Plant & Cell Physiol. 22(6): 953–967 (1981)

Freezing Avoidance Mechanisms by Supercooling in Some *Rhododendron* flower Buds with Reference to Water Relations[1,2]

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Excised florets of some hardy *Rhododendron* species did not tolerate freezing at −5°C when ice-inoculated due to intracellular freezing. Florets in intact December buds, however, could be supercooled to about −30°C. When flower buds of *R. japonicum* were slowly cooled with daily decrements of 5°C to temperatures ranging from 0 to −20°C, the exotherm temperatures of the florets drastically decreased. This was accompanied by a decrease in water content of florets and peduncle and an increase in that of scales. The water in florets and the peduncle is thought to migrate to scales and other tissues during the early stages of freezing; the dehydrated floret has a lower freezing point which enhances its supercooling ability and the dehydrated peduncle helps to maintain the supercooled state of the florets. This hypothesis would explain the dependence on the cooling rate of supercooling in *Rhododendron* flower buds. Water migration within flower buds was observed in other hardy *Rhododendron* species with some variation in ice formation site and the quantity of migrated water. The exotherm temperature of excised florets was inversely proportional to their water content. Dehydration of flower buds by wind at 0°C also enhanced their supercooling ability. Mechanisms of freezing avoidance by supercooling in *Rhododendron* flower buds and the relationship of supercooling to freezing tolerance are discussed.

Key words: Cold hardiness — Extraorgan freezing — Freezing avoidance — *Rhododendron* — Supercooling — Water relations.

Plant tissues seem to develop various physiological strategies against cold stresses (Levitt 1972). One strategy is to avoid freezing stress by losing freezable water and tolerating the desiccated state as seen in seeds (Levitt 1972, Junttila and Stushnoff 1977, Ishikawa and Sakai 1978). Tolerance for freezing by extracellular freezing, however, appears to be the most common strategy in hardy tissues. Alternatively, freezing avoidance by deep supercooling has been found in xylem ray parenchyma cells of many temperate broad-leaved trees (Quamme et al. 1972a, George and Burke 1976, Kaku and Iwaya 1978, Sakai 1978), seeds (Junttila and Stushnoff 1977, Ishikawa and Sakai 1978), conifer buds (Dereuddre 1978, Sakai 1979a), and in overwintering flower buds. *Prunus* (Quamme 1974, Rajashekar and Burke 1978, Burke

Abbreviations: DTA, differential thermal analysis; HTE, high temperature exotherm; LTE, low temperature exotherm; SEM, standard error of the mean; FW, fresh weight; DW, dry weight.

[1] Contribution No. 2254 from the Institute of Low Temperature Science.

[2] This is a revised form of the master's thesis of the senior author (M.I.) which is cited in the present and previous papers (Sakai 1979a, b, etc.).

and Stushnoff 1979, Proebsting and Sakai 1979), grape (Pierquet et al. 1977), blueberry (Quamme et al. 1972), and some *Rhododendron* species (George et al. 1974, Graham and Mullin 1976a, Graham and Mullin 1976b, Ishikawa 1979) have been shown to have their flower primordia avoid freezing by deep supercooling. In addition, flower buds of some fifty species in Ericaceae were recently found to employ supercooling as a strategy against cold stress by the present senior author (M. I., unpublished data). Further surveys may show that flower buds of more genera and families also display this type of cold resistance. Thus, an accurate understanding of the nature of freezing avoidance in flower buds seems necessary when the importance of flower buds in horticulture and in the life cycle of plants is considered.

Mechanisms of supercooling in flower buds have been studied by several workers (George et al. 1974, Graham and Mullin 1976b, Quamme 1978, Ishikawa 1979). Dorsey (1934) observed that ice crystals formed in peach flower bud scales before any appeared in the florets. Quamme (1978) has suggested that the mechanism of supercooling in peach flower buds involves migration of water from the bud axis to the scales, thus, forming a dry region which may function as a barrier against ice growth from the stem while water in the floret does not migrate during the cooling of the flower buds. Graham and Mullin (1976b) have suggested that the water content of *Rhododendron* florets is related to their exotherm temperature and that florets in hardier cultivars have a greater ability to lose water from their tissues as air temperature decreases and that scales may function as an ice sink for reduction of water content in the florets. However, few reports have ever shown that scales really act as an ice sink for drawing water from florets and little is known about precise water relations in *Rhododendron* flower buds cooled to subzero temperatures.

The cooling rate dependence of supercooling in flower buds of *Prunus* and *Rhododendron* is a well-known phenomenon (George et al. 1974, Ishikawa 1979, Burke and Stushnoff 1979); the slower the cooling rate, the greater the extent of supercooling. However, little is known about the mechanism of this phenomenon either.

The present study was undertaken to clarify the properties and mechanisms of supercooling in *Rhododendron* flower buds with special reference to water relations during the process of freezing, as a step to understanding the physiological adaptation of plants to cold stress.

