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Regular paper

Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*

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Abstract

The role of the xanthophyll cycle in regulating the energy flow to the PS II reaction centers and therefore in photoprotection was studied by measurements of light-induced absorbance changes, Chl fluorescence, and photosynthetic O_2 evolution in sun and shade leaves of *Hedera canariensis*. The light-induced absorbance change at 510 nm (ΔA_{510}) was used for continuous monitoring of zeaxanthin formation by de-epoxidation of violaxanthin. Non-radiative energy dissipation (NRD) was estimated from non-photochemical fluorescence quenching (NPQ).

High capacity for zeaxanthin formation in sun leaves was accompanied by large NRD in the pigment bed at high PFDs as indicated by a very strong NPQ both when all PS II centers are closed (F'_m) and when all centers are open (F'_o). Such F_o quenching, although present, was less pronounced in shade leaves which have a much smaller xanthophyll cycle pool.

Dithiothreitol (DTT) provided through the cut petiole completely blocked zeaxanthin formation. DTT had no detectable effect on photosynthetic O_2 evolution or the photochemical yield of PS II in the short term but fully inhibited the quenching of F_o and 75% of the quenching of F_m, indicating that NRD in the *antenna* was largely blocked. This inhibition of quenching was accompanied by an increased closure of the PS II reaction centers.

In the presence of DTT a photoinhibitory treatment at a PFD of 2000 μmol m^{-2} s^{-1}, followed by a 45 min recovery period at a low PFD, caused a 35% decrease in the photon yield of O_2 evolution, compared to a decrease of less than 5% in the absence of DTT. The F_v/F_m ratio, measured in darkness showed a much greater decrease in the presence than in the absence of DTT. In the presence of DTT F_o rose by 15–20% whereas no change was detected in control leaves.

The results support the conclusion that the xanthophyll cycle has a central role in regulating the energy flow to the PS II reaction centers and also provide direct evidence that zeaxanthin protects against photoinhibitory injury to the photosynthetic system.

Abbreviations: F, F_m, F_o, F_v – Fluorescence yield at actual degree of PS II center closure, when all centers are closed, when all centers are open, variable fluorescence; NPQ – non-photochemical fluorescence quenching; NRD – non-radiative energy dissipation; PFD – photon flux density; Q_A – primary acceptor PS II

Introduction

In recent years the following question has attracted much interest: how do plants regulate the excitation energy reaching the photosynthetic reaction centers so that overexcitation of these centers and resulting photoinhibitory damage are avoided? Various kinds of mechanisms have been proposed. These include:

1. cyclic electron flow around PS II, perhaps mediated by Cyt b_{559} (Falkowski et al. 1988)
2. conversion of the PS II centers into a form that is able to accept and to dissipate excitation energy as heat but has a low photochemical yield (Weis and Berry 1987)
3. a cycle in which hydrogen peroxide acts as an electron acceptor (Neubauer and Schreiber 1989) and
4. non-radiative energy dissipation in the pigment bed (Björkman 1987a, Demmig and Björkman 1987).

The last process is thought to be mediated by the carotenoid zeaxanthin (see below). In the following our main focus will be placed on recent evidence for a role of the xanthophyll cycle as a possible mechanism for dissipation of excess energy and photoprotection.

In an extensive series of studies, Demmig-Adams and co-workers have provided strong indirect evidence in support of the hypothesis that zeaxanthin mediates non-radiative dissipation of excitation energy (Demmig et al. 1987, 1988, Demmig-Adams et al. 1989a,b,c; see also Demmig-Adams and Adams, this volume).

Further indirect evidence in support of this hypothesis recently has been provided by Thayer and Björkman (1990) who found that the pool size of the xanthophyll cycle components (violaxanthin + antheraxanthin + zeaxanthin) is greatly promoted by a high light regime during leaf development in leaves of a number of different species. This increase is specific to the components of the xanthophyll cycle; other major leaf xanthophylls were little, or not at all, affected by growth light regime. Moreover, sudden transfer of plants from weak to strong light caused several-fold increases in the sum of the xanthophyll cycle components (Demmig-Adams et al. 1989d). These results indicate that the xanthophyll-cycle pool size adjusts to an excess of excitation energy such that there will be a sufficiently large pool available for zeaxanthin formation.

Another forward step in the study of the role of zeaxanthin in relation to energy dissipation and photoprotection was made possible by our discovery that dithiothreitol (DTT), fed to leaves through the cut petiole, completely blocked zeaxanthin formation in photosynthesizing cotton leaves with no detectable short-term effects on any aspect of photosynthetic gas exchange characteristics (Bilger et al. 1989). DTT has long been known to block zeaxanthin formation in vitro by inhibiting the activity of violaxanthin de-epoxidase (Yamamoto 1979). Our studies further showed that a large component of 'non-photochemical' fluorescence quenching in cotton leaves was abolished by DTT treatment, and that the reduction state of Q_A, the primary electron acceptor of PS II, estimated from the degree of 'photochemical' fluorescence quenching, increased in the presence of DTT (Bilger et al. 1989). Subsequent studies on spinach leaves by Demmig-Adams et al. (1990) confirmed these results.

The main objective of the present study was to provide direct evidence for or against a photoprotective role of zeaxanthin. A high reduction state of Q_A may be considered to promote the generation of triplet excited states of Chl and/or singlet oxygen and therefore also to promote photoinhibitory damage to the PS II reaction center complex. However, conclusive evidence that DTT treatment induces an increased susceptibility to photoinhibitory damage, as assessed by a decrease in the photon yield exposure to a high PFD, was lacking at the time the present study was initiated. In this study, mainly conducted on leaves of *Hedera canariensis*, we compare the effects of high-light treatment on sun and shade leaves in the presence and absence of DTT. We are taking advantage of the finding that de-epoxidation of violaxanthin to zeaxanthin in intact leaves can be readily monitored simultaneously with fluorescence quenching by measuring light-induced absorbance changes in the 505–510 nm region (Bilger et al. 1989). We will also briefly consider the light-induced absorbance change which has a broad peak around 535–540 nm and has been thought to be associated

with a build-up of a proton gradient across the thylakoid membrane (Heber 1969, Kobayashi et al. 1982).

