Plant Physiol. (1989) 89, 1372–1379
0032-0889/89/89/1372/08/$01.00/0

Received for publication August 3, 1988
and in revised form December 9, 1988

Relative Sensitivity of Photosynthesis and Respiration to Freeze-Thaw Stress in Herbaceous Species[1]

Importance of Realistic Freeze-Thaw Protocols

Kenneth L. Steffen[2], Rajeev Arora, and Jiwan P. Palta*

Department of Horticulture, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

The relative effect of a freeze-thaw cycle on photosynthesis, respiration, and ion leakage of potato leaf tissue was examined in two potato species, *Solanum acaule* Bitt. and *Solanum commersonii* Dun. Photosynthesis was found to be much more sensitive to freezing stress than was respiration, and demonstrated more than a 60% inhibition before any impairment of respiratory function was observed. Photosynthesis showed a slight to moderate inhibition when only 5 to 10% of the total electrolytes had leaked from the tissue (reversible injury). This was in contrast to respiration which showed no impairment until temperatures at which about 50% ion leakage (irreversible injury) had occurred. The influence of freeze-thaw protocol was further examined in *S. acaule* and *S. commersonii*, in order to explore discrepancies in the literature as to the relative sensitivities of photosynthesis and respiration. As bath cooling rates increased from 1°C/hour to about 3 or 6°C/hour, there was a dramatic increase in the level of damage to all measured cellular functions. The initiation of ice formation in deeply supercooled tissue caused even greater damage. As the cooling rates used in stress treatments increased, the differential sensitivity between photosynthesis and respiration nearly disappeared. Examination of agriculturally relevant, climatological data from an 11 year period confirmed that air cooling rates in the freezing range do not exceed 2°C/hour. It was demonstrated, in the studies presented here, that simply increasing the actual cooling rate from 1.0 to 2.9°C/hour, in frozen tissue from paired leaflet halves, meant the difference between cell survival and cell death.

While the effects of freezing on a number of essential cellular and subcellular processes have been extensively studied (11, 13, 14), there has been little effort to relate systematically the sequential development of injury to several of these processes during the imposition of realistic freezing and thawing stress on an intact tissue system. To the contrary, many of the investigations in this area rely solely on results obtained with isolated systems, such as chloroplasts or thylakoid membranes, which can lead to conclusions that are often non consistent with those derived from intact tissue (8, 9). Although one can gain valuable information from such studies, regarding some aspects of freeze-induced impairment of photosynthetic functions, one can clearly not gain an understanding of the interactions among the various cellular compartments within an intact tissue during freeze-thaw stress. For example, Singh *et al.* (27) have demonstrated that fully functional plant mitochondria can be isolated from cells which have been lethally damaged by freezing stress. Prolonged exposure of mitochondria to the altered cellular environment, however, was found to result in the loss of function. Little work has been done on the effect of the reversible or transitory changes in the cellular environment on organellar function.

The general preoccupation with dramatic events during experimental freezing tests, such as the rather sudden loss of cellular functions within a very narrow temperature range, has led most investigators to overlook very early events in the initial stages of freezing injury. The results of Palta *et al.* (7, 18, 19, 23) have shown that freeze-induced functional alterations of the cellular membrane transport proteins are gradual and reversible. Alterations in membrane transport properties would clearly result in an altered cellular environment. In spite of this altered environment, no changes in the ultrastructure of the chloroplasts nor the mitochondria are observed at incipient (reversible) injury, while swelling and ultrastructural disorganization are observed following a lethal (irreversible injury) freeze-thaw stress (17, 21, 22). The protocols frequently used to study freezing tolerance often produce stresses which have little relevance to freezing stress in the natural environment and tend to obscure subtle changes occurring during incipient injury. Levitt (11) noted (but provided no data) that air cooling rates in nature are usually less than 1 to 2°C/h and that ice nucleation generally occurs before tissue temperature reaches −2°C. In spite of this common observation, some recent studies investigating the mechanism of freezing injury have used cooling rates of 6 to 20°C/h (10, 25) and 60°C/h (3, 24). In some studies there is no indication that the nucleation temperature was controlled (9, 10, 25). Many studies investigating the mechanism of freezing injury to the photosynthesis apparatus using isolated membrane preparations have utilized cooling rates of 20 to 150°C/h (4, 5, 26).

The studies on freezing stress presented here were designed to investigate the alterations in cell membrane, chloroplast, and mitochondrial function following a realistic, freeze-thaw

[1] This work was supported by the College of Agriculture and Life Sciences, University of Wisconsin, Madison.

[2] Present address: Department of Agronomy, University of Kentucky, Lexington, KY 40546.

stress. The approach utilized here allowed investigation of the sequential development of injury to these three cellular functions. Our results demonstrate that (a) chloroplast, mitochondrial, and cell membrane function differ in their sensitivity to a realistic, freeze-thaw stress; (b) the conclusions on the sensitivity of these three functions are highly dependent upon the freeze-thaw protocol utilized; and (c) slight increases in freezing rates above those normally occurring in nature can mean the difference between cell survival and cell death.

