Developmental History Affects the Susceptibility of Spinach Leaves to \textit{in Vivo} Low Temperature Photoinhibition$^1$

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ABSTRACT

Room temperature chlorophyll a fluorescence was used to determine the effects of developmental history, developmental stage, and leaf age on susceptibility of spinach to \textit{in vivo} low temperature (5°C) induced photoinhibition. Spinach (\textit{Spinacia oleracea} cv Savoy) leaves expanded at cold hardening temperatures (5°C day/night), an irradiance of 250 micromoles per square meter per second of photosynthetic proton flux density, and a photoperiod of 16 hours were less sensitive than leaves expanded at nonhardening temperatures (16 or 25°C day/night) and the same irradiance and photoperiod. This differential sensitivity to low-temperature photoinhibition was observed at high (1200) but not lower (500 or 800 micromoles per square meter per second) irradiance treatment. In spite of a differential sensitivity to photoinhibition, both cold-hardened and nonhardened spinach exhibited similar recovery kinetics at either 20 or 5°C. Shifting plants grown at 16°C (day/night) to 5°C (day/night) for 12 days after full leaf expansion did not alter the sensitivity to photoinhibition at 5°C. Conversely, shifting plants grown at 5°C (day/night) to 16°C (day/night) for 12 days produced a sensitivity to photoinhibition similar to control plants grown at 16°C. Thus, any resistance to low-temperature photoinhibition acquired during growth at 5°C was lost in 12 days at 16°C. We conclude that leaf developmental history, developmental stage, and leaf age contribute significantly to the \textit{in vivo} photoinhibitory response of spinach. These characteristics must be defined clearly in studies of plant susceptibility to photoinhibition.

Photoinhibition has been reported in many olympic plant species that have been exposed to light that exceeds that required for photosynthesis (14). This is manifested as a reversible reduction in the quantum yield and light-saturated rates of CO$_2$ uptake, CO$_2$-dependent O$_2$ evolution, or decrease in the room temperature Chl \textit{a} fluorescence ratio of \( F_v/F_m \) (3, 14). Photoinhibition measured \textit{in vivo} appears to be prevalent even under moderate light conditions when plants are subjected to environmental stresses such as chilling (1, 4–9, 11–13, 15, 18–23) and freezing (12, 20, 21, 23).

Cold-tolerant plants develop a decreased susceptibility to photoinhibition at 5°C when exposed to cold-hardening conditions (4, 5, 13, 15, 18–22). Somersalo and Krause (18) were the first to show that spinach cold hardened under controlled (19, 21, 22) or field conditions (20) exhibited a decreased susceptibility to low-temperature-induced photoinhibition. Öquist and Huner (13) have shown that the susceptibility of rye leaves to photoinhibition at low temperature depends on the leaf orientation and the cold-hardened state of the leaf material. In this report, we summarize the results of experiments designed to detail the impact of growth temperature, photoinhibitory treatment conditions, developmental history, developmental stage, and leaf age on the susceptibility of spinach leaves to low-temperature-induced photoinhibition.

MATERIALS AND METHODS

Plant Material

Spinach (\textit{Spinacia oleracea} L. cv Savoy) was grown at 5, 16, or 25°C as described previously (4, 5) at an irradiance of 250 \( \mu\text{mol m}^{-2} \text{s}^{-1} \) and a 16-h photoperiod. The second pair of leaves was utilized either at full leaf expansion or 12 d after full expansion. The second leaves reached full expansion at 27 or 87 DPE for 16 or 5°C grown plants, respectively (4, 5). Plants grown at 5°C are referred to as cold hardened, whereas those grown at 16 or 25°C are referred to as nonhardened.

Subsequently, plants were shifted from 5 to 16 or 16 to 5°C for 12 d following full expansion of the second leaf pair. Control plants were maintained at the initial growth temperature for an additional 12 d past full expansion of the second leaves to determine the effects of aging during the 12-d shift period.

Photoinhibitory Treatments

Leaf discs, 10 cm$^2$, were cut from the midportion of leaves and floated on a thin layer of water in a Petri dish that was placed on a tray of ice. Dark controls were prepared in the same way, and the Petri dish was wrapped in foil. All sampling occurred at the growth temperature 2 to 3 h after the beginning of the photoperiod. Immediately after sampling, the Petri dishes were placed under high light at 5°C. Irradiance of 500, 800, and 1200 \( \mu\text{mol m}^{-2} \text{s}^{-1} \) PPFD) provided by high-pressure sodium vapor lamps over an 8-cm layer of circulating cold water was achieved by varying the distance between the light source and the samples. Leaf disc temperature never exceeded 6°C during the photoinhibitory

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$^1$ This research was supported by an operating grant from the National Science and Engineering Research Council of Canada to N.P.A.H.