Materials and methods

Plant material

Leaves of *Hedera canariensis* Willd. (Algerian Ivy) were taken from plants growing outdoors at Stanford, CA (37° north latitude). Experiments were conducted during the summer months. The shade plants grew on the north-facing side of a building and received direct sunlight only in the early morning. The sun plants grew on the south-facing side of a building and received direct sunlight from 8:30 to 18:30 Pacific Standard Time. The daily photon receipts were 5–7 and 50–55 mol m^{-2} d^{-1} for the shade and the sun plants, respectively. All plants were irrigated daily.

Measurements of light-induced absorbance changes

Light-induced absorbance changes were measured by means of a custom-built apparatus. The measuring beam was chopped at a frequency of 2 kHz by an optical chopper (Model OC 4000, Photon Technology International, Princeton, NJ, USA) and guided by bifurcated glass fiber optics to the leaf. The PFD of the measuring beam was *ca.* 3 μmol m^{-2} s^{-1}. Different wavelengths were obtained by interference filters (Ditric Optics Inc., Hudson, MA, USA) with maximal transmission at 510 nm (half-bandwidth = 7.7 nm) or 540 nm (half-bandwidth = 8.7 nm). Although the absorbance change caused by zeaxanthin formation has a peak at 505 nm, we chose 510 nm to avoid causing slow changes in leaf absorbance due to chloroplast rearrangements within the leaf that may be induced even by a weak measuring beam at 505 nm, especially in shade leaves. The overlap at 510 nm with the slowly reversible light-scattering change which peaks at 530–540 nm, although larger than at 505 nm, is still very small at 510 nm (Bilger et al. 1989). The modulated measuring beam transmitted through the leaf and filtered through a short-pass filter (Ditric 15-21640 plus OCLI Detector Trimmer) was detected by a photodiode (model S-1227–66BR, Hamamatsu, Middlesex, NJ, USA), positioned under the leaf. The signal from the photodiode was pre-amplified by a small low-noise AC amplifier. The amplified signal was then filtered by a passive electronic bandpass filter (TTE, Inc., Series KB3, peak frequency = 2 kHz, bandwidth = 120 Hz at 3 dB). The filtered signal was demodulated and after further amplification the DC signal was detected by a data logger (model M-700, Metrabyte Corp., Taunton, MA, USA) connected to a lap-top computer (Toshiba T1000). The signal was converted to ΔA values (25 data points per min) which were continuously displayed on a video monitor and stored on disk.

The measuring and the actinic beams were provided from two cold mirror halogen lamps (Type ENL, 50 W, General Electric Multimirror), connected to stabilized DC power supplies (Model LJS-12–12-OV Lambda Electronics, Melville, NY, USA). The actinic beam was filtered through a hot mirror (OCLI, Santa Rosa, CA, USA) and through a light-red dichroic filter (OCLI; 50% transmittance at 585 nm) and adjusted to the desired PFD by neutral density filters (FNG series, Melles Griot, Irvine, CA, USA). The actinic light was guided through the second branch of the fiber optic to the common end which was fitted with a small lens pair to collimate the beam. The leaf was held steady during the measurements by a small aluminum leaf holder and exposed to room air which was kept at 25.5 ± 1°C. Leaf temperature ranged from 25 to 30°C depending on the PFD.

Fluorescence measurements

Fluorescence measurements were made on a spot on the leaf adjacent to the one used for absorbance-change measurements, using a PAM chlorophyll fluorometer (Walz, Effeltrich, FRG), modified as previously described by Schäfer and Björkman (1989). Actinic light was provided from a lamp-filter arrangement identical to that used for the absorbance-change measurements. The PFD was adjusted to match the same value as used for absorbance-change measurements by varying the combination of neutral

density filters and, for fine tuning, by slightly adjusting the voltage of the lamp power supply. The saturating light pulses had a PFD of 5000 μmol m^{-2} s^{-1}. As in the absorbance change measurements the common end of the fiber optic was fitted with a small collimating lens pair.

The approximate reduction state of Q_A, $1\text{-}q_P$, was calculated as described by Bilger and Schreiber (1986). Non-photochemical quenching (NPQ) was calculated from the Stern–Vollmer relationship as $F_m/F'_m - 1$, where F_m is the maximal fluorescence yield after dark adaptation, and F'_m is the maximal yield in a saturation pulse during actinic illumination. F_o determinations were made in the presence or absence of low-level far-red radiation as described by Schäfer and Björkman (1989).

Other methods

Measurements of photosynthetic oxygen evolution were conducted at 5% CO_2 as described by Bilger et al. (1989), using a gas-phase O_2 electrode system operated in open-flow mode. Leaf temperature was 29.0°C during the photon yield measurements and 32–34°C during the high-light treatments and during measurements of light-saturated photosynthesis.

Determinations of violaxanthin, antheraxanthin and zeaxanthin were made as described in detail by Thayer and Björkman (1990).

PFD measurements were made with quantum sensors (model LI-190B, Li-Cor, Lincoln, NE, USA). For determinations of daily photon receipts in the field these sensors were connected to a data logger (CR7, Campbell, Logan, UT, USA).

Experimental procedure and protocol

Leaves were cut under water in the field with a sharp razor blade in the morning, before the sun leaves were exposed to direct sunlight. During a following preincubation period of 2 to 3 h, leaves subjected to DTT treatment were allowed to take up a DTT solution (3–5 mM) through their petioles. The petioles of control leaves were kept in water. All leaves were kept at a PFD of 35 μmol m^{-2} s^{-1} (cool white fluorescent light) during the preincubation period. With DTT-treated leaves, measurements of light-induced absorbance changes and fluorescence were begun when an average DTT concentration of 4 to 5 mM in the bulk leaf water had been reached.

