THESIS

RESPONSE OF 'RED LAKE' CURRANT AND RED-OSIER DOGWOOD TO COLD ACCLIMATION

Submitted by

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In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY M. DAVID HANNA ENTITLED RESPONSE OF 'RED LAKE' CURRANT AND RED-OSIER DOGWOOD TO COLD ACCLIMATION BE ACCEPTED AS FULFILLING PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

RESPONSE OF 'RED LAKE' CURRANT AND RED-OSIER DOGWOOD TO COLD ACCLIMATION

Twigs of 'Red Lake' currant (*Ribes rubrum* L.) and Red-osier dogwood (*Cornus sericea* L.) were collected at the beginning of each month starting October 1992, and ending the first of April 1993. Twig samples were subjected to controlled temperature treatments: 0^0C, 10^0C, -5^0C, 0^0C for 12 hr/ 10^0C for 12 hr, and -5^0C for 12 hr/ 10^0C for 12 hr for one month. Treated and field samples were evaluated for hardiness by freeze tests and glucose, sucrose, raffinose and stachyose content in cortical tissue. A study of hardiness was repeated the following year beginning in September and ending the first of April. Cold tolerance was promoted best by fluctuating temperatures in the fall with greater acclimation achieved in the colder temperature treatments as the season progressed until mid-winter and into the spring months when de-acclimation occurred. Hardiness was best retained by the cooler steady temperatures. The endogenous content of raffinose was strongly associated with cold hardiness in both plant materials. Raffinose and glucose levels of field samples from both currant and dogwood were significantly correlated with cold hardiness. Stachyose was also associated with hardiness of currant taken from outdoors and treated at 0^0C. Sucrose was associated with hardiness in dogwood stored at $-5C^0/10^0$C. The best association between sugars and cold tolerance in either plant species was seen in samples taken directly from the field with no treatment. Storage at 0^0C/10^0C increased raffinose as hardiness increased in both species. Storage at

0^0C and at -5^0C increased raffinose as hardiness increased in dogwood, but not in currant. Warm temperature storage as 10^0C and $-5^0/10^0C$ destroyed the relationship between hardiness and raffinose levels in both plant materials.

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CHAPTER ONE

INTRODUCTION

Tolerance to below freezing temperatures in woody plants is partly influenced by their ability to respond to temperature conditions in the environment. Response to any given temperature or temperature changes may also vary depending on the developmental stage of the plant. Freeze damage occurring at temperatures that would not ordinarily injure the same plants during full mid-winter hardiness because of some environmental factor prior to injury is testimony to these facts. Although it is known that temperature conditions in the field can produce changes in cold tolerance, the range and variability in temperature that occurs in the field and influences cold tolerance must be further characterized under controlled conditions so that cause and affect relationships between hardiness and environmental conditions can be better understood.

Changes in cold tolerance are the result of physiological changes that occur within plant tissues. Physiological changes in plants can take place as a result of the temperature environment. Because of the complex nature of physiological adaptations to cold stress, changes in the physiology and degree of cold tolerance in plant tissue as the result of any temperature environment must be studied together. Physiological changes that coincide with changes in cold tolerance have been observed under field conditions. However, the association between cold hardiness and any physiological change must be observed under a more controlled environment than what is afforded in the field before a direct relationship can be assumed.

By observing the accumulation of compounds in plant tissues, certain aspects of physiological changes can be observed. Observed changes in compound concentrations that are consistent with changes in cold tolerance may be indicative of a physiological response within a plant acclimating to its environment. Compounds whose levels can be associated with hardiness under a variety of conditions in a number of plant taxa are most likely to have a direct relationship with cold tolerance. It is this stage of understanding cold tolerance which this study addresses. There are many reports of associations between soluble sugars and cold tolerance in many species of plants which suggests that soluble sugars may play a role in cold tolerance. The purpose of this study is to observe changes in cold tolerance and the levels of four soluble sugars in two hardy woody plant species: Red Lake currant (*Ribies rubrum* L.) and Red-osier dogwood (*Cornus sericea* L.) under controlled temperature environments during the winter season, beginning in October and ending in April of the following year.

Once a direct association can be made between cold tolerance and a given compound, work towards the identification of metabolic pathways and ultimately the genes that control physiological responses which cause changes in cold tolerance may be more easily approached. Although compounds such as soluble sugars acting as endogenous cryopotectants may only be part of the mechanism that affords cold tolerance in living plants, the positive identification of such compounds playing this role may also give a better understanding of the other mechanisms involved in cold tolerance. The implications of such an understanding could be improved screening and breeding methods as well as direct genetic manipulation of ornamental and food crop plants that can tolerate cold stress in areas where this has been a limiting factor.

CHAPTER 2

THE EFFECTS OF CONTROLLED TEMPERATURE TREATMENTS ON HARDINESS IN DORMANT TWIGS OF 'RED LAKE' CURRANT AND RED-OSIER DOGWOOD

2.1 INTRODUCTION AND REVIEW OF LITERATURE

2.1.1 Overview

The distribution of plant species may largely be attributed to their adaptation to environmental stresses which enable them to grow, develop, and complete a life cycle in a given environment (Junttila 1989, Weiser 1970, Woodward 1990). Minimum winter temperatures also place northern limits on where cultivated plants can be grown (Sakai, 1982). In some cases, adaptation to cold stress may be so specific to survival in a given location, that plants moved from harsh locations to warmer ones can sustain frost injury in the new locations (Koski 1985). From sub-tropical to boreal regions of North America, resistance to freezing injury in food and timber crops has become the focus of much research in recent decades although several documented studies on this topic can be found dating back to the earlier part of this century and interest in this subject has probably been on the minds of many for centuries (Weiser 1970). Most of the work done toward achieving an understanding of tolerance toward sub-freezing temperatures has been through observations of stress in temperature thresholds where plastic or irreversible changes to plant tissue occur and are manifested as injury or death. Much less attention has been focused on the elastic changes in plants due to freezing stress where the condition of the plant prior to stress is quickly regained after stress has subsided. Such elastic changes within plants due to cold temperature stress may play no less an important role in how the plant responds to cold stress (Levitt 1980).

Since woody perennial plants in non-tropical regions do not regulate their own temperatures, they must survive cold temperature stress by mechanisms of tolerance or avoidance (Levitt 1980). As the temperatures change in these regions throughout the year, so does the lowest temperature in which these plants are able to survive. For this discussion, a change in low temperature tolerance will be referred to as acclimation when there is a decrease in lowest survival temperature and de-acclimation when there is an increase in lowest survival temperature. Plants or plant tissues that have the potential of attaining very low minimum survival temperatures will be referred to as hardy. Olien (1967) advanced the idea that when determining the lowest survival temperature, the highest limiting temperature must be defined where any subsequent injuries that may occur at lower temperatures have no bearing on the survival of the plant. How the lowest survival temperature is determined is rather complicated and has been the focus of much study in its own right (Burke & Stushnoff 1979, Burr et. al. 1986, 1988, 1989, 1990, Dexter et. al. 1932, Junttila 1989, Levitt 1980 ,Quamme & Stushnoff 1984, Wilner 1961).

2.1.2 Acclimation

Work done in recent decades to characterize the acclimation process in a variety of different plants with respect to low temperature tolerance has produced a number of different findings, many of which have proved to be difficult to explain in terms of the physiology occurring within the plant (Quamme & Stushnoff 1983). There is certainty that acclimation involves metabolic activities which must have an energy source in order to take place (Olien 1967). Therefore, the onset of cold acclimation in hardy plants requires adequate health and vigor as well as the fulfillment of water, light, and temperature requirements before and during acclimation (Quamme & Stushnoff 1983, Levitt 1980, Weiser 1970). Acclimation can be characterized as two primary stages (Levitt 1980, Sakai & Larcher 1987, Van Huystee et. al. 1967, Weiser 1970). However, a third stage of acclimation in some plants before the full extent of acclimation has been achieved in some species has been advanced by some of those working with species of willow, pine, birch, and currant (Sakai & Larcher 1987). This third stage of acclimation is intermittent in nature and occurs in very hardy woody plants at temperatures below -30^{0}C (Weiser 1970). Experiments by Kohn in 1957 indicated that there can be as many stages as desired in work with cabbage (Sakai & Larcher 1987). For this discussion, the two stages of acclimation in woody plants will be of primary concern.

The first stage mentioned can be characterized by discontinuation of vegetative growth and the beginning of a rest stage that occurs during a time of decreasing day length (Koski 1985, Sakai & Larcher 1987). The relationship between rest and acclimation is not clear (Kiboyashi et. al. 1983). At the first stage of acclimation, growth inhibitors may increase in abundance and a relative decrease in growth promoters may occur within the plant (Van Huystee et. al. 1967). In Red-osier dogwood, decreasing day length alone was not sufficient to induce acclimation (Van Huystee et. al. 1967). It was also shown in this same experiment with dogwood that a gradually decreasing photo-period was more effective in inducing acclimation than was a sudden decrease in photo-period.

Light may be necessary for metabolic processes within the acclimating plant. Experiments with wheat indicated that light was an essential element in promoting cold tolerance (Levitt 1980). It is believed that the acclimation factor occurs first in the leaves and spreads into the rest of the plant over a period of time (Sakai & Larcher 1987, Weiser 1970). In controlled experiments using light exposure with wheat plants, the translocation of an acclimation factor or factors from acclimated tissues in older leaves to less acclimated tissues in younger leaves was shown (Levitt 1980). The translocation of an acclimation factor or factors has also been shown in grape by grafting (Weiser 1970).

Temperatures during the first stage of acclimation may range from 10^0C to 20^0C for highs (Sakai & Larcher 1987) and 0^0C to 5^0C for lows (Levitt, 1980). In this first stage however, lower temperatures alone have a limited affect in promoting acclimation in Red-osier dogwood (Van Huystee et. al.1967) suggesting that the effects of decreasing day length and decreasing temperatures are both necessary for the first stage of the acclimation process to occur in some taxa. Work with conifers showed a slower rate of acclimation in seedlings when they where kept in a warmer environment where temperatures were fluctuating between 15^0C and 20^0C indicating that lower temperatures are necessary for onset of a full and timely acclimation in some species before the arrival of cooler seasonal temperatures (Burr and Tinus 1988). Other work with *Betula, Picea,* and *Pinus* have indicated that low temperature was not necessary for induction of acclimation prior to the onset of cooler seasonal temperatures. This same study also suggested a large margin of safety with adequate levels of cold tolerance attained in time to survive upcoming seasonal cold temperatures (Koski 1985). The requirements for induction of acclimation appear to be varied and depend on the taxa and the region and climate where they evolve.

The second stage of acclimation usually occurs at temperatures from 5^0C to below 0^0C with no light required by the plant, with perhaps the exception of some coniferous species (Levitt 1980). Van Huystee et. al. (1967) found the most profound increases in acclimation occurred in this second stage with Red-osier dogwood. Tolerance to temperatures below -40^0C by dogwood acclimated in controlled temperature environments was also shown in this experiment (Van Huystee et. al. 1967). In other work with Red-osier dogwood, an increase in the acclimation rate occurred during the second stage of acclimation where the minimum survival temperature went from 0^0C to -50^0C over a 7 day period (Li et. al. 1965). In this stage, frost may be an essential element in the acclimation process (Quamme &Stushnoff 1983, Weiser 1970). Short photoperiod alone will not induce this stage of acclimation (Sakai & Larcher 1987).

Temperature ranges where acclimation occurs most readily have been the subject of investigation. Previous work with dogwood, cabbage, and English ivy has shown that acclimation can be induced under artificial conditions to the same extent as when under natural conditions (Levitt 1980). Controlled quantitative studies by Gay & Engles (1991) indicated that acclimation in *Lolium* occurs over a finite temperature range. In work with Red-osier dogwood, a woody species, maximum acclimation rates occurred between 5^0C and 20^0C. It was also found from this same study with dogwood that the fastest rates of acclimation occurred at full rest (Kobayashi 1983).

Differences in lowest temperature survival may exist between two parts or tissues in the same plant (Weiser 1970). Work with apple by Tumanov and others has shown differential acclimation between roots and shoots on the same plant. The root tissue which was less acclimated, was shown to have the same low temperature survival potential after exposing roots to air and light for 3-5 months prior to the seasonal acclimation period (Levitt 1980). This work suggests that cold acclimation may be an independent expression of modular units within a plant to a degree as well as a translocatable phenomenon.

The timing and extent of low temperature survival are both essential factors in the survival of plants to seasonal cold stress (Junttila 1989, Koski 1985) and may very well be adaptations that insure survival in a given region. The degree of acclimation attained at any given time may be in good agreement with short term fluctuations in temperature (Sakai & Larcher 1987, Kobayoshi et. al.1983) but, the extent of low temperature survival that can be attained by a plant at a given time of the year may also depend on its growth stage (Van Huystee et. al. 1967). Work with *Betula pubescens* Ehrh., *Picea abies* L., and *Pinus sylvestris* L. suggest that fluctuations in temperature may only modify the extent of acclimation and that the annual trend in acclimation may remain largely unchanged (Koski, 1985). Regional differences in the timing and rate of acclimation and extent of low temperature survival have been shown within a single species (Weiser 1970). Work with *Acer rubrum* L. showed slower acclimation and less cold tolerance in southern cultivars than those found farther north (Linstrom & Dirr 1989).

Alternating temperatures in controlled environments have been used to simulate outdoor conditions in several experiments involving a number of plant species. It has been found in recent work with lentil, that a fluctuation in temperature between 0^0C and 10^0C every 12 hours with a photo period of 10 hours was most conducive in promoting cold hardiness in these plants (Ali 1994). If cool temperature is needed to induce acclimation in a majority of hardy plants and acclimation is an energy driven process, it is possible that fluctuating temperatures may be conducive to the acclimation process in many species.

2.1.3 The cold tolerant state and de-acclimation

The retention of the low temperature tolerant state in some plants has proven hard to explain in terms of dormant and non-dormant stages or temperature exposures alone. Plants have also been shown to lose acclimation if subjected to temperatures for prolonged periods of time that originally hardened them (Olien 1967). De-acclimation occurred in Red-osier dogwood at temperatures in the range of 7^0C to 20^0C; these same plants had originally started to acclimate at temperatures in the range of 5^0C to 20^0C (Kobayashi et. al. 1983).

Some coniferous species have been shown to de-acclimate upon exposure to warm temperature even when acclimating in the fall (Burr et. al. 1989) suggesting that the findings by Koski (1985) may not be true of all plants. Work with dogwood has shown that great amounts of cold tolerance can be retained even after the seasonal rest stage in the plant had passed (Levitt 1980). There have been similar findings with flower buds of saskatoon-berry in February and March where these buds where not dormant and still had considerable frost tolerance (Junttila et. al. 1983). Red-osier dogwood has also been found to have a lower rate of de-acclimation when compared with other taxa (Kobayashi et. al. 1983), and some degree of frost tolerance is retained year-round in bark tissue of this species (Van Huystee et. al. 1967).

Guy & Engles (1991) demonstrated in *Lolium*, that plants in a de-acclimating condition are less cold-tolerant when growing in any given temperature environment, than are plants in an acclimating condition growing in the same given temperature. De-acclamation is primarily a response to increasing temperatures (Juntilla 1989) but may also be strongly linked to genotype and the stage of plant growth (Kobayashi et. al. 1983, Koski 1985).

2.1.4 Testing Acclimation

Field testing is the ideal test of a plant for cold survival. However, this is also an unreliable method that requires much provision in the way of time, money and space. Outdoor tests should be done over a period of at least two years (Karam & Sullivan 1991). Many methods have been attempted to determine the limit of low temperature tolerance under controlled environments. Step-down freezing of whole plants or plant parts is common to many such tests with assessment of injury being subject to different methods of determination. Whole plant freeze tests can be highly reliable but are labor intensive, require large chambers, are destructive of whole plants, do not immediately yield results, and although they are accurate, they are not very precise (Burr et. al. 1986, 1990).

Working with plant parts affords convenience, but differences in lowest survival temperatures between different plant parts may exist. Work with coniferous species by Burr and others (1986) found that there was greater cold tolerance in stems than in needles or buds. This difference in acclimation between above ground parts may also be observed in other hardy taxa (Weiser 1970).

Rate of change in temperature is a very important element that determines the lowest survival temperature of a plant or plant tissue. In work with lentils, 2^0C/hr or 3^0C/hr cooling rates were shown to be best for separating hardy and non-hardy lines. Faster rates stressed all the lines to a degree where no distinction could be made between them, and while slower rates enabled less hardy lines to survive lower temperatures, and again made separation difficult (Ali 1994). Very hardy species in highly cold tolerant states were found to be tolerant of temperatures down to -196^0C, but had to cool from 0^0C to -30^0C at a very slow rate before this could be achieved (Weiser 1970).

There are many methods of assessing freeze injury in plant tissue. Some of these methods have the advantage of being less subjective and more quantitative; some have the advantage of making large sample sizes practical, and some have advantages of yielding results in a short order of time while others may take months before any certainty of results is attained (Purcell & Young 1963). Some plant species do not yield reliable data when certain methods are employed and so the necessity of utilizing other perhaps less desirable methods of assessing injury is presented.

Electrolyte leakage and visual examination of oxidative browning are two methods that have been reported to be reliable in assessing freeze injury in woody plant tissues (Burr et. al. 1986, Purcell & Young 1963, Li et. al. 1965, Wilner 1961) and were used in this study. Previous work with Red-osier dogwood has shown injured tissues to become soft and brown and oftentimes covered by fungal hyphae when kept at ambient room temperature for 4 days (Li et. al. 1965). Though visual examination of oxidative browning has been shown to be a reliable indicator of freeze injury, recorded results of visual observations can vary between observers. There does tend to be less variability among observations as the observers in question gain more experience (Purcell & Young 1963).

