Supercooling ability of *Rhododendron* flower buds in relation to cooling rate and cold hardiness

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The freezing process and supercooling ability in flower buds of 11 native *Rhododendron* species were examined with reference to the cooling rate and cold hardness by differential thermal analysis. The freezing patterns of the excised whole buds varied with the season: in autumn, buds froze as whole units, while in winter, freezing was initiated in the scales and propagated to each floret. The supercooling ability of florets was enhanced during winter. The freezing patterns in winter buds were strongly influenced by the cooling rate (1 to 30°C/hr). Although the first exotherm in scales occurred at —5 to —10°C and was rate-independent, the occurrence of several floret exotherms shifted considerably to lower subzero temperatures at slower rates. The most reliable cooling rate for testing maximum supercooling ability was 1°C/hr. The exotherm in florets of hardier species occurred at —20 to —25°C and at —7 to —20°C for less hardy ones, and were well correlated with their killing temperatures. Water relations within bud tissues in response to freezing are briefly discussed.

**Key words:** Cold hardiness — Cooling rate — *Rhododendron* flower buds — Supercooling ability.

Deep supercooling in xylem ray parenchyma and flower buds of woody plants in the range —20 to —40°C has recently attracted attention due to its physiological and ecological implications as a frost avoidance mechanism (2, 3, 7, 9, 10, 15). The occurrence of deep supercooling in flower buds was first found in the excised floret of a hardy deciduous *Rhododendron* species (*R. mollis* and its hybrids), with the degree of supercooling in excised florets being highly correlated with their moisture content (6). Although the importance of the cooling rate for the supercooling ability of florets in excised whole buds has not been considered (3), George et al. (4) have found that the DTA profile and exotherm temperatures of floral primordia in excised whole buds vary considerably with an increase in the cooling rate. Recently, Ishikawa (7) has stated that there is a marked increase of the supercooling ability of florets in *R. japonicum* buds during overnight cooling from 0 to —5°C at which bud scales are frozen. They surmised the floret water is withdrawn into the scales and freezes, and the lowering of exotherm temperatures is highly correlated with freezing point depression as a result of water withdrawal from the floret. Although the effect

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**Abbreviations:** DTA, Differential thermal analysis; KT, killing temperature.
of the cooling rate upon supercooling of flower buds has been fragmentarily studied for several genera such as Prunus (12), Vaccinium (1) and Vitis (11), but not for Rhododendron, little is known because cooling rate studies were not the main objective of these works.

The probability of ice nucleus formation in a supercooled liquid increases with time and falling temperature and both factors function together as the rate of cooling. In biological systems such as insect larvae (16) and plant leaf pieces (8), the mean freezing temperature rises as the rate of cooling decreases. Thus, the enhancement of supercooling ability in florets in excised whole buds occurring at very slow cooling rates is remarkable, and further extensive studies are needed.

Rhododendron species used by previous workers (4–7) for deep supercooling in winter buds belong taxonomically to the section Rhodora. Their bud morphology, especially floret-scale arrangement, is not the same as the section, such as Tsutsusi and Brachycalyx, including more dominant species of natural vegetation in Japan (cf. Fig. 6). In the present work, the section Tsutsusi is mainly used as material. The objectives of the study were to characterize the freezing process of flower buds in these species in terms of the cooling rate and water relation within bud tissues, and to determine whether exotherm temperatures of florets are associated with their freezing resistance and their different ecological habitats.

Materials and methods

Plant materials

Rhododendron species used as materials comprise 11 taxa, of which R. weyrichii is exceptionally deciduous, three are semi-deciduous (R. kiusianum, R. ripense and R. macrosepalum) and the remainder are evergreen. Two- or three-year-old twigs having flower buds were sampled. Most of the samples were collected from outdoor plantings at the Karume Branch, Vegetable and Ornamental Research Station, Ministry of Agriculture, Forestry and Fisheries and at our University campus. Some of the samples for R. sataense, R. tashiroi var. lasiophyllum and R. eriocarpum were collected from natural habitats in Kagoshima Prefecture. Although the natural ranges of the species tested are mainly Kyushu and the southwestern part of Honshu, their natural habitats are quite characteristic: the range for R. kiusianum is restricted to mountain regions about 1000 m above sea level in Kyushu and that for R. sataense is confined to mountain areas of the most southern part of the Kyushu mainland, while R. eriocarpum, R. tashiroi and R. tashiroi var. lasiophyllum occur in the Ryukyus and also on Satunan Is. In addition, an azalea cultivar [R. × akebono] was used for some observations. This species is a bud mutation of R. × oomurasaki and is regarded as the hybrid, R. scabrum × R. ripense.

