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Assay of Chilling Injury in Wild and Domestic Tomatoes Based on Photosystem Activity of the Chilled Leaves

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ABSTRACT

Tomato leaves were detached and stored at 0 C for various periods of time. Chloroplasts were isolated from the leaves and their photoreductive activities were determined. Comparisons were made between two altitudinal forms of the wild tomato *Lycopersicon hirsutum* Humb. and Bonpl. (a tropical lowlands form and a highlands form adapted to growth at 3,100 meters), and two cultivars of the domestic tomato *L. esculentum* Mill. In each case the capacity of the isolated chloroplasts to photoreduce ferricyanide declined linearly with time of storage of the leaves at 0 C, but not at 10 C. This injury developed more slowly in the high altitudinal form of the wild tomato compared with the low altitudinal form and the two domestic cultivars indicating an enhanced resistance toward chilling injury in the tomato from 3,100 meters. Chloroplast activity declined in green tomato fruit held at 0 C, at about the same rate as in the chilled leaves.

Measurements of photochemical activities in the isolated chloroplasts and *in vivo* measurements of cytochrome-554 photooxidation in chilled leaves showed that the site of action of the chilling effect was water donation to photosystem II.

The chilling-induced impairment of photoreductive activity in chloroplasts provides a useful assay for detecting and measuring differences in the susceptibility of plants to chilling injury.

Plant physiological studies of chilling resistance in plants, as well as the breeding and selection of varieties with enhanced cold tolerance, are hampered by the lack of assays which can detect and measure chilling-induced cellular changes well before obvious tissue damage occurs. Cellular membranes appear to be especially sensitive to chilling temperatures (6) and indeed the membrane-localized Hill reaction activity of chloroplasts declines rapidly in chilled leaves of chilling-sensitive plants (3, 5, 7, 8). This suggests the possibility of using measurements of Hill activity to detect early changes caused by chilling and to measure differences in resistance to chilling injury in closely related species.

Tomatoes, in common with most plants of tropical origin, are damaged by prolonged exposure to chilling temperatures. All stages of the plant's development are affected by chilling temperatures, including germination, growth, and fruit set (4). The harvested fruit are also susceptible to chilling injury and low temperatures can disrupt the normal ripening process. We have examined changes in the photoreductive activity of chloroplasts isolated from chilled, detached leaves of two cultivars of the domestic tomato, *Lycopersicon esculentum*, and high and low altitudinal forms of the wild tomato, *L. hirsutum*. In all cases activities declined linearly with the time that the leaves were exposed to a chilling temperature of 0 C. There was a clear distinction between the rate of decline in the high and low altitudinal forms of the wild tomato indicating a considerable adaptation to low temperature. A chilling-induced decline in photoreductive activity also took place in green fruit of the cultivar Tiny Tim.

MATERIALS AND METHODS

Cultivars of the domestic tomato (*L. esculentum* Mill.) used were Rouge de Marmande and Tiny Tim. A high and a low altitudinal form of the wild tomato (*L. hirsutum* Humb. and Bonpl.) derived from seed collected by Professor C. M. Rick (Department of Vegetable Crops, University of California, Davis) were also used. Seed of *L. hirsutum* hirsutum (designated AF, 3,100 m in text) was collected from wild populations growing at an altitude of 3,100 m in the Alta Fortaleza river valley, Ancash, Peru (9°S). Seed of *L. hirsutum* glabratum (designated MG, 30 m in test) was collected from plants growing at 30 m near Mirador Guayaquil, Guayas, Ecuador (2°S).

In comparing the different tomatoes, the plants were raised from seed in a temperature-controlled glasshouse (25 C day, 18 C night) under natural daylength. All were grown hydroponically using Hoagland solution as the source of nutrient.

Leaves were detached and then chilled by placing them in Petri dishes containing filter paper moistened with water, sealing the dishes with Gladwrap, and packing the dishes in melting ice. At least 10 fully expanded leaves were used for each sample. Control batches of leaves were stored in Petri dishes in a room maintained at 10 C.

