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COLD HARDINESS OF GRAPEVINES:

-UTILITY OF AMBIENT TEMPERATURES AND TISSUE WATER CONTENT TO ESTIMATE COLD HARDINESS.
-EFFICACY OF CHLOROPHYLL FLUORESCENCE AS AN OBJECTIVE VIABILITY TEST FOR WOODY TISSUES

By

Hongying Jiang

AN ABSTRACT OF A DISSERTATION

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ABSTRACT

COLD HARDINESS OF GRAPEVINES

-UTILITY OF AMBIENT TEMPERATURES AND TISSUE WATER CONTENT TO ESTIMATE COLD HARDINESS
-EFFICACY OF CHLOROPHYLL FLUORESCENCE AS AN OBJECTIVE VIABILITY TEST FOR WOODY TISSUES

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Freezing stress causes losses of millions of dollars in agricultural production each year in the US. Cold hardiness is a complex phenomenon and is affected by many environmental and physiological factors. Conventional methods of evaluating cold hardiness of woody vegetative tissues are presently limited by time consuming, subjective methods: the tissue browning and regrowth tests. Studies were conducted on two components of this complex using Concord grapevines in MI: 1) Evaluating cold hardiness changes influenced by air temperatures and tissue water content during the dormant season with the purpose of using readily available data to estimate vine cold hardiness in the field; 2) Determining the efficacy of chlorophyll fluorescence (CF) technique as an effective method for evaluating cold hardiness and viability of woody vegetative tissues.

Conventional cold hardiness and water content measurements as well as chlorophyll fluorescence measurements were conducted regularly on 1-year-old canes of bearing Concord grapevines during the 1997-1998, 1998-1999, and 2000 dormant seasons. Results showed that: 1) cold hardiness, expressed as T_{50} (temperature at which 50% of the sample is injured), of buds and canes was closely correlated to cane water

content, and can be predicted from minimum air temperatures 1 to 2 d before each cold hardiness evaluation, and a series of best-fit equations were evaluated; 2) a protocol was developed for assessing CF as a viability test for grape canes and wood; Fv/Fm (ratio of variable fluorescence to maximum fluorescence) was demonstrated to be a good estimator of tissue injury after a controlled freeze-stress; 3) the Fv/Fm temperature inflection points (temperature at which no and 100% injury occurred) were closely related to T_{50} calculated from a regrowth test; 4) Fv/Fm injury thresholds $Fv/Fm_{0,}$ $Fv/Fm_{50,}$ and Fv/Fm_{100} (the Fv/Fm values when 0%, 50%, and 100% of the tissue were injured, respectively) are proposed for different woody tissues (1-year vs. 2-year) at three dormant periods (cold acclimation, midwinter, and deacclimation); and 5) preliminary applications to other grape varieties and field measurement were also demonstrated.

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KEY TO SYMBOLS AND ABBREVIATIONS

CA: cold acclimation

CF: chlorophyll fluorescence

d: day(s)

DCA: deacclimation

Fo: initial or minimal chlorophyll fluorescence

Fm: maximal chlorophyll fluorescence

Fv: variable chlorophyll fluorescence (Fm - Fo)

Fv/Fm: ratio of variable to maximum fluorescence.

hr: hour(s)

LTE: low temperature exotherm

min: minute(s)

MW: midwinter

n.a.: not available

PS II: photosystem II

s: second(s)

Tmax: maximum ambient air temperature

Tmin: minimum ambient air temperature,

Tmean: mean ambient air temperature.

wk: week(s)

LITERATURE REVIEW

OUTLINE

1. Overview of the Problem (Introduction)
   - Types of low temperature stress
   - Severity of freezing injury (survival and economic loss)
   - Cold hardiness index
2. The mechanisms of plant injury by freezing temperatures

   Intercellular ice formation and extracelluar freezing
3. The mechanisms of plant survival after freezing stress
   - Avoidance and tolerance (supercooling and dehydration)
   - Evolutionary mechanisms (natural process)
4. Cold hardiness of perennials woody plants and grapevines
   - Annual cycle of the dormant season
   - Physiological and metabolic changes accompanying hardiness dynamics
5. Techniques of cold hardiness measurement
   - Sampling criteria and methods
   - Field assessment
   - Controlled freezes
   - Viability assessment
   - Differential thermal analysis (DTA)
   - Infrared video thermography
   - Chlorophyll fluorescence (CF)
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INTRODUCTION

During their life cycles, plants maybe exposed to periods of environmental stresses, such as low or high temperatures, drought, flooding, and pollution. Low temperature (LT) is one of the most important factors limiting plant growth, distribution, and production of fruit crops in many regions of the world (Alberdi and Corcuera, 1991; Palonen and Buszard, 1997; Parker, 1963). Improving cold hardiness, the ability of a plant or plant part to survive/resist freezing conditions (Fuchigami, 1996), has been a major objective in many breeding and physiological research efforts (Palonen and Buszard, 1997).

Types of low temperature stress

There are two main types of low temperature stress: chilling stress and freezing stress. Tropical and subtropical plants at temperatures between –1 and 10 °C usually experience chilling stress. Freezing stress occurs at temperatures below –1 °C. Since most of the world's major crop-growing regions are subject to freezing stress (Guy, 1990b), this review will focus on freezing stress to woody perennials, and specifically on grapevines (*Vitis* sp.). Understanding how grapevine and other perennial woody plants respond to freezing stress is critical for developing approaches to mitigating these losses.

In the course of evolution, plants have developed complicated mechanisms to protect against freezing stress. A dynamic change in cold hardiness occurs during the year. Tissues from a plant that are killed a few degrees below zero in the active growing season may survive -196 °C (tested in the lab) in the winter (Sakai and Weiser, 1973; Weiser, 1970a, 1970b). The increase in cold hardiness from fall to winter (known as cold acclimation, CA, hardening) is followed by a spring decline in cold hardiness (deacclimation, DCA, dehardening).

Severity of freezing injury (survival and economic loss)

Freezing damage can occur at any time during the year. The impact of extremely low temperatures can be severe. For example, on 19 January 1994, the minimum air temperature (-47 °C) in Amasa, Michigan was the lowest recorded in the last 100 years, and the maximum temperature on the same date was only -15 °C (Michigan Agricultural Statistics, 1994). In Southwest Michigan where most grapes are grown, temperatures reached as low as –27 °C (Howell, 1994a). These severe freeze episodes caused considerable damage to fruit trees and grapevines; the collective economic loss exceeded $22 million, and the production of *Vitis vinifera* wine grapes was reduced 65% (MI agricultural Statistics, 1995). Most *vinifera* grapes in southwestern MI were killed to the snow line (Howell, 1994a).

Even when there are no extremely low temperatures in the winter, plants may be injured by unseasonably cold temperatures or temperature fluctuations in the fall and/or spring, transitional periods of hardening and dehardening. Subfreezing temperatures during the blossom period result in greater losses of production for fruit crops than any other environmental and biological hazard in the US (USDA, 1988). Spring freeze injury is a problem endemic to many states in the US, and no viticultural area in the United States is completely free from spring freeze injury (Howell and Wolpert, 1978).

Cold hardiness is a complex phenomenon and is affected by both external factors (e.g. air temperature, day length) and internal factors (e.g. genotype, physiological age, maturity, dormancy status, development stages, water content, and nutrients) (Stushnoff, 1972; Weiser, 1970a, 1970b). Even within a single plant (e.g. 'Concord' grapevines), the difference in cold hardiness can be considerably different (up to 12 °C for 'Concord'

grapevines) among adjacent tissues or positions within the same tissue or organ (Howell and Shaulis, 1980; Sakai and Weiser, 1973; Weiser, 1970a, 1970b; Wolpert and Howell, 1985a). Understanding of the nature and responses of individual species and their commercially important varieties/cultivars is also important (Chandler, 1954). 'Concord' (*V. labruscana* Bailey) is the major grape cultivar in Michigan, Pennsylvania and New York. Although 'Concord' cold hardiness and vine physiology have been studied extensively in the last 20 years (Fennell and Hoover, 1991; Howell and Shaulis, 1980; Stergios and Howell, 1977; Wample and Wolf, 1996; Wolf and Cook, 1992; Wolpert and Howell, 1984, 1985a, 1985b, 1986a, 1986b), unanswered questions remain. These include: 1) How can changes in cold hardiness be predicted over the dormant season with easily measured environmental and physiological parameters? And, 2) how can cold hardiness be evaluated objectively and more efficiently than current time consuming, subjective methods of tissue browning and regrowth tests? Therefore, my research has focused primarily on these topics utilizing 'Concord' grapevines.

Cold hardiness index

Percentage of killing was originally used in cold hardiness research (Proebsting and Fogle, 1956). However, since the percentage data are not easy to analyze and compare, probit analysis was employed (Proebsting and Fogle, 1956). Probits are transformations of percentage data following a sigmoid curve to values with a straight-line function. The relationship between subfreezing temperatures and the killing percentage is usually a sigmoid curve. Therefore, temperatures of specific killing responses were used as cold hardiness index.

A killing point is the temperature that the plant or tissue is injured beyond recovery. Hardiness T_{50} is the temperature at which 50% of the samples are injured (Bittenbender and Howell, 1974). T_{50} is modified from LD_{50} in toxicology, which represents the insecticide dosage that is lethal to 50% of the population (Proebsting and Fogle, 1956). The same tissue of the same cultivar may have different killing points or T_{50}s during different periods of dormant season. T_{50} has been used widely now as a statistically valid and logical hardiness index.

THE MECHANISMS OF PLANT INJURY BY FREEZING TEMPERATURES

Freezing injury occurs when ice forms in the tissues of tender or actively growing plants, regardless of the temperature at which freezing is initiated (Chen et al, 1995). In contrast, hardy plants can tolerate some ice formation in their tissues after acclimation in the fall (Weiser, 1970a, 1970b). While injury is closely related to cooling rate, site of ice nucleation, ice nucleation temperature, rate of ice growth, and duration of exposure to freezing, Levitt (1980) classified freezing injury into three types: (1) primary direct injury due to intracellular freezing, (2) secondary, freeze-induced dehydration injury due to extracellular freezing, and (3) injury due to other secondary or tertiary freeze-induced stresses.

Intracellular ice formation

Rapid freezing (5-20 °C hr^{-1}, Levitt, 1980) favors ice formation within the cell, rupturing the plasmalemma, and causing immediate cell death. Intracellular ice crystal formation causes cell death because of its mechanical destruction of the membrane system (Burke et al., 1976; Levitt, 1980; Sakai, 1973). However, some cells can survive intracellular freezing when freezing is extremely fast (>100 °C s^{-1}), during which only fine ice crystals

or no crystals are formed (known as vitrification, Levitt, 1980). If the thawing process is also extremely fast, ice crystals cannot grow large enough to cause injury; this is the mechanism for cryopreservation (Chen et al, 1995; Levitt, 1980). Even non-hardy cells can survive vitrification to extremely low temperatures (Weiser, 1970a, 1970b). Thus, it is the ice crystal (not the low temperature) that causes the mortality (Levitt, 1980; Weiser, 1970a, 1970b).

Extracelluar freezing

Slow freezing (<5 °C hr^{-1}), typical in the field, results when water in the extracellular spaces freezes first producing lower water potential. Consequently, intracellular water moves across the plasma membrane to extracellular ice nucleation points as temperatures slowly decline (Levitt, 1980; Sakai, 1982). When this process is slow enough, the volume of cytoplasm decreases gradually and the cell sap is concentrated (dehydrated). This dehydration reduces the freezing point of the intracellular water (Burke et al, 1976) and enhances the supercooling (avoidance of ice formation at its theoretical freezing point) ability (Ishikawa and Sakai, 1981). Equilibrium is reached when the water potential of the cell water equals that of the extracellular ice at a given temperature.

Length of frozen period

There are no conclusive results on the impact of length of freezing duration on plant survival (Levitt, 1980). Injury may occur upon freezing. However, once equilibrium is reached, the injury seems to be independent of freezing duration over a relative short period of time (2-24 hr). However, for longer periods (1-30 d), injury appears to increase with the length of time being frozen (Levitt, 1980), although plant cold hardiness

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increases with increasing days in sub-zero chambers (Howell and Weiser, 1970a). Increased hardiness does not necessarily guarantee no injury.

Freeze and thaw cycle

Repeated freezing and thawing (temperature fluctuation) can be more harmful than a single freezing episode because thawing can raise the killing temperature (deharden) dramatically (15 °C) within 24 hr (Howell and Weiser, 1970a). Dehardened plants/tissues are killed more easily if a freezing episode follows warm temperature exposure. This is the mechanism behind sunscalding of tree trunks in the winter.

Freezing injury (≤ -30 °C) in woody stems is not a continuous process; rather the injury is associated with a distinct freezing point (Weiser, 1970a, 1970b). Tumanov and Krasavtsev (1959) and Weiser (1970a, 1970b) proposed two hypotheses (a second-supercooling-point and vital water exotherm), respectively, to explain how freezing stress causes cell death. The key points of "second-supercooling-point" are: during freezing, a point is reached when the free movement of water out of the cell is restricted by the protoplast and/or plasma membrane. The intracellular ice formation occurs suddenly resulting in death. "Vital water exotherm" hypothesis stated that during freezing, a point is reached when all readily available water has frozen extracellularly and only 'vital' water remains in the protoplasm. As the temperature declines, vital water is removed from the protoplasm and freezes extracellularly. These chain reactions cause the death. Levitt (1980) proposed that a critical freezing zone existed for each hardy plant. Only in the critical zone are the rates of freezing and thawing important to the death of the plant.

Extracellular freezing is not lethal to hardy plants. However, the ice formation could also result in dehydration (drought stress) and injury when tissues are cooled below

a critical temperature, or if they are held frozen for a prolonged period of time at temperature slightly higher than the killing temperature (Chen et al. 1995). Dehydration would cause structural transitions of many temperature-dependent cell substances, such as protein aggregation and membrane phase transition (Burke and Stushnoff, 1979).

In all, freeze stress kills the plant by ice formation within the protoplasm, not the low temperature essentially.

THE MECHANISMS OF PLANT SURVIVAL AFTER FREEZING STRESS

Avoidance and tolerance are two major types of resistance to freezing stress (Levitt, 1980). Before lethal low temperatures occur, annuals and some herbaceous perennials partially or completely end life cycles of the above ground tissues to avoid ice formation. Woody perennials, however, can tolerate the stress of ice formation in the tissues (Levitt, 1980). Based on the above freezing injury mechanisms, it appears that hardy plants survive freezing stress by either avoiding ice formation (supercooling/deep supercooling) or tolerating its presence in the tissue (Levitt, 1980).

Supercooling

Supercooling is the process by which a solution remains in the liquid state several degrees below its theoretical freezing point (Taiz and Zeiger, 1998). In plant tissues, it can be expressed as either supercooling or deep supercooling. Supercooling can prevent ice formation until -10 to -15 °C (Burke et al., 1976), and deep supercooling can be as low as the homogeneous nucleation temperature of the aqueous solution (approximate –38 to –40 °C for plant solutions) (Becwar et al., 1981; Burke et al., 1976; Quamme, 1995). Homogeneous nucleation point refers to the temperature (c.a. –38 °C) at which

pure water automatically crystallize in the absence of nucleators (particles that act as a core/initiator for ice to form (Burke et al., 1976).

Tumanov et al. (1969) first reported supercooling on cherry flower buds, but Graham (1971) was the first to demonstrate that the killing temperature of azalea flower buds was correlated with the tissue supercooling temperature. Since this report, extensive research has been conducted on buds and stems of many species (Burke et al.,1976; Graham and Mullin, 1976a, 1976b; Ketchie and Kammereck, 1987; Quamme, 1978, 1991 and 1995; Quamme et al., 1983). Supercooling to –3 to –10 °C has also been measured *in situ* on leaves of herbaceous plants under field conditions (Anderson et al., 1982, Lindow et al 1978).

Deep supercooling, however, was primarily observed in flower primordia, vegetative and compound buds, and xylem ray parenchyma cells in many shrubs and hardwood/fruit trees native to the Eastern deciduous forest, USA (George et al., 1974; Rajashekar and Burke, 1978; Quamme, 1991). It was also found in bark tissues (phloem) of grapes and pears (Pierquet and Stushnoff, 1980; Rajashekar et al., 1982). Quamme (1995) summarized that deep supercooling was found in most fruit crops, including apple, plum, apricot, grape, cherry, blueberry, raspberry, current, and pear.

When a liquid (solution) becomes a solid (phase change), heat is released resulting in a temperature increase known as an exotherm. Usually, deep supercooled tissues exhibit one high temperature exotherm (HTE, around -5 to -10°C) and several low temperature exotherms (LTE, –20 to –48 °C). The HTE is generally a result of ice formation in the apoplast. This is not lethal to woody tissues of hardy perennials (Graham and Mullin, 1976 a; Ishikawa and Sakai, 1981). By contrast, a single LTE, resulting from

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ice formation within the primordium of the tissue (buds), coincides with the killing temperature for that tissue (Andrews and Proebsting, 1987; George et al., 1974; Graham and Mullin, 1976a; Ishikawa and Sakai, 1981; Pierquet and Stushnoff, 1980; Quamme, 1991, 1995). The maximum (lowest) LTE varies with species: peach flower buds ranged from -20 to -30 °C (Quamme, 1978, 1991; Quamme et al., 1983), while apple flower buds from -30 to -40 °C (Ketchie and Kammereck, 1987; Quamme et al, 1983). Compound grape buds supercool and produce LTEs from -20 to -30 °C for tender cultivars (Quamme, 1986), and -30 to -42 °C for hardy cultivars. The xylem ray parenchyma cells of hardy grape canes (*V. riparia*) supercool to near -48 °C (Pierquet et al, 1977), or near the homogeneous nucleation point as reported by Burke et al. (1976).

Deep supercooling reduces tissue-killing temperatures and is an important avoidance mechanism for plant winter survival. It can be used for breeding cold-hardy cultivars (Burke and Stushnoff, 1979; George et al., 1974; Quamme, 1978; Rajashekar and Burke, 1978). It also appears to limit the northern extension or high elevation for survival (-40 to -47 °C) of timberline flora and many important horticultural trees and shrubs. This is because ice formation would automatically initiate when temperatures are below the homogenous point (Becwar et al., 1981; Rajashekar and Burke, 1978). One piece of evidence in support of this suggestion is the lack of deep supercooling in stem tissues of tree species native to the Northern Boreal Forest. These trees are able to survive to temperatures below -45 °C (George et al. 1974).

Although there has been extensive research on supercooling, it is still not clear how and why supercooling is initiated and maintained (Chen et al, 1995; Quamme, 1991). There are three primary requirements for supercooling to occur: 1) the absence, or low

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activity, of ice nucleators on, or within, the tissue when temperatures are above -40 °C (Ashworth and Rowse, 1982; Burke et al. 1976); 2) a lower freezing temperature due to the presence of solutes, which lowers the freezing point (freezing point depression); and 3) the inhibition of ice propagation from adjacent tissues (Quamme, 1995).

Barriers to ice propagation and supercooling

Barriers to ice propagation were first observed by Weigand (1906); ice was formed preferably at some sites not the adjacent places on over-wintering leaves and flower buds. Further, Dorsey (1934) noted that ice was found in the bud scales and axis, but not in the primordial regions at -29.5 °C. Ishikawa and Sakai (1981) reported that vascular tissues at the attachment region of the floret of *Cornus officinalis* and *Rhododendron japonica* might serve as a barrier to ice propagation. Quamme (1978) found similar results with peach flower buds and proposed two structural barriers operating below -10 °C that prevented external nucleation of the supercooling flower primordium. The structural features (e.g. the cell wall of the xylem ray parenchyma) are involved in supercooling of both xylem ray parenchyma and the flower buds (Quamme, 1991).

Supercooling of the flower buds and xylem ray parenchyma appears to differ in temperate broad-leaved trees. Burke and Stushnoff (1979) and Ishikawa and Sakai (1981) observed that the exotherm temperature of the florets declined dramatically (< -40 °C) if they were gradually prefrozen, allowing water to move out from flower primordia to scales or other adjacent tissues. This extra-organ freezing resulted from slow freezing and caused dehydration of the floret, which lowered the freezing point. However, xylem ray parenchyma supercooling was not affected by cooling rate, nor was there any water migration (Quamme et al., 1972; Sakai, 1979, 1982).

Ice nucleation

In the field, supercooling may not occur because there are many ice nucleators available (bacteria, fungi, morning dew, and intrinsic nucleators) that can initiate the freezing process at temperatures close to 0 °C (Chen et al., 1995). Freezing of small amounts of supercooled water in the stem can result in injury to the whole stem (Rajashekar et al., 1982).

Dehydration (Freezing point depression)

Under field conditions, the rate of temperature decline is usually a few degrees per hour (Weiser, 1970a, 1970b). Tissue freezing is initiated at a few ice nucleation sites on the tissue surface, and then ice spreads quickly to the apoplast via plant vessels (Chen et al, 1995; Levitt, 1980) if there are no physical barriers (Quamme, 1991). This process prevents supercooling and reduces intracellular tissue water (dehydrated). Tumanov et al. (1969) suggested that freeze resistance was obtained by several different means, and one of these was by timely and sufficient dehydration of cells.

However, when apple twigs were frozen at a slow rate of 10 °C per day, there was a large amount of water remaining unfrozen below -30 °C (Tumanov and Krasavtsev, 1959). While some of this unfrozen water could freeze extracellularly to -30 °C (Quamme, 1972), other researchers suggest that the rate of temperature decline was critical. Hardy species (birch and willows) prefrozen to -20 °C can survive in liquid nitrogen because cells are dehydrated by removal of freezable water. In the cortex of very hardy twigs, freezable water appears to freeze extracellularly at temperatures of -30 °C or above and no injury is observed during subsequent slow or rapid cooling (Sakai, 1973).

In other words, when the tolerance of freeze-induced dehydration is great, the plant can acclimate markedly and thus increase freezing tolerance.

Using red-osier dogwood (*Cornus stolonifera* Michx), Burke et al. (1974b) also concluded that surviving a freezing temperature exposure depended on its ability to tolerate physiological drought. Chen et al. (1977) observed increased hardiness in water-stressed plants. Thus, dehydration caused by extracellular ice-formation is suggested to be the major mechanism for woody tissues in hardy plants to tolerate freezing stress (Levitt, 1980).

Clearly, both supercooling and dehydration can exist in hardy plants. Levitt (1972, 1980) and others (Burke et al., 1974a, 1974b; Burke and Stushnoff, 1979; Chen et al., 1977) suggested that freezing resistance was a combination of tolerance and avoidance of tissue ice formation. Research at the molecular level showed that freezing and drought tolerance might have certain mechanism in common, e.g. functions encoded by common genes (Hajela et al., 1990).

Some researchers have also suggested that CA could be induced by dehydration. This may be an important winter survival mechanism of supercooling trees (freeze automatically at homogenous nucleation point) native to Canada and Alaska (where temperatures could be lower than homogenous nucleation point) (Becwar et al., 1981; Burke and Stushnoff. 1979; Sakai, 1979). Sakai (1982) demonstrated that the killing temperature of extracellularly frozen cells varies depending on their ability to withstand freeze-dehydration and its stresses when cooled slowly. However, the inherent ability of temperate plants to acclimate in the fall, the time and rate of acclimation, and the stability

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of cold hardiness greatly determined their low temperature survival (Weiser, 1970a, 1970b; Chen et al, 1995).

COLD HARDINESS OF PERENNIAL WOODY PLANTS AND GRAPEVINES

Annual cycle of the dormant season

Most temperate woody perennial plants, including grapevines, have evolved the ability to tolerate harsh winters by dynamic changes in cold hardiness over the year. Generally, there are three stages during dormancy (Howell, 1988, 1994b; Proebsting, 1970; Wolf and Cook, 1992): 1) cold acclimation (CA, hardening), a dramatic increase in cold hardiness beginning in early fall; 2) mid-winter (MW), the achievement and maintenance of the maximum hardiness; and 3) deacclimation (DCA, dehardening), the decrease in cold hardiness in the spring. It is important to mention which period when discussing cold hardiness problems and/or results because the difference/ranking in cold hardiness among species or cultivars varies over the season (Howell et al., 1997). The general profile of 'Concord' cold hardiness (indicated by T_{50}) is shown in Figure.1.

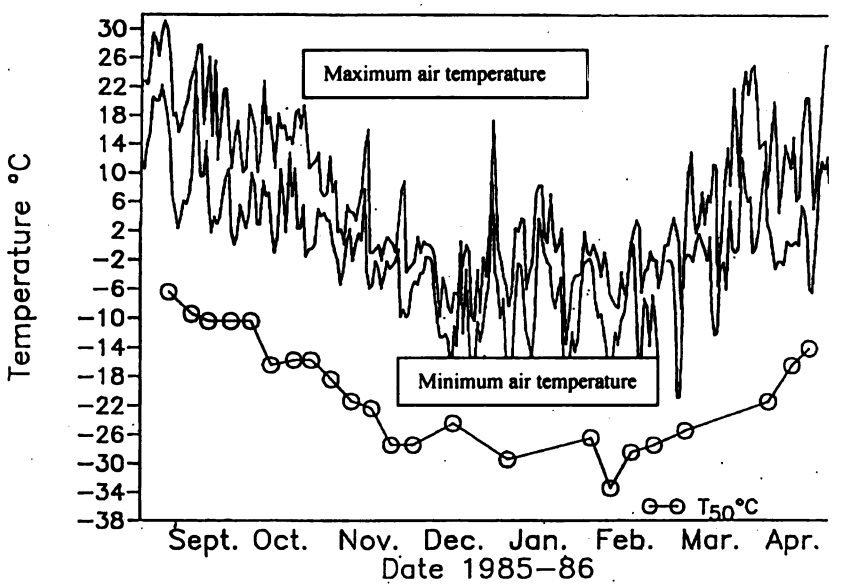

Figure 1. Cold hardiness of Concord grapevines during 1985-1986 dormant season

(Howell, 1994b)

(1) Cold Acclimation

Acclimation progresses in two stages in most woody species (Howell and Weiser, 1970b; Lu and Rieger, 1990; van Huystee et al. 1967; Weiser, 1970a; 1970b). In the first stage, cold hardiness increases from a few degrees below zero to about –10 to –20 °C as CA proceeds. Plants do not get hardier until the second stage is triggered. As the second stage begins, hardiness increases dramatically until maximum hardiness is reached for a specific species.

Under natural conditions, the first stage of acclimation is primarily triggered by short days (SD, actually long nights; Weiser, 1970a, 1970b), but other factors can also be effective. Low temperature (LT) under long days, or high temperature under short days can also achieve the first stage of acclimation to a limited extent (Howell and Weiser, 1970b; Irving and Lanphear, 1967a). Low temperatures (often the first frost, a moderate sub-freezing temperature) seem to be the prerequisite for the second stage of acclimation, which leads to maximum hardiness. A hardiness plateau separated the two stages at about -20 °C for *Cornus stolonifera* (Weiser, 1970a, 1970b).

