DISSERTATION

INHERITANCE OF COLD STRESS TOLERANCE IN GRAPES

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

Fall 1989

COLORADO STATE UNIVERSITY

NOVEMBER 2,1989

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY VIRGIL D. ESENSEE ENTITLED INHERITANCE OF COLD STRESS TOLERANCE IN GRAPES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

INHERITANCE OF COLD STRESS TOLERANCE IN GRAPES

Cold hardy 'Valiant' was crossed to the lesser hardy cultivars 'Zinfandel' and 'French Colombard' to produce progeny that were later planted in the field for evaluation. In January and February, 1989, buds with subjacent stem tissue were evaluated by differential thermal analysis (DTA). The results were compared with those obtained from tests of browning that were performed following controlled freezing of buds in a methanol bath. DTA was found to be an accurate means to determine the mean midwinter killing temperature of grape buds. The apparatus used cooled the experimental tissues at a smooth linear rate (-15.8 C/hr) and produced low variances within replicates. An analysis of variance indicated that there were true differences between progeny means. Field evaluations in June, 1989, also verified the midwinter DTA data. These tests substantiate recommending DTA as a breeding tool for evaluating midwinter grape cold hardiness.

Broad sense heritability was calculated using grape bud DTA data for both 'Valiant'x'Zinfandel' and 'Valiant'x 'French Colombard' crosses. All calculations produced heritabilities near unity, indicating that of the progeny

tested, the phenotypes were due primarily to expression of the genotypes.

It was also found that poor seed germination commonly found in grapes can be ameliorated through the use of embryo rescue techniques. Data showed that low germination of grape seeds is not necessarily due to inviability, since rescued embryos grew and developed when placed on Nitsch's medium.

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ACKNOWLEDGEMENTS

I thank Dr. Harrison G. Hughes for his guidance as my major professor and for his letting me "do my own thing" on this research project. I also thank those who served on my graduate committee: Drs. Duane Johnson, Chi Won Lee and Leigh Towill. Stephen Wallner served on the committee through my preliminary examinations and for that I am grateful.

The students in horticulture and agronomy who have helped me by way of inspiration or encouragement are too many to list here, but you know who you are and I thank you. Likewise, my family and friends have helped me stay with the task, through thick and thin.

Special thanks are due to LaRue Wentz who typed the majority of this text and "cracked the whip" when I got too slow. Extra special thanks go to Linda Esensee for helping pull this entire dissertation together. Phil Chapman helped with statistics.

Thank you everyone!

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Introduction

Grapes survive extreme cold temperatures by a stress avoidance mechanism known as deep supercooling. Some grapes perform better in this respect than do others. The process of winter survival is complex in that plants must acclimate and deacclimate during fall and spring respectively, as well as survive the deep cold temperatures of midwinter. The ability to deep supercool fluctuates in magnitude in response to the ambient environment. The testing of plants to determine to what extent they are cold hardy at any given time has involved several different techniques, the efficacy of each being somewhat species dependent. Each of these techniques must ultimately indicate viability of the tissues under study following a cold stress of known magnitude. To underscore the importance of the evaluation of stress hardiness, it is well to note that the Science Priorities Committee of the American Society for Horticultural Science (ASHS) has recently listed environmental stress as the top priority for research. They cite that crop productivity is most severely limited by environmental stress (Faust, 1986), such as extreme cold.

Part of the problem in measuring cold stress survival in grapes is that the underlying processes are poorly

understood. The previously mentioned complexity during acclimation and deacclimation has given rise to the speculation that the inheritance of these characteristics, which allow for winter survival, must be multigenic in nature. Few researchers have looked at cold hardiness in terms of heritability. What is needed are studies of heritability of cold stress tolerance, as well as accurate and rapid techniques for measuring and evaluating hardiness, so that heritability of cold hardiness can be estimated. This could enhance the overall breeding and selection processes involved in advancing the range of grape production in cold climates.

Viability

The fundamental nature of plant cold resistance has been studied for over 200 years (Chandler, 1954). During this time several methods have been developed in the search for ways to rapidly and accurately determine the degree of cold that plants will withstand during the various stages of their growth. Some of these procedures have value in determining not only the degree of cold stress which a plant can withstand, but also the response to other stresses, such as heat and drought. This discussion will focus on the use of these techniques in evaluating cold hardiness only.

When considering grape cold hardiness, one must keep in mind the unique qualities of grapes, which will determine the direction that evaluation of cold survival should take.

Some perennial plant species are able to survive extreme cold temperatures that kill the buds, but not the stems (Layne, 1983). Other species may be able to withstand liquid nitrogen temperatures (-196 C) and below, as in many conifers (Burke, et al., 1976). Grapes, however, will generally not survive when all of the above ground buds have been killed (Wu, 1981). The stems, which generally are hardier than the buds, do not have latent buds. Plant survival alone depends upon growth of any protected buds which have laid dormant below the soil or possibly below the snowline during extreme cold periods, but commercially, it is disastrous when the majority of the above ground buds have been killed or injured. It is for these reasons that the study of cold hardiness of grapes should necessarily focus on the survival of the buds.

In the past, evaluation of a plant's abilities to survive deep cold stress has been by the use of test winters. The use of the method is simple, since the plants are grown outdoors and subjected to whatever nature provides in terms of cold stress (Clore, et al., 1974). There are several obvious problems with this procedure as pointed out by Christiansen (1979). The stress, provided it does occur, may be too severe, too mild, or altered by other environmental factors. Quantification of survival can be quite difficult and tends to be largely subjective.

A number of laboratory procedures have been developed to ameliorate this situation. The ultimate test of

hardiness is that a plant or organ be subjected to a cold stress of known magnitude and then be able to regrow. Frequently, a procedure to determine the temperature required to kill 50% of plants or organs (T_{50} or LT_{50}) is determined. This can be quite laborious and time consuming, since numerous plant samples are required. Plant samples are subdivided in a manner which will allow for equal numbers of them to be subjected to known cold temperatures. A count of survivors which regrow, following each individual stress, is made and the LT_{50} is derived by regression or probit analysis. Plant samples are rarely, if ever, subjected to the true LT_{50}. This procedure has been used on a large number of plant species and plant parts, including grape seedlings (Conrod, 1987), grape buds (Johnson and Howell, 1981; Proebsting, et al., 1980), grape buds and stems (Damborska, 1978), Jerusalem artichoke tubers (Ishikawa and Yoshida, 1985), peaches and nectarines (Layne, 1983), and Chrysanthemum cells grown in culture (Steponkus and Bannier, 1971). Regrowth has been the standard by which other hardiness procedures are judged for accuracy.

A test of browning of tissues has also been used. Tissues that have been killed will typically turn brown after a few days and visual observation of dissected tissues will distinguish whether dead (brown) or viable (green) tissues are obtained from any given treatment. Count data are taken and used in a manner like that of regrowth, where the actual killing temperature is derived by a statistical

procedure. Grape (Clark, 1936; Howell and Shaulis, 1980; Howell, et al., 1978; Wolpert and Howell, 1985a), filbert (Hummer, et al., 1986) and many other species, have been tested for cold hardiness using this technique. As with regrowth, browning is frequently used to verify other means of stress survival determination.

Vital staining is a laboratory procedure for determining the viability of plant cells. The concept is that only live cells will stain, while dead ones only to a limited extent or not at all. The most common of these procedures is based on the reduction of 2, 3, 4 - triphenyltetrazolium chloride (TTC) to produce formazan, a water-insoluble red dye. The tissue in question may be visually observed, or the formazan may be extracted and the absorbance measured at approximately 485 to 530nm (Irving and Lamphear, 1967; Steponkus and Lamphear, 1967; Towill and Mazur, 1975). Quantification of the hardiness temperature is derived statistically, as was noted with previous procedures. Steponkus and Bannier (1971) reported that correlation of TTC reduction and regrowth of Chrysanthemum callus cells was not entirely satisfactory. Many other researchers have used this method, though not with callus.