Increased permeability in cell membranes and increased electrical conductivity in injured tissue were well-known concepts prior to the 1930's. Theories pertaining to plant hardiness at that time, evolved around the concept of plant water relations and that structural, osmotic, or colloidal protection from water withdrawn due to freezing was presumed to be the basis for the extent of cold tolerance. One method of determining injury is through the measurement of electrolytes that are exuded from plant tissues. Conductivity is proportional to the loss of semipermeability of cell membranes and the amount of damage to tissue incurred by cold stress (Dexter et. al. 1932). Cell lysing may take place in plant cells at the killing temperature (Burke & Stushnoff 1979). In such a case, a substantial increase in measured electrical conductivity should be present in tissues or leakage from tissues subjected to killing temperatures (Burke & Stushnoff 1979). Burr and others found an agreement between whole plant freeze tests and electrolyte leakage in stems of coniferous tree species (Burr et. al. 1986). Work with apple found that electrical conductivity and electrical resistance were both good methods of assessing freeze injury (Wilner 1961). Obtaining numerical values that represent tissue damage in a non-subjective way make the application of this concept of electrical conductivity or electrolyte leakage an attractive way to obtain data (Dexter et. al. 1932). However, this proportional relationship must be calibrated to the actual extent of injury or amount of recovery by the plant tissues form the stress imposed (Dexter et. al. 1932, Burr et. al. 1986). Work by Dexter and others in 1932 on alfalfa, Red clover , and 30 varieties of small grains found electrical conductivity to be useful in separating plants by degrees of cold tolerance. However, extent of damage in plants possessing different degrees of cold tolerance could not be differentiated once they had all been subjected to temperatures well below their lowest tolerable temperature. Fermentation in tissues was also listed by Dexter et. al. (1932) as a factor that may interfere with obtaining good results using these methods.

2.2 OBJECTIVES

The objectives of this study are to investigate the seasonal acclimation responses in dormant twigs of two hardy woody taxa when placed under five controlled temperature environments including two fluctuating regimes where one involves a freezing temperature and the other does not. Little has been published in the literature pertaining to the effects of fluctuating temperatures in contrast to constant temperatures. This study is an attempt to address some aspects of this issue. Also to be assessed is the reliability of two methods to determine injury in freeze-stressed plant tissues. These methods are electrolyte leakage by measurement of electrical conductivity and visual inspection of oxidative browning.

2.3 MATERIALS AND METHODS

Twigs of current season's growth from Red-osier Dogwood (*Cornus sericea* L.) and Red Lake Currant (*Ribes rubrum* L.) were collected at the beginning of each month from plants growing on the Colorado State University W. D. Holley Plant Environmental Research Center facility in Fort Collins, CO and placed in controlled temperature environments with no lighting for approximately 30 days. This experiment was performed from the months of October 1992 through March 1993, and from September 1993 through March 1994. Controlled temperature environments included three constant temperatures: -5°C, 10°C, and 0°C and two alternating temperatures between -5°C and 10°C and between 0°C and 10°C where temperatures alternated every twelve hours by automatic controllers. Constant temperatures were provided by two refrigerators and one chest freezer. Two of these constant temperature units were regulated by Omega BS 5001 T3 (Omega Engineering Inc. Stamford, CT) controllers operating plumbing heater cable. A refrigerator was used to provide a constant 0°C environment, and was not equipped with a controller. The two fluctuating temperature regimes were provided by two refrigerated growth cabinets with controlled temperature fluctuation capability. The unit used for the 0^{0}C/10^{0}C regime was a Freas 818 (Precision Scientific Co. Chicago, IL) and the unit used for the -5^{0}/10^{0}C was a Percival model I-35 LLVL, (Percival MFG Co. Boone, IA).

Collected twig specimens were wrapped in moist paper toweling and sealed inside plastic bags before being placed inside each of the five chambers. Samples at the beginning of September and October had not yet undergone leaf senescence. Leaves where cut off 1/4" to 1/2" from the base of the petiole. Several specimens collected in the field were also placed in a step down-freezer within 2 hours after collection and evaluated for lowest temperature survival using oxidative browning and electrolyte leakage methods, as were the treated samples after the approximate 30-day duration in one of the five controlled temperature environments. Samples collected at the beginning of each month and subjected to lowest temperature survival testing with no treatment are referred to as the 'original' for that month and as the 'field' treatment for the previous month. These samples served as a reference to compare treated samples with the plant material in the field.

Low temperature survival testing was performed using one of three step-down freezers: Tenny Jr. (Tenny Engineering Corp. Union, NJ), liquid nitrogen operated cooling chamber (Quamme et. al. 1972), or Revco UTL 1490 D-N-P deep-freeze (Rhem MFG. Asheville, NC) with a temperature controlled compartment equipped with air circulation operated by an Omega series CN-2010 controller (Omega Engineering Inc. Stamford, CT.) operating a plumbing heat cable. Step-down freezing units were programmed to decrease from 0°C to a predetermined set point at 5°C/hr. This rate of decrease in temperature was chosen because it was deemed to be slow enough so as not to cause stress due to excessively rapid cooling rates based on past experience and yet it allowed experiments to be performed in a feasible length of time. Specimens were prepared for this procedure by wrapping a minimum of five twig samples with each twig having at least one bud in moist paper toweling to prevent drying and provide external ice nuclei, and sealing them in a plastic bag. One such preparation to serve as a control for each species and treatment was not subjected to step-down freezing. An identical preparation for each species and treatment was provided for each temperature at which they were to be tested. Three or four temperatures at 10°C intervals were accommodated in this manner in an effort to bracket the lowest survival temperature. Specimens were withdrawn from the step-down freezer after having reached their designated test temperature and placed at ambient room temperature of approximately 22°C for a period of approximately 10 days before oxidative browning and electrolyte leakage methods where used to assess low temperature injury and survival.

Data from oxidative browning was taken visually by using a scoring method to rate each sample as to the degree of injury it had sustained. A bud from each stem was removed as shown in Figure 1.1.

Figure 1.1.

Data were recorded as a score from 0 to 4 with '0' indicating no browning and a score of 4 indicating fullest extent of browning. The scoring of injury was done as shown in figures 1.2 though 1.6.

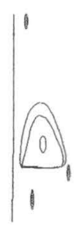

Figure 1.2. A score of (0): No observable browning. Cortical tissue is light green and woody tissue is light tan in color.

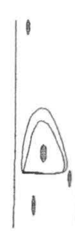

Figure 1.3. A score of (1): Over all appearance is similar to (0) with the exception of oxidative browning present in bud xylem tissue.

Figure 1.4. A score of (2): Oxidative browning has extended beyond the periphery of bud xylem area into surrounding secondary xylem tissue.

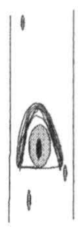

Figure 1.5. A score of (3): Discoloration of cortex is evident and tending toward olive or drab green in color. All tissue in sample was assumed dead.

Figure 1.6. A score of (4): Sample displays browning in all tissues. More extensive damage to tissues is indicated than those scored as a three.

The visual evaluation of oxidative browning was set up as a completely randomized design experiment. Data from visual rating of specimens was analyzed using the Spearman-Kärber method to determine the temperature lethal to 50% of the samples or LT_{50}. The following equation was used to estimate the variance (S) where:

$$S=\frac{d}{n^2(n-1)}[b_i(n_i-b_i)]$$

d = temperature interval in ^{0}C between treatments

n = number of samples in each treatment

b = number of injured or dead samples in each treatment

The standard error was the calculated by dividing S by the square root of the sample number or :

$$\frac{S}{\sqrt{n}}$$

The equation used to calculate variance is used where *n* is constant. In this experiment, $n = 5$ (Brittinbender and Howell 1974).

A score rating of (3 or 4) was used as the criterion of a completely killed sample in using the Spearman-Kärber method. The Spearmen-Kärber method was also used to calculate the T_{50} at which other degrees of injuries occurred so that comparisons between treatments could be made if the lethal temperature was not successfully bracketed and so a range of numbers could be provided for correlation computations with chemical analysis in another part of this experiment. All scores included the differentiation between a score of 3 and 4 so that this data could be analyzed for correlation with data obtained from electrolyte leakage.

Buds removed during visual scoring were placed in a cell of a tray containing 2 ml of de-ionized water. Each tray contained 100 cells allowing for 100 bud samples to be tested for electrolyte leakage at one time. Electrolyte leakage was measured using a Neogen ASAC 1000-B (Neogen Food Tech Corp., Lansing, MI) seed analyzer. The parameters used in this experiment were 4 volts and a baud rate setting of 9600. Measurements were taken every ten minutes for the first 2 hours and every 30 minutes for the next 2 hours with the final measurement being taken at the end of 4 hours. The measurement at the end of four hours was chosen to calculate leakage percent. After measurements for electrolyte leakage had been taken, cell trays were placed in a freezer set at -70°C to kill and store samples before a second single measurement was taken. During the colder months of the year when samples were predicted to have a very low survival temperature, samples were placed in an autoclave for 1 minute at 100°C at 15 psi. to insure complete death of tissues. This was done by moving each bud with a pair of clean forceps to porcelain tiles with pockets corresponding to cells in the plastic leakage tray. A cover was firmly placed over the pockets to insure buds would not be lodged out of pockets during autoclaving. Autoclaving was limited to 1 minute as a precaution against prolonged boiling of cell contents and subsequent loss of electrolytes from tissue that could result in experimental error. Samples where allowed to set at room temperature (22°C) for 3 hours after thawing or autoclaving before a single electrolyte leakage measurement was taken.

The leakage experiment was set up as a completely randomized design. Five replications of each treatment and each of the two species were performed. Data for each test sample were expressed as a percent of the total leakage when completely killed. Percent leakage = (E initial/ E killed) x 100, where E initial is the conductivity reading after the sample had been subjected to a given temperature in the step-down freezing process, and "E killed" is the conductivity of a completely killed sample.

2.4 RESULTS AND DISCUSSION

2.4.1 Electrolyte leakage Results and discussion

Results from the electrolyte leakage data were not satisfactory (Table 2.1). Linear regression analysis revealed a very poor relationship between data from oxidative browning and electrolyte leakage for both Red-osier dogwood and Red Lake currant in both years. There was also a high degree of variability in the leakage data between replications indicating none of the precision that was observed by Burr et. al.

(1986) while working with coniferous species. Explanations for the unreliable data may include bud size, microbial contamination, and experimental error due to the instrument as well as human error.

The buds of dogwood and currant are relativity small when compared to the buds of other woody plants such as apple and conifers (Burr et. al. 1986, Wilner 1961) where good results have been documented. This difference in size may mean a difference in surface to volume ratio such that any differences in conductivity due to loss of semi-permeability in membranes may have been obscured by the conductivity afforded by residues present on surfaces of bud scales. The percent leakage of these samples was measured over a period of time and a uniform increase in electrolytes into the surrounding solution was shown indicating that all electrolytes originated from the bud tissue, but whether this was due to loss of semipermeability of the cell membranes or artifacts such as the liberation of compounds from surfaces of bud scales is not clear. Contamination due to microbial growth is another possibility for unreliable results. Because of the limited amount of plant material, time, equipment, and other resources, the same buds excised from stems in the visual evaluation were then placed into cells to measure leakage of electrolytes. Since these samples were in a high humidity environment for a period of about 10 days, significant microbial growth was possible. Many of the heavily damaged samples in visual observations were often covered by hyphae of fungi just as described by Li et. al. (1965). While this fungi was useful as a visual indicator of death, its presence may have had undesirable affects on the electrolyte leakage data. The problems of microbial contamination have been mentioned by other researchers using this technique (Dexter et. al. 1932). Although there may be many causes for the poor reliability of the results in this portion of the experiment, the use of the technique for evaluation of hardiness on Red Lake currant and Red-osier dogwood in future experiments is not recommended, especially when considering that this experiment involved an estimated 3000 or more buds over a two-year period where no significant relationship could be found between electrolyte leakage data and visual observations of oxidative browning.

2.4.2 Visual Results

Observations of oxidative browning provided the clearest indications of damage in the less acclimated states with damage being the most easily discernable in the early fall. During mid-winter months, it became harder to distinguish the first signs of damage as the secondary xylem produced from the previous year's growth began to slightly darken in both dogwood and currant species. More cold-tolerant tissues in mid-winter months generally displayed greater differentiation in hardiness between tissues within an individual sample. Hardiness in tested samples often exceeded the predetermined range of temperatures that were to be tested and a less extensive degree of damage was used as a basis of comparison between treatments in these cases. Lethal T_{50} for some samples during midwinter exceeded -70^{0}C to a degree where no accurate estimation was possible.

Dogwood and currant collected at the beginning of September 1993 both had a lethal T_{50} of -15^{0}C. All samples survived month-long treatments with the exception of current which was treated at -5^{0}C. Remaining portions of leaf petioles could be cleanly broken with little effort at the abscission layer in all treatments except the -5^{0}C treatment while samples in the field still had leaves present on them. Portions of remaining petioles on samples of -5^{0}C treatment were soft and water-soaked in appearance and did not readily break from stems at the abscission layer. The calculated lethal T_{50} for all but one of the treatments at the end of September 1993 was not obtained due to extraneous circumstances at the time of the freeze test. The lethal T_{50} temperatures for these samples is known to be above -20^{0}C. The exception was dogwood with the constant 0^{0}C treatment which had a lethal T_{50} of -21^{0}C.

Samples collected at the beginning of October in both years were tolerant of low temperatures in the range from -15^{0}C to -25^{0}C with dogwood showing a greater degree of cold tolerance than currant at this time for both years (Tables 2.2-2.4). Leaves were present on field plants at the beginning of October, but abscised during the month as did samples in all treatments except -5^{0}C, where petioles of both species did not abscise and were similar in appearance to those in September 1993. Freeze testing to -40^{0}C was performed at the end of October. Alternating treatments and samples collected at the beginning of November showed the greatest amount of cold tolerance when compared with lethal T_{50} temperatures of other treatments in both years for currant and in 1993 for dogwood (Tables 2.2-2.4). Data for October 1992 dogwood in the 0^{0}C and 10^{0}C treatments also indicated increased hardiness from time of collection in the field at the beginning of October. Relative comparisons of lethal T_{50} temperatures could not be made on these more acclimated samples because these temperatures were below the lowest temperature tested. Currant stems tested at 0^{0}C/10^{0}C in 1992 acclimated to the extent that there was not even enough injury to estimate a non-lethal T_{50} value using a score of 1 as the criteria (Table 2.4). Samples of this treatment appeared to exceed cold tolerance in the field at that time. October samples having the -5^{0}C treatment with the exception of currant in 1992 displayed some injury and were less cold tolerant than samples having other treatments. Cold tolerance of currant was the same whether treated by either of the fluctuating temperatures or collected form the field. None of the treatments were as effective in acclimating dogwood as field conditions and constant freezing temperature was detrimental to survival of dogwood in October of 1992.

At the end of November 1992, freeze testing was performed down to -40^{0}C, but this was not low enough to obtain lethal T_{50} values. November 1993 data showed that currant treated at -5^{0}C/10^{0}C acclimated below -70^{0}C exceeding cold tolerance achieved under field conditions while all other treatments displayed some acclimation since their collection out in the field (Table 2.5). The -5^{0}C and 0^{0}C/10^{0}C treatments approached the degree of acclimation achieved by field conditions. In dogwood, acclimation was occurring in all treatments, but not to the extent found in field samples. Both species in November of 1993 displayed slightly less cold tolerance in samples with the 10^{0}C treatment than with other treatments, but these still displayed an increase in cold tolerance from the beginning to the end of November in both years.

During December of both years, none of the treated currant samples acclimated to the extent of those in the field with the exception of the -5^{0}C treatment in 1993 (Tables 2.8-2.10). Both currant and dogwood samples from the 10^{0}C and 0^{0}C/10^{0}C treatments appeared to have lost some cold tolerance from time of collection. In both species, the -5^{0}C treatment was similar in hardiness to those samples from the field. Although lethal

T_{50} for samples from the field and the end of November 1992 was not obtained, data from 1993 showed no appreciable gain in cold tolerance from the beginning to the end of December under field conditions.

Slight decreases in cold tolerance occurred under field conditions in January with the exception of a lower lethal T_{50} temperature in currant during January 1994 (Table 2.11). Decreases in hardiness were most evident in 10^0C treatments with currant showing greater loses than dogwood under these conditions. Both the fluctuating treatments suggested a trend of de-acclamation and were less cold tolerant than samples under field conditions. No data was obtained from the 0^0C/10^0C treatment in January of 1994 due to the failure of the heating element in the growth chamber allowing the temperature to plummet from 10^0C to -23^0C in less than an hour resulting in complete death of all samples in that treatment. One increase in acclimation was observed in currant having the 0^0C treatment in 1994, but a loss of cold tolerance was observed with this same treatment on currant in January of the previous year. (Tables 2.11-2.13).

In February of both years, both species indicated a general decrease in hardiness from beginning to end of the month under field conditions (Table 2.14). Retention cold tolerance from beginning to end of the month was evident in both species with the -5^0C treatment in 1994. Samples in fluctuating temperature treatments lost hardiness. Samples of dogwood in 1994 under the -5^0C treatment retained hardiness with a lethal T_{50} that was 10^0C lower than those from field conditions.

In March of both years, lethal T_{50} temperature increased over 10^0C for both species (Table 2.17). Samples tested at -5^0C increased in hardiness with the exception of currant in March 1994. In general, acclimation of currant samples other than the -5^{0}C treatment was similar to those in the field for both years. This trend was true for dogwood in 1993, but all treated samples of dogwood in March of 1994 were more cold tolerant than those in the field at the end of that month.

2.4.3 Discussion of visual results

Linear regression was used to evaluate the agreement between the data for the two years. Only data where a T_{50} could be calculated using the Spearmen-Kärber method was used. This analysis was performed at the three injury levels indicated by Tables 2.2 through 2.19. The r^2 values for dogwood for both years, for injury levels of 1, 2, and (3&4) were 0.217, 0.329, and 0.788 respectively. The same calculations performed on currant data for injury levels of 1, 2, and (3&4) were 0.627, 0.622, and 0.618. These calculations indicate that there is strong agreement between years in dogwood with an identical treatment at the same time of year if only lethal T_{50} or samples injured to score level of 3 or 4 is the basis of comparison. No consistency in results can be expected if lesser degrees of injury are considered in dogwood. However, currant data appeared to be somewhat consistent between the two years regardless of which level of injury was used as the criterion for comparison.

Although injury to tissues was apparent, these visual evaluations were not studied relative to regrowth of whole plants, and thus these results are in no way intended to portray the kind of losses that may be sustained by plants grown commercially. The fact that visual observations are just that, makes them subject to personal bias in evaluating how much injury has been sustained. Although the same observer was used throughout this experiment, the relative degree of experience that the observer had with this plant material changed through the course of the experiment suggesting that earlier observations may not have been as consistent or as reliable as later observations in the second year.