For the photoinhibitory treatments initial F_v/F_m ratios were determined on that area of the leaf which would later be exposed to high PFD. Another spot on the leaf was used for measurements of the absorbance change at 510 nm, induced by a PFD of 2190 μmol m^{-2} s^{-1} to verify that zeaxanthin formation was inhibited. After these measurements the leaf was enclosed in the leaf chamber of the O_2 measurement system and kept at a PFD of 100 to 200 μmol m^{-2} s^{-1} until a stable and maximal photon yield was obtained. The PFD was then increased to 2050 ± 50 μmol m^{-2} s^{-1} and maintained at this level for 3 h. Following this high-light treatment the PFD was reduced to 150 μmol m^{-2} s^{-1} and the photon yield determined every 10 min. Throughout these measurements the leaf was kept in an atmosphere of 4.5–5% CO_2, 19.8–20.3% O_2, balance N_2. After some degree of recovery of the photon yield during the first 30 min little further recovery was observed. After 45 min at low PFD the leaf was transferred to the fluorescence apparatus and the F_v/F_m ratio determined. The period in darkness between the last illumination in the leaf chamber and the fluorescence measurements was 5–10 min.

Results and discussion

Figure 1 shows light response curves of photosynthesis for *Hedera* shade and sun leaves similar to those used in the experiments described below. In both kinds of leaves all of the light energy absorbed by the leaves is dissipated by photosynthesis up to a PFD of *ca.* 200 μmol m^{-2} s^{-1}. As the PFD is increased further, photons continue to be absorbed in proportion to the initial slope of the light curve indicated by the dotted line. However, a portion of this light energy is not used in photosynthesis and this portion represents the excessive light. Since photosynthesis in the shade leaf reaches saturation at much lower PFDs than in the sun leaf the amount of excessive light at any given

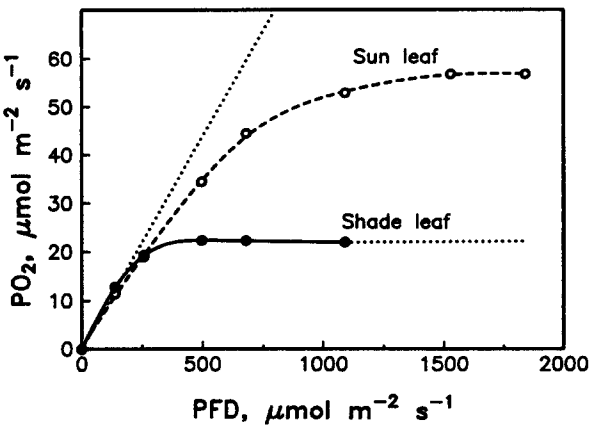

Fig. 1. Rate of CO_2 saturated photosynthesis (+ dark respiration) at a leaf temperature of 30–33°C as a function of incident PFD in a shade and a sun leaf of *Hedera canariensis*. The slope of the dotted line depicts the maximum photon yield (φ_i). The PFD was increased in steps. Each data point depicts the value obtained *ca.* 15 min after each increase in PFD. The xanthophyll cycle pool sizes (V + A + Z) were 26 and 126 μmol m^{-2} or 38 and 165 mmol mol^{-1} Chl for the shade and sun leaf, respectively.

incident PFD above 250 μmol m^{-2} s^{-1} is much greater in the shade than in the sun leaf. Moreover, the size of the V + A + Z pool which represents the maximum amount of zeaxanthin that can be formed in response to excessive light, was only about one-fourth as large in the shade leaf as in the sun leaf. Therefore the sun leaf is able to dissipate a greater fraction of the excitation energy via photosynthesis as well as via a zeaxanthin-mediated dissipation process than the shade leaf.

Light-induced absorbance changes and zeaxanthin formation

The time course and amplitude of the light-induced absorbance changes at 510 nm, caused by zeaxanthin formation in *Hedera* shade and sun leaves, are shown by the solid traces in the upper panels of Fig. 2. The dashed traces show the absorbance changes in leaves in which zeaxanthin formation was prevented by DTT treat-

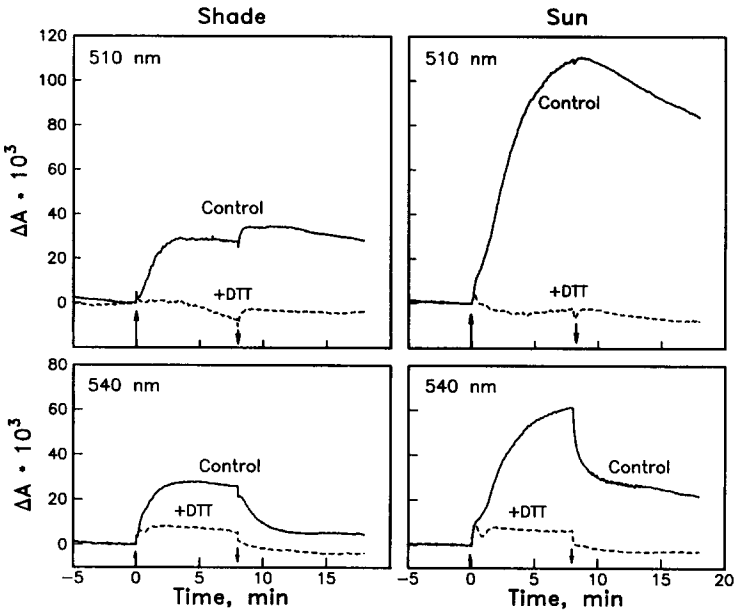

Fig. 2. Kinetics of absorbance changes at 510 and 540 nm induced by a PFD of 2190 μmol m^{-2} s^{-1} in sun and shade leaves of *Hedera canariensis*. Where indicated the leaves had taken up a DTT solution through the petiole. Upward and downward arrows indicate actinic light on and off, respectively. The leaves were in air and reached a temperature of 29 to 32°C during illumination. Before illumination, the leaves were preincubated at low light as described in Materials and Methods and in addition predarkened for at least 30 min. The absorbance change at 540 nm was measured on the same leaf at an adjacent spot to the one used for the measurement at 510 nm.

