INVESTIGATIONS OF COLD HARDINESS OF BLACKBERRY SEEDLINGS AND METHODS FOR EVALUATING HARDINESS OF MATURE BLACKBERRY CANES

by

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Introduction

Lack of winterhardiness is a major concern for blackberry (Rubus subgenus Eubatus) production in much of North America (15,24,27). Shoot dieback and a reduction in flowering are the primary expressions of winter damage (16). Consistant productivity and vigor of blackberry are dependent on cane hardiness and retention of enough uninjured canes over winter to enable the plants to bear a full crop the next season (16, 28).

Although many efforts have been made to improve plant cold hardiness, little is known about the genetic mechanisms of this trait (36). It is known that plant cold hardiness is a very complex trait with many genes involved, which makes genetic analysis very difficult (10). In thornless blackberry breeding for winterhardiness, Knight et al. (15) acheived some progress in incorporating cold hardiness into thornless material by hybridization with hardy cultivars from Wisconsin. Evaluations conducted by Moore and Brown (25) showed that the blackberry cultivars `Darrow' and `Hedrick' were the most hardy of 12 cultivars tested. Because of the genetic complexity of the winterhardiness trait and its low heritability, progress from selection for improved cold tolerance has been slow compared to selection for other traits (9). However, studies of some morphological and physiological responses related to cold hardiness may provide an opportunity to further analyze this character.

Cold hardiness is not a static entity and is influenced by a number of variables including plant species, climate of origin, physiological tissue age, moisture content, rate of temperature change and duration of low temperature (36). Rajashekar and Hellman (31) indicated that many blackberry cultivars are not well adapted to Kansas conditions. They are frequently subjected to winter injury because of premature spring growth in late winter or early spring. As temperature increases in late winter and early spring, plants begin to lose hardiness and they are easily injured by spring frost. Marked seasonal changes in plant cold hardiness have been reported by many investigators (12, 16, 23, 31, 38). Even plants that survive the most extreme freezing during midwinter may be killed by very slight freezing during spring (18).

In this study, artificial acclimation of blackberry seedlings and freezing tests were conducted to determine the amount and timing of acclimation required and the minimum temperatures that seedlings can tolerate. Cane samples from field-grown mature plants of 7 blackberry cultivars were also tested to observe differences in cold hardiness and stability of this character during late winter and early spring. Three evaluation methods (conductivity, tissue browning, bud viability) were used to measure cold hardiness, and the results of the tests were compared.

Literature Review

1. Nature of freezing injury

Freezing injury in plants is caused by ice formation. There are two types of ice formation in plant cells and tissues; the freezing process that occurs in the intercellular space is termed extracellular freezing; ice formation inside plant cells is termed intracellular freezing. Under laboratory conditions, plants may be induced to freeze either intracellularly or extracellularly. Yet, it is still not known with certainty whether or not intracellular freezing occurs in nature. Circumstantial evidence indicates that sunscald injury which occurs on south-facing branches of many tree species may be a cause of intracellular freezing caused by rapid temperature changes (20,39). When such intracellular freezing occurs, death almost inevitably results.

In nature, air temperature typically decreases very slowly, only a few degrees per hour. At such slow rates of freezing, in most cases, ice forms in the intercellular space. As ice crystals form, they can withdraw water from the protoplast because vapor pressure in the intercellular space drops below that of the protoplast. Generally, the lower the temperature, the greater the proportion of water that freezes in the tissue and the faster the speed of freezing. This leads to dessication and dehydration of the plant cell.

Extracellular ice formation may or may not cause injury depending on the freezing temperature and hardiness of the tissue, according to the observation of Asahina (1) on isolated cultured cells. During very slow extracellular freezing, if the cells are cooled beyond a certain limit of tolerable low temperature, unhardy or moderately hardy cells are easily injured irreversibly without any ice formation within the cells. Death occurs in these cells under dehydrated and contracted conditions. Extracellular ice in hardy tissues, on the other hand, appears to form continuously, withdrawing water from cell interiors resulting in a remarkable growth of ice crystals on the cell surface. Cells frozen in this way consequently undergo a severe dehydration and contraction. If the cells have not been seriously injured by the previous freezing, the contracted cells absorb water after thawing as surrounding ice melts and expands to recover a normal appearance and activity. No "pseudoplasmolysis" is observed in most hardy cells after freeze-thawing. One hypothesis to explain injury by ice formation is that the withdrawal of water from cells results in dehydration of the protoplast. Dehydration in turn induces various disorganizing consequences such as coagulation of certain layers of the protoplasm which seems most destructive. Another explanation is that the formation of crystals in the intercellular space results in mechanical deformation of the protoplasm.

Lyons et al. (21) indicated that membrane damage is a universal manifestation of freezing in biological systems and is commonly inferred to be the primary cause of injury. The flaccid, water-soaked appearance of various plant tissues and organs following thawing strongly suggests that exposure to lethal low temperatures results in membrane disruption. According to Steponkus and Wiest (34), freeze-thaw injury to isolated protoplasts is the result of two major strains: a freeze- or contraction-induced membrane alteration which decreases maximum critical surface area of the plasma membrane, and a thaw- or expansion-induced dissolution of the plasma membrane which occurs when the maximum critical surface area is exceeded. These two strains interact during a freeze-thaw cycle and results in lysis of the cell. Steponkus and Wiest (33) suggested that freezing injury to protoplasts is the result of alteration in the resilience of the protoplast. While the altered resilience is the result of alteration in the plasma membrane that occurs during contraction, it is not manifested until the protoplasts are induced to expand, either during osmotic dilution or thawing, when disruption of inter-molecular forces in the membrane causes protoplast lysis.

Markhart (22) indicated that membrane changes may be secondary to other cellular alterations. A major component of the cell cytoskeleton, the microtubules, have been shown to depolymerize when exposed to low temperatures. Breakdown

of the cytoskeleton could have a wide range of effects on cell metabolism and membrane function.

There is a great divergence in hypotheses on the mechanism of freezing damage. For example, dehydration of water from protoplasts has several consequences which include: a reduction in cell volume and surface area, an increase in concentration of solutes, precipitation of some buffering salts resulting in pH changes, and removal of water of hydration of macromolecules. Since freezing results in a multitude of stresses, it is reasonable to assume that the overall mechanism of freezing injury is a composite of the many hypotheses put forth and they should not be considered as mutually exclusive (21).