Photoperiodic induction of CA is mediated by phytochrome (McKenzie et al., 1974a). The status of phytochrome in the plant is determined by the length of the dark period and/or by the light quality received at the end of the day (Shropshire, 1972). Under controlled environments, short days or end-of-day far red light exposure after long days, induced growth cessation and CA (McKenzie et al., 1974a). The site of light perception is the leaf. Leaves grown under SD (or LD) produce translocatable CA promoters (or inhibitors) that move to the over-wintering stem via phloem (Fuchigami et al., 1971b;

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Howell and Weiser, 1970b). So, leaf removal can promote or inhibit CA depending on the day/night length (Howell and Weiser, 1970a, 1970b; Irving and Lanphear, 1967b). However, the LT-induced second stage of CA was not associated with a translocatable substance(s) (Howell and Weiser, 1970b; Weiser, 1970a, 1970b).

Isolation and identification of photoperiodically induced hardiness promoting- or inhibiting-factors has become an interesting research area and hormones seem to be likely candidates. A breakthrough in this area would help in finding chemical control of CA (Howell and Weiser, 1970b; McKenzie et al., 1974b; Weiser, 1970a, 1970b), however, it remains to be accomplished.

Internal plant factors determine CA patterns. Different climatic races of red osier dogwood grown at the same location had different autumnal phenological events, including the onset of rest, leaf abscission and change of bark color. The timing of CA also differed, but the maximum hardiness of all climatic races was almost identical (Smithberg and Weiser, 1968).

The time of initiation and the rate of CA could be crucial for the survival of a species (McKenzie et al., 1974a). Some dogwood races could resist extreme low temperatures in mid-winter, but could not survive the first severe autumn frosts because of the delay in CA. The best CA pattern is the closest to the annual climatic changes; and it appears that races evolve to be in conformity with the local climatic pattern.

Grapevine cold hardiness

Bourne et al. (1991) compared cold hardiness of the primary buds of four grape genotypes in Arkansas. The two genotypes with the greatest genetic component of *V. vinifera* acclimated more slowly in the fall and had less maximum hardiness in mid-

winter than the other two with more percentage of *V. labrusca* in their parents. Wolpert and Howell (1985b, 1986a, 1986b) found that CA of canes and primary buds of 'Concord' in Michigan started in early August. Hardiness increased in an almost linear fashion until mid-November, except for one year, when the hardiness of the primary buds did not change during 4 wk from September to October.

The evidence for photoperiodic effect on cold acclimation of grapevines is not conclusive. Fennell and Hoover (1991) reported that two species, *V. labrusca* and *V. riparia*, responded to photoperiod to a limited extent and with different sensitivities. Short days increased periderm development and the onset of bud dormancy. However, there was little cold hardiness response to photoperiod in *V. vinifera and V. labrusca* (Schnabel and Wample, 1987; Wolpert and Howell, 1986b). Wolpert and Howell (1986b) did show that night interruption significantly delayed cessation of shoot growth and speculated that the intensity of the night interruption was insufficient (although it is comparable to that used in other species). It seems that SD, combined with decreasing temperatures, increased acclimation in *Vitis* while *V. labruscana* is less responsive than other *Vitis* species (Fennell and Hoover, 1991; Schnabel and Wample, 1987).

(2) Midwinter

As CA proceeds, cold hardiness reaches a maximum (the lowest killing temperature) for that plant, and is maintained during MW. This is typically the coldest period of the winter (late December-late February) depending on species or cultivars and environmental conditions. Maximum cold hardiness of apple trees, grapevines, and red-osier dogwood can reach -55 °C, -36 °C and -196 °C, respectively (Howell and Weiser, 1970b; Howell and Shaulis, 1980; Smithberg and Weiser, 1968).

The level of maximum cold hardiness mainly depends on grape genotype. Bourne et al. (1991) reported that cv. 'Mars' (a *V. labrusca* type) is hardier than 'Saturn' (a *V. vinifera* type). Cultural practices (e.g. crop load, pruning, rootstock selection, fertilization) and other environmental stresses (water stress) can also influence the maximum cold hardiness (Hamman et al., 1990; Miller et al., 1988; Wolf and Pool, 1988; Wolpert and Howell, 1984). Factors that improved sunlight penetration into the canopy during the growing season generally favored cold hardiness (Byrne and Howell, 1978; Howell, 1988; Striegler and Howell, 1991), although some researchers found canopy treatment or pruning date did not affect bud hardiness (Hamman et al, 1990; Wample, 1994; Wolf et al., 1986). This discrepancy could be explained by the difference in treatment severities, climates, cultivars, sampling procedures, or other unspecified factors. Superior hardiness can occur only when culture allows the vine to express its maximum genetic potential (Howell, 1988).

Brierley and Landon (1946) and Proebsting (1963, 1970) suggested that dormancy or rest period is the major mechanism by which plants maintain cold resistance. According to results on peaches and cherries, Proebsting (1963, 1970) proposed a concept of minimum hardiness level (MHL), a limiting temperature above which plant hardiness would not decrease even under warm weather (daily minimum temperature > -1 °C). This value was constant until the end of the rest period, i.e. during mid-winter and before deacclimation, or before the chilling requirement has been satisfied. Proebsting et al (1980) also reported similar results of grape buds, i.e. after acclimation, cold hardiness index T_{50} (temperature at which 50% of the sample was injured) was constant until deacclimation.

(3) Deacclimation

Temperature and DCA

Cold hardiness decreases after MW. In contrast with cold acclimation, air temperature (and resulting tissue temperatures) seems to be the primary factor controlling cold hardiness during the DCA (Bittenbender and Howell, 1975a; Edgerton, 1954; Howell and Weiser, 1970a; Proebsting, 1963).

Correlations between hardiness and the preceding air temperatures (e.g. mean or minimum temperatures) were statistically significant, but the r^2 values were less than 0.5 or 0.75 (Bittenbender and Howell, 1975a; Howell and Weiser, 1970a; Proebsting, 1963; Proebsting et al., 1980; Wolf and Cook, 1992). After mid-winter when the plant was still dormant, apple T_{50} fluctuated as much as 15 °C in response to air temperature changes in a day (Howell and Weiser, 1970a). The low temperature exotherm (killing point) of cherry flower buds decreased as quickly as 1 °C hr^{-1} when thawed at 0 °C or above (Andrews and Proebsting, 1987).

Rate of DCA

Temperature exposure and its duration influenced the rate and magnitude of hardiness loss. Deacclimation rate tended to increase linearly with the duration of exposure to warm temperatures (Bittenbender and Howell, 1975a; Howell and Weiser, 1970a; Proebsting, 1963; Wolf and Cook, 1992). Partially dehardened tissues (apple twigs, raspberry canes, and peach flower buds) rehardened upon exposure to cold temperatures (Brierley and Landon, 1946; Howell and Weiser, 1970a; Proebsting, 1963). However, dehardening was a more rapid process than re-hardening and was only partially reversible (Brierley and Landon, 1946; Howell and Weiser, 1970a; Irving and Lanphear,

1967c; Proebsting, 1963). Therefore, the ability of plants to deharden slowly or regain hardiness rapidly after dehardening is an important characteristics for survival. Daily exposure to freezing temperature was necessary for apple shoots and raspberry canes to reharden (Brierley et al., 1952; Howell and Weiser, 1970a).

Spring phenology

Woody perennial plants resume growth in the spring. This phenological development is closely related to plant hardiness loss (Bittenbender and Howell, 1975b; Johnson and Howell, 1981a, 1981b; Proebsting, 1963). The greater (more advanced) development of the plant, the faster the plant tissue deacclimates and the less ability to reharden after dehardening. Cultivar differences also affected both the time and rate of DCA, which was probably due to their effects on spring growth rate (Johnson and Howell, 1981b). Once at bud burst, the bud's ability to reharden was lost.

Deacclimation has not been studied as much as acclimation (Howell and Weiser, 1970a). The reason may be due to complex responses to fluctuating air temperatures and their effects on plant physiology. It deserves more research on both individual plant and interactions of environmental (temperature) and plant morphological, physiological, biochemical, cytological, or molecular changes. Although rehardening of dehardened tissues occurred at the temperature of -15 °C, it has not been demonstrated that the transition is not a metabolic process since low or warm temperature induced enzymes might be involved (Howell and Weiser, 1970a). Elucidation of hardening and dehardening transition cycles after endodormancy must be accomplished if one is to fully understand the plant hardiness mechanism.

Thus, plants undergo dynamic cold hardiness changes in response to low temperatures and other environmental factors. Cold acclimation can be initiated by more than one environmental factor, and can achieve extremely cold resistant by MW. This indicates the complexity and flexibility of plant winter survival mechanism.

Physiological and metabolic changes accompanying hardiness dynamics

The dynamic changes in cold hardiness over the season are accompanied by a series of physiological and metabolic changes. Correlations have been found among plant growth and development, water relations, carbohydrates, hormones and growth regulators, nutrients, membranes and fatty acids, proteins and enzymes, amino acids, etc. Extensive reviews on freezing injury and cold hardiness have been written from different perspectives since 1970. Interested readers are referred to reviews of Weiser (1970 a, 1970b), Burke et al., (1976), Burke and Stushnoff (1979), Levitt (1980), Steponkus (1984), Chen et al., (1995), Quamme (1995), Palonen and Buszard (1997), and Pellett and Carter (1978). Selected topics are discussed in this review.

Vegetative growth and maturity

Cessation of vegetative growth and tissue maturity begins in the fall prior to the onset of CA. Actually, the first stage of CA in dogwood was associated with vegetative maturity of shoots (Fuchigami et al., 1971a). Weiser (1970a, 1970b) summarized that growth cessation was the key factor in the induction of acclimation in woody plants. However, this did not indicate that plants must be physiologically dormant (Howell and Weiser, 1970b; Weiser, 1970a, 1970b). In fact, Howell and Weiser (1970b) found actively growing apple trees in the greenhouse still acclimated slightly.

When trees (e.g. apples) stop growth, a terminal bud is formed at the twig apex. Cold acclimation begins at the shoot apex and progresses to the base of the twig (Howell et al., 1997). In grapevines, however, the acclimation process is different; growth cessation is not accompanied by the formation of a terminal bud. It acclimates from the base to the apex and CA was closely related to decreases in tissue water content as stems achieved vegetative maturity (brown periderm) (Wolpert and Howell, 1985b, 1986a).

Water content

Water plays essential roles as a constituent, a reactant in various biochemical processes, and in the maintenance of cell turgidity. Its physiological importance is reflected in its ecological importance: plant distribution on the earth is controlled by the availability of water wherever temperature permits growth (Kramer, 1983). As mentioned earlier, cell water content, distribution and status are very crucial for plant survival at low temperatures.

Under natural conditions in the fall, cold acclimation (CA, the process of dramatic hardiness increase) occurs with decreasing water content (McKenzie et al, 1974b; Wolpert and Howell, 1984, 1985, 1986a). Utilizing nuclear magnetic resonance (NMR) methods, Burke et al. (1974) found that cold-acclimated stems of red osier dogwood had less mobile/liquid water; and this water loss was partially from the loss of water from the central part of the stem (pith). In 1974, McKenzie et al. (1974b) also reported that stem water loss of red-osier dogwood during CA was related to water loss from pith cells; and the decrease in stem water content during CA was the result of decreased stomatal resistance and increased root resistance to water flow. Similar results were reported for grape roots (Wolpert and Howell, 1986b).

This negative relationship between cold hardiness and water content was also demonstrated in a significant hardiness enhancement in response to artificial dehydration treatments in red-osier dogwood in the fall and spring (Chen et al., 1977; Li and Weiser, 1971) and azaleas in the fall (Anisko and Lindstrom, 1996). On the contrary, hardiness decreased after either re-watering or an application of artificial water spray on fruit buds of apple, peach, apricot and grape (Anisko and Lindstrom, 1996; Hewett et al., 1978; Johnson and Howell, 1981a). Limin and Fowler (1985) reported that temperature and tissue water content explained a large part of the variation in hardiness index T_{50} during CA, and tissue moisture content reduction was a natural response to cold-acclimating temperatures.

Wolpert and Howell (1984, 1985) reported that the relationship between tissue (canes and buds) hardiness and water content existed only during early CA (from Aug until late Sept in Michigan) on Concord grapevines. However, a close inverse relationship was observed between peach flower bud hardiness and water content throughout the dormant season (Johnston, 1923); and hardiness of apple twigs fluctuated with preceding air temperatures and tissue moisture content during both CA and spring deacclimation (DCA) periods (Coleman and Estabrooks, 1988). Others reported that hardiness loss or the extent of deep supercooling ability of flower buds (peach, cherry and azalea) and grape buds was closely related to preceding air temperatures during the whole dormant season, as was the fluctuation of water content in the whole, or parts of, the flower bud (Andrews and Proebsting, 1987; Graham and Mullin, 1976; Ishikawa and Sakai, 1981; Johnson and Howell, 1981a, 1981b; Proebsting et al., 1980; Quamme, 1983; Wolf and Cook, 1992).

However, there were three limitations in these efforts: 1) most research was conducted only during the cold acclimation period and the period was different in length and duration (early stage or the whole period of cold acclimation); 2) only a few researchers used multiple-regression analysis to determine the major factor(s) contributing to the main effect (Coleman and Estabrooks, 1988; Pool et al., 1992); and 3) most researchers correlated hardiness with only a few specific preceding air temperatures, such as average maximum and minimum of 3 d before each sampling (Coleman and Estabrooks, 1988; Pool et al., 1992), mean temperatures of preceding 2, 3, 5, and/or 7 d (Proebsting, 1963; Proebsting et al., 1980; Quamme, 1983; Wolf and Cook, 1992), and average maximum, minimum, and mean temperatures between two adjacent sampling dates (Hubácková, 1996).

Cold injury can occur at any time during the year and can be economically devastating. Therefore, it is helpful to predict cold hardiness changes for a given crop over the season with easy available environmental information, such as local weather records and forecasts. With this information, one can predict plant hardiness status and allow for crop protection to avoid or minimize the possible winter damage.

Therefore, the objectives of study in Chapter I were to: 1) monitor seasonal changes of cold hardiness and tissue water content of canes and buds during the whole dormant season in Michigan; and 2) use simple and multiple linear regression analyses to determine the most significant (predictive) air temperature factor(s) affecting cold hardiness and tissue water content assuming that a regression relationship exists.

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TECHNIQUES OF COLD HARDINESS MEASUREMENT

An accurate and precise methodology is required for any sound and effective research. Consistent and standardized methods are needed if one is to compare different results from similar research. However, the cold hardiness literature is characterized by different methodologies being used on many research subjects.

For example, the canes of both dogwood and grapevine mature from the base to the apex and the tissue maturity is related to its cold hardiness. Therefore, there could be a considerable variance in cold hardiness among different samples. Mckenzie et al (1974a, 1974b) numbered the stem nodes from the apex to the base in dogwood, while Wolpert and Howell (1985b, 1986a, 1986b) counted the opposite way in grapes. Li and Weiser (1971) used stem diameter as the only sampling criterion for dogwood. Irving and Lanphear (1967a, 1967b) did not even mention the sampling procedure. Moreover, by freeze-drying the stems, Li and Weiser (1971) might have caused unpredictable variation due to the low-temperature effects of dehydration (Bittenbender and Howell, 1975a).

In terms of cold hardiness index, Irving and Lanphear (1967a, 1967b) used killing points (temperature) calculated from TTC (triphenyl tetrazolium chloride) viability test and tissue browning; Fennell and Hoover (1991) used killing temperature only from tissue browning, and many others used T_{50} (Bittenbender and Howell, 1974, 1976; Howell and Shaulis, 1980; Schnabel and Wample, 1987; Wolpert and Howell, 1986a, 1986b) or use it as a standard (Gu, 1999; Wample et al., 1990; Wolf and Pool, 1987). All these suggest a need for a standard methodology in cold hardiness research (Bittenbender and Howell, 1976; Howell and Shaulis, 1980; Wolpert and Howell, 1986a, 1986b).

Sampling criteria and methods

The characteristics of winter damage (e.g. freezing and frost), and plant survival mechanisms involved, determine the research methodology. Under similar ecological conditions, plant cold hardiness varies with species, cultivars and phenology. Even within one plant, the hardiness of different organs, tissues and adjacent parts of the same tissue varied greatly (Howell and Shaulis, 1980; Weiser, 1970a, 1970b). This demonstrates that sampling is potentially the biggest source of variability and a standard sampling procedure is the prerequisite for any cold hardiness experiment.

In a series of experiments on grapevines, Howell and Shaulis (1980) found that cold hardiness variations are not random; conditions favoring greatest cold resistance were exposure to sunlight, dark-colored periderm, medium cane diameter, medium internode length, and lack of persistent lateral canes. They suggested a stratified random sampling procedure with sampled canes possessing similar diameter, medium internode length, and dark brown periderm sampled from the exterior of the canopy. After this report, many researchers adopted their sampling procedure (Fennell and Hoover, 1991; Jiang et al., 1999; Wolf and Cook, 1992; Wolpert and Howell, 1986a, 1986b).

Field assessment

Hardiness evaluations were once based on field assessment under natural winter conditions. After a severe winter or a specific freeze episode, the injury to tissues was determined by observing the growth in the following spring or summer (Brierley et al., 1950). This method relied heavily on the weather patterns from year to year. Thus, it is not efficient due to unpredictable weather, the episodic natures of the freeze events and their infrequency.

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Controlled freezes

About 50 years ago, researchers began to use controlled-freeze tests to simulate field conditions. Plant tissues, or the whole plant, can be put in the freezer, where temperatures can be controlled manually or automatically. Controlled-freeze methods have been conducted at different cooling rates, sub-zero temperature ranges, and prefreeze treatment (hardening or none). For example, Sakai (1973, 1979, 1982; Sakai and Weiser, 1973) preferred to give hardening treatment before applying controlled-freeze stress, and used different cooling rates and duration at each temperature. However, most recent researchers have used cooling rates ranging from 1-20 °C hr^{-1} (constant within each experiment) on grapevine research (Bourne et al., 1991; Rajashekar et al., 1982; Wolf and Cook, 1992; Wolf and Pool, 1987; Wolpert and Howell, 1985a, 1985b, 1986a, 1986b). Cooling rates can have significant effect on freeze injury as previously mentioned, therefore, slow cooling rate (1-5 °C hr^{-1}), similar to field episodes, is suggested for general cold hardiness research.

During the controlled-freeze stress, tissue temperature was monitored by insertion of a thermal couple inside the tissue of each replicate bundle or bottle. One of the replicated samples was taken out at each of the several test temperatures and allowed to thaw gradually. The range of temperatures was chosen to allow the warmest temperature to produce no injury and the coldest temperature to kill all tissues (Wolpert and Howell, 1985a). The range varies with species, cultivars and different stages of cold hardiness and dormancy. Thawed samples were then subjected to different viability tests to estimate or measure tissue injury.

Viability assessment

A good method for measuring tissue, organ or plant viability should be quantitative, reliable, convenient, and capable of predicting the future performance of the plant (Howell and Shaulis, 1980; Steponkus and Lanphear, 1967a). The absolute value of hardiness is less important than a relatively precise and consistent value that can differentiate cold hardiness, especially survival or mortality among treatments.

When freezing injury occurred, the tissue lost healthy green color, became water soaked due to cell membrane leakage of cytoplasmic constituents (e.g., electrolytes, ninhydrin-reaching compounds, reductants), produced a temporary heat release (exotherm), and turned dark brown or black. Viability tests based on tissue browning, specific conductivity (electrical conductivity or electrolyte leakage), ninhydrin method (leakage of amino acids or proteins), and TTC (based on red color of formazan derivatives produced when H^+ ions are accepted by triphenyl tetrazolium chloride), and direct measurement of DTA (differential thermal analysis) are based on the above injury responses, respectively.

Regrowth test

The growth-recovery test (regrowth test) may be the first viability test developed. For this, cold-stressed samples are planted on a propagating mist bench in a greenhouse under a frequent water mist spray. Tissues were considered alive if there is any visible growth, callusing or green appearance (Stergios and Howell, 1973). While accurate, it is slow; grapevines and other woody species usually take 3-6 wk to obtain visible growth.

Tissue browning

Tissue browning is the most widely used viability test. Stressed samples are incubated in a plastic humidity chamber at room temperature for 5-7 d. Tissues are recorded as dead if xylem and/or cambium/phloem layer were brown, and alive if they are green (Stergios and Howell, 1973). Some researchers have also used injury ranking in visual browning (Wample and Wolf, 1996; Jiang et al., 1999).

The results from the above regrowth and tissue browning tests were calculated as a percentage of the stems surviving at each temperature, and T_{50} can be calculated by the Spearman-Karber equation (Bittenbender and Howell, 1974). These two methods are the most common methods of cold hardiness measurement on perennial woody tissues and T_{50} has been widely reported as a cold hardiness index (Bittenbender and Howell, 1974, 1975a, 1976; Gu, 1999; Howell and Shaulis, 1980; Schnabel and Wample, 1987; Wample et al., 1990; Wolf and Pool, 1987; Wolpert and Howell, 1986a, 1986b). However, these tests were flawed because they were subjective (based on visual browning), time consuming (tissue browning takes at least 5 d, and the regrowth test usually takes 4 wk after the controlled freeze-stress episode), and could not allow rapid vineyard decisions (such as pruning, winter protection etc.).

Quantitative tests, such as specific conductivity, triphenyl tetrazolium chloride (TTC) and ninhydrin method were developed to provide unbiased measurement. However, they all require trained experimental skills and more labor (Stergios and Howell, 1973).

Electrical conductance/resistance

As mentioned earlier, freezing injury induces compound leakage from the cell. Dexter et al. (1932) described the method of measuring specific conductance (corresponding to the electrolyte concentration of the tissue solutions) and correlating with levels of low temperature injury in plant tissues. Wilner (1955, 1959, 1960, 1961, 1967) has improved this method, developing electrolytic resistance (impedance, reciprocal of conductance), and reported extensively on its use in woody plants. He demonstrated that there was a significant correlation between conductivity (resistance) and tissue injury.

This method has been used mainly in shoots/stems, buds, and needles of woody plants (Evert and Cooley, 1979; Ketchie et al., 1972; Pellett and Heleba, 1998; Zhu and Liu, 1987). However, Palta et al. (1977) reported that electrical conductance was a poor estimation of cellular death because it was not a direct measurement of the cellular death *per se*. Boorse et al. (1998) also found that the T_{50} estimation from electrical conductance was significantly higher than other methods used; and it did not correspond with field observations of chaparral needle dieback.

Triphenyl tetrazolium chloride (TTC)

Triphenyl tetrazolium chloride was refined and practically used as a viability test by Steponkus and Lanphear (1967a). They found that there was a high correlation between cold injury and TTC reduction (the appearance of red color). Cold hardiness was expressed as optical density of solutions from stressed tissue times 100 and divided by the optical density of solutions from the control. Thus, higher percentage of TTC reduction (redness) indicated living tissues, vice verse (Stergios and Howell, 1973).

Triphenyl tetrazolium chloride does not require a large sample of tissues, but it requires considerable labor and has large variance among samples. This may explain why it has not been found in the more recent literature.

Differential thermal analysis (DTA)

During the controlled-freeze stress, water in tissues that supercool gives off exotherms in the presence of ice nucleator and ice formation. The low temperature exotherm (LTE) is always associated with death of florets or buds and xylem tissues (Andrews et al., 1987; Biermann et al., 1979; Pierquet and Stushnoff, 1980; Quamme 1978, 1986, and 1991; Rajashekar and Burke, 1978). Based on these characteristics, differential thermal analysis (DTA) was developed by Graham (1971), Quamme (1978), and Proebsting and Sakai (1979), and improved by Andrews et al. (1983), Wample et al. (1990), and Wolf and Pool (1986, 1987). Differential thermal analysis, detected by thermo-electric modules, measures the exothermal temperature at which the tissue is killed by recording relative temperature changes in a plant sample and a non-living reference. A computer system, including freeze control, data acquisition, and analysis system, was developed (Wample et al. 1990; Wolf and Pool, 1986). This technique has been widely used and shown to be a useful and reliable means of determining cold hardiness of buds of grape, peach, and cherry, and woods of apple and pear (Andrews et al., 1983, 1984; Clark et al., 1996; Ketchie and Kammereck, 1987; Montano et al., 1987; Proebsting and Sakai, 1979; Rajashekar et al., 1982; Wample et al. 1990; Wolf and Pool, 1987). It also correlates well with field survival (Wolf and Cook, 1994).

However, DTA is applicable only to tissues that survive via supercooling, such as buds and xylem ray parenchyma (Rajashekar and Burke, 1978). Important hardiness

issues involve the survival of vegetative structures of mature woody plants. The cambium, the regenerative organ in the wood, avoids supercooling. Further, observations by Wample and Wolf (1996) indicated that differences in bud hardiness are not indicative of vegetative tissue or whole plant hardiness status.

Infrared video thermography

Exotherm can also be visualized by thermal imaging equipment (infrared video thermography). The equipment videotapes water activity (ice nucleation and propagation) in plants during freezing, and information on the videotape can be accessed and analyzed by infrared image analysis software (Wisniewski et al., 1997). Experiments on apple (*Malus domesica*), peach (*Prunus persica*), and jojoba (*Simmondsia chinensis* (link) Schneider) showed that this is an accurate, non-destructive, informative method compared to radiometer and DTA (Ceccardi et al., 1995; Wisniewski et al., 1997). However, this equipment is expensive and requires a special designed transparent freezer cover.

Chlorophyll fluorescence (CF)

When light strikes on a leaf surface, chlorophyll molecules absorb most of the energy (~ 80-90%). The excited molecules are not stable and have four ways to release the energy: energy transduction (for photochemistry reactions), resonance energy transfer (energy is transferred to non-photosynthetically active molecules), heat loss, or light re-emission (the transition from the lowest excited singlet state to the ground state) (Taiz and Zeiger, 1998). *In vivo*, only a small portion (at a maximum of 3-5%) of the energy is re-emitted with a wavelength of about 680-720 nm (Lichtenthaler, 1990). This re-emitted light is chlorophyll fluorescence (CF). In general, almost all fluorescence is emitted from

chlorophyll *a* associated with photosystem II (PS II) since chlorophyll *b* transfers the excitation energy to chlorophyll *a* (Bolhàr-Nordenkampf et al., 1989; Lichtenthaler, 1990).

The fluorescence emission can be detected in a non-destructive fashion, and the spectroscopic characteristics of CF reflect the properties of the chlorophyll molecules and their environment. Therefore, CF measurement of photosynthetic organisms has rapidly become a powerful tool to study the structure and functions of photosynthetic apparatus and many environmental stresses that could cause photosynthetic disturbances (Binder et al., 1997; Boorse et al., 1998; Buwalda and Noga, 1994; Jimenez et al., 1997; Karukstis, 1991; Larcher and Neuner, 1989; Mohammed et al., 1995; Percival and Dixon, 1997; Rütten and Santarius, 1992; Scarascia-Mugnozza et al., 1996; Schreiber and Bilger, 1993; Strand and Öquist, 1988). In these researches, Fv/Fm (the ratio of variable fluorescence to maximum fluorescence) has been shown to be a rapid (~ 0.8 s), sensitive, reliable and non-destructive method to detect and quantify environmental stress-induced disturbances.

