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Assessment of Freeze Injury in 'Boskoop Giant' Black Currant Buds

Fumiomi Takeda[1], Rajeev Arora[2], Michael E. Wisniewski[2], and Glen A. Davis[3]

U.S. Department of Agriculture, Agricultural Research Service, Appalachian Fruit Research Station, 45 Wiltshire Road, Kearneysville, WV 25430

Michele R. Warmund[4]

Department of Horticulture, University of Missouri, Columbia, MO 65211

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Abstract. **A seasonal study was conducted to assess the freezing injury of 'Boskoop Giant' black currant *(Ribes nigrum* L.) samples from Oct. 1991 through Mar. 1992. Buds were subjected to either differential thermal analysis (DTA) or one of a series of temperatures (0 to -36C). Freeze injury was then assessed either visually or with TTC. Results indicated that black currant floral buds have multiple low-temperature exotherms (LTE). Freeze injury in intact buds could not be visually quantified because of the lack of visible browning, nor assayed with TTC reduction. Excised floral primordia incubated in TTC, however, developed colored formazan following exposure to nonfreezing and sublethal freezing temperatures, but remained colorless when exposed to lethal temperatures. The percentage of floral primordia that were colored and colorless were tabulated and a modified Spearman-Karber equation was used to calculate the temperature at which 50% of floral primordia were killed (T_{50} The T_{50} temperature was correlated with the temperature at which the lowest LTE was detected (R^2 = 0.62). TTC reduction assay using excised floral bud primordia was a good indicator of viability in frozen blackcurrant buds. Chemical name used: 2,3,5-triphenyltetrazolium chloride (TTC).**

Differential thermal analysis (DTA) has been used to study response to freezing temperatures in floral buds of several fruit genera (Wisniewski and Arora, 1992). In buds containing a racemose inflorescence, such as *Rubus* spp., *Ribes nigrum* L., and *R. sativum* Rchb. (Syme), low-temperature exotherms (LTE) correspond to lethal freeze-injury of individual flowers (Warmund et al., 1988) or the entire floral region (Kraut et al., 1986; Warmund et al., 1991). Warmund et al. (1991) reported that freezing of supercooled water in the floral primordia in black and red currants caused tissue injury. None of the floral bud primordia, however, exhibited visual browning when thawed. It was unclear whether LTEs detected were related to freeze-injury of individual floral primordia or some other portion of the inflorescence tissue.

A quick, reliable method to determine tissue viability in *Ribes* would be useful to evaluate bud survival under low-temperature stress. Attempts to quantify floral injury in thawed intact buds of black currant using TTC were unsuccessful (Warmund et al., 1991). TTC did not diffuse into floral bud primordia tissues through bud scales or from the bud axis base. In the present study, we determine the suitability of TTC reduction assay (Cook and Stanley, 1960) to assess excised black currant floral primordia viability following controlled freezing. This method was compared with fluorescein diacetate (FDA) staining technique (Rotman and Papermaster, 1966). In addition, we determined the seasonal pattern of cold hardiness using a TTC reduction assay and compared it with the deep supercooling seasonal pattern in 'Boskoop Giant' blackcurrant floral buds.

Bud collection and handling. Bud samples were collected from 2-year-old 'Boskoop Giant' black currant plants growing at the Univ. of Missouri Horticultural Research Station, New Franklin. One-year-old shoots were cut 20 to 50 cm above the soil surface in Oct. and Dec. 1991, and in Jan., Feb., and Mar. 1992; sealed in plastic bags containing a moist paper towel; packed on ice; and shipped by overnight mail to the Appalachian Fruit Research Station, Kearneysville, W.Va., for DTA and freeze tests.

Freeze test. Seven or eight subsamples, each containing seven to 15 single-node cuttings, were subjected to a series of temperatures to bracket the one at which 50% of the buds would be killed (T_{50}). One subsample was a nonfrozen control. Buds were held at SC for 6 to 10h, cooled at 3C/h to-3C, held at–3C for 3 to 7 h, and cooled further to eight test temperatures at the rate of 3C/h. The temperature range selected for freeze tests was based on the results of our previous study (Warmund et al., 1991) and within the range estimated to result in injury. Frozen samples were held over ice for 24 to 28 h and then stored at 4C for 16 h before viability tests. Individual primordia were excised and incubated in FDA (Arora and Palta, 1988) or in 0.16% TTC for 22 h in darkness (Towill and Mazur, 1974). Primordia incubated in FDA were examined for fluorescein with a Zeiss (Aixophot photomicroscope; Eastern Microscope Co., Raleigh, N. C.) microscope equipped with epifluorescence optics (excitation filter =485 nm; barrier filter = 520 nm). Following incubation in TTC, the number of colored and colorless floral primordia were recorded under a dissecting microscope at ×8, and the percentage of viable floral primordia within each bud was determined. The viability data were used in a modified Spearman-Karber equation (Bittenbender and Howell, 1974) to calculate T_{50} values for buds at each sampling date. Previously, we had determined that nonfrozen primordia incubated in TTC developed colored formazan, whereas primordia exposed to –50C remained colorless.

