_M}$; $0 < \Phi_P < 1$	(14)
Φ_2 LAS	Effective quantum yield of photochemical energy conversion in PS2: $\Phi_2 = \frac{F'_M - F_S}{F'_M} = \frac{\Delta F}{F'_M} = q_P\, \Phi_P$; $0 \leq \Phi_2 < 1$	(15)
NPQ LAS	Non-photochemical ChlF quenching: $\mathrm{NPQ} = \frac{F_M - F'_M}{F'_M} = \frac{F_M}{F'_M} - 1$; $0 \leq \mathrm{NPQ} < +\infty$	(16)
q_{CN} LAS	Complete non-photochemical quenching of Chl FY: $q_{CN} = \frac{F_M - F'_M}{F_M} = 1 - \frac{F'_M}{F_M}$; $0 \leq q_{CN} < 1$	(17)
q_{TQ} LAS	Total quenching of Chl FY: $q_{TQ} = \frac{F_M - F_S}{F_M} = 1 - \frac{F_S}{F_M}$; $0 \leq q_{TQ} < 1$	(18)
ETR LAS	Effective photosynthetic electron transport rate ($f_1 = 0.5, f_2 \cong 0.84$) $\mathrm{ETR} = \mathrm{PFD}\, \Phi_2 f_1 f_2$; $0 \leq \mathrm{ETR} \leq$ limit	(19)
Rfd LAS	Ratio of Chl FY decrease; "vitality index" $\mathrm{Rfd} = \frac{F_P - F_S}{F_S} = \frac{F_P}{F_S} - 1$; $0 \leq \mathrm{Rfd} < +\infty$	(20)

have to be used instead of F_M', F_V', F_S, and F_0', respectively. Changes of above Chl FPs, measured at the specific time, indicate alterations in the photosynthetic apparatus of plants under varying laboratory or environmental conditions. Thus, values of FPs can be taken as the quantitative measure of those changes. Some examples are shown in the following section.

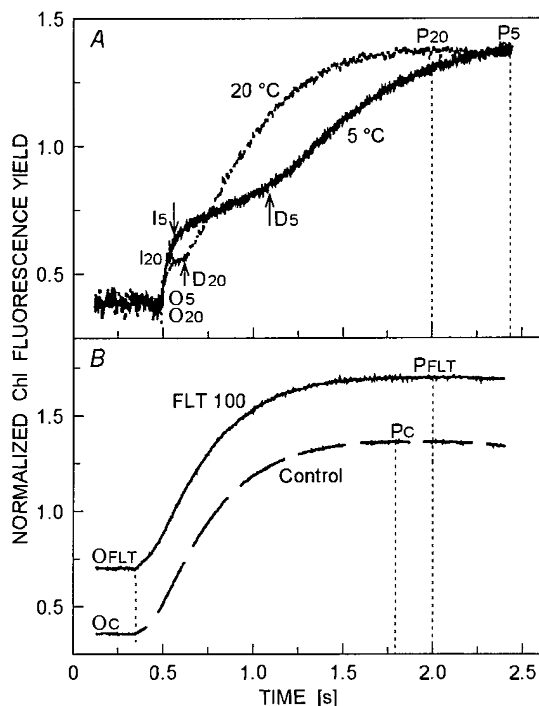

Fig. 6. (*A*) Fast chlorophyll (Chl) FIKs recorded using a *PAM-2000* fluorometer on a leaf of *Lolium perenne* at 20 °C and, after the 40-min-lasting lowering of air temperature, at 5 °C (for details see Barták *et al.* 1998). The effect of temperature drop is seen as much higher I-, D-Chl FY levels (indicated by arrows: I – ↓, D – ↑) and a shift of the times at which I, D, P points are reached (cf. X5 *vs*. X20, X = I, D, P). (*B*) Fast Chl FIK recorded on control (C) and fluoranthene-treated (FLT) leaves of *Zea mays* (Kummerová and Barták, unpublished). The effect of fluoranthene is seen as a pronounced increase of F_0 (cf. OC *vs*. OFLT), and a decrease of absolute Chl FY at the P point (non-normalized values, not shown). The measurements were made on 15-min dark-adapted *Z. mays* leaves, under room temperature (22 °C) using 2 s flash of AR (60 μmol m^{-2} s^{-1}). FLT 100 – plants treated for 4 weeks with 100 mmol m^{-3} fluoranthene in a root medium. The FIK records presented are normalized to the F_V values.

