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How to correctly determine the different chlorophyll fluorescence parameters and the chlorophyll fluorescence decrease ratio R_{Fd} of leaves with the PAM fluorometer

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Abstract

This contribution is a practical guide to the measurement of the different chlorophyll (Chl) fluorescence parameters and gives examples of their development under high-irradiance stress. From the Chl fluorescence induction kinetics upon irradiation of dark-adapted leaves, measured with the PAM fluorometer, various Chl fluorescence parameters, ratios, and quenching coefficients can be determined, which provide information on the functionality of the photosystem 2 (PS2) and the photosynthetic apparatus. These are the parameters F_v, F_m, F_0, F_m', F_v', NF, and ΔF, the Chl fluorescence ratios F_v/F_m, F_v/F_0, $\Delta F/F_m'$, as well as the photochemical (q_P) and non-photochemical quenching coefficients (q_N, q_{CN}, and NPQ). q_N consists of three components ($q_N = q_E + q_T + q_I$), the contribution of which can be determined *via* Chl fluorescence relaxation kinetics measured in the dark period after the induction kinetics. The above Chl fluorescence parameters and ratios, many of which are measured in the dark-adapted state of leaves, primarily provide information on the functionality of PS2. In fully developed green and dark-green leaves these Chl fluorescence parameters, measured at the upper adaxial leaf side, only reflect the Chl fluorescence of a small portion of the leaf chloroplasts of the green palisade parenchyma cells at the upper outer leaf half. Thus, PAM fluorometer measurements have to be performed at both leaf sides to obtain information on all chloroplasts of the whole leaf. Combined high irradiance (HI) and heat stress, applied at the upper leaf side, strongly reduced the quantum yield of the photochemical energy conversion at the upper leaf half to nearly zero, whereas the Chl fluorescence signals measured at the lower leaf side were not or only little affected. During this HL-stress treatment, q_N, q_{CN}, and NPQ increased in both leaf sides, but to a much higher extent at the lower compared to the upper leaf side. q_N was the best indicator for non-photochemical quenching even during a stronger HL-stress, whereas q_{CN} and NPQ decreased with progressive stress even though non-photochemical quenching still continued. It is strongly recommended to determine, in addition to the classical fluorescence parameters, *via* the PAM fluorometer also the Chl fluorescence decrease ratio R_{Fd} (F_d/F_s), which, when measured at saturation irradiance is directly correlated to the net CO_2 assimilation rate (P_N) of leaves. This R_{Fd}-ratio can be determined from the Chl fluorescence induction kinetics measured with the PAM fluorometer using continuous saturating light (cSL) during 4–5 min. As the R_{Fd}-values are fast measurable indicators correlating with the photosynthetic activity of whole leaves, they should always be determined *via* the PAM fluorometer parallel to the other Chl fluorescence coefficients and ratios.

Additional key words: adaxial leaf side; chlorophyll fluorescence decrease ratio; continuous saturating irradiance; *Ficus*; high irradiance stress; quenching coefficients; R_{Fd}-ratio; *Schefflera*.

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Abbreviations: AL – "actinic light"; Car – carotenoids; Chl – chlorophyll; cSL – continuous saturating light; F_0 – ground fluorescence in the dark-adapted state; F_0' – ground fluorescence in the light-adapted state; F_d – fluorescence decrease from F_p to F_s; F_m – maximum Chl fluorescence at a saturating radiation pulse in the dark-adapted state; F_m' – maximum Chl flurorescence in the light-adapted state; F_p – maximum Chl fluorescence at a non-saturating light pulse in the dark-adapted state; F_s – steady state Chl fluorescence; ΔF – fluorescence spike at the end of the induction kinetic on top of the AL-induced kinetics ($F_m' - F$); F_d/F_s – ratio of Chl fluorescence decrease F_d to steady state Chl fluorescence F_s; HI – high irradiance (high light); ML – measuring light; NF – Chl fluorescence parameter related to non-photochemical quenching ($F_m - F_m'$); P_N – net CO_2 assimilation rate; PAR – photosynthetically active radiation; PPFD – photosynthetic photon flux density; R_{Fd} – variable chlorophyll fluorescence decrease ratio; SL – saturating light.

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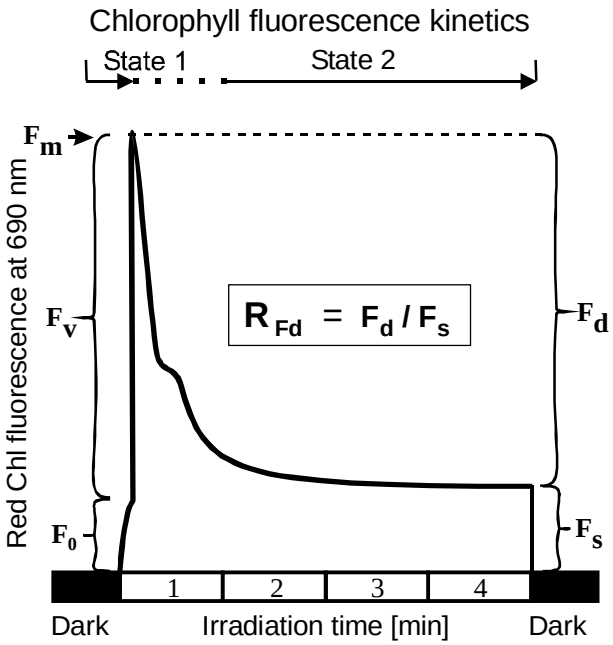
General introduction

In irradiated leaves, the two photosystems, PS2 and PS1, in series perform the photosynthetic light reactions and associated electron transport in a strict, highly coordinated cooperation. In dark-adapted green leaves, which had been placed into darkness for 20 min or longer, the cooperation of the two photosynthetic photosystems is impaired. In the dark, the photosynthetic apparatus is in its non-functional 'state 1'. Upon irradiation of dark-adapted leaves it takes a few minutes to induce again the cooperation of both photosystems and to bring about the joint photosynthetic electron transport reactions that lead to proper water splitting, oxygen evolution, as well as $NADP^+$ reduction and ATP formation. The latter are required to guarantee the photosynthetic net CO_2 assimilation in the Calvin-Benson cycle. This light-triggered induction period of the photosynthetic apparatus to its functional 'state 2' ('state 1'→ 'state 2' transition), which is caused by several changes, *e.g.* also by a phosphorylation of the LHC2 (see below), can easily be detected and measured *via* the chlorophyll (Chl) fluorescence induction kinetics, a transient in Chl fluorescence yield that had first been detected and described in 1931 (Kautsky and Hirsch 1931) as fully reviewed in Lichtenthaler (1992). In photosynthetic literature the dark-adapted non-functional state of the photosynthetic apparatus with open PS2 reaction centres is usually designated as "state 1", whereas the irradiance-adapted, functional state is defined as "state 2" (*e.g.* Fork and Satoh 1986, Haldrup *et al.* 2001). Also, in the new Chl fluorescence book (Papageorgiou and Govindjee 2004) most authors stick to this nomenclature. Govindjee (personal communication, 2003) had proposed to proceed the other way around, as done by Lichtenthaler and Babani (2004), but this is confusing since other authors did not follow his proposal. Thus, we return here to the generally accepted way of using state 1 for the dark-adapted and state 2 for the light-adapted photosynthetic apparatus.

This irradiance-induced Chl fluorescence induction kinetic is characterized by a fast rise of Chl fluorescence *via* the initial level F_0 (also termed ground fluorescence) to a maximum fluorescence level, F_m, at saturating light (SL) within 100–200 ms as indicated in Fig. 1. With the non-saturating 'actinic light' AL, applied in the PAM fluorometer, one does not obtain the F_m but the F_p level reached within 300–800 ms depending on the AL. In this Chl fluorescence rise only PS2 is involved. Thereafter, PS1 starts to work and drains off electrons from PS2, then the cooperation of PS2 and PS1 as well as the photosynthetic electron transport go into full operation and the net CO_2 assimilation and oxygen evolution are triggered. This is seen in a gradual decline of the Chl fluorescence intensity from F_m within 3–5 min to a much lower steady state F_s (Fig. 1). The greater this Chl fluorescence decrease F_d, from F_m to F_s, the higher the net photosynthetic rate (P_N) of the leaf examined (Lichtenthaler and Rinderle 1988a, Lichtenthaler and Miehé 1997, Lichtenthaler and Babani 2004). In fully or partially sun-exposed leaves of outdoor plants this decrease is mainly caused by the photosynthetic quantum conversion



F_0 = initial fluorescence
F_v = variable fluorescence
F_m = maximum fluorescence
F_d = fluorescence decrease
F_s = steady state fluorescence
R_{Fd} = fluorescence decrease ratio