Materials and Methods

Plant materials—Flower buds of *Rhododendron japonicum* [Section Sinenses (Kitamura and Murata 1971)] were primarily used in this study. *R. dilatatum* (Sect. Sciadorhodin), *R. mucronatum* (Sect. Tsutsusi), *R. obtusum* var. *kaempferi* (Sect. Tsutsusi), *R. brachycarpum* (Subgenus Hymenanthes), and *R. dauricum* (Subgen. Rhodorastrum), each of which had different internal flower bud morphologies (Fig. 1) were also used to check variations in supercooling properties in the genus *Rhododendron*. The used species were cold hardy and successfully overwintered in Sapporo. Twigs with flower buds of *R. japonicum* and *R. dauricum* were collected from 6- to 9-year-old outdoor plantings at the Agricultural Center of Sapporo City and the remainder were obtained from the Botanical Garden of Hokkaido University during the autumn to spring periods from 1977 to 1980. Twigs were stored at 0°C in polyethylene bags until used. Each experiment was replicated at least twice in a

winter, but the data of different sampling dates or years were not averaged because of differences in the water contents of the samples. The data shown in the present paper are mainly those obtained during the seasons of 1978 to 1979. Similar results were obtained in the other experimental years.

Differential thermal analysis (DTA)—Exotherm responses were detected with 0.2-mm Cu-const. thermocouples, amplified 40 times, and recorded on potentiometric recorders in the 10 to 2 mV range. The thermocouple junction was inserted between the scales of a flower bud with a 5-mm twig. Temperature of the sample was recorded with another thermocouple. Samples were cooled either by placing the vacuum flask which contained the samples in a freezer with programmable temperature or by placing samples in a DTA chamber with a freezing attachment (DTA-1500 L-S Shinkuriko Co.). With these systems, cooling rates of 0.5 to 40°C/hr were obtained.

Water content determination—To determine the water content of each part of a flower bud, the bud was separated into the following components: outer scales, inner scales, florets, primordial peduncle, bud axis which is the base of the primordial peduncle (peduncle and axis represent the part of the bud where the florets and scales are attached), leaf buds and a twig of 2 cm from the flower bud axis (Fig. 1). Samples were dried in an oven at 70°C for 24 hr and the water content was expressed as the percentage of dry weight, unless otherwise noted.

Cold treatment—To know the effect of cold treatment on exotherm temperatures and the water content of flower buds, twig pieces of 15 cm with flower buds were enclosed in polyethylene bags containing a little snow to prevent desiccation and cooled at 5°C decrements daily (0 to −20°C). Then flower buds in the frozen state were excised at each temperature and usually three to nine of the buds were used for water content measurement. Eight buds were cooled further to −45°C and exotherm temperatures were recorded. To determine the water content of the frozen buds, each bud was dissected into its components in cold rooms at −5 to −20°C.

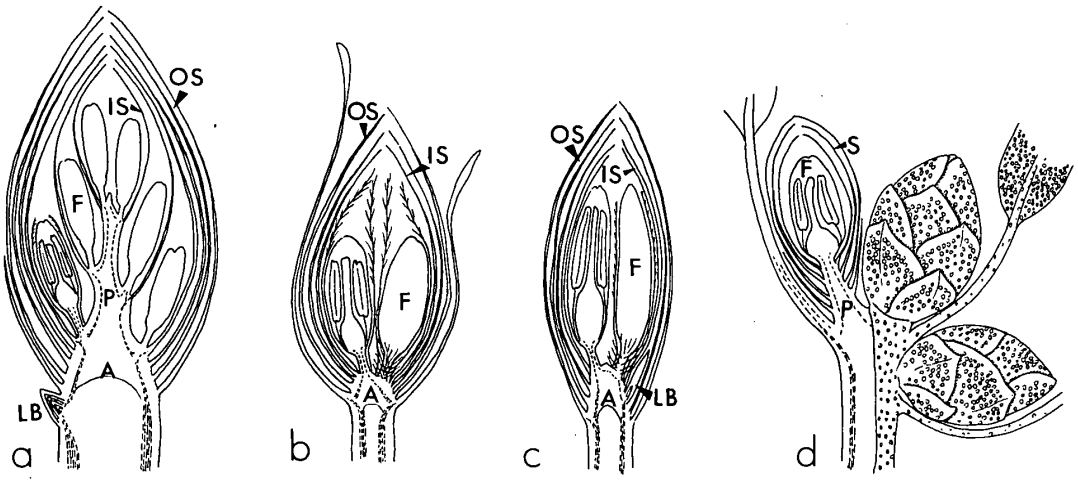

Fig. 1 Diagrams illustrating flower buds of (a), *R. japonicum*; (b), *R. obtusum* var. *kaempferi*; (c), *R. dilatatum*; (d), *R. dauricum*. (A), bud axis; (F), floret; (LB), leaf bud; (P), primordial peduncle; (IS), inner scale; (OS), outer scale; (S), scale. Bracts are not shown in some cases in (a), (c), and (d). Buds of *R. mucronatum* and *R. brachycarpum* are omitted, as they are similar to (b) and (a), respectively.

Then each part was wrapped with aluminium foil and put into small polyethylene bags allowing as little space as possible within it to minimize tissue water loss during rewarming. After the bags had been brought to room temperature and their temperature had reached equilibrium, tissue weights were measured.

Dehardening and rehardening treatment—To deharden flower buds, 15-cm-long twig pieces of *R. japonicum* with flower buds were put in water in a vase in early December and incubated in a greenhouse (10 to 30°C) for a month. On the 11th day of dehardening, a group of twig pieces was rehardened by being placed at −3 to −5°C for 2 days and another group was further cooled slowly to −15°C in 8 days. DTA and water content determination were done with these rehardened and dehardened flower buds.