Applications

Applications of fast Chl fluorescence induction kinetics: Parameters derived from the fast kinetics of Chl FY reflect changes in the structure and functioning of the photosynthetic apparatus and are very sensitive indicators of plant stress. If PS2 is negatively affected by any stress it usually leads to a change in the F_0 level.

Increasing air temperature leads to a significant increase in F_0 (Terzaghi *et al.* 1990). Thus, all parameters defined on the basis of F_0 (see Table 3) are affected and can be used as a measure of heat stress. Under stress, the Chl FY rise is usually faster than in unstressed plants which results in a decrease in the area A, and the half-time $t_{1/2}$ (Fig. 3). Similarly, in stressed plants, the area B is smaller than in unstressed plants. Therefore, the lowering of A and/or B indicates a negative effect on photochemical reactions of PS2. Generally, when proper parameters are chosen, the fast Chl FIK can be used to describe a negative impact of the excessive radiation (*e.g.*, Srivastava and Strasser 1996), drought stress (van Rensburg *et al.* 1996), chemical elements and compounds (Ouzounidou *et al.* 1997), and many other stressors to the PS2 functioning. This flexibility is the main advantage of the method. Another advantage is the low time consumption (several minutes *per* sample). It enables a large number of repetitive measurements. Last but not least, it is a laboratory as well as field method. Two examples of the fast Chl FIK experiment are shown below.

Temperature effect: The effect of a sudden (40 min lasting) fall in air temperature from 20 to 5 °C on fast Chl FIK was studied on intact leaves of *Lolium perenne* using a *PAM-2000* fluorometer (Barták *et al.* 1998). The FIKs (Fig. 6*A*) show that the risetimes of the I and D points characterising the redox state of Q_A were markedly affected by lowering the air temperature. At 5 °C, both the I and D points were reached later and at higher FYs than at 20 °C. Also the time of rise from I to D was prolonged at 5 °C. Significant increase was found in the area A over the rising part of the ChlF curve taken at 5 °C. The changes in fast Chl FIK and the parameters derived indicate the altered functioning of PS2 at low temperature. Mechanisms causing the increased I-D time at 5 °C have not yet been fully elucidated but a slowed down turnover of quinones (quinone reduction) at low temperatures might be the reason. Also the rate of electron transport from Q_B to the PQ pool might be altered at these conditions. These effects could be associated with lower fluidity of the thylakoid membrane at low temperature due to desaturation of fatty acids in the membrane (Vigh *et al.* 1995), resulting in lowered PQ migration and reoxidation of Q_A (Chow *et al.* 1989). In this example, however, one must consider only short-distance PQ migration within a small domains of about 3–5 PS2 RCs. The between-domain or stroma-to-grana migration of PQs requires more time (in terms of seconds - Lavergne and Leci 1992).

Effect of chemicals: Toxic effects of many chemical compounds (*e.g.*, fluoranthene, a member of a large group of polycyclic aromatic hydrocarbons, PAHs) on PS2 functioning have also been estimated using fast Chl FIK. PAHs enter plants either directly *via* stomata or indirectly by root system. Negative effect of fluoranthene-treated (FLT) plants is associated with the membrane damage caused by photosensitized lipid oxidation (Girotti 1990). In the thylakoid membrane, it usually inhibits the electron transport through PS2 (Karukstis *et al.* 1990) and also the PS1 functioning (Huang *et al.* 1996). Compared to control (Fig. 6*B*), the FLT plants showed an increase in F_0 (both in absolute, +18 %, and F_V-normalized, +98 %, values), and higher rate of a Chl FY rise to the P point. In F_V-normalized FIKs, higher F_P was found also for FLT plants (+31 %). Fluoranthene affected negatively

