Survival of Plant Tissue at Super-Low Temperatures III. Relation between Effective Prefreezing Temperatures and the Degree of Frost Hardiness[1]

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It has been reported (5, 6, 7) that twigs of woody plants from which almost all of the freezable water in the cells has previously been withdrawn by sufficient extracellular freezing can survive immersion in liquid nitrogen or in liquid helium (8), and that if the living materials can withstand freezing at temperatures below −30°, they are able to survive freezing at extremely low temperatures with the prefreezing method. In addition, if the materials are previously prefrozen at −30°, the percentage of survival is very little affected by the rates of cooling to and rewarming from super-low temperatures with this prefreezing method.

These findings suggest that there is a definite temperature at which almost all of the easily freezable water in a cell may be withdrawn from the cell by extracellular freezing, and that cells and tissues in this state are not injured, even when exposed to extremely low temperatures. In the case of plants and animals, this temperature may be around −30°.

Preliminary experiments demonstrated, however, that in winter prefreezing temperatures below −15° were sufficient to enable willow twigs to withstand liquid nitrogen, while in early spring and in early winter prefreezing temperatures below −30° were required. This suggests that the effective prefreezing temperature may vary with the degree of frost-hardiness in plants examined.

As most twigs which can survive freezing at −70° or lower are not injured even when exposed to extremely low temperatures, it is impossible to determine the degree of their frost-hardiness by the usual freezing methods. To clarify the mechanism of frost-hardening in these hardy plants, it is essential to find a suitable method of determining of degree of frost-hardiness. If it is confirmed that the greater the frost-hardiness, the higher the effective prefreezing temperatures, the effective prefreezing temperature may be used as a reliable index of the degree of frost-hardiness in hardy plants.

To determine the mechanism of survival in woody plants at super-low temperatures and to find a suitable method for determining the degree of frost-hardiness in hardy plants, the relation between the effective prefreezing temperature and the degree of frost-hardiness was studied for 2 years using various woody plants.

Materials and Methods

Various hardy woody plants growing on the campus of Hokkaido University were used as experimental materials. The complete list is as follows: willow (*Salix sachalinensis* Fr. Schm., *Salix koriyanagi* Kimura, *Salix ezoensis* Kimura, *Salix Sieboldiana* Blume), poplar (*Populus nigra* L. var. *italica* Muenchh., *Populus maximowiczii* Henry, *Populus simonii* Carr., *Populus glaubka*), birch (*Betula Tauschii* Koidz., *Betula Ermani* Cham. var. *Communis* Koidz.), Alpine rose (*Rosa pendulina* L.), black alder tree (*Alnus hirsuta* Trucz.), larch tree (*Larix dahurica* Turcz. var. *Japonica* Maxim.), apple tree (*Malus pumira* Mill.), creeping pine (*Pinus pumira* Regel.), Todo fir (*Abies sachalinensis* Masters), Ezo spruce (*Picea glehni* Masters).

In the same series of experiments, only 1-year-old twigs from the same tree in every species were used, and in every experiment, 5 pieces (0.6–0.7 cm in a diameter, 10 cm in length) from these twigs were used. In almost all of the experiments, there was no applicable difference in degree of frost-hardiness or effectiveness of frost-hardening in the 5 pieces.

The pieces of twig were put in a small polyethylene bag and cooled in chambers at −5°. After 1 hour, they were inoculated with ice and then were gradually transferred at 1-hour intervals to chambers maintained at temperatures graded at 5° intervals from −5° to −30°. After the twig pieces were cooled to the desired temperature, they were kept in temperatures for a length of time, and then slowly thawed in air at 0°.

To determine the effective prefreezing temperature, 5 of the pieces of twig were tied with a thread and prefrozen at temperatures between −10° and −30° for 6 hours. A sinker was then attached to the prefrozen twig pieces and they were immersed directly in liquid nitrogen (−196°). The cooling rate of a twig piece immersed in liquid nitrogen following prefreezing at −30° was almost 20° per second. After 30 minutes, the twigs were transferred to −10° for 1 hour and then to approximately 0° for 16 hours.

The viability of an entire twig piece cannot be judged on the basis of a plasmolysis test or a tetra-

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zolium test in parenchymal cells of the cortex just subsequent to or even after many days of thawing. These tests only indicate the relative viability of certain tissues in the twig, and that the inner cortex, pith and xylem tissues are less resistant to freezing than the parenchymal cells of the cortex. In order to determine the viability of the entire twig piece, it was planted in water or in sand in a green house and its capacity to continue normal development for 2 months was tested. In coniferous trees, browning of the leaves, buds and xylem tissues were taken as a sign of injury. Judging the viability of twigs of willow and poplar is very easy and sure, because these twigs frozen at the temperatures below −10° are able to put forth buds and send out roots in any season, provided that they are not damaged. The degree of frost-hardiness in a twig piece was represented by the lowest temperatures at which it could withstand freezing without injury.