Fig. 1. Light-induced chlorophyll (Chl) fluorescence induction kinetics (Kautsky effect) in 20 min pre-darkened green, photosynthetically active leaves measured at saturation irradiance >1 500 µmol(photon) m^{-2} s^{-1}. Upon irradiation, the Chl fluorescence rises *via* F_0 to the maximum F_m (within 100–200 ms) and then declines with the onset of photosynthetic CO_2-fixation, within 3–5 min, to a low steady state fluorescence, F_s, which in fully photosynthetically active leaves is slightly above the level of F_0. F_d is the Chl fluorescence decrease from F_m to F_s. The Chl fluorescence decrease ratio R_{Fd}, defined as ratio F_d/F_s, when measured at saturation irradiance, correlates with the potential CO_2 fixation rate P_N of leaves as shown for several plants as well as sun and shade leaves (Lichtenthaler and Rinderle 1988a, Lichtenthaler and Babani 2004, Lichtenthaler *et al.* 2005). The ratio R_{Fd} can be expressed either by F_d/F_s or by $(F_m/F_s) - 1$. The 'state 1' of the dark-adapted photosynthetic apparatus, where F_m is reached after a few hundred ms of irradiation, is in the light gradually turned into the functional 'state 2' of the light-adapted photosynthetic apparatus.

process, however, also including some non-photochemical processes. This relationship can be quantified by the Chl fluorescence decrease ratio, R_{Fd}, defined as ratio F_d/F_s or $(F_m/F_s) - 1$, usually measured at a saturating "white light" of 2 000 µmol m^{-2} s^{-1} or a short wavelength red He/Ne-laser radiation (632.8 nm) of *ca.* 700 µmol m^{-2} s^{-1}. The R_{Fd} is higher in sun leaves of trees (values of 3–5) than in shade leaves (values of 1.0–2.5) reflecting their higher photosynthetic capacity and CO_2 fixation rates (Lichtenthaler and Burkart 1999, Lichtenthaler and Babani 2004). In fact, the R_{Fd}-values being measured at the saturation irradiance of photosynthesis exhibit a highly significant linear correlation to P_N as shown in Lichtenthaler *et al.* (2005). This correlation particularly applies to outdoor plants that are much less affected by photoinhibition in comparison to leaves of greenhouse plants. In leaves from the extreme shade or from greenhouse plants one should check by repetition of the induction kinetic measurement whether the applied irradiance for measuring the R_{Fd} values might already cause a certain photoinhibition of the photosynthetic apparatus. If so, the excitation radiation can be reduced to *ca*. 1 000–1 200 µmol m^{-2} s^{-1}. Using the red He/Ne laser we did not see any significant photoinhibition by the excitation radiation during the performance of the Chl fluorescence induction kinetics.

Photochemical processes during Chl fluorescence induction kinetic: The major part (>90 %) of the Chl fluorescence of leaves at room temperature emanates from PS2 (see, *e.g*. Gitelson *et al.* 1998). Hence, the Chl fluorescence rise and decrease upon irradiation of darkened leaves reflect the processes taking place in the light-harvesting antenna LHC2 and in the reaction centre RC2. The initial or ground fluorescence F_0 apparently originates exclusively from the LHC2, whereas in the rise from F_0 to F_m the RC2 is involved as well (Govindjee 2004, Lichtenthaler and Babani 2004). In dark-adapted leaves the RC2 is "open", *i.e.* the primary acceptors Q_A and Q_B are in their oxidized form. With the onset of irradiation, the absorbed energy of photons is transferred *via* excitons from LHC2 to RC2 where charges are separated, Q_A and consecutively also Q_B are reduced, resulting in a "closed" reaction centre RC2. It can no longer separate charges and the absorbed energy is emitted to a higher proportion as Chl fluorescence, which, at this state, rises to the maximum level F_m. At the same time also the deactivation of absorbed photon energy by heat emission is increased. Then several additional processes simultaneously occur, the spill-over of excitation energy from PS2 to PS1, a pH gradient is built up over the thylakoid membranes, light-harvesting Chl proteins are phosphorylated, and PS1 is starting its activity and drains off electrons from Q_A, Q_B, and the plastoquinone-9 pool. As a consequence, RC2 becomes open again and continuously functions as a trap for further excitation energy. Hence, the Chl fluorescence declines from F_m to a very low steady state level F_s as a result of the full activation of photochemical quantum conversion including non-photochemical processes as well. In the light-adapted steady state the photosynthetic apparatus is in its functional "state 2" and PS2 works in the photosynthetic quantum conversion which is quantified by the photochemical quench q_P defined as $\Delta F/F_v'$ (as indicated below).

The Chl fluorescence induction kinetics (Fig. 1) can be measured with many different self-made or commercially available Chl fluorometers. Some of them cannot resolve the Chl fluorescence rise (into a differentiation of F_0 and F_v level), but all allow determining the maximum Chl fluorescence F_m and the steady state level F_s. Hence, it is possible to determine the Chl fluorescence decrease ratio R_{Fd}, as an indicator of CO_2 fixation, with most Chl fluorometers. This is valid also for the pulse amplitude modulated PAM fluorometer which is available in many laboratories nowadays. One has to keep in mind, however, that the Chl fluorescence yield strongly depends on the quality and quantity of the excitation radiation and on the wavelength range and sensitivity of the fluorescence sensor. Thus, the relative height of the individual Chl fluorescence parameters and ratios differ from instrument to instrument. Chl fluorescence emission spectra of leaves are characterized by a maximum in the red (near 690 nm) and far-red region (near 730–740 nm) as shown by Lichtenthaler and Rinderle (1988a) and Lichtenthaler and Babani (2004). Measurements with the laser-induced two-wavelengths Chl flurometer *LITWaF* showed that R_{Fd} values determined in the red Chl fluorescence band F_{690} exhibit higher values than those determined in the far-red fluorescence band F_{735} (Buschmann and Schrey 1981, Haitz und Lichtenthaler 1988, Lichtenthaler and Rinderle 1988a). This is due to the fact that the amplitude of the Chl fluorescence changes during the light-triggered induction kinetics is higher in the F_{690} than the F_{735} band. These lower R_{Fd} values at the 735 nm band as compared to the 690 nm band may be due to a relatively higher ground fluorescence of F_0 because the far-red PS1 Chl fluorescence contributes, at room temperature, also to this F_{735} band (and rather little to the F_{690} band) as has been shown by Pfündel (1998) and Franck *et al.* (2002).

Due to the selected wavelength region of the excitation radiation and the special filter systems applied, the PAM fluorometer measures the Chl fluorescence kinetics in the range of *ca*. 705–740 nm, *i.e*. in the range of the far-red Chl fluorescence emission band (Haitz and Lichtenthaler 1988). Moreover, blue and red radiation penetrates the leaf mesophyll to a different degree and depth. Thus, blue excitation radiation is not only absorbed by Chls but also by carotenoids (Cars), and therefore passes into the leaf mesophyll to a lower degree than the red excitation radiation being absorbed only by the Chls. For this reason the blue and red radiation induced Chl fluorescence emission spectra possess a different shape (Lichtenthaler and Rinderle 1988b). The reason is

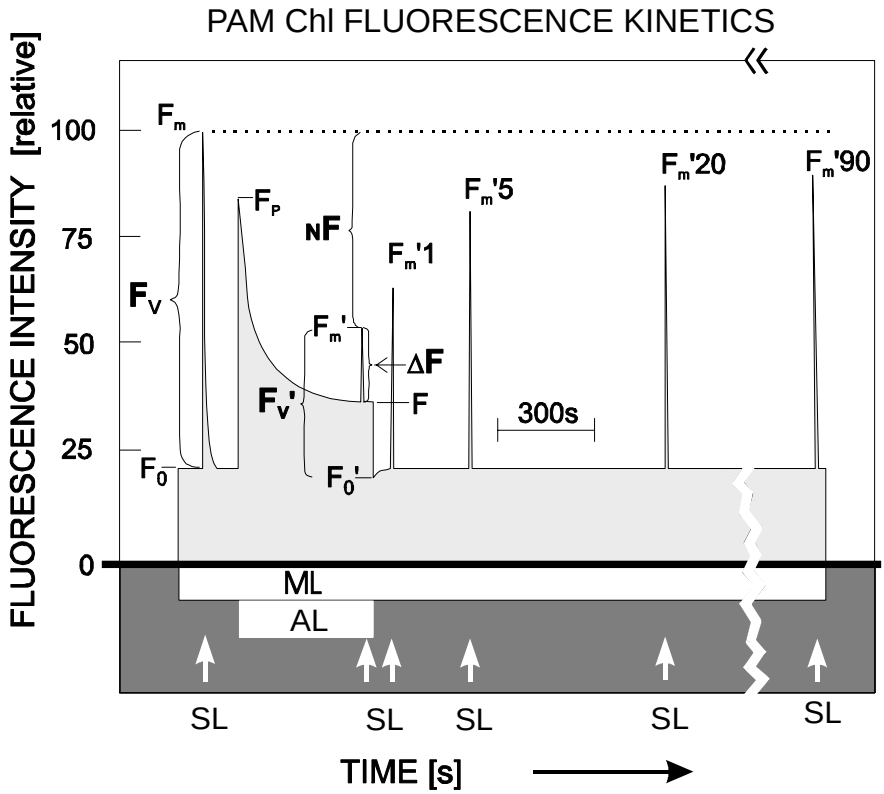
that the red radiation induced Chl fluorescence band F_{690}, also emitted in the deeper mesophyll parts of the lower leaf half, is reabsorbed by the absorption bands of the *in vivo* Chl *a* to a higher degree than the blue radiation induced Chl fluorescence which primarily emanates from the upper outer leaf half (Lichtenthaler and Rinderle 1988a, Buschmann and Lichtenthaler 1998). In addition to this, one has to take into consideration the excitation irradiance. If this irradiance is low, it reaches the green leaf mesophyll only at the upper outer mesophyll range next to the upper epidermis. Hence, the Chl fluorescence signals and information obtained by non-saturating excitation radiation is principally representative for a small chloroplast layer in the upper outer leaf half. Even at saturation irradiance of Chl fluorescence the latter still appears to emanate to a somewhat higher proportion from the upper as opposed to the lower leaf half.