Another problem with this technique is that the correct moisture environment must be provided for the samples during the browning process. In handling of hundreds of sample preparations, it is possible that differential levels of moisture may have surrounded the samples as they were undergoing the oxidative browning process. It is possible that injury was not readily seen because inadequate browning occurred on injured tissue. This can be caused by excessive water content or lack of moisture during browning.

Cold tolerance appeared to increase somewhat in the fall in both species regardless of the treatment with the exception of the -5^{0}C treatment. Some treatments were more conducive to acclimation in early fall than other treatments. This would support the idea presented by Koski (1985) that acclimation is an annual cycle that is modified only by environmental conditions. Acclimation of currant occurred in the -5^{0}C treatment of currant in October of 1992, but was injurious to dogwood that same year and to both species the following year. The -5^{0}C treatment appeared to injure samples early in the fall and did not appear to have an acclimating affect on them except for currant in 1992. The 0^{0}C/10^{0}C treatment worked well in October and compared favorably with hardiness achieved in the field for currant. Work by Ali (1994) with lentils along with these findings would suggest that acclimation is accommodated well by a 0^{0}C/10^{0}C treatment.

The 0^0C treatment proved to be conducive to acclimation, and with dogwood October 1992, it proved more conducive than the 0^0C/10^0C treatment (Table 2.2-2.4). Samples responded similarly to the -5^0C/10^0C treatment as they did with the 0^0C/10^0C treatment with regard to lethal T_{50}, but injuries at higher temperatures occurred more readily with this treatment during October. This finding along with the injuries observed in the -5^0C treatments would suggest that frost exposure too early in the growing season is not conducive to acclimation and can be detrimental to plant survival when exposure is prolonged. The observation of the 10^0C treatment, less conducive than some of the other treatments, would also indicate that while excessive frost exposure can be detrimental, low temperatures are still needed to promote acclimation as indicated by studies presented in Levitt (1980).

Dogwood did not acclimate as much under any of the controlled conditions in October as it did in the field (Tables 2.2-2.4). Since these samples retained their leaves at the time of collection, there is a likelihood that they may have been in the first stage of acclimation where more light would have been beneficial to the acclimation process as discussed by Levitt (1980) and Van Huystee et. al. (1967). These observations may also suggest that currant may not be as dependent as dogwood on presence of light during the first stage of acclimation, or that the two species were at a different level of progression in acclimation at the time of collection. In November, all samples increased in cold tolerance and indicate that the findings of Koski (1985) may be partly true of currant and dogwood. Freezing treatments in no way hurt any of the samples in November (Tables 2.5-2.7). In November, 1993, dogwood, the -5^0C treatment showed the greatest amount of cold tolerance of all treatments with dogwood. In November 1993 the -5^{0}C/10^{0}C treatment promoted cold tolerance of currant that exceeded all treatments of both species, including samples from the field. These observations would indicate that these samples were in or could be induced into the second stage of acclimation as described by Levitt (1980) and that freezing temperature may have played a role in increased acclimation found in samples exposed to treatments with freezing temperatures, as well as those samples found out in the field as mentioned by Quammie & Stushnoff (1983) and Weiser (1970).

By December, samples had already demonstrated the ability to de-acclimate. In the 10^{0}C treatment, there was a decrease in cold tolerance from levels found in field samples; where in November, there was a gain in cold tolerance from time of collection in the field. The greatest cold tolerance was indicated by those samples having the -5^{0}C treatment. This would indicate that as a woody plant becomes more acclimated, increased frequencies with and longer durations of below freezing temperature become necessary to promote increased cold tolerance.

A decrease in cold tolerance from the beginning to the end of January was found in field samples, with the exception of an increase in January 1993 with currant. Most treated samples also indicated a general decline in cold tolerance. The best retention of cold tolerance in January was from the -5^{0}C and 0^{0}C treatments which would indicate that stable low temperatures are most effective at retaining cold tolerance. Greater retention of cold tolerance was shown in January with dogwood than with currant with the 10^{0}C treatment confirming findings of other workers about the relatively slow rates of de-acclimation found in dogwood (Kobayashi et. al.1983).

A continued decrease in cold tolerance was observed in the field between the beginning and end of February. The slower rate of de-acclimation in dogwood than in currant when subjected to the 10^0C treatment was more evident in February than in January. Fluctuating treatments indicated some loss of cold tolerance and there was little difference between the -5^0C/10^0C treatment and the 0^0C/10^0C treatment confirming claims by Olien (1967) that acclimation can be lost under conditions that had originally acclimated them. The best retention of cold tolerance was found in the 0^0C and -5^0C treatments, but the 0^0C treatment was not as affective as it was the previous month. Samples in the -5^0C treatments actually displayed increased acclimation from time of collection (Tables 2.14-2.16). Similar trends were observed in March.

It would appear that fluctuating temperatures in the fall may be important in the promotion of acclimation because they afford the stimulus needed to induce acclimation while providing intermittent heat needed to drive metabolic pathways that produce changes that increase low temperature tolerance. As tissues become more acclimated, the need for below-freezing temperature for further acclimation becomes apparent. As the season progresses and plants have gone though the rest stage, the need for continuous cool temperatures becomes apparent for retention of acclimation. This is not to say that a plant can not regain some acclimation after the rest stage, but that low temperatures like those of the -5^0C and 0^0C treatments appeared to be more conducive to acclimation retention over a month-long period in February and March. While fluctuating regimes that include these two temperatures and a warmer temperature like 10^{0}C are not as effective in causing retention of acclimation.

2.5 SUMMARY

Low temperature tolerance was induced under controlled conditions using above freezing temperatures in October. As the season progressed into November, cold tolerance was further increased with temperature regimes that included a freezing temperature. Fluctuations in temperature promoted acclimation in the first and second stages of acclimation. Cold tolerance in mid-winter and early spring months was best retained by steady cold temperatures. Regimes that originally were conducive to acclimation in the fall months were not as effective at retaining cold tolerance over a period of time. The electrolyte leakage method for assessing freeze injury was not satisfactory in experiments with Red-osier dogwood or Red Lake currant. Visual observations of oxidative browning work well, but are best done by experienced observers. Careful attention must also be paid to maintaining the correct environment for oxidative browning to occur. As with all freeze tests, a liberal range of temperatures with a low-end temperature sufficient to kill all samples should be used if possible.

Table 2.1. Correlation coefficient r^2 between visual ratings and percent leakage.

	September	October	November	December	January	February	March
92/93 Currant		0.21	0.27	0.21	0.15	0.03	0.04
93/94 Currant	0.48	0.49	0.22	0.52	0.25	0.09	0.32
92/93 Dogwood		0.2	0.13	0.37	0	0.03	0.01
93/94 Dogood	0.38	0.28	0.01	0.01	0.04	0	0

Table 2.2. T_{50} temperatures for October Samples with injury score of 3 or 4.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-15°C	> -20°C	-25°C	-22±0.2°C
0°C	-33±0.2°C	-25°C	< -40°C	-31±0.3°C
10°C	-25°C	-25°C	< -40°C	-29±0.3°C
-5°C	-37°C	-29±0.4°C	Injured	-29±0.3°C
0°C / 10°C	< -40°C	-35°C	< -40°C	-33±0.2°C
-5°C / 10°C	< -40°C	-35°C	< -40°C	-33±0.2°C
Field	< -40°C	-35°C	< -40°C	-35°C

Table 2.3. T_{50} temperatures for October Samples with injury score of 2 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-15°C	> -20°C	-25°C	> -20°C
0°C	-25°C	-25°C	< -40°C	-25°C
10°C	-23±0.2°C	-23±0.2°C	< -40°C	-23±0.2°C
-5°C	-31±0.3°C	-11±0.3°C	Injured	-17±0.3°C
0°C / 10°C	< -40°C	-35°C	< -40°C	-25°C
-5°C / 10°C	-27±0.4°C	-35°C	< -40°C	-25°C
Field	-27±0.2°C	-35°C	< -40°C	-31°C

Table 2.4. T_{50} temperatures for October Samples with injury score of 1 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-13±0.2°C	> -20°C	-25°C	> -20°C
0°C	-25°C	-25°C	-29±0.3°C	-19±0.4°C
10°C	-19±1.3°C	-17±0.2°C	-35±0.3°C	-15°C
-5°C	-21±0.4°C	injured	injured	injured
0°C / 10°C	< -40°C	-29±0.3°C	-29±0.4°C	-23±0.2°C
-5°C / 10°C	-23±0.5°C	-27±0.2°C	-27±0.2°C	-15±0.5°C
Field	-25°C	-21±0.4°C	-27±0.4°C	-27±0.2°C

Table 2.5. T_{50} temperatures for November Samples with injury score of 3 or 4.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	< -40°C	-35°C	< -40°C	-35°C
0°C	< -40°C	-45°C	< -40°C	-55°C
10°C	< -40°C	-43±0.3°C	< -40°C	-49±0.2°C
-5°C	< -40°C	-47±0.3°C	< -40°C	-57±0.2°C
0°C / 10°C	< -40°C	-47±0.2°C	< -40°C	-47±0.4°C
-5°C / 10°C	< -40°C	< -70°C	< -40°C	-57°C
Field	< -40°C	-49±0.3°C	< -40°C	-65°C

Table 2.6. T_{50} temperatures for November Samples with injury score of 2 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-27±0.2°C	-35±0.3°C	< -40°C	-31°C
0°C	< -40°C	-45°C	< -40°C	-49±0.3°C
10°C	< -40°C	-37±0.2°C	< -40°C	> -30°C
-5°C	-31±0.3°C	est. -39°C	est. -37°C	-51±0.3°C
0°C / 10°C	< -40°C	> -40°C	< -40°C	est. -37°C
-5°C / 10°C	< -40°C	< -70°C	< -40°C	< -40°C
Field	< -40°C	-43±0.2°C	< -40°C	-55°C

Table 2.7. T_{50} temperatures for November Samples with injury score of 1 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-25°C	-21±0.4°C	-27±0.4°C	-27±0.2°C
0°C	-31±0.3°C	-43±0.2°C	-33±0.5°C	-37±0.2°C
10°C	-25°C	-31±0.3°C	-35±0.5°C	> -30°C
-5°C	injured	-35°C	-23±0.4°C	-39±0.3°C
0°C / 10°C	-37±0.4°C	> -40°C	< -40°C	> -40°C
-5°C / 10°C	< -40°C	-54±0.5°C	< -40°C	> -40°C
Field	< -40°C	-37±0.2°C	< -40°C	-43±0.5°C

Table 2.8. T_{50} temperatures for December Samples with injury score of 3 or 4.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	< -40°C	-49±0.3°C	< -40°C	-65°C
0°C	-55°C	-50°C	-55°C	-61±0.3°C
10°C	-45°C	-37±0.2°C	> -40°C	< -50°C
-5°C	-55°C	-63±0.2°C	est. -61°C	< -70°C
0°C / 10°C	-51±0.3°C	-45°C	-41±0.4°C	< -50°C
-5°C / 10°C	-53±0.2°C	-47±0.2°C	-53±0.2°C	< -70°C
Field	-59±0.2°C	-51±0.3°C	< -60°C	< -70°C

Table 2.9. T_{50} temperatures for December Samples with injury score of 2 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	< -40^{0}C	< -40^{0}C	< -40^{0}C	-55^{0}C
0^{0}C	-51±0.3^{0}C	>-40^{0}C	-51±0.3^{0}C	-51±0.3^{0}C
10^{0}C	-45^{0}C	-35^{0}C	> -40^{0}C	-35^{0}C
-5^{0}C	-55^{0}C	-55^{0}C	-53±0.2^{0}C	-49±0.3^{0}C
0^{0}C / 10^{0}C	-49±0.3^{0}C	-41±0.2^{0}C	-43±0.5^{0}C	-33±0.3^{0}C
-5^{0}C / 10^{0}C	-51±0.3^{0}C	-57±0.3^{0}C	>-40^{0}C	-43±0.2^{0}C
Field	-55^{0}C	-49±0.2^{0}C	-53±0.2^{0}C	-59±0.3^{0}C

Table 2.10: T_{50} temperatures for December Samples with injury score of 1 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	<-40°C	-37±0.2°C	< -40°C	-43±0.5°C
0°C	-41±0.4°C	> -40°C	-39±0.3°C	-37±0.2°C
10°C	-43±0.2°C	-33±0.2°C	> -40°C	-29±0.3°C
-5°C	-53±0.2°C	-45±0.4°C	-47±0.4°C	-37±0.2°C
0°C / 10°C	-47±0.2°C	-31±0.4°C	> -40°C	-23±0.3°C
-5°C / 10°C	-47±0.4°C	-39±0.3°C	> -40°C	-43±0.4°C
Field	-49±0.4°C	-43±0.2°C	-45±0.5°C	-41±0.2°C

Table 2.11. T_{50} temperatures for January Samples with injury score of 3 or 4.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-59±0.3°C	-51°C	< -60°C	< -70°C
0°C	-41±0.3°C	-65°C	-51±0.2°C	< -60°C
10°C	> -40°C	-37°C	-51±0.3°C	-49±0.3°C
-5°C	-53±0.4°C	-55±0.3°C	-51±0.3°C	-63±0.2°C
0°C / 10°C	-47±0.3°C	Dead	< -60°C	Dead
-5°C / 10°C	-47±0.2°C	-55°C	-55°C	-59±0.3°C
Field	-55°C	-59±0.3°C	-59±0.3°C	-63±°C

Table 2.12. T_{50} temperatures for January Samples with injury score of 2 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-55°C	-49±0.3°C	-53±0.2°C	-59±0.3°C
0°C	> -40	-55°C	> -40°C	-43±0.4°C
10°C	> -40	-35°C	> -40°C	-43±0.5°C
-5°C	-51±0.4°C	-51±0.3°C	-41±0.3°C	-55±0.3°C
0°C / 10°C	-45°C	Killed	-51±0.3°C	Killed
-5°C / 10°C	-43±0.2°C	-47±0.2°C	-51±0.3°C	-47±0.2°C
Field	-53±0.2°C	-47±0.2°C	-51±0.3°C	-51±0.3°C

Table 2.13. T_{50} temperatures for January Samples with injury score of 1 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-49±0.4°C	-43±0.2°C	-45±0.5°C	-41±0.2°C
0°C	> -40°C	-39±0.3°C	> -40°C	-35°C
10°C	> -40°C	-29±0.3°C	> -40°C	> -30°C
-5°C	-49±0.3°C	-43±0.2°C	> -40°C	-39±0.4°C
0°C / 10°C	-41±0.3°C	Dead	> -40°C	Dead
-5°C / 10°C	-39±0.3°C	-41±0.3°C	-41±0.3°C	-37±0.4°C
Field	-47±0.2°C	-43±0.4°C	-37±0.8°C	-45°C

Table 2.14. T_{50} temperatures for February Samples with injury score of 3 or 4.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-55°C	-59±0.3°C	-59±0.3°C	-63±0.2°C
0°C	-47±0.3°C	-45°C	< -50°C	-59±0.3°C
10°C	-35±0.5°C	-35°C	-39±0.3°C	-41±0.3°C
-5°C	< -50°C	-57±0.2°C	< -50°C	-65°C
0°C / 10°C	< -50°C	-45°C	< -50°C	-45°C
-5°C / 10°C	< -50°C	-41±0.3°C	< -50°C	-53°C
Field	< -50°C	-47±0.2°C	< -50°C	-49±0.2°C

Table 2.15. T_{50} temperatures for February Samples with injury score of 2 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-53 ± 0.2^oC	-47 ± 0.2^oC	-51 ± 0.3^oC	-51 ± 0.3^oC
0^oC	-39 ± 0.3^oC	-45^oC	$< -50^oC$	-49 ± 0.3^oC
10^oC	$> -30^oC$	-35^oC	$> -30^oC$	-31 ± 0.3^oC
-5^oC	-45 ± 0.3^oC	-47 ± 0.3^oC	$< -50^oC$	-55^oC
0^oC / 10^oC	est. -45^oC	-43 ± 0.2^oC	-37 ± 0.2^oC	-45^oC
-5^oC / 10^oC	-45^oC	-39 ± 0.3^oC	-37 ± 0.3^oC	-45 ± 0.2^oC
Field	-45^oC	$> -40^oC$	$< -50^oC$	-45^oC

Table 2.16. T_{50} temperatures for February Samples with injury score of 1 or more

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-47±0.2°C	-43±0.4°C	-37±0.8°C	-45°C
0°C	-33±0.2°C	-39±0.3°C	-39±0.3°C	-43±0.2°C
10°C	> -30°C	-33±0.3°C	> -30°C	> -30°C
-5°C	-35±0.5°C	-43±0.4°C	< -50°C	-49±0.4°C
0°C / 10°C	-33±0.5°C	-39±0.3°C	> -30°C	-39±0.3°C
-5°C / 10°C	-35±0.5°C	-37±0.4°C	-33±0.2°C	> -40°C
Field	-41±0.3°C	> -40°C	-43±0.2°C	-41±0.3°C

Table 2.17. T_{50} temperatures for March Samples with injury score of 3 or 4.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	< -50°C	-47±0.2°C	< -50°C	-49±0.3°C
0°C	-39±0.3°C	> -40°C	< -50°C	-61±0.3°C
10°C	-41±0.3°C	-31±0.3°C	-35°C	-45°C
-5°C	< -50°C	-51±0.2°C	< -50°C	-61±0.3°C
0°C / 10°C	-45°C	-35°C	-43±0.2°C	-53±0.2°C
-5°C / 10°C	<-50°C	-35°C	-43±0.2°C	-47°C
Field	-43±0.3°C	-35°C	-43±0.2°C	-35°C

Table 2.18. T_{50} temperatures for March Samples with injury score of 2 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-45±0.3°C	> -40°C	< -50°C	-45°C
0°C	-35°C	> -40°C	-39±0.2°C	-49±0.3°C
10°C	-39±0.3°C	-39±0.3°C	-31±0.3°C	-39±0.3°C
-5°C	< -50°C	-49±0.3°C	< -50°C	-43±0.2°C
0°C / 10°C	-43±0.2°C	-31±0.3°C	-35±0.2°C	-45°C
-5°C / 10°C	-39±0.2°C	-35°C	-37±0.4°C	-43±0.3°C
Field	-39±0.3°C	-33±0.2°C	-37±0.3°C	-35°C

Table 2.19. T_{50} temperatures for March Samples with injury score of 1 or more.