MATERIALS AND METHODS

Plant Material

Two wild, tuber-bearing potato species were used in this study; *Solanum acaule* Bitt. (PI 472659) and *Solanum commersonii* Dun. (PI 472834), which when grown under the conditions described below, can survive frosts down to −6.0 and −4.5°C, respectively (2, 12). Clonal material from each species was maintained in aseptic culture on MS media (16). Single node stem sections were grown in culture tubes for 3 weeks under a continuous photoperiod with 70 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAR from cool-white fluorescent lamps (Sylvania/GTE, Danvers, MA). Rooted plantlets were then transferred to 8 L pots containing 1:1 (v/v) sphagnum and vermiculite (Jiffy Mix, JPA, West Chicago, IL). Plants were grown at 20°C/15°C (light/dark) for 6 to 8 weeks with a 14 h photoperiod of 350 to 450 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAR from cool-white fluorescent lamps. RH varied between 55 to 70% in the different experiments. Plants were irrigated four times daily with one-half strength modified Hoagland nutrient solution (6), containing an additional 2.5 mM $Ca(NO_3)_2$. To prevent salt accumulation the nutrient solution was supplied in excess at each watering.

Freezing Procedures

Experiment 1. Simulation of Natural Freeze-Thaw Stress

Leaves that were nearly 'fully expanded' (80–90% of the largest leaves on the plant) and which had been exposed to an incident light level of 375±25 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAR under controlled environment conditions (see "Plant Material") were excised from *S. acaule* or *S. commersonii* at the time of the experiment. The terminal and two subtending lateral leaflets of *S. acaule* were removed as a unit, still attached to the rachis and tissue was then exposed to a freeze-thaw stress using the procedure similar to that of Sukumaran and Weiser (30). Freezing was initiated between −0.5 and −1.0°C with ice crystals and tissue was then cooled at a rate of 1.0°C/h. The tissue temperature was monitored with 0.1 mm diameter copper-constantan thermocouples. Samples were held for 30 min at the minimum temperature before thawing for 2 h on ice. In our preliminary experiments we found that both healthy and injured leaf tissue will maintain its initial photosynthetic state when held on ice in the dark, for at least 10 h (28).

Experiment 2. Comparison of Protocols

To compare our results with those obtained by Klosson and Krause (9) we used their cooling rates in our system and simulated the possible early and late ice nucleation extremes that could occur if ice nucleation was left uncontrolled. Leaflets were selected and prepared for freezing as in experiment 1. (A) Slow freeze: This was the same procedure as used in experiment 1. (B) Fast freeze: Bath temperature was lowered from 5°C at a continuous rate of 3 or 6°C/h to −3.5 or −4.5°C and freezing was initiated at −1.0°C by nucleation with ice crystals. Bath temperature was maintained at the minimum temperature for 2 h, followed by rewarming to 5°C at the same rate as cooling. Tissue was then held on ice in the dark for up to 2 h until assays could be conducted. (C) Flash freeze: This procedure was similar to the fast freeze with the only difference being that ice nucleation was initiated when the tissue reached the minimum temperature of −3.5 or −4.5°C rather than −1.0°C. (D) Supercooling: The procedure followed here was identical to the two previous protocols except that no ice nucleation was initiated. Any samples that spontaneously nucleated, as determined by the release of latent heat, were discarded.

Experiment 3. Comparison of Two Freezing Rates on a Single Leaflet

To reduce errors due to tissue variability, the effects of two different freezing rates were studied on tissue from a single leaflet. In this approach, terminal leaflets of *S. commersonii* were divided down the midvein. Tissue from each of these paired leaflet halves were then subjected to one of two freezing rates, either slow freezing similar to experiment 1 or fast freezing similar to the fast freeze protocol in experiment 2. Freezing was initiated in leaf tissue at −0.5°C with ice crystals and then held for 30 min until tissue had frozen and equilibrated at this temperature. Frozen tissue then was cooled at the slow (1.0°C/h) or fast (2.9°C/h) rate to −3.5°C and held for 1 h once tissue had reached this minimum temperature. Tissue was then thawed on ice in the dark for at least 2 h before assays were conducted. Freshly harvested controls and chilled controls which were held on ice in the dark for the duration of the freezing and thawing treatments were also assayed for comparison.

Photosynthesis and Respiration Measurements

Assays of photosynthetic oxygen evolution and respiratory oxygen uptake were conducted in a similar manner to the procedures used in earlier studies (29). In experiment 1, four discs (13 mm diameter) were cut from two terminal leaflets which had been frozen in separate tubes. Leaf discs were infiltrated with buffer, consisting of 50 mM Hepes-KOH and 0.5 mM $CaSO_4$ (pH 7.2), and then sliced into pieces approximately 1.5 × 3.0 mm for measurement of photosynthesis and respiration in a Clark-type oxygen electrode (model 53, Yellow Springs Instruments, Yellow Springs, OH). A single leaf disc was used in experiments 2 and 3. Assay buffer was 50 mM Hepes-KOH, 0.5 mM $CaSO_4$, and 20 mM $NaHCO_3$ (pH 7.2). All light was excluded from the chamber and linear rates of respiratory oxygen uptake at 20°C were recorded for 8 min. Photosynthetic oxygen evolution was then measured on the same tissue for 15 min by supplying either 2000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAR (experiment 1) or 1750 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAR (exper-

iments 2 and 3) from a 250 W tungsten-halogen lamp (29). Chl was extracted in 96% ethanol from tissue pieces and determined spectrophotometrically according to Wintermans and Demots (31).

Ion Leakage

Tissue from the same leaflet or leaf used in the previous assays was utilized for ion leakage measurements. Leaflet midribs and margins were removed in all cases and about 0.5 g of tissue was excised. In *S. commersonii*, tissue for ion leakage measurements was taken from the terminal leaflet, whereas in *S. acaule*, tissue was taken from the subtending lateral leaflets. This tissue was infiltrated in distilled water and then shaken in 20 mL of distilled water for 1 h at 20 to 22°C. Electrical conductivity of the leachate was measured using a conductivity salt bridge (YSI conductivity meter, Yellow Springs Instruments, Yellow Springs, OH). Results were expressed as a percentage of total leakage following heat killing of tissue.