Desiccation treatment—In desiccation experiments, twigs with flower buds were placed for 16 days at 0°C in a chamber where a gentle wind was blowing while other twigs were put in polyethylene bags at 0°C as the control. DTA and water content determination were done with these buds. DTA was also conducted on excised florets which had been rehydrated after being killed in an oven at 70°C, to check the supercooling ability of dead florets.

Microscopic observations—Microscopic observations of frozen buds were conducted in a cold room held at −5 or −10°C to determine the localization of ice.

Freezing injury of flower buds or florets was observed with a binocular microscope and browning was rated after a week of incubation at room temperature or at 5°C.

Some excised florets were ice-inoculated by being placed in a mixture of water and ice and cooled slowly to −5 or −10°C to examine their ability to tolerate extracellular freezing. This freezing process was observed with floret petal and receptacle tissues under a cryomicroscope.

Freezing point determination—Freezing points were measured by the freezing curve method. A homogenate of florets was placed in a small glass tube (5 mm in diameter) and cooled at 0.6 to 2.9°C/min. The temperature of the sample was measured with a thermocouple. The sample was ice-seeded at various temperatures (T_i) with a touch of a thin copper wire which had been cooled in liquid nitrogen. When the ice-inoculation temperature (T_i) was higher than the freezing point (T_{fp}) of the sample, no exotherm was observed (T_i in this case was termed T_{ih}). When T_i was lower than T_{fp}, supercooling was broken and the temperature of the homogenate rose and then remained constant (T_e) for a few minutes, forming a plateau on the freezing curve (T_i in this case was termed T_{il}). Thus,

$$0°C > T_{ih} > T_{fp} > T_e > T_{il}$$

The freezing point, which was unknown, was expressed as the temperature range between the lowest T_{ih} (min T_{ih}) and highest T_e (Max T_e) obtained with a sample.

Results

Basic features of supercooling of florets

Fig. 2A shows a typical DTA profile of a flower bud of *R. japonicum* cooled at 5°C/hr. The first exotherm (HTE) at −5.1°C is considered to represent the freezing

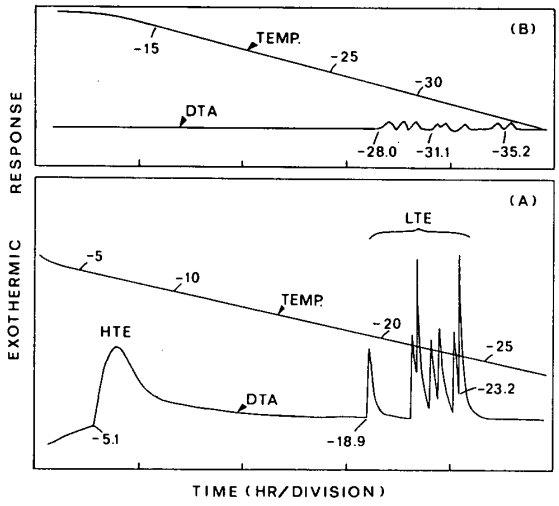

Fig. 2

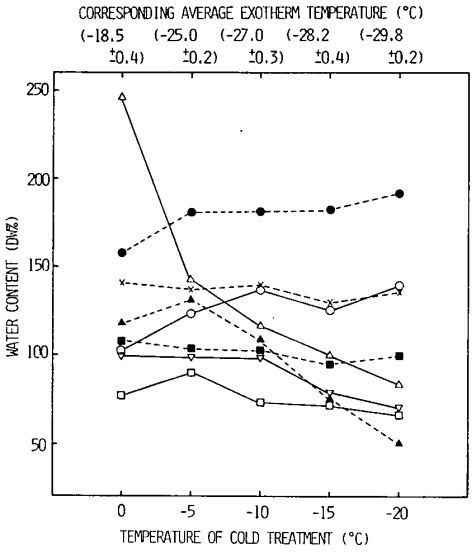

Fig. 3

Fig. 2 (A) A typical DTA profile of a flower bud of *R. japonicum* collected on Nov. 22, 1979. For details, see text. (B) A DTA profile of a flower bud of *R. japonicum* stored at −15°C for 50 days and cooled to lower temperature without thawing. Since scales and other tissues were frozen, no HTE was observed. Supercooling of the florets was not broken during the storage at −15°C for 50 days. Exothermic responses in (B) were scaled up 5 times compared to those in (A). This is an example of 8 replicates.

Fig. 3 Water content of flower bud parts of *R. japonicum* of December 1978 and corresponding exotherm temperature of florets cooled at 5°C decrements to different temperatures at daily intervals. ○, outer scale; ●, inner scale; △, floret; ▲, primordial peduncle; ▽, bud axis; □, leaf bud; ■, twig; ×, whole flower bud including scales, florets, peduncle and bud axis. The corresponding average exotherm temperature of about 80 florets (8 flower buds) cooled at the rate of 4 to 6°C/hr, is given in parenthesis at the top of the figure (Mean±SEM). Three to four buds were used for water content analysis at each temperature. The data are for one example out of five replicates.

of scales and other tissues which tolerate extracellular freezing, and the sharp ones (LTE), ranging from −18.9 to −23.2°C, the freezing of florets. When the bud was taken from the freezer after the fifth LTE and rewarmed, five florets died while two remained alive. This confirms that the LTE indicates the killing temperature of florets as shown by Graham and Mullin (1976a). Supercooling of florets was stable and at −15°C, they remained supercooled without nucleating for periods as long as 50 days (Fig. 2B). The supercooling ability of florets indicated by LTE showed seasonal changes (George et al. 1974, Graham and Mullin 1976b, Ishikawa 1979); from autumn to winter LTE shifted to lower temperatures and became smaller (data not given).

