Photoinhibition of Intact Attached Leaves of C₃ Plants Illuminated in the Absence of Both Carbon Dioxide and of Photorespiration

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ABSTRACT

When leaflets of bean and leaves of other species of C₃ plants are illuminated in the absence of CO₂ and at low O₂ partial pressure, the capacity for CO₂ assimilation at saturating light and its efficiency at low light intensities are inhibited. This photoinhibition is dependent on leaflet age and period of illumination. In young leaflets and following short exposure to these photoinhibitory conditions, some recovery of CO₂ assimilation capacity is observed immediately after treatment. Following substantial (70 to 80%) photoinhibition of CO₂ assimilation, recovery in fully expanded leaflets is observed only after 48 hours in normal air. The photoinhibition is largely prevented by providing CO₂ at partial pressures equivalent to the CO₂ compensation point, or by >210 millibars O₂ which permits internal CO₂ production by photorespiration. If leaflets are illuminated in 60 microbars CO₂ and 210 millibars O₂ (the CO₂ compensation point in air), no photoinhibition is observed. Electron transport processes and fluorescence emission associated with photosystem I are inhibited in chloroplast thylakoids isolated from leaflets after illumination in zero CO₂ and 10 millibars O₂. These studies support the hypothesis that CO₂ recycling through photorespiration is one means of effectively dissipating excess photochemical energy when CO₂ supply to illuminated leaves is limited.

Excess light energy can have deleterious effects on photosynthetic organisms. This phenomenon has been termed photoinhibition (13). Algae exposed to light intensity greatly in excess of that required to saturate photosynthesis show marked reduction in photosynthetic capacity (13, 16, 21, 22). Shade plants exposed to high light intensities show reduction in rates of light-saturated and light-limited CO₂ assimilation (2, 3). Chloroplast membrane fragments (11, 12, 20) and intact isolated chloroplasts (14) suffer photoinhibition when illuminated at high light intensities or in the absence of an electron acceptor. In the above experiments it has been proposed that the light energy absorbed exceeds the capacity for electron transport and CO₂ fixation so that a large part of the excitation energy is not dissipated in an orderly fashion and the excess excitation energy may result in the inactivation of the photochemical reaction centers (2, 13).

Recently, we reported a similar photoinhibition phenomenon in intact bean leaflets following exposure to normal light intensities (19), under conditions in which the metabolism of the integrated photosynthetic carbon reduction cycle and the photorespiratory carbon oxidation cycle (1) is largely prevented. When leaflets were illuminated under conditions which prevent photorespiration and CO₂ cycling (3 h zero CO₂, 10 mbar O₂, irradiance 2,000 μE/m²·s), substantial reduction in the light-saturated and the light-limited CO₂ assimilation rate was observed (19). Cornic (6, 7) noted a similar decline in light-saturated CO₂ assimilation with Sinapis leaf fragments illuminated in a low CO₂ and O₂ atmosphere. In both sets of experiments photoinhibition was markedly dependent upon the length of exposure and the CO₂ and O₂ partial pressures throughout the photoinhibitory treatment.

Here, we report experiments with developing and with fully expanded bean leaflets and with expanded leaves of other C₃ species. These results demonstrate that the reduction in both the light-saturated and the light-limited rate of CO₂ assimilation following exposure to a photoinhibitory treatment is a general response. We also describe some aspects of the short and long term recovery from this photoinhibition. Some properties of electron transport in chloroplast thylakoids were measured after photoinhibition which show that this phenomenon is associated with substantial damage to PSI and lesser damage to PSII.

MATERIALS AND METHODS

Plant Material. Plants were grown from seed in a naturally lit and temperature-controlled glasshouse during the winter and spring months in 15-cm pots of soil. They were well watered and fertilized daily with nutrient solution (24 mM NO₃⁻, 4 mM K⁺, 4 mM Ca²⁺, 1.5 mM Mg²⁺, 13.33 mM Na⁺, 1.5 mM SO₄²⁻, and 1.33 mM PO₄³⁻). Irradiation during growth regularly reached quantum fluxes (400–700 mm) of 1,800 μE/m²·s. Plants that developed during periods of extended cloudy days were not used. The day/night temperature regime in the glasshouse was approximately 30/18 C and the RH was between 60 and 75%.

