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Chilling-Enhanced Photooxidation[1]

THE PEROXIDATIVE DESTRUCTION OF LIPIDS DURING CHILLING INJURY TO PHOTOSYNTHESIS AND ULTRASTRUCTURE

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ABSTRACT

Chilling-induced photooxidation was studied in detached leaves of chilling-sensitive (CS) cucumber (*Cucumis sativus* L.) and chilling resistant (CR) pea (*Pisum sativum* L.). The rates of photosynthesis and respiration, measured as O_2 exchange, were found to be comparable in the two species over a temperature range of 5 to 35°C. Chilling at 5°C for 12 hours in high light (1000 microeinsteins per square meter per second) decreased CO_2 uptake 75% in detached pea leaves whereas CO_2 uptake by cucumber was reduced to zero within 2 hours. Respiration was unaffected in either species by the chilling and light treatment. Although ultrastructural alterations were apparent in chloroplasts of both species, cucumber's were affected sooner and more severely. The mechanism of photooxidative lipid peroxidation was investigated by following the production of ethane gas under a variety of conditions. Maximum ethane production occurred in the CS cucumber at low temperature (5°C) and high light (1000 microeinsteins per square meter per second). Atrazine, an inhibitor of photosynthetic electron transport, almost completely halted this chilling- and light-induced ethane production. These data, taken with those reported in an accompanying article (RR Wise, AW Naylor 1986 Plant Physiol 83: 278–282) suggest that the superoxide anion radical is generated in cucumber chloroplasts (probably via a Mehler-type reaction) during chilling-enhanced photooxidation. Parallel experiments were conducted on pea, a CR species. Detached pea leaves could only be made to generate ethane in the cold and light if they were pretreated with the herbicide parquat, a known effector of O_2^- production. Even so, pea showed no lipid peroxidation for 6 hours, at which time ethane production began and was at a rate equal to that for the chilled and irradiated cucumber leaves. The results indicate that pea has an endogenous mechanism(s) for the removal of toxic oxygen species prior to lipid peroxidation. This mechanism breaks down in pea after 6 hours in the cold, light, and the presence of paraquat.

Low temperatures and high light can cause photooxidation (a light-and oxygen-dependent bleaching [18]) in the leaves of chilling-sensitive plants (24, 30). In addition to chlorosis (24), other symptoms of chilling injury in the light include the rapid appearance of photosynthetic dysfunction (9, 19, 20, 23, 28), altered chloroplast ultrastructure (22, 27, 31), and cellular lipid degradation (5, 26).

It is reasonable to assume that chloroplasts are a primary site for photooxidative injury because these organelles absorb roughly 70% of the PAR entering the leaf mesophyll layer (11) and are oxygenic. In addition, lipid peroxidation in thylakoids is an ever-present problem. Approximately one-half of the thylakoid dry weight is composed of lipid and lipid soluble components (16) with about 20% of this total being the photosynthetic pigments (14). Thus, significantly less than half of the membrane's dry weight represents the structural lipids responsible for influencing membrane fluidity. Linolenic acid (trebly unsaturated at 18:3) accounts for as much as 90% of the esterified fatty acids in the thylakoids of some species (12) and such polyunsaturated fatty acids are particularly susceptible to peroxidation (8).

In the present study, the subcellular site of chilling-enhanced photooxidation has been localized to the chloroplast. Some of the factors required to obtain chilling-induced lipid peroxidation have also been characterized and a role for the production of the superoxide anion radical via a Mehler-type reaction is postulated. The effects of chilling and light on photosynthetic pigments and endogenous antioxidants are considered in an accompanying article (30).

MATERIALS AND METHODS

Growth and Treatment Conditions. Cucumber (*Cucumis sativus* L., cv Ashley) and pea (*Pisum sativum* L., cv Early Alaska) plants were raised from seed in the Duke University Phytotron and treated as described elsewhere (30).

