THESIS

COLD HARDINESS AND CRYOPRESERVATION OF SMALL FRUITS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY MIN-TZE WU ENTITLED COLD HARDINESS AND CRYOPRESERVATION OF SMALL FRUITS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS OF THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

COLD HARDINESS AND CRYOPRESERVATION OF SMALL FRUITS

This study examined cold hardiness and the occurrence of deep supercooling in stem with an attached bud of 4 cv. of grape and 4 of raspberry. Cryoprotectants were also tested for their influence on cold hardiness of raspberry vegetative bud and stem.

Differential thermal analysis (DTA) of 4 cv. of grapes, *Vitis* species hybrids, 'Concord', 'Beta', 'Valient', and 'Rougeon' showed that the stem and bud pieces of all cv. deep supercool in winter. They all showed a bud exotherm at approximately -25 to -30°C, and a stem tissue exotherm at approximately -40°C. The temperature at which the bud exotherm occurs may be the killing point of the bud which in turn could lead to the death of the above-ground canes. Evidence of this was the observation of the death of 'Rougeon' buds, bud exotherm observed at approximately -25°C, during the winter of 1980-1981 when the lowest temperature was -25.6°C.

DTA profiles of stem with attached bud samples of the 4 raspberry cv., 'Heritage', *Rubus idaeus* L., 'Black Hawk', *R. occidentalis* L., 'Amethyst', *R. neglectus* Peck, and 'Darrow', *R. allegheniensis* Porter, suggest that these do not deep supercool during winter. The mechanism of freezing resistance of these may be tolerance of extracellular freezing. Survival, indicated by sprouting, on January 30, 1981 showed that the LT_{50} (lethal temperature for at least 50% bud

sprouting) was -55°C, -50°C, and -45°C for 'Black Hawk', 'Amethyst', and 'Heritage' respectively. This supports the idea that at least these 3 cv. resist freezing by tolerance of extracellular freezing. Therefore minimum temperature may not be the major limiting factor in their northernly distribution. In descending order the observed degree of hardiness was 'Black Hawk', 'Amethyst', and 'Heritage'.

Survival tests of 'Heritage' samples treated with cryoprotectants showed no benefit of cryoprotectants for enhancing the resistance of acclimated buds and stem to cold injury. However, there were some implications that cryoprotectants may enhance the resistance of nonacclimated samples to lower temperatures.

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INTRODUCTION TO CRYOPRESERVATION

INTRODUCTION

The loss of 15% of the corn crop due to the Southern Crop Leaf Blight in the U.S. in 1970 was a recent reminder of the narrow genetic base of our major crop plants (45). This blight was actually beneficial in that it focused national and international attention on this problem. This was the impetus for the formation of national and international germplasm boards to investigate germplasm preservation.

In order to broaden our genetic base as well as provide genes for future breeding it is necessary to collect and preserve germplasm. Seed propagated crops have been preserved for many years by means of low temperature and low moisture. However, vegetatively propagated tissues must generally be maintained in the field. Even seed stored by conventional means has its drawbacks (4, 6). Seed storage problems include loss of viability over a period of time which necessitates growing these seeds at intervals. During these grow-out periods the material is subject to genetic drifts with the possible loss of potentially valuable genes. Mutation may also occur during the storage of seeds or during the grow-out periods.

Studies with animals and recent studies with some plant tissues have shown that cryopreservation offers a means of preserving tissues in an unaltered state indefinitely (13). This cryptobiosis as described by Keilen (16) occurs when the state of an organism shows no visible signs of life and when metabolic activity becomes hardly measureable, or comes reversibly to a standstill.

Cryopreservation is accomplished at -196°C, the temperature of liquid nitrogen, or lower. Liquid nitrogen is most commonly employed because it: 1) has the low-temperature of -196°C; 2) has no reaction with plant materials; 3) is relatively safe; and 4) is relatively inexpensive.

Bajaj (4) and Frankel (9) as well as others proposed the development of a genetic bank system for plant breeders. The purpose of such a system would be to store plant genotpyes for the use of future breeders. Bajaj (4, 5) has particularly emphasized the use of shoot tip and tissue culture systems for the preservation of vegetatively propagated plant species.

The purpose of this work was to investigate the possibility of cryopreservation of *Rubus* vegetative buds. Cold hardiness and some pretreatments were examined for their influence on cryopreservation.

PREVIOUS WORK

Many plant parts have been tried in cryopreservation. These include seeds, shoots, apical meristem, cell and callus cultures, and pollen.

SEED

Lipman and Lewis, in 1934, were the first to report survival of seeds of higher plants in liquid nitrogen for 60 days (19). Lipman (18) later reported survival of seed held at near absolute zero for 44 hours. Lipman proposed that the seeds are capable of survival because water is bound so tightly that it does not freeze at ultralow temperatures.

Three factors have been found to influence seed survival in cryopreservation. These are moisture content, storage temperature, and oxygen pressure (25). Moisture content and storage temperature are considered of prime importance.

In cryopreservation, observation has shown that the more hydrated the material the more susceptible it is to injury during the cooling and rewarming process. Seeds can be separated into 2 groups based on their ability to survive drying without injury. Orthodox seeds may be dried to a relative low moisture content (4 to 7%) without injury. In cryopreserving these seeds, few moisture-associated problems during the freezing and thawing process have been observed (25). Recalcitrant seeds are more like animal systems in that they are normally damaged by desiccation (25). In any attempts to freeze them to

ultralow temperatures they will experience injury during the freezing and thawing process. No information concerning liquid nitrogen storage of recalcitrant seeds is available (42).

SHOOTS

Conventional methods of maintaining asexually propagated plants have involved growth of wild species and cultivars in large nurseries. This requires extensive space and labor.

Recent work by Sakai et al. (33) indicates that freeze storage of shoots, especially in liquid nitrogen, may be a suitable method to maintain the variability of biological materials. Sakai and co-workers have successfully frozen and thawed shoots of some 20 trees and shrubs such as apple, pear, and numerous ornamental species (26, 27, 28, 29, 30). Although this saves considerable space and labor as compared to conventional methods it is somewhat more expensive of space compared to the use of apical meristems.

APICAL MERISTEM

The apical meristem region and/or shoot tips seem an ideal material for freeze preservation of vegetatively propagated plants (4). This is because they are genetically stable, rather easy to grow on simple media, and may be pathogen-free. Their small, highly cytoplasmic, and thin-walled cells lend themselves to freeze preservation.

Sun (44) showed evidence of the value of apical meristems for survival in liquid nitrogen with pea seedlings. He observed survival of meristems in seedlings, previously dried to 72 to 77.1% their weight, immersed in liquid nitrogen. Small seedlings showed survival of the entire seedling, while intermediate and large seedlings showed only meristem survival. This suggests that the absence of large vacuoles and the consequently denser protoplasm in the small cells of the root and stem meristems are related to the capacity of young pea seedlings to survive desiccation and freezing.

Survival to -196°C of meristem tips of potato (3, 10), tomato (11), pea (12, 14), strawberry (15, 37), and carnation (39, 40, 41) has been observed. Survival rates were generally low, 20 to 30%, with the exception of 55% in strawberry (15), 60% in pea (14), 80% in carnation (41) and strawberry (37). Therefore, it seems reasonable that further work with other species will give similar high survival rates.

CELL AND CALLUS CULTURES

Cell cultures are usually maintained by methods involving repeated subculturing. This continuous subculturing poses potentially serious problems such as contamination, chromosomal aberrations, mutations, and ploidy changes. Therefore, a method which allows for their long-term preservation under conditions that would prevent or minimize such problems would be valuable.

Quatrano (24) was the first to succeed in maintaining viability of flax and *Haplopappus gracilis* cell cultures after treatment at a subfreezing temperature of -50°C in the presence of dimethylsulfoxide (DMSO). Latta (17) reported that *Daucus carrota* cells grown in a conventional medium containing 20 g/l of sucrose survived freezing and a short period of storage at -196°C in the presence of a suitable protective agent (10% DMSO/5% glycerol). *Ipomoea* sp. cell cultures, however, survived freezing only if they were adapted to growth in a medium containing 65 g/l of sucrose followed by treatment with a protective agent.

Nag and Street (20) demonstrated that the morphogenic potential of carrot cells was unimpaired by liquid nitrogen freezing. Dougall and Wetherall (7) reported that actively dividing cells from 22 lines of wild carrot maintained in suspension culture have been successfully frozen and stored in the vapor of liquid nitrogen for 7 to 12 months. Sugawara and Sakai (43) also successfully immersed sycamore suspension cells in liquid nitrogen. Prior to immersion in liquid nitrogen, the cells were prefrozen to a temperature of -30 to -50°C in the presence of DMSO and glucose. Nag and Street (22) later ascertained that carrot cultures which had been stored for 10 months at -196°C showed no change in cell morphology and growth potential in culture and retained embryogenic potential. Cell and callus cultures of several other species have also survived liquid nitrogen freezing (5). Thus, it may be possible to establish cell and tissue banks of particular plant genotypes using this approach.

Although cells recovered from freeze-preservation appear cytologically and physiologically intact according to the above investigators, few of these have been regenerated. Regeneration of plants from thawed cells has been reported only in tobacco and carrot callus (1). It is most important to regenerate whole plants from cryopreserved cells for use in breeding programs.

POLLEN

Cryopreservation of pollen has less obvious benefits as a tool for long-term genetic preservation than do seeds or apical meristems. This is primarily due to complications involved in reconstituting the diploid material from which the pollen originated. Pollen could, however, provide the necessary gene source for crossing species in which maternal effects are of litte consequence. It would not be necessary to grow plants to maturity in order to obtain the needed pollen for crosses. This is an important consideration in the breeding of tree crops. There are serious storage problems that are similar to those for seeds, e.g. temperature, moisture content, and oxygen pressure (25). The pollen of many species are sensitive to even slight changes in moisture content.

In studies with corn and lily pollen, a lipid pigment has been implicated in resistance to freezing damage (23). Lipid pigment content and freezing resistance were inversely related.

FACTORS INFLUENCING CRYOPRESERVATION

MORPHOLOGICAL STAGE AND PHYSIOLOGICAL CONDITION OF PLANT MATERIALS

The absence of large vacuoles has been related to the capacity of cells to survive desiccation and freezing. Sun's work (44) indicated that, at least in pea seedlings, small cells containing highly dense protoplasm survived desiccation and freezing while highly vacuolated cells did not. Others working with tissue or cell cultures have reported that morphology and physiology of the cells, in particular cell age, size and degree of vacuolation clearly affect survival in the freezing and thawing treatments (1, 21, 41, 43, 46, 47). In carrot and sycamore tissue cultures, young suspensions in an exponential phase of growth withstood cold treatment much better than relatively old cultures (6, 43). Cells in the exponential phase differ from the older cultures which are characterized by larger thick-walled cells, limited cytoplasm and larger vacuoles.

Results with somatic embryos and pollen embryos at various stages of development have been similar (2). The young globular embryos showed higher survival when compared with the heart-shaped and fully differentiated embryo. This was also generally related to smaller thin-walled cells, highly dense cytoplasm, and smaller vacuoles.

The physiological aspects, especially as related to seasonal changes, have been implicated in differential survival of plant tissues exposed to extreme temperatures. Increases in the level of protein, sugars, and polyhydric alcohols which may be related to increased resistance of plants to extreme temperatures have been

reported (32). The effectiveness of pretreatments such as the application of cryoprotectants, prefreezing temperature, and even the cooling and thawing rate used, depends on the degree of cold hardiness of the plant materials. Sakai (30) reported that, in mid-winter, prefreezing temperature below -15°C enabled willow twigs to withstand immersion in liquid nitrogen. However, in mid-November, prefreezing temperatures below -20°C were reuqired.

The density of cell suspensions has been indicated as an important criteria for cell survival when frozen at liquid nitrogen temperatures (1, 6). Packed suspensions invariably showed higher rates of survival than ones with lower cell density. Filtered suspensions of single cells only showed low survival rates while actively growing suspensions containing clumps in colonies of small highly cytoplasmic cells showed survival to 70% in carrot (6).