2. Mechanisms of freezing tolerance

Levitt (20) indicated that freezing survival requires the prevention and repair of both intracellular and extracellular freezing injury, which induces both avoidance and tolerance.

Some tropical alpine plants such as Dendrosenecio brassica can avoid freezing by bending their leaves inward upon cooling to form a so-called night-bud. The multilayer of the many rosette leaves and the styrofoam-like structure of their mesophyll result in a substantial delay of cooling of the central leaf-bud. Therefore, the temperature of growing leaves in the cone rarely drops below the freezing

point (5). This kind of mechanism of avoidance also depends on many environmental factors; on sunny days the plant can increase its temperature several degrees above that of air. Plants that pass winter under a snow cover are able to avoid low air temperatures of the above ground atmosphere. Due to the poikilothermic nature of plants, this avoidance mechanism is rare and of limited value to the plant, occuring only when the freezing temperature persists for short periods of time (20).

Deep supercooling is another mechanism of freezing avoidance which has been observed in dormant buds of a number of species including azalea, blueberry, grape, flowering dogwood, forsythia, and several Prunus species (2). Pure water, with an absence of extraneous nucleators, can be cooled to -38°C without ice formation. This is the empirical limit called the homogeneous ice nucleation temperature. Plants that supercool have a temperature limit around -40°C (32). If water within these tissues remains supercooled, no injury occurs and , presumbly, cells are killed by intracellular ice formation when ice formation is initiated (3). Wisniewski et al. (40) observed that in stem tissue of peach (Prunus persica), bark tissues exhibits extracellular freezing, whereas xylem parenchyma exhibits deep supercooling in response to low temperature. It is still not clear how these specialized tissues succeed in remaining supercooled for hours, days, or months, in the

same plant in which the vast majority of cells and tissues freeze extracellularly (20).

Freezing tolerance, in other words, is tolerance of extracellular freezing, which is by definition a freeze-dehydration. Levitt (20) indicated that plants may survive the freezing dehydration in two ways: 1) avoidance of dehydration; and 2) tolerance of freeze-dehydration. The former can be explained because it has long been known that the amount of solutes (primarily sugar) accumulated is very often charateristic of the hardening process. Tolerance of freeze-dehydration is more complicated and poorly understood. It is believed to depend on membrane properties, and more specifically properties of membrane proteins.

Plant hardiness changes considerably with season. We know that plants usually have little hardiness during the growing season and increase hardiness after growth ceases in autumn. The development of cold hardiness is termed acclimation and the loss of hardiness is termed deacclimation (29). Howell and Weiser (12) found that acclimation of `Haralson' apple occurred in two stages which are induced by short days and frost (or low temperature), respectively. According to Wisniewski and Ashworth (40), cold hardiness of xylem and bark tissues of peach begins to increase markedly in the fall, gradually reaches a peak in midwinter, and sharply decreases in early spring. During acclimation, cortical cells with a large central vacuole and

a thin band of peripheral cytoplasm gradually develop a more homogeneous distribution of cytoplasm, a centrally located nucleus, and many small vacuoles. The reduction in size of vacuoles most likely results from decrease in tissue water content associated with increased cold hardiness. A disappearance of starch grains occurred in xylem parenchyma. Modification of plasmalemma occurs during acclimation, which allows it to withstand stresses incurred during freezing and prevents either loss of osmotic responsiveness or an inflexibility that leads to membrane leakage or lysis on rehydration. Because fluctuation of temperature in the environment leads to changes in plant cold hardiness, hardiness tests conducted on a single date may give misleading results, e.g. `Siberian C' peach rootstock may rank the most hardy in midwinter, but it is less hardy in the spring than other cultivars because it deacclimates earlier. It seems advisable to test cultivar hardiness at more than one time when there is a possibility that cultivar rank changes with season (29).

3. Measurement of Cold Hardiness

In early plant breeding efforts, selection for cold hardiness was usually conducted in the field following a "test winter". However, selection for cold hardiness in the field does not always occur because plants may be protected by snow cover in northern regions of the United States. In addition, environmental factors often change year by year;

sometimes, low temperature stress does not occur, is too severe, or the effect is altered by another factor (7). All of these factors make screening plants for cold hardiness in the field difficult and time-consuming.

Because there are certain disadvantages to the use of field empirical methods for isolating cold hardy genotypes, artificial freezing tests in the laboratory have been examined to separate hardy and unhardy plants. According to Levitt (19), this method was first introduced by Harvey in 1918. By freezing a series of apple cultivars at a previously determined temperature it was possible to obtain a graded series of injuries, depending on the hardiness of cultivars. Through decades of development, artificial freezing tests have been used for many different kinds of plants and found to give excellent agreement with winter survival in the field.

The use of artificial freezing techniques for either evaluating existing cultivars or selecting seedlings demands that plant material be subjected to some sort of preconditioning treatment, either naturally or artificially (36). Young and Hearn (43) observed that `Clementine' tangerine seedlings did not segregate for cold hardiness when unhardened, but did segregate after hardening in a plant growth chamber. Fear and Stushnoff (8) studied cold tolerance of six-week-old strawberry seedlings as part of an investigation to determine the value of early selection and

mass screening for improved cold tolerance. First, they hardened seedlings for periods of 1, 2 and 4 weeks at 2°C and an 8-hour photoperiod. Frost treatments were given after 7 days hardening by holding seedlings at 3°C for 18 hours. Hardening for 1 week was sufficient to increase cold tolerance over control seedlings held in a greenhouse. However, 2 to 4 weeks of hardening increased survival of seedlings to -11°C. 'Valencia' orange trees withstood -6.7°C for 4 hr without injury following 5 weeks of cold hardening at 10° with continuous light. Cold hardening was faster in stems than in the leaves (42). Auld et al. (4) studied winter hardiness of peas under both field and laboratory conditions. Screening at -9°C in the laboratory was significantly correlated with 4 of 8 field environments, and they concluded that laboratory procedures could greatly facilitate the development of new cultivars with high levels of winter hardiness.

When plant tissue is cooled below 0°C, freezing does not usually occur immediately, but the tissue remains supercooled. In conducting freezing tests, it is advisable to inoculate plant tissues with ice crystals to prevent excessive supercooling because detached parts tend to supercool more than whole plants (19).

