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Fluorescence as a Tool in Photosynthesis Research: Application in Studies of Photoinhibition, Cold Acclimation and Freezing Stress [and Discussion]

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Fluorescence as a tool in photosynthesis research: application in studies of photoinhibition, cold acclimation and freezing stress

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Chlorophyll fluorescence induction (at 20 °C and 77 K) and quenching were analysed in relation to effects of environmental stresses imposed by chilling in high light and by freezing and thawing of spinach (*Spinacia oleracea* L.) leaves. The data indicate that cold acclimation of spinach plants, which leads to increased frost tolerance of the leaves, results in decreased susceptibility to photoinhibition of photosynthesis at chilling temperatures.

When plants acclimated to 18 °C and 260–300 μmol quanta m^{-2} s^{-1} were exposed to higher light (550 μmol quanta m^{-2} s^{-1}) at 4 °C, they developed strong photoinhibition, as characterized by decreased quantum yield of O_2 evolution and decreased ratio of variable: maximum fluorescence (F_V/F_M) of photosystem II. The decrease in F_V/F_M resulted from a decline in F_V and an increase in F_0. The F_V/F_M ratio was lowered to a significantly greater extent when induction was recorded at 20 °C, as compared with 77 K. The effects related to photoinhibition were fully reversible at 18 °C in dim light. Plants that had been cold-acclimated for 10 days exhibited slightly decreased quantum yield and lowered F_V/F_M ratio. However, they did not show further photoinhibition on exposure to 550 μmol quanta m^{-2} s^{-1} at 4 °C. The reversible photoinhibition is discussed as a protective pathway serving for thermal dissipation of excessive light energy. It is hypothesized that such a mechanism prevents destruction of the photosynthetic apparatus, until other means of protection become effective during long-term acclimation to high light.

Inhibition of photosynthetic carbon assimilation caused by freezing and thawing of leaves in the dark was closely correlated with inhibition of photochemical fluorescence quenching (q_Q). As a sensitive response of the thylakoid membranes to freezing stress, the energy-dependent quenching, q_E, was inhibited. Only more severe impact of freezing caused a significant decline in the F_V/F_M ratio.

It is concluded that measurements of fluorescence induction signals (F_V/F_M ratios) provide a sensitive tool with which to investigate photoinhibition, whereas freezing damage to the photosynthetic system can be detected more readily by the quenching coefficients q_Q and q_E than by F_V/F_M ratios.

Introduction

Chlorophyll *a* fluorescence is frequently used to assess effects of environmental stress on plants (see Krause & Weis 1984; Renger & Schreiber 1986). As fluorescence emission is influenced, directly or indirectly, by various parameters of photosynthesis, the fluorescence signal from intact leaves is extremely complex and requires careful interpretation for each stress phenomenon under investigation. Often, low-temperature (77 K) fluorescence measurement is applied, which excludes the effects of whole-chain electron transport and carbon metabolism on fluorescence. PSI fluorescence can be analysed at 77 K, as well as emission from PSII.

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Recording at 77 K allows routine determination of the variable:maximum fluorescence (F_V/F_M) ratio of PSII, which is viewed as a measure of potential primary photochemical efficiency (see Butler 1977). The F_V/F_M ratio at 77 K has been found to be remarkably constant (0.832 ± 0.004 for C_3 plants) among many species and ecotypes (Björkman & Demmig 1987). At room temperature, fluorescence is emitted almost only from PSII, and F_V/F_M ratios very similar to those at 77 K can be obtained, provided that conditions are chosen to eliminate or minimize effects of carbon metabolism (high light after dark incubation). The peak (F_P) of the Kautsky signal is then very close to F_M.

Stress factors that primarily affect the function of PSII will be reflected by a decrease in the F_V/F_M ratio. This is the case for excess light causing photoinhibition of photosynthesis. It has been shown for 77 K fluorescence that, upon photoinhibition treatments of leaves, the F_V/F_M ratio of PSII decreases linearly with the quantum yield of photosynthetic CO_2 assimilation (Demmig & Björkman 1987; Demmig *et al.* 1987). In this paper, a reversible photoinhibition of spinach (*Spinacia oleracea* L.) leaves at chilling temperature and related effects on fluorescence, recorded at 77 K and 20 °C, are investigated in detail. Our results confirm the linear relation between the F_V/F_M ratio and quantum yield of photosynthesis, but show quantitatively different changes in fluorescence at 77 K and 20 °C. Furthermore, it is shown that the chilling-induced photoinhibition in non-acclimated ('unhardened') plants is correlated with a reversible increase in the initial fluorescence, F_0. The data also indicated a role of cold acclimation ('hardening') in decreasing the susceptibility to photoinhibition.