Ability to tolerate freezing

Excised florets of overwintering flower buds in *R. japonicum* did not tolerate freezing at −5°C when ice-inoculated in contrast to the high ability to avoid freezing of those in whole flower buds (Table 1). Similar results were obtained with florets of *R. dilatatum, R. obtusum* var. *kaempferi* and *R. dauricum*. Observation of this freezing process in petal and receptacle tissues of a floret under a cryomicroscope showed

Table 1 Survival of excised florets of *R. japonicum* with different treatments after freezing by ice inoculation at 0 to −5°C (Dec. 1978)

Treatment	Survival[a] (%)		
	0°C	−5°C	−10°C
Florets of buds stored at 0°C	100	0 (100)[b]	0 (90)[b]
Florets of buds stored at −15°C	—	57	0
Florets desiccated at 0°C	100	50	0

[a] 15 to 20 florets derived from different flower buds were used for each survival determination.
[b] Survival of excised florets at each temperature when not ice-inoculated is given in parenthesis.

that most of the cells did not freeze extracellularly even when massive ice had formed around them, but froze intracellularly, resulting in death of the florets. Nearly half of the florets with lower water content, due to either storage of flower buds at −15°C or drying at 0°C in air, resisted −5°C. But this was probably due to freezing avoidance by freezing point depression since the freezing point turned out to be about −11°C withflorets stored at −15°C (Table 5). Floret tissue did not seem to have the capability to freeze extracellularly. This is very unusual because in usual overwintering plant tissues, ice inoculation at high subzero temperatures induces extracellular freezing. Interestingly, floret tissues which did not tolerate freezing at −5°C when ice-seeded, could resist about −30°C (Fig. 3) in intact buds by avoiding freezing by supercooling. These tissues did not seem to employ two physiological strategies, freezing avoidance and tolerance, at the same time.

Cooling rate dependency of supercooling of flower buds

Although exotherm temperatures for excised florets were not greatly affected by the cooling rate (George et al. 1974), those for excised whole buds shifted markedly to lower temperature as the cooling rate decreased, −10.2 to −19.2°C in *R. japonicum* and −11.6 to −19.4°C in *R. dauricum* (Table 2).

Table 2 Effect of cooling rate on the exotherm temperatures of excised flower buds and excised florets of *R. japonicum* and *R. dauricum* (1978)

Excised florets		Excised whole buds			
R. japonicum (Dec. 6)[a]		*R. japonicum* (Oct. 25)		*R. dauricum* (Dec. 6)	
Cooling rate (°C/hr)	ET[b] (°C)	Cooling rate (°C/hr)	ET[c] (°C)	Cooling rate (°C/hr)	ET[d] (°C)
2.2 to 2.8	−16.5±0.6	0.7 to 2.2	−19.2±0.3	2.1 to 2.2	−19.4±0.4
		3.5 to 4.7	−12.3±0.4		
34.0 to 41.0	−14.7±0.6	6.7 to 9.5	−10.2±0.2	9.0 to 11.1	−11.6±0.8

[a] Date of collection.
[b] Exotherm temperature (mean±SEM), mean of 30 to 40 florets.
[c] Mean of 4 to 8 buds (about 10 florets in a bud).
[d] Mean of 20 to 30 buds (1 floret in a bud).

Effect of cold treatment on LTE and water content of flower buds

To investigate the effect of cold treatment on supercooling ability, twigs with flower buds of *R. japonicum* were subjected to temperatures decreasing 5°C a day from 0°C to −20°C. Exotherm temperature of the florets markedly shifted to lower temperature, especially from −18.5 to −25.0°C when they were cooled from 0 to −5°C at which the scales and twigs froze, and finally to −29.8°C. The size of the exotherm became smaller, accompanied by a continuous decrease in water content of floret and peduncle (Fig. 3). The water content of florets decreased from 246 to 142% during freezing at −5°C for a day, while that of the outer scales increased from 102 to 123% and that of the inner scales from 157 to 182%.

Since twigs with flower buds were put in polyethylene bags which were water saturated with a small amount of snow, there was no significant change in the water content of whole flower buds during the cold treatment. Therefore, the decrease in water content in florets and primordial peduncles was not due to desiccation, but to migration of water within the flower bud. The combined dry weight of inner and outer scales in an average flower bud used in this experiment was 94 mg, which was about four times greater than that of florets (22 mg). That of the peduncle (2.3 mg) was one-tenth that of florets. The decrease in water content of florets during cooling from 0 to −5°C was about four to five times greater than the increase in scale water content (Fig. 3). These calculations indicate that the estimated absolute decrease of water in florets coincided with the increase in scales. Thus, water in the floret and peduncle seemed to have moved to the scales during cooling from 0 to −5°C in a day. When the same cold treatment was conducted on twigs with flower buds whose florets were excised, causing as little damage as possible to scales and other tissues, no significant increase was observed in scale water content. This is indirect evidence that floret water migrated to the scales.