Several methods are used for determining injury in plant material after it has been subjected to freezing stress. The most direct method of measuring freezing injury

is the recovery rating; evaluating growth of plants after freezing stress. Lapins (17) indicated that the recovery test was much more sensitive than the conductivity test in differentiating between 2 apple cultivars and among 3 seedling progenies.

The conductivity test is also an effective method for measuring freezing injury of plants (11,14,30,41). Freezing stress results in membrane damage and leakage of cellular electrolytes. Cell leakage, and thus cellular damage, can be determined by measuring the conductivity of water extracts of the tissue. Blazich et al. (6) evaluated electrolytic, visual, and electrical impedence methods and indicated that the electrolytic method was more closely associated with the visual method and better separated the effects of freezing temperatures.

Another evaluation method is to use the degree of tissue browning after incubation as an estimate of freezing injury. Stergios et al. (35) conducted 5 viability tests: growth, tissue browning, triphenyl tetrazolium chloride (TTC) reduction, specific conductivity, and double freezing point tests on 4 different species: `Montmorency' sour cherry, `Concord' grape, `Latham' raspberry and `Midway' strawberry, and found that even though growth and tissue browning tests were slow and qualitative, these 2 tests were the most reliable and could be used as a control for other tests.

Materials and Methods

1. Seedling test

Open-pollinated seedlings of the blackberry cultivar `Shawnee' were used in freezing tests to determine an effective period of cold acclimation and evaluate seedling hardiness after freezing stress. The seeds were collected from field-grown plants in Manhattan, Kansas in 1986. Because of problems of low seed germinability and erratic emergence, the seeds were scarified with concentrated sulphuric acid for 30 min. in an ice bath to prevent heat damage of embryos. Following acid treatment, the seeds were rinsed with running water for 5 min., neutralized by placing seeds in an excess solution of sodium bicarbonate for 5 min., and then rinsed again for 5 min. in running water according to the method of Moore et al. (26). Following scarification treatment, the seeds were air-dried for 24 hr. and wrapped with moist paper towels, placed in perforated polyethyene bags and stored at 2°C for more than 4 months.

The seeds were sown on February 20, 1987 in a flat filled with a 1:1 peat and perlite mixture and placed in a greenhouse. Germination began on March 13; the individual seedlings were transplanted at the 2 true-leaf stage into speeding styrofoam flats having chambers of 3.8 x 3.8 cm. Each flat contained 21 seedlings and a total of 9 flats of seedlings were used in the freezing test.

After six weeks of growing in the greenhouse, the seedlings were moved into a walk-in cooler and maintained at $4^{o}C$, 10 hr. photoperiod for 1, 2 or 3 weeks of acclimation.

Following one week of acclimation, three flats of seedlings were put into a freezing chamber and cooled from 0^{o} to $-3^{o}C$ at a rate of $2^{o}C$ per hour. One 24 gauge copper-constantan thermocouple was inserted into the soil and another was placed in the canopy of seedlings for temperature monitoring. When the temperature reached $-2^{o}C$, seedlings were inoculated with ice to avoid supercooling. The temperature was maintained at $-3^{o}C$ for 15 hr. to ensure that nucleation occurred in the seedlings. One flat of seedlings was removed from the freezer at $-3^{o}C$, then the temperature was decreased to $-6^{o}C$ at the same cooling rate and kept constant for 4 hr. A second flat of seedlings was removed at this time and the temperature was then dropped to $-9^{o}C$ and kept constant for 3 hr., then the final flat of seedlings was removed. The seedlings were moved directly into the walk-in cooler operating at approximately $4^{o}C$ for a 24 hr. period of slow thawing, and then moved back to the greenhouse. The individual injury ratings were recorded 2 weeks after the freezing test. Seedlings hardened for 2 and 3 weeks were tested with the same procedure described above. The following rating categories were used for plant injury evaluation:

1 = no serious leaf injury occurred.

2 = half of leaves killed.

3 = most leaves and upper shoot killed, but stem was alive.

4 = whole plant killed.

Data from the seedling freezing test was analyzed with a two-way analysis of variance. Mean difference of seedlings at different acclimation and temperature treatments were compared by Duncan's multiple range test.

2. Field-grown mature plant test

Conductivity, tissue browning and bud viability methods were used to evaluate hardiness of cold-stressed cane sections of 7 blackberry cultivars: `Hull', `Cherokee', `Chester', `Cheyenne', `Shawnee', `Darrow' and `Navaho'. All samples were obtained from plants under field cultivation at Ashland Horticulture Farm, Manhattan.

Blackberry samples were collected on Jan. 25, 27, and Feb. 6 of 1988. Cane sections approximately 20 cm long were removed from the middle portion of lateral canes and, following return to the laboratory, were immediately cut into 4 cm pieces with each piece containing at least one bud. Temperature treatments consisted of 10 levels from -4^{o} to -40^{o}C with a 4^{o} interval between each level. On the first sampling date, 4 cane pieces from each cultivar were wrapped in a moistened paper towel for each temperature level; 2

cane pieces were used for the conductivity test and the other 2 were used for tissue browning and bud viability ratings. Each cultivar had 2 untreated cane pieces used as a control for each evaluation method. On the second and third sampling dates, 3 cane pieces of each cultivar were used at each test temperature for each evaluation method.

The freezing tests were performed in a Tenney Benchmaster freezing chamber controlled by a computer and programmed at a cooling rate of 5°C per hour. Samples were seeded with ice at -2°C to prevent supercooling. A 24 gauge copper-constantan thermocouple was inserted in the samples for temperature monitoring. Four cane pieces (six on second and third date) of each cultivar were removed from the freezer at 4° intervals from -4° to -40°C and placed in a refrigerator for 2 hours, then moved to room temperature (approximately 23°C) for another 2 hours of thawing. One half of samples were used for the conductivity test and the remaining samples were used for tissue browning and bud viability tests.

Electrolytic conductance. The method used was similar to that used by Ketchie et al. (14) with the following modifications. Following freezing stress, samples were cut into 1 cm sections, halved longitudinally and placed in 20 x 150 mm test tubes with 15 ml distilled water and covered with aluminum foil. The samples were held for 24 hours at room temperature for diffusion of electrolytes. Conductivity

measurements were made with a YSI model 32 conductivity meter. Following the measurement, samples were put into a water bath at 80°C for 40 minutes to kill the tissues. After an additional 24 hours of electrolyte diffusion, final readings on the conductivity meter were obtained. The results were expressed as the percentage of electrolytes, as reported by Ketchie et al. (14).