DTA. DTA were carried out to determine deep supercooling. The basal end of a cutting

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[1]Research Horticulturist.

[2]Plant Physiologist.

[3]Biological Laboratory Technician.

[4]Associate Professor.

was dipped in a colony of an ice nucleation-active strain of *Pseudomonas syringae* van Hall to initiate ice nucleation at warmer, subfreezing temperatures (–2to–3C). Each sample was placed in an aluminum foil cup and attached to a thermocouple. Exotherms were detected using previously described techniques (Ashworth, 1984 Wisniewski et al., 1990).

Flower bud development. By October, 'Boskoop Giant' black currant buds had developed one or more inflorescence axes. The number of floral primordia ranged from three to as many as 22 per inflorescence (Fig. 1A). Throughout the sampling period (October to March), the average number of floral primordia was 10 ± 2 primordia per bud, which indicated that floral primordia initiation was essentially complete by fall. In an earlier study (Warmund et al., 1991), both 'Danka' black and 'Red Lake' red currant floral primordia had developed sepal, petal, stamen, and carpel primordia by November. Little or no development occurred during the winter in our study. With higher temperatures in mid-March, floral organs enlarged rapidly, and pedicels and inflorescence axes elongated.

Viability tests. Regardless of collection date, none of the buds examined exhibited visible browning after exposure to lethal temperatures (Fig. lB). Floral primordia generally appeared turgid, but some buds from March samples exhibited signs of water soaking of tissues, especially those exposed to below –30C.

FDA was unsuitable for assessing cold injury (Fig. lE-H). Control buds, as well as buds exposed to temperatures as low as –36C, fluoresced after incubation in FDA (Fig. 1 E and F). The fluorescence was clearly limited to the floral tissue in buds exposed to nonlethal temperatures (Fig. 1 E). In buds exposed to lethal temperatures, fluorescence was observed first in the floral tissue and then in the incubation medium (Fig. lF). In cold-injured buds, esterase enzymes apparently retained the capacity to hydrolyze fluorescein diacetate to yield fluorescein, although the cell membrane was unable to confine fluorescein in the cytoplasm (Fig. lG). This view is supported further by the fact that extraneous fluorescence amounts decreased after rinsing stained samples (Fig. 1 H). Using FDA to determine viability also limited the number of samples that could be evaluated, because only a few primordia (0.5 to 1.0 mm in diameter) could be viewed with the epifluorescent microscope at the magnification (×100) needed to assess the release of fluorescence into the medium.

Following incubation in TTC, viable and injured floral primordia could be clearly distinguished. Viable primordia developed colored formazan, and injured primordia remained colorless (Fig. 1 C and D). Within an axillary