Gas Exchange Techniques. CO₂ exchange and leaf conductance measurements were made with an open system gas analysis apparatus, utilizing an IR CO₂ analyzer (model 865 Beckman Instruments, Fullerton, Calif.) and a humidity sensor (model HM-111, Weather Measure Corp., Sacramento, Calif.). The apparatus used was as previously described (19) except for the gas-mixing system. A high flow rate (10 liters/min) was required to permit measurements with large leaves (130 cm²). To achieve this a precision micro metering valve (SS-22R52 Whitey Company, Oakland, Calif.) was used to bleed 5% CO₂ in N₂ into a CO₂-free gas stream. By using a differential manometer and an electronic sensing device it was possible to maintain a constant pressure difference between the 5% CO₂ and the CO₂-free gas stream. The IR CO₂ analyzer (in the absolute mode) was used to monitor the CO₂ partial pressure.
Experiments were carried out during the photoperiod corresponding to that of growth conditions. Intact attached terminal leaflets of the first trifoliolate leaf of Phaseolus vulgaris L. (Hawkesbury Wonder) were used 25 to 30 days after sowing (average leaflet area was 90–120 cm²). Experiments with other C₃ species were conducted with large mature leaves.

Response of CO₂ Assimilation Rate to Low Irradiance (Apparent Quantum Yield). An attached leaf was exposed to a CO₂ partial pressure of 330 μbars in air at an irradiance of 160 μE/m²·s. After a constant CO₂ assimilation rate had been achieved the irradiance was reduced in four equal steps and the CO₂ assimilation was determined at least 5 min after each change. Leaf temperature was maintained at 30 ± 0.5 C and leaf to air vapor pressure difference between 12 and 20 mbars. Gas flow rate through the leaf chamber was 4 liters/min. The response of CO₂ assimilation to CO₂ partial pressure was then measured on this same leaf.

Response of CO₂ Assimilation Rate to CO₂ Partial Pressure. After measurement of the low irradiance response the irradiance was increased to 2,000 μE/m²·s. CO₂ partial pressure was maintained at 330 μbars and leaf temperature was held at 30 ± 0.5 C. After a constant CO₂ assimilation rate was achieved the CO₂ partial pressure was reduced in three steps, and after each step CO₂ assimilation was allowed to attain a steady-state. Gas flow rate through the leaf chamber was maintained at 8 liters/min and the ambient vapor pressure difference was from 12 to 16 mbars. The leaf was then exposed to a photoinhibitory treatment following which the light response (quantum yield) and CO₂ response curves were again measured in the same sequence as described above. The results of these experiments are expressed as a function of C³. C³ is calculated as:

\[ C³ = C⁰ - 1.6 \frac{AP}{G} \]

where C⁰ and C³ are the intercellular and ambient CO₂ partial pressures respectively, A is the assimilation rate, P is the atmospheric pressure, and G is the leaf conductance to H₂O.

Photoinhibitory Treatments. Photoinhibitory treatments were applied by replacing the air with CO₂-free N₂ containing the desired partial pressures of O₂ and CO₂ and illuminating leaves at 2000 μE/m²·s. The temperature of leaflets or leaves was maintained at 30 C, the gas flow rate at 8 liters/min, and vapor pressure difference at 12 to 16 mbars. The standard photoinhibitory treatment used in these experiments was a 3-h exposure to CO₂-free N₂ containing 10 mbars O₂. Further details of different treatments are given for each experiment.

Studies with Chloroplast Thylakoids. PSI and PSII activities were measured in chloroplast thylakoids isolated from one of the subterminal bean leaflets before exposure of the terminal leaflet to photoinhibitory treatments. Chloroplast thylakoids were also prepared from the treated leaflet immediately after exposure to photoinhibitory conditions. The midvein was removed and leaflets were cut into strips (2–3 mm wide) and blended in a Sorvall Omni-Mixer for 10 s (25% line voltage). The extraction buffer (50 ml) contained 0.33 M sorbitol, 30 mM Hepes (pH 7.8), 1 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM DTT, 0.5% BSA, and 2% PVP, mol wt 10,000. Cell debris was removed by filtration through two layers of Miracloth and the filtrate was then centrifuged for 5 min at 1,000g. The pellet was resuspended in 10 ml of resuspension medium (0.3 M sorbitol, 10 mM KH₂PO₄, 1 mM MgCl₂) and recentrifuged for 5 min at 1,000g. Chloroplasts and chloroplast fragments were resuspended in 2 ml of 10 mM phosphate buffer (pH 7.8) with 1 mM MgCl₂. All steps were carried out at 4 C.