RESULTS

Experiment 1. Simulation of Natural Freeze-thaw Stress

Three important cellular functions, namely photosynthesis, respiration, and membrane permeability, differed markedly in their sensitivity to a freeze-thaw stress when intact tissue was used (Fig. 1). With increasing stress (lower temperature), an inhibition of net photosynthetic oxygen evolution was apparent much earlier than was the inhibition of respiratory oxygen uptake. Moderate declines in photosynthetic capacity, of 19 and 13% of freshly harvested controls, were seen already in tissues subjected to −2.5 and −3.0°C, respectively (Fig. 1).

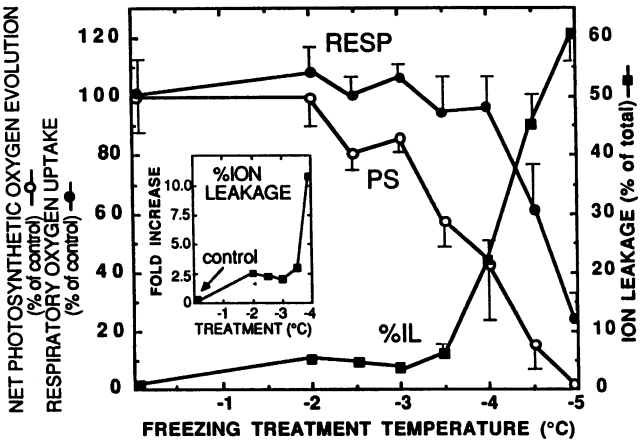

Figure 1. Effect of slow cooling and controlled ice nucleation in intact leaflet tissue from *S. acaule* on net photosynthesis, respiration, and ion leakage. All values are shown as the mean of three replications ± SD, except where the SD is less than the symbol. Values for photosynthesis and respiration are expressed as a percentage of the mean of three fresh controls. Chilled tissue behaved as freshly harvested controls in all measured parameters. Control values ± SD are: 63.4 ± 8.22 μmol oxygen evolved·(mg Chl)$^{-1}$·h^{-1} for photosynthesis; 5.7 ± 0.82 μmol oxygen uptake·(mg Chl)$^{-1}$·h^{-1} for respiration; and 1.9 ± 0.24 percentage of total solutes leaked.

At a freezing stress of −4.0°C, photosynthetic capacity had declined to 43% of control. In contrast, tissue respiration was not significantly impaired at −4.0°C (96% of control) and in fact appeared to be slightly stimulated in tissues subjected to temperatures at or above −3.5°C. A significant inhibition of respiration was first observed at −4.5°C (to 67% of control) with a concomitant drop of photosynthesis to 16% of control in the same tissue. In tissues subjected to −5.0°C, photosynthesis was completely inhibited while respiration rates were sustained at about one-third of control (Fig. 1). Similar trends were observed in *S. commersonii* (data not shown).

Ion leakage which is an indirect measurement of the integrity of cellular membranes showed a somewhat different response to increased freezing stress than did photosynthesis and respiration (Fig. 1). There was an initial doubling in the amount of ion efflux from the tissue (representing only 5% of the total solutes) at −2.0°C where no adverse effect was seen on photosynthesis and respiration (Fig. 1 and inset). Photosynthetic rates began to decline as ion leakage remained relatively stable between −2.0 and −3.5°C. However, respiration did not begin to decline until over 50% of the total solutes had leaked from the tissue (between −4.0 to −4.5°C). The chilling of freshly harvested leaf tissue on ice for 24 h caused no increase in ion leakage over fresh controls (Fig. 1) and generally gave a slight stimulation of respiration and a slight inhibition of photosynthesis (28).

Experiment 2. Comparison of Protocols

The approach in this set of experiments was to explore the various scenarios that could result from the freezing protocols used by Klosson and Krause (9). In the absence of ice formation (supercooling treatments), at cooling rates of 3.2°C/h (Fig. 2A) or 6.4°C/h (data not shown), tissue and bath temperatures were nearly identical during both cooling and rewarming. Ice formation caused tissue temperature to lag behind bath temperature during both the freezing and the thawing of leaf tissue water (Fig. 2, B and C). Ice nucleation between −0.5 and −1.0°C resulted in tissue cooling rates that were less than bath cooling rates, while the ice nucleation of supercooled tissue at −3.5 or −4.5°C, resulted in tissue cooling rates that exceeded bath cooling rates. In experiments in which ice formation was initiated between −0.5 to −1.0°C, leaf tissue cooled at 2.9°C/h when bath temperature was lowered to −3.5°C at a cooling rate of 3.2°C/h (Fig. 2B). Tissue froze at 5.1°C/h, when bath temperature was lowered to −4.5°C at a cooling rate of 6.4°C/h (data not shown). When ice nucleation was initiated in supercooled tissue at −3.5 or −4.5°C, leaf tissue froze at 3.6°C/h (Fig. 2C) and 7.1°C/h (data not shown), respectively.