Twig samples collected in fall were kept in a vase at room temperature until exotherm analysis. Twigs collected during the winter season were enclosed in polyethylene bags saturated with water vapor to prevent desiccation and stored at 2 to 3°C until used for exotherm analysis (1 to 2 weeks).

Exotherm analysis

DTA measurement, revealing the heat released at the temperature at which the
samples were frozen, was used to determine the freezing process in flower buds. The DTA apparatus has been described by Kaku and Iwaya (9, 10) in detail and is only briefly outlined here. An excised whole bud with 1–2 mm axis was secured in a hollow on a cork board (4 mm thick) by cotton threads with the tip of the thermocouple junction touching the central part of the sample surface. Contact between the sample and the thermocouple junction was maintained by the tension of the thermocouple leads, and the tip of the thermocouple was not inserted into the sample. To regulate the cooling rate, the assembly was enclosed within a glass vial (4.5 × 11 cm) and placed concentrically in thermos flasks of various sizes of 5 to 7 cm in diameter and 14 to 22 cm high. The flask was transferred to a freezer which could be cooled to as low as —90°C.

Cooling rates of 10 to 40°C/hr were obtained by manually controlling the temperature of the freezer. The cooling rate of 1°C/hr was obtained by using a programmatic regulator (Chino BF-121) attached to the freezer.

Water content determination

The water content of the bud scale, axis and floret was determined with the same buds used for DTA before freezing (+3°C) and after freezing to about —30°C and expressed as the percentage of dry weight. Each bud tissue in the frozen bud was excised as quickly as possible with tweezers, cooled to —30°C, on a dissecting plate, also cooled to —30°C. Room temperature was kept at about 10°C. Five determinations were made.

Hardiness determination

Five twig pieces (5 to 15 cm long) with 5 to 15 flower buds were used for the hardiness determination. Natural cold hardiness at the time of collection was determined as for the spring and autumn buds, while the maximum hardiness was evaluated for the materials collected from January to February: twig pieces enclosed in polyethylene bags saturated with water vapor were subjected to 2 to 3°C for 3 weeks for preconditioning. Next, they were cooled in 5°C increments manually each day (—10 to —40°C for the Kurume collection and —10 to —30°C for the Kagoshima collection). After equilibration had been established for 1 day at the selected test temperature, the bags were removed from the freezer and thawed in a chamber at 2 to 3°C. Browning was used as a criterion for rating injury after 2 days of thawing. The hardiness was expressed as KT—the lowest test temperature at which about half of the florets survived without any injury.

Results

Freezing patterns and freezing process in autumn and winter buds

The freezing patterns between autumn and winter buds were compared at relatively fast cooling rates, 30 to 40°C/hr. As described in detail later, the freezing pattern at a fast cooling rate is not ideal but has the advantage that many species can be tested within short periods of time, thus offering some useful information.

Freezing patterns in excised whole buds differed for autumn and winter buds in most of the species tested. In general, a single large exotherm occurred in autumn
buds, while midwinter buds had multiple exotherms of which the first one or two appeared usually as a small rounded step with sharp major ones. This freezing pattern was the most typical found in seven out of the ten species examined (Fig. 1A). However, in buds of *R. weyrichii* and *R. sataense*, multiple exotherms occurred throughout fall and midwinter (Fig. 1B) and a single large exotherm in autumn and midwinter buds was found only in *R. tashiroi* (Fig. 1C).

These freezing patterns are in a sense a generalized pattern and each freezing pattern was by no means uniform for the same species. For example, most autumn buds in *R. kiusianum* and *R. ripense* had a single large exotherm but multiple sharp exotherms were occasionally found and multiple ones occurred in almost all winter buds. On the other hand, in winter buds of *R. tashiroi*, multiple exotherms were exceptional at relatively fast cooling rates.