The Hill reaction, in the resuspended thylakoids, was measured as O₂ evolution with ferricyanide as an electron acceptor. The assay contained 20 mM Tris-HCl (pH 7.8), 20 mM NaCl, 0.5 mM K₂Fe(CN)₆, 5 mM methylamine-HCl, and thylakoids (18–25 μg Chl) in a total of 3 ml. Ferricyanide-dependent O₂ evolution was ablished by 3.3 μM DCMU, inhibited 60% by 1 μM DBMIB. Rates of O₂ evolution using dimethylenzoxoquinone were identical to those obtained with ferricyanide.

PSI activity was measured by the methylviologen-Mehler reaction using ascorbate-DCPIP as electron donor and measuring O₂ uptake with a Rank O₂ electrode. The assay contained 20 mM Tris-HCl (pH 7.8), 33 μM DCPIP, 3.3 μM Na-ascorbate, 3.3 μM DCMU, 5 mM methylamine-HCl, and 33 μM methylviologen with chloroplasts (18–25 μg Chl) in 3 ml. No catalase activity was detected in the preparations used in these experiments. The assay was insensitive to DBMIB and cyanide.

Fluorescence emission spectrum was recorded at 77 K after freezing chloroplast fragments in 63% glycerol containing 50 mM Hepes, 100 mM sorbitol, 20 mM NaCl, and 4 mM MgCl₂. The Chl concentration was approximately 2 μg/ml and the fluorescence was recorded with a spectrophotometer incorporating automatic correction for photomultiplier and monochromator responses, and variation in output of the light source as described previously (4, 5).

Chl concentrations were calculated from spectra of aqueous acetone extracts. Samples of the same area were taken from the bean leaflet subjected to the photoinhibitory treatment and were compared with samples taken from the two lateral leaflets that were not treated. In other C₃ species samples were taken from the photoinhibitory leaf and with samples taken from a leaf of a similar age on the same plant. Fresh tissues were extracted with 80% (v/v) acetone/H₂O in a glass homogenizer. One volume of chloroplast suspension was extracted in 4 volumes of acetone. Spectra were recorded with a Varian 465 spectrophotometer.

RESULTS

Photoinhibition in Bean Leaflets Illuminated in CO₂-free N₂ Containing 10 mbars O₂. The CO₂ assimilation rate of the terminal leaflet of first trifoliolate leaves of bean was measured at intervals from 5 days after unfolding (rapid leaf expansion) through to 50 days from unfolding (early senescence). The CO₂ assimilation rate was at a maximum (16–21 μmol/m²·s at 200 μbar intercellular CO₂) in leaflets 10 to 14 days after unfolding, as leaves reached full expansion. It declined to 11 and to 2.5 μmol/m²·s at 30 and 50 days after unfolding. Unless otherwise stated, experiments with beans were carried out with leaflets which had just reached full expansion.

The effect of a standard photoinhibitory treatment on the CO₂ assimilation rate of a recently unfolded leaflet (10 days after unfolding) is shown in Figure 1. The light-saturated CO₂ assimilation rate was markedly reduced following treatment as observed previously (19). Figure 1A shows the response of CO₂ assimilation rate to different intercellular CO₂ partial pressures before and after the standard photoinhibitory treatment. In all experiments the response of CO₂ assimilation rate to increasing intercellular CO₂ partial pressure (C₃) was linear to above 120 μbars; this partial pressure of CO₂ was thus chosen as a basis for comparing rates of light-saturated CO₂ assimilation within and between experiments. Reduction of the light-saturated CO₂ assimilation rate, at an intercellular CO₂ partial pressure of 120 μbars, in the present experiment (Fig. 1A) was 66%.