the F_V/F_M ratio (0.733 in control, 0.587 in FLT plants) probably by inactivation of a part of PS2 population. The inactivation may take place mainly in a water-splitting complex. Similar results were obtained by Huang *et al.* (1996) on *Brassica napus* after the foliar application of other PAHs, benzo(a)pyrene and anthracene. The shape of F_V-normalized FIKs (Fig. 6*B*) is similar for control and FLT plants but Chl FY in the latter is constantly higher throughout the whole time interval. Thus the functioning of a remaining fraction of the active PS2 complexes in FLT plants is practically unaffected by fluoranthene, whereas the inactive complexes contribute to the non-radiative dissipation of the excitation energy demonstrated as a pronounced increase of the F_0 level.

Applications of slow Chl fluorescence induction kinetics: Slow Chl FIK enables to study both photochemical and non-photochemical processes associated with the energy conversion in chloroplasts. A set of Chl FPs (Table 4) allows to extract a complex information on processes acting in plants under different external and internal conditions. Numerical values of FPs reflect the interaction of plants with their environment, metabolism of chloroplasts, regulatory mechanisms in thylakoid membrane under stress, excitation energy transfer, *etc.* (*e.g.*, Krause and Weis 1991, Govindjee 1995, Owens 1996). Parameters associated with the non-photochemical quenching of Chl FY (q_N, NPQ, q_{CN}) can be used to estimate a share of NRD within thylakoid membranes (*i.e.*, thermal dissipation, LHC2 phosphorylation, pH-gradient build-up, zeaxanthin formation) on the overall excitation energy consumption (Horton and Hague 1988, Björkman and Demmig-Adams 1995, Horton 1996, Demmig-Adams *et al.* 1996, Buffoni *et al.* 1998).

If the F_M and F_0 levels are not fully relaxed in DAS due to the photoinhibition caused to PS2 by high irradiance or by other stressors, not only the F_V/F_M ratio is negatively affected (Demmig-Adams *et al.* 1996) but also all FPs based on F_M and F_0. Likewise, the experimental conditions and instrumental settings under which FIKs are measured (*i.e.*, PFD of ambient irradiation, ambient temperature, CO_2 concentration, time of dark adaptation, PFDs of device sources, *etc.*) should always be well defined since variations of these physical parameters lead to large results' scattering (*e.g.*, Papageorgiou 1975, Renger and Schreiber 1986, Schreiber *et al.* 1995). As seen, the employment of Chl FPs has own limitations. If the parameters are used mechanically, misleading interpretations can be drawn. The following example should demonstrate a capability of Chl FPs to reflect changes in the photosynthetic apparatus of plants.

Effect of external conditions – the circadian cycle: Effect of the ambient temperature and irradiance on values of FPs calculated from the records of slow Chl FIK is demonstrated using results of the 24-h experiment with conifers (Roháček and Šiffel 1995). Four-year-old seedlings of Norway spruce (*Picea abies* [L.] Karst.) were grown and measured in a clima box *Sherer* (Kysor, USA) under controlled conditions: the day/night temperatures of a spruce needle surface (T_S) were held at 21/11 °C, relative humidity varied between 65–75 %, the light period with maximum PFD of 470 $\mu mol\, m^{-2}\, s^{-1}$ (08:00–19:00 h) alternated periodically with darkness