Since light can affect chilling-induced changes in some plants (2, 3, 5), leaves stored at both 0 and 10 C were kept in total darkness. After various periods of storage, chloroplast thylakoids were isolated following the procedure described by Nolan and Smillie (9). The chilled leaves were transferred directly from the Petri dishes into ice-cold medium and blended immediately.

Mature green fruit of *L. esculentum* cv. Tiny Tim were treated in the same way except that the fruit were put in plastic bags which were then covered with melting ice.

Chloroplast activities were assayed at 23 C using an Aminco DW-2 spectrophotometer operated in the dual wavelength mode. Photoreduction of ferricyanide was measured at 420 nm minus 450 nm in a reaction mixture (1.5 ml) consisting of chloroplast thylakoids (4 μg Chl ml^{-1}), 0.05 M Sørensen's phosphate buffer (pH 7.5), 0.05 M NaCl, 0.05% (w/v) BSA, 0.34 mM $K_3Fe(CN)_6$ and gramicidin D (4 μg ml^{-1}). Photoreduction of DCIP[1] was measured at 575 nm minus 540 nm using the same reaction mixture except that 42 μM DCIP replaced the ferricyanide and gramicidin D was omitted. For both assays, red actinic light (11 $\times$ 10^4 ergs cm^{-2} s^{-1}) was provided by light from a 150-w tungsten lamp filtered through heat filters (Calfex C and Corning 1-75) and a Corning 2-60 red cut-off filter. Photoreduction of NADP using

[1] Abbreviations: DCIP: 2,6-dichloroindophenol; DPC: 1,5-diphenylcarbazide.

reduced DCIP as the electron donor (PSI activity) was measured at 350 nm minus 370 nm using a reaction mixture (1.5 ml) containing chloroplast thylakoids (8 μg Chl ml^{-1}), 0.03 M Sørensen's phosphate buffer (pH 7.5), 0.03 M NaCl, 0.03% (w/v) BSA, 0.67 mM NADP, 2.5 mM ascorbate, 63 μM DCIP, 1.4 μM ferredoxin from *Anacystis nidulans* (1), 6.7 μM DCMU, and gramicidin D (4 μg ml^{-1}). The intensity of red actinic light was 3×10^4 ergs cm^{-2} s^{-1}. The photoreduction of NADP involving PSI and PSII was measured in the same way except that the ascorbate, DCIP, and DCMU were omitted from the reaction mixture.

The photooxidation of Cyt-554 was measured in a section of leaf (1.2 × 2.5 cm) positioned at 45° to the measuring and actinic light beams. *A* changes were recorded at 554 nm with the reference wavelength set at 541 nm. Cross-illuminating actinic light was provided using the filter system described above for red light (4.8×10^4 ergs cm^{-2} s^{-1}), and the addition of a 705 nm interference filter for far red light (3.8×10^3 ergs cm^{-2} s^{-1}).

RESULTS

Chilling-induced Injury in Leaves. Experiments carried out with a number of chilling-sensitive plants including cucumber, tomato, and several tropical *Passiflora* and cereals showed that their chloroplast photoreductive activity, using ferricyanide as the electron acceptor, declined linearly with the time the detached leaves were kept at 0 C (C. Critchley and R. M. Smillie, unpublished results). Strictly chilling-resistant plants, on the other hand, showed no decrease in this activity during prolonged storage at 0 C. In these plants, chloroplast activity eventually declined in leaves stored at 10 C before there was any decrease at 0 C, whereas the opposite was true of the plants known to be susceptible to chilling injury. This distinct difference in behavior between the chilling-resistant and -susceptible plants and the linear decline in activity of the latter at 0 C, suggested that it should be possible to use chloroplast activity measurements as a way of detecting degrees of susceptibility to chilling injury in closely related chilling-sensitive species.

Figure 1 shows changes in the activities of chloroplasts isolated from chilled leaves of a low altitudinal form (MG, 30 m) of the wild tomato. Photoreductive activity declined linearly in leaves kept at 0 C but not in leaves kept at 10 C. The reaction became completely inactivated after 45 h at 0 C. The extent of the variation in the method can also be seen. Each point in the figure represents activity determinations carried out on a separate preparation of chloroplasts obtained using a minimum of 10 fully expanded leaves. The time of the day when the leaves were harvested had little effect on the assay, but to minimize possible diurnal influences on the changes subsequently induced by chilling, leaves were harvested routinely at 4 PM and stored at 10 C in the dark for 16 to 24 h before placing them in ice.