Recent studies have applied chlorophyll fluorescence to buds (Düring et al., 1990) and fruits (Tijskens et al., 1994; Song et al., 1997) to detect bud injury and fruit quality. However, to our knowledge, there is no report on use of chlorophyll fluorescence to assess viability of woody vegetative non-photosynthetic tissues.

In the tissue-browning test, the survival of the cambium layer is estimated by the green color, indicating the presence of chlorophylls. For this reason, a series of experiments was conducted in this dissertation with the purpose of determining if CF could be used as a new objective and effective viability test.

Plant cold hardiness is not easy to study because of its great variation influenced by plant internal and external, direct and indirect factors. Researchers need to consider all the related factors, and then make a decision on the methodology according to the problems of interest. Generally, browning and/or regrowth test is recommended at least as a control for other tests because they correlate well with field responses (Stergios and Howell, 1973). Differential thermal analysis is recommended for measuring supercooling tissues, such as flower buds and xylem ray parenchyma tissues under a standard procedure (Wolf and Pool, 1987). Chlorophyll fluorescence has been suggested to be a possible new rapid viability test (Palonen and Buszard, 1997).

QUESTIONS ADDRESSED IN THE DISSERTATION

Chapter 1

The key question is whether it is possible to predict cold hardiness dynamic changes with easy available data, such as ambient air temperatures and tissue water content.

1) What are the seasonal changes of cold hardiness and tissue water content of canes and buds during the whole dormant season?
2) What is the major contributing part of the cane to overall water changes over the dormant season?
3) What are the influence of ambient air temperatures and water content on cold hardiness of canes and buds?
4) What is the most significant factor(s) influencing seasonal cold hardiness and tissue water content variations?

Chapters 2 and 3

The main questions concern whether and how CF can be used as an objective and effective viability test for woody vegetative tissues. In order to answer this critical question, a series of questions were addressed:

1) Can CF be used to measure the viability of freeze-stressed woody vegetative tissues? If so, what is the appropriate protocol for CF measurement? Is it the same as leaf measurement?
2) How may CF measurement be used to quantify tissue viability? Is it related to conventional methods (browning and/or regrowth tests)?
3) How quickly can a predictive CF measurement be obtained after a controlled freeze-stress? How does the CF time compare with browning or regrowth tests (5-7 d or 4 wk)?
4) Can CF measurement assess hardiness difference among different positions of the canes and wood? 1-year-old vs. 2-year-old; apical vs. basal?
5) Is the CF response consistent across the whole dormant season? If not, what are the differences? And how should we use it during different periods of dormancy?
6) Can CF be used to evaluate cold hardiness among different species or cultivars?
7) Can CF be used directly in the field?
8) Are there any differences between different CF florometers?

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CHAPTER I

THE RELATIONSHIPS AMONG AIR TEMPERATURES, WATER CONTENT, AND COLD HARDINESS OF CONCORD GRAPEVINES

ABSTRACT

Cold hardiness and water content were measured weekly or biweekly on one-year-old cane cuttings of bearing Concord grapevines in East Lansing, Michigan from September 1998 to April 1999 and January to April of 2000. The cold hardiness index T_{50} (temperature at which 50% of the sample is injured) was determined by a regrowth test after laboratory controlled sub-freezing treatments. The water content of buds and the overall cane as well as its periderm, pith, and wood were measured by the difference between tissue fresh weight and dry weight. Daily and averaged daily maximum, minimum, and mean air temperatures of 1 to 7 d prior to each field sampling were correlated with the T_{50} and water content of the canes and buds. Results showed that: 1) among all air temperatures analyzed, the averaged minimum temperatures of 2 d before each sampling date were most significantly correlated with T_{50} of canes and buds during cold acclimation, while the minimum air temperature on the day before each sampling date was most significantly correlated with T_{50} of canes and buds during deacclimation; correlation coefficient during midwinter was not significant or less than that during CA and DCA. 2) cane water content was most significantly correlated to air temperatures of 7 d before each sampling date during cold acclimation, and averaged maximum air

temperatures 4 and 5 d during deacclimation, while there was no significant factor during midwinter. Bud water content was correlated with mean/minimum temperatures the day before and 7 d before each sampling date during CA and MW, while none significant correlation during DCA. 3) the total water content of the cane was significantly related to its wood water content, while the water content of periderm and pith of the cane was not. 4) multiple linear regression analysis showed that the cane water content had greater r^2 values to the T_{50} of canes and buds than any air temperature tested during the whole dormant season.

Key words: Concord (*Vitis labruscana* Bailey), cold hardiness, multiple linear regression

INTRODUCTION

Seasonal changes in cold hardiness and/or plant tissue water content have long been correlated with preceding air temperatures. Correlations among these parameters have been reported on grapevines (Hamman et al., 1990; Hubácková, 1996; Johnson and Howell, 1981; Pool et al., 1992; Proebsting et al., 1980; Wolf and Cook, 1992; Wolpert and Howell, 1984, 1985, 1986a) and other woody species (Anisko and Lindstrom, 1996; Bittenbender and Howell, 1975; Coleman and Estabrooks, 1988; Grossnickle, 1992; Vertucci and Stushnoff, 1992). In general, cold hardiness, to a certain extent, increases with exposure to non-lethal low temperatures and/or decreasing tissue water content.

Under natural conditions in the fall, cold acclimation (CA, the process of dramatic hardiness increase) proceeds with decreasing water content (McKenzie et al., 1974; Wolpert and Howell, 1984, 1985, 1986a). Utilizing nuclear magnetic resonance (NMR) methods, Burke et al. (1974) found that cold-acclimated stems of red-osier dogwood (*Cornus stolonifera* Michx.) had less mobile/liquid water; and this water loss was partially from the loss of water from the central part of the stem (pith). In 1974, McKenzie et al. also reported that stem water loss in red-osier dogwood during CA was related to water loss from pith cells; and the decrease in stem water content during CA was the result of decreased stomatal resistance and increased root resistance to water flow. Similar results were reported for grape roots (Wolpert and Howell, 1986b).

This negative relationship between cold hardiness and water content was also demonstrated in a significant hardiness enhancement in response to artificial dehydration treatments in red-osier dogwood in the fall and spring (Chen et al., 1977; Li and Weiser, 1971) and azaleas in the fall (Anisko and Lindstrom, 1996). In contrast, hardiness

decreased after either re-watering or an application of artificial water spray on fruit buds of apple, peach, apricot and grape (Anisko and Lindstrom, 1996; Hewett et al., 1978; Johnson and Howell, 1981). Limin and Fowler (1985) reported that temperature and tissue water content explained a large part of the variation in hardiness index T_{50} during CA, and tissue moisture reduction was a natural response to cold-acclimating temperatures.

Wolpert and Howell (1984, 1985) reported that the relationship between tissue (canes and buds) hardiness and water content existed only during early CA (from Aug until late Sept in Michigan) on Concord grapevines. However, a close inverse relationship was observed between peach flower bud hardiness and water content throughout the dormant season (Johnston, 1923); and hardiness of apple twigs fluctuated with preceding air temperatures and tissue moisture content during both CA and spring deacclimation (DCA, the process of cold hardiness decrease) periods (Coleman and Estabrooks, 1988). Others reported that hardiness loss or the extent of deep supercooling ability of flower buds (peach, cherry and azalea) and grape buds was closely related to preceding air temperatures during the whole dormant season, so was the fluctuation of water content in the whole, or parts of, the flower bud (Andrews and Proebsting, 1987; Graham and Mullin, 1976; Ishikawa and Sakai, 1981; Johnson and Howell, 1981; Proebsting et al., 1980; Quamme, 1983; Wolf and Cook, 1992).

However, there were three limitations in previous research: 1) most research was conducted only during the cold acclimation period and the period was different in length and duration (early stage or the whole period of cold acclimation); 2) only a few researchers used multiple-regression analysis to determine the major factor(s)

contributing to the main effect (Coleman and Estabrooks, 1988; Pool et al., 1992); and 3) most researchers correlated hardiness with only a few specific preceding air temperatures, such as average maximum and minimum of 3 d before each sampling (Coleman and Estabrooks, 1988; Pool et al., 1992), mean temperatures of preceding 2, 3, 5, and/or 7 d (Proebsting, 1963; Proebsting et al., 1980; Quamme, 1983; Wolf and Cook, 1992), and average maximum, minimum, and mean temperatures between two adjacent sampling dates (Hubácková, 1996).

Cold injury can occur at any time during the year and can be economically devastating. Therefore, it is helpful to predict cold hardiness changes for a given crop over the season with easy available environmental information, such as local weather records and forecasts. With this information, one may predict plant hardiness status and allow for crop protection to avoid or minimize the possible winter damage.

Therefore, the objectives of this study were to: 1) monitor seasonal changes of cold hardiness and tissue water content of canes and buds during the whole dormant season in Michigan; and 2) use simple and multiple linear regression analyses to determine the most significant (predictive) air temperature factor(s) affecting cold hardiness and tissue water content assuming that a regression relationship exists.

MATERIALS AND METHODS

Plant Material: Bearing (28-year-old) Concord grapevines (*Vitis labruscana* Bailey) at the Horticultural Teaching and Research Center at Michigan State University were trained to a top-wire (1.7m), bilateral cordon (Hudson River Umbrella). Vines were vigorous and high yielding (6 to 8 tons/acre), and were balanced pruned using a 20+20 formula (i.e. twenty buds retained for each 0.45 kg of cane pruning) (Wolpert and

Howell, 1985).

Sampling Procedure: One-year-old canes were sampled on clear mornings (0800 hr to 0900 hr) regularly (weekly or biweekly), beginning in 6 September 1998 to 22 April 1999 (monthly from January to April), and 27 January to 18 April 2000. Canes with similar diameter (5.5-6.5 mm), medium internode length, and dark brown periderm were sampled from the exterior of the canopy (Howell and Shaulis, 1980). Samples were sealed in plastic bags held at ambient field temperature and transferred to the laboratory within 1 hr after sampling. Nodes 3 to 12 from the base of the cane were used for assessment. Canes with intact buds were separated randomly into four groups: one group was used for water measurement, and the other three were cut into basal, middle and apical portions for controlled sub-freezing stress evaluation (Wolpert and Howell, 1985).

Freezing Procedure: The freezing procedure was similar to that used by Wolpert and Howell (1985). Segments of 2-3 cm-long canes were cut from each portion of the cane (basal, middle and apical). The base of each cane segment was wrapped with moist cheesecloth to provide ice inoculation and avoid supercooling (McKenzie and Weiser, 1975), then wrapped with aluminum foil to facilitate heat exchange. The freezer temperature was programmed to decrease at 3 °C h^{-1}. Tissue temperatures were monitored via a 40-gauge copper-constantan thermocouple inserted into the pith of a representative cane segment in each foil bundle. Three or five replicate foil bundles were removed at each of several test temperatures and allowed to warm slowly overnight in a 3 °C cooler, where the control bundles had been placed. The test temperatures (usually 7-9 temperatures each time) were chosen so that the highest temperature produced no injury and the lowest was lethal to all tissues.

Viability (Regrowth) Test: Samples, thawed overnight, were placed in a propagation mist bench (bench temperature 25 ± 2 °C, ambient air temperature 23 ± 2 °C) for 4 wk (regrowth test), after which they were sectioned and injury was rated by visually evaluating tissue browning of the primary bud and phloem/cambium layer. In this experiment, buds were rated dead or alive, while cane tissues were rated into 5 injury categories based on visual browning of the cane surface with the periderm removed (Jiang et al., 1999). Hardiness was calculated as T_{50} by means of the modified Spearman-Karber equation (Bittenbender and Howell, 1974).

Water Content: Canes were also separated into basal, middle and apical sections. In each section, twelve to fifteen excised buds and eight to ten cane pieces (2 cm long) were dissected into three parts (periderm, pith, and wood) and each part was placed into airtight glass vials with ground glass stoppers for fresh weight determination. These were weighed in a 3 °C walk-in cooler within 1.5 hrs after field sampling, then oven-dried for 5 d at 70 °C (vials open) and weighed again (dry weight was not changed after 3-5 d) (Wolpert and Howell, 1984, 1985). Water content was expressed as the water content (the difference between fresh and dry weights) in each tissue dry weight (Wolpert and Howell, 1984). The overall cane as well as its periderm, wood and pith were measured separately at the same time.

Air Temperature: Daily maximum (Tmax) and minimum (Tmin), and hourly ambient air temperatures were recorded by an automatic agricultural weather station at the Horticultural Teaching and Research Center on Michigan State University campus. Air temperatures were measured with a platinum resistance thermometer, which was protected in a standard radiation shield at 150 cm height. Daily mean temperature was

calculated by the average of hourly air temperatures from 0100 to 2400. Daily and averaged daily maximum, minimum, and mean air temperatures of 1 to 7 d before each field sampling (total 39 temperatures) were calculated and designated as $Tmax_1$, $Tmin_1$, $Tmean_1$, ..., $Tmax_7$, $Tmin_7$, and $Tmean_7$; $AvgTmax_2$, $AvgTmin_2$, $AvgTmean_2$, ..., $AvgTmax_7$, $AvgTmin_7$, and $AvgTmean_7$ (the subscript numbers denotes the days prior to each sampling date). For example, $Tmax_2$ is the maximum temperature 2 d before each sampling date, $AvgTmax_2$ is the average of maximum temperatures 1 and 2 d before each sampling date.

Statistical Analysis: Simple linear correlation/regression analyses were conducted by analysis of variance of SimgaPlot program; while multiple regression analysis was determined by analysis of variance of SAS/STAT software (V7.01) (SAS Institute Inc., Cary, NC.) and the stepwise and R square method (Sokal and Rohlf, 1995) were used to select the best-fit equation.

RESULTS AND DISCUSSION

Air temperature and cold hardiness: The dormant season was separated into CA, midwinter (MW), and DCA periods based on cane T_{50}. Cold hardiness increased dramatically from the non-hardy condition during CA. The DCA was the period of hardiness loss (dehardening) from the maximum hardiness until spring when the non-hardy condition was achieved. MW was the dormant period between CA and DCA. Maximum hardiness (lowest T_{50}) occurred in mid- to late-February during the dormant season of 1998-1999 (September to April) and the late winter of 2000 (January to April) (Figures 1 and 2).

Cold hardiness T_{50} was correlated with 39 daily and averaged daily Tmax, Tmin,

and Tmean. Multiple linear regression analysis and stepwise selection showed that T_{50} of canes and buds were highly correlated with $AvgTmin_2$ during CA ($R^2/r^2 = 0.97$), while best correlated with $Tmin_1$ during the DCA period. During MW, the Tmean3 and AvgTmax4 correlated significantly with cane T_{50}, while no temperature had significant effects on bud T_{50} (Table 1). All the coefficient of determination (R^2/r^2) values were significant at a probability (*Pr*) less than 0.05. The difference between real measured and estimated T_{50} of canes and buds (calculated from best-fit equations) were no more than 1.5 °C during CA (Figure 2); difference could be 5.9 °C during the other two dormant periods.

Cold temperature has been widely reported to contribute to cold acclimation. Therefore, it is not surprising to see that minimum temperatures correlated most to hardiness changes during CA. Based on our data, the Tmin had a big influence on the T_{50}s of the following 1-2 d. Previous research showed that warm temperature and its duration after mid-winter was the most significant factor contributing to hardiness loss (Coleman and Estabrooks, 1988; Edgerton, 1954; Proebsting, 1963; Proebsting et al., 1980; Proebsting and Mills, 1972). However, little research has defined which temperatures, either daily maximum, daily minimum, or daily mean temperatures, had the major contribution. Many authors reported correlation with either daily or averaged mean temperatures (Proebsting, 1963; Proebsting et al., 1980; Quamme, 1983; Wolf and Cook, 1992). Proebsting (1963, 1970) proposed that after mid-winter or after chilling requirement had been satisfied, warm temperatures (above –1.1 °C or –2.2 °C) would cause hardiness loss. Our results suggested that it was the daily minimum temperature that most significantly influenced the dehardening process (Table 1). The poor correlation

during MW may due to the fact that vines are under deep dormancy and are not responsive to external factors, such as air temperatures.

Our data differed from those of Hubácková (1996) on grapes and Coleman and Estabrooks (1988) on apples. Using *Vitis vinifera* cultivars of White Riesling and Muller-Thurgau, Hubácková (1996) found that bud T_{50} was correlated well with preceding average maximum and mean temperatures during the whole dormant season; but correlation with the minimum temperature was poor, except during mid-winter. One possible explanation about the discrepancy might be species based, *Vitis vinifera* grapes may have responded differently than *Vitis labruscana* grapes. Secondly, the average maximum, minimum, and mean temperature were calculated differently. Hubácková (1996) used the average maximum, minimum and mean temperatures between two adjacent sampling dates, which were not constant ranging from 7 to 24 d. With apples, Coleman and Estabrooks (1988) correlated percent injury with preceding air temperatures. The results were difficult to compare because percent injury resulting from a specific degree of freezing (-35 °C) was not the same as our cold hardiness index (T_{50}), although they were closely (inversely) related.

Air temperature and tissue water content: Tissue water content was significantly correlated with accumulated maximum and minimum temperatures several days before each field sampling (Table 2). For cane water content, the maximum and minimum temperatures 7 d before each sampling date were predictive during CA, the average maximum temperature of 4- and 5-d before sampling was better for DCA, while no variable could be predictive during MW. Bud water content was mostly correlated with $Tmean_7$ and $Tmin_1$ during CA and MW, and was not correlated with any air temperatures

during DCA.

Our data supported those previous reports, which showed that seasonal changes in cold hardiness were partially due to changes in tissue water content (Andrews and Proebsting, 1987; Coleman and Estabrooks, 1988; Grossnickle, 1992). Tissue water content is a result of integrated internal and external physiological processes, including 1) root absorption; 2) tissue transport; 3) tissue transpiration; 4) precipitation; and 5) internal distribution. It seems that air temperatures have longer term and accumulative effects on tissue water content.

Dissection of the cane showed that wood water content was the major contributor to the overall cane water content during the whole dormancy (Table 3). This is not due to the fact that the wood is the primary part of the cane because the water content is expressed as water in the dry weight of each cane portion. Simple linear regression of wood water content (W1) with overall cane water content (Wc) produced an equation as: $Wc = 0.029 + 0.93W1$ ($r^2 = 0.97$, $P < 0.0001$) (Figure 3). This result was similar to those on Seyval (*Vitis hybrid*) grapes (Howell, 1988), but differed from those on red-osier dogwood (Burke et al., 1974; McKenzie et al., 1974;). The difference might due to different anatomy structures of two species.

The relationship among air temperatures, tissue water content and cold hardiness: When cold hardiness T_{50} was correlated with all 39 preceding air temperatures and tissue water content during dormancy, cane water was the most significant contributing factor to the seasonal changes of both cane and bud hardiness (R^2 ranging from 0.92 to 0.99 during CA and DCA) (Table 4). Simple linear regression analysis showed that cane water content explained 85% to 90% of the cane T_{50} variation over 1998-1999 and 2000

dormant seasons; Quadratic regression improved r^2 from 85% to 96% for DCA, while not much for CA (from 90% to 91%) (Figure 4).

With apples, Coleman and Estabrooks (1988) found different responses by using similar multiple linear regression analysis. They found the percent injury at a stress temperature of –35 °C appeared to be primarily related to the previous average maximum 3-d air temperatures (i.e.Tmax3), and to a lesser extent, percent moisture content of the twigs during the whole dormant season. The discrepancy could be due to differences in species, climatic conditions, viability tests (they used electrolytic conductivity method), and expressions of water content (theirs was on the basis of twig fresh weight).

Weather (temperature) patterns change from year to year even in the same location, and plants integrate all the environmental and biological conditions. Our data showed that it is possible to predict hardiness from preceding air temperatures. However, research needs to be conducted for several years with a precise definition of local conditions (environmental and biological factors, such as climate, crop species, field management etc.) (Pool et al., 1992). A vineyard weather station is suggested as an important tool to forecast severe weather events and allow one to take protective actions accordingly. For example, overhead sprinklers have been used successfully to protect many horticultural crops from frost (Rieger, 1989). The system uses a temperature activated switch to turn on the over-tree sprinkler whenever the temperature drops to a critical value (usually 0 °C to –2 °C) in orchards and vineyards (Kidder and Davis, 1914; Lombard et al., 1980).

CONCLUSIONS

Canes and buds of Concord grapevines started acclimation at least in early September, reached the maximum hardiness level in February, and deacclimated quickly after late

February in each of the two dormant seasons (Figures 1 and 2). Similarly, the water content of canes and buds decreased from fall to midwinter and then started increasing after late February (Figure 1). Cane T_{50} correlated well with cane water (negatively) and preceding air temperatures (positively), while cane water had slightly higher r^2 than air temperatures during CA and much higher during DCA and MW (Tables 1 and 4). The inverse relationship between cold hardiness and water content expanded to the whole CA period, not just early CA (Wolpert and Howell, 1984, 1985). It seems that air temperatures affect cold hardiness via short-term direct effect (AvgTmin2, Tmin1) and long-term ($Tmax_7$, $Tmin_7$, $AvgTmax_4$, and $AvgTmax_5$) indirect effects through variation in tissue moisture content.

Tissue water content is important in cold hardiness fluctuations and tissue injury. Our data showed similar result as those previously reported, the cold hardiness and tissue water content had inverse relationship (Figure 1, Table 4). While it is noteworthy that an increase in cold hardiness does not necessarily guarantee adequate hardiness and no freeze injury or damage. If the increase in hardiness was not enough to withstand the upcoming freezing episode, protective strategies should be taken. In the spring, the increase in tissue water content would speed up the dehardening process. Thus, when using over-tree sprinkling to delay bud burst or bloom in late winter or early spring in some areas, it was suggested to ensure the tissue surface dried before the freezing episode came because wet flower buds were more susceptible to low temperature injury than dry buds (Hewett et al., 1978). Prevailing weather conditions such as dew point, wind speed, and precipitation and bud development status are also important for predicting freezing injury in the spring (Johnson and Howell, 1981; Rieger and Myers, 1990).

This paper again demonstrated that multiple regression analysis is a useful tool in handling multiple factors in agriculture research (Coleman and Estabrooks, 1988; Pool et al., 1992). However, a significant correlation coefficient does not necessarily prove a cause and effect relationship, and statistical significant probability can still have long-term bias in the predicted curves (R. Heins, personal communication, Michigan State University, East Lansing, MI; O. Shabenberger, personal communication, Michigan State University, East Lansing, MI). More data (other environmental and genetic factors) are needed to test these regression models and help understand the physiological and anatomical bases for the responses.

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Table 1. The correlation between cold hardiness (T_{50}, Y) and preceding daily and averaged daily maximum, minimum, and mean air temperatures[z] before each sampling date during the dormant seasons of 1998-1999 and 2000.

Dormant Period	T_{50}	Equation	R^2/r^2	*Pr*	Major Factor(s)
CA[y]	Cane	Y= -20.88+0.86AvgTmin2	0.97	<0.0001	AvgTmin2
	Bud	Y= -19.20+1.30Avg Tmin2-0.46Tmin4	0.97	0.0008	AvgTmin2
DCA[x]	Cane	Y= -19.02+1.05Tmin1-0.18AvgTmin2	0.78	0.0273	Tmin1
	Bud	Y=-11.08+2.27Tmin1-1.19Tmean2	0.78	0.0100	Tmin1, Tmean2
MW[w]	Cane	Y= -22.39+1.30Tmean3-1.04AvgTmax4	0.75	0.0163	Tmean3, AvgTmax4
	Bud	Y=-20.77+0.54AvgTmin2	0.40	0.0677	None

[z] Daily and averaged daily maximum, minimum, and mean air temperatures of 1 to 7 days before each sampling date were designated as $Tmax_1$, $Tmin_1$, $Tmean_1$, ..., $Tmax_7$, $Tmin_7$, and $Tmean_7$; $AvgTmax_2$, $AvgTmin_2$, $AvgTmean_2$, ..., $AvgTmax_7$, $AvgTmin_7$, and $AvgTmean_7$ (the subscript numbers denotes the days prior to each sampling date). For example, $Tmax_2$ is the maximum temperature two days before each sampling date, $AvgTmax_2$ is the average of maximum temperatures one and two days before each sampling date.

[y] CA: cold acclimation during 1998-1999 dormancy

[x] DCA: deacclimation during 1998-1999 and 2000 dormancy

[w] MW: midwinter during 1998-1999 and 2000 dormancy

r^2: coefficient of determination for two variables, percentage of variance associated with the relationship expressed as correlation coefficient (r)

R^2: multiple coefficient of determination (for two and more than two variables)

Table 2. The correlation between tissue water content (g. g^{-1} dry wt, Y) and daily and averaged daily maximum, minimum, and mean air temperatures[z] before each sampling date during the dormant seasons of 1998-1999 and 2000.

Dormant Period	Tissue	Equation	R^2/r^2	*Pr*	Major Factor(s)
CA[y]	Cane	Y= 0.75+0.0075Tmax7-0.0027Tmin7	0.93	<0.0001	Tmax7, Tmin7
	Bud	Y= 0.65+0.014Tmin7+0.0086Tmean1	0.96	<0.0001	Tmean1, Tmin7
DCA[x]	Cane	Y=0.63-0.022AvgTmax4+0.035AvgTmax5	0.67	0.0069	AvgTmax4, AvgTmax5
	Bud	Y=0.84+0.059Tmean4	0.35	0.0441	None
MW[w]	Cane	Y= 0.76+0.0033Tmin6	0.27	0.0476	None
	Bud	Y=0.62+0.0038Tmin7+0.0088Tmean1	0.92	<0.0001	Tmean1, Tmin7

[z] Daily and averaged daily maximum, minimum, and mean air temperatures of 1 to 7 days before each sampling date were designated as $Tmax_1$, $Tmin_1$, $Tmean_1$, ..., $Tmax_7$, $Tmin_7$, and $Tmean_7$; $AvgTmax_2$, $AvgTmin_2$, $AvgTmean_2$, ..., $AvgTmax_7$, $AvgTmin_7$, and $AvgTmean_7$ (the subscript numbers denotes the days prior to each sampling date). For example, $Tmax_2$ is the maximum temperature two days before each sampling date, $AvgTmax_2$ is the average of maximum temperatures one and two days before each sampling date.

[y] CA: cold acclimation during 1998-1999 dormancy

[x] DCA: deacclimation during 1998-1999 and 2000 dormancy

[w] MW: midwinter during 1998-1999 and 2000 dormancy

r^2: coefficient of determination for two variables, percentage of variance associated with the relationship expressed as correlation coefficient (r)

R^2: multiple coefficient of determination (for two and more than two variables)

Table 3. Best-fit multiple linear equations relating cane water content (g. g^{-1} dry wt) to different parts of the cane during the dormant seasons of 1998-1999 and 2000.

