DISSERTATION

PHYSIOLOGICAL RESPONSES OF GRAPEVINES TO ENVIRONMENTAL STRESSES

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 1997

COLORADO STATE UNIVERSITY

April 2, 1997

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY IMED EDDINE DAMI ENTITLED PHYSIOLOGICAL RESPONSES OF GRAPEVINES TO ENVIRONMENTAL STRESSES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PHYSIOLOGICAL RESPONSES OF GRAPEVINES TO ENVIRONMENTAL STRESSES

The field response of several grapevine (*Vitis vinifera* L) cultivars, grown at the Orchard Mesa Research Center, to methanol application on foliage and trunks was investigated. Sublethal methanol doses were determined as 90% for leaves and 100% for trunks. The application of these concentrations on grapevines during mid-summer did not affect sugar accumulation, photosynthesis, transpiration, stomatal resistance, yield components, nor bud cold hardiness as compared to untreated vines.

Cold hardiness and endogenous levels of soluble sugars were monitored during the dormant season for Chardonnay and Riesling dormant buds and stem cortical tissues. Endogenous levels of glucose, fructose, raffinose, stachyose, but not sucrose were strongly associated with cold hardening, increasing from the onset of cold acclimation in August to maximum cold hardiness in December and January. During dehardening in March and April, levels of these sugars dropped as temperature increased. A high ratio of glucose and fructose to sucrose coincided with maximum cold hardiness, and a low ratio was associated with the dehardened condition in fall and spring. Neither cold hardiness nor soluble sugars of grape tissues were influenced by late harvest compared to harvest at normal fruit maturity.

Various alginate- and sucrose-based cryoprotective treatments were tested under laboratory and field conditions for their capacity to increase freezing resistance by slowing deacclimation and delaying bud break of dormant grapevines. Early application of the treatments in mid-winter did not have a significant effect on cold hardiness of buds nor canes. However, over three years late winter and early spring field applications consistently increased freezing resistance of treated primary bud and cane tissues by up to 5C and 7C, respectively, as compared to untreated grapevines. Under laboratory conditions, the bud break of treated cuttings was delayed by at least 4 phenological stages when compared to the control. In the spring, similar treatments were applied on ecodormant grapevines. Visual evaluations, on a weekly basis, resulted in bud break delay of several days (up to 10 days) of the treated vines as compared to the control. At harvest, the yield components and fruit composition were not affected in treated vines. These findings appear promising to reduce injury in viticultural areas where spring frost is a threat.

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ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Cecil Stushnoff for the opportunity to work with him and benefit from his exceptional guidance, availability, excellent advice and suggestions on this work, and especially for his friendly attitude. Special thanks are also extended to Dr. Harrison Hughes for his continual academic support and encouragement throughout my graduate studies at CSU. I would also like to thank Dr. Danny Smith and Dr. Jack Morgan for being part of my graduate committee and for their constructive comments and suggestions on this work.

Rick Hamman, viticulturist of the state, deserves special recognition and thanks for his outstanding support during my work at OMRC; for giving me the chance to join his viticulture program; for answering my endless questions and for sharing his expertise on grape growing; and most importantly for the friendship that made my stay in Grand Junction enjoyable. Also, I thank the rest of the "crew" at OMRC, including Dr. H. Larson, Dr. M. Rogoyski, Joyce Sexton, Susan Baker, John Wilhelm and David Neil for being friendly and making my work go very smooth.

I am grateful to all faculty, headed by Dr. Steve Wallner, staff, and students of the Department of Horticulture whom I have interacted with the last few years for making me feel like a family member and for their assistance and friendship. I would also like to thank Dr. Ken Brink for his encouragement and support during the course of my study.

I am also grateful to the Tunisian Ministry of Education and Sciences for the fellowship award and the opportunity to study abroad and make friends from all over the world. My deepest fondness and gratitude go especially to my best friend, Cory Rasia Defenbaugh, for her continuous encouragement, exceptional support and motivation, great sense of humor, and most of all her unlimited love which gave me joy and confidence to persevere and complete this task despite occasional difficult times.

Most importantly, I cannot thank enough my family in Tunisia, especially my father Moncef, my mother Fatima, my brothers Samir, Faouzi, Sami, and my sisters Monia and Olfa, and my aunt Latifa for their patience, unconditional support and love throughout the course of my study abroad.

This dissertation is dedicated

in memory of my best friend

Moez Dahmani

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

In nature, plants are often exposed to several environmental stresses at any given time. Environmental stresses impact not only established crops but also the introduction of new crops into non-traditional areas. A significant problem for agriculture in the world is the major yearly variation in crop yields due to variations in environmental stresses such as flooding, drought, salinity, and high and low temperatures. To emphasize the importance of studying environmental stresses, it is well to note that the Science Priorities Committee of the American Society for Horticultural Science has listed environmental stress as the top priority for research. The committee concludes that environmental stresses represent the most limiting factors to agricultural productivity, causing actual average yields of crops to fall three to seven times below potential yields (Faust 1986). Environmental stresses and weed competition account for 90% of this shortfall, diseases for 6%, and insects for 4%. According to Boyer (1982), plants growing in natural environments are often prevented from expressing their full genetic potential for reproduction and are considered "stressed". He adds that the genetic potential for very high productivity is present in the crops of today, and productivity falls far short of the potential. Hence, improvements in plant productivity need not rest solely on increases in genetic potential but should also emphasize ways of bringing

productivity closer to the existing genetic potential.

Environmental stresses come in many forms, including low temperatures stress. Low temperature stresses are divided into chilling temperature (above 0C) and freezing temperature (below 0C). Plants of tropical and subtropical origins, such as banana and corn are usually stressed under chilling temperatures. However, plants of temperate origin, such as deciduous woody plants e.g. grapes have the ability to develop true freezing resistance. Nevertheless, injury could occur and freezing injury is a major cause of crop loss. Low temperature injury to perennial crops, whether from fall or spring frosts or mid-winter conditions, accounts for greater crop loss than all other environmental stresses combined (Flore and Howell 1987).

Grape is the world's biggest and most widespread deciduous fruit crop. According to the U.S. Department of Commerce, world vineyard acreage accounts for about 20.3 million acres in 1994, with a production of 61.6 million tons, which exceeds that of any other fruit crop (Wines and Vines 1996). Therefore, severe economic losses may result from freeze injury to grapevines especially in northern areas. The dormant bud is usually the most susceptible part of the grapevine, frequently exhibiting injury while other tissues of the vine survive.

MECHANISMS OF FREEZING INJURY

Introduction

For decades, the study of plant cold hardiness and freezing stress injury has had two primary goals. The first is to describe the mechanisms during a freeze-thaw cycle that leads

to cell injury and death (Burke et al 1976, Steponkus 1984). Researchers believe that once the mechanisms of injury are understood, the second goal may be achieved and it should be a relatively straightforward matter to determine the cellular and biochemical changes necessary for conferring a higher level of freezing tolerance. This approach is supported by the fact that some plants are hardier than others, and that in certain tissues of many plants cold hardiness changes seasonally. However, the mechanisms involved in freezing injury of plants have turned out to be exceedingly complex (Levitt 1980, Sakai and Larcher 1987). Nevertheless, researchers have developed a general understanding of the basic elements of the formation of ice in plant tissues (Levitt 1980) and identified the major probable site of lethal injury (Palta and Weiss 1993, Steponkus 1984). Hence, the second and parallel goal, to catalog and understand the biochemical and physiological changes occurring during cold acclimation, has been viewed as a viable alternative approach.

Factors contributing to freezing injury

While some species are always killed at the moment they freeze, others can tolerate extremely low temperatures of liquid nitrogen (-196C)(Sakai and Larcher 1987). The low temperature responses of most plants fall between these extremes, and freezing resistance may change markedly with season and stage of development (Burke et al 1976). The mechanisms of freezing damage have been studied for a long time and different hypotheses and theories have been proposed (Levitt 1980). Levitt (1980) reported that early workers believed the principal cause of freezing injury was physical damage caused by ice crystals. The volume increase associated with the freezing of water was thought to rupture plant cells, thus destroying their cellular structure. This early explanation was rejected when it was observed

that cells were collapsed rather than expanded during freezing and did not rupture while in a frozen state. Semi-permeable membranes permit compartmentalization of ice allowing extracellular freezing, leading to cell collapse as water moves to extracellular ice masses.

To understand freezing injury, it is important to consider both freezing and thawing since it is really the plant condition after a freeze-thaw cycle that is relevant. Furthermore, in freezing injury the secondary stress caused by dehydration upon freezing of extracellular water might be of greater significance than the low temperature per se. Several factors, including ice nucleation temperature, freezing (cooling) rate, duration of exposure to ice, thawing rate, and post thaw conditions, contribute to the degree of injury from a given freeze-thaw event.

A. Ice nucleation

Ice nucleation temperature depends largely on the prevalence of ice nucleators such as dust, ice nucleating active bacteria, wind, and agitation (Levitt 1980, Lindow et al 1982). Presence of ice nucleating substances within the tissue has been also reported (Kaku 1973). In addition, the size of the tissue has a significant influence on the nucleation temperature, i.e. the larger the tissue the warmer the ice nucleation temperature (Anderson and Ashworth 1985). Duration of the freezing temperature also influences the ice nucleation temperature. The longer the duration, the warmer the temperature at which nucleation would occur.

Nucleation temperature can greatly influence plant survival from a frost episode. Ice nucleation at a warm temperature, e.g. -1C or -2C, allows slow ice growth in extracellular space, so that plant is able to survive by tolerance of ice. However, as the nucleation temperature is lowered, the chance of ice formation in the intracellular space increases and

the ice growth is relatively rapid. Intracellular ice is always lethal to the cell (Levitt 1980, Sakai and Larcher 1987).

B. Cooling rates

During typical spring and fall frosts, air temperatures usually drop at the rate of 1C to 2C/h (Levitt 1980). This suggests that experimental tests, conducted to stimulate the impact of freeze-thaw stress on plants, should use a realistic freezing process, i.e. cooling rates of 1C to 2C/h. However, several studies investigating the mechanism of freezing injury have utilized very fast cooling rates (above 10C/h) (Dowgert and Steponkus 1984, Krause et al 1982). Rapid cooling rates allow for many samples to be tested in succession, but high rates are not typically found in nature and can lead to erroneous results. Studies conducted by Steffen et al (1989) have shown that a slight increase of cooling rate from 1C to 2.9C/h meant the difference between cell survival and death in herbaceous plants. Furthermore, it has been demonstrated that slower freezing rates facilitate the redistribution of water in buds and stems of woody plants so that freezing occurs outside of particularly vulnerable tissues (Ashworth and Davis 1987, Pierquet and Stushnoff 1980).

Slow cooling results in ice initiation in the extracellular water. This is because extracellular water has a lower solute concentration than the intracellular water and because ice nucleators such as dust and bacteria are prevalent in the extracellular environment (Levitt 1980, Sakai and Larcher 1987). As the air temperature drops further, ice grows in the extracellular space, withdrawing water from the cell and resulting in collapse of the cell walls (Palta 1990). Water moves from the inside to the outside of the cell because the vapor pressure of extracellular ice is lower than the vapor pressure of vacuolar and cytoplasmic

water at the same temperature. This behavior of the cell during freezing stress is well documented (Levitt 1980, Sakai and Larcher 1987). Recent direct microscopic examination of frozen tissues supports this conclusion (Palta and Weiss 1993, Pearce 1988). These studies confirm that there is no separation of cell membranes from the cell wall during extracellular freezing stress.

C. Thawing rates

Thaw rate can have an impact on the degree of injury (Levitt 1980). The speed of rehydration is directly influenced by the thaw rates. If the ice melts relatively slowly, the water will go back into the cell at a slower speed. As ice melts and water enters the cell, the cell wall expands. In order to maintain viability, the membrane and cytoplasm may not move rapidly enough as the cell wall expands. Such rapid movement tear the membrane from the cell wall. This condition could give rise to the well described "frost plasmolysis" as a microscopic symptom of cell death in early studies (Levitt 1980). Levitt (1980) has described a narrow freezing temperature range, or "critical zone", in which plants are sensitive to freezing and thawing rates.

D. Secondary freeze-induced stresses

A common feature among several environmental stresses is dehydration. The process of dehydration and stresses that result from it are similar for both water and extracellular freezing stresses (Palta 1990). However, for osmotic stress, these dehydration and accompanying stresses are very different. Therefore, the use of protoplasts by several studies as a model to investigate the mechanism of freezing injury could be erroneous (Dowgert and Steponkus 1984, Steponkus 1984, Steponkus and Weist 1978). The freezing stress is

essentially an osmotic stress in these systems. The use of protoplasts eliminates the role of cell wall in freezing stress. This is an important consideration because freezing stress imposes additional mechanical stress due to the collapse of the cell wall that is absent in osmotic stress. Tao et al (1983) have reported a large difference in the freezing tolerance of intact cells (LT_{50} = -12C) and their protoplasts (LT_{50} = -21.5C).

Freeze injury via mechanical stress has been proposed by Iljin since 1933 (Levitt 1980); however, it has not been widely accepted probably because of the difficulty in distinguishing between freeze-induced dehydration stress and mechanical stress. During extracellular freezing, ice begins to form on the outer surface of the cell wall (Levitt 1980). As the temperature drops further, the ice grows and at the same time the protoplasm becomes dehydrated and the cell becomes deformed. Ice exerts increasing pressure on deformed cells and protoplasts as it continues to grow, and cell collapse results from non-uniform mechanical stresses imposed by extracellular ice (Tao and Li 1986). The adhesion between the cell wall and the plasma membrane and the sliding movement along the interface between the cell wall and the plasma membrane may also cause mechanical injury during freeze-thaw cycles. Since freeze-induced dehydration, excess salting concentration, and mechanical stress develop concomitantly during freezing, it is extremely difficult to distinguish their differential effects on cell injury. Nevertheless, Tao et al (1983) were able to demonstrate that at equal osmolality, cultured potato cells were killed at about -3C in PEG 6000 solution, while cells in PEG 1000 solution survived down to -8C. They attributed the death of cells in PEG 6000 solution to mechanical rather than osmotic stress.

Another source of freezing injury observed in woody plants is called cavitation. During freezing, cells are exposed to a large dehydrating force (George and Burke 1977), and cell wall rigidity may prevent loss of intracellular water to extracellular ice. The rigid cell walls, however, can prevent dehydration only as long as cohesion of cell water and its adhesion to the cell components are not exceeded by the dehydrative force (Weiser and Wallner 1988). When limiting tensions are exceeded, cavitation may occur, resulting in migration of cellular water to extracellular ice and expansion of gas bubbles (Tyree and Dixon 1986). Cell injury manifested by fragmented protoplasm has been observed following cavitation (Ristic and Ashworth 1993), which is measured as emission of ultrasonic acoustic sounds (Weiser and Wallner 1988).

Membrane as a site of injury

Biomembranes have been the main targets of freezing stress and the plasma membrane has attracted most interest. Steponkus et al (1990) have stressed the importance of specific lipid components in the plasma membrane that may prevent or reduce injury during freezing. Possible causes of membrane injury during freezing could be the loss of semipermeability, loss of active ion transport, degradation of phospholipids, redistribution of proteins due to lateral displacement, irreversible endocytotic vesiculation of the plasma membrane, and a dehydration-induced phase transition in biological membranes (Burke et al 1976, Levitt 1980, Sakai and Larcher 1987, Steponkus et al 1990). Nevertheless, the nature of freeze-induced injury to the plasma membrane is still open to debate.

A. Ion leakage

Cell membrane was recognized over 80 years ago as a site of freeze-thaw injury. However, the nature of injury at a more fundamental level has been investigated only recently. This has been due in part to the assumption that freezing injury, as assessed by solute leakage, results in a complete loss of membrane semipermeability or membrane rupture. Studies by Palta and Li (1978, 1980) have shown that injured yet living cells exhibit enhanced ion leakage and water-soaked appearance following a freeze-thaw event. The major cation that leaks out of the cell has been found to be K^+ whose transport is coupled to the activity of membrane pumps, H^+-ATPase (Sze 1985). Palta and Weiss (1993) conclude that increased ion leakage from the cells following freeze-thaw injury does not necessarily result from membrane rupture, but rather a specific alteration in the transport properties of the cell membranes. They add that plasma membrane ATPase is a sensitive and early site of injury by freeze-thaw stress and that its regulation and activity are controlled by the intracellular concentration of Ca^{2+} (which acts as a "second messenger") and/or by change in the membrane lipid composition.

B. Membrane lipids

A change in the physical state of membrane lipids from a flexible liquid crystalline to a solid gel structure has been proposed as one of the primary responses leading to low temperature-induced injury. This hypothesis was first proposed to explain chilling injury in chilling-sensitive plants and has been now extended to explain freezing injury (Lyons et al 1979). It is believed that if significant areas of the membrane are in the gel state, then membrane function can be significantly impaired. Membrane-bound enzymes clearly could

be impacted from the transition of membrane lipids to the gel state. Electron microscopic observations of freeze-fractured membrane preparations suggest that following phase transition, proteins are squeezed out of the gel-crystalline domains, resulting in protein-free areas in the membrane (Gurr and Harwood 1991). Such displacement could cause changes in functions of membrane proteins. Membrane lipids may also directly modulate the activity and functions of the membrane proteins. Features of lipids that can alter membrane protein functions include lipid head groups, lipid acyl chain length, lipid backbone, and lipid fluidity (Palta and Weiss 1993). In addition, a large body of evidence has been reviewed by Burke et al (1976) to demonstrate protein denaturation following a freeze-thaw cycle. Protein denaturation is expressed as loss of solubility and protein dissociation into subunits; and it has been attributed to change in pH, increased salt concentration, oxidation of sulfhydryl groups, protein concentration, and loss of water which maintains essential conformation.

MECHANISMS OF PLANT FREEZE RESISTANCE

Introduction

Unlike animals, plants are unable to escape from adverse environmental conditions. They may be able to avoid the consequences of stress by restricting growth to favorable environmental conditions. For example, an annual plant avoids the most extreme seasons, although its seed has to survive if further generations are to result. Similarly, trees in the temperate zone are exposed to a variety of environmental stresses in nature to which they must adapt or die. Thus, woody species annually undergo rhythmic growth cycles that are synchronized with seasonal environmental changes. These plant species have evolved

mechanisms that allow them to acclimate to a variety of environmental stresses (Levitt, 1980). Cold temperature is one such condition. When temperate plant species are exposed to low, but non-freezing temperatures and/or short day length, they become tolerant to extreme sub-freezing temperatures. This process of transition from cold tender to cold hardy is termed cold acclimation or cold hardening (Levitt 1980). Cold acclimation is a complex developmental process that occurs in winter annuals, biennials, and perennials in which the ability to withstand freezing temperatures increases during the fall and winter seasons (Levitt 1980, Sakai and Larcher 1987). The increased freezing tolerance is perhaps the most dramatic manifestation of cold acclimation. There is a wide range in the increase in freezing tolerance that occurs, ranging from a few degrees in herbaceous species to tens of degrees in winter cereals to over 100 degrees in some extremely hardy deciduous trees (Sakai and Larcher 1987). In this review, because of the complexity and variety of cold acclimation only woody species of temperate origin, especially grapes, will be discussed.

Stages of cold acclimation

Weiser (1970) has shown that acclimation is a multi-step process which requires a combination of both changes in daylength and temperature to invoke the necessary alterations in plant metabolism. Nevertheless, several authors agree that cold acclimation primarily involves two stages (Levitt 1980, Sakai and Larcher 1987). In temperate zones, the first stage of cold acclimation occurs in late summer or early fall, well before any frost events (Weiser 1970). The results of controlled environment studies of many woody plant species have indicated that the first stage is induced by short days (Fuchigami et al 1971, Howell and Weiser 1970, Irving and Lanphear 1967, Van Huystee et al 1967). Those plants that respond

to short days require reduced day length prior to low temperature stimulus to achieve maximum cold hardiness. At this stage, deciduous trees still have their leaves which are the sites of reception of the photoperiod stimulus (Fuchigami et al 1971, Hurst et al 1967). These leaves have been found to carry a translocatable and unidentified factor which moves to the bark tissues and initiates cold acclimation processes (Fuchigami et al 1971, Howell and Weiser 1970, Irving and Lanphear 1967). This has been further demonstrated by grafting studies. These studies have revealed that the acclimation-promoting factor from the leaves of one genotype can enhance the cold acclimation in the bark tissues of another genotype (Fuchigami 1970, Weiser 1970). The active metabolic processes result in substantial changes of woody plants, including growth cessation, terminal bud set, onset of dormancy, and a substantial degree of cold acclimation (Fuchigami et al 1971, Howell and Weiser 1970). This developmental stage is usually associated with vegetative maturity of shoots (Fuchigami et al 1982, Nissila and Fuchigami 1978). Examples of plant species reacting to short days include apple (Howell and Weiser 1970), Red-osier dogwood (Fuchigami et al 1971), *Viburnum* and box elder (Irving and Lanphear 1967). During the first stage of acclimation, temperatures may range from 10C to 20C for highs (Sakai and Larcher 1987) and 0C to 5C for lows (Levitt 1980), and cold hardiness may reach levels ranging from -10C to -25C (Howell and Weiser 1970, Weiser 1970).

The second stage is induced by low temperatures (usually 5C to below 0C) and does not involve translocatable factors nor requires light. Cold temperature induces the completion of cold acclimation. Increases in cold hardiness during the second stage occur very rapidly and normally coincide with the first freezing temperatures of the fall (Howell and Weiser

1970). The temperature most effective in increasing hardiness depends on species, tissues and developmental stage of the plant. In nature, hardiness of trees increases remarkably when the daily minimum temperature falls to sub-zero (Howell and Weiser 1970, Sakai and Larcher 1987). Howell and Weiser (1970) have shown that frost is a prerequisite to trigger the second stage of acclimation at which apple twigs reached their full hardiness at -50C. In the absence of frost, plants did not acclimate beyond the levels attained in the first stage, i.e. -25C to -30C. In other words, short photoperiod alone will not induce the second stage of acclimation. Other authors, however, disagree and believe that frost is not necessary for the second stage of acclimation, but its occurrence may increase the rate of acclimation (Sakai and Larcher 1987). These authors have reported that very hardy woody plants such as willow species survived -196C after hardening at 0C for 14 days in mid-October. Similar results have been reported of potted plants of *Populus* x *euramericana* which survived -196C after hardening for two weeks at 15C followed by two weeks at 0C.

Under natural conditions, plants are exposed to fluctuating temperatures. Thus, alternating temperatures in controlled environments have been used to simulate outdoor conditions in several experiments. Studies reported by Sakai and Larcher (1987) indicate that alternating temperatures are as effective for cold acclimation as constant temperatures. Others studies have failed to obtain hardy plants with alternating temperatures. A recent study by Hanna (1995) indicates that cold acclimation of Red-osier dogwood and 'Red Lake' currant is best achieved by constant (0C) and alternating above-zero temperatures (0C/10C), respectively, in the fall.

The response of grapevines to photoperiod and low temperature is different from other woody plants in that vine shoots do not set terminal buds as an indication of growth cessation and initiation of cold acclimation. Wolpert and Howell (1986b) have demonstrated that *V. Labruscana* cv. Concord is responsive to photoperiod. While vines grown under natural decreasing photoperiod stopped growing, vines under photoperiod supplemented with artificial lights continued active growth of their shoot tips. Another study with a better control over temperature and photoperiod supported the results of Wolpert and Howell. Fennel and Hoover (1991) have shown that both *V. labruscana* Bailey and *V. riparia* Michx. have a decrease in cane growth in response to short days. Furthermore, they demonstrated that short days promote periderm development (vegetative maturity of canes), onset of bud dormancy, and small but significant bud cold acclimation. A report by Shnabel and Wample (1987) on *V. vinifera* cv. White Riesling has indicated that short days and low temperatures, when administered separately, have a small effect on cold acclimation. However, when short days and low temperature are combined, a greater increase in hardiness occurs, indicating a synergistic effect of these two environmental factors.

Several researchers have been hesitant in concluding that cold acclimation of grapes involves two stages as in other woody plants (Fennel and Hoover 1991, Salzman et al 1996, Shnabel and Wample 1987, Wolpert and Howell 1985b). The reason for that is probably the weak cold acclimation (about -5C to -15C) achieved via short days alone (first stage of acclimation) as compared to other woody plants (-25C to -30C) (Howell and Weiser 1970). Nevertheless, authors almost unanimously agree that the combination of short days and low temperatures are prerequisites for maximum cold acclimation.

Mechanisms of cold acclimation

After elucidating what induces cold acclimation, the next step is to find how the plant responds to these environmental factors as a whole and at the cellular level. A classical problem in studies of stress and adaptation in plants, as induced by environment cues such as low temperature, is the difficulty in causally relating molecular changes at enzyme and membrane levels to functional properties of physiology at the cell and organism level. In other words, although good correlations may be found between biophysical, biochemical changes and physiological responses, a causal relationship is rarely established. The establishment of causal relationships is the ultimate goal of all stress research.

Cold acclimation is a very complex process and genetic experiments suggest that the inheritance of the capacity for cold acclimation-induced freeze tolerance is a quantitative trait controlled by a number of additive genes (Guy 1990, Thomashow 1990). For these reasons cold acclimation has been associated with multiple major changes at the molecular, cellular and organ levels. All these changes almost always lead to a substantial increase in freezing tolerance.

A. Physiological changes

Every season, temperate woody plant species undergo dormancy, a necessary but not sufficient phase as part of cold acclimation. Dormancy can be divided primarily into three types (Lang et al 1987). Paradormancy or correlative inhibition, endodormancy or rest, and ecodormancy or quiescence. Following summer growth, lateral buds in woody plants first undergo paradormancy which is regulated by physiological factors outside the affected structure (i.e. terminal buds) such as apical dominance and photoperiodic responses (Lang et

al 1987, Fuchigami et al 1982). Woody plants which set terminal buds may resume growth after leaf or terminal bud removal (Fuchigami et al 1971, 1982). When leaf removal no longer stimulates dormant buds to resume growth, the tree is considered to have reached vegetative maturity (Fuchigami et al 1982). Vegetative maturity is an extremely crucial phase for achieving maximum cold acclimation and it is an indication of the initiation of endodormnacy. Vegetative maturity also indicates the beginning of the first stage at which cold acclimation can occur (Friesen and Stushnoff 1989). Maximum rest which is usually associated with maximum cold hardiness corresponds to the point at which buds require the longest chilling to resume growth (Fuchigami et al 1982). When the chilling requirement is fully satisfied, dormant buds will rapidly grow when exposed to favorable environmental conditions; otherwise they remain ecodormant (Fuchigami et al 1982).

Early stages of cold acclimation have been always associated with a decrease in tissue water content (Bray and Parsons 1981, McKenzie et al 1974). It has been speculated that pith senescence and dehydration, increase in root resistance, and decrease in stomatal resistance may have led to a decline in stem water content of Red-osier dogwood (McKenzie et al 1974, Parsons 1978). A decline in water content in both primary buds and canes has also been observed in Concord grapes and it was inversely related to cold hardiness (Wolpert and Howell 1985a, b). Furthermore, the decrease in water content and hardiness of grape canes is acropetal, thus basal tissues are hardier than apical tissues (Wolpert and Howell 1985b). Specific reasons for the positional effects are not known but it has been hypothesized that tissue maturation, or periderm formation, is involved. Progression of acclimation is closely related to periderm formation, which like acclimation, is acropetal in its development

(Wolpert and Howell 1986a). Unlike other woody plants, periderm formation in grapes is easily seen because of the color change of the shoot from green to brown (Pratt 1974).