	92/93 Currant	93/94 Currant	92/93 Dogwood	93/94 Dogwood
Original	-41±0.3°C	> -40°C	-43±0.2°C	-41±0.3°C
0°C	-33±0.2°C	> -40°C	-37±0.2°C	-37±0.2°C
10°C	-35°C	-23±0.4°C	-27±0.2°C	-35°C
-5°C	-43±0.5°C	-43±0.2°C	-49±0.3°C	-39±0.3°C
0°C / 10°C	-35°C	-29±0.3°C	-27±0.2°C	-37±0.5°C
-5°C / 10°C	-33±0.3°C	-33±0.2°C	-29±0.3°C	-39±0.3°C
Field	-33±0.2°C	-33±0.2°C	-25°C	-31±0.3°C

Chapter Three

THE EFFECT OF CONTROLLED TEMPERATURE TREATMENTS ON ENDOGENOUS SUGAR CONTENT IN CORTICAL TISSUE IN DORMANT TWIGS OF 'RED LAKE' CURRANT AND RED-OSIER DOGWOOD

3.1 INTRODUCTION AND REVIEW OF LITERATURE

3.1.1 Overview

The search for mechanisms that enable plants to tolerate cold winter temperatures dates back before the turn of the century (Sakai 1962). Although no single factor has been singled out as a primary reason why plant cells have been found to survive freezing stress, several mechanisms and combinations of these mechanisms have been suggested. Chemical and structural changes within the living cell that impart cold tolerance to plants have been implicated by many studies over the past several decades and the total number of factors in freezing tolerance is still unknown (Levitt 1980). Much study has focused on various compounds, called compatible osmolytes, found within living cells suspected of being protective agents to cellular components against the catastrophic events caused by formation of ice crystals.

3.1.2 Implication of sugars in cryoprotection

In general, cell sap increases with total solutes as cold tolerance increases. This does not hold true in many cases making any direct cause and effect relationship between the two occurrences questionable. Compounds that have most commonly been investigated are those where accumulation has been correlated with hardening (Levitt 1980). The concept of sugars playing a role as endogenous cryoprotectants though not universally accepted, has had considerable support in many studies (Weiser 1970). Sugars have also been implicated as a translocateable hardiness factor (Steponkus and Lanphear 1967, Weiser 1970). It has been observed that neither cold hardiness nor increased sugar accumulation can be induced in many woody species in mid-summer, while during other times of the year, sugar concentrations have been observed to increase during hardening, and to decrease as plants de-harden. This relationship when it does occur, is most pronounced from late fall to late winter (Levitt 1980). Temperature has an important influence on the sugar content as well as on cold tolerance within a plant (Rease et. al. 1978).

As early as 1882, work by Müller and Turgau indicated that there was a correlation between sugar content and frost hardiness in some plants (Sakai 1962). Work by Sakai (1962) showed a strong correlation between sugar content and frost hardiness in mulberry twigs. Frost hardiness in twigs of willow and poplar was closely related to sugar content especially in xylem tissue (Sakai 1965). Work with apple showed an increase in fructose, glucose, sucrose, and sorbitol in most cases (Rease 1977, 1978). Sugar accumulation in the leaves of citrus trees was also associated with hardening (Young 1969). Conditions under artificially controlled environments, as well as in nature, that hardened and de-hardened plants also affected sugar concentrations. Sugar content has been shown to increase upon hardening in cabbage under artificial conditions in experiments done by Le Saint and Frottes in 1972 (Levitt 1980). Drought stress on dogwood also resulted in increased sugars and cold tolerance (Chen and Li 1976).

3.1.3 Proposed Mechanisms

Although increases in total sugar concentration have been correlated with increases in cold tolerance, there has been other evidence that implicates sugars in the association with cold hardiness more favorably than other solutes found in the cell. The proposed mechanisms by which sugars protect living cell components are both colligative and interactive in nature (Sakai and Larcher 1987, Steponkus 1979). Sugars may play a role in freeze tolerance by an osmotic affect or freeze point depression (Levitt 1980, Sakai and Larcher 1987, Chen and Li 1976). There are chemical properties of sugars that make them likely to have an interactive role in cold tolerance based on current theories of how freezing stress damages plant cells. Sugars have been implicated as water binding agents (Levitt 1980). The formation of glass as the result of binding of water and the resulting inhibition of ice crystal formation has been the subject of major concern in studies of cold tolerance in plants (Levitt 1980, Sakai and Larcher 1987). The possibility of sugar substituting for water at the surface of a membrane may also exist (Caffrey et al. 1988). In work with spinach, Steponkus (1979) found that glucose, sucrose, and raffinose all played a protective role in osmotic responsiveness due to membrane protection. Herber and Santarius (1964) have suggested that sugars play a role in protection against freezing injury by preventing uncoupling of phosphorylation.

3.1.4 Sucrose

While sugars in general have been implicated as cryoprotective agents in living cells, sucrose in particular has drawn considerable attention. Work with apple showed highest levels of sucrose coincided with increased cold tolerance in plants stored at -8^{0}C during mid-winter (Rease et. al. 1978). Frost hardiness in parenchyma cells of mulberry also was found to be proportional to the amount of sucrose (Sakai 1962). Sucrose was shown to markedly change the characteristics of phospholipid membranes causing them to retain characteristics of a hydrated membrane in the absence of water (Caffrey et al. 1988). Leaf feeding experiments with *Hedera helix* L. Var. Thorndale showed hardiness increasing only after floating leaves on sucrose solutions. This same study also implicated sucrose in donor-receptor relationships between leaves and relative cold tolerance (Steponkus and Lanphear 1967).

3.1.5 Other Factors

Other seasonal cell composition changes may occur within the cell as cold hardiness increases. Such changes include the degree of saturation in lipid membranes (Lynch and Steponkus 1987, Steponkus et al. 1988, Yoshida 1975, Sakai and Yoshida 1968). These changes in living cells may be an integral part in how well sugars may protect against cold stress. Plants can vary in their response to sugars in feeding experiments depending on the season (Sakai and Yoshida 1968). Cold tolerance increased by a greater proportion in hardened plants with each given increase in sugar concentration than non-hardy plants in sugar feeding experiments (Levitt 1980). In spinach, lower concentrations of sucrose were needed to protect thylakoid membranes of acclimated plants than those of non-acclimated plants (Steponkus 1979). From late October to November, total sugars in dogwood increased only 3.45% while cold resistance increased from 0^0C to -70^0C during that same time period (Li et al. 1965). Although sugars have been correlated with cold tolerance, they may only be part of the mechanism that give living plant tissue the ability to survive freezing temperature (Rease et. al. 1978).

3.1.6 The Role of Sugars in Question and Proposals of Other Important Chemical Changes

Although total sugar or sucrose concentration has been correlated with hardiness in many plants, this has not held true in all cases leading some researchers to doubt that these compounds played a role in cold tolerance. Poor correlations between sugars and hardiness have been found in wheat (Sakai and Larcher 1987). Work with poplar seedlings showed little increase in sugars while showing a large increase in hardiness (Sakai and Yoshida 1968). Feeding experiments by Fuchigami failed to increase hardiness in dogwood by feeding plants with glucose and sucrose (Weiser 1970). Compounds other than sugars have been proposed as having a role in cold tolerance. The role of water-soluble proteins in cold hardiness was suggested by Siminovitch and Briggs in work with black locust the early 1950's. However, later work by Sakai and Yoshida (1968) with this same species showed that there was no such relationship because of poor associations found between cold hardiness and water-soluble proteins in artificial hardening tests and because under field conditions, these proteins remained at high concentrations in the spring after plants had de-acclimated.

In the fall, a decrease in starch and an increase in sugar has been observed (Sakai and Larcher 1987). In dogwood, starch was observed to decrease in the late summer and early fall during the onset of cold acclimation (Li et al. 1965, Ashworth et al.1993). Work with grapefruit suggested that low starch to sugar conversions may restrict maximum cold hardening (Yelenosky and Guy 1977). The depletion of starch during the onset of cold acclimation in black locust has led some to suggest that cold tolerance may have as much to do with the depletion of starch as it does with the accumulation of sugars (Weiser 1970, Rease et. al.1978).

3.1.7 Specific Sugars and Raffinose Family Oligosaccharides (RFO)

Much of the doubt over whether sugars played a role in cold tolerance may be attributed to the sugars that were being investigated and the circumstances in which they were being investigated. Different sugars may vary in their effects as a cryoprotectant and may also depend on the taxa. While Steponkus (1979) found no differences in the effectiveness between sugars in protecting membrane responsiveness in spinach, he did find that the most protection of coupling factor one (CF_1) in thylakoid membranes was afforded by raffinose followed by sucrose with glucose affording the least amount of protection. With gardenia leaves, pentose appeared to impart more hardiness in feeding tests than hexose, disaccharides, or raffinose (Sakai 1962). In work with eight different taxa grown in the field, Stushnoff et al. (1993) found a poor correlation between total sugar content and seasonal low temperatures, but found a strong correlation between low seasonal temperatures and content of raffinose family oligosaccarides. Work with dogwood showed the largest increase of any sugar during acclimation was that of raffinose (Li et al. 1965). Raffinose in *Ajuga* was found to be low in the summer and high in the winter and RFO greatly increased when this species was treated by a $3^0C/10^0C$ regime. (Bachmann et al. 1994). Raffinose and stachyose were found to increase greatly in six coniferous species during the late fall (Parker 1959). In winter, there is an increase in raffinose and other raffinose family oligosaccharides (RFO) in leaves and seeds of legumes (Castillo et al. 1990). The study by Castillo et al. (1990) also showed an increase in galactinol synthase activity after exposures of 4^0C to both leaves and seeds of young soy bean and kidney bean plants further suggesting the role of RFO in cold tolerance (Dey 1985). Koster and Lynch (1992) found that increases of total sugar during acclimation of rye plants were primarily due to increases in raffinose and sucrose.

Later work noting the possible importance of raffinose in cold tolerance has led to speculation about possible mechanisms that may take place inside plant cells. One idea was that the presence of an oligosaccharide such as raffinose may prevent the crystallization of sucrose (Caffrey et al. 1988). However, there is no research to date that further validates the claim of crystal inhibition in sucrose by raffinose as it pertains to living plant cells. This work also maintains that sucrose is the primary cold hardiness factor in woody plants which has not been well supported in some of the previously mentioned studies.

3.2 OBJECTIVES

The objectives of this experiment were: (1) to observe the effects that controlled temperature environments have on carbohydrate content in twigs of dogwood and currant, (2) to determine if there is a correlation between the content of a given sugar and the degree of cold tolerance associated with each controlled temperature treatment, and (3) to evaluate two different chromatography techniques. The soluble compounds of greatest interest were glucose, sucrose, raffinose, and stachyose.

3.3 MATERIALS AND METHODS

Twigs of current season's growth from Red-osier dogwood (*Cornus sericea* L.) and Red Lake Currant (*Ribes rubrum* L.) were collected at the beginning of each month from plants growing on the Colorado State University W. D. Holley Plant Environmental Research Center facility in Fort Collins, CO and placed in unlit, controlled temperature environments for approximately 30 days. This experiment was performed from the months of October 1992 through March 1993. Controlled temperature environments included three constant temperatures: -5°C, 10°C, and 0°C and two alternating temperatures between -5°C and 10°C and between 0°C and 10°C where temperatures alternated every twelve hours. Constant temperatures were provided by two refrigerators and one chest freezer. Two of these constant temperature units were regulated by Omega BS 5001 T3 (Omega Engineering Inc. Stamford, CT) controllers operating plumbing heater cable. A refrigerator was used to provide a constant 0°C environment, and was not equipped with a controller. The two fluctuating temperature regimes were provided by two refrigerated growth cabinets with controlled temperature fluctuation capability. The unit used for the 0^0C/10^0C regime was a Freas 818 (Precision Scientific Co. Chicago, IL) and the unit used for the -5^0/10^0C was a Percival model I-35 LLVL, (Percival MFG Co. Boone, IA).

A minimum of five twigs for each of four replications per treatment were wrapped in moist paper toweling, and sealed inside plastic bags before being placed inside each of the five chambers. The experiment was set up as a completely randomized design. Samples at the beginning of October had not yet undergone leaf senescence. Leaves were removed from twigs, 1/4" to 1/2" from the base of the petiole. Several twig specimens collected in the field were placed in a nylon stocking and plunged into liquid nitrogen within 2 hours after collection as were the treated samples after the approximate 30 day duration in one of the five controlled temperature environments. Following submersion in liquid nitrogen, twigs were immediately placed in a freeze-dryer and lyophilized for a period of 7-10 days. Samples collected at the beginning of the month are referred to as the 'original' for that month and as the 'field' treatment for the previous month. These samples served as a reference to compare treated samples with the plant material in the field.

Following lyophilization, cambium tissue was removed from twigs by scraping with a scalpel using short firm strokes held at a right angle to the axis of twigs to avoid slicing of unwanted secondary xylem tissue. Removed cambium tissue was placed directly into a mortar where it was pulverized and then passed though a #100 mesh screen. Ground material was stored in vials at -20^{0}C until chemical analysis was performed either by Gas chromatography (GC) or high performance liquid chromatography (HPLC).

Soluble carbohydrate analysis of currant was done by high performance liquid chromatography (HPLC), using a Dionex DX-300 series chromatography system equipped with a PA-100 ion exchange column and a pulsed electrochemical detector (Dionex Inc., Sunnyvale, CA). Samples between 1 and 2 mg were weighed to the nearest 0.001 mg and placed in a 1 ml centrifuge vile. One ml of 0.1 M NaOH solution was added to the vile and shaken by hand for approximately 3 minutes to insure thorough dispersion of dried sample particles in the NaOH solution, before centrifugation at 10,000 RPM for five minutes at 4^0C. The liquid phase was loaded into a syringe by passing though a 2 μm pore size nylon filter prior to injection. Quantitation of carbohydrate content was calculated by comparing samples with standard concentration calibration curves from standard solutions. The concentration of each sugar was automatically computed for the 1 ml solution injected. Carbohydrate content the of dry sample was calculated by dividing the concentration of sample solution by measured weight of sample and expressed as moles/gram of dry weight.

. Chemical analysis of carbohydrate content in dogwood was determined by derivitizing all samples by trimethalsilylation of hydroxyl groups and analyzing the derivatives of the sugars with gas chromatography. A 25 μl aliquot of internal standard solution containing 1 gram α-D-glucopyranoside/1 ml H_2O was injected into individual 13 x 80 mm test tubes. The internal standard solution was blown dry and a measured amount of dried ground sample was added. Sample weights were limited between 0.8 and 1.5 mg and weighed to the nearest 0.001 mg. Derivitization was done by adding 0.4 ml of pyridine, 80 μl hexamethyldisilylazane, and 40 μl of chlorotrimethylsilylazane to each tube containing weighed sample and dried internal standard, placing a screw top firmly on each tube and placing tubes in a heating block maintained at 80^0C for 20 minutes. After removing tubes from heating block, screw-caps were removed and samples were blown down until only non-volatile residues remained. 0.5 ml of hexane was added to each tube and sugar derivatives were phase-separated from remaining residue. New tubes containing the transferred hexane solution were blown dry and 0.2 ml of hexane was added to attain a desired sample concentration for use in the gas chromatograph (Sweeley et al. 1963, Stushnoff et al. 1993). The gas chromatograph used was a Hewlet-Packard 5840-A chromatograph equipped with a flame ionization detector (Hewlet-Packard Corp., Avondale, PA). The column used was a DB1-30W capillary column (J & W Scientific, Folsom, CA).

Sample sugar content was expressed as moles/gram of dry weight and was calculated by C x (SA/ISA/SWT) where C is a constant expressed as moles and is established for a particular derivatized sugar by a standard mix run, SA is the area of the peak in the chromatogram corresponding to a particular derivatized sugar in the sample, ISA is the area of the peak corresponding to the internal standard derivative, and SWT is the sample weight (Stushnoff et al. 1993).

3.4 RESULTS AND DISCUSSION

3.4.1 The Effect of Controlled Temperature Treatments on Endogenous Sugar Composition

3.4.1.1 Glucose in currant

Glucose content in Red Lake currant increased significantly in all treatments and in the field during October (Table 3.1). In November, decreases occurred in all treated samples with the greatest loss occurring in the 0^0C/10^0C treatment. No significant change in glucose occurred in the field during the month of November.

In December, no change occurred in glucose content in any of the treated samples. However, a highly significant increase did occur in the field during this time and was the highest level attained by currant.

All controlled treatments in January showed highly significant losses, but no significant loss occurred in the field. A similar trend occurred in February as in January except that losses were less significant in the treatments and no loss occurred at all in the 10°C treatment.

In March, significant losses in glucose occurred in the field and in the 10°C, 0°C/10°C, and -5°C/10°C treatments. No significant losses in glucose occurred in the 0°C and -5°C treatments during March.

3.4.1.2 Glucose in dogwood

In October, samples of Red-osier dogwood glucose increased significantly in the 0°C and 0°C/10°C treatments, decreased in the -5°C treatment (Table 3.2). No significant change in glucose level occurred in the 10°C treatment or in the field during October.

In November, no losses occurred in any of the treatments and significant increases occurred in the 0°C, 0°/10°C, and -5°C/10°C treatments and in the field samples. No significant changes occurred in the 10°C or -5°C/10°C treatments during this period.

In December, a highly significant increase in glucose occurred in the field with no changes occurring in any of the treatments with the exception of the -5°C treatment which showed a significant decrease.

In January, glucose decreased in all treatments with the exception of the -5°C/10°C treatment, where the decrease was not significant. The greatest decrease was in the 10°C treatment. There was no significant change in the January field samples.

In February, decreases in glucose occurred in field and all controlled treatments. The best retention of glucose in February was in the -5°C treatment where the decrease was not significant.

In March, glucose decreased significantly in the field and in all treatments with the exception of 0°C. The samples from the 10°C treatment and the field lost glucose equally and were the greatest for that month.

3.4.1.3 General affects on glucose content by temperature

It appears that glucose content of both species under field conditions increased by varying amounts up to the month of January and was maintained at a certain level and then decreased in March (Figures 3.1 & 3.2). With a few exceptions, controlled treatments were generally not as effective as field conditions at promoting glucose accumulation and retention through February. In March, the 10°C treatment had the greatest depletion of glucose in both species, which would be expected as cells break dormancy and become most metabolically active under the warmest temperature conditions, and may begin to use these reserves at faster rates (Tables 3.1 & 3.2). Consistent affects on glucose levels due to any given treatment throughout the season were not obvious.

