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Freezing Avoidance of Primordial Shoots of Very Hardy Conifer Buds[1]

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Abstract Most of the water in primordial shoots of hardy conifers which belong to *Abietoideae* and *Laricoideae* of *Pinaceae* freezes out through the crown as temperature decreases, which enables primordial shoots to supercool to very low temperatures. In buds of Alaskan spruces, no low temperature exotherm was detected on the DTA profile to −45°C at the cooling rate of 0.8°C/min, unlike other hardy conifers which are native to lower latitudes. However, exotherms of the excised primordial shoots occurred above −17°C, resulting in death. In Alaskan spruces, ice segregation outside the primordial shoot was also observed. Thus, it may be postulated that ice segregation outside the primordial shoots proceeds too rapidly to produce intracellular freezing during cooling to about −40°C, unlike other hardy conifers. The freezing avoidance mechanism in which substantial decrease in water content exists during slow freezing, could reasonably explain why primordial shoots of conifer which are marginally hardy to −15°C or above, can winter in Alaska and Siberia where the air temperature cools down to as low as −60°C.

Introduction

The leaves and twigs of very hardy conifers, distributed in sub alpine and sub-cold climates tolerated freezing to −70°C or below, while primordial shoots of their buds preferentially sustained injury between −30 and −35°C (13, 15), except Alaskan and Siberian conifers (14), when cooled at 5°C increments at 2 hr intervals (Fig. 1). Primordial shoots of some conifers avoid lethal freezing to around −30°C by supercooling (16). George and Burke (4) reported that a low temperature exotherm in the xylem of several angiosperm species results from the freezing of cellular water in a manner predicted for supercooled dilute aqueous solution and that the freezing avoid-

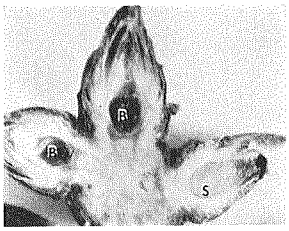

Fig. 1. Frost injury of primordial shoots of winter buds of *Abies homolepis* which were cooled down to −30°C
B: Injured bud
S: Surviving bud ×4.7

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ance mechanism by supercooling has a low temperature limit at around −40°C (2), which corresponds to the homogeneous nucleation temperature for water. We reported that winter buds of Alaskan spruce survived freezing to −80°C when cooled very slowly at 5°C increments each day (14). In this study the mechanism whereby winter buds avoid freezing on exposure to environmental temperature to −40°C or below was investigated.

Materials and Methods

Dormant one year twigs were mainly collected in the nursery of our Institute and in the Tokyo University Forest in Hokkaido(Yamabe). Spruce twigs wintering in Alaska were sent from Fairbanks by air. These twigs were stored at about −5°C in polyethylene bags containing snow to prevent desiccation. Five twig pieces, 5 to 10 cm long, were cut from each twig sample, enclosed in polyethylene bags and cooled at 5°C increments at about 2 hr or daily intervals to successively colder temperatures. After thawing at 0°C twig pieces were placed at room temperature for about 2 weeks to evaluate viability. Excised primordial shoots were placed on a watchglass in a petridish with a little water. Browning was used as the criterion for rating injury. Freezing resistance was expressed by the lowest temperature at which little or no injury was sustained. Apical buds were excised at the base and the ability of excised buds or primordial shoots to supercool was investigated by differential thermal analysis (DTA). Exotherms were detected with thermocouples placed in the central part of several excised buds wrapped with aluminum foil. The temperature of the bud was determined with an additional thermocouple and recorded. Each excised primordial shoot was wrapped separately with aluminum foil. Most exotherm analyse are based on one bud per thermocouple. In this study the number of buds per thermocouple was increased to 4 to 6. Then that number was doubled by

