FREEZING STRESS IN POTATO

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The potato[1] is an ideal crop to exploit in an effort to meet the increasing food demand of the world because of its economic value among the world major food crops (9), high energy production per unit of land (36), and high nutritive value compared with other food crops (18). It provides not only a good source of carbohydrates, but also a high quality of proteins (19), minerals and vitamins. More than 100 countries at present grow potatoes. However, it has been primarily cultivated in the Temperate Zone of North America and Europe, and tropical Andean highlands of South America where frost is often a major factor in reducing production or resulting in crop failure (22). Breeding for better adapted clones to frost is promising by genetic manipulation of existing potato germplasms (8). A better understanding of potato frost resistance (2), its hardening characteristics (1, 5), and the establishment of a laboratory screening method (34) have contributed to the recent successful breeding efforts (8).

Furthermore, the development of frost tolerant clones will greatly expand to the areas much of which is currently marginal due to low temperature. By the same token, the potato production can be increased in the presently cultivated areas by simply extending the growing season.

[1] *'Potato' in this paper is referred to the tuber-bearing Solanum species in addition to the S. tuberosum potato.*

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I. FROST HARDINESS AND FREEZING INJURY

A. *Frost Hardiness*

Frost hardiness is defined as the resistance of foliage to a freezing temperature above which no injury occurs in the tissue. During freezing, water moves from the cells (mainly vacuole) to the intercellular ice that results in cell dehydration. As freezing proceeds, ices cause cellular contraction. Thus, during freezing, besides the low temperature, a plant experiences mainly three types of stresses: a dehydration stress due to extracellular ice formation, an osmotic stress due to removal of water from vacuole and a mechanical stress due to ice accumulation and cell contraction. The mechanisms of frost hardiness in herbaceous plants have been reviewed by Olien (24) and Levitt (21). Levitt (21) concluded that the tolerance of dehydration and other stresses listed above due to extracellular freezing and the avoidance of intracellular freezing are mechanisms of frost hardiness. In general, herbaceous plants cannot withstand temperatures below -20°C.

The difference in frost hardiness between resistant and sensitive potatoes is only about 3 to 4°C. In some plant species, a small variation in tissue water content (g H_2O/g dry weight) could result in such a difference in a plant's capacity to survive. A high cell sap concentration may increase slightly the ability of tissue to supercool; thus, the tissue could survive greater stress by avoiding freezing. However, the tissue water content and the cell sap concentration have not correlated to the frost hardiness in potato leaves (2).

Using freezing curve analysis, Sukumaran and Weiser (35) found that the leaf tissue of *S. acaule* potato could tolerate a greater amount of ice formation than the sensitive species, *S. tuberosum*. Nuclear magnetic resonance spectroscopic studies have shown that the resistant potato species can tolerate more freeze-induced dehydration than the susceptible ones (2). For example, at the killing temperatures the amount of unfrozen tissue water averaged to about 20 and 45% of total liquid water in *S. acaule* and *S. tuberosum*, respectively. Thus it appears that the major difference in frost hardiness between resistant and susceptible potatoes is due to the ability of the hardy potato to tolerate more frozen water than the susceptible one.

B. *Freezing Injury*

Freezing injury has been thought to involve cell membranes. One of the most common signs of freezing injury is the intercellular space infiltered with water, leading to a soaked appearance and loss of cell turgor. Freezing injury also often causes leakage of ions from the cells. The efflux of ions and the infiltration with water following a freezing injury have been assumed due to the breakdown of semipermeable properties of cell membrane (21, 35). Recently, Palta *et al.* (26, 27)

have shown that during the progress of freezing injury (reversible or irreversible), semipermeable properties (membrane lipids) remain intact whereas the active transport properties (membrane intrinsic proteins) are damaged.

TABLE I. Percent Ionic Leakage[a] of Two Potato Species at Two Time Intervals after Freezing-thawing (T_1 = 12 hr., T_2 = 60-120 hr.).