3.4.1.4 Sucrose in currant

In October, significant increases in sucrose occurred in the 0°C, 0°C/10°C, and -5°C/10°C treatments with the greatest increase occurring in the -5°C/10°C treatment

(Table 3.3). There were no significant changes in sucrose content in samples from the field or from the 10^0C and -5^0C treatments.

In November, the -5^0C/10^0C treatment had the highest increases in sucrose. There was also a lesser, but significant increase in the 0^0C treatment. No significant changes in sucrose levels were detected in other treatments or in samples from the field at the end of November.

In December, sucrose levels increased in samples from the field and the 0^0C, 10^0C, and -5^0C/10^0C treatments with the -5^0C/10^0C treatment showing the highest increase. Samples from the -5^0C and 0^0/10^0C treatments showed no significant changes.

In January, sucrose decreased significantly in the 0^0C/10^0C and -5^0C/10^0C treatments, but a sightly significant increase occurred in samples from the field and the -5^0C treatment. Little change occurred in the 0^0C and 10^0C treatments during this period.

In February, there were no significant changes in sucrose levels in the treatments except for the 10^0C treatment where there was a slight but significant loss. A significant increase did occur in the field during February.

In March, samples from the field and all treatments except the 0^0C treatment lost sucrose. No significant change occurred in samples from the 0^0C treatment. The greatest decreases occurred in samples from the 10^0C treatment and the field.

3.4.1.5 Sucrose in dogwood

In October, dramatic increases in sucrose occurred in samples from the 0^0C, 10^0C, and -5^0C/10^0C treatments (Table 3.4). No significant changes occurred in the 10^0C and -5^0C treatments or in the field.

In November, increases in sucrose occurred in the field and in all treatments with the exception of the 10^{0}C treatment where no significant change took place. The greatest increase during November, was in the -5^{0}C/10^{0}C treatment as was the case in currant at this time.

In December, increases occurred in samples from the treatments and the field. All samples had similar increases in sucrose with no differential changes occurring as a result of the different treatments.

In January, decreases in sucrose occurred in samples from the -5^{0}C/10^{0}C and -5^{0}C treatments and the field with the greatest decrease in the field. Samples from the other three treatments, 0^{0}C, 10^{0}C, and 0^{0}C/10^{0}C, showed little change in sucrose levels from the beginning to the end of January.

In February, field conditions produced another significant decrease. All treatments, however, produced varying degrees of increases in sucrose levels with the greatest increase occurring in the 10^{0}C treatment and the smallest increases occurring in the -5^{0}C and 0^{0}C/10^{0}C treatments.

In March, highly significant increases were detected in samples from all treatments and from the field. The greatest increase occurred in the -5^{0}C/10^{0}C treatment. Increases in the field and the other treatments were approximately the same.

3.4.1.6 General affects of temperature on sucrose content

In October and November, neither of the two species had significant increases under field conditions while highly significant, and the largest increases for these two months, occurred in the $-5^0C/10^0C$ treatment. However, neither the -5^0C or 10^0C treatments alone showed significant changes in sucrose content during these two months with the exception of dogwood in the -5^0C treatment in November where the increase was only moderately significant. The combination of freezing temperature with warm temperature was most favorable to sucrose accumulation in cortical tissue by these two species. The $0^0C/10^0C$ treatment had an effect similar to the $-5^0C/10^0C$ treatment in both species in October, but not in November. The 0^0C treatment had consistently high levels most months for both species when compared to other treatments proving this temperature to be favorable for increases in, or retention of sucrose levels depending on the time of the year.

Sucrose levels in the field began to reach maximum levels in dogwood at the end of December and decreased during January and February until they were the same as they were in the beginning of October (Figure 3.4). In currant, maximum levels of sucrose were not reached until the beginning of March (Figure 3.3), when sucrose levels decreased from the beginning to the end of March in currant, and began to rise again in dogwood. The high or increased levels of sucrose in both of these species during warmer periods of the year such as March, does not support the concept of sucrose playing a direct role in cold tolerance in either of these two plant species.

3.4.1.7 Raffinose in currant

In October, a large increase in raffinose occurred in the 0^0C treatment (Table 3.5). Samples from the field and the 10^0C treatment also showed highly significant increases in raffinose. A small, but significant increase occurred in the $0^0C/10^0C$ treatment.

In November, no significant increases in raffinose occurred in any of the treatments while a moderately significant increase occurred in the field. A highly significant decrease in raffinose occurred in samples from the -5^{0}C/10^{0}C treatment.

In December, a highly significant increase in raffinose occurred in the -5^{0}C treatment, while a highly significant decrease occurred in the -5^{0}C/10^{0}C treatment. Other decreases occurred in the 10^{0}C and 0^{0}C/10^{0}C treatments. There was no change in raffinose levels in the field.

In January, no significant increases in raffinose occurred in the field or in the treatments. An increase was apparent in the -5^{0}C treatment as was the case in December, but this increase did not prove statistically significant based on the experimental data. Slight, but significant decreases occurred in the 10^{0}C, 0^{0}C/10^{0}C, and -5^{0}C/10^{0}C treatments.

In February, decreases in raffinose occurred in all the treatments and under field conditions. Samples in the 10^{0}C treatment had the greatest decrease.

In March, no changes in raffinose levels occurred in the treatments with the exception of the 10^{0}C treatment which displayed approximately the same very large and significant loss that occurred under field conditions.

3.4.1.8 Raffinose in dogwood

In October, highly significant increases in raffinose occurred in samples from the field and all treatments with the exception of the -5^{0}C treatment where a highly significant decrease in raffinose was evident (Table 3.6). The smallest increase in raffinose occurred in the 10^{0}C treatment. A similar pattern was repeated in November, except that a significant loss from the original did not occur in the -5°C treatment. Raffinose levels in samples from the field at the end of November and December were the highest recorded for the season in dogwood.

Whereas the highest recorded levels of raffinose in dogwood occurred in samples from the field at the end of December, significant losses occurred during this time in samples from all treatments. This same pattern was repeated in January where significant losses occurred in all treatments A significant decrease also occurred in the field in January.

In February, a highly significant loss of raffinose was shown in samples from the field. Significant decreases occurred in three treatments with the greatest decrease shown in the 10°C treatment, but none of these losses were equal to that in the field during this period. Decreases in raffinose in the treatments during February were generally not as great as in January.

In March, no significant change in raffinose occurred under field conditions, but highly significant increases occurred in all treatments, except the 10°C treatment, which had a significant decrease.

3.4.1.9 General affects of temperature on raffinose content

Consistent increases in raffinose level increases in the fall and decreases in the spring were evident in both species in the field (Figures 3.5 & 3.6). Greater increases in currant than in dogwood were detected in the field during October. Decreases in the field occurred later in the spring in currant than in dogwood. The lowest raffinose levels attained in both species during February and March were in samples from the 10°C treatment which may indicate that an increased state of metabolic activity associated with its depletion were induced by this treatment during February. Samples of both species in the 0^0C treatment during all months had consistently high levels of raffinose indicating that this temperature has a favorable affect on the production of raffinose in October and retention of raffinose during the other months. It is possible that the 0^0C treatment is optimal for lower utilization or expenditure of raffinose while allowing synthesis to take place. Decreases occurred in the -5^0C treatment during the month of October in both species although the decrease was not statistically significant in currant. This decrease may be due to the injury of tissue in these samples as a result of the -5^0C treatment prior to adequate hardening, particularly in dogwood during October of 1992 as indicated by freeze tests (Chapter 1). While the trend in raffinose levels in the field during the season was the most consistent with relation to seasonal temperature in both species, the increases observed in raffinose were not necessarily the greatest increase of any sugar in the field in dogwood as was the case with work by Li et al (1965).

3.4.1.10 Stachyose in currant

No statistical analysis was done on effects of treatments in stachyose levels during October because stachyose levels in samples collected at the beginning of the month were below measurable amounts in the 'Dionex' HPLC system. The samples from the 0^0C treatment sample did have significantly higher levels of stachyose than samples with other treatments where data were taken (Table 3.7). No significant differences existed between the other treatments.

In November, only the -5^{0}C treatment had stachyose levels that were below the level of detection in the 'Dionex' HPLC system. A significant increase occurred in the 0^{0}C and 10^{0}C treatments. No change occurred in the field during November.

In December, increases in stachyose levels were evident in the 0^{0}C/10^{0}C treatment and in the field. There were no significant changes in any of the other treatments.

In January, no changes in stachyose levels occurred in the field or in any of the treatments. This pattern was again repeated in February with the exception of a decrease in the 10^{0}C treatment during that period.

In March, a highly significant decrease in stachyose was found in the 10^{0}C treatment and a moderately significant decrease occurred in the field. No significant change was shown in the other treatments during March.

3.4.1.11 Stachyose in dogwood

In October, significant increases occurred in the 10^{0}C, -5^{0}C, 0^{0}C/10^{0}C, and the 0^{0}C treatments(Table 3.8). The greatest increase in stachyose was in the 0^{0}C treatment. No significant changes were detected in the -5^{0}C/10^{0}C treatment or in the field during October.

In November, the -5^{0}C and -5^{0}C/10^{0}C treatments did not show any increases in stachyose levels while significant increases were shown in other treatments and the field. The greatest increases in stachyose during November were shown in samples from the 0^{0}C treatment and from the field.

In December, decreases took place in samples from the 0^0C, -5^0C, and -5^0C/10^0C treatment and from the field. No significant change occurred in the 0^0C/10^0C treatment and an increase occurred in the 10^0C treatment.

In January, increases of stachyose levels were evident in the 0^0C and 0^0C/10^0C treatments while decreases took place in all the other treatments. No significant change occurred in the field during this time.

In February, decreases in stachyose levels occurred in the field and all treatments except the 10^0C treatment where no significant change occurred. Almost the reverse of this pattern took place in March where increases were shown in the field and in all treatments except 10^0C and -5^0C.

3.4.1.12 General affects of temperature on stachyose content

The patterns of stachyose levels in the field are not consistent between currant and dogwood and would not be supportive of the concept that stachyose has a major role in cold tolerance in these two species (Figures 3.7 & 3.8). Dogwood has a plausible trend of increases and decreases through the winter season to suggest that cold hardiness and stachyose levels could be correlated, but an inexplicable increase was shown at the end of March. Increases of stachyose levels in treated dogwood samples during March often exceeded amounts found in samples from the same treatments in February, yet did not prove to be more hardy in freeze tests (Chapter 1). This may raise further doubts about a possible association between stachyose levels and cold tolerance.

The trend in stachyose levels in currant in the field does not suggest that there is a correlation because significant increases were not shown in this species until the end of December while samples of this species were shown to attain maximum levels of hardiness at the end of November in freeze tests (Chapter 1).

The fact that the 'Dionex' HPLC system was working at the lower limits of its useful detection capabilities for stachyose should be taken into account. Better measurement of stachyose levels may have been obtained in this system if there was better separation of stachyose from raffinose by the column.

3.4.2 Correlation between Cold Tolerance and Sugars

3.4.2.1 Correlation between sugar and cold tolerance among all treatments

Correlation analysis was done by comparing the level of a sugar in the cortical tissue of twig samples with the killing temperature for that particular sample. Analysis was only done on samples where a definitive T_{50} temperature was obtained and did not include samples where the T_{50} temperature could only be roughly estimated. As a result, points for 19 out the 37 treatment and field categories in Red-osier dogwood were plotted together for correlation analysis for each of the four sugars. In Red Lake currant, 22 of these 37 categories could be plotted with the exception of stachyose analysis where 21 points were plotted. The T_{50} values where injury level criteria was death of the cortical tissue (Chapter 1) were used since the cortex was the tissue from which the sugars were extracted.

Raffinose levels in dogwood correlated with T_{50} temperatures in regression analysis as indicated by a significant ($P<0.0001$) r^2 value of 0.710 (Table 3.9 & Figure 3.9). A slight correlation between glucose and T_{50} temperatures was also indicated in dogwood by a r^2 value of 0.575 ($P = 0.0002$) (Figure 3.10). The correlation between raffinose and T_{50} temperatures in currant was indicated by an r^2 value of 0.385 and a level of significance ($P = 0.0027$), suggesting little association, but this was the highest r^2 value shown for currant (Figure 3.11). No other associations between the sugars studied and T_{50} temperatures in either of the two species were indicated when including all field and controlled temperature treatments together in correlation analysis. Although the P value (0.0096) for sucrose in dogwood was highly significant, there was no relationship indicated by the r^2 value. The high probability may be explained by the strong association between hardiness and sucrose levels in dogwood samples from $-5^0C/10^0C$ treatment.

Sugar levels were generally higher in currant than in dogwood and may have been in excess of what was needed to serve as a cryoprotectant if any of the sugars studied in fact serve this purpose. As a result, a response in sugar levels to environmental conditions may not be as well defined in currant and other factors may play a more important role in response to cold temperature in this species. Since the highest possible association between hardiness and sugar content in currant was indicated with raffinose and this sugar did show a significant correlation in dogwood, it may be possible that raffinose does play a role as an endogenous cryoprotectant in these two plant species.

3.4.2.2 Correlation between sugar and cold tolerance within treatments

In currant, the highest association between a sugar and hardiness was shown by raffinose levels in those samples having the $0^0/10^0C$ treatments as indicated by significant ($P = 0.022$) r^2 of 0.767 (Table 3.10). Another significant association indicated by an r^2 of 0.731 ($P = 0.030$) was stachyose levels in the 0^0C treatment. Currant samples from field conditions showed r^2 and P values of 0.631($P = 0.033$), 0.622 ($P = 0.035$), and 0.620 (P

= 0.036) for glucose, raffinose, and stachyose respectively indicating some degree of association between hardiness and levels of these sugars in the field. No such association was shown with sucrose and hardiness in field samples for any other sugar in currant samples from any of the other treatments not mentioned above.

Raffinose was highly correlated with hardiness in dogwood samples from the 0^0C, -5^0C, and 0^0C/10^0C treatments and field conditions, but not from the 10^0C and -5^0C/10^0C treatments (Table 3.11). Glucose was associated with hardiness in dogwood samples from the field as shown by an r^2 of 0.726 (P = 0.015). Sucrose was associated with hardiness in dogwood samples from the -5^0/10^0C treatment by an r^2 of 0.780 (P = 0.020). A slight association between hardiness and stachyose was found in dogwood samples from the -5^0C treatment as shown by an r^2 of 0.675 (P = 0.045). No other associations were found between sugars and hardiness in samples from other treatments.

Both currant and dogwood samples from field conditions showed closer relationships between more sugars than did samples from any of the treatments. This was shown with three sugars in currant and two in dogwood, suggesting there are factors in the field environment that have not been duplicated in any of the controlled environments. Exposure to solar radiation and attachment of samples to intact plants prior to sampling may be factors other than temperature contributing to differences observed between twig samples kept in controlled environments and those collected directly from the field.

In both currant and dogwood, raffinose in field samples was shown to have the strongest association with cold hardiness of all the sugars. In the case of dogwood, raffinose was associated with hardiness in samples from three of the five treatments as well as from the field. These findings would suggest that raffinose may be of particular importance to cold tolerance in dogwood. While a similar relationship between raffinose levels and cold tolerance may exist in currant, it is less obvious based on the results of this experiment.

3.4.3 Chromatographic Methods

'Dionex' High performance liquid chromatography for carbohydrates worked well for analyzing Red Lake currant samples. However, running samples of Red-osier dogwood resulted in increased pressures within the HPLC system to the point where flushing the column became necessary after as few as five sample injections. Because of this, chemical analysis of dogwood samples was done exclusively by the gas chromatography method.

Separation of sugars using the 'Dionex' high performance liquid chromatography (HPLC) had the advantage of each sugar eluting as a single peak. Derivatives of certain sugars eluted as several peaks using the gas chromatographic (GC) method. Fructose, although not a concern in this study, was an example of a sugar that eluted as a single peak in the un-derivatised form using HPLC, but its derivatives were detected and graphed as multiple peaks in GC analysis. This would indicate that such sugars may attain differential derivatisation with the trimethylsilation method used and that HPLC analysis or a different method of derivatisation prior to CG analysis for these particular sugars may be preferred. The sugars of interest in this study, glucose, sucrose raffinose, and stachyose, were identified as single distinct peaks in the chromatograph-print out and consistent results were obtained.

The 'Dionex' HPLC system has the advantage over the GC used in this experiment of comparing data against a calibration curve rather than assuming a linear relationship between sample concentration and internal standard. Ideally, this would make measurement of compounds that occur in varying amounts more precise. However, the internal standard method is not as prone to variability due to changes in the instrument.

Gas chromatography separated the raffinose derivative from the stachyose derivative better than 'Dionex' HPLC. The poor separation of raffinose and stachyose became most apparent when stachyose levels were very low and the integrator could not detect a change in slope on the graph as stachyose eluted after raffinose. Attempts to correct this problem by changing eluant gradients were not successful. Since the amounts of stachyose were very small before this was encountered, accurate measurement of raffinose was not adversely affected.

Analysis for carbohydrates with the 'Dionex' HPLC system and sample preparation method used in this experiment would be preferred when the plant material poses no problem to the operation of the equipment and high degrees of separation between raffinose and stachyose are not critical. The HPLC method used holds the distinct advantage of not having the derivatisation step which can be labor intensive and can create opportunities for experimental error to occur.

Better methods of sample preparation prior to HPLC analysis are clearly needed so that a greater variety of plant materials can be successfully analyzed for carbohydrates using this equipment. The derivatisation of samples prior to GC analysis eliminates problems associated extraneous compounds being introduced and possibly complicating analysis, giving GC a distinct advantage with respect to dependability in this experiment.

Mechanical parts such as valves and pumps, frequent opening of the system to add eluant, and eluant preparation pose distinct disadvantages for 'Dionex' HPLC when compared with GC equipment which has no moving parts with the exception of the dropping needle injector and where opening the system to replace gas supplies is done very infrequently. Wear and failure of moving parts may result in variability and introduction of leaks in the system. Frequent disassembly of lines introduces an increased risk of contamination and leaking in the system. The introduction of leaks in either case may result in variability.