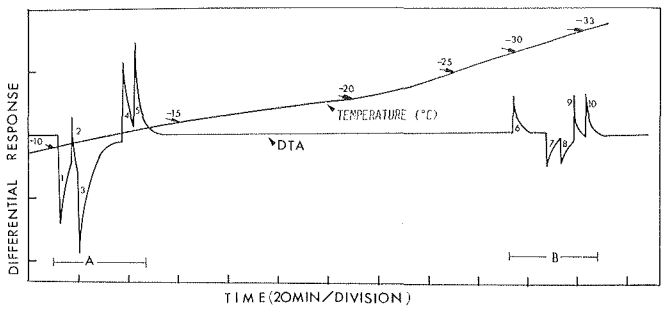

Fig. 2. DTA profile of excised winter buds of *Picea glauca*

Two and three excised buds were separately wrapped with two aluminum foil vessels and the positive and the negative thermocouples were placed in the central part of each aluminum vessel. DTA: Differential thermal analysis. A: Exotherms (1–5) correspond to freezing of water in the bud scales and the stem axis. B: Exotherms (6–10) correspond to freezing of primordial shoots. Cooling rate: 0.06°C/min from −10 to −20°C. Seed origin of *Picea glauca*: Northern Wisconsin

using both the positive and the negative junctions (9, 17). This method saves much time and allows a valid estimation of the occurrence of exotherms, especially in material in which only one low temperature exotherm appears in each bud (Fig. 2).

Results and Discussion

Primordial shoots of excised winter buds of very hardy conifers supercooled to around −32°C (Fig. 2), while these excised primordial shoots (freezing point of homogenate: about −5.5°C, water content: 55% fresh weight base) supercooled only to around −12°C. DTA profiles of excised primordial shoots revealed that the supercooling ability increased with decreasing water content and that no exotherm was detected in the primordial shoots with water content below 20% (17). Also, it was recently observed by the present author that air dried primordial shoots with water content as low as 20% remained alive (17). The exotherm temperature of the primordial shoot in excised buds was greatly affected by the cooling rate (17). A similar shift of the low temperature exotherm has been observed in azalea (7) and peach flower buds (9) after slow cooling. As shown in Fig. 3, in the excised winter buds which were cooled at 5°C increments at daily intervals from −5 to −20°C, no

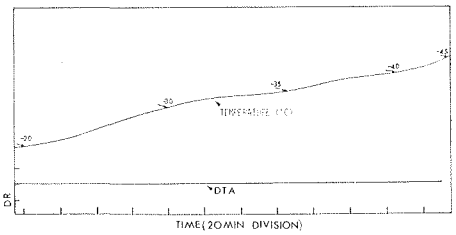

Fig. 3. DTA profile of excised buds of *Picea glauca* (February 2)

Excised buds enclosed in a small polyethylene bag were cooled at 5°C increments at daily intervals from −5 to −20°C and then DTA was performed with a cooling rate of about 0.06°C/min. Seed origin of *Picea glauca*: Northern Wisconsin

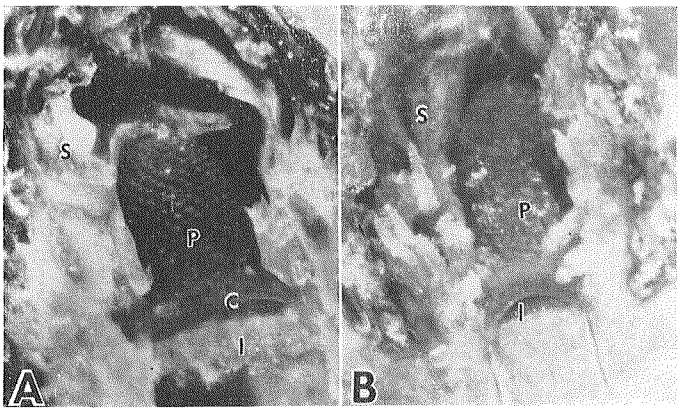

Fig. 4. Ice segregation from primordial shoots of *Abies homolepis* which were held at −5 and −10°C for 1 day, respectively

A: Buds held at −10°C; B: Buds held at −5°C; P: Primordial shoot; C: Crown; I: Ice; S: Bud scale. A: ×17, B: ×12

exotherm was detected even after cooling to −45°C (Fig. 3). These results suggest that most of the water in primordial shoots may be gradually removed from primordial shoots during slow freezing of whole buds.