The exotherm of the excised scale and floret occurred always as a single one, and that of scales was smaller than that of florets. They also occurred at higher temperatures than the latter (Table 1). The number of large sharp exotherms generally corresponded to the number of florets within the flower bud. Thus, it is evident that the first one or two small exotherms in the excised whole bud results from the freezing of water in the scales, and succeeding multiple exotherms are a consequence of freezing in each successive floret. Several workers have shown that the freezing of scales preceded that of florets in deciduous azalea (4, 5, 7). The initiation tem-
Table 1  Mean exotherm temperatures of excised scales and florets, and initiation temperatures of 1st round and 2nd sharp exotherms for excised whole buds

<table>
<thead>
<tr>
<th>Axis</th>
<th>Scale</th>
<th>Floret</th>
<th>Whole bud</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wt (mg)</td>
<td>Exotherm temp. (°C)</td>
<td>Wt (mg)</td>
</tr>
<tr>
<td><strong>R. kiussianum</strong></td>
<td>2-3</td>
<td>-12.1±1.7*</td>
<td>5-7</td>
</tr>
<tr>
<td><strong>R. wuyrichii</strong></td>
<td>5-10</td>
<td>-13.4±2.0</td>
<td>25-30</td>
</tr>
</tbody>
</table>

* Mean and standard deviation of five samples.

Table 2  Changes of water contents within flower bud tissues in response to freezing (cooling rate: 1°C/hr)

<table>
<thead>
<tr>
<th>Axis</th>
<th>Scale</th>
<th>Floret</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R. kiussianum</strong></td>
<td>73.8±6.6*</td>
<td>58.6±9.4</td>
</tr>
<tr>
<td><strong>R. xakehono</strong></td>
<td>113.4±9.0</td>
<td>46.5±5.7</td>
</tr>
<tr>
<td><strong>R. tashiroi var. lasiophyllum</strong></td>
<td>173.5±13.3</td>
<td>133.3±54.7</td>
</tr>
</tbody>
</table>

* Mean and standard deviation of 7 samples.
temperatures of the first small rounded and the succeeding large sharp exotherms in whole buds were much higher than those of excised scales and florets (Table 1).

**Effect of cooling rate upon the supercooling of excised winter buds**

The effect of cooling rates upon the supercooling of excised whole buds in winter was investigated at four different rates, 22 to 30, 16 to 20, 10 to 13 and 1°C/hr using *R. kiusianum*, *R. × akebono* and *R. tashiroi* var. *lasiophyllum*. DTA profiles of excised whole buds varied considerably with an increase in the cooling rate (Fig. 2). The first small rounded exotherm which resulted from the freezing of water in bud scales occurred at almost the same temperature range, about —5 to —10°C, and was rate-independent, while the large sharp exotherms which resulted from the freezing of the florets varied with the rate of cooling. At fast rates (20 to 30°C/hr) several floret peaks followed the first rounded exotherm very closely, and slower rates produced "spread out" profiles of the appearance of several floret exotherms.

The effect of changing the cooling rate on exotherm temperature distribution for florets is shown in Fig. 3. Floret exotherms for slower rates occurred at lower subzero temperatures than those for faster rates. In *R. kiusianum*, exotherm temperatures occurred at higher subzero temperatures when cooling was fast than when it was slow. The range gradually shifted downwards, to about —20 to —25°C, at slower rates (10 to 13 and 1°C/hr). At the slowest rates, 1°C/hr, the exotherm temperatures concentrated in a narrow range of lower subzero temperatures. In *R. × akebono* and *R. tashiroi* var. *lasiophyllum*, on the other hand, exotherm temperature distributions ranged over about the same subzero temperatures with wide ranges

![Fig. 2. DTA profile in excised whole bud for *R. kiusianum* affected by changing cooling rates.](http://pcp.oxfordjournals.org/Downloaded from at Penn State University (Paterno Lib) on September 17, 2016)
Supercooling of Rhododendron flower buds

Fig. 3. Effect of changing cooling rate on exotherm temperature distribution of florets in excised whole buds. X, exotherm temperature of bud scales; O, exotherm temperature of first floret in a bud; ●, exotherm temperature of second or succeeding florets in a bud.

of cooling rates (10 to 30°C/hr). However, the exotherm temperatures at the slowest cooling rate, 1°C/hr, occurred in a narrow range of temperatures in both species as similarly observed in R. kiusianum. Thus, it is apparent that the most reliable cooling rate for testing maximum supercooling ability was 1°C/hr, and this rate was used in subsequent trials.