CRYOPROTECTANT

Cryoprotectants have been shown in many experiments to impart resistance to cold treatments and even survival in liquid nitrogen (4, 5, 6). The most common cryoprotectants used are glycerol, dimethylsulfoxide (DMSO), and various sugars.

A cryoprotectant, to be effective, must be easily miscible with water, easily washed away, capable of rapid infusion into the cells, and have low toxicity. Several studies have indicated that high concentrations of cryoprotectants cause toxicity and/or lethal plasmolysis (4). Therefore, it is generally adviseable to use lower

concentrations of cryoprotectants, in the range of 5 to 15% (4). There is some supporting evidence to show that a gradual increase in concentration permits the use of high concentrations of cryoprotectants with minimal injury. Since high concentrations of cryoprotectants have been reported to interfere with RNA and protein synthesis (2) it is recommended that two or more cryoprotectants (at lower concentrations of each) be used in order to avoid these toxic effects. *Ipomoea* cells could not be successfully revived after freezing with only 5% DMSO. However, a mixture of DMSO (2.5%), glycerol (2.5%), and sucrose (6.5%) enabled the cells to resume growth after thawing (20). Finkle et al. (8) reported that a combination of two or three cryoprotectants gave a higher percent survival than single cryoprotectants with sugarcane cells.

Many different types and concentrations of cryoprotectants have been tested by researchers (2, 7, 11, 15, 17, 20, 22, 32, 41, 43). DMSO has been reported to be excellent for preventing freezing injury in cell and tissue cultures (7, 15). Glycerol was less effective as a cryoprotectant, in particular in working with strawberry meristems (15). The reason may be that, although glycerol is of low molecular weight, it is much slower in its penetration of cells than is DMSO.

PREFREEZING TREATMENT

Prefreezing of certain plant tisuses prior to subsequent exposure to much lower temperature enables them to withstand immersion

in liquid nitrogen. Sakai (26), working with twigs of various species, has shown that they did not survive if immersed directly into liquid nitrogen for one hour without prefreezing. However, if the twigs were prefrozen below -20°C they survived immersion in liquid nitrogen for 20 days and subsequently produced normal buds. In studies with willow, he showed that a prefreezing temperature of -15°C was sufficient for the survival of twigs tested in mid-winter (29). However, a prefreezing temperature below -30°C was required for survival when testing early spring twigs in liquid nitrogen. In studies with apple shoots (37) he found that temperatures between -40 and -50°C were required for the prefreezing treatment in order to obtain survival in liquid nitrogen.

The implication here is that there is a definite temperature zone over which almost all of the freezeable water may be drawn from the cell by extracellular freezing (26, 27, 28, 30, 33). The cells in this condition are then not injured even when exposed to an extremely low temperature. This freeze dehydration also results in a notable increase in sugar concentration in the cells. This in turn may contribute to the decreased growth rate of intracellular crystals which are formed in the course of rapid cooling (32).

Sakai (34, 35, 43) noted that sufficiently dehydrated cells were not damaged by rapid immersion in liquid nitrogen even with subsequent slow rewarming. In partially dehydrated cells, the intracellular crystal nuclei formed in the course of rapid cooling in liquid nitrogen may be small enough to be innocuous. However, their size likely increases and causes damage during the subsequent slow

rewarming in air. Rapid rewarming on the other hand may not allow sufficient time for crystal nuclei to grow to a damaging size. If plant tissues can survive a prefreezing temperature lower than -30°C it is likely that they will be able to survive freezing at extremely low temperatures (28). Those plant tissues incapable of surviving to -30°C may, through the use of a cryoprotectant such as glycerol, be made to survive freezing at -30°C and thus survive in liquid nitrogen (28).

Effective prefreezing temperatures are variable according to the cold hardiness of the plant itself. The greater the cold hardiness of the plant, the more effective a higher prefreezing temperature will be (29, 30, 33).

COOLING RATE

The cooling rate of cells during freezing to -196°C has a major effect on their survival. Sakai and coworkers as well as others have taken a critical look at this phenomenon. Sakai et al. (36, 38) used cortical cells of mulberry in their studies of freezing rate. Slow freezing rates (1 to 15°C/min) resulted in survival regardless of thawing rates, but rates of 50 to 2,000°C/min resulted in decreased survival. At 2,000°C/min there was no survival; however, a very high cooling rate (10,000°C/min) resulted in an increase in viable cells.

Apparently at very high cooling rates, such as 10,000°C/min, any ice crystals that form within the cells may be small enough to be innocuous. After mulberry cells were rapidly cooled by direct

immersion into an isopentane bath below -60°C, Sakai et al. observed no ice crystal formation under a light microscope (34). At intermediate cooling rates, the ice crystals that form in most of the cells are large enough to be immediately damaging.

Nath and Anderson (23) recommended rapid cooling rates based on their study of lily and corn pollen frozen to -196°C. They observed greater germination in pollen frozen at rates above 150°C/min than in those frozen at 0.5° to 100°C/min. They proposed that small ice crystals formed at rapid freezing rates which caused less structural damage to the subcellular organelles and membrane systems in the pollen grain than slow freezing which resulted in larger ice crystals causing greater structural damage.

However, Grout (11) observed that vapor phase cooling at 20° to 55°C/min gave better survival than did 800°C/min cooling. Very slow cooling, 2°C/min, was totally ineffective in allowing survival of thawed plants. Nag and Street (20, 22) reported 65 to 68% survival of carrot cells in 5% DMSO using a cooling rate of 1.8°C/min during freezing to -196°C. This slow rate of cooling was considered essential for high survival. They reported optimum freezing rates from 10°C/min to 50°C/min in carrot, belladonna and sycamore suspensions. Bajaj (1), working with carrot, datura, tobacco, and soybean cells, and Kartha (15), working with strawberry apical meristems, reported greater survival at slow cooling rates of from 0.5° to 3.0°C/min. Rapid freezing by direct immersion into liquid nitrogen resulted in lower survival rates.

It seems apparent from the previous discussion that a very wide range of cooling rates may be necessary in order to successfully freeze different plant tissues. The optimum cooling rate for maximum survival is dependent upon the structure and physiology of cells concerned, the cryoprotectant applied, and the thawing rate used. Apparently different sized ice crystals form during the cooling phase depending on the cooling rate and this may affect the physical behavior and thus survival during the thawing phase of the tissues. An effective cryoprotectant may serve to decrease the growth rate of intracellular ice crystals thus negating the need for ultra-fast cooling rates. The observation that shoots and subsequently plants can be obtained from surviving shoot apices means that the morphogenic potential of plant tissues are not necessarily destroyed by the freezing process (39).

STORAGE TEMPERATURE

The storage temperature for long-term preservation of plant tissues must be low enough to stop metabolic activity and thus prevent biochemical injury. Nath and Anderson (23) reported that a significant loss of germinability was observed in lily and corn pollen when the storage temperature was above -50°C; best results were observed when temperatures were below -50°C.

REWARMING RATE

The influence of the rewarming rate on survival of plant tissues is significant. Early work with thin sections of cortical tissue of mulberry twigs showed survival differences with varying rewarming rates (26, 27, 31). Those cells which were rewarmed slowly showed low survival while those rewarmed rapidly in water at 30°C showed a high percentage of survival.

Tissue sections mounted between cover glass with water were studied to examine the influence of prefreezing treatments and subsequent rewarming on survival of the cells (27, 31, 36). In these studies the sections were prefrozen at temperatures low enough to dehydrate the tissues. With the freeezeable water removed the cells were able to survive immersion in liquid nitrogen. The survival was very little affected by the rates of cooling to and rewarming from ultralow temperatures (31, 36). However, the survival of the cells was influenced by the rate of rewarming when they were immediately frozen at ultralow temperatures. Slow rewarming in air destroyed all cells while rapid rewarming in water at 30°C did not affect them.

A series of experiments were conducted to determine the range of temperatures in which the greatest damage occurred when rewarming insufficiently prefrozen mulberry twigs (38). Sakai et al. froze tissues in liquid nitrogen and then transferred them to different isopentane baths ranging from -10° to -70°C. After holding at these temperatures for various times, tissues were rapidly rewarmed in a 30°C water bath. Observations indicated that the critical range in which cells were damaged was from -50° to -2.5°C for insufficiently

prefrozen cells. Thus it is most important to pass through this range of temperatures rapidly to prevent damage.

Fast rewarming at -30°C or -37°C led to greatest survival in cell suspensions of carrot (7, 20); carrot and belladonna (22); sycamore (43); pollen of lily and corn (23); and carnation shoot apices (40). Evidently slow thawing, particularly in insufficiently dehydrated tissues, allows growth of ice crystals. This leads to disruption of internal structures of the cells primarily through a physical effect.

OBJECTIVE OF THE STUDY

The objective of this study was to investigate cold hardiness and deep supercooling of buds of *Vitis* and *Rubus* and the influence of cold hardiness and pretreatments on cryostorage of vegetative buds of *Rubus*. Cold hardiness and deep supercooling were investigated at various times during the growing season and dormant period. Cryoprotectants were tested to determine if an increase in resistance to cold damage could be obtained.

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CHAPTER 2

COLD HARDINESS AND LOW TEMPERATURE EXOTHERMS IN STEM AND BUD TISSUES OF 4 *VITIS* CULTIVARS

ABSTRACT

Stem cuttings with attached buds of 'Concord', 'Beta', 'Valient', and 'Rougeon' were collected on 5 dates from October to April in Fort Collins. Differential thermal analysis (DTA) detected 3 exotherms in fully acclimated tissues. DTA of stem pieces only and of excised buds indicated that the first exotherm (at approximately -8°C) and third exotherm (at approximately -43°C) originated in the wood. The second exotherm (at approximately -28°C) originated in the bud. Shifts in the temperature of the second and third exotherm were noted as tissues acclimated and deacclimated.

Evidence suggests the temperature at which the bud exotherm occurs may be the killing point of the above ground canes of 'Rougeon'. Further research is necessary to determine whether desiccation on low temperatures (below the bud exotherm temperatures) cause the death of the tissues.

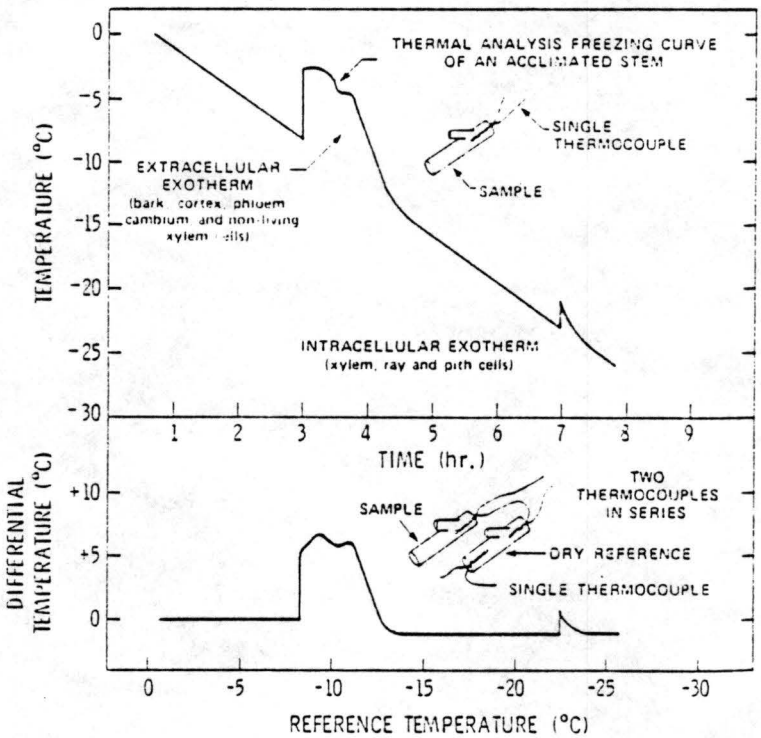
INTRODUCTION

Freezing avoidance by supercooling is one important mechanism of freeze resistance for many woody plants during midwinter (16). Due to the extensive work at the University of Minnesota and elsewhere, involving differential thermal analysis (DTA), supercooling has been shown to occur in stems (13, 18, 24, 25) and flower buds (2, 11, 12, 13, 20, 29) of many woody plants. However, little information concerning the supercooling of mixed or vegetative buds has appeared, except that of grape (18) and Pyrus (27).

