Undercooling Points and Frost Resistance in Mature and Immature Leaf Tissues of Some Evergreen Plants

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It is well known that living organisms can be undercooled to certain low temperatures. Most of them are not injured by undercooling itself, but when freezing once occurs within their tissues they suffer from frost-injury. The degree of such an injury is various according to the kinds of organisms. The great majority of works on the relation between capacity for undercooling and cold resistance have dealt with animal materials, especially with insects. Salt showed that a high capacity of undercooling is regarded as the essential factor of cold resistance in the non-frost-resistant insects. According to Aoki, the value of undercooling point can not be an index of frost resistance because it does not differ in insects with different frost resistance. In a potato tuber it was shown by Terumoto that undercooling point was lowered during the hardening process. However, whether the undercooling is an important factor in frost resistance or not is still uncertain. The purpose of the present investigation is to clarify the relation between degree of undercooling and frost resistance in leaf tissue.

Since the undercooling point is, as a rule, affected by several physical factors, especially by the rate of cooling, the effect of cooling rate on the undercooling points in leaf tissues is also reported here.

Materials and Methods

The present experiment was carried out mainly from June to July in the leaf tissues of Aspidistra elatior and Viburnum odoratissimum. In winter season from November to December some experiments were also made in these two plants and Fatsia japonica. From spring to summer these plants have leaves different in the stage of maturity, i.e., the mature (old) leaves remaining from the last year and the immature (young) ones unfolded in the spring of the present year. The leaf pieces used were cut off from the leaf blade excluding the middle main vein. The size of each leaf piece was about 30 mm × 10 mm in Aspidistra and 25 mm × 10 mm in Viburnum and Fatsia. The sap flowed out from the cut face of the leaves was blotted off with filter paper. The undercooling point was determined by the freezing curve (temp. vs. time) of each piece. The temperature of leaf pieces was measured by means of a thermocouple consisting of copper and constantan wires (0.25 mm in diameter). The tip of thermocouple was kept in contact with the central part of the surface of leaf piece, where a bit of silicon oil (Toshiba TS 951–350 c. s.) was dropped to increase the heat conduction between thermocouple and leaf. The thermoelectric potential was measured with a galvanometer (Yokogawa, D-2 LD) and recorded every 15 seconds. In the experiments after September it was automatically recorded with

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** The undercooling point means the temperature at which undercooled state in the system is spontaneously broken without any artificial ice seeding.
a galvanometer (Toa Electronics AD-7) and a polyrecorder (Toa Electronics EPR-2T). The cooling of samples was done as follows: the leaf piece which had been fixed on a supporting wire by a rubber band, was placed in a protecting glass tube with the thermocouple and the glass tube was cooled in a refrigerator (Toshiba icecream stocker—cooling temperature $-20^\circ$). The cooling rate was regulated by changing the size of protecting glass tube or multiplying the layers of air mantle around the protecting glass tube. As the rate of cooling of leaf pieces, $1^\circ$ to $7^\circ$ per minute at $0^\circ$ was employed. The osmotic value of the leaves was measured by the plasmolytic method using KNO$_3$ as a plasmolyte in upper epidermis which was stained by neutral red solution (0.01%). Water content was determined by drying the leaf pieces excluding the main vein at $95^\circ$ for 48 hours and expressed in percentage of the fresh weight.

**Results and Discussion**

*Comparison of the mature and immature leaf tissues in frost resistance:* Figs. 1 and 2 show the typical freezing curves in mature and immature leaves of *Aspidistra* and *Viburnum*. The letters along the curves indicate the various stages of the freezing process in leaf tissue; $b$ and $b'$ indicate the undercooling points, and $c$ and $c'$ the rebound points. The ice formation in freezing tissue is most active between $c$ and $d$ or $c'$ and $d'$, and seems to be almost finished at the stage of $e$ or $e'$. Afterward the tissue piece is gradually cooled down to the temperature of refrigerator. Comparing the shape of freezing curves of mature leaves with those of immature ones, the rise in temperature after release from the undercooled state was more gradu-

![Fig. 1](image1.png) ![Fig. 2](image2.png)

Fig. 1. A typical freezing curve in the mature and immature leaves of *Aspidistra*. Cooling rate 2.5$^\circ$/min. at $0^\circ$ in the mature leaf and 3.0$^\circ$ in the immature one (on June 13). The letters along the curve indicate the various stages of the freezing process. See the text for the explanation.