Freezing Temperature (°C)	Days in cold hardening conditions[b] 0		7		14	
	T_1	T_2	T_1	T_2	T_1	T_2
			Solanum tuberosum			
-2.0	40.5	20.2	-	-	-	-
-2.5	89.0	57.7	45.2	24.7	16.7	14.7
-3.0	90.9	68.1	59.0	38.0	33.1	37.4
-3.5	92.5	93.0	54.2	59.5	55.4	68.1
Control	11.3	8.3	13.7	7.1	5.6	4.1
			Solanum acaule			
-4.0	23.5	13.7	10.0	5.6	-	-
-5.0	54.0	41.3	34.6	21.6	12.5	9.5
-6.0	93.0	92.9	90.8	92.6	52.5	39.1
-7.0	94.0	92.3	93.0	93.8	59.3	70.6
-8.0	-	-	-	-	93.5	91.9
Control	9.5	6.1	7.1	5.4	5.9	4.6

[a] *These data were presented at the Society for Cryobiology Annual Meeting (28).*

[b] *Cold hardening conditions (1).*

1. Reversibility of Membrane Damage after Freezing. Plants of *S. acaule* and *S. tuberosum* were subjected to cold hardening conditions (for details see Ref. 1). At 0, 7, 14 and 21-day intervals, leaflets of both species were frozen from -2°C to -9°C at a cooling rate of 1°C/hr. They were then thawed slowly over ice. During the 60 to 120 hours following the thaw, measurements were made on the conductivity of the effusate. In the unhardened plants (0 day, Table I) the leaflets frozen to -2.0 and -2.5°C for *S. tuberosum*, and -4 and -5°C for *S. acaule* recovered from freezing injury during the post-thaw period, while at the lower temperatures, injury increased or remained unchanged with time until final death of the tissue. After 14 days of hardening, the leaflets frozen to -3°C in *S. tuberosum* and -6°C in *S. acaule* also recovered during the post-thawing periods (Table I). In both species, a recovery in injury was followed by disappearance of the infiltration with water, increase in turgidity and a decrease in the conductivity of the effusate (Table I). The freezing temperatures at which injury was reversible or irreversible varied by 0.5 or 1.0°C depending upon the age of plant materials. These results suggest that the initial freezing injury may be to the active transport systems of the cell membranes because only the recovery of such process can lead to an active uptake of effusates, such as K^+, against the cell gradient. These observations are in agreement with earlier reports (26, 27).

2. Microscopic Observations of Freezing Injured Cells. As discussed, a freeze injured cell may or may not have the ability to recover completely. In order to further study the nature of injury, microscopic observations were carried out on injured cells (22, 29). Leaflets of both resistant *(S. acaule)* and sensitive species *(S. tuberosum)* were slowly frozen down to -7°C from -2°C. Immediately after slowly thawing, segments from the middle portion of the leaflets were collected and fixed for ultrastructural studies. Although the tissue became soaked and flaccid various organelle appeared normal at a low degree of freezing stress. Abnormalities at the subcellular level seemed to start with swelling of the protoplasm (29). With increasing stress, swelling of the mitochondria and chloroplast was apparent. Separation of plasma membrane and cell wall (frost plasmolysis) and coagulation of the protoplasm were observed in dead cells (22). In these cells coagulation of the protoplasm and disruption of the tonoplast and plasma membrane were observed. Possible sequence of freezing injury in potato leaf cells appears to initiate with some disturbances in the cell membrane leading to swelling of protoplasm, and then swelling of mitochondria and chloroplasts, followed by breakdown of the tonoplast and plasma membrane system and coagulation of protoplasm resulting in cell death.

3. Membrane Permeability after Freezing Injury. In spite of leakage of ions and loss of turgor due to freezing injury, microscopic observations revealed that injured yet living cells were visually intact in cell membrane. The leakage of ions appears due to alterations in the cell

membrane properties rather than the membrane rupture. Furthermore, freeze injured cells could be plasmolyzed in a hypertonic mannitol solution (0.8 osm), and they remained plasmolyzed for several days similar to the unfrozen control cells (29). Even the irreversibly injured cells that showed a swelling of the protoplasm could be plasmolyzed in hypertonic mannitol solution just like the unfrozen control or reversibly injured cells (29). These observations indicate that the semipermeable properties of these cell membranes are still intact.

During the post-thaw period, an injured yet living cell may have the ability to recover from freezing injury (reversible injury) or may eventually die (irreversible injury). In order to examine the nature of alterations in membrane properties, the transport of urea, methyl-urea and potassium across the cell membrane were studied in freezing injured yet alive cells (29). A plasmometric method described by Stadelmann (33) was used in measuring the cell membrane permeability. In these experiments, the plasmolyzed protoplasts were allowed to expand in response to the passive uptake of permeable solute (equimolar solution of KCl, urea and methyl-urea). The protoplast expansion was measured directly with a microscope with an eyepiece micrometer.