Dormant Period	Equation	R^2	*Pr*	Major Factor
CA[z]	Wc[y]=0.092+0.71W1[x]+0.02W2[w]+0.14W3[v]	0.99	<0.0001	W1
DCA[u]	Wc=0.11+0.71W1+0.03W2	0.99	<0.0001	W1
Whole dormancy[t]	Wc=0.054+0.77W1+0.02W2+0.17W3	0.99	<0.0001	W1

[z] CA: cold acclimation during 1998-1999 dormancy

[y] Wc: cane water content

[x] W1: wood water content

[w] W2: pith water content

[v] W3: periderm water content

[u] DCA: deacclimation during 2000 dormancy

[t] Whole dormancy included data of 1998-1999 and 2000

R^2: multiple coefficient of determination (for more than two variables)

Table 4. Best-fit multiple linear regression among cold hardiness (T_{50}, Y), water content of canes and buds, and preceding air temperatures[z] during the dormant seasons of 1998-1999 and 2000.

Dormant Period	Tissue T_{50}	Equation	R^2/r^2	*Pr*	Major Factors
CA[y]	Cane	Y= -75.56+69.73Wc[x]	0.98	<0.0001	Wc
	Bud	Y= -76.15+71.16Wc	0.99	<0.0001	Wc
DCA[w]	Cane	Y= -78.45+0.45Tmax7+72.09Wc	0.92	0.0227	Wc
	Bud	Y= -83.31-0.36Tmin3+85.24Wc	0.93	0.0190	Wc
MW[v]	Cane	Y= -78.19+70.26Wc	0.83	0.0042	Wc
	Bud	Y= -80.96+75.76Wc	0.68	0.0234	Wc

[z] Daily and averaged daily maximum, minimum, and mean air temperatures of 1 to 7 days before each sampling date were designated as $Tmax_1$, $Tmin_1$, $Tmean_1$, ..., $Tmax_7$, $Tmin_7$, and $Tmean_7$; $AvgTmax_2$, $AvgTmin_2$, $AvgTmean_2$, ..., $AvgTmax_7$, $AvgTmin_7$, and $AvgTmean_7$ (the subscript numbers denotes the days prior to each sampling date). For example, $Tmax_2$ is the maximum temperature two days before each sampling date, $AvgTmax_2$ is the average of maximum temperatures one and two days before each sampling date.

[y] CA: cold acclimation during 1998-1999 dormancy

[x] Wc: cane water content (g. g^{-1} dry wt)

[w] DCA: deacclimation during 2000 dormancy

[v] MW: midwinter during 1998-1999 and 2000 dormancy

r^2: coefficient of determination for two variables, percentage of variance associated with the relationship expressed as correlation coefficient (r)

R^2: multiple coefficient of determination (for two and more than two variables)

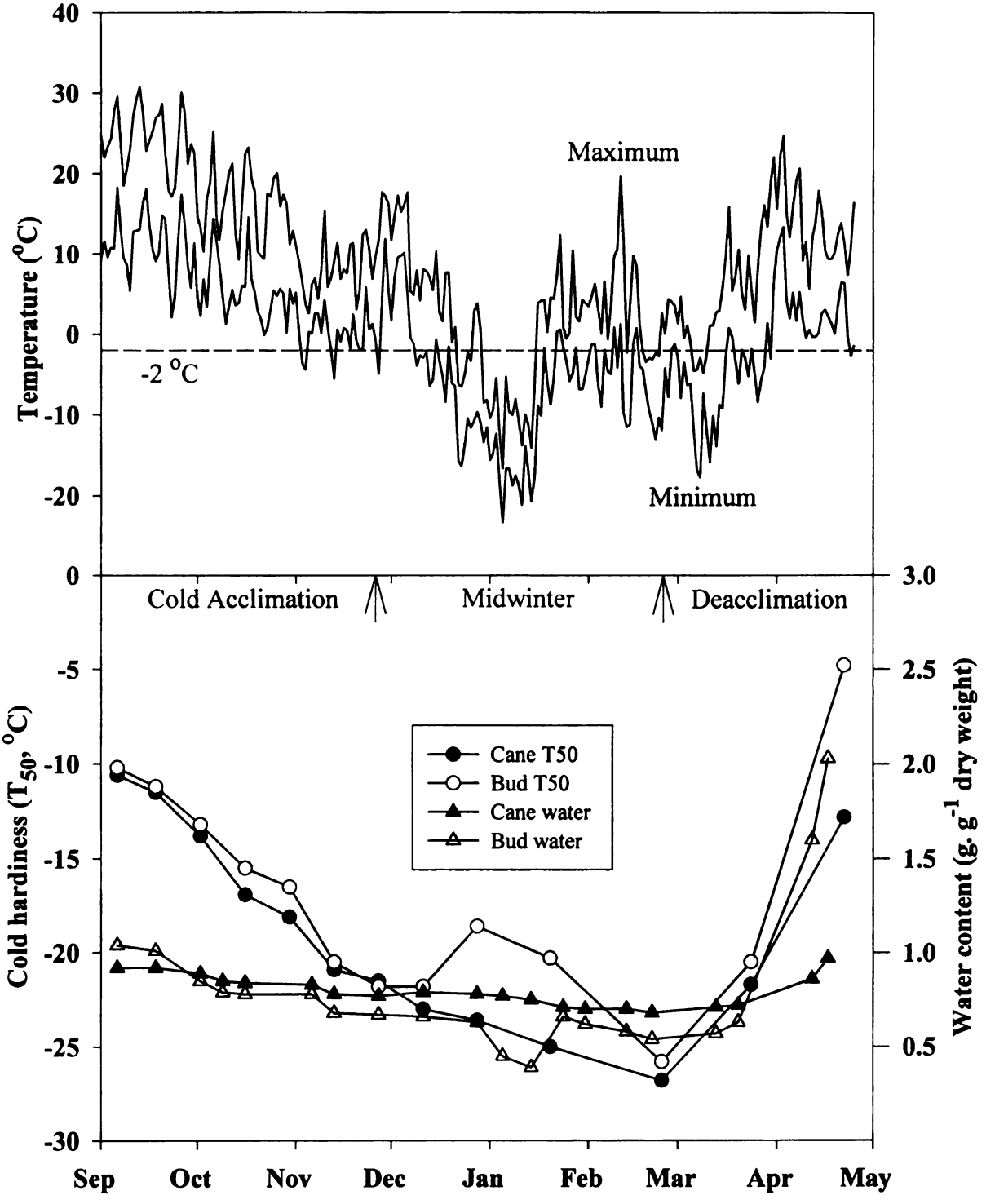

Figure 1. The maximum and minimum air temperatures, cold hardiness (T_{50}) and water content of Concord grape canes and buds during 1998-1999 dormant season. Each point is the average of nine replicates of apical, middle, and basal portions of the cane. Dormancy was separated (marked by arrows) into cold acclimation, midwinter, and deacclimation.

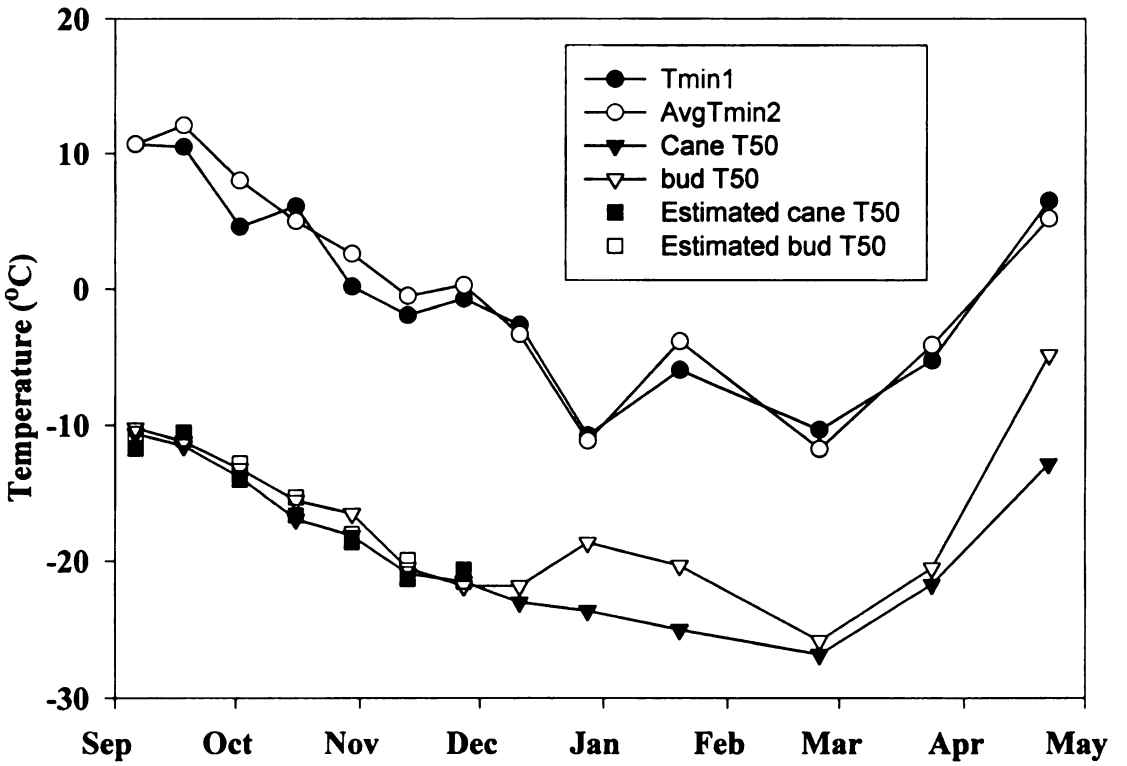

Figure 2. Air temperatures and cold hardiness (T_{50}) of canes and buds during 1998-1999 dormant season. $Tmin_1$ is the minimum air temperature the day before each sampling date; $AvgTmin_2$ is the average minimum air temperature of 1 and 2 d before each sampling date. Estimated T_{50} during cold acclimation was calculated from regression equations listed in table 1.

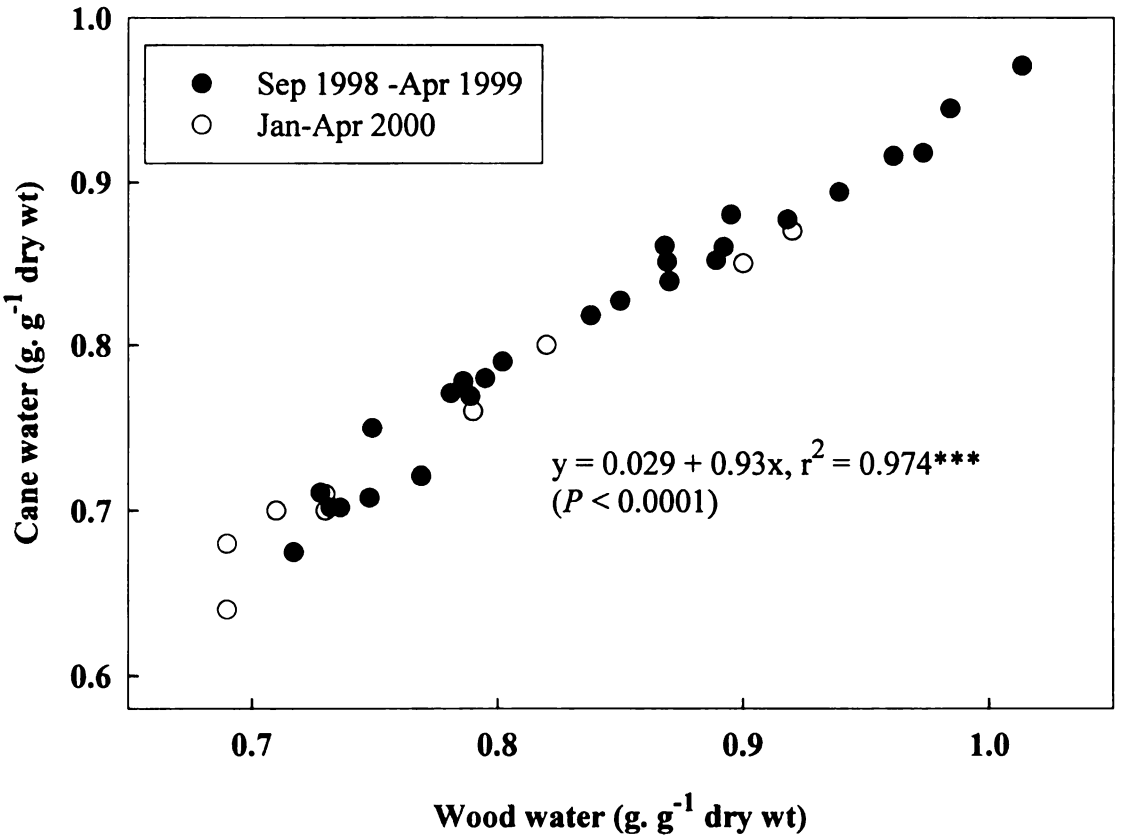

Figure 3. Regression between wood and overall cane water content of Concord grape canes during 1998-1999 dormant season and early spring of 2000. Each point is the average of six (in 2000) or nine (in 1998 and 1999) replicates of apical, middle, and basal portions of the canes.

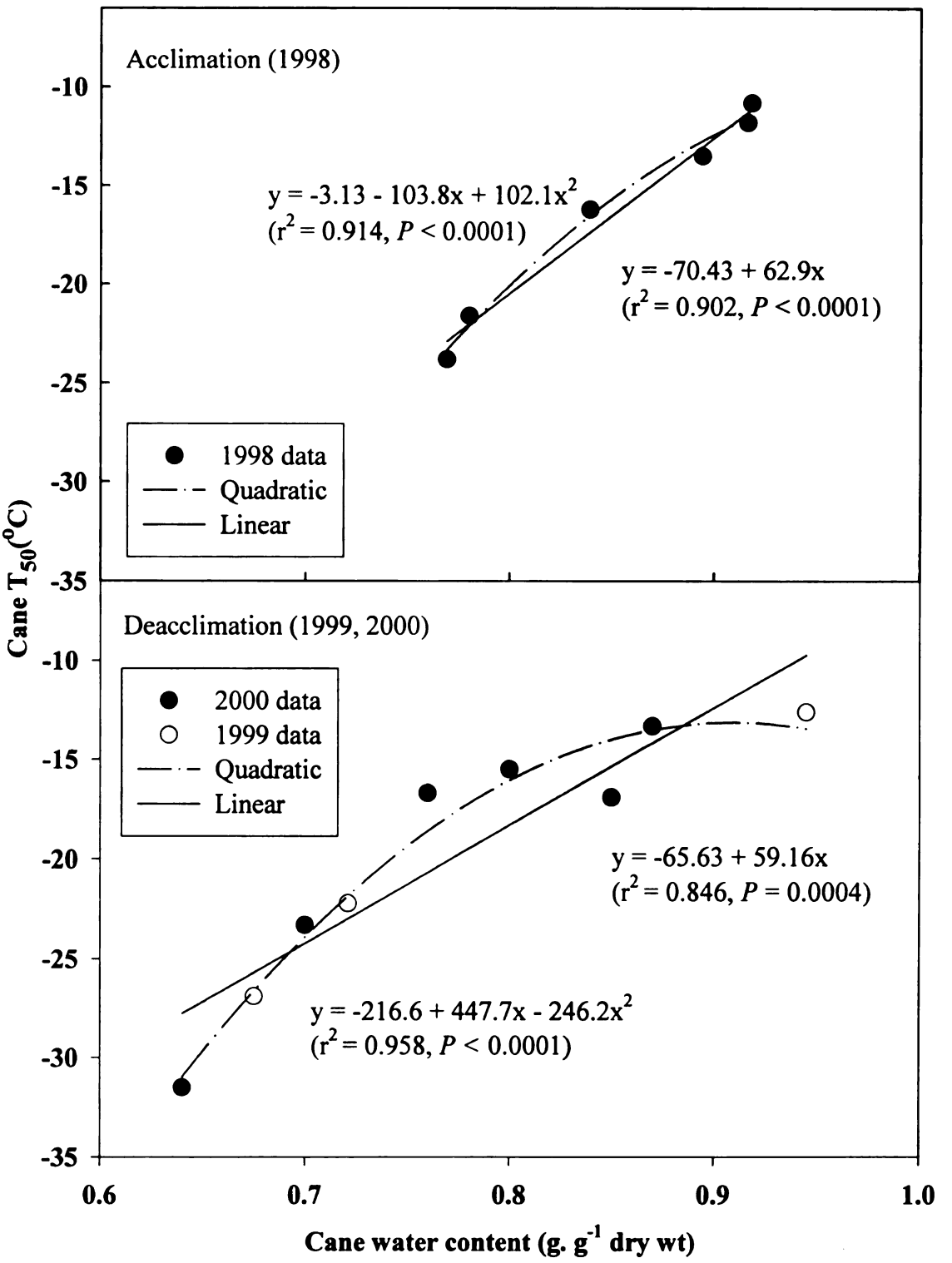

Figure 4. Correlations between water content and T_{50} of Concord grape canes during acclimation and deacclimation periods in 1998-1999 and 2000. Each point is the average of all the replicates of apical, middle, and basal portions of the cane.

CHAPTER II

EFFICACY OF CHLOROPHYLL FLUORESCENCE AS A VIABILITY TEST FOR FREEZE-STRESSED WOODY GRAPE TISSUES

ABSTRACT

Woody tissue cold hardiness and chlorophyll fluorescence of Concord (*Vitis labruscana* Bailey) grapevines were measured every 2 wk from late September to mid-December 1997. Plant Efficiency Analyser (Hansatech Ltd., England) was used to measure chlorophyll fluorescence after a laboratory-controlled freeze-stress. From five preliminary experiments, valid chlorophyll fluorescence measurements were obtained from the cane surface with periderm removed, under ~ 2700 μmol m^{-2} s^{-1} and 10 min dark adaptation after 24-30 hr post-stress. For large samples, dark adaptation was not necessary if only Fv/Fm (ratio of variable fluorescence to maximum fluorescence) was of interest. This ratio was positively correlated with freeze-stress temperatures, and negatively correlated with tissue injury. The Fv/Fm temperature-inflection point was positively correlated with T_{50} (temperature at which 50% of the sample is injured) calculated from the regrowth test. Fv/Fm was a reliable, objective, and rapid method of assessing woody tissue viability after a freeze-thaw episode during cold acclimation. An Fv/Fm_{50} was proposed as an indicator of the injury threshold.

Key words: Concord (*Vitis labruscana*), cold hardiness, Fv/Fm, regrowth test

INTRODUCTION

Effective use of laboratory freezing tests for determining cold hardiness of woody perennial plants requires: 1) a freeze stress regime that is similar to natural freeze episodes in the field, and 2) an accurate and precise method of determining tissue viability.

A mechanism by which dormant buds survive is supercooling -- the maintenance of the liquid state of water at temperatures well below the freezing point (Burke et al., 1976; Quamme, 1995). Heat is given off when supercooled water freezes as indicated by an exotherm. The low-temperature exotherm in a bud gives a direct, physical, and objective determination of the temperature at which the bud is killed (Andrews et al., 1987; Quamme, 1978, 1986, and 1991; Rajashekar and Burke, 1979). Based on these characteristics, differential thermal analysis (DTA) was developed by Graham and Mullin (1976), Quamme (1978), and Proebsting and Sakai (1979). This technique has been widely used for more than 20 years and has made the assessment of bud cold hardiness simpler, more rapid, and accurate (Andrews et al., 1987; Quamme, 1986, 1991; Wample et al., 1990; Wolf and Cook, 1994; Wolf and Pool, 1987).

However, DTA is applicable only to tissues that survive via supercooling, such as buds and xylem ray parenchyma (Rajashekar and Burke, 1979). Severe hardiness tissues are involved in the survival of vegetative structures of mature woody plants. Observations by Wample and Wolf (1996), confirmed in our laboratory, indicate that differences in bud hardiness are not indicative of vegetative tissue or whole plant hardiness status.

Another prerequisite for useful cold hardiness measurements is an ability to assess

hardiness levels of the critical tissue of the plant, one whose mortality causes plant death (i.e., cambium layer). T_{50} is defined as the lethal temperature at which 50% of the sample is injured (Bittenbender and Howell, 1974). T_{50} of the cambium/phloem layer, as determined by a controlled freeze-stress and a subsequent viability test (tissue browning or regrowth test) has been widely accepted as a practical cold hardiness index for woody vegetative tissues (Stergios and Howell, 1973; Wolf and Pool, 1987; Wolpert and Howell, 1985). However, tissue browning and regrowth tests are subjective and time-consuming (the browning and regrowth tests normally require at least 5 d and 4 wk, respectively, after a controlled freeze-stress). Just as the development of DTA enhanced our understanding of bud hardiness, a rapid and objective estimation of vegetative tissue viability should encourage more intense effort on woody plant cold hardiness.

Chlorophyll fluorescence is very sensitive to many parameters that affect the photosynthetic processes, which are responsive to environmental stresses (Binder et al., 1997; Schreiber and Bilger, 1993). Chlorophyll fluorescence, especially Fv/Fm (ratio of variable to maximum fluorescence) has proven to be a useful estimator of photosystem II (PS II) quantum efficiency and an indirect measurement of plant physiological status. Chlorophyll fluorescence has been used to determine physiological injury in tree leaves and needles (photosynthetic organs) under low temperature and water stress conditions. Fv/Fm is a rapid ($\leq$ 0.8 s), sensitive, reliable and non-destructive method to detect and quantify environmental stressed-induced disturbances (Kooten and Snel, 1990; Larcher and Neuner, 1989; Mohammed et al., 1995; Peeler and Nayler, 1988; Rütten and Santarius, 1992; Scarascia-Mugnozza et al., 1996; Schreiber and Bilger, 1993; Smillie and Heterrington, 1984; Strand and Öquist, 1988). Recent studies have applied

chlorophyll fluorescence to buds (Düring et al., 1990) and fruits (Tijskens et al., 1994; Song et al., 1997). However, to our knowledge, there are no reports on use of chlorophyll fluorescence to assess viability of freeze-stressed woody vegetative, non-photosynthetic and low chlorophyll content, tissues.

Woody vegetative tissues are important in cold hardiness research for both physiological and economic reasons. The survival of the cambium layer has been estimated by the green color, indicating that chlorophyll fluorescence might be used to measure viability of stressed tissues objectively and quantitatively. Although there are a lot of leaf and needle chlorophyll fluorescence studies, there is no systematic documentation on standardization of sampling or experimental protocols for accurate and repeatable measurements (Mohammed et al., 1995). Moreover, leaf-measuring protocols may not be suitable for woody vegetative tissues that have lignified periderms. The objectives of our experiments were: 1) to determine an appropriate protocol for chlorophyll fluorescence use as an objective assessment of differentiated woody tissue viability after a freeze-stress episode, 2) to determine whether chlorophyll fluorescence was correlated with T_{50}, as calculated from browning and/or regrowth tests (Bittenbender and Howell, 1974; Stergios and Howell, 1973; Wolpert and Howell, 1985), and 3) to determine whether the chlorophyll fluorescence method might produce information more rapidly than current, subjective methods.

MATERIALS AND METHODS

Plant material: Twenty six-year-old Concord grapevines (*Vitis labruscana* Bailey) at the Horticultural Teaching and Research Center at Michigan State University were trained to a top-wire (1.7m) bilateral cordon (Hudson River Umbrella). Vines were vigorous and

high yielding, and were balanced pruned using a 20+20 formula (Wolpert and Howell, 1985).

Sampling Procedure: 1- and 2-year-old canes and wood, respectively, were sampled every 2 wk beginning from late September to mid-December 1997. Cane sampling was based on similar diameter, periderm color and maturity status, and from exterior of the canopy (Howell and Shaulis, 1980). Nodes 3-12 from the base of the cane were used for assessment. Canes were separated randomly into three groups as replicates, and each cane was cut into basal, middle and apical portions for stress evaluation (Wolpert and Howell, 1985). The terms 1B, 1M and 1A refer to 1-year-old basal, middle and apical canes respectively, while 2B and 2A refer to second-year wood. A paired sample was produced by splitting each cane or wood segment into half longitudinally.

Freezing Procedure: The freezing procedure was similar to that used by Wolpert and Howell (1985). Segments of 2-3 cm long were cut from each part of the cane position (1A, 1M, 1B, 2A and 2B). Each cane segment was wrapped with moist cheesecloth at its base to provide ice inoculation and avoid supercooling (McKenzie and Weiser, 1975), then wrapped with aluminum foil to facilitate heat exchange. Freezer temperatures were programmed to decrease at 3 °C h^{-1}. Tissue temperatures were monitored via a 40-gauge copper-constantan thermocouple inserted into the pith of a representative cane segment in each foil bundle. Three replicate foil bundles were removed at each of several test temperatures and allowed to warm slowly overnight in a 3 °C cooler, where the control bundles stayed. The test temperatures were chosen so that the highest produced no injury and the lowest was lethal to all tissues.

Viability Tests: TISSUE BROWNING IN BROWNING AND REGROWTH TESTS. Samples,

thawed overnight, were placed into humidity chambers for 5-7 d (browning test) or in a propagation mist bench for 4 wk (regrowth test), after which they were sectioned and injury was rated by visually evaluating tissue browning of the phloem and cambium layer (Stergios and Howell, 1973). In this experiment, tissues were rated into 5 injury categories based on visual browning of the cane surface with the periderm removed (Wample and Wolf, 1996). Hardiness was calculated as T_{50} by means of the modified Spearman-Karber equation (Bittenbender and Howell, 1974).

CHLOROPHYLL FLUORESCENCE MEASUREMENT. Chlorophyll fluorescence measurements were made at ambient temperature (25 °C) and room light intensity (5 μmol m^{-2} s^{-1}) by a Plant Efficiency Analyzer (PEA) (Hansatech Instruments Ltd., England, 1995, available online at http://www.ppsystems.com/pppea.html). The periderm of the thawed cane or wood segments was peeled off and the exposed cane or wood was inserted into the sample clip (4 mm diameter) for measurement.

The fluorescence illumination was provided by an array of 6 high intensity, light emitting diodes (LEDs), which were focused onto the sample surface to provide even illumination over the exposed area of tissues. Red actinic light of a peak wavelength of 650 nm was provided and readily absorbed by the chloroplast. Maximum light intensity (100%) was 3000 μmol m^{-2} s^{-1}. An algorithm was used to determine the line of best fit through the initial 8-24 data points at the onset of illumination. This line of best fit was then extrapolated the time zero to determine Fo (initial or minimal fluorescence), the Fm (maximum fluorescence) was obtained from the same light intensity when the primary electron acceptor from PSII (Q_A) became fully reduced. Since the fluorescence signal was proportional to the excitation light intensity, the saturating light was determined to

obtain an accurate reading. The fully dark tissue sample was also required for absolute Fm measurement. The Fv (variable fluorescence) was calculated by subtracting Fo from Fm. The ratio of Fv/Fm was calculated from the PEA (Hansatech Instruments Ltd, 1995, 1996).