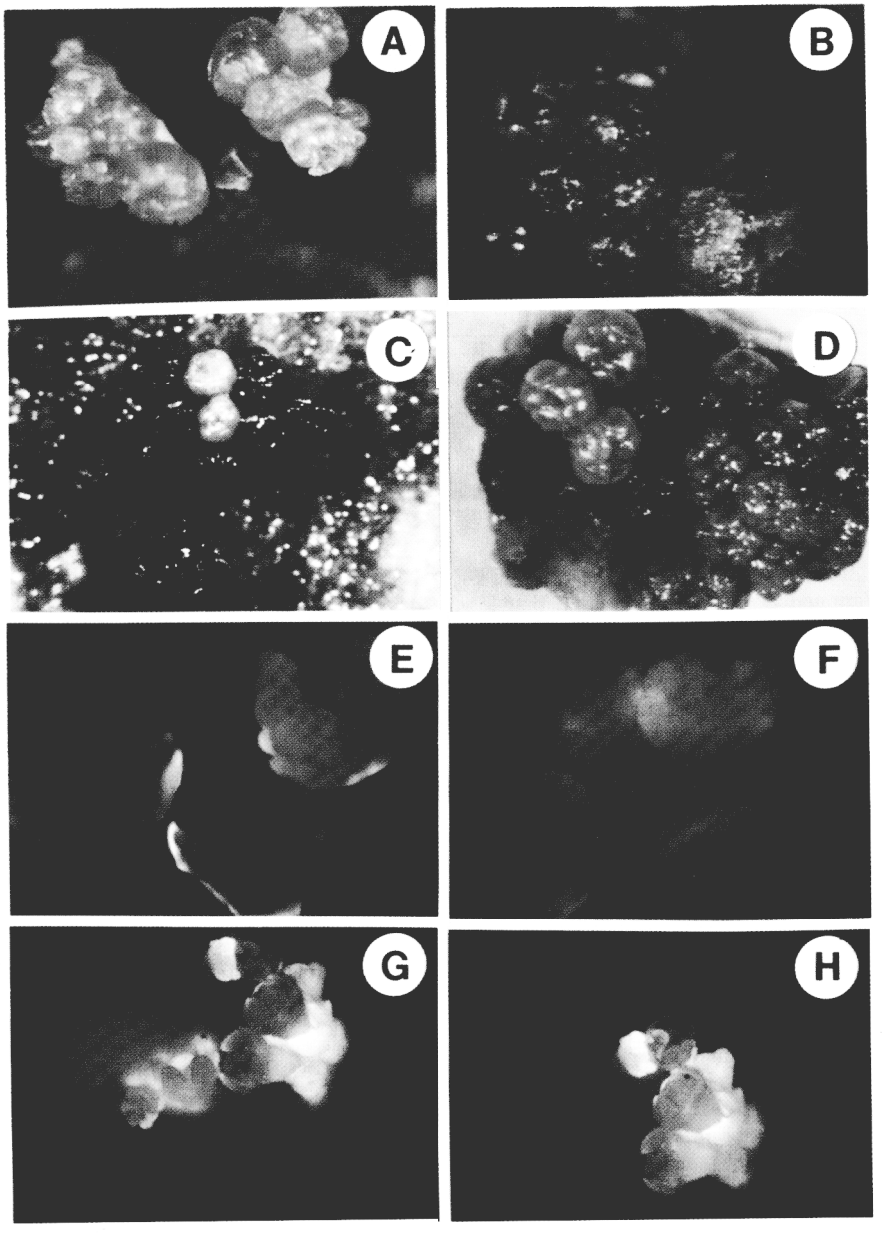

Fig. 1. Photomicrographs of 'Boskoop Giant' black currant floral primordia before and after incubation with TTC or fluorescein diacetate (FDA). Excised floral primordia of **(A)** nonfrozen control bud and **(B)** bud exposed to a lethal temperature and with no oxidative browning. **(C)** Floral primordia of nonfrozen control bud after 22 h incubation in TTC. All but two primordia reduced the colorless TTC to red formazan. **(D)** Floral primordia of nonfrozen bud exposed to a lethal temperature and with no TTC reduction. **(E)** Epifluorescence microscopy of floral primordia after adding FDA. The fluorescence is limited to the tissue. **(F)** Epifluorescence microscopy of floral primordia exposed to a subfreezing temperature. Some fluorescein is detected outside the tissue. **(G)** Fluorescence in floral primordia after 10-min exposure to excitation light. Note the diffusion of fluorescein out of the tissue. **(H)** Floral primordia after two rinses with distilled H_2O. Note the amount of diffused fluorescein.

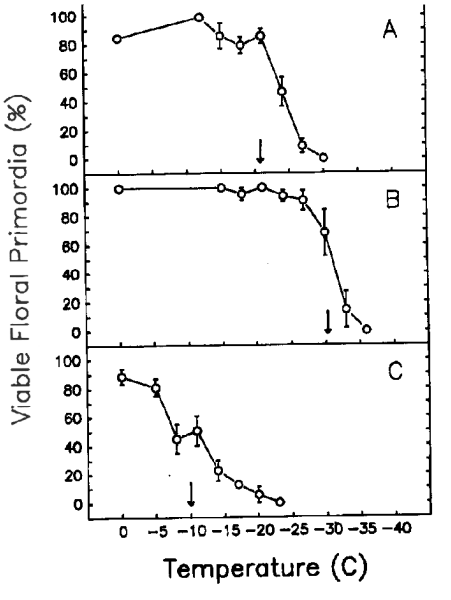

Fig. 2. Relationship between temperature and floral tissue viability as determined by a TTC reduction assay for October **(A),** early February **(B),** and late March **(C)** samples. The T_{50} value (arrow) for each sampling date was calculated using a modified Spearman-Karber equation. Vertical bars represent SE. Missing SE bars were smaller than graph symbols.

Table 1. Individual floral primordia viability in each bud sample after controlled freezing as determined by a TTC reduction assay. The percentage of floral primordia surviving at a given temperature was either high (>80%) or low (<20%). The first number in each pair represents the percentage of buds in which >80% of floral primordia were alive and the second number represents the percentage of buds in which <20% of floral primordia were alive.