The same cold treatment was also conducted on twigs with flower buds of *R. dilatatum, R. mucronatum, R. obtusum* var. *kaempferi, R. brachycarpum* and *R. dauricum,* each of which has different internal flower bud morphology (Fig. 1). Water content of the florets of the species sampled generally decreased with the cold treatment; the extent of the decrease tended to be greater when the initial floret water content was higher as in early winter and spring (Table 3). Florets of *R. dauricum* of April 12 froze during cold treatment down to −15°C and the decrease in the floret water content was not great. Water content of the peduncle and the axis decreased during the cold treatment in *R. japonicum, R. dilatatum* and *R. obtusum* var. *kaempferi* while in the other species, it did not change so much. The extent of the decrease tended to be greater with higher initial peduncle and axis water content. Scale water content generally increased during the treatment, but there were some variations; the increase was larger in inner scales than outer ones in *R. japonicum,* an increase in outer scales and a slight one in inner scales occurred in *R. dilatatum* and *R. obtusum* var. *kaempferi,* and increases in both inner and outer scales were found in the other species. The inner scales of *R. dilatatum* on April 3 even showed a decrease in water content. Microscopic observation of frozen flower buds at −15°C generally supported these increases or decreases in scale water content, which will be mentioned later.

Table 3 Effect of cold treatment on the water content of flower bud parts of different *Rhododendron* species[a]

Species	Collection date	Water content of flower bud parts[b] (%)												Ice formation[c]	
		Outer scale			Inner scale			Floret			Peduncle and axis			Outer scale	Inner scale
		0°C	−15°C	Dif.	0°C	−15°C	Dif.	0°C	−15°C	Dif.	0°C	−15°C	Dif.		
R. japonicum	Dec. 6	102[d]	138[e]	+36[f]	157	200	+43	246	110	−136	106	88	−18	+	+
	Jan. 18	57	61	+4	74	85	+11	95	77	−18	81	67	−14	+	+
	April 12	79	90	+11	107	144	+37	269	172	−97	128	76	−52	+	+
R. dilatatum	Dec. 16	78	97	+19	66	73	+7	159	138	−21	100	77	−23	+	−
	April 3	84	103	+19	41	32	−9	179	159	−20	103	69	−34	+	−
	April 12	106	135	+29	74	—	—	237	202	−35	157	86	−71	+	−
R. obtusum var. *kaempferi*	Dec. 16	95	106	+11	78	85	+7	129	103	−26	81	60	−21	+	+
	April 3	86	106	+20	77	83	+6	155	125	−30	—	78	—	+	+
	April 12	112	134	+22	84	92	+8	207	155	−52	111	65	−46	+	+
R. mucronatum	Dec. 16	109	123	+14	103	129	+26	157	97	−60	79	80	+1	+	+
	April 3	73	112	+39	70	103	+33	144	94	−50	58	53	−5	+	+
R. brachycarpum	Dec. 16	82	98	+16	86	95	+9	134	84	−50	106	105	−1	+	+
	Feb. 10	78	89	+11	80	93	+13	71	64	−7	80	86	+6	+	+
	April 12	91	110	+19	94	112	+18	129	92	−37	121	102	−19	+	+
R. dauricum[g]	Dec. 6	72	98	+26				164	102	−62	—	—	—	+	
	Jan. 18	61	84	+23				126	66	−60	59	61	+2	+	
	April 12	156	195	+39				262	234[h]	−28	132	117	−15	+	

[a] The flower buds were cooled slowly in 5°C decrements at daily intervals from 0 to −15°C.

[b] Average of 3 to 4 buds for *R. japonicum* and *R. brachycarpum*, 15 to 20 for *R. dauricum*, and 6 to 9 for the others.

[c] Localization of ice within the scales of flower buds at −15°C are listed: +, ice was observed; −, ice was not observed.

[d,e,f] f=e−d

[g] The scales of *R. dauricum* were difficult to classify into outer or inner scales and are listed as outer scales.

[h] Florets in this case had already frozen between −5 and −8°C.

Table 4 Effect of dehardening and rehardening on water content and exotherm temperature of flower buds of *R. japonicum* of December 1978

Treatment[a]	Water content of flower bud parts[b]					LTE of flower buds[c]
	Outer scale	Inner scale	Florets	Peduncle	Bud axis	
Control (stored at 0°C)	102	157	246	118	100	−16.7±0.5
Dehardened for 11 days	96	215	305	207	150	−10.7±0.3
Dehardened for 25 days	139	302	415	333	221	−9.0±0.7
Rehardened for 2 days	140	236	285	69	78	−16.1±0.6
Rehardened for 8 days	186	310	253	105	104	−20.7±0.4

[a] For details, refer to **Materials and Methods.**
[b] Mean of 3 to 4 buds.
[c] Mean±SEM, mean of 5 to 6 flower buds; cooling rate was about 9 to 12°C/hr.

Effect of dehardening and rehardening on water content and LTE of flower buds

When flower buds of *R. japonicum* were dehardened by incubation in a greenhouse, the water content of each part of the flower buds increased, especially that of the primordial peduncle (Table 4). As the incubation time increased, LTE shifted to higher temperature becoming difficult to recognize because it overlapped with HTE, as florets, scales and other tissues tended to freeze at the same time. After 11 days of dehardening, some twig pieces with flower buds were rehardened by being placed at subzero temperature. After 2 days of rehardening, florets supercooled to the same extent as the control. At the same time, water content of the scales increased and that of the floret and peduncle decreased. The decrease in water content of peduncle and bud axis was drastic and might have played an important role in enhancing the supercooling ability of florets as well as the reduction in floret water content.