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$^3$ Abbreviations: \( F_v \), variable fluorescence (\( F_v - F_d \)); DPE, days postemergence; \( F_m \), maximum fluorescence yield after dark adaptation with all PSII reaction centers closed; \( F_m' \), minimum fluorescence yield after dark adaptation with all PSII reaction centers open.
treatment. The photoinhibitory response at 5°C was measured repeatedly over time on the same samples or by using different samples for each time period. Comparable results were obtained with both methods.

**Measurement and Recovery**

Photoinhibition was measured as a reduction in room temperature $F_v/F_m$ using a PAM Chl fluorometer (Heinz-Walz) (2, 16, 17) as described in detail previously (5). Samples photoinhibited at 5°C and 1200 μmol·m$^{-2}$·s$^{-1}$ during a period of 9 h were maintained subsequently at either 5 or 20°C with 20 μmol·m$^{-2}$·s$^{-1}$ for recovery. At prescribed times, samples were dark adapted for 1 h, and the $F_v/F_m$ was determined at room temperature.

**Apparent Quantum Yield**

$O_2$ evolution of spinach leaf discs under 5% CO$_2$ was measured between 0 to 90 μmol·m$^{-2}$·s$^{-1}$ PPFD using a Hansatech LD2 system as described in detail elsewhere (5). Apparent quantum yields were estimated using the Leaf Disk software package (Hansatech) (24).

**RESULTS**

**Effects of Irradiance and Time**

Preliminary experiments verified an earlier experiment (4, 5) that photoinhibitory reduction in apparent quantum yield for CO$_2$-dependent $O_2$ evolution was correlated with a concomitant reduction in $F_v/F_m$ obtained after dark adaptation of leaf samples. Concomitantly, a 30% reduction in PSII photochemistry (680–695 nm) relative to PSI (730–740 nm) (10) was also observed at 77K for thylakoids isolated from 16°C leaf discs exposed to 1200 μmol·m$^{-2}$·s$^{-1}$ for 6 h at 5°C (data not shown). The apparent quantum yield for $O_2$ evolution appeared to be more sensitive to photoinhibition than $F_v/F_m$, and, thus, as reported previously (3), a nonlinear relationship was observed (data not shown) (4).

Leaves grown at high temperature were significantly more susceptible to photoinhibitory treatment than the leaves grown at low temperature (Fig. 1). Even the cold-hardened spinach leaves exhibited a reduction in $F_v/F_m$ but to a much lesser extent than spinach grown at nonhardening temperatures (Fig. 1). The results were similar regardless of whether attached or detached leaves were assayed. Our results for rye (13) and wheat (V.M. Hurry, N.P.A. Huner, unpublished results) are consistent with this observation. We have never observed complete resistance to photoinhibition at 5°C as reported by Somersalo and Krause (18, 19).

The extent of photoinhibition at 5°C, measured as a reduction in the $F_v/F_m$, was dependent upon treatment irradiance and duration of exposure (Fig. 2). The $F_v/F_m$ for control discs ($F_v/F_m = 0.78 ± 0.02$ for 5°C discs and 0.79 ± 0.04 for 16°C discs) kept in the dark at 5°C was not reduced even after 9 h of treatment, which confirms that the reduction in $F_v/F_m$ was a light-dependent phenomenon. Exposure to irradiances of 500 and 800 μmol·m$^{-2}$·s$^{-1}$ for 9 h at 5°C resulted in similar reductions of 24 and 28%, respectively, in $F_v/F_m$ (Fig. 2) for leaves expanded at either 5 or 16°C. However, a differential reduction in $F_v/F_m$ was observed when samples were subjected to 1200 μmol·m$^{-2}$·s$^{-1}$ (PPFD) at 5°C. After only 3 h, the $F_v/F_m$ values were reduced by 24 and 40%, respectively, for leaves expanded at 5 and 16°C. After 9 h, the reductions were 40 and 60%, respectively.

The differential reduction in $F_v/F_m$ after treatment at 1200 μmol·m$^{-2}$·s$^{-1}$ (PPFD) for 9 h resulted principally from a differential decrease in $F_v$ (54% for 5°C discs and 71% for 16°C discs). No significant changes in $F_o$ were observed between 5 or 16°C controls and treated leaf discs (Table 1).