B. Biophysical changes - Deep supercooling

1. Introduction

The primary obstacle to plant survival at low temperatures is coping with ice formation and the accompanying dehydrative stress. Consequently, plants have evolved a variety of mechanisms to physically resist freezing temperatures. Some survive by avoiding rather than tolerating freezing, such as the annual plants with little or no frost resistance which survive by means of dehydrated seeds that are very hardy. Roots, crowns, and even crops of some herbaceous biennials and perennials survive because snow and soil moderate extremes of air temperatures (Burke et al 1976, Levitt 1980, Sakai and Larcher 1987). Other plants which are tolerant to freezing generally undergo extracellular freezing which is not lethal. These plants are usually very hardy and can tolerate the presence of extracellular ice. This results in dehydration of the cells, and plants basically tolerate desiccation during extreme subfreezing temperatures (Levitt 1980, Sakai and Larcher 1987). Examples of plants that survive freezing by tolerating freeze-induced desiccation range from herbaceous plants, e.g. winter wheats (survive at -25C) to very hardy woody plants native to the Boreal Forest of North America such as birch, Red-osier dogwood, willow and aspen (survive liquid nitrogen at -196C) (Burke et al 1976).

Rather than tolerating freeze-induced cellular dehydration, some plant tissues avoid freezing by "deep supercooling". Examples of plant tissues and organs that deep supercool include xylem ray parenchyma of numerous temperate trees (George and Burke 1977,

Ashworth 1993, Quamme et al 1982), reproductive, vegetative, and mixed buds of many species (Quamme 1995), and hydrated seeds (Stushnoff and Juntilla 1978). Examples of woody plants that deep supercool include azalea (Graham and Mullin 1976), forsythia (Nus et al 1981), blueberry (Bierman et al 1979), blackberry (Warmund et al 1988), apricot (Ashworth et al 1981, Quamme 1974), peach (Ashworth 1982, Quamme 1978), apple (Quamme et al 1982), pear (Rajashekar et al 1982), pecan (Rajashekar and Reid 1989), sweet cherry (Andrews and Proebsting 1987) and grape (Andrews et al 1984, Pierquet and Stushnoff 1980, Pierquet et al 1977, Quamme 1986). Only the mechanism of deep supercooling in deciduous woody plants will be discussed as it is relevant to grapes.

Deep supercooling, also called deep undercooling, is a mechanism of freeze avoidance and can be defined as the ability of a population of cells to retain cellular water in a liquid phase at subfreezing temperatures (below 0C) by remaining free from internal heterogeneous ice nuclei and isolated from the nucleating effect of extracellular ice (Burke 1979). In these instances, cellular water is not in equilibrium with extracellular ice and will supercool despite the presence of ice in adjacent tissues (Burke et al 1976). Since supercooled water is in a metastable condition, it will form intracellular ice in response to heterogeneous nucleating agents (e.g. presence of ice nucleating bacteria or dust particles), or spontaneously when homogenous nucleation temperature of pure water is reached (-38C)(Rasmussen and Mackenzie 1972). Furthermore, colligative properties of the cell sap may further depress the freezing point beyond the homogeneous nucleation point by several more degrees (George and Burke 1977, Burke et al 1976).

The geographic distribution of woody plant species that exhibit the deep supercooling characteristic has been shown to be limited in their northern distribution by the -40C isotherm, which approximately represents the temperature of homogenous ice nucleation of water (Burke et al 1976, George et al 1974a, Gusta et al 1983). This characteristic also appears to influence the agricultural distribution of fruit trees and other horticultural crops (Quamme 1976, Rajashekar et al 1982). Since the most cold hardy species do not exhibit the deep supercooling characteristic, it has been proposed that breeding to eliminate this trait may enhance freezing resistance (Burke and Stushnoff 1979).

2. Detection of deep supercooling and injury assessment

Detection of freezing of plant tissues that deep supercool can be monitored using differential thermal analysis (DTA)(Quamme et al 1972). Thermocouples are used to detect the latent heat released by water (80 cal/g H_2O) in the samples as it undergoes a liquid to solid phase change or, conversely, the heat absorbed as a result of ice melting. Sample temperatures are compared to a reference (freeze-dried tissue) undergoing the same rate of cooling. This produces a flat baseline on the thermogram until the water in the sample undergoes a phase change, resulting in a difference in temperature between the sample and the reference. The sample/reference differential is recorded as a peak on the thermogram and represents an exotherm during freezing or an endotherm during melting (Burke et al 1976). Other methods of detection such as differential scanning calorimetry and nuclear magnetic resonance (Burke et al 1976, George and Burke 1977, Quamme et al 1982), and acoustic emissions (Weiser and Wallner 1988) have been used to lesser extent. Some studies have incorporated other methods of assessing injury with DTA in order to test the reliability and

accuracy of the latter. Examples of these methods include tissue browning (Wolf and Pool 1987, Hamman et al 1996), staining (Stergios and Howell 1973), and electrolyte leakage (Stergios and Howell 1973). Unlike DTA which measures the actual killing temperature, in the other methods the killing temperature is estimated as the temperature that kills 50% of plant tissues (T_{50}) by a statistical procedure.

During controlled freezing several authors, using DTA, have typically observed two distinct exotherms in xylem and bud tissues of many temperate woody plants (Burke et al 1976). The first peak, also called "high temperature exotherm" (HTE), has been associated with extracellular ice formation resulting from the freezing of bulk water. This freezing event does not appear injurious and it often occurs at temperatures between -5C and -10C (Quamme 1995). The following peak(s), also called "low temperature exotherm" (LTE), has been associated with intracellular ice formation resulting from the freezing of supercooled water. This exotherm occurs between -10C and -45C, depending on the plant species and anatomical structure, and it is correlated with tissue injury (Quamme 1995).

Early research on deep supercooling established a correlation between LTE and tissue injury (George et al 1974a, George and Burke 1977, Quamme et al 1972). It is believed that the correlation of the LTE with tissue injury is due to the formation of intracellular ice within living cells, which results in lysis of the cell (Burke and Stushnoff 1979). Hong and Sucoff (1980), in a study of eight species of woody plants, have observed a linear relationship between the number of dead xylem ray cells and the amount of supercooled water that had frozen. Pierquet and Stushnoff (1980) first described a relationship between LTEs and injury in grapevine dormant buds. Death of the primary bud was consistently observed when

excised buds of *V. riparia* were removed immediately after occurrence of an LTE. Removal of buds before the occurrence of LTEs resulted in no injury. The relationship between LTE and grapevine bud injury was reexamined in subsequent studies on *V. vinifera* and showed similar results (Andrews et al 1984, Wolf and Pool 1987). Additional evidence relies on the peculiar structure of grape buds. Unlike other deciduous fruit trees, grapevines have complex or compound buds containing primary, secondary, and tertiary buds, each of which may contain an apical meristem and flower primordia. Typical DTA thermograms show up to three LTEs which are thought to correspond to the freezing and consequently death of each bud type (Andrews et al 1984, Quamme 1986, Pierquet and Stushnoff 1980, Wolf and Pool 1987).

Although much of the circumstantial evidence suggests that the LTE represents the lethal freezing of intracellular water, there is no direct evidence that intracellular ice formation is responsible for injury. The LTE clearly represents the freezing of supercooled water, and its occurrence is correlated with death of xylem and bud tissues (Burke and Stushnoff 1979, Burke et al 1975, 1976, Quamme et al 1972, 1973). However, whether water within the xylem and bud primordia cells supercools and whether intracellular ice formation is the cause of cell death has not been established. Only recently, this issue has been resolved using freeze substitution and transmission electron microscopy techniques. Ristic and Ashworth (1993) have shown the first microscopic evidence that the intracellular ice formation is the source of freezing injury of supercooling xylem ray parenchyma cells of flowering dogwood. Another possible source of freezing injury in supercooling tissues, besides intracellular ice, has been associated to cavitation but results were inconclusive (Ristic and Ashworth 1993).

3. Mechanisms of deep supercooling

Although supercooling was discovered over 30 years ago and studied in a number of species, its mechanism is still not fully understood. It is not known how the supercooled state of the plant tissue is maintained or how freezing of supercooled tissue is initiated. Nevertheless, researchers have come up with several hypotheses to elucidate the mechanisms and/or properties that regulate supercooling. According to George and Burke (1977), in order for deep supercooling to occur, the targeted cell must be free of heterogeneous nucleating substances active at "warm" subzero temperatures; a barrier must be present that excludes the growth of ice crystals into a cell; concomitantly a barrier to water movement must exist that prevents a "rapid" loss of cellular water to extracellular ice in the presence of a strong vapor pressure gradient; finally, cell walls must have sufficient tensile strength to counteract the negative hydrostatic pressures that result from the large vapor pressure deficit.

The possible role of cell wall, its porosity and permeability, in deep supercooling has been investigated (George and Burke 1977, George 1983, Ashworth and Abeles 1984, Wisniewski et al 1987). Water within small diameter pores will freeze at lower temperatures than bulk water, and small diameter pores (less than 60 to 100 nm) within the cell wall could inhibit the loss of cellular water and the propagation of ice (Ashworth and Abeles 1984, George 1983, George and Burk 1977). The effectiveness of cell wall microcapillaries as a barrier would be limited by the diameter of the largest capillary. Wisniewski and his group (1993, 1995) have demonstrated an important role for pit membrane structure in regulating deep supercooling. The pit structure of xylem ray parenchyma appears to be an important constraint to water permeability and ice propagation. They have also implicated pectin and

the interaction of pectin with other cell wall constituents as playing a role in defining the porosity and/or permeability of this region of the cell wall. However, differences in components of cell wall structure between species that do supercool and those that do not have not been depicted.

Properties of the plasma membrane have also been implicated in deep supercooling. Treatment of grapevine wood tissue with nystatin caused a shift in the LTE to warmer temperatures (Bakradze et al 1985). Nystatin treatment was believed to increase the size of pores in the plasma membrane, leading the authors to conclude that the diameter of plasma membrane pores dictated the extent of supercooling. Unfortunately, no measurements of pore size were made in this study to confirm the effect of nystatin on grape membranes. A study by Quamme et al (1973) has shown that xylem tissue of apple kept its properties of deep supercooling after being killed by steam, chloroform or oven drying (with rehydration). However, when the tissue was ground up it did not deep supercool. The authors concluded that structural feature in deep supercooling tissue does not involve the protoplasm.

It has also been proposed that water may supercool within xylem ray parenchyma cells because tension prevents water from being withdrawn to extracellular ice (George and Burke 1977, Quamme et al 1982). The development of negative cell turgor would be a function of the rigidity of the cell wall. Cells with noncompressible walls would retain water against a large water potential gradient and would therefore survive until either intracellular ice nucleation or cavitation occurs. The concept of negative pressure facilitating the supercooling of water in wood tissues is consistent with observation on wood structure and cell wall response during freezing. Xylem ray parenchyma cells have thick cell walls and often

share common walls with adjacent cells. No evidence of cell wall disruption or separation following a freeze stress was apparent in chemically fixed tissues (Malone and Ashworth 1991, Wisniewski and Ashworth 1985). A corollary of the negative pressure hypothesis would be that species that do not exhibit deep supercooling would have less rigid cell walls, and that cell walls would collapse during freezing. This was not observed (Malone and Ashworth 1991).

Although buds do deep supercool like xylem ray parenchyma in stems, the former behave somewhat differently. Several authors have demonstrated that anatomical structure of the bud plays an extremely important role in the acquisition of the deep supercooling capacity. The removal of the basal tissue from excised shoot primordium of fir raises the LTE from -25C to -15C (Sakai 1978). The basal zone is formed of dense collenchyma cells present at the base of the shoot primordium and may act as a barrier to ice propagation from the subtending tissue into the shoot primordium (Sakai 1978). Dormant peach flowers also have a basal zone of cells that lacks intercellular spaces (Quamme 1995). The flower can be induced to freeze by ice nucleation above the basal zone but not below it (Quamme 1995). Other supporting evidence of the existence of a barrier came from the failure of water soluble dyes to move from the peach bud axis tissue into the floral primordium (Ashworth 1982, Quamme 1978). Quamme (1986) also observed similar phenomenon in grape buds and concluded "...In both species (i.e. peach and grape), excising the bud to include a piece of the node was required to achieve full expression of supercooling".

In anatomical studies of a number of *Prunus* species, a lack of xylem continuity between floral primordia and bud axes was a common feature of species which deep

supercool (Ashworth 1984). During deacclimation in the spring, the flower buds lose the capacity to deep supercool. The loss of supercooling has been associated with the development of functional xylem between the bud and the stem (Ashworth 1984). Therefore, fully differentiated xylem may act as a conduit for ice propagation into the flower (Proebsting and Andrews 1982). Similar histological observations have, for the first time, been made in grape buds where the differentiation of vascular tissues between the apical and basal parts of the compound bud is correlated with loss of supercooling (Barka et al 1995). The authors have also demonstrated that, in the compound bud of Chardonnay, freezing is not an instantaneous event but rather a gradual one which initiates from the base of the bud, to the bud axis, and finally to the apical part (LTEs at -11C, -17C, -22C, respectively).

Other features that modify supercooling includes rate of cooling or freezing. In nature, cooling rates usually are about 1C/hr to 2C/hr (Levitt 1980, Sakai and Larcher 1987). Rapid cooling rates, however, are used under artificial freeze testing. The main reason for using fast cooling rates is to allow for many samples to be tested in succession, but high rates are not typically found in nature and can lead to erroneous results. The use of non-uniform or standardized cooling rate for each species has generated many conflicting reports on the effects of cooling rate on survival of plant cells. For example, the LTEs of buds from many species such as conifers (Sakai and Larcher 1987), *Prunus* spp (Ashworth 1982, Burke and Stushnoff 1979), *Rhododendron* spp (Kaku et al 1980), azalea (George et al 1974b), blueberry (Biermann et al 1979), and grape (Pierquet et al 1977) occur at warmer temperature with increasing cooling rates. A report by Quamme (1986), on the effect of cooling rate on European and hybrid grapes, has indicated no significant differences in bud LTEs at cooling

rates ranging from 1.5C/hr (slow) to 40C/hr (fast). Wolf and Pool (1987), on the other hand, have seen small but statistically significant differences in LTEs of Chardonnay buds cooled at 2C/hr and 5.6C/hr. The examples of grape buds clearly indicate that there are differences in response to cooling rate among species within the same genus, and may be among cultivars within the same species. This emphasizes the statement made long ago by Stushnoff (1972) that whenever artificial freezing is used, the researcher should develop a standard cooling rate by testing the effects of different rates on survival.

Fast cooling rate has been speculated to preclude the migration of water to preferred sites of freezing. For example, rapid cooling (above 15C/hr) of peach flower buds disrupts the water migration to preferred sites such as bud scales, and thus results in loss of supercooling (Ashworth 1982, Quamme 1983). Furthermore, if the water content of flower bud decreases, then LTE occurs at a colder temperature. This has been demonstrated in the following species: blueberry (Biermann et al 1979), sweet cherry (Andrews and Proebsting 1987), flowering dogwood (Ishikawa and Sakai 1985), peach (Quamme 1983) and Rhododendron (Kaku et al 1981). It is not known exactly how dehydration decreases the temperature of the LTE, but Ishikawa and Sakai (1981) observed that after drying, the freezing point of sap decreased. Presumably, cell solutes were concentrated by drying and this decreased the nucleation temperature of water.

Sakai (1979) termed the segregation of ice within plant tissues "extraorgan freezing". As a consequence of extraorgan freezing, water is withdrawn from one tissue to freeze in another where it is less injurious. Ishikawa and Sakai (1982) added that new definitions should be made to distinguish between "true deep supercooling" and "extraorgan freezing".

They have emphasized that true supercooling does not involve water movement and is not affected by cooling rate. In contrast, the supercooling occurring concomitantly with extraorgan freezing does involve water migration and is cooling rate dependent. Extraorgan freezing is further classified on the basis of its effect on tissue survival into three possible consequences. First, the organ completely dehydrates and is tolerant to severe desiccation at temperatures as low as -196C. Second, the organ completely dehydrates during slow cooling but is killed by the extreme dehydration. Third, some dehydration can occur but freezable water remains in the organ and breaks supercooling at fairly warm temperatures.

C. Biochemical changes

1. Introduction

Cold acclimation in higher plants is a complex process involving changes in gene expression, protein synthesis, and altered cell metabolism (Guy 1990). Indeed, during the exposure of plants to low temperatures, several biochemical changes have been observed and have been associated with cold acclimation. These changes include modified levels and activities of enzymes from various metabolic pathways, accumulation of soluble carbohydrates, amino acids, nucleic acids, proteins, phospholipids, and altered lipid composition of cell membranes (Levitt 1980, Sakai and Larcher 1987). These complex changes indicate that cellular metabolism and membrane structures undergo considerable modifications at low temperatures, of which some may be related to frost survival and some to improved performance at low temperatures. Nevertheless, in most cases, the role that a given change has in freezing tolerance remains poorly understood. In this section, the focus will be on the role of soluble carbohydrates in freezing tolerance as it relates to the thesis.

2. Endogenous changes in soluble carbohydrates

The accumulation of soluble sugars during cold acclimation has been documented for more than a century by Muller-Thurgau (Sakai and Larcher 1987), and the significance of sugar accumulation has been debated ever since. Several reports have clearly indicated that soluble sugars in herbaceous and woody plants increase from the fall to winter when plants are subjected to low temperatures, and decrease in spring as plants begin to deacclimate (Levitt 1980, Sakai and Larcher 1987). The accumulation of soluble sugars is usually associated with a decrease in the insoluble carbohydrate reserve, starch, by hydrolysis and vice versa (Sakai and Larcher 1987).

Herbaceous monocots such as gramineae accumulate fructose polymers called fructans when exposed to low temperatures (Pollock 1986). The accumulation of fructans in these temperate grasses is generally correlated with better winter survival (Pollock 1986). Fructans accumulate in the crown which contains the overwintering shoot meristem (Levingston 1991). Cereals with distinct freezing resistance accumulate fructans with different degree of polymerization (Levitt 1980). Hardy species such as wheat, barley and rye accumulate high-molecular-weight fructans, whereas oats (less winter hardy) accumulate low-molecular-weight fructans.

Woody plants, unlike temperate grasses, do not accumulate fructans but other sugars such as mono- and oligosaccharides during cold acclimation. Acclimating plants may accumulate monosaccharides or simple sugars such as glucose and fructose, disaccharides such as sucrose, tri- and tetrasaccharides such as raffinose and stachyose, or sugar alcohols such as sorbitol (Sakai and Larcher 1987). The special group of oligosaccharides, also called

raffinose family oligosaccharide (RFO), has been frequently observed to change exclusively with cold acclimation. The accumulation of RFO during cold acclimation has been documented in a wide variety of plants, including algae (Salerno and Pontis 1989), needles of conifer species (Hinesley et al 1992), leaves of Puma rye (Koster and Lynch 1992), cabbage (Santarius and Milde 1977), Ajuga (Bachmann et al 1994), and alfalfa (Castonguay et al 1995). Accumulation of RFO has also been reported in woody plants, including 'Dolgo' crabapple, 'Red Delicious' apple, 'Red Lake' currant, Western sandcherry, 'Valiant' grape, red-osier dogwood and 'Schubert' choke cherry (Stushnoff et al 1993). It has been generally observed that RFO levels increase in the fall, as a response to low temperature, reach maximum levels during the coldest months in mid-winter, and decrease in the spring (Stushnoff et al 1993).

3. Proposed mechanisms of role of soluble carbohydrates in freeze tolerance

Although the physiological significance of sugar accumulation has not been established, circumstantial evidence suggests that sugar accumulation may play a role in increased freezing resistance. Consequently, several schools of thought have proposed different mechanisms on possible role of soluble sugars in general and RFO in particular on increasing freezing tolerance of plants. Levitt (1980) suggests that sugars may act as soluble solutes and their accumulation leads to a decrease in osmotic potential which results in a depression of the freezing point of cell water. Protection of plant tissues with such mechanism is the result of colligative effect, i.e. the sugars non-specifically change the bulk properties of the solution by, for example, contributing to the freezing point depression (Burke et al 1976) and/or diluting potentially toxic compounds (Santarius 1982). Thus, given

equal osmolalities of different sugars, the same amount of cryoprotection by each sugar should be observed.

The observation that different sugars provide differing amounts of cryoprotection indicates that non-colligative effects of sugars also play a role in preventing plant injury during freezing (Santarius and Bauer 1983). The nature of the non-colligative effects is not fully understood; however, direct interactions between sugars and the targeted tissue, cell, molecule, or membrane have been demonstrated. In vitro studies have demonstrated that soluble sugars can function as cryoprotectants by stabilizing proteins and membranes during both a freeze-thaw cycle, and during freeze-drying (Strauss and Hauser 1986, Strauss et al 1986, Crowe et al 1990, Carpenter et al 1990). Soluble sugars are known to form hydrogen bonds and thus may substitute for water, during desiccation, in maintaining hydrophilic structures in their hydrated orientation, even when water is no longer present (Crowe et al 1988). Water replacement by soluble sugars has been demonstrated in model systems (Crowe et al 1988). Hoekstra et al (1989) have also proposed that soluble sugars help stabilize membranes during severe desiccation imposed by extracellular ice formation, also called freeze-induced desiccation. Withdrawal of water molecules from the phospholipids can lead to membrane phase transitions which coincide with membrane leakage and cell death (Crowe et al 1989, Hoekstra et al 1989). Membrane stabilization via soluble sugars is achieved by preventing phase separation and membrane fusion; thus preventing leakage of cellular solutes during freeze-induced dehydration (Hoekstra et al 1991). In addition to their role in membrane protection, soluble sugars are involved in the stabilization of proteins and retention of enzymatic activity during dehydration (Carpenter and Crowe 1988).

Another mechanism by which soluble sugar accumulation may act to protect the cell is by the formation of intracellular glass (Burke 1986). As a solution becomes concentrated during freeze-drying, the solutes may crystallize, or the solution may become supersaturated with an accompanying increase in viscosity (Franks 1985). Glass formation, or vitrification, takes place at the glass transition temperature Tg. A glass is an undercooled liquid with the viscosity of a solid. Its existence is temperature dependent, i.e. a solution that exists as a glass at one temperature will melt at a higher temperature giving rise to a liquid and the possibility of crystallization (Franks 1985). Glass formation in an organism would preclude chemical reactions requiring diffusion, thus ensuring stability during a period of dormancy. The viscous flow in a glass is of the order of mm/century (Franks 1985). During freeze-induced desiccation, glasses may prevent further dehydration, stabilize cellular components, and permit the continuance of hydrogen bonding at the interface between the glass and hydrophilic surfaces in the cell (Burke 1986). Therefore, these properties would help ensure the survival of an organism during freeze-induced desiccation. Glass formation has been implicated in survival of some extremely desiccation tolerant organisms (Koster and Leopold 1988, Williams and Leopold 1989, Koster 1991, Bruni and Leopold 1991), and the survival of *Populus* at low temperatures (Hirsh et al 1985, Hirsh 1987).

Not all sugars are equally effective cryoprotectants. On an equal molar basis, disaccharides are more effective than monosaccharides (Crowe et al 1990, Carpenter et al 1990). This is most likely associated with Tg, i.e. the higher the molecular weight of the sugar, the higher the Tg (Franks 1985). As a result, RFOs such as stachyose and raffinose (tetra- and trisaccharides, respectively) form a glass more readily (higher Tg) than the

disaccharide sucrose which in turn forms glass more readily than the monosaccharides fructose and glucose (Franks 1985). Hirsh et al (1985) have provided the first evidence of glass formation in a very winter hardy woody plant, *Populus*, at Tg = -28C. Hirsh (1987) subsequently attributed the relatively warm subzero glass transition to the presence of stachyose and raffinose. Although RFOs exhibit cryoprotective activity (Seufferheld 1995, Tada et al 1990), their effectiveness in combination with other carbohydrates is not known. RFOs have been shown to allow sucrose availability in dehydrated cells by preventing its crystallization (Caffrey et al 1988). Smithe (1967) has reported that among many sugars and other organic compounds tested, raffinose and stachyose were the most effective inhibitors of sucrose crystal growth. He has also demonstrated that small amounts of these RFOs suffice to suppress the sucrose crystal growth rate. Hincha (1990) suggests that the cryoprotective efficiency of RFO could be the result of complex structural interactions with lipid head groups.

Although the accumulation of soluble carbohydrates is well established in cold acclimating plants, their role as cryoprotectants is still unclear. In order for sugars to play a role as cryoprotectants, rather than storage reserves, they must accumulate in the cytoplasm and in the vicinity of plasma membrane rather than in the vacuole. In other words, the knowledge of intracellular compartmentation is an extremely important clue about the potential cryoprotective effects of soluble sugars. Unfortunately, very few studies have been conducted to test this hypothesis probably because of experimental difficulties. Koster and Lynch (1992) have demonstrated that during cold acclimation of Puma rye, most of the sugar (84% w/w) in the protoplast is located in the extravacuolar space. Of this extravacuolar

sugar, sucrose and raffinose account for the greatest part. The authors conclude that the accumulation of RFO outside the vacuole contribute more to cryoprotection rather than storage.

To date there is increasing evidence that single sugars correlate more significantly than the total soluble sugars with cold acclimation. Moreover, expression of the accumulation of specific sugars as ratios has been shown to be more meaningful in elucidating composition changes that are otherwise not detected if expressed as absolute sugars. For example, Stushnoff et al (1993) have demonstrated that expressing specific sugars in ratios like raffinose/sucrose and raffinose + stachyose/sucrose results in the highest correlation with cold acclimation of seven taxa. These ratios reach a maximum when outdoor temperatures are minimal and thus cold hardiness is maximum. In the spring, the ratios decrease to their lowest levels which coincide with loss of hardiness (Stushnoff et al 1993). In lesser hardy species such as European grapes (Hamman et al 1996), and cereals (Olien and Clark 1995), ratios of monosaccharides to disaccharides correlate best with cold acclimation, although these species have completely distinct mechanisms of surviving freezing temperatures. Furthermore, monitoring at close time intervals of cold acclimation and sugar accumulation has indicated that the timing and the magnitude of the accumulation do not always coincide with increases in freezing tolerance. For example, Koster and Lynch (1992) have observed that soluble sugars have reached their maximal levels before the LT_{50} of Puma rye has reached its lowest temperature. Another example comes from a recent study by Sauter et al (1996) in which early and full cold acclimation (LT_{50} = -50C) of ray parenchyma cells of poplar wood correlate very closely with the accumulation of RFOs; however, during deacclimation RFOs

reach their lowest levels, while cold hardiness remains relatively high (LT_{50} = -25C). This discrepancy does not negate the possibility of a cryoprotective role for sugars. It does emphasize the fact that freezing is a complicated phenomenon, and that no one, or few, factors can be singled out as conferring freezing tolerance to an organism.