Although the minimum tissue temperatures attained were identical in each set of experiments (Fig. 2, A, B, and C), the various freeze-thaw protocols had dramatically different effects on photosynthesis, respiration, and ion leakage. Photosynthesis was slightly stimulated in tissue supercooled to −3.5°C (Fig. 3A). While the slow freeze resulted in a 40% inhibition of photosynthetic capacity, the fast freeze and flash freeze treatments resulted in more dramatic inhibitions of about 80 and 90%, respectively, compared to control (Fig. 3A). The flash freezing of tissue supercooled to −3.5°C re-

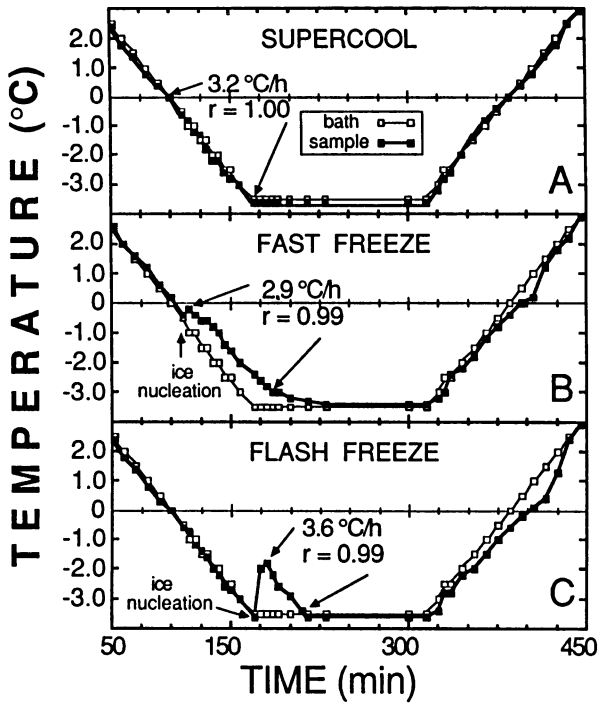

Figure 2. Time course of cooling bath and tissue temperatures using the protocols of Klosson and Krause (see "Materials and Methods" for descriptions). A representative sample is shown from each treatment. Bath temperature is indicated with open squares and tissue temperature is indicated with solid squares. The point at which ice nucleation was initiated with ice crystals is indicated. Values used to calculate the regressions for the cooling rates include all points between the arrows.

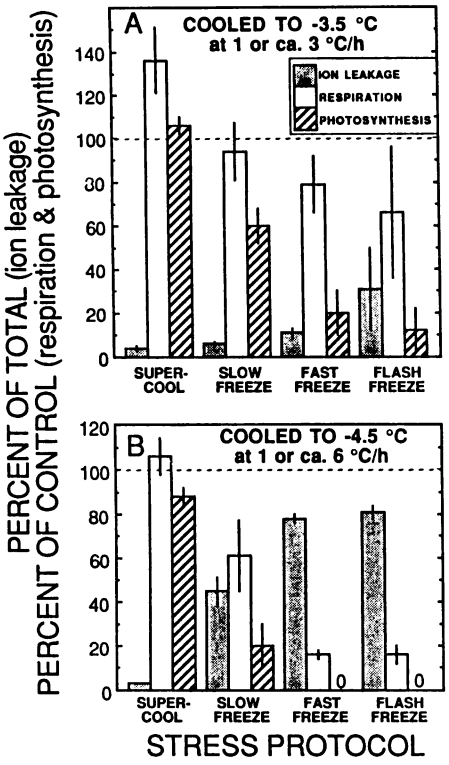

Figure 3. Comparison of the effects of freeze-thaw protocols on total photosynthetic oxygen evolution, respiratory oxygen uptake, and ion leakage. A, Leaf tissue from *S. acaule* cooled to −3.5°C; B, leaf tissue from *S. acaule* cooled to −4.5°C. Treatments are as follows: Supercool—cooling at 3°C/h (A) or 6°C/h (B) without ice formation in the tissue; Slow Freeze—cooling at 1°C/h and ice nucleation at −1.0°C (A and B); Fast Freeze—cooling at 3°C/h (A) or 6°C/h (B) with ice nucleation at −1.0°C (A and B); Flash Freeze—cooling at 3°C/h (A) or 6°C/h (B) with ice nucleation at −3.5°C (A) or −4.5°C (B). Values for total photosynthesis and respiration are percentages of freshly harvested controls ± SD, n=6. Control values ± SD are: 73.8 ± 7.47 μmol oxygen evolved·(mg Chl)$^{-1}$·h^{-1} for total photosynthesis; 7.1 ± 1.08 μmol oxygen uptake·(mg Chl)$^{-1}$·h^{-1} for respiration; and 4 ± 0.4% of total ions leaked for ion leakage.

sulted in the fastest rate of freezing (Fig. 2C) and the greatest damage (88% inhibition) to photosynthesis (Fig. 3A). Increasing the level of stress to −4.5°C and the cooling rates to 6°C/h resulted in more severe damage to photosynthesis, although the same trends among protocols were observed (Fig. 3B). Photosynthesis was inhibited 12% when tissue was supercooled to −4.5°C at 6°C/h, and inhibited by 80% when tissue experienced a slow freeze (Fig. 3B). However, photosynthetic oxygen evolution was totally eliminated in the tissue exposed to the fast and flash freeze treatments at this temperature.

In tissue supercooled to −3.5°C, respiration was markedly stimulated (136% of control), whereas the slow freeze treatment to this temperature had little effect (Fig. 3A). The fast freeze and flash freeze treatments resulted in much greater declines in respiration rates than the slow freeze treatment, with 21 and 34% reductions, respectively. In supercooled tissue, increasing the stress level to −4.5°C with a 6°C/h cooling rate, still resulted in respiration rates that were slightly higher than controls (Fig. 3B). Slow freezing to −4.5°C, gave a large decline in respiration of about 40%, whereas in the fast freeze and flash freeze treatments, respiration was reduced by about 85%. Thus, as with photosynthesis, the sensitivity of respiration to a given low temperature was determined by the protocol.