**Recovery Kinetics**

Recovery of $F_v/F_m$ at 20°C and 20 μmol·m$^{-2}$·s$^{-1}$ exhibited similar biphasic kinetics in both the 5 and 16°C leaf samples.

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**Figure 1.** Effects of growth temperature on the response of $F_v/F_m$ to photoinhibitory treatment at 5°C. Fully expanded second leaves of spinach grown at 5, 16, or 25°C were exposed to 1200 μmol·m$^{-2}$·s$^{-1}$ (PPFD) at 5°C for the durations indicated. Samples were dark adapted for 1 h before determination of $F_v/F_m$. Data are the means of four replicate measurements from one experiment ± SD. Where bars do not appear, the SD was less than the symbol size.

**Figure 2.** Effects of irradiance and duration on the degree of photoinhibition measured as a reduction in $F_v/F_m$. Fully expanded second leaves of spinach grown at 5 or 16°C were subjected to 1200, 800, or 500 μmol·m$^{-2}$·s$^{-1}$ (PPFD) at 5°C for the times indicated and then dark adapted for 1 h at room temperature before measurement. Data are means of four replicate measurements from one experiment ± SD.
were dark adapted SD.

Where 3. Resulted

rye (13) leaves, values after of at great reduced nonhardened and (Fig. 3). Within 1.5 h at 20°C, the F/Fm had recovered to 79 and 86% of the original values for the 16 and 5°C expanded leaves, respectively. Samples had recovered to 90% of their pretreatment values by 3 h and were fully recovered by 24 h (Fig. 3). Recovery resulted principally from an increase in Fm.

Recovery at 5°C also proceeded with biphasic kinetics but at greatly reduced rates. Both 5 and 16°C leaf samples exhibited 44% recovery of F/Fm after 10 h and full recovery after 55 h (4). The similar recovery kinetics for cold-hardened and nonhardened spinach are consistent with our results for rye (13) and wheat (V.M. Hurry, N.P.A. Huner, unpublished results).

Effects of Temperature Shifts

As indicated above, 1200 µmol·m⁻²·s⁻¹ (PPFD) at 5°C resulted in greater inhibition in 16°C expanded leaves than those expanded at 5°C. We wished to determine whether exposure of nonhardened, fully expanded, second-leaf pairs to low temperature (16 → 5°C) could induce a decreased susceptibility to low-temperature photoinhibition. Nonhardened 16°C plants shifted to 5°C for 12 d (16 → 5°C) exhibited a similar 50% reduction in F/Fm after treatment as observed for 16°C control plants (Table I). Again, this was due to a decrease in Fm with no significant change in Fv. Thus, fully expanded leaves of nonhardened plants do not acquire any resistance to low-temperature photoinhibition within 12 d at 5°C.

When fully expanded second-leaf pairs of cold-hardened spinach plants were shifted to warm temperatures (5 → 16°C) (Table I) for 12 d, they exhibited F/Fm values similar to those observed for nonhardened leaves. Thus, the resistance to photoinhibition acquired during growth at 5°C is lost in 12 d at 16°C.

Developmental age may affect susceptibility to photoinhibition. Thus, cold-hardened (5°C) spinach plants with fully expanded second-leaf pairs after 87 DPE were shifted to 16°C for an additional 12 d. Because 5°C plants at 87 DPE and 16°C plants at 27 DPE were at a similar developmental stage before the shift, we used 16°C plants at 39 DPE as a control. Age of development had little effect because both treatments produced similar susceptibilities to low-temperature photoinhibition (Table I). Conversely, nonhardened, 16°C plants at 27 DPE were shifted to 5°C for 12 d. Thus, 5°C plants at 99 DPE were used as controls. The plants shifted from 5 → 16°C exhibited similar susceptibilities to low-temperature photoinhibition as did plants shifted from 16 → 5°C for an additional 12 d (Table I). Thus, it appears that developmental aging during the 12-d shift period at either 5 or 16°C can account for most of the observed increase in susceptibility to photoinhibition as a result of a temperature shift.

