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Minimization of the photon energy absorbed by 'closed' reaction centers of photosystem 2 as a photoprotective strategy in higher plants

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Abstract

Photoinactivation of photosystem 2 (PS2) results from absorption of so-called "excessive" photon energy. Chlorophyll *a* fluorescence can be applied to quantitatively estimate the portion of excessive photons by means of the parameter $E = (F - F_0')/F_m'$, which reflects the share of the absorbed photon energy that reaches the reaction centers (RCs) of PS2 complexes with Q_A in the reduced state ('closed' RCs). Data obtained for cotton (*Gossypium hirsutum*), bean *(Phaseolus vulgaris*), and arabidopsis (*Arabidopsis thaliana*) suggest a linear relationship between the total amount of the photon energy absorbed in excess (excessive irradiation) and the decline in PS2 activity, though the slope may differ depending on the species. This relationship was sensitive not only to the leaf temperature but also to treatment with methyl viologen. Such observations imply that the intensity of the oxidative stress as well as the plant's ability to detoxify active oxygen species may interact to determine the damaging potential of the excessive photons absorbed by PS2 antennae. Energy partitioning in PS2 complexes was adjusted during adaptation to irradiation and in response to a decrease in leaf temperature to minimize the excitation energy that is trapped by 'closed' PS2 RCs. The same amount of the excessive photons absorbed by PS2 antennae led to a greater decrease in PS2 activity at warmer temperatures, however, the delay in the development of non-photochemical and photochemical energy quenching under lower temperature resulted in faster accumulation of excessive photons during induction. Irradiance response curves of EF suggest that, at high irradiance (above 700 μmol m^{-2} s^{-1}), steady-state levels of this parameter tend to be similar regardless of the leaf temperature.

Additional key words: *Arabidopsis*; chlorophyll *a* fluorescence; excessive photons; *Gossypium*; lincomycin; methyl viologen; non-photochemical quenching; *Phaseolus*; photoinhibition.

Introduction

The ability of plants to minimize photosystem 2 (PS2) photoinhibition depends on the efficiency of photon energy utilization in metabolic reactions and safe dissipation of this energy as heat. Demmig-Adams *et al.* (1996) employed chlorophyll (Chl) fluorescence parameter E [$E = (1 - q_p) F_v'/F_m' = (F - F_o')/F_m'$] to estimate the portion of photons absorbed by PS2 antennae that was excessive, *i.e.* the portion that was not used for electron transport nor dissipated in non-photochemical processes. Weis and Lechtenberg (1989) discussed the possible significance of this parameter even earlier. It was suggested that E also estimated the share of excitation energy that reaches 'closed' reaction centers (RCs) of PS2 complexes. In our previous reports, we introduced the derived parameter, time-dependent averaged E, that estimates the total amount of excessive photons absorbed during irradiation (Kornyeyev *et al.* 2001, 2002). This term was later replaced by 'excessive light exposure' (Kornyeyev *et al.*

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Abbreviations: Chl, chlorophyll; E, excess; EF, excessive photon flux; F, actual level of fluorescence; F_m and F_v, maximal and variable chlorophyll fluorescence yield for dark-acclimated samples, respectively; F_0', F_m', and F_v', minimal, maximal, and variable chlorophyll fluorescence yield during irradiation, respectively; J_e, rate of electron transport; NPD, rate of non-photochemical dissipation; NPQ, non-photochemical chlorophyll fluorescence quenching coefficient; PPFD, photosynthetic photon flux density; PS, photosystem; Q_A, primary quinone acceptor of PS2; q_p, photochemical quenching coefficient; RC, reaction center; Φ_2, quantum yield of electron transport through PS2; Φ_N, effective quantum yield of non-photochemical processes in PS2.
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2003). A linear relationship between the total amount of excessive photons and the extent of PS2 photoinactivation was observed for cotton leaves pretreated with lincomycin, an inhibitor of PS2 repair. Moreover, the correlation between the magnitude of excess energy (the rate of excess energy absorption) and the rate constant of PS2 photodamage was demonstrated by Kato *et al.* (2003) for leaves of *Chenopodium album*. Taken together, these results imply that a connection exists between the photon energy that reaches 'closed' RCs of PS2 and the level of PS2 inactivation.

Despite these findings, little is known about the regulatory changes in E during short-term acclimation to different environmental conditions, such as occurs with a change in temperature. The aim of the present work was to determine the levels of electron transport, non-photochemical dissipation, and the parameter E at different irradiances and temperatures in order to examine the hypothesis that the regulation of energy partitioning in PS2 complexes in response to changing environment results in a minimization of the photon energy absorbed by PS2 complexes with Q_A in a reduced state. We also investigated the possible influence of oxidative stress on the relationship between the excessive irradiation and PS2 damage.

Materials and methods

Plants: Cotton (*Gossypium hirsutum* L. cv. Coker 312) and bean (*Phaseolus vulgaris* L. cv. Topnotch Golden Wax) plants were grown in 8 000 cm^3 pots in a greenhouse at ~30/26 °C (day/night) with a natural photoperiod. *Arabidopsis thaliana* L. (cv. Columbia) plants were grown in smaller (500 cm^3) pots under the same conditions. Plants were fertilized with Hoagland's solution twice a week. The youngest fully expanded leaves of 5- to 8-week-old plants were used for all analyses. Fluorescence measurements were performed on the upper fully expanded leaves.

Experimental treatments: For most analyses, leaves were pre-treated with lincomycin to inhibit chloroplast protein synthesis and PS2 repair processes. For this treatment, the leaves were harvested before sunrise by cutting their petioles under water. They were then immediately transferred to microfuge tubes containing 1 g m^{-3} lincomycin (863 units mg^{-1}) and kept in the dark for 3–4 h at room temperature. At the end of this dark incubation period, the concentration of lincomycin in the bulk leaf tissue (C_I) was 0.9 to 2.3 mM as estimated from the formula: $C_I = C_S(W_S/W_L)$, where C_S is the inhibitor concentration in the solution, W_S is the mass of the solution taken up by a leaf, and W_L is the fresh mass of the leaf (Bilger and Björkman 1994). The same procedure was used to treat *Arabidopsis* leaves with methyl viologen. The concentration of methyl viologen in the leaf tissue was 7.9±0.1 μM.