The fluorescence quenching, observed in the long-term induction signal at room temperature, is predominantly caused by the photochemical (q_Q) and energy-dependent (q_E) mechanisms (Krause *et al.* 1982). Other components of quenching may result from photoinhibition and from phosphorylation of the light-harvesting complex of PSII (see Horton & Hague 1988). As q_Q and q_E are strongly influenced by the utilization of reducing equivalents and ATP in photosynthesis, the quenching depends on metabolic activities (see Krause & Weis 1984; Dietz *et al.* 1985; Briantais *et al.* 1986; Krause & Laasch 1987; Weis & Berry 1987; Krause *et al.* 1988). Stress factors that primarily inhibit the carbon reduction cycle are therefore expected to become manifest by altered quenching coefficients, particularly of q_Q. The effects on quenching will be demonstrated here for the case of freezing stress in spinach leaves.

Materials and methods

Material

Spinach plants (*Spinacia oleracea* L.), five weeks old, were acclimated for ten days to 18 °C (unhardened plants) or were hardened by decreasing the temperature in two-day intervals: 18–15 °C, 15–10 °C, 10–3 °C, 3–1 °C, 1–1 °C (day–night) as described by Klosson & Krause (1981). In both cases, the photon flux density (PFD) was 260–300 μmol quanta m^{-2} s^{-1} and the light period 8 h.

Photoinhibition and recovery treatment

Detached spinach leaves were exposed to white light, PFD 550 μmol quanta m^{-2} s^{-1}, in normal air cooled to 4 °C. The leaf temperature, measured from the lower side of the leaf with a copper–constantan thermocouple, was kept at 5.5–6 °C. Recovery from the photoinhibition was followed at 18 or 4 °C and a PFD of 2.5–5.0 μmol quanta m^{-2} s^{-1}.

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Frost treatment

Detached spinach leaves placed into a cryostat were cooled in darkness at the rate of 6 °C h^{-1} from 4 °C to different minimum temperatures, at which the leaves were kept for 2 h and thereafter warmed up at the same rate. Control leaves were kept at 4 °C in darkness during the frost treatments. The temperature limit of frost tolerance (minimum temperature causing 50 % damage of leaf tissue) was −6.5 to −8 °C for unhardened and −10 to −12.5 °C for hardened leaves.

Quantum yield

Rates of photosynthetic O_2 evolution were measured at 20 °C and CO_2 saturation with a leaf-disc electrode (Hansatech, Kings Lynn, Norfolk, U.K.). Light was provided from a set of photodiodes (λ_{max} 660 nm). Optimal quantum yield was calculated according to Björkman & Demmig (1987). Absorptance was 85 %.

Fluorescence at 20 °C

Fast fluorescence-induction kinetics were measured in the far-red region from leaf discs with a Hansatech fluorometer attached to the leaf-disc chamber. Exciting PFD was 220 μmol quanta m^{-2} s^{-1}, red light (λ_{max} 660 nm). Leaf discs were kept for 10 min in darkness in CO_2-free air before measurements.

Components of fluorescence quenching (q_Q and q_E) were determined with a pulse-amplitude modulation fluorometer (PAM 101, H. Walz, Effeltrich, F.R.G.) according to Schreiber *et al.* (1986). CO_2 fixation was monitored simultaneously with an infrared gas analyser (UNOR 5N1, Maihak, Hamburg, F.R.G.).

Fluorescence at 77 K

Fluorescence induction at liquid nitrogen temperature was measured with blue exciting light (7.5 μmol quanta m^{-2} s^{-1}) from leaf discs at 694 nm and 735 nm (half bandwidth 11.5 and 7.0 nm, respectively) similar to the method of Ögren & Öquist (1984). The leaf discs were kept for 5 min in darkness at room temperature before freezing.

Results and discussion

Photoinhibition of unhardened spinach leaves at chilling temperature

Figure 1 depicts fluorescence induction signals at 20 °C from leaves of spinach (a chilling-resistant plant), acclimated to 18 °C and a PFD of 260–300 μmol quanta m^{-2} s^{-1}. It can be seen that at 4 °C a moderate increase in the PFD to 550 μmol quanta m^{-2} s^{-1} caused strong photoinhibitory fluorescence quenching of F_V, accompanied by a considerable increase in F_0 (curve (*b*)). No indication of photoinhibition was observed in the same light at 18 °C (not shown). Full restoration of the original F_V and F_0 levels took place within 1–3 h upon return to 18 °C and dim light (curve (*c*)). Similar phenomena have been observed with the chilling-resistant *Lemna gibba* (Ögren & Öquist 1984) and chilling-sensitive *Phaseolus vulgaris* (Greer *et al.* 1986). An increase in F_0 has been discussed as indication of permanent damage to PSII (Demmig & Björkman 1987). However, in view of the capacity for total recovery, this interpretation does not seem valid in the present case.