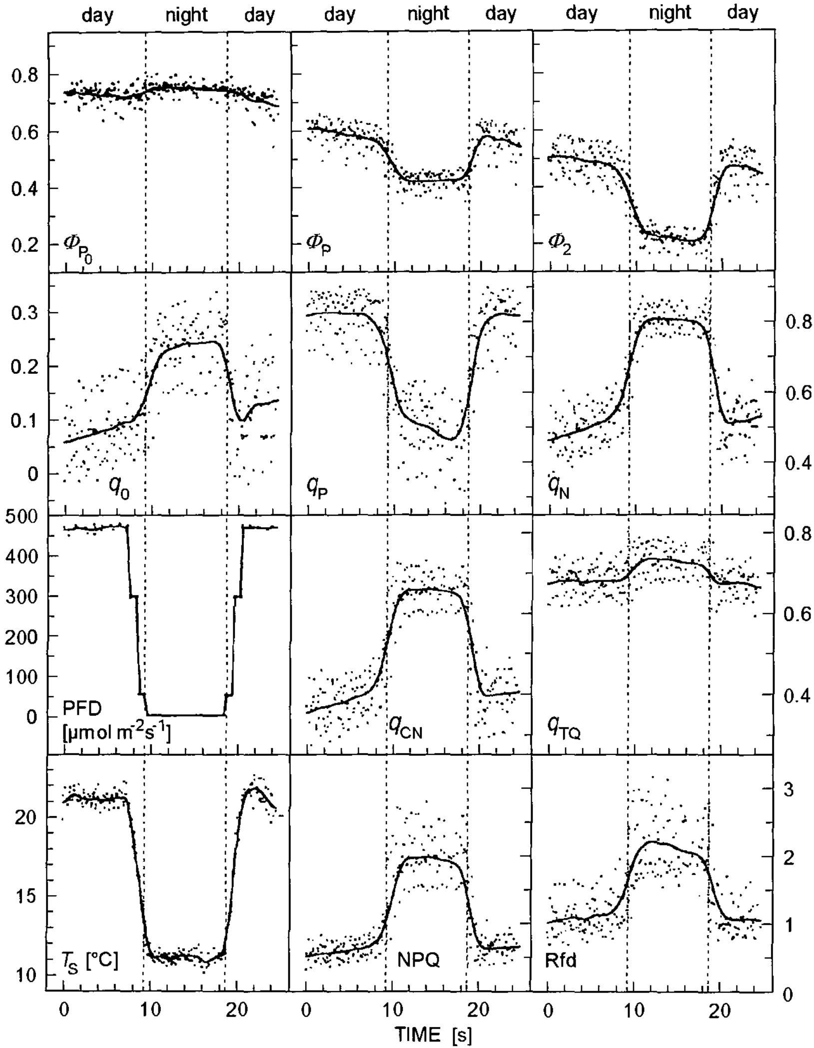

Fig. 7. Diurnal courses of ten chlorophyll (Chl) FPs induced by simultaneous changes of the ambient irradiance (PFD) and surface temperature of spruce needles (T_S). Each point in the graphs corresponds to one FIK recorded *in vivo* using a *PAM101-103* fluorometer (*H. Walz*, FRG) on 4-year-old seedlings of Norway spruce (*Picea abies* [L.] Karst.) under the following conditions: PFD of MR < 0.2 μmol m^{-2} s^{-1} (λ = 655 nm, *PAM*-frequency of 1.6 kHz), PFD of AR ≅ 170 μmol m^{-2} s^{-1} (λ = 655 nm, *PAM*-frequency of 100 kHz), switched on for 5 min (to the F_M', F_S levels' determination) and 5.5 min (to the F_0' determination), PFD of SR ≈ 9000 μmol m^{-2} s^{-1} (halogen lamp, pulse duration of 0.8 s, *PAM*-frequency of 100 kHz). The current-year spruce branch of a top whorl with intact needles was fixed in a small chamber and pre-darkened herein for 15 min before the F_M level was determined. Branches were changed clockwise for each seedling, T_S was measured in the chamber using a Ni/NiCr-thermocouple, and PFD in the surroundings of branches using a *Lambda*-sensor. Experiments started at 11:30 h (time zero) and were performed on 6 different spruce seedlings. Solid curves in graphs are results of the 20-times repeated (except PFD) non-equidistant smoothing from 3 points. For definitions of Chl FPs see Table 4.

(21:00–06:00 h). A dawn and nightfall were simulated using sets of bulbs and fluorescent tubes switched on and off progressively in three steps: 50 - 300 - 470 $\mu mol\ m^{-2}\ s^{-1}$ (06:00–08:00 h) and 300 - 50 - 0 $\mu mol\ m^{-2}\ s^{-1}$ (19:00–21:00 h), respectively. In these time periods, the changeover between day and night temperature regimes was performed. Seedlings were watered and dewed with distilled water during the nightfall and their positions were changed with the period of 5–7 d. Plants were adapted for 4 weeks to the above conditions before measurements started.