The mechanism of deep supercooling is still not clear. There are generally two approaches used to explain the freezing phenomena exhibited in a thermal analysis profile (Figure 1). First, as temperature drops, ice forms first in the extracellular spaces and grows as water from inside the cells migrates to extracellular sites. This migration is in response to the vapor pressure gradient between the unfrozen solution inside and ice outside the cell. This extracellular freezing many explain the first exotherm in the DTA profile. As the plant tissues continue supercooling, the supercooled water may freeze spontaneously. This results in intracellular ice formation. This intracellular freezing may response with a sudden heat release, causing the second exotherm in the DTA profile, as shown in Figure 1.

In most woody plant stems which have been studied the deep supercooled fraction of tissue water has been indicated to occur in xylem and pith parenchyma (10, 25, 26). In flower buds the deep supercooled water is primarily in primordial tissue (9, 11, 12, 22). Therefore, the second explanation arises: As temperature drops, extracellular

Figure 1. Comparison of thermal analysis and differential thermal analysis (DTA). The peaks or exotherms indicate freezing points. (From Burke et al., 1976).


THERMAL ANALYSIS FREEZING CURVE
OF AN ACCLIMATED STEM
SINGLE
THERMOCOUPLE
SAMPLE
EXTRACELLULAR
EXOTHERM
(bark, cortex, phloem
cambium, and non-living
xylem cells)
INTRACELLULAR EXOTHERM
(xylem, ray and pith cells)
TEMPERATURE (°C)
0
-5
-10
-15
-20
-25
-30
1
2
3
4
5
6
7
8
9
TIME (hr.)
DIFFERENTIAL
TEMPERATURE (°C)
+10
+5
0
SAMPLE
TWO
THERMOCOUPLES
IN SERIES
DRY REFERENCE
SINGLE THERMOCOUPLE
0
-5
-10
-15
-20
-25
-30
REFERENCE TEMPERATURE (°C)

freezing occurs in the bark, cortex, phloem, and cambium in stems or the scales in flower buds. This may explain the first exotherm in Figure 1. These tissues are more hardy than the xylem and pith parenchyma tissue in stem or primordial tissue in flower buds. They can tolerate the extracellular freezing and do not deep supercool as freezing proceeds. The xylem and pith parenchyma in stem or primordial tissue in flower bud are generally the only part of the tissue water that deep supercools. The freezing of this deep supercooled tissue water results in intracellular freezing of these tissues and is responsible for the second exotherm in Figure 1.

A liquid is in a supercooled state when its temperature falls below its freezing point without crystallization (17). The degree of supercooling in water depends on droplet size in the absence of external nucleation. However, spontaneous nucleation invariably occurs when supercooled water is cooled below a critical temperature. Fine droplets of pure water in the absence of nucleating substances can be cooled to $-38.1^{\circ}C$ without ice formation (14). This temperature is the homogeneous nucleation temperature, i.e., the temperature at which spontaneous ice nucleation occurs in the absence of nucleating substances. The presence of solute in the tissue water can further depress this homogeneous nucleation temperature (10). The homogeneous nucleation temperature of deep supercooled tissue water is around $-40^{\circ}C$. However, the capacity of supercooling is not uniform in plants. For example, freezing temperature of deep supercooled xylem water is much higher in autumn and spring than in midwinter (25); a similar phenomena is also true for azalea flower buds (7).

So far, the lowest freezing point of supercooled tissue water reported is -47°C in xylem of *Fraxinus nigra* Marsh (10).

While the supercooled state is metastable, tissue water can persist as liquid for long periods at temperatures only slightly above the spontaneous nucleation point (8). It is thought that the sudden freezing of deep supercooled water results in intracellular ice formation that causes the death of the deep supercooled tissues (3). Supercooling therefore may limit tree species native to the Eastern Deciduous Forest of North America to latitudes where the minimum winter temperature does not drop below -40°C (10). Similar relationships of the low temperature exotherm to limits of commercial apple and pear production in North America have also been shown (21). The northern limits are the isotherms at approximately -34° to -40°C for apple and -29° to -34°C for pear (21). Becwar et al. (1) also found a similar relationship between tissue freezing pattern and plant distribution in mountainous flora of the Rocky Mountains. Woody species collected from the Rocky Mountains of Colorado which exhibited supercooling were no hardier than about -40°C. The climatological data from this region shows that average minimum temperatures at treeline coincided with the hardiness limit of these species. In contrast, some hardy woody species that do not supercool extend to regions where sub -40°C occurs (1, 10, 21). These species are found to be extremely hardy and survive very low temperatures by tolerating extracellular freezing.

This study was designed to examine the deep supercooling characteristic of four grape cultivars of diverse species background as related to acclimation.

MATERIALS AND METHODS

DTA

DTA was conducted on buds (attached to a stem segment) of 4 cultivars of grape (Table 1) harvested at intervals from early September to April. Samples of stem segments were from 1 year old wood and approximately 1 cm in length with 1 node per segment. They were split longitudinally and the half with the bud was used for DTA. Each sample was placed into aluminum foil with a thermocouple in direct contact with the bud; three samples from different stems were used for each cultivar. DTA was performed using a Cryomed (Model 1000) attached to a Data acquisition system (Model HP 3052A), run by a Hewlett-Packard desk computer (Model 9845A). Computer regulation of the cooling rate was at 0.3^{o}C/minute.

Table 1. Species origin of cultivars used in the study with years grown in field.

Cultivar	Years in Field at Fort Collins	Species Origin
'Rougeon' (31)	2	*Vitis vinifera* x Some American species
'Concord' (15)	3	Primarily *Vitis labrusca* X some *Vitis vinifera*
'Beta' (32)	3	'Concord' *Vitis labrusca* X *Vitis* (unknown species)
'Valient' (30)	1	*Vitis raparia* (Montana source) X 'Fredonia' *Vitis labrusca*

VIABILITY ASSAY

Determination of survival of freezing temperatures was based on measurements of electrolyte leakage, an approach commonly used by others (1, 27) Stems from the previous season's growth of *Vitis labrusca* Bailey cv. 'Concord' were collected from the field on April 3, 1981. Stems were immediately cut into 7 cm long segments, each containing a bud. These were wrapped in lots of 5 segments with cheesecloth which was then moistened with distilled water, wrapped in aluminum foil, and placed in weighed glass test tubes (3 x 20 cm).

Tubes were placed in a methanol bath at $0^{\circ}C$ and were cooled at $3.3^{\circ}C/hr$; at $-3^{\circ}C$ the samples were nucleated with ice pieces. As temperatures reached -15°, -25°, -35°, $-45^{\circ}C$, and $-55^{\circ}C$ samples were removed and thawed in a cold room at $5^{\circ}C$. The cheesecloth was removed from the samples 1 hour after removal from the methanol bath, the samples however were left in the test tubes at $5^{\circ}C$ for 5 days. After 5 days, the samples were removed and dissected into bud only, dissected stem, (half of the sample minus the bud), and dissected wood (the other half of the sample minus bud and bark). Each part was then placed into test tubes (2.5 x 15 cm) with 1 ml of distilled water and held in a $25^{\circ}C$ water bath for 24 hours. Electrolyte leakage (C_i) was measured. Each dissected part was then moved to a water bath at $80^{\circ}C$ and held for 30 minutes. Electrolyte leakage (C_t) was measured again. Electrolyte loss was determined by

$$\frac{C_t - C_i}{C_t} \times 100\%.$$

DTA was conducted to correlate survival with low temperature exotherm.

RELATIVE WATER CONTENT

Stems from the previous season's growth were collected from 'Concord' on April 8, 1981 and cut into 3 to 4 cm segments with 1 bud attached. Each stem segment was then divided into an excised bud, a 1 cm half section of stem and a corresponding 1 cm half section of stem minus bark, termed 'wood'. Buds were carefully excised at point of attachment to the stem. Stem and wood samples were taken from a segment 1 cm below the bud. Fresh weights (FW) of each were measured and the samples were then dried in an oven at 103°C for 24 hours. Dry weights (DW) were then measured and relative water content (RWC %) was computed. This was expressed as

$$\frac{FW - DW}{DW} \times 100\%.$$

RESULTS AND DISCUSSION

A summary of the observed DTA with replications for each of the 4 grape cultivars are presented in Table 2. Figures 2 through 5 show the change in the exotherms, particularly the bud and stem tissue exotherm, with seasonal changes.

In order to clearly determine the tissue origin of exotherms, an experiment involving the dissection of acclimated samples was

Table 2. Variation of first exotherms, bud exotherms and stem tissue exotherms in 4 cultivars of grape at different acclimation stages.

Cultivars	Sample Collection Date	Stage of Acclimation	Midpoint of First Exotherm	Midpoint of Bud Exotherm	Range of Stem Tissue Exotherm	
'Concord'	10/17	Acclimating	- 8.35 ± 0.49	-13.75 ± 0.21, -14.0, -18.1	[y]	
	11/28	Acclimated	- 9.7 ± 0.7	-25.17 ± 2.40	[y]	
	01/05	Acclimated	-10.53 ± 0.50	-29.1 ± 0.95, -29.2	[y]	
	02/13	Acclimated	- 9.6 ± 1.44	-28.8 ± 3.11	-38.57 ± 0.72	-45.63 ± 0.81
	04/09	Deacclimating	- 9.75 ± 1.63	-13.8 ± 0.28, -17.5 ± 0.99, -19.65 ± 0.07	-26.3 ± 1.56	-42.2 ± 0.57
'Valient'	10/17	Acclimating	- 7.53 ± 0.72	-14.33 ± 0.81	[y]	
	11/28	Acclimated	- 8.7 ± 1.13	-26.7 ± 1.27	[y]	
	01/05	Acclimated	-10.3 ± 0.0	-28.85 ± 0.35	[y]	
	02/13	Acclimated	- 8.8 ± 1.06	-31.13 ± 0.46	-40.17 ± 0.75	-47.17 ± 1.31
	04/09	Deacclimating	- 8.4 ± 0.85	-10.1 ± 1.27, -14.0 ± 0.28, -19.2 ± 1.70	-21.4 ± 0.0	-40.9 ± 1.27
'Beta'	10/17	Acclimating	- 7.23 ± 0.59	-11.8, -16.2 ± 0.26, -23.97 ± 0.21	[y]	
	11/28	Acclimated	- 9.17 ± 0.42	-28.0 ± 0.85	[y]	
	01/05	Acclimated	-10.73 ± 1.18	-20.27 ± 0.47	[y]	
	02/13	Acclimated	- 9.8 ± 0.92	-30.13 ± 1.10	-30.13 ± 0.45	-46.77 ± 0.70
	04/09	Deacclimating	- 8.2 ± 3.11	-9.2, -13.9 ± 0.14, -15.6	-21.8 ± 1.98	-42.25 ± 1.91
'Rougeon'	10/17	Acclimating	- 7.57 ± 0.67	-10.9 ± 1.84, -19.8 ± 1.13	[y]	
	11/28	Acclimated	-10.07 ± 2.71	-24.75 ± 0.07	[y]	
	01/05	Acclimated	- 9.2 ± 0.28	-25.85 ± 1.20	[y]	
	02/13	Acclimated	- 8.8 ± 0.0	[z]	-38.35 ± 0.83	-45.60 ± 0.56
	04/09	Deacclimating	[z]	[z]	[z]	

[y] Not determined.

[z] No exotherms observed possibly due to death of tissues.

Figure 2. Differential thermal profiles at different stages of acclimation of 'Concord' grape stem with bud attached. DTA profiles are plotted as the differential temperature between the sample and the reference on the ordinate vs. the reference temperature on the abscissa.