Freezing injury and ultimately the death of cells results in the affected cells losing all or part of their contents into the surrounding milieu. Any one temperature will not kill all cells, so it becomes possible to determine the percentage of damage incurred to any given tissue by

measuring the amount of cell contents lost. Measurement of the contents leached from cells has been accomplished with considerable success by monitoring the change in electrical conductivity. The ions leached will increase the conductivity of the liquid into which it diffuses, usually water, since ions leached from cells improve electrical conductance. This method has been used with grapes (Edgerton and Shaulis, 1953) and other species. Xiu-wu, et al. (1987) compared electrical conductivity to TTC reduction and found the former to be superior. Flint, et al. (1967) proposed an index of injury. It rates injury due to freezing damage by considering not only the conductivity of leachate from frozen cells, but also the electrical conductivity of leachate from uninjured and dead (boiled) cells.

Campbell and Ghosheh (1957) used conductivity in a different way; by measuring the conductivity of grape cane segments six inches long, they were able to differentiate relative cold hardiness of several cultivars. This method was used successfully by Evert and Weiser (1971) on Cornus stolonifera Michx. However, they found the use of two frequencies gave better estimates of hardiness than did one.

Improvements have been made to the electrical conductivity method since it was first used. Research reported by Zhang and Willison (1987) indicated that the electrical conductivity method is improved by looking at the amount of cell contents leaked between one and 18 hours in

de-ionized water. This was referred to as differential percent leakage (DPL) and was highly correlated to the results obtained from vital staining using fluorescein diacetate. Recent work by Chalker-Scott, et al. (1989) has shown that results obtained from electrolyte leakage, visual browning and TTC reduction are all highly correlated to spectrophotometric measurement of leached phenolic compounds.

Another procedure quantifies the amount of amino acids and other alpha-amino compounds leached from killed or injured cells. Ninhydrin is used as a reactant to label these compounds so that a colorimetric determination of concentration may be made (Siminovitch, et al., 1964; Tamassy and Zayan, 1982b). Thus far, this procedure has not been used commonly in cold hardiness research.

Differential thermal analysis (DTA) has been used in a considerable amount of research dealing with cold hardiness in plants. This procedure is based on the fact that water releases latent heat of fusion upon freezing (exotherms). The freezing of live tissues is compared with the freezing of dead dehydrated tissues of similar mass. Many plant species are able to avoid freezing of their water through mechanisms which allow supercooling of selected tissues to a temperature well below 0 C. This is most common in deciduous perennials which typically survive to temperatures near -40 C. At this temperature, the water in the tissues freezes due to ice nucleation mainly caused by spontaneous

homogeneous nucleation. It has been proposed that supercooling as a method of freezing avoidance limits the spread of some species into regions where the average annual low temperature isotherm goes below -40 C (George, et al., 1974; Gusta, et al., 1983). DTA has been used to evaluate cold hardiness in many species, including apple (Quamme, et al., 1982), azalea (Graham and Mullin, 1976b), blackberry (Warmund, et al., 1988), blueberry (Biermann, et al., 1979), peach (Ashworth, 1982; Ashworth and Davis, 1984; Ashworth et al., 1983; Quamme, 1978), pear (Rajashekar, et al., 1982), pecan (Rajashekar and Reid, 1989), sweet cherries (Andrews and Proebsting, 1987), and grapes (Pierquet and Stushnoff, 1980; Pierquet, et al., 1977; Quamme, 1986).

Andrews, et al. (1987) have produced a conceptual model of seasonal changes in deep supercooling based on DTA. The model calculates the average low temperature exotherm (LTE_{50}) on an hourly basis and uses DTA to start the model and update it through the season. Differential thermal analysis is rapid, reliable and has been shown to accurately estimate the mean freezing point of tissues, especially when exotherms are measured with thermoelectric modules (Andrews, et al., 1983a). This makes DTA unique with respect to the viability tests discussed earlier in that mean cold hardiness can be measured directly rather than estimated by a statistical method. Wolf and Pool (1986) and Hamman (1988) have used microcomputer-based systems to automate the procedure.

A recently reported method for determining cold hardiness (Weiser and Wallner, 1988) is acoustic emissions measurement (AE). Sounds are emitted from cells in plant stems during freezing events. It is believed that these acoustic emissions are the result of cavitation taking place in water of ray parenchyma cells. The results of AE experimentation are not clear, thus further investigations into the use of this method are required before it will be accepted.

Several papers have evaluated some of the methods used for viability tests. Some of these studies have made comparisons relating other methods to the procedure under study, for example in Chen and Gusta (1983). Other reports have been more direct in their comparisons (Palta, et al., 1978; Stergios and Howell, 1973; Stushnoff, 1972) in that the comparisons have been the objective of the research. Based on these efforts, one may choose a procedure or select one on the basis of their own empirical determinations.

A number of possible selection procedures have been noted and these consist primarily of tests of viability after a cold stress has occurred. In addition to these, Dickson and Petzoldt (1987) suggested the use of a greenhouse for screening and selection to alleviate the wide temperature fluctuations found outdoors. Their work was with beans, and the use of greenhouses may be ideal for annual crops, but would be more difficult and expensive for use with perennials. Perennials generally require more

space per plant and would require a longer period of time before the plants might be evaluated.

Cooling Rate

No matter which viability test is chosen, a controlled laboratory procedure is essential for obtaining accurate and timely evidence. Much of the accuracy of viability procedures depends upon the cooling rate that has been chosen. 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. It is presumed that the redistribution of water in buds and stems occurs so that freezing of this relocated water occurs outside of cells and outside of particularly vulnerable tissues. A slower freezing rate facilitates the redistribution of water (Ashworth and Davis, 1987; Pierquet and Stushnoff, 1980). Weiser (1970) made the point that it is the formation of ice crystals within cells which kills them and not the low temperatures.

There have been various conflicting reports on the effects of cooling rates on survival of plant cells. McLeester, et al. (1969) observed that Cornus stolonifera twigs frozen at 200 C per hour were not injured. Biermann, et al. (1979), however, observed that blueberry buds exhibited a single low temperature exotherm (LTE), which was rate dependent and correlated to death at cooling rates that were slow (6.5 - 9.0 C/hr). Some reports have also shown

that faster cooling rates caused freezing to occur at higher temperatures (Graham and Mullin, 1976b; Pierquet, et al., 1977) whereas other reports have noted freezing at lower temperatures (Quamme, 1986; Wolf and Pool, 1987).

Stushnoff (1972) reiterated that whenever artificial freezing is used, the researcher should develop a standard cooling rate by testing the effects of different rates on survival. To complicate matters, low temperature exotherms have been observed in tissues which had previously been killed (Pierquet and Stushnoff, 1980). This was thought to indicate that ice formation does not necessarily kill, rather it may destroy the protection system (Graham and Mullin, 1976a). That "protection system" has since been shown to be the plasma membrane (Steponkus, 1984), which is like any protection system, having extreme limits beyond which effectiveness is destroyed.

Water Content

Water content of plant tissues is a critical factor in cold stress survival during deep cold, but also during times of wide temperature fluctuation, as during acclimation and deacclimation. Water content is usually measured by weighing both fresh and oven-dried samples. Water content of plant samples as measured by nuclear magnetic resonance (NMR) has given similar results to the oven drying method in azalea (Kaku, et al., 1984), apples and peach (Quamme, et al., 1982).