Results

To investigate effective prefreezing temperatures, the twigs of willow in midwinter were prefrozen for 16 hours at temperatures ranging from −10° to −30° and were then immersed directly in liquid nitrogen. After 15 minutes, these twigs were transfered to −10° for 1 hour and then to approximately 0° for 16 hours. The twigs which were prefrozen at temperatures below −15° all budded, while the twigs prefrozen at −10° showed no sign of development (fig 1).

In midwinter, prefreezing temperatures below −15° were sufficient to enable willow twigs to withstand immersion in liquid nitrogen, while in the middle of November, prefreezing temperatures below −20° were required. This suggests that the effective prefreezing temperature may vary with the degree of frost-hardiness in the twigs used.

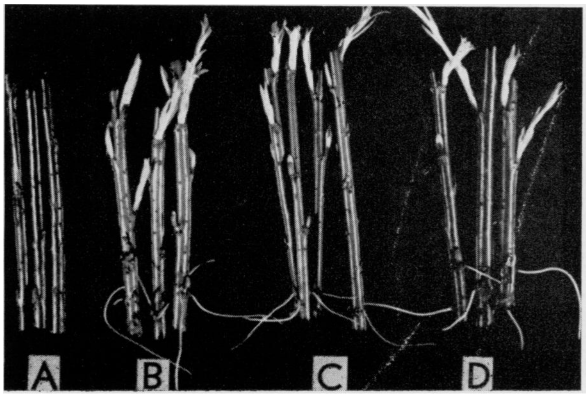

FIG. 1 Development of willow twigs immersed in liquid nitrogen following prefreezing at various temperatures. Material: *Salix sachalinensis* (January 15). A, twigs prefrozen at −10°; B, −15°; C, −20°; and D, −30°. Period of growth: 2 months after thawing.

The prefreezing temperatures which are effective in maintaining the viability of willow twigs following immersion in liquid nitrogen or liquid helium were examined. The experiments showed that in both nitrogen and helium, the effective prefreezing temperature was below −20° (table I). After 2 years, willow twigs immersed in liquid nitrogen or helium following prefreezing at −30° have grown to about 2 m in height.

When twigs of white birch were immersed in liquid nitrogen following prefreezing at temperatures of −10°, −15°, —20°, —30°, −40°, −50°, and —70°, the twigs prefrozen at temperatures below —15° normally put forth their buds and showed no browning in the xylem or the cortical tissues, while those prefrozen at —10° were all destroyed.

Identical experiments were conducted with twigs of the Alpine rose. Alpine rose buds can survive freezing at −30° and −70°, but the xylem and pith are damaged by freezing at temperatures below about —40°. As is shown in figure 2, buds on twigs pre-

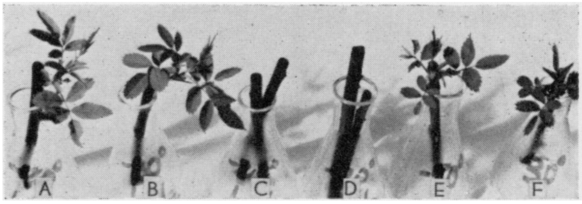

FIG. 2. Development of twigs of Alpine rose immersed in liquid nitrogen following prefreezing at various temperatures. Date of experiment: Jan. 20. Period of growth: 1 month after thawing. A, twigs of Alpine rose immersed in liquid nitrogen following prefreezing at −70°; B, −30°; C, −25°; and D, −20°. E and F, twigs frozen at −70° (E) and −30° (F) for 16 hours without subsequent immersion in liquid nitrogen.

frozen at temperatures below —30° sprouted normally, although browning was observed in the xylem tissues, while those prefrozen at the temperatures higher —25° showed no sign of development.

The effective prefreezing temperatures for twigs of various woody plants in midwinter are summarized in table II. In every species, all of the twigs prefrozen at —5° were destroyed by immersion in liquid nitrogen. The effective prefreezing temperature differed considerably in each species; in the willows, *Salix sachalinensis* and *Salix ezoensis,* and in the

Table I. *Effective Prefreezing Temperature for Willow Twigs Immersed in Liquid Nitrogen or Helium*

These experiments were done on February 12.