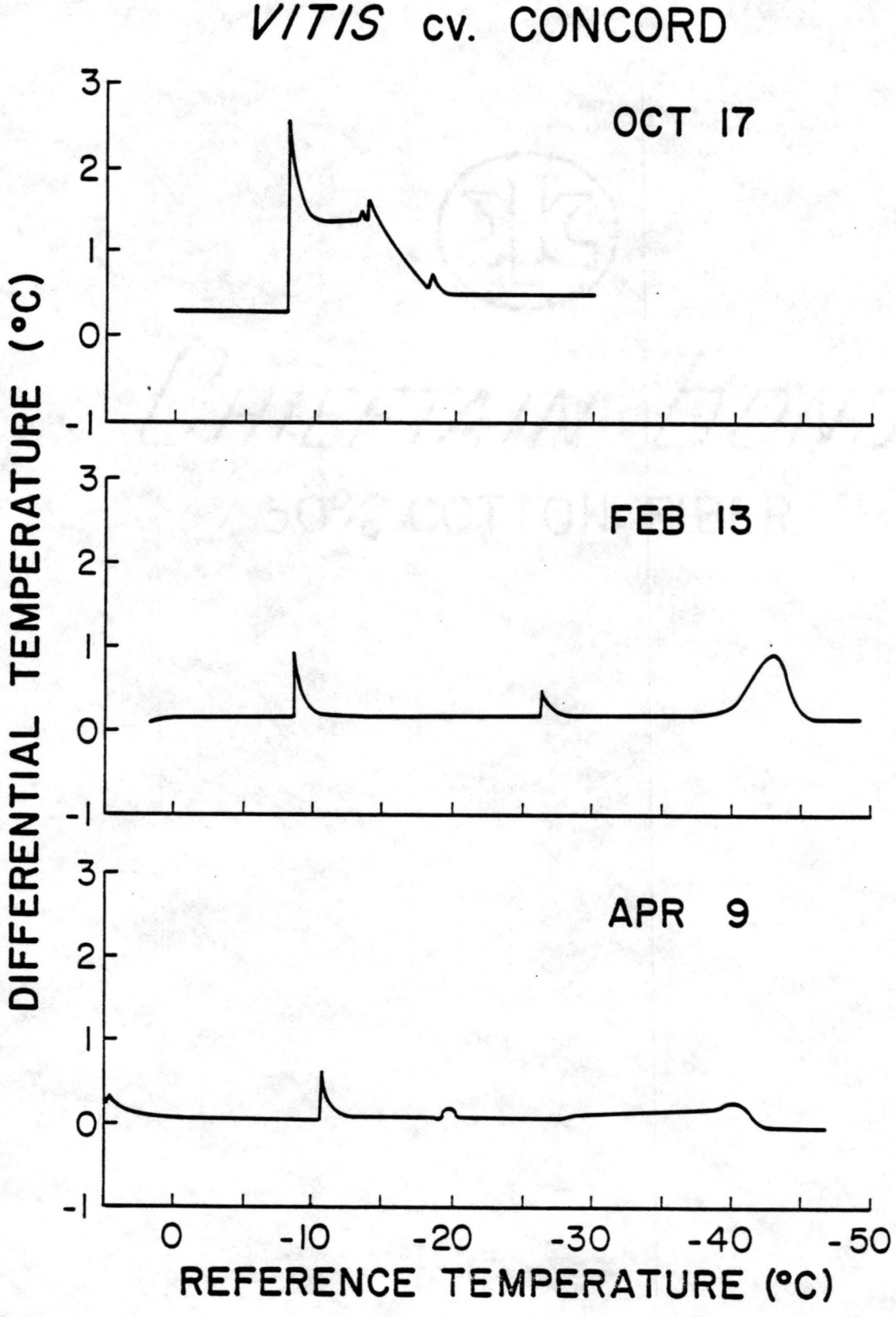

VITIS cv. CONCORD
OCT 17
FEB 13
APR 9
DIFFERENTIAL TEMPERATURE (°C)
3
2
1
0
-1
0
-10
-20
-30
-40
-50
REFERENCE TEMPERATURE (°C)

Figure 3. Differential thermal profiles at different stages of acclimation of 'Valient' grape stem with one bud attached. DTA profiles are plotted as the differential temperature between the sample and the reference on the ordinate vs. the reference temperature on the abscissa.

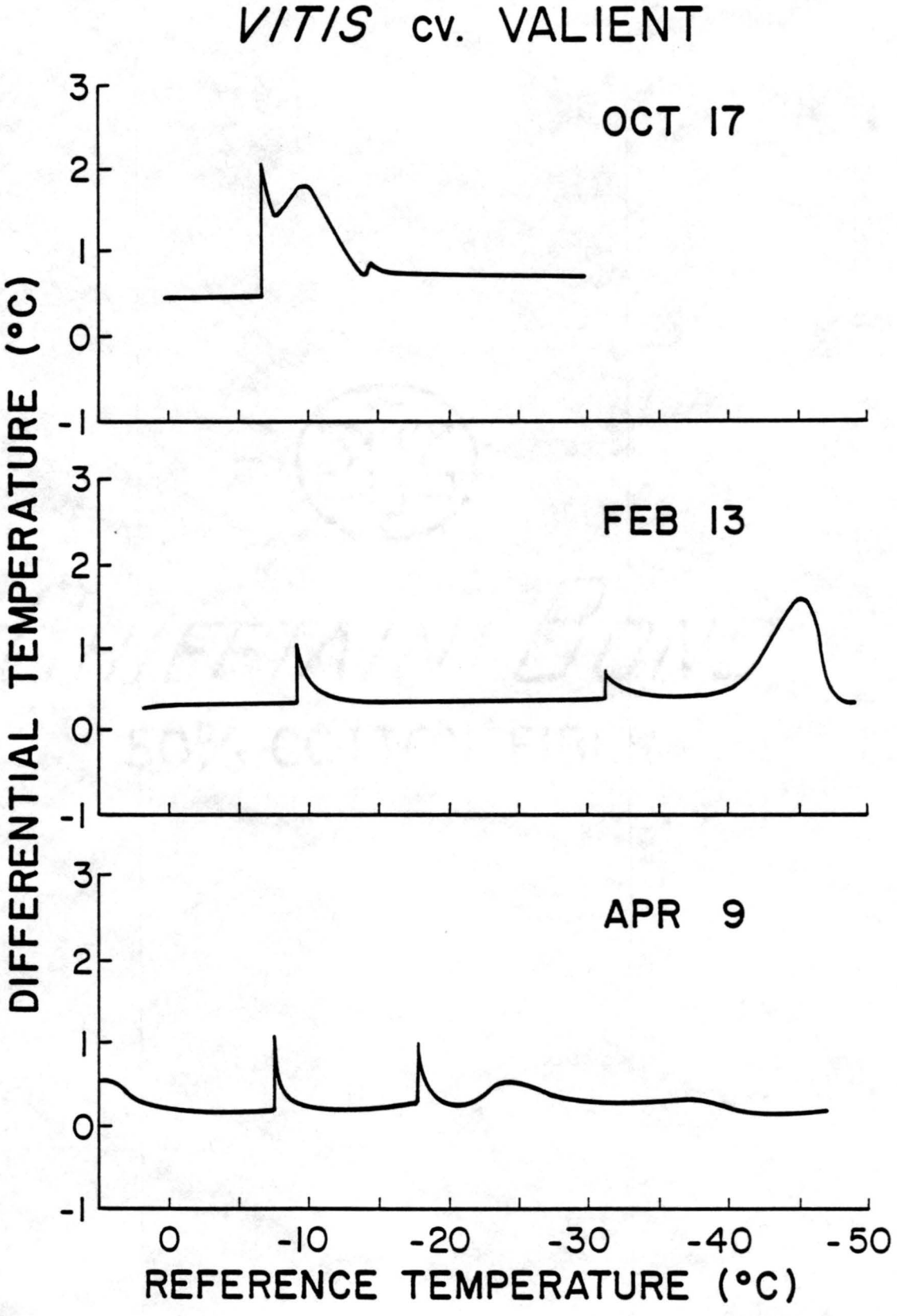

VITIS cv. VALIENT
OCT 17
FEB 13
APR 9
DIFFERENTIAL TEMPERATURE (°C)
3
2
1
0
-1
0
-10
-20
-30
-40
-50
REFERENCE TEMPERATURE (°C)

Figure 4. Differential thermal profiles at different stages of acclimation of 'Beta' grape stem with one bud attached. DTA profiles are plotted as the differential temperature between the sample and the reference on the ordinate vs. the reference temperature on the abscissa.