The photoreduction of NADP using ascorbate-reduced DCIP as the electron donor involves PSI but not PSII. This reaction did not change in leaves stored at 0 C for up to 80 h (Fig. 1). Both PSI activity and the photoreduction of ferricyanide showed only small changes in leaves stored at 10 C.

Figure 2 shows results obtained with the high altitudinal form (AF, 3,100 m) of the wild tomato and the domestic tomatoes. For purposes of comparison the dashed line in the figure indicates the decrease in activity shown by MG, 30 m during chilling (Fig. 1). Both domestic cultivars, Rouge de Marmande and Tiny Tim, showed a susceptibility to chilling injury comparable to the lowland wild tomato. This activity decreased more slowly in chilled leaves of AF, 3,100 m compared with the other tomatoes. Whereas AF, 3,100 m is chilling-sensitive, it shows greater resistance to a 0 C stress than either MG, 30 m or the domestic tomatoes. Chloroplast activity did not decline in leaves of AF, 3,100 m or the *L. esculentum* cultivars kept at 10 C for 80 h.

The intersection of the *x* axis with the line showing the decline in photoreductive activity can be used as a measure of relative susceptibility to chilling injury. This point appeared to coincide with the development of massive tissue injury when the chilled leaves were returned to room temperature, even under conditions of high humidity. When leaves of MG, 30 Rouge de Marmande or Tiny Tim were kept at 0 C for 2 days and then warmed to 22

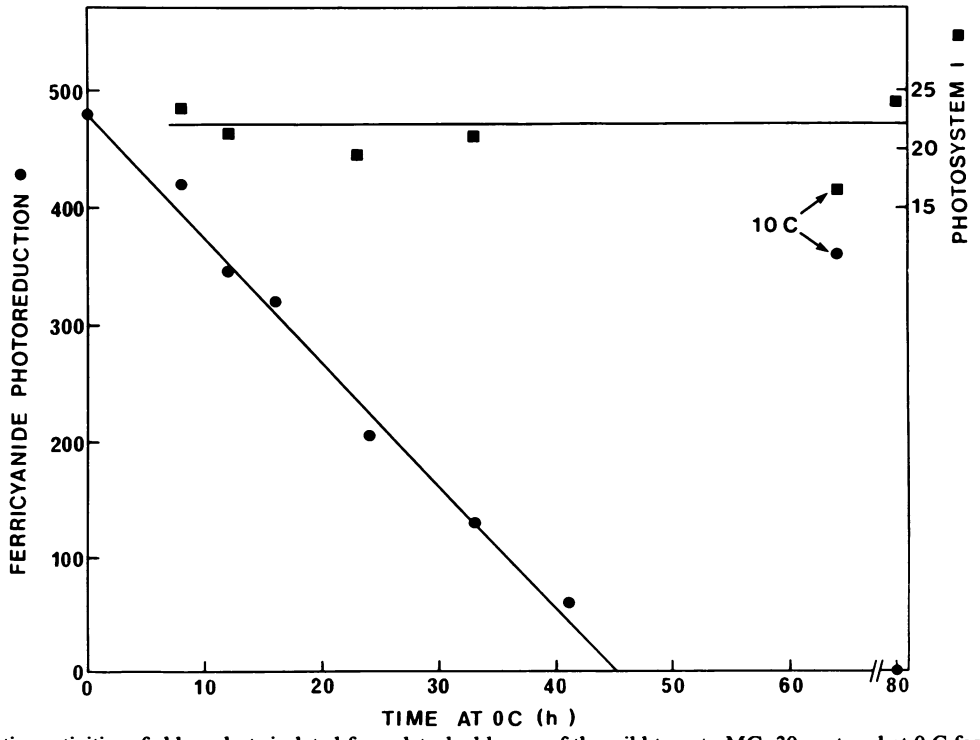

FIG. 1. Photoreductive activities of chloroplasts isolated from detached leaves of the wild tomato MG, 30 m stored at 0 C for various periods. (■): PSI activity in μmol NADP photoreduced h^{-1} (mg Chl)$^{-1}$ (●): photoreduction of ferricyanide in μmol ferricyanide reduced h^{-1} (mg Chl)$^{-1}$. Values obtained with leaves stored at 10 C are indicated on the graph.