The course of F_V/F_M decline and F_0 increase caused by photoinhibition treatment is shown in figure 2. Data are from recordings at 20 °C and 77 K. The changes of F_V (not shown),

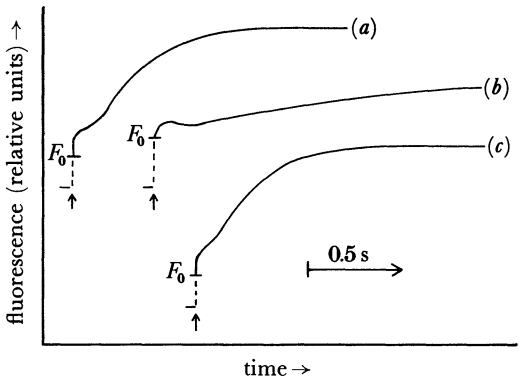

FIGURE 1. Examples of fluorescence-induction curves of unhardened spinach leaves recorded at 20 °C. (*a*) Control leaf: $F_V/F_M = 0.838$. (*b*) Leaf after 3 h photoinhibition treatment (exposure to 550 μmol quanta m^{-2} s^{-1} at 4 °C): $F_V/F_M = 0.463$. (*c*) Leaf after 3 h photoinhibition treatment (as for (*b*)) followed by 100 min recovery at 18 °C, 2.5–5 μmol quanta m^{-2} s^{-1}: $F_V/F_M = 0.808$.

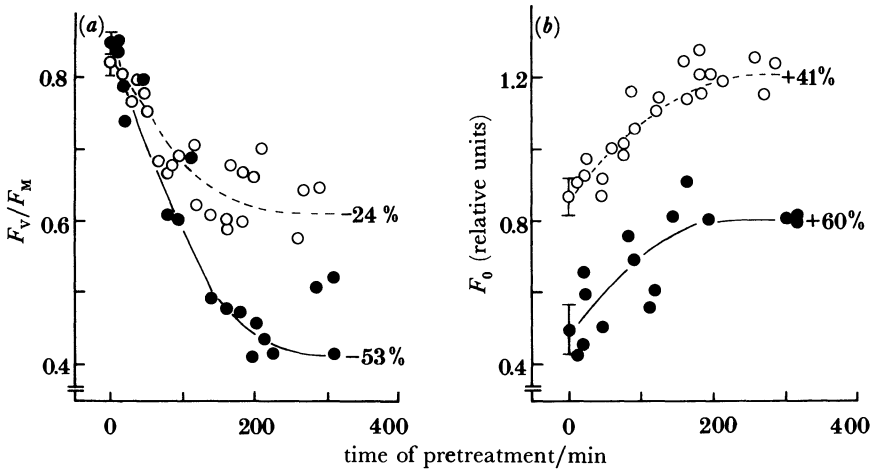

FIGURE 2. Fluorescence induction characteristics of PSII measured at room temperature (closed symbols) and at 77 K (694 nm) (open symbols) of unhardened spinach leaves exposed to 550 μmol quanta m^{-2} s^{-1} at 4 °C for different times. (*a*) F_V/F_M ratio. (*b*) F_0 (relative units). Standard deviations of the control values are indicated ($n = 8$–9).

F_V/F_M and F_0 were considerably smaller at 77 K than at 20 °C. If the quantum yield of photosynthesis is plotted against the F_V/F_M ratio, a linear relation is obtained for both temperatures (figure 3). The relatively strong scattering of data can be explained by the fact that each measuring point represents an individual leaf. Notably, the slopes of the two plots are very different. Only the 20 °C data extrapolate to the origin, and thus the F_V/F_M ratio at 20 °C can be viewed as the more suitable measure of quantum yield. A similar relation between quantum yield and F_V/F_M at 20 °C that extrapolates to the origin has been found by Leverenz & Öquist (1987) for *Pinus sylvestris* exposed to 'winter stress' (low temperatures and high light). For 77 K fluorescence, Demmig & Björkman (1987) and Demmig *et al.* (1987) also observed deviation (however, smaller than in figure 3*b*) of the extrapolation from the origin of such plots. The differences between 77 K and 20 °C data can partly be explained by different contributions of upper, more strongly inhibited, and lower layers of the leaf in the two techniques. Figure 4 shows that at 20 °C much stronger photoinhibition is apparent from the