Tissue browning and bud viability. Following freezing treatment and thawing, 2 (3 on second and third sampling date) cane pieces from each temperature treatment were wrapped with moist paper towel, placed in a plastic bag and incubated at 100% relative humidity, 24°C for 10 days. Buds and phloem were then examined for tissue discoloration as an indicator of viability. Buds were rated 1 if alive, 2 if killed. Phloem was rated on a scale of 1 to 3 for tissue browning (1 = no browning, 2 = moderate browning, 3 = severe browning and dead). Observation of callus formation at the ends of cane sections was also used in tissue evaluations. There was no callus formation from dead cane sections.

All data were subjected to analyses of variance. The model included the main effects of cultivar, temperature, and sampling date, and interactions among the main effects. Differences in cold hardiness of the 7 cultivars were compared by Duncan's multiple range test. Correlations between different factors and evaluation methods were also calculated.

Results and Discussion

1. Seedling acclimation and freezing treatment

A. Acclimation

Following acclimation for 1, 2 or 3 weeks, 63 seedlings from each acclimation treatment were subjected to freezing at 3 test temperatures.

Analysis of variance showed a highly significant variation in seedling injury associated with acclimation period (Table 1). There was no significant difference between 1 and 2 week acclimation treatments, but they were both significantly different from the 3 week acclimation

Table 1. Analysis of variance obtained from seedling injury evaluation following freezing test.

Source	df	M S	F value
Temperature	2	144.640	610.2**
Acclimation	2	2.958	12.5**
T x A[z]	4	2.394	10.1**
Error	180	0.237	

**Significant at 1% level.
[z]T=temperature, A=acclimation.

treatment (Table 2). The reason why seedlings receiving 3 weeks of acclimation treatment were less hardy is not clear. Because seedlings were still in an active growth stage, extended exposure to low temperature (and low light conditions) might have adverse effects on physiological and biochemical activities of seedlings.

Table 2. Mean injury ratings of blackberry seedlings subjected to 3 acclimation periods and 3 freezing temperature treatments.[z]

Temperature treatment (°C)	Acclimation period (weeks)		
	1	2	3
-3	1.00	1.00	1.00
-6	1.19	1.05	2.19*
-9	3.79	3.86	3.90
Mean	2.02	1.97	2.37*

*Within the same row, significant at 5% level.
[z]1 = no serious leaf injury occurred.
2 = half of leaves killed.
3 = most leaves and upper shoot killed but stem was alive.
4 = whole plant killed.

B. Cold resistance evaluation of seedlings

Seedling injury was significantly affected by temperature of the freezing treatments (Table 1). During freezing tests, all seedlings from the 3 acclimation treatments were alive and no injury occurred at $-3^{o}C$ (Table 2). At $-6^{o}C$, most seedlings survived and various degrees of injury began to occur. A significant difference was found at $-6^{o}C$ among seedlings with different acclimation treatments, i.e., seedlings with a 3 week acclimation period had more injury than seedlings receiving 1 or 2 week acclimation treatments. Most seedlings died at -9^{o} and the few surviving seedlings exhibited serious injury, such as death of leaves and the upper shoot. These seedlings were still alive and adventitious buds eventually emerged from the stem. There were no significant differences at $-9^{o}C$ for different acclimation treatments. This may be due to the large number of seedlings that died, which may have masked any differences in cold hardiness of seedlings with different acclimation treatments.

The presence of a highly significant temperature x acclimation interaction indicates that blackberry seedlings responded differently to different temperature treatments depending on the length of their acclimation treatment.

In this study, signs of injury in six-week old `Shawnee' seedlings began to occur at $-6^{o}C$ and they did not

survive temperatures as low as $-9^{o}C$. In future seedling freezing tests, a temperature range between -6^{o} and $-9^{o}C$ should be choosen as the critical temperature range to test blackberry seedlings for cold hardiness.

During freezing tests, thermocouples in the soil and canopy of seedlings indicated that the initial temperature of the soil was much higher than that of the air temperature in the freezing chamber. The differences between soil and air temperature became smaller as the temperature was lowered to $-9^{o}C$. The validity of whole-plant freezing tests for identifying hardy blackberry seedlings may be complicated by variable hardiness of different tissues and organs. Roots are generally less hardy than the aerial parts of plant, therefore, injury and death of seedlings might have been partly due to injury of root systems. Furthermore, root hardiness is not considered to be an important criterion for selection because blackberry root injury in the field is rare. Since there were no more seedlings available for further tests, the above questions remain to be investigated.

2. Laboratory tests for field-grown plants

Multiple variance analyses were performed on percentage electrolytes, tissue browning, and bud viability data from three sampling dates.

A. Conductivity test.

Analysis of variance indicated a significant variation in percentage electrolytes associated with temperature treatments (Table 3). This is expected because test temperatures from -4^{o} to -40^{o}C ranged from nonlethal to 100% lethal to blackberry cane tissues. In addition, percentage electrolytes extracted is closely associated with injury to plant tissues subjected to low temperature stress (14). Percentage electrolytes extracted from the 7 blackberry cultivars was fairly constant until -24^{o}C (Table 4). Temperatures below -24^{o} caused a sharp increase in percentage electrolytes extracted, which reflected the occurrence of tissue injury as indicated by tissue browning ratings (Fig.1). Because plant tissue injury began to occur at -24^{o}C and all plant samples were killed at -36^{o}C (as indicated by tissue browning test), the critical temperature range for the testing of cane tissues of the 7 blackberry cultivars was between -24^{o} and -32^{o}C.

The temperature chosen for analysis of plant hardiness should have the capacity of distinguishing degrees of hardiness among cultivars. Because no injury or only slight

Table 3. Analysis of variance of percentage electrolytes extracted from freeze-treated cane sections of 7 blackberry cultivars over 3 sampling dates.