Carbon Dioxide Exchange. Flat-sided flasks (model T-15, Bellco Glass Inc., Vineland, NJ) containing 12.5 cm^2 of leaf tissue were flushed for 5 min with precooled air containing 365 ppm CO_2 then placed in the cold (5°C) and light (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$). Exactly 5 min later, 1 ml of air was withdrawn from the flasks and injected into a Beckman model IR215A IRGA. The difference between the initial CO_2 level (365 ppm) and the concentration thus measured was used to calculate the average CER.[3] The IR gas analyzer was calibrated using air with known CO_2 concentration (Linde Specialty Gases) and connected to a Hewlett Packard model 3900A reporting integrator. The flasks were returned to the cold and light conditions until the next sampling period when the above flushing and sampling procedure was repeated.

Oxygen Evolution and Uptake. Oxygen exchange from leaf discs was measured in an aqueous buffer using a Clark-type electrode at various temperatures as described in Wise and Naylor (29). Measurements were usually completed within 3 min after tissue was sampled.

Electron Microscopy. Leaf tissue was prepared for EM using a rapid protocol adapted from Bain and Gove (1) and Tagaki

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[3] Abbreviations: CER, carbon exchange rate; CR, chilling-resistant plant; CS, chilling-sensitive plant; PPFD, photosynthetic photon flux density.

and Sato (21). A 15 min fixation of 2 mm² leaf pieces in 3% (v/v) glutaraldehyde in 100 mM Na cacodylate buffer (pH 7.2) was followed by two 5 min buffer rinses then a 15 min osmium post-fix (2% OsO_4 in buffer), all on ice. Dehydration was conducted at room temperature using a graded ethanol series prior to embedding in Ladd Ultra Low epoxy (Ladd Res. Ind., Inc., Burlington, VT). Over 30 separate fixation runs were performed, and all showed well preserved ultrastructural detail in spite of the abbreviated protocol.

Gas Chromatography. At appropriate time intervals (Figs. 7 and 8), the air inside the glass flasks was sampled for ethane as described elsewhere (30).

Application of Herbicides. Paraquat (100 μM) or atrazine (87 μM) were applied either separately or in the same solution to pea and cucumber by laying excised leaves abaxial-side down on an aqueous solution of the herbicide (plus 0.05% Tween 80) in the dark at room temperature for 30 min. A Brancker model SF20 (Richard Brancker Ind., Ottawa, Canada) fluorimeter was used to check Chl fluorescence at that time to ensure that the herbicides had reached their site of action. *In situ* Chl fluorescence traces are characteristically altered by the application of either paraquat or atrazine. The leaves were then blotted dry, sealed in the flat-sided glass flasks, and treated with cold and light as described previously (30). Ethane production was measured after 8 h with a gas chromatograph as outlined in Wise and Naylor (30).

RESULTS

The rapid imposition of low temperatures did not reveal significant differences between the photosynthetic capabilities of cucumber and pea leaf discs (Fig. 1). The Q_{10} values for photosynthesis between 30 and 5°C were close to 3 for both cucumber and pea. Respiration in both species declined steadily as a function of decreasing temperature (35–5°C) (Fig. 1), showing Q_{10} values of slightly less than 2.

Pea leaves were able to maintain net, albeit low and declining, CO_2 uptake rates for up to 12 h at 5°C and high light (1000 μE·m^{-2}·s^{-1}). Cucumber leaves, however, rapidly lost photosynthetic competence, falling to zero within 3 h (Fig. 2). The values for CO_2 uptake when measured immediately upon imposition of cold (Fig. 2, $t = 0$ h) are about 10% of the CER at 25°C for these plants (T Peeler, unpublished data). The sustained cucumber respiration rate from 4 to 12 h (2.5 nmol CO_2·cm^{-2}·min^{-1}, Fig. 2) is similar (given the SE) to the instantaneous rate of dark oxygen uptake measured at 5°C (Fig. 1). Indeed, neither cucumber nor pea respiration were affected by up to 12 h of chilling in the light (Fig. 3).