To study the changes in Chl *a* fluorescence characteristics in response to different photosynthetic photon flux densities (PPFD), previously dark-acclimated attached leaves of greenhouse-grown plants were exposed to increasing PPFD using different neutral density filters under natural irradiation. The leaves were allowed 20 min of acclimation to each PPFD before fluorescence measurements were commenced, and measurements continued until a steady state was reached before changing the PPFD. For the other fluorescence analyses, leaf discs (10 cm^2) or whole detached leaves (in the case of *Arabidopsis* plants) acclimated to darkness for at least 1.5 h were exposed to the specified temperature for 20 min prior to irradiation in the chamber of an oxygen electrode (*Hansatech*, King's Lynn, Norfolk, UK). CO_2 was supplied by flow of humidified ambient air.

Chl fluorescence emission from leaf discs in the laboratory was measured with a pulse amplitude-modulated fluorometer (*PAM 101/103*, *H. Walz*, Effeltrich, Germany) through a port in the oxygen electrode chamber at various times during the treatment. The Chl *a* fluorescence measurements of attached leaves were conducted using the *PAM 101/103* through the window in a temperature-controlled *PLC4* leaf chamber of an *LCA-4* portable photosynthesis system (*ADC*, Hoddesdon, U.K.) at ambient CO_2 concentration under natural irradiation. Prior to the fluorescence measurements, the attached leaves were acclimated to the specified temperature and PPFD. The experimental protocol described by Schreiber *et al.* (1986) and the nomenclature of van Kooten and Snel (1990) were used. Measurements of F_0 and F_0' were performed after a 5-s low-far-red irradiation. Saturating pulses of 2-s duration were provided by a *KL 1500* light source (*Schott*, Wiesbaden, Germany).

The decline in dark-adapted F_v/F_m value was calculated to assess PS2 photoinactivation since a close correlation exists between this parameter and the amount of functional PS2 RCs (Park *et al*. 1995, Lee *et al*. 2001).

NPQ and q_N, the parameters assessing the level of non-photochemical quenching of Chl fluorescence, were calculated as $F_m/F_m' - 1$ and $1 - F_v'/F_v$, respectively. The effective quantum yield of non-photochemical processes (Φ_N) was calculated as $\Phi_N = 1 - F_v'/F_m'$ where F_v'/F_m' is the ratio of variable to maximal fluorescence for light-acclimated leaves (Roháček 2002). The quantum yield of electron transport through PS2 was determined as $\Phi_2 = (F_m' - F)/F_m'$ (Genty *et al.* 1989). The rates of electron transport (J_e) and non-photochemical dissipation (NPD) in PS2 complexes were calculated as:

$$J_e = \Phi_2 \text{ PPFD Abs } I_{PS2} \quad (1)$$

$$NPD = \Phi_N \, PPFD \, Abs \, I_{PS2} \quad (2)$$

where I_{PS2} is the fraction of absorbed photons directed to PS2. Assuming an equal distribution of photon energy between PS1 and PS2 (Krall and Edwards 1992), I_{PS2} value of 0.5 was used for our calculations. Even if some deviations from an equal distribution of the photon energy between PS1 and PS2 occurred it would not affect the relationship between J_e and NPD. Abs is an absorbance coefficient determined by means of an integrating sphere ($Abs = 1 - C_R - C_T$, where C_R and C_T are reflectance and transmittance coefficients, see Pickering *et al*. 1996 for details). In some cases (Figs. 2 and 3), a value of 0.75 was used as Abs for cotton leaves (Björkman and Demmig 1987).

The following parameters were calculated to estimate the portion of total absorbed photon energy that was excessive (E), the excessive photon energy flux (EF), and the total excessive irradiation:

$$E = F_v'/F_m'(1 - q_P) = (F - F_o')/F_m' \quad (3)$$

$$EF = E \, PPFD \, Abs \, I_{PS2} \quad (4)$$

$$\text{Excessive irradiation} = \sum_{i=2}^{n} t\left(EF_i + EF_{i-1}\right)/2 \quad (5)$$

where t is the time between EF measurements; EF_i and EF_{i-1} are the levels of EF measured at the current and previous time-points, respectively; n = total number of time-points. F_v/F_m was used as the level of E at t = 0, because at the beginning of the chilling treatment (i = 1), q_P was assumed to be 0. The average level of EF, calculated as $(EF_i + EF_{i-1})/2$ for each time period, was multiplied by the duration of this period and the results obtained for all periods were summed to obtain the estimation of total of excessive photons absorbed during the irradiation.

Instantaneous EF reflects the rate of the delivery of the photon energy to the RCs of PS2 complexes with Q_A in reduced state. Kato *et al.* (2003) used the term "rate of excess energy production" for this parameter. Eq. (5) is analogous to equations used for the calculation of the rates of electron transport and non-photochemical processes in PS2 complexes (Eqs. 1 and 2). Excessive irradiation estimates the total amount of photons captured by 'closed' but potentially active PS2 complexes with Q_A in the reduced state.

Results

To validate the use of $\Phi_N (1 - F_v'/F_m')$ as a parameter for comparing non-photochemical energy dissipation and photochemistry, we examined the relationship between Φ_N and other parameters traditionally applied to estimate non-photochemical energy quenching (Fig. 1). A non-linear relationship was observed between Φ_N and q_N for cotton, bean, and *Arabidopsis* leaves. Assuming that the relationship can be described with the equation $q_N = A \ln(\Phi_N) + B$, the following r^2 values were obtained: 0.926 for cotton [$q_N = 0.7 \ln(\Phi_N) + 1.13$], 0.976 for bean [$qN = 0.53 \ln(\Phi_N) + 1.12$], and 0.949 for *Arabidopsis* [$q_N = 0.52 \ln(\Phi_N) + 1.08$]. A correlation between F_v'/F_m' [$\Phi_N = 1 - F_v'/F_m'$] and q_N was previously demonstrated by Linger and Brüggemann (1999) for tomato leaves. A strict linear relationship between Φ_N and NPQ ($F_m/F_m' - 1$) was detected for leaves of all species used in the present experiment ($r^2 = 0.918$ for cotton, $r^2 = 0.944$ for bean, and $r^2 = 0.881$ for *Arabidopsis*). This relationship did not depend on the leaf temperature. Thus, Φ_N can be used instead of NPQ, especially in situations when it is impossible or impractical to determine the level of Chl fluorescence for dark-acclimated leaves (F_m) before a photoinhibitory treatment. Indeed, the calculation of $\Phi_N = 1 - F_v'/F_m'$ does not require knowledge of the dark-acclimated F_m, thereby significantly simplifying Chl fluorescence analysis in the field. Since thermal dissipation is not the only process involved in non-photochemical energy dissipation in PS2 complexes, we used an integrating term 'effective quantum yield of non- photochemical processes' to define Φ_N (Roháček 2002).