The unfrozen cells did not take up K^+ in two hours and remained plasmolyzed at a constant volume. The injured cells, on the other hand, expanded immediately upon transfer to KCl solution and gradually deplasmolyzed. The rates of K^+ uptake varied for individual cells in the same tissue because the extent of injury was different in different cells. Although freeze injury resulted in a rapid increase in the rate of K^+ transport across the cell membranes, no change in rates was detected for nonelectrolytes such as urea and methyl-urea. The permeability of a nonelectrolyte has long been known as a direct function of its lipid solubility (6). Therefore, a change in the nonelectrolyte permeability should reflect the alterations in the lipid component of the membrane. No change in the permeability constants of these nonelectrolytes indicates that the freezing injury does not alter the lipid portion of the cell membranes. The finding that water permeability constants remain unchanged during freezing injury further supports this conclusion (26).

The increase in K^+ permeability provides evidence that the protein components of the membrane are possible targets of freezing injury. Membrane proteins are of two types, intrinsic and peripheral. Some of the intrinsic proteins pass entirely through the bilayer (31). This type of intrinsic proteins has been proposed as being sites for active transport of ions (32). Sublethal freezing temperature can lead to the denaturation of membrane proteins resulting in an inactivation of the active transport systems. When inactivated, these intrinsic proteins could serve as channels for passive ion transport, giving very high K^+ permeability values and swelling of the protoplasm. A large passive efflux of ions could also occur through these channels in the direction of the concentration gradient from the vacuole to the extracellular solution. A repair of the inactivation leads the tissue to recover from freezing injury (reversible) and failure to do so leads ultimately to the death of cells (irreversible).

II. COLD HARDENING

Many herbaceous plants undergo increase in frost hardiness when subjected to a specific environmental condition such as low temperatures, short daylength, changes in quality and quantity of light, reducing water supply, etc. Some species can harden (increase in frost hardiness) extensively in response to these environmental changes, while others will harden only a few degrees and some do not harden at all.

A. Species, Varieties and Tissues

Prior to 1976, there was some dispute as to whether the tuber-bearing *Solanum* potatoes can be cold hardened. Hayden *et al* (14) indicated that potatoes possess a stable frost hardiness level and do not harden. Richardson and Estrada (30) reported that 2 to 3 weeks of cool temperatures could differentiate hardy from nonhardy potatoes. Chen and Li (1) confirmed that some noncultivated species like *Solanum acaule, S. chomatophilum, S. commersonii and S. multidissectum* could harden, while the cultivated *S. tuberosum* (varieties of "Red Pontiac," "Kennebec," "Norland" and "Norchip") failed to harden.

Recently, additional tuber-bearing *Solanum* species were screened for their frost hardiness and cold hardening ability. Based on existing information, we can classify them into four groups in terms of their frost hardiness and cold hardening (Table II). These groups are (I) the species which possess frost hardiness (survive to -4.0°C or colder) and are able to cold harden, (II) the species which possess frost hardiness but are unable to cold harden, (III) the species which possess no frost hardiness (survive to -3.0°C or warmer) but are able to cold harden, and (IV) the species which possess no frost hardiness and are unable to cold harden. Example species for each group are listed in Table II. *Solanum Tuberosum*, the commonly grown potatoes, falls into the last category.

The potato is a cool season grown crop and is usually chilling resistant. We observed chilling injury in *S. trifidum* (PI 25541) when plants were grown in 2/2°C, day/night, regime. It is possible that additional tuber-bearing *Solanum* species, which are chilling sensitive, exist.

Stem-cultured plants of *S. tuberosum* and *S. commersonii* grown in agar medium showed similar hardening patterns as the potted plants (5). Leaf callus tissues of *S. tuberosum*, "Atlantic," "Kennebec" and "Red Pontiac," showed no hardening ability and were killed at -3°C. The leaf callus tissues of *S. acaule*, however, can be hardened to -9°C after 15 days at 3°C in darkness, a 3°C increase in frost hardiness (5). These results agreed with observations from potted plants previously reported (1).