	Prefreezing temp				
	−10°	−15°	−20°	−30°	−70°
Liquid nitrogen	●*	●	○	○	○
Liquid helium	●	●	○	○	○

*○, normal; ●, killed.

poplars, *Populus maximowichi, Populus nigra,* and *Populus simonii,* and in the white birches, it was below —15°, but in the poplar, *Populus glaubka,* in the willow, *Salix koriyanage,* it was below —20°. In the less hardy willow, *Salix Sieboldiana,* and club apple, black alder, and *Sachalin* larch trees, only twigs prefrozen at temperatures below —30° put forth normal buds, although in the other species, with exception of the *Salix Sieboldiana* and the creeping pine, the xylem tissues were damaged. In the creeping pine, needle leaves prefrozen at temperatures higher —25° turned brown within a few days after thawing from liquid nitrogen, while those prefrozen at temperatures below —30° remained green for at least one month.

The results reported above suggest that the effective prefreezing temperature may vary with the degree of frost-hardiness of the plants examined. To further clarify this, the seasonal fluctuation in the effective prefreezing temperature of willow twigs was investigated during the period ranging from the middle of November to the end of March. The experiments showed that the effective prefreezing temperature varied with the seasonal fluctuation in frost-hardiness, being higher from the middle of November to midwinter, as natural frost-hardening proceeded,

Table II. *Prefreezing Temperatures which are Effective in Maintaining the Viability of Various Woody Plants Following Immersion in Liquid Nitrogen*

All experiments were made in the middle of January.

Species	Prefreezing temp −5°	−10°	−15°	−20°	−25°	−30°	−40°	−50°	−70°
Salix sachalinensis	●*	△	○	○	○	○	○	○	○
Salix yezoensis	●	△	○	○	○	○	○	○	○
Populus maximowichi	●	△	○	○	○	○	○	○	○
Populus simonii	●	△	○	○	○	○	○	○	○
Populus nigra	●	●	○	○	○	○	○	○	○
Betula taushii	●	●	○	○	○	○	○	○	○
Betula Ermani	●	●	○	○	○	○	○	○	○
Salix koriyanagi	●	●	△	○	○	○	○	○	○
Populus glaubka	●	●	●	○	○	○	○	○	○
Salix Sieboldiana	●	●	●	●	△	○	○	○	○
Pinus pumira	●	●	●	●	●	○	○	○	○
Rosa pendulina	●	●	●	●	●	△	△	△	△
Alnus hirsta	●	●	●	●	●	△	△	△	△
Larix dahurica	●	●	●	●	●	△	△	△	△
Club apple tree	●	●	●	●	●	△	△	△	△

* ○, normal; △, injured; ●, killed.

Table III. *Relation of the Effective Prefreezing Temperature to the Degree of Frost-hardiness in* Salix sachalinensis

Date of expt		Prefreezing temp −5°	−10°	−15°	−20°	−25°	−30°	−40°	−50°	−70°	Frost-hardiness of twigs (°C)
Nov. 15	N**	●***	●	●	●	○	○	○	○	○	−70
	H	●	●	●	○	○	○	○	○	○	−70
Dec. 5	N	●	●	●	○	○	○	○	○	○	−70
	H	●	●	○	○	○	○	○	○	○	−70
Jan. 5	N	●	●	○	○	○	○	○	○	○	−70
	H	●	△	○	○	○	○	○	○	○	−70
Feb. 28	N	●	●	●	●	○	○	○	○	○	−70
	H	●	●	△	○	○	○	○	○	○	−70
Mar. 11	N	●	●	●	●	●	●	●	●	●	−25
	H	●	●	●	△	○	○	○	○	○	−70
Mar. 20	N	●	●	●	●	●	●	●	●	●	−25
	H	●	●	●	△	○	○	○	○	○	−70
Mar. 25	N	●	●	●	●	●	●	●	●	●	−20
	H	●	●	●	●	△	○	○	○	○	−70

* The degree of frost-hardiness of a twig is represented by the minimum temperature at which the twig survived freezing for 16 hours without injury.

** N, normal control; H, following frost-hardening for 5 days at −5°.

*** ○, normal; △, injured; ●, killed.

Table IV. *Frost Damage in Twigs Frozen at the Temperatures below −30°*
Date of experiment; January 20. The symbols 0, 1, 2, and 3 represent the degree of frost damage.