The PPFD responses of the estimated rates of electron transport (J_e) and non-photochemical energy dissipation (NPD) in PS2 complexes of attached cotton leaves (Fig. 2) were similar to those described in earlier studies (*e.g.* Demmig-Adams *et al.* 1996, Gorbunov *et al.* 2001). As PPFD increased, electron transport was saturated and non-photochemical energy quenching became the major mechanism employed to minimize E, especially at 10 °C. The saturated electron transport was considerably lower at 10 °C in comparison to that at 25 °C, while the non-photochemical energy dissipation was higher at the lower temperature. Interestingly, the combined influence of these two processes resulted in only a slightly greater portion of E during exposure to 10 °C in comparison to 25 °C at PPFDs below 700 μmol $m^{-2} s^{-1}$ (Fig. 3). The magnitude of E increased with incident PPFD increase and then became saturated at about 0.3 for both 10 and 25 °C (Fig. 3). The relationship between incident PPFD and EF was essentially linear and did not differ substantially between the two temperatures, especially between 500 and 1 500 μmol $m^{-2} s^{-1}$.

For bean plants, the magnitude of EF was determined for leaf discs at two different PPFDs during a dark-to-light transition (Fig. 4A). Steady-state levels of EF obtained for the moderately irradiated leaf discs (PPFD = 500 μmol $m^{-2} s^{-1}$) were higher at lower temperatures. When the irradiance was elevated up to 1 000 μmol $m^{-2} s^{-1}$, steady state levels of EF determined at 10 and 25 °C were similar (Fig. 4*B*). Therefore, in bean and cotton leaves the temperature-dependent differences in EF are not evident at high irradiance. A comparison of

the time-courses for EF obtained at different temperatures (Fig. 4) implies that the initial decrease in EF is substantially delayed by chilling temperature, leading to faster accumulation of excessive energy (the excessive irradiation is the area under each curve). At PPFD of 500 μmol m^{-2} s^{-1}, a steady-state EF was reached in approximately 2 h at 10 °C, while it took only 20 min at 25 °C. The greatest differences in EF between temperatures were observed at the beginning of the acclimation to PPFD. Similar delay in EF decline caused by low temperature was observed for *Arabidopsis* leaves (data not shown) and was previously reported for cotton leaves (Kornyeyev *et al.* 2003).

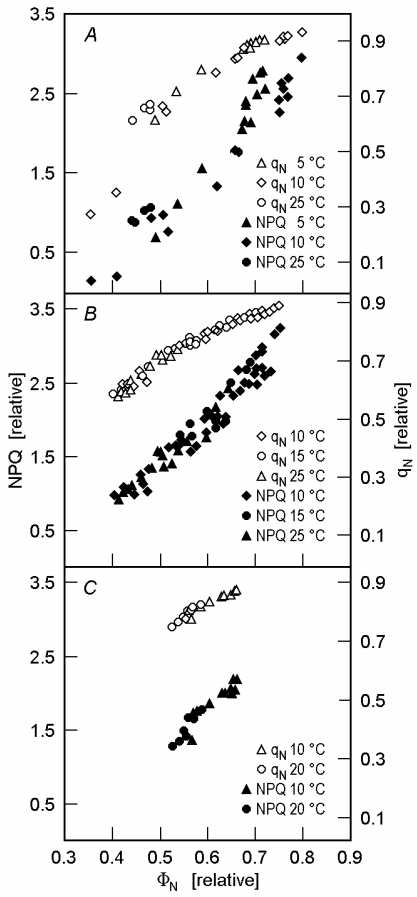

Fig. 1. The relationship between Φ_N and other parameters used for assessing the intensity of non-photochemical energy dissipation in photosystem 2 complexes (NPQ and q_N) of (*A*) cotton, (*B*) bean, and (*C*) *Arabidopsis*. The measurements were conducted during irradiance of 500 μmol m^{-2} s^{-1} and different temperatures as indicated on the graph.

We also investigated the changes in energy partitioning in PS2 complexes occurring in bean leaves as a result of a change in temperature during irradiation. An abrupt decrease in the temperature of PPFD-acclimated leaves led to a sharp decline in the quantum efficiency of electron transport along with a more gradual increase in non-photochemical energy quenching (Fig. 5). Low temperature caused a momentary rise in the magnitude of E followed by its slow decrease down to the level close to that observed at the warmer temperature. Similar to the steady-state data obtained on cotton at 10 and 25 °C (Fig. 3), the steady-state level of E did not change substantially with the shift to the lower temperature. Increased non-photochemical energy quenching almost fully compensated for the diminishing of the quantum efficiency of electron transport.

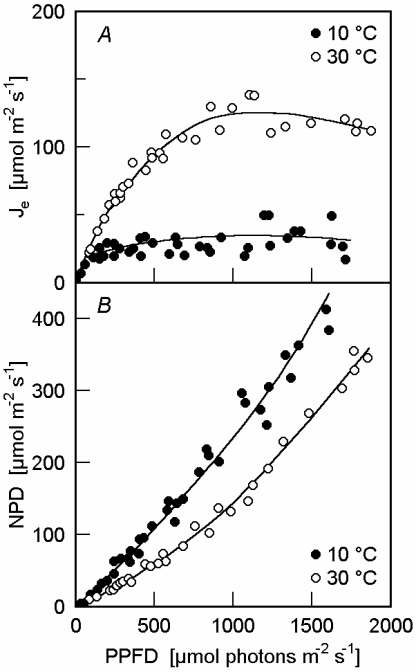

Fig. 2. Photosynthetic photon flux density (PPFD) dependence of estimated rates of electron transport (J_e) (*A*) and non-photochemical dissipation (NPD) (*B*) in photosystem 2 complexes at leaf temperatures of 10 and 25 °C. Chlorophyll *a* fluorescence was measured on attached cotton leaves (not treated with lincomycin) through the window of a temperature-controlled *PLC4* leaf chamber of an *LCA-4* portable photosynthesis system at ambient CO_2 concentration (360–380 μmol mol^{-1}) under natural irradiation. Neutral density filters were used to vary the PPFD.

During the treatment described in previous paragraph, smaller leaf discs were periodically removed and placed into a Petri dish with wetted filter paper for dark adaptation and further determination of the levels of PS2 inactivation using the F_v/F_m parameter. Dark adaptation was used to allow the relaxation of the regulatory decrease in the PS2 activity (down-regulation), which usually occurs within 1.5 h of darkness at room temperature (Vavilin *et al.* 1995). In our preliminary experiments no significant changes in F_v/F_m were observed between the 3rd and 6th hours of dark adaptation, implying that the 3-h dark period we used was sufficient to eliminate the effects of the down-regulation of PS2 activity.