ment. Thus the differences between the solid and dashed traces represent the absorbance change caused by zeaxanthin formation. (For an explanation of the rapid transients seen upon light on and light off see Bilger et al. 1989.) It is obvious that the maximum amplitude of the ΔA_{510} is much larger in the sun than in the shade leaf. This difference in amplitude roughly corresponds to the amount of zeaxanthin formed during the illumination (zeaxanthin data not shown; see Bilger et al. 1989 for data on the relationship between zeaxanthin content and ΔA_{505} in cotton leaves). Figure 2 also shows that zeaxanthin formation is quite rapid; the estimated half-times of the rise in ΔA_{510} were *ca.* 1 and 2 min for the shade and sun leaf, respectively. Although much slower than the rise, the fall in ΔA_{510} after light off was still considerable in both the shade and the sun leaves. After a lag of 1–2 min ΔA_{510} fell at a rate corresponding to a re-epoxidation rate of 25–30% of the zeaxanthin formed during illumination over a 10 min period in near darkness (measuring beam only; PFD $\approx$ 3 μmol m^{-2} s^{-1}). A similar rate of decline in ΔA_{510} was observed at a PFD of 920 μmol m^{-2} s^{-1} in *Hedera* leaves treated with DTT after accumulation of zeaxanthin had taken place in the absence of DTT (data not shown). This indicates that in untreated leaves the de-epoxidation and re-epoxidation reactions occur simultaneously in the presence of excessive light.

The time course and amplitude of the light-mediated absorbance change at 540 nm for these shade and sun leaves are shown in the lower panels of Fig. 2. In the control leaves this absorbance change has two distinct components: one is rapidly reversible upon light off whereas the other is only slowly reversible. The slowly reversible component was totally abolished in the presence of DTT and follows the same kinetics as ΔA_{510}. Like ΔA_{510} this component of ΔA_{540} is undoubtedly caused by changes in the zeaxanthin level whereas the rapidly reversible component most probably is associated with changes in selective light-scattering (Heber 1969, Thorne et al. 1975). The light-scattering change has been thought to be caused by conformational changes of the thylakoid membrane and has been used as an indicator of membrane energization (Heber 1969, Kobayashi et al. 1982). However, as in the study on cotton leaves by Bilger et al. (1989), the major part of this rapidly reversible absorbance change in *Hedera* leaves was abolished by DTT even though a highly energized membrane is to be expected at a PFD of 2190 μmol m^{-2} s^{-1}. Selective scattering is dependent on the presence of one or more pigments absorbing at a wavelength about 10 nm lower than the observed scattering (Latimer and Rabinowitch 1959). The inhibition of the scattering change by DTT suggests that zeaxanthin might be such a pigment. This would mean that although the conformational changes were not inhibited by DTT, they cannot be detected as light-scattering changes. Yamamoto and Bangham (1978) showed that when zeaxanthin was incorporated into liposome membranes it had a considerable absorbance at 520 to 530 nm. This was also true for β-carotene. It is therefore possible that the presence of β-carotene is responsible for the 20–30% of the light-scattering change remaining in the presence of DTT.

Fluorescence quenching in relation to zeaxanthin formation

Figure 3 shows the light-dependence of zeaxanthin formation (measured as ΔA_{510}), NPQ (expressed as $F_m/F'_m - 1$), the reduction state of Q_A $(1 - q_P)$ and F_o in *Hedera* shade leaves in the absence and presence of DTT. Figure 4 shows the same parameters for *Hedera* sun leaves. We have chosen to calculate non-photochemical quenching from the Stern–Vollmer equation rather than expressing it in terms of the coefficient, q_{NP}.

In the absence of DTT, NPQ increased with increasing PFD in roughly the same manner as ΔA_{510} in both shade and sun leaves. This is consistent with the hypothesis that there is a close relationship between zeaxanthin and non-radiative energy dissipation in the *antenna* of PS II. This relationship is further demonstrated by the finding that DTT present in sufficiently high concentrations to inhibit zeaxanthin formation also inhibited a very large part of the non-photochemical quenching. The main difference between the two kinds of leaves is that in control shade leaves, partial saturation of both ΔA_{510} and non-photochemical quenching occurred at