The initial morphological reactions of pea chloroplasts to chilling and high light were marked thylakoidal and stromal changes (*cf.* Figs. 4b and 5a), but there was no evidence of lipid peroxidation (Fig. 7B). They became misshapen and contorted by 6 h and many showed peripheral vesicles attached to the inner

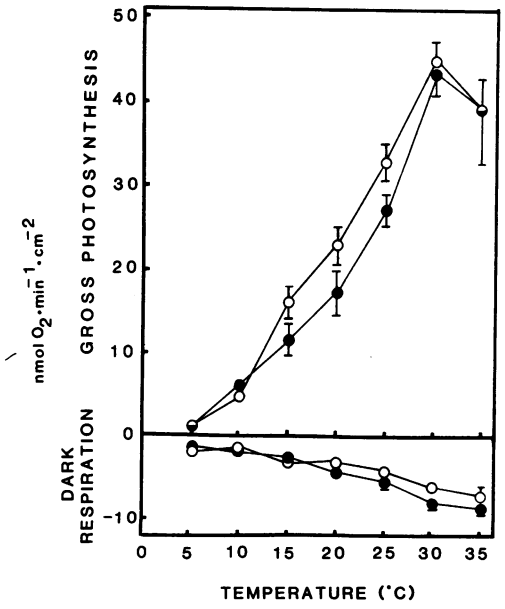


FIG. 1. Temperature dependence of O_2 evolution and uptake by leaf discs of pea (●) and cucumber (○). Gross photosynthesis was defined as light-dependent O_2 evolution plus the absolute value of dark respiration. Leaf discs (4 × 1.8 cm²) were removed from warm grown plants and immediately subjected to measurement at the indicated temperature. The results are the mean ± SE for six replicates.

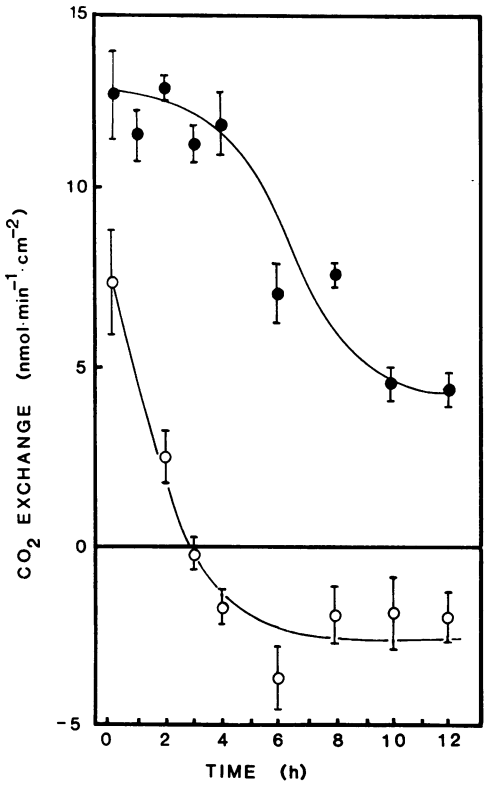


FIG. 2. Time course of CO_2 exchange by leaf segments of pea (●) and cucumber (○) maintained at 5°C and 1000 μE·m^{-2}·s^{-1}. Rates were determined hourly and leaves were held in the cold and light between measurements. The results are the mean ± SE of 4 replicates. Lines were fitted by eye.