A cell that is highly hydrated is less likely to survive low temperature stresses. This also applies to tissues and organs as a whole. As previously indicated, the movement of water from vulnerable tissues into extracellular spaces may be one mechanism of freezing avoidance for those cells. In several species increased water content is highly correlated to decreased cold tolerance. This has been shown in such diverse species as blueberry (Biermann, et al., 1979), peach (Quamme, 1983; Quamme and Gusta, 1987), wheat (Andrews, et al. 1984b), and grape (Hamman, 1988; Isaenko, 1977; Wolpert and Howell, 1985a; 1985b; 1986a).

Much of the ability to survive during changes of environment in spring or fall depends upon water status of the plant and the ability to supercool. This was demonstrated in sweet cherry buds, which were able to survive freezing in mid-August by supercooling (Andrews and Proebsting, 1986). In grape, basal cane tissues were seen to acclimate earlier than apical tissues. These differences were attributed to an inverse relationship of hardiness to water content (Wolpert and Howell, 1985b). This inverse relationship was seen in sweet cherry flower buds (Andrews and Proebsting, 1987) and in Amelanchier alnifolia (Kaurin, et al. 1984) as an increase in LTE_{50} during deacclimation. Grape buds were also reported to have shown this same inverse relationship to water content and cold hardiness (Hamman, 1988; Wolpert and Howell, 1985b; 1986a).

Acclimation and Deacclimation

Climates of various extremes through selection have given rise to the different ecotypes of a species. The existence of ecotypes is common within the plant kingdom. This is especially evident in grapes, Vitis sp., with the many species and cultivars that are extant (Wagner, 1974). Survival of winter environs is an important aspect of plant selection, whether that selection is natural or by man. The ability to survive the cold winters of high latitudes is dependent upon the ability to attain a deep cold hardy state, as well as to deacclimate in a timely manner. These are very complex physiological processes about which very little is known. According to Stushnoff (1972), acclimation and deacclimation can be broken down into several general components. These components consist of timing, rate, intensity and retention of the development of cold tolerance during acclimation. It is also important that the timely loss of tolerance at an appropriate rate should occur, as well as the ability to regain cold tolerance during deacclimation. Temperatures may fall rather than rise over an extended time period in early spring.

The biochemical complexities of the systems that regulate acclimation or deacclimation are still largely unknown and cold acclimation is yet to be adequately explained on the cellular level. Studies have recently focused on cell wall and plasmalemma changes which result from induced cold acclimation. Limin and Fowler (1984)

helped to substantiate this focused interest when they reported that, "...the cytoplasm has little direct effect on cold hardiness, or on the nuclear expression of cold hardiness." It would appear that in some plants which supercool, maintenance of the individual cell's abilities to withstand severe desiccation stresses during supercooling may be functions naturally associated with the cell wall and plasma membrane, although cells that supercool may not necessarily suffer desiccation stress (Wisniewski and Ashworth, 1986a; Tao, et al., 1983). Physiological changes take place in the plasmalemma, which allow for cellular collapse during these periods. Membrane invaginations have been shown to be one mechanism by which this is accomplished (Steponkus, 1984,; Wisniewski and Ashworth, 1986b). Associated with these behavioral changes in the plasmalemma are possible losses of membrane proteins (Tamassy and Zayan, 1982b) and changes in lipid composition and content (Ishiwaka and Yoshida, 1985; Lynch and Steponkus, 1987; Novitskaya and Mashchanova, 1983). Wallner, et al. (1986) have evidence of increased deposition of callose or other B-1,3-glucans at the surface of cells during cold acclimation, when these events occur.

Plants must necessarily take cues from the environment. 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. Those plants that respond

to short days require reduced daylength prior to low temperature stimulus to induce maximum cold hardiness. Hardiness is a photoperiodic response and the short day stimulus could be reversed by long days in Acer negundo and Viburnum plicatum (Irving and Lamphear,1967). Light treatments of grapes produced minor differences in hardiness and acclimation was not prevented by night interruption (Wolpert and Howell, 1986b). Schnabel and Wample (1987) conclude the acclimation of grape was a synergistic effect of light and temperature. Kobayashi, et al., (1983) showed that acclimation and deacclimation of red-osier dogwood was related to plant developmental stage and to temperature. Increasing acclimation was promoted by decreasing temperatures. These reports, as well as others, indicate that species responses to certain cold acclimating stimuli are not necessarily common to all.

One investigation concluded that abscisic acid (ABA) treated wheat suspension cultures were able to bypass the cold requirement to attain cold acclimation (Chen and Gusta, 1983). It was suggested that ABA may be responsible for inducing the hardening process by triggering the genetic system involved.

Whether or not ABA is the key hormone in the response to temperature induced cold acclimation does not detract from the many observations that cold temperatures play a major role in influencing plant acclimation responses. It has been shown that grape buds increase in hardiness slowly,

while they are frozen, and that this continues regardless of how cold they are (Proebsting, et al., 1980). The LT_{50} became lower as long as temperatures were below -1 C. Field studies of apples showed that short term changes in hardiness were closely correlated to ambient air temperatures of the previous day (Howell and Weiser, 1970) and in grapes, air temperatures during dormancy affected hardiness (Damborska, 1978). This had also been previously seen in peach (Proebsting, 1963). Damborska (1978) also reported that the rate of dehardening in grapes is dependent upon grape variety plus the duration and magnitude of positive temperatures experienced.

Ashworth (1982) proposed that in peach a mechanism in bud cold survival is that of extracellular frozen water causing migration of water into preferred sites in the bud scales. This opinion was also promulgated by Quamme (1983). It is after this migration from the primordia, that the remaining water deep supercools, facilitating freezing avoidance. The structural, physiological and morphological features of the buds are critical components in this mechanism. Ashworth and Abeles (1984) proposed that micropores in cell walls not only alter the freezing temperature of water, but impede the spread of ice and facilitate deep supercooling in cells of various tissues. This would tend to mean survival is on a cell by cell basis provided that the tissue is mature (Howell and Shaulis, 1980). As was reported for grape buds by

Ahmedullah, et al. (1986), midwinter pruning has little influence on the ability of a whole tissue to supercool and survive, since each cell survives cold stress independently.

It has also been shown that xylem continuity is broken as cold hardiness increases in some plants. Ashworth and Rowse (1982) and Ashworth (1984) observed that the spread of ice in Prunus is prevented by the lack of xylem continuity and this, in turn, facilitates supercooling of water in the flower buds. Deep supercooling in xylem parenchyma occurs with individual cells being the unit of freezing, while freezing is at the tissue level in buds (Ashworth, 1986). Prunus buds have also been observed supercooling even during the early stages of flower development in the spring (Andrews, et al., 1983b).

The ability of grapes to survive the winter has been linked to hydrolysis of starch to sugars in the roots during October to December (Warmund, et al., 1986); however, there is no evidence of translocation of carbohydrates during this time (Winkler and Williams, 1985). This could be reconciled by the observation that grape vines that were the least stressed during the growing season (fewer fruit, thinned) were the most hardy (Howell, et al., 1978), implying that carbohydrate reserves were greater. This situation obtains until the canes become over vigorous in later years and are unable to cease growth, mature and acclimate at the proper time. This again points to the complexity of the mechanism of hardiness and its seeming unpredictability.