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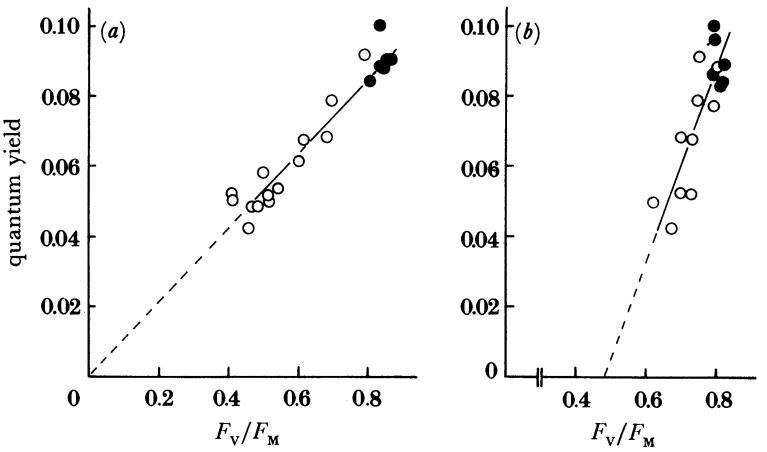

FIGURE 3. Correlation between quantum yield of O_2 evolution and F_V/F_M ratio of unhardened spinach leaves. Fluorescence measured at (*a*) room temperature and (*b*) 77 K (694 nm). Closed symbols represent the untreated control leaves and open symbols the photoinhibited leaves (exposure for different times to 550 μmol quanta m^{-2} s^{-1} at 4 °C).

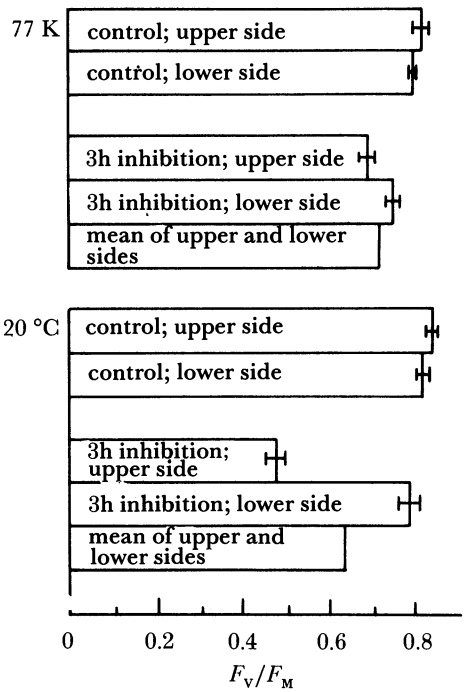

FIGURE 4. F_V/F_M ratio of PSII at 77 K (694 nm) and 20 °C of unhardened spinach leaves. Fluorescence was excited and measured from the upper and lower leaf sides, respectively. Inhibition treatment (3 h) was carried out at 4 °C and 550 μmol quanta m^{-2} s^{-1}. Standard deviations are indicated ($n = 3$–5).

decrease in F_V/F_M on the upper, as compared with the lower, side of the leaves. The more severely inhibited upper chloroplast layers should also contribute relatively more to the quantum yield, which is measured in strictly limiting light. The difference between the two leaf sides is much smaller for 77 K fluorescence. The mean values of F_V/F_M of upper and lower sides show a smaller but still significant difference between 20 °C and 77 K fluorescence. This may be based on yet another effect, possibly inhibition of electron donation to PSII, that becomes manifest only at 20 °C.

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It should be noted that at 77 K, the fluorescence yield of PSI (F_V, F_M and F_V/F_M ratios of the 735 nm band) also declined in relation to photoinhibition (not shown). This is in agreement with published data (Powles & Björkman 1982; Ögren & Öquist 1984; Demmig & Björkman 1987) and indicates that the decrease in PSII fluorescence yield is not caused by increased energy transfer to PSI but rather by increased thermal de-excitation of pigments.

All changes of quantum yield and fluorescence parameters shown in figures 2–4, as well as all changes in PSI fluorescence at 77 K, were fully reversible in dim light at 18 °C within about 3 h. Data on the 'recovery' of quantum yield and 20 °C fluorescence are presented in table 1.

TABLE 1. COMPARISON OF EFFECTS OF IRRADIATION (3 h, 550 μmol QUANTA m^{-2} s^{-1} AT 4 °C) ON FLUORESCENCE CHARACTERISTICS AND QUANTUM YIELD OF UNHARDENED AND HARDENED SPINACH LEAVES

(For unhardened leaves the values after 2.5–3.5 h recovery at 18 °C, PFD 2.5–5 μmol quanta m^{-2} s^{-1}, are also given. Fluorescence was recorded at 20 °C from the upper leaf sides. Standard deviations are indicated, $n = 3$–6.)

		unhardened	hardened
F_V	control	2.79 ± 0.32	1.08 ± 0.09
	irradiated	0.64 ± 0.07	1.00 ± 0.26
	recovered	2.81 ± 0.07	—
F_0	control	0.50 ± 0.08	0.46 ± 0.05
	irradiated	0.77 ± 0.03	0.53 ± 0.03
	recovered	0.53 ± 0.05	—
F_V/F_M	control	0.84 ± 0.02	0.70 ± 0.04
	irradiated	0.45 ± 0.04	0.65 ± 0.05
	recovered	0.85 ± 0.01	—
quantum yield	control	0.094 ± 0.007	0.076 ± 0.04
	irradiated	0.048 ± 0.004	0.069 ± 0.01
	recovered	0.086 ± 0.006	—