Fig. 2. A typical freezing curve in the mature and immature leaves of *Viburnum*. Cooling rate 2.5$^\circ$/min. at $0^\circ$ in both leaves (on June 18). For the letters along the curve, see Fig. 1.

al in the former than in the latter, and the grade of temperature rise was smaller in the mature leaves than in the immature ones. The length of the plateau ($c$ to $d$) on the freezing curve in the mature leaves was longer than that ($c'$ to $d'$) in the immature ones (Figs. 1 and 2).
The leaf pieces were rewarmed at the predetermined stages of the freezing process and the survival rate was determined as follows: the upper epidermis was quickly cut off from the leaf piece immediately after the rewashing and stained with neutral red solution (0.01%). The numbers of epidermal cells capable of apparent plasmolysis (in isotonic concentration ×1.5) and deplasmolysis (in isotonic concentration ×0.5) in KNO₃ solution were counted in each tissue section. The percentage survival in epidermal cells of leaf tissue is shown in Fig. 3. The letters along the curves indicate the percentage survival of the epidermal cells rewarmed at the points of the same letters on the freezing curve shown in Figs. 1 and 2. As is shown in Fig. 3, the percentage survival in the immature leaf cells was high when the leaf piece was rewarmed before reaching the stage of d', but if the cooling proceeded further, it suddenly decreased and almost all the epidermal cells were killed at the stage of e'. In the mature leaves, on the other hand, no epidermal cells were killed till they were cooled to the stage of e, and then were gradually injured. In other words, no epidermal cells in mature leaves suffered from frost-injury until the freezing of the leaf tissue was nearly completed. It seems therefore reasonable to assume that the epidermal cells in mature leaves are more frost-hardy than those in immature ones.

The mature and immature leaves in both *Aspidistra* and *Viburnum* were compared in water content and osmotic value (Table 1). As

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<thead>
<tr>
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<th>Aspidistra</th>
<th>Viburnum</th>
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<tbody>
<tr>
<td><strong>Water content (%)</strong></td>
<td>Mature 68.0</td>
<td>Immature 84.4</td>
</tr>
<tr>
<td><strong>Osmotic value (KNO₃ M)</strong></td>
<td>0.60</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Water content (%)</strong></td>
<td>Mature 68.2</td>
<td>Immature 74.4</td>
</tr>
<tr>
<td><strong>Osmotic value (KNO₃ M)</strong></td>
<td>0.41~0.45</td>
<td>0.27~0.31</td>
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is shown in Table 1, the mature leaves had a lower water content and a higher osmotic value than the immature ones in both plants.

*Comparison of the undercooling points in mature and immature leaf tissues*: The undercooling points of mature and immature leaves in *Aspidistra* and *Viburnum* were measured at various cooling rates. As is shown in Fig. 4, in *Aspidistra* the undercooling points of the mature leaves were always lower than those of the immature ones, while in *Viburnum*, on the other hand, the immature leaves showed lower undercooling points than the mature ones. This was commonly observed at the cooling rate of 1° to 7° per minute. The difference of undercooling points between the mature and immature leaves were more remarkable in *Aspidistra* than in *Viburnum*. 
Such a tendency in the property of leaf tissue of these two species was also observed in water contents and osmotic values (Table 1). Though epidermal cells in mature leaves are harder than in immature leaves in both plants, the relation of undercooling points of the mature leaf to that of the immature one is quite inverse in both plants. Therefore, it seems that there is no direct interrelation between frost resistance and the undercooling points in leaf pieces.