Breeding and Selection

The culture of grapes is widespread throughout the world, but is limited to those areas of temperate climes which have plenty of sunshine during the growing season. Vitis vinifera is the most popular of the grape species with thousands of cultivars. The center of origin for grapes has been placed in the vicinity of the Caspian Sea (Wagner, 1974), although there are many species found in the Americas and elsewhere, which are presumed to have been indigenous in pre-Columbian times. Early in man's history, grapes were carried from place to place, as new habitats were explored. The ease of asexual propagation facilitated the process of transporting grapes. Natural selection thinned out the weaker genotypes, and men chose among the results.

In the 1800's, grapes taken to Europe from America inadvertently carried the phylloxera insect, which feeds on grape roots. The vines of Europe were devastated and it took years of labor, breeding and experimentation to find compatible American rootstocks which carried resistance to these insects. At this time, hybridization was also performed in the hopes of producing grapes that had the fruit qualities of V. vinifera, the hardiness and resistance to phylloxera of the American types. The results were improvements in hardiness and resistance, but fruit quality was inferior.

Cold hardiness of V. vinifera has been a major limiting factor in the spread of these grapes into higher latitudes. It has been pointed out that the limits of grape cold hardiness are set by the limits of supercooling, which is about -40 C (Pierquet, et al., 1977). It has been estimated that 10% of the United States' grape crop is lost due to cold weather each year (Johnson and Howell, 1981). This is associated with the limits of hardiness for V. vinifera at relatively higher temperatures, typically around -15 C (Oberle, 1943; Clore, et al., 1974). Extreme cold temperature tolerance is not the sole determining factor in survival of grapes, as was previously pointed out, but in the case of vinifera grapes it is probably the most prominent. In muscadine grapes, Muscadinia rotundifolia, one strategy to avoid freezing stress is to commence growth relatively late in the spring, offering greater security against late frosts (Olmo, 1986).

Cane maturity is important, in that those canes that are shaded may fail to obtain the hardiness levels of those canes which receive full sunlight (Howell and Shaulis, 1980; Johnson and Howell, 1981). This issue of cane maturity was also of concern to Conrod (1987) and she rejected the idea of being able to accurately select one year grape seedlings for cold hardiness, since some otherwise hardy seedlings may be unwittingly rejected.

A difficulty with grape breeding for cold hardiness is that there is differential hardiness between the stems and

buds, with the buds being less hardy (Clark, 1936; Wu, 1981). This was noted in peach by Layne (1983), and he indicated that the hardiness of buds and stems needed to be assessed independently. In grape, which has a multiple bud system, the primary bud is the most fruitful and the least cold hardy. This primary bud may be killed, with only secondary or tertiary buds surviving (Ashworth, 1986). Shoots arising from secondary or tertiary buds are usually sterile, that is, bearing few, if any, flowers (Clark, 1936).

Little work has been done to quantify the inheritance of cold hardiness, especially in perennial plants. The main strategy has been to perform crosses and then select on the basis of performance. Mowry (1964) reported that the inheritance of cold hardiness in peach flower buds was difficult to predict. He did indicate, however, that hardy parents are able to transmit greater mean bud-hardiness to their offspring than nonhardy parents.

Peach wood cold hardiness showed a high broad-sense heritability (Cain and Anderson 1980); however, those hybrid progeny expressing the greatest cold tolerance also bore the poorest quality fruit. The work of Hildreth and Powers (1940) found that winter hardiness in strawberries was a simple dominant trait. In the case of apple, the inheritance is more complex; both dominance and epistasis have been ruled out as being major components of variance in the inheritance of cold hardiness (Watkins and Spangelo,

1970). Cytoplasmic inheritance plays a key role in the inheritance of cold tolerance in apples (Wilner 1965). In a study of blueberry, Fear, et al. (1985) suggested that additive variance may be more important than non-additive variance in the inheritance of cold tolerance. These few are the major reports on quantitative heritability studies of cold tolerance in perennial plants. The major deterrent to making progress in understanding heritability has been the long time between generations and the inability to accurately determine cold hardiness through the early screening of young progeny.

The inheritance of cold tolerance in grape has not been determined. It has been pointed out that grape is an inconvenient plant for genetic analysis due to the long life cycle, high number of chromosomes (n=19 and n=20), generally low germination of seeds and partial sterility of the ovules (Einset and Pratt, 1975). The New York State Agricultural Experiment Station program, which began in 1888 with emphasis on combining vinifera fruit quality with cold hardiness found in the American grapes, has not published a report on quantification of cold acclimation inheritance. However, superior cold hardy types have been released from the Geneva station (Hedrick, 1908; Oberle, 1943). Cold hardy types have been reported from the USSR (Gorodea, 1983). However, Isaenko (1977) stated that, "It is impossible by means of breeding to develop varieties with high and stable frost resistance in grape,..." This

pessimistic view is not shared by all researchers. What is required for breeding cold hardy genotypes is that there be suitable parents, that cold hardiness is a heritable trait and that seedlings can be obtained (Young and Hearn, 1972). These conditions have been met in grape heretofore. Care should be taken in parental selection, however, as Dorsey and Bushnell (1925) have pointed out with plum, where both parents are hardy, the offspring are hardy. When both parents are tender, the progeny are tender, and when the parents show extremes of hardiness, the progeny are intermediate. When mating the hardiest parents, which have been screened for other characters, one may rely on additive variance for cold hardiness breeding (Stushnoff, 1972). Unfortunately, the most desirable fruit characteristics in grape tend to be found in seedlings which lack hardiness (Oberle, 1943).

Chromosome studies of grape have been few. Two chromosome numbers have been reported, n=19 for vinifera and n=20 for the muscadines (Olmo, 1937). The chromosomes appear as small short rods with homologous chromosomes being difficult to distinguish. Varietal differences were reported to have gone undetected because the chromosomes were so small. At one time there was an unsubstantiated belief that polyploids were generally more cold hardy than diploids, although this was dispelled by Bowden (1940a; 1940b). Roberts (1986) in studying wheat reported that chromosomes 2A, 5A and 5B carry loci affecting cold

hardiness after 8 weeks in a 6 C/ 4 C regime, while chromosomes 6A, 3B, 5B and 5D were involved when plants were grown at 0.8 C followed by -5 C. It was suggested that two different genetic and biochemical bases were the reasons for these differences. One mitigating factor in all these confusing and scant reports is the heterozygous nature of perennial fruit crops (Firoozabady and Olmo, 1987). These researchers recommend the use of 'mass selection' methods, once a suitable selection procedure has been determined for the particular trait. Their report also noted another difficulty with grapes; there is strong inbreeding depression, indicating to them that most gene action is probably non-additive.

Meza-Basso, et al. (1986) suggested that one might measure varietal differences via the changes in proteins which may occur as a result of exposure to cold temperatures. This is similar to the work of Siminovitch, et al. (1964) and the use of ninhydrin to measure amounts of amino acids leached from injured cells. Tamassy and Zayan (1982a) proposed that in cold hardy apricot tissues, hardiness increases with increasing sugar content, but that non-hardy tissues do not improve in hardiness with an increase in sugars.

Morphological observations may also be employed as a means of selection for cold hardiness. Xui-wu, et al. (1987) observed that the root cortex cells of grape vary in size with respect to cold hardiness. They imply that the

hardier the tissue, the smaller the cells will be and the more compacted together. Isaenko (1983) correlated cold hardiness of grape root cold hardiness to shoot cold hardiness in one-year seedlings, and also reported compacted cells possess greater cold tolerance. Another approach would be by the observation of Yeager (1936) that hardy grapes tend to have glabrous (or nearly) leaves. All of these procedures need to be substantiated for development of useful screening procedures.