Ice crystals formed in frozen buds were observed under a dissecting microscope at different temperatures. In the buds frozen at −10°C for one day, outer scales and axial pith remained frozen, while primordial shoots still remained supercooled. Much needle ice formed beneath the crown and outside the basal area of the primordial shoot (Fig. 4-A). The needle ice formed below the crown was also visible at −5°C (Fig. 4-B). It increased

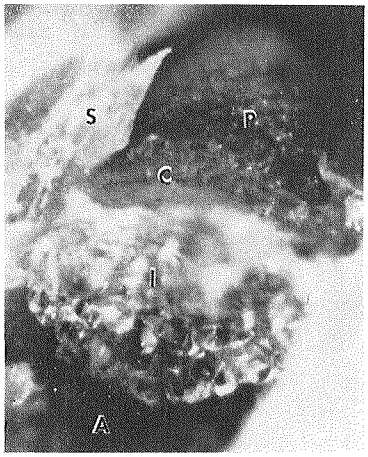

Fig. 5. Ice segregation outside primordial shoot of *Abies homolepis* which was held at −20°C for 20 days

Ice was formed beneath the crown of primordial shoot in the pith cavity between crown and head of axial pith.
P: Primordial shoot; C: Crown; I: Ice; A: Pith cavity; S: Bud scale. ×30

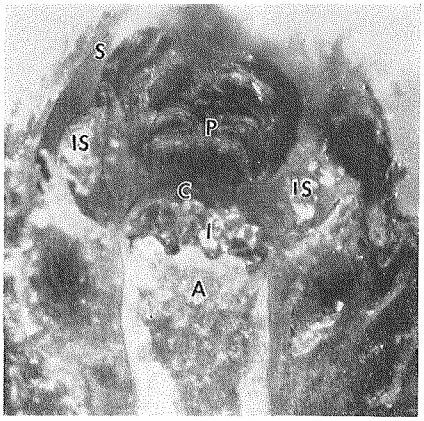

Fig. 7. A longitudinal section of winter bud of *Larix sibirica* which was held at −20°C for 1 day

The ice labeled (I) is underneath the crown and the ice surrounding outside the basal part of the primordial shoot is labeled (IS). C: Crown; P: Primordial shoot and leaves; S: Bud scale; A: Axial pith. ×21

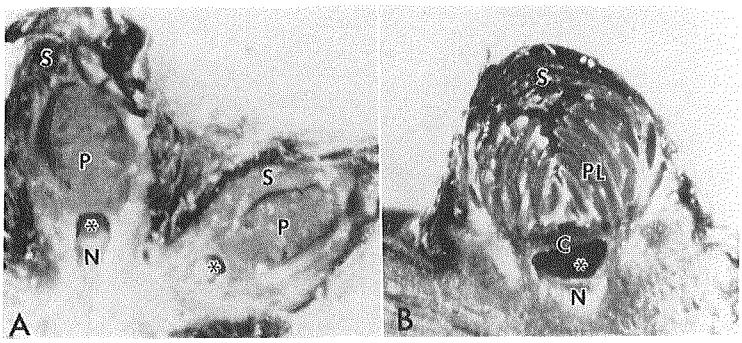

Fig. 6. Longitudinal scetions of winter buds

A: *Abies homolepis*; B: *Larix sibirica*; ∗: Pith cavity; P: Primordial shoot; PL: Primordial leaves; C: Crown; N: Axial pith; S: Bud scale. A: ×8, B: ×15

with decreasing temperatures to about −20°C at least and with the length of time held there (17, Fig. 5). In the buds held at −20°C for 30 days, the primoridal shoots still remained greenish and elastic which are characteristic of unfrozen primordial shoots. In some cases, primordial shoots were observed to turn suddenly from green to whitish opaque at the time of incision of the primordial shoot before inoculation with ice. It thus appears that supercooled water in the primordial shoot migrates outside through the crown and freezes out due to a vapour pressure gradient between supercooled water in the primordial shoot and the ice crystals outside.

In the genera which belong to *Abietoideae* and *Laricoideae* of *Pinaceae*, the pith cavity (Fig. 6) located between the crown of the primordial shoot and the head of bud axial pith is formed in mid November which effectively prevents ice spreading from the bud axis into the primordial shoot.

The present author has recently demonstrated that freezing resistance of primordial shoots of winter hardy buds differed greatly depending upon the cooling rates (17). And winter buds of *Abies balsamea* frozen at −20°C for 20 days survived down to −60°C or even immersion in liquid nitrogen after prefreezing (11, 12) to −30°C (17).