Here we inform how to determine Chl fluorescence induction kinetics with the PAM fluorometer, an instrument with world-wide distribution, and show the different measurable Chl fluorescence parameters and ratios including the determination of the Chl fluorescence decrease ratio R_{Fd}. In addition, we present the differential changes in Chl fluorescence parameters and ratios at combined high irradiance (HI) and heat stress treatment. This is implemented separately for the upper and lower leaf sides, since both respond differently to the HI-stress applied *via* the upper leaf side. Moreover, we introduce a new parameter NF, *i.e*. the difference between F_m to F_m' in the irradiance-adapted state, that permits to quickly determine the non-photochemical quenching coefficients q_N, q_{CN}, and NPQ. This is needed to simplify and standardize the calculation of the Chl fluorescence ratios q_N, q_{CN}, and NPQ, for which quite different equations are found in literature. The different equations are rather confusing not only for beginners but also for experts.

Performance of the Chl fluorescence induction kinetics with the PAM fluorometer

A pulsed measuring low red light (ML) is used to determine F_0 of dark-adapted leaves. The irradiance must be low enough, not to induce a Kautsky-type fluorescence induction kinetic. A low frequency of 1.6 kHz is applied. Then a 1-s saturation flash (SL) of "white light" [*e.g.* 3 000 µmol(photon) m^{-2} s^{-1}] is applied and F_m is determined. When this fluorescence spike has decreased again to the F_0 level, the continuous red "actinic light" (AL) is started (Fig. 2). One has to keep in mind that the AL is and must be a non-saturating radiation which cannot reach all chloroplasts especially those in the lower half of the green leaf mesophyll. The AL that induces the Chl fluorescence kinetics, seen as a rise to the momentary maximum fluorescence level F_p and the subsequent slow decline (within 3–4 min) to the much lower level 'F' as shown in Fig. 2. F_p is always lower than F_m because AL is a non-saturating irradiance. During the AL induced fluorescence kinetic saturating flashes SL can be applied in order to obtain and keep track of the fluorescence parameters F_m' (the maximum fluorescence level in the light-adapted state 2). At the end of the induction period (after several minutes of AL), when a constant low F is reached (state 2), a last SL flash is given, then AL is turned off and a far-red radiation activating only PS1 is switched on during a few seconds. This yields the ground fluorescence level F_0' which in sun-exposed outdoor plants is often identical to F_0; however, in low-irradiance plants it is usually clearly lower than F_0. During the induction kinetic induced by AL the ML is automatically switched to a higher frequency of 100 kHz in order to achieve a better signal to noise ratio and time resolution. In ML, while measuring F_0, the frequency of 100 kHz is replaced by 1.6 kHz. A frequency of 100 kHz of ML would induce a slight Chl fluorescence induction kinetic, which has to be avoided when F_0 is measured. The characteristics of the different irradiation sources applied in the PAM fluorometer are summarized in Table 1 and the individual Chl fluorescence parameters obtained from such a Chl fluorescence induction kinetic are listed in Table 2 and Fig. 2.

By calculating the difference between these basic parameters, several other Chl fluorescence parameters can be determined, *e.g*. F_v, F_v', NF, and ΔF, which are used to calculate particular Chl fluorescence ratios applied as indicators of particular aspects of the photosynthetic apparatus (Lichtenthaler and Rinderle 1988b, Buschmann 1999, Roháček 2002). One has to consider that the absolute height of the different Chl fluorescence parameters depends on the Chl content of leaves, the angle of excitation, sensing the Chl fluorescence, and whether they are measured from straight, smooth leaf parts or such with a ruffled surface. Moreover, they are different in leaf veins and inter-costal fields and also when measured at the upper and lower leaf side. Thus, in the PAM fluorometer one should avoid to measure in the leaf vein region and always excite and detect the Chl fluorescence at the same leaf side. In addition, the comparison of the absolute values of Chl fluorescence parameters, *e.g*. between controls and stressed plants, can only be done from mean values when several leaves and different leaf parts of the same leaf have been measured. By determination of Chl fluorescence ratios, however, it is better to be on the safe side of the judgment, since the ratios are much less dependent on the factors mentioned above for the absolute Chl fluorescence values. The usually applied Chl fluorescence ratios are the maximum quantum yield of PS2 photochemistry (F_v/F_m), its more sensitive form F_v/F_0, the actual quantum yield of PS2 photochemical energy conversion in the light-adapted state of leaves ($\Delta F/F_m$'), as well as the photochemical quenching coefficient q_P and the three non-photochemical quenching coefficients q_N, q_{CN}, and NPQ; these are listed



ML = measuring light (weak red pulses)
AL = actinic light (continuous, non-saturating red light)
SL = saturating "white light" flashes (1 s, *ca.* 3 000 μmol m^{-2} s^{-1})

Parameters:	Quenching coefficients:
$\mathbf{F_v} = F_m - F_0$	$q_P = \Delta F / F_v'$
$\mathbf{F_{v'}} = F_m' - F_0'$	$q_N = NF / F_v$
$\mathbf{NF} = F_m - F_m'$	$q_{CN} = NF / F_m'$
$\mathbf{\Delta F} = F_m' - F$	$NPQ = NF / F_m$

Fig. 2. Scheme of the chlorophyll (Chl) fluorescence induction kinetics as measured in dark-adapted leaves with the PAM fluorometer. The ground Chl fluorescence F_0 is induced by the weak ‚measuring light' (ML), and the maximum Chl fluorescence F_m is obtained by a 'saturating white light' flash (SL) of 1 s. The Chl fluorescence induction kinetic is induced by continuous, non-saturating red ‚actinic light' (AL) yielding the level F_p that declines after 3–5 min to the steady state level F. When AL is turned off, the F_0' level is obtained after a fast re-oxidation of the reduced PQ-pool using far-red radiation. SL flashes during the AL-induced kinetic show the maximum level F_m' in the light-adapted state of the leaf. From these kinetics several Chl fluorescence parameters are calculated such as F_v, F_v', NF, and ΔF which are used to determine the photochemical (q_P) and non-photochemical quenching coefficients (q_N, q_{CN}, and NPQ). Moreover, the SL flashes given during the subsequent dark period (after AL has been turned off) at 1, 5, or 20 min (F_m'1, F_m'5, and F_m'20, respectively) indicate the fluorescence levels F_m' of the initially fast, then intermediate, and finally rather slow relaxing components of F_m' towards the original F_m (see paragraph on dark relaxation kinetics).

and defined in Table 3 and/or Fig. 2. It is an essential requirement and a standard condition of PAM measurements that the Chl fluorescence induction kinetic in the PAM fluorometer is induced by AL. If it were SL, one would not obtain the particular fluorescence parameter ΔF, and F_v', or F_m' and the latter would be identical with F (cf. Fig. 2). Then one could neither determine the essential Chl fluorescence ratio $\Delta F/F_m$' nor the photochemical quench $\Delta F/F_v$', which would result in zero.