A different approach was suggested by Olien (1974), who proposed that one might improve cultivars for cold tolerance by breeding for factors that modify the dynamics of the freezing of water. This would ultimately mean measuring the freezing of water in tissues, which can be accomplished by observing the release of the latent heat of fusion. This is essentially differential thermal analysis (DTA). The procedure has been recommended by Quamme (1985) as a method to screen and select woody plants in general, and has been used for azalea (Graham and Mullin, 1976b), blueberry (Biermann, et al., 1979) and Pyrus sp. (Rajashekar, et al., 1982). Cold hardiness is, however, temporal, and as such is relative in terms of the seasonal acclimation as well as location (latitude, elevation) of the plants. Breeding material should be chosen not only for maximum cold hardiness, but for acclimation and deacclimation characteristics (Hummer, et al., 1986).

Wild plants have been used to impart cold hardiness in breeding programs. Hildreth and Powers (1940) collected about 42,000 strawberry plants in the Rocky Mountains for their breeding program in Wyoming. Strawberry, black currant, blueberry, raspberry and sea buckthorn breeding programs were substantially enhanced by the use of wild germplasm in Finland (Hiirsalmi, 1985). Wild grapes have been used for decades to improve grape hardiness (Hedrick, 1908).

There have been problems in breeding grapes and other small fruits, but there have been solutions. One impasse was the short storage time of grape pollen. Ganeshan (1985) has shown that grape pollen may be stored in liquid nitrogen (-196 C) for more than a year with no significant decrease in viability compared to fresh pollen. Another problem has been poor germination of grape seeds once the crosses have been made and the fruit harvested. Usually, the seeds are held at 10 C. for 3 months in moist peat or sphagnum (Einset and Pratt 1975) as a stratification treatment. The germination after that has been shown to be quite variable. Ellis, et al. (1983) in a test of seven grape genotypes, saw germination range from 0% in two cultivars to a maximum of 24% in the rest. They also showed that alternating the temperature on a 30/20 C regime proved to give the best germination of those seeds tested.

Part of the low germination problem may lie in the choice of parents in any cross. The choice of the maternal

genotype is critical in grape (Olmo, 1936) and in strawberry (Powers, 1945). Olmo (1936) also notes that seed from grape hybrids germinate better than do those from self-pollination. Thus heterosis seems to play an important role.

Several researchers have approached the problem of poor germination with various degrees of success. Manivel and Weaver (1974) reported that all of the heat treatments attempted (27 - 54 C) failed to break rest, while ethephon slightly enhanced germination of grape seeds. Soaking seeds in indolebutyric acid (IBA), indoleacetic acid (IAA) or ethrel at several concentrations prior to sowing failed to produce normal seedlings, although germination was improved (Selim, et al., 1981). Gibberellic acid (GA_3) at 250 ppm improved germination of early muscat grape seeds to 74% (Pal, et al., 1976). This is to be contrasted with the poor germination of untreated cultivar 'Cot'. Of the 1278 seeds collected, 17 germinated, yielding six established seedlings (Ottenwaelter, et al., 1974).

The work to achieve seedlings from crosses of 'seedless' grapes has improved the chances of getting viable offspring from difficult crosses. Cain, et al., (1983) were able to obtain normal embryos and seedlings from seeded and seedless cultivars when the embryos were extracted 38 to 52 days postanthesis; all of the 'rescued' embryos survived. Seeds of 'Cot' that were stratified and germinated in the

usual way produced only 1% germination of the 4000 seeds extracted.

Growth of ovules is induced by fertilization, yet the embryo is limited to very little growth until the endosperm has become cellular. This occurs approximately 20 to 21 days after anthesis (Kassemeyer and Staudt, 1983). It is not until 75 days postanthesis that grape embryos attain maximum size (Nitsch, et al., 1960; Staudt, et al., 1986). There are two media which are common to grape embryo rescue (Gray, et al., 1987) of which Nitsch's medium is the most frequently used (Nitsch and Nitsch, 1969). Nitsch's medium is solid and was found to be superior to liquid media used with filter paper supports or bridges (Gray, et al., 1987). It is presumed that the liquid medium may provide inadequate aeration when compared with the solid.

Grape cells will also grow in suspension culture (Arya, et al., 1962). Adventitious embryos are produced from excised flower clusters, leaf, stem or petiole segments (Krul and Worley, 1977). A possible use for this procedure would be to screen grapes for cold hardiness using stem callus culture. Sakai and Sugawara (1973) reported that poplar callus may be acclimated to survive temperatures around that of liquid nitrogen (-196 C). They proposed that the study of cold acclimated callus would be an effective way to study cold acclimation mechanisms in plants. Ogolevets (1976) was able to harden isolated callus tissue of sour cherry, but pointed out that isolated tissues

require the same acclimating conditions as whole plants. He also noted that young (seven day old) cultures were hardier than old (42 day old) cultures.

Deng (1987) has suggested that grape stem calli may be used as a means to study cold hardiness mechanisms. She found that abscisic acid (ABA) enhanced grape callus growth. It has also been shown that ABA can induce freezing resistance in cell suspension cultures (Chen and Gusta, 1983; Johnson-Flanagan and Singh, 1987). This use of ABA to enhance the production of grape calli in culture might also generate misleading cold hardiness data. Although their work was with cell suspension cultures, Dix and Street (1976) point out that individual cells in culture differ from one another, and when exposed to cold will be arrested in various phases of growth. Response would then be expected to be highly variable.

Research Objectives

The mechanisms that allow for the survival of grapes in midwinter are poorly understood. The inheritance of these mechanisms has rarely been investigated. Part of the problem has been the lack of a rapid and reliable means to estimate cold hardiness. Another problem has been the long generation time of those species that over-winter. The purpose of the research reported here is two-fold: (1) to investigate the inheritance of midwinter hardiness in grapes and, (2) to define and establish the efficacy of using DTA

as a screening method in breeding grapes for midwinter cold hardiness.

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THE INHERITANCE OF MIDWINTER COLD HARDINESS IN GRAPES

Introduction

Grapes survive winter in cold environments by freezing avoidance through supercooling. The buds are the most vulnerable tissues at this time and have higher freezing points than do the stems. Bud survival is the most critical, since the production of a crop, as well as the survival of the plant, depends upon the buds being able to grow in the spring. Hardiness also is a function of acclimation events in the fall, as well as deacclimation events in the spring. Acclimation and deacclimation involve complex mechanisms such as the movement of water, physiological changes in membranes, and shifts in overall cell metabolism. Midwinter cold hardiness appears to be more stable than acclimation/deacclimation responses and would, therefore, be easier to quantify. This is largely due to the stability of the stress responses during midwinter, since environmental fluctuations tend to be minimized during this time.

Methods used to quantify cold hardiness which involve test winters are unpredictable. The use of laboratory procedures allows for controlled freezing of known magnitude. Most of the laboratory methods previously used

arrive at the estimated mean low killing temperature (LT_{50}) by way of regression and do not directly measure this value. The use of differential thermal analysis (DTA) appears to be a superior method; however, the relationship to viability needs to be established. DTA use has previously been suggested by several authors for use as a screening method in breeding for cold hardiness, although it has yet to be reported in the literature as having been used as such.

Another difficulty in breeding grapes, besides screening, is the poor germination of seeds that result from crosses, or from self-fertilization. It may be possible to increase the number of seedlings obtained from breeding experiments through the use of 'embryo rescue' procedures. This has been successfully used in breeding seedless grapes which abort ovules early in their development.

This study was done to quantify the inheritance of cold tolerance by making crosses between grapes, which have been reported to be cold hardy in midwinter at contrasting temperature levels. Embryo rescue was studied in order to improve the number of seedlings obtained from the crosses.