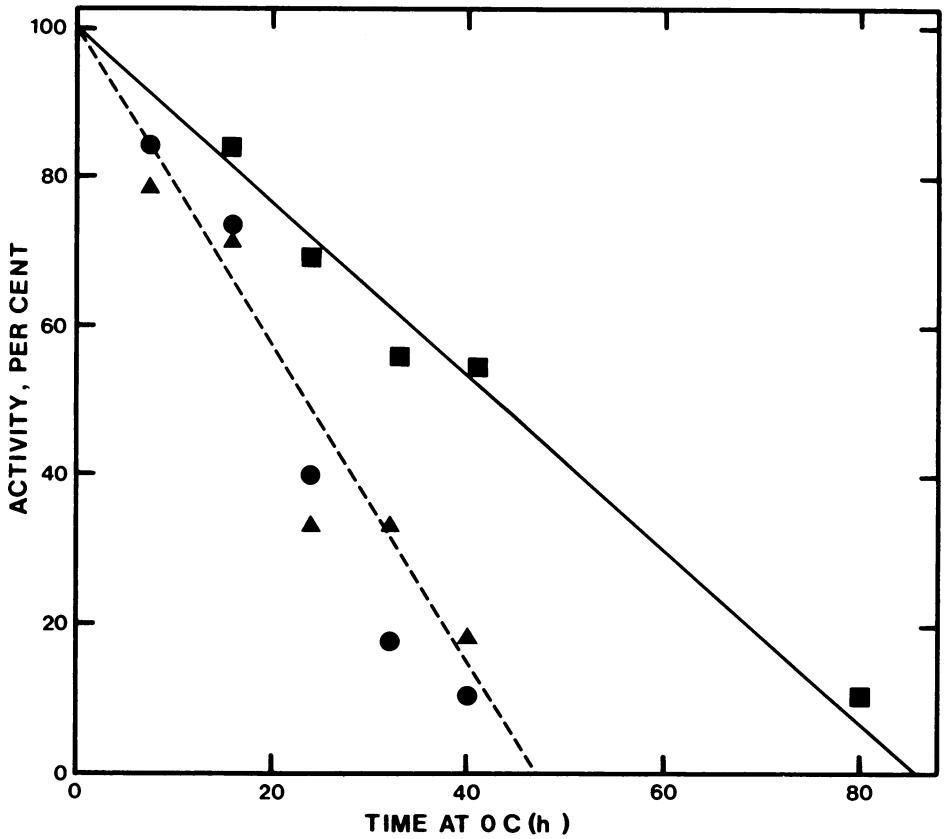

FIG. 2. Photoreductive activities of chilled leaves of the wild tomato AF, 3,100 m and two cultivars of domestic tomato. The photoreduction of ferricyanide was measured in chloroplasts isolated from detached leaves kept at 0 C for the times indicated. (■): AF, 3,100; (●): *L. esculentum* cv. Rouge de Marmande; (▲): *L. esculentum* cv. Tiny Tim. Dashed line indicates decline in activity for MG, 30 m (Fig. 1).

C they developed obvious symptoms of chilling injury over the next 2 to 3 days and most of the leaves died. In contrast all AF, 3,100 m leaves treated in the same way survived and showed little if any evidence of injury. Reliable assays for chilling injury in leaves based on visible symptoms, do not appear to be available and we have been also unable to develop satisfactory measurements of chilling injury based on the appearance of symptoms of injury at 0 C or upon rewarming. The lack of suitable assays for chilling injury was the main stimulus for looking at the chloroplast activities (see introductory section).