Preliminary Experiment 1: Comparison of different light intensities

Since 80% of maximum light intensity (~ 3000 μmol m^{-2} s^{-1}) had been used for leaf photosynthesis measurement (J. A. Flore, personal communication, Michigan State University, East Lansing, MI), four light levels of 70% (~ 2100 μmol m^{-2} s^{-1}), 80% (~ 2400 μmol m^{-2} s^{-1}), 90% (~ 2700 μmol m^{-2} s^{-1}) and 100% (~ 3000 μmol m^{-2} s^{-1}) were tested on three different days. On October 3, three light levels (70, 80 and 90%) were used as subplots in a split-plot design with four different dark adaptation times as the main-plot factor (see preliminary experiment 2) on 36 basal control twigs. On October 24, paired basal samples (6 controls and 22 injured) were used to compare 80 and 90% light intensities. On November 8, a split-plot experiment was used with main-plot factor of three temperature treatments (3, -24 and –36 °C) and sub-plot factor of the above four different light intensities. Twenty-four twigs were used: 8 for each temperature treatment.

Preliminary Experiment 2: Comparison of different dark adaptation times

Leaf chlorophyll fluorescence was normally measured after 15 min dark-adaptation (Hansatech Instrument Ltd. 1996; J. A. Flore, personal communication, Michigan State University, East Lansing, MI). Therefore, we tested four dark-adaptation times (5, 10, 15 and 20 min) on October 3 with 36 control samples. On November 8, a total of 24 tissues were tested by three temperatures (3, -24 and -36 °C) as the main-plot factor and same four dark adaptation times as sub-plot factor. Previous chlorophyll fluorescence

applications on fruit suggested that measurements could be done without dark adaptation (Song et al., 1997) because the absolute value was not necessary if one could separate the differentiated samples in the range of interest. Therefore, comparison between non-dark adapted and 10 min dark-adapted was conducted on November 22 (26-pair control samples) and December 22 (23-pair both control and freeze-stressed samples). All the samples were 1M and 1B.

Preliminary Experiment 3: Comparison of different tissue preparations

Three sample preparations (see Figure 1) were tested. They were: 1) Pd, direct measurement on the cane/wood surface with intact periderm, 2) -Pd, cane/wood surface with periderm removed, and 3) -Pd (sl), with -Pd surface shallowly sliced to expose the phloem and cambium layers, the critical position for tissue browning (Stergios and Howell, 1973). To minimize the variation from different cane pieces, paired samples were used to compare Pd versus -Pd (seven control and three injured pairs) and -Pd versus -Pd (sl) (six control and four injured pairs) respectively.

Preliminary Experiment 4: Comparison of partly and fully filled measurement area

Measurement on smaller diameter cane tissues (i.e. 1A and 1M) was often of concern because they could not fill the clip area completely. Further, Fm and Fv/Fm measurements have different requirements regarding tissue fillings of the measurement clip area of PEA. Ten paired 1A and 1M control samples were used to fill the clip with one half cane segment (~ 50-90% partly filled) and two half segments to fill the area completely.

Preliminary Experiment 5: Comparison of different times after controlled freeze stress

Since injured tissues may partially or fully recover over time after freeze-stress, and

measurements on samples immediately removed from the freezer exhibited large variations (M. C. Vasconcelos, personal communication, Oregon State University, Corvallis, OR), a preliminary assessment of the thawing times after freeze-stress was conducted on 72 twigs. Three thawing times (3, 6 and 27 hr) were investigated on September 27. Since the controlled freeze-stress took 7 hr, it was of no practical importance to evaluate time periods between 6 and 17 hr (during the night). Studies on chaparral needles showed that valid chlorophyll fluorescence measurement was obtained 24 hr after controlled freeze-stress (Boorse et al., 1998); therefore, 27 hr was evaluated in this experiment.

Main Experiment: Using Fv/Fm as an objective viability test during cold acclimation The preliminary work defined the precise potential and limits of the method for freeze stressed tissue viability test. Consistency among different measurements is a necessity to obtain meaningful results (Hansatech Instruments Ltd., 1996; Mohammed et al., 1995). Based on the above five experiments and other references, -Pd samples (half of the cane/wood segment) were dark adapted for 10 min and measured at 90% (~ 2700 μmol m^{-2} s^{-1}) light intensity. On each date, three measuring times were compared: they were 3 hr, 5-7 d (browning test) and 4 wk (regrowth test) post stress before the end of October; after October, 3 hr was switched to 24-30 hr and the other two remained the same. There were three replicates for each measurement.

Statistical Analysis and Calculation: Analysis of variance was conducted by SAS/STAT software (V6.12) (SAS Institute Inc., Cary, NC.) using proc mixed procedure, and proc univariate for paired sample *T*-test. LSD was used for multiple mean separation. The coefficient of variation was used to compare the relative amount of

variation in populations having or possibly having different means (Sokal and Rohlf, 1997).

Temperature Records: Maximum and minimum air temperatures were recorded at the weather station at the Horticultural Teaching and Research Center on Michigan State University campus.

RESULTS AND DISCUSSION

Preliminary Experiment 1: Light intensities affected Fo and Fm significantly, but not Fv/Fm. This held true for all three tests (Table 1). Compared to the other two times that used only control samples, the lower Fv/Fm values of October 24 were due to using both control and freeze injured samples. Light level of 70% (~ 2100 μmol m^{-2} s^{-1}), similar to the light intensity used by Song et al (1997), did not give enough saturation light to obtain the maximum fluorescence response for grape woody tissues. Light intensities of 90% (~ 2700 μmol m^{-2} s^{-1}) had the highest Fm compared to either 70% (~ 2100 μmol m^{-2} s^{-1}) or 80% (~ 2400 μmol m^{-2} s^{-1}), and was not significantly different from 100% (~ 3000 μmol m^{-2} s^{-1}). Therefore, 90% (~ 2700 μmol m^{-2} s^{-1}) was selected for the main experiment.

Preliminary Experiment 2: There was no significant difference among 5, 10, 15, and 20 min dark adaptations on either control samples or injured samples (data not shown). Also, the non-dark adaptation treatment was not significantly different from the 10 min dark-adaptation on chlorophyll fluorescence measurement (data not shown). This result significantly simplified and shortened sample preparation and the chlorophyll fluorescence measurement. However, the 10 min dark-adapted samples had lower coefficient of variation that could be important for small, accurate sample measurement.

A possible reason for dark adaptation being unnecessary is that the tissues had been exposed to low light conditions (room light of 5 μmol m^{-2} s^{-1}) for at least 2 d (during the controlled freeze-stress set up and run) before the chlorophyll fluorescence measurement was taken.

Preliminary Experiment 3: Nondestructive tissue measurement has been a positive feature of the chlorophyll fluorescence technique. However, this was not possible for woody tissue measurement. Direct measurement on the cane surface (Pd) had much lower readings on Fo, Fm, and Fv/Fm compared to -Pd measurement, although the two measurements had a significant positive correlation (r = 0.851) (Table 2). The Fv/Fm difference between seriously injured and non-injured control canes was much narrower from Pd measurement (0.14-0.39) than from -Pd (0.37-0.75 or 0.01-0.83 depending on experiments). The Fv/Fm measurements from -Pd control samples (0.75-0.83) were similar to those in most references (Düring et al., 1990; Tijskens et al., 1994; Song et al., 1997).

The -Pd and -Pd (sl) were not different in any chlorophyll fluorescence parameter and they were closely correlated (r = 0.790) (Table 2). This supported the suggestion that chlorophyll fluorescence can be measured at a distance rather than at the surface of the critical tissues (Tijskens et al., 1994; Song et al., 1997). By microscope measurement, -Pd surface was less than 0.5mm to the phloem/cambium layer (F. W. Ewers, personal communication, Michigan State University, East Lansing, MI). This also supported the modified approach to tissue browning method used by Wample and Wolf (1996), who estimated the tissue injury by simply looking at the color of -Pd surface instead of traditional -Pd (sl) cambium layer (Stergios and Howell, 1973).

Preliminary Experiment 4: Using paired comparisons, partly and fully filled measuring areas did not show any significant differences in Fo, Fm or Fv/Fm, although the fully filled sample had lower coefficient of variation in Fo and Fm (Table 3). If Fv/Fm was the main parameter of interest, the partly filled clip area was as good as the fully filled. This information is very useful in practice because the diameters of apical and some middle cane segments were less than 4 mm, and even those with larger diameters could not fully cover the clip area due to the rounded cane surface.

Preliminary Experiment 5: There was no significant difference between thawing 3 and 6 hr, but the same tissues had much lower Fv/Fm after thawing 27 hr in the 3 °C cooler (Table 4). Measurements from browning test (5-7 d post stress) and from regrowth test (4 wk post stress) were not significantly different from the measurement of 27 hr post-stress, but the former two were different from the 3 hr post stress measurement (Figure 2). The interactions between freezing temperatures and thawing hours showed that temperatures above -18 °C had no significant difference from the control, but –33 °C samples caused tissue injury and the injury was most significant after 27 hr. Thus, this technique could accurately and objectively define tissue viability 4-6 d earlier than the normal browning test and almost 4 wk earlier than the regrowth test.

Main Experiment: The objective of the above five preliminary experiments was to determine if and how one could obtain a valid, reproducible chlorophyll fluorescence measurement from mature woody tissues. The important application for cold hardiness research was to determine whether chlorophyll fluorescence, mainly using Fv/Fm, could separate a range of different hardiness levels, to correlate Fv/Fm with T_{50}, and to determine the earliest time after freeze-stress that one could obtain reliable data.

From the experiment during cold acclimation in 1997, we found that there was a positive relationship between Fv/Fm and freeze-stress temperatures (Figures 2 and 3). As temperature decreased after a certain freeze-stress temperature (e. g. –12 °C in Figure 3), Fv/Fm decreased greatly indicating a decreased photochemical efficiency of PS II. This curve was similar to the Arrhenius plot in chilling-injury response reported by Lyons and Raison (1970) and McMurchie (1979). The turning-point temperature was defined as Fv/Fm temperature-inflection point, the lowest temperature at which Fv/Fm was not significantly different from the control. Linear and quadratic regressions both showed significant relationships. Fv/Fm had a negative relationship with tissue-injury levels (Figure 4). Linear and quadratic regressions were obtained from five measurement dates during cold acclimation and both regressions showed significant relationships. In most cases, quadratic regressions had higher r^2 than linear regressions.

Fv/Fm separated the cold-hardiness differences among different positions on the same vine. Generally, in agreement with known cold-hardiness differences within vine (Howell and Shaulis, 1980; Wolpert and Howell, 1985), 2-year-old wood had higher Fv/Fm values (i.e. they were more hardy) than 1-year-old canes and the basal segments were similarly higher (i.e. more hardy) than the apical segments (Figure 5). Fv/Fm value increased from October to November given same freezing temperatures on the same portion of the cane or wood (Figure 2). Fv/Fm also accurately separated the tissue-injury differences at 3 °C intervals in October and November samples (data not shown). A possible reason for Fv/Fm differences over different tissue positions and dates during cold acclimation may be the result of differences in chlorophyll concentration and/or activity. This requires further investigation by chlorophyll content measurement.

The Fv/Fm temperature-inflection point decreased linearly with T_{50} calculated from the regrowth test, and both decreased as minimum ambient temperature decreased (cold hardiness increased) (Figure 6). However, the Fv/Fm inflection point temperatures were generally higher than T_{50}, which may be a result of differences in tissue injury and/or the 3 °C freezing temperature intervals of each controlled freeze-stress regime.

The Fv/Fm_{50}, which we proposed to be an Fv/Fm injury threshold, was defined as the Fv/Fm value when 50% of the tissue were injured and was calculated from the linear regression function. During the cold-acclimation period, Fv/Fm_{50} increased slightly (less than 0.2) (Figure 7). This trend is similar to the study on needles of Scots pine (Strand and Öquist, 1988). According to this analysis in 1997, 0.538 and 0.588 were suggested as the possible thresholds for 1- and 2-year-old basal canes/wood mortality, respectively.

Chlorophyll fluorescence, especially the ratio of Fv/Fm, was shown to be a reliable, rapid, and objective method of assessing woody tissue viability of Concord grapevines after controlled freeze-stress. However, this work was conducted on one cultivar of one genus during the acclimation period. Further work is underway to assess the applicability and limits of this method for other grape cultivars, other woody species, and at other periods of time during dormancy, including mid-winter and deacclimation.

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Table 1. Effect of light intensity (% of maximum 3000 μmol m^{-2} s^{-1}) on chlorophyll fluorescence of grape cane tissues

Light	Oct. 3[z]			Oct. 24[y]			Nov. 8[x]		
(%)	Fo	Fm	Fv/Fm	Fo	Fm	Fv/Fm	Fo	Fm	Fv/Fm
70	241.4*b*	1316.3*b*	0.814*a*	n.a.	n.a.	n.a.	418.3*d*	1055.2*b*	0.766*a*
80	282.6*a*	1586.0*a*	0.821*a*	550.9*b*	855.0*b*	0.261*a*	496.8*c*	1137.5*ab*	0.720*a*
90	306.3*a*	1620.8*a*	0.810*a*	599.9*a*	945.7*a*	0.268*a*	546.5*b*	1329.3*a*	0.724*a*
100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	609.8*a*	1476.7*a*	0.737*a*
P[w]	0.0136	0.0339	0.2070	0.0082	0.0030	0.1223	0.0001	0.0095	0.7783

[z] On Oct. 3, three intensity levels (70%, 80%, 90%) were compared by four different dark-adaptation times on 36 basal control (non-stressed) tissues ($r = 3$).

[y] On Oct. 24, six control and 22 freeze-injured paired basal samples were used to compare 80% and 90% light intensities.

[x] On Nov. 8, four different light intensities were tested by split-plot experimental design with three different temperatures (3, -24 and –36 °C). Total: 24 cane pieces with 8 twig samples for each temperature treatment.

[w] Probability (P) > | T | in paired T-test or P > F in split-plot experiment designs.

a-d Means with the same letter within the column do not significantly differ (P<0.05) according to LSD or pared T-tests.

Table 2. Effect of tissue preparations[z] on chlorophyll fluorescence of grape cane tissues

Tissue preparation	Fo			Fm			Fv/Fm		
	Mean	$P>\|T\|$	CV(%)[y]	Mean	$P>\|T\|$	CV(%)	Mean	$P>\|T\|$	CV(%)
Pd vs. -Pd									
Pd	93	0.0001	14.6	127	0.0001	21.1	0.259	0.0001	36.3
-Pd	534		26.2	1498		17.0	0.618		28.1
-Pd vs. -Pd (sl)									
-Pd	546	0.7675	35.0	1455	0.2805	18.7	0.625	0.8498	19.5
-Pd (sl)	555		22.5	1607		22.9	0.630		17.7

[z] Three ways of tissue preparation: Pd, direct measurement on cane surface with intact periderm; -Pd, surface with cane periderm removed; -Pd (sl), -Pd surface was shallowly sliced to expose the cambium/phloem layer. Paired comparisons were conducted on Pd vs. -Pd and -Pd vs. -Pd (sl), respectively. Each comparison was presented by the average of 10 paired 1-year-old middle and (or) basal control and injured cane samples on Nov. 8, 1997. *P* value was calculated by SAS univariate procedure.

[y] CV (%, coefficient of variation) is the percentage of the standard deviation over the mean, which measured the relative variance in populations having different means (Sokal and Rohlf 1997).

Table 3. Effect of partly versus fully filled clip (measurement) area[z] on chlorophyll fluorescence of grape cane tissues

Clip area filled	Fo		Fm		Fv/Fm	
	Mean	CV (%)[y]	Mean	CV (%)	Mean	CV (%)
Partly	312	30.3	1487	42.7	0.768	9.6
Fully	345	19.6	1515	21.5	0.762	9.8
$P>\|T\|$	0.3849		0.8569		0.5554	

[z] Data presented were the average of 10 paired one-year-old apical and middle control cane samples on Oct. 3, 1997. A half segment was used to partly fill (50-90%) the clip area and two half segments were used to fill the clip area completely. The *P* value was calculated using SAS univariate procedure.

[y] CV (%, coefficient of variation) is the percentage of the standard deviation over the mean, which measured the relative variance in populations having different means (Sokal and Rohlf 1997).

Table 4. Effects of controlled freeze-stress temperatures, thawing hours post stress, and plant-tissue positions on Fv/Fm measurement of grape cane tissues[z]

Single effect	Fv/Fm	Interactions[y]	Fv/Fm
Temperature (°C)		$T_0 \times H_1$	0.727*a*
3 (T_0)	0.705*a*	$T_0 \times H_2$	0.687*a*
-6 (T_1)	0.704*a*	$T_0 \times H_3$	0.700*a*
-18 (T_2)	0.681*a*	$T_1 \times H_1$	0.707*a*
-33 (T_3)	0.483*b*	$T_1 \times H_2$	0.726*a*
P > *F*	0.0001	$T_1 \times H_3$	0.679*a*
Post-stress hour		$T_2 \times H_1$	0.676*a*
3 (H_1)	0.663*a*	$T_2 \times H_2$	0.671*a*
6 (H_2)	0.653*a*	$T_2 \times H_3$	0.697*a*
27 (H_3)	0.613*b*	$T_3 \times H_1$	0.543*b*
P > *F*	0.0041	$T_3 \times H_2$	0.527*b*
Tissue position		$T_3 \times H_3$	0.378*c*
Apical	0.607*a*	*P* > *F*	0.0005
Basal	0.679*b*		
P > *F*	0.0001		

[z] Split-split-plot experimental design was conducted on Sept. 27, 1997. The temperature (°C), post-stress hour, and plant-tissue positions were the main-plot factor, sub-plot factor, and sub-sub-plot factor, respectively. Total sample size was 72 twigs and 3 replicates for each measurement.

[y] Only two-factor interactions between temperature and post-stress hours are shown in this table because of our research objectives in this paper.

a-c Means with the same letter in each category do not significantly differ ($P<0.05$) according to LSD test.

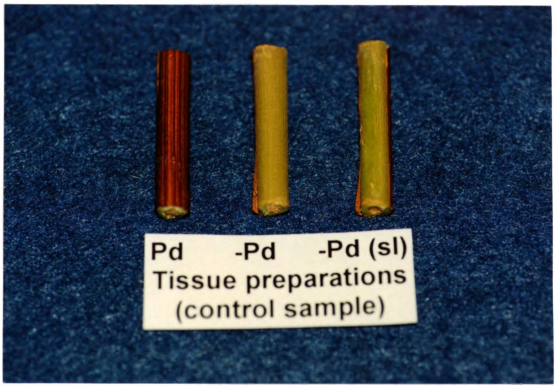

Figure 1. Comparison of three different cane-preparation methods used for measuring chlorophyll fluorescence. (1) Pd, cane with intact periderm; 2) -Pd, cane with periderm removed; and (3) -Pd (sl), cane with periderm removed and a shallow slice to expose phloem and cambium layers.

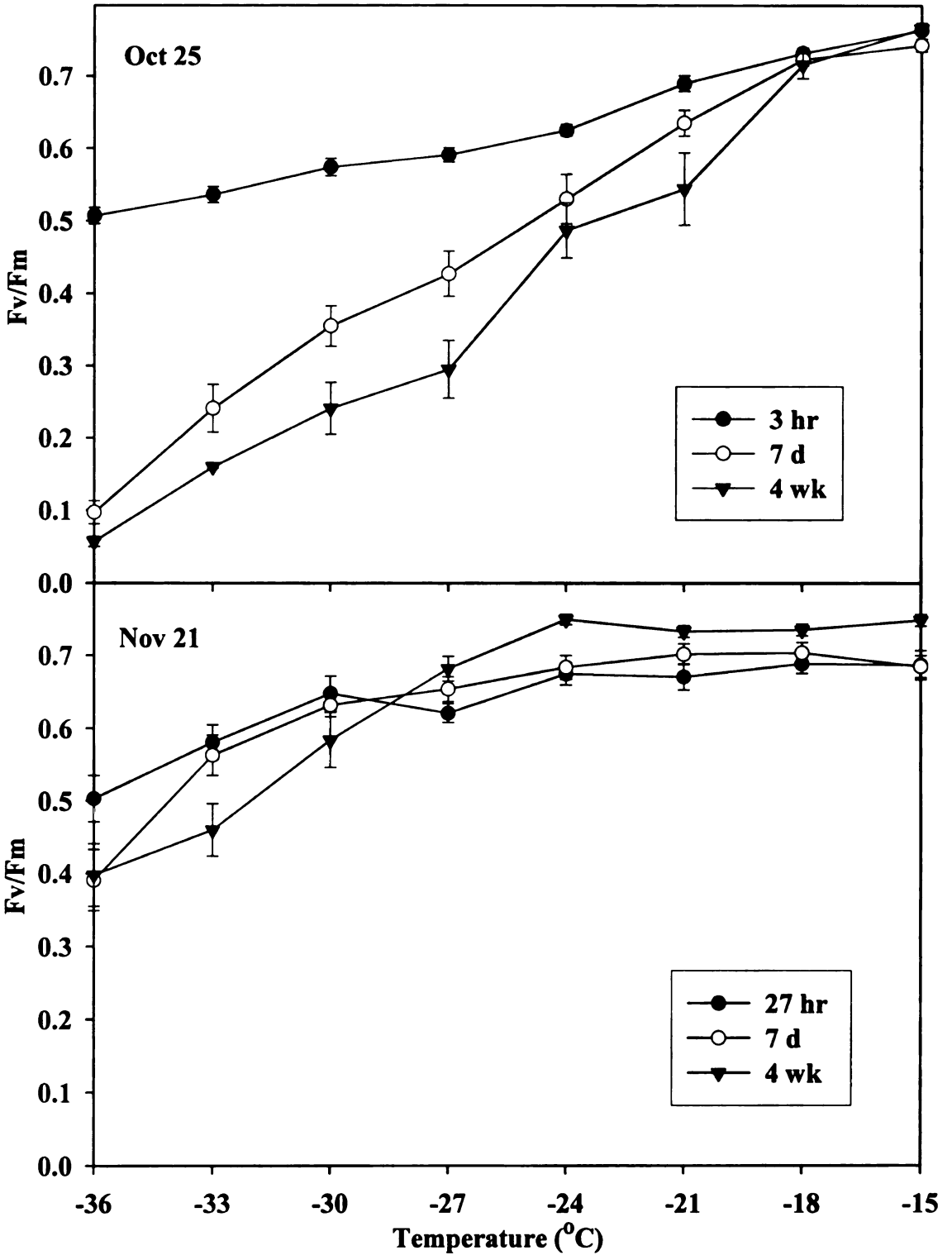

Figure 2. Fv/Fm comparison of three measuring times after woody tissues were exposed to controlled freeze-stress on Oct. 25 and Nov. 21, 1997. Three measuring times were 3 hr, 7 d, and 4 wk on Oct. 25; 27 hr, 7 d and 4 wk on Nov. 21. Data presented were the average of the five portions of the grapevine woody tissues tested. Three replicates were used for each measurement. Error bars are ±SE.

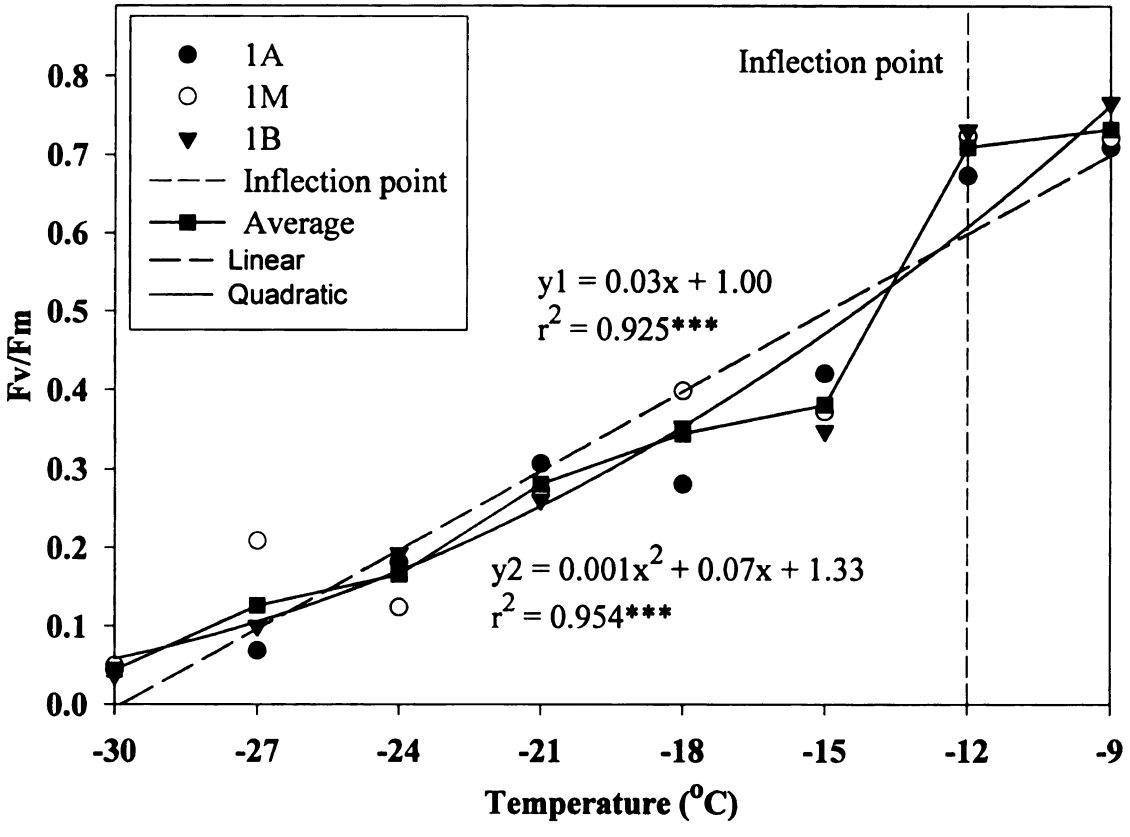

Figure 3. The relationship between Fv/Fm and controlled freeze-stress temperatures of 1-year-old canes on Sept. 27, 1997; 1A, 1M and 1B refer to 1-year-old apical, middle and basal canes, respectively. Inflection point was defined as the lowest temperature at which Fv/Fm was not significantly different from the control. Analysis of variance was done by SAS proc mixed procedure. ***significant at $P = 0.001$.