Materials and Methods

Plant Materials

Grape crosses were made between cultivars that are very different in terms of known cold hardiness. In late May, 1985, a series of crosses were made with 'Valiant', a cold hardy cultivar which had been growing in the vineyard at

Colorado State University, Fort Collins, Colorado, since 1981. This site is at 1524 meters elevation and has a growing season that averages 145 days. The soil type at this location is a clay loam with an unamended pH of 8.2. Fresh pollen was air-expressed 338 linear kilometers to Fort Collins from Grand Junction, Colorado, elevation 1397 meters.

Pollinations were performed with fresh pollen from Vitis vinifera L. 'French Colombard' and V. vinifera L. 'Zinfandel'. Fruit from these crosses were harvested August 16, 1985, when the fruit were fully ripe. Using a hand refractometer, fruit sugars were measured and the average was 16.5%. Seeds were extracted from the fruit and stratified at 4.5 C for eight months in moist sphagnum. There were 106 seeds from the 'Valiant'x'Zinfandel' crosses, of which 31 floated and were discarded. Of the 111 seeds from the 'Valiant'x'French Colombard' crosses, 28 floated and were discarded. The seeds were then soaked in a 10% Chlorox (sodium hypochlorite) solution for ten minutes and rinsed in de-ionized water. Twenty seeds from each cross were placed on moist blotter paper in plastic germination boxes. They were then placed in a germination chamber set for 30/24 C day/night. One month later, none of the 40 seeds had germinated. All were then scarified by notching each seed with a small metal file until the endosperm was just visible. Only two seedlings resulted. In an attempt to improve germination, the rest of the seeds, 55 'Valiant'

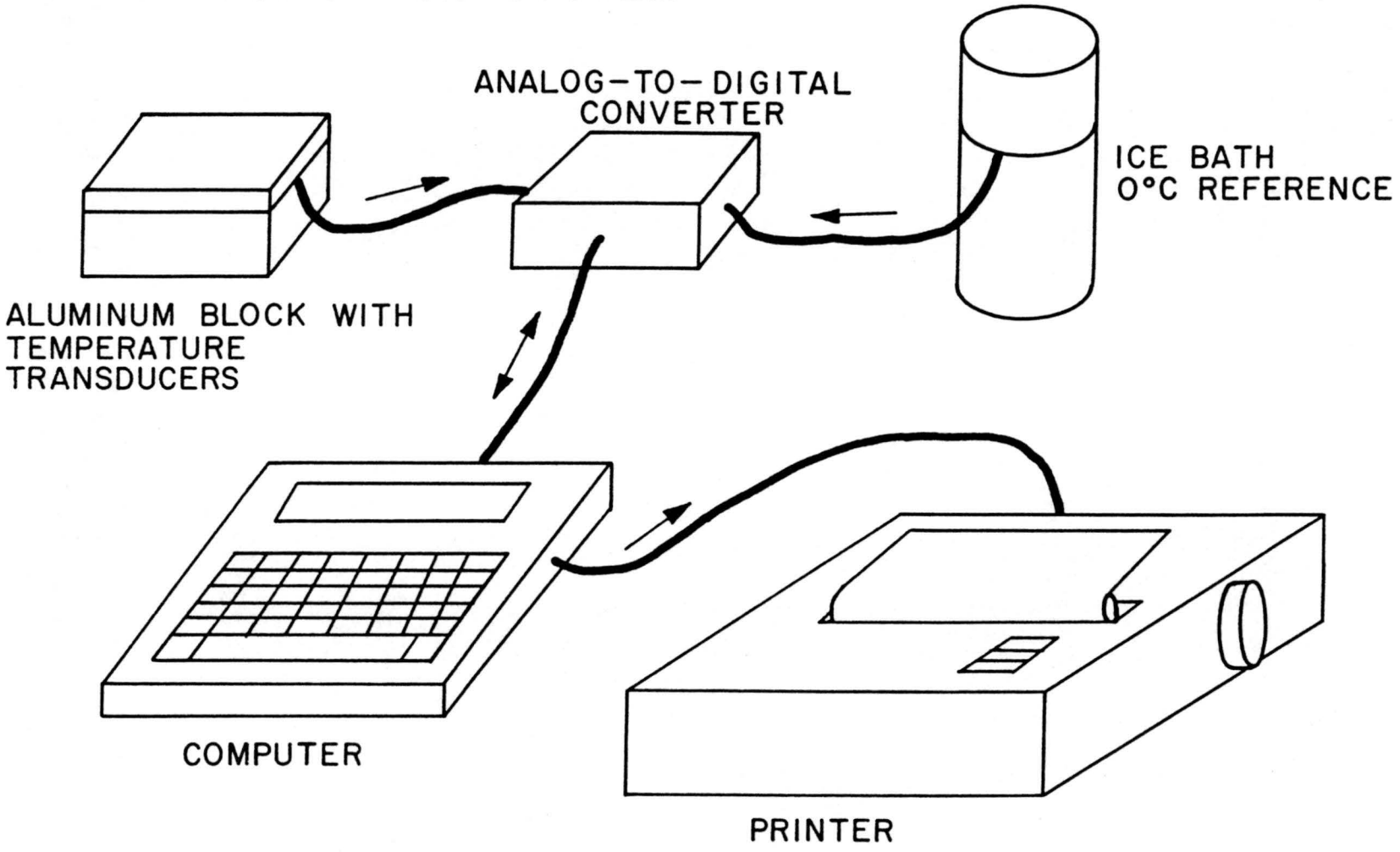
x'Zinfandel' and 65 'Valiant'x'French Colombard' were planted in plastic cell-pacs with Fison's No. 3 ™ seedling mix as the growth medium. The temperature regime was the same, 30/24 C. Upon germination, the seedlings were transferred to a greenhouse and eventually re-potted to larger pots as needed. In May and June, 1987, all of the seedlings were transplanted to the vineyard at 10 foot within-row intervals and 10 feet between rows. A four-arm kniffin system of training was established.

Differential Thermal Analysis

The apparatus used for DTA was quite similar to that of Hamman (1988). The arrangement is laid out in schematic form in Fig. 1. The sample holder was a 0.75 kg aluminum block, which had been cut to form a main body and a lid. The main body had ten equally spaced holes drilled 15 mm in diameter and 37 mm deep, into which 15 mm diameter Nalgene centrifuge tubes, cut to 37 mm length, were placed. The lid had 10 equally spaced 3.17 mm holes drilled to match the centers of the holes in the main body. The body and lid were held together by two stud bolts and wing nuts. Wires were placed in eight of the 10 lid holes to connect and support the thermoelectric modules (TEM's). The TEM's were the temperature transducers used to detect the latent heat of fusion given off when water in each sample froze. One hole allowed the entrance of the thermocouple used to measure the internal block temperature, while the tenth hole

Fig. 1. Schematic of the DTA apparatus used. The computer controlled the analog-to-digital converter and the printer. The ice bath was used as a reference temperature to compare with readings obtained from the thermocouple inside the aluminum sample block. Arrows denote the flow of information.

DATA ACQUISITION SYSTEM



became blocked during construction and was not used. Prior to the construction of this device, a similar block was made, but thermocouples were used as transducers. Thermocouples were found to be much less sensitive than the TEM's and could not be effectively used for small grape buds. The same problem and remedy were reported by Andrews, et al. (1983). The TEM's are manufactured by Melcor (Trenton, NJ) and are intended to be used as miniature heat pumps. In the model Fco 6-18-06L used here, the role is reversed and the 18 semiconductor thermocouples act as heat sensors to detect heat given off when sample water freezes.