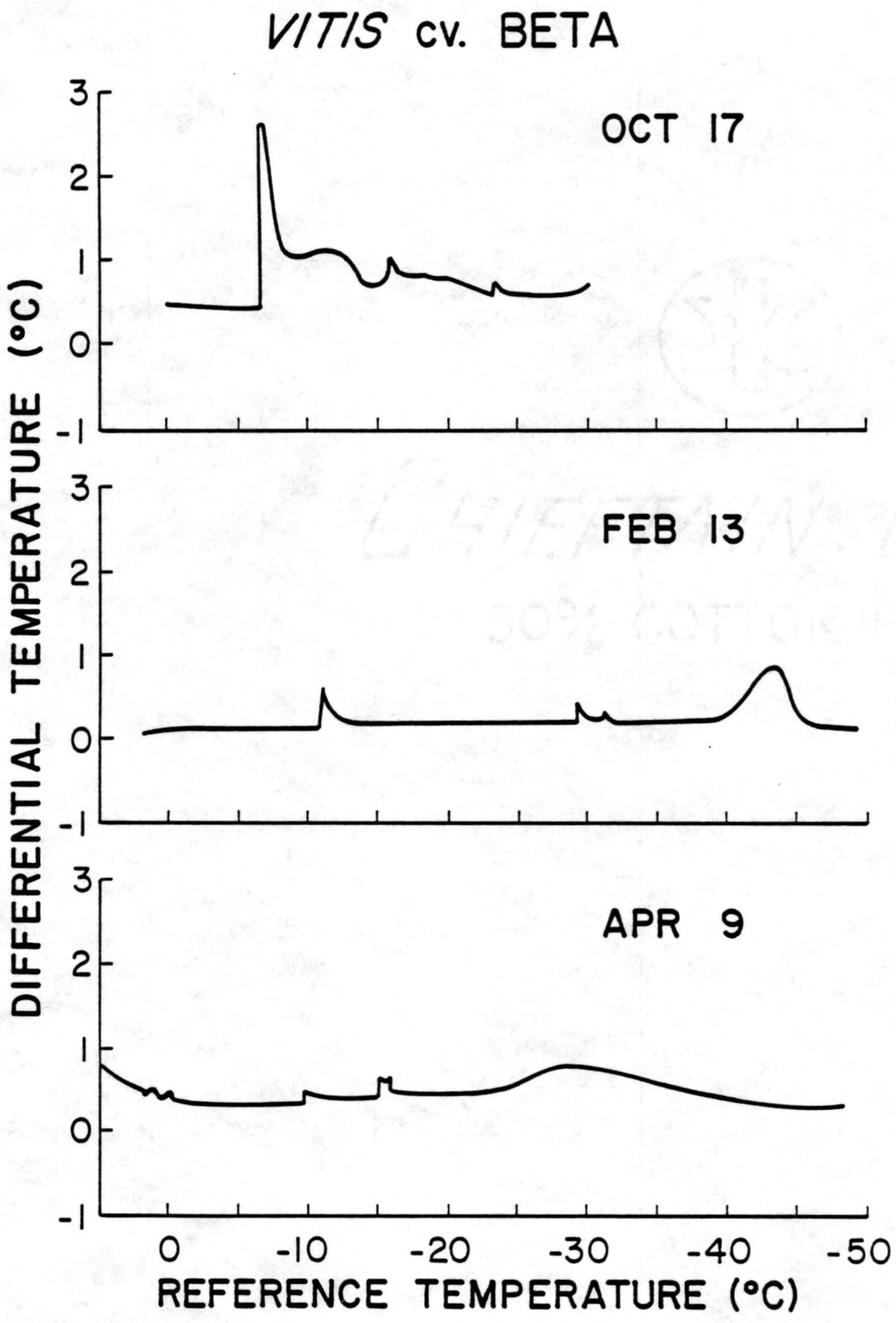
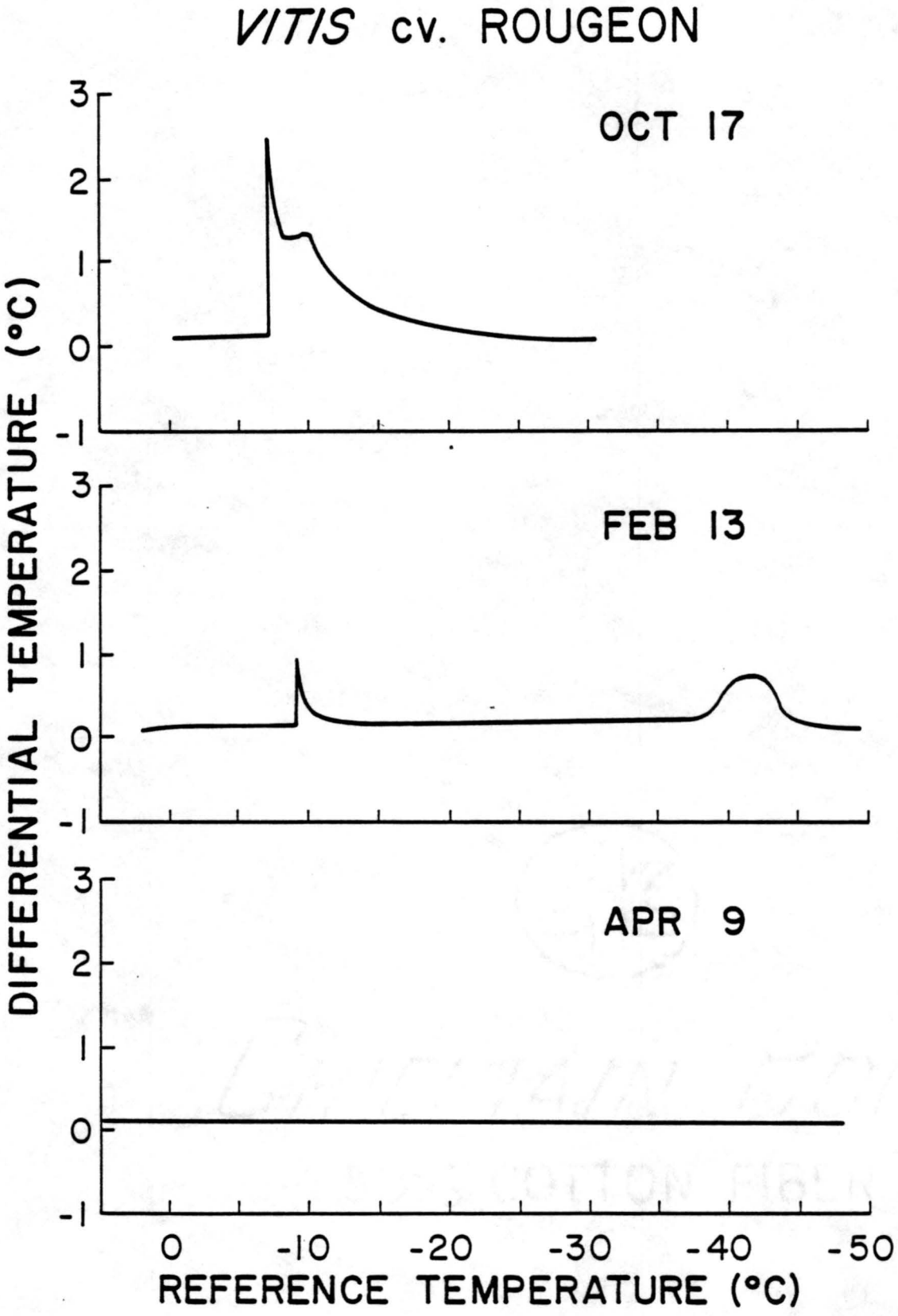

VITIS cv. BETA
OCT 17
FEB 13
APR 9
DIFFERENTIAL TEMPERATURE (°C)
3
2
1
0
-1
0
-10
-20
-30
-40
-50
REFERENCE TEMPERATURE (°C)

Figure 5. Differential thermal profiles at different stages of acclimation of 'Rougeon' grape stem with one bud attached. DTA profiles are plotted as the differential temperature between the sample and the reference on the ordinate vs. the reference temperature on the abscissa.

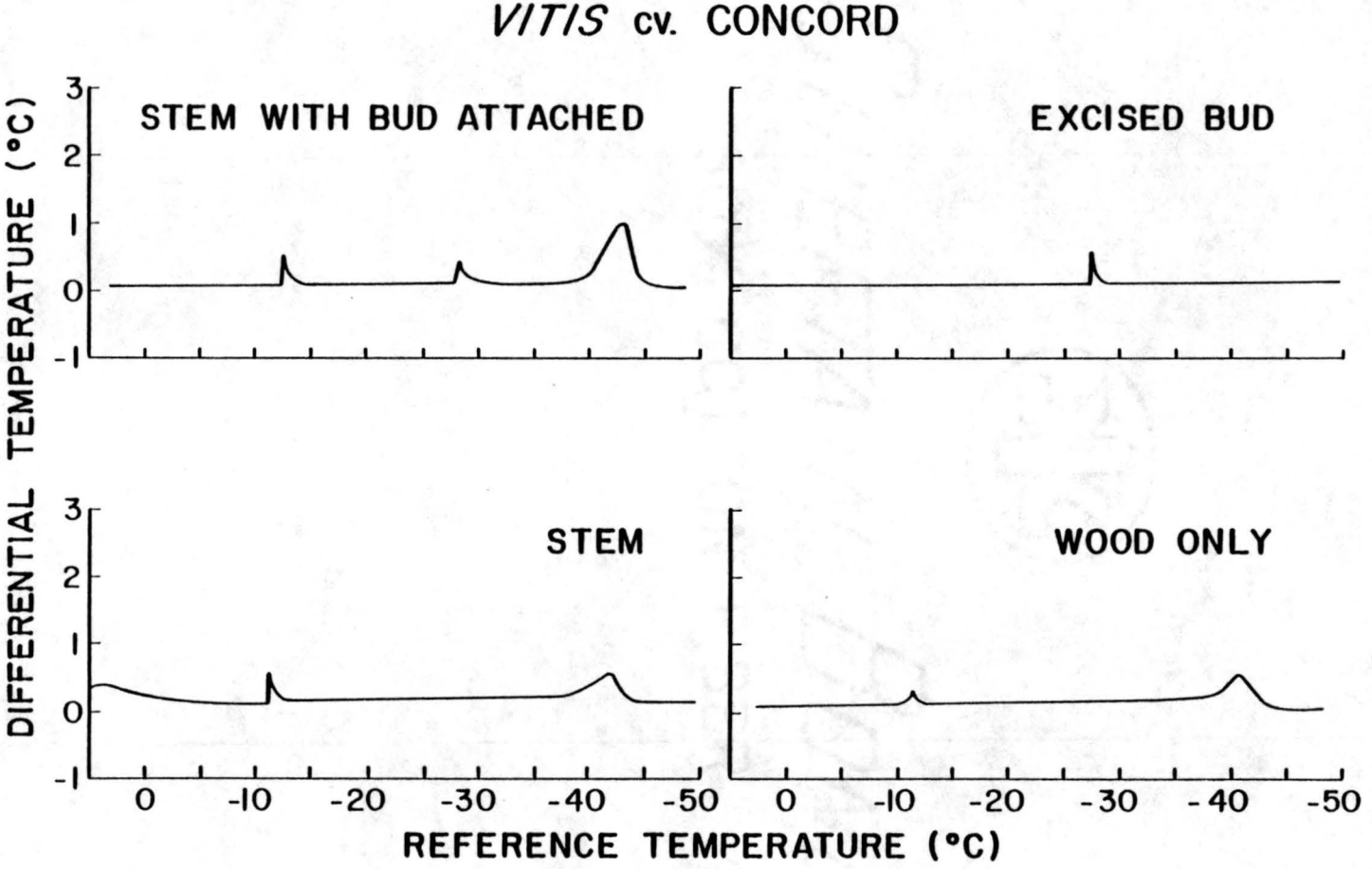

VITIS cv. ROUGEON
OCT 17
FEB 13
APR 9
DIFFERENTIAL TEMPERATURE (°C)
3
2
1
0
-1
0
-10
-20
-30
-40
-50
REFERENCE TEMPERATURE (°C)

performed. DTA was conducted on the stem segment with one bud attached and dissected (Figure 6). The DTA profile of the excised bud DTA showed only a single exotherm, at approximately -28°C. This corresponded to the second exotherm of the stem segment with attached bud. The stem segment without bud and the wood segment showed 2 exotherms. These corresponded to the first and third exotherm in the stem segment with the attached bud.

Exotherm temperatures of the 4 grape cultivars showed a transition from October to April (Figures 2 through 5). DTA data for all 4 cultivars conducted during the study are summarized in Table 2. Both bud exotherm and stem tissue exotherm of samples collected on October 17, 1980 appeared at much higher temperatures than in the samples collected on November 28, 1980, January 5, 1981, and February 13, 1981. More than 1 bud exotherm in a sample appeared as the tissue was acclimating (October 17, 1980). A similar occurrence was observed as the tissue was deacclimating (April 9, 1981). The low temperature exotherms of the stem tissue occurred over a wide range of temperatures for these non-acclimated samples. The range was approximately -10° to -20°C for acclimating samples and -20° to -40°C for deacclimating samples.

Electrolyte leakage was used to test the killing point of bud, stem, and wood tissues harvested on March 10, 1981. Due to the small change in percentage of electrolyte leakage (10%) the experiment was repeated on April 3, 1981. The small percentage of electrolyte leakage was likely due to insufficient time at 5°C for samples to develop injury. Therefore, samples from the repeated experiment were left for 5 days instead of 2 days at 5°C after