METHODS OF FREEZE PROTECTION

Introduction

Low temperature injury to perennial crops, whether from fall or spring frosts or mid-winter deep freezes, accounts for greater crop loss than all other environmental stresses combined (Flore and Howell 1987, USDA 1986). The primary cause of crop loss in the nursery industry also is freeze damage, which occurs largely to roots of overwintering container-grown plants (van de Werken 1987). Rieger (1989) reported that the annual losses of agricultural crops due to freezing injury were estimated at over 800 million dollars in the United States. Approximately half of the estimated losses occurred in orchard crops. He added that estimation of economic losses due to freezing is a complex matter which may explain the lack of recent and accurate data. The price of horticultural crops is relatively elastic, increasing greatly in freeze years when supplies are reduced. Therefore, freezes affect individual growers, the local economy of major horticultural crop growing regions, and consumers due to higher prices paid for commodities in freeze years (Kiker 1979). Hence, implementation of freeze protection methodology and related research efforts can have tremendous benefits.

Efforts to protect crops from freezing injury began at least 2000 years ago, when

Roman farmers protected grapes by burning scattered piles of dead vines and prunings (Blanc et al 1963). Theoretically any crop can be protected during any freeze, but economic and logistic factors limit the number of methods and the conditions under which each can be used. Ultimately, profitability becomes the dominant factor determining whether freeze protection can be practiced and the method chosen in a given situation. Fully covering and heating a crop as in a greenhouse is one of the best and also one of the most expensive cold protection systems, but it is hardly practical for large areas of orchards, vineyards and many other small fruit and vegetable crops. The question of how, where, and when to protect a crop must be addressed by each grower after considering crop value, expenses, and cultural management practices (Evans 1991).

Before describing methods of freeze protection common types of frosts frequently mentioned below, should be defined. There are basically two dominant types of frost situations which could be encountered. These are radiation frost and advection frost. A radiation frost is characterized by calm or light winds, clear skies, temperature inversions, relatively high subfreezing temperatures and dew points, and daytime temperatures above OC. Below-freezing temperatures largely result from radiant heat loss from vegetation and earth-bound objects to the sky and subsequent cooling of the air at the surface by conduction and free convection. An important feature of radiation frost is a temperature inversion, which is a layer of warm air floating over a layer of cold air next to the ground surface (Wallace and Hobbs 1977). In other words, inversion results from the physical properties of cold air (which is heavier than warm air) to remain at ground level or flow to lower areas, whereas warm air dissipates into the sky. Advection frost occurs when subfreezing temperatures from

an influx of an arctic or polar air mass moves into a region displacing warmer air that was present before the frost occurrence and cools the vegetation and earth-bound objects. Advection frosts are characterized by moderate to high wind speed, low dew point, freezing air temperature even during daylight, no inversion, and unlike radiation frosts may have cloudy skies. In some cases, the temperature can remain below freezing for several successive days. Advection frosts are difficult to protect against and, fortunately, are not frequent in occurrence (10 to 30 year-cycle depending on the region where fruit crops are grown). Radiation frosts are much more common than advection frosts and protection methods/systems described below are usually effective against the former.

Active freeze protection

Active (or direct) freeze protection involves methods that are implemented just before or during a freeze event in order to prevent plant temperatures from dropping to damaging levels or inhibit ice formation in plant tissues (Rieger 1989). Examples of active freeze protection include heating, sprinkler irrigation, wind machines, fogging, and insulation. Only the currently most popular protection methods used for fruit crops, including grapes, will be discussed below.

A. Heating

Heating has been employed for centuries as a method of freeze protection, and is still widely used throughout the world. Heating is considered as one of the most reliable methods of freeze protection (Martsolf 1979). However, rising fuel costs late in the 1970's have precluded the use of heating for freeze protection, except for high cash-value crops or as a supplement or back up system to other devices such as wind machines. With the lower cost

of oil and propane in the last few years, heaters have made a minor come back, especially in large fruit-growing areas like Washington (Evans 1991). However, should fuel costs increase in the future, use of heaters will most likely diminish.

Heating provides freeze protection by raising air temperature in the orchard or the vineyard through radiant and convective heat transfer from the heaters directly to plant surfaces (Perry et al 1977). The degree of air temperature increase in a heated planting depends largely upon wind speed and inversion strength (Valli 1970). Wind not only removes heated air from plantings but also prevents inversions from forming. Strong inversions increase the efficiency of heating by preventing the heated, buoyant air from rising far above the level of the plants. Several types of heaters are being used with the most common probably being the cone and return-stack oil burning varieties (Evans 1991). Other fuel types include propane and wax.

Heating seems to be economically not profitable for several reasons. The use of heaters requires a substantial investment in money and labor. Additional equipment is needed to move the heaters in and out of the orchards and vineyards as well as refill the heaters with fuel. Furthermore, a fairly large labor force is needed to properly light and regulate the heaters in a timely manner (Evans 1991). This system has also proven to be inefficient since only 10% of the radiant energy is captured by plants; the rest of the heat is either lost due to radiation to the sky, convection above the plants or to the wind drift moving the warmed air out of the orchard (Evans 1991). Added to that, the smoke liberated by the heaters causes air pollution, thus it is environmentally unacceptable unless heaters meet air pollution standards regulated in each fruit-growing district.

Nowadays, heaters are almost always used in conjunction with wind machines. The combination of heaters with wind machines not only produces sizeable savings in heater fuel use (up to 90%), but increases the overall efficiency of both components. The number of heaters per acre is reduced by at least 50%, the heat normally lost by rising above the tree canopy may be mixed back into the orchard by the wind machines, and additional heat is added from the inversion (Evans 1991).

B. Wind machines

The idea of protecting crops from freeze temperatures by mixing stratified air dates back to the early 1900's and was first put into practice with California citrus orchards in 1920's (Gerber 1979). However, they were not generally accepted by fruit growers in the U.S. until the 1950's, when more powerful and effective machines were produced. A growing interest in this approach to frost protection in recent years can be directly attributed to the cost of fuel. Orchard heaters consume a great deal of fuel and are expensive to operate even for short periods.

The theory behind the development of wind machines for frost protection is based on one simple assumption. Temperatures in and above orchards and vineyards are assumed to increase with height during nights with little cloud cover and light surface winds; i.e. typical inversion conditions. The purpose of a fan is to mix air vertically and transport it horizontally in order to displace cold air at tree height in the orchard with warmer air brought down from above (Gerber 1979). Effective operation of fans or propellers requires that a sufficiently strong temperature inversion exist (Renquist 1985). With a strong inversion, temperature could be increased up to 50% of the temperature difference (inversion strength) between

ground level and the 15-20 m height (Reese and Gerber 1969). Also, the degree of warming is proportional to the distance from the fan (Reese and Gerber 1969), thus several wind machines need to be installed in very large orchards or vineyards. There is an additional requirement that surface winds be light (upper limit 3-4 m/s) to ensure safe mechanical fan operation (Doesken et al 1989).

Wind machines have been introduced and proved to be effective in Colorado only since last decade (Renquist 1985). The fruit growing region of western Colorado includes the highest elevation commercial orchards in the U.S. and the effectiveness of wind machines against frost was considered uncertain in such high elevation orchards. However, to date, wind machines are the primary method for frost protection in western Colorado's fruit-growing district.

Helicopters are an expensive and sometimes dangerous variation of wind machines which can also be used under radiation frost conditions. They can be very effective since they can adjust to the height of an inversion and move to cold spots in the orchard (Evans 1991). However, due to the large standby and operational costs, the use of helicopters for frost protection is limited to special cases or emergencies.

Advantages of wind machines over other freeze protection methods include reduced labor requirement, low operating cost, low fuel input per degree C of protection, and proven effectiveness under radiative conditions for over 70 years. Disadvantages include high initial costs and lack of effectiveness under advective freeze conditions or unusually cold radiative freeze conditions.

C. Water sprinkler systems

Overhead or overtree sprinkling is the field system which provides the highest level of protection at a very reasonable cost. The initial investment and operating costs per acre of overhead sprinklers are about half that of a combination of wind machines and heaters (Kasimatis et al 1982). The principle is based on covering plants with an ice-water coating to maintain plant parts above killing temperatures during a frost event (Gerber and Martsolf 1979). When applied water freezes and consequently releases heat (heat of fusion) keeping the temperature of an ice-water mixture at about OC. By constantly rewetting the surfaces of the tree leaves, shoots and fruits, it is possible to maintain a protective film of ice-water at OC. The application rate required to maintain an ice-water coating depends largely on wind speed, air temperature, humidity, and the crop; generally rates of 0.15-0.18 inches per hour are required (Evans 1991, Perry 1979). Clear ice formation and icicles are field indicators of sufficient application rates (Gerber and Hendershott 1963).

The advantages of this method are that it does not rely on the inversion strength for its effectiveness and it is the only method that can provide some protection under advective frost conditions (Evans 1991). Furthermore, overhead sprinklers do not require extensive labor to operate, they are clean and quiet in operation, do not use large amounts of fossil fuels and can be used for other cultural purposes such as irrigation, application of fertilizers and pesticides, heat suppression, and evaporative cooling to delay bloom (Evans 1991, Kasimatis et al 1982, Rieger 1989). The disadvantages of this method include the very large amounts of water required to cover a significant portion of any one orchard. Thus, this method may not be feasible where water is scarce or not available during a frost event like in Western

Colorado because the irrigation canals are closed at that time of the year (Hamman, personal communication). Large amounts of water may also waterlog heavy soils which are common in Colorado. In addition, sprinkling for frost protection can be beneficial or deleterious because freezing of water on the plant releases heat, while evaporation extracts heat which will cause evaporative cooling. This system, as reported by Rieger (1989), has been shown to cause severe damage to fruit crops as a result of system failure or insufficient application rates especially during an advective frost. Sprinkled citrus trees sustained more damage than non-irrigated trees due to evaporative cooling and elevation of tissue killing temperatures by the presence of water. Finally, some fruit trees (e.g. stone fruits) may not be able to support the ice loads and branch breakages result (Evans 1991). Overhead sprinklers have been successfully used in fruit districts where water is not scarce like Washington (apples), Florida (citrus), and Northern California (wine grapes); also this method has been most effective in Minnesota, Wisconsin, and Canada for strawberry protection, where limb breakage is not a problem and frost temperature at soil level are most severe.

Not long ago, overhead systems were being replaced in Washington with undertree systems due to disease (e.g. Fireblight, Coryneum blight, and scab) rather than frost protection related problems (Evans 1991). The principle of operation of undertree sprinkling differs from overhead system and resembles more closely the flood irrigation system. The objective is to apply enough water to the ground using sprinklers mounted on low risers to warm the air at ground level which then rises up through the canopy and warms plant tissues. The transfer of heat to the frosty buds is by radiation, convection and by any condensation which occurs on the coldest plant tissues (Evans 1991). With this system, the problem of limb

breakage associated with overhead irrigation is avoided. Also, there is less risk and less disease problems since water does not come in contact with the buds (Evans 1991). Warner (1987) also added that the cost of installation of undertree and overtree systems are comparable. Sutherland et al (1981) found that the undertree sprinkling increased temperature by about 2C in citrus under radiative conditions, comparable to increases obtained by a less energy efficient heater/wind machine combination. However, unlike overhead system, undertree sprinkling is effective for protection of deciduous fruit trees only when a temperature inversion was present (Davies et al 1987). Evans (1987) added that tall cover crops are beneficial for undertree sprinkling since they provide more freezing surface area and insulation from the ground. In Washington, the use of undertree sprinklers has increased rapidly during the last 10 years. Many of the systems are now being used in conjunction with wind machines. This combination has worked well since heat from the inversion is supplied plus any heat and humidity from the sprinkling that rises above the canopy is captured and recirculated back through the orchard (Evans 1991)

Passive freeze protection

Passive (or indirect) freeze protection results from practices which decrease the probability or severity of freezes at a given site. Passive methods are implemented well in advance of a freeze as preventive measures. Examples of passive freeze protection include selection of hardy cultivars that develop later in the spring, good site selection which has adequate cold air drainage and free of freezes during the growing season, cultural practices, and effective passive protection methods. Passive management practices can minimize or eliminate the need for active freeze protection and thus reduce expenses (Rieger 1989).

A. Site selection

To date, the best frost protection technique is good site selection. Generally, low areas are warmer than adjacent hill tops under advective freeze conditions, but much colder than hilltops under radiative conditions (Bartholic and Martsolf 1979). The latter effect is due to drainage of cold air. Because cold air is more dense than warm air, it flows downhill much like water. Thus, low spots or frost pockets where cold air will collect should be avoided. Temperatures near the soil surface in these frost pockets can be 8C lower than those on adjacent hilltops (Blanc et al 1963). Furthermore, sites close to heat sources like large bodies of water moderate extremes in temperature. The moderation of temperature also called the lake effect may reduce the probability of freeze damage by warming cold air during the winter, and retards bud break by cooling air during the spring (evaporative cooling effect).

Other site characteristics such as row orientation, windbreaks and cover crops can influence the drainage of cold air, and therefore, influence the risk of freezing injury. South-facing walls of buildings absorb solar radiation during the day, emit radiant heat at night and thereby provide protection to nearby crops. Alternatively, winter injury can be accentuated in evergreen landscape plants with southern exposure due to rapidly fluctuating temperatures (White and Weiser 1964). Further, warm temperatures tend to promote bloom or bud break, thus, planting on a north-facing slope can delay bloom and/or bud break and reduce the probability of frost damage. Windbreaks and tall cover crops between rows can enhance the performance of active freeze protection methods by reducing convective heat loss from the site, but also act to impede drainage on calm radiative frost (Martsolf et al 1986, Krezdorn and Martsolf 1984).

B. Cultural practices

Spring frosts can be modified by few degrees by improving the daytime absorbing potential of the soil. This is achieved by storing more of the heat radiated by the sun during the daytime for release at night. A moist, compact, and deep soil, dark in color and without vegetation will absorb more radiation during the day and release more heat at night than loose, cultivated, shallow dry soil. Irrigation applied to the soil prior to a freeze increases soil heat storage and thermal conductivity, and may provide protection down to -4C (Rieger 1989).

Pruning as a common cultural practice for woody plants has generated several publications with conflicting results regarding its effect on cold hardiness and bud break delay. However, recent reports indicate no effect of pruning on cold hardiness nor on bud break of *V. vinifera* either pruned in mid-winter (Wample 1994) or in early spring (Hamman et al 1990). The application of such findings is that growers have a wider time-window to prune large vineyards or orchards to insure completion in time and permit good crop development. A common practice for perennial crops that require pruning is to delay pruning as much as possible to provide the opportunity to compensate for winter injury and to avoid spring frosts (Flore and Howell 1987, Hamman et al 1996). This is especially important in more northern latitudes with shorter growing seasons and earlier fall frost like in Colorado.

The effect of nutritional status on cold hardiness of plants has been reviewed by Pellet and Carter (1981) and indicates that high nitrogen fertilizer rates reduce cold hardiness. However, most of the literature is inconclusive and inconsistent due, in part, to the variability in genetic, environmental and cultural factors (Pellet and Carter 1981, Wample et al 1993).

A recent report by Wample et al (1993) indicated that the widely held view that high nitrogen fertilization of grapevines is detrimental to bud cold hardiness may not be correct.

Other management practices include unintentional delayed harvest of perennial fruit crops. This is caused by factors outside the grower's control, such as environmental stress, or labor shortages. As a consequence, delayed harvest has been reported to be linked with poor cold hardiness (Flore and Howell 1987, Shaulis et al 1968, Stergios and Howell 1977). It has been speculated that late-harvested fruit accumulate carbohydrate reserves at the expense of the stem and bud tissues, thereby, altering cold hardiness. However, these reports provide little evidence of the cause-effect relationship. Recent reports have shown that early, normal, or delayed harvest have no effect on cold hardiness nor on soluble carbohydrate reserves of several *V. Vinifera* cultivars (Hamman et al 1996, Wample and Bary 1992). These reports conclude that under sufficient photosynthetic conditions to provide for fruit development and storage reserves, no limiting growth factors, and good management practices, there is no adverse effect of a delayed fruit harvest on bud and cane hardiness nor on soluble carbohydrate reserves.

C. Evaporative cooling to delay bud break

Cold hardiness is known to decrease with advancing phenological development of dormant buds especially following the accumulation of heat units above a given threshold after the chilling requirements are completed (Levitt 1980, Sakai and Larcher 1987). Hence, evaporative cooling of buds by sprinkling during the period of heat unit accumulation in late winter could slow the rate of bud development and delay bud break or bloom sufficiently to reduce the risk of freezing injury. Overtree or overhead evaporative cooling applies water

above the crop. As the water evaporates it extracts heat and thus cools the trees and the surrounding environment (Evans et al 1995). Evaporative cooling has been extensively used in apple and peach orchards during the pre-bloom period and delay in bloom has resulted (Alfaro et al 1974, Lipe et al 1977, Unrath 1972, Evans et al 1995). Microjet sprinkling of Chardonnay and Cabernet Sauvignon resulted in delay in deacclimation and bud break (Lipe et al 1992). Although this system seems to be effective in delaying bloom and bud break, reports indicated in some cases no gain in bud hardiness and, thus, no frost benefit (Evans 1991). Evans reasoned that when buds of woody plants are imbibed with water they lose the ability to supercool. In addition, sprinkling may result in excessive soil moisture which lead to flooding stress and crop loss (Stang et al 1978). Other adverse effects include delayed fruit maturity (Anderson et al 1975), reduced fruit set and color observed in apples (Stang et al 1978), and increased disease incidence (Collins et al 1978, Spotts et al 1976). Finally, the additional costs of water, system setup and energy consumed make this system very costly and preclude its widespread use for frost protection.

D. Exogenous application of protective products

The use of chemical products to increase cold hardiness and/or delay spring development of crop plants has been carried out for a long time (Rieger 1989). This method is attractive because of the low cost and ease of application. Inconsistent results, however, due to climate, location, or genetic factors and deleterious effects of chemicals on plants and environment have precluded widespread acceptance of chemical freeze protection. Nevertheless, it is established that these chemicals have the potential to provide freeze protection and prevent crop damage in herbaceous and woody plants (Rieger 1989). These

products usually consist of plant growth regulators, antitranspirants, dormant oils, and cryoprotectants.

1. Plant growth regulators

Both dormancy and the following hardening processes are believed to be hormonally regulated, thus, their regulation can be manipulated by exogenous application of growth regulators (ASHS 1994). Abscisic acid (ABA) has been suggested to play a major role in dormancy mediation in herbaceous and woody plants, as it is the case for seeds (ASHS 1994). During dormancy, ABA accumulates in plant tissues, growth is inhibited and in the case of trees bud dormancy ensues (Guy 1990, Levitt 1980). In grapes, the onset of dormancy is associated with an increase in the content of cis-ABA, while the dormancy break is associated with a decrease (Koussa et al 1994). Studies on exogenous ABA application to delay dormancy release and increase hardiness in grapes and other fruit trees have been minimal and unsuccessful (Considine 1983, Emmerson and Powell 1978, Holubowicz et al 1982). Other studies have established that exogenously added ABA can substitute in many plant species for the low temperature stimulus in inducing the acclimation response both with suspension culture cells (Chen and Gusta 1983, Dhindsa et al 1993) and whole plants (Dhindsa et al 1993, Lalk and Dorffling 1985). With whole plants, best results in inducing freezing tolerance have been obtained by application of ABA to the root system (Dhindsa et al 1993, Robertson et al 1993). Robertson et al (1993) suggest that ABA is effective in enhancing freezing tolerance only in species of limited cold hardiness, but not in hardy species. For these reasons the role of ABA still remains controversial.

The application of ethylene-releasing compounds has usually been successful in increasing bud hardiness and/or delaying bloom in several woody species (Rieger 1989). Ethephon increases the freezing tolerance of sweet cherry (Proebsting and Mills 1976), apple (Raese 1977), pear (Ketchie and Murren 1976) and peach (Durner and Gianfagna 1988). Furthermore, application of Ethephon especially in the fall has caused a delay in bloom or bud break of almond (Browne et al 1978), apricot (Dennis 1976), sweet cherry (Proebsting and Mills 1973), sour cherry (Dennis 1976), peach (Crisosto et al 1989), and grape (Considine 1983, Weaver et al 1974). The rate and time of application are critical factors that determine the effectiveness of Ethephon or Ethrel (Gianfagna 1989). High concentrations are phytotoxic and, with peaches, they caused excessive gumming, bud drop, and poor fruit set resulting in low yields (Dennis 1976). With grapes, weak growth in the following season and increased susceptibility to winter injury were observed with such treatment (Considine 1983). Although the mechanism for delayed bud break and increased hardiness is not known, it has been suggested that Ethephon delays bloom by delaying flower differentiation and development during dormancy (Crisosto et al 1989), and by increasing the heat requirement of non-dormant flower buds for growth in the spring (Gianfagna 1989).

Gibberellic acid (GA) has been less successful in delaying bloom and increasing cold hardiness than ethylene-releasing compounds. In fact, it has been demonstrated that GA causes a decrease rather than an increase in cold hardiness presumably because of its growth--promoting characteristics (Irving and Lanphear 1967, Proebsting and Mills 1974, Dennis 1976). Delay in bud break has been more obvious in grapes especially seeded *V. Vinifera* than any other fruit species when treated with GA (Patterson and Howell 1995, Weaver et

al 1961). Anti-gibberellin compounds, or growth retardants, however, have a positive effect on cold hardiness. These compounds have presumably been expected to increase cold hardiness because growth and cold hardiness are inversely related (Howell and Dennis 1981). Examples of growth retardants are Cycocel, Mefluidide, Daminozide, and Paclobutrazol. Cycocel has increased the freezing tolerance of box elder (Irving 1969), potato (Chen and Li 1976), winter wheat (Gusta et al 1988), alfalfa (Paquin et al 1976) and cabbage (Marth 1965). Daminozide has increased cold hardiness in apple (Raese 1977) and delayed its bloom (Sullivan and Widmoyer 1970). Mefluidide has increased the freezing tolerance of spring wheat and potato (Li 1991). Paclobutrazol has increased cold hardiness of sour orange and rough lemon seedlings slightly (Yelenosky et al 1987), but has been ineffective with apricot, peach and sweet cherry (Proebsting and Mills 1985) and grape (Ahmedullah et al 1986). Paclobutrazol has delayed bud break of Concord grapevines (Ahmedullah et al 1986) but has advanced it with peach, sweet cherry and apricot (Proebsting and Mills 1985).

2. Antitranspirants/ Dormant oils

Antitranspirants (also called antidesiccants) are widely used in ornamental horticulture to reduce desiccation-related injury. Winter injury of ornamental landscape or containerized evergreens is caused when transpiration rates are high and soil water is frozen (Pair and Still 1982). Several antitranspirants are available in the market with different trade names such as Wilt-Pruf, Protec, and Vapor guard. Their active ingredient is usually pinolene, a terpenic polymer, which when sprayed on plant surfaces forms flexible and thin films reducing water loss and preventing desiccation. In addition, antitranspirants are hypothesized to act as barriers to external nucleators (Levitt 1980). The antitranspirant film on the surface of the

leaves is thought to impede the frost that forms on the surface from providing a nucleator for water inside the plant (Perry et al 1992). The degree of success has been inconsistent and variable from one plant species to another, and among the products used. Pair and Still (1982) have reported that antitranspirants reduced transpiration of *Ilex* x *attenuata,* but effectively reduced winter injury in only the first year of plant establishment. Call and Seeley (1989) reported that the antitranspirant Wilt-Pruf significantly reduced T_{50} for peach flower buds by delaying dehardenning. In other reports, antitranspirants were ineffective for increasing freeze survival or cold hardiness of developing peach fruits (Matta et al 1987), young citrus trees (Burns 1970), tropical foliage plants (Fitzpatrick et al 1986), and tomatoes and peppers (Perry et al 1992); and increased mortality of almond and plum flowers after exposure to -4.4C (Rieger and Krewer 1988).

Dormant oils have also been used to increase freeze resistance and/or delay bloom. Application of dormant oil at non-phytotoxic concentrations on 'Johnson Alberta' peach trees has been effective in delaying bloom by up to 5 days and increasing flower hardiness by 4C (Call and Seely 1989). Myers et al (1996) have confirmed these results using soybean oil and have suggested that this oil could also be used as a bloom thinner. They have shown that dormant sprays of soybean oil modified internal $C0_2$ concentration of peach shoots and reduced respiration, thus bloom delay resulted.

3. Cryoprotectants

Cryoprotectants are penetrating or non-penetrating compounds that provide protection against freezing (Levitt 1980). Cryoprotectants must be nontoxic, biodegradable, easily absorbable, and should have the capacity to penetrate membranes, low volatility,

limitless solubility in water, in addition to ability to form multiple hydrogen bonds, and ability to dissolve in electrolyte and non-electrolyte solutes (Robertson et al 1993). Cryoprotectants include surfactants, polyhydric alcohols, sugars, and water soluble polymers e.g. ethylene glycols. Cryoprotectants are particularly useful for cryopreservation of plant tissues at ultra low temperatures (-196C) (Sakai, 1984). The use of cryoprotectants in field testings has not been as successful as for cryopreservation and their effectiveness has varied among products and species.

Early work by Ketchie and Murren (1976) showed an increase in cold hardiness in young apple trees using sprayed or injected cryoprotectants such as glycerol, ethylene glycol, polyvinyl-pyrolidone (PVP) and dimethylsulfoxide (DMSO). They concluded that these cryoprotectants were only effective under short day conditions. 'Minus F', a commercially formulated mixture of glycol and buffering agents, promotes supercooling and increases freeze survival of young citrus trees but is phytotoxic to leaves (Yelenosky et al 1987). 'Frost Free', a commercial cryoprotectant made out of the following surfactants: 50% propylene block copolymer of polyoxyothylene and 50% propylene glycol, has been ineffective to protect early blooming *Prunus* species (peach, plum, and almond) during spring frost (Matta et al 1987, Rieger and Krewer 1988), nor tomatoes and peppers (Perry et al 1992). Another surfactant, called dodecyl ether of polyethylene glycol or DEPEG, has shown cryoprotective effects on black currant flowers when sprayed before flower emergence (Wilson and Jones 1980, 1983) and on 'Concord' grapevines when sprayed in the Fall (Himelrick et al 1991). This product, however, is no longer available. Finally, 'Frost Guard' a commercial product with undisclosed composition, but with claims contains cryoprotective agents, heavy metals,

and nitrogen has not been effective on freeze protection of bud and leaf tissues of grapes (Himelrick et al 1991, Gardea et al 1993).

4. Alginates

a. Introduction

Seaweeds, the algal plants, are classified into four principal groups: Chlorophyceae, the green algae; Phaeophyceae, the brown algae; Rhodophyceae, the red algae; and Cyanophyceae, the blue-green algae (Chapman 1970). The brown and red algae are salt--water plants and are important commercially because of their polysaccharide content. Agar and carrageenan are extracted from various types of the red algae, and algin is derived from the brown seaweeds. The giant kelp, *Macrocystis pyrifera*, which grows in abundance along the coasts of North and South America, New Zealand, Australia and Africa, is one of the principal sources of the world's algin supply. Alginate was first described by the British chemist E. E. C. Stanford in 1881. Algin exists in the brown algae as the most abundant polysaccharide comprising up to 40% of the dry matter (Skjak-Braek 1992). It is located in the intercellular matrix as a gel containing sodium, calcium, magnesium, stronium, and barium ions. Its main function is believed to be skeletal, giving both strength and flexibility to the algal tissue. Because of its ability to retain water, and its gelling, viscosifying and stabilizing properties, alginate is widely used by many industries.

b. Structure and properties

Alginate is a family of unbranched binary co-polymers of D-mannuronic and L--guluronic acid, of which there are three types: homopolymers of mannuronic acid (MM),or guluronic acid (GG), and a heteropolymer with an alternating sequence (MG) (Skjak-Braek

et al 1989). The differences in composition and fine structure of mannuronic and guluronic acid polymers and alternating segments account for the differences in properties and functionality of alginates isolated from different species of brown algae.