TABLE II. Classification of Tuber-bearing Solanum Potatoes in Terms of Frost Resistance and Cold Hardening

Categories	Species (examples)	Killing Temp (C^o) Before Treatment[a]	After Treatment[b]
Group I: frost resistance and able to cold harden	*S. acaule* (Oka 3885)[c]	-6.0	-9.0
	S. commersonii (Oka 5040)	-4.5	-11.5
Group II: frost resistance but unable to cold harden	*S. sanctae-rosae* (Oka 5697)	-5.5	-5.5
	S. megistacrolobum (Oka 3914)	-5.0	-5.0
Group III: frost sensitive but able to cold harden	*S. oplocense* (Oka 4500)	-3.0	-8.0
	S. polytrichon (PI 184773)	-3.0	-6.5
Group IV: frost sensitive and unable to cold harden	*S. tuberosum*	-3.0	-3.0
	S. stenotomum (PI 195188)	-3.0	-3.0

[a] Plants were grown in a regime of 20/15°C day/night, 14 hrs.
[b] Plants were grown in a regime of 2°C day/night, 14 hrs, for 20 days.
[c] Identification number at Potato Introduction Station, Sturgeon Bay, Wisconsin.

B. Optimum Hardening Conditions

Low temperatures and short days are the two major environmental factors in inducing frost hardiness. Some species can be hardened by low temperatures with a sufficient amount of light, regardless of the photoperiod (20). Potatoes belong to this category. Low temperature conditioning for maximum hardiness varies from species to species. For example, winter rape (17) and cabbage (20) required freezing temperature during the hardening to achieve the maximum hardiness. The optimum hardening temperatures for *Solanum* potatoes are about 1 to 2°C above freezing.

Cold hardening of potato can be achieved by directly exposing plants to constant day/night low temperatures (5) or stepwise lowering the temperature (1). The hardiness level which can be achieved is dependent upon the temperatures used. Lower temperatures result in greater

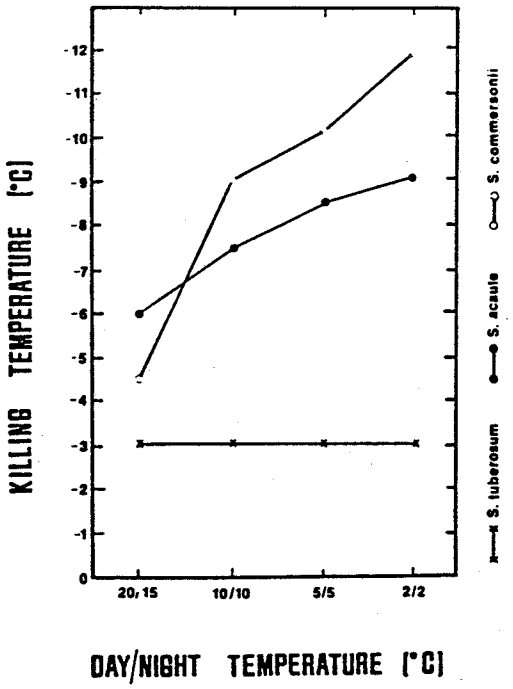

FIGURE 1. Changes in frost hardiness of three Solanum species at constant low day/night temperatures with 14 hr light for 2 weeks.

degrees of hardiness (Fig. 1). Under constant low temperatures, plants appeared to reach their maximum hardiness at about 15 days, while plants that were subjected to stepwise hardening conditions continued to harden even after 20 days (5).

C. *Growth Regulators*

The application of growth regulators to induce frost hardiness has long been considered but the results have been contradictory (21). Most studies were conducted by exogenous application to the foliage or the soil. Foliar applications raise certain problems because leaf surfaces have a tendency to restrict the uptake of exogenously applied chemicals. Microbiological degradation causes problems with the soil application. For studying the effect of growth regulators on potato hardiness, *Solanum* stem-cultured plants grown in the agar medium were therefore used (5). Preliminary results indicated that the exogenously applied abscisic acid (ABA) can increase potato frost hardiness. No increase in frost hardiness was found with gibberellic acid treatments. The increase

and change in hardiness with ABA treatments are the same in both warm and low temperature regimes. It appears that the exogenous ABA may substitute the functional role of the low temperature in inducing frost hardiness. However, different levels of ABA produced the same results. As suggested by Kacperska-Palacz (17), ABA may serve as a driving force to shift the metabolic pathway in favoring cold hardening. Possibly higher levels of ABA have an effect similar to the lower levels once the endogenous ABA reaches the functional level. Apparently, ABA can induce hardiness not only in those species which are able to harden but also in *S. tuberosum* which is a species that is unable to harden at low temperature.