**Exotherm temperature and KT of flower buds in species native to different ecological habitats**

The distributions of exotherm temperatures of florets and KTs in excised whole flower buds are shown in Fig. 4. Exotherm temperatures of florets for R. kiusianum occurred between about -19 to -26°C and those for R. macrosepalum, R. ripense, R. mucronatum and R. sataense were at about -14 to -25°C. Otherwise, the KTs for R. kiusianum were at -26°C and those for R. macrosepalum, R. ripense, R. mucronatum and R. sataense were at -21 to -23°C. Exotherm temperatures of florets for more southern species such as R. tashiroi, R. tashiroi var. lasiophyllum and R. eriocarpum occurred at higher subzero temperatures, -7 to -20°C, and KTs were at -13 to -18°C. These results indicate that hardier species have generally lower exotherm temperatures and lower KTs than less hardy ones.
Fig. 4. Exotherm temperature distributions of florets and KT\textsuperscript{s} of flower buds for 8 species native to different ecological habitats. The species are arranged according to their distribution, with northern or high mountain species toward the top and southern ones toward the bottom. Symbols (O, •) as in Fig. 3. (†): KT\textsuperscript{e}. * indicates the material collected at natural habitats in Kagoshima.

Fig. 5. Seasonal variations of exotherm temperature distribution of florets and KT\textsuperscript{s} of flower buds in spring. Symbols as in Fig. 3 and 4.
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Seasonal variation of exotherm temperatures and KT's during cold-deacclimation

In March, the ranges of exotherm temperatures of florets in *R. × akebono* were drastically shifted to higher subzero temperatures, —6 to —20°C, and the KT's were about —16°C, while those for *R. kiussianum* remained in the same temperature range as in winter. However, they shifted upwards, from —23 to —12°C in April and KT's rose to about —13°C (Fig. 5). The decrease of supercooling ability accompanying cold-deacclimation was clearly observed for the florets of flower buds in both species, and the timing of cold-deacclimation for *R. × akebono* was one month earlier than that for *R. kiussianum*.

Changes of water content within bud tissues in response to freezing

The moisture content of each bud tissue, i.e., the axis, scale and floret, was determined for *R. kiussianum*, *R. × akebono* and *R. tashiroi* var. *lasiophyllum* to investigate the water movement within the flower bud in response to freezing (Table 2).

After freezing at the slowest cooling rate, 1°C/hr, a marked decrease in the moisture content was found in the axes of all species, while no significant changes were observed in bud scales for *R. kiussianum* and *R. × akebono*, despite the decrease for *R. tashiroi* var. *lasiophyllum*. The water contents in florets decreased in each species.

In almost all bud tissues except for the scales of *R. kiussianum*, the water content decreased after freezing, unlike the decrease of water content in florets and its increase in scales in studies of *R. japonicum* by Ishikawa (7).

Discussion

On the basis of nucleation theory (16, 17), the mean freezing temperatures in biological materials as well as supercooled liquids rise as the rate of cooling decreases. However, recent studies showed that a decreased cooling rate enhances the supercooling ability of florets in excised whole flower buds and primordial shoots in some genera of conifers, and that this might be related to the decrease of water content in these tissues in response to freezing (7, 15).