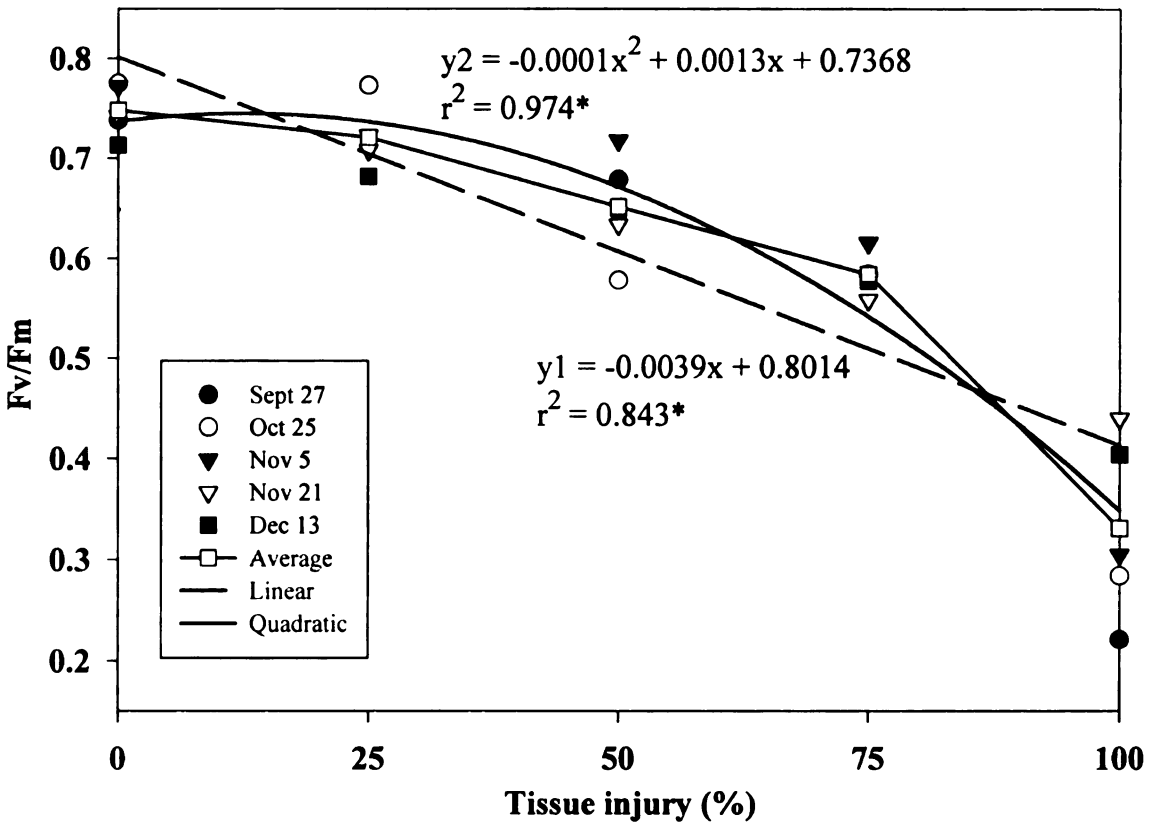

Figure 4. The relationship between Fv/Fm and tissue injury (%) during cold acclimation. Data presented were the average of the five portions of the woody tissue tested from September to December, 1997. The curve was plotted from the average of the data at each injury level.
* significant at $P = 0.05$.

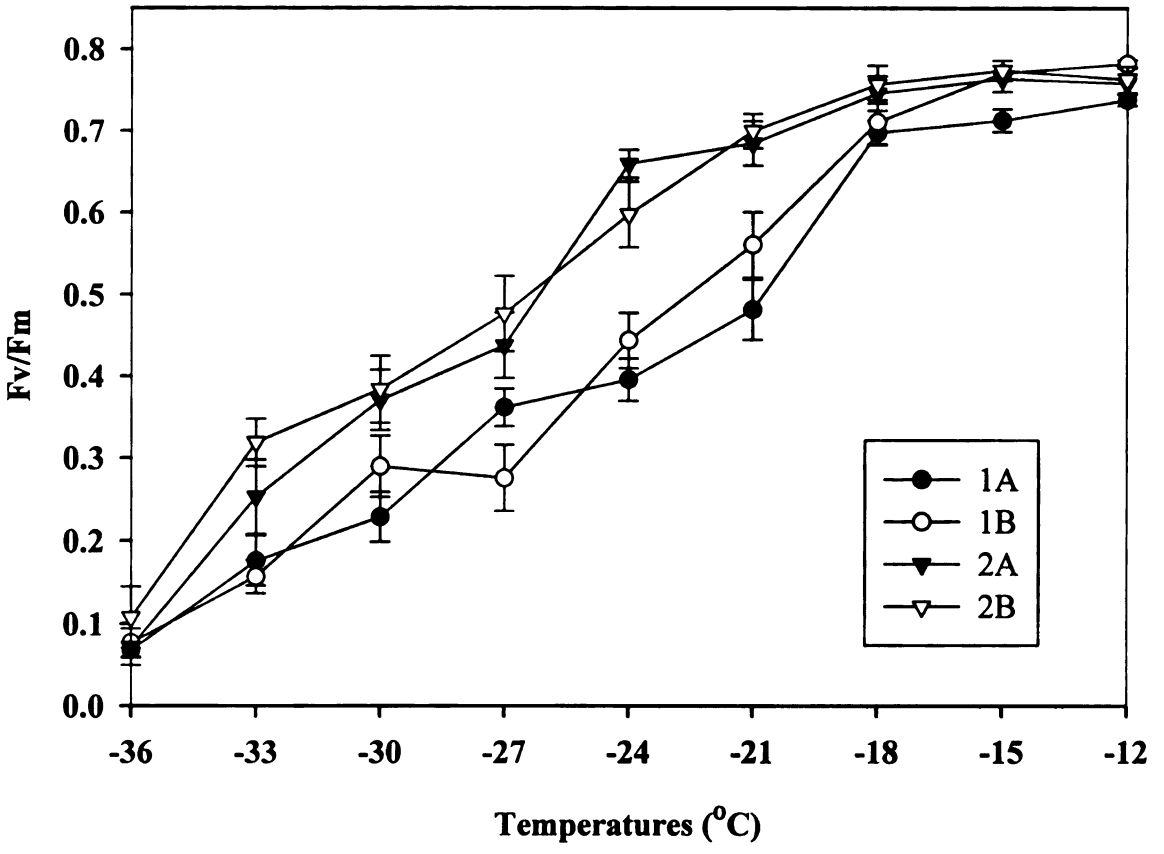

Figure 5. Fv/Fm comparisons of 1A, 1B, 2A, and 2B after exposure to controlled freeze-stress on Oct. 25, 1997. 1A and 1B refer to 1-year-old apical and basal canes, respectively; similarly, 2A and 2B refer to 2-year-old apical and basal wood, respectively. Data presented were the average of measurements from 7 d and 4 wk of each grapevine woody tissue tested. Error bars are ±SE.

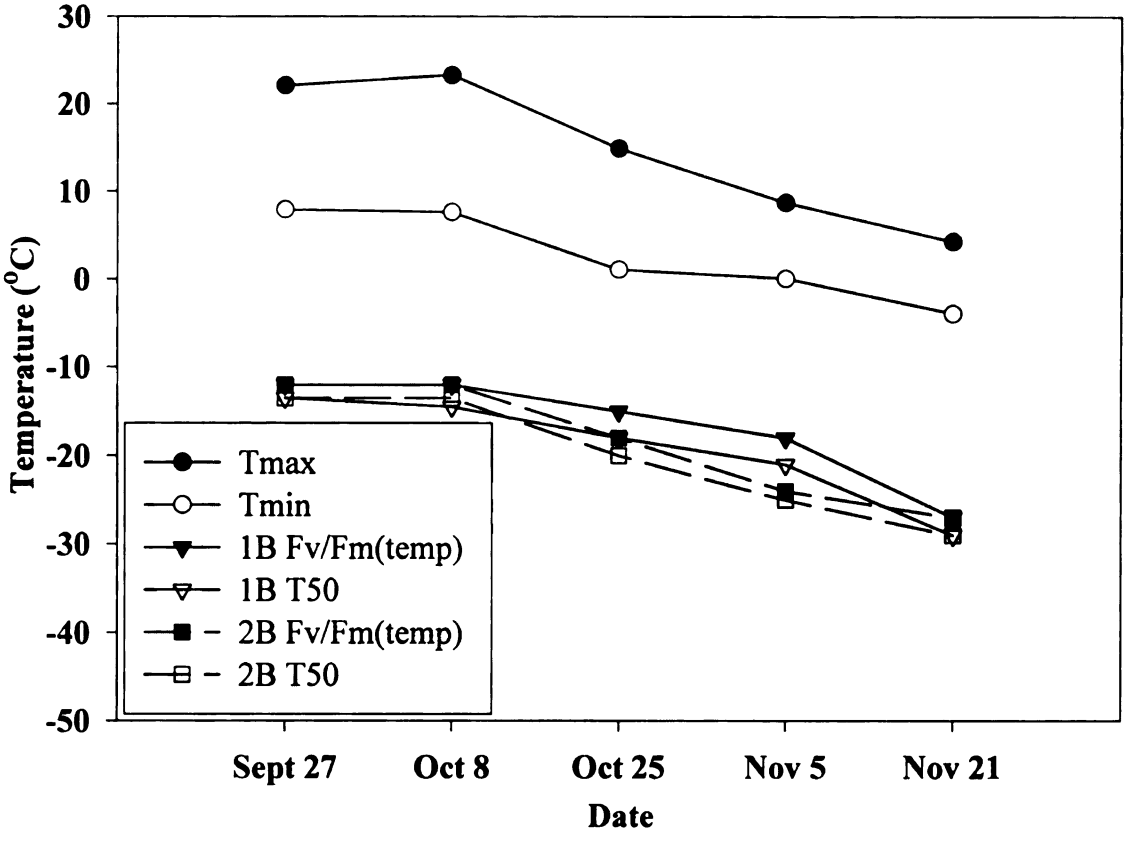

Figure 6. The relationships among average maximum and minimum air temperatures (Tmax and Tmin), Fv/Fm temperature-inflection point (Fv/Fm(temp)), and T_{50} during cold acclimation in 1997. The Tmax and Tmin were determined by the average of temperatures 10 d before each sampling date. Fv/Fm(temp) was the lowest temperature at which Fv/Fm was not significantly different from the control; T_{50} was calculated from regrowth test; 1B and 2B refer to 1- and 2-year-old basal cane and wood, respectively.

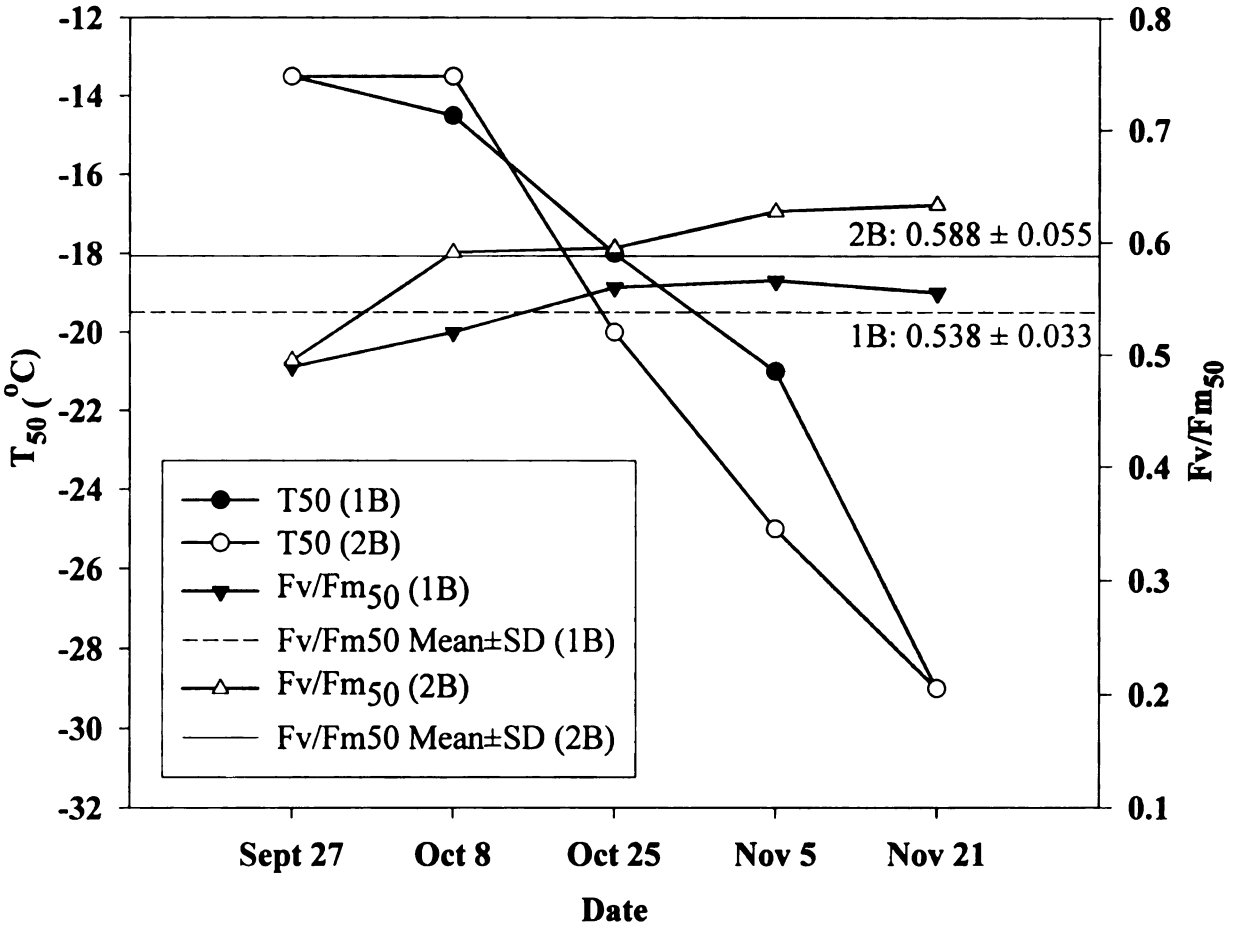

Figure 7. The relationship between T_{50} and Fv/Fm_{50} during cold acclimation in 1997. T_{50} was calculated from regrowth test; Fv/Fm_{50}, calculated from regression function at each sampling date, was the value of Fv/Fm when 50% of the tissue was injured; 1B and 2B refer to 1- and 2-year-old basal cane/ wood, respectively. SD: standard deviation.

CHAPTER III

APPLYING THE CHLOROPHYLL FLUORESCENCE TECHNIQUE TO COLD HARDINESS STUDIES OF GRAPEVINES DURING THE DORMANT SEASON

ABSTRACT

Woody tissue cold hardiness and chlorophyll fluorescence of Concord (*Vitis labruscana* Bailey) grapevines were measured regularly during the dormant seasons in 1997-1998, 1998-1999, and late winter of 2000. A Plant Efficiency Analyser (Hansatech Ltd., England) was used to measure chlorophyll fluorescence after a controlled freeze-stress. The Fv/Fm was used as an indicator of viability. It is shown that Fv/Fm is an objective, sensitive, and efficient viability test for grape woody tissues; the response magnitude varies with each portion of the dormant season and the type of woody tissues (1- and 2-year-old). The Fv/Fm temperature inflection points (temperature at which no and 100% tissue injury occurred) are closely related with T_{50} (temperature at which 50% of the sample is injured) calculated from a regrowth test. Injury thresholds Fv/Fm_0, Fv/Fm_{50}, and Fv/Fm_{100} (the Fv/Fm values when no, 50% and 100% of the tissue were injured, respectively) are proposed for different woody tissues at three basic dormant periods. Preliminary application on different grape cultivars, two fluorometers, and field measurement are also demonstrated.

Key words: Concord (*Vitis labruscana*), chlorophyll fluorescence, Fv/Fm temperature inflection points, Fv/Fm injury thresholds, viability test.

INTRODUCTION

When sunlight strikes on a leaf surface, chlorophyll molecules absorb 80-90% of the energy. The excited molecules are not stable and have four ways to release the energy: energy transduction (for photochemistry reactions), resonance energy transfer (energy is transferred to non-photosynthetically active molecules), heat loss, or light re-emission (the transition from the lowest excited singlet state to the ground state) (Taiz and Zeiger, 1998). *In vivo*, only a small portion (at a maximum of 3-5%) of the energy is re-emitted with a wavelength of about 680-720 nm (Lichtenthaler, 1990). This re-emitted light is chlorophyll fluorescence (CF). In general, almost all fluorescence is emitted from chlorophyll *a* associated with photosystem II (PS II) since chlorophyll *b* transfers the excitation energy to chlorophyll *a* (Bolhàr-Nordenkampf et al., 1989; Lichtenthaler, 1990).

The fluorescence emission can be detected repeatedly in a non-destructive fashion, and the spectroscopic characteristics of CF reflect the properties of the chlorophyll molecules and their environment. Therefore, CF measurement of photosynthetic organisms has rapidly become a powerful tool to study the structure and functions of photosynthetic apparatus and many environmental stresses that could cause photosynthetic disturbances (Binder et al, 1997; Boorse et al, 1998; Buwalda and Noga, 1994; Jimenez et al, 1997; Karukstis, 1991; Larcher and Neuner, 1989; Mohammed et al., 1995; Peeler and Naylor, 1988; Percival and Dixon, 1997; Rütten and Santarius, 1992; Scarascia-Mugnozza et al., 1996; Schreiber and Bilger, 1993; Strand and Öquist, 1988). In these researches, Fv/Fm (the ratio of variable fluorescence to maximum fluorescence) has shown to be a rapid (~ 0.8 s), sensitive, reliable and non-destructive method to detect

and quantify environmental stressed-induced disturbances.

Recent studies have applied chlorophyll fluorescence to buds (Düring et al., 1990) and fruits (Tijskens et al., 1994; Song et al., 1997) to detect bud injury and fruit quality. However, to our knowledge, there is only one report (Jiang et al., 1999) on use of chlorophyll fluorescence to assess viability of freeze-stressed woody vegetative non-photosynthetic tissues.

Cold hardiness is important because most of the world's crop-growing regions are subject to varying degrees of freezing stress (Guy, 1990). There has been significant advance in research on bud hardiness of woody fruit trees. Most important among them is the use of differential thermal analysis (DTA) to determine killing temperatures (mortality) of the buds (Graham, 1971; Quamme, 1978; Andrews et al., 1983; Wample et al., 1990; Wolf and Pool, 1986, 1987). Differential thermal analysis, however, cannot be directly applied to woody tissues because woody tissues and bud primordium survive freeze episodes by different physiological mechanisms. Buds supercool for survival, while the cambium, the regenerative organ in the wood, avoids supercooling. Further, the hardiness of buds is not predictive of wood hardiness (Wample and Wolf, 1996). The key fact is that there is greater economic loss associated with the damage to woody tissues, e.g. trunk sunscald and split, blackheart in stems of shrubs and trees and mortality (Weiser, 1970), than with seasonal flower buds loss.

An effective use of laboratory freezing tests for determining cold hardiness of woody perennial plants requires: 1) a freeze stress regime that is similar to natural freeze episodes in the field, and 2) an accurate and precise method of determining tissue viability. It is easy to achieve the first requirement with a programmable freezer.

However, the current viability tests are still frequently employed as wither tissue browning or the regrowth test. These two tests are subjective and time-consuming (the browning and regrowth tests normally require at least 5 d and 4 wk, respectively, after a controlled freeze-stress).

In the tissue-browning test, the survival of the cambium layer is estimated by the green color, indicating the presence of chlorophylls. For this reason, we recently reported a series of initial experiments on grape woody tissues demonstrating that: 1) non-destructive leaf measuring protocols are not suitable for woody vegetative tissues that have lignified periderms; 2) chlorophyll fluorescence, especially Fv/Fm, was a sensitive, reliable, objective, and rapid (27 hr instead of 5-7 d) method of assessing woody tissue viability after a controlled freeze-stress; 3) The Fv/Fm temperature inflection point (the lowest temperature at which Fv/Fm was not significantly different from the control) was positively correlated with T_{50} (temperature at which 50% of the sample is injured) calculated from the regrowth test.

However, those results represented only the cold acclimation (CA) period of the annual dormant period in 1997, and were for only one grape cultivar. Different portions of the dormant periods (CA, midwinter, and deacclimation) are suggested to involve different physiological and biochemical activities (Howell, 1988). Thus, it is not clear whether the reported results could be similarly applied to other periods of dormant season (midwinter and deacclimation), to other grape cultivars, and to field use.

Therefore, the objectives of this study were: 1) to determine whether the protocol for chlorophyll fluorescence measurement used during CA is valid during the whole dormant period; 2) to determine whether chlorophyll fluorescence was correlated with

T_{50}, as calculated from a conventional viability test (e.g. the regrowth test) during the whole dormant season; and 3) to determine whether and how the chlorophyll fluorescence method might be used in the field. In order to answer these questions, a series of experiments were conducted to compare the responses among the dormant periods, different parts of the vine (cane vs. wood; apical, middle vs. basal canes), measurements in different post freeze-stress times (i.e. viability tests), different grape cultivars, and different fluorometers.

MATERIALS AND METHODS

Plant Material: Seven mature, bearing grape cultivars were tested during the dormant periods of 1997-1998, 1998-1999, and 2000. These include Concord (*Vitis labruscana*), Niagara (V. *labruscana),* Delaware *(V. labruscana*), and Frontenac (V. hybrid) grapevines from the Horticultural Teaching and Research Center (HTRC) at Michigan State University, East Lansing, MI. Pinot noir (*V. vinifera*), White Riesling (*V. vinifera*), and Chardonnay (*V. vinifera*) from Southwest Michigan Research and Extension Center (SWMREC) near Benton Harbor, MI.

All vines at HTRC were trained to a top-wire (1.7m) bilateral cordon (Hudson River Umbrella), while those at SWMREC were trained to a low head (0.8m) with horizontal canes (modified Guyot) with vertical shoot positioning.

Sampling Procedure: All sampling was based on the precepts of Howell and Shaulis (1980). Canes were sampled in the morning (0800-0930am) from the canopy exterior and possessed similar diameter, dark periderm color and medium internode length within each cultivar. Cuttings were sealed in a plastic bag and brought to the laboratory within

an hour except samples from SWMREC. A paired sample was made in the laboratory by splitting each cane or wood segment into half longitudinally.

Experiment 1: This experiment was conducted only on Concord grapevines.

Sample Processing: One- and two-year-old canes and wood, respectively, were sampled every 2 wk beginning in early September to April 1997-98, and 1998-99, and late January to mid-April of 2000. Nodes 3-12 from the base of the cane were used for assessment. Canes were separated randomly into three (in 1997-1999) or five (in 2000) groups as replicates, and each cane was cut into basal, middle and apical portions for stress evaluation (Wolpert and Howell, 1985). The terms 1B, 1M and 1A refer to 1-year-old basal, middle and apical canes, respectively, while 2B and 2A refer to 2-year-old basal and apical wood. During the 1998-99 dormant season, only 2A was sampled for two-year wood due to an inadequate quantity of the plant material. No two-year-old wood was tested in 2000.

Freezing Procedure: The procedure is similar to that of Wolpert and Howell (1985). Segments of 2-3 cm long woody tissues were cut from each portion of the cane (1A, 1M, 1B, 2A and 2B). The base of each cane segment was wrapped with moist cheesecloth to provide ice inoculation and prevent supercooling (McKenzie and Weiser, 1975), then wrapped with aluminum foil to facilitate heat exchange. Freezer temperatures were programmed to decrease at 3 °C hr^{-1}(5 °C hr^{-1} during mid-winter of 1999). Tissue temperatures (ranging from –9 °C to –42 °C) were monitored via a 40-gauge copper-constantan thermocouple inserted into the pith of a representative cane segment in each foil bundle. Three (1997-1999) and five (2000) replicate foil bundles were removed at each of several test temperatures and allowed to warm slowly overnight in a 3 °C cooler,

where the control bundles stayed. The test temperatures were chosen so that the highest temperature produced no injury and the lowest was lethal to all tissues.

Viability Tests: TISSUE BROWNING AND REGROWTH TESTS. Freeze-stressed samples, thawed overnight, were placed into humidity chambers for 1 d (24-30 hr), 5-7 d or in a propagation mist bench for 4 wk (regrowth test), after which they were sectioned and injury was rated by visually evaluating tissue browning of the phloem and cambium layer (Stergios and Howell, 1973). Two additional replicate samples of 1d (24-30 hr) and 5-7 d (browning tests) were also planted in the same greenhouse as those regrowth samples on 29 Feb, 27 Mar, 3 Apr and 11 Apr in 2000. The mist bench was sprayed with water every 5 min (no pesticide control) during dormant seasons of 1997-1998 and 1998-1999, while a vapor pressure deficit was controlled at 0.3 Kpa at all times via steam injection in 2000. Weekly pesticide (Duraguard+Truban+Clearys for the first week, and Heritage for the rest of the 3 wk) spray was also used in the greenhouse in 2000. Tissues were ranked from 1 to 5 injury levels based on visual browning of the cane surface with the periderm removed (Wample and Wolf, 1996). Hardiness was calculated as T_{50} by means of the modified Spearman-Karber equation (Bittenbender and Howell, 1974).

CHLOROPHYLL FLUORESCENCE MEASUREMENT. Chlorophyll fluorescence measurements were made at ambient temperature (25 °C) and room light intensity (5 μmol m^{-2} s^{-1}) by a Plant Efficiency Analyzer (PEA) (Hansatech Instruments Ltd., England, 1995). The periderm of the thawed cane or wood segments was peeled off and the exposed cane or wood was inserted into the sample clip (4 mm in diameter) for measurement (Jiang et al, 1999).

The fluorescence illumination was provided by an array of 6 high intensity, light

emitting diodes (LEDs), which were focused onto the sample surface to provide even illumination (650 nm) over the exposed area of tissues. Maximum light intensity (100%) was 3000 μmol m^{-2} s^{-1}. An algorithm was used to determine the line of best fit through the initial 8-24 data points at the onset of illumination. This line of best fit was then extrapolated to zero to determine Fo (initial or minimal fluorescence). Fm (maximum fluorescence) was obtained from the same light intensity when the primary electron acceptor from PSII (Q_A) became fully reduced. Fv (variable fluorescence) was calculated by subtracting Fo from Fm. The ratio of Fv/Fm was calculated from the PEA (Hansatech Instruments Ltd, 1995 and 1996). Based on the previous experiment (Jiang et al., 1999), -Pd samples (periderm removed cane/wood segment) were measured at 90% (~ 2700 μmol m^{-2} s^{-1}) light intensity without dark adaptation.

Chlorophyll fluorescence was measured each time that tissue browning or regrowth tests were conducted in each of the three time periods post-stress: 1 d (24-30 hr), 5-7 d and 4 wk.

Chlorophyll Content Measurement: The chlorophyll content of Concord woody tissues was conducted on 9 Feb, 3 Mar, 1 Apr and 15 of Apr, 1999. The extraction method was modified from Hiscox and Israelstam (1979). A cane or wood (internode) of 4.0 g was weighed, the periderm was peeled off, and placed into a flask containing 20 ml DMSO. Extraction was conducted in a 65 °C water bath for 6 hr. Then a 2.0ml extraction sample was transferred to a cuvette, and the OD (Absorbance) values at 645 and 663nm were measured. Arnon equations were used for calculation: Total chlorophyll Concentration (mg. L^{-1}) = 20.2 D_{645} + 8.02 D_{663}.

Experimental Design: A split-split-plot experimental design using the three

measurement times (24-30 hr, 5-7 d and 4 wk) as main-plot, controlled freezing temperatures were the sub-plot factor, and different portions of the cane or wood positions (1A, 1M, 1B, 2A, 2B) were the sub-sub-factor. Three or five replicates were used for each measurement.