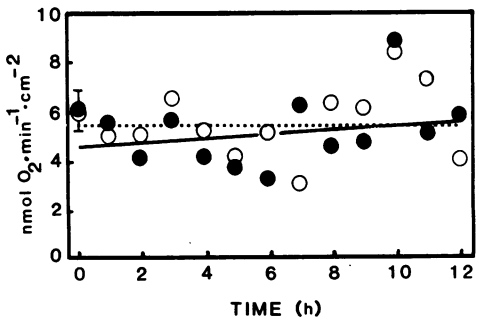
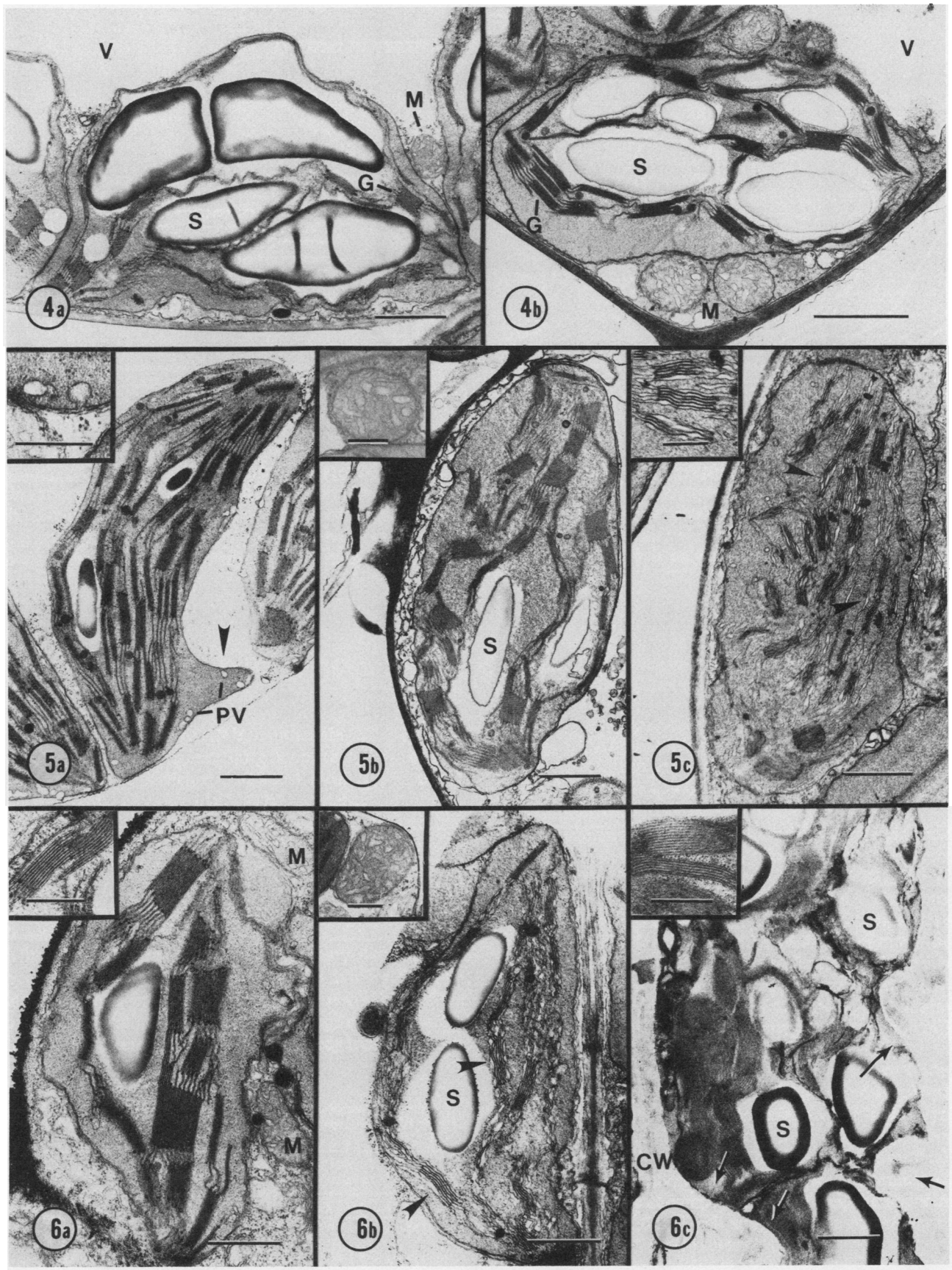


FIG. 3. Time course of dark respiration by pea (●, ——) and cucumber (○, – – –) measured polarographically in leaf discs treated at 5°C and 1000 μE·m^{-2}·s^{-1}. The measurements were taken at 25°C in the dark immediately after removal from the cold and light. Results are the mean of 4 replicates. Representative SE are given for the 0 h samples.



FIGS. 4–6. Electron micrographs of pea and cucumber chloroplasts from control and treated (5°C, 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) plants. CW, cell wall; G, granum; M, mitochondria; PV, peripheral vesicle; S, starch; V, vacuole. Bar = 1.0 μm for figures and 0.25 μm for insets.