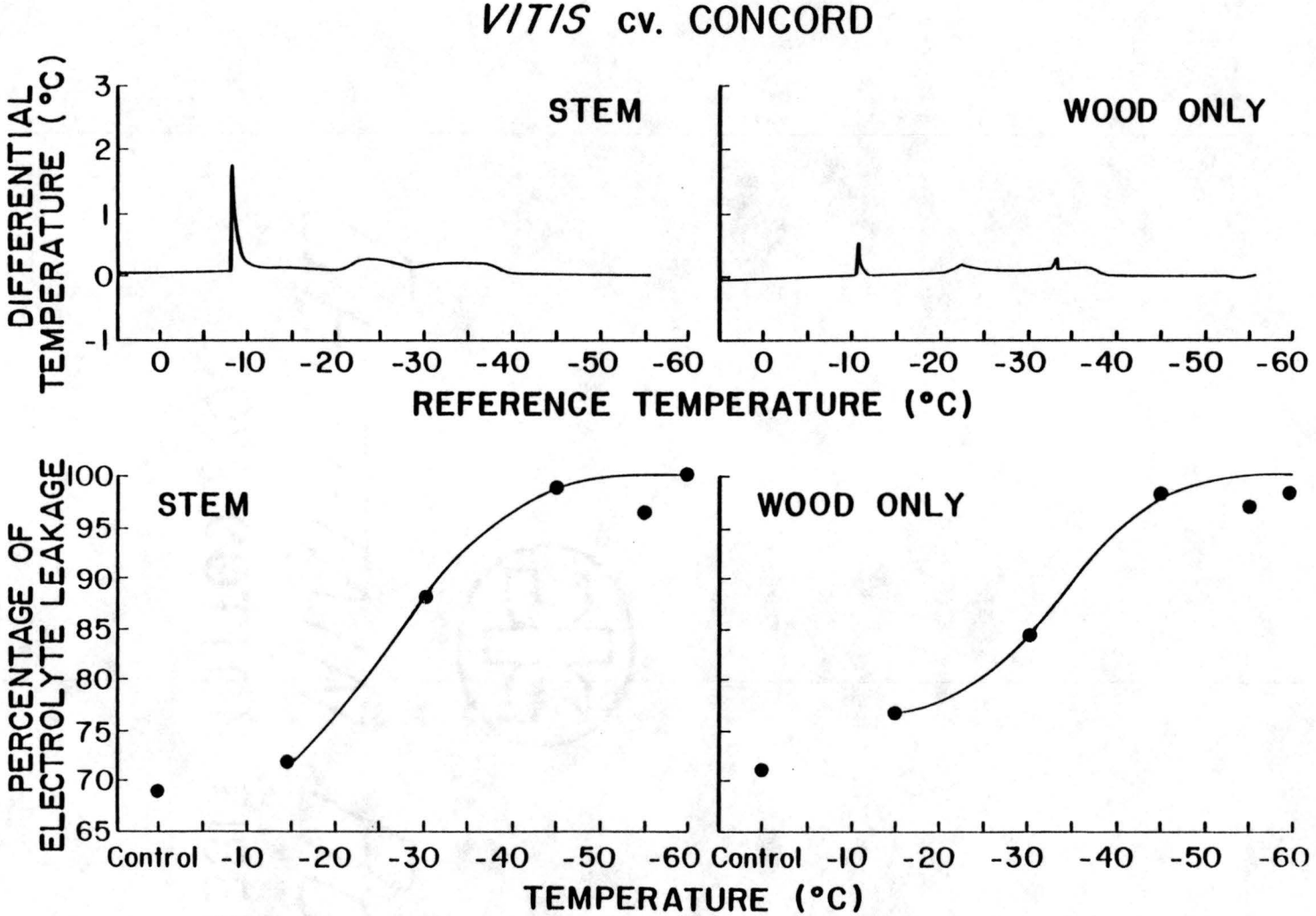
Figure 6. Differential thermal profiles of 'Concord' grape stem with one bud attached and its dissected parts. Samples were collected on March 8, 1981. DTA profiles are plotted as the differential temperature between the sample and the reference on the ordinate vs. the reference temperature on the abscissa.


VITIS cv. CONCORD
STEM WITH BUD ATTACHED
EXCISED BUD
STEM
WOOD ONLY
DIFFERENTIAL TEMPERATURE (°C)
3
2
1
0
-1
0
-10
-20
-30
-40
-50
REFERENCE TEMPERATURE (°C)

treatment. DTA was conducted on some samples in order to compare the low temperature exotherm with the killing point. As shown in Figure 7, low temperature exotherms of stem and wood tissue were correlated with death of tissues as indicated by electrolyte leakage. This implies that avoidance of freezing by deep supercooling may play an important role in the survival of grape wood tissue at low temperatures. The bud tissue did not show a good relationship between the bud exotherm and the killing point as determined by electrolyte leakage. This may have been due to the time that the samples were collected as the buds were deacclimating. Therefore, this experiment should be repeated when the buds are fully acclimated.

The bud exotherm temperature may have shifted to higher temperatures faster than did the stem during dehardening. This shift was observed by Pierquet et al. (18) in DTA of field collected stem and bud samples when compared frozen and thawed for various lengths of time at 4.5°C. They proposed that the appearance of the bud exotherms, which was not apparent in the frozen state, was most likely due to the freezing of water which moved from the stem tissue into the bud tissue during thawing. This thawing and associated water movement is a dehardening process which may lead to the death of the bud at higher freezing temperatures than for the stem. Field observations of grapes have indicated that wood tissues of grapes seemed less susceptible to injury than buds in winter (5, 19, 29). Field observation of the 2 'Rougeon' plants, located in Fort Collins, used in this study showed the death of all the above ground canes in May. This may partly explain the DTA results of 'Rougeon'

Figure 7. Differential thermal profiles and relative electrolyte loss of 'Concord' grape stem and wood. Samples were collected on April 3, 1981. DTA profiles are plotted as the differential temperature between the sample and the reference on the ordinate vs. the reference temperature on the abscissa.


VITIS cv. CONCORD
DIFFERENTIAL TEMPERATURE (°C)
3
2
1
0
-1
STEM
WOOD ONLY
0 -10 -20 -30 -40 -50 -60
REFERENCE TEMPERATURE (°C)
PERCENTAGE OF ELECTROLYTE LEAKAGE
100
95
90
85
80
75
70
65
STEM
WOOD ONLY
Control -10 -20 -30 -40 -50 -60
TEMPERATURE (°C)

observed in February and April (Figure 5 and Table 2). No bud exotherms were observed in 3 samples of 'Rougeon' on February 13, although stem tissue exotherm were observed in these samples. The lack of bud exotherm at that time indicated that the buds were dead. This is likely as bud exotherms were observed in the other 3 cultivars which survived through the winter.

This apparent death of 'Rougeon' buds is most interesting in light of the minimum temperatures observed in Fort Collins. The state climatological records (6) showed a minimum temperature during the winter of 1980-1981 of -25.6°C on February 11. This compares to a bud exotherm of -25.85°C $\pm$ 1.20 observed on January 5 for 'Rougeon'. Bud exotherms of the other 3 cultivars were all lower than -28°C on the same date (Table 2). Since the other cultivars showed only a small percentage of bud death while 'Rougeon' died back entirely, it seemed likely that the minimum temperature observed on Feburary 11 resulted in the death of 'Rougeon' buds.

It seems likely that the bud exotherm in these 4 cultivars is the point at which freeze-induced death occurs. Bud exotherms have been related to killing point by Quamme et al. (23) working with peach flower buds as well as by other workers (2, 9, 11). The compound bud of grape thus may be killed as the ambient air falls below the bud exotherm temperature. However, one can not eliminate the possibility of desiccation as a cause of the death of 'Rougeon' bud and stem.

Northern limits of the commercial production of apple and pear as well as the distribution of some native woody species in North American (10, 21) has been correlated with the deep supercooling

capacity of their xylem water. This is a reflection of xylem tissues greater susceptibility to cold injury than other tissues. Since buds (or the associated wood in the bud) apparently is the most susceptible tissue in grape, it is proposed that this may be the limiting factor in culture of those commercial cultivars with a mixture of *labrusca riparia*, and *vinifera* genes.

Suggested research to further support the hypothesis would include survival tests of excised buds at several times during the year, especially the fully acclimated buds.

In related work on grape by Pierquet et al. (18), it was proposed that water moved into buds from the stem when tissues are thawed from the frozen state. In order to test this hypothesis, the percent of water to dry weight was determined on stem and bud before and after freezing for 8 days. No conclusions could be drawn from a comparison of these relative water contents. It is suggested that a more precise experiment could be performed through the use of nuclear magnetic resonance or differential scanning calorimetric methods to determine water content before and after freezing. If the buds contained less water when frozen than when thawed, this would support Pierquet et al.'s hypothesis.

Our DTA results with bud and stem tissue exotherms are in general agreement with the idea that 'Valient' is the most hardy of these cultivars while 'Beta', 'Concord', and 'Rougeon', are less hardy.

CONCLUSIONS

Results indicated that at least some cultivars of grape show 3 exotherms when DTA is conducted on fully acclimated stem segments with attached buds. The bud supercools to -25° to -30°C when fully acclimated. It is possible that the temperature at which the bud exotherm occurs is the killing point of the above ground stems. Desiccation may or may not be a factor in the death of the stems.

Bud and stem tissue exotherms shift lower in the fall as they acclimate and higher in the spring as they deacclimate.

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CHAPTER 3

COLD HARDINESS OF *RUBUS* VEGETATIVE BUDS AND CRYOPROTECTANT EFFECT

ABSTRACT

Differential thermal analysis of stem with attached bud samples of the 4 *Rubus* cv. 'Heritage', *R. idaeus* L., 'Black Hawk', *R. occidentalis* L., 'Amethyst', *R. neglectus* Peck, and 'Darrow', *R. allegheniensis* Porter, suggest that these do not deep supercool during winter. Survival tests to determine cold hardiness of these 4 cv. were conducted at 5 times from September 20, 1980 to April 11, 1981. These preliminary data indicated that the order of degree of hardiness of the three cultivars may be 'Black Hawk', 'Amethyst', and 'Heritage', respectively. Survival data on April 11, 1981 as samples were deacclimating, indicate that 'Black Hawk' and 'Heritage' may deacclimate earlier than 'Darrow' and 'Amethyst'.

Survival tests of 'Heritage' samples treated with cryoprotectants showed reduced survival in the acclimated stage. There were some results indicating that cryoprotectants may enhance the resistance of nonacclimated samples to lower temperatures.

INTRODUCTION

A prefreezing treatment has been proven to be critical for plant tissues to survive at -196°C by many investigators (20, 22, 23, 26, 27, 28, 29, 30, 31, 34). According to Sakai (26), less hardy samples require a lower prefreezing temperature for survival at -196°C. That is, the degree of cold hardiness is correlated with the prefreezing temperature requirement for survival. Therefore, cold hardiness is one factor requiring study for successful cryopreservation.

In temperate regions, plants show different degrees of frost resistance in different seasons. They are most tender in summer and enter into acclimation in late summer or fall depending on the species; during winter they are most hardy. Even plants that survive the most extreme freezing during midwinter may be killed by very slight freezing during spring (11). The relationships involved in the transition from non-acclimated to acclimated tissues have been associated with changes in concentration of proteins (5, 21, 32, 33), sugars (14, 21, 25, 32, 33), polyhydric alcohols (24) and the membrane lipids (39).

Deep supercooling of tissue water has been proposed to be a means of frost injury avoidance in many plants (35). Freezing of the supercooled water is known to result in intracellular ice formation (35) and has been associated with tissue injury (7, 17). The seasonal transition of the low temperature exotherm has been described by Quamme (16) in peach flower buds; George et al. (8) in azalea flower buds; and Quamme et al. (17) in apple stem segments.

Cryoprotectants have been used to successfully impart resistance to cold treatments and even survival in liquid nitrogen (1, 2, 4, 10). Effective cryoprotectants include dimethylsulfoxide, glycerol, and various sugars (12).

The resistance to low temperature injury imparted by cryoprotectants may be due to their colligative effects and/or protection of cell membranes against denaturation (19). In one study using glucose, sucrose, and raffinose with spinach chloroplast thylakoids (13), the cryoprotective effect of these sugars was largely due to their colligative capabilities. However, the increase in -OH groups due to the presence of the sugars was also noted. This has been postulated to be a stabilizer of membrane structure.

High concentrations of cryoprotectants may plasmolyze the plant cells and reduce respiration and inhibit RNA and protein synthesis in isolated plant cells and tissues (3). Gradual increases in cryoprotectant concentration may overcome the plasmolysis problem (9). For example, strawberry meristems were precultured on agar-solidified medium supplemented with either 5% DMSO for 2 days or different concentrations (5-10%) of glycerol for 1 to 3 days. Meristems were then subjected to step-wise increases of cyroprotectant concentrations in liquid medium. No plasmolysis was observed.

Finkle and Ulrich (6) showed that mixtures of glucose and dimethylsulfoxide gave as much as a 100% increase in cryoprotection of sugarcane cells, as compared to either compound alone. Much of the effect of mixing cryoprotectants was due to decreased toxicity, i.e. little reduction in respiration or inhibition of RNA and protein synthesis.

Besides dimethylsulfoxide, glycerol, and various sugars, many other cryoprotectants such as ureas, amino acids, and amides have been tested in both animal and plant systems. Recently, Withers and King (38) reported that proline is an effective cryoprotectant for the storage of cultured cells of *Zea mays* L. in liquid nitrogen.

This study described here was designed to gain information for use in eventual cryopreservation of *Rubus*. Cold hardiness and possible deep supercooling behavior in the DTA profile of vegetative buds of *Rubus* were studied at different seasons. Cryoprotectants were also tested for their effect on cold hardiness.