Internal observation of frozen flower buds

R. japonicum flower buds in early December, kept at −5°C for one day, showed a large amount of ice within both outer and inner scales (Fig. 4). The distribution of ice within a scale was not even; more ice had formed in the basal part of the scale. Also, less ice was found in scales of more desiccated buds in midwinter. When the scales were observed in the thawed state, they looked double-layered or bag-shaped since an air cavity had developed within them. An interesting fact is that winter flower buds have much larger air space in the scales than early autumn buds which had not been subjected to severe frosts. As flower buds experience subfreezing temperatures, a large air cavity seems to form in the scales as a result of ice accumulation. Ice crystals were also observed within the outer and inner scales of flower buds of other *Rhododendron* species except for the inner scales of *R. dilatatum*, which were brown-colored and had little air space (Table 3). The amount of ice varied with the species, but was approximately proportional to the increase in the water content of the scales. Inner scales of *R. obtusum* var. *kaempferi* exhibited a slight increase in water content during the cold treatment, but ice crystals were observed

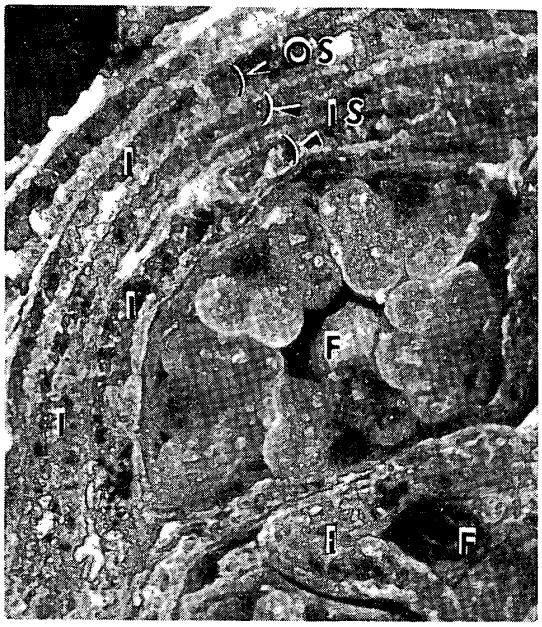

Fig. 4 A cross section of a flower bud of *R. japonicum* (Dec. 1978) cooled slowly to −10°C in 3 days, then cut and photographed under a dissecting microscope at −10°C. The ice labeled (I) is within the inner scales (IS) and outer scales (OS) of the flower bud. (F) is the floret. ×20. Similar ice was also observed in the buds cooled to −5°C in a day.

within the inner scales. On the other hand, in the case of *R. dilatatum*, the inner scales did not function as an ice sink.

Freezing points and exotherm temperature of excised florets

During the cold treatment, the freezing point of florets markedly decreased (Table 5), probably due to the decrease in floret water content. The supercooling ability of the florets was enhanced with the increase in their freezing point depression.

The exotherm temperature of the excised florets was in inverse proportion to their water content (Fig. 5). A similar relationship was observed in florets desiccated in air at 0°C and rehydrated florets which had been killed at 70°C. Dead florets seemed to retain their ability to supercool. No exotherms were detected in florets with 10 to 30% (fresh weight basis) water. These results show that the floret itself has the potential to supercool.

Table 5 Effects of cold treatment on freezing point of homogenized florets and exotherm temperature of excised florets and whole buds of *R. japonicum* in December 1978

Treatment	Freezing point[a] (°C) min T_{ih}～Max T_e[b]	Exotherm temperature (°C)	
		Excised floret[c]	Whole bud[d]
0°C storage	−1.2～ −1.8	−18.6±0.7[e]	−18.5±0.4
−10°C storage	−6.0～ −7.8	—	−27.0±0.3
−15°C storage	−9.8～−11.5	−30.3±1.1	−28.2±0.4

[a] At least 6 measurements were made with each sample.
[b] For details, refer to **Materials and Methods**.
[c] Cooling rate, 40°C/hr. Mean of 6 to 8 florets.
[d] Cooling rate, 4 to 6°C/hr. Mean of 8 flower buds.
[e] Mean±SEM.