FIG. 4. Control chloroplasts. Note normal, nondilated granal thylakoids and absence of peripheral vesicles. a, Cucumber. b, Pea.

FIG. 5. Pea chloroplasts during chilling and light stress. a, 6h. Chloroplast envelope is contorted (arrowhead) and peripheral vesicles bud from the inner envelope membrane (inset). b, 9 h. Chloroplast and mitochondria (inset) look fairly normal. c, 12 h., Thylakoids are dilated (arrowheads and inset).

FIG. 6. Cucumber chloroplasts during chilling and light stress. a, 6 h. Chloroplast has a misshapen periphery but thylakoids are not dilated (inset). Mitochondria appear normal. b, 9 h. Thylakoids are dilated (arrowheads). Mitochondria appear normal (inset). c, 12 h. Cells are collapsed, cell walls are folded (arrows), thylakoids are compressed and practically undetectable (inset).

membrane of the chloroplast envelope. Some, but fewer, peripheral vesicles were evident in the 3 h collections (not shown). These vesicles seemed to be a transient feature as none were apparent after 9 h of treatment. In contrast, pea chloroplasts after 9 h at 5°C in the light had few symptoms of injury other than a slight swelling of many plastids (Fig. 5b). By 12 h, many pea chloroplasts had an extensively dilated thylakoid system (Fig. 5c) although an equal number (not shown) remained identical to control chloroplasts. Pea mitochondria (Fig. 5b, inset) remained unaffected by the treatment. Cucumber chloroplasts subjected to 5°C and 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 6) showed a more rapid decline in ultrastructure than pea. Three h of treatment (not shown) caused most chloroplasts to swell to roundness whereas by 6 h the envelope had become quite irregular (*cf.* Figs. 4a and 6a). Within 9 h thylakoid dilation was wide spread (Fig. 6b) and by 12 h some of the cucumber tissues were completely destroyed (Fig. 6c). A gradation in the rate of plastid degradation evidently occurs because other areas of the leaf collected at 12 h (not shown) were similar in appearance on Figure 6b. Peripheral vesicles were not seen in cucumber chloroplasts at any of the sampling times. Mitochondria (Fig. 6b, inset) appeared unaltered by the cold and light treatment.

Cucumber and pea leaves were subjected to several combinations of irradiance, temperature, and herbicide application in order to characterize the factors responsible for chilling-enhanced photooxidation. Ethane gas, a commonly used measure of lipid peroxidation (4), was monitored as an indicator of photooxidative damage. Cucumber leaves generated the maximum amount of ethane when they were chilled in the presence of high light (Fig. 7A). At 25°C and high light, most of this ethane production was eliminated. Reducing the PPFD to 200 or 0 $\mu E \cdot m^{-2} \cdot s^{-1}$ during a chilling treatment also resulted in greatly diminished ethane production. Pea leaves, on the other hand, did not generate ethane under any combination of light and temperature used in this study (Fig. 7B).

Incubating cucumber leaves with paraquat, a known inducer of superoxide production (7), prior to a 12 h chilling and light treatment (Fig. 8A) did not increase ethane production above that seen in the cold and light without paraquat (Fig. 7A). Blocking photosynthetic electron flow with atrazine, however, substantially reduced ethane levels (Fig. 8A). Atrazine and paraquat applied simultaneously to cucumber leaves yielded the same response as atrazine alone. Pea leaves, which could not be made to generate ethane by cold and light (Fig. 7B), did produce this gas during paraquat treatment (Fig. 8B). Of significance is the finding that C_2H_6 generation by pea leaves in the presence of paraquat was not measurable for the first 6 h of treatment. Once ethane production began in pea (Fig. 8B) it continued at the same rate as in cucumber (Figs. 7A and 8A). The simultaneous treatment of pea leaves with paraquat and atrazine resulted in no ethane production (Fig. 8B).