3.5 SUMMARY

Levels of glucose, sucrose, raffinose, and stachyose in the cortical tissue of Red Lake currant and Red-osier dogwood change under field conditions from the beginning of October to the end of March. Modifications in the level of these four sugars in twig samples of either plant species can be achieved throughout the season by placing them in controlled temperature environments. The effect on the level of any given sugar by a controlled temperature treatment depends on the sugar, time of year, conditions prior to sample collection, species, and temperature treatment. A closer association between more than one type of sugar and cold tolerance as determined by freeze tests was shown in samples of both species from field conditions than those samples treated by controlled temperature treatments. A significant association between raffinose levels and cold tolerance in Red-osier dogwood samples from three controlled temperature treatments and the field throughout the season was also shown. Carbohydrate analysis can be done using either gas chromatography (GC) or 'Dionex' high performance liquid chromatography (HPLC), with both techniques having advantages and disadvantages. Investigation of dogwood using the HPLC method did present a complication. The most preferable method depends on the carbohydrate being investigated and the type of plant material studied.

Table 3.1 Glucose content in 'Red Lake' currant expressed as $1x10^{-5}$ M/g dry wt. ± standard error of the mean.

	October	November	December	January	February	March
Original	**9.47±0.13**	**13.76±0.23**	**12.75±0.44**	**23.17±0.90**	**18.79±1.40**	**21.20±0.59**
0°C	12.48±0.65*	11.12±0.14*	14.26±0.29 ns	12.28±1.03***	12.42±0.17*	19.99±0.28 ns
10°C	13.58±0.42**	10.25±0.47**	11.11±0.55 ns	12.41±1.05***	19.81±1.50 ns	7.33±0.29***
-5°C	13.25±0.61*	9.98±0.72**	14.60±0.63 ns	11.56±0.04***	11.82±0.41*	18.76±1.07 ns
0°C/10°C	13.37±0.46*	8.84±0.09***	13.67±0.65 ns	12.82±0.10***	9.85±0.65**	12.39±0.08***
-5°C/10°C	14.01±0.84**	10.27±0.42**	9.98±0.07 ns	10.74±0.67***	13.31±0.87*	12.53±0.62***
Field	13.76±0.23**	12.75±0.63 ns	23.17±0.90***	18.79±1.40 ns	21.20±0.59 ns	14.99±0.45**

Level of significance; difference from "original" in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

Table 3.2 Glucose content in Red-osier dogwood expressed as $1x10^{-5}$ M/g dry weight ± standard error of the mean from triplicate samples from GC analysis.

	October	November	December	January	February	March
Original	**5.37±0.15**	**5.77±0.05**	**13.86±0.42**	**17.39±0.57**	**19.46±0.25**	**16.39±0.37**
0°C	11.81±0.31***	12.88±0.30***	14.90±0.49 ns	14.78±0.96*	13.94±0.55***	14.99±0.63 ns
10°C	4.89±0.20 ns	5.12±0.12 ns	12.98±0.27 ns	10.65±0.39***	13.30±0.56***	7.06±0.25***
-5°C	3.97±0.30*	4.77±0.20 ns	10.56±0.36**	14.21±0.43*	17.68±0.20 ns	12.40±0.76***
0°C/10°C	8.94±0.23***	9.83±0.23***	13.36±0.54 ns	13.43±0.43**	14.30±0.39***	12.04±0.29***
-5°C/10°C	5.80±0.29 ns	11.61±0.53***	12.93±0.36 ns	15.88±0.40 ns	16.66±0.67**	13.43±0.27**
Field	5.77±0.05 ns	13.86±0.42***	17.39±0.57***	19.46±0.25 ns	16.39±0.37**	7.67±0.40***

Level of significance; difference from "original" in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

Table 3.3 Sucrose content in 'Red Lake' currant expressed as $1x10^{-5}$ M/g dry weight ± standard error of the mean from duplicate samples from HPLC analysis.

	October	November	December	January	February	March
Original	**14.50±0.33**	**15.55±0.44**	**16.00±0.82**	**22.00±0.75**	**27.58±1.24**	**38.62±1.41**
0°C	19.76±1.06**	19.18±0.83*	23.11±0.26**	24.91±0.65 ns	22.27±0.80 ns	36.82±0.68 ns
10°C	13.39±0.01 ns	12.82±0.25 ns	21.21±0.42**	19.86±0.27 ns	19.36±1.05*	16.79±0.80***
-5°C	12.35±0.21 ns	15.78±0.24 ns	17.47±0.71 ns	26.44±0.73*	21.59±0.61 ns	31.58±0.12**
0°C/10°C	18.75±0.48**	16.64±0.15 ns	16.14±0.55 ns	17.19±0.45*	23.39±0.55 ns	27.26±0.65**
-5°C/10°C	24.74±0.06***	25.73±0.90***	26.92±0.69***	15.20±0.62**	32.06±2.01 ns	30.87±0.57***
Field	15.55±0.44 ns	16.00±0.82 ns	22.00±0.75**	27.58±1.24*	38.62±1.41**	15.41±0.79***

Level of significance; difference from "Original" in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

Table 3.4 Sucrose content in Red-osier dogwood expressed as $1x10^{-5}$ M/g dry weight ± standard error of the mean from triplicate samples from GC analysis.

	October	November	December	January	February	March
Original	**3.96±0.10**	**5.55±0.20**	**7.99±0.41**	**11.29±0.32**	**7.25±0.06**	**3.63±0.10**
0°C	11.90±0.20***	11.58±0.51***	11.86±0.32***	11.99±0.54 ns	11.42±0.49***	9.68±0.19***
10°C	3.73±0.15 ns	6.31±0.13 ns	11.92±0.27***	11.26±0.25 ns	13.96±0.47***	9.70±0.22***
-5°C	3.07±0.11 ns	8.18±0.22***	11.42±0.59***	8.88±0.25**	8.56±0.28 ns	5.91±0.14***
0°C/10°C	15.49±0.35***	7.53±0.16**	11.31±0.48***	12.97±0.42*	8.50±0.33 ns	10.23±0.11***
-5°C/10°C	17.81±0.73***	12.38±0.37***	11.49±0.43***	9.63±0.28*	9.13±0.33*	14.11±0.09***
Field	5.55±0.20 ns	7.99±0.41**	11.29±0.32***	7.25±0.06***	3.63±0.10***	10.67±0.42***

Level of significance; difference from "Original" in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

Table 3.5 Raffinose content in 'Red Lake' currant expressed as $1x10^{-5}$ M/g dry weight ± standard error of the mean from duplicate samples from HPLC analysis.

	October	November	December	January	February	March
Original	**2.50±0.03**	**9.28±0.52**	**12.78±0.51**	**12.60±0.35**	**12.05±0.84**	**9.85±0.25**
0°C	11.50±0.38***	8.45±0.28 ns	12.48±0.02 ns	12.39±0.40 ns	8.21±0.06**	9.85±0.45 ns
10°C	6.91±0.01***	9.38±0.17 ns	10.75±0.24*	9.56±0.25*	5.41±0.25***	3.50±0.31***
-5°C	1.09±0.13 ns	10.31±0.01 ns	16.90±0.41***	14.45±0.65 ns	8.87±0.03**	9.67±0.34 ns
0°C/10°C	4.13±0.24*	10.23±0.06 ns	10.17±0.40**	8.47±0.42**	7.96±0.16***	10.60±0.18 ns
-5°C/10°C	1.04±0.01 ns	5.56±0.19***	8.07±0.12***	9.15±0.19*	9.14±0.19**	8.54±0.10 ns
Field	9.28±0.52***	12.78±0.51**	12.60±0.35 ns	12.05±0.83 ns	9.85±0.25*	3.18±0.13***

Level of significance; difference from "Original" in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

Table 3.6 Raffinose content in Red-osier dogwood expressed as $1x10^{-5}$ M/g dry weight ± standard error of the mean from triplicate samples from GC analysis.

	October	November	December	January	February	March
Original	**2.00±0.02**	**3.77±0.17**	**9.92±0.56**	**10.29±0.28**	**7.54±0.16**	**3.67±0.11**
0°C	4.56±0.02***	7.24±0.18***	7.60±0.22**	7.46±0.28***	7.16±0.34 ns	7.24±0.22***
10°C	2.99±0.11**	4.94±0.01*	5.16±0.28***	5.50±0.24***	5.51±0.19***	2.37±0.08***
-5°C	0.77±0.12***	4.58±0.17 ns	5.60±0.08***	6.47±0.32***	7.39±0.29 ns	6.52±0.16***
0°C/10°C	4.48±0.08***	5.16±0.08***	6.53±0.32***	8.27±0.18***	5.87±0.09**	6.25±0.10***
-5°C/10°C	3.93±0.13***	7.05±0.33***	5.42±0.08***	5.99±0.06***	6.25±0.29*	7.13±0.10***
Field	3.77±0.17***	9.71±0.42***	10.29±0.28 ns	7.54±0.16***	3.67±0.11***	4.14±0.11 ns

Level of significance; difference from "Original in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

Table 3.7 Stachyose content in 'Red Lake' currant expressed as $1x10^{-5}$ M/g dry weight ± standard error of the mean from duplicate samples from HPLC analysis.

	October	November	December	January	February	March
Original	†	**0.59±0.01**	**0.57±0.08**	**1.49±0.08**	**1.98±0.24**	**1.75±0.14**
0^{0}C	2.73±0.11	0.909±0.04*	1.09±0.07 ns	2.08±0.10 ns	1.61±0.03 ns	2.16±0.11 ns
10^{0}C	1.00±0.09	1.37±0.01***	0.82±0.09 ns	2.01±0.06 ns	1.09±0.04**	0.58±0.12***
-5^{0}C	†	†	0.58±0.04 ns	1.59±0.09 ns	1.58±0.06 ns	2.23±0.15 ns
0^{0}C/10^{0}C	0.88±0.05	0.78±0.08 ns	1.64±0.08 **	1.77±0.02 ns	1.78±0.01 ns	1.86±0.01 ns
-5^{0}C/10^{0}C	†	0.52±0.01 ns	1.12±0.23 ns	1.52±0.06 ns	1.48±0.05 ns	1.59±0.05 ns
Field	0.59±0.01	0.57±0.08 ns	1.49±0.08**	1.98±0.24 ns	1.75±0.14 ns	0.75±0.04**

Level of significance; difference from "original" in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

(†) indicates levels below detectable limits of instrument.

Table 3.8 Stachyose content in Red-osier dogwood expressed as $1x10^{-5}$ M/g dry weight ± standard error of the mean from triplicate samples from GC analysis.

	October	November	December	January	February	March
Original	**0.314±0.023**	**0.396±0.074**	**0.865±0.067**	**0.661±0.026**	**0.628±0.037**	**0.280±0.012**
0°C	0.967±0.034***	0.851±0.010***	0.514±0.009***	0.813±0.006*	0.397±0.044**	0.541±0.014***
10°C	0.464±0.021**	0.604±0.010**	1.045±0.051*	0.454±0.051**	0.765±0.048 ns	0.385±0.024 ns
-5°C	0.053±0.006***	0.308±0.011 ns	0.376±0.012***	0.512±0.022*	0.392±0.032**	0.350±0.013 ns
0°C/10°C	0.562±0.018***	0.532±0.015*	0.691±0.025 ns	0.881±0.021**	0.401±0.036**	0.447±0.015*
-5°C/10°C	0.321±0.020 ns	0.401±0.009 ns	0.528±0.027***	0.448±0.020**	0.421±0.051***	0.740±0.073***
Field	0.396±0.017 ns	0.865±0.067***	0.661±0.026*	0.628±0.037 ns	0.280±0.012***	1.039±0.002***

Level of significance; difference from "original" in a given month, according to Tukey's mean separation test.
(ns) P value > 0.05
(*) P value < 0.05
(**) P value < 0.01
(***) P value < 0.001

Table 3.9. Correlation coefficient r^2 between sugar content in cortical tissue and hardiness as indicated in freeze tests (Chapter 1). Probability is in parenthesis.

	Red Lake currant	Red-osier dogwood
Glucose	0.074 (0.219)	0.575*** (0.0002)
Sucrose	0.130 (0.0992)	0.334** (0.0096)
Raffinose	0.385** (0.0027)	0.710*** (<0.0001)
Stachyose	0.005 (0.769)	0.128 (0.132)

** Highly significant ($P<0.01$)

*** Extremely significant ($p<0.001$)

Table 3.10. Correlation coefficients r^2 between sugar content in cortical tissue and hardiness as indicated by freeze tests (Chapter 1) in all monthly currant samples from different treatments. Probability is in parenthesis.

	Glucose	Sucrose	Raffinose	Stachyose
Field	0.631* (0.033)	0.199 (0.315)	0.622* (0.035)	0.620* (0.036)
0^0C	0.013 (0.830)	0.025 (0.764)	<0.001 (0.987)	0.731* (0.030)
10^0C	0.472 (0.345)	0.098 (0.546)	0.077 (0.596)	0.035 (0.725)
-5^0C	0.003 (0.919)	0.298 (0.262)	0.616 (0.065)	0.383 (0.190)
$0^0C/10^0C$	0.024 (0.768)	0.037 (0.716)	0.767* (0.022)	0.220 (0.348)
$-5^0C/10^0C$	0.636 (0.057)	0.041 (0.702)	0.004 (0.905)	0.037 (0.716)

* Significant ($P<0.05$)

Table 3.11. Correlation coefficients r^2 between sugar content in cortical tissue and hardiness as indicated by freeze tests (Chapter 1) in all monthly dogwood samples from different treatments. Probability is in parenthesis.

	Glucose	Sucrose	Raffinose	Stachyose
Field	0.726* (0.015)	0.320 (0.186)	0.868** (0.002)	0.226 (0.281)
0^0C	0.570 (0.083)	0.268 (0.293)	0.825* (0.012)	0.582 (0.078)
10^0C	0.224 (0.421)	0.049 (0.672)	0.405 (0.174)	0.016 (0.813)
-5^0C	0.408 (0.173)	0.588 (0.075)	0.855** (0.008)	0.675* (0.045)
0^0C/10^0C	0.361 (0.207)	0.184 (0.798)	0.726* (0.031)	0.402 (0.176)
-5^0C/10^0C	0.556 (0.089)	0.154 (0.385)	0.372 (0.199)	<0.001 (0.974)

* Significant (P<0.05)

** Very significant (P<0.01)

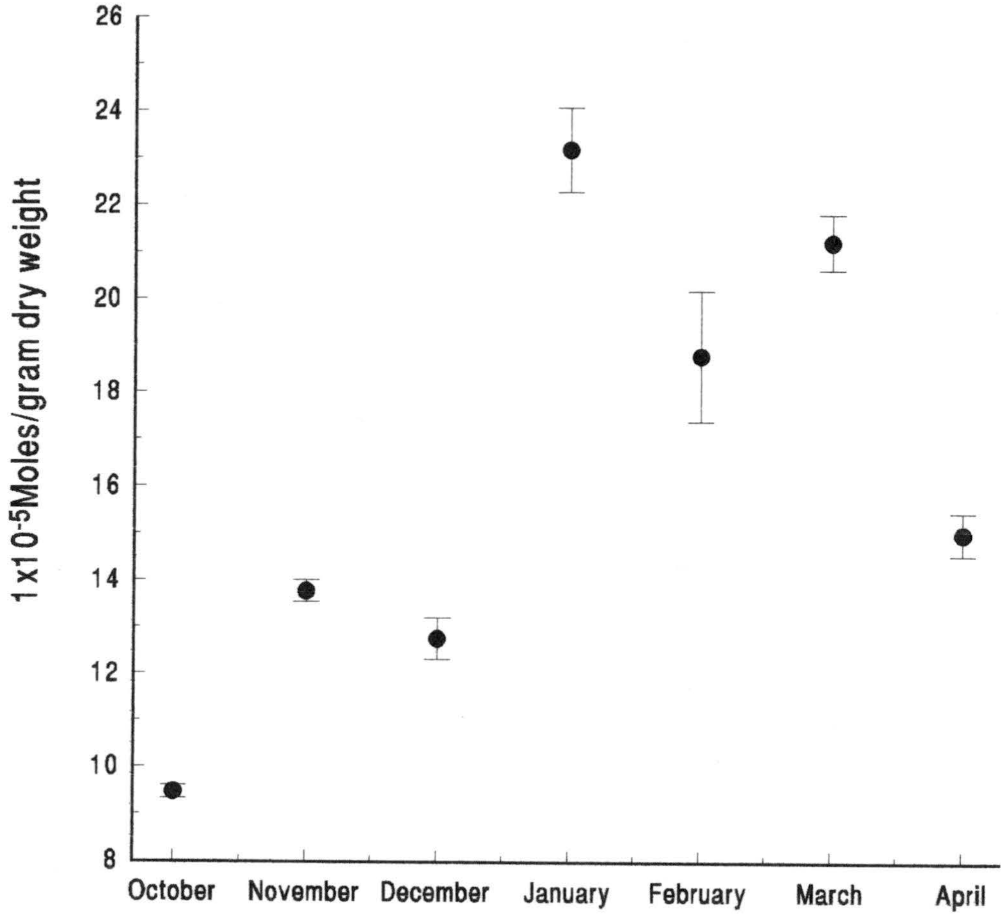

Figure 3.1. Glucose content in cortical tissue in 1992-93 field samples of 'Red Lake' currant. Circle with vertical bar represents mean ± standard error of duplicate samples from 'Dionex' HPLC analysis.