One of the most important and useful properties of alginates is the ability to form gels by cross-linkage reaction with divalent or trivalent cations (with the exception of magnesium and mercury ions)(Redenbaugh et al 1993). The gelling is a result of the formation of an "egg-box" structure consisting of GG blocks in parallel being held together by a cation source (Grant et al 1973). Gelling characteristics depend on the alginate sources and the guluronic to mannuronic acid ratio (M/G). The alginate from *Laminaria hyperborea*, with a small M/G ratio (high G) forms rigid, brittle gel with high porosity that is heat stable and tends to undergo syneresis, or loss of bound water. In contrast, alginate from *Macrocystis pyrifera* with a large M/G ratio (high M) forms soft, elastic gel with low porosity that is less heat stable, but exhibits more freeze-thaw stability and has a markedly reduced tendency toward syneresis (Skjak-Braek 1992). The polyvalent cation most used to change the viscosity and gel characteristics of algin solution is calcium. Calcium may also be used as the precipitating ion for the formation of insoluble films, which are not water-repellent and swell in water (Kelco 1987). Alginates are readily available in the sodium salt form and when the sodium ion is substituted with the divalent cation, calcium, ionic cross-linkages are formed and gelation occurs to yield calcium alginate as indicated by the following reaction:

$$2\text{Na-alginate} + CaCl_2 \rightharpoonup \text{Ca-alginate} + 2\ NaCl$$

Sodium alginate forms solutions of unusually high apparent viscosity even at low concentrations because of its high molecular weight and the rigid nature of the molecules

(Kelco 1987). Furthermore, alginates in solutions are compatible with a wide variety of materials including preservatives (formaldehyde), thickeners (guar gum), plasticizers (glycerol), organic solvents, water-soluble resins, latices, sugars, oils, fats, waxes, pigments, and surfactants (Kelco 1987).

c. Applications

Because of their unique properties and lack of toxicity, alginates have many applications in food and industrial products including frozen foods, bakery jellies, salad dressings, beer, fruit juice, textile printing and dyeing, toys, and ceramics (Kelco 1987).

In recent years, alginates have been used more in biomedicine and biotechnology. Entrapment in calcium alginate gel beads has become the most widely used technique for immobilizing living cells (Skjak-Braek and Martinsen 1991). These include bacteria, yeasts, cells from higher plants, and animal cells such as pancreas cells (Skjak-Braek et al 1989). The use of alginate encapsulation in plants was first attempted on somatic embryos to form the so-called synthetic seeds (Redenbaugh et al 1986). The alginate capsules were used as seed coats to protect the embryos while allowing for germination and conversion of synthetic seeds (Redenbaugh et al 1987). The alginate-encapsulation technique was further extended from propagation purposes (Bapat et al 1987) to cryopreservation in liquid nitrogen at -196C. The first successful cryopreservation, using the encapsulation-dehydration technique, was realized by a french group (Dereuddre et al 1990) who attempted to cryopreserve alginate-coated axillary shoot tips excised from in vitro pear plantlets. This method became very attractive to researchers throughout the world because it is relatively simple; it does not require the use of toxic cryoprotectants; and recalcitrant plants or plant parts are amenable to

cryopreservation. Successful cryopreservation via encapsulation-dehydration included somatic embryos such as coffee (Hatanaka et al 1994), carrot (Dereuddre et al 1991 a, b), and oilseed rape (Uragami et al 1993) and shoot tips of the following micropropagated species: carnation (Tannoury et al 1991), potato (Fabre and Dereuddre 1990), strawberry (Seufferheld 1995), apple (Niino and Sakai 1992), pear (Niino and Sakai 1992, Scottez et al 1992), mulberry (Niino and Sakai 1992), eucalyptus (Poissonier et al 1991) and grape (Plessis et al 1991, 1993). Furthermore, to bypass the in vitro culture procedure, our group was able to cryopreserve woody plant organs, specifically dormant apple and grape buds using the encapsulation-dehydration technique (Stushnoff, personal communication). The wide array of alginate applications led to the first laboratory and field trials of alginate in an attempt to enhance freezing resistance and delay bud break of *V. Vinifera* grapevines. The experimental designs and results will be discussed in more details in Chapter 4.

RESEARCH OBJECTIVES

The response of grapevines to environmental stresses have been studied in this thesis with emphasis on low temperatures. Grapevines are perennial woody plants which undergo seasonal changes to overcome unfavorable growing conditions by entering into a dormant state. These changes are both genotype- and environment-dependent and result in the acquisition of higher levels of cold resistance of the exposed-above the ground plant parts. Cold resistance (or tolerance) of plant parts such as buds and stems has been associated with timed multiple biochemical changes, including the accumulation of non-structural carbohydrate reserves. This may suggest that soluble sugars may play a role in cold

resistance. In Chapter 2, the objective of the study was to increase carbohydrate pool within grapevines during the growing season using methanol as a carbon-source. The goal was to gain few more degrees in cold hardiness as a result of higher accumulation of carbohydrate reserves. Another hypothesis in this study was to evaluate whether methanol protects grapevines from heat stress during the hot summers of the Grand Valley in Grand Junction, Colorado. Chapter 3 examines the correlation between seasonal changes in bud cold hardiness and soluble sugars of two *V. vinifera* cultivars, Chardonnay and Riesling. Another objective of this study was to evaluate whether cold-tender grapevines accumulate the same soluble sugars (quantity and quality) as the very cold hardy woody plants. Once a close correlation is established between the soluble sugar(s) and cold hardiness, the next step is to identify the metabolic pathway(s) that lead(s) to sugar accumulation, the enzymes involved and the gene(s) that control(s) all these processes. The ultimate goal is to isolate these genes and use them in gene transfer studies to elucidate key steps in cold acclimation. Genetic manipulation to improve cold hardiness seems possible but not feasible in the near future, primarily due to the complexity of the trait. An alternate, practical, and near-term technique to improve cold hardiness of grapevines has been developed and discussed in Chapter 4. Chapter 2 and 3 have been published and reprints are included in the Appendix section.

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CHAPTER 2

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF *VITIS VINIFERA* CULTIVARS TO METHANOL

INTRODUCTION

Improving quality and yield of cultivated plants have been major goals of growers and scientists during past decades. Manipulation of the environment and the genome have resulted in substantial progress toward attaining these goals. Application of methanol recently produced impressively high yields, surpassing expectations with several crops of economic importance (Nonomura and Benson 1992a, b). Because of the ease and low cost of application, methanol was considered by some to be a breakthrough with potential to revolutionize agricultural production of several C_3 crops, but the concept needed further research investigation with untested crops.

Capacity to utilize methanol by bacteria, fungi, algae, animals and plants was reported many decades ago. Large et al (1961) were the first to document that bacteria could grow on methanol as a sole source of carbon and energy in an aerobic environment. Other reports indicated that methanol is rapidly metabolized and converted to amino acids under dark conditions by plants (Cossins 1964). A Russian group led by Doman and Romanova (1962) demonstrated that plant leaves are capable of photosynthetic assimilation of methanol. They added that bean and barley leaves assimilated vapors of formic acid, formaldehyde and

methanol with the formation of products of about the same qualitative composition and about the same sequence of appearance as observed in CO_2 assimilation.

Nonomura and Benson's conclusions (1992a, b) were not limited to the assimilation of methanol per se, but went one step beyond. They discovered that, if properly applied to C_3 crops grown in areas of intense sunlight, methanol can substantially boost crop production and shorten the maturity cycle, hence potentially conserving water by reducing irrigation requirements (Table 2.1). Since C_4 plants did not respond to methanol, Nonomura and Benson (1992a) suggested that methanol may affect the photorespiratory pathway, which is functional only in C_3 plants. They proposed that methanol may inhibit photorespiration in C_3 plants, thus rendering the latter to act like the more efficient C_4 plants (Benson and Nonomura 1992, Nonomura and Benson 1992a). Another possible mechanism is the incorporation of methanol into the photorespiratory pathway, leading to the production of more amino acid such as serine, and ultimately doubling carbohydrate (i.e., sucrose) synthesis (Nonomura and Benson 1992b).

The objective of this study was to evaluate whether the application of methanol on grapevines is beneficial under conditions of high temperature and high light intensity relevant to the Grand Valley in the Western slope of Colorado. These conditions are presumably prerequisites for the most effective methanol treatment.

MATERIALS AND METHODS

The experiments were conducted at the Orchard Mesa Research Center, Grand Junction, Colorado (trickle irrigated), and two other private vineyards at Palisade, Colorado,

Table 2.1. Effects of methanol on a wide variety of C_3 plant species. Data are summary of results published by Nonomura and Benson (1992a).

Plant species	Yield increase (%)	Growth increase (%)	Early maturity
Tomato	50	50	Yes
Cabbage	60	50	Yes
Strawberry	65	60	N/A
Eggplant	63	60	N/A
Cotton	N/A	50	Yes
Watermelon	36	N/A	N/A
Wheat	100	N/A	Yes
Rose	45	40	Yes
Palm	N/A	75	N/A

seven kilometers from the research center, both furrow irrigated. *Vitis vinifera* L. mature vines (5-12 years old), both white and red cultivars, were used in this study. They were all own-rooted, trained to bilateral cordons, spur-pruned, and shoots were vertically positioned with a wire trellis system. The three vineyard sites were regularly irrigated and all normal production practices were followed. The experimental design was a randomized complete block, replicated four or five times at each site, with a single vine per replicate.

The first part of the study consisted of determining the sublethal dose of methanol for leaves and trunks as described by Nonomura and Benson (1992a). Assessment of methanol-toxicity symptoms was based on visual observations of necrosis, chlorosis or any other form of methanol-related injury on different parts of the sprayed vine. Solutions of methanol, ranging from 10 to 100% (v/v) in 10% increments, were each mixed with a surfactant, 0.1% (v/v) Triton X100 (Sigma, St. Louis). Hand-held (7.5 liters, Optimum Model 1402 RL Co., Lowell, Michigan) and backpack (15 liters, Solo Model 425, Solo Inc., Newport News, Virginia) sprayers were used for all applications. The spray was applied on clear-sky days between 1300 hr and 1600 hr, when temperatures ranged between 27C and 35C. The developmental stages at which vines were sprayed ranged between full bloom and berry set. Cultivars sprayed to runoff included Chardonnay, Semillon, Riesling, Muscat blanc, Pinot blanc, Sauvignon blanc, Gewurztraminer, Aligote, Sylvaner, French Colombard, Gamay Beaujolais, Pinot noir, Merlot, Cabernet Sauvignon, Malbec, and Carmine.

Once sublethal doses were determined, cultivars were selected to evaluate the effect of further applications of methanol on physiological and biochemical characteristics of

grapevines. The treated vines consisted of multiple applications (2-3) of methanol. Sprays included foliage (90% methanol + 0.1% Triton X100) or trunk (100% methanol + 0.1% Triton X100) application. Control vines were sprayed with water + 0.1% Triton X100. The volume of methanol solution sprayed to runoff was estimated to range between 0.2 L and 0.7 L per vine, depending on the vigor and the part of the grapevine sprayed (trunk or foliage). Treatments were applied under the same conditions mentioned above. Since the results among several cultivars between foliage and trunk treatments were similar, only the foliage treatments are discussed with the following cultivars: Muscat blanc, Chardonnay and Merlot. Dates of application (A), veraison (V) and harvest (H) for each cultivar were as follows. (1) Muscat blanc: 27 July, 3 August, 23 August (A), 13 August (V), 21 September (H); (2) Chardonnay: 29 July, 22 August (A), 14 August (V), 13 September (H); (3) Merlot: 29 July, 22 August (A), 18 August (V), 1 October (H).

Total soluble sugars (TSS) were monitored following the first application until harvest. Ten to twenty berries on a replicate vine were taken on each sampling date at about seven-day intervals, and a laboratory refractometer (Abbe-3L Milton Roy, Rochester, NY) was used to measure TSS (°Brix) from the squeezed juice. At harvest, the clusters from each vine were counted, the fresh weight per vine recorded and the average cluster weight calculated. Also, berries were randomly collected from clusters and the average weight of ten berries recorded, and their size measured with a caliper (Max-Cal Jensen Tools Inc., Phoenix, AZ).

In an attempt to confirm the results of Nonomura and Benson regarding the effect of methanol on photosynthesis, measurements of CO_2 uptake, transpiration and stomatal

resistance were taken in an open system configuration. The photosynthesis rate was determined by measuring the rate of CO_2 concentration assimilated by a known leaf area in a given time. Transpiration rate is based on measuring the water vapor flux per one-sided leaf area. Stomatal resistance was obtained by measuring transpiration and leaf surface temperature and applying the appropriate equation for calculation. These parameters were simultaneously taken on a single leaf clamped inside a leaf chamber connected to a CO_2 gas analyzer Model CI-301 (CID Inc., Vancouver, WA). Readings were from fully expanded, but not senescent leaves in the middle part of the vertically-trained shoots. The leaves were also fully exposed and in almost a perpendicular position to the sunlight. Data were collected in the morning between 0900 hr and 1100 hr under saturating light > 1000 $\mu molm^{-2}s^{-1}$ PPF. Measurements on Muscat blanc were taken 24 hours after the first and the second spray. Measurements on Chardonnay and Merlot were taken 24 hours and one week after the first spray. No further measurements were taken because of technical problems encountered with the equipment. Three to five leaves were measured per vine and four to five vine replicates were used in this experiment.

In the winter of the same year, buds were collected from dormant vines of Muscat blanc which were sprayed with methanol in the summer of 1993. Bud cold hardiness was monitored from 2 December 1993 until 5 April 1994, using differential thermal analysis described elsewhere (see Chapter 3). The low temperature exotherms (LTE) for the overwintering buds were recorded as the killing temperatures. It was hypothesized that if methanol altered sugar metabolism and translocation, it might also alter bud cold hardiness, since the latter has been shown to be strongly correlated with endogenous sugar metabolism

(Hamman et al 1996).

All data are subjected to analysis of variance where applicable and are presented as treatment means. Means were separated using T-tests.

RESULTS AND DISCUSSION

Sublethal dose

None of the concentrations between 10 and 90% for leaves and 60 to 100% for trunks showed any toxicity symptoms during the six-week visual observation period. The only damage observed was on vines sprayed on foliage with 100% methanol. The effect was almost immediate; within a few hours after spray application, young leaves and shoot tips curled and turned necrotic. Also, the small green berries discolored and turned purple at the surface of contact with methanol. The effect of absolute methanol on berries was irreversible since the latter never recovered and remained small in size and cracked by the time of ripening. The shoot tips, however, resumed growth normally. It was, therefore, concluded that the sublethal doses for foliage and trunk application were 90 and 100%, respectively. These concentrations were used for subsequent experiments.

Effect of methanol on TSS

The TSS in the berries, monitored every week until harvest, showed significant differences in Muscat blanc during veraison. The methanol-treated vines had about 1 °Brix more than the control on 19, 24 and 30 Aug (Fig. 2.1). This difference, however, disappeared as fruit maturity approached, and at harvest the TSS for both treatments were almost the same. On the other hand, Chardonnay and Merlot showed no significant differences in TSS

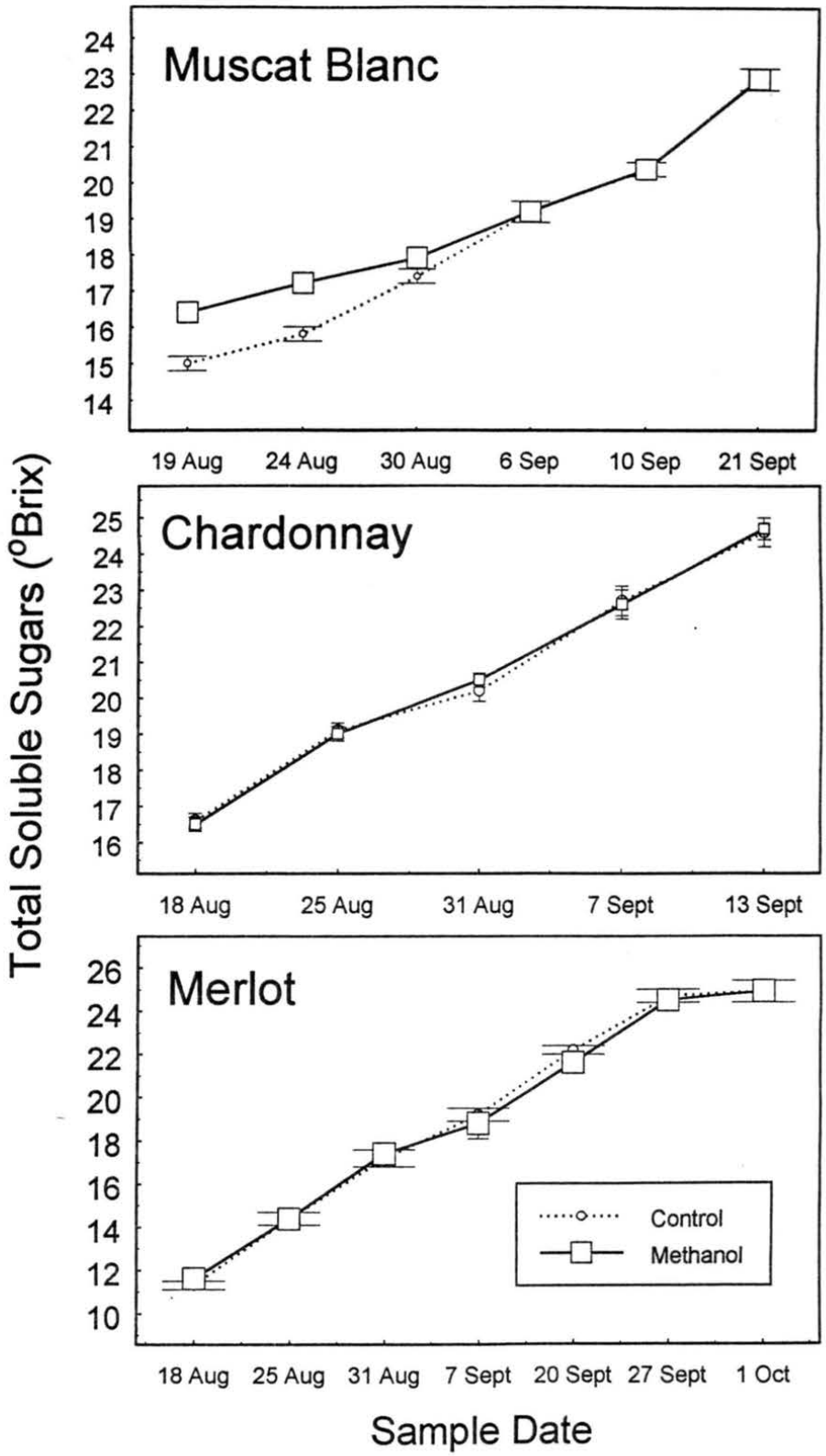

Figure 2.1. Changes in berry total soluble solids following methanol applications on Muscat blanc, Chardonnay, and Merlot. Data points are means ± SEM. Control treatment: water + 0.1 % Triton X100. Methanol treatment: 90% methanol + 0.1% Triton X100.

between the two treatments throughout the fruit development period (Fig. 2.1). A late ripening cultivar, Riesling, exhibited a rather negative effect of methanol where TSS was even lower in treated vines than that of control (data not shown). Similar conclusions were drawn by Wutscher (1994), where no significant differences in juice content or soluble solids in orange fruits were observed between control and methanol-treated trees. These results do not support the positive effect of methanol on sugar accumulation reported for tomato plants (Nonomura and Benson 1992a).

Effect of methanol on photosynthesis

Methanol stimulation of growth was attributed to reduced photorespiration by Nonomura and Benson (1992a, b). They did not, however, test the direct effect of methanol on photosynthesis. In this experiment, readings of photosynthesis, transpiration and stomatal resistance were taken in three cultivars. we tested a short-term response (readings taken 24 hours after spray) and relatively long-term response (readings taken 1 week after spray) of grapevines to methanol. In all cases, no statistically significant differences between control and methanol-treated vines were observed (Table 2.2), indicating no effect of methanol on photosynthesis, at least in the cultivars studied. Furthermore, the results in this study contradict the findings by Nishio et al (1993). These authors reported that 20% methanol treatment of spinach plants stimulated photosynthetic CO_2 gas exchange as quickly as three hours after treatment, with a continued positive response, compared to the control, for two weeks. Nishio et al (1993), however, could not explain the perplexing long-term stimulatory effect of methanol on photosynthesis.

Table 2.2. Effect of methanol-foliar application on photosynthesis, transpiration and stomatal resistance. Data are means ± SEM.

Time of reading	Treatments	Photosynthesis rate: CO_2 uptake ($\mu molm^{-2}s^{-1}$)	Transpiration rate: H_2O vapor ($mmolm^{-2}s^{-1}$)	Stomatal resistance to H_2O vapor (m^2smol^{-1})
Muscat blanc				
24 hrs after 1st application	control	12.6 ± 0.8	2.1 ± 0.2	15.3 ± 0.8
	90% methanol	12.8 ± 0.4	2.0 ± 0.1	16.8 ± 0.9
24 hrs after 2nd application	control	13.0 ± 0.3	2.1 ± 0.3	15.4 ± 1.1
	90% methanol	13.0 ± 0.4	2.2 ± 0.2	15.1 ± 1.1
Chardonnay				
24 hrs after one application	control	7.5 ± 0.5	1.1 ± 0.2	26.0 ± 2.6
	90% methanol	8.4 ± 0.8	1.2 ± 0.1	23.7 ± 2.0
1 week after one application	control	5.3 ± 0.7	1.0 ± 0.2	21.4 ± 1.4
	90% methanol	5.4 ± 0.3	1.2 ± 0.1	25.0 ± 2.7
Merlot				
24 hrs after one application	control	11.5 ± 0.8	2.1 ± 0.2	13.8 ± 1.5
	90% methanol	11.4 ± 0.7	2.4 ± 0.1	12.2 ± 0.4
1 week after one application	control	12.1 ± 0.3	2.5 ± 0.2	13.5 ± 0.8
	90% methanol	12.2 ± 0.6	2.7 ± 0.2	12.8 ± 0.9

Effect of methanol on yield components

The effect of methanol on yields, as reported by Nonomura and Benson (Table 2.1), was the most interesting response, attracting researchers throughout the world to test the hypothesis. Unfortunately, in this study methanol had no effect whatsoever on any component of yield in all three cultivars (Table 2.3). Numerous studies had similar disappointing results with a wide range of crop species such as orange (Wutscher 1994), okra, chili pepper, and eggplant (Valenzuela et al 1994), water melon, creeping bentgrass, lemon, savoy cabbage, carrot, romaine lettuce, radish, wheat, corn and pea (McGiffen et al 1995), tomato and melon (Hartz et al 1994), peppermint (Mitchell et al 1994), cotton (Mauney and Gerik 1994), snapbean (Lee and Rowland 1994), and potato (Feibert et al 1995).

Effect of methanol on bud cold hardiness

LTEs of buds collected from treated and control Muscat blanc vines revealed no significant differences for any sample dates (Table 2.4). Data comparing specific soluble sugars in cane tissues between the two treatments also showed no differences (data not shown).

CONCLUSIONS

Even though we attempted to follow a similar protocol to that used by Nonomura and Benson (1992a), there were no responses to methanol in any of the parameters measured in grapevines. Other experiments comparing single vs. multiple applications showed no influence of methanol (data not shown). The methanol formulation used in this study was modified from that reported by Nonomura and Benson (1992a) since we did not include any

Table 2.3. Effect of methanol on yield components of Muscat blanc, Chardonnay and Merlot. Data are means ± SEM[x].

Cultivars	Treatments	Cluster weight (g)	Berry weight[y] (g)	Berry size (mm)
Muscat Blanc	control	155.3 ± 10.7	20.4 ± 1.0	15.4 ± 0.2
	90% methanol	152.5 ± 14.1	18.4 ± 0.4	15.4 ± 0.1
Chardonnay	control	73.1 ± 6.3	12.3 ± 0.3	12.1 ± 0.1
	90% methanol	72.6 ± 11.5	11.9 ± 0.4	12.3 ± 0.2
Merlot	control	108.6 ± 12.3	13.3 ± 0.2	13.1 ± 0.1
	90% methanol	104.1 ± 12.5	13.2 ± 0.2	12.9 ± 0.1

[x] = Treatments are foliar applications as follows: control = water + 0.1% Triton X100; methanol = 90% methanol + 0.1% Triton X100

[y] = average weight of 10 berries

nutrients, amino acids or nitrogen fertilizer. The reason was to exclude any extra- or side-effect(s) that may interfere or override the methanol action per se. Nevertheless, we can conclude that methanol had no physiological or practical effect on the C_3 species, *V. vinifera*, and we do not recommend its use in a commercial production scale. Most of the published studies agree that there is no measurable response of methanol to enhance crop quality or quantity, and our study is another to support that claim.

Table 2.4. Effect of methanol on bud cold hardiness of Muscat blanc determined by differential thermal analysis and expressed as low temperature exotherm (LTE, C) at different dates of collection. Data are means ± SEM[x].

Sample date	LTE (C)	
	Control	Methanol
2 Dec 1993	-23.7 ± 0.3	-21.8 ± 1.6
13 Jan 1994	-22.2 ± 0.9	-21.9 ± 1.0
8 Feb 1994	-23.0 ± 0.8	-23.2 ± 0.5
2 Mar 1994	-21.9 ± 0.3	-21.5 ± 0.6
16 Mar 1994	-12.6 ± 1.0	-13.5 ± 0.6
22 Mar 1994	-13.9 ± 1.2	-12.9 ± 0.5
5 Apr 1994	-14.2 ± 1.0	-12.3 ± 0.8

[x] = Treatments are foliar applications as follows: control = water + 0.1% Triton X100; methanol = 90% methanol + 0.1% Triton X100

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CHAPTER 3

SEASONAL CARBOHYDRATE CHANGES AND COLD HARDINESS OF CHARDONNAY AND RIESLING GRAPEVINES

INTRODUCTION

Grand Junction, Colorado, has an ideal growing-season climate for the production of high quality wine grape cultivars. Although abundant sunshine, low relative humidity, cool night and hot day temperatures, and a reasonably long frost-free season are ideal for must and wine quality, high-elevation sites can experience damaging winter temperatures with unpredictable frequency (Hamman 1993). Accordingly, physiological and biochemical information, providing insight into mechanisms associated with resistance to freezing injury, is essential for the development and implementation of management practices which may minimize injury. This study was designed to characterize the cold hardiness response of Chardonnay and Riesling grapevines relative to seasonal environmental changes and to determine if the metabolism of one or more soluble sugars may be associated with cold hardiness.