We analyzed the functional activity of PS2 and did not examine D1 protein dynamics, because the decline in

D1 protein content and PS2 inactivation are not believed to be the same processes (Ottander *et al.* 1993, Salonen *et al.* 1998). The loss of D1 protein lags behind the loss of PS2 activity during photoinhibition at low temperature (Aro *et al.* 1990, Schnettger *et al.* 1994) and is not in all studies correlated with PS2 photoinactivation (Aro *et al.* 1994). In addition, also other PS2 proteins experience photodamage (Anderson and Barber 1996, Henmi *et al.* 2004).

Smaller leaf discs collected during the abrupt temperature transition (Fig. 5) were used to analyze the relationship between the excessive irradiation and PS2 activity (F_v/F_m) for bean leaves pre-treated with lincomycin. These data were compared with results obtained for cotton (Kornyeyev *et al.* 2003). In bean, the relationship was linear at both 10 and 20 °C, with different slopes at each temperature (Fig. 6*A*). As was previously shown for cotton, the same excessive irradiation resulted in a smaller decline in PS2 activity at cooler temperatures.

Fig. 6*B* represents experiments in which the leaves of cotton, bean, and *Arabidopsis* were subjected to the same photoinhibitory treatment (PPFD = 500 µmol m^{-2} s^{-1}, 20 °C). The relationship between PS2 activity and excessive irradiation was linear for all plants, but with different slopes for each species (Fig. 6*B*). The calculated slopes were [mol^{-1} m^2]: –70.6±3.7 % for cotton, –125.8±7.7 % for bean, and –91.5±7.3 % for *Arabidopsis* (means±S.D., *n* = 3). Since the production of reactive oxygen species is associated with PS2 damage, we conducted an experiment in which oxidative stress was induced artificially using methyl viologen (MV). *Arabidopsis* leaves were

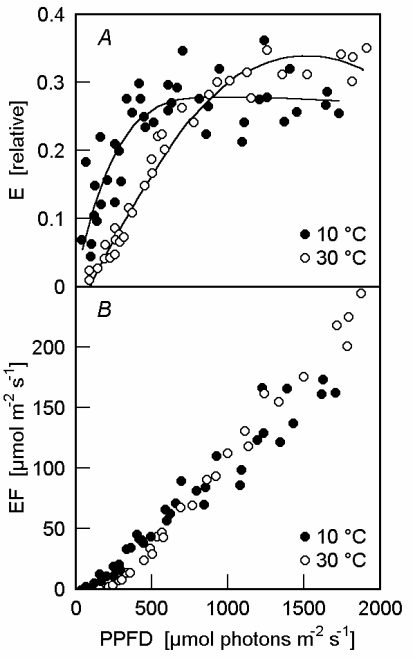

Fig. 3. Photosynthetic photon flux density (PPFD) dependence of E, the portion of photon energy trapped by photosystem 2 complexes with 'closed' reaction centers [$E = (F_m' - F)/F_m'$] (*A*), and EF, "excessive" photon flux (*B*), at leaf temperatures of 10 and 25 °C. For chlorophyll *a* fluorescence measurements see Fig. 2.

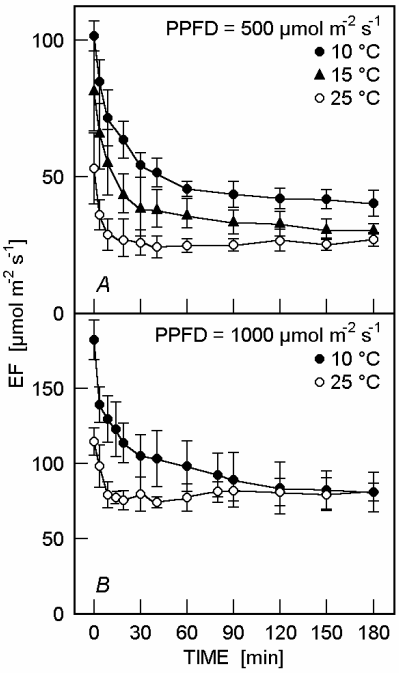

Fig. 4. The changes in EF, "excessive" photon flux, during dark-to-light transitions at different leaf temperatures for bean leaf discs in a *Hansatech* oxygen electrode chamber. Leaf discs were not treated with lincomycin. PPFD was 500 (*A*) or 1 000 (*B*) µmol m^{-2} s^{-1}. *Error bars* represent ± standard deviation (*n* = 3–4).

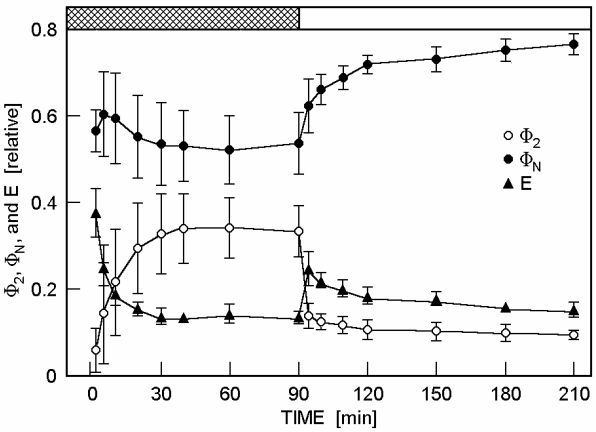

Fig. 5. Time-course of the changes in Φ_2 (quantum efficiency of electron transport), Φ_N (quantum yield of non-photochemical processes), and E [$E = (F_m' - F)/F_m'$] upon irradiation of dark-acclimated bean leaf discs at a PPFD = 500 µmol m^{-2} s^{-1} in a *Hansatech* oxygen electrode chamber. A temperature of 20 °C was maintained for 90 min and then decreased to 10 °C within 5 min. The leaf discs were obtained from leaves that were treated with lincomycin. *Error bars* represent ± standard deviation. When not visible, they are smaller than the symbol (*n* = 6).

pre-treated with both lincomycin and MV. Then they were irradiated. At the same excessive irradiation, the samples treated with MV exhibited a considerably greater PS2 inactivation in comparison to untreated samples (Fig. 6*C*).

Discussion

There is a necessity to develop a numerical equivalent for the term "excessive irradiation" to be able to estimate quantitatively the level of the "excess" energy absorbed and the damage potential of excessive irradiation. Absorbed photon energy that does not enter photochemistry (electron transport) can be considered as "excessive photon energy" that may eventually lead to destruction of photosynthetic apparatus. However, much of the absorbed energy that does not enter photochemistry is dissipated in non-photochemical processes and does not possess damage potential. Therefore, it would be more logical to express "excessive irradiation" as the portion of the absorbed photon energy that cannot be used in electron transport nor dissipated non-photochemically. Chl fluorescence analysis offers the possibility to estimate this portion of the photon energy absorbed by PS2 antennae (E) (Demmig-Adams *et al.* 1996). This approach reveals how the photosynthetic apparatus of different plant species is capable of regulating energy partitioning in PS2 complexes to minimize E (the portion of the energy absorbed that is excessive) and, thereby, minimize damage to these complexes.

