林木の耐寒性に関する研究(II)

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論文

Studies on the Cold Resistance of Forest Trees (II)
The Freezing Mode of Frost Hardy Mesophyll Cells of Sugi in the Neighborhood of their Lethal Point

Akimasa TAKEDA*

林木の耐寒性に関する研究 (II)
高い耐凍性を得たスギの針葉の耐凍限界温度付近における，その葉肉細胞の凍結様式

武田明正*

要　旨: スギの葉肉細胞の耐凍性と凍結様式との関係については，すでに述べた[2]。しかし，高い耐凍性を獲得したその細胞の，耐凍限界温度付近における凍結様式は観察できなかった。そこで，凍結置換によって，耐凍性の高いスギの葉肉細胞の，耐凍限界温度付近における凍結様式を観察し，凍害の生じる凍結様式について検討した。

1) 急速に凍結した耐凍性の低い未成熟葉において，その凍結置換による像は，細胞内凍結の結果と考えられる原形質の混乱を示した（写真-2）。

2) －22℃ に凍結した耐凍性の高い葉肉細胞では，全体に，その原形質体が収縮していた（写真-4, 6）。

3) 凍結した耐凍性の高い葉肉細胞のうちで，無凍結の対照と同じように，ほとんど収縮しない細胞が観察された（写真-4, 矢印）。しかし，その細胞が，細胞内凍結をしたものかどうかは，はっきりしなかった。

以上の結果から，スギの針葉の耐凍限界温度付近においては，その葉肉細胞の多くが細胞外凍結をしており，それらの凍結による害は，おもに，細胞外凍結のもとで生じると推察される。

Summary: In order to obtain the basic information related to the freezing mode causing frost injury, the freezing mode of hardy mesophyll cells of Sugi (*Cryptomeria japonica* D. DON) was observed at their lethal point with freeze-substitution.

1) Frozen unhardy cells showed disorder of their protoplasts that suggested ice formation in intracellular space.

2) The protoplast of frozen hardy cells shrank and separated from cell wall more remarkably than that of unfrozen hardy cells.

3) Some of the frozen hardy cells did not shrink. Even in these cells, the disorder of their protoplasts such as in the case of unhardy cells was not observed.

From these observations, the freezing mode of hardy mesophyll cells of Sugi may be mainly extracellular freezing at their lethal point. So, frost injury of hardy mesophyll cells may be caused under the extracellular freezing.

Many of the past observations on the freezing mode of plant cells suggested that intracellular freezing caused the fatal damage and extracellular ice formation was not lethal for hardy cells.[1] Therefore, it is important to observe the freezing mode of cells from seedlings of Sugi for the purpose of studying their frost hardiness and frost injury.

As shown in the previous paper,[2] frost hardy mesophyll cells of Sugi were frozen extracellularily and unhardy cells frozen intracellularily at high subzero-temperature. The freezing mode of hardy cells in the neighborhood of their lethal point, however, could not be observed. So, in this paper, the freezing mode of mesophyll cells of Sugi at their lethal point were observed with freeze-substitution method.

Materials and Methods

Experiment 1. Estimation of the lethal point of needles

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Needles of two year old seedling of Sugi (*Cryptomeria japonica D. Don*) which had been potted in the previous year and grown in a glass house were investigated at the beginning of February 1970.

Five excised branches were placed on the wetted filter paper with distilled water in a Petri dish. This Petri dish was put in a cooling apparatus and cooled at −15°C. To avoid super-cooling of the materials, the wetted cotton thread was extended from the frosted wall of the apparatus to the branches in a PETRI dish. Temperature in the PETRI dish was measured with a small thermojunction (Copper and Constantan, 0.3 mm in diameter). The cooling velocity at this experiment was approximately 6°C/hr. This process was repeated at −20°, −25° and −30°C.

After two hours at each temperature, the cooling was stopped and the apparatus was allowed to warm up to room temperature. The rate of temperature rise, resulting from this procedure was approximately 10°C/hr. After thawing, the Petri dish was removed from the apparatus and placed in an incubator at 25°C with illumination of white fluorescent lamp at 2,000 lux.