Statistical Analysis and Calculation: Analysis of variance was conducted by SAS/STAT software (V7.1) (SAS Institute Inc., Cary, NC.) using a proc glm or proc mixed procedure, and proc univariate for paired sample T-test. Fisher's LSD was used for multiple mean separations. Regression analysis was conducted by ANOVA using SigmaPlot (v4.0) program.

Temperature Records: Maximum and minimum air temperatures were recorded at the weather station at the Horticultural Teaching and Research Center on Michigan State University campus.

Experiment 2: Comparison among different grape cultivars

Different grape cultivars were compared only on control (non-freeze stressed) samples, and CF was measured in the same way as in experiment I by PEA.

Delaware, Frontenac, and Marechal Foch were sampled together with Concord on Nov 19, 1998 in HTRC. Thirty basal cane pieces were measured. Pinot noir was sampled on Mar 31, 2000; White Riesling and Chardonnay were samples on Apr 5, 2000 from SWMREC. Ten basal cane pieces were used for measurement of each cultivar.

Experiment 3: Comparison between two fluorometers

There are different kinds of fluorometers available in the market. Pulse-modulated fluorometers have been used more often in recent years. Therefore, a pulse-modulated fluorometer (PMF, model OS-500, OptiSciences, Tyngsboro, Mass.) was tested on the

same samples of Oct 16 and Nov.4, 1998 in addition to PEA measurement. Paired control and injured samples (55 and 36 pairs, respectively) were used to minimize the variation from plant materials, i.e. half of the same cane piece was measured by PEA and the other half was measured by PMF.

The pulse-modulated fluorometer was used similarly to that of Song et al (1997). The fluorometer was operated in the "Fv/Fm" mode and fluorescence was measured using a photodiode in the 710-760 nm range. The excitation (modulated) light intensity (660 nm) for the Fo measurement was ~ 0.15 μmol m^{-2} s^{-1}, which was sufficient for accurate measurement of Fo. After Fo was determined, a saturation light (660 nm) of pulse was supplied via the light guide by a halogen lamp. The light intensity striking on the tissue surface was estimated to be 2400 μmol m^{-2} s^{-1}. The Fm was determined during each pulse, which lasted 0.8s. Fv/Fm was calculated by the fluorometer. Cane or wood samples were clamped by the cuvettes, the same way as of PEA.

Experiment 4: Measurement in the field

Field CF measurement was conducted during Sept 16-19, 1999. Liquid nitrogen was applied to the cane/wood surface to cause artificial freeze injury (Pratt and Pool, 1981). Copper-constantan thermal couples were used to monitor the temperature on the tissue surface. Half of the cane surface was dark acclimated (wrapped with aluminum foil) for 15 min, and the other half was not. PEA measurement was conducted several times (15-20 min, 5 hr and 51 hr) after the artificial freeze stress.

RESULTS AND DISCUSSION

In this paper, dormancy was separated into CA, MW, and DCA each year according to

T_{50} from the regrowth test, i.e. T_{50} decreased (hardiness increased) dramatically from early fall until reaching maximum hardiness during midwinter, and then T_{50} increased (hardiness decreased) starting in late winter or early spring.

1. **Fv/Fm had a negative relationship with tissue injury, and positive relationship with sub-freezing temperatures during the whole dormant season**

As injury increased or freezing temperatures decreased, Fv/Fm decreased. These relationships were demonstrated during CA of 1997-1998 (Jiang et al., 1999). Similar results were obtained during dormant seasons of 1998-1999 (Figures 1 and 2) and 2000 (Figure 3).

The Fv/Fm decrease may indicate a decrease in chlorophyll content/activity and the photochemical efficiency of PS II. These curves were similar to the Arrhenius plot in chilling injury response reported by Lyons and Raison (1970) and McMurchie (1979). Linear and quadratic regressions were obtained from 6 different measurement dates during CA in 1998 and 9 dates in 2000 after mid-winter. Both regressions showed significant at least at 0.05 level. Generally, quadratic regressions had higher r^2 (Figures 1 and 3) than linear regressions.

2. **Fv/Fm separated the cold hardiness differences among different positions on the same vine**

In agreement with cold hardiness variations within vine (Howell and Shaulis, 1980; Wolpert and Howell, 1985), two-year-old wood generally had higher Fv/Fm values and lower injury (i.e. they were more hardy) than one-year-old canes during CA (Jiang et al., 1999) and DCA in 1997 (Figure 4), and the basal segments were similarly higher (i.e. more hardy) than the apical segments at each sub-freezing temperature (Figure 5). Fv/Fm

could sometimes separate the tissue injury differences at 3 °C intervals during the dormant season (Table 1).

A possible reason for Fv/Fm differences over different tissue positions and dates during dormancy may be the result of differences in chlorophyll concentration and/or activity. Our preliminary measurement during DCA in 1999 showed that 1) chlorophyll content (mg. L^{-1}) decreased as injury increased (control samples had significant higher chlorophyll content than injured samples); 2) Basal tissues had higher content than apical ones, and 1-year-old canes had higher content than 2-year-old canes (Table 2). This is consistent with general Fv/Fm measurement in the same time period. However, the incubation time affects the chlorophyll measurement (Table 2). Further complete measurement on chlorophyll and other pigments is necessary to obtain more understanding.

3. Early viability test is possible during CA, but care must be taken during MW and DCA

Jiang et al (1999) reported that measurements from the browning test (5-7 d post stress) and from the regrowth test (4 wk post stress) were not significantly different from the measurement of 1 d (24-30 hr) post-stress during CA. No significant difference was also found during MW and DCA of 1997-1998, and CA and MW in 1998-1999 (Tables 1 and 3). However, during MW and DCA of 2000 and DCA of 1998-1999, 1 d measurement was not significantly different from 5-7 d browning test, but different from that of the regrowth test (Tables 3 and 4).

One of the possible reasons could be the dehydrated woody tissues, which had less chlorophyll activities, during MW and DCA. Therefore, measurements were compared at

the same post-stress times (24-30 hr and 5-7 day) on tissues recovered in the humidity chamber in the lab and those in the propagating mist-bench in the greenhouse, where tissues had higher moisture. The Fv/Fm measured in the greenhouse was higher than those in the lab in both post-stress times; however, they were still significant lower than the 4-wk regrowth test.

Fluorescence (Fv/Fm) changed over the dormant season (Figure 6). The general trend was that it was higher during CA, then decreased and stayed at the low level during midwinter, and 1-year-old canes increased again during late spring (April in Michigan). The seasonal changes of the chlorophyll and the involvement of other pigments in the cane/wood may be able to explain some of these changes.

4. Suggested Parameters

(1) Fv/Fm temperature inflection point (Fv/Fm(temp))

The turning point of Fv/Fm, defined as the lowest temperature at which Fv/Fm was not significantly different from the control (Jiang et al., 1999). Fv/Fm(temp) of each sampling date was determined by analysis of variance. For example, Figure 7 showed that -12°C was the Fv/Fm(temp) of measurements during DCA in 2000. Also, we can see that although three measurement times were significantly different in the absolute Fv/Fm values at each sub-freezing temperature, the Fv/Fm(temp) was the same for all three tests (Figure 7). This was the case in most of the measurements in the last three dormant seasons.

Fv/Fm (temp) defined above was actually calculated as the temperature when no injury occurred, i.e. Fv/Fm (temp)_0. Another interesting temperature inflection point was when compete (100%) injury occurred, i.e. Fv/Fm (temp)_100. These two

temperature inflection points were closely related to T_{50} as expected (Figure 8), i.e. T_{50} fell in the middle of them. The trends of the three curves were similar in all the tests (data not shown). There were several times of measurement where three curves were not paralleled so well; the reason may be the limitation of artificial freeze temperature regimes and temperature intervals (3 or 5 °C).

(2) Fv/Fm injury thresholds

Fv/Fm_0, Fv/Fm_{50}, and Fv/Fm_{100} (the Fv/Fm value when no (0%), 50% and 100% of the tissue were injured) were calculated from the corresponding Fv/Fm readings at each injury level on each test date. The results showed that the three thresholds varied with different dormant periods, tissue positions, and years (Table 5).

Three Fv/Fm injury thresholds were all higher during CA than MW and DCA, while the latter two were not significantly different from each other. The result mentioned above were also demonstrated in this table, i.e. in the increasing order of the Fv/Fm threshold: 1A < 1M < 1B, and 1B was almost the same as 2A. It was also shown that as injury increased (from 0 to 100%), the Fv/Fm decreased almost in every category in the table.

Attention should be paid to the variation among years. Year 2000 had significantly higher readings than the previous two dormant seasons in all of the injury levels. One possible reason was the difference in placement of the greenhouses for the regrowth tests. As mentioned in the materials and methods, the greenhouse was poorly managed (no pesticide control and too often of the spray-every 5 min) in 1997-1999, when some rot was observed on the samples. Bruce (1999) also observed lower Fv/Fm on rotted samples. This may indicate the necessity of doing one set of experiments in each dormant

period each year under the same condition.

5. Comparison among different grape cultivars

Basal control samples of four cultivars (V. *lubruscana* and V. hybrid) from HTRC showed significant differences in all CF parameters (Table 6). Among these, Concord (V. *lubruscana*) had the highest Fo, Fm, and Fv, but medium Fv/Fm; Delaware (V. *lubruscana*) had the lowest Fm and Fv/Fm.

Three vinifera cultivars from SWMREC did not show any significant difference in Fm, Fv, nor in Fv/Fm; however, Fo was significantly different from each other in early spring control samples (data not shown). In decreasing order of Fo, they were: Riesling, Pinot Noir, and Chardonnay. This order is in agreement with field observation of spring frost resistance in Michigan (Howell et al., 1998). This may indicate that Fo can be used as a better indicator than Fv/Fm under some circumstances.

6. Comparison between two fluorometers

Commonly used fluorometers can be divided into two categories: pulse modulated (e.g. OptiScience) and non-pulse modulated (e.g. Plant Efficiency Analyser, Hansatech). Pulse-modulated OptiScience has an advantage that no dark acclimation is needed in both indoor and outdoor conditions. Therefore, it has been used widely in recent years (Binder et al., 1997; Buwalda and Noga, 1994; Scarascia-Mugnozza et al., 1996; Song et al., 1997; Tijskens et al., 1994). Our results showed that pulse-modulated measurement had lower Fo, Fm and Fv, but significantly higher Fv/Fm of 0.03 than those of Hansatech on paired samples. Our research showed that no dark acclimation was needed for Hansatech when measurement was conducted indoors (Jiang, et al., 1999), but was needed in the field. The pulse-modulated OptiScience may have an advantage in the field; however, the

larger diameter (~ 1cm) of the end of the fiber probe (where samples were enclosed to be measured) may not be suitable for measuring thin and round shoot/cane tissues.

7. Chlorophyll fluorescence field test

Field CF measurement was conducted during CA in 1999. Within 1 min of pouring liquid nitrogen onto the tissue surface, surface temperature dropped from physiological temperature to –25 to -30°C. Results showed that dark acclimation (15 min) is needed on both exposed and shaded canes for each measurement, and artificially freeze-injured tissues had significantly lower Fm and Fv/Fm, which was below the threshold of Fv/Fm_{100} (less than 0.48). Injury could be detected in 5 hr after the injury occurred (no significant difference from that of measurement in 51 hr). The occurrence of the injury needs was not detected minutes after the artificial freezing.

SUMMARY

Chlorophyll fluorescence (Fv/Fm) is an objective, sensitive, and reliable method of assessing woody tissue viability during the whole dormancy. It correlates with results from conventional viability tests, and therefore can be used as a predictor to evaluate tissue injury and cold hardiness after a controlled freeze-stress. However, Fv/Fm may not be a good predictor to evaluate cold hardiness among grape cultivars using only field control samples (Table 6). Stressed-controlled samples of different cultivars and other CF parameters (e.g. Fo) should be tested as well.

The Fv/Fm responses and thresholds vary with portions of the dormant season and types/positions of woody tissue evaluated. In another words, different thresholds should be used to evaluate the viability of the tissues in different dormant periods. Field

application is possible at least under extreme freeze-stress conditions (liquid nitrogen freezing).

It is recommended to run at least one test in each portion of the dormant season to quantify the thresholds for each species of interest using the same florometer, although it is possible to use the same thresholds over the years given the same environmental conditions.

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Table 1. Effects of controlled freeze-stress temperatures, cane and wood positions, and measuring times after freeze-stress on Fv/Fm of Concord grapevines during 1997-1998 dormancy[z]

Fv/Fm	Cold acclimation	Midwinter	Deacclimation
Temperature (°C)			
3 (control)	0.71 AB	0.60 A	0.54 A
-12	0.79 A	n. a.[y]	0.56 A
-15	0.70 AB	0.59 A	0.46 BC
-18	0.69 B	0.59 A	0.48 BC
-21	0.66 B	0.60 A	0.41 CD
-24	0.63 BC	0.58 A	0.37 DE
-27	0.56 CD	0.56 A	0.32 E
-30	0.50 DE	0.47 B	0.19 F
-33	0.44 E	0.43 BC	0.11 FG
-36	0.35 F	0.38 C	0.07 GH
-39	0.34 F	0.26 D	0.02 H
Pr>F	0.0001	0.0001	0.0001
Tissue position[x]			
1A	0.52 D	0.36 D	0.24 E
1M	0.55 C	0.43 C	0.32 D
1B	0.58 B	0.45 B	0.34 C
2A	0.62 A	0.47 A	0.37 B
2B	0.62 A	0.46 AB	0.41 A
Pr>F	0.0001	0.0001	0.0001
Measuring time			
24-30 hr	0.58	0.42	0.36
5-7 d	0.57	0.43	0.34
4 wk	0.58	0.47	0.31
Pr>F	0.9569	0.1444	0.8643

[z] Dormancy was separated as cold acclimation (Sept-Dec.), midwinter (Dec-Feb), and deacclimation (Feb-Apr).

[y] n.a. data not available.

[x] 1A, 1M, 1B, 2A, and 2B refer to 1-year-old apical, middle, basal and 2-year-old apical and basal wood.

Multiple mean separations were conducted by Fisher's LSD using SAS/STAT software (v8.0). The same letter following values indicates that respective values are not significantly different within columns.

Table 2. Total chlorophyll content (mg. L^{-1}) of Concord canes and wood on Apr.3 and Apr. 11, 1999.

Effects	Chlorophyll content (mg. L^{-1})
Tissue viability[Z]	
Control (1B)	5.06
Injured (1B)	4.32
Pr>F	0.0001
Tissue position[y]	
1B	6.65 A
1M	5.17 B
2M	4.46 C
Pr>F	<0.0001
Incubation (Extraction) time	
2 hr	3.77 D
4 hr	5.48 C
6 hr	5.95 B
8 hr	6.51 A
Pr>F	<0.0001

[Z] Control refer to samples that were not exposed to freeze-stress, injured samples were almost 100% injured.

[y] 1B, 1M, and 2M refer to 1-year-old basal, middle, and 2-year-old middle wood.

Multiple mean separations were conducted by Fisher's LSD using SAS/STAT software (v8.0). The same letter following values indicates that respective values are not significantly different within columns.

Table 3. Effects of controlled freeze-stress temperatures, cane and wood positions, and measuring times after freeze-stress on Fv/Fm of Concord grapevines during 1998-1999 dormancy[z]

Fv/Fm	Cold acclimation	Midwinter	Deacclimation
Temperature (°C)			
3 (control)	0.77 AB	0.58 A	0.68 A
-9	0.79 A	0.57 A	0.67 A
-12	0.82 A	0.56 AB	0.66 A
-15	0.70 B	0.59 A	0.53 B
-18	0.59 C	0.55 B	0.48 B
-21	0.51 D	0.54 B	0.41 C
-24	0.44 E	0.47 C	0.38 C
-27	0.37 F	0.43 CD	0.25 D
-30	0.37 F	0.39 D	0.21 DE
-33	0.32 G	0.33 DE	0.15 E
-36	0.19 H	0.31 E	n.a.[y]
Pr>F	<0.0001	<0.0001	<0.0001
Tissue position[x]			
1A	0.47 C	0.32 C	0.40 C
1M	0.51 A	0.40 B	0.47 B
1B	0.52 A	0.43 A	0.51 A
2A	0.49 B	0.42 A	0.52 A
Pr>F	<0.0001	<0.0001	<0.0001
Measuring time			
24-30 hr	0.53	0.39	0.44 B
5-7 d	0.50	0.37	0.46 B
4 wk	0.49	0.42	0.52 A
Pr>F	0.0555	0.2281	0.0001

[z] Dormancy was separated as cold acclimation (Sept-Dec.), midwinter (Dec-Jan), and deacclimation (Feb-Apr).

[y] n.a. data not available.

[x] 1A, 1M, 1B, 2A refer to 1-year-old apical, middle, basal and 2-year-old apical wood.

Multiple mean separations were conducted by Fisher's LSD using SAS/STAT software (v8.0). The same letter following values indicates that respective values are not significantly different within columns.

Table 4. Effects of controlled freeze-stress temperatures, cane and wood positions, and measuring times after freeze-stress on Fv/Fm of Concord grapevines from Jan-Apr, 2000[z]

Fv/Fm	Midwinter	Deacclimation
Temperature (°C)		
3 (control)	0.52 A	0.58 A
-9	n.a.[y]	0.58 A
-12	n.a.	0.59 A
-15	n.a.	0.54 B
-18	n.a.	0.42 C
-21	n.a.	0.32 D
-24	0.52 A	n.a.
-27	0.51 A	n.a.
-30	0.52 A	n.a.
-33	0.46 B	n.a.
-36	0.42 C	n.a.
-39	0.31 D	n.a.
Pr>F	<0.0001	<0.0001
Tissue position[x]		
1A	0.32 C	0.44 C
1M	0.41 B	0.49 B
1B	0.52 A	0.59 A
Pr>F	<0.0001	<0.0001
Measuring time		
24-30 hr	0.32 B	0.43 B
5-7 d	n.a.	0.46 B
4 wk	0.53 A	0.63 A
Pr>F	0.0191	0.0020

[z] Dormancy was separated as midwinter (Jan-Feb) and deacclimation (Mar-Apr).

[y] n.a. data not available.

[x] 1A, 1M, and 1B refer to 1-year-old apical, middle, and basal canes.

Multiple mean separations were conducted by Fisher's LSD using SAS/STAT software (v8.0). The same letter following values indicates that respective values are not significantly different within columns.

Table 5. Comparison of Concord woody tissue Fv/Fm readings at 0, 50% 100% injury ratings (Fv/Fm_0, Fv/Fm_{50}, and Fv/Fm_{100}, respectively) during cold acclimation (CA), midwinter (MW), and deacclimation (DCA) from 1997-2000.

Main effects	Fv/Fm_0	Fv/Fm_{50}	Fv/Fm_{100}	*Pr*>F
Dormant period				
CA	0.75 A	0.68 A	0.48 A	<0.0001
	A	B	C	
Midwinter	0.67 B	0.62 B	0.45 B	<0.0001
	A	B	C	
DCA	0.66 B	0.62 B	0.45 B	<0.0001
	A	B	C	
Pr>F	<0.0001	<0.0001	<0.0001	
Tissue				
1A	0.63 C	0.57 C	0.43 B	<0.0001
	A	B	C	
1M	0.68 B	0.63 B	0.47 A	<0.0001
	A	B	C	
1B	0.73 A	0.68 A	0.49 A	<0.0001
	A	B	C	
2A	0.70 AB	0.66 AB	0.43 B	<0.0001
	A	A	B	
Pr>F	<0.0001	<0.0001	0.0063	
Year				
1997-98	0.64 B	0.63 AB	0.43 B	<0.0001
	A	A	B	
1998-99	0.70 A	0.60 B	0.42 B	<0.0001
	A	B	C	
2000	0.71 A	0.65 A	0.54 A	<0.0001
	A	B	C	
Pr>F	<0.0001	<0.0001	<0.0001	

Multiple mean separations were conducted by Fisher's LSD using SAS/STAT software (v8.0). Letters following values indicate significance within columns and letters below values indicate significant within rows. The same letter within a row and column indicates that respective values are not significantly different. Interactions were ignored.

1A, 1M, 1B, and 2A refer to 1-year-old apical, middle, basal and 2-year-old apical wood.

Table 6. Comparisons of chlorophyll fluorescence among four cultivars on 11/19/1998. Thirty control samples were used for each cultivar. Plant Efficiency Analyser measurement was conducted as described in materials and methods.

Cultivar	Fo	Fm	Fv	Fv/Fm
Concord	459 A	1988 A	1529 A	0.764 BC
Delaware	423 B	1683 C	1260 B	0.742 C
Frontenac	364 C	1747 BC	1383 AB	0.778 B
Marechal Foch	336 D	1872 AB	1537 A	0.819 A
Pr	<0.0001	0.0016	0.0029	<0.0001

Multiple mean separations were conducted by Fisher's LSD using SAS/STAT software (v8.0). The same letter following values indicates that respective values are not significantly different within columns.

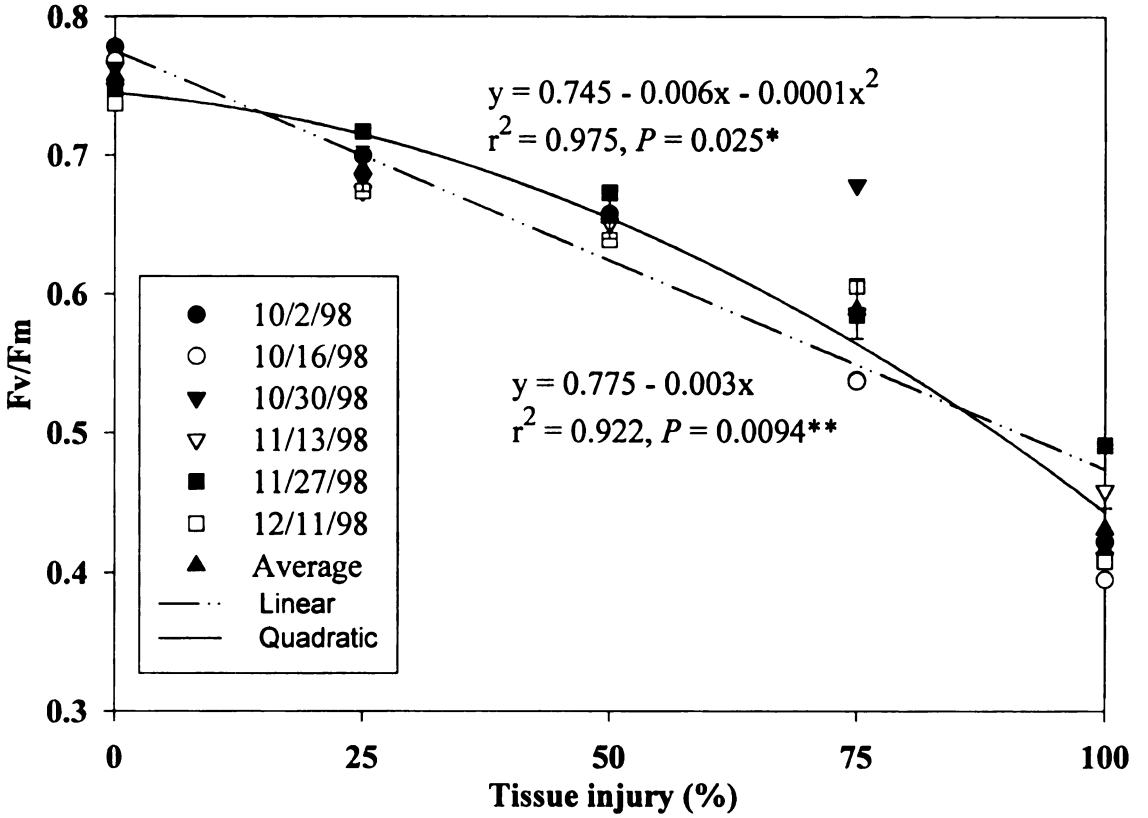

Figure 1. The relationship between Fv/Fm and tissue injury (%) during cold acclimation. Data presented are the average of the three portions of 1-year -old canes tested from Oct 2 to Dec 11, 1998. Fv/Fm was regressed with the average of the data at each injury category. The regression analysis was conducted by analysis of variance in Sigma Plot (4.0). Error bars are standard errors for the mean data.

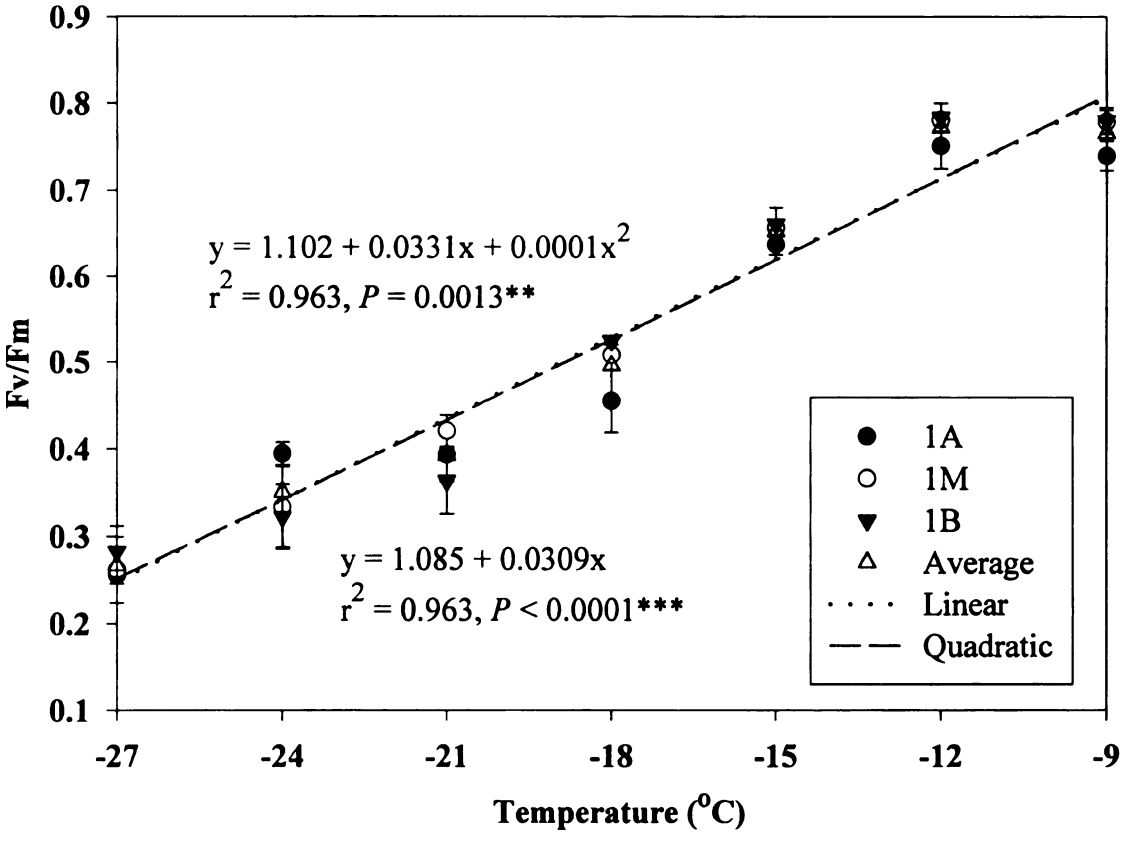

Figure 2. The relationship between Fv/Fm and controlled freeze-stress temperatures of 1-year-old canes on 2 Oct, 1998. 1A, 1M, and 1B refer to 1-year-old apical, middle, and basal canes, respectively. Data presented were the average of the measurements from three measuring times (27 hr, 7 d and 4 wk). Error bars are standard errors of the mean. Fv/Fm was regressed with the mean of each point. **, *** significant at $P = 0.01, 0.001$, respectively.