Ion leakage from supercooled tissue was similar to control following cooling to either −3.5 or −4.5°C (Fig. 3, A and B). However, a slow freeze to −3.5°C gave a slight but significant increase in electrolyte leakage compared to control (Fig. 3A). Tissue treated with fast and flash freeze treatments resulted in a marked increase in electrolyte leakage over control, 3- and 8-fold, respectively (Fig. 3A). The ion leakage at −3.5°C by fast freezing was 11%; however, this leakage was 31% in the flash freeze protocol.

An increase in the freezing stress from −3.5°C to −4.5°C resulted in a large increase in electrolyte leakage from 6 to 45% of the total solutes in the slow freeze treatment. This level of leakage corresponded to large declines in both photosynthesis (to 39% of control) and respiration (to 55% of control). The leakage observed in the fast and flash freezing treatments with cooling and rewarming rates increased to 6.4°C/h was about 80% and corresponded to almost total inhibition of both photosynthesis and respiration (Fig. 3B).

Experiment 3. Comparison of Two Freezing Rates in Divided Leaflets

In paired sections of a leaflet (Fig. 4) the slow freeze (1.0°C/h) to −3.5°C resulted in 17% ion leakage, whereas the fast

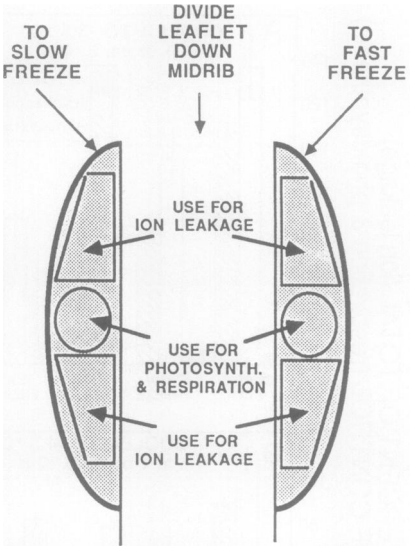

Figure 4. Diagrammatic representation of a *S. commersonii* terminal leaflet showing paired sections of tissue used for the slow (1.0°C/h) and fast (2.9°C/h) freeze-thaw tests and particular regions of leaflet halves used for the various assays.

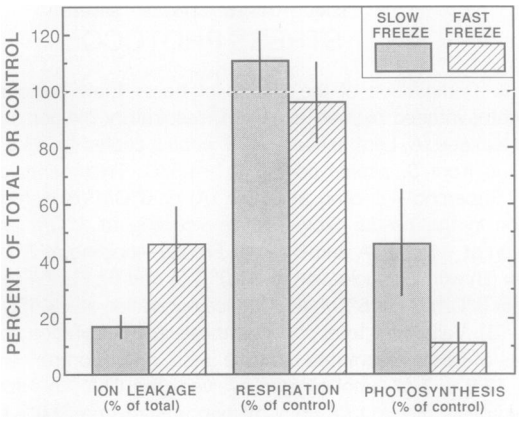

Figure 5. Direct comparison of the effect of increasing the cooling rate from 1.0 (Slow Freeze) to 2.9°C/h (Fast Freeze) in frozen tissue from the same leaflet on net photosynthetic oxygen evolution, respiratory oxygen uptake and ion leakage. Values are means ± SD. Rates for freshly harvested controls are: 80.7 ± 6.05 μmol oxygen evolved·(mg Chl)$^{-1}$·h^{-1} for total photosynthesis; 6.9 ± 0.32 μmol oxygen uptake·(mg Chl)$^{-1}$·h^{-1} for respiration; and 4.5 ± 1.29% of total ions for ion leakage.

freeze (2.9°C/h) to the same temperature resulted in 46% ion leakage (Fig. 5). Respiration was largely unaffected by the two treatments, slightly stimulated by the slow freeze (111% of control), and slightly inhibited by the fast freeze (96% of control). Photosynthesis was the most dramatically altered by the two treatments, inhibited 54% by the slow freeze and 89% by the fast freeze (Fig. 5). Respiration was nearly normal following both treatments in spite of substantial alterations in membrane permeability (enhanced ion leakage). However, the same alteration produced a dramatic inhibition of photosynthesis. Furthermore, the two treatments produced clear differences in sensitivity of membrane and photosynthetic functions. The only difference in these two treatments was in the rates at which nearly identical tissue (Fig. 4) was frozen.

DISCUSSION

Differential Sensitivity of Photosynthesis and Respiration to a Simulated Freeze-Thaw Stress

In the studies presented here, photosynthesis was clearly much more sensitive to a freeze-thaw stress than respiration (Figs. 1; 3, A and B; 5). The first change observed in cellular function was a small but significant increase in ion leakage at incipient freezing injury (Fig. 1). This level of ion leakage which remained constant at freezing temperatures, between −2.0 and −3.5°C represented a two- to threefold increase over control values (Fig. 1, inset), and was seen consistently from experiment to experiment. However, in this range a consistent decline in photosynthesis was observed while respiration was unaffected or slightly stimulated in some cases (Fig. 1). Furthermore, the ion leakage at −3.5°C was only 10% of the total, indicative of nonlethal, reversible injury (17). These data demonstrate that the freezing-induced impairment of photosynthesis during realistic freezing stress is not a cataclysmic phenomenon and occurs in a relatively intact cell compartment (*i.e.* low ion leakage and respiration unaffected). A sharp decline in photosynthesis, at this stress level, suggests that the chloroplast environment, possibly both externally and internally has been altered during the early stages of freezing injury. It is important to note that alteration in the photosynthetic and cell membrane functions (ion leakage) can be observed (Fig. 1, stress levels −2.0, −2.5, and −3.0°C) even in reversibly injured (*i.e.* able to fully recover) tissue (17, 28).