The light-limited CO₂ assimilation rate was also markedly reduced after the photoinhibitory treatment (Fig. 1B). The apparent quantum yield (A was not measured in these experiments) can be calculated from the linear response of CO₂ assimilation to low irradiance and used to compare the efficiency of the light-harvesting photosynthetic system both within and between experiments. In this experiment (Fig. 1B) photoinhibition reduced the

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1 Abbreviations: C₃: intercellular CO₂ partial pressure; DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone.
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Apparent quantum yield from 0.060 to 0.027 mol CO₂ (einstein of incident light⁻¹) (55% reduction). The rate of CO₂ fixation in air remained inhibited at saturating light intensities, as previously shown (19). The dark respiration rate was not affected in any experiment.

Photoinhibition also resulted in a 17% decrease in the Chl content of the leaflet in this experiment. In most experiments where exposure to a photoinhibitory treatment caused marked inhibition of the CO₂ assimilation rate there was a small decline (approximately 10%) in the Chl content of the leaflet; however, in some experiments Chl destruction did not accompany photoinhibition.

Leaflet Age and Photoinhibition in Beans. The inhibition of CO₂ assimilation rate following exposure to a standard photoinhibitory treatment was dependent on leaflet age. Figure 2 shows that the inhibition of the light-saturated CO₂ assimilation rate and reduction in apparent quantum yield was least in a young rapidly expanding bean leaflet (6 days from unfolding) but similar in a recently fully expanded leaflet and a senescing leaflet (50 days from unfolding). Careful attention to leaf age is important in the comparative and recovery experiments described below.

Time and Temperature Dependence of Photoinhibition. The inhibition of both the light-saturated CO₂ assimilation rate (at a C₅ of 120 μbars), and the apparent quantum yield, increased to about 70% after 3- to 5-h exposures to CO₂-free N₂ containing 10 mbars O₂ at an irradiance of 2,000 μE/m²·s (Fig. 3). This time course is quite similar to experiments with leaf fragments treated at much lower light intensities (7). Exposure to the photoinhibitory treatment for 1.5 h or longer resulted in a reduction in the leaflet Chl content.

Bean leaflets were exposed for 3 h to zero CO₂ in N₂ containing 10 mbars O₂ (irradiance 2,000 μE/m²·s) at a leaflet temperature of 15, 18, and 30 C. The light-saturated rate of CO₂ assimilation was inhibited by 73, 70, and 70%, respectively. This indicates that photoinhibition was not affected by treatment temperature within this range.

In a previous communication (19) we noted that very young bean leaflets showed some recovery of CO₂ assimilation rate after initial photoinhibition. In recently fully expanded bean leaflets used in the experiments shown in Figure 3, partial recovery of CO₂ assimilation rate was also observed (Fig. 4). The percentage inhibition of CO₂ assimilation rate was greater when measured immediately after the photoinhibition treatment than when measured 1.5 h later, when a steady state was obtained. This partial recovery was observed only after relatively short photoinhibitory treatments (to 1.5 h); after longer treatments no partial recovery was observed within several hours of treatment. In other experiments we have noted that if low O₂ partial pressures (10 mbars) are used during assay of the CO₂ assimilation rate, partial recovery following photoinhibition is more pronounced. These experiments suggest the recovery process is itself O₂-sensitive. The characteristics and mechanisms of the short term recovery of CO₂ assimilation capacity in photoinhibited leaflets require further investigation.

Fig. 1. Effect of standard photoinhibitory treatment (3-h exposure to CO₂-free N₂ containing 10 mbars O₂, irradiance 2,000 μE/m²·s) on (A) light-saturated CO₂ assimilation rate at different intercellular CO₂ partial pressures; and (B) apparent quantum yield. Apparent quantum yield (mol CO₂ assimilated per incident E) is shown on the curve. (●): Before treatment; (○): after treatment.
Bean leaflets subjected to a standard 3-h photoinhibitory treatment recovered their former CO₂ assimilation rate over a longer period. A recently fully expanded bean leaflet (10 days after unfolding) showed almost complete recovery of light-saturated CO₂ assimilation rate (Fig. 5A) and apparent quantum yield (Fig. 5B) when returned to the glasshouse for 48 h. A similar potential for recovery of CO₂ assimilation capacity within 24 to 48 h was observed in both young and senescing leaflets exposed to the photoinhibitory treatment. However, with these leaflets it was not possible to separate recovery after treatment from the ontogenetic change in CO₂ assimilation rate over the recovery period. Exposure to a photoinhibitory treatment for 3 h usually caused a decline in the Chl content of the leaflet, but there was no increase in leaflet Chl content in any recovery experiment.