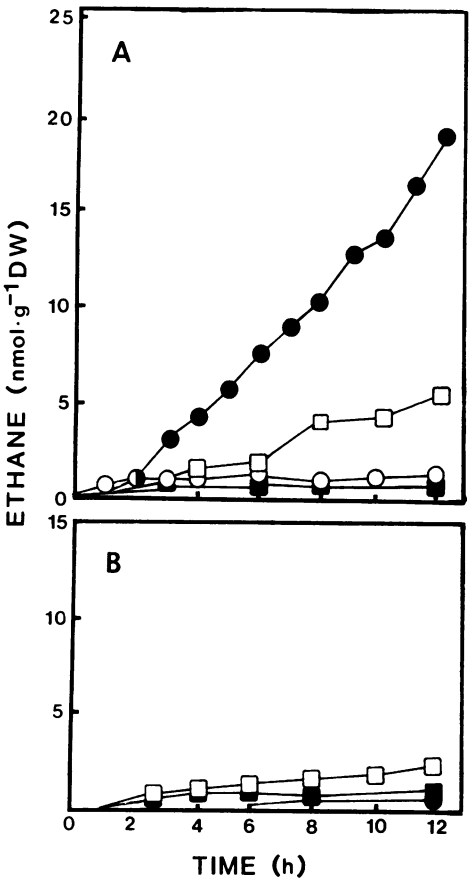


FIG. 7. Time course of ethane production by cucumber (A) and pea (B) leaf segments. Leaf segments were maintained at either 5°C and 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (●), 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ (○), or darkness (■) or at 25°C and 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (□). The results are the mean ± SE of 4 samples. SE bars were frequently smaller than symbol.

DISCUSSION

Photosynthesis, when measured in terms of O_2 evolution, declined equally in pea and cucumber as the temperature decreased from 30 to 5°C (Fig. 1). Moll and Steinback (13) obtained essentially the same result using rice (CS) and barley (CR). Longer (*i.e.* up to 12 h) exposure to chilling and light, on the other hand, did allow a discrimination between cucumber and pea in terms of their ability to maintain net CO_2 uptake. A temperature of 5°C immediately reduced both plants' CER to 10% of the rate observed using warm, attached, control leaves (T Peeler, unpublished data). Cucumber's photosynthetic rate continued to decline rapidly, whereas the rate in pea declined only 50% (Fig. 2). Thus, low temperature- and light-induced injury to photosynthesis in cucumber may not simply be a direct result of the lowered temperature but, rather, a secondary consequence of some process(es) initiated in the light. Other workers have reported a similar, rapid loss of photosynthesis during chilling in the light (9, 19, 20, 23).

Dark respiration monitored at 25°C was not altered after 12 h at 5°C and high light (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) in either pea or cucumber (Fig. 3). Following complete inhibition of CO_2 uptake in cucumber by exposure to high light at 5°C, CO_2 production continued for some 8 h (Fig. 2) at the same rate as instantaneous dark O_2 uptake at 5°C (Fig. 1). The lack of change in respiration indicated that light-induced chilling injury in cucumber was most likely restricted to metabolic processes associated with the chloroplast (and not the mitochondrion).

Ultrastructural injury was restricted to the chloroplast (Figs. 5 and 6) which is in agreement with longer term, lower irradiance studies involving other chilling-sensitive plants (15, 22, 27, 31). Of possible significance in the present study is the finding that pea chloroplasts showed signs of ultrastructural alterations (Fig. 5) in the absence of any indication of lipid peroxidation (Fig. 7). The decline of the CO_2 uptake rate in pea (Fig. 2) roughly paralleled the observed time course of thylakoid dilation and chloroplast swelling, suggesting a possible relationship between these phenomena. As anticipated, cucumber chloroplasts exhibited an earlier and more severe response than pea's when chilled in the light (Fig. 6); by 12 h approximately 50% were destroyed. Although ultrastructure during dark chilling was not followed in this study, Wise *et al.* (31) have shown that irradiance greatly enhances chilling injury to chloroplasts of cotton, bean and collard but not to other organelles. A relevant observation was made by van Hasselt (27) who achieved substantial protection of