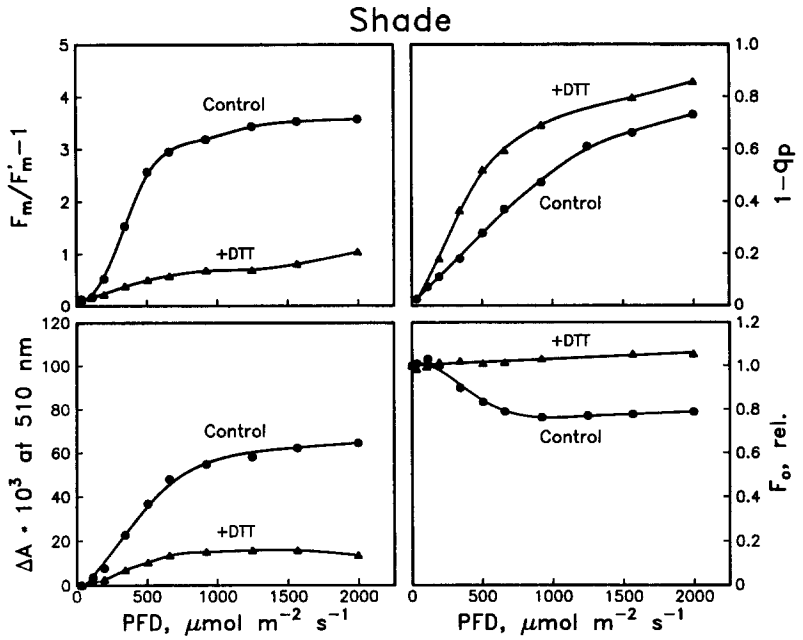

Fig 3. Light-dependence of ΔA_{510}, NPQ (expressed as $F_m/F'_m - 1$), the reduction state of Q_A (determined as $1\text{-}q_P$), and the relative yield of F'_o in shade leaves of *Hedera canariensis* in the presence or absence of DTT. Fluorescence and absorbance were measured simultaneously on two adjacent spots of the same leaf. F'_o is the fluorescence yield in the measuring light obtained directly after switching off the actinic light. The leaves were kept in air at 25–29°C. Each measurement was taken after 16–20 min at each PFD after a steady state had been reached.

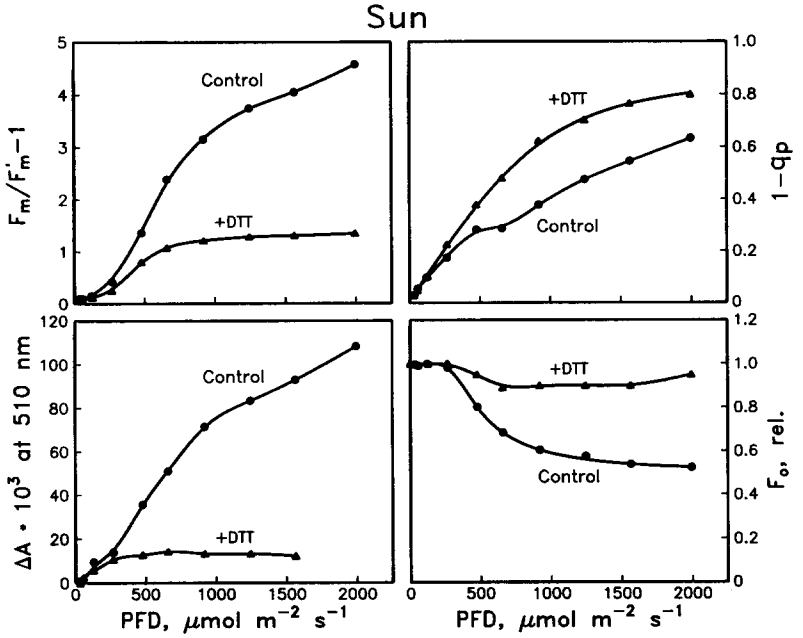

Fig. 4. Light-dependence of ΔA_{510}, NPQ (expressed as $F_m/F'_m - 1$), the reduction state of Q_A (determined as $1\text{-}q_P$), and the relative yield of F'_o in sun leaves of *Hedera canariensis* in the presence or absence of DTT. Measurements were as in Fig 3.

much lower PFDs than in control sun leaves. In fact, the latter did not approach saturation even at a PFD equivalent to full sunlight. These results are in agreement with the finding that the pool size of xanthophyll cycle components, available for zeaxanthin formation in the sun leaves, was *ca.* 3 times larger than in the shade leaves.

Another observation that supports the hypothesis that much of zeaxanthin-associated nonradiative energy dissipation occurs in the *antenna* (e.g., Björkman 1987a) is that a strong F_o quenching developed in the control leaves as the PFD was increased (Figs. 3 and 4; cf. Kitajima and Butler 1975). This F_o quenching was especially pronounced in sun leaves in which F_o was quenched by almost 50%. In *sun* leaves F_o quenching was largely prevented by DTT at any PFD and in DTT treated *shade* leaves, F_o exhibited a small apparent increase at high PFDs.

Such differences in quenching behavior between shade and sun leaves in the presence or absence of DTT are also evident from the induction curves shown in Fig. 5. In these experiments the leaves were suddenly exposed to a PFD of 2190 μmol m^{-2} s^{-1}. At this high PFD, F (continuous trace) was nearly equal to F'_m. Strong quenching of F_m took place within the first minute in all leaves. In the shade leaves F'_m fell to a fairly steady plateau within 2 min but this plateau was some 6 times higher in the DTT treated than in the control leaf. Upon light off, F_o was initially quenched but recovered during the next 3 min. No such transient F_o quenching was detected in the DTT-treated leaf. In both treated and untreated shade leaves the F_m levels obtained after a 5-min period in darkness were quenched by *ca.* 30%. In control sun leaves a different response was obtained: After 2 min in