**Dependence of Photoinhibition on O₂ and CO₂ Partial Pressures.** The inhibition of both light-saturated and light-limited CO₂ assimilation rates in recently fully expanded bean leaflets (illuminated at 2,000 μE/m²/s for 3 h in 10 mbars O₂) was dependent on the intercellular CO₂ partial pressure maintained throughout the treatment period. Maximum inhibition of the light-saturated CO₂ assimilation rate (Fig. 6A) and the apparent quantum yield (Fig. 6B) occurred when the intercellular CO₂ partial pressure was near zero. In 10 mbars O₂ at an intercellular CO₂ partial pressure of 70 μbars (approximately equal to the CO₂ compensation point in 210 mbars O₂) there was only a small reduction in the light-saturated and light-limited CO₂ assimilation rate following a photoinhibitory treatment. In other experiments with younger leaflets (19) no photoinhibition was observed when CO₂ partial pressure was maintained at 60 to 70 μbars.

The degree of photoinhibition resulting from a 3-h exposure to zero CO₂ and irradiance of 2,000 μE/m²/s was dependent on the O₂ partial pressure maintained throughout the treatment period. Maximum inhibition of the light-saturated CO₂ assimilation rate occurred when the O₂ partial pressure was lowest (Fig. 7A) and the degree of photoinhibition decreased with increase in the O₂ partial pressure. Even in the presence of atmospheric partial pressures of O₂ (210 mbars) there was reduction of the light-saturated CO₂ assimilation rate after treatment. O₂ was less effective in preventing reduction of the apparent quantum yield (Fig. 7B); about 40% inhibition remained after treatment at zero CO₂ and 210 mbars O₂. Reduction in leaflet Chl content (about 10%) occurred regardless of the O₂ partial pressure used in these experiments.
One interpretation of the data in Figure 7 is that high levels of O₂ permit internal CO₂ production via photorespiration. However, the actual intercellular CO₂ partial pressure maintained is dependent upon the CO₂ and O₂ partial pressures and the stomatal conductance. In the experiments shown in Figure 7, high stomatal conductances, high flow rates, and zero external CO₂ ensured that the intercellular CO₂ partial pressure did not rise above 6 μbars CO₂.

There are discrepancies between the data reported here and those reported earlier (7, 19) which may be ascribed to differences in technique and leaf age. In the younger bean leaflets used previously (leaflet area 35 cm²), the time course of photoinhibition and the O₂ requirement for prevention of photoinhibition differ (19). Younger leaflets are clearly less sensitive to photoinhibition (Fig. 2) and show more rapid recovery. We suspect that such processes in younger leaves, and the very different techniques used by Cornic (7) may be responsible for the above discrepancies.

In another series of experiments the interactions of O₂ and CO₂ partial pressures were examined. Leaflets were provided with 210 mbars O₂ (normal atmospheric partial pressure) and with different partial pressures of CO₂ during the 3-h photoinhibitory period. When leaflets were illuminated at 2,000 μE/m²·s for 3 h in the presence of 210 mbars O₂ and an intercellular CO₂ partial pressure of 45 μbars or greater, no inhibition of the light-saturated CO₂ assimilation rate or of the apparent quantum yield was observed. These treatments correspond to the internal CO₂ and O₂ partial pressures prevailing at the compensation point in C₃ plants. If leaflets were treated at intercellular CO₂ partial pressures below that of the compensation point photoinhibition resulted (data not shown). These data show that illumination of leaves under conditions equivalent to the normal intercellular CO₂ and O₂ partial pressures at the CO₂ compensation point provides complete protection against photoinhibition.