Experimental values fall into two major bands corresponding to the day and night steady-state (Fig. 7). The transition between both states is clearly connected with the three-step regulation of ambient PFD and day/night variation of T_S. The slightly descending or ascending day trend (see Φ_{P_0}, Φ_2, q_0, q_N) is probably caused by repetitive positioning of the chamber to the same place on the branches measured. The courses of FPs can be divided in three categories considering effect of the changing external conditions: no significant influence (Φ_{P_0}, q_{TQ}), pronounced *decrease* in the night period (Φ_P, Φ_2, q_P), and pronounced *increase* in the night period (q_0, q_N, q_{CN}, NPQ, Rfd).

The maximum quantum yield of PS2 photochemistry (Φ_{P_0}) remained almost constant all over the 24-h period. It means that both the growth temperature and PFD did not injure PS2 or stress the plants. The time of pre-darkness (15 min) was sufficient to induce DAS in the sample during the day period as follows from comparison with the night results. In the case of excessive external PFD, Φ_{P_0} would be lowered owing to effect of a long-living component of the non-photochemical quenching (q_I; Walker 1987, Horton and Hague 1988). Moreover, the night temperature (11 °C) did not affect markedly Φ_{P_0} in PS2 complexes.

Other graphs in Fig. 7 document a principal difference between the day (optimum LAS) and night (optimum DAS) physiological regimes of plants. The experimental settings of the PAM-fluorimeter were identical during both periods. Therefore, interpretation of observed changes has to be sought in a changing redistribution of the excitation energy between photochemical and non-photochemical pathways during the adaptation of a spruce photosynthetic apparatus to actual PFD and T_S. The fall in the actual efficiency of photochemistry in PS2 (Φ_P, Φ_2) and photochemical capacity of PS2 (q_P) corresponds to the pronounced increase of NRD (heat dissipation) within thylakoid membranes (see q_N, q_{CN}, NPQ; cf. Oberhuber and Edwards 1993, Demmig-Adams *et al.* 1996). Both the excitation energy consuming processes are synchronized and act in opposite (compare q_P with q_N). It explains why no significant changes were observed for q_{TQ} which combines both trends.

The courses of q_N and q_{CN} are very similar to NPQ (Fig. 7). This confirms the statement made in the above section that all these parameters can be used to quantify NRD processes within thylakoid membranes. In addition, FPs q_0 and Rfd are clearly connected with the non-photochemical processes (compare with q_N, NPQ). From this view, a direct relation of Rfd to the "vitality" of plants, which should be logically the

same for day and night conditions, is controversial. Of course, a more detailed analysis of these experimental results exceeds a frame of this paper.

References

Anonymous: Portable fluorometer PAM-2000 and data acquisition software DA-2000. Handbook of Operation, Ed. 2. - Heinz Walz, Effeltrich 1993.

Barták, M., Nijs, I., Impens, I.: The susceptibility of PSII of *Lolium perenne* to a sudden fall in air temperature - response of plants grown in elevated CO_2 and/or increased air temperature. – Environ. exp. Bot. **39**: 85-95, 1998.

Bilger, W., Björkman, O.: Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. – Photosynth. Res. **25**: 173-185, 1990.

Bilger, W., Schreiber, U.: Energy-dependent quenching of dark-level chlorophyll fluorescence in intact leaves. – Photosynth. Res. **10**: 303-308, 1986.

Björkman, O., Demmig, B.: Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. – Planta **170**: 489-504, 1987.

Björkman, O., Demmig-Adams, B.: Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. – In: Schulze, E.-D., Caldwell, M.M. (ed.): Ecophysiology of Photosynthesis. Pp. 17-47. Springer-Verlag, Berlin - Heidelberg 1995.

Bolhàr-Nordenkampf, H.R., Öquist, G.: Chlorophyll fluorescence as a tool in photosynthesis research. – In: Hall, D.O., Scurlock, J.M.O., Bolhàr-Nordenkampf, H.R., Leegood, R.C., Long, S.P. (ed.): Photosynthesis and Production in a Changing Environment: A Field and Laboratory Manual. Pp. 193-206. Chapman & Hall, London - Glasgow - New York - Tokyo - Melbourne - Madras 1993.