Site of Action of the Chilling Effect. Storage of tomato leaves at 0 C appears to inhibit specifically the capacity for electron donation from water to PSII. This is shown in Figure 3 using chloroplast thylakoids isolated from leaves of MG, 30 m and AF, 3,100 m stored for 80 h at 0 C. By this time photoreductive activity for ferricyanide has been lost in MG, 30 m and reduced by about 90% in AF, 3,100 m. Photoreduction of DCIP also could not be detected in MG chloroplasts, but after the addition of 0.5 mM DPC, which can donate electrons to PSII, a rapid photoreduction of DCIP occurred. This reduction was inhibited 87% by 6.7 μM DCMU. (DCIP is substituted for ferricyanide in this experiment because DPC chemically reduces ferricyanide rapidly but DCIP only slowly). The chloroplast thylakoids of AF showed some reduction of DCIP which was increased 8-fold by the addition of DPC. Both preparations showed high PSI activity. Thus, both PSII and PSI activity remained intact in these chloroplasts after 80 h of storing the leaves at 0 C, but the ability to use water as donor for PSII was either lost or greatly reduced. The site of action of the chilling stress in tomato chloroplasts is the same as in bean (7, 8) and cucumber (2, 5) leaves. This is not a universal phenomenon inasmuch as we have found in similar experiments with leaves of the yellow passion fruit (*Passiflora edulis forma flavicarpa*) that the chilling-induced decrease in activity for photoreduction of ferricyanide is accompanied by a general decline in the activities of both photosystems (unpublished experiments).

Chilling-induced Changes in the Intact Leaf. Evidence that the changes obtained with isolated chloroplasts also occur in the intact leaf was sought by measuring the photooxidation of Cyt-554 (Cyt *f*) in chilled leaves. This Cyt lies between the two photosystems and is reduced by reductants produced by PSII and oxidized by PSI. The oxidation and reduction of Cyt-554 can be monitored spectrally in intact leaves and such measurements should indicate the states of PSI and PSII in chilled leaves.

When intact and freshly detached leaves of MG and AF were illuminated with red light there was a decrease in *A* at 554 nm (Table I). The *A* changes measured at wavelengths from 540 to 570 nm indicated that these changes were due to photooxidation of Cyt-554. Illumination with far red light at 705 nm, which activates predominantly PSI, resulted in a slightly smaller *A* change and a much slower rate of reduction of the Cyt following a period of illumination. This rate of dark reduction of the Cyt can be used as a measure of PSII activity in the intact leaf since differences in the "light-off" rate reflect differences in the amount of excess reductant produced by PSII during the period of illumination.

The rate of Cyt reduction in chilled leaves of MG, 30 m was the same regardless of whether leaves were illuminated with red or far red light. This rate was also the same in fresh leaves following illumination with the far red light. These data indicate that the *A* changes in the chilled leaves were brought about by PSI alone. In chilled AF, 3,100 m leaves after illumination with red light, the rate of dark reduction was intermediate between the rates obtained with fresh leaves following illumination with red or far red light. This indicated that PSII activity was present in the chilled leaves but reduced in amount.