Species	Tissues tested	Freezing temp				
		−30° (1 day)	−30° (2 days)	−40° (6 hr)	−50° (6 hr)	−70° (6 hr)
Club apple tree	Bud	0	0	0	0	0
	Cortex	0	0	0	0	0
	Xylem	0	0	1	2	3
	Pith	1	1	2	3	3
Alpine rose	Bud	0	0	0	0	0
	Cortex	0	0	0	0	0
	Xylem	0	0	1	2	3
	Pith	1	1	2	3	3
Black alder tree	Bud	0	0	0	0	0
	Cortex	0	0	0	0	0
	Xylem	0	0	1	2	3
	Pith	1	1	2	3	3

Table V. *Frost Damage in Willow Twigs at the Temperatures below −30°*
The symbols 0, 1, 2, and 3 represent the degree of frost damage.

Date of expt	Tissues tested	Freezing temp			
		−30°	−50°	−70°	−30° L.N.*
Nov. 10	Bud	0	0	0	0
	Xylem	0	2	3	3
Nov. 15	Bud	0	0	0	0
	Xylem	0	1	2	2
Nov. 20	Bud	0	0	0	0
	Xylem	0	0	0	0

* Twigs treated in liquid nitrogen following prefreezing at −30°.

and lower from the middle of February to May (table III). In addition, in the twigs artificially hardened for 5 days at —5°, the effective prefreezing temperature ranged from —15° to −30°, according to the degree of frost-hardiness (table III). To eliminate the possibility of hardening in the process of cooling and prefreezing in this experiment, twigs frozen at −5° were cooled to the desired temperature within 2 hours, and were then prefrozen at that temperature for only 4 hours.

The author has previously reported that at the temperatures below —30°, the intensity of cold seems not to exert any important effect upon woody plants (5, 7). A preliminary experiment demonstrated, however, that the Todo fir can survive freezing at —30° without any damage, for at least 2 days, but cannot do so at temperatures below —40°. To investigate this more fully twigs of club apple, alpine rose and black alder were frozen at temperatures ranging from —30° to —70°. The experiments showed that the xylem and pith can survive freezing at −30° without any damage for at least 2 days, but cannot survive at temperatures below —40°; the frost damage in the xylem and pith increased with decreasing temperature to −70°. However, the buds survived and grew following thawing from —70° as well as from −30° (table IV).

In addition, in the middle of November, willow twigs with varying frost-hardiness were frozen for one day at temperatures below −30°. As is shown in table V, results similar to those in table IV were obtained. From these results, it seems apparent that from −30° to —70°, frost damage increases with decreasing temperatures.

Twigs immersed in liquid nitrogen following prefreezing for 16 hours at —20° or −30° were allowed to stand for one day in cold chambers maintained at —70°, —30°, −20°, −10° and 0°, and then transfered to about 0° for 1 day. This experiment revealed that the twigs prefrozen at —20° or —30° budded and rooted normally, irrespective of the temperatures at which they had been maintained following immersion in liquid nitrogen.

Discussion

It has been well known that various organisms can be cooled to super-low temperatures in liquid gases without injury, provided that they have previously sufficiently desiccated (1). Extracellular freezing is also considered to be an effective method for dehydrating living cells. In previous papers (5, 7), it was demonstrated that, following sufficient dehydration by extracellular freezing at the temperatures below —30°, twigs from woody plants can even survive rapid cooling to super-low temperatures. Also twig pieces prefrozen at −30° were immersed in liquid nitrogen for 10 minutes, and then kept at −30° for 1 hour. The procedure was repeated 5 times with no effect upon the survival rate (5, 7).

The results published previously (5, 7) indicate that if twigs have been previously dehydrated by extracellular freezing at —30°, fatal intracellular freezing barely occurs even at extremely low temperatures, and that the survival rate is barely affected by the rate of cooling to and rewarming from a super-low temperature, if the material can withstand freezing at —30° without any damage.

It has been demonstrated in many insects and plants, that the amount of water crystallizing at —30° is more than nine-tenths of total water content, or nearly all of the freezable water in these insects and plants (9, 10, 11). On the basis of these facts, the author concluded that the effective prefreezing temperature in both animals and plants is around —30° (5, 6, 7). The effective prefreezing temperature reported here ranged from —15° to —30° according to the degree of frost-hardiness. Besides, in every experiment, it was observed that the greater the frost-hardiness, the higher the effective prefreezing temperature. From these facts, it seems unlikely that the effective prefreezing temperature is around —30° irrespective of species and the degree of frost-hardiness.