In the present work, the cooling rate dependence of the supercooling ability in florets was also ascertained in excised whole buds of *Rhododendron* species belonging to the sections *Tsutsutsi* and *Brachycalyx*. Although patterns for exotherm temperature distributions of florets at the cooling rate ranging from 10 to 30°C/hr varied with each species tested, the largest supercooling ability was observed when the slowest cooling rate, 1°C/hr, was employed in each species (Fig. 3). Water movement within bud tissues, causing increased supercooling ability in florets of section *Rhodora* has been demonstrated by previous workers (6, 7). They assumed that water in florets appeared to migrate into bud scales when the temperature fell slowly, and the scales thus served as an ice sink. In the present work, the water contents of florets and axes in all species examined decreased after freezing, while those of scales for *R. kiussianum* and *R. × akebono* did not change significantly, despite the decrease for *R. tashiroi* var. *lasiophyllum* (Table 2).

There is a noticeable difference in flower bud morphology, especially the arrangement of floret and scale between these two sections; *Rhodora*, which was used
Fig. 6. Diagrams illustrating the patterns of the scale and floret arrangement in flower buds for *R. kiusianum* (A), *R. × akebono* (B) and *R. japonicum* (C). The former two belong to section Tsutsutsi and the latter section Rhodora. a: axis, s: scale, is: inner scale, os: outer scale, f: floret, lb: leaf bud.

by Graham and Mullin (6) and Ishikawa (7), and Tsutsutsi and Brachycaulix, which were used in this work. In the former, every inner scale forms alternate layers between each floret inside several outer scales, while in the latter several florets concentrated in the middle part of the bud are covered with several scales as a whole (Fig. 6). Therefore, the water movement among florets, scales and axes during freezing must be different for these two sections, and changes in moisture contents between florets and scales would be more directly manifested in buds of section Rhodora. Moreover, another possibility for inconspicuous changes (increase) of water in bud scales in the present work is that water loss (transpiration) from the scales might occur during long periods of cooling and freezing at a very slow cooling rate. Although we found no clear evidence for water migration between florets and other bud tissues in the present work, it is likely that the enhancement of the supercooling ability in florets of *R. kiusianum* and *R. × akebono* depends on a similar water movement from floret to bud scale as suggested for *R. japonicum* by Ishikawa (7). However, the result found with *R. tashiroi* var. *lasiophyllum* still remains to be explained: water contents in axis and scale in this species decreased markedly after freezing but a narrow decrease was found in florets (Table 2). This species is one of the less cold-hardy ones distributed in the Ryukyu Islands and the most southern part of Kyushu. Therefore, less hardy species may have lower capability for water migration within bud tissues and lower supercooling ability in florets in comparison with hardier species such as *R. kiusianum*. Clearly, further investigation along this line is needed.

Flower bud low temperature exotherms occur at the killing point of the floral primordia in the flower buds of *Rhododendron*, *Vitis* and *Prunus* species (4–6, 11, 13,
Supercooling of *Rhododendron* flower buds

The mean flower bud exotherm temperatures in *Prunus* species are within ±1°C of visually determined 50% injury temperature of the buds, and in common commercial *Prunus* cultivars, the extent of supercooling is about −25°C. This temperature approximates the average annual minimum temperature in the northern range of their production (13). In the studies of Burke and Stushnoff (2) on native and cultivated members of genus *Prunus*, however, the buds were always considerably harder than indicated by the exotherm temperature. For the native species, there is no apparent correlation between the geographic distribution of the species and the presence or absence of the flower bud low temperature exotherm, unlike the twig exotherms, which are well correlated with geographic distribution. The slowest cooling rate used for their differential scanning calorimeter technique was 18°C/hr and this is much faster than the cooling rates used by Quamme (13). In our preliminary experiment for the *Rhododendron* species at fast cooling rates, about 30 to 40°C/hr, the exotherm temperatures and KTs of flower buds occurred at about −10 to −20°C regardless of their different potentialities for northern distributions. The cooling rate, 1°C/hr, used in this work appears to simulate fairly closely rates occurring in nature, and thus seems to be a reliable rate for testing maximum supercooling ability. In conclusion, much hardier species distributed in colder habitats or higher elevations showed a larger supercooling ability and lower KTs than less hardy ones, and also the supercooling ability of florets markedly decreased accompanying cold deacclimation during spring. These results suggest that exotherm temperature measurement of flower buds (florets) at the slow cooling rate of 1°C/hr is useful for testing freezing resistance and evaluating the potentiality for northern ranges of distribution.

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References