D. Biochemical Changes

Many studies have been reported regarding biochemical changes in herbaceous plants during cold-hardening (7, 11, 12). We also examined and compared some of the biochemical changes in leaves of *S. acaule* and *S. tuberosum* during cold hardening (4). The results are summarized below. Since DNA in matured leaf cell is maintained at a constant level during growth (25) metabolite changes during hardening would be better expressed on unit DNA than on dry weight basis. Following discussions relative to the changes in sugars, starch, nucleic acids, proteins and lipids are therefore based on unit DNA (same numbers of cells).

1. Sugars and Starch. Sugar increase was found in both *S. acule* and *S. tuberosum* during hardening. Total sugar content more than doubled in both species after 15 days hardening. Levitt (21) reviewed the protective role of sugars against frost injury. However, such a role in potato frost hardiness is questionable because *S. tuberosum* fails to harden even when its sugar content increases more than twofold during hardening. Huner and Macdowall (16) found that the cold adaptive rye plants increased the stability of RuBP carboxylase in chloroplasts under cold stress. This would enable plants to maintain photosynthetic ability under cold hardening condition. Therefore, sugars can accumulate in leaf tissues.

Under hardening condition, leaf starch content was also increased in both *S. acaule* and *S. tuberosum*. Hatano (13) reported an increase of starch in *Chlorella* during cold hardening under light. Heldt *et al* (15) reported that isolated spinach chloroplasts from hardy winter materials showed higher starch synthesis rate than from nonhardy winter materials. The decrease in starch content during cold hardening was almost always found in nonphotosynthetic tissues such as bark (23) and root (10) which possess very little or no photosynthetic activity. The cold hardening is a process requiring energy. The energy source in nonphotosynthetic tissues seems to be the stored food such as starch. On the other hand, leaves which can maintain their photosynthetic activity at low temperature should be able to directly supply photosynthate as the

energy source for hardening. This may be an explanation for both starch and sugar increases in potato leaves during cold hardening.

2. *Ribonucleic Acids.* In *S. acaule*, plants grown under hardening environment always maintained at a higher level of rRNA than plants grown under unhardening environment. In *S. tuberosum*, there was no different change in rRNA content between hardened plants and controls. It has been reported that the temperature had a marked influence on rRNA metabolism while the photoperiodic response was not as great in potato plants (25). The higher level of rRNA in *S. acaule* plants during hardening is paralleled with higher level of soluble protein and the increase of hardiness. The difference in rRNA levels between *S. acule* and *S. tuberosum* in response to hardening suggests that the hardening process likely initiates at the transcription and/or translation levels.

3. *Proteins.* In *S. acaule*, soluble proteins were maintained at a much hjgher level in cold hardened plants than in controls. The soluble protein fraction also increased at a much higher rate in the former than in the latter during hardening. No significant protein changes were observed in both controls and hardened plants of *S. tuberosum*, which failed to harden. Since the increase in hardiness is always parallel with the increase in soluble protein content, several researchers have discussed the role of soluble protein in cold hardening (17, 21). Cox *et al* (7) concluded that only plants that were able to conduct active protein synthesis at low temperature had the capability to harden. This conclusion was supported by Hatano's work (13) in which cyclohexamide, an inhibitor for protein synthesis, could inhibit the hardening in *Chlorella.*

4. *Lipids.* During hardening, total lipid increased in *S. acaule* and a more or less constant level was maintained in *S. tuberosum*. The increase in lipid in *S. acaule* during hardening supported the EM observations of lipid bodies accumulations in hardened chloroplasts (3). The phospholipid content in hardened *S. acaule* plants was much higher than in controls, while lower level of phospholipid was observed in *S. tuberosum* hardened plants than in controls during hardening. Cold hardening alters membrane properties and increases its stability during freezing as suggested (17). The development of hardiness in *S. acaule* is probably associated with alterations in membrane properties as evidenced in phospholipid increase.