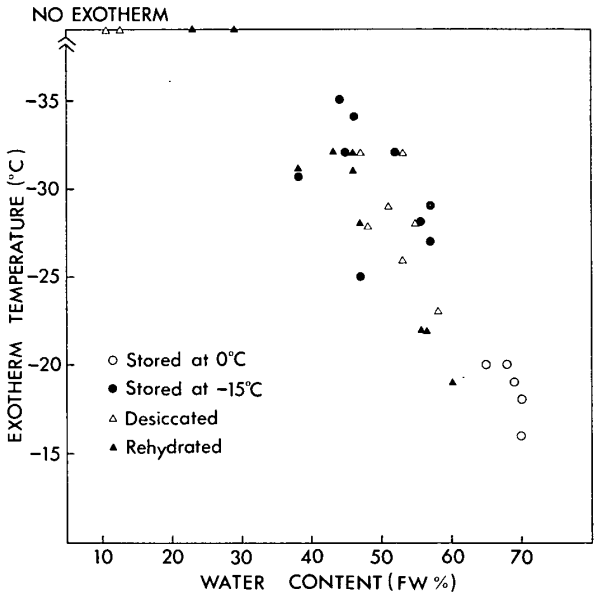

Fig. 5

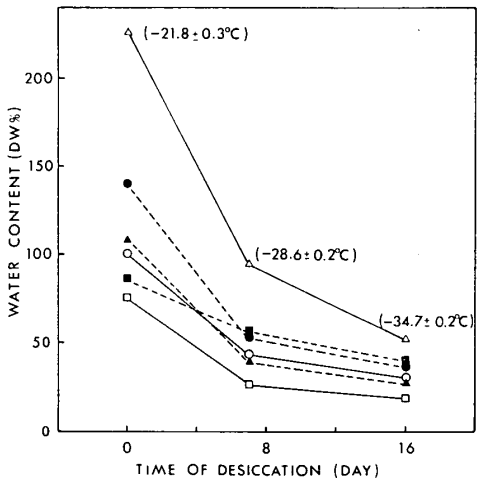

Fig. 6

Fig. 5 Relationship between water content and exotherm temperature of excised florets of *R. japonicum* (Dec. 1978). ○, florets excised from flower buds stored at 0°C; ●, florets from those stored at −15°C; △, florets desiccated in air at 0°C; ▲, rehydrated florets after having been heated at 70°C and killed. Exotherms were not observed in florets with 10 to 30% water content on fresh weight basis.

Fig. 6 Water content of flower bud parts of *R. japonicum* (Dec. 1979) and corresponding exotherm temperature of florets desiccated at 0°C for different lengths of time. ○, outer scale; ●, inner scale; △, floret; ▲, peduncle and bud axis; □, leaf bud; ■, twig. Corresponding exotherm temperature of florets cooled at the rate of 5°C/hr is given in parenthesis (Mean±SEM).

Effect of desiccation on water content and exotherm temperature in florets

Fig. 6 shows the changes in water content and floret exotherm temperature of flower buds of *R. japonicum* during desiccation treatment. The water content of each part of the flower bud decreased, especially that of the florets, but remained higher than that of the other tissues even after 16 days of treatment. The mean exotherm temperature of the florets decreased from −21.8 to −34.7°C. The lower water content in the florets caused by desiccation resulted in increased supercooling ability, similar to that observed in the cold treatment.

Discussion

Migration of water in florets and peduncles

The present results with *Rhododendron* flower buds seem to substantiate the idea that scales serve as an ice sink for drawing water from florets (Graham and Mullin 1976b), and the following process can be postulated. During freezing of a flower bud, cell water migrates from the floret mainly to the scales or to other freezing tissues, resulting in a rise in cell sap concentration which in turn causes freezing point depression and further enhances the supercooling ability of the florets. The

reduced water content in the peduncle and bud axis might play an important role in maintaining the supercooled state of the florets. This hypothesis seems to reasonably account for the following features of supercooling of florets.

The cooling rate dependency of the exotherm temperature of florets in intact buds, which is well known (George et al. 1974, Ishikawa 1979, Burke and Stushnoff 1979), might be explained as follows. As an intact flower bud is cooled, scales and other freezing-tolerant tissues freeze first as represented by HTE in DTA. If the bud remains longer at these temperatures, then the water in the florets and the peduncle might have enough time to be withdrawn into the scales and other tissues. But if the cooling rate is fast, enough floret water might remain to break supercooling at a higher temperature and the high water content in the peduncle and bud axis might make it difficult to maintain the supercooled state of the florets. Thus, the seemingly unique character of supercooling in florets of intact buds can now be understood; the faster the cooling rate, the less they supercool, which is unlike the usual property of plant tissues (Aoki 1950, Kaku 1964) where the faster the cooling rate, the lower the temperature is to which they supercool.

Burke and Stushnoff (1979) observed that prefreezing for *Prunus* flower buds shifts the exotherms of flower primordia to lower temperature and reduces their size. This phenomenon seems also to be explainable by water migration from flower primordia to scales or other tissues as observed in *Rhododendron* flower buds. On the other hand, Quamme (1978) postulated that in supercooling of peach flower buds, water in flower primordia does not migrate and instead water in the bud axis is withdrawn into the scales, forming a dry region which may function as a barrier against ice propagation from the stem during cooling of the buds. However, we think that if the peach flower bud is placed at a low temperature like -10 or -15°C for a long time, the flower primordia water would migrate to the scales or other tissues as in the case of *Rhododendron* flower buds. One exception is that in the case of *Prunus* flower buds, the effect of prefreezing disappears at the moment they are thawed (Burke and Stushnoff 1979), that is, a rapid return of withdrawn water seems to occur. In *Rhododendron* flower buds, the water withdrawn during prefreezing took 3 days to return to the florets (data not given).

The increase in the supercooling ability of florets in *Rhododendron* flower buds from autumn to winter (Graham and Mullin 1976b, Ishikawa 1979) can be explained by a combination of water movement from the florets and the peduncle to other tissues during the stages of freezing and to the atmosphere by wind desiccation. Florets in midwinter, well-adapted to subfreezing temperature, seem to have already lost a considerable amount of their water and cold treatment down to -15°C does not appear to cause a great water migration from the florets and peduncle to the scales (Table 3). More extensive water migration seems to occur in autumn to early winter and in spring than in midwinter; when the water content in the floret and the peduncle is rather high, a sudden attack of frost might be overcome by the migration of water from the florets and peduncle to the scales and other tissues. This seems to be a unique adaptation of *Rhododendron* flower buds to subfreezing temperatures.