Cooling Rate and Ice Nucleation in Nature

The true frost tolerance of a plant species is ultimately its ability to survive a frost episode in the natural environment. It is therefore extremely important that experimental tests assessing relative tolerance are designed to provide an accurate simulation of the type and magnitude of stresses associated with frost damage. Examination of climatological data demonstrates that air cooling rates in the subzero range generally do not exceed 2°C/h (Table I) even during extreme drops in temperature and are usually 1–2°C/h during agriculturally relevant frosts of annual crop plants in Wisconsin (Fig. 6). Levitt (11) noted a cooling rate of about 1°C/h for plants experiencing freezing stress in nature. We have demonstrated that tissue temperature follows air temperature quite closely except during ice formation when the release of the latent heat of crystallization causes tissue temperature to lag considerably behind air (28) or bath temperatures (Fig. 2) for approximately 1 to 2 h. Clearly, freeze-thaw protocols which use cooling rates of 20 to 60°C/h (3, 10, 24, 25) do not accurately represent the conditions of a natural freeze-thaw stress. We have shown that the point at which ice formation is initiated *in situ* varies within a relatively narrow temperature range, between −1.2 and −3.1°C (28). This agrees quite closely with the findings of Ashworth *et al.* (1) who observed in a variety of plant materials under field conditions that ice formation

Table I. *Air Cooling Rates Below 0°C, during the Last Spring Minimum or First Fall Minimum of −4.4°C or Below from 1975 through 1985, Inclusive, in Madison, WI*

Also included are two of the greatest historic declines in air temperature over a 24 h period in Wisconsin.

Season	Date (prefrost)[a]	Day/Night Temperature Range (°C)[b]	Maximal Cooling Rate below 0°C (°C/h)[c]
Historic	11-11-11	21.1 to −10.0	1.7
	11-11-40	10.0 to −12.2	1.8
Spring	05-08-77	25.0 to −3.9	1.1
	04-20-83	13.3 to −3.3	1.9
	04-06-84	15.0 to −5.6	1.8
Fall	10-13-79	8.3 to −5.6	1.4
	10-22-81	7.2 to −5.6	1.4
	10-20-82	4.4 to −5.6	1.7
	1975–1985	Mean ± SD	1.2 ± 0.36
		Range	0.6–1.9

[a] Date which includes the highest daily temperature preceding the frost. [b] The range includes the maximum daily temperature preceding the frost to the minimum temperature reached during the frost. [c] Temperature data was obtained from the National Weather Service in which hourly readings had been rounded to the nearest Fahrenheit degree. In order to minimize rounding influences, three consecutive hourly temperatures in the freezing range (covering 2 h) were regressed to determine the midpoint freezing rate.

was initiated between −1.0 and −3.7°C with a mean temperature of −2.6°C.

Influence of Freeze-Thaw Protocol on the Sensitivity of Photosynthesis and Respiration

In spite of these common observations, investigations into the mechanisms of frost tolerance frequently use very fast cooling rates, commonly 6 to 60°C/h (3, 10, 24, 25). In addition, little attention is given to the point at which ice nucleation is initiated. Even if tissue temperature is lowered at rates approaching realistic values, the failure to initiate freezing in the experimental protocol (9, 10) can result in the potential flash freezing of supercooled tissue at much faster rates than anticipated (Fig. 2, A and B). These protocols also tend to obscure subtle changes occurring during incipient injury. Furthermore, such cooling rates can produce stresses which may have little relevance to natural freeze-thaw stress experienced by herbaceous plants, such as intracellular freezing or extremely rapid rates of desiccation.

Intracellular freezing is almost always lethal to the cell, and in nature occurs rarely in herbaceous plants (11). The extracellular water which is relatively pure as compared to vacuolar water has a much higher freezing point and therefore freezes first. Rapid desiccation during extracellular ice formation can cause greater injury since molecular adjustments during cellular dehydration appear to be necessary. In support of this explanation, slower dehydration and rehydration rates have been reported to be less damaging to onion epidermal cells (20). In our study, the increase in damage to cell membrane and chloroplast functions when the rate of cooling was increased from 1 to 2.9°C/h, can very likely be explained by the rapid rate of desiccation occurring at 2.9°C/h (Fig. 5). In tissue frozen to −4.5°C at the relatively fast cooling rate (6 *versus* 1°C/h), the dramatic increase in injury in all three cell functions suggests intracellular freezing in the fast and flash treatments (Fig. 3B). Respiration is reduced to a residual level at the faster freezing rates, suggesting total cellular disruption by intracellular ice formation. It is clear that the rate and/or temperature at which tissue freezing occurs has a dramatic effect on the degree of inhibition of chloroplast and mitochondrial functions. As the stress became more extreme with respect to freezing rates and minimum temperatures, the differences in the relative sensitivity of these two organelles was obscured (Fig. 3, A and B).