Changes in the fluorescence level at the 'inflection' of the Kautsky signal (F_I) differed from those of other parameters. As shown in figure 5*a*, F_I first increased and then decreased with progressing photoinhibition. This phenomenon is even more pronounced for the variable part of fluorescence at the inflection, $F_I - F_0$ (figure 5*b*). The initial increase in $F_I - F_0$ could possibly result from an inhibition of electron flow from Q_A to the plastoquinone pool (cf. Lavergne 1974). This would be consistent with an inactivation of the PSII reaction centre at the Q_B site (see Kyle 1987). The later decline in $F_I - F_0$ probably results from the general loss of variable fluorescence, in agreement with the assumed transformation of PSII reaction centres to photochemically inactive fluorescence quenchers (Cleland & Critchley 1985; Cleland *et al.* 1986; Cleland & Melis 1987). Further experimental evidence is, however, needed to prove this hypothesis. It can be seen from figure 5*b* that at the end of the recovery phase, the $F_I - F_0$ values were higher than in the control leaves (see also figure 1). The significance of this effect is unclear, but because the F_V/F_M ratio and quantum yield had been fully restored (table 1), it does not indicate a photoinhibition.

A further phenomenon of photoinhibition is seen in long-term fluorescence quenching. Figure 6 demonstrates that in control leaves the terminal fluorescence level (F_T) measured in CO_2-free air was always higher than F_0. In contrast, F_T became lower than F_0 (represented by negative $F_T - F_0$ values) in the more strongly inhibited leaves. Thus an 'F_0 quenching' (cf. Bilger & Schreiber 1986) occurred during prolonged illumination, particularly in those

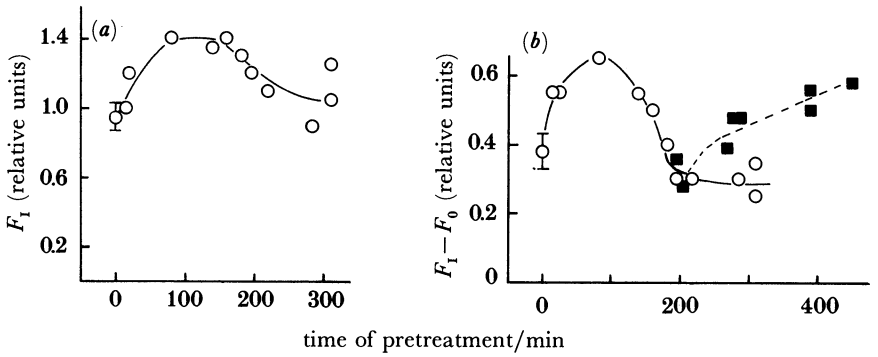

FIGURE 5. Changes in the 'inflection' (F_I) of the fluorescence-induction signal (20 °C) caused by photoinhibition and recovery treatments of unhardened spinach leaves. (*a*) F_I (relative units) as a function of irradiation time (550 μmol quanta m^{-2} s^{-1} at 4 °C). (*b*) $F_I - F_0$ (relative units) as a function of irradiation time (–○–) and following recovery treatment at 18 °C and 2.5–5 μmol quanta m^{-2} s^{-1} (--■--); recovery was started after 3 h photoinhibition treatment. Standard deviations of the controls are indicated ($n = 5$).

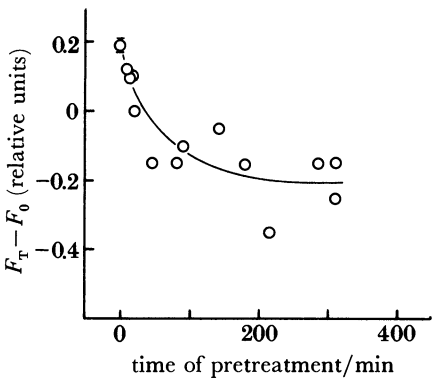

FIGURE 6. Difference ($F_T - F_0$) between the levels of steady-state ('terminal') fluorescence (F_T) and initial fluorescence (F_0) in the induction signal of unhardened spinach leaves exposed to 550 μmol quanta m^{-2} s^{-1} at 4 °C for different times. F_T was reached after about 3 min in exciting red light of 220 μmol quanta m^{-2} s^{-1}. Standard deviation of the controls is indicated ($n = 4$).

leaves that initially exhibited a strongly increased F_0. As quenching in the absence of CO_2 is dominated by the q_E mechanism (Krause *et al* 1982), a q_E-related lowering of F_0 (Weis & Berry 1987) seems to be enforced in photoinhibited leaves. The effect requires more detailed investigation.