The relationship of E $[(1 - q_P)\, F_v'/F_m']$ with other parameters used in Chl fluorescence analysis of PS2 photoinactivation: The close correlation between the total amount of the excessive photon energy to which a plant is exposed and PS2 photoinactivation obtained for several species of higher plants (Fig. 6) supports the suggestion that the calculation of E can be used to estimate the potential for damage to PS2. In previous studies, the susceptibility of PS2 to photoinactivation has been related to both the redox status of Q_A, the primary quinone acceptor of PS2 (Öquist *et al.* 1992, Osmond *et al.* 1993, Ottander *et al.* 1993, Melis 1999), and the level of thermal energy dissipation (Demmig-Adams and Adams 1992). The derivation of E combines the reduction state of Q_A $(1 - q_P)$ and the efficiency of non-photochemical energy dissipation (the primary factor leading to a decline in F_v'/F_m' during acclimation to PPFD) [$E = (1 - q_P)\, F_v'/F_m'$, which simplifies to Eq. 3]. Parameter $1 - q_P$ is also known as "excitation pressure" (Maxwell *et al.* 1995).

The parameters estimating photochemical and non-photochemical quenching of Chl *a* fluorescence have been applied to predict the level of PS2 photoinactivation (Ögren 1991, Osmond 1994, Park *et al.* 1996). The susceptibility of PS2 to PPFD stress has been estimated by means of the ratio $(1 - q_P)$/NPQ, where NPQ is non-photochemical Chl *a* fluorescence quenching $(F_m/F_m' - 1)$ (Osmond 1994, Park *et al.* 1995, 1996). This parameter and E are closely related, since NPQ and F_v'/F_m' are

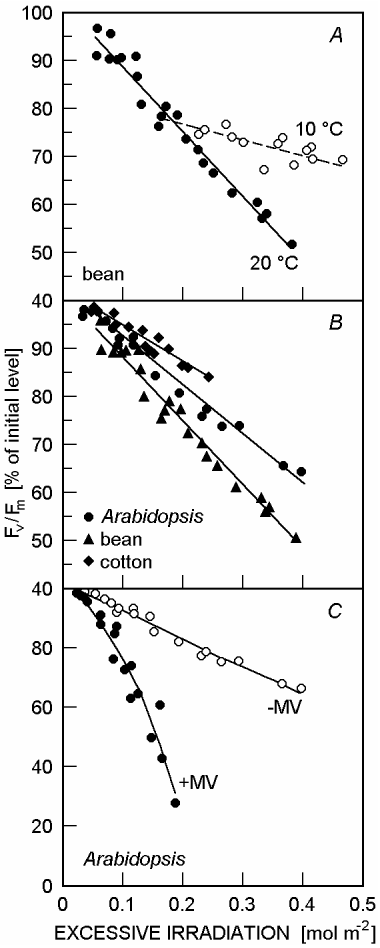

Fig. 6. Values of F_v/F_m [%] of initial, dark-acclimated values measured before the photoinhibitory treatment *versus* excessive irradiation. Leaves were pre-treated with lincomycin to inhibit chloroplast repair processes. The initial F_v/F_m values detected for lincomycin-treated leaves were as follows (mean±S.D.): 0.787±0.010 for bean, 0.773±0.003 for cotton, and 0.774±0.006 for *Arabidopsis*. *A*: Leaf discs were irradiated at a PPFD = 500 μmol m^{-2} s^{-1} and 20 °C in a *Hansatech* oxygen electrode chamber for 90 min, then they were subjected to either 10 °C (*open symbols*) for an additional 120 min or maintained at 20 °C (*closed symbols*). Smaller leaf discs (1 cm^2) were removed during irradiation in order to determine F_v/F_m after 3 h of incubation in darkness at 25 °C. *B*: Detached leaves (*Arabidopsis*) or leaf discs (cotton and bean) were exposed to 20 °C for different time periods before samples were removed for F_v/F_m determinations. The portion of excessive irradiation was determined periodically during irradiation and calculated as described in Materials and methods. *C*: The procedure was similar as described above. *Arabidopsis* leaves were treated with both lincomycin and methyl viologen, MV (*closed symbols*) or only with lincomycin (*open symbols*). Irradiance of 500 μmol m^{-2} s^{-1} and 20 °C. Mean F_v/F_m for *Arabidopsis* leaves treated with both lincomycin and MV was 0.770±0.012.

inversely correlated and primarily responsive to changes in the level of non-photochemical dissipation in PS2. The calculation of E does not require the knowledge of dark-acclimated, maximal fluorescence (F_m) and, therefore, may be applied to situations when the determination of NPQ is difficult, as in the field.

In addition, E can be directly compared to the quantum yields for electron transport (Φ_2) and non-photochemical processes (Φ_N) in PS2 complexes. The portion of the absorbed photon energy that is utilized in photochemistry can be estimated as F_v'/F_m' (Harbinson *et al.* 1989). Therefore, $\Phi_N (1 - F_v'/F_m')$ should reflect the relative amount of absorbed energy consumed (dissipated) in non-photochemical processes. The ratio F_v'/F_m' is commonly used as an indicator of non-photochemical energy quenching. For this purpose, it can be replaced by $1 - F_v'/F_m'$, which makes possible the direct comparison of the quantum efficiency of electron transport and non-photochemical dissipation in PS2 complexes. The foundations for such characterization of the distribution of absorbed energy between different de-excitation pathways were described in Weis and Lechtenberg (1989). According to a recent review by Roháček (2002), $\Phi_N = 1 - \Phi_P$ and $\Phi_2 = q_P \Phi_P$, where Φ_N is the effective quantum yield of non-photochemical processes in PS2 (including thermal dissipation and Chl fluorescence), Φ_P = effective quantum yield of PS2 photochemistry (F_v'/F_m'), Φ_2 = effective quantum yield of photochemical energy conversion in PS2 (the quantum yield of non-cyclic electron transport in PS2 complexes, Genty *et al*. 1989), and q_P = photochemical quenching of variable Chl fluorescence. The first equation can be rewritten using the second:

$$\Phi_N + \Phi_P = 1 \quad (6)$$

$$\Phi_N + q_P \Phi_P + (1 - q_P) \Phi_P = \Phi_N + \Phi_2 + (1 - q_P) \Phi_P = 1 \quad (7)$$