The results obtained with intact leaves are consistent with those

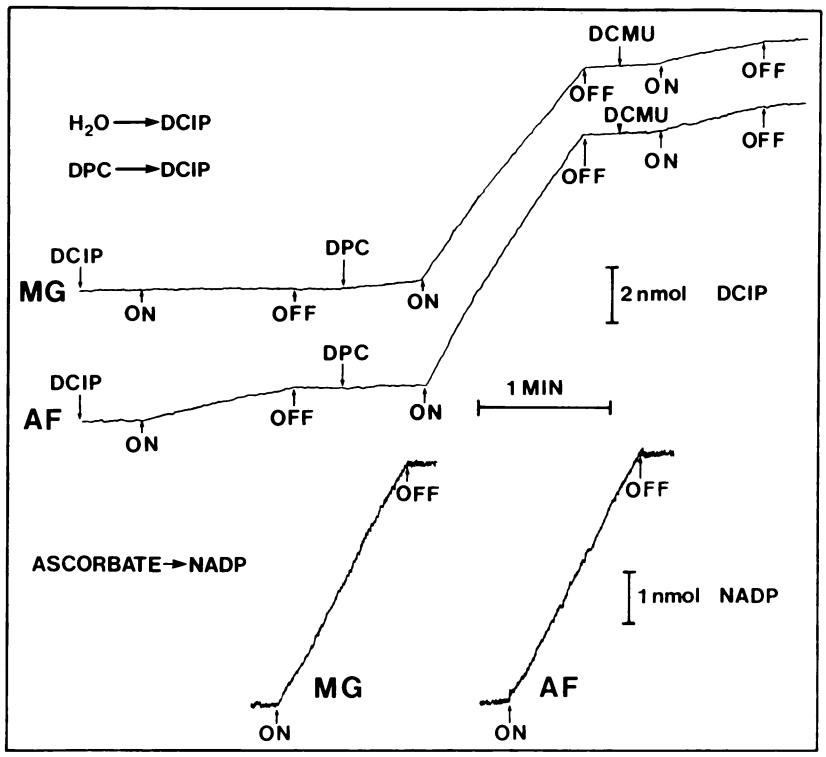

FIG. 3. Activities of chloroplasts isolated from detached leaves of the wild tomatoes MG, 30 m and AF, 3,100 m kept at 0 C for 80 h. Top portion of figure compares measurements of the photoreduction of DCIP with MG, 30 m and AF, 3,100 m chloroplasts. Additions of 0.5 mM DPC and 6.7 μM DCMU to the reaction mixtures are indicated. Bottom portion of figure compares measurements of PSI activity made with the two chloroplast preparations.

Table I. *Photo-oxidation of Cytochrome-554 in Fresh and Chilled Detached Tomato Leaves*

Tomato species	Absorbance change, light on		Initial rate, light off	
	Red light	Far red light	Red light	Far red light
	$A_{554-541} \times 10^3$		$A_{554-541} \times 10^2$ per min	
MG, 30 m				
Fresh	1.4	1.1	14.8	1.5
O C, 84h	1.3	0.8	1.6	1.6
AF, 3100 m				
Fresh	2.0	1.6	7.2	1.0
O C, 84h	1.8	1.2	3.5	1.4

obtained with the isolated thylakoids (Figs. 1–3) and indicate that the latter reflect a chilling-induced change taking place in the intact tissues.

Chilling Injury in Tomato Fruit Chloroplasts. The possibility was investigated that the assays used to follow the development of chilling injury in leaves might be applied directly to the green fruit. Chloroplasts isolated from the mature green fruit of *L. esculentum* cv. Tiny Tim were comparable in activity with chloroplasts isolated from leaves of the same plant. When the fruit was stored at 0 C, photoreductive activity, using ferricyanide as the acceptor, declined at about the same rate as was found for the leaves (Fig. 4). The same result was obtained using the PSII acceptor *p*-phenylenediamine, except that the activity did not decline to zero and a small residual activity persisted (Fig. 4).

As with the leaves, the PSI activity of the fruit choloroplasts was unaffected by the chilling stress (Table II). The photoreduction of NADP with water as the donor (involves PST and PSII, had not declined in chloroplasts isolated from fruit stored at 0 C for 1 day. Presumably the decrease in water-splitting capacity was insufficient to cause it to limit the over-all reaction, but with longer storage times a rapid decline in NADP photoreduction ensued.

DISCUSSION

The linear decrease in photoreductive activity of chloroplasts in leaves subjected to a chilling stress of 0 C provides a measure of the development of subcellular chilling injury well in advance of visible symptoms of chilling injury in the tissue itself. In order to obtain consistent results, leaves should be protected from light during chilling and any effects from rewarming (3) avoided by not allowing the temperature of the leaf samples to rise during transfer to the isolation medium. Maximum (uncoupled) rates should be measured and failure to include an uncoupler like gramicidin D in the reaction mixture can produce misleading results in chilling studies with chloroplasts as the degree to which