In *Camellia sinensis*, the undercooling points of old leaves are lower than those of the young leaves\(^9\), while in *Rhododendron ripense* the undercooling points showed no clear difference between old and young leaves\(^6\). Thus the relation between undercooling point and degree of leaf maturity is not the same in various plants.

It has been considered that undercooling is one of the important factors in avoiding ice formation in plants\(^6\). According to Terumoto\(^3\), the undercooling of potato tubers stored at a low temperature becomes large. Ecologically a stable undercooled state in a field plant has a more important significance in preventing frost-injury than the lowering of the freezing point of cell sap. The seasonal periodicity of undercooling points was observed both in an insect\(^7\) and the twig of a mulberry tree\(^8\). In these investigations the undercooling points lowered in winter season, and at least in an insect, it was correlated to the increase of frost resistance. On the other hand, in a non-hardy prepupa of ruby-tailed wasp, *Crysis* (*Pentacrysis*) shanghaiensis, which was parasitic on a slug moth grown at a warm district, the undercooling point was found to fall in the same temperature range as that of the prepupa of slug moth, a remarkably frost-resistant insect\(^9\). From these results, Aoki considers that the value of the undercooling point itself merely shows a relative capacity of undercooling under a definite cooling condition. This is in agreement with the present result. From these facts it may be concluded that the relation between frost resistance and the undercooling point is not the same in different materials, and that it is impossible to regard the undercooling point alone as an index of frost resistance.

**Effect of the cooling rate on the undercooling points in leaf pieces**: The effect of the cooling rate on the undercooling points was studied in the leaf pieces. The materials used were the mature and immature leaves of *Aspidistra* and *Viburnum* in summer (from June to July), and the mature leaves of the above two plants and *Fatsia* in winter (from November to December). The undercooling points determined at the cooling rates ranging from 1° to 7° per minute are shown in Figs. 4 and 5. According to Aoki and Shinozaki\(^10\) the undercooling points in the prepupae of slug moth showed only a relatively small fluctuation under any definite cooling condition. Such a tendency was also recognized in the present experiments. In summer, the fluctuation of undercooling points in mature leaves was smaller than that in immature
ones. This may probably be based on the fact that the mature leaves were in almost the same stage of maturity. In summer the fluctuation of the undercooling points was, as a rule, small at a slow cooling rate, but large at a rapid cooling rate. This tendency could not be observed in winter. The undercooling points at different cooling rates showed a falling tendency with increasing cooling rates. In other words, at slow cooling rates the undercooled states in leaf pieces were broken at high temperature, while at high cooling rates initiation of freezing occurred at low temperatures. Such a tendency was equally observed in experiments both in summer and winter. However, in each plant the gradual lowering in undercooling points with increasing cooling rates was different in degree. This appeared more clearly in the following order: Fatsia > Aspidistra > Viburnum (Fig. 5).

Aoki and Shinozaki studied the effect of the cooling rate, ranging from 0.7° to 13° per minute, on the undercooling points in the prepupa of slug moth. They found the undercooling points at a slow cooling rate, i.e., 1° per minute were scattered only within a relatively narrow temperature range around −20°, but when the cooling rate gradually increased, the highest value of undercooling points was raised to high temperatures though the lowest values did not change. They concluded therefore that the undercooled state of the prepupa became unstable with the increasing cooling rate. In the present experiment, only in summer the undercooled state in leaf pieces was rather stable at a slow cooling rate, but this was not the case in winter. The leaf tissue was found to be quite different from the prepupa of slug moth in the behaviour of the undercooling point. The undercooling points of leaf pieces were gradually lowered with increasing cooling rates, while the temperature range of the fluctuation of undercooling points was nearly the same at any cooling rate (Fig. 5). The gradual lowering of undercooling point with increasing cooling rates may conceivably be explained by the fact that the higher...
is the rate of cooling, the shorter is the time spent before spontaneous freezing occurs in the leaf piece (Fig. 6), because the probability of ice nucleation increases in any system as the duration of undercooling is lengthened\textsuperscript{11,12}.