The most frequently applied Chl fluorescence ratio is F_v/F_m (regular value 0.74–0.85) since it is easy and fast to determine. A decline in F_v/F_m indicates a decline in the quantum yield of PS2 photochemistry and a disturbance in or damage to the photosynthetic apparatus. If this decline in F_v/F_m is due to photoinhibition of PS2 units or to other causes cannot really be judged from the F_v/F_m

ratio as such. However, it has been done so by many young scientists without any evidence for such an assumption. Whether the cause is really a photoinhibition or not can only be determined *via* the photoinhibitory quench q_I, which is one of three constituents of the non-photochemical quenching coefficient q_N (see below). Moreover, F_v/F_m is a relatively inert ratio where stress-induced changes are detected rather late. A much more sensitive ratio is F_v/F_0, as has first been recommended by Lichtenthaler and Buschmann (1984), see also Babani and Lichtenthaler (1996). That ratio contains the same basic information but exhibits higher values and a higher dynamic range than F_v/F_m. The ratio F_v/F_0 shows higher amplitude at stress conditions, since all changes of F_v and/or F_0 are immediately reflected in it. Thus, in leaves with partial photoinhibition the values of F_v/F_m changed very little, whereas F_v/F_0 exhibited already a large significant decline (Lichtenthaler *et al.* 1992) as well as F_m/F_0 which is equivalent to $F_v/F_0 + 1$.

Another point to be cautious with conclusions from the Chl fluorescence ratio F_v/F_m is the fact that this ratio indicates the maximum quantum yield of PS2 photochemistry in the dark-adapted state, which is the non-functional 'state 1' of the photosynthetic apparatus.

Table 1. Characterization of the different irradiation sources applied in the PAM fluorometer to determine Chl fluorescence kinetics and the Chl decrease ratio R_{Fd}. Data are based on the written instructions given by the producer company *Walz* (Effeltrich, Germany), and Schreiber *et al*. (1986).

Pulsed 'measuring light' (ML): weak red pulses LED (wavelength of 650 nm)
(*a*) for F_0 measurement: 1.6 kHz
(b) during "actinic" or saturating light: 100 kHz
pulse duration: 1 µs
Irradiance: *ca*. 0.01 µmol(photon) m^{-2} s^{-1} (at 1.6 kHz)

'Actinic light' (AL): non-saturating continuous red radiation (LED of 650 or 655 nm) of a chosen medium irradiance
Duration: several minutes
Irradiance: 80–300 µmol(photon) m^{-2} s^{-1} (low to medium photon flux density)
AL induces some photosynthesis, but it must be weak enough not to saturate the photosynthetic process. It should not exceed 30–40 % of the irradiance for saturating net photosynthetic CO_2 assimilation activity, P_N. (Note: this low AL cannot be used for the determination of R_{Fd}-values.)

'Saturating light' (SL): strong "white light" given as a saturating flash (SL) or as continuous irradiation (cSL):
(*a***)** as saturating flash of 1 s at an irradiance of *ca*. 3 000 µmol(photon) m^{-2} s^{-1}. The flash needs to be strong enough to fully saturate photochemistry. All Q_A molecules in the reaction centres of photosystem 2 are reduced (the reaction centres are closed), the energy of ML is then exclusively transferred to Chl fluorescence and heat;
(*b***)** as continuous irradiation (cSL) of *ca*. 2 000 µmol(photon) m^{-2} s^{-1} for 4 to 5 min to fully saturate photosynthetic CO_2 fixation. This allows to determine the Chl fluorescence decrease ratio, $R_{Fd} = F_d/F_s$, which is a straight-forward indicator of the P_N of leaves.

PS1 irradiation: far-red radiation (LED with peak emission at 735 nm),
duration: 5 s [*ca*. 6 µmol(photon) m^{-2} s^{-1}]
This low far-red irradiation is only absorbed by photosystem (PS) 1 and takes away the electron from the acceptor side of the reaction centre of PS2. Thus, all Q_A molecules at the PS2 acceptor side become fully oxidized (the reaction centre of PS2 is open again). This far-red radiation is used to correctly determine F_0' after AL is removed.

Table 2. Chlorophyll (Chl) fluorescence parameters determined from the Chl fluorescence induction kinetics measured with the PAM fluorometer. For the nomenclature of Chl fluorescence parameters, see also van Kooten and Snel (1990).

F_0	ground Chl fluorescence yield induced by ML in the dark-adapted state
F_m	maximum Chl fluorescence (induced by a saturating pulse, SL) in the dark-adapted state
F_p	maximum Chl fluorescence induced by a non-saturating irradiation (*e.g.* AL)
F_m'	maximum Chl fluorescence in the light-adapted state of leaves (induced by an SL flash)
F	Chl fluorescence level induced by non-saturating irradiation (AL)
F_0'	ground fluorescence (minimum yield) after AL is turned off (usually measured after a far-red pulse)
F_v	variable fluorescence in the dark-adapted state ($F_m - F_0$)
F_v'	variable fluorescence in the light-adapted state ($F_m' - F_0'$)
$_N$F	decrease of the maximum fluorescence level in the light-adapted state ($F_m - F_m'$) or ($F_v - F_v'$) (related to q_N)
ΔF	fluorescence spike on top of the AL-induced fluorescence kinetic ($F_m' - F$) induced by an SL flash
F_m'1, F_m'5, F_m'20, and F_m'90	maximum fluorescence at the dark relaxation period, measured within one minute or after 5, 20, and 90 min after removal of AL

Table 3. Definition of particular chlorophyll (Chl) fluorescence ratios based on the signals measured during the induction kinetics (Kautsky effect) of leaves with the PAM fluorometer (compare Figs. 1 and 2). For some of the Chl fluorescence ratios in this table also other equations are found in the literature, all these are, however, fully equivalent to the ones presented here. Such additional equations are *e.g.* for $q_P = (F_m' - F)/F_v'$; for $q_N = (F_v - F_v')/F_v$ or $1 - (F_v'/F_v)$ or $(F_m - F_m')/F_{v;}$; for $q_{CN} = (F_m - F_m')/F_m$ or $1 - (F_m'/F_m)$; for $NPQ = (F_m - F_m')/F_m'$ or $(F_m/F_m') - 1$, for $\Delta F/F_m' = 1 - (F/F_m')$, and for $R_{Fd} = (F_m/F_s) - 1$. In order to simplify the calculations of the different non-photochemical quenching coefficients q_N, q_{CN}, and NPQ and to understand their relationship we have introduced the fluorescence parameter NF (= $F_m - F_m'$) which is related to non-photochemical fluorescence quenching and which is required to determine the three quenching coefficients q_N, q_{CN}, and NPQ.

Parameter and definition	Name	Reference
(*A*) Kinetics measured with non-saturating AL		
$q_P = \Delta F/F_v'$	photochemical quenching of variable Chl fluorescence	Bilger and Schreiber (1986)
$q_N = NF/F_v$	non-photochemical quenching of variable Chl fluorescence	Bilger and Schreiber (1986)
$q_{CN} = NF/F_m$	complete non-photochemical quenching of Chl fluorescence	Roháček (2002)
$NPQ = NF/F_m'$	Non-Photochemical Quench	Bilger and Björkman (1990)
F_v/F_m	maximum quantum yield of PS2 photochemistry (in the dark-adapted state)	Kitajima and Butler (1975)
F_v/F_0	maximum quantum yield of PS2 photochemistry (more sensitive than F_V/F_m)	Lichtenthaler and Buschmann (1984), Babani and Lichtenthaler (1996)
$\Delta F/F_m'$	actual or effective quantum yield of photochemical energy conversion in PS2 (in the light-adapted state), or actual quantum yield Φ_2 (sometimes also PS2 electron quantum yield)	Genty *et al.* 1989
(B) Kinetics measured with continuous 'saturating light', cSL		
$R_{Fd} = F_d/F_s$	Chl fluorescence decrease ratio (vitality index)	Lichtenthaler *et al*. (1983) Lichtenthaler and Rinderle (1988b) Lichtenthaler and Miehé (1997)

Examples of measurements: values of several Chl fluorescence parameters and ratios measured under different physiological conditions are found, *e.g.* in the papers of Demmig-Adams *et al.* (1989), Lichtenthaler *et al.* (1992), Schindler and Lichtenthaler (1994, 1996), Buschmann (1995, 1999), Ruban and Horton (1995), Babani and Lichtenthaler (1996), Lichtenthaler and Burkart (1999), Lichtenthaler and Rinderle (1988a), Roháček (2002).

If a leaf can use this quantum yield of PS2 (*i.e*. the potential photochemical activity of PS2) under continuous irradiation in the light-adapted state (functional 'state 2'), can not be deduced from the measurement of F_v/F_m with dark-adapted leaves. Therefore, measurements of Chl fluorescence levels, *e.g.* F_s in the light-adapted 'state 2', must be included as well. Thus, as an example we found that under various stress conditions F_v/F_m was not yet changed, whereas the R_{Fd}-values had already considerably decreased indicating a decline in photosynthetic quantum conversion. Therefore, measuring only the F_v/F_m may lead to the wrong conclusion that the photosynthetic apparatus was undisturbed which was not or is not yet the case. In contrast, the R_{Fd}-values and P_N measurements indicate already an early decline in photosynthetic activity. For this reason, it is highly recommended to determine with the PAM fluorometer not only the ratios F_v/F_m and F_v/F_0, but also R_{Fd} (under the conditions given below) because the latter is a straight-forward indicator of P_N of leaves (Lichtenthaler *et al*. 2005). Some authors argue that also the effective quantum yield of photochemical energy conversion in PS2, the ratio $\Delta F/F_m'$, determined with the PAM fluorometer would correlate with P_N of leaves. This may partially be the case in light-green leaves with low Chl content but we cannot confirm this for green and dark-green leaves. In fact, fully green maple leaves exposed to full sunlight for several hours exhibited extremely low values of $\Delta F/F_m'$, as explained below in detail, yet still possessed 70 % of their P_N (Schindler and Lichtenthaler 1996).