In most hardy coniferous genera such as *Pices, Abies, Larix, Tsuga, Pseudotsuga, Cedrus*, which belong to *Abietoideae* and *Laricoideae* of *Pinaceae*, winter survival of the buds mainly appears to be related to the capability of supercooling (17). It was also confirmed that the same ice segregation outside the primordial shoots through the crown occurred in these genera (Fig. 7).

In buds of Alaskan spruces *P. glauca* and *P. mariana*, which are characterized by small primordial shoots, about 1.2 mm long and about 1 mm wide at the base, no low temperature exotherm below −15°C was detected at a cooling rate of 0.8°C/min (Fig. 8). However, exotherms of excised primordial shoots of *Picea glauca*, enveloped separately with aluminum foil, occurred above −17°C, resulting in death (Fig. 9). Excised primordial shoots suspended in a small water drop at 0°C were frozen at −5°C with ice inoculation and then cooled to −10 and −15°C. The primordial shoots frozen to −10°C sustained freezing injury. Thus, it seems that the primordial shoots of Alaskan spruces are marginally hardy to −10°C or above, though

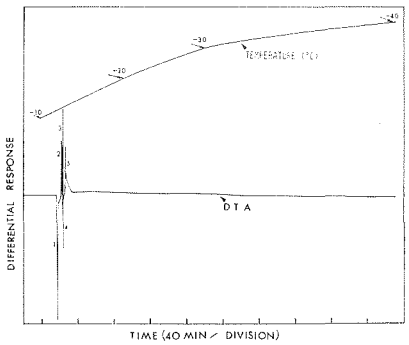

Fig. 8. DTA profile of excised buds of *Picea glauca* from Alaska

Two and three excised buds were separately wrapped with two aluminum foils, and the positive and the negative thermocouples were placed in each aluminum vessel. Exotherms (1–5) correspond to freezing of water in the bud scales and stem axis. No low temperature exotherm was observed on the DTA profile. Cooling rate: 0.8°C/min from −10 to −35

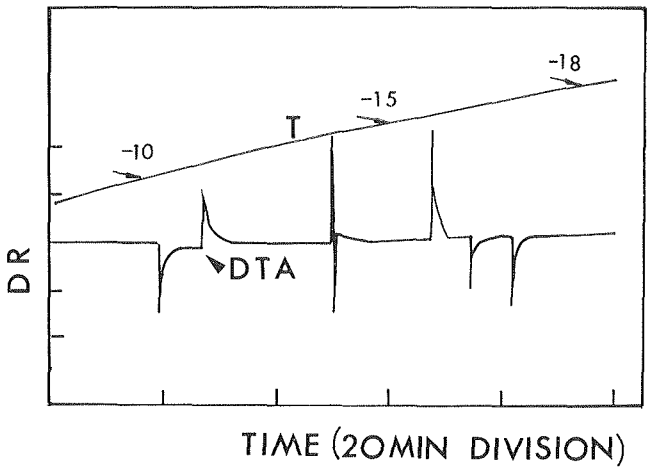

Fig. 9. DTA profile of excised primordial shoots of *Picea glauca* from Alaska

Six excised primordial shoots were individually wrapped in small aluminum vessels. Two groups of three vessels were then wrapped in another aluminum vessel. The positive and the negative thermocouples were placed in the central part of each aluminum vessel. DTA was performed with a cooling rate of about 1.0°C/min.
T: Cooling curve; DR: Differential response

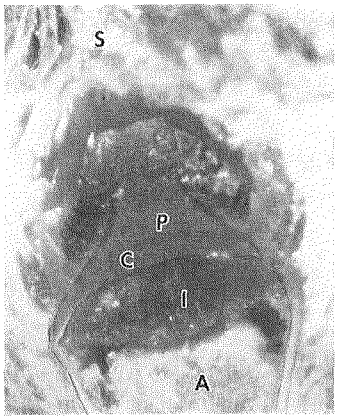

Fig. 10. A longitudinal section of the winter bud of *Picea glauca* from Alaska which was held at −20°C for 1 day

Ice segregation underneath the crown was clearly observed. The crown was characteristically curved, resulting in the increase of the surface area. The size of the primordial shoot is about 1.2 mm long and about 1 mm in diameter at the base.
A: Head of axial core pith; C: Crown; I: Ice segregation underneath the crown; P: Primordial shoot; S: Bud scale. ×25

the winter buds survived to −50°C or below, depending upon the cooling rate (17). In Alaskan spruces, ice segregation outside the primordial shoot was also observed (Fig. 10). From these results, it may be postulated that ice segregation outside the primordial shoots in Alaskan spruces proceeds too rapidly to produce intracellular freezing during cooling to about −40°C, unlike other hardy conifers.