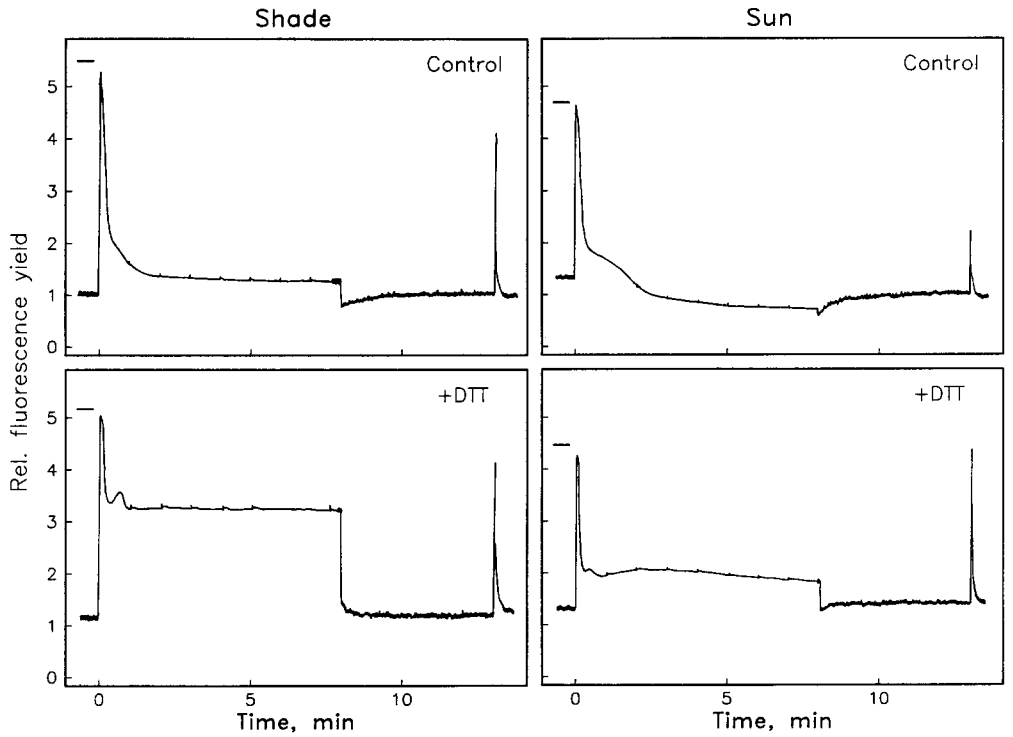

Fig. 5. Kinetics of fluorescence induction in shade and sun leaves of *Hedera canariensis* in the presence or absence of DTT. In each panel, the horizontal mark and the trace preceding the first fluorescence peak indicate the F_m value (obtained in a saturating light pulse) and the F_o value before illumination, respectively. At time 0 actinic light with a PFD of 2190 μmol m^{-2} s^{-1} was switched on and saturating pulses to obtain F'_m were given at 1-min intervals during the exposure. These pulses are indicated by the small blips. After 8 min the actinic light was switched off. Fluorescence was then recorded continuously in the dark to obtain F_o. At 13 min. another saturating pulse was given. The curves were measured simultaneously with the ΔA_{510} determinations shown in Fig. 2. For other conditions, see Fig. 2.

the light, the F_m level fell below the initial F_o level, indicating a very strong quenching of both F_m and F_o. Upon light off, F_o rose slightly but did not recover to the initial F_o value; instead it remained quenched. In the DTT-treated sun leaf F_m remained well above the initial F_o level and no sustained F_o quenching was observed after light off. Moreover, the F_m value measured after 5 min in darkness was similar to the initial F_m showing that little, if any, sustained quenching of F_m took place. This is in sharp contrast to the response of the control sun leaf which exhibited a strong (~80%) quenching of F_m.

As is apparent from Figs. 3, 4 and 5, NPQ decreased in the presence of DTT, both in shade and in sun leaves. Consequently the reduction state of Q_A, or the fraction of PS II centers that were in the 'closed' state was increased by DTT treatment. This difference between DTT treated and control leaves persisted at all moderate and high PFDs (Figs. 3 and 4, top right panels).

The PFD dependence of the efficiency of PS II photochemistry is shown in Fig. 6. This efficiency is presented in two ways:

1. $(F'_m - F'_o)/F'_m$ which is the efficiency if all reaction centers remained open (top panel) and
2. $(F'_m - F)/F'_m$, i.e., the efficiency under the actual degree of reaction center closure at each PFD (bottom panel).

Evidently, by preventing non-radiative energy dissipation in the *antenna*, DTT causes a strong increase in the photochemical yield of the 'open' PS II centers. In contrast to the yield with 'open' centers, the photochemical yield with 'actual' degree of center closure was not affected by DTT (Fig. 6, bottom panel). This is not surprising since neither the photosynthetic rate nor its light dependence was affected by DTT in the short term in any of the three species examined so far (*Hedera*, cotton and spinach). It is well known that electron transport in photosynthesizing leaves is limited by reactions on the acceptor side of the plastoquinone pool. Therefore, when in the absence of regulation by non-radiative energy dissipation the energy flow to the PS II centers exceeds the rate of the limiting step, an increased fraction of the centers simply close and the yields of PS II photochemistry and photosynthesis remain the same as in the presence of non-radiative dissipation. Results similar to those shown in Fig. 6 for *Hedera* shade leaves were also obtained with *Hedera* sun leaves and with cotton leaves, grown under the conditions described by Bilger et al. (1989). In each case the curve for $(F'_m - F)/F'_m$ vs. PFD of DTT-treated leaves was indistinguishable from that for the control leaves (data not shown).

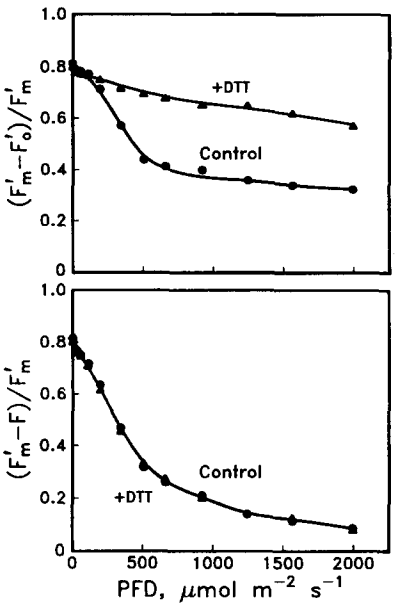

Fig. 6. Light-dependence of the efficiency of PS II if all reaction centers remained open $[(F'_m - F'_o)/F'_m]$ and the efficiency under the actual degree of closure $[(F'_m - F)/F'_m]$ in shade leaves of *Hedera canariensis* in the presence or absence of DTT. These values were calculated from the fluorescence determinations obtained in the experiment shown in Fig. 3. F is the steady state fluorescence in the actinic light, F'_o the yield obtained directly after switching off the actinic light, and F'_m the maximum value obtained in a saturating light pulse.