One can notice that $\Phi_P = q_P \Phi_P + (1 - q_P) \Phi_P = \Phi_2 + (1 - q_P) \Phi_P$. In this way, the third component of energy partitioning in PS2 complexes, namely $(1 - q_P) \Phi_P$, can be realized. $(1 - q_P) \Phi_P$ estimates the portion of photon energy not used for electron transport (Φ_2) nor dissipated in non-photochemical processes (Φ_N), so $(1 - q_P) \Phi_P = 1 - \Phi_N - \Phi_2$. Since $\Phi_P = F_v'/F_m'$ (Roháčcek 2002), $(1 - q_P) \Phi_P = (1 - q_P) (F_v'/F_m') = E$. Potentially, the symbol E may be replaced with Φ_E for the reason of analogy to Φ_N and Φ_2. The quantitative characteristics (quantum yields) of the processes mentioned above are determined by means of *in vivo* Chl fluorescence analysis and are related to the pool of the PS2 complexes in the sample not to a single PS2 complex.

According to Genty *et al.* (1989), the parameter $q_P (F_v'/F_m') (\Phi_2)$ reflects the portion of photon energy trapped by 'open' RCs of PS2 complexes with Q_A in the oxidized state. Following this logic, one may say that $(1 - q_P) (F_v'/F_m')$ is the portion of the photon energy that reaches 'closed' RCs. Absorption of the photon energy by PS2 complexes with 'closed' RCs can lead to double reduction of Q_A, the formation of triplet P680, and oxygen reduction (Melis 1999, Oxborough and Baker 2000, Noguchi 2002), the reactions detrimental to PS2 complexes. $(1 - q_P) (F_v'/F_m')$ may reflect the portion of photon energy that was consumed in such 'side reactions'. However, Weis and Lechtenberg (1989) suggest that $1 - \Phi_N - \Phi_2$ (equals E) is the proportion of energy distributed to 'closed' RCs and dissipated as 'radiative' de-excitation. More theoretical studies are needed to improve our understanding of the meaning of this parameter. Nevertheless, E can be successfully applied as an empirical parameter to estimate the damaging potential of the absorbed photon energy under various stresses (Roberts *et al.* 1998, Martin *et al.* 1999, Fleck *et al.* 2000, Olivera and Penuelas 2001, Lima *et al.* 2002, Manter 2002, Kato *et al.* 2003, Shirke and Pathre 2003, Tsonev and Hikosaka 2003) including comparison of the sensitivity of different genotypes to photoinhibitory treatment (Niinemets and Kull 2001).

Excessive irradiation and PS2 damage: A linear relationship between total amount of the excessive irradiation and the decline in PS2 activity, initially observed in cotton (Kornyeyev *et al.* 2001, 2002), was also observed in bean and *Arabidopsis* (Fig. 6*B*). However, the slopes for this relationship differ among species, implying that these species have different sensitivities to the excessive absorbed photons. At the present time, it is unclear what factors control this sensitivity. One may assume that the structure of PS2 complexes (including the content of xanthophylls), the possible phosphorylation of PS2 proteins, and the efficiency of scavenging of the reactive oxygen species in the vicinity of PS2 complexes can affect the damaging potential of excessive photon energy. The data obtained for *Arabidopsis* leaves treated with MV, which sensitizes the production of reactive oxygen species in chloroplasts, support the last of these assumptions (Fig. 6*C*).

Despite less sensitivity of PS2 complexes to excessive irradiation at low temperatures than at warm temperatures, EF decreases slowly during PPFD acclimation at low temperature and, for a long time, remains well above that at warmer temperatures (Fig. 4). This leads towards much greater excessive irradiation. It is unclear why the same amount of excessive photons causes less reduction in PS2 activity at low temperature (Fig. 5*A*; for data obtained from cotton see Kornyeyev *et al*. 2003). The higher tolerance of PS2 complexes to photoinhibitory treatment as temperature falls has been demonstrated previously by various researchers (Aro *et al*. 1990, Schnettger *et al*. 1994, Kruse *et al*. 1997) and could be associated with phosphorylation of PS2 core proteins (Salonen *et al*. 1998).

In contrast to the well-known exponential decline in PS2 activity in response to incident irradiation

(Tyystjärvi and Aro 1996), the relationship between PS2 activity and the dose of excessive photons is linear. The explanation for such differences could be the fact that only the photon energy trapped by active PS2 complexes with 'closed' RCs is taken into account, while incident PPFD accounts for total irradiation including the photon energy absorbed by Chls associated with damaged PS2 complexes. Therefore, the damage potential of the incident photons declines with the time because of the accumulation of photoinactivated PS2 complexes, which still absorb the photon energy (Lee *et al.* 2001).

The relationship between the decrease in PS2 activity and exposure to excessive photons can be used to predict the extent of PS2 inactivation on the basis of the levels of the Chl fluorescence measured during photoinhibitory treatment. However, the dependence of the slope of this relationship on the leaf temperature should be taken into account. Since PS2 repair occurs in the leaves not treated with an inhibitor of protein synthesis in chloroplasts, the extent of PS2 decline in those leaves will be lower than calculated PS2 inactivation. The difference between predicted and visible levels of PS2 inactivation gives an estimation of PS2 repair (Kornyeyev *et al.* 2003).

The relationship between PPFD and EF, first described in the present paper (Fig. 3), is similar to the relationship between PPFD and the rate constant of PS2 photoinactivation (Tyystjärvi and Aro 1996, Melis 1999). Recently, Kato *et al.* (2003) showed directly that the rate constant of the PS2 photoinactivation was proportional to the rate of excessive energy production, or EF, as presented here. However, as shown in Fig. 4, the value of EF changes during acclimation to PPFD due to the development of photochemical and non-photochemical energy quenching. Therefore, in comparison to EF, the total excessive irradiation gives a more accurate estimation of the damage effect of the photoinhibitory treatment, especially at low temperatures when the development of photochemical and non-photochemical energy quenching is delayed during photosynthetic induction (Fig. 4). In fact, our experiments revealed that steady-state EF is relatively insensitive to temperature between PPFDs of 700 and 1 500 μmol m^{-2} s^{-1} (Figs. 3*B*, and 4*B*), though longer times were required to reach the steady state at lower temperatures. One may speculate that at low PPFD the mechanisms responsible for the regulation of the energy partitioning in PS2 complexes may not be as effective as at stronger PPFD.