Source	df	M S	F value
Temperature	9	4563.88	528.6**
Sampling date	2	961.26	111.3**
T x S[z]	18	27.79	3.2**
Cultivar	6	357.50	41.4**
C x T	54	32.68	3.8**
C x S	12	61.03	7.1**
C x T x S	108	30.31	3.5**
Error	350	8.63	

**Significant at 1% level.
[z]T=temperature, S=sampling date, C=cultivar.

Table 4. Mean percentage electrolytes extracted from cane sections of 7 blackberry cultivars from 3 sampling dates subjected to freezing temperatures.[z]

	Temperature (°C)									
Cultivar	0	-4	-8	-12	-16	-20	-24	-28	-32	-36
Chester	27.2	27.2	27.1	27.2	28.8	27.5	30.5	34.4	44.4	55.1
Cheyenne	27.5	30.9	29.1	30.6	31.5	31.1	32.1	36.8	48.2	51.6
Hull	28.3	27.1	27.5	24.6	27.9	26.4	32.3	39.1	49.9	54.6
Navaho	28.6	29.3	27.6	30.2	30.9	30.2	34.3	39.7	48.1	60.5
Cherokee	28.1	31.4	30.1	31.4	29.6	31.7	33.4	39.8	45.8	55.7
Darrow	30.3	38.6	33.1	30.9	31.2	35.4	44.2	42.2	50.9	57.5
Shawnee	30.1	30.4	30.6	30.2	35.2	33.8	33.5	42.3	50.0	53.6

[z]Means of 3 sampling dates.

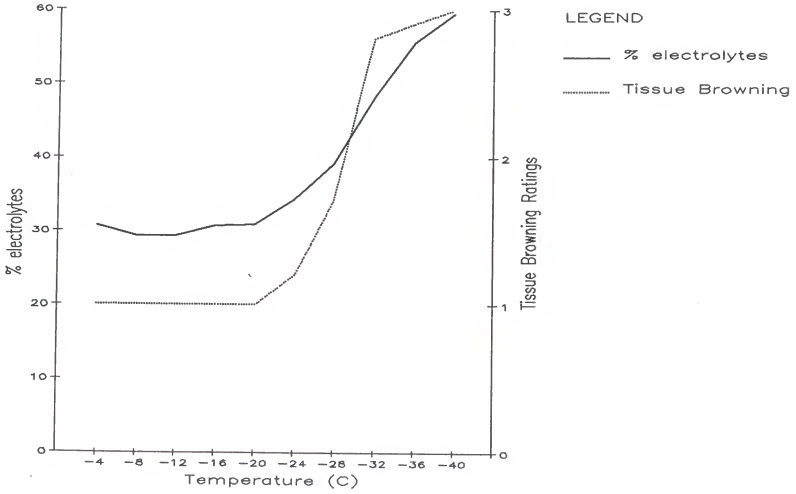

Fig. 1. Changes of percentage electrolytes and tissue browning ratings of blackberry cultivars at different test temperatures.

injury occurred at -24°C, little separation of cultivars was possible at or above this temperature. The optimum differential conductivity occurred at -28°C as indicated by separate Duncan's multiple range tests of cultivar means at -28° and -32°C.

The highly significant mean square for sampling date indicates that conductivity of blackberry cane sections varied over the 3 sampling dates (Table 3). This strongly suggests that plant cold hardiness was affected by environmental conditions. Howell and Weiser (13) studied the relationship of environmental temperature to the cold resistance of apple bark tissue during spring and observed that plant cold hardiness fluctuated day to day by dehardening and rehardening. These short term changes in cold resistance were closely related to the air temperature of the preceding day.

Environmental temperature in Manhattan fluctuated greatly every day through January to February 1988 (Fig. 2). On the first sampling date (Jan.25), the minimum temperature of the preceding day was -6.7°C, and -14.4°C one day before the second sampling date (Jan.27). Temperature continuously decreased from the first to the second sampling date (Fig.2). During this period, the blackberry plants may have undergone the process of rehardening, based on the significantly decreased percentage electrolytes obtained on the second date (Fig. 3). Before the third sampling date,

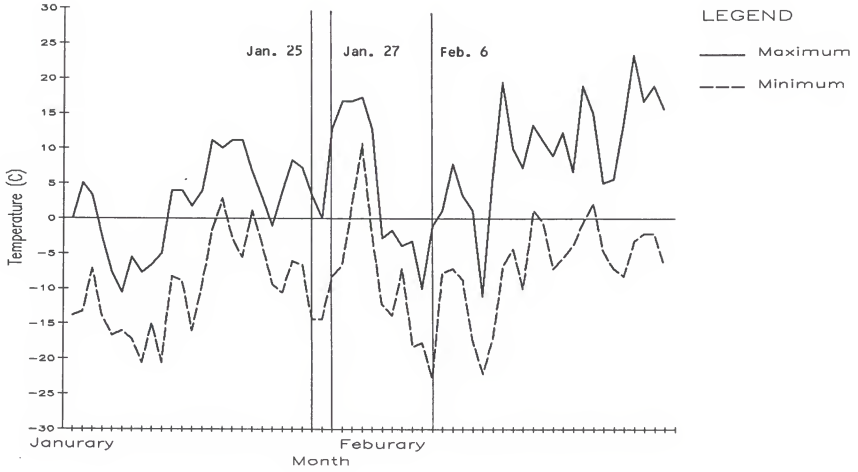

Fig. 2. Daily maximum and minimum temperatures in Manhattan, Kansas during January and February 1988.

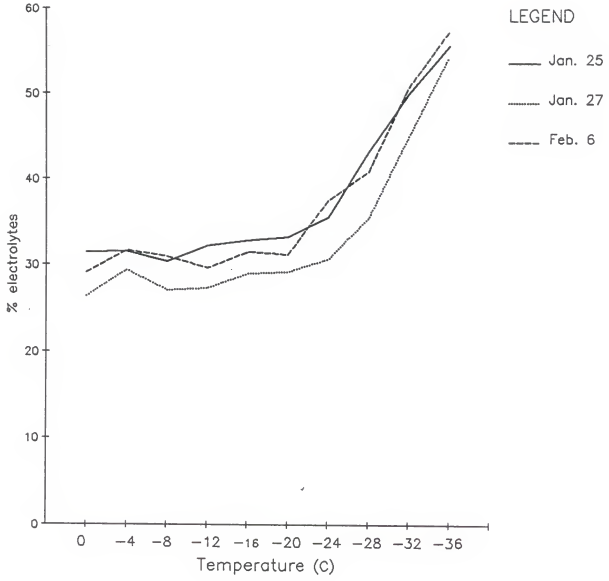

Fig. 3. Percentage electrolytes extracted from freeze-treated blackberry cane sections on three sampling dates.

air temperature began to increase after Jan. 27 and reached a maximum temperature of 17.2° and minimum temperature of 10.6°C on Jan. 30; then decreased again to -17.8°C on Feb. 5. During this period, blackberry plants may have been dehardening and had not fully rehardened by the third sampling date (Feb. 6). Dehardening may explain the significantly higher percentage electrolytes from cane samples from Feb. 6 compared to Jan. 27. Statistical analysis indicated that the mean percentage electrolytes extracted from the cane sections of the 7 blackberry cultivars on January 27 was significantly lower from that on January 25 or February 6 (not shown).