Evidence for photoprotection

Although all results in which the fluorescence characteristics of DTT-treated leaves were compared indicate that DTT treatment should lead to an increased susceptibility to photoinhibition, our early experiments with cotton leaves and *Hedera* shade leaves failed to show conclusively that a photoinhibitory treatment in the presence of DTT leads to a greater sustained decrease in the photon yield of O_2 evolution than the same treatment in the absence of DTT. Invariably, the

F_v/F_m ratio determined after photoinhibitory treatments similar to those shown below, was slightly lower in the presence than in the absence of DTT but the difference in photon yield was within the experimental error (data not shown). However, the results obtained with *Hedera* sun leaves show that DTT, at concentrations sufficient to prevent zeaxanthin formation, caused a pronounced increase in the susceptibility to photoinhibition. These experiments will be discussed in detail since we believe that they provide *direct* evidence for a photoprotective role of the xanthophyll cycle.

The mean photon yield values (φ_i) determined immediately before each photoinhibitory treatment were 0.0706 and 0.0707 O_2 per incident photon for DTT-treated and control *Hedera* sun leaves, respectively. Since these leaves absorbed 83–84% of the light, the photon yield on an absorbed light basis would be 0.085 O_2 photon^{-1}. The mean F_v/F_m ratios were 0.713 and 0.715 for the DTT-treated and control leaves, respectively. Both the photon yield and the F_v/F_m values are somewhat low (compare Björkman and Demmig 1987), indicating that the rate constant for non-radiative energy dissipation in these *Hedera* sun leaves was relatively high, even after overnight recovery. This is consistent with the presence of a significant amount of zeaxanthin in these leaves (*ca.* 8% of the xanthophyll cycle pool). However, it is also possible that the leaves were slightly photoinhibited. The mean xanthophyll cycle pool sizes and mean light-saturated photosynthetic rates were similar in the DTT-treated and control leaves although there were significant differences between different leaf pairs (Table 1). The capacity for zeaxanthin formation (ΔA_{510}) was high in all control leaves but completely absent in the DTT-treated leaves (Table 1).

Photon yields and F_v/F_m ratios were measured again after exposing each leaf to a PFD of 2000 μmol m^{-2} s^{-1}, followed by a 45 min recovery period at a low PFD. As shown in Fig. 7, in each pair of control and DTT-treated leaves the inhibition of both the photon yield and the F_v/F_m ratio was considerably greater in the DTT-treated leaf. The average inhibition of the photon yield was 34.9% in the DTT-treated leaves, compared with 4.7% in control leaves. Corrresponding average values for inhibition of F_v/F_m, measured after an additional 5–10 min dark period, were 20.6% and 3.8%. In all DTT-treated leaves the inhibition of F_v/F_m was accompanied by a 15–21% rise in F_o, whereas no change in F_o was detected in the control leaves. Hence, a significant part of the decline in F_v/F_m in the DTT-treated leaves is attributable to the rise in F_o. Such a rise in F_o has been considered as indicative of photoinhibitory injury to the PS II reaction centers (e.g., Björkman 1987a). It may be significant that among the DTT-treated leaves, the inhibition of the photon yield of O_2 evolution and F_v/F_m was smallest in leaf no. 2B which had the highest light-saturated photosynthetic rate (and thus would be subject to the least amount of excess light); largest in leaf no. 3B which had the lowest photosynthetic rate; and intermediate in leaf no. 1.

A probable reason why the effect of DTT treatment on the susceptibility to photoinhibition was much more evident in *Hedera* sun leaves than in *Hedera* shade leaves is that the xanthophyll cycle (V + A + Z) pool size in the shade leaves is quite small (one-third to one-fourth of the sun leaf pool size). Moreover, only some

Table 1. Light-saturated rates of photosynthetic O_2 evolution (PO_2), xanthophyll cycle pool sizes (V + A + Z) and light-induced absorbance changes at 510 nm (ΔA_{510}) in individual leaves used for photoinhibitory treatments shown in Fig. 7. PO_2 was measured 11–13 min after the start of each high-light treatment. Leaves nos. 1A-3A were controls, leaves nos. 1B-3B were DTT-treated

Parameter	Leaf no.					
	1A	1B	2A	2B	3A	3B
PO_2, μmol m^{-2} s^{-1}	44.2	38.5	41.9	50.8	33.1	36.1
V + A + Z, μmol m^{-2}	123	106	111	128	108	86
V + A + Z, mmol mol^{-1} Chl	197	175	180	200	218	205
$\Delta A_{510} \times 10^3$	113	0	105	0.9	95	−7