Fluorescence characteristics of hardened leaves

After long-term cold acclimation under a controlled PFD (260–300 μmol quanta m^{-2} s^{-1}) the spinach leaves exhibited a substantially increased frost tolerance (cf. Klosson & Krause 1981). These hardened leaves showed phenomena of a weak photoinhibition, characterized by a slightly decreased F_V/F_M ratio and quantum yield of photosynthesis (table 1). Raising the temperature to 18 °C in dim light (35 μmol quanta m^{-2} s^{-1}) caused within 3–4 h an increase in F_V and F_V/F_M ratios in fluorescence signals from the upper leaf side (table 2).

Exposure of hardened leaves to higher light (550 μmol quanta m^{-2} s^{-1}) did not induce any further photoinhibition (table 1). Only above 800 μmol quanta m^{-2} s^{-1}, slight inhibition effects (lowered F_V/F_M, but no increase in F_0) were observed (not shown). Thus hardened leaves, acclimated to the same PFD as unhardened controls, exhibited a substantially increased

TABLE 2. CHARACTERISTICS OF FLUORESCENCE INDUCTION (20 °C) OF HARDENED SPINACH LEAVES EXCITED AND MEASURED FROM THE UPPER AND LOWER SIDES OF THE LEAVES, RESPECTIVELY, BEFORE AND AFTER 3 h TREATMENT AT 18 °C AND 35 μmol QUANTA m^{-2} s^{-1}

(Percentage changes induced by this treatment are given in parentheses. Standard deviations are indicated, $n = 3$–6.)

	F_0	F_V	F_V/F_M
upper side			
before treatment	0.46±0.05	1.08±0.01	0.70±0.04
after 3 h at 18 °C	0.44±0.00	1.55±0.03 (+44%)	0.78±0.003 (+11%)
lower side			
before treatment	0.35±0.05	1.11±0.11	0.76±0.02
after 3 h at 18 °C	0.37±0.03	1.32±0.05 (+19%)	0.78±0.01

resistance towards photoinhibition at chilling temperature. We are presently investigating whether this is based on increased rates of recovery (or 'repair') processes and/or on enforcements of reaction systems that prevent damaging side reactions. Both ways may, in fact, contribute to the altered response of the leaves to excess light. Data of Greer *et al.* (1986) indicate that chilling temperatures enhanced photoinhibition in *Phaseolus* owing to decreased recovery rates. Lowered susceptibility to photoinhibition observed in *Anacystis nidulans* upon acclimation to high light was found to be based predominantly on increased repair rates (Samuelsson *et al.* 1987). Long-term acclimation to low temperatures may possibly lead to an increased capacity for recovery in chilling-resistant plants. On the other hand, our results (S. Schöner and G. H. Krause, unpublished data) indicate that by cold acclimation the activities of scavenger systems for active oxygen species are increased.

Fluorescence as an indicator of freezing damage

Previous studies on the effects of freezing stress (in the absence of additional light stress) showed that CO_2 assimilation is more readily affected than the electron-transport system in the thylakoids (Klosson & Krause 1981; Strand & Öquist 1985; Rumich-Bayer & Krause 1986; Bauer & Kofler 1987). Apparently, below a certain tolerated temperature limit the light-activation system of the carbon reduction cycle becomes impaired (Rumich-Bayer & Krause 1986). Figure 7 shows that for hardened spinach leaves the F_V/F_M ratio at 20 °C is indeed only little affected at the temperature of frost pretreatment that strongly inhibits CO_2 fixation. The figure also demonstrates that the capacity of the thylakoids to undergo energy-dependent fluorescence quenching (q_{Emax}) is considerably more susceptible to freezing stress than the F_V/F_M ratio; however, it is less so than CO_2 fixation. This quenching is caused by the light-induced transthylakoid proton gradient via an unknown membrane alteration that supposedly leads to an increase in the rate-constant of thermal de-excitation in PSII (see Krause *et al.* 1983). Previous experiments with isolated mesophyll protoplasts have indicated that freezing stress affected q_E considerably more than the proton gradient (Rumich-Bayer & Krause 1986). Thus the inhibition of q_E has to be viewed as a loss of the response of the membrane to the build-up of a high ΔpH. The q_E effect is supposed to indicate a dynamic property of the thylakoids that regulates thermal energy dissipation in excess light (Krause & Behrend 1986; Krause & Laasch 1987; Weis & Berry 1987; Krause *et al.* 1988). Loss of the capacity to form q_E after impact of freezing would therefore be expected to enhance photoinhibition.