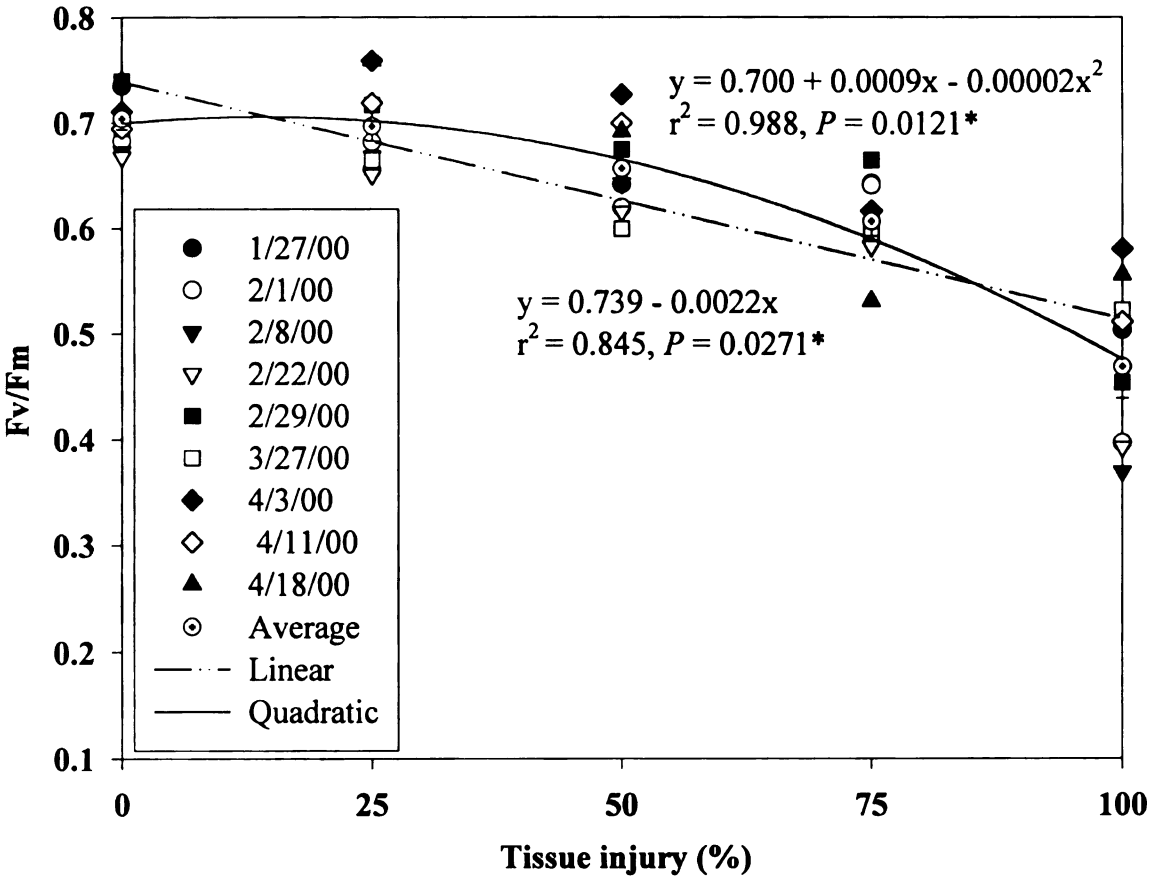

Figure 3. The relationship between Fv/Fm and tissue injury (%) after mid-winter. Data presented are the average of the three portions of 1-year-old canes from regrowth test during Jan 27 to Apr 18, 2000. Fv/Fm was regressed with the average of the data at each injury category. Error bars are standard errors of the average. * significant at $P = 0.05$.

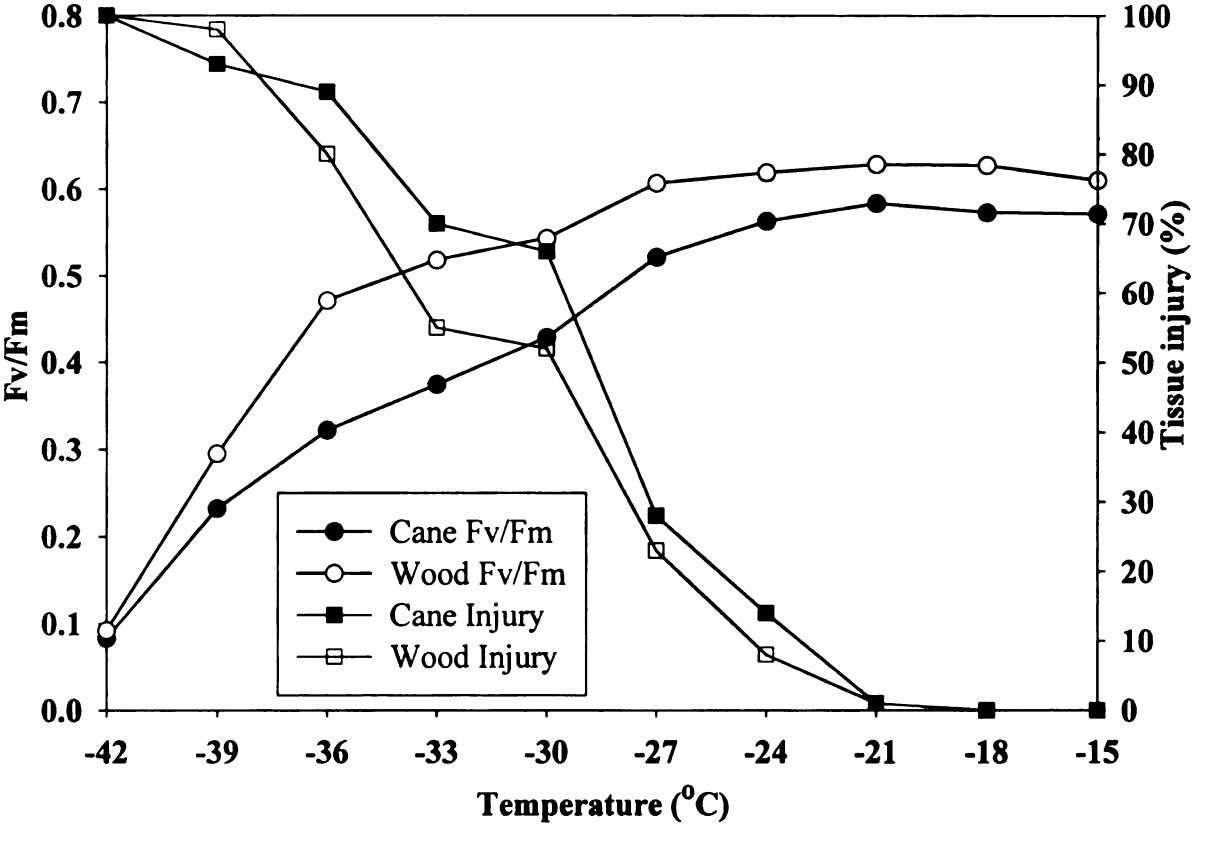

Figure 4. The relationships among Fv/Fm, injury level, and stress temperature of 1-year-old canes and 2-year-old wood of Concord grapevines during deacclimation in 1997.

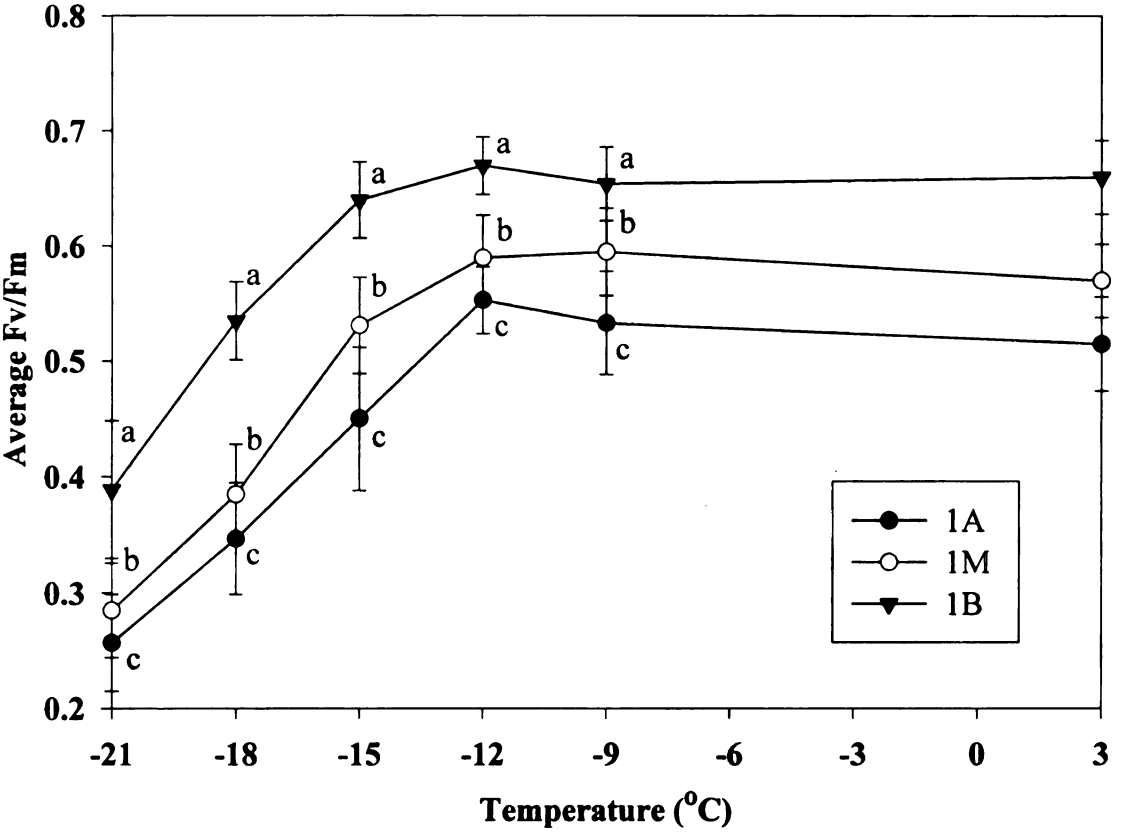

Figure 5. Average Fv/Fm of 1A, 1M and 1B after exposure to controlled freeze-stress during deacclimation in 2000. Data presented were the average of three measurement times (26 hr, 5-7 d, and 4 wk) on three dates (3/27/00, 4/3/00, and 4/11/00); each time there were five replicates of each portion. Error bars are standard errors. Letters next to each point indicate significant difference (P = 0.0001) in Fv/Fm of 1A, 1M, and 1B at each stress temperature by LSD test.

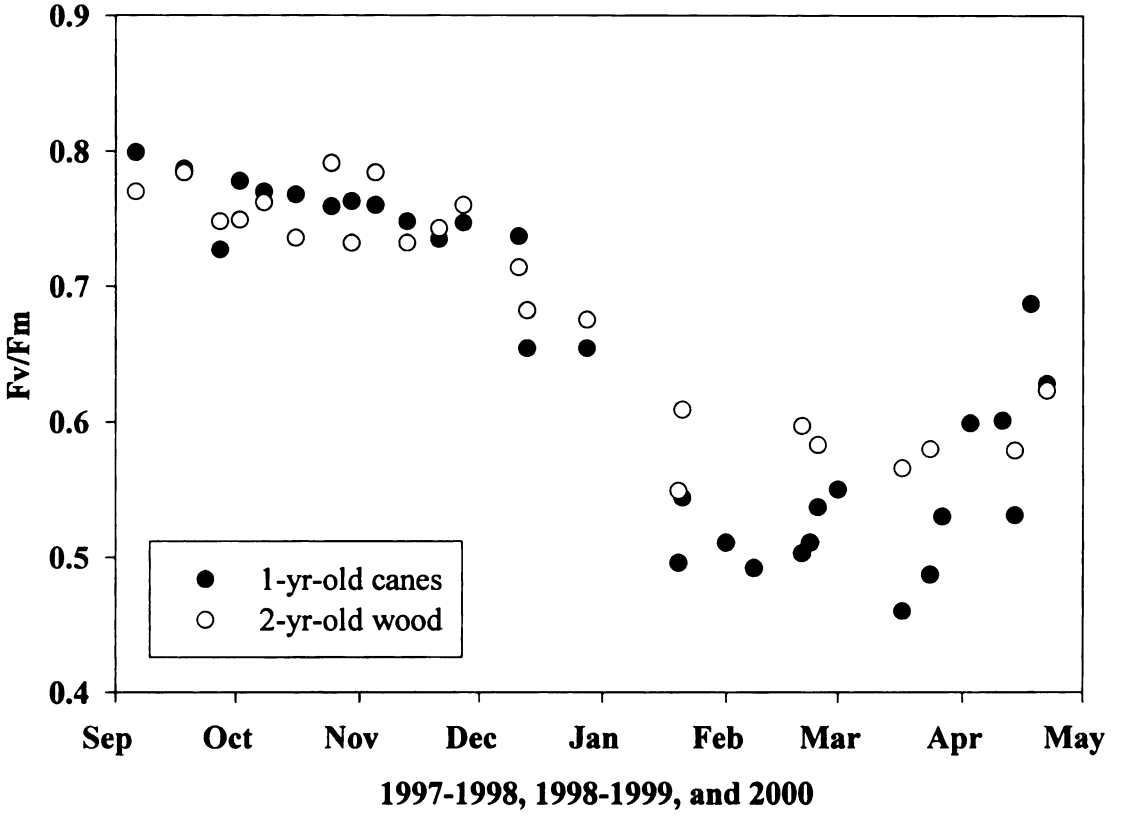

Figure 6. Seasonal changes of Fv/Fm (control samples) during dormancy of 1997-1998, 1998-1999, and 2000.

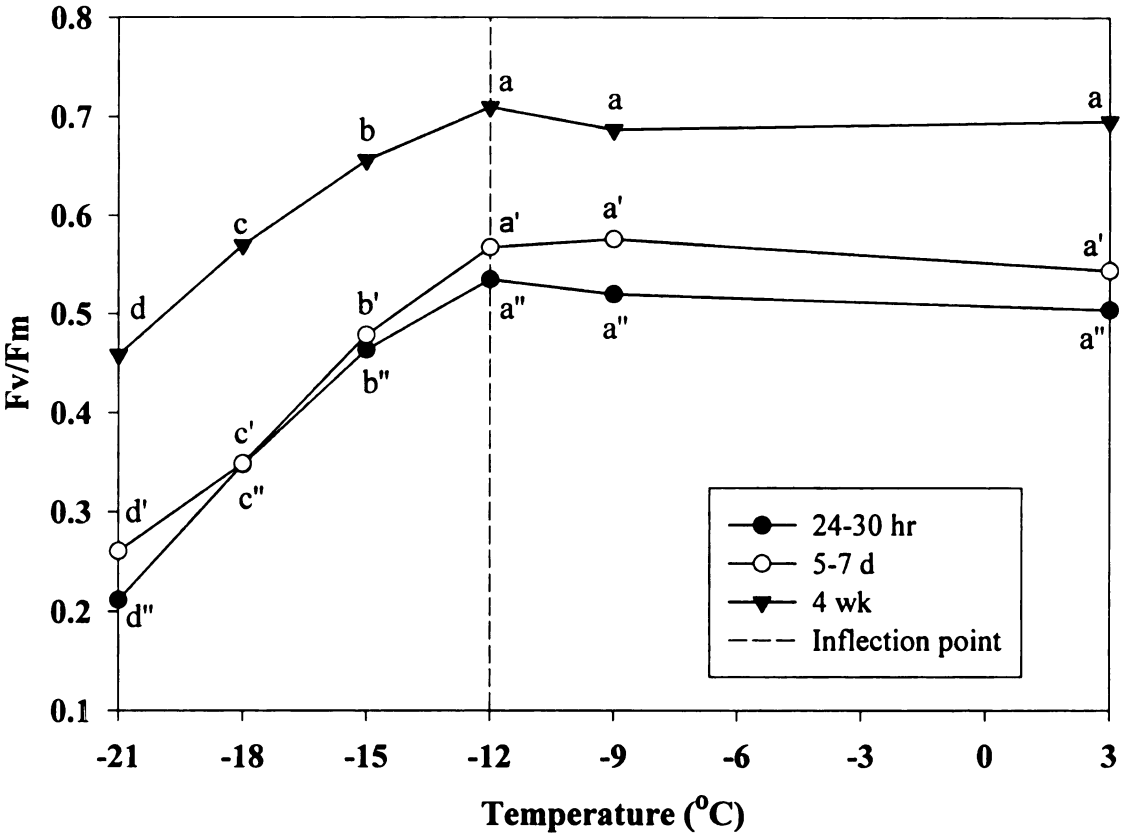

Figure 7. Fv/Fm comparison of three measuring times of 1-year-old canes before and after exposure to controlled freeze stress during DCA, 2000. Five replicates were used for each measurement. Letters next to each point indicate significant difference (P = 0.05) in Fv/Fm within each measuring time by LSD test.

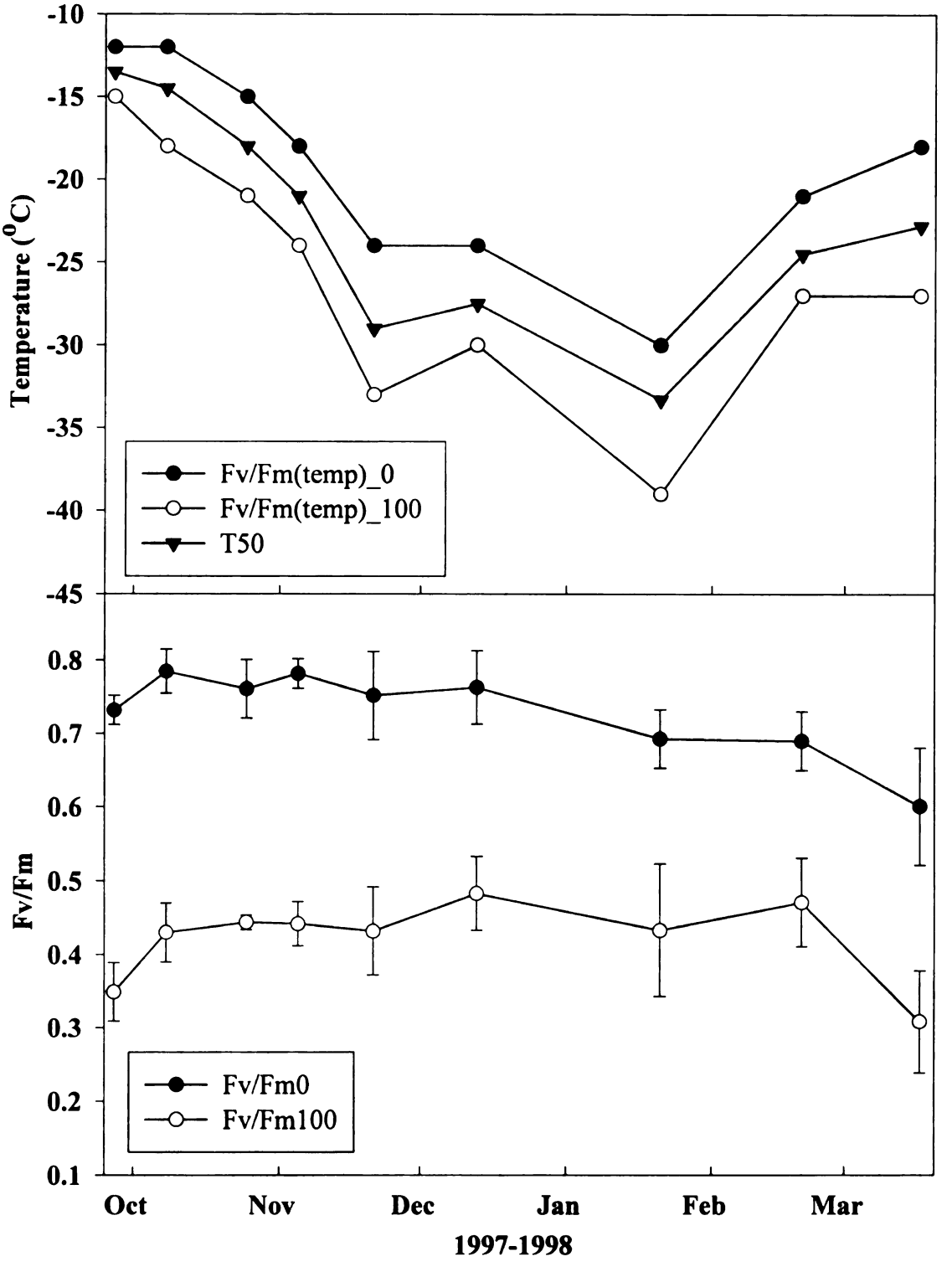

Figure 8. Comparison among T_{50}, Fv/Fm(temp)_0 and Fv/Fm(temp)_100 of 1B during 1997-1998 from regrowth test (Fv/Fm(temp)_0 and Fv/Fm(temp)_100 refer to the temperature at which no (0%) and 100% injury. Fv/Fm_0 and Fv/Fm_{100} are Fv/Fm values of corresponding Fv/Fm(temp)_0 and Fv/Fm(temp)_100). Error bars are standard errors.

SUMMARY

Cold hardiness of grapevines is complex and economically significant. One aspect of cold hardiness that limits our understanding of the process in woody species like grapevines is the lack of a rapid, objective viability test for woody tissues (canes, cordons, and trunks). The economics of crop protection and cultivar choice are problematic as it is impossible to accurately predict bud and wood hardiness status. Both of these topics have been addressed in this dissertation.

In the final paragraph of the literature review, a number of questions related to the above topics were posed. Those questions and the answers provided by the dissertation are as follows.

In Chapter I, the key question was whether it is possible to predict dynamic changes in cold hardiness with readily available data, i.e. ambient air temperatures and tissue water content. Questions addressed were: 1) what are the seasonal changes of cold hardiness and tissue water content of canes and buds during the whole dormant season? 2) what cane structure (wood, pith, periderm) contributes most to overall water changes during dormancy? 3) what are the influences of ambient air temperatures and water content on cold hardiness of canes and buds? 4) which of these two is more significant in influencing seasonal cold hardiness? Results from 1998-1999 and 2000 showed that:

1) Canes and buds of Concord grapevines began cold acclimation in early September, reached the maximum hardiness level in February, and deacclimated dramatically after late February. Similarly, the water content of canes and buds decreased from fall to midwinter and then started increasing after late February.

2) Among the varying expressions of air temperature evaluated, the averaged minimum temperatures of the 2 d before each sampling date were most significantly correlated with T_{50} of canes and buds during cold acclimation, while the minimum air temperature on the day before each sampling date was most significantly correlated with T_{50} of canes and buds during deacclimation; correlation coefficient during midwinter was not significant or less than that during CA and DCA.

3) Cane water content was most significantly correlated to air temperatures of 7 d before each sampling date during cold acclimation, and averaged maximum air temperatures 4 and 5 d during deacclimation, while no significant factor during midwinter. Bud water content was correlated with mean temperatures the day before and 7 d before each sampling date during cold acclimation and midwinter, while none significant correlation during DCA.

4) The total water content of the cane was significantly correlated to its wood water content, while the water content of periderm and pith of the cane was not.

5) Multiple linear regression analysis showed that the cane water content had more significant correlation with the T_{50} of canes and buds than any air temperature tested during the whole dormant season.

The primary questions addressed in Chapters II and III concerned methods to viability evaluation, i.e. whether and how CF might be used as an objective and effective viability test for freeze-stressed woody vegetative tissues. In order to accomplish this goal, a series of questions were addressed. 1) Can CF be used to measure the viability of freeze-stressed woody vegetative tissues? If so, what is the appropriate protocol for CF measurement? 2) Once a protocol was established, how could CF measurement be used

to quantify tissue viability? How is CF related to conventional viability assessment for vegetative tissue cold hardiness (browning and/or regrowth tests)? 3) How quickly can a CF based viability assessment be obtained after a controlled freeze-stress? How does this compare with commonly used browning or regrowth tests (5-7 d or 4 wk)? 4) Can CF measurement demonstrate the hardiness difference among varying positions on the canes and 2-year-old wood? 1-year-old vs. 2-year-old, apical vs. basal? 5) Are CF values consistent across the whole dormant season? If not, what are the differences? and how should we modify the use of CF during different periods of the dormant season? 6) Can CF be used to evaluate cold hardiness among different species or cultivars? 7) Can CF be used directly in the field to assess *in situ* cold injury? 8) Do different CF florometers require changes in the viability assessment protocol or simply different threshold?

Data from the last three dormant seasons leads to the following conclusions:

1) Valid chlorophyll fluorescence measurements can be obtained from the cane surface with periderm removed, under ~ 2700 μmol m^{-2} s^{-1} and 10 min dark adaptation. For large tissue samples, dark adaptation was not necessary if only Fv/Fm was of interest.

2) The Fv/Fm was positively correlated with freeze-stress temperatures, and negatively correlated with tissue injury. So, it can be used as an indicator of viability.

3) The Fv/Fm can separate hardiness differences among different positions on the canes and wood (basal tissues are more hardy than apical tissues, and 2-year-old tissues are hardier than 1-year-old tissues, generally).

4) The Fv/Fm could detect woody tissue viability in 24-30 hr post-stress during CA; much faster than the conventional methods. However, during midwinter and deacclimation, results from the 24-30 hr detection produced significantly lower values

than that from the regrowth test. There was no difference between 24-30 hr and the conventional tissue-browning test.

5) The Fv/Fm temperature inflection point was positively correlated with T_{50} calculated from the regrowth test.

6) The Fv/Fm response magnitude varied with each portion of the dormant season. It was generally higher during CA, and lower during MW and DCA.

7) The Fv/Fm injury thresholds Fv/Fm_0, Fv/Fm_{50}, and Fv/Fm_{100} (the Fv/Fm values when no, 50% and 100% of the tissue were injured, respectively) are proposed for different woody tissues at three basic dormant periods.

8) Preliminary evaluations of different grape cultivars, two fluorometers, and field measurement were promising. However, complete experiments must be conducted before the final conclusions can be made.

Finally, The Fv/Fm was demonstrated to be an objective and effective method of assessing woody tissue viability after a controlled freeze-stress.