Soluble sugars are generally known to increase, and starch to decrease at the onset of cold acclimation in a number of woody plant species (Levitt 1980, Sakai and Larcher 1987). Decreases in starch and/or increases in soluble sugars of cane tissues have also been associated with acclimation and cold hardiness in grapes (Gorozova 1978, Pickett and

Cowart 1941, Richey and Bowers 1924, Schrader 1924, Wample et al 1993, Winkler and Williams 1945). Recently, Wample and Bary (1992) demonstrated that fructose, glucose and sucrose were highest during the dormant, cold hardy state in buds of Cabernet Sauvignon grown in Washington state. Contrary to prevailing opinion, total soluble carbohydrates have not been found to indicate the state of cold hardiness in many plant species; i.e. high levels of total soluble sugars do not necessarily coincide with increases in cold hardiness (Stushnoff et al 1993). On the other hand, some specific sugars, especially the galactosides of sucrose such as raffinose and stachyose, have been observed to change in accordance with acclimation to cold temperatures (Hinesley et al 1992, Sauter and Cleve 1991, Sauter and Kloth 1987, Stushnoff et al 1993). Stushnoff et al (1993) found that raffinose and stachyose were the only specific soluble sugars which were statistically associated with season-long cold hardiness status in the cold hardy cv. Valiant (*Vitis labrusca* x *riparia)* and that total soluble sugars were not related to cold hardiness.

Raffinose family oligosaccharides (RFO) have been shown to be strongly associated with cold hardiness of several woody species; wherein, endogenous concentrations of raffinose and stachyose, but not sucrose, increase during cold acclimation and decrease with loss of hardiness (Stushnoff et al 1993). However, the plant species studied (*Amelanchier alnifolia* Nutt., *Cornus sericea* L., *Malus baccata* x *domestica, Prunus besseyi* Bailey, *Prunus virginiana* L., *Ribes rubrum* L., and *Vitis riparia* x *labrusca*) are extremely cold hardy (LT_{50} below -40C), and it is possible that cold-tender plants such as European wine grapes have different cryoprotectant mechanisms or lack regulation of biosynthetic pathways necessary for optimal development of cryoprotection.

In this investigation, we evaluated cold hardiness of two relatively cold-tender European grape cultivars from August 1992 to April 1993. The soluble sugars were monitored individually during this period to examine the relationship between cold hardiness and the most predominant soluble carbohydrates. Another objective was to determine the influence of crop load on cold hardiness and sugar production by comparing tissues from vines harvested during late September, with fruit at 22° Brix, with those from vines with fruit at 28° Brix harvested after the first frost of 1 November 1992. It was hypothesized that late-harvested fruit might accumulate sugar at the expense of the cane and bud tissues, thereby, altering cold hardiness.

MATERIALS AND METHODS

Plant materials

Dormant buds and cortical tissues were collected from canes of healthy six-year-old vines of *V. vinifera* cv. Chardonnay and Riesling grown at Orchard Mesa Research Center, Grand Junction, Colorado. The cordon-pruned vines were trained to a six-wire vertical, shoot positional trellis system, grown under clean cultivation with a fall cover crop of winter wheat sown 20 September 1992, and irrigated to maintain 75% field capacity as determined by a neutron probe. All normal production practices were followed. In this experiment, the normal harvest date was based on fruit sugars attaining about 22° Brix. The normal harvest date for Chardonnay and Riesling was 11 September 1992 and 7 October 1992, respectively. The late harvest date was 3 November 1992, following the first fall frost (-3C) of 1 November 1992. Late harvested fruits of Chardonnay and

Riesling attained 28° and 26° Brix, respectively.

Sample collection of plant material and fruit was from treatments assigned at random to four replications of vines grown in a randomized block design. Samples were collected at approximately two-week intervals, from 1 September to 6 October 1992, and thereafter at three- to four-week intervals until 18 April 1993. Bud break was considered to be 21 April 1993 for Chardonnay and 23 April 1993 for Riesling.

Determination of bud cold hardiness

A. Lowest survival temperature by visual examination (LST_{100})

Dormant buds were harvested with a 20-mm long section of stem on each harvest date. These buds were placed in plastic bags to prevent moisture loss and subjected to gradual freezing by lowering the temperature 2C/hr. Four samples were removed at 2.5C intervals, at each of four stress temperatures chosen to span the probable lethal temperature at a particular sampling date. The test buds were thawed and maintained at room temperature in sealed plastic bags for seven days, at which time they were sectioned free-hand and examined for oxidative browning, an indicator of lethal freeze injury (Stergios and Howell 1973). The temperature at which no injury (100% survival) was detected, was recorded as the lowest survival temperature (LST_{100}) for the sample date.

B. Low temperature exotherm (LTE)

Cold hardiness was also determined by monitoring the low temperature exotherms (LTE) detected at the ice nucleation temperature for overwintering buds. Copper constantan, 36 gauge, thermocouples were attached to the outside surface of each bud with parafilm. Seven test buds and a dried reference bud of approximately equal weight were

subjected to controlled-rate freezing at 2C/hr in an aluminum block placed in a Tenny Jr. programmable freezer (Tenney Inc., South Brunswick, NJ). Temperature data were recorded every second from 0C to -30C using an Omega WB-AAIB high resolution interface card (Omega, Stamford, CT). Differential thermal analysis freezing curves were plotted from stored data using Axum graphics software (Trimetrix Inc., Seattle, WA). LTEs for the overwintering buds were recorded as the killing temperatures (Fig. 3.1).

Quantitative determination of soluble sugars

On each sample date (eleven), mid-cane internode segments, approximately 10 cm long, were frozen and shipped by overnight delivery with dry ice in insulated styrofoam containers from Grand Junction to Fort Collins. Unexpended dry ice was always present upon arrival. The samples were immediately plunged into liquid nitrogen, lyophilized in a freeze-dryer, then stored at -20C in a desiccated environment until chemical analysis was performed either by gas chromatography (GC) or high performance liquid chromatography (HPLC)(see example of chromatograms in Fig. 3.2). In this study, HPLC analysis was used, but in other studies (see chapter 4) GC analysis was used. Both methods are described in the following sections.

A. High Performance Liquid Chromatography (HPLC)

Three to five internodal stem segments (with the bark removed) were combined in each replicate and ground with a Wiley Mill using a 40-mesh screen, further pulverized with a mortar and pestle and passed through a 100-mesh stainless steel screen. About 1 mg of screened sample was solubilized in 1 mL, 100 mM NaOH, centrifuged for five

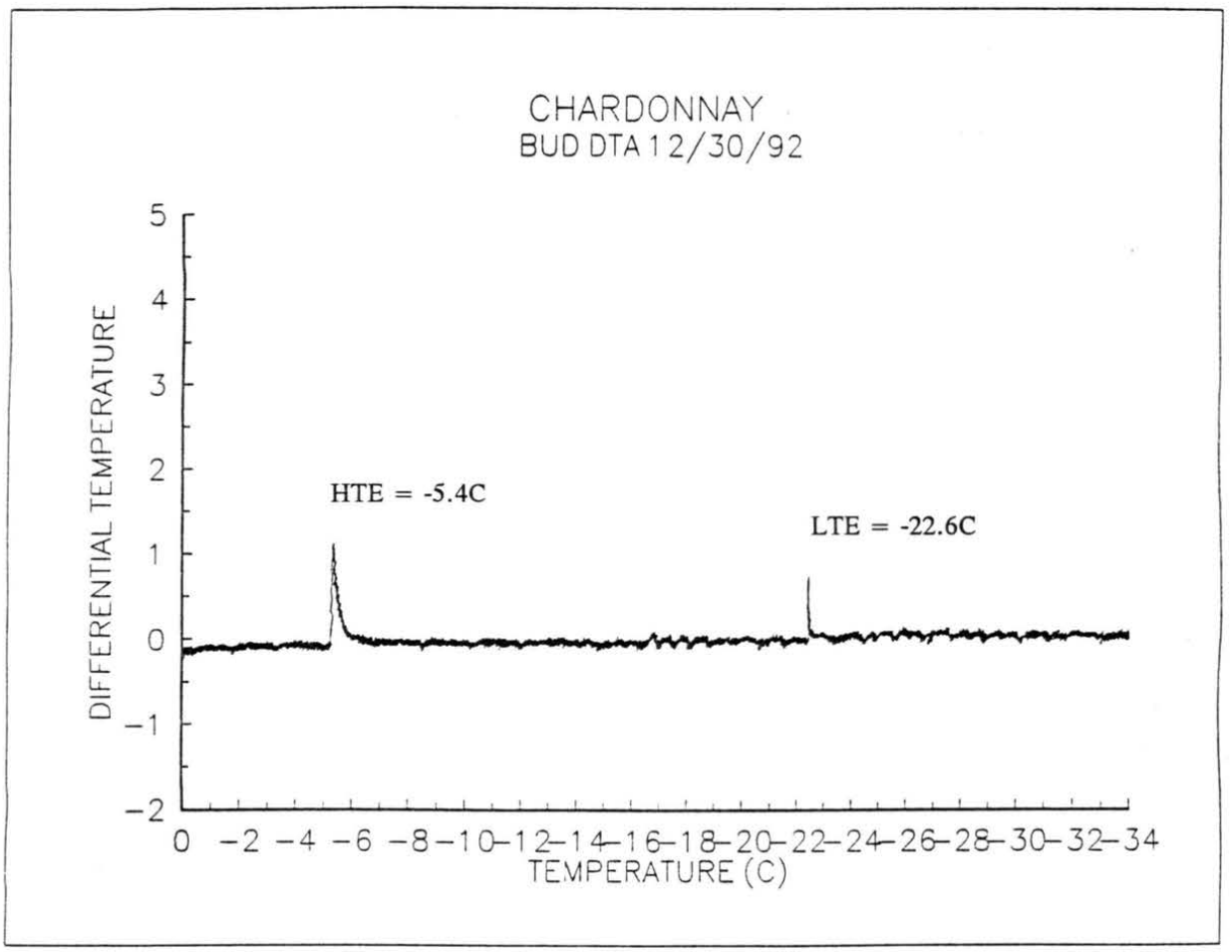

Figure 3.1. Differential thermal analysis profile of Chardonnay primary bud collected on 30 December 1992, and frozen at a rate of 2C/hr. The peak occurring at warmer temperature is the high temperature exotherm, or HTE which represents a freezing event at the extracellular level (no injury). The peak occurring at lower temperature is the low temperature exotherm, or LTE which represents a freezing event at the intracellular level (injury).

minutes at 4C, at 10 000 rpm, and filtered through a 0.22 μM Whatman Nylon 66 syringe filter. The filtrate was analyzed using a Dionex DX-300 series HPLC system (Dionex Co, Sunnyvale, CA), equipped with a 25-μL injection loop. Oligosaccharides were separated on a Carbopac PA-100 ion exchange column (4.6 x 250 mm) using a flow rate of 1 mL/min at ambient temperature, equipped with a Dionex guard column (3 x 25 mm). A pulsed electrochemical detector was used for detection of oligosaccharides. An eluant gradient was used to optimize the oligosaccharides separation. NaOH concentration was linearly increased from 70 mM to 120 mM in 18 minutes, combined with sodium acetate concentration held at 3 mM for 10 minutes, then increased to 35 mM for the last 8 minutes. Quantitation of carbohydrate content was calculated by comparing samples with standard concentration calibration curves from authentic standard sugar solutions. The concentration of each sugar was automatically computed and expressed on a moles per gram of dry weight basis. Two to three extractions were analyzed for each of the four replicates.

B. Gas Chromatography (GC)

Unlike HPLC analysis, soluble sugars in plant samples must be derivitized (rendered volatile) prior to injection into the GC and an internal standard is used. A 25-μL aliquot of internal standard containing 1g/mL of α-D-glucopyranoside was injected into individual 13 x 80 mm test tubes. The internal standard solution was blown dry and measured amount of dried ground sample (about 1mg) was added. Derivitization was done by adding 0.4 mL of pyridine, 80 μL of hexamethyldisilylazane, and 40 μL of chlorotrimethylsilylazane to each tube containing weighed sample and dried internal

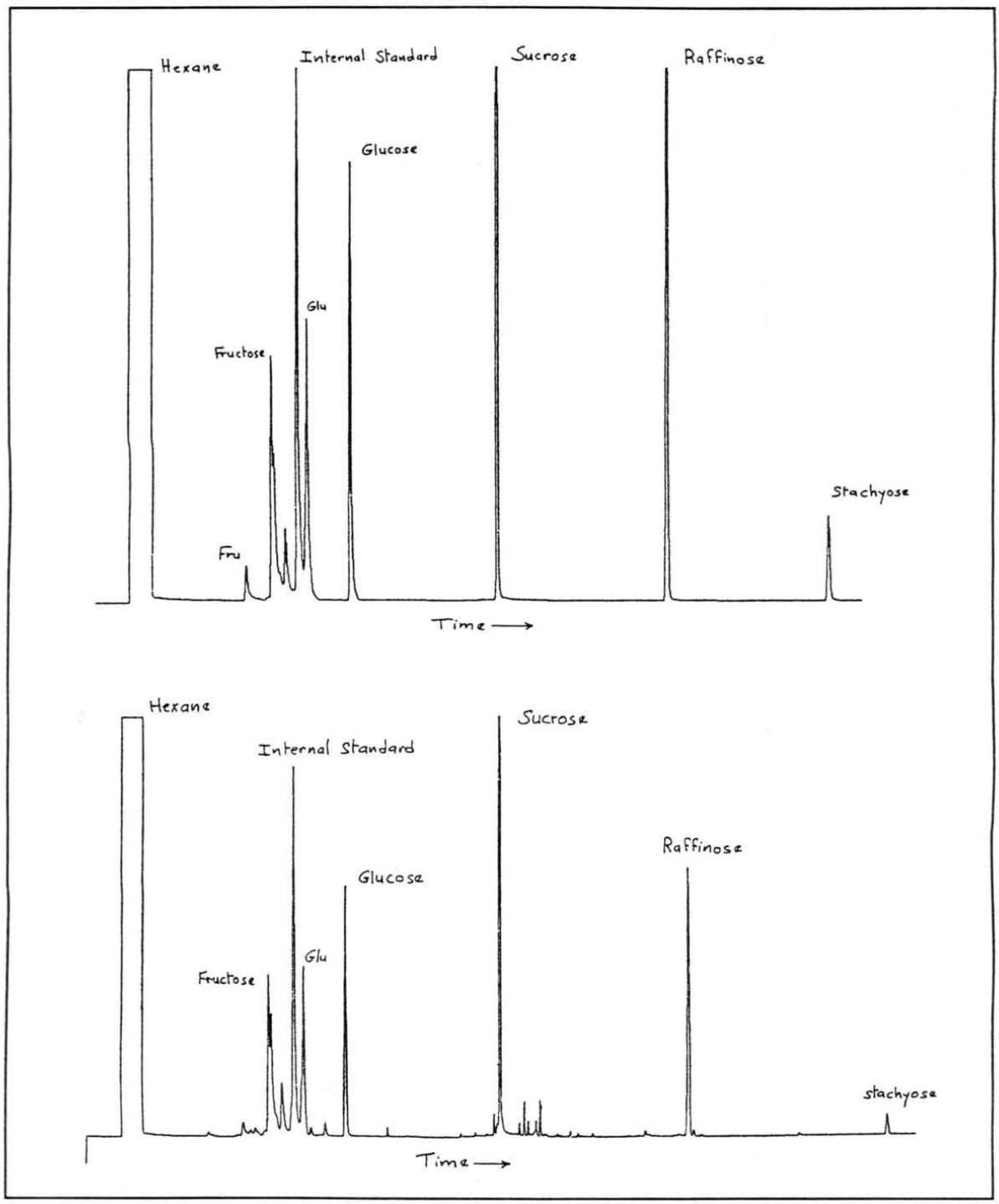

Figure 3.2. Chromatograms illustrating separation of authentic standard sugars plus internal standard, all dissolved in hexane (top) and separation of the same sugars present in internode tissue samples of Chardonnay (bottom).

standard, placing a screw top firmly on each tube and placing tubes in a heating block maintained at 80C for 20 minutes. After removing tubes from heating block, screw caps were removed and samples were blown down until only non-volatile residues remained. Hexane was added (0.5 mL) to each tube and sugar derivatives were phase-separated from remaining residue. New tubes containing the transferred hexane solution were blown dry and 0.2 mL of hexane was added to attain a desired sample concentration to inject into the GC (Sweeley et al 1963). The GC used is an HP-5890-Series II, equipped with a flame ionization detector (Hewlett-Packard Corp, Avondale, PA), and a fused-silica microcapillary column, DB1-30W (Length = 30m; internal diameter = 0.25 mm; film thickness = 0.25 μm, J & W Scientific, Folsom, CA). The carrier gas is helium (99.999% purity) with a flow rate of 2 mL/min. The separation of the sugar derivatives of interest with a wide range of boiling points requires programmed oven temperatures with multiple steps set up as follows: (1) initial T1 = 180C; (2) time at T1 = 2 min; (3) rate 1 = 6C/min; (4) final T1 = 215C; (5) rate 2 = 40C/min; (6) final T2 = 325C; (7) time at T2 = 20 min. The concentration of each soluble sugar was computed and expressed in moles per gram of sample dry weight.

Statistical analyses

Correlation analyses were conducted to determine the association among the following factors: injury estimated by assessment of visual browning for LST_{100} and LTE for overwintering buds; mean outdoor minimum temperature for 2, 4, 7, 10, and 15 days preceding sampling date and LST_{100} of buds and endogenous levels of fructose, glucose, sucrose, raffinose and stachyose in internodes. An unpaired t-test was used to compare

differences in bud cold hardiness based on LST_{100} and on LTE for canes taken from vines on the normal and late harvest dates.

RESULTS AND DISCUSSION

Cold hardiness and the environment

Cold hardiness of Chardonnay and Riesling dormant buds, for both normal- and late-harvested vines, was closely associated with the mean minimum temperature preceding determination of cold hardiness (Table 3.1). The lowest temperature experienced was -17C on 26 December 1992, and according to results of the controlled freezing tests, at no time in 1992-1993 did outdoor minimum temperatures drop low enough to injure the buds of either cultivar (Fig. 3.3). Bud injury, evaluated by tissue browning (LST_{100}), produced results similar to evaluation of injury by monitoring LTEs for the buds in both cultivars (Fig. 3.3, 3.4).

For both normal- and late-harvested vines, endogenous levels of fructose, glucose, raffinose and stachyose, but not sucrose, were directly related to low temperatures from 2 to 15 days preceding analyses (Table 3.1) and to cold hardiness status 25 August 1992 through 30 March 1993 (Table 3.2). We cannot imply from this study that increasing accumulation of any one of these sugars in internodes causes an increase in bud cold hardiness; however, low temperature and endogenous levels of fructose, glucose, raffinose and stachyose in internodes are very strongly associated with bud cold hardiness in these two cold-tender cultivars.

Table 3.1. Linear correlation coefficients (r) for soluble sugars (moles/g dw) in internodes and bud cold hardiness (determined by visual browning, lowest survival temperature with no injury [LST_{100}]) and the mean minimum temperature (C) for 2, 4, 7, 10, 15 days preceding sampling for Chardonnay and Riesling grapevines. Data are based on means of four replicates for each collection date.

Cultivars	Days preceding sampling	Glucose	Fructose	Sucrose	Raffinose	Stachyose	LST_{100}
Chardonnay	2	-0.76*	-0.81**	-0.47 ns	-0.77**	-0.88***	0.93***
	4	-0.82**	-0.88***	-0.58 ns	-0.84**	-0.92***	0.96***
	7	-0.84**	-0.91***	-0.60 ns	-0.83**	-0.92***	0.97***
	10	-0.83**	-0.90***	-0.60 ns	-0.84**	-0.92***	0.97***
	15	-0.82**	-0.89***	-0.59 ns	-0.82**	-0.90***	0.97***
Riesling	2	-0.71*	-0.73*	-0.51 ns	-0.81**	-0.82**	0.93***
	4	-0.80**	-0.83**	-0.62 ns	-0.88***	-0.88***	0.95***
	7	-0.83**	-0.85**	-0.64*	-0.88***	-0.89***	0.96***
	10	-0.83**	-0.86**	-0.64*	-0.89***	-0.90***	0.96***
	15	-0.80**	-0.84**	-0.61 ns	-0.87**	-0.87***	0.96***

* = P(.05), ** = P(.01), *** = P(.001)

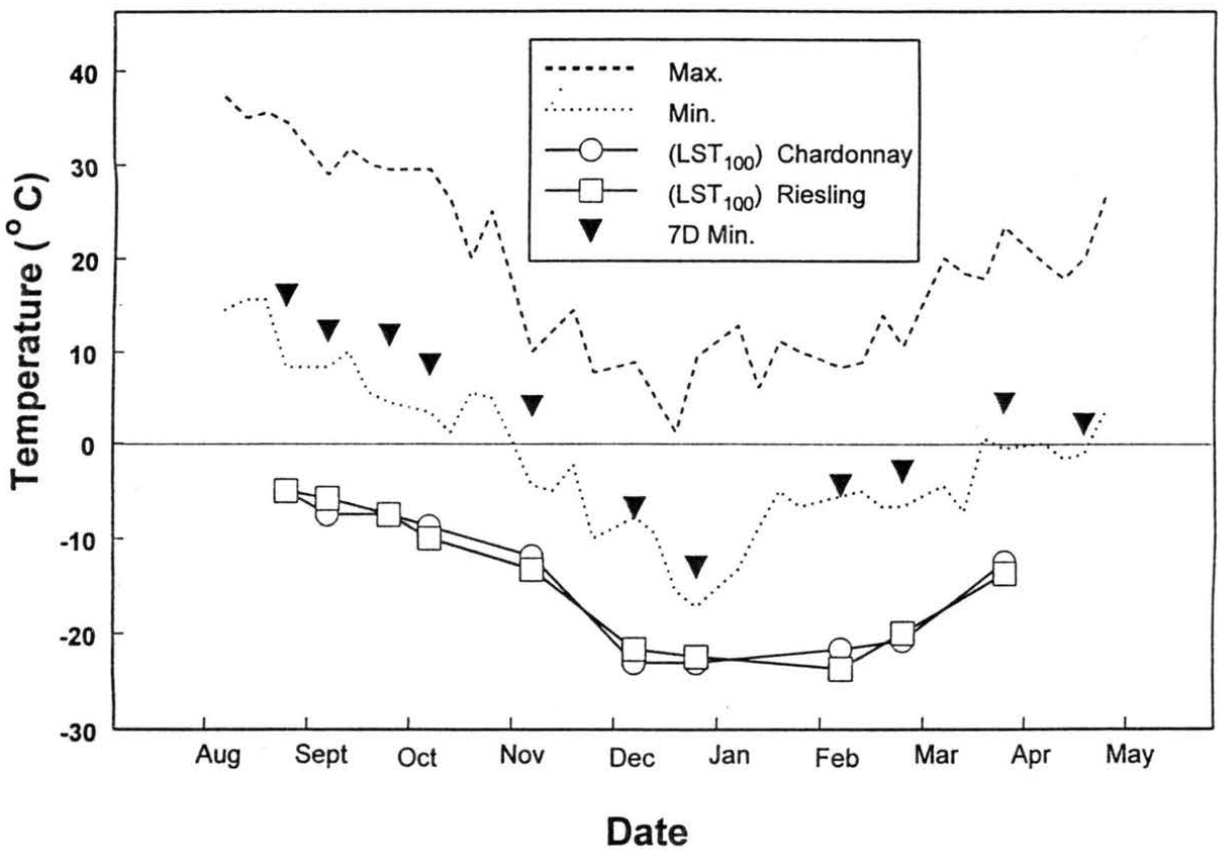

Figure 3.3. Seasonal changes in bud cold hardiness in Chardonnay and Riesling grapevines, based on lowest survival temperature (LST_{100}), relative to weekly maximum and minimum temperatures (C). ▼ denotes the average minimum temperature for seven days prior to the sample date of cold hardiness tests and biochemical analysis.

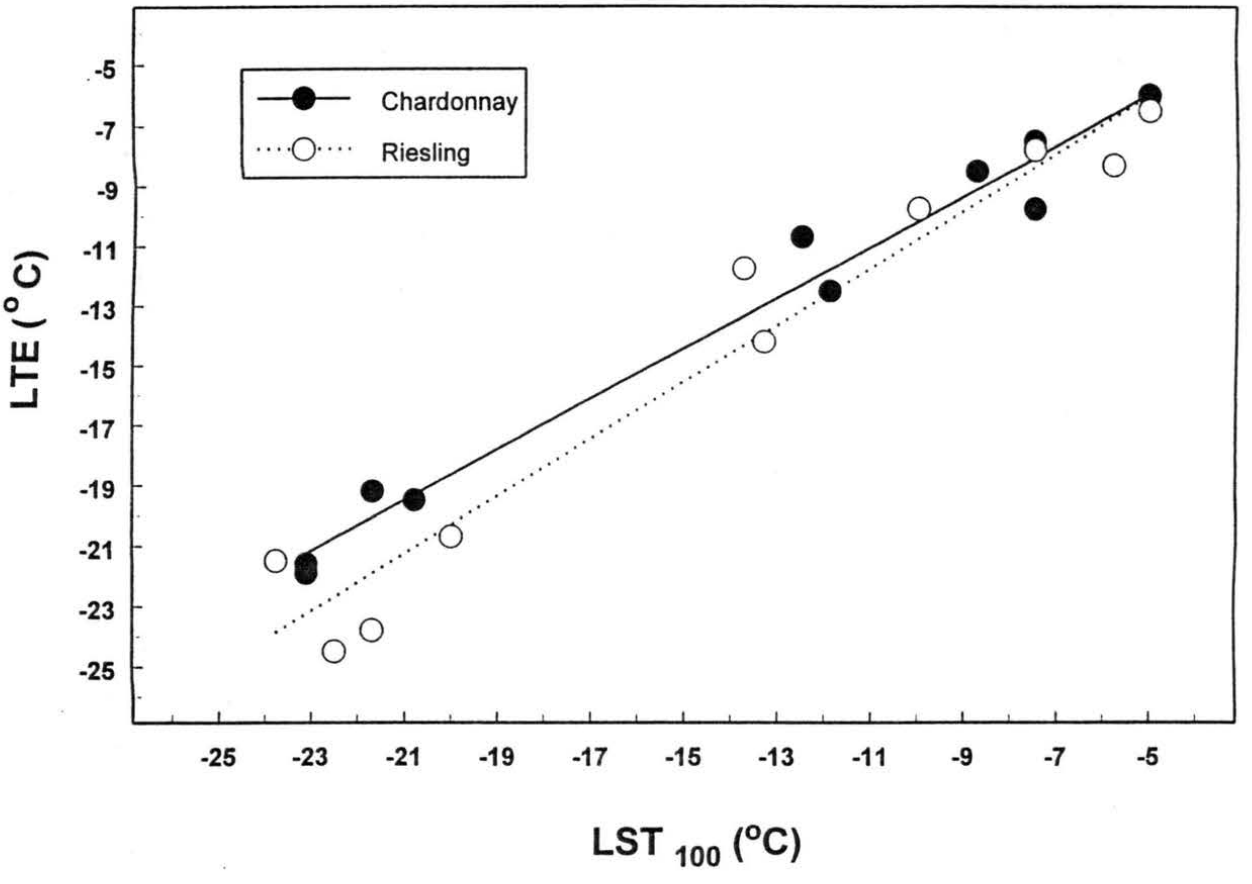

Figure 3.4. Relationship of bud cold hardiness in Chardonnay and Riesling grapevines, based on lowest survival temperature (LST_{100}), and low temperature exotherms (LTE), determined by regression analysis. Data are based on means of ten dates from August through March.

Table 3.2. Linear correlation of bud cold hardiness (lowest survival temperature with no injury [LST_{100}]), 25 August 1992 to 30 March 1993, with internode soluble sugars (moles/g dw) for Chardonnay and Riesling grapevines. Data are based on means of four replicates for each collection date.