MATERIALS AND METHODS

SURVIVAL TEST

Canes of 4 *Rubus* species; red raspberry, *Rubus idaeus* L. cv. 'Heritage', black raspberry, *Rubus occidentalis* L. cv. 'Black Hawk', purple raspberry, *Rubus neglectus* Peck, cv. 'Amethyst', and blackberry, *Rubus allegheniensis* Porter, cv. 'Darrow' were collected from the field in Fort Collins, Colorado at 5 times during 1980-1981 (Tables 1 through 5). The canes, approximately 60 cm in length and 0.6 cm in diameter, were immediately cut into pieces of 2-3 cm in length, each containing 1 vegetative bud; the terminal 15 cm was excluded.

Table 1. *Rubus* bud survival (percent), collected September 30, 1980, as indicated by observation of bud break 20 days following treatment.

	Control[x]	-5°C	-10°C	-15°C	-20°C	-25°C
'Darrow'	75	85	0	0	0	0
'Black Hawk'	90	95	0	0	0	0
"Amethyst'	35	100	0	0	0	0
'Heritage'	100	90	0	0	0	0
'Heritage' (10% DMSO)	85	95	15	0	0	0
'Heritage' (10% Glyercol)	75	95	15	0	0	0
'Heritage' (5% DMSO + 5% Sucrose)	85	90	0	0	0	0

[x] Control was planted directly from field without cold treatment.

Table 2. *Rubus* bud survival (percent), collected December 18, 1980, as indicated by observation of bud break 50 days following treatment.

	Control[x]	-5°C	-10°C	-15°C	-20°C	-25°C
'Darrow'	0	0	0	5	10	5
'Black Hawk'	55	60	70	65	55	50
'Amethyst'	80	95	80	70	95	15
'Heritage'	85	90	95	80	60	65
'Heritage' (10% DMSO)	65	80	55	70	55	45
'Heritage' (10% Glycerol)	65	75	60	45	15	5
'Heritage' (5% DMSO + 5% Sucrose)	70	20	35	50	40	15

[x] Control was planted directly from field without cold treatment.

Table 3. *Rubus* bud survival (percent), collected January 17, 1981, as indicated by observation of bud break 50 days following treatment.[y]

	Control[x]	-5°C	-10°C	-15°C	-20°C	-25°C	-30°C	-35°C	-40°C
'Darrow'	80	70	60	70	55	60	35	15	0
'Black Hawk'	90	75	80	75	70	55	55	55	50
'Amethyst'	70	50	55	55	65	70	70	55	50
'Heritage'	90	60	45	70	85	65	75	55	20
'Heritage' (10% DMSO)	70	30	25	25	15	5	10	5	10
'Heritage' (10% Glyercol)	70	55	10	5	10	15	5	10	5
'Heritage' (5% DMSO + 5% Sucrose)	50	15	20	30	50	5	10	15	10

[x] Control was planted directly from field without cold treatment.

[y] Observations of 'Darrow' were made 65 days after treatment.

Table 4. *Rubus* bud survival (percent), collected January 30, 1981, as indicated by observation of bud break 50 days following treatment.

	Control[x]	-45°C	-50°C	-55°C	-60°C
'Darrow'[y]	-	-	-	-	-
'Black Hawk'	95	90	70	50	15
'Amethyst'	100	85	85	35	35
'Heritage'	100	50	35	40	25
'Heritage' (10% DMSO)	80	25	15	5	5
'Heritage' (10% Glycerol)	85	15	0	0	0
'Heritage' (5% DMSO + 5% Sucrose)	60	50	0	0	0

[x] Control was planted directly from field without cold treatment.

[y] Bud survival was not tested for 'Darrow' at this time.

Table 5. *Rubus* bud survival (percent), collected April 11, 1981, as indicated by observation of bud break 20 days following treatment.

	Control[x]	-5°C	-10°C	-15°C	-20°C	-25°C	-30°C
'Darrow'	100	100	100	95	95	60	5
'Black Hawk'	90	90	85	85	80	10	5
'Amethyst'	95	95	100	95	85	75	25
'Heritage'	100	100	100	85	55	5	0
'Heritage' (10% DMSO)	80	75	80	35	25	0	0
'Heritage' (10% Glycerol)	80	85	75	65	25	0	0
'Heritage' (5% DMSO + 5% Sucrose)	100	95	95	80	55	35	0

[x] Control was planted directly from field without cold treatment.

Lots of 20 pieces were wrapped with cheesecloth, moistened with distilled water, and then wrapped with aluminum foil. The wrapped samples were then placed into weighted test tubes (3 x 20 cm) closed with a rubber stopper.

Samples of each species, (1 test tube of 20 segments per treatment) were subjected to freezing temperatures. Samples were placed into a methanol bath at 0°C and then cooled at a rate of 2.4°C/hr to temperatures as noted in Tables 1 through 5. At -3°C the tissues in the test tubes were nucleated by the placement of a small piece of ice in contact with the cheesecloth. As the treatment temperatures were reached, tubes were pulled from the bath and warmed at room temperature. The segments were then planted in a 1:1 peat:perlite mix under a timed mist system of 30 seconds per 15 minutes.

Observations of survival as indicated by bud break were made at 20, 30, 40, 50, and 60 days.

DIFFERENTIAL THERMOAL ANALYSIS (DTA)

DTA was conducted on the 4 *Rubus* species at the beginning of each month from September, 1980 to April, 1981. Canes were cut into small segments approximately 1 cm in length and containing 1 vegetative bud. Then each segment was cut longitudinally and the half segment with the bud used for DTA. Each sample was placed in aluminum foil with the thermocouple in direct contact with the bud; three samples from different canes were used for each cultivar. DTA was performed using a Cryo-med (Model 1000) attached to a Data

acquisition system (Model HP 3052A), run by a Hewlett-Packard desk computer (Model 9845A). Computer regulation of the cooling rate was at 0.3^{o}C/minute.

CRYOPROTECTANTS

Survival tests were made using 10% dimethosulfoxide (DMSO), 10% glycerol, and 5% DMSO + 5% sucrose, with samples of 'Heritage'. Canes were collected and cut into segments as described above. Samples were placed into test tubes (2.5 x 15 cm) covered by cryoprotectant for 1 hour. The cryoprotectant was then decanted and samples removed and blotted dry with Kim-wipes. Following this, samples were temperature-stressed and survival assessed as described previously.

RESULTS AND DISCUSSION

Bud break was used as the criteria to indicate survival. If the buds failed to sprout they were classified as dead. A summary of the percentage of bud break (hereafter termed survival) with time of bud and stem collection and cryoprotectant treatment is presented in Tables 1 through 5. Table 6 is a summary of Tables 1 through 5

presenting LT_{50}, the lowest temperature at which 50% of the buds sprouted. Figure 1 is a graphic display of the percentage of survival of 'Heritage' with time of collection and cryoprotectants used.

An examination of Tables 1 through 5 and their summary in Table 6 shows differences in hardiness between cultivars at different times of the year. All cultivars showed an LT_{50} at $-5^{\circ}C$ on September 20, 1980. This would be expected as plants were still in active growth. Death of woody plant tissues in active growth is generally assumed to occur at the moment of freezing, usually between -2 and $-8^{\circ}C$ (37).

Observations of survival of the 4 cv., Tables 2, 3, 4, and 6, on December 18, January 17, and January 30 indicated a general increase in hardiness to January 30. This was expected as plants have been observed to increase in hardiness as ambient air temperatures decrease through midwinter (15). LT_{50} was at $-55^{\circ}C$, $-50^{\circ}C$, and $-45^{\circ}C$ for 'Black Hawk', 'Amethyst', and 'Heritage', respectively. 'Darrow's lowest LT_{50} was not determined on January 30, so its extreme hardiness cannot be compared to the other cultivars.

It is surprising to note that 'Black Hawk' appeared to be more hardy than 'Heritage'. The red raspberries are normally considered more hardy than black raspberries (37). The plant material used in this study may be atypical of their types. Perhaps a survival test in February would have indicated greater hardiness of 'Heritage' than 'Black Hawk'. More survival tests over several years and at monthly or biweekly intervals would give more convincing evidence of the hardiness relationship between the 4 cultivars studied.

Table 6. Lethal temperature for 50 percent kill (LT_{50}).

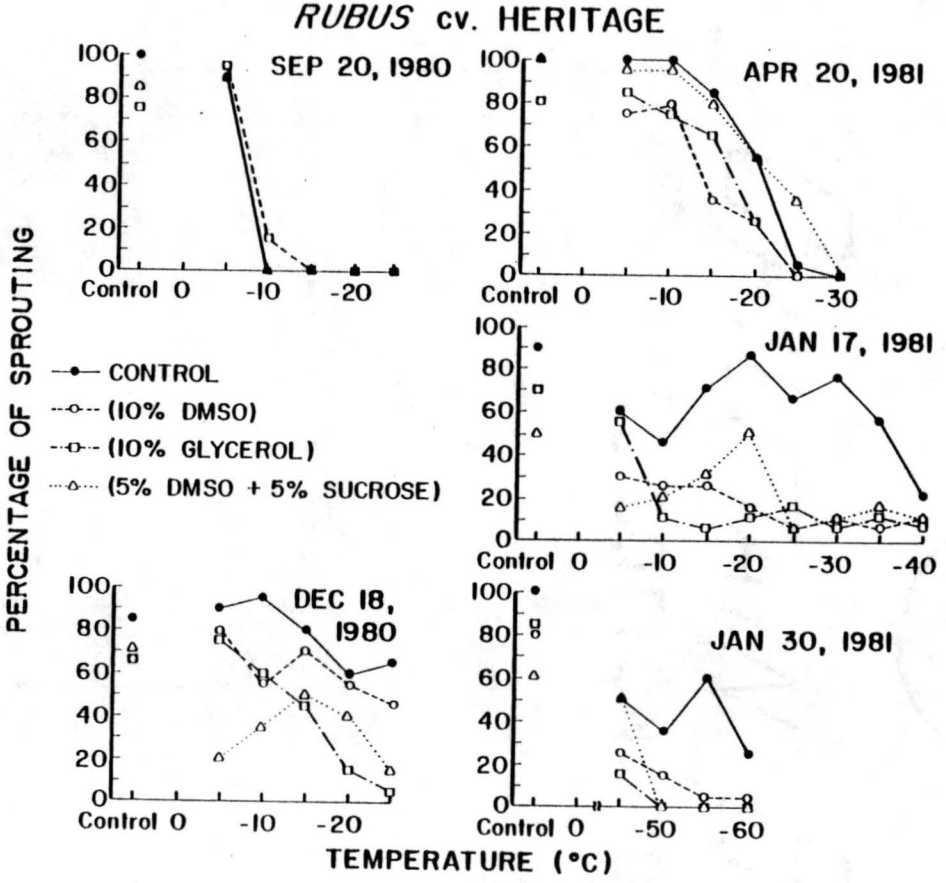
	'Darrow'	'Black Hawk'	'Amethyst'	'Heritage'
9/20	- 5°C	- 5°C	- 5°C	- 5°C
12/10	ND[x]	below -25°C[y]	-20°C	below -25°C[y]
1/17	-25°C	below -40°C[y]	below -40°C[y]	-35°C
1/30	ND[z]	-55°C	-50°C	-45°C
4/11	-25°C	-20°C	-25°C	-20°C

[x] Undetermined due to failure of bud break although observations of tissues indicated survival.

[y] LT_{50} is at or below -25°C/-40°C as indicated, due to limited testing temperature in that experiment.

[z] This was not completed.

Figure 1. Percentage of bud break of 'Heritage' vegetative bud treated with 10% DMSO, 10% glycerol, or 5% DMSO + 5% sucrose prior to cold stress at different acclimation stages. Percentage of sprouting was calculated from the observation of 20 samples and is plotted on the ordinate versus the testing temperatures plotted on the abscissa. Control was without cold treatment.