The temperature inside the sample block, at which each sample freezes, is measured by a single thermocouple inside the block. This thermocouple (type T) is wired in series with a reference thermocouple which is in a 0 C ice bath. The TEM's, although larger than thermocouples, are 6.8 x 8.3 x 2.7 mm in size. There are two ceramic faces to each device and the slightly smaller face is used for the sample and the larger face for the oven-dried (70 C, 72 hrs.) reference piece. Each reference piece was a small section of oven-dried grape stem cut longitudinally and then trimmed to be close to the mass of the sample. Both the sample and dry reference were attached to the respective TEM by wrapping them with a thin strip of Parafilm™ (American National Can, Greenwich, CT).

Bud samples were taken by making a straight longitudinal cut down the stem, removing the bud and a small

portion of subjacent stem tissue. This flat surface was placed against the ceramic plate for maximum heat exchange. Analog data were read from the temperature transducers and converted to 12-bit digital format by an ADC-1 (Remote Measurement Systems, Inc., Seattle, WA) analog-to-digital converter. The ADC-1 used here had an amplifier option which allowed for a resolution of two microvolts per channel. These digital data were relayed every three seconds to a portable computer (Radio Shack, Ft Worth, TX; TRS-80 model 100). The computer was programmed in BASIC (Fig. 13) and sent a microvolt temperature reading for each channel to a printer (Epson FX-80, Epson Corporation, Japan). These data were later converted to temperatures by a computer program, written in BASIC, which utilized an algorithm (Fig. 14) for type T thermocouples given in thermocouple reference tables (Omega Engineering, Inc., 1986; Powell, et al., 1974).

Once the samples and reference pieces were attached to the TEM's, the assembled block was placed inside a cardboard box filled with styrofoam packing chips and this was sealed with tape. The sample assemblage was placed in a Warren Sherer (Marshall, WI) model CF-2 chest freezer set at -70 C. The cooling rate averaged -15.8 C per hour with a standard deviation of less than 0.5 C per hour. Quamme (1986) had reported that there was little difference in low temperature exotherm values of grape buds over a cooling rate range of 1.5 to 40 C per hour. One freezing run with eight samples

typically took four hours and was terminated at -45 C. The data were then analyzed to determine the mean bud exotherm for each of the samples.

The Use of DTA for Cold Hardiness Determination of Grapes

The use of DTA to evaluate cold hardiness of grapes and to use the resultant data to select progeny by this technique has been suggested (Quamme, 1985; Graham and Mullin, 1976b; Bierman, et al., 1979; Rajashekar, et al., 1982). However, it has not been reported as having been used to this end. It was, therefore, important to evaluate the suggested use of DTA for this. Progeny from both crosses, 'Valiant'x'Zinfandel' and 'Valiant'x'French Colombard' were sampled during midwinter and the buds tested for hardiness using the DTA apparatus previously described. Both populations were evaluated separately and the data analyzed by analysis of variance to determine if the variance among progenies was large enough to provide evidence that this technique significantly distinguished differences among them.

A second approach was used to compare the mean bud survival temperature, as determined by DTA, with a different procedure. A test for browning was performed on 'Valiant' buds by placing the buds in weighted glass test tubes, one bud per tube, and holding these in a methanol bath. DTA was performed the day before on eight separate buds and the

cooling rate and mean bud hardiness determined. The following day, the methanol bath experiment was performed using the cooling rate of the previous day's DTA (-15.5 C/Hr). Buds were cooled in the bath and the temperature held at the mean, plus approximately two and four standard deviations either side of the mean, for five minutes each. Sample buds were removed at each temperature and later held at 4 C for 16 hours in the glass tubes. The buds were held at 30 C for seven days in the same sample tubes. The buds were then examined for damage by dissection and the color noted. Buds that received no treatment, except the 4 C and 30 C holding temperatures, served as control treatments.

The linearity of the DTA cooling rate of the apparatus used was also examined. Temperature and time were noted on one DTA run of eight buds. These data were then analyzed using a standard linear regression technique of least squares (Snedecor and Cochran, 1967).

Since the bud samples for DTA were removed in a manner that left some subjacent stem tissue attached, it was important to establish that exotherms measured were actually separated, as assumed, into bud and stem components. Eight buds of 'Valiant' were subjected to DTA consisting of two buds with stem and the rest stem sections without buds.

Estimation of the Inheritance of Cold Tolerance in Grapes

Canes were taken from 'Valiant' and the progeny of both crosses, 'Valiant'x'Zinfandel' and 'Valiant'x'French Colombard', from plants growing in the vineyard at Colorado State University in Fort Collins. 'Zinfandel' and 'French Colombard' canes came from plants grown at the Orchard Mesa Research Center, Grand Junction, Colorado. Only canes that were mature, as denoted by a brown or tan periderm, were evaluated. These canes were judged to have been hardened, since they were grown in the field prior to the start of testing in January, 1989. Typically, the time from field to laboratory was less than 10 minutes and another 20 minutes was required to prepare the samples for testing. This time for sample preparation was the same as that of Montano, et al. (1987). Preparation consisted of removing the buds and subjacent stem tissue with a sharp surgical knife. Samples and dry reference pieces were tied onto TEM's and the block assembly sealed in an insulated box, as previously described. This was placed into the -70 C freezer and the four hour run begun. Accumulated data were later analyzed by analysis of variance and z-tests to determine variance of parents and progeny, as well as the relationships of progeny means to parents and mid-parent values. Heritability was estimated by the procedure of Cain and Anderson (1980).

Field observations were made to confirm the survival or death of parents and progeny in June, 1989. This was

compared with the recorded extreme low winter temperature for the 1988-89 season and the data obtained from DTA. The winter of 1988-89 was severe and thus made field observations important.

Leaf Pubescence as a Selection Criterion for Cold Hardiness

Since leaf abaxial pubescence might be inversely correlated to cold hardiness of grapes (Yeager, 1936), leaves of nine French grape cultivars, the parents of the progeny being studied and the progeny were collected and observed for degree of pubescence. It was hypothesized that there may be some genetic linkage involved with these two traits.

Embryo Rescue of Grape

In February, 1986, 100 'Valiant' grape seeds that had received four months of moist stratification in sphagnum were placed in warm moist sand (30.5 C) for germination. The sand was later kept in an 24/18 C day/night germination chamber and the seedlings removed and counted as they emerged.

In August, 1987, seeds were taken from the open-pollinated fruit of 'Valiant' grapes, approximately 65 days after anthesis. The seeds, which still had soft white seed coats, were rinsed in de-ionized water for 10 minutes, then emersed in 10% Chlorox (sodium hypochlorite solution) for 10

minutes. They were then rinsed three times in sterile water. Thirty-two embryos were extracted and placed individually in tubes containing 20 ml of Nitsch's medium (Nitsch and Nitsch, 1969).

Extraction of the embryos was performed on sterile petri plates under a dissecting microscope. The seeds were initially scored from the micropilar end to the chalazal end using a sterile surgical blade, while held in place with sterile forceps. The point of the blade was then positioned on the petri plate just in front of the micropilar end. Keeping the point on the plate, slight pressure was brought to bear on the seed coat with the knife blade until a small fracture was initiated. The point of the blade was then positioned on the seed 2/3 of the distance of the seed from the micropilar end. Pressure was applied until the knife penetrated the seed coat down to the plate, making sure that the knife cut only the 1/3 of the seed near the chalazal end. A slight twisting motion of the blade usually laid the seed open, exposing the undamaged embryo, which was then extracted and placed on the medium for culture.