Analysis of variance showed there was a significant variation in percentage electrolytes from cane sections of 7 blackberry cultivars (Table 3). Mean separation by Duncan's multiple range test indicated that `Chester' and `Cheyenne' cane sections produced the lowest percentage electrolytes and therefore appear to be more hardy (Table 5). `Hull',`Navaho' and `Cherokee' comprised an intermediate hardiness group, while `Darrow' and `Shawnee' were in the less hardy group.

`Darrow' at one time was considered to be among the more hardy blackberry cultivars, as reported by Moore and Brown (25). Blackberry cultivars used in their study were totally different with ours except cultivar `Darrow'. The results from our study, however, showed that percentage

electrolytes of `Darrow' cane sections was greater than that of other cultivars, except `Shawnee', at each test temperature, indicating that more injury occurred in the cane tissues of `Darrow' during the freezing tests (Table 4). This suggests that `Darrow' lost its hardiness earlier than other cultivars after mid-winter. Because there was not

Table 5. Mean comparison of percentage electrolytes of 7 blackberry cultivars from 3 sampling dates at -28°C.

Cultivar	Mean percentage electrolytes[z]
Chester	34.4 a
Cheyenne	36.8 ab
Hull	39.1 bc
Navaho	39.7 cd
Cherokee	39.8 cd
Darrow	42.2 d
Shawnee	42.3 d

[z]Means followed by different letter or letters are significantly different at 5% level.

snow cover throughout the entire 1987-1988 winter season in Kansas, blackberry hardiness was likely affected by the fluctuation of environmental temperature. `Chester' seems

better adapted to the Kansas environment than `Darrow' based on hardiness evaluation by the conductivity test.

Analysis of variance (Table 3) also showed a highly significant variation in cultivar x temperature and cultivar x sampling date interactions. The presence of a cultivar x temperature interaction is expected because the 7 blackberry cultivars varied in hardiness and should have different responses at different temperature treatments. The highly significant cultivar x sampling date interaction indicates that the cultivars responded differently on different sampling dates, resulting in different hardiness rankings. This may reflect differing dehardening and rehardening patterns among the cultivars. Results from a single sampling date, therefore, can be misleading and freezing tests should be conducted over several sampling dates to provide a better overall evaluation of cold hardiness.

B. Tissue browning test.

Blackberry cane samples were rated for tissue injury after they had been incubated at 100% humidity for 10 days following low temperature treatment. Tissue browning ratings for each cultivar at different test temperatures are shown in Table 6.

Analysis of variance indicated a highly significant difference in tissue browning ratings among temperature

treatments (Table 7). Tissue browning followed the same pattern as the conductivity test (Fig. 1); no difference from -4° to -20°C and sharp increase begining at -24°C, when signs of injury first appeared in `Shawnee', `Navaho',

Table 6. Mean tissue browning ratings of cane sections of 7 blackberry cultivars from 3 sampling dates subjected to freezing temperatures.[z]

	Temperature (°C)								
Cultivar	-4	-8	-12	-16	-20	-24	-28	-32	-36
Chester	1.0	1.0	1.0	1.0	1.0	1.0	1.1	2.6	3.0
Cheyenne	1.0	1.0	1.0	1.0	1.0	1.0	1.3	2.8	3.0
Cherokee	1.0	1.0	1.0	1.0	1.0	1.0	1.6	2.8	3.0
Shawnee	1.0	1.0	1.0	1.0	1.0	1.3	1.8	2.9	3.0
Navaho	1.0	1.0	1.0	1.0	1.0	1.1	2.0	2.9	3.0
Darrow	1.0	1.0	1.0	1.0	1.0	1.6	2.0	2.6	3.0
Hull	1.0	1.0	1.0	1.0	1.0	1.3	2.3	3.0	3.0

[z]Means of 8 cane sections per cultivar at each temperature. 1 = no browning, 2 = moderate browning, 3 = severe browning and dead.

Table 7. Analysis of variance of tissue browning ratings of freeze-treated cane sections of 7 blackberry cultivars over 3 sampling dates.

Source	df	M S	F value
Temperature	9	32.847	792.85**
Sampling date	2	.690	16.66**
T x S[z]	18	.248	5.99**
Cultivar	6	.316	7.64**
C x T	54	.181	4.36**
C x S	12	.443	10.70**
C x T x S	108	.217	5.24**
Error	350	.041	

**Significant at 1% level.
[z]T = temperature, S = sampling date, C = cultivar.

`Darrow' and `Hull'. Severe injury and death occurred at -32°C and all samples were killed at -36°C. Therefore, the critical temperature range for the 7 blackberry cultivars was between -24° and -32°C, and -28°C was chosen as the critical temperature for cultivar comparisons. Separation of cultivars was poorer at -32°C, probably due to the more severe injury and death that occurred at this temperature, which may have masked differences in hardiness among cultivars.

A highly significant difference in tissue browning due to sampling date indicates that blackberry cane sections varied in tissue injury, probably due to varying degrees of dehardening and rehardening over the time period of testing (Table 7). Tissue browning ratings indicated that cane samples collected on Jan. 27 were more hardy than samples collected on Jan. 25 and Feb. 6 (Fig. 4.). Tissue browning ratings of all 3 sampling dates were significantly different from each other. Fluctuation of environmental temperatures was the probable cause of significant changes in the hardiness of cultivars over time.

Significant variability in cane tissue browning of the 7 blackberry cultivars was evident from the freezing treatments (Table 7). Mean separation placed `Chester' and `Cheyenne' in the more hardy group, `Hull', `Darrow' and `Navaho' in the less hardy group, and `Cherokee' and `Shawnee' in an intermediate group (Table 8).