Dark relaxation measurements of quenching coefficients

The non-photochemical quench q_N is based on three major constituents: (*1*) the energy quench q_E (related to the built-up of a pH gradient), (*2*) the state transition quench q_T (related to 'state 1'→'state 2' transitions of the photosynthetic apparatus including phosphorylation of the mobile light-harvesting protein LHC2), and (*3*) the photoinhibitory quench q_I caused by a photoinhibition of PS2 units. The relative proportions of these three components of q_N can be determined *via* dark relaxation measurements after AL has been turned off. For this purpose SL is applied immediately after turning off AL (one SL-flash within the first min of darkness), and then every 2 min or only once at 5 and 20 min of the dark period as indicated in Fig. 2. The ML remains switched on

throughout this dark relaxation measurement of q_N. With the SL flashes applied, *e.g.* within 1 min and after 5 or 20 min of the dark period, F_m'1, F_m'5, and F_m'20 are obtained that can be used to calculate the NF values (= F_m – F_m') which are NF1, NF5, and NF20 or any NF values in between. In this respect it has to be taken into consideration that F_m' consists of the two components F_v' and F_0'. Whereas F_0' recovers very fast within a few minutes, the relaxation of F_m' takes a much longer time, especially when the proportion of the photoinhibitory quench q_I is high. The NF-parameter allows the calculation of q_N (NF/F_v) and its individual components including q_I (see Table 4). The non-photochemical quench q_N continuously relaxes in the dark which is visible *via* the rising values of F_m' with an increasing dark period up to the original F_m measured in the dark-adapted state. Its three components relax, however, with different speeds. q_E relaxes very fast within 2–5 min, followed by q_T with a slower speed between 12–20 min. The dark relaxation of the photoinhibitory quench q_I takes several hours or even days (depending on the degree of photoinhibition), since the damaged PS2 units have first to be repaired. To obtain the relative proportions of q_E, q_T, and q_I one determines q_N after every SL flash in the dark period. When plotting these q_N values, the fast relaxation component q_E becomes evident, followed by q_T relaxing at an intermediate rate. The slow relaxation that occurs after 20 min is regarded as the photoinhibitory quench q_I. In a first approximation one can determine the three components of q_N applying SL flashes after 4 or 5 min in the dark (q_E), and after 20 min (q_T). All non-relaxed parts of q_N after 20 min are regarded to correspond to q_I. For a more detailed analysis of the three components the first two relaxation rates are determined by plotting the various q_N forms after each SL flash. For further details see Quick and Stitt (1989), Walters and Horton (1991), and Lichtenthaler and Burkart (1999). We do not want to go into all details of the formulas to precisely calculate the different proportions of q_E, q_T, and q_I. Here our intention is to give a short practical indication of how to quickly estimate the approximate relative amounts and the contribution of the photoinhibitory quenching coefficient q_I which in many stress investigations is the desired essential fluorescence quenching information.

Table 4. Separation of the three major components of the non-photochemical quench q_N by measuring the F_m' level during the dark relaxation kinetics (Quick and Stitt 1989, Walters and Horton 1991, Lichtenthaler and Burkart 1999). The quench q_N consists of the fast (1–5 min) relaxing component q_E (energy quench), the intermediate (10–20 min) relaxing component q_T ('state 1'→'state 2' transitions), and the very slow relaxing photoinhibitory quench q_I (>20 min up to several hours or 1 or 2 d).

$$q_N = q_E + q_T + q_I$$

$$q_E = (NF1 - NF5) / F_V$$
$$q_T = (NF5 - NF20) / F_V$$
$$q_I = NF20 / F_V$$

NF1 = directly after or within 1 min after turning off the actinic light

NF5 = within 5 min after turning off the actinic light

NF20 = at 15 to 20 min after turning off the actinic light

For the actual proportions of the individual components of the total q_N, that, at a low overall q_N (*e.g.* at normal or low stress physiological conditions), amounts to 28 % for q_E, to 30 % for q_T, and to 42 % for q_I after a 20 min treatment of leaves at a medium to high photosynthetic photon flux density, PPFD (see Lichtenthaler and Burkart 1999). Only at a low q_N value below 0.3 the relative proportion of q_T can be that high. With increasing PPFD the total q_N increases considerably. The relative proportions of q_I and q_E then rise to a somewhat higher percentage, whereas the relative proportions of q_T decrease to a percentage of near 6 % of the total q_N. Only after the determination of the q_I level it is certain that a particular plant treatment, resulting in a decline in F_v/F_m, F_v/F_0, or $\Delta F/F_m$' has actually been caused by photoinhibition.

Most laboratory plants (indoor plants) that grow at or near optimum physiological conditions in the laboratory or greenhouse are very sensitive to SL flashes. Thus, SL flashes given during the Chl fluorescence induction kinetics can already induce a distinct photoinhibition, which is seen in a rather clear percentage of the photoinhibitory quench q_I. In contrast, outdoor plants, repeatedly exposed to direct sunlight, are less or not at all affected by these SL flashes given during the induction kinetics.

Measurements of R_{Fd} with the PAM fluorometer

R_{Fd} (= F_d/F_s) is determined from the Chl fluorescence induction kinetics recorded at continuous SL of *ca*. 2 000 µmol m^{-2} s^{-1} applied for about 4–5 min as indicated for the PAM fluorometer (Fig. 3). Applying the usual ML one first determines F_0 in the PAM fluorometer. Then continuous saturating "white light" (cSL) is applied yielding the fluorescence maximum F_m. By determination of F_0, before starting the saturation kinetics for the R_{Fd}-value determination, one can determine from the same Chl fluorescence induction kinetics not only the R_{Fd}-values but also the ratios F_v/F_m and F_v/F_0. The Chl fluorescence then declines from F_m to the steady state fluorescence F_s.

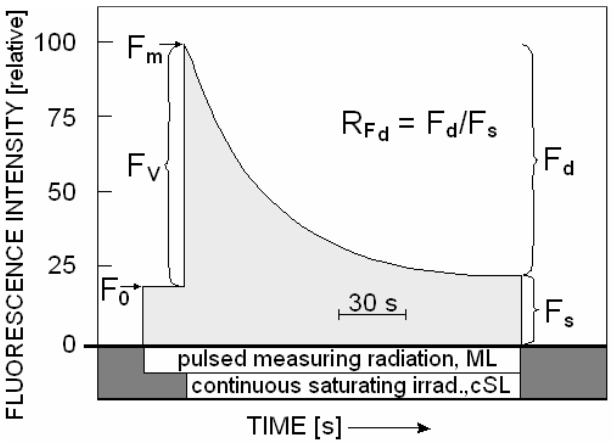


Fig. 3. Scheme for the determination of the Chl fluorescence decrease ratio R_{Fd} from the measurement of the Chl fluorescence induction kinetic at continuous saturation irradiance (cSL) using the PAM fluorometer. An irradiance of *ca*. 1 000 (shade plants and shade leaves) and 2 000 µmol(photon) m^{-2} s^{-1} (sun plants and sun leaves) is applied as cSL.

The latter is reached also in HI-adapted plants and sun leaves within 4–5 min. The steady state Chl fluorescence can somewhat further decline or change upon further irradiation, but these are then slow adaptive responses of the photosynthetic apparatus not related to the induction kinetics. The higher the fluorescence decrease F_d from F_m to F_s, the better the photosynthetic quantum conversion of the leaf. The R_{Fd}-values, when determined at SL in the conventional Chl fluorometers using orange-red excitation radiation (He/Ne laser, 632.8 nm), are representative of all or at least of most of the chloroplasts of the palisade and the spongy parenchyma cells of the irradiated leaf area (of the upper and lower leaf half), since this radiation penetrates deep into the green leaf mesophyll (Lichtenthaler and Rinderle 1988b, Babani and Lichtenthaler 1996). For this reason, the R_{Fd}-values correlate with the P_N of leaves (Lichtenthaler and Babani 2004, Lichtenthaler *et al.* 2005). Although the measuring principles in the PAM fluorometer are different from those of conventional Chl fluorometers, one can determine R_{Fd}-values with the PAM instrument at cSL of 1 500–2 000 µmol(photon) m^{-2} s^{-1}, and we found that these R_{Fd}-values also correlate to P_N of green leaves. The R_{Fd}-values measured with conventional fluorometers and the PAM fluorometer were practically the same (Haitz and Lichtenthaler 1988). In contrast to the non-saturating AL, the cSL applied saturates the photosynthetic quantum conversion of nearly all leaf chloroplasts. Using the cSL, the ML then yields a different Chl fluorescence kinetic with a somewhat lower F value than that obtained with the continuous AL. This lower F level has been termed F_s by Lichtenthaler and Rinderle (1988b) (see also Lichtenthaler and Miehé 1997), since it is the steady state level of the Chl fluorescence induction kinetic determined at or near to SL, and the use of F_s should be restricted to the R_{Fd}-measurements using cSL. The use of 'F_s' for the F level measured in the PAM fluorometer applying the non-saturating AL should be avoided, because the AL-induced Chl fluorescence induction kinetics cannot be used for the determination of the R_{Fd}-values as by applying AL the R_{Fd}-values were much too low, neither representative of the leaf chloroplasts nor do they correlate to the P_N of leaves.