Cultivars	Coefficient and probability	Glucose	Fructose	Sucrose	Raffinose	Stachyose
Chardonnay	Correlation (r)	-0.76	-0.88	-0.55	-0.83	-0.92
	Probability (p)	.01	.0007	.09	.003	.0001
Riesling	Correlation (r)	-0.71	-0.75	-0.50	-0.84	-0.82
	Probability (p)	.02	.01	.1	.002	.003

Soluble sugars

At the end of the growing season (August, September, October), sucrose was the most predominant reserve sugar for Chardonnay and Riesling internodes (Fig. 3.5, 3.6). Glucose and fructose were also found in lesser amounts. RFO were present in even lower quantities in the stems of both cultivars. As the mature shoots began to cold acclimate, RFO levels increased slowly but steadily. Sucrose, glucose and fructose, however, remained at almost the same levels until the first frost.

About one month after the first frost (1 November 1992), there was a major change in the sugar content of internodes (Fig. 3.5, 3.6). Glucose and fructose increased markedly and peaked in late December. The rise in monosaccharides was accompanied by an increase in RFO. These sugars reached their maximum levels during mid-winter, then started to decline with the other sugars as the spring season approached. Furthermore, despite the presence of the same sugars throughout this period, their proportions relative to total soluble sugars changed substantially, especially the monosaccharides and disaccharide. In mid-winter (December, January, February), the monosaccharides (glucose + fructose) and sucrose represented 60-70% and 25-33% of the total soluble sugars, respectively (Table 3.3). These proportions were reversed during the de-acclimation season (late spring and summer), when monosaccharides ranged between 27% and 47% of the total soluble sugars, and disaccharides 50-70%. The ratio of the monosaccharides to disaccharide [(Glucose + Fructose) / Sucrose] indicates, in a striking manner, the sugar changes during the season (Table 3.3). For example, the ratio in Chardonnay was lowest in late August (0.53), early September (0.38) and again in March

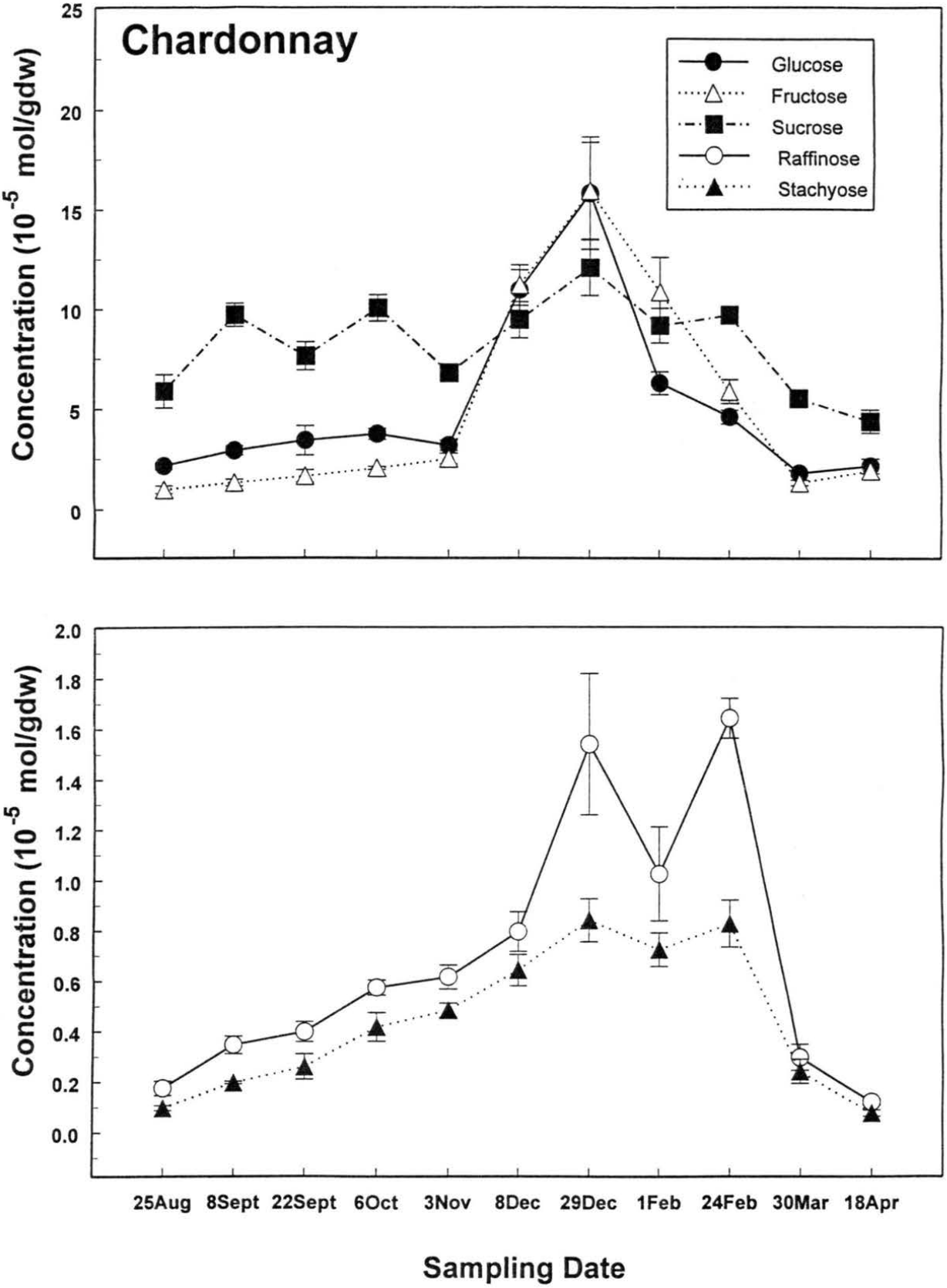

Figure 3.5. Seasonal changes in soluble carbohydrates derived from cane internode tissues of Chardonnay grapevines, 25 August 1992 to 18 April 1993 at Grand Junction, Colorado. Data are means ± SE from four replicates.

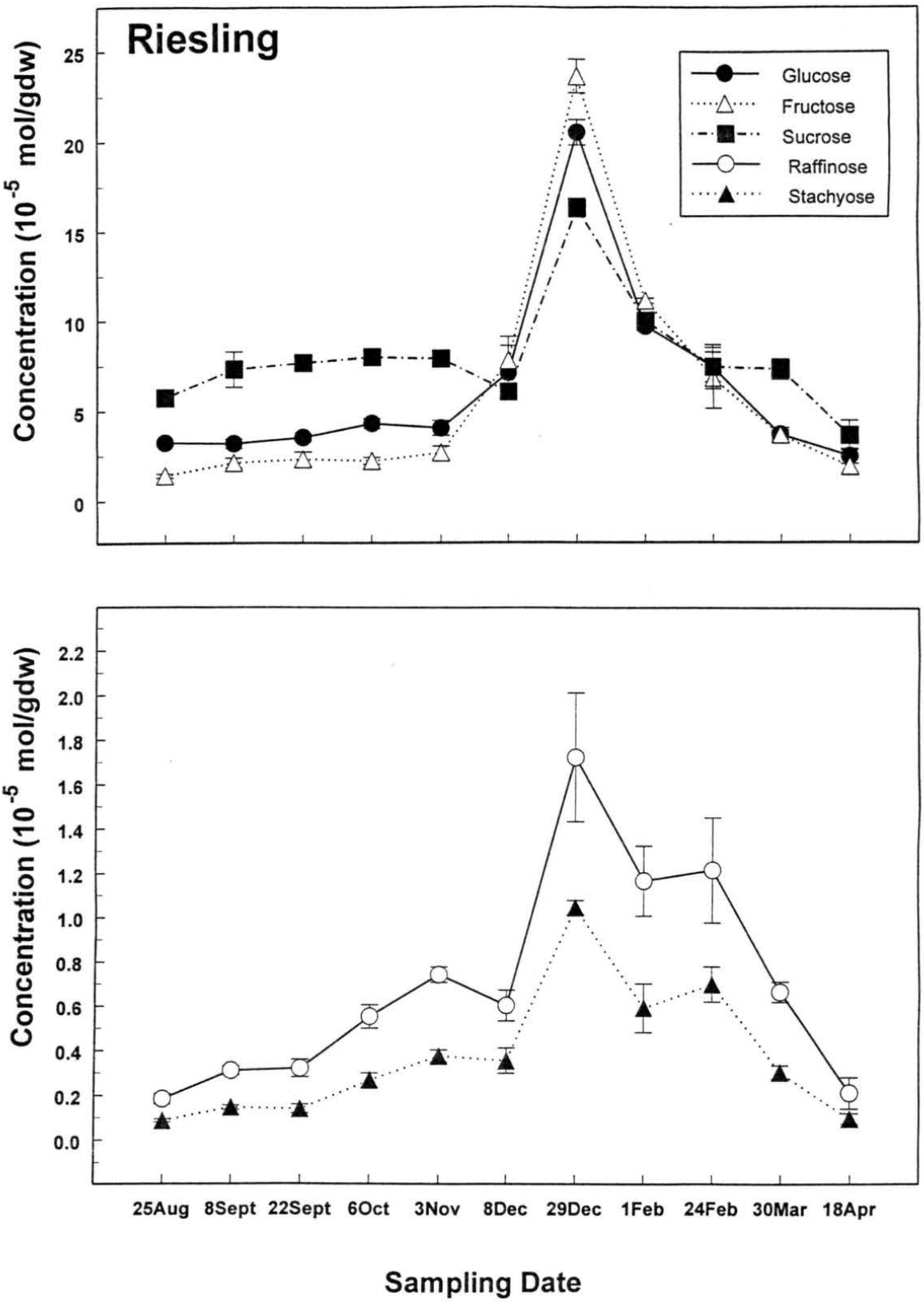

Figure 3.6. Seasonal changes in soluble carbohydrates derived from cane internode tissues of Riesling grapevines, 25 August 1992 to 18 April 1993 at Grand Junction, Colorado. Data are means ± SE from four replicates.

Table 3.3. Proportion of monosaccharides and sucrose to total soluble sugars in internode tissues of Chardonnay and Riesling grapevines from August 1992 through March 1993.

Date of collection	Chardonnay				Riesling			
	Glucose (%)	Fructose (%)	Sucrose (%)	(Glu + Fru)/ Sucrose	Glucose (%)	Fructose (%)	Sucrose (%)	(Glu + Fru)/ Sucrose
25 Aug	23.1	10.5	63.4	0.53	30.4	13.2	53.9	0.81
8 Sept	18.4	8.4	69.7	0.38	24.4	16.2	55.9	0.72
22 Sept	25.5	12.3	57.2	0.66	25.3	16.6	54.8	0.76
6 Oct	22.3	12.2	59.7	0.58	28.0	14.5	52.1	0.81
3 Nov	23.4	18.6	49.9	0.84	24.5	15.7	52.6	0.76
8 Dec	33.1	34.0	28.6	2.34	32.4	35.6	27.6	2.46
29 Dec	34.3	34.5	26.1	2.63	32.4	37.4	25.8	2.70
1 Feb	22.4	38.7	32.7	1.86	29.8	34.1	30.7	2.08
24 Feb	20.3	26.0	42.8	1.08	31.4	28.9	31.5	1.91
30 Mar	19.6	14.4	60.0	0.57	23.5	23.4	46.8	1.00

(0.57), when the buds and stems were most cold sensitive. This ratio reached its maximum (2.63) in late December when the buds were the hardiest.

Extremely cold hardy species such as currant, dogwood and chokecherry (LT_{50} from -60C to -80C) produce substantial amounts of RFO, but undetectable amounts of monosaccharides during winter (Stushnoff et al 1993). Amounts of RFO correlate strongly with cold hardiness in cold hardy taxa as measured by LT_{50} (Stushnoff et al 1993). In addition, the cold hardy cultivar, Valiant, accumulated raffinose, stachyose, sucrose and glucose but not fructose (Stushnoff et al 1993); however, only raffinose and stachyose correlated significantly with cold hardiness, whereas sucrose and glucose did not.

Although other studies have implicated RFO as cryoprotectants (Hincha 1990, Koster and Lynch 1992, Stushnoff et al 1993), it is not clear whether glucose and fructose play a significant role as cryoprotectants. This study suggests they may play such a role because of their abundance and association with hardiness. Another interpretation is that their accumulation pattern may interfere with RFO synthesis and thus limit cold hardiness. This may explain the differences in cold hardiness potential observed between European grapes, Chardonnay and Riesling (about -25C), compared to American grapes such as Valiant (about -40C). The monosaccharide synthesis pathway may utilize sucrose and interfere with the RFO pathway, which uses sucrose as a substrate for the synthesis of raffinose and stachyose (Dey 1990). This shift may result in reduced synthesis of RFO, providing only limited cryoprotection, such as found in *V. vinifera* dormant buds. This hypothesis also assumes that the monosaccharides do play a limited role in freeze protection.

Normal versus late harvest

No consistent or insightful trends were detected in vines subjected to a late harvest after the first killing frost on 1 November 1992. Comparisons for each sampling date and cultivar, using an unpaired t-test, revealed only a few significant differences (Table 3.4). There were no meaningful trends relating soluble carbohydrate levels to cold hardiness of either cultivar for normal versus delayed harvest. LTEs for both Chardonnay and Riesling were significantly lower from buds of late-harvested vines tested 3 November 1992, and for Riesling tested 24 February 1993, but there were no corresponding differences using LST_{100} to evaluate injury. Wample and Bary (1992) concluded that neither delaying harvest nor failing to harvest Cabernet Sauvignon vines adversely influenced cold hardiness of vines grown at Prosser, Washington from 1985 to 1988.

Grand Junction, Colorado is a very suitable high-elevation site for high quality wine grape production. It has high light intensity, high daytime temperatures and cool night temperatures. Unfortunately, the high elevation is also accompanied by a significant risk of low temperature injury to *V. vinifera* during the dormant season. Growers could benefit significantly if a single production practice, such as avoiding a late harvest, would influence winter hardiness, as suggested by observations in several other growing areas. On the basis of these data and three years of data from Prosser, Washington (Wample and Bary 1992), we conclude that with normally high photosynthetic conditions and no other apparent limiting growth factors, there is no adverse influence of a delayed fruit harvest on bud hardiness nor on accumulation of soluble carbohydrates in cane internodes.

Table 3.4. Endogenous sugars (10^{-5} mol/g dry weight) and cold hardiness (LTE temperature) of Chardonnay and Riesling dormant buds from vines harvested at 22 °Brix compared to vines late-harvested at 28° Brix. Non-significant data for other sugars are not shown.

Sample date	Glucose		Sucrose		LTE (C)	
	N[x]	L[y]	N	L	N	L
Chardonnay						
3 Nov	3.18	3.27	6.79	8.08	-12.5	-17.8**
8 Dec	10.95	11.41	9.44	10.57	-21.9	-23.5
29 Dec	15.85	20.15	12.08	14.72	-21.6	-18.7
1 Feb	6.28	7.07	9.12	9.15	-18.8	-21.9
24 Feb	4.60	5.66	9.58	9.68	-19.5	-18.7
30 Mar	1.80	1.91	5.51	7.11*	-10.7	-9.9
Riesling						
3 Nov	4.03	4.22	7.87	7.14	-14.2	-16.7**
8 Dec	-	-	-	-	-23.8	-24.8
29 Dec	20.48	24.22*	16.29	14.94	-24.5	-23.3
1 Feb	9.64	8.07	9.93	11.01	-21.5	-19.5
24 Feb	7.37	7.31	7.39	9.44	-20.7	-25.5**
30 Mar	3.65	4.07	7.27	7.35	-11.7	-9.3

* = P(.05), ** = P(.01)
x = Normal harvest (Chardonnay: 11 Sept 1992; Riesling: 7 Oct 1992)
y = Late harvest (Chardonnay and Riesling: 3 Nov 1992)

CONCLUSIONS

A strong association between cold hardiness and endogenous content of several sugars was found. This may be a good starting point to search for biochemical mechanisms that may be responsible for stabilization of grape tissues during freezing stress. Because the monosaccharide synthesis pathway is stimulated during dormancy as cold hardiness increases, it probably interferes with the RFO pathway, which uses sucrose as a substrate for the synthesis of raffinose and stachyose. This 'competition' may lead to restricted synthesis of RFO, limiting protection to the extent of that found in *V. vinifera* dormant buds, in contrast to much higher levels of RFO found in cold hardy Valiant cultivar and other woody plant species. With the cold-tender cultivars such as Chardonnay and Riesling, a high monosaccharides to disaccharide ratio is a better indicator of cold hardiness than RFO levels per se.

If good conditions exist for growth, such as optimal photosynthesis and no apparent abiotic or biotic stress, there is no adverse influence of a delayed fruit harvest on bud hardiness nor on endogenous accumulation of soluble carbohydrates.

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CHAPTER 4

EFFECT OF ALGINATE ON COLD DEACCLIMATION AND BUD BREAK OF GRAPEVINES

INTRODUCTION

During spring, deciduous fruit trees undergo a dehardening or deacclimation phase, which corresponds to a seasonal transition from a frost-resistant to a frost-susceptible condition (Weiser et al 1979). It is at this stage that plants are the most vulnerable to even mild subfreezing temperatures. Freezes during or after bud break and flower bloom often cause complete crop loss. These losses have occurred more frequently the last few decades for several reasons. Growers have increased the risk of frost injury by extending crops to sites where frost injury is a threat. Furthermore, fruit growers have turned to early maturing cultivars in order to capitalize on high prices for early products in the market. Unfortunately, most early maturing cultivars bloom, or break buds when spring frost is common.

To alleviate these problems, several methods and practices have been applied. Traditional methods have involved the use of wind machines, heaters and irrigation (Rieger 1989). Recently, evaporative cooling has been successfully used on grapevines grown in areas where early deacclimation is a problem (Lipe et al 1992). Evaporative cooling by sprinkling during the period of degree-hour accumulation in late winter slowed the rate of bud development of Chardonnay and Cabernet Sauvignon sufficiently to reduce the risk of

freezing injury (Lipe et al 1992). These methods, however, usually are prohibitively expensive and thus are not widely used by growers who seek low cost-effective means of frost protection.

The use of chemicals is a more attractive method because of the low cost and ease of application, but also poses a threat to the environment. Several chemical products such as growth regulators, anti-transpirants, dormant oils and cryoprotectants have been used in order to increase cold hardiness and/or delay bud break of horticultural crops (Rieger 1989). Although these chemical products have the potential to provide freeze protection and prevent crop damage, their effectiveness has been inconsistent. Growth regulators such as ethylene-releasing compounds, gibberellic acid, naphthalene acetic acid and paclobutrazol have been reported to play a more significant role in delaying flower bloom or bud break than increasing freezing resistance of several deciduous species (Ahmedullah et al 1986, Crisosto et al 1989, Patterson and Howell 1995, Proebsting and Mills 1985). The use of anti-transpirants has been unsuccessful in providing frost protection in peach, plum and almond (Rieger and Krewer 1988) and tomato and pepper (Perry et al 1992), while the application of dormant oils has been effective in delaying bloom and/or increasing cold hardiness of peach (Call and Seeley 1989, Myers et al 1996). The effectiveness of commercially available cryoprotectants has varied according to the product, plant species, site and time of application (Gardea et al 1993, Himelrick et al 1991, Perry et al 1992). The consensus from literature is that commercial cryprotectants have not been as effective as advertised, and they usually have little or no effect in field trials.

The objective of this study was to develop a new product and evaluate its effectiveness under laboratory and field conditions. The attempt was to delay bud break and/or slow down deacclimation of grapevines, including *Vitis vinifera* cv. Chardonnay. A delay in the deacclimation and bud break of grapevines by a few days in the spring could mean a difference between a full crop and no crop at all.

MATERIALS AND METHODS

Effect of alginate before deacclimation (94/95 season)

Alginate treatment was first tested in the field during the 1994/1995 season. Two treatments were used, control and alginate [3% alginate (w/v) + 0.5 M sucrose]. The alginic acid used in this experiment is in a salt form, sodium alginate of medium viscosity and extracted from *Macrocystis pyrifera* (Kelp) (Sigma Co, St. Louis, MO). The treatments were tested on five-year-old bilateral-cordon-trained Chardonnay vines (*V. vinifera*), growing at the Orchard Mesa Research Center, Grand Junction, CO. Vertically-positioned canes were cut back to 15-20 buds per cane before application. This was not typical pruning, but a procedure undertaken in order to avoid excessive application of materials. The alginate treatment was first sprayed with a 1-quart hand-paint-sprayer driven by a tractor-mounted-compressor, followed by another spray of 3% calcium chloride using a 15-Liter Solo backpack sprayer (Model 425, Solo Inc., Newport News, Virginia). Calcium chloride was used to harden the viscous solution by cation cross-linking reaction. The experiment consisted of three treatment applications on 16 November 1994, 4 January 1995, and 7 February 1995. Three collections were made 2, 4, and 6 weeks following each application.

Samples consisted of single-bud cuttings collected from the basal parts of the vertically-positioned canes. A differential thermal analysis (DTA), described previously, was conducted on each collection date.

Effect of alginate during deacclimation (95 season)

The purpose of this experiment was to investigate whether the alginate treatments had an effect on slowing bud and cane cold deacclimation or dehardening in late winter and early spring, after the grapevines had satisfied their chilling requirements (ecodormancy). The experiment consisted of treatment application at dates following maximum cold hardiness, which is usually reached in the month of January (Hamman et al 1996). Spray dates and data collected at each date are described as follows.

Experiment 1 consisted of 7 treatments applied on 9 February 1995. Six treatments contained the basic two elements, alginate and sucrose, to which adjuvants (NuFilm, Triton X100) or cryoprotectant (glycerol) were added. In one treatment, Latex paint was added to the alginate and sucrose mix. These treatments were compared to control vines with no treatment. Samples were taken 2 weeks later and consisted of cutting at the base of the cane at bud position 3 or 4. A freezing test was conducted on these twigs to evaluate the killing temperatures of the primary buds and canes (phloem and cambium tissues). Evaluation was based on tissue browning and expressed as LST_{100} (lowest survival temperature for no injury to 100% of buds). The plant tissue was considered alive if it remained green after the freeze test and dead if it turned brown (Stergios and Howell 1973). Visual evaluation of oxidative browning was set up as a completely randomized design. Analysis of variance was run on the data collected and computed means were compared using a Tukey's test at $p = 0.05$.

Experiment 2 evaluated the effect of single vs. multiple application of the best treatment from the previous experiment. An experiment was conducted to determine whether multiple applications were needed to maximize the effectiveness of the treatment. A 'single spray' treatment was applied on 24 February 1995 and 'multiple sprays' were applied weekly on 24 February, 8 March, and 15 March 1995. Samples were collected from the field on 22 March 1995 in order to conduct a freezing test. The killing temperatures of the buds were expressed as LT_{50} (temperature that kills 50% of the samples). LT_{50} were estimated from the Spearman-Karber method, which was used every time the sample size was relatively small. Water content of the twigs from each treatment was also measured. Three replicates were used in this experiment in a randomized complete block.

Experiment 3 consisted of applying the same best treatment from the previous two experiments in the spring on 25 March 1995. The attempt was to evaluate whether an application in the most critical period, when spring frost injuries are likely to occur, may increase the freezing resistance of bud and cane tissues by a few crucial degrees. Samples were collected on 10 April 1995 to determine LST_{100}, water content and both qualitative and quantitative analyses of the main soluble carbohydrates in the cortical tissues (including the cortex, the phloem and the cambium but not the bark). All parameters were compared to control vines with no treatment. The computed means were compared using an unpaired T-test.

Effect of alginate before and during deacclimation (96 season)

In 1996, the experiment was repeated with the following treatments: Control, no treatment; Alg 1, 3% alginate + 1M sucrose; Alg 2, 3% alginate + 1M sucrose + 1% Triton

X100; Alg 3, 3% alginate + 1M sucrose + 10% latex; Alg 4, 3% alginate + 1M sucrose + 20% WiltPruf; Alg 5, 3% alginate + 1M sucrose + 1% Triton X100 + 10% latex; Alg 6, 3% alginate + 1M sucrose + 1% Triton X100 + 5% latex + 20% WiltPruf. All treatments except control were subsequently sprayed with 3% calcium chloride. A few modifications have been made in some treatments to improve the stability and performance of the gels. Triton X100 is a surfactant which makes the alginate solution less viscous and easier to apply on vines. Latex provides a white surface which reflects incident light, and thus reduces the accumulation of heat which may cause deacclimation of canes and buds. WiltPruf is an antidesiccant used to reduce peeling and breakdown of the gels. The application of alginate on Chardonnay was repeated on the following dates: 29 January, 4 March, and 21 March 1996. Samples were collected two weeks after each application date. Also, application of Alg 6 was made on Merlot and Riesling on 21 March 1996.

Effect of alginate on bud break under laboratory and field conditions

The objective of this experiment was to study another aspect of the effect of alginate on bud break. It was hypothesized that if alginate influenced the freezing habits of the dormant buds, this may change its intrinsic characteristics and eventually its physiological responses to the environment. One of the desired responses is a delay in bud break, which would be beneficial in areas with a threat of spring frost such as the Grand Valley in Colorado.

A. Laboratory testing

Canes were collected at random on the following dates: 14 December 1994, 4 January 1995, and 8 March 1995. Samples were packed with ice and sent by overnight delivery to

Fort Collins, where they were placed in plastic bags and kept at -2 ± 1C until use. Single bud cuttings, about 3 cm in length, were excised from canes with bud positions between 4 and 15. Each twig was encapsulated with a gel-like solution of 0.5M sucrose and 3% alginate. The encapsulated twigs were then dipped in a solution of 0.1M calcium chloride for fifteen minutes in order to harden the gel by cation cross-linking reaction. Controls consisted of untreated twigs. The twigs were then stuck in trays that hold individual cells filled with premoistened peat moss. Each tray was covered with a clear humidity dome to keep relative humidity high (above 90%). The trays were placed in a bench top Percival growth chamber set at 25C/20C as day/night temperatures and a 16hr photoperiod. The twigs were incubated for 5-6 weeks before they were evaluated for bud break. Bud break evaluation was based on visual quantification or scoring of bud stage development according to Eichhorn and Lorenz (1977). High scores indicate further advanced phenological stage and vice versa. A score of 5 indicates bud break or bud burst (green shoot first clearly visible through the scales). Twenty to thirty single bud cuttings were used for each treatment. Visual evaluations of buds were subjected to an analysis of variance and mean comparisons were made using an unpaired t-test or student-Newman-Keuls multiple comparisons test at $p = 0.05$.

Another experiment was conducted to evaluate several products and treatments, in addition to the basic alginate and sucrose treatment. An additional goal of this experiment was to evaluate whether delayed bud break of field-treated vines could be detected by laboratory testing. Eight treatments were applied to single-bud cuttings in the laboratory and on intact vines in the field. Twigs for lab treatment were collected on 9 February 1995. Field treatments were applied on 9 February 1995 and collected for incubation in the growth

chamber on 12 February 1995. The same incubation procedures and evaluations were carried out as in previous experiments. Scores were subjected to ANOVA and the means were compared using Tukey's multiple comparison test at $p = 0.05$.