Minimization of excessive irradiation as a photoprotection strategy: Maintaining E within safe limits depends on both the capacity of photosynthetic electron transport and the level of non-photochemical dissipation of photon energy absorbed by PS2 complexes (Roberts *et al.* 1998). The role of electron transport in consuming absorbed photons is limited by the capacity for consumption of reducing power, primarily *via* the Calvin cycle, which can be affected by the kinetic constraints imposed by chilling temperatures. Therefore, modulation in the level of non-photochemical energy dissipation is of considerable importance in maintaining low E under high PPFD, especially at chilling temperatures. When cotton and bean leaves are chilled, increased non-photochemical dissipation compensates for low rates of electron transport to prevent a large increase in E (Figs. 2 and 5).

Although steady-state E is not greatly affected by temperature, chilled leaves sustain considerably higher excessive energy flux at the beginning of irradiation (Fig. 4), because activation of electron transport and non-photochemical dissipation exhibit slow induction kinetics (see Kornyeyev *et al.* 2002). Thus, the photosynthetic apparatus can adjust photon energy partition to prevent PS2 photoinactivation during chilling, but these adjustments occur slowly, rendering plants more vulnerable at the beginning of irradiation. In the field in winter, chilling-tolerant plants retain de-epoxidized members of the xanthophyll cycle (zeaxanthin and antheraxanthin) and maintain high thermal energy dissipation overnight (Adams *et al.* 1994, Logan *et al.* 1998). This obviates the need for induction of photoprotection and reduces exposure to excessive irradiation in the mornings when these plants would otherwise be most vulnerable to PS2 photoinactivation.

The suggestion that the regulation of energy partitioning in PS2 complexes is aimed towards keeping the amount of excitation energy reaching 'closed' RCs as low as possible can be used to explain the data on the diurnal changes in E values collected by other researchers. Despite the dramatic changes in PPFD accompanied by considerable redistribution of photon energy between electron transport and thermal dissipation, the midday increase in E value is relatively low (Demmig-Adams *et al.* 1996, Roberts *et al.* 1998, Morales *et al.* 2000, Shirke and Pathre 2003). The highest E occurring around midday does not usually exceed 20 % of total photon energy absorbed by PS2 antennae. This demonstrates the ability of plants to control the damaging potential of the absorbed photons under natural conditions.

References

Adams, W.W., III, Demmig-Adams, B., Verhoeven, A.S., Barker, D.H.: 'Photoinhibition' during winter stress: involvement of sustained xanthophyll cycle-dependent energy dissipation. – Aust. J. Plant Physiol. **22**: 261-276, 1994.

Anderson, B., Barber, J.: Mechanisms of photodamage and protein degradation during photoinhibition of photosystem II. – In: Baker, N.R. (ed.): Photosynthesis and Environment. Pp. 101-121. Kluwer Academic Publ., Dordrecht 1996.

Aro, E.-M., Hundal, T., Carlberg, I., Andersson, B.: *In vitro* studies on light-induced inhibition of Photosystem II and D_1-

protein degradation at low temperature. – Biochim. biophys. Acta **1019**: 269-275, 1990.

Aro, E.-M., McCaffery, S., Anderson, J.M.: Recovery from photoinhibition in peas (*Pisum sativum* L.) acclimated to varying growth irradiances. Role of D1 protein turnover. – Plant Physiol. **104**: 1033-1041, 1994.

Bilger, W., Björkman, O.: Relationships among violaxanthin deepoxidation, thylakoid membrane conformation, and non-photochemical chlorophyll fluorescence quenching in leaves of cotton (*Gossypium hirsutum* L.). – Planta **193**: 238-246, 1994.

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.

Demmig-Adams, B., Adams, W.W., III: Photoprotection and other responses of plants to high light stress. – Annu. Rev. Plant Physiol. Plant mol. Biol. **43**: 599-626, 1992.

Demmig-Adams, B., Adams, W.W., III, Baker, 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.

Fleck, I., Aranda, X., Omari, B.L., Permanyer, J., Abadia, A., Hogan, K.P.: Light energy dissipation in *Quercus ilex* resprouts after fire. – Aust. J. Plant Physiol. **27**: 129-137, 2000.

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.

Gorbunov, M., Kobler, Z.S., Lesser, M.P., Falkowski, P.G.: Photosynthesis and photoprotection in symbiotic corals. – Limnol. Oceanogr. **46**: 75-85, 2001.

Harbinson, J., Genty, B., Baker, N.R.: Relationship between the quantum efficiencies of photosystems I and II in pea leaves. – Plant Physiol. **90**: 1029-1034, 1989.

Henmi, T., Miyao, M., Yamamoto, Y.: Release and reactive-oxygen-mediated damage of the oxygen-evolving complex subunits of PSII during photoinhibition. – Plant Cell Physiol. **45**: 243-250, 2004.

Kato, M.C., Hikosaka, K., Hirotsu, N., Makino, A., Hirose, T.: The excess light energy that is neither utilized in photosynthesis nor dissipated by photoprotective mechanisms determines the rate of photoinactivation in photosystem II. – Plant Cell Physiol. **44**: 318-325, 2003.

Kornyeyev, D., Holaday, A.S., Logan, B.A.: Predicting the extent of photosystem II photoinactivation using chlorophyll *a* fluorescence parameters measured during illumination. – Plant Cell Physiol. **44**: 1064-1070, 2003.

Kornyeyev, D., Logan, B.A., Holaday, A.S.: A chlorophyll fluorescence analysis of the allocation of radiant energy absorbed in photosystem 2 antennae of cotton leaves during exposure to chilling. – Photosynthetica **40**: 77-84, 2002.

Kornyeyev, D., Logan, B.A., Payton, P., Allen, R.D., Holaday, A.S.: Enhanced photochemical light utilization and decreased chilling-induced photoinhibition of photosystem II in cotton overexpressing genes encoding chloroplast-targeted anti-oxidant enzymes. – Physiol. Plant. **113**: 324-332, 2001.

Krall, J.P., Edwards, G.E.: Relationship between photosystem II activity and CO_2 fixation in leaves. – Physiol. Plant. **86**: 180-187, 1992.

Kruse, O., Zheleva, D., Barber, J.: Stabilization of photosystem two dimers by phosphorylation: implication for the regulation of the turnover of D1 protein. – FEBS Lett. **408**: 276-280, 1997.

Lee, H.-Y., Hong, Y.-N., Chow, W.S.: Photoinactivation of photosystem II complexes and photoprotection by non-functional neighbours in *Capsicum annuum* L. leaves. – Planta **212**: 332-342, 2001.

Lima, A.L.S., DaMatta, F.M., Pinheiro, H., Totola, M.R., Loureiro, M.E.: Photochemical responses and oxidative stress in two clones of *Coffea canephora* under water deficit conditions. – Environ. exp. Bot. **47**: 239-247, 2002.