Changes in Photochemical Properties of Chloroplast Thylakoids Isolated from Bean Leaflets following Photoinhibition. The inhibition of CO₂ assimilation rate and the apparent quantum yield is associated with substantial changes in photochemical properties of the chloroplast thylakoids. In thylakoids from bean leaflets the Hill reaction was consistently reduced by about 60% following a 3-h photoinhibitory treatment, but was practically unchanged if leaflets were maintained at conditions that approximate the CO₂ compensation point during illumination (Table 1). The Mehler
reaction in the same thylakoids was less consistently reduced by the photoinhitory treatment. The rates of electron transport obtained in these chloroplast thylakoid preparations were substantially lower than the light-saturated rates of CO2 fixation in intact leaflets in air (3.6 μmol/mg Chl-min). Further experiments are in progress to evaluate the changes in electron transport capacity in these preparations.

Clear evidence of substantial changes in the photochemical processes associated with PSII, following these photoinhitory treatments, was obtained from fluorescence emission spectra of thylakoids at liquid N2 temperature. Following a 3-h illumination at 2,000 μE/m2·s in zero CO2 at 10 mbar O2 the fluorescence emission at 683 nm, characteristic of PSII, is depressed by about 60% when spectra were normalized in terms of PSI fluorescence at 735 nm (Fig. 8A). If leaflets were maintained at conditions that approximate the CO2 compensation point (210 mbar O2 and 60 μbar CO2) while illuminated, no changes in the relative intensity of PSIII and PSI fluorescence (735 nm) were observed (Fig. 8B).

**DISCUSSION**

These experiments show that when the supply of external CO2 to illuminated leaves is removed, and when photorespiration (a major internal source of CO2 in the light) is prevented, intact leaflets of C3 plants are susceptible to photoinhibition. This photoinhibition is manifest as substantial reduction in the capacity for light-saturated CO2 assimilation and apparent quantum yield, as noted previously (19). The reduced apparent quantum yield in intact leaflets is reflected in a reduction of PSII activity of chloroplast thylakoids isolated from treated leaflets (Table I and Fig. 8). Further experiments are required to determine if the changes in photochemical properties are due to primary damage to the reaction centers, interference with the transfer of excitation energy to the reaction centers, or to inhibition of specific steps in electron transport. Such primary changes in the properties of thylakoid membranes may affect the coupling of photophosphorylation and/or the capacity of chloroplasts to maintain enzymes such as ribulose bisP carboxylase in a fully active state (10). One or all of these processes would result in CO2 assimilation remaining depressed following photoinhibition. The mechanism of the photoinhibition evident in these experiments is unknown.

The inhibition of CO2 assimilation following illumination in zero CO2 at low partial pressures of O2 is a general phenomenon in C3 plants. Photoinhibition can be largely prevented by increased partial pressures of CO2 and/or O2 (Figs. 6 and 7) and is prevented if the leaf is illuminated at conditions that approximate the CO2 compensation point. Evidently, carbon flux through the integrated carbon reduction and oxidation cycles, at the CO2 compensation point, allows adequate dissipation of photochemically generated energy which might otherwise result in photoinhibition. The potential contributions of additional CO2 evolving reactions in the photorespiratory carbon oxidation cycle (8) or continued CO2 production in the light via the tricarboxylic acid cycle, remain to be assessed. The possibility of additional energy dissipation in O2 uptake by a Mehler-type reaction cannot be excluded (9). However, if this reaction occurs in vivo, and is coupled to ATP synthesis, a sufficiently active sink for the ATP generated (in the absence of CO2 assimilation) is not immediately evident.

Speculations on the importance of these and other CO2-recycling processes in photosynthetic metabolism under stress conditions have been developed elsewhere (17, 18). The prevailing partial pressure of O2 in the atmosphere, and the oxygenase activity of ribulose bisP carboxylase make it inevitable that in C3 plants, photorespiration provides protection against photoinhibition in most terrestrial habitats when external CO2 supply is limited by stomatal closure in the light. This is not true of freezing tolerant C3 species in which carbon turnover is limited by very low temperatures. It is not surprising that substantial photoinhibition is observed following illumination of these leaves in the vicinity of zero C (15).

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