Effects of Developmental Age

The results of the shift experiments indicate that plant age may significantly affect susceptibility to low-temperature photoinhibition. To clarify this, susceptibility of second-leaf

<table>
<thead>
<tr>
<th>Treatment</th>
<th>F/Fm</th>
<th>Fv/Fm</th>
<th>n</th>
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<tr>
<td>5°C Control</td>
<td>0.79 (0.03)</td>
<td>2.22 (0.38)</td>
<td>8.54 (1.54)</td>
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<tr>
<td>Treated</td>
<td>0.58 (0.10)</td>
<td>2.58 (0.55)</td>
<td>3.89 (1.48)</td>
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<td>16°C Control</td>
<td>0.80 (0.02)</td>
<td>2.08 (0.39)</td>
<td>8.44 (1.37)</td>
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<tr>
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<td>2.44 (1.28)</td>
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<tr>
<td>5 → 16°C</td>
<td>0.77 (0.02)</td>
<td>2.71 (0.48)</td>
<td>9.16 (0.81)</td>
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<tr>
<td>Treated</td>
<td>0.34 (0.08)</td>
<td>2.77 (0.24)</td>
<td>1.42 (0.51)</td>
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<tr>
<td>16 → 5°C</td>
<td>0.79 (0.02)</td>
<td>2.20 (0.35)</td>
<td>8.38 (1.05)</td>
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<tr>
<td>Treated</td>
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<td>2.74 (0.50)</td>
<td>1.75 (0.82)</td>
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<tr>
<td>5°C, 99 DPE</td>
<td>0.78 (0.07)</td>
<td>2.18 (0.40)</td>
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<td>2.09 (0.13)</td>
<td>1.57 (0.19)</td>
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<td>16°C, 39 DPE</td>
<td>0.78 (0.02)</td>
<td>2.44 (0.11)</td>
<td>8.75 (0.77)</td>
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<tr>
<td>Treated</td>
<td>0.24 (0.07)</td>
<td>2.20 (0.42)</td>
<td>0.73 (0.35)</td>
</tr>
</tbody>
</table>

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Table I. Effects of Growth Temperature and Temperature Shifts on the Response of Chl a Fluorescence Parameters to Photoinhibition

Data were collected from fully expanded second leaves that were measured before and after a 6-h photoinhibitory treatment (treated) at 5°C and 1200 µmol·m⁻²·s⁻¹ (PPFD). Controls represent data from leaves grown at either 5 or 16°C; 5 → 16°C, leaves grown at 5°C and then shifted to 16°C for 12 d; 16 → 5°C, leaves grown at 16°C and then shifted to 5°C for 12 d; 5°C, 99 DPE, leaves grown at 5°C for 99 DPE; 16°C, 39 DPE, leaves grown at 16°C for 39 DPE. Numbers in parentheses, ± standard deviation (sd), n, number of replicate.

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Figure 3. Time course of photoinhibition at 5°C with 1200 µmol·m⁻²·s⁻¹ (PPFD) and recovery at 20°C with 20 µmol·m⁻²·s⁻¹ (PPFD). Samples were dark adapted for 1 h before the determination of F/Fm. All data are the means of four replicate measurements from one experiment ± so. Where bars do not appear, the so were less than the symbol size.
pairs to low-temperature photoinhibition was examined as a function of time during growth at 16°C (Fig. 4A) and 5°C (Fig. 4B). Second-leaf pairs at 16°C were fully expanded at 27 DPE, and those at 5°C were fully expanded at 87 DPE. Susceptibility to low-temperature-induced photoinhibition was least at or before full leaf expansion at either 16°C (compare 27 and 20 DPE) or 5°C (compare 82 and 55 DPE). However, as leaves aged after full leaf expansion had been attained, a significant reduction in resistance to photoinhibition was observed for both the 5 and 16°C second-leaf pairs. These results verify that developmental age is an important factor affecting susceptibility to low-temperature photoinhibition.

**DISCUSSION**

Growth temperature (Fig. 1) and photoinhibitory irradiance (Fig. 2) have a significant impact on low-temperature photoinhibition of spinach. In general, this is consistent with the reports of Somersalo and Krause (18–22). After 9 h, consistent with gas exchange data (5), the Fv/Fm of both cold-hardened and nonhardened spinach declined to a similar extent (25%) for the 500- and 800-µmol·m⁻²·s⁻¹ (PPFD) photoinhibitory treatments. Leaves fully expanded at 5 or 16°C and 250 µmol m⁻² s⁻¹ PPFD had identical light response curves for both O₂ evolution and CO₂ exchange at 5 and 16°C even at an irradiance as high as 800 µmol·m⁻²·s⁻¹ (PPFD) (5). Furthermore, the pigment contents per unit area were identical in the two sets of leaves (5). Thus, spinach leaves grown under the same light environments but at different temperatures possess similar abilities to adjust photosynthetically to incident light energies that are two- to threefold higher than the growth irradiance. However, when the photoinhibitory irradiance was increased to 1200 µmol·m⁻²·s⁻¹ (PPFD), i.e., fivefold greater than the growth condition, leaves expanded at 5°C were less susceptible to photoinhibition than those expanded at 16°C. We have never observed complete resistance to photoinhibition induced by low temperature.