Some reports suggested that the function of the flower bud scales was to accommodate ice which was formed from water derived from the flower primordium (1, 11, 7, 6, 10). To know the function of outer and inner scales in freezing avoidance by primordial shoots of conifer buds, DTA was performed on excised buds from which all bud scales except a few inner ones had been stripped off and the results were compared with those of untreated buds. As a result, only a slight difference was observed in the exotherm temperatures between them. It was also confirmed that in the excised buds from which the crown of primordial shoots had been removed, no low temperature exotherm below −15°C could be detected, while low temperature exotherms appeared around −25°C in untreated buds. Low temperature exotherms below about −20°C were exclusively observed in the genera which belong to *Abietoideae* and *Laricoideae* of *Pinaceae* but not in the other genera of conifer such as *Pinus*, *Cryptomeria*, *Taxus*, *Thujopsis*,

etc. These genera don't have a crown in the primordial shoot and bud axial tissues directly connected with the primordial shoots. From these results, it may be therefore concluded that most of the water in the primordial shoot freezes out through the crown, and that the crown plays the major role in freezing avoidance of primordial shoots of conifer buds. It appears that the main function for outer and inner scales of conifer buds is to prevent water loss to the air through the scales due to transpiration.

Needle ice is the result of diurnal ice segregation near the soil surface. Ice segregation results from the increase in the water (ice) volume fraction of soil layer produced by the upward migration of soil water to the freezing place after nucleation occurs and the freezing place remains at a fixed level in an initially unforzen soil (8). Ice segregation of primordial shoots through the crown appears to be a phenomenon similar to needle ice near the soil surface. To understand fully the mechanism of freezing avoidance in conifer buds, an analysis of needle ice growth through the crown and the morphological properties, especially pore size of the crown would seem to be neccessary.

Ishikawa (7) recently demonstrated that in the azalea flower buds cooled to different temperatures in 5°C increments at daily intervals exotherm temperature was highly correlated with freezing point depression as a result of water migration from flower primordia to scales. In the present study similar results were obtained in conifer buds.

Winter buds of Alaskan spruces survived freezing to −60°C, though these primordial shoots were marginally tolerant to freezing to −10°C or above. No low temperature exotherm was detected on the DTA profile to −45°C, unlike other hardy conifers native to lower latitudes. In Alaskan spruce buds frozen to −20°C, the primordial shoots remained supercooled and masses of ice were observed mainly beneath the crown. These facts suggest that in Alaskan spruce ice segregation outside the primordial shoot proceeds more rapidly than with other conifers, which is probably due to the small size of the primordial shoot and the small ratio of the length/surface area at the base and/or the morphological properties of the crown (the most suitable pore size for ice segregation through the crown probably being 10 to 15 μ).

In a finely dispersed state, pure water can be cooled to as low as approximately −40°C, which corresponds to the homogeneous nucleation temperature of water. George *et al* (3) proposed that supercooling of the azalea flower primordium would be maintained by very small nucleation barriers such as at the cell wall or plasma membrane level, which would isolate each individual cell. In peach buds, Quamme (10) proposed that the barriers appear to be organized at the tissue level rather than at the cellular level. Xylem ray parenchyma appears to be capable of supercooling to the same degree as dispersed water systems, which has a lower limit around −40°C (2, 4, 5). This is not, however, the case in primordial shoots of conifer

buds. The freezing avoidance mechanism in which some substantial decrease in water content exists, could reasonably explain why primordial shoots of conifer and/or flower primordia which are marginally hardy to −10 or above, can winter in Alaska and Siberia where the air temperature cools down to as low as −60°C. In this mechanism, the ability of winter buds to survive very low temperature depends on the ability to withstand diminished quantities of liquid water and the rate of water removal from primordial shoots during freezing.

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