Figure 8 shows that in steady-state photosynthesis in limiting light, inhibition of

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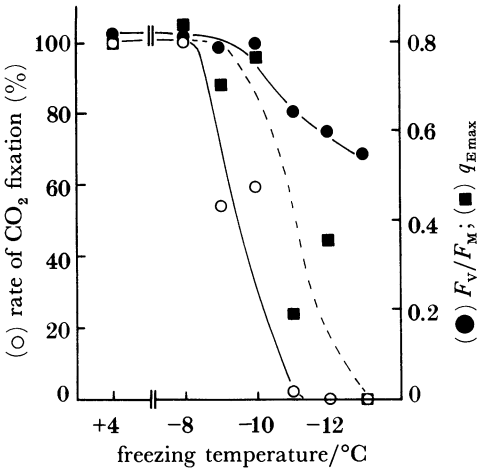

FIGURE 7. Rate of CO_2 fixation (percentage of control), F_V/F_M ratio and maximal energy-dependent quenching (q_{Emax}) as a function of minimum temperature of frost treatment. Measurements were made at 20 °C after freezing and thawing of hardened leaves. Dark-adapted leaves were irradiated in normal air with 260 W m^{-2} white light. F_V/F_M was determined after about 1 s, maximal q_E was reached after 1.5 min illumination. Light was limiting for CO_2 fixation, which was measured in the steady state (control rate 40 μmol CO_2 per milligram chlorophyll per hour).

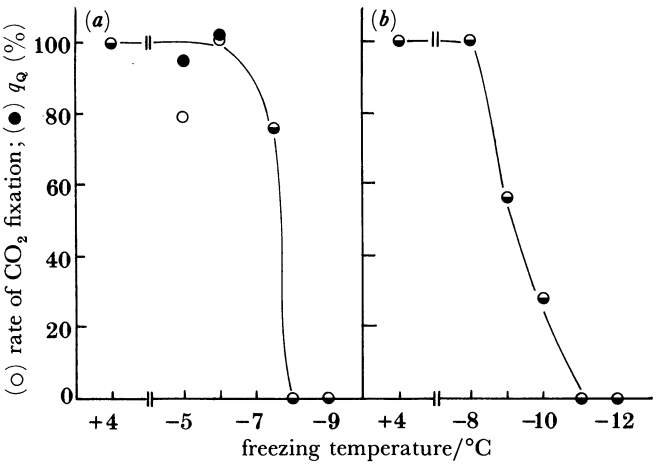

FIGURE 8. Rate of CO_2 fixation and photochemical quenching, q_Q (percentage of controls), as function of minimum freezing temperature. (*a*) Unhardened, (*b*) hardened spinach leaves. After freezing/thawing, measurements were carried out in the steady state at 20 °C (200 W m^{-2}, white light, normal air). CO_2 fixation rates of the controls were (*a*) 67 and (*b*) 53 μmol CO_2 per milligram chlorophyll per hour. Control values for q_Q were 0.65 (*a*) and 0.72 (*b*).

photochemical quenching (q_Q) by frost pretreatment is closely related to inhibition of CO_2 fixation, which occurs at lower pretreatment temperatures in hardened than unhardened leaves. Such correlation can be understood from the role of the carbon reduction cycle as the major acceptor for photosynthetically transferred electrons. Inhibition of the carbon cycle would increase the proportion of reduced Q_A and thereby inhibit q_Q. Thus measurements of q_Q may serve as a convenient, sensitive means to detect freezing damage in plant leaves.

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Conclusions

In our investigation, chlorophyll-fluorescence analyses were applied to characterize various responses of leaves of a chilling-resistant plant to low temperature and excess light. We confirm that even a moderate light flux can cause photoinhibition at chilling temperature. This may be explained by decreased 'repair' rates and/or by lowered utilization of photosynthetic energy in carbon metabolism, which imposes conditions of excess light energy. The observation of full recovery upon return to favourable conditions led us to the view that this photoinhibition does not represent damage, but rather a controlled protective mechanism. Its physiological function would be to increase thermal dissipation of excitation energy and thereby to prevent gross destruction in the thylakoid system. This mechanism allows a response of the photosynthetic apparatus within minutes to hours after changes in temperature and light conditions; however, it causes a transient decrease in the efficiency of photosynthesis.

An even faster response, occurring within seconds, is the q_E-related mechanism that also results in enhanced thermal de-excitation. In high light, the q_E effect becomes saturated (Krause & Laasch 1987; Weis & Berry 1987). Then reversible photoinhibition seems to be induced as an additional dissipative pathway. Long-term acclimation to chilling temperature and high light, occurring within days, supposedly introduces other means of protection, so that reversible photoinhibition takes place only under more extreme light conditions. Furthermore, the mechanism of photoinhibition occurring after long-term acclimation might be different from that seen in the non-acclimated state. This is indicated by different responses of F_0. Thus the photosynthetic cell is apparently capable of a dynamic adjustment to excess light in various ways and timescales.