With the prefreezing method, if any freezable water remains in a cell following prefreezing, the intracellular crystallization nuclei originally formed in the course of very rapid cooling in liquid nitrogen (20°/sec) will probably recrystallize during the subsequent slow rewarming and will damage the cells (3). However, in sufficiently prefrozen twigs, no harmful effects were observed, irrespective of the rewarming velocity which followed removal from the liquid nitrogen. It may be, therefore, that hardly any water crystallizes in the tissue cells of these twigs if they have been sufficiently prefrozen, even if they are subsequently cooled very rapidly to extremely low temperatures. However, no direct evidence has been presented to confirm this.

Twigs which can survive freezing at approximately —70° are not injured, even when exposed to extremely low temperatures. In Sapporo, twigs taken from willows, poplars, white birches, etc. in the middle of November, can survive freezing at —70°. Therefore, during the period from the middle of November to early March, seasonal fluctuations in frost-hardiness and the effectiveness of the hardening temperature when the twigs are artificially exposed to various temperatures, cannot be determined in these twigs by the usual freezing tests.

In midwinter, it is also impossible to compare the degree of frost-hardiness in twigs of different species in these highly hardy plants. It is therefore essential to find a suitable method of determining the degree of frost-hardiness in plants which can withstand freezing below —70°. As has been previously observed, the greater the frost-hardiness, the higher the effective prefreezing temperature and therefore, the effective prefreezing temperature may be used as a reliable indication of the degree of frost-hardiness in highly hardy plants. This method may also be applicable to hardy animals.

Tumanov et al. (2, 12) have reported that frost damage increased with decreasing temperatures to —70° in all of the trees that they examined, and that the freezable water in the cells decreased with decreasing temperatures to —70°. They assumed therefore that fatal intracellular freezing may occur in cells cooled rapidly at temperatures below —30°, even if the cells had been cooled slowly to —30°. Therefore, they used slow rates of cooling to bring the twigs to —70° before immersing them in liquid nitrogen or hydrogen. Parker (4) has also reported that frost resistance in several conifers may range from a few degrees below zero to more than —180°, and that such plants are unable to survive immersion in liquid nitrogen unless they are first cooled slowly to −70°. Tumanov and Parker have not, however, reported any attempts to immerse twigs in liquid nitrogen which had been prefrozen at any temperatures higher than —70°.

In the present experiments, the degree of frost-hardiness in willow twigs was determined in detail during the process of natural frost-hardening. The experiments showed that frost damage increased with decreasing temperatures to —70°, in twigs which had just become resistant to —30°, although about 1 week later, these twigs could resist freezing at —70°. Therefore, in the hardiest plants, such as willow, birch, poplar etc., there appears to be far less difference in the degree of frost-hardiness between —30° and —70° than between −20° and −30°. The same trend has been observed in hardy tissues such as the cortical parenchymal cells and winter buds of less hardy woody plants.

In previous papers (5, 6), the author had suggested that plants which withstand freezing at −30° are not injured by temperatures far lower than this. The results of the present experiments indicate that this hypothesis is in error. It is reasonable, however, to assume from these results, that at temperatures below approximately —70°, the intensity of the cold has no further effect, it is not of long duration, and that if living materials withstand freezing at temperatures of approximately −70°, they can withstand immersion in liquid nitrogen, or even liquid helium, following prefreezing at temperatures ranging from —15° to −30° according to the degree of frost-hardiness in materials examined. The mechanism of frost injury in plants at temperatures below −40° is not yet clear.

Summary

Effective prefreezing temperatures differed considerably in different species in the temperature range from −15° to −30°. In the same species, it varied with seasonal fluctuation in frost-hardiness. Also, in twigs artificially hardened or dehardened, the effective prefreezing temperature ranged from −15° to −30°, according to the degree of frost-hardiness. In every experiment, it was observed that the greater the frost-hardiness, the higher the effective prefreezing temperature. Therefore, the effec-

tive prefreezing temperature may be used as a reliable indication of the degree of frost-hardiness in hardy plants which can withstand freezing below −70°.

The xylem and pith of twigs of some trees can survive freezing for 2 days at −30° without any damage, but at temperatures below −40°, the xylem and pith are damaged, although the buds survive and put forth bud after thawing from −70°. Moreover, the frost damage in xylem and pith increases with decreasing temperatures to −70°. It is therefore reasonable to assume that hardy plants are not damaged, even when exposed to extremely low temperatures, provided that they can withstand freezing at approximately −70°. The mechanism of frost damage below −40° is not yet clear.

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