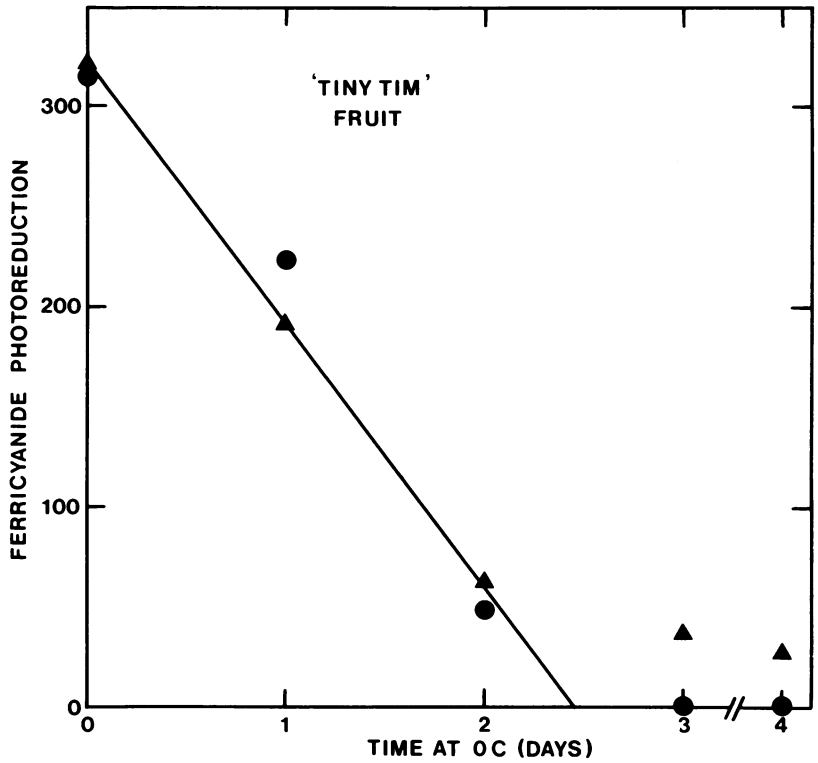

FIG. 4. Photoreductive activities of chloroplasts isolated from fruit of *L. esculentum* cv. Tiny Tim stored at 0 C for various periods. (●): photoreduction of ferricyanide in μmol h^{-1} (mg Chl)$^{-1}$. Activity was also measured in the presence of the PSII acceptor *p*-phenylenediamine (▲). In this case 1 mM *p*-phenylenediamine was included in the reaction mixture and the gramicidin D was omitted.

Table II. *Activities of Chilled Tomato Fruit*

The activities were measured on chloroplasts isolated from mature green fruit of *L. esculentum* cv. Tiny Tim stored at 0 C for the times indicated.

Days at 0 C	Photosystems I & II	Photosystem I
	μmol NADP h^{-1} (mg chl)$^{-1}$	
0	27	25
1	32	25
2	13	31
3	5	34
10	0	26

the chloroplasts are coupled changes during chilling. The feasibility of using chloroplast activity changes for comparing differences in susceptibility to chilling injury was tested using two closely related forms of the wild tomato *L. hirsutum* which differed in their chilling sensitivity. This species is found naturally over a wide range of altitudes, from near sea leavel in Ecuador and up to 3,300 m in neighboring regions of Peru (12). Of the two forms of *L. hirsutum* selected for study, AF, 3,100 m is adapted for growth at around 3,000 m whereas MG, 30 m is a lowlands form. Compared with the latter form, AF, 3,100 m shows a higher rate of protoplasmic streaming at low temperatures (10), a higher germination rate, a greater ability to synthesize Chl at low temperatures, and a higher survival rate among seedlings after exposure to 0 C (11).

Figure 2 shows that the assay based on chilling-induced decrease in photoreductive activity clearly distinguished between the two forms of *L. hirsutum* and suggests that the method could have a wide application in assaying susceptibility to chilling injury in a variety of plants. Since the skin and sometimes the underlying pulp tissues of many tropical fruits are green at some stage in their development, the results obtained with tomato fruit (Fig. 4) suggest that the assay could be adapted for making comparisons of the susceptibility of many fruits to chilling injury.

Knowledge of one subcellular location of the action of the applied chilling stress (Fig. 3) coupled with the *in vivo* demonstration of chilling injury based on changes in Cyt oxidation (Table I) also give rise to the hope that it should be possible to develop direct and nondestructive assays of the course of chilling injury in intact leaf and fruit tissues based on *in vivo* changes in such parameters as Chl fluorescence and absorption and light-induced oxidation of Cyt.

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