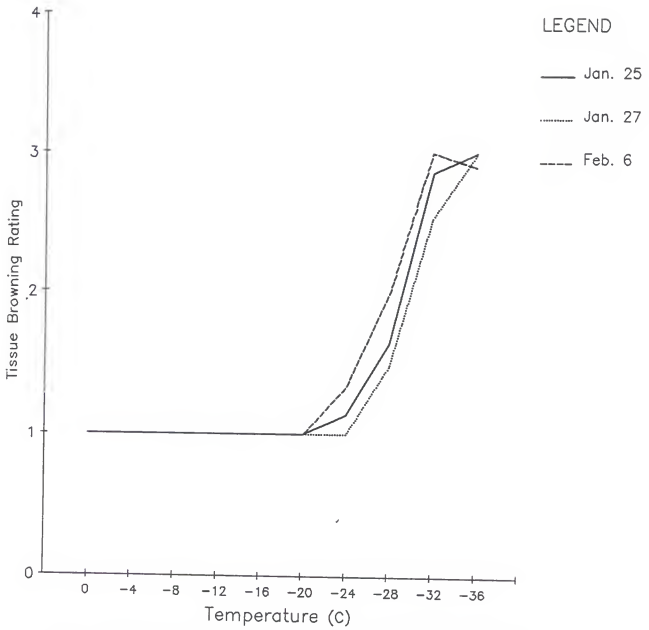

Fig. 4. Mean tissue browning ratings of blackberry cultivar cane sections from 3 sampling dates.

Table 8. Mean comparison of tissue browning ratings of 7 blackberry cultivars from 3 sampling dates at -28°C.

Cultivar	Mean tissue browning rating[z]
Chester	1.1 a
Cheyenne	1.3 ab
Cherokee	1.6 bc
Shawnee	1.8 cd
Navaho	2.0 de
Darrow	2.0 de
Hull	2.3 e

[z]Means followed by different letter or letters are significantly different at 5% level. Means of injury ratings for 8 cane sections.

Mean squares for cultivar x temperature and cultivar x sampling date interactions were highly significant (Table 7). Response of cultivars was different at different temperature treatments (Table 6). Significant cultivar x sampling date interaction indicates that tissue injury of blackberry cultivars varied on different sampling dates. Therefore, blackberry samples should be collected on several dates in order to get a better evaluation of plant performance and obtain a correct ranking in hardiness among cultivars over a time period.

C. Bud viability test.

Bud viability was evaluated on the same cane samples used for the tissue browning test. Each cane section contained at least one bud.

Analysis of variance indicated a highly significant variation in bud viability due to the temperature treatments (Table 9). Temperatures between -4^{o} and $-16^{o}C$ had no significant effect on the buds of all 7 cultivars (Table 10). Bud injury first appeared at $-20^{o}C$ in `Hull' and `Darrow'. All buds of the 7 cultivars were killed at -32^{o} and $-36^{o}C$. Therefore, the critical temperature range for the testing of bud hardiness was from -20^{o} to $-28^{o}C$.

The presence of highly significant variation in sampling date indicates that bud hardiness varied over the sampling times. These results correspond to our tissue browning and conductivity data.

Although the mean square for cultivar effect is highly significant (Table 9), only two groups were separated by the freezing test. `Cheyenne', `Chester' and `Shawnee' were in the more hardy group, the remaining cultivars were in the less hardy group (Table 11).

Warmund et al. (37) reported that `Darrow' had the greatest bud survival compared with that of other cultivars such as `Cheyenne' and `Shawnee'. This is quite different from our results that indicated `Darrow' was in the least

Table 9. Analysis of variance of bud viability ratings of freeze-treated cane sections of 7 blackberry cultivars over 3 sampling dates.

Source	df	M S	F value
Temperature	9	9.980	551.55**
Sampling date	2	.248	13.76**
T x S[z]	18	.157	8.66**
Cultivar	6	.239	12.66**
C x T	54	.107	5.92**
C x S	12	.115	6.35**
C x T x S	108	.075	4.03**
Error	350	.018	

**Significant at 1% level.
[z]T = temperature, S = sampling date, C = cultivar.

Table 10. Mean bud injury ratings of cane sections of 7 blackberry cultivars from 3 sampling dates subjected to freezing temperatures.[z]

Cultivar	Temperature (°C)								
	-4	-8	-12	-16	-20	-24	-28	-32	-36
Cheyenne	1.0	1.0	1.0	1.0	1.0	1.0	1.4	2.0	2.0
Chester	1.0	1.0	1.0	1.0	1.0	1.3	1.4	2.0	2.0
Shawnee	1.0	1.0	1.0	1.0	1.0	1.3	1.5	2.0	2.0
Cherokee	1.0	1.0	1.0	1.0	1.0	1.3	1.9	2.0	2.0
Navaho	1.0	1.0	1.0	1.0	1.0	1.4	2.0	2.0	2.0
Hull	1.0	1.0	1.0	1.0	1.3	1.3	2.0	2.0	2.0
Darrow	1.0	1.0	1.0	1.0	1.1	1.6	2.0	2.0	2.0

[z]Eight cane sections per cultivar were examined at each temperature. Rating system: 1 = alive, 2 = dead.

Table 11. Mean comparison of bud viability ratings of 7 blackberry cultivars from 3 sampling dates at -28°C.

Cultivar	Mean bud viability rating[z]
Cheyenne	1.4 a
Chester	1.4 a
Shawnee	1.5 a
Cherokee	1.9 b
Navaho	2.0 b
Hull	2.0 b
Darrow	2.0 b

[z]Means followed by different letter are significantly different at 5% level. Means of 8 buds per cultivar. 1 = alive, 2 = dead.

hardy group in bud hardiness ranking, while `Cheyenne' and `Shawnee' were in the more hardy group. Procedures used in their study may have contributed to the discrepency with our results, they collected blackberry cane samples on only one date. In addition, the samples were stored at -7°C for 24 days prior to freezing treatments and this might have changed hardiness ranking of cultivars. From the analysis of our results, particularly the significant effect of sampling date, it is not surprising that blackberry samples collected from different areas and seasons should produce different results, especially for a quantitative trait such as cold

hardiness. Therefore, blackberry cultivars should be evaluated with replicated tests for hardiness in the environment where they will be grown.

Analysis of variance also showed a highly significant difference in cultivar x temperature and cultivar x sampling date interactions. All of these interactions indicate that plant cold hardiness was not only affected by the individual factors studied, but also by mutual effects between these factors. In order to get more accurate cold hardiness evaluations, increasing the number of sampling dates and improving evaluation techniques should be emphasized.