About 20 hours after the freezing treatment, the viability of treated needles was estimated. As the criteria of viability of needles, impedance (a.c. resistance) of needles was measured with a.c. bridge (YOKOGAWA, BV-Z-13A) and a probe of our own making. In the probe, two electrodes (0.16 mm in diameter, 1.0 mm in length) were mounted. Distance between the electrodes was 6 mm.

The results are illustrated in Figure 1. Accompanying with the drop of temperature at which needles were frozen, impedance of needles decreased. From these results and preliminary correlation studies between impedance and visual observation of tissue viability, it was estimated that the lethal point of materials existed in the range from −20° to −25°C.

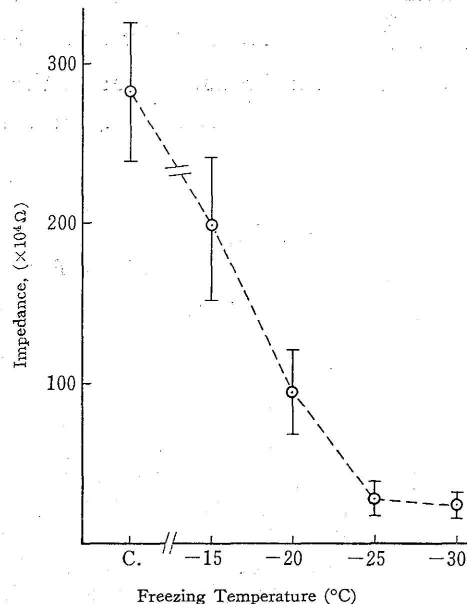


Fig. 1. Relation between impedance of needles measured 20 hours after thawing and freezing temperature. C: unfrozen needles as control. Vertical lines represent standard deviation

Experiment 2.

1) Freeze-substitution of hardy needles

Needles were cut to the length of approximately two millimeters, and were put in a test tube. The cooling condition and other procedures were the same as in Experiment 1. The freezing temperature was −22°C. Two hours after the beginning of freezing treatment, the test tube was quickly removed from the cooling apparatus in a thermos. This thermos had been cooled with dry ice and ethanol below −70°C, to avoid marked development of ice crystals during the freeze-substitution.

As fixing agent, cooled ethanol below −70°C which contained one percent picric acid was poured in the test tube. This test tube was kept in a thermos below −70°C. About two weeks after, the thermos was warmed up to room temperature and needles were washed with absolute ethanol repeatedly. Lastly, these needles were kept in ethanol which contained lithium carbonate.

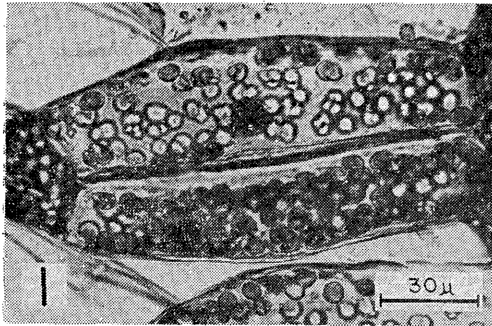
Some pieces of unfrozen needles which were fixed with the same fixing agent at room temperature over-night were prepared as control to compare with the frozen needles. These pieces of frozen or unfrozen needles were washed again with ethanol, embedded in Carbowax and sectioned with the microtome. Observed sections were stained with DELAFIELD's hematoxylin solution and were mounted with glycerin-water.

2) Freeze-substitution of unhardy needles

This experiment was accomplished at the end of September 1970. The unhardy immature needles in a test tube were frozen at −23°C rapidly (approximately 3°C/min). After two hours freezing, the fixing agent which had been cooled at the same temperature was poured in the test tube and this test tube was kept at that temperature over-night. The following day, the test tube was warmed up to room temperature and fixed needles were washed, embedded and observed as previously mentioned.