Are Chl fluorescence signals indicators of the photosynthetic behaviour of leaves?

In contrast to the R_{Fd}-values, F_v/F_m and F_v/F_0 determined in the dark-adapted state or the ratio $\Delta F/F_m'$ using AL for the induction kinetics, usually do not correlate with the P_N of leaves. In fact, under stress the values for R_{Fd} and $\Delta F/F_m'$ develop independently of each other. At many stress constraints there occurs a strong decline in $\Delta F/F_m'$ whereas R_{Fd} is little or much less affected. The reason is that $\Delta F/F_m'$, determined with the non-saturating AL, reflects the photochemical quantum conversion function of a rather small layer of chloroplasts at the upper outer leaf half, whereas the R_{Fd}, determined at cSL, represents the Chl fluorescence information of the major portion of chloroplasts of the upper and lower leaf halves. This difference between those two Chl fluorescence ratios is emphasized by the fact that leaves from sun exposed maple trees, where the ratios $\Delta F/F_m'$ (decline from 0.64 to 0.06), F_v'/F_m' (decline from 0.76 to 0.17), and the photochemical coefficient q_P (decline from 0.84 to 0.08) had almost fully declined, still exhibited *ca*. 70 % of their maximum P_N (Schindler and Lichtenthaler 1996) as summarized in Table 5. Also R_{Fd} dropped, similar to P_N, only to 73 % of the control value indicating that R_{Fd} and P_N developed in the same way.

The high increase in q_N and NPQ of these maple leaves was, in fact, associated (and apparently caused) with a strong increase in the light-induced transformation

Table 5. Changes in chlorophyll fluorescence ratios, photosynthetic rates, and percentage of xanthophyll cycle carotenoids of fully developed green maple leaves (*Acer platanoides* L.) from the morning at 08:00 until noon (12:00) on an extremely clear sunny early summer day with a PPFD of >2 000 µmol(photon) m^{-2} s^{-1}. The Chl fluorescence induction kinetics were measured with a PAM fluorometer; excitation and sensing of Chl fluorescence was performed on the upper adaxial leaf side. The photosynthetic CO_2-fixation rates, P_N [µmol(CO_2) m^{-2} s^{-1}], were determined with a CO_2/H_2O porometer (*Walz*, Effeltrich, Germany). The R_{Fd}-values (measured near 690 nm) were determined with the Karlsruhe laser-induced two-wavelength fluorometer *LITWaF* using a He-Ne laser [632.8 nm, 650 µmol(photon) m^{-2} s^{-1}] as excitation source. Mean of 4 determinations, maximum deviation ±3 %. (Data based on Schindler and Lichtenthaler 1996.)

	08:00	12.00
F_v/F_m	0.772	0.631
F_v/F_0	3.33	1.71
F_m/F_0	4.33	2.71
$\Delta F/F_m'$	0.64	0.03
F_v'/F_m'	0.76	0.30
q_P ($\Delta F/F_v'$)	0.84	0.10
q_N (NF/F_v)	0.05	0.75
q_{CN} (NF/F_m)	0.035	0.470
NPQ (NF/F_m')	0.036	0.880
R_{Fd690}	3.35	2.46
P_N	11.2	7.9
% violaxanthin	83	6
% antheraxanthin	7	4
% zeaxanthin	10	90

of violaxanthin into its de-epoxidized derivative zeaxanthin which can only be determined for the whole leaf blade section (Table 5) indicating that the xanthophyll cycle functioned efficiently. The Chl *a+b* and total Car content of these maple leaves were 490 mg(*a+b*) m^{-2} and 109 mg(*x+c*) m^{-2} leaf area, respectively. Chl *a/b* was 2.88 and the ratio *(a+b)/(x+c)* amounted to 4.49. The three xanthophyll cycle Cars made up *ca*. 24 % of the total leaf Cars.

These results demonstrate that the Chl fluorescence signatures and ratios determined at the adaxial leaf side are not representative of all leaf chloroplasts, but only of the behaviour (and photoinactivation) of the chloroplasts of the palisade parenchyma cells that are close to the sun-exposed upper leaf epidermis. The strategy of plant leaves to survive *e.g*. an excessive HI apparently is to form a screen of photoinactivated or photoinhibited chloroplasts below the adaxial epidermis cells as a shield to protect the chloroplasts that are deeper inside the leaf mesophyll. Some scientists call this a down-regulation of PS2 activity. These inner chloroplasts, protected against excessive HI by photoinhibited chloroplasts, then can perform their normal photosynthesis process.

From the results with maple leaves, reviewed here, one can conclude that, if Chl fluorescence signatures had been measured at the non-treated lower leaf side of these leaves, these might have shown different and fairly normal values of the Chl fluorescence parameters and ratios. This would then explain that, despite the almost full decline of the photosynthetic quantum conversion as judged from measurements of the upper leaf side, 70 % of the maximum P_N were actually measured for these HI-stressed maple leaves. To test this assumption that the Chl fluoresecence signatures measured of the upper and lower leaf side are different, especially under stress, we comparatively determined these in leaves of control plants and those treated with a HI-stress.

Examples of measurement

Chl fluorescence induction kinetics were measured with a pulse amplitude modulated fluorometer, the PAM fluorometer *101/102/103* (*Walz*, Effeltrich, Germany). Dark-green leaves of C_3 plants, the Indian fig tree (*Ficus elastica* Roxb.) and the parasol plant (*Schefflera arboricola* Hayata), were investigated at the adaxial and abaxial leaf sides. These plants were grown and kept in the greenhouse. On bright sunny days, when the greenhouse was not shaded, the leaves could show a partial photoinhibition of the chloroplasts of the upper leaf side during midday and early afternoon as monitored by PAM fluorometer measurements. Chl (*a+b*) and Cars (*x+c*) were extracted with 100 % acetone and determined spectrophotometrically using the re-determined extinction coefficients and equations as given in Lichtenthaler (1987) and Lichtenthaler and Buschmann (2001).

Differences in Chl fluorescence signatures of the upper and lower leaf side: The measurable Chl fluorescence signals (excitation and sensing) at the upper adaxial leaf side were different from those measured at the abaxial lower leaf side. The standard Chl fluorescence parameters such as F_0, F_v, F_m, F_m', ΔF, and NF, *etc*. showed *ca*. 10–22 % higher values (relative fluorescence units) when measured at the lower as compared to the upper leaf side (see Table 6). Thus F_0 [relative units] increased from 9 to 10 (*Schefflera*) and from 8.5 to 10.0 (*Ficus*), and F_m from 39 to 42 and from 42 to 51, respectively, when excited and sensed at the lower leaf side. This is because most plants have a bifacial leaf structure with densely packed long green palisade parenchyma cells (with high chloroplast numbers) in the adaxial leaf half and a much lower number of green cells separated by large aerial interspaces in the spongy parenchyma cells of the abaxial lower leaf half. Hence, the Chl fluorescence excited and emitted in the leaf cells at the adaxial leaf side is reabsorbed at the adaxial leaf side during its passage through the leaf to the upper leaf surface, where it is sensed, to a higher degree than the Chl fluorescence excited and sensed at the lower leaf side which contains

much less total Chl *a*+*b*. Due to the lower Chl content and re-absorption rate at the lower leaf half, the Chl fluorescence signals are higher when measured at the abaxial leaf side. The absorption bands of the *in vivo* Chl *a* forms overlap with the Chl fluorescence emission bands, whereby the re-absorption mainly affects the red Chl fluorescence band between 670–710 nm and then decreases towards the longer far-red wavelengths (>720 nm) (Lichtenthaler and Rinderle 1988b, Gitelson *et al.* 1998, 1999). Due to the special filter systems applied in the PAM fluorometer, the Chl fluorescence is measured starting from *ca*. 705 nm to longer wavelengths and thus is affected by the re-absorption process. Hence, the R_{Fd}-values determined with the PAM fluorometer are somewhat lower than the R_{Fd690}-values determined with a two-wavelength fluorometer in the red 690 nm fluorescence band. In fact, they correspond to the R_{Fd735}-values measured with the two-wavelength fluorometer in the far-red F_{735} band (Haitz and Lichtenthaler 1988).