D. Comparison between different methods.

All three cold hardiness evaluation techniques separated more hardy and less hardy types of blackberry cultivars during late winter and early spring. Electrolyte leakage and tissue injury ratings corresponded well, both exhibited sharp changes begining at $-24^{o}C$ (Fig. 1). Correlation analysis indicated a high degree of correlation between the 3 evaluation methods (Table 12), although there were some difference in cultivar rankings among the three test results. For example, `Hull' belongs to the more hardy group as the result of the conductivity test, but it was the least hardy in the tissue browning and bud viability tests (Table 13). `Shawnee' was the least hardy in the conductivity test, moderately hardy in the tissue browning

test, and in the most hardy group by the bud viability test. Complete agreement among the 3 tests should not be expected since they are not evaluating the same tissues in the case of bud viability and tissue browning. Generally, the tests performed well, consistently identifying `Chester' and `Cheyenne' as the most hardy cultivars.

Table 12. Correlation coefficients between 3 cold hardiness evaluation methods and test temperatures.[z]

	Conductivity	Tissue browning	Bud viability
Temperature	.74	.73	.78
Conductivity	---	.85	.86
Tissue browning	---	---	.79

[z]All correlation coefficients are significant at 1% level.

The conductivity test provides quicker results than the other two methods, usually requiring only 3 days of work. However, this method does not measure plant injury or death directly. If this method is accompanied by the analysis of tissue browning, the results will be more accurate. Tissue

Table 13. Comparison of 7 blackberry cultivars by 3 different cold hardiness evaluation methods.

Evaluation method [z]					
Conductivity		Tissue Browning[y]		Bud Viability[x]	
Cultivar	Percentage electrolytes	Cultivar	Mean rating	Cultivar	Mean rating
Chester	34.4 a	Chester	1.1 a	Chester	1.4 a
Cheyenne	36.8 ab	Cheyenne	1.3 ab	Cheyenne	1.4 a
Hull	39.1 bc	Cherokee	1.6 bc	Shawnee	1.5 a
Navaho	39.7 cd	Shawnee	1.8 cd	Cherokee	1.9 b
Cherokee	39.8 cd	Navaho	2.0 de	Navaho	2.0 b
Darrow	42.2 d	Darrow	2.0 de	Hull	2.0 b
Shawnee	42.3 d	Hull	2.0 e	Darrow	2.0 b

[z]Means followed with different letter or letters within the same column are significant at 5% level.
[y]1 = no browning, 2 = moderate browning, 3 = severe browning dead.
[x]1 = alive, 2 = dead.

browning and bud viability tests take more time to obtain results, usually about 10 days after the freezing test. Evaluation of cane injury and death, however, is relatively easy and quick following the incubation period, compared to the laborious procedures of the conductivity test. If time is a factor of concern for hardiness evaluation, the conductivity method would be preferred over the other two methods. The bud viability test showed less varistion in hardiness than the other methods, so it may not be selective enough to be useful.

Conclusion

Blackberry seedling tests indicated that one week of acclimation at 4°C was sufficient for six-week old greenhouse-grown seedlings to survive a freezing temperature of -6°C. The results were similar to those reported by Fear et al. (8) in strawberry seedling freezing tests. Blackberry seedlings acclimated for 3 weeks were less hardy than those receiving 1 or 2 weeks of acclimation treatment. This result suggests that extended artificial cold acclimation may have an adverse effect on blackberry seedlings. The critical temperature range for the testing of blackberry seedlings was between -6° and -9°C, suggesting that future blackberry seedling tests for cold hardiness can be conducted in this range.

The question remaining is: are the results of the seedling recovery test accurate? Roots of container-grown seedlings occupy only a small amount of soil which can not isolate temperature effctively. Therefore, seedling injury in our freezing tests may actually have been due to root damage from low temperature, resulting in inaccurate cold hardiness evaluations. This problem requires further investigation, possibly utilizing evaluation methods such as conductivity and tissue browning.

In freezing tests of mature, field-grown blackberry cultivars, results from the conductivity and tissue browning

methods were well correlated. Both tests consistently identified `Chester' and `Cheyenne' as the more hardy cultivars. Therefore, either method could be used effectively for evaluation of blackberry cold hardiness. The conductivity method would be preferred if results are needed quickly. However, if conductivity and tissue browning are used together, it is likely that more accurate results can be obtained. The bud viability test indicated that blackberry buds had less variation in cold hardiness among cultivars. Therefore, this method is not recommended for the comparison of cold hardiness between blackberry cultivars.

Analyses of variance of data from the three evaluation methods showed highly significant effects of temperature treatment, sampling date, and cultivar, as well as interactions between thses factors. This indicates that plant cold hardiness is a very complicated trait and is easily affected by environmental factors. Different cultivars responded differently to those factors. Therefore, blackberry cultivars grown in different regions and environmental conditions should be evaluated with replicated tests for cold hardiness under local conditions. In order to get more accurate results, sampling over a period of time is required.

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INVESTIGATIONS OF COLD HARDINESS OF BLACKBERRY SEEDLINGS AND METHODS FOR EVALUATING HARDINESS OF MATURE BLACKBERRY CANES

by

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AN ABSTRACT OF A THESIS

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ABSTRACT

Six-week old seedlings from open-pollinated seeds of the blackberry cultivar `Shawnee' were treated for 1, 2 and 3 week periods of artificial cold acclimation. Freezing tests demonstrated that seedlings receiving 1 or 2 week acclimation treatments were more hardy at colder temperatures than seedlings receiving the 3 week acclimation treatment. Therefore, a one week period was sufficient for seedling acclimation. The results indicate that the temperature between -6^{o} and -9^{o}C can be used as the critical temperature range to test blackberry seedlings for cold hardiness.

In mature plant freezing tests, cane sections of 7 blackberry cultivars were collected during late winter and early spring and subjected to controlled freezing treatments. Three methods of evaluating cold hardiness were compared; conductivity, tissue browning, and bud viability. The combined results of 3 sampling dates indicated that `Chester' and `Cheyenne' were consistently more hardy than the other cultivars by all three evaluation methods. The three methods were well correlated with each other in this study. Multiple variance analysis showed significant differences among main factors such as cultivars, temperature treatments, sampling dates and interactions between individual factors. This indicates that plant hardiness is not a static trait and is easily affected by these factors.