Linger, P., Brüggemann, W.: Correlation between chlorophyll fluorescence quenching parameters and photosynthesis in a segregating *Lycopersicon esculentum×L. peruvianum* population as measured under constant conditions. – Photosynth. Res. **61**: 145-156, 1999.

Logan, B.A., Grace, S.C., Adams, W.W., III, Demmig-Adams, B.: Seasonal differences in xanthophyll cycle characteristics and antioxidants in *Mahonia repens* growing in different light environments. – Oecologia **116**: 9-17, 1998.

Manter, D.K.: Energy dissipation and photoinhibition in Douglas-fir needles with fungal-mediated reduction in photosynthetic rates. – J. Phytopathol. **150**: 674-679, 2002.

Martin, C.E., Tuffers, A., Herppich, W.B., Willert, D.J. von: Utilization and dissipation of absorbed light energy in the epiphytic crassulacean acid metabolism bromeliad *Tillandsia oinantha*. – Int. J. Plant Sci. **160**: 307-313, 1999.

Maxwell, D.P., Falk, S., Huner, N.P.A.: Photosystem II excitation pressure and development of resistance to photoinhibition. – Plant Physiol. **107**: 687-694, 1995.

Melis, A.: Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage *in vivo*? – Trends Plant Sci. **4**: 130-135, 1999.

Morales, F., Belkhodja, R., Abadía, A., Abadía, J.: Photosystem II efficiency and mechanisms of energy dissipation in iron-deficient, field-grown pear trees (*Pyrus communis* L.). – Photosynth. Res. **63**: 9-21, 2000.

Niinemets, Ü., Kull, O.: Sensitivity of photosynthetic electron transport to photoinhibition in a temperate deciduous forest canopy: Photosystem II center openness, non-radiative energy dissipation and excess irradiance under field conditions. – Tree Physiol. **21**: 899-914, 2001.

Noguchi, T.: Dual role of triplet localization on the accessory chlorophyll in the photosystem II reaction center: photoprotection and photodamage of the D1 protein. – Plant Cell Physiol. **43**: 1112-1116, 2002.

Ögren, E.: Prediction of photoinhibition of photosynthesis from measurements of fluorescence quenching components. – Planta **184**: 538-544, 1991.

Olivera, G., Penuelas, J.: Allocation of absorbed light energy into photochemistry and dissipation in a semi-deciduous and an evergreen Mediterranean woody species during winter. – Aust. J. Plant Physiol. **28**: 471-480, 2001.

Öquist, G., Chow, W.S., Anderson, J.M.: Photoinhibition of photosynthesis represents a mechanism for the long-term regulation of photosystem II. – Planta **186**: 450-460, 1992.

Osmond, C.B.: What is photoinhibition? Some insights from comparisons of shade and sun plants. – In: Baker, N.R., Bowyer, J.R. (ed.): Photoinhibition of Photosynthesis: from Molecular Mechanisms to the Field. Pp. 1-24. Bios Scientific Publ., Oxford 1994.

Osmond, C.B., Ramus, J., Levavasseur, G., Franklin, L.A., Henley, W.J.: Fluorescence quenching during photosynthesis

and photoinhibition of *Ulva rotundata* Blid. – Planta **190**: 97-106, 1993.

Ottander, C., Hundal, T., Andersson, B., Huner, N.P.A., Öquist, G.: Photosystem II reaction centres stay intact during low temperature photoinhibition. – Photosynth. Res. **35**: 191-200, 1993.

Oxborough, K., Baker, N.R.: An evaluation of the potential triggers of photoinactivation of photosystem II in the context of a Stern-Volmer model for downregulation and the reversible radical pair equilibrium model. – Phil. Trans. roy. Soc. London B **355**: 1489-1498, 2000.

Park, Y.-I., Chow, W.S., Anderson, J.M.: Light inactivation of functional photosystem II in leaves of peas grown in moderate light depends on photon exposure. – Planta **196**: 401-411, 1995.

Park, Y.-I., Chow, W.S., Anderson, J.M.: Chloroplast movement in the shade plant *Tradescantia albiflora* helps protect photosystem II against light stress. – Plant Physiol. **111**: 867-875, 1996.

Pickering, J.W., Moes, C.J.M., Sterenborg, H.J.C.M., Prahl, S.A., van Gemert, M.J.C.: Two integrative spheres with an inverting scattering sample. – J. opt. Soc. Amer. A **9**: 621-631, 1992.

Roberts, A., Borland, A.M., Maxwell, K., Griffiths, H.: Ecophysiology of the C_3-CAM intermediate *Clusia minor* L. in Trinidad: seasonal and short-term photosynthetic characteristics of sun and shade leaves. – J. exp. Bot. **49**: 1563-1573, 1998.

Roháček, K.: Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationships. – Photosynthetica **40**: 13-29, 2002.

Salonen, M., Aro, E.-M., Rintamäki, E.: Reversible phosphorylation and turnover of the D1 protein under various redox states of Photosystem II induced by low temperature photoinhibition. – Photosynth. Res. **58**: 143-151, 1998.

Schnettger, B., Critchley, C., Santore, U.J., Graf, M., Krause, G.H.: Relationship between photoinhibition of photosynthesis, D1 protein turnover and chloroplast structure: effects of protein synthesis inhibitors. – Plant Cell Environ. **17**: 55-64, 1994.

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.

Shirke, P.A., Pathre, U.V.: Diurnal and seasonal changes in photosynthesis and photosystem 2 photochemical efficiency in *Prosopis juliflora* leaves subjected to natural environmental stress. – Photosynthetica **41**: 83-89, 2003.

Tsonev, T.D., Hikosaka, K.: Contribution of photosynthetic electron transport, heat dissipation, and recovery of photoinactivated photosystem II to photoprotection at different temperatures in *Chenopodium album* leaves. – Plant Cell Physiol. **44**: 828-835, 2003.

Tyystjärvi, E., Aro, E.-M.: The rate constant of photoinhibition, measured in lincomycin-treated leaves, is directly proportional to light intensity. – Proc. nat. Acad. Sci. USA **93**: 2213-2218, 1996.

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

Vavilin, D.V., Tyystjärvi, E., Aro, E.-M.: In search of a reversible stage of photoinhibition in a higher plant: No changes in the amount of functional Photosystem II accompany relaxation of variable fluorescence after exposure of lincomycin-treated *Cucurbita pepo* leaves to high light. – Photosynth. Res. **45**: 239-247, 1995.

Weis, E., Lechtenberg, D.: Fluorescence analysis during steady-state photosynthesis. – Phil. Trans. roy. Soc. London B **323**: 253-268, 1989.