The photoinhibition at chilling temperature is well reflected by the F_V/F_M ratio. In our experiments, the decrease in quantum yield of O_2 evolution was more closely correlated with the F_V/F_M ratio obtained at 20 °C, as compared with 77 K. A large part, but not all, of this difference can be explained by the higher contribution of upper leaf layers to fluorescence emission at 20 °C.

In contrast, the F_V/F_M ratio is not a sensitive indicator of freezing damage to the photosynthetic apparatus. Freezing and thawing of leaves leads to an inactivation of the carbon-reduction cycle before the energy-conserving system in the thylakoids is affected. Thus photochemical quenching (q_Q) is inhibited as a first effect of freezing damage. This is followed by a decrease in the capacity for energy-dependent quenching ($q_{E\,max}$), which serves as a sensitive indicator of thylakoid dysfunction. Only more severe freezing stress leads to a strong decline in F_V/F_M, which is related to an impairment of the electron transport system.

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Discussion

C. B. Osmond, F.R.S. (*Department of Botany, Duke University, Durham, U.S.A.*). Professor Krause's data confirm our experience with low (chilling) temperature photoinhibition in rice and in spinach. In rice it may be of interest that varieties differ in their response to exposure to bright light (700 μmol quanta m^{-2} s^{-1}) at 10 °C. Varieties with a short life cycle (chilling avoiders) are less sensitive to low-temperature inhibition, as indicated by changes in room temperature and 77 K fluorescence. They also show more rapid recovery from these treatments when returned to 25 °C in low light, and seem to correspond to the hardened spinach leaves described in his paper.

With respect to the greater responsiveness of F_V/F_M measured at room temperature, does Professor Krause think this might be a consequence, not only of the leaf geometry described, but also of the fact that it measures many biochemical properties of PSII energy transfer, whereas 77 K fluorescence does not?

G. H. Krause. Our data on fluorescence characteristics of upper and lower leaf sides explain only part of the difference between F_V/F_M ratios measured at 20 °C and 77 K. We have speculated that this difference might result in part from an inhibition of electron donation to PSII (which would be manifested in 20 °C fluorescence only). However, additional biochemical evidence is needed to prove this.

C. B. Osmond. Concerning Professor Krause's speculation about photochemical and biochemical consequences of low-temperature photoinhibition, we have used this treatment with unhardened spinach leaves and thylakoids isolated from them in an effort to demonstrate that PSII photochemistry can be impaired (decreased concentration of active PSII centres shown by flash-yield measurements; increased F_0 at 77 K; decreased F_V/F_M at 77 K) before changes in the concentration of the D-1 protein can be detected (Chow *et al.* 1989). Are his data consistent with the notion that at low temperatures in the light, impaired photochemistry at PSII makes the D-1 protein associated with a damaged reaction centre disposable? Is it reasonable to propose that whether or not this protein then remains in the membrane depends on the effect of temperature on the biochemistry of both protease activity (removal of D-1) and protein synthesis (replacement of D-1)?

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G. H. Krause. Further experiments are required to clarify the relation of inactivation, degradation and resynthesis of the D-1 (Q_B-binding) protein to photoinhibition under various environmental conditions. We assume that there are different mechanisms of photoinhibition; e.g. in our experiments, the increase in F_0 was fully reversible at 4 °C, whereas F_V recovered completely only at 18 °C. It is feasible that the D-1 protein is not directly involved in all cases of photoinhibition and recovery.

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J.-M Briantais (*CNRS I.P.V.*, *Gif-sur-Yvette*, 91198 *Cedex*, *France*.) Professor Krause showed a linear relation for quantum yield of electron flow versus F_V/F_M that extrapolates to zero when F_V/F_M is measured at room temperature but crosses the F_V/F_M axis when fluorescence is measured at 77 K. I suggest this indicates that the stress affects a PSII electron donor required at room temperature but does not affect (or affects less) an accessory donor that donates at 77 K.

He showed that the slope of this relation is higher at 77 K than at room temperature. Can this result from a larger dead fluorescence from PSI at 77 K?

U. Schreiber (*Lehrstuhl Botanik* 1, *Universität Würzburg*, *F.R.G.*). I completely agree with Dr Briantais that the differences in F_V/F_M observed at room temperature and 77 K are caused by a limitation at the donor side of PSII. Actually, this becomes quite clear when looking at the fluorescence-induction kinetics in saturating light: photoinhibition causes a suppression of the 'thermal' rise phase I_1–I_2, which we have shown to be dependent on rapid electron donation from watersplitting. At room temperature, PSII acceptors are continuously reoxidized either by linear electron transport or by a PSII cycle, and full reduction of Q_A can be expected only when a functional donor side provides a continuous flow of electrons from water splitting. At 77 K, Q_A reoxidation is not possible and F_M is reached even with a defective donor side as long as P^+_{680} receives one electron from a secondary donor.

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