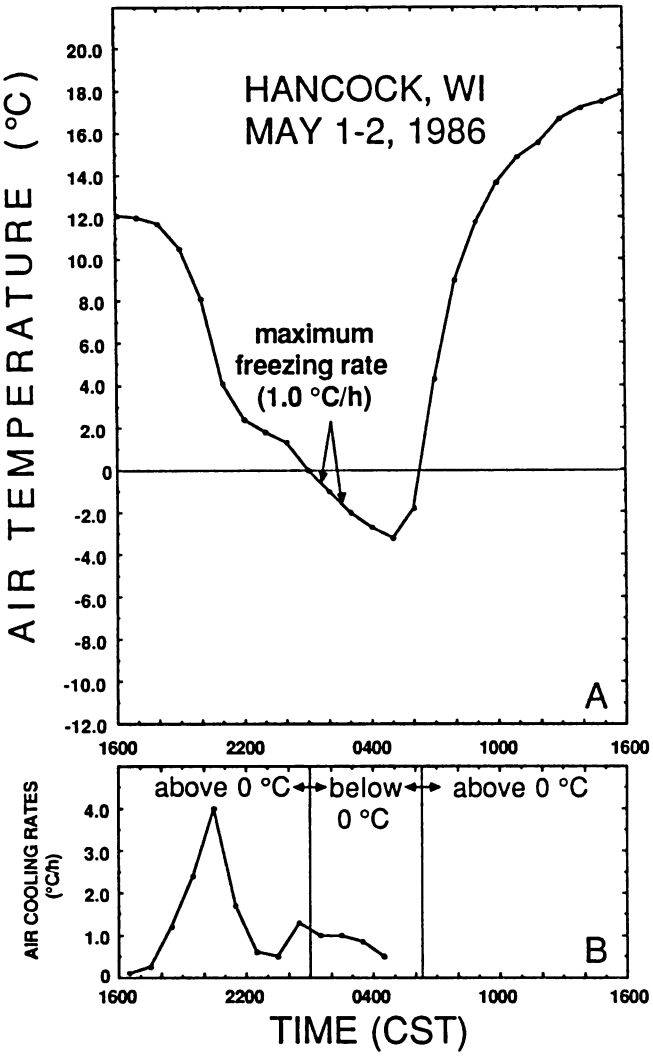

Figure 6. A typical spring frost in central Wisconsin. A, Hourly air temperature collected by a Wisconsin Automated Agricultural Weather Station on the University of Wisconsin Agricultural Experiment Station at Hancock, WI. Data was collected on 60 s intervals and reported as a mean at the end of a hourly period ending on the hour. B, Air cooling rates. Rates were calculated from two consecutive hourly readings and reported here as a midpoint cooling rate on the half-hour.

Results reported here are in disagreement with those of

Klosson and Krause (9), who have found an equal sensitivity of photosynthesis and respiration to a freezing stress. The present study shows that the discrepancy can possibly be explained by important differences in freezing protocols (very rapid freezing rates and possibly flash freezing due to lack of controlled ice nucleation) used by these researchers. The protocols of Klosson and Krause (9) are nearly identical to the fast and flash treatments used in experiment 2. It is important to emphasize that simply increasing the cooling rate in frozen tissue, from 1 to 2.9°C/h can mean the difference between nonlethal (reversible) and lethal (irreversible) injury (Fig. 5). Palta *et al.* (17) have shown that in *S. commersonii* leaf tissue there is nearly complete recovery from a level of freezing injury that causes 30% or less ion leakage while irreversible damage begins to appear as ion leakage exceeds this level. Ultimately, tissue in each freezing rate treatment experienced the same level of dehydration stress since adjacent tissue from the same leaflet (Fig. 4) would have approximately the same initial osmotic concentration and thus reach nearly identical levels of tissue water content at −3.5°C (2). The mechanism by which the rate of dehydration affects cell functions is not clear; however, one important factor may be the amount of time required for various domains within the cell to equilibrate to the drastically changing cellular environment.

This study also shows that the stress associated with ice formation, not simply tissue temperature is an important factor in freezing injury. The tissue samples that were supercooled showed essentially no inhibition (Fig. 3). These results are in agreement with those of Lindstrom and Carter (15) who found no increase in ion leakage following short term supercooling to various freezing temperatures.

Summary

Results of this study show that during the progressive removal of cellular water by freezing stress, the impairment of various cellular functions occurs. The data suggests that cell membrane functions are altered very early on, followed by a nearly simultaneous effect on photosynthetic function. Furthermore, the respiratory function seems to be much more buffered to cellular perturbations than is chloroplast function. It is important to note that the initial substantial alterations in cell membrane and chloroplast function are reversible (17, 28) indicating subtle changes rather than the dramatic effects which have been the subject of many other investigations. Our results also clearly demonstrate that only slight deviations in the experimental freezing protocol can result in dramatic differences in the type and magnitude of the stress produced. These different stresses produce very different results, yet in far too many studies in the area of frost tolerance, little effort is made to ensure that stresses produced in the laboratory approximate those occurring in the natural environment.

ACKNOWLEDGMENTS

Plant material for these studies was provided by the interregional potato introduction station, Sturgeon Bay, WI. We would like to thank Ms. Laurie S. Meade for assistance with the culture and maintenance of plant materials and Dr. D. R. Clark, State Climatologist of the Wisconsin Geological and Natural History Survey and the University of Wisconsin-Extension System for assistance in the collection of climatological data.