Winter rye (13), winter and spring wheat (V.M. Hurry, N.P.A. Huner, unpublished results), and spinach (Figs. 1–4, Table I) are partially susceptible to photoinhibition at low temperatures regardless of developmental history. However, developmental history does affect the extent of this susceptibility, with plants grown at cold-hardening temperatures exhibiting a significantly greater resistance than plants grown at nonhardening temperatures.

We have noted some interesting differences between our results and those of Somersalo and Krause (18–22) even though the same plant species was used. First, Somersalo and Krause (18, 19) reported a 46% decline in the Fv/Fm of their nonhardened plants after only 3 h at 550 µmol·m⁻²·s⁻¹ (PPFD) and 4°C. The Fv/Fm of leaves fully expanded at 16°C used in our study was reduced by only 26% after 9 h at either 500 or 800 µmol·m⁻²·s⁻¹ (PPFD) and 5°C with no differential response observed between cold-hardened and nonhardened spinach. They also reported that the cold-hardened plants exhibited complete resistance to a low-temperature photoinhibitory treatment at 550 µmol·m⁻²·s⁻¹ (PPFD) at 4°C. However, the cold-hardened samples had an initial Fv/Fm of 0.70 compared with the 0.84 of their nonhardened samples. In our case, cold-hardened and nonhardened spinach controls exhibited similar values of Fv/Fm (0.78–0.80). Clearly, spinach grown and measured under our conditions was generally less sensitive to photoinhibition at low temperature and irradiance between 500 and 800 µmol m⁻² s⁻¹ than those reported by Somersalo and Krause (18, 19).

Second, the decline in Fv/Fm after our low-temperature photoinhibitory treatment is the result of a decrease in the Fo, with no significant change in Fm (Table I) regardless of the growth condition. Thus, our results indicate that low-temperature photoinhibition of spinach grown at either cold-hardening or nonhardening temperatures is probably due to a decreased efficiency of PSII reaction centers (1). In contrast, Somersalo and Krause (22) reported that photoinduction of cold-hardened spinach at low temperature was due to a different mechanism than that observed in their nonhardened spinach. Fv remained constant but Fm decreased in cold-hardened spinach, whereas nonhardened spinach exhibited an increase in Fv and a decrease in Fm (19, 22).

Third, our results indicate that cold-hardened and nonhardened spinach exhibited comparable rates of recovery when measured at low light at either 20°C (Fig. 3) or 5°C. In contrast, Somersalo and Krause (22) reported that cold-hardened spinach exhibited a greater capacity for recovery from

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**Figure 4.** Effects of leaf age on the response of Fv/Fm to photoinhibitory treatment at 5°C. The second leaves were harvested from plants grown at 16°C (A) or at 5°C (B) at different DPE. Leaf discs were exposed to 1200 µmol·m⁻²·s⁻¹ (PPFD) at 5°C for up to 9 h. Samples were dark adapted for 1 h at room temperature before determination of Fv/Fm. Data are the means of four replicate measurements from one experiment ± SD.
and D. The differences between the results of Somersalo and Krause (18–22) may, in part, be due to the different cold-hardening protocols used by these authors compared to our laboratory. In the former, mature, nonhardened plants were exposed to a combination of a stepped decrease in temperature from 18 to 1°C and an 8-h photoperiod during a 10-d period. The independent and combined effects of photoperiod and temperature on susceptibility to photoinhibition at 5°C are being investigated presently in our laboratory.

We conclude that leaf developmental history and leaf age must be clearly defined and carefully considered in studies of susceptibility to photoinhibition. Discrepancies between our results and those of Somersalo and Krause (18–22) are probably due to differences in the developmental state of leaf tissue utilized as well as photoperiod and the minimum temperature during cold hardening. The relationship among photoperiod, temperature, and changes in the susceptibility to photoinhibition will be the subject of a forthcoming paper.

ACKNOWLEDGMENTS

The helpful discussions with Drs. M. Krol, L. Lapointe, V. Hurry, and D. Campbell are gratefully acknowledged.

LITERATURE CITED