**Summary**

The undercooling points of leaf tissues in *Aspidistra*, *Viburnum* and *Fatsia* were determined by the freezing curve of leaf pieces. The relation between the undercooling points and frost resistance both in mature and immature leaves, and the effect of the cooling rate on the undercooling points were investigated.

1. In *Aspidistra* and *Viburnum*, the epidermal cells in mature leaves were more frost-hardy than those in the immature ones. In immature leaves, the injury caused by freezing occurred with the beginning of freezing in the tissues, while in mature leaves no injury appeared till the freezing of tissues was nearly completed.

2. The undercooling points in leaf pieces showed a relatively small fluctuation under any definite cooling condition. The undercooling points of the mature leaves of *Aspidistra* were always lower than those of the immature ones, while in *Viburnum*, the undercooling points of immature leaves showed lower values than those of the mature ones. This was observed at any cooling rates, ranging from 1\(^\circ\) to 7\(^\circ\) per minute.

3. The effect of the cooling rate on the undercooling points was studied under the cooling conditions of 1\(^\circ\)~7\(^\circ\) per minute at 0\(^\circ\). In summer the temperature range of the fluctuation of undercooling points was small at a slow cooling rate, and became larger with the increasing cooling rate, but in winter it was nearly the same at any cooling rates.

4. The undercooling points were gradually lowered with increasing cooling rates, that is, the undercooled state was broken at higher temperatures in slowly cooled leaf pieces than in rapidly cooled ones.

5. The relation between frost resistance and the undercooling point in leaf piece is various according to the species of plants. It is therefore not adequate to regard the value of the undercooling point alone as an index of frost resistance in plants.

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**References**

賛来章録：数種の常緑植物の成熟および未熟葉組織における過冷却点と耐凍性との関係

ハラン・サンゴジュおよびヤッデの葉組織における過冷却点（過冷却が自動的に破れする限界温度）を各組織の凍結曲線から測定し、成熟葉（前年葉）と未熟葉（当年葉）における過冷却点と耐凍性との関係、ならびに過冷却点に及ぼす冷却速度の影響をしらべた。

1. ハランとサンゴジュの成熟および未熟両葉組織片について、凍結曲線をとりながら、その凍結過程の任意の時期に冷却を中断し、その際における表皮組織の生存率から耐凍性を比較すると、両植物いずれにおいても成熟葉は未熟葉より高い耐凍性を示す。すなわち、未熟葉では組織の凍結開始後まもなく生存率が低下しはじめるのに対し、成熟葉では組織の凍結がほとんど終了するまで生存率は高い。

2. 葉組織の過冷却点は、一定の冷却条件下では、植物の種類・組織の成熟度に応じてそれぞれ比較的狭い温度域にあつまる安定した値を示す。ハランおよびサンゴジュの成熟葉は未熟葉にくらべ耐凍性が高いか、過冷却点については、ハランでは成熟葉が未熟葉より低く、サンゴジュでは逆に未熟葉の方が成熟葉より低く、したがって、葉組織の耐凍性と過冷却点との関係は、植物の種類によってこととなり、過冷却点の値を直ちに耐凍性判定の指標にすることはできない。

3. 過冷却点に及ぼす冷却速度の影響を、0℃において1℃/分の冷却条件でしらべると、夏期のハランおよびサンゴジュでは、冷却温度が小さいときほど過冷却点の温度域は狭く、冷却速度が大きくなるにつれて次第にその幅は拡大する。しかし、このような傾向は冬期には認められず、冷却速度において温度域の幅はほとんど等しい。

4. 冷却速度が大きくなるにつれて、過冷却点は次第に下降する。すなわち、急速に冷却すると、よく過冷却するが、この状態は不安定で早く破れやすく、ゆっくり冷却すると過冷却の状態は時間的に長く続く高い温度で破れる。（九州大学教養部生物学教室）