RUBUS cv. HERITAGE
SEP 20, 1980
APR 20, 1981
JAN 17, 1981
DEC 18, 1980
JAN 30, 1981
CONTROL
(10% DMSO)
(10% GLYCEROL)
(5% DMSO + 5% SUCROSE)
PERCENTAGE OF SPROUTING
TEMPERATURE (°C)
Control 0 -10 -20
Control 0 -10 -20 -30
Control 0 -10 -20 -30 -40
Control 0 -50 -60
0 20 40 60 80 100

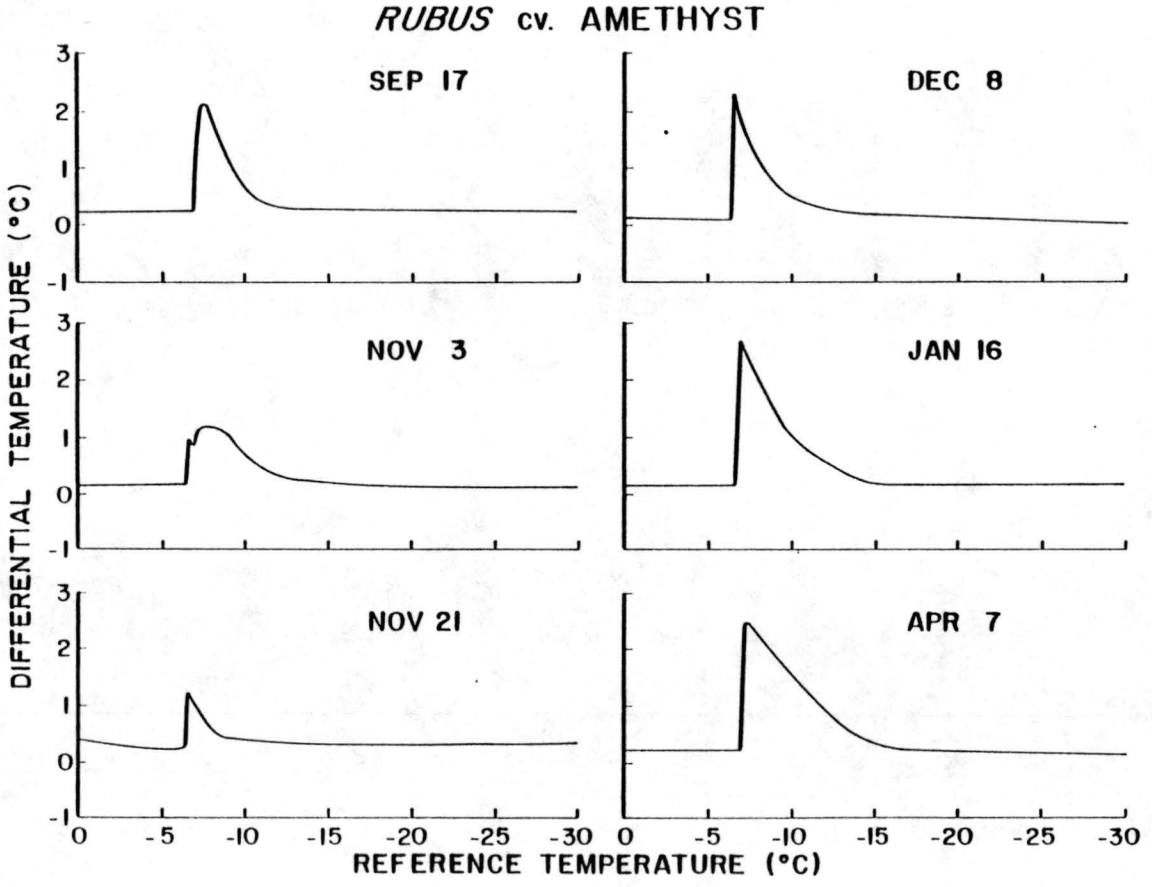
Proesbting (15) and Van Adrichem (36) observed plants over 4 years to determine the degree of cold hardiness.

'Amethyst' is a purple raspberry which is a first generation offspring of black and red raspberry parents (18). The LT_{50} of 'Amethyst' was -50°C on January 30, midway between the hardiness of the red and black raspberries. General observations normally categorize purple raspberries as similar to black raspberries in hardiness (37).

Survival of samples collected on April 11, 1981 showed an LT_{50} of -25°C in 'Darrow' and 'Amethyst' and -20°C in 'Black Hawk' and 'Heritage' (Table 6). These lower LT_{50} values would be expected as plants normally are deacclimating at this time as warmer temperatures are occurring. It is interesting to note the differences in the LT_{50} of the cultivars. The differences may indicate that the 2 cv. 'Black Hawk' and 'Heritage' deacclimate earlier in the spring. This would make them more susceptible to late spring frosts.

DTA of the *Rubus* cv., example in Figure 2, showed evidence of only 1 exotherm. Although the reference temperatures shows only -30°C in the figure, many samples were tested to -40°C and -60°C on January 6 and April 7, respectively. No low temperature exotherms were observed. It seems possible that these *Rubus* cv. do not deep supercool. Further evidence of this is seen in the LT_{50} of stem with attached bud down to -50 and -55°C in 'Amethyst' and 'Black Hawk'. This is well below the minimum observed deep supercooling of -47°C in *Fraxinus nigra* Marsh (8). However, further studies are needed to determine if xylem is killed at the -50°C temperatures. It seems possible that bud break may occur with

Figure 2. DTA profiles of 'Amethyst' stem with one vegetative bud attached at different acclimation stages. DTA profiles are plotted as the differential temperature between the sample and the reference on the ordinate versus the reference temperatures on the abscissa.


RUBUS cv. AMETHYST
SEP 17
DEC 8
NOV 3
JAN 16
NOV 21
APR 7
DIFFERENTIAL TEMPERATURE (°C)
3
2
1
0
-1
0
-5
-10
-15
-20
-25
-30
REFERENCE TEMPERATURE (°C)

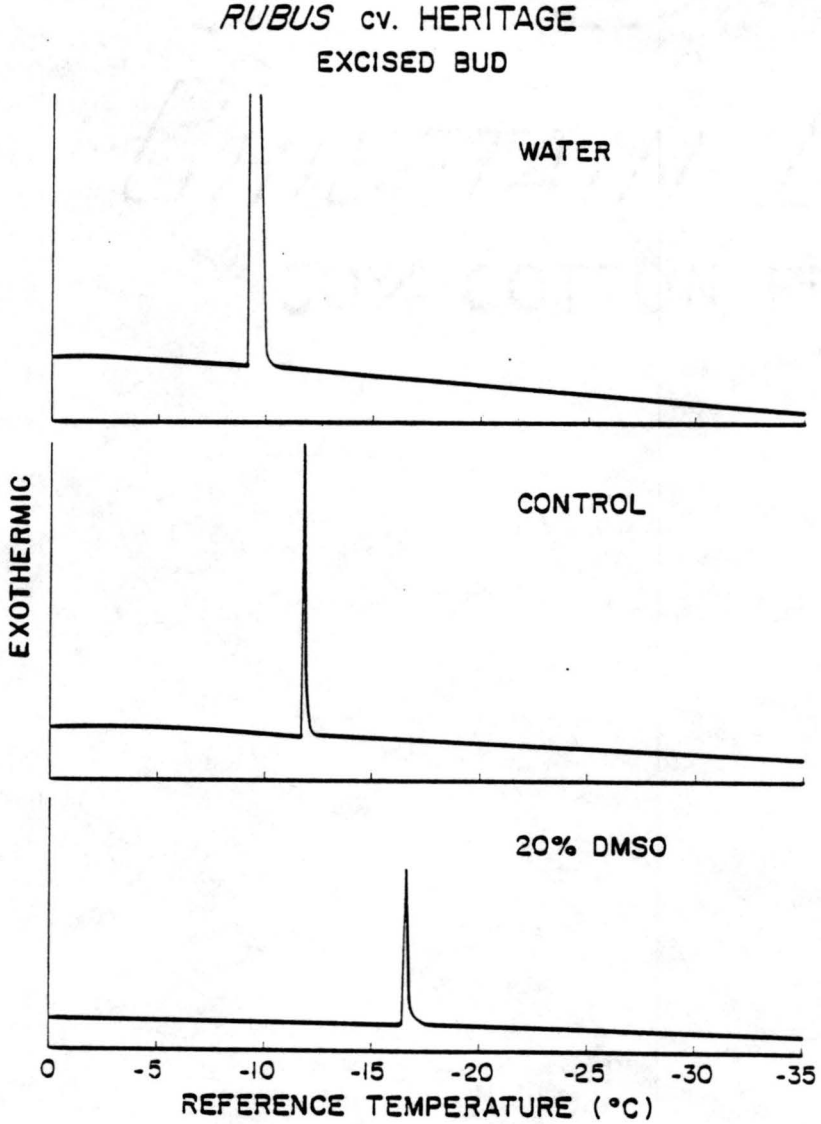
survival of the shoot under mist even when the xylem is dead in the stem. The definitive experiment would involve browning tests of xylem of the acclimated *Rubus* cv. at -50°C.

In general, cryoprotectants showed no obvious benefit in increasing resistance to cold injury of stem with attached buds of 'Heritage' samples collected at 5 times during this study. Samples collected on September 5, 1981 (non-acclimated) showed only a slight increase in survival when treated with 10% DMSO and 10% glycerol (Table 1 and Figure 1). The slight increase in survival of treated samples over untreated samples (15% at -10°C versus no survival at -10°C) may be due to freezing point depression. Evidence of this may be noted in Figure 3. Freezing point depression was noted in 'Heritage' excised buds treated with 20% DMSO for 1 hour (-16.4°C) as compared to untreated bud (-11.7°C) and those soaked in water for 1 hour (-9.1°C).

Treatment of acclimated tissues actually decreased survival with all 3 cryoprotectants at all 3 times collected (Tables 2, 3, 4, and Figure 3). A possible explanation is that the use of the cryprotectant solutions resulted in a rehydration of the samples making them more susceptible to cold injury.

Deacclimating samples (collected on April 20, 1981) treated with 10% DMSO or 10% glycerol showed slightly lower survival than untreated samples. Samles treated with 5% DMSO + 5% sucrose showed similar results except that at -25°C when 35% bud break was noted as compared to 5% of untreated samples.

Figure 3. DTA profiles of 'Heritage' excised bud treated with water or 20% DMSO for 1 hour prior to DTA run. Control is the fresh sample without treatment prior to DTA run. DTA profiles are plotted as the relative temperature difference between the sample and the reference on the ordinate versus the reference temperature on the abscissa.


RUBUS cv. HERITAGE
EXCISED BUD
WATER
CONTROL
20% DMSO
EXOTHERMIC
0
-5
-10
-15
-20
-25
-30
-35
REFERENCE TEMPERATURE (°C)

There is an indication that tissues not in an acclimated stage may survive to lower temperatures if treated with cyroprotectants (Table 1 and 5, and Figure 1). Therefore, it may be of value to conduct more extensive experiments subjecting unacclimated tissues to mixtures of cryoprotectants for longer periods of time.

CONCLUSION

Survival tests of *Rubus* stem segments with an attached bud conducted on 4 cultivars collected at 5 times from September 20, 1980 to April 11, 1981, indicated differences between cultivars with time of collection. Preliminary information indicated 'Black Hawk' to have the greatest degree of hardiness followed by 'Amethyst' and 'Heritage'. Bud break of all 3 cultivars was observed after -55^{o}C treatment in the samples collected on January 30, 1981. This observation and the lack of a second exotherm around -40^{o}C indicated that *Rubus* may not deep supercool during winter. 'Black Hawk' and 'Heritage' appeared to deacclimate earlier than 'Amethyst' and 'Darrow' based on survival of samples collected on April 11, 1981.

Acclimated 'Heritage' stem segments with bud attached showed a decrease in survival when treated with any of 3 cryoprotectants as compared to the untreated control. There was some indication

that nonacclimated or deacclimating samples may be protected to lower temperatures with cryoprotectants. The use of different concentrations of, or mixtures of, or prolonged exposure to cryoprotectants may enhance the resistance of plant samples to cold injury.

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