E. *Electron Microscopic Observations*

Comparative ultrastructural studies were undertaken between hardy *(S. acaule)* and tender *(S. tuberosum)* potatoes grown under warm and cold temperature regimes. In less than 10 days during cold hardening, the hardy species of *S. acaule* showed a drastic increase in starch grains in chloroplasts (Fig. 2B). Such an increase, however, was

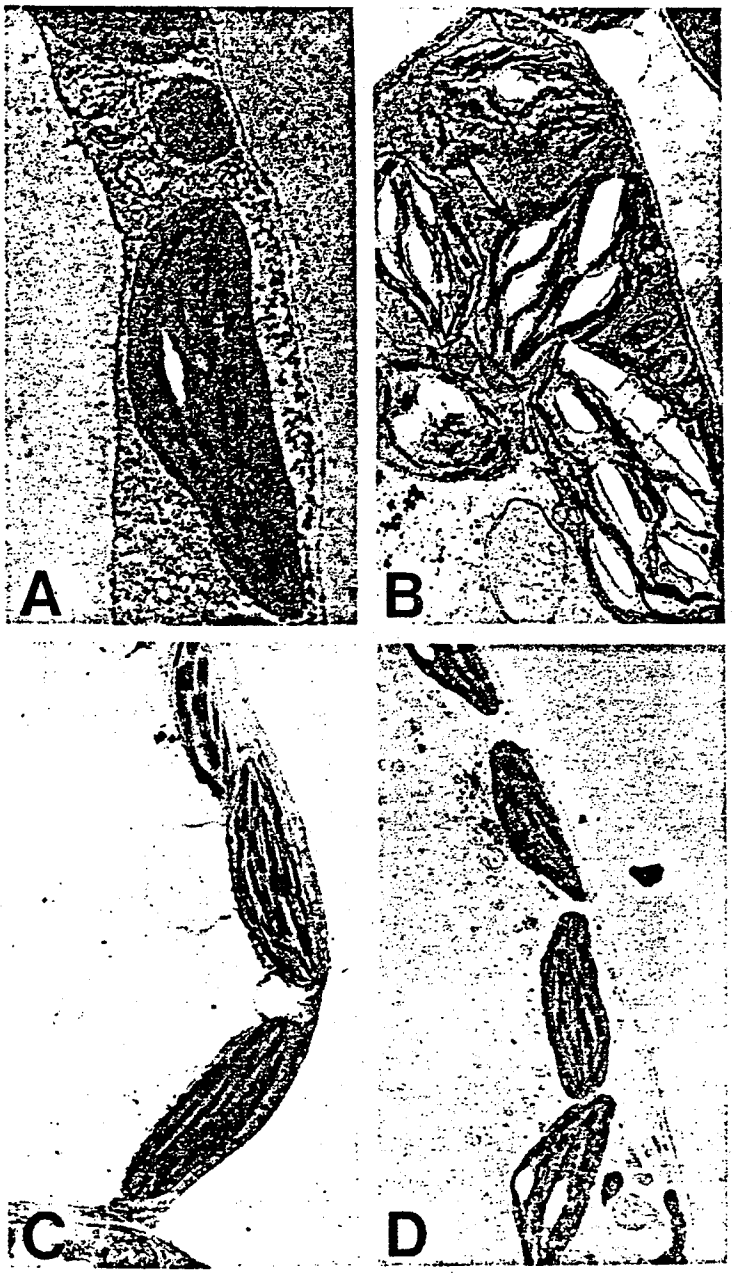

FIGURE 2. Electron microscopic observations of leaf cross-sections showing the accumulation of starch grains (→) in chloroplasts after 8 days cold hardening (5/2°C day/night temp., 14 hr light). A,B: Solanum acaule; C,D; Solanum tuberosum; A & C: before hardening; B & D: after hardening.

not observed in chloroplasts of the tender species of *S. tuberosum* (Fig. 2D) grown under the same regime. It appears that energy sources for cold hardening in potato came directly from photosynthate rather than from starch to sugar transformation. Chen *et al* (3) have reported the swelling and irregularity of thylakoid membranes and large patches of stroma in chloroplasts of hardened *S. acaule*, while no significant changes were observed in cold treated *S. tuberosum* chloroplasts.

III. SUMMARY

The difference in frost hardiness between a resistant and sensitive type of potato is about 3 to 4°C. This difference is not due to the avoidance of intracellular freezing because cell sap concentration and tissue water content have shown no correlation to the variations of frost hardiness among different species; rather, it is due to the tolerance of freeze-induced dehydration (extracellular freezing). During the initial stage of freezing injury, semipermeable properties of the cell membranes remain intact. Freezing injury, due to extracellular ice formation, results in alterations of membrane transport properties, most likely the intrinsic membrane proteins which involve in active transport systems. Freezing injury can be reversible (leading to complete recovery) or irreversible (leading to death). The mechanisms of frost hardiness and cold hardening appear to be independent. Increases in ribosomal RNA, soluble proteins and phospholipids are associated with the increase of frost hardiness during cold hardening.

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