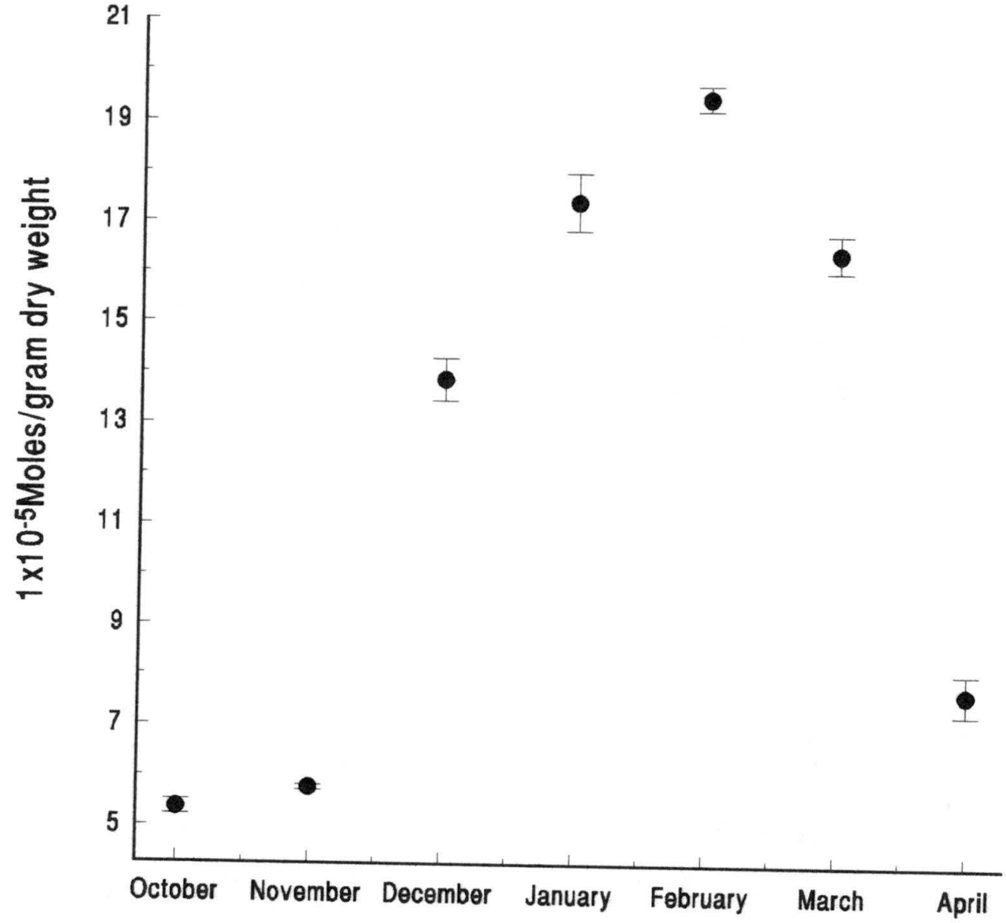

Figure 3.2. Glucose content in cortical tissue in 1992-93 field samples of Red-osier dogwood. Circle with vertical bar represents mean ± standard error of triplicate samples from GC analysis.

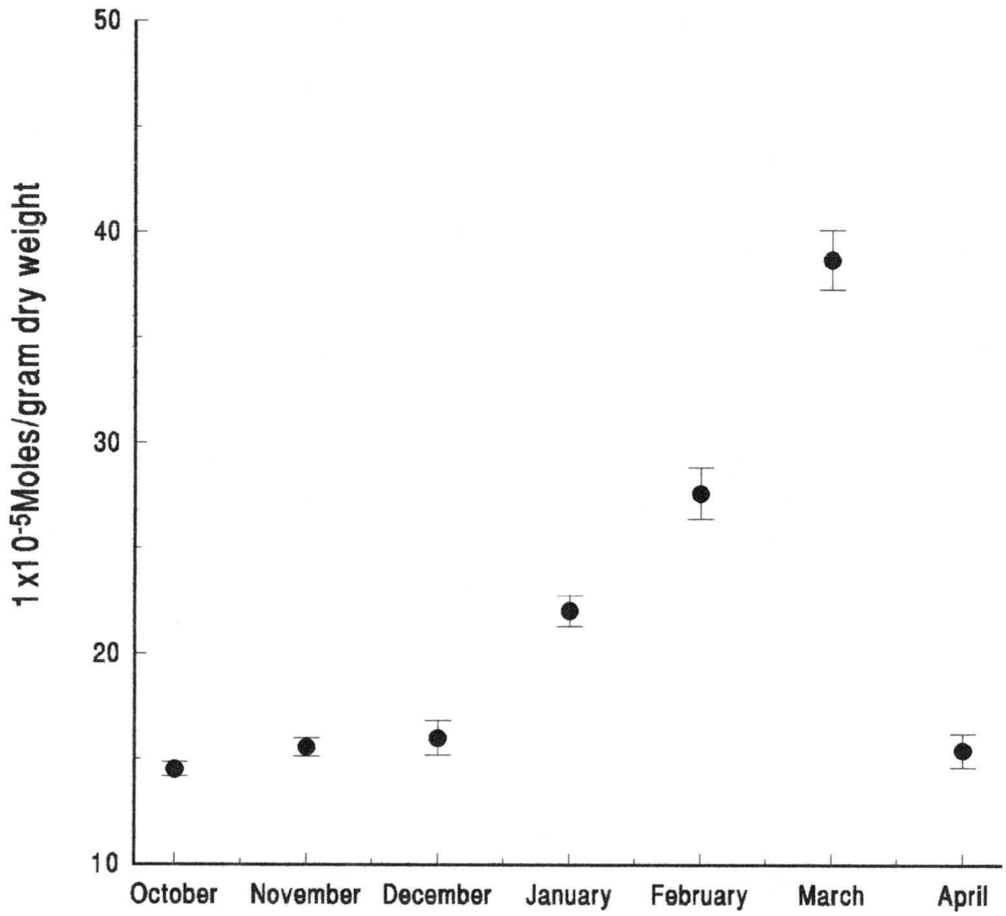

Figure 3.3. Sucrose content in cortical tissue in 1992-93 field samples of 'Red Lake' currant. Circle with vertical bar represents mean ± standard error of duplicate samples from 'Dionex' HPLC analysis.

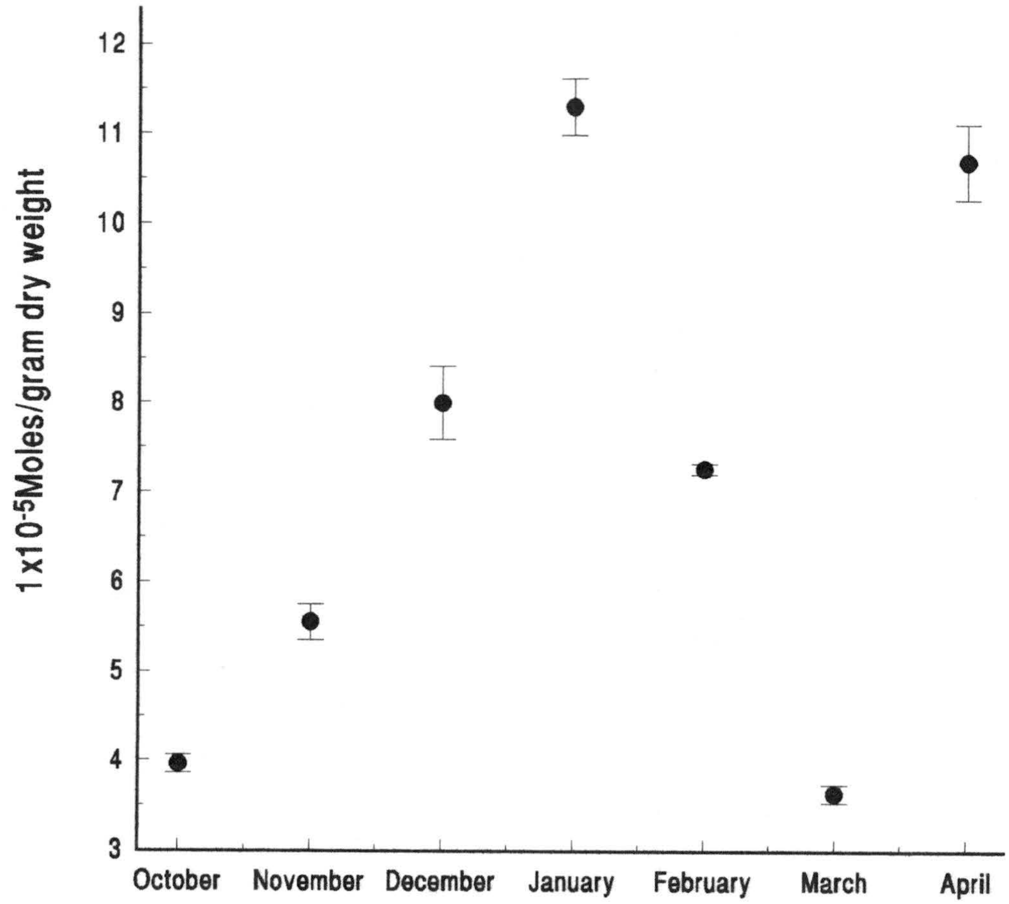

Figure 3.4. Sucrose content in cortical tissue in 1992-93 samples of Red-osier dogwood. Circle with vertical bar represents mean ± standard error of triplicate samples from GC analysis.

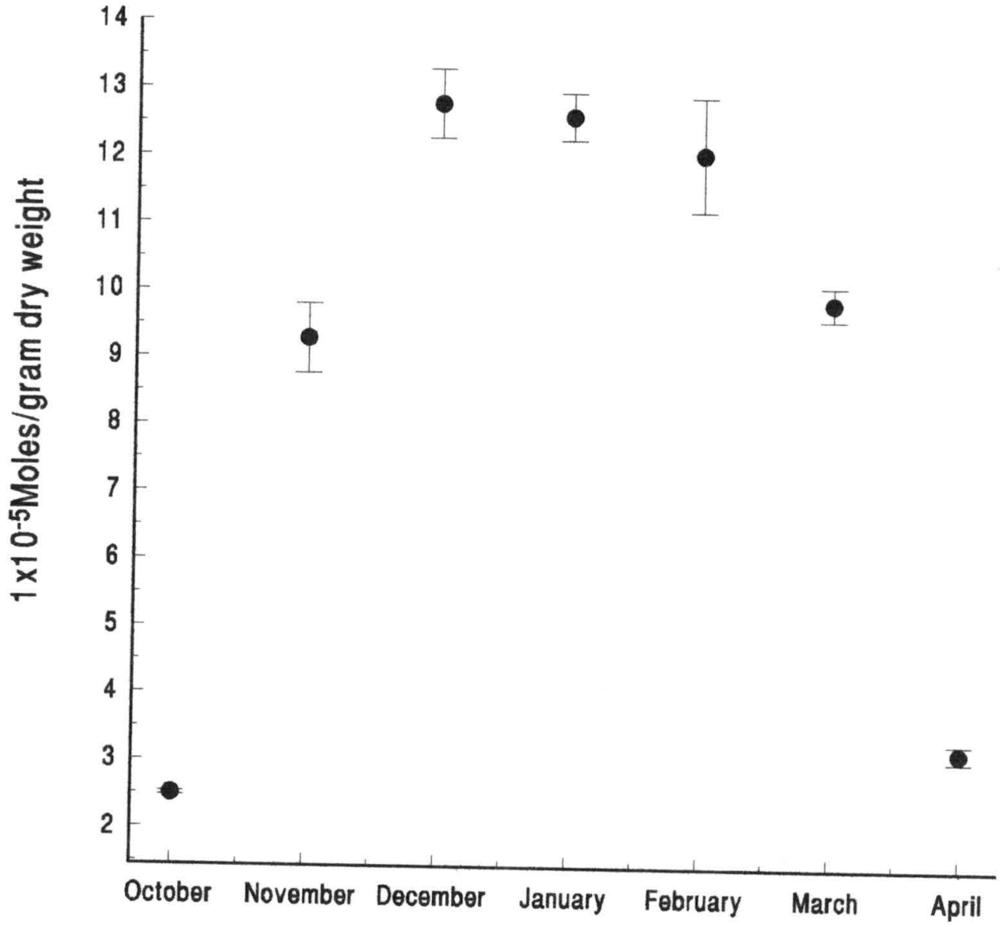

Figure 3.5. Raffinose content in cortical in tissue in 1992-93 field samples of 'Red Lake' currant. Circle with vertical bar represents mean ± standard error of duplicate samples from 'Dionex' HPLC analysis.

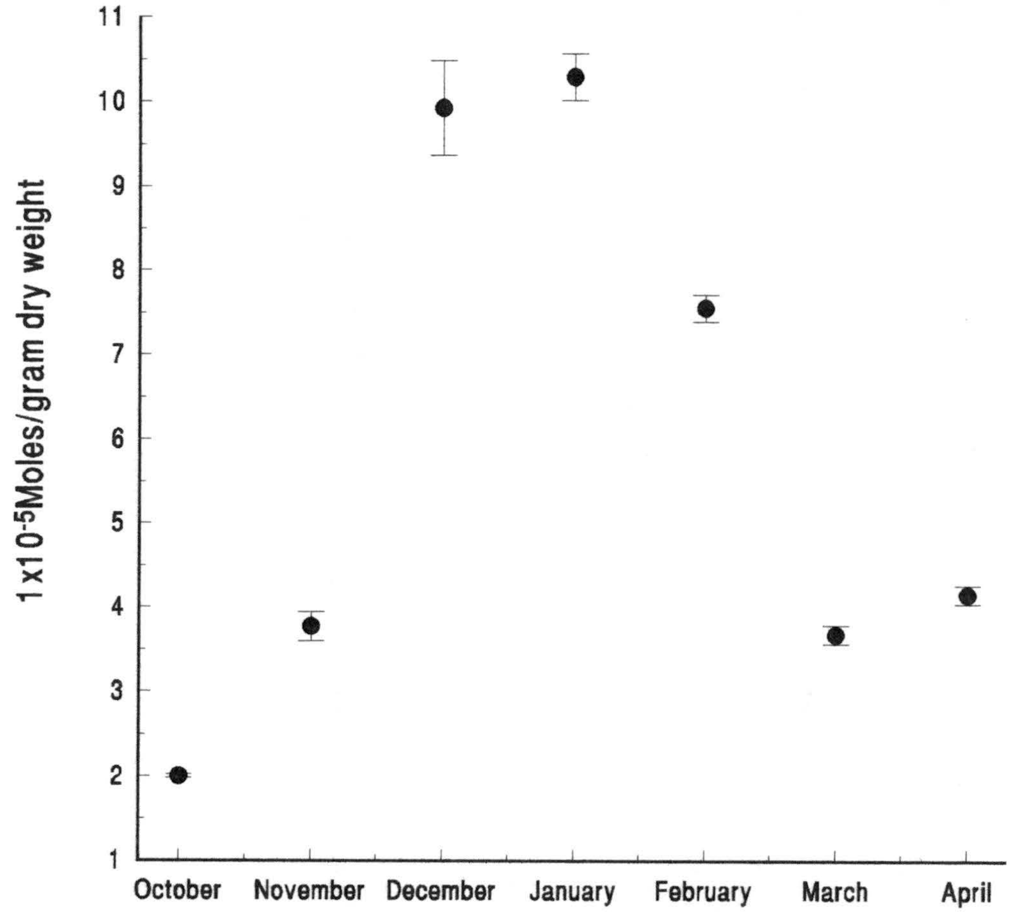

Figure 3.6. Raffinose content in cortical tissue in 1992-93 filed samples of Red-osier dogwood. Circle with vertical bar represents mean ± standard error of triplicate samples from GC analysis.

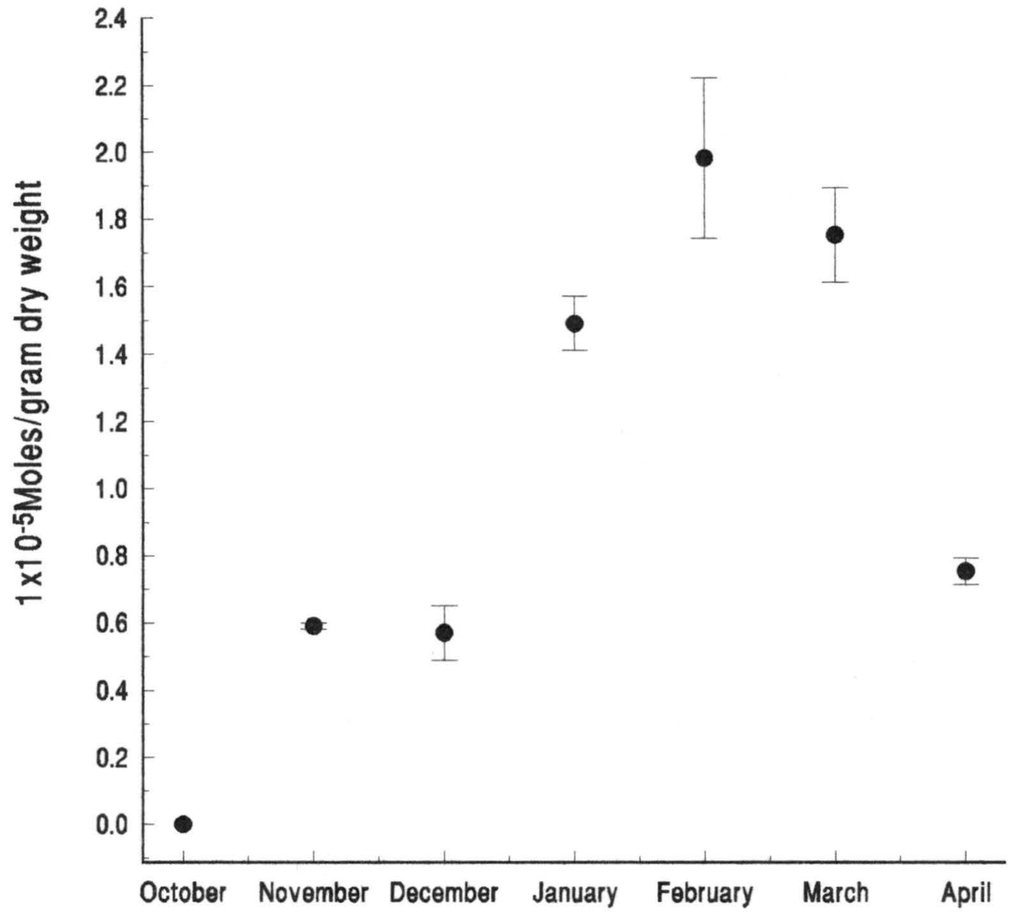

Figure 3.7. Stachyose content in cortical tissue in 1992-93 field samples of 'Red Lake' currant. Circle with vertical bar represents mean ± standard error of duplicate samples from 'Dionex' HPLC analysis.