Bradbury, M., Baker, N.R.: Analysis of the slow phases of the *in vivo* chlorophyll fluorescence induction curve. Changes in the redox state of photosystem II electron acceptors and fluorescence emission from photosystems I and II. – Biochim. biophys. Acta **635**: 542-551, 1981.

Bradbury, M., Baker, N.R.: A quantitative determination of photochemical and non-photochemical quenching during the slow phase of the chlorophyll fluorescence induction curve of bean leaves. – Biochim. biophys. Acta **765**: 275-281, 1984.

Brown, J.S.: Fluorometric evidence for the participation of chlorophyll *a*-695 in system 2 of photosynthesis. – Biochim. biophys. Acta **143**: 391-398, 1967.

Buffoni, M., Testi, M.G., Pesaresi, P., Garlaschi, F.M., Jennings, R.C.: A study of the relation between CP29 phosphorylation, zeaxanthin content and fluorescence quenching parameters in *Zea mays* leaves. – Physiol. Plant. **102**: 318-324, 1998.

Cao, J., Govindjee: Chlorophyll *a* fluorescence transient as an indicator of active and inactive photosystem II in thylakoid membranes. – Biochim. biophys. Acta **1015**: 180-188, 1990.

Chow, W.S., Osmond, C.B., Huang, L.K.: Photosystem II function and herbicide binding sites during photoinhibition of spinach chloroplasts *in-vivo* and *in-vitro*. – Photosynth. Res. **21**: 17-26, 1989.

Demmig-Adams, B., Adams, W.W., III, Barker, D.H., Logan, B.A., Bowling, D.R., Verhoeven, A.S.: Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. – Physiol. Plant. **98**: 253-264, 1996.

Genty, B., Briantais, J.-M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim. biophys. Acta **990**: 87-92, 1989.

Gerst, U., Schönknecht, G, Heber, U.: ATP and NADPH as the driving force of carbon reduction in leaves in relation to thylakoid energization by light. – Planta **193**: 421-429, 1994.

Gilmore, A.M.: Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. – Physiol. Plant. **99**: 197-209, 1997.

Girotti, A.W.: Photodynamic lipid peroxidation in biological systems. – Photochem. Photobiol. **51**: 497-509, 1990.

Govindjee: Sixty-three years since Kautsky: Chlorophyll *a* fluorescence. – Aust. J. Plant Physiol. **22**: 131-160, 1995.

Govindjee, Govindjee, R.: Introduction to photosynthesis. – In: Govindjee (ed.): Bioenergetics of Photosynthesis. Pp. 1-50. Academic Press, New York - San Francisco - London 1975.

Havaux, M., Strasser, R.J., Greppin, H.: A theoretical and experimental analysis of the q_P and q_N coefficients of chlorophyll fluorescence quenching and their relation to photochemical and nonphotochemical events. – Photosynth. Res. **27**: 41-55, 1991.

Horton, P.: Nonphotochemical quenching of chlorophyll fluorescence. – In: Jennings, R.C., Zucchelli, G., Ghetti, F., Colombetti, G. (ed.): Light as an Energy Source and Information Carrier in Plant Physiology. Pp. 99-111. Plenum Press, New York - London 1996.

Horton, P., Hague, A.: Studies on the induction of chlorophyll fluorescence in isolated barley protoplasts. IV. Resolution of non-photochemical quenching. – Biochim. biophys. Acta **932**: 107-115, 1988.

Horton, P., Ruban, A.V.: Regulation of Photosystem II. – Photosynth. Res. **34**: 375-385, 1992.

Huang, X.-D., Zeiler, L.F., Dixon, D.G., Greenberg, B.M.: Photoinduced toxicity of PAHs to the foliar regions of *Brassica napus* (canola) and *Cucumbis sativus* (cucumber) in simulated solar radiation. – Ecotox. environ. Safe. **35**: 190-197, 1996.