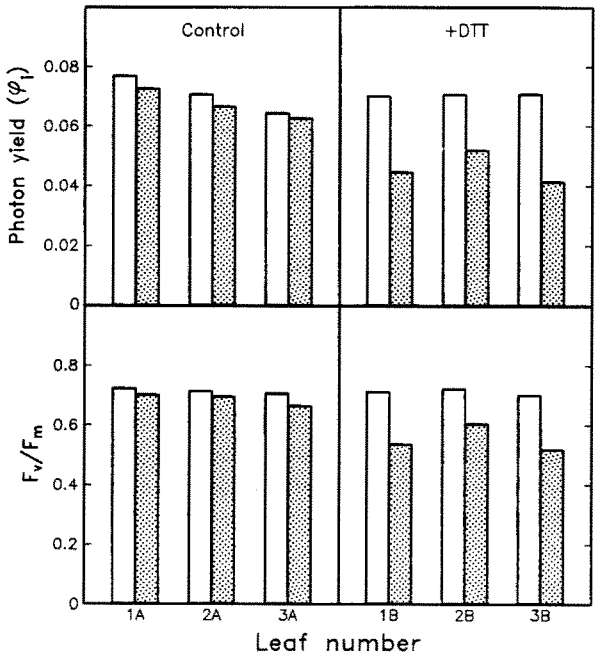

Fig. 7. Effects of 3-h photoinhibitory treatments at a PFD of 2000 μmol m^{-2} s^{-1} on the photon yield of the O_2 evolution (φ_i) and the F_v/F_m ratio in sun leaves of *Hedera canariensis* in the absence (control) or in the presence of 4–5 mM DTT. Open bars denote the photon yield or F_v/F_m before treatment; stippled bars denote the value of the parameters after treatment plus 45 min recovery at an average PFD of 130 μmol m^{-2} s^{-1}. φ_i measurements were made at strictly rate-limiting PFDs (0–150 μmol m^{-2} s^{-1}); F_v/F_m ratios were determined without actinic light. Leaf numbers (1A, 1B, etc.) denote leaf pairs that were matched to have similar leaf angles and developmental stage and comparable light-saturated rates of O_2 evolution. Compare Table 1.

65–75% of this pool may be available for zeaxanthin formation. Thus, prevention of zeaxanthin formation by DTT treatment of shade leaves had a much smaller effect on the amount of zeaxanthin formed than in sun leaves (compare Fig. 2). The ratio between V + A + Z pool size and light-saturated photosynthetic capacity is also likely to have an influence on the extent to which DTT treatment will increase the susceptibility to photoinhibition. The cotton leaves used in our experiments were grown under controlled conditions, using artificial light. In these leaves the V + A + Z pool size was only about one-half that of the *Hedera* sun leaves but the light-saturated photosynthetic rate was almost twice that of the *Hedera* sun leaves. Thus, the energy dissipation via photosynthesis was larger and the potential for zeaxanthin formation was smaller than in *Hedera* sun leaves.

Conclusions

In this paper we have attempted to accomplish two objectives. The first was to provide examples of the usefulness of combining different non-destructive methods such as measurements of light-induced absorption changes, fluorescence, and gas-exchange in studies aimed at gaining an improved understanding of how plants cope with the stress imposed by the great variation in the light environment that occurs in most field situations. We believe that these combinations of methods will be routinely used in future field studies. At present, the main obstacle to a wide use of these methods in field-related research is not technical but rather caused by difficulties in data interpretation.

Our second and main objective was to provide further insight into the mechanisms that leaves use to regulate the energy flow to the reaction centers in a manner that permits the light energy absorbed by the chlorophyll to be transferred to these centers with highest possible efficiency when light is limiting, while at the same time prevent overexcitation of the centers when it becomes excessive. When such overexcitation occurs, as is often the case when shade leaves are suddenly exposed to direct sunlight, photoinhibitory damage occurs. Although there may well be more than one way in which the excessive excitation energy can be dissipated it now seems highly probable that most of this energy is dissipated by non-radiative dissipation in the pigment bed. The studies of Demmig-Adams and co-workers brought our attention to the specific involvement of the carotenoid zeaxanthin in this process (Demmig et al. 1987, 1988, Demmig-Adams et al. 1989a,b,c).

In contrast to the content of other major leaf carotenoids such as lutein, neoxanthin, and β-carotene, zeaxanthin content rapidly adjusts to the need for dissipation of excessive light and also decreases, although less rapidly, when the light becomes rate-limiting to photosynthesis (Fig. 2). Moreover, as recently shown by Thayer

and Björkman (1990) the xanthophyll cycle pool size increases in response to a sustained high irradiance so that it does not become limiting to zeaxanthin formation. This pool size decreases again if the plant finds itself in a prevailingly low light environment. Such a dynamic response to the light environment is what one should expect from a mechanism that serves to regulate the energy flow to the photosynthetic reaction centers.

Another feature of the xanthophyll cycle that strongly suggested that it serves such a regulatory function is the close correlation that exists between zeaxanthin formation and non-photochemical fluorescence quenching. As mentioned in the Introduction, Demmig-Adams and coworkers have shown that this relationship holds, irrespective of the means used to induce an excess of light energy. Increasing the light, imposing water stress, or depriving the leaf of CO_2, all led to similar results. Good correspondance between zeaxanthin formation and non-photochemical quenching was obtained also in the present short-term experiments, both in terms of time course (Figs. 2 and 5) and dependence on photon flux density (Figs. 3 and 4).

Furthermore, the results presented in this paper, together with the very recent results of Bilger et al. (1989) and Demmig-Adams et al. (1990) on the effect of DTT, further strengthen the evidence for the involvement of zeaxanthin in non-radiative energy dissipation. Not only did prevention of zeaxanthin formation by DTT inhibit some 75% of non-photochemical quenching but also fully inhibited the strong quenching of F_o present in untreated leaves (Figs. 3, 4 and 5). These results are in full agreement with those that one would expect from non-radiative energy dissipation in the pigment bed, mediated by zeaxanthin. DTT also caused an increased closure of the PS II reaction centers, thereby presumably setting the stage for photoinhibition caused by overexcitation of these centers.

Finally, even though in the absence of excessive light DTT did not have any detectable effect on the photon yield or photosynthetic rate, nor on the yield of PS II photochemistry, prevention of zeaxanthin formation led to a pronounced increase in the susceptibility to photoinhibition in *Hedera* sun leaves (Fig. 7). The conclusion therefore seems justified that zeaxanthin does indeed function as a photoprotective agent and that it does so by mediating energy dissipation in the pigment bed.

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Note added in proof

Since the submission of this paper it has been reported that DTT also increases the susceptibility to photoinhibition of *Nerium oleander* leaves (Winter K and Königer M (1990), Planta 180: 24–31)

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