The calculated Chl fluorescence ratios: From the Chl fluorescence signals various Chl fluorescence ratios can be calculated, *e.g.* F_v/F_m, F_v/F_0, R_{Fd}, $\Delta F/F_m'$, q_P, and q_N. These are for leaves of non-stressed plants somewhat higher when measured at the upper as compared to the lower leaf side (as seen for *Schefflera* in Table 6). These differences in Chl fluorescence ratios always exist on sunny and cloudy days in leaves of outdoor and greenhouse plants. On bright days with full sunshine, a midday decline in F_v/F_m, F_v/F_0, R_{Fd}, and $\Delta F/F_m'$ caused by partial photoinhibition may occur. In such cases these Chl fluorescence ratios measured at the adaxial leaf side can be somewhat lower than those determined at the abaxial leaf side, as is shown for *Ficus* leaves (Table 6).

Influence of HI-stress in *Ficus* leaves on the Chl fluorescence signals: We submitted the leaves of *F. elastica* to a 1-h exposure of 2 000 µmol(photon) m^{-2} s^{-1} PAR applied on the adaxial leaf side. Despite of the fact that the applied lamp was a "cold light mirror lamp", this exposure not only meant HI-stress but simultaneously some heat stress. The total radiation input was 850 W m^{-2} (measured in the range of 400–800 nm) and 1 200 W m^{-2} when measured with a thermopile optical detector between 380–40 µm. Due to the filter system of the lamp, UV radiation (<395 nm) was excluded.

Change of Chl fluorescence signals at the upper leaf side: After a 1-h HI exposure time at room temperature, F_0, F_v, F_m, ΔF, and NF considerably declined to very low values when measured at the HI-exposed upper leaf side (Table 7, adaxial leaf side). A very strong decline also showed up for F_v/F_m, F_v/F_0, R_{Fd}, and $\Delta F/F_m'$. The photochemical quenching coefficient q_P ($\Delta F/F_v'$), an indicator of open PS2 reaction centres, declined much less, demonstrating that it is a less indicative parameter for stress events or a decline in photosynthetic activity than the other Chl fluorescence ratios. In contrast, q_N (ratio NF/F_v) had increased to a higher value, as anticipated, whereas the non-photochemical quenching coefficients q_{CN} (ratio NF/F_m') and NPQ (ratio NF/F_m) unexpectedly had declined as compared to the initial control value. This is due to the differential behaviour of the Chl fluorescence parameters F_v or F_v' and F_0 or F_0'. F_m consists of $F_v + F_0$ and F_m' of $F_v' + F_0'$. When NF is divided by F_v at a stress induced decline of F_v, the quenching coefficient q_N ($=NF/F_v$) is increased. However, when NF is divided by F_m or F_m' as in q_{CN} and NPQ, respectively, the change in F_0 and F_0' plays also an important role in the stress-induced development of q_{CN} and NPQ. At mild stress, F_0 and F_0' do not change, even though F_v may decline. Under these conditions the values of q_N, q_{CN}, and NPQ increase. At a high stress load, however, F_v and F_v' decline and the relative proportions of F_0 and F_0' increase, since they decline at a much lower rate than F_v and F_v'. Then the coefficients q_{CN} and NPQ decline whereas q_N remains high. This has been demonstrated below (cf. Table 9). During the 1-h HI-stress treatment of some of the *Ficus* leaves, the Chl fluorescence signals and ratios in the non-treated control *Ficus* leaves [kept at 300 µmol(photon) m^{-2} s^{-1}] had not changed, neither at the adaxial nor at the abaxial leaf side.

Table 6. Differences in the chlorophyll (Chl) fluorescence parameters and ratios of dark-green bifacial leaves of the parasol plant (*Schefflera arboricola* Hayata) and the Indian fig tree (*Ficus elastica* Roxb.) when excited and measured at either the adaxial upper leaf side or the abaxial lower leaf side. The measurements were performed with a PAM fluorometer. Standard deviation <4 % (Chl fluorescence signals) and <3 % (ratios); mean values of six determinations for each plant and leaf side.

	Schefflera		*Ficus*	
	upper	lower	upper	lower
F_0	9.0	10.0	8.5	10.0
F_v	39	42	33.5	41
F_m	48	52	42	51
F_m'	44.5	49.0	37.0	44.5
ΔF ($F_m' - F$)	32.5	32.0	26.0	28.5
NF ($F_m - F_m'$)	3.5	3.0	5.0	6.5
F_v/F_m	0.813	0.808	0.798	0.804
F_v/F_0	4.33	4.20	3.94	4.20
R_{Fd}	2.10	1.48	1.47	1.55
$\Delta F/F_m'$	0.730	0.653	0.703	0.640
q_P ($\Delta F/F_v'$)	0.916	0.821	0.912	0.826
q_N (NF/F_v)	0.090	0.071	0.149	0.159
q_{CN} (NF/F_m)	0.073	0.058	0.119	0.127
NPQ (NF/F_m')	0.079	0.061	0.135	0.146

Chl fluorescence signals at the lower leaf side: The 1-h HI-stress applied to *Ficus* leaves upon the upper epidermis resulted only in rather small changes in most of the Chl fluorescence parameters and ratios when these were excited and sensed *via* the lower untreated leaf

Table 7. Differential changes of chlorophyll (Chl) fluorescence parameters [relative] and ratios in the upper (adaxial) and the lower (abaxial) leaf side of dark-green leaves of the Indian fig tree (*Ficus elastica* Roxb.) during a 1-h high irradiance exposure (HI-stress) of 2 000 µmol(photon) m^{-2} s^{-1}. Means of six determinations per condition. Standard deviation <4 % (Chl fluorescence parameters) and <3 % (Chl fluorescence ratios). The measurements were performed with a PAM fluorometer.

	Adaxial leaf side			Abaxial leaf side		
	control	HI-stress	[% change]	control	HI-stress	[% change]
F_0	8.5	4.5	–47	10.0	10.5	+5
F_v	33.5	1.3	–96	41.0	37.0	–10
F_m	42.0	5.8	–86	51.0	47.5	–7
F_m'	37.0	5.5	–85	44.5	34.0	–25
NF ($F_m - F_m$')	5.0	0.3	–94	6.5	13.5	+108
ΔF (F_m' – F)	26.0	1.0	–96	28.5	20.0	–30
F_v' (F_m' – F_0')	28.5	1.5	–95	34.5	24.0	–30
F_0' (F_m' – F_v')	8.5	4.0	–53	10.0	10.0	±0
F_v/F_m	0.798	0.217	–73	0.804	0.779	–3
F_v/F_0	3.94	0.28	–93	4.20	3.52	–16
R_{Fd}	1.47	0.05	–97	1.55	1.47	–5
$\Delta F/F_m$'	0.703	0.182	–74	0.640	0.588	–8
q_P ($\Delta F/F_v$')	0.912	0.667	–27	0.826	0.823	±0
q_N (NF/F_v)	0.149	0.231	+55	0.159	0.365	+130
q_{CN} (NF/F_m)	0.119	0.052	–56	0.127	0.284	+124
NPQ (NF/F_m')	0.135	0.055	–59	0.146	0.397	+172

epidermis. The parameter F_0 slightly increased from 10.0 to 10.5 in relative units (and always found upon repetition) whereas F_v, F_m, and F_m' only decreased by 7–25 % (Table 7, abaxial leaf side). The two parameters ΔF (the Chl fluorescence spike on top of the induction kinetic) and F_v' decreased somewhat stronger (–30 %), whereas the parameter NF (which is indicative of non-photochemical quenching) exhibited a high increase (+108 %).

F_v/F_m, F_v/F_0, R_{Fd}, and $\Delta F/F_m$' showed a small decline of 3–16 %, whereby the ratio F_v/F_m (–3 %) was much less an indicator of changes than the ratio F_v/F_0 (–16 %) (Table 7, abaxial leaf side). There occurred no change in q_P, whereas both q_N and NPQ exhibited a more than 2-fold increase (+130 and +124 %, respectively). This again demonstrates that q_P as a measure of the fraction of open PS2 reaction centres changes little under HI-stress and much less than the other Chl fluorescence ratios. In contrast, the parameter NF and the non-photochemical quenching ratios q_N and NPQ are, under the milder stress effects at the lower leaf half of the HI-exposed *Ficus* leaf, excellent indicators of the increasing non-photochemical quenching processes.