	Sampling date						
Temp (°C)	28 Oct.	9 Dec.	19 Jan.	7 Feb.	27 Feb.	14 Mar.	23 Mar.
Control	78/00	93/00	90/00	100/00	100/00	95/00	83/00
–6	---[z]	---	---	---	---	80/00	67/20
–9	---	---	---	---	71/00	100/00	17/33
–12	100/00	83/00	---	---	86/00	100/00	14/57
–15	80/10	86/00	90/00	100/00	71/14	100/00	09/64
–18	90/05	77/08	78/22	89/00	31/62	50/30	00/67
–21	60/20	71/21	90/10	100/00	23/77	64/40	00/83
–24	60/20	100/00	67/22	89/00	00/100	00/100	00/100
–27	00/80	38/62	00/92	90/00	00/100	---	---
–30	00/100	33/67	11/78	67/33	00/100	---	---
–33	---	00/100	00/100	13/87	---	---	---
–36	---	---	00/100	00/100	---	---	---

[z]Not determined.

Table2. Viability (T_{50})of floral primordia based on TTC assay and the data from differential thermal analyses (DTA). The number of multiple low-temperature exotherms (LTE) and the temperatures at which the highest, lowest, and median LTE occurred were determined from DTA Profiles of five buds at each sampling date. Only those spikes that were sharp and ≥2 mm were tabulated.

		LTE			
Sampling date	T_{50} (°C)	No.	Highest	Lowest [temp (°C)]	Median
28 Oct.	–22.2	1.8 ± 0.5[z]	–12.5	–22.5	–19.5
9 Dec.	–25.0	1.4 ± 0.2	–21.0	–27.0	–26.5
9 Jan.	–23.1	1.4 ± 0.2	–22.0	–28.5	–26.5
7 Feb.	–31.2	1.2 ± 0.2	–20.9	–28.2	–26.5
27 Feb.	–18.3	3.8 ± 0.4	–3.5	–22.6	–21.0
14 Mar.	–19.0	5.4 ± 0.6	–6.5	–20.0	–15.0
23 Mar.	–10.3	7.6 ± 2.3	–4.5	–20.5	–17.0

[z]Mean ± SE.

bud, the percentage of floral bud primordia surviving at a given temperature was usually >80% or <20% (Table 1). At most sampling dates, there was a temperature range above and below which either a high or a low bud survival percentage was observed (Table 1, Fig. 2). The SE of mean values was small above and below this temperature range, whereas within the temperature range, the SE values were high (Fig. 2). The results suggest that floral bud primordia in cold-acclimated buds are killed over a narrow temperature range (6C), as is true of other species. The large SE in this temperature range may have been due to a high degree of variability in the number of living vs. injured floral primordia. This suggestion agrees with another report that cited electrolyte leakage (Ashworth et al., 1983). In reacclimated buds, low-temperature injury occurred over a wide range of temperatures and at moderate temperatures (Fig. 2), as shown in 23 Mar. samples. As many as half the buds exposed to–9to–18C temperatures had a high degree of variability in individual floral primordia viability and did not fall into either the >80? or <20% category. Bud hardiness increased from October to February, when maximum hardiness of –3lC was reached (Fig. 2). Thereafter, buds reacclimated rapidly.

DTA. One to 16 LTEs were detected as sharp spikes on the thermograms from bud samples cooled at 3C/h (data not presented). In addition to these sharp, narrow LTEs, Warmund et al. (1991) reported that low, broad exotherms between –14 and –20C were detected in black currant bud samples. The latter were thought to have resulted from freezing of supercooled water in the outer nonliving region of the periderm of attached cane tissue. No broad exotherms were observed in our study, a result which maybe due to differences in sample preparation or DTA protocols.

The number of LTEs obtained from bud samples at any collection date were highly variable. From October to February, they averaged 1 to 3; in March, they increased to five to eight per bud and occurred over a wider range of temperatures (Table 2). At all sampling dates, both small (<2 mm in height) and large LTEs were present. The additional LTEs detected in March may be related to isolated nucleation events in individual and enlarging floral organs. However, the number of floral primordia on the inflorescence axis did not correlate with the number of LTEs per bud (r^2 = 0.09). Similarly, in 'Danka' black and 'Red Lake' red currants, the LTE detected from DTA samples did not correspond to the number of floral bud primordia (Warmund et al., 1991).

Regression analyses were performed to determine if any set of DTA data could be associated with the calculated T_{50} values obtained from the TTC assay of excised primordia. The best correlation was obtained by regressing T_{50} values to temperatures at which the lowest LTE (Table 2) was detected (r^2 = 0.62). The correlation between T_{50} and the median LTE (Table 2) was even lower (r^2 = 0.56). These results indicate that until more is known about the sources of multiple LTEs detected from DTA, the use of DTA alone for assessing freeze injury in black currant floral buds would not be reliable.

Since oxidative browning does not occur in floral tissues of currant buds as a result of low-temperature injury, an alternative staining technique to assess bud viability was developed. Using TTC to evaluate flower bud viability provided a quick and reliable technique in which viability and cold hardiness could be determined within 2 days as compared with more than 7 days for regrowth tests. Excised individual floral primordia are necessary to determine *Ribes* viability with TTC reduction assay after freeze tests.

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