Results and Discussion

Some observations on the freezing mode of plant



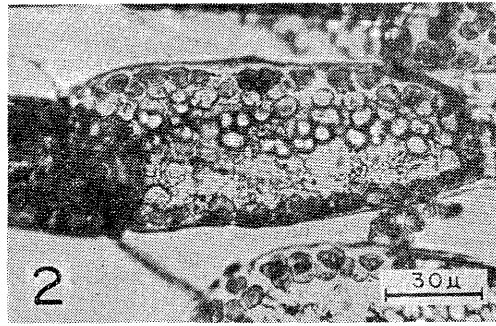
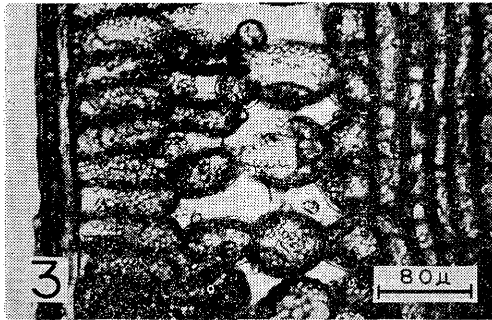


Photo. 1–2. Unhardy mesophyll cells 1: unfrozen cells and 2: frozen cells



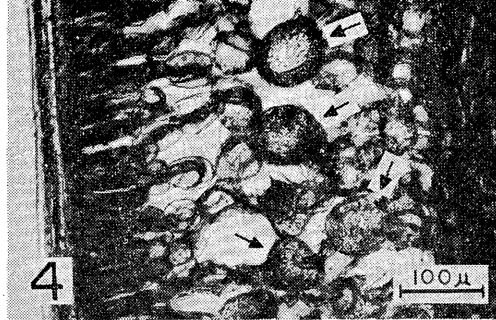
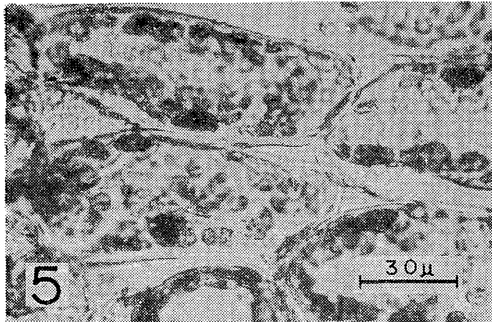


Photo. 3–4. Cross section of hardy needles 3: unfrozen needle and 4: frozen needle



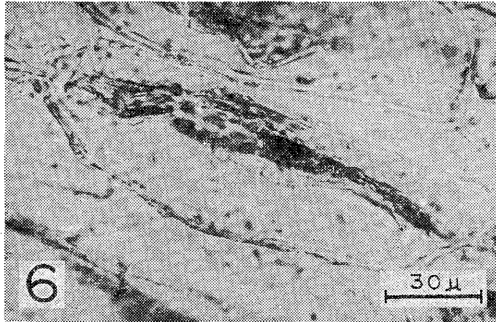


Photo. 5–6. Hardy mesophyll cells 5: unfrozen cells and 6: frozen cells

cells with freeze-substitution method had been made to date[3~6]. In conifers, the freezing mode of the mesophyll cells of blue spruce was extraprotoplasmic at −25°C in winter[5]. In the mesophyll cells of Sugi, protoplasts of frozen unhardy cells shrank a little. These shrinkage of protoplasts may be an artifact by fixation, for unfrozen cells also showed the same phenomenon.

In comparison with the figure of the protoplasts of unfrozen cells (Photo-1), that of frozen unhardy cells indicated the disorder of their protoplasts which suggested the intracellular freezing (Photo-2).

On the other hand, the protoplasts of frozen hardy cells, shrank more markedly (Photo-4, 6) than that of unfrozen hardy cells (Photo-3, 5). Some protoplasts of the frozen hardy cells did not shrink (Arrows in Photo-4). Even in these cells, the disorder of protoplasts which suggested the ice formation in the intracellular space like in the case of unhardy cells was not observed. From these observations, the mesophyll cells of Sugi appeared to be frozen extracellularily in the neighborhood of the lethal point.

These hardy mesophyll cells of Sugi, however, were frozen to death at below their lethal temperature. Many factors appeared to be responsible for the freezing injury that occurred in the absence of intracellular freezing. The marked dehydration of

protoplasm was a widely known factor associated with lethal injury[7]. So, the frost injury of hardy mesophyll cells of Sugi may be accounted for by this factor. This view is strengthened by the fact that the difference in the frost hardiness among varieties of Sugi is closely related to that of desiccation resistance[8].

But, the freezing injury of hardy Sugi may have been caused by intracellular ice formation following extracellular freezing below their lethal temperature and these ice crystals may have been too small to be detected under the optical microscope. Further studies on these problems are required.

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