LITERATURE CITED

1. **Ashworth EN, Davis GA, Anderson JA** (1985) Factors affecting ice nucleation in plant tissues. Plant Physiol **79:** 1033–1037
2. **Chen HH, Li PH** (1980) Characteristics of cold acclimation and de-acclimation of tuber-bearing Solanum species. Plant Physiol **65:** 1146–1148
3. **Dowgert MF, Steponkus PL** (1984) Behavior of plasma membrane of isolated protoplasts during a freeze thaw cycle. Plant Physiol **75:** 1139–1151
4. **Grafflage S, Krause GH** (1986) Simulation of *in situ* freezing damage of the photosynthetic apparatus by freezing *in vitro* of thylakoids suspended in complex media. Planta **168:** 67–76
5. **Hincha DK, Heber U, Schmitt JM** (1985) Antibodies against thylakoid membrane proteins as molecular probes to study chemical and mechanical freezing damage *in vitro*. Biochim Biophys Acta **809:** 337–344
6. **Hoagland DR, Arnon DI** (1950) The water-culture method for growing plants without soil. Calif Agric Exp Stn Cir **337**
7. **Iswari S, Palta JP** (1988) Plasma membrane H^+-ATPase as a site of functional alteration during cold acclimation and freezing injury. *In* PH Li, ed, Low Temperature Stress Physiology in Crops. CRC Press, Boca Raton, FL, pp 123–137
8. **Kaiser WM, Kaiser G, Schoner S, Neimanis S** (1981) Photosynthesis under osmotic stress: differential recovery of photosynthetic activities of stroma enzymes, intact chloroplasts, protoplasts, and leaf slices after exposure to high solute concentrations. Planta **153:** 430–435
9. **Klosson RJ, Krause GH** (1981) Freezing injury in cold-acclimated and unhardened spinach leaves. I. Photosynthetic reactions of thylakoids isolated from frost-damaged leaves. Planta **151:** 339–346
10. **Krause GH, Klosson RJ, Tröster U** (1982) On the mechanism of freezing injury and cold acclimation of spinach leaves. *In* PH Li, A Sakai, eds, Plant Cold Hardiness and Freezing Stress. Mechanisms and Crop Implications. Academic Press, New York, pp 55–75
11. **Levitt J** (1980) Responses of Plants to Environmental Stresses, Vol 1, Chilling, Freezing, and High Temperature Stresses. Academic Press, New York
12. **Li PH, Huner NPA, Toivio-Kinnucan M, Chen HH, Palta JP** (1981) Potato freezing injury and survival, and their relationships to other stresses. Am Potato J **58:** 15–29
13. **Li PH, Sakai A,** eds (1978) Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications, Vol 1. Academic Press, New York
14. **Li PH, Sakai A,** eds (1982) Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications, Vol 2. Academic Press, New York
15. **Lindstrom OM, Carter JV** (1985) Injury to potato leaves exposed to subzero temperatures in the absence of freezing. Planta **164:** 512–516
16. **Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497
17. **Palta JP, Jensen KG, Li PH** (1982) Cell membrane alterations following a slow freeze-thaw cycle: Ion leakage, injury and recovery. *In* PH Li, A Sakai, eds, Plant Cold Hardiness and Freezing Stress. Mechanisms and Crop Implications. Academic Press, New York, pp 221–242
18. **Palta JP, Levitt J, Stadelmann EJ** (1977) Freezing injury in onion bulb cells: I. Evaluation of the conductivity method and analysis of ion and sugar efflux from injured cells. Plant Physiol **60:** 393–397
19. **Palta JP, Levitt J, Stadelmann EJ** (1977) Freezing injury in onion bulb cells: II. Post-thawing injury or recovery. Plant Physiol **60:** 398–401
20. **Palta JP, Levitt J, Stadelmann EJ, Burke MJ** (1977) Dehydra-

tion of onion cells: a comparison of freezing vs. desiccation and living vs. dead cells. Physiol Plant **41:** 273–279

21. **Palta JP, Li PH** (1978) Cell membrane properties in relation to freezing injury. *In* PH Li, A Sakai, eds, Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications. Academic Press, New York, pp 93–115
22. **Palta JP, Li PH** (1978) Examination of ultrastructural freeze-injury in the leaf cells of tender and hardy potato species. Hort Science **13:** 387
23. **Palta JP, Li PH** (1980) Alterations in membrane transport properties by freezing injury in herbaceous plants. Evidence against rupture theory. Physiol Plant **50:** 169–175
24. **Pihakaski K, Steponkus PL** (1987) Freeze-induced phase transitions in the plasma membrane of isolated protoplasts. Physiol Plant **69:** 666–674
25. **Rumich-Bayer S, Krause GH** (1986) Freezing damage and frost tolerance of the photosynthetic apparatus: studies with isolated mesophyll protoplasts of *Valerianella locusta* L. Photosynth Res **8:** 161–174
26. **Santarius KA** (1986) Freezing of isolated thylakoid membranes in complex media III. Differences in the pattern of inactivation of photosynthetic reactions. Planta **168:** 281–286
27. **Singh J, de la Roche I, Siminovitch D** (1977) Relative insensitivity of mitochondria in hardened and nonhardened rye coleoptile cells to freezing in situ. Plant Physiol **60:** 713–715
28. **Steffen KL** (1987) Response of the photosynthetic process to freezing injury and cold acclimation in tuber-bearing *Solanum* species. PhD thesis, University of Wisconsin, Madison
29. **Steffen K, Palta JP** (1986) Effect of light on photosynthetic capacity during cold acclimation in a cold-sensitive and a cold-tolerant potato species. Physiol Plant **66:** 353–359
30. **Sukumaran NP, Weiser CJ** (1972) Freezing injury in potato leaves. Plant Physiol **50:** 564–567
31. **Wintermans JFGM, Demots A** (1965) Spectrophotometric characteristics of chlorophyll and their pheophytins in ethanol. Biochim Biophys Acta **109:** 448–453