Karukstis, K.K., Berliner, M.A., Jewell, C.J., Kuwata, K.T.: Chlorophyll fluorescence measurements to assess the competition of substituted anthraquinones for the Q_B binding site. – Biochim. biophys. Acta **1020**: 163-168, 1990.

Kautsky, H., Hirsch, A.: Chlorophyllfluoreszenz und Kohlensäureassimilation. I. Das Fluoreszenzverhalten grüner Pflanzen. – Biochem. Z. **274**: 423-434, 1934.

Kitajima, M., Butler, W.L.: Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. – Biochim. biophys. Acta **376**: 105-115, 1975.

Klinkovský, T., Nauš, J.: Sensitivity of the relative F_{pl} level of chlorophyll fluorescence induction in leaves to the heat stress. – Photosynth. Res. **39**: 201-204, 1994.

Knox, R.S.: Excitation energy transfer and migration: theoretical considerations. – In: Govindjee (ed.): Bioenergetics of Photosynthesis. Pp. 183-221. Academic Press, New York - San Francisco - London 1975.

Krause, G.H., Weis, E.: Chlorophyll fluorescence and photosynthesis: the basics. – Annu. Rev. Plant Physiol. Plant mol. Biol. **42**: 313-349, 1991.

Lavergne, J., Leci, E.: Properties of inactive photosystem II centres. – Photosynth. Res. **38**: 323-343, 1992.

Lichtenthaler, H.K.: Applications of chlorophyll fluorescence in stress physiology and remote sensing. – In: Steven, M.D., Clark, J.A. (ed.): Applications of Remote Sensing in Agriculture. Pp. 287-305. Butterworth-Heinemann, Stoneham 1990.

Lichtenthaler, H.K., Buschmann, C., Rinderle, U., Schmuck, G.: Application of chlorophyll fluorescence in ecophysiology. – Radiat. environ. Biophys. **25**: 297-308, 1986.

Lichtenthaler, H.K., Meier, D., Buschmann, C.: Development of chloroplasts at high and low light quanta fluence rates. – Israel J. Bot. **33**: 185-194, 1984.

Mitchell, P.: Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. – Biol. Rev. Cambridge philos. Soc. **41**: 445-502, 1966.

Oberhuber, W., Edwards, G.E.: Temperature dependence of the linkage of quantum yield of photosystem II to CO_2 fixation in C_4 and C_3 plants. – Plant Physiol. **101**: 507-512, 1993.

Öquist, G., Wass, R.: A portable, microprocessor operated instrument for measuring chlorophyll fluorescence kinetics in stress physiology. – Physiol. Plant. **73**: 211-217, 1988.

Ouzounidou, G., Moustakas, M., Strasser, R.J.: Sites of action of copper in the photosynthetic apparatus of maize leaves: Kinetic analysis of chlorophyll fluorescence, oxygen evolution,

absorption changes and thermal dissipation as monitored by photoacoustic signals. – Aust. J. Plant Physiol. **24**: 81-90, 1997.

Owens, T.G.: Processing of excitation energy by antenna pigments. – In: Baker, N.R. (ed.): Photosynthesis and the Environment. Pp. 1-23. Kluwer Academic Publ., Dordrecht - Boston - London 1996.

Oxborough, K., Baker, N.R.: Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of qP and F_v'/F_m' without measuring F_0'. – Photosynth. Res. **54**: 135-142, 1997.

Paillotin, G.: Movement of excitations in the photosynthetic domains of photosystem II. – J. theor. Biol. **58**: 237-252, 1976.

Papageorgiou, G.: Chlorophyll fluorescence: an intrinsic probe of photosynthesis. – In: Govindjee (ed.): Bioenergetics of Photosynthesis. Pp. 319-371. Academic Press, New York - San Francisco - London 1975.

Prosser, V. *et al.*: Experimentální metody biofyziky. [Experimental Methods of Biophysics.] - Pp. 386-387. Academia, Praha 1989. [In Czech.]

Renger, G., Schreiber, U.: Practical applications of fluorometric methods to algae and higher plant research. – In: Govindjee, Amesz, J., Fork, D.C. (ed.): Light Emission by Plants and Bacteria. Pp. 587-619. Academic Press, Orlando - San Diego - New York - Austin - Boston - London - Sydney - Tokyo 1986.