B. Field testing

In this experiment, both application of treatments and evaluations were conducted in the field. The vines were spur-pruned before treatment on 25 March 1995, leaving 2-3 buds per spur and about 30 buds per vine (15 buds on each cordon). Twenty treatments were applied and compared to 2 controls (no treatment and a water-spray treatment). The spray application was directed at the spurs only. The dates of application were 26 and 27 March 1995. Visual evaluations of bud break were made on 10 April 1995. The visual evaluations were categorized in five groups as follows: group 1, number of vines with 0% bud break; group 2, number of vines with 25% bud break; group 3, number of vines with 50% bud break; group 4, number of vines with 75% bud break; group 5, number of vines with 100% bud break. Eight to twelve vine replicates were used in a completely randomized design. Treatments were divided into 5 groups and ANOVA procedures with orthogonal contrasts were used to analyze the data (SAS Institute Inc., Cary, NC). The means were compared to control (untreated) using Dunnett's test at $p = 0.05$.

In the spring of 1996, the experiment was repeated with 16 treatments. The date of application was on 4 April 1996. All treatments containing alginate were subsequently sprayed with 3% calcium chloride. The method of data collection of bud break was modified from that of the previous year. Instead of only one observation as in the spring of 1995, nine observations were made throughout the spring of 1996. Developmental stage 5 was again

used as indication of bud break, and bud break was counted in each vine replicate (5 replicates) on a weekly basis until 100% of bud burst had occurred. Also, treatment application was made on cultivars other than Chardonnay including Pinot noir, and Vignoles. ANOVA was used to compare all treatments and the means were separated from the control mean using Dunnett's test at $p = 0.05$.

RESULTS

Effect of alginate before and during deacclimation

A. Fall 1994/ Winter 1994-1995

No significant differences in cold hardiness were detected between the buds of control and treated vines for 6 sampling dates from 30 November 1994 through 17 February 1995 (Table 4.1). The treatment did not affect hardiness under field testing. There may be at least two reasons; the first, is that the treatment does not affect the freezing capacity of buds during endodormancy; the second, because the treatment peeled rapidly and lost its efficacy. Peeling was observed during this period of time but periodical observations were not made to confirm the problem.

During the last application on 7 February 1995, differences in freezing resistance between the control and treated buds began to appear on the last three sampling dates. On 22 February, the low temperature exotherm (LTE) of control buds averaged -17.9C, while LTE of treated buds averaged -19.3C (Table 4.1). On 8 March, mean LTE of treated buds was about 3C lower than that of control buds. Finally, on 23 March the LTE of treated buds was again lower than that of control (Table 4.1, Fig. 4.1). Although, there are differences in

Table 4.1. Effect of alginate on freezing resistance of primary buds in Chardonnay. Data are means of LTE (low temperature exotherm) ± SD. Data in parentheses are means of LST_{100} (lowest survival temperature with no injury). No statistically significant differences were detected in LTEs between control and treated vines using an unpaired T test at $p = 0.05$; except on 8 Mar 1995, when significant difference was detected in LST_{100}.

Application Date	Collection Date	Control (LST_{100})	Treated*(LST_{100})
16 Nov 1994	30 Nov 1994	-23.3 ± 0.5 (-22.8)	-23.1 ± 0.6 (-22.8)
	14 Dec 1994	-22.4 ± 1.9 (-22.8)	-22.6 ± 2.0 (-22.5)
	4 Jan 1995	-23.4 ± 1.6 (-23.1)	-23.6 ± 1.2 (-23.1)
4 Jan 1995	12 Jan 1995	-22.4 ± 1.0 (-21.2)	-22.4 ± 1.2 (-21.8)
	24 Jan 1995	-22.8 ± 1.2 (-21.6)	-23.4 ± 1.1 (-22.8)
	17 Feb 1995	-21.6 ± 1.5 (-22.4)	-21.5 ± 0.7 (-21.2)
7 Feb 1995	22 Feb 1995	-17.9 ± 2.4 (-17.8)	-19.3 ± 1.4 (-18.7)
	8 Mar 1995	-13.4 ± 3.6 (-14.8)	-16.4 ± 1.1 (-18.4)
	23 Mar 1995	-6.5 ± 1.4 (-6.2)	-8.2 ± 1.9 (-8.7)

* 3% alginate + 0.5 M sucrose, cross-linked with 3% calcium chloride

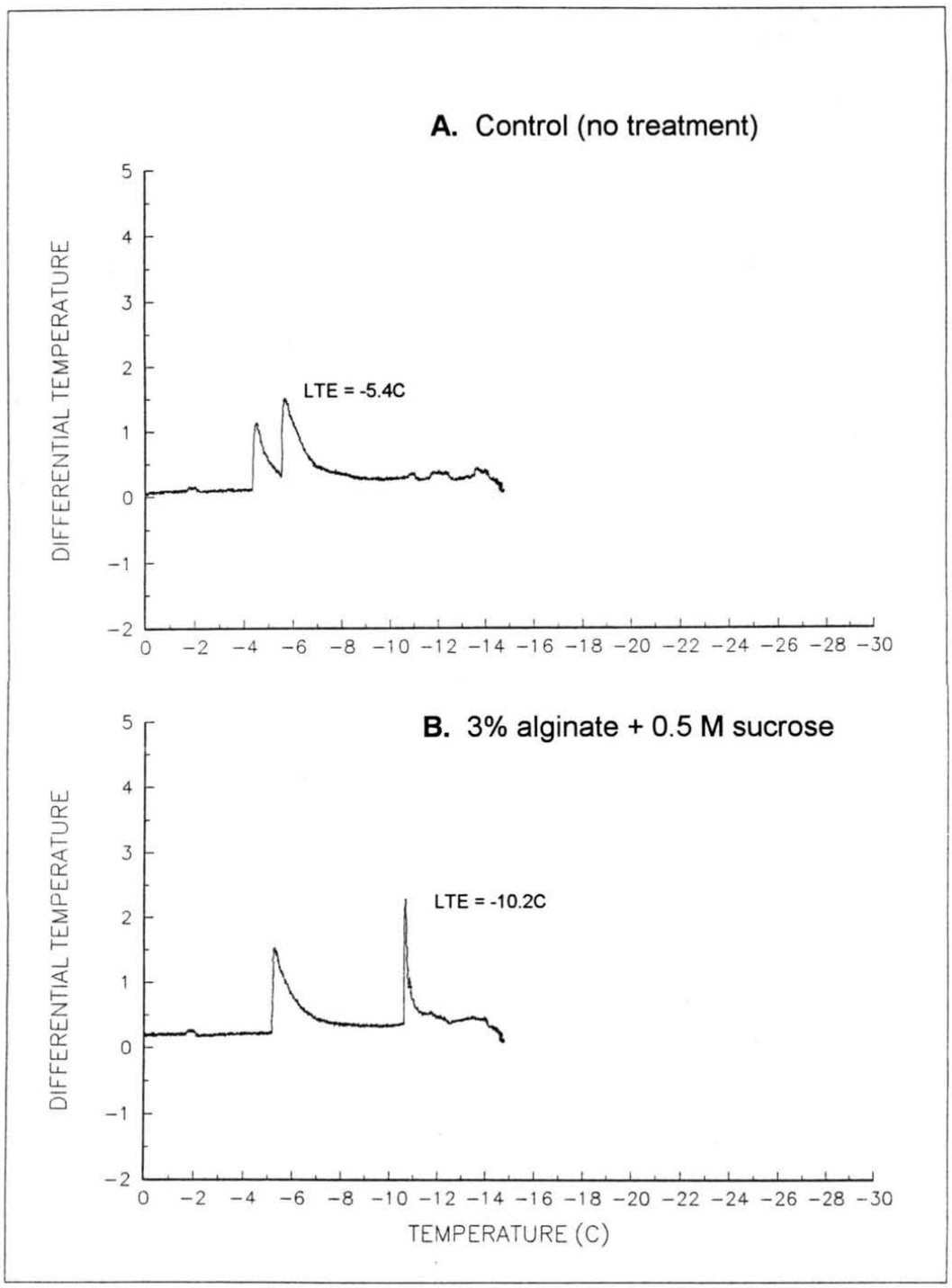

Figure 4.1. Differential thermal analysis profiles of primary buds from control (A) and treated (B) Chardonnay vines collected on 23 Mar 1995 (treatment application on 7 Feb 1995). The first peak at warmer temperature represents the high temperature exotherm; the second peak at cooler temperature represents the low temperature exotherm, or LTE.

LTEs for all three dates between control and treated vines, these differences were statistically nonsignificant ($p = 0.05$) (Table 4.1). This was somewhat surprising, especially when 3C difference is observed between the 2 means. It was noticed that the standard deviations of control samples are much higher than those of treated samples for all three dates. For example, on 8 March LTEs of control buds ranged between -10C and -17C (7C difference, $n = 6$), whereas treated buds have LTEs between -15C and -17.8C (less than 3C difference, $n = 6$). It is not common to see such a large variation in freezing resistance within the same population of grapevines. It is tempting to suggest that the treatment may have reduced this variation within the population by acting as a buffer and preventing large fluctuations in freezing resistance. However, this needs further investigation. The statistical analysis of the same data using LST_{100} of primary buds instead of LTE indicates significant differences between treated and control vines on 8 March 1995 (Table 4.1).

B. Winter 1995 - Experiment 1

Significant differences were observed between the control and treatments in both bud and cane tissues (Table 4.2). Buds treated with 3% alginate + 1M sucrose had the coldest survival temperature, whereas control buds had the warmest survival temperature. This treatment lowered the killing temperature of buds by about 5C as compared to that of control (Table 4.2). Other treatments, 3% alginate + 1M sucrose + 10% latex and 3% alginate + 0.5M sucrose + 1% Triton X100, also had a lower LST_{100} than the control. The rest of the treatments had no noticeable effect on LST_{100}.

Similar results were observed in canes. The canes treated with 3% alginate + 1M sucrose had the lowest LST_{100}, about 7C lower than that of controls (Table 4.2).

Table 4.2. Effect of alginate treatments on LST_{100} of primary buds and canes of Chardonnay. Data are means ± SEM. Mean differences are separated by Tukey's test, and same letters within each column indicate no significant differences at p = 0.05[x].

Treatment[y]	LST_{100} (C) buds	LST_{100} (C) canes
control (no treatment)	-16.6 ± 0.8 b	-17.7 ± 0.2 b
3% alg + 0.5M suc	-16.7 ± 0.8 b	-21.7 ± 0.8 a
3% alg + 1M suc	-21.7 ± 1.4 a	-24.2 ± 0.8 a
3% alg + 1M suc +10% latex	-19.2 ± 1.4 a	-21.7 ± 0.8 a
3% alg + .5M suc + 50% gly	-16.7 ± 0.8 b	-18.3 ± 0.8 b
3% alg + .5M suc + 5% NuF	-16.2 ± 1.2 b	-18.3 ± 0.7 b
3% alg + .5M suc + 1%X100	-20.0 ± 1.5 a	-21.7 ± 0.9 a

[x]= Application date: 9 Feb 1995; collection date: 23 Feb 1995.
[y]= Abbreviations: alg: alginate; suc: sucrose; gly: glycerol; NuF: NuFilm; X100: Triton X100.

Furthermore, other treatments including 3% alginate + 0.5M sucrose, 3% alginate + 1M sucrose + 10% latex, and 3% alginate + 0.5M sucrose + 1% Triton X100 had significantly lowered LST_{100} as compared to controls. Another observation was the mean LST_{100} of canes were usually lower than those of the buds as previously reported (Pierquet et al 1977).

C. Winter/Spring 1995 - Experiment 2

Since the treatment with 3% alginate +1M sucrose gave the best results in the previous experiment, it was used for further studies to compare single vs. multiple applications. The treatment again showed its effectiveness, compared to the control, by lowering the killing temperatures of the buds and canes regardless if one or three sprays were applied (Table 4.3). Buds that were sprayed three times had the lowest LT_{50}, but it was not significantly different than that of buds sprayed only once. The LT_{50} of canes was expectedly lower than LT_{50} of buds. In this experiment only LT_{50} of controls was computed, but not the mean LT_{50} of the alginate treatment. The killing temperature of 50% of cane tissues was not reached in this freezing trial, and 100% of samples were still alive at temperature of -15C, which did kill the controls (LT_{50} = -13.3C). Therefore, the comparison of LT_{50}s of single and multiple application treatments was not possible.

The water content of twigs from the three treatments was also determined. There was no significant difference between the water content of control and single application treatment. However, the water content of the twigs treated three times showed a significant decrease of about 8% as compared to the other treatments (Table 4.3).

Table 4.3. Effect of single and multiple applications on LT_{50} and water content of Chardonnay buds and canes. Data are means ± SEM and means are separated using Tukey's test (p = 0.05). Same letters within each column indicate no significant differences*.

Treatment	LT50 buds (C)	LT50 canes (C)	Water content (%)
control (no spray)	-6.6 ± 0.1 b	-13.3 ± 0.8	45.4 ± 0.4 b
Single spray	-8.4 ± 0.2 a	<-15	45.6 ± 0.8 b
Multiple sprays	-9.1 ± 0.5 a	<-15	37.4 ± 0.9 a

*Single spray on 24 Feb 1995; multiple sprays on 24 Feb, 8 Mar, and 15 Mar 1995; collection date: 22 Mar 1995.

D. Spring 1995 - Experiment 3

The freeze test was conducted on 10 April 1995, which corresponded to 2 days before bud break (12 April 1995). The results once again show the effectiveness of the treatment by lowering the mean LST_{100} of treated buds and canes by about 5C as compared to those of control (Table 4.4). These results imply that if a spring frost took place on 10 April 1995 and temperature had reached a low of -8C, for example, all buds of non-treated vines would have been killed (LST_{100} = -6.2C) but not the ones treated with 3% alginate + 1M sucrose (LST_{100} = -11.2C). In this experiment, the water content of treated twigs was significantly lower than that of the control even though the vines were sprayed only once (Table 4.4). It seems that lower water content plays a complementary role in enhancing bud and cane freezing resistance.

The levels of specific sugars in both treatments were very low (Table 4.4). This was expected since soluble carbohydrate concentrations are usually low during deacclimation of bud and cane tissues as demonstrated in a previous study (Hamman et al 1996). Furthermore, the sugar analysis revealed no significant differences in all specific sugars, except sucrose between control and treated twigs. The sucrose concentration in treated twigs was 43% higher than that of the control. Although precautions were taken during sample preparation, it is possible that high levels of sucrose might have originated from contamination of the sample with sucrose from the alginate treatment (i.e. 3% alginate + 1M sucrose). Another speculation for higher levels may be that metabolism during the onset of growth had stimulated the sucrose pool. Whether sucrose accumulation in treated canes plays a role in growth and development or in freezing resistance is unknown. The levels of stachyose were

Table 4.4. Effect of spring-applied alginate on LST_{100} of primary buds and canes, water content of nodal cuttings and soluble sugars of cortical tissues in Chardonnay. Data are means ± SEM, and comparison of control and treatment is based upon an unpaired T test ($p = 0.05$). Same letters within each row indicate nonsignificant differences[x].

Parameters		Control	Treated[y]
LST_{100}	Bud	-6.2 ± 0.7 b	-11.2 ± 0.7 a
(C)	Cane	-11.2 ± 0.7 b	-15.6 ± 0.6 a
Water Content		48.7 ± 0.3 b	44.7 ± 0.4 a
(%fw)			
Soluble sugars	Fructose	1.6 ± 0.3 a	1.9 ± 0.4 a
($x10^{-5}$mol/gdw)	Glucose	1.6 ± 0.1 a	1.6 ± 0.4 a
	Sucrose	10.0 ± 0.5 a	14.3 ± 1.1 b
	Raffinose	1.0 ± 0.1 a	1.3 ± 0.1 a
	Stachyose	<0.1	<0.1

[x] = Application date: 25 Mar 1995; collection date: 10 Apr 1995.
[y] = 3% alginate + 1M sucrose, cross-linked with 3% calcium chloride.

very small (less than 1μmol / g dry weight) and out of the detectable range of the gas chromatograph. Thus, no comparison of stachyose levels were made.

E. Winter/Spring 1996

Freezing resistance of treated vines was not enhanced when treatments were applied in mid-winter. Buds and canes of samples collected on 12 February 1996 have statistically similar values of LT_{50} and water content (Table 4.5). These observations confirm the results obtained previously (94/95), i.e. cold hardiness of canes and buds of Chardonnay was unaffected by alginate treatments applied in the fall or early winter. On 19 March 1996, some treatments were worse than the controls. For example, the treatments Alg 2 and Alg 4 caused bud injury at warmer temperatures compared to the control (Table 4.5). The negative effect may be associated with breakdown of the gels and/or phytotoxicity of the additives such as WiltPruf. The treatment Alg 2, which contains Triton X100 peeled the most at both collection dates, 12 Feb and 19 Mar (data not shown). For this reason, WiltPruf was added as another treatment to prevent or reduce peeling. Although WiltPruf has substantially reduced peeling, it has a negative effect on freezing resistance. The negative effect may be attributed to the high concentration (20%) used in the experiment.

Significant differences in LT_{50} between control and alginate treatments were observed on 10 April. The treatment, Alg 1, lowered the killing temperatures of buds and canes the most. This again indicates that alginate treatment is most effective in lowering LT_{50} during deacclimation. In addition, lower LT_{50}s have been associated with lower water contents (Table 4.5). The treatment Alg 6 was formulated in the spring of 1996 to include all additives, i.e. Triton X100, latex, and WiltPruf. Although this treatment lowered LT_{50} of

Table 4.5. Effect of alginate treatments on LT_{50} and water content of buds and canes in Chardonnay, Merlot and Riesling. Data are means separated by Tukey's test or an unpaired T test at p = 0.05. Same letters within columns in each date indicate no significant differences.

Application Date	Collection Date	Cultivar	Treatment [y]	LT_{50} Buds (C)	WC[z] Buds (%)	LT_{50} Canes (C)	WC[z] Canes (%)
29 Jan	12 Feb 96	Chardonnay	Control	-24.0 a	40.5 a	-24.2 a	46.0 a
			Alg 1	-24.8 a	38.9 a	-26.4 a	45.0 a
			Alg 2	-24.1 a	39.4 a	-26.2 a	45.9 a
			Alg 3	-24.6 a	39.6 a	-25.3 a	45.3 a
4 Mar	19 Mar	Chardonnay	Control	-25.7 a	41.2 b	-25.1 b	47.8 a
			Alg 1	-25.4 a	39.1 a	-26.2 ab	46.0 a
			Alg 2	-23.2 b	38.6 a	-24.0 bc	46.6 a
			Alg 3	-25.8 a	39.8 ab	-26.5 ab	45.9 a
			Alg 4	-22.7 b	41.4 b	-25.2 b	47.1 a
21 Mar	10 Apr	Chardonnay	Control	-11.4 c	61.9 b	-15.4 a	47.9 a
			Alg 1	-13.6 a	46.0 a	< -16	46.1 a
			Alg 4	-12.3 bc	54.4 ab	< -16	45.9 a
			Alg 5	-13.1 ab	48.0 a	-16.2 a	45.7 a
			Alg 6	-12.4 b	52.6 ab	-15.9 a	45.9 a
21 Mar	9 Apr	Merlot	Control	-11.3 b	56.3 b	-14.3 b	51.0 b
			Alg 6	-13.7 a	48.0 a	-16.3 a	47.9 a
21 Mar	9 Apr	Riesling	Control	-13.7 a	69.0 b	-18.7 a	49.9 b
			Alg 6	-14.1 a	46.3 a	-18.9 a	44.9 a

[y] **Control**, no treatment; **Alg 1**, 3% alg + 1M suc; **Alg 2**, 3% alg + 1M suc + 1% Triton X100; **Alg 3**, 3% alg + 1M suc + 10% latex; **Alg 4**, 3% al g+ 1M suc + 20% WiltPruf; **Alg 5**, 3% alg + 1M suc + 1% Triton X100 + 10% latex; **Alg 6**, 3% alg + 1M suc + 1% Triton X100 + 5% latex + 20% WiltPruf

[z] WC, water content on a fresh weight basis.

buds in Chardonnay, Merlot and Riesling as compared to the controls (-12.4C vs. -11.4C; -13.7C vs. -11.3C; -14.1C vs. -13.7C, respectively), it did not outperform the original Alg 1 treatment (see on 10 Apr, LT_{50} of Alg 1 = -13.6C and LT_{50} of Alg 6 = -12.4C, Table 4.5). Finally, in almost all treated vines, especially the ones treated on 21 Mar, the moisture content of buds and canes was substantially reduced (sometimes as much as 33%) as compared to that of controls (Table 4.5).

Effect of alginate on bud break

A. Laboratory testing:

There were significant differences between control and treated cuttings for all three sample dates. For all dates, the treated cuttings averaged a bud score less than 5, which indicated that the buds were still closed (dormant) (Fig. 4.2a, 4.2b, 4.2c). In the meantime, the means of control bud scores ranged between stage 9 and 12, which indicate a more advanced stage of development than the treated cuttings which had mean scores of less than 5 (Table 4.6). This suggests that the treatment had a profound effect in delaying bud break and retarding development and growth by at least 4 stages, according to Eichhorn and Lorenz (1977) chart.

On the last sample date (8 March 1995), another treatment was added to single out whether the effect was due to sucrose or alginate. The visual scoring indicated no significant differences between 3% alginate alone and 3% alginate + 0.5M sucrose (Fig 4.2c). The alginate alone seems to be as effective as alginate with sucrose at least in this experiment and under controlled environment conditions.

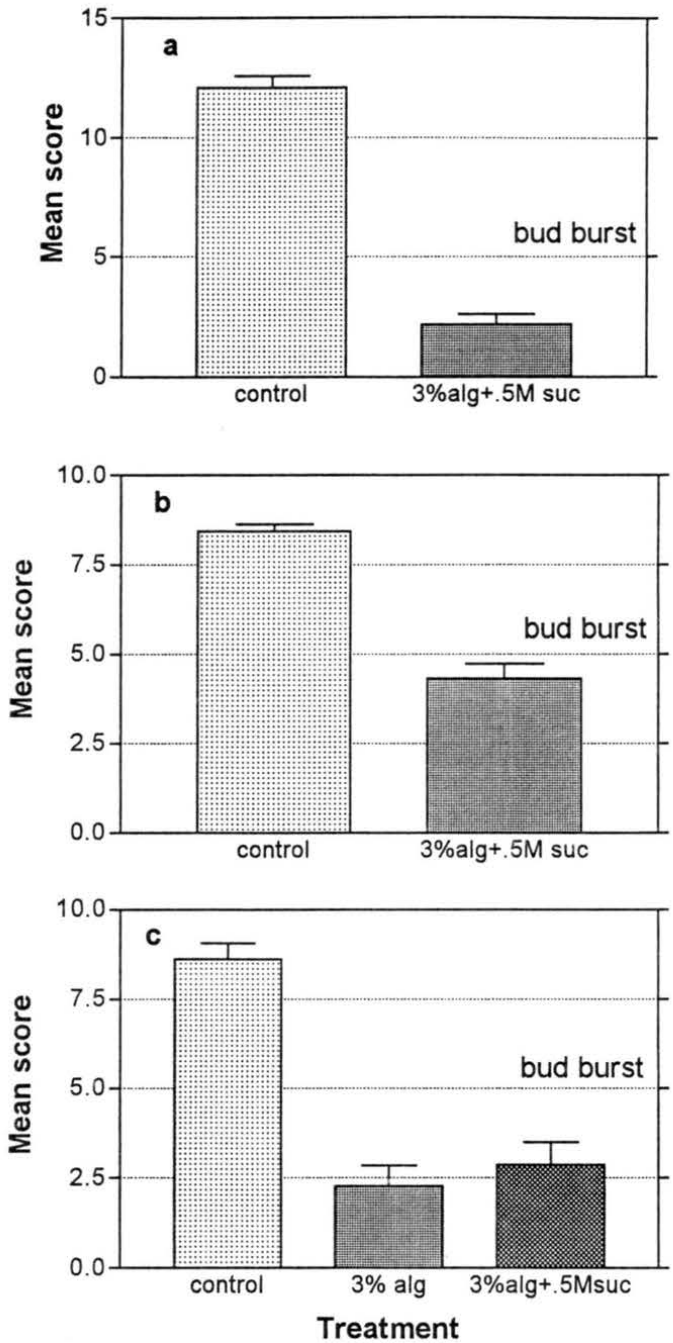

Figure 4.2. Effect of alginate treatments on bud break of Chardonnay single-bud cuttings grown in a controlled environment for 5-6 weeks. Sample dates: a. 14 Dec 1994, b. 4 Jan 1995 and c. 8 Mar 1995. Score of 5 indicates bud burst.

Table 4.6. Effect of alginate treatments on bud break of Chardonnay single-bud cuttings grown under controlled environment for 5-6 weeks. Data are means of visual scores ± SEM. Significance was tested with an unpaired T test compared to the control, $p = 0.05$. Same letters within column at each sample date indicate no significant differences.

Sample date	Treatment	Visual score
14 December 1994	control	12.1 ± 0.5 a
	3% alginate + .5M sucrose	2.2 ± 0.4 b
4 January 1995	control	8.4 ± 0.2 a
	3% alginate + .5M sucrose	4.3 ± 0.4 b
8 March 1995	control	8.6 ± 0.5 a
	3% alginate	2.3 ± 0.6 b
	3% alginate + .5M sucrose	2.9 ± 0.6 b

The addition of other components to alginate and sucrose showed different effects on bud break. First, many treatments had a positive effect on delaying bud break, whether the treatment was applied in the field or in the laboratory (Fig. 4.3). Second, the treatments with 3% alginate + 0.5M sucrose and 3% alginate + 1M sucrose had similar scores regardless of type of application (about 5 and 4, respectively). These scores were significantly lower than those of controls (Fig. 4.3). Third, the best treatment that gave the lowest mean score (<2) was an application of Triton for both field and lab tests. This means that the buds were still closed at the time of evaluation, 5 to 6 weeks after start of incubation. Although few buds were excised and looked alive, we cannot conclude whether the rest of the buds from the Triton treatment were dormant, injured or simply dead. The addition of other adjuvants such as NuFilm and Penetrator did not improve the effectiveness of alginate and sucrose when used alone. Finally, the treatments which included glycerol and latex were less effective under field conditions than in the lab (Fig. 4.3).