The results with the 1-h HI-stress exposure of the dark-green *Ficus* leaf demonstrated that the HI-exposure damaged and inactivated the photosynthetic apparatus of the chloroplast of the upper leaf half close to the upper leaf surface to a very high degree, whereas the chloroplasts in the middle to lower leaf half (as sensed *via* the lower leaf epidermis) were rather little affected by the HI-stress. The most prominent aspect was the high increase in NF, q_N, q_{CN}, and NPQ, which helps to protect the chloroplasts of the middle to lower half of the leaf blade against a photoinactivation or photoinhibition.

Despite of the strong inactivation of the chloroplasts of the upper outer layer of the adaxial leaf half, we could not detect a destruction of Chls or Cars during the 1-h HI-stress treatment. The contents of Chls *a*+*b* and Cars *x*+*c*, which can only be determined for the whole leaf blade section, as well as the pigment ratios Chl *a*/*b* and (*a*+*b*)/(*x*+*c*) remained the same, lying before and after the treatment within the general range of green plants given by the standard deviation, *i.e.* <5 % for pigment values and <3 % for the pigment ratios (see Table 8, *Ficus*). Although a very slight breakdown of Chls *a*+*b* during the HI-stress can not fully be excluded, it must have been minor, if it occurred at all. The inactivation of the chloroplasts of the upper palisade parenchyma cells (next to the upper epidermis) by the HI-stress, as demonstrated here, was predominantly caused by photoinhibition. This can be concluded from dark relaxation kinetics (cf. Lichtenthaler and Burkart 1999), since the recovery of the extremely low F_m' after the HI-stress to the high previous F_m signal proceeded very slowly and was not yet complete after 24 h.

Effect of HI-stress in *Schefflera* leaves: Dark-green leaves of the parasol plant *Schefflera* had similar Chl, Car, and water contents as the *Ficus* leaves (Table 8). An essential difference was that the *Schefflera* leaves were less thick (60 % of the fairly thick *Ficus* leaves). As a response to the HI-stress we found similar differences in the Chl fluorescence parameters and ratios at the adaxial and abaxial leaf sides (data not presented here) in *Schefflera* and *Ficus* leaves. The decline of the Chl fluorescence parameters and ratios was stronger at the upper compared to the lower leaf half. The percent decline of the

signals and ratios at the adaxial leaf side was somewhat higher than that found at the abaxial side of *Ficus* leaves (see Table 8).

Change of q_N and NPQ at progressing HI-stress: The unexpected low quenching coefficients q_{CN} and NPQ under the 1-h HI-stress of 2 000 µmol(photon) m^{-2} s^{-1}, when measured at the adaxial leaf side, whereas q_N increased to a fairly high value, prompted us to check the development of all three quenching coefficients at low, medium, and high HI-stress at both leaf sides. Dark-green leaves of *Schefflera* were exposed to 1 200, 1 500, and 2 000 µmol(photon) m^{-2} s^{-1} for 1 h applied to the adaxial upper leaf side.

At 1 200 µmol(photon) m^{-2} s^{-1}, q_N, q_{CN}, and NPQ, measured at the abaxial leaf sides, increased relatively little (Table 9). When 1 500 µmol(photon) m^{-2} s^{-1} was applied, all three coefficients were strongly enhanced on both leaf sides, but more when measured on the lower compared to the upper leaf side. At the strong HI-stress of 2 000 µmol(photon) m^{-2} s^{-1}, however, the q_N value remained on both leaf sides as high as with 1 500 µmol(photon) m^{-2} s^{-1}. In contrast, the coefficients q_{CN} and NPQ (where NF is divided by F_m and F_m' including F_0 and F_0', respectively) considerably declined, especially when measured at the upper leaf side (Table 9). These results indicate that q_{CN} and NPQ are not suitable parameters to measure and describe the non-photochemical quenching of absorbed photon energy at stronger stress conditions. In contrast, q_N also indicates at the strong HI-stress [2 000 µmol(photon) m^{-2} s^{-1}] that at the upper adaxial leaf half a strong non-photochemical quenching still proceeds.

All three non-photochemical quenching coefficients are mathematically related to each other. However, they do not show the same development with increasing HI-stress. The values of q_{CN} and NPQ decrease at higher stress exposure, whereas q_N remains high also under a stronger stress load. For these reasons one should always determine q_N which is a more reliable indicator than the coefficients q_{CN} and NPQ.

Table 8. Content of photosynthetic pigments, chlorophylls (Chl) *a*+*b* and total carotenoids (Car) *x*+*c*, as well as pigment ratios, Chl *a*/*b* and (*a*+*b*)/(*x*+*c*), in dark-green leaves of the Indian fig tree (*Ficus elastica* Roxb.) and the parasol plant (*Schefflera arboricola* Hayata). The pigment contents are expressed on a leaf area basis [mg m^{-2}] and a dry mass basis [g kg^{-1}(DM)]. The percent water content and specific leaf area [cm^2 kg^{-1}(DM)] are also indicated. Means of 5 determinations; standard deviation <5 % (leaf thickness, pigment contents), <3 % (pigment ratios), <2 % (specific leaf area), and <1 % (water content).

	Ficus	*Schefflera*
Leaf thickness [mm]	0.78	0.35
Specific leaf area	102	213
Water content [%]	81.5	78.3
Chl *a*+*b* [mg m^{-2}]	808	913
Car *x*+*c* [mg m^{-2}]	156	173
Chl *a*+*b* [g kg^{-1}(DM)]	8.3	19.6
Car *x*+*c* [g kg^{-1}(DM)]	1.6	3.7
Chl *a*/*b*	2.71	2.65
(*a*+*b*)/(*x*+*c*)	5.21	5.32

Table 9. Changes in the chlorophyll (Chl) fluorescence quenching coefficients q_N, q_{CN}, and NPQ in the upper (abaxial) and lower (adaxial) leaf side of dark-green *Schefflera* leaves after a one hour high irradiance (HI) stress of 1 200, 1 500, and 2 000 µmol(photon) m^{-2} s^{-1}. Means of six determinations per condition. The measurements were performed with a PAM fluorometer. Standard deviation <3 %.

		q_N	q_{CN}	NPQ
Adaxial side	**control**	**0.090**	**0.073**	**0.079**
	HI-stress			
	1 200	0.107	0.081	0.088
	1 500	0.409	0.300	0.428
	2 000	**0.429**	**0.158**	**0.185**
Abaxial side	**control**	**0.071**	**0.058**	**0.061**
	HI-stress			
	1 200	0.108	0.085	0.093
	1 500	0.643	0.474	0.900
	2 000	**0.636**	**0.424**	**0.737**

Conclusion: The results of this application of HI-stress to *Ficus* and *Schefflera* demonstrate that the differential response of the chloroplasts of the palisade parenchyma cells near the upper leaf epidermis are mainly affected by HI-stress administered *via* the adaxial leaf side, *i.e.* the upper epidermis. The strong decrease of all Chl fluorescence parameters and ratios in *Ficus* and *Schefflera* shows a high rate of photoinhibition and damage when measured at the upper leaf side. In contrast, the chloroplasts of the middle to lower leaf half are much less affected and still guarantee a certain photosynthetic quantum conversion and photosynthesis rate, even after HI-stress as can be seen from the R_{Fd}-values which decline less. The chloroplasts of the lower leaf half are mainly protected against excess photon energy and photodamage by high rates of non-photochemical quenching as seen in the several fold increase in NF and the related ratios q_N, q_{CN}, and NPQ. This again applies to the leaves of both plants. That the chloroplasts of the middle to lower leaf side of *Schefflera* were more affected by the HI-stress than those of the *Ficus* leaves is due to the fact that *Schefflera* leaves are considerably thinner than *Ficus* leaves. The thinner *Schefflera* leaves heated up faster and were more heat-affected under the given HI + heat in comparison to the thicker *Ficus* leaves.

The most essential finding was that the adaxial and abaxial leaf sides not only deliver different Chl fluorescence signals (individual parameters and ratios) but also greatly differ in their sensitivity to HI-stress. As a

consequence, one can not judge the functionality of the photosynthetic apparatus of a leaf by Chl fluorescence measurements solely performed at the adaxial leaf side; one has to measure the Chl fluorescence signals of the lower leaf side as well. Only then can one judge the effective photosynthetic behaviour of leaves and their chloroplasts in leaves of control and stressed plants. In other words, a presumable full photoinhibition of the photosynthetic apparatus by an exposure to excess HI, as one would conclude from Chl fluorescence measurements at the adaxial leaf side, does not mean at all that a photosynthetic quantum conversion does no longer proceed in this leaf. Thus, Chl fluorescence measurements at the abaxial leaf side provide information if the photosynthetic apparatus of the middle to lower leaf chloroplasts is still functional or not. The observation of Schindler and Lichtenthaler (1994) that the presumably fully photoinactivated maple leaves, as concluded from Chl fluorescence measurements performed at the upper leaf side, still possessed 70 % of their maximum P_N is then understandable, when the chloroplasts of the middle to lower leaf half were still functional in photosynthetic quantum conversion.

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