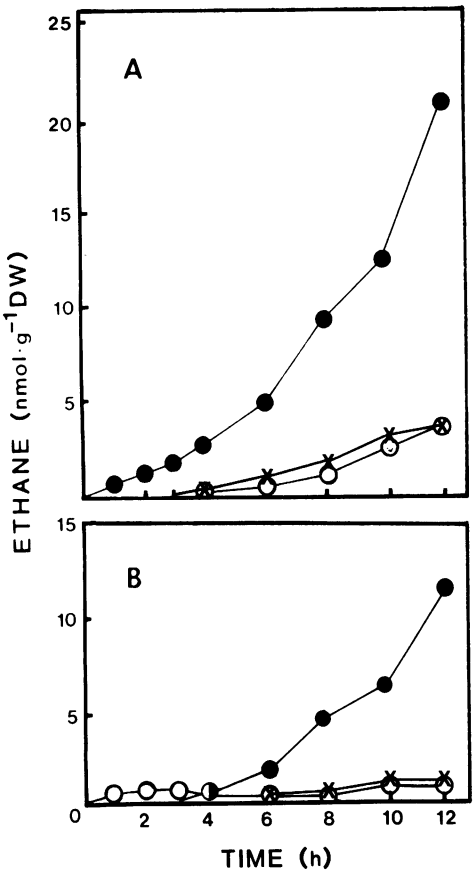


FIG. 8. Time course of ethane production by cucumber (A) or pea (B) leaf sgements maintained at 5°C and 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ following a 30 min preincubation with either paraquat (●), atrazine (○), or both paraquat and atrazine (X). Results are the mean ± SE of 4 samples. Standard error bars were frequently smaller than symbol.

cucumber chloroplast ultrastructure during chilling in the light under 100% N_2.

The morphological changes that occurred in the pea chloroplast envelope, the appearance of peripheral vesicles and irregular protrusions (Fig. 5a), are intriguing. Such changes have been reported previously in chilling-sensitive plants such as *Phaseolus vulgaris* and *Gossypium hirsutum* given a moderate stress of 5°C, 100%RH, and 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ (31) and *Glycine max* given 10°C, 85% RH, and 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ (15). The peripheral vesicles are similar in appearance to components of the chloroplast peripheral reticulum, a system of anastomosing tubules connected to the inner envelope membrane and thought to be involved in metabolite transport (10). What role peripheral vesicles play in chilling stress remains obscure. Likewise, the significance of the chloroplast envelope extensions, although also reported previously (15), is not known.

Ethane generation was monitored during chilling- and light-induced injury to photosynthesis and ultrastructure for these reasons: (a) Low temperatures and high light produce the classic symptoms of photooxidation in plants (5, 18, 20, 24–29). (b) Photooxidation caused by known inducers of free radicals (2, 3, 17) results in ethane production. (c) Ethane can be used as a measure of both *in vitro* and *in vivo* lipid peroxidation (4, 6, 8). Therefore, by monitoring ethane levels in the cold and light in both CS and CR plants, we sought to better understand the biochemical mechanism of environmentally induced photooxidation.

We found that maximum ethane production occurred in the CS cucumber under low temperature (5°C) and high light (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Fig. 7A) and was almost completely prevented by pretreatment with atrazine (Fig. 8A), an inhibitor of photosynthetic electron transport (8). In an accompanying article, we report that ethane production is greatly reduced under an atmosphere of 100% N_2 (30). These results are consistent with a scheme in which the photosynthetic generation of the superoxide anion radical, probably via the Mehler reaction (8), plays an important intermediate role in chilling-enhanced photooxidation. The involvement of singlet oxygen, as well, has been demonstrated elsewhere (30).

The mechanism(s) by which pea, a CR species, can avoid chilling-enhanced photooxidation (Fig. 7B) is largely unknown. However, the fact that it can prevent ethane generation for up to 6 h during treatment with paraquat (a known inducer of O_2^- [7]) in the cold and light (Fig. 8B) demonstrates that it can successfully detoxify free radicals prior to the lipid peroxidation stage. After 6 h, however, pea's protective mechanism fails and ethane is produced at a rate (Fig. 8B) similar to that for cucumber (Fig. 7A). We do not know if it is this ability to detoxify O_2^- at low temperatures or a lower level of free radical production (as suggested by the work in [13] that plays a role in pea's chilling resistance.

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