B. Field testing

No significant differences were observed between the control without any spray and the control with water spray. Therefore, all treatments were compared to the control with no application using Dunnett's test at $p = 0.05$. Among all treatments, eleven showed significant differences of bud break delay with the control. Treatment responses and the corresponding mean percent of bud break per vine are given in Table 4.7. On the evaluation date, 10 April 1995, the two treatments of interest (3% alginate + 0.5M sucrose, and 3% alginate + 1M sucrose) had 7.5% and 8.3% of bud break per vine, respectively, as compared to 45% for the control. Substantial differences in phenological stages of bud and shoot

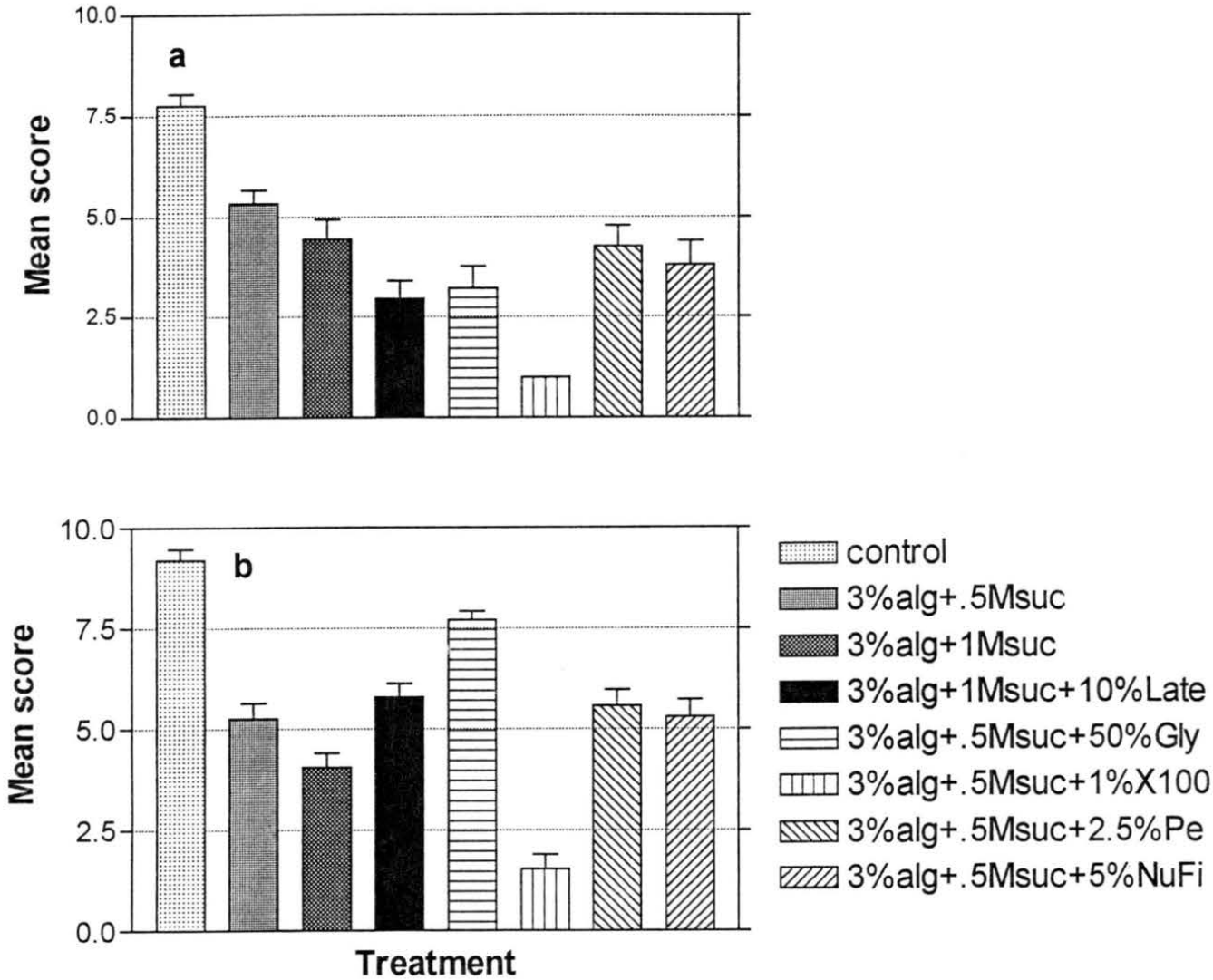

Figure 4.3. Effect of alginate treatments on bud break of Chardonnay single-bud cuttings grown under controlled environment. a. Lab treatment and b. Field treatment. Score of 5 indicates bud burst.

Table 4.7. Effect of alginate treatments on bud break. Visual evaluation was taken on 10 April 1995. Data are percent means of bud break per vine. Significant differences between the treatment and the control (no spray) were computed by Dunnett's test ($p = 0.05$) and indicated by *.

Treatment	Bud break (%)
control, no spray	45.0
control, water spray	32.5
3% alginate	28.1
3% alginate + .5M sucrose	7.5 *
3% alginate + 1M sucrose	8.3 *
4% alginate	15.6 *
4% alginate +.5M sucrose	18.7
4% alginate + 1M sucrose	14.6 *
3% alginate + 1M sucrose + 80% latex	25.0
3% alginate +1M sucrose + 50% latex	12.5 *
3% alginate + 1M sucrose + 10% latex	9.4 *
100% latex	25.0
3% alginate + 1M sucrose + 1% Triton X100	10.4 *
3% alginate + 1M sucrose + .5% Triton X100	5.0 *
1% Triton X100	5.0 *
.5% Triton X100	35.0
3% alginate + 1M sucrose + 50% glycerol	32.5
3% alginate + 1M sucrose + 25% glycerol	25.0
3% alginate + 1M sucrose + 5% glycerol	25.0
50% glycerol	32.5
25% glycerol	2.5 *
5% glycerol	2.5 *

development were readily detectable between the control and the treatment of 3% alginate + 1M sucrose (Fig. 4.4). When Triton or latex was added to the basic mix (i.e. 3% alginate + 1M sucrose), bud break percent was significantly lower than that of control. Higher concentrations of latex, however, were not effective and peeling of the mix was observed on sprayed spurs (data not shown).

The use of alginate alone was not effective at 3% but was significant at 4% concentration. Once again the problem of peeling and cracking of the alginate at lower concentrations (such as 3%) may explain the inefficacy of 3% alginate in delaying bud break. Field observations also revealed that alginate formed a hard and durable gel film around spurs only when mixed with sucrose, especially high concentration such as 1M.

The effect of Triton and glycerol when sprayed alone was surprising (Table 4.7). The treatments with glycerol concentrations of 5% and 25% gave the lowest bud break percent of 2.5%, followed by the treatment of 1% Triton with 5% bud break. In this study, it was unknown whether these treatments had a toxic effect on grapevines or negative effect on crop quantity and quality. For these reasons, the experiment was repeated in 1996.

In 1996, fifteen treatments were compared to control Chardonnay vines at each observation date. Then, the number of days to different levels of bud break percent were estimated using probit analysis (Fig. 4.5). The dates of different levels of bud break (25%, 50%, 75% and 80%) of each treatment are presented in Table 4.8. All treatments which contain 3% alginate + 1M sucrose significantly delayed bud break as compared to the control (Table 4.8). Treated vines delayed bud break by 4 to 10 days, with Alg 6 and Alg 8 being the most effective (Table 4.8). Alg 6 is described previously and contains 3% alginate + 1M

Figure 4.4. Effect of treatment, 3% alginate + 1M sucrose, cross-linked with 3% calcium chloride, on bud break of Chardonnay grapevines. Treatment was applied on 26-27 March 1995. Picture was taken on 10 April 1995 illustrating bud burst and leaf unfolding in control vine (right spur), and closed buds in treated vine (left spur).

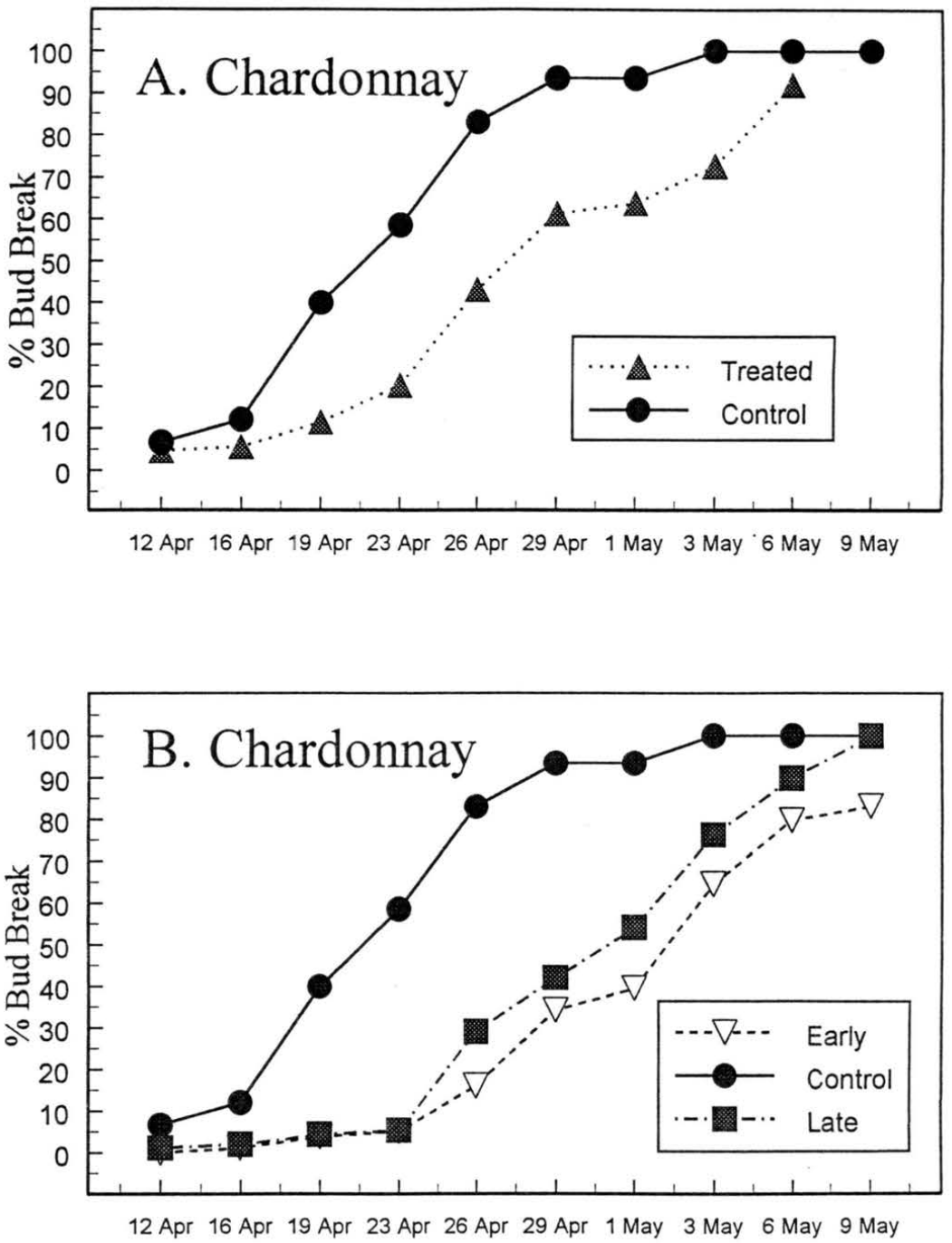

Figure 4.5. Bud break pattern of Chardonnay grapevines in spring 1996. A. control (no treatment) vs. treated (Alg 1: 3% alginate + 1M sucrose, cross-linked with calcium chloride). B. Early application of treatment Alg 6 (3% alginate + 1M sucrose + 1% Triton X100 + 5% latex, cross-linked with calcium chloride) on 19 March vs. Late application on 4April vs. Control.

Table 4.8. Effect of alginate treatments on dates of different levels of bud break of Chardonnay vines in spring 1996. Data are dates derived from probit analysis. Significant differences between treatments and the control were computed by Dunnett's test (p = 0.05) and indicated by *.

Treatment[y]	Percent Bud Break			
	25 %	50%	75%	80%
Control	17 Apr	20 Apr	23 Apr	24 Apr
Alg 1	23 Apr*	27 Apr*	2 May *	3 May *
Alg 2	24 Apr*	28 Apr*	3 May *	4 May *
Alg 4	25 Apr*	28 Apr*	2 May *	3 May *
Alg 6	26 Apr*	29 Apr*	3 May *	4 May *
Alg 7	21 Apr	25 Apr*	29 Apr *	30 Apr *
Alg 8	27 Apr*	30 Apr*	3 May *	4 May *
Alg 9	25 Apr*	28 Apr*	2 May *	3 May *
Alg 10	25 Apr*	28 Apr*	1 May *	2 May *
1% X100[z]	20 Apr	23 Apr	27 Apr	28 Apr *
20% Pruf[z]	20 Apr	23 Apr	27 Apr*	28 Apr*
5% Gly[z]	19 Apr	22 Apr	25 Apr	26 Apr
5% Sun[z]	16 Apr	21 Apr	25 Apr	26 Apr
10 ppm GA[z]	18 Apr	21 Apr	24 Apr	25 Apr
100 ppm PB[z]	16 Apr	20 Apr	24 Apr	24 Apr
3% $CaCl_2$	19 Apr	23 Apr	26 Apr	27 Apr

[y] **Control**, no treatment; **Alg 1**, 3% alg + 1M suc; **Alg 2**, 3% alg + 1M suc + 1% X100; **Alg 4**, 3% alg+ 1M suc + 20% Pruf; **Alg 5**, 3% alg + 1M suc + 1% X100 + 10% latex; **Alg 6**, 3% alg + 1M suc + 1% X100 + 5% latex + 20% Pruf; **Alg 7**, 3% alg + 1M suc + 5% Gly; **Alg 8**, 3% alg + 1M suc + 5% Sun; **Alg 9**, 3% alg + 1M suc + 10ppm GA; **Alg 10**, 3% alg + 1M suc + 100ppm PB.

[z] Abbreviations: Alg, alginate; Suc, sucrose; X100, Triton X100; Pruf, WiltPruf; Gly, glycerol; Sun, sunflower oil; GA, gibberellic acid; PB, paclobutrazol.

sucrose + 1% Triton X100 + 5% latex + 20% WiltPruf. Alg 8 contains 3% alginate + 1M sucrose + 5% sunflower oil. The latter treatment was used for the first time and was included in this study because sunflower oil was used in a separate trial for bio-control of powdery mildew. The basis of adding sunflower oil to alginate is to complement the effect of bud break delay with early spring control of powdery mildew in one spray application. Other treatments include growth regulators such as gibberellic acid and paclobutrazol. When additives are used with alginate and sucrose , they showed significant delay in bud break as compared to the control. However, when they are used alone, their effect is minimal or non-existent (Table 4.8). These results indicate that additives play little or no role in delaying bud break when they are applied in the spring. Furthermore, calcium chloride, the hardening agent has no effect on bud break when sprayed alone (Table 4.8).

Another study consisted of comparing vines treated with Alg 6 at earlier date, 19 March 1996 vs. later treatment application on 4 April 1996. These treatments are compared to control vines. The results indicate that early treatment application delay bud break further as long as the gel does not break down (Fig. 4.5b). This study, however, needs further field trials. The Alg 6 treatment was also used in other cultivars and successfully delayed bud break of Pinot noir and Vignoles as compared to the controls (Fig 4.6a, 4.6b).

There is strong evidence from the data collected in both years that alginate and sucrose are the main contributors to bud break delay. Additional compounds may enhance the effectiveness of alginate somewhat, most likely by increasing its stability and durability. To test whether alginate treatments have an effect on fruit quality and quantity, data were taken at harvest on 12 September 1996. Yield components and fruit composition of treated

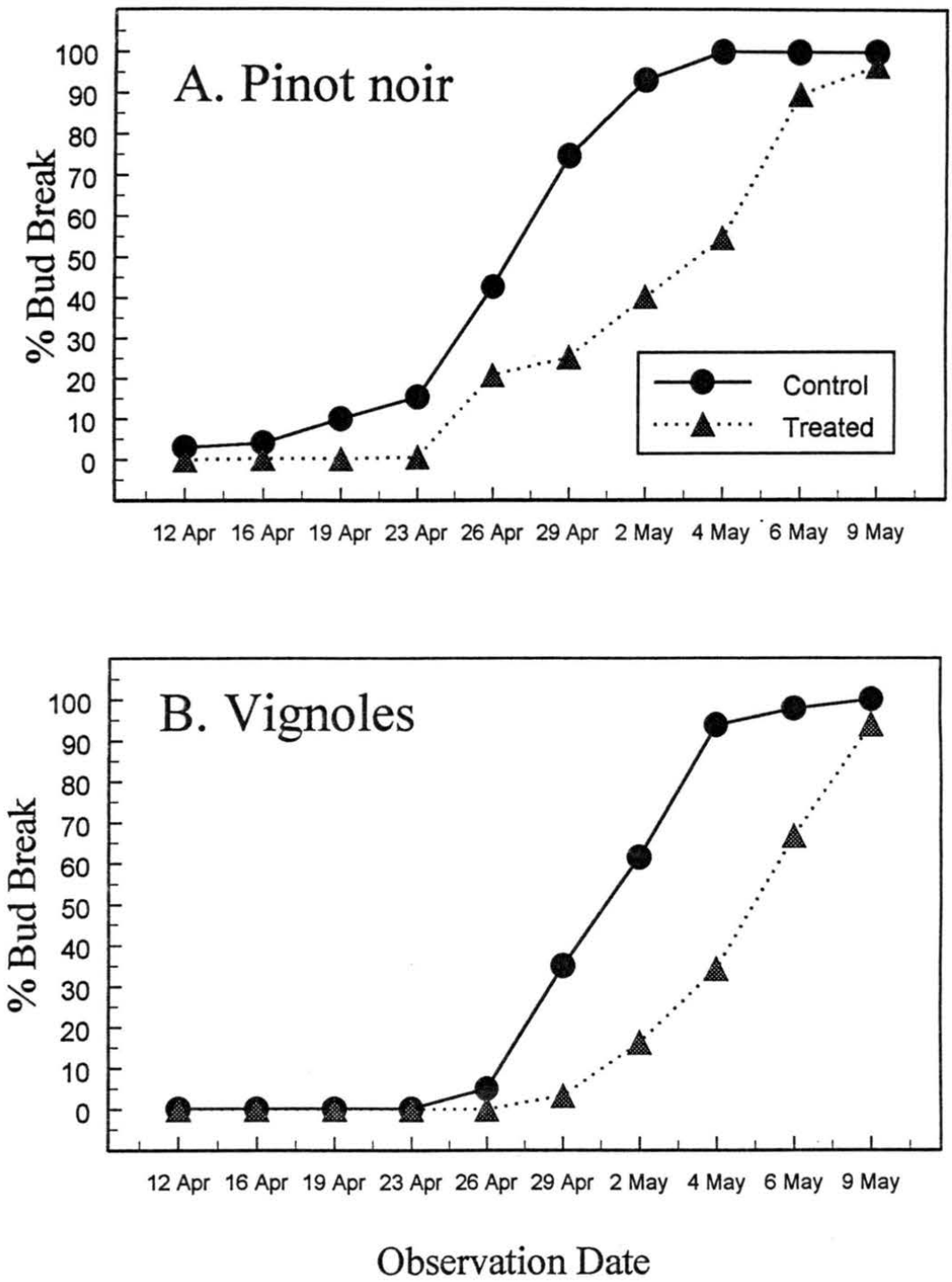

Figure 4.6. Bud break pattern of A. Pinot noir and B. Vignoles grapevines in spring 1996. Control vines are not treated; treated vines are painted with Alg 6, which contains 3% alginate + 1M sucrose + 1% Triton X100 + 5% latex, cross-linked with calcium chloride. Treatment application was on 4 April 1996.

Table 4.9. Yield components and fruit composition of alginate treatments compared to control in Chardonnay grapevines. Data are means ± SEM. No significant differences in all treatments using Tukey's test at p = 0.05.

Treatment*	Cluster Weight (g)	Total weight/vine (Kg)	°Brix	pH	Total Acid (g/L)
Control	119.6 ± 11.9	6.1 ± 0.8	24.8 ± 0.6	3.45 ± 0.02	7.5 ± 0.3
Alg 1	124.2 ± 6.7	5.8 ± 0.5	25.4 ± 0.3	3.46 ± 0.03	7.4 ± 0.2
Alg 6	128.7 ± 8.2	6.5 ± 0.5	25.1 ± 0.2	3.43 ± 0.02	7.3 ± 0.4
Alg 8	133.2 ± 15	5.9 ± 0.9	24.9 ± 0.3	3.44 ± 0.03	7.6 ± 0.2

* Control, no treatment; **Alg 1**, 3% alginate + 1M sucrose; **Alg 6**, 3% alginate + 1M sucrose + 1% Triton X100 + 5% latex + 20% WiltPruf; **Alg 8**, 3% alginate + 1M sucrose + 5% Sunflower oil.

vines are statistically similar to controls (Table 4.9). Moreover, although treated vines break buds 4 to 10 days later than control vines, their growth seems to catch up with that of untreated vines by mid-season and eventually they all mature at the same time (data not shown).

DISCUSSION

Effect of alginate on deacclimation

Freezing resistance of bud and cane tissues of Chardonnay grapevines was enhanced by using the alginate- and sucrose-based product. Although the results were positive and very promising, the mechanism and mode of action of alginate mixed with other compounds are unknown. The application of alginate in the field was the first ever documented trial, and thus possible mechanisms are subject to speculations. It was originally hypothesized that if preservation of dormant woody plant tissues was successful at cryogenic temperatures using a similar protocol, then improving the freezing resistance of grape tissues under field conditions by few degrees might be possible. This hypothesis appears to be correct. It has been demonstrated that the combination of alginate and sucrose plays a cryoprotective role in apple (Seufferheld 1995) and grape buds (unpublished data) when preservation in liquid nitrogen (-196C) is attempted. Furthermore, the role of non-structural carbohydrates in plant tissues as cryoprotectants has been widely documented (Santaurius 1973, Stushnoff et al 1993). To be effective, these cryoprotectants must come in contact with the bio-molecules or cell membranes in order to stabilize their integrity and/or function. In this experiment, although enhancement of freezing resistance was observed, it is unlikely that it was achieved

via cryoprotection since there was no significant change in sugars, except sucrose, in the cortical tissues between control and treated vines (Table 4.4). Previous study has indicated that sucrose does not correlate well with cold hardiness of Chardonnay buds throughout the acclimation and deacclimation season (Hamman et al 1996).

It is known that grape tissues avoid intracellular freezing by deep supercooling (Pierquet and Stushnoff 1980, Andrews et al 1984). This experiment showed that treated vines had more supercooling capacity (lower killing temperature) than untreated vines in late winter and early spring (deacclimation season). Furthermore, the buds and canes with the lowest LST_{100} or LT_{50} had the lowest water contents, respectively, on a fresh weight basis (Tables 4.3, 4.4, 4.5). It is tempting to conclude that this type of freezing resistance relies, at least in part, on water status of the plant tissue. In other words, the supercooling of treated tissues was depressed because there was less freezable water available. The coating of grapevine canes with alginate and sucrose has caused the dehydration of the tissues. This dehydration may be the result of an osmotic effect due to the extremely high solute concentrations created by sucrose and alginate. Therefore, the rate of water movement between tissues and/or their surroundings may have been altered and thus affected the level of tissue freezing resistance.

In one instance, the water contents of both tissues (treated and untreated) were statistically the same, though the supercooling temperature of treated buds and canes was lower than controls (Table 4.3). This may seem to contradict the mechanism explained above, since the water content did not seem to play a role in this case. This phenomenon may be explained as follows. The gel entrapping the twigs continuously contracts and expands

according to the relative humidity, which fluctuates between day and night. When there is a cold spell and the gel freezes, it will form a protective coat around the bud and the cane tissues. The frozen state of the gel would create an ice sink which, with slow freezing, would withdraw water vapor from the plant tissues due to vapor pressure differential between cell water and ice at the same temperature (Sakai and Larcher 1987). This phenomenon may be compared to extra-organ freezing (Sakai and Larcher 1987), where water is redistributed during slow freezing.

In summary, a gain in freezing resistance of the treated vines may be the result of an osmotic dehydration of the bud and cane tissues and/or the redistribution of water during slow cooling that may have caused further dehydration. Sugar levels with the exception of sucrose in the cortical tissues were not affected by the treatment and seem to have little or no effect on changing the freezing behavior of bud and cane tissues. High levels of sucrose in treated vines might be an artifact or the result of an induction of metabolism pathway associated with the onset of growth. However, further investigations are needed in this area since the data are limited and inconclusive.

Effect of alginate on bud break

This study showed that delay of bud break for Chardonnay was successful on cuttings and live vines. The treatment with 3% alginate + 1M sucrose, which increased freezing resistance, also caused bud break delay. In laboratory testing, the study comparing the alginate treatments with and without sugar minimized the effect of sucrose since no difference in bud break was detected (Fig. 4.2c). Although alginate seems to be effective without any additives under laboratory testing, field trials were unsuccessful since alginate breaks down

readily and quite rapidly (data not shown). This was not the case when sucrose was added primarily at high concentrations. It seems that sucrose improves the stability of the alginate matrix and may act like glue by strengthening the assembly of the polymers.

How does the alginate treatment have such a profound effect on bud break only two weeks after application? The study discussed above indicated that treated canes had less water than the control. This could contribute to slowing the rate of metabolic activity inside the buds prior to burst. Cane moisture content has been reported to increase during spring deacclimation in *V. vinifera* cv. Merlot (Hamman et al 1990). This could be a factor but no water content data were taken in this experiment.

Another speculation relies on the intrinsic properties of the alginate. Alginate may play a double role on dormant buds to prevent their normal burst, mechanical and physiological. The mechanical effect of alginate results from the strength and flexibility of the gel (Skjak-Braek 1992). By tightly entrapping the bud, the gel may hinder the opening of bud scales and the extension of the shoot primordia through them. This mechanical effect of the gel has been previously reported in artificial seeds which exhibited low percent of embryo conversion (Onishi et al 1992). The physiological effect of the alginate may involve a retardation of bud development due to an accumulation of CO_2 or a deficiency of O_2 resulting from entrapment of the bud with alginate. On one hand, alginate has been documented to have very low permeability to oxygen (Hulst et al 1989). On the other hand, respiratory activity, measured as heat of metabolism and evolution of CO_2, steadily increased from ecodormant to bud break stage in *V. vinifera* cv. Pinot noir (Gardea et al 1994). In this experiment the alginate was applied on 26 and 27 March, which corresponds approximately

to the ecodormant-bud swelling stage. According to Gardea et al (1994), respiratory activities are very high during that stage. Therefore, during this period the lack of oxygen in the vicinity of the buds is very crucial and hence may prevent or substantially decrease respiratory activity. Another possibility is that the interference of alginate, with the escape of respiratory CO_2 from the bud, may increase internal CO_2 concentrations, which may in turn cause a decrease in respiratory rates as a result of feedback inhibition (Isenberg 1979). In a recent study, application of soybean oil to dormant peach trees delayed the date of bloom (Myers et al 1996). The authors found that the delay was caused by increasing levels of internal CO_2 in flower buds that resulted in low respiration rates. The alginate treatment may have induced the so-called atmospheric ecodormancy (Lang et al 1987). This means that the dormancy of buds is controlled by environmental factors specifically by O_2 and/or CO_2 levels.

The addition of other compounds to alginate and sucrose were equally effective in some cases. Some additives have enhanced the effectiveness of alginate further and improved its stability. Finally, alginate treatments have been effective in other grape cultivars, and they have no side effects on the quantity or the quality of the crop.

CONCLUSIONS

The application of alginate and sucrose on ecodormant Chardonnay grapevines had a dual effect. During spring, the cold hardiness of treated buds and canes was maintained about 2C to 5C higher than that of untreated buds and bud break was delayed by about 4 to 10 days when compared to the control. This study was successful in integrating research findings into agricultural application. In fact, these findings may have an important economic

impact in viticultural areas where spring frost injuries are common. Over the past 50 years, hundreds of chemicals have been marketed and, unfortunately, most showed inconsistent results due either to climate, location or genetic factors. Furthermore, the deleterious side effects of most chemicals have precluded their wide-spread since growers have to trade off phytotoxicity of these chemicals with beneficial effects of increased hardiness and/or bloom or bud break delay. The alginate product is not phytotoxic since it is basically a polysaccharide derived from seaweed; also, it has an environmental advantage by being biodegradable and non-polluting unlike other chemicals available in the market. Finally, alginate is an excellent matrix that also could be used as a delivery system to enhance absorption of growth regulators, pesticides, and nutrients during the dormant-season or pre-bud burst application.

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APPENDIX: REPRINTS

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