Roháček, K., Šiffel, P.: Temperature and light dependences of chlorophyll fluorescence *in vivo* for Norway spruce seedlings. – In: Mathis, P. (ed.): Photosynthesis: from Light to Biosphere. Vol. V. Pp. 937-940. Kluwer Academic Publ., Dordrecht - Boston - London 1995.

Schreiber, U.: Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer. – Photosynth. Res. **9**: 261-272, 1986.

Schreiber, U., Bilger, W., Neubauer, C.: Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. – In: Schulze, E.-D., Caldwell, M.M. (ed.): Ecophysiology of Photosynthesis. Pp. 49-70. Springer-Verlag, Berlin - Heidelberg - New York 1995.

Schreiber, U., Klughammer, C., Neubauer, C.: Measuring P700 absorbance changes around 830 nm with a new type of pulse modulation system. – Z. Naturforsch. **43c**: 686-698, 1988.

Schreiber, U., Schliwa, U., Bilger, W.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. – Photosynth. Res. **10**: 51-62, 1986.

Sinclair, J., Spence, S.M.: Heterogeneous photosystem-2 activity in isolated spinach chloroplasts. – Photosynth. Res. **24**: 209-220, 1990.

Srivastava, A., Strasser, A.: Stress and stress management of land plants during a regular day. – J. Plant Physiol. **148**: 445-455, 1996.

Strasser, B.J., Strasser, R.J.: Oscillations of the chlorophyll *a* fluorescence related to the S-states of the oxygen evolving complex. – In: Garab, G. (ed.): Photosynthesis: Mechanisms and Effects. Vol. V. Pp. 4325-4328. Kluwer Academic Publ., Dordrecht - Boston - London 1999.

Strasser, R.J., Tsimilli-Michael, M.: Activity and heterogeneity of PSII probed in vivo by the chlorophyll *a* fluorescence rise O-(K)-J-I-P. – In: Garab, G. (ed.): Photosynthesis: Mechanisms and Effects. Vol. V. Pp. 4321-4324. Kluwer Academic Publ., Dordrecht - Boston - London 1999.

Terzaghi, W.B., Fork, D.C., Berry, J.A., Field, C.B.: Low and high temperature limits to PS2. A survey using *trans*-parinaric acid, delayed light emission, and F_0 chlorophyll fluorescence. – Plant Physiol. **91**: 1495-1500, 1990.

van Grondelle, R., Dekker, J.P., Gillbro, T., Sundstrom, V.: Energy transfer and trapping in photosynthesis. – Biochim. biophys. Acta **1187**: 1-65, 1994.

van Kooten, O., Snel, J.F.H.: The use of chlorophyll fluorescence nomenclature in plant stress physiology. – Photosynth. Res. **25**: 147-150, 1990.

van Rensburg, L., Krüger, G.H.J., Eggenberg, P., Strasser, R.J.: Can screening criteria for drought resistance in *Nicotiana tabacum* L. be derived from the polyphasic rise of the chlorophyll *a* transient (OJIP)? – South Afr. J. Bot. **62**: 337-341, 1996.

Vigh, L., Los, D.A., Murata, N., Glatz, A., Kovács, E., Horváth, I.: Is the membrane the primary target in the biological perception of temperature? Effect of membrane physical state on the expression of stress-defence genes. – In: Kader, J.-C., Mazliak, P. (ed.): Plant Lipid Metabolism. Pp. 369-371. Kluwer Academic Publ., Dordrecht 1995.

Walker, D.: Fluorescence. – In: Walker, D. (ed.): The Use of the Oxygen Electrode and Fluorescence Probes in Simple Measurements of Photosynthesis. Pp. 17-46. Oxgraphics, University of Sheffield, Sheffield 1987.

Weis, E., Berry, J.A.: Quantum efficiency of photosystem II in relation to 'energy'-dependent quenching of chlorophyll fluorescence. – Biochim. biophys. Acta **894**: 198-208, 1987.