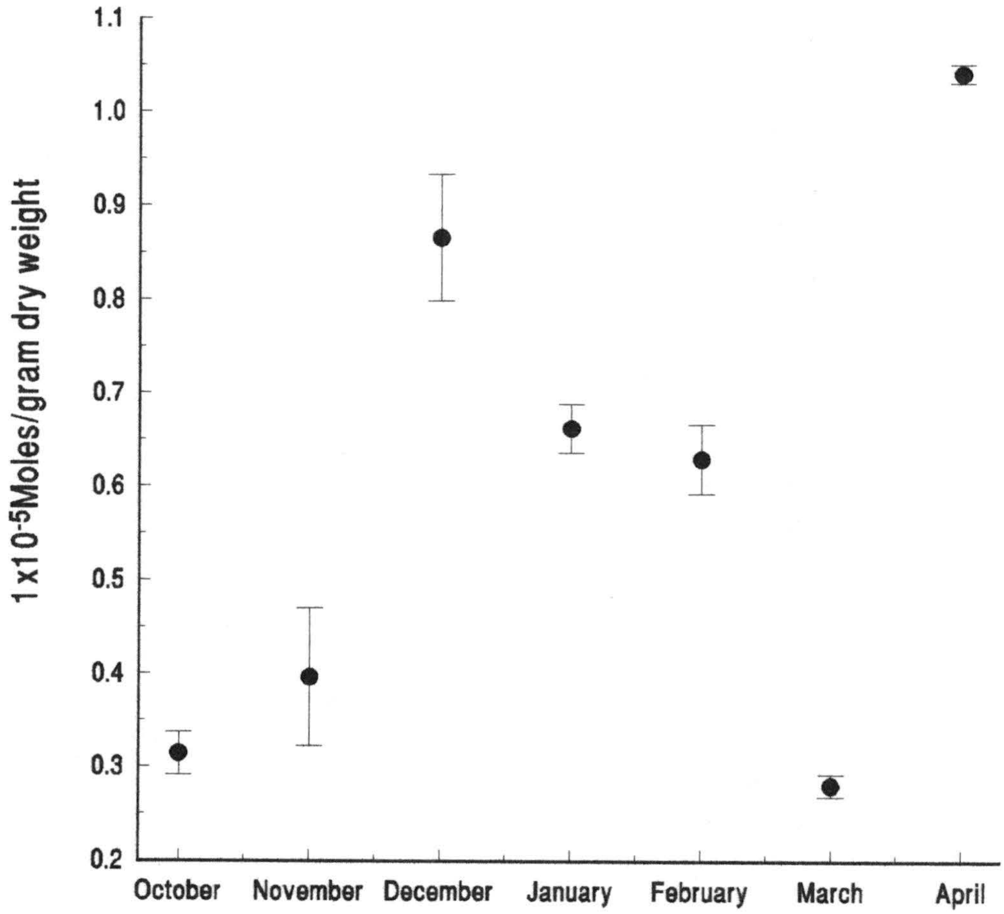

Figure 3.8. Stachyose content in cortical tissue in 1992-93 samples of Red-osier dogwood. Circle with vertical bar represents mean ± standard error of triplicate samples from GC analysis.

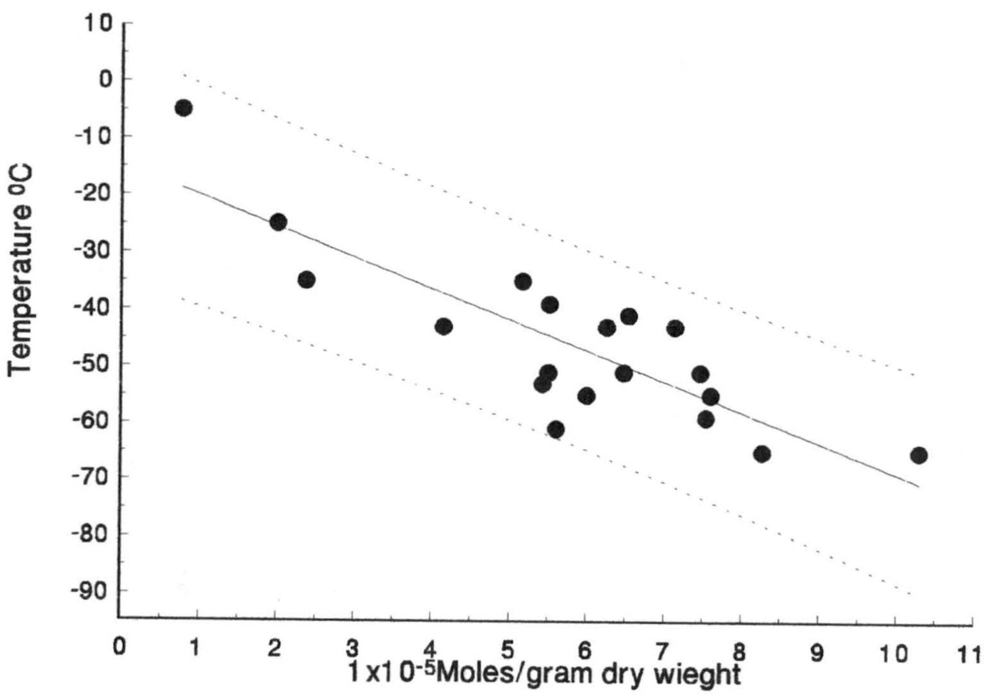

Figure 3.9. The relationship between raffinose content in cortical tissue and hardiness as indicated by freeze tests (Chapter 1) in Red-osier dogwood, 1992-93. Solid line indicates linear regression, and dotted lines indicate 95% confidence interval.

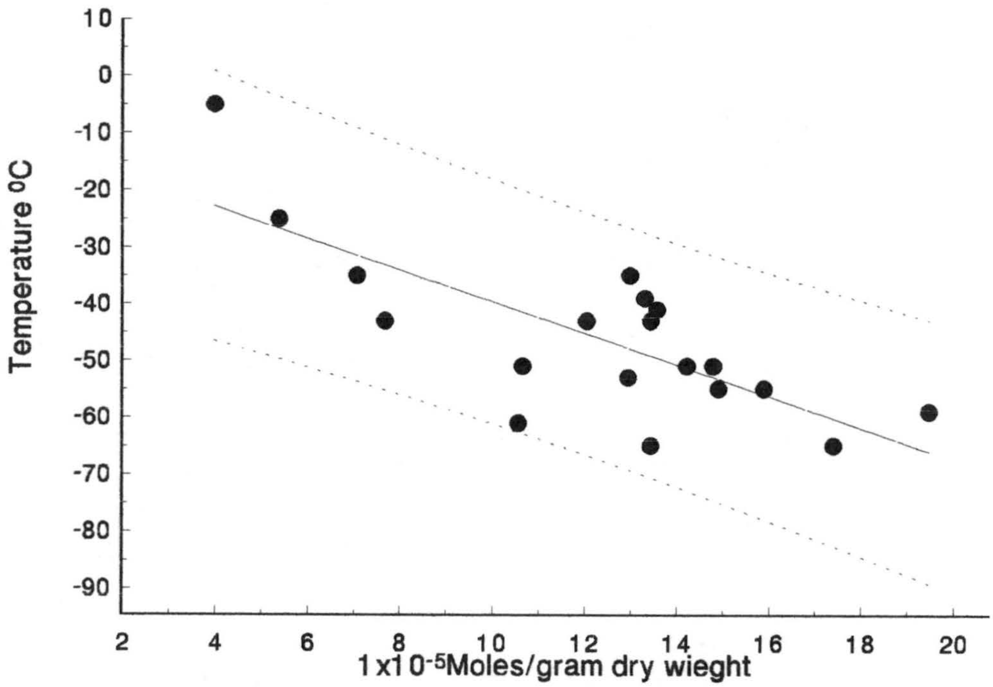

Figure 3.10. The relationship between glucose content in cortical tissue and hardiness as indicated by freeze tests (Chapter 1) in Red-osier dogwood, 1992-93. Solid line indicates linear regression, and dotted lines indicate 95% confidence interval.

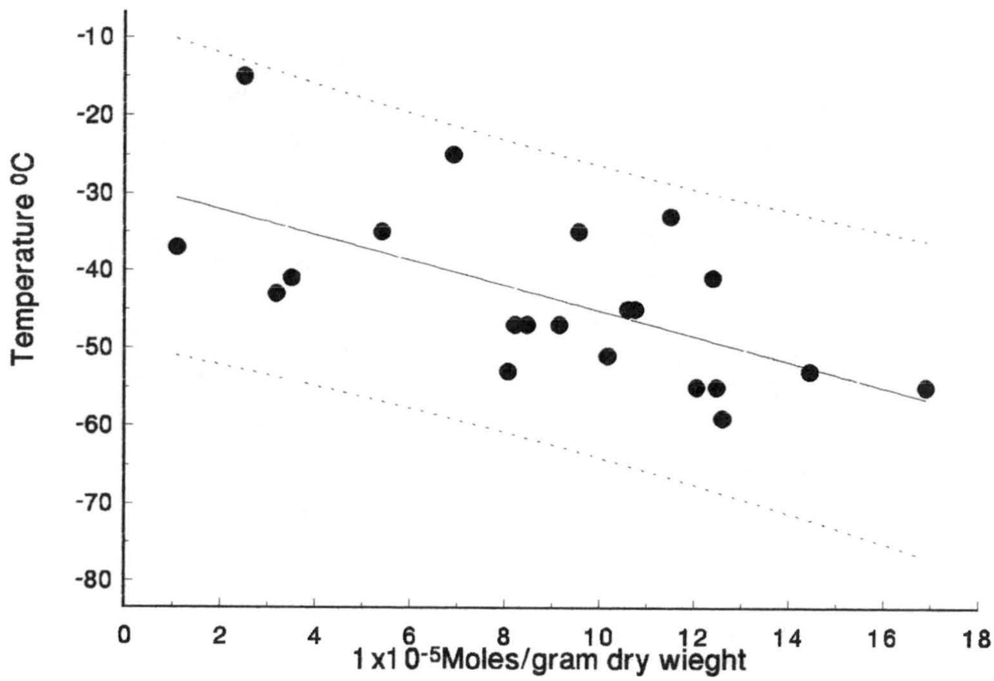

Figure 3.11. The relationship between raffinose content in cortical tissue and hardiness as indicated by freeze tests (Chapter 1) in 'Red Lake' currant, 1992-93. Solid line indicates linear regression, and dotted lines indicate 95% confidence interval.

LITERATURE CITED

Ali, A., D. 1994. Heritability and Screening for Cold Hardiness in Lentil. Ph. D. Thesis, Colorado State University. 75p.

Ashworth, E. N., V. E. Stirm, and J. J. Volenec. 1993. Seasonal variations in soluble sugars and starch within woody stems of *Cornus sericea* L.. Tree Physiology 13: 379-388.

Bachmann, M., P. Maltile, and F. Keller. 1994. Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga repatans* L. Plant Physiology 105: 1335-1345.

Bittinbender, H. C., and G. S. Howell.1974. Adaptation of the Kärber method for estimating the T_{50} of cold Stressed flower buds. J. Amer. Hort. Sci. 99:187-190.

Burke, M. J.,L. V. Gusta, H. A. Quamme, C. J. Weiser, and P. H. Li. 1976. Freezing and injury in plants. Ann. Rev. Plant Physiology 27:507-528.

Burr, K. E., R. W. Tinus, S. J. Wallner, and R. M. King. 1990. Comparison of three cold hardiness tests on conifer seedlings. Tree Physiology 6: 351-369.

Burr, K. E., R. W. Tinus, S. J. Wallner, and R. M. King. 1989. Relationships among cold hardiness , root growth potential, and bud dormancy in three conifers. Tree Physiology 5: 291-396.

Burr, K. E. and R. W. Tinus. 1988. Effect of the timing of cold storage on cold hardiness and root growth potential of douglas fir. General Technical Report. Rocky Mountain Forest Range Station, U. S. D. A. Forest Service. December 1988. 167: 133-138.

Burr, K. E., R. W. Tinus, S. J. Wallner, and R. M. King. 1986. Comparison of four cold hardiness tests on three western conifers. General Technical Report. Rocky Mountain Forest and Range Station, U.S.D.A. Forest Service. December 1986. 137: 87-95.

Caffrey, M., V. Fonseca, and A. C. Leopold. 1988. Lipid-sugar interactions; relevance to anhydrous biology. Plant Physiology 86: 754-758.

Castillo, E. M., B. O. de Lumen, P. S. Reyes, and H. Z. de Luman. 1990. Raffinose synthase ad galactinol synthase in developing seeds and leaves of legumes. J. Agric. Food Chem. 38: 351-355.

Chen, P. M., and P. H. Li. 1976. Induction of frost hardiness in stem cortical tissues of *Cornus stolonifera* Michx. by water stress. Plant Physiology 59: 240-243.

Dexter, S. T., W. E. Tottingham, and L. F. Graber. 1932. Investigations on the hardiness of plants by measurement of electrical conductivity. Plant Physiology 7: 63-78.

Dey, P. M.. 1985. D-Galactose Containing Oligosaccharides . In Biochemistry of Storage Carbohydrates in Green Plants: Dey, P. M. and R. A. Dixon, Eds.: Academic Press, New York.

Gay, A. P. and C. F. Eagles. 1991. Quantitative analysis of cold hardening and dehardening in *Lolium*. Annals of Botany 67: 339-345.

Herber, U. W. and K. A. Sentarius. 1964. Loss of adenosine triphosphate synthesis caused by freezing and it's relationship to frost hardiness problems. Plant Physiology 39: 712-719.

Junttila, O.. 1989. Physiological responses to low temperature. Annals of Forest Science. 46: 604-613.

Junttila, O., C. Stuffnoff, and L. Gusta. 1983. Dehardening in flower buds of saskatoon-berry, *Amelanchier alnifolia,* in relation to temperature, moisture content, and spring bud development. Canadian Journal of Botany. 61: 164-170.

Karam, F. H., and J. A. Sullivan. 1991. A rapid method for detection of cold hardiness in roses. Hort Science 26: 59-60.

Kobayashi, K. D., L. H. Fuchigami, and C. W. Weiser. 1983. Modeling cold hardiness of Red-Osier dogwood. J. Amer. Soc. Hort. Sci. 108: 376-381.

Koster, K. L., and D. V. Lynch. 1992. Solute accumulation and compartmentation during the cold acclimation of puma rye. Plant Physiology 98: 108-113.

Koski, V. 1985. The timing of hardening and dehardening of forest trees. Acta Horticulturae 168: 117-124.

Levitt, J. 1980. Responses of plants to environmental stresses. Vol. 1 Chilling, Freezing, and high temperature stresses. Academic Press Inc. Orlando, Florida.

Li, C.H., C. J. Weiser, and R. van Huystee. 1965. Changes in metabolites of Red-osier dogwood during cold acclimation. Amer. Soc. Hort. Sci. 86: 723-730.

Linstrom, O. M., and M. A. Dirr. 1989. Acclimation and low-temperature tolerance of eight woody taxa. Hort Science 24: 818-820.

Lynch, D. V., and P. L. Steponkus. 1987 Plasma Membrane Lipid Alterations Associated with Cold Acclimation of Winter Rye Seedlings (*Secale cereale*L. cv. Puma). Plant Physiology 83: 761-767.

Olien, C. R.. 1967. Freezing stress and survival. Ann. Res. Plant Physiol. 19: 387-408.

Parker, J.. 1959. Seasonal variations in sugars of conifers with some observations on cold resistance. Forest Science 5: 56-63.

Purcell, A. E., and R. H. Young. 1963. The use of tetrazolium in assessing freeze damage in citrus trees. Amer. Soc. Hort. Sci. 83: 352-358.

Quamme, H. A., and C. Stushnoff. 1983. Resistance to environmental stress. Methods in Fruit Breeding, Chapter 15. Editors: J. N. Moore and J. Jansick. Purdue University Press. West Lafayette, Indiana.

Rease, J. T., M. W. Williams, and H. D. Billingsley. 1978. Cold hardiness, sorbitol, and sugar levels of apple shoots as influenced by controlled temperature and season. J. Amer. Hort. Sci. 103: 796-801.

Rease, J. T.. 1977. Induction of cold hardiness in apple tree shoots with epthephon, NAA, and growth retardants. J. Amer. Hort. Sci. 102: 789-792.

Sakai, A. and W. Larcher. 1987. Frost Survival in Plants. Springer-Verlag. New York, N. Y.

Sakai, A.. 1982. Freezing resistance of ornamental trees and shrubs. J. Amer. Soc. Hort. Sci. 107:572-581.

Sakai A., and S. Yoshida. 1968. The Role of sugar and related compounds in variations of freezing resistance. Cryobiology 5: 168-174.

Sakai, A.. 1965. Studies of frost hardiness in woody plants. II. Effect of temperature hardening. Plant Physiology 41: 353-359.

Sakai, A.. 1962. Studies on the frost-hardiness of woody plants I. The causal relation between sugar content and frost hardiness. Contrib. Instit. Low Temp Sci., Sapporo, Japan. Series B. 11: 1-40.

Steponkus, P. L., M. Uemura, R. A. Belsamo, T. Arvinte, and D.V. Lynch. 1988. Transformation of the plasma membrane lipid composition. Proc. Natl. Acad. Sci. 85, 9026-9030. December 1988.

Steponkus, P. L., 1979. Effect of freezing and cold acclimation on membrane structure and function. P. 143-158. In H. Mussell and R. C. Staples editors. Stress Physiology of Crop Plants. Wiley Interscience Publishers. New York, N.Y.

Steponkus, P. L., and F. O. Lanphear. 1967. Light stimulation of cold acclimation: production of a translocatable promoter. Plant Physiology 42: 1673-1679.

Stushnoff, C., R. L. Remmele Jr., V. Essensee, and M. McNeil. 1993. Low temperature induced biochemical mechanisms: Implications for cold acclimation and de-acclimation. In M. B. Jackson and C. R. Black editors. Interacting Stresses on Plants in a Changing Climate. Springer-Verlag Berlin Heidelberg.

Sweely, C. C., R. Bentley, M. Makita, and W. W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Amer. Chem. Soc. 85: 2497.

Van Huystee, R. B., C. J. Weiser, and P. H. Li. 1967. Cold acclimation in *Cornus stolonifera* under natural and controlled photoperiod and temperature. Botanical Gazette 128: 200-205.

Weiser, C. J.. 1970. Cold resistance and acclimation in woody plants. Hort Science 5: 403-409.

Wilner, J.. 1961. Relationship between certain methods and procedures of testing for winter injury of outdoor-exposed shoots and roots of apple trees. Can. J. Plant Sci. 41: 309-315.

Woodward, F. I.. 1990. The impact of low temperatures in controlling the geographical distribution of plants. Phil. Trans. R. Soc. Lond. B 326: 585-593.

Yelenosky, G., and C. L. Guy. 1977. Carbohydrate accumulation in leaves and stems of 'Valencia' orange at progressively colder temperatures. Botanical Gazette 138:13-17.

Young, R.. 1969. Cold Hardening in Citris Seedlings as related to artificial hardening conditions. J. Amer. Soc. Hort. Sci. 94: 612-614.

Yoshida, S.. 1975. Changes in microsomal enzymes and phospholipid during dehardening in stem bark of black locust. Plant Physiology 57: 710-715.