Mechanisms of supercooling in florets of Rhododendron

In considering the mechanisms of supercooling in *Rhododendron* florets, one important factor is the potential ability of the florets to supercool (Fig. 5), as suggested

by George et al. (1974). Also, water migration from florets and peduncle to other tissues may play a role in further enhancing the supercooling ability. Thirdly, florets require some barriers against external ice inoculation since they did not tolerate freezing even at −5°C when seeded with ice. Scale and floret surfaces look glossy due to cuticle and both the cuticle and the scales probably protect the florets from external ice crystals. Barriers against internal ice propagation at the attachment region of the florets are also needed. Some morphological structure of vascular tissues might be involved in the prevention of internal ice inoculation. Another possibility is that decreasing water content in a primordial peduncle or bud axis during the cold treatment might create a dry region which would serve as a barrier against internal ice propagation. In the case of supercooling of wheat, nodes are considered to delay ice propagation (Single 1964), but the barriers in flower buds of *R. japonicum* are expected to stop ice propagation completely since florets can remain supercooled at −15°C for 50 days (Fig. 2B). The expected barriers might also play a part in withdrawal of water from the florets to the other tissues. Still unknown is whether the water migration occurs in the form of vapor through the surface of the florets or as liquid water by way of vascular systems.

As shown in Table 3, some species did not exhibited a great decrease in the water content of the peduncle and the axis during the cold treatment. However, there is a possibility that a dry region which might help the florets to supercool forms within the peduncle and the axis if the necessary critical dry region occupies such a small part of the peduncle and the axis that formation of it does not greatly change the water content of the peduncle and axis. Generally, the postulated water migration from florets and peduncle to scales seems to be applicable to the hardy species investigated in the present study. The variations in the decrease of the peduncle and axis water content or in the ice accumulation site, however, indicate that a more detailed analysis regarding bud morphology is necessary to elucidate the barriers against internal ice inoculation and to understand the differences in supercooling properties among species. Also, further research is required to see if the phenomena observed here is applicable to less hardy species of *Rhododendron*.

Comparison of freezing avoidance by supercooling in florets and freezing tolerance

In comparing freezing avoidance by deep supercooling in *Rhododendron* florets with tolerance of extracellular freezing in other hardy tissues, one important feature is that water is withdrawn from cells or tissues to some other space or cavity where it freezes and consequently they are more or less dehydrated. In the case of extracellular freezing, ice is usually formed just outside the cell. On the other hand, with freezing avoidance in florets, ice forms in more distant regions and no ice crystals appear within the floret tissues. In this sense, supercooling in florets could be called extraorgan freezing and extracellular freezing would be intraorgan or intratissue freezing. Even in extracellular freezing, water sometimes moves to some special space where ice masses are formed as seen in the vascular bundle ring region in frozen root of table beet (Terumoto 1960), but in that case, ice is eventually formed within the root and the phenomenon could be intraorgan or extratissue freezing.

Whether there is a limit to the ability of florets to avoid freezing is another interesting point. In an attempt to check this, DTA and water content determination were done with flower buds of *R. japonicum* planted in Shotoshibetsu in inland

Hokkaido where minimum air temperature was −32°C in Feb. 1980 and the twigs protruding above the snow level were highly desiccated because of the winter sun and frozen soil. The floret water content decreased to as low as 24% (fresh weight basis) in late February, and the florets in situ survived such severe cold and winter drought. In DTA of these florets, no exotherms were detected down to −50°C in agreement with the result shown in Fig. 5, but the florets did not survive the artificial cooling to −50°C. The reason for their death is considered to be that the very little free water remaining in the florets caused intracellular freezing and the amount was too small to be detected by DTA or that the florets were not able to tolerate further dehydration caused by water migration from them to the scales upon cooling to −50°C. In the case of supercooling in rice and wheat seeds (Ishikawa and Sakai 1978), seeds with water content below 17 to 19% (on a fresh weight basis) seemed to have no freezable water and consequently showed no exotherm in DTA and survived immersion in liquid nitrogen. Likewise, if the florets of *R. japonicum* could lose all freezable water (water content being below about 20%) and tolerate the consequent extremely dehydrated state, they would survive even immersion in liquid nitrogen as seen in seeds, prefrozen hardy twigs (Sakai 1965), and prefrozen hardy conifer buds (Dereuddre 1978, Sakai 1979b).

Considering these features of freezing avoidance in *Rhododendron* florets, supercooling of the florets seems considerably different from that of xylem ray parenchyma cells in temperate broad-leaved trees. The supercooling ability of the latter is unaffected by the cooling rate (Quamme et al. 1972a), thus probably involving no water migration, and has a lower limit of around −40°C (Quamme et al. 1972a), which corresponds to the homogeneous nucleation temperature of pure water in a finely dispersed state. Therefore the term "supercooling" seems appropriate in xylem ray parenchyma cells, but not necessarily for the precise description of the strategy employed by florets to survive cold stress since it involves water migration and subsequent dehydration as in extracellular freezing.

As demonstrated in Fig. 5 and 6, the degree of desiccation of florets is an important factor in cold hardiness of *Rhododendron* flower buds, that is, the cold hardiness is easily affected by environmental factors like cooling rate, humidity in air and snow coverage. These factors must be taken into consideration in estimating the cold hardiness of *Rhododendron* flower buds. The potential hardiness of flower buds of *Rhododendron* seems to lie in the ability to lose water from florets and peduncle or bud axis in response to temperature drop and to tolerate extreme dehydration.

The present authors wish to express their sincere thanks to Mr. Y. Hanazono and Mrs. K. Nakahira of the Botanical Garden of Hokkaido University and to Mr. H. Sanpei of the Agricultural Center of Sapporo City for providing the plant materials.

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(Received August 11, 1980; Accepted June 1, 1981)