Midwinter Water Content of Grape Buds and its Relationship to Cold Hardiness

Many investigators have reported that there is an inverse relationship between water content of over-wintering tissues and cold hardiness (Graham and Mullin, 1976a; Gusta, et al., 1983; Kaurin, et al., 1984; Pierquet and Stushnoff,

1980; Quamme, 1983; Wolpert and Howell, 1985a; 1985b). In mid-January, 1989, buds of the parents and 'Fredonia' were removed from hardy mature canes with virtually no stem tissue attached. Fresh weights of ten buds per cultivar were recorded and the buds placed in a 70 C oven for 72 hours. Dry weights were then taken and percent moisture determined.

The same procedure was followed for six progeny from both crosses in early March. The progeny were chosen so that the broadest range of cold hardiness among the offspring would be represented. The canes had been in cold storage (-23.9 C), individually wrapped in plastic wrap, since February. The moisture content was determined in the same manner as with the parents.

Results and Discussion

The Use of DTA for Cold Hardiness Selection of Grapes

Data taken from DTA's of 'Valiant'x'Zinfandel' progeny, as well as 'Valiant'x'French Colombard' progeny, were used in an analysis of variance. F-test values of 23.73 (Table 1) and 80.30 (Table 2) indicate that there are true differences between progeny means. The highly significant results of these tests suggest that DTA may be a valid way to determine mean bud low temperature killing points.

The efficacy of DTA was also verified by a browning test of 'Valiant' grape buds. The cooling rate was the same as that used when the experimental mean of -35 C was

Table 1. Analysis of variance of the estimated mean freezing temperatures of progeny ('Valiant' x 'Zinfandel') as determined by DTA.

Source	DF	SS	MS	F	F Prob.
Between	11	304.71	27.701	23.73	.0000
Within	72	84.05	1.167		
Total	83	388.76			

Table 2. Analysis of variance of the estimated mean freezing temperatures of progeny ('Valiant' x 'French Colombard') as determined by DTA.

Source	DF	SS	MS	F	F Prob.
Between	9	933.47	103.719	80.3	.0000
Within	64	82.67	1.292		
Total	73	1016.14			

determined by DTA on the previous day. Buds subjected to the control treatment, -31 C, and -33 C showed no browning of the compound bud (primary, secondary and tertiary buds). Three of the buds subjected to -33 C did show browning of the primary bud, while the secondary and tertiary buds were green. This is noted in the column 'Both' in Table 3. At -35 C, two buds were totally brown, one totally green, while four had both brown and green buds. Of these four, two had both primary plus secondary brown buds, the tertiary being green, while the other two had only brown primary buds, with green secondary and tertiary buds. At temperatures of -37 C and -39 C all buds were brown. These data support the idea that DTA is a valid method for the estimation of the mean killing temperature of grape buds, since -35 C was determined to be the mean killing temperature by both DTA and browning.

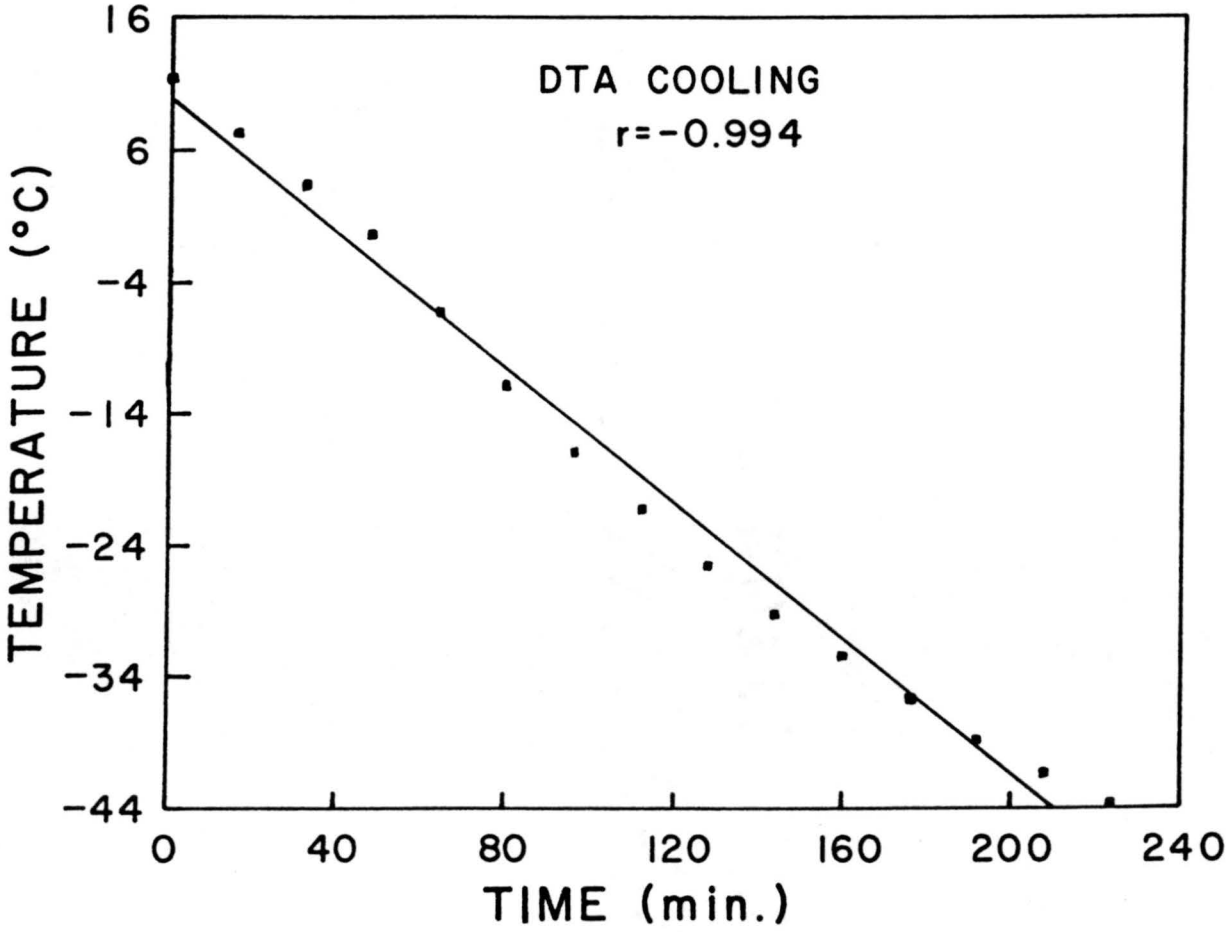
It is important that the sample cooling rate be at a constant rate from initiation to the end of the experiment. Since DTA's were to be performed with a previously untested sample block, the temperature inside the block was plotted over time for one randomly selected DTA run. The plot is shown in Fig. 2. The linear regression yielded an $r = -0.9940$. These data indicate that the DTA cooling rate using this apparatus is linear over the temperature range that the samples were subjected to.

During the sampling process, buds that were taken from canes had some subjacent stem tissue attached. The bud

Table 3. The effect of temperature on the survival of 'Valiant' grape buds as determined by a test for tissue browning. The number of grape buds of each color class is given for each of the six temperature treatments.

Temperature	Color of Bud		
	Green	Brown	Both
control	4	0	0
-31	5	0	0
-33	5	0	3
-35	1	2	4
-37	0	8	0
-39	0	8	0

Fig. 2. Regression of temperature vs. time of one DTA run. The cooling rate was a constant linear rate (-15.5 C/Hr) during the entire experiment. (r=-0.994)


DTA COOLING
r=-0.994
TEMPERATURE (°C)
16
6
-4
-14
-24
-34
-44
0
40
80
120
160
200
240
TIME (min.)

exotherms of 'Valiant' were occurring at the mean temperature previously established (-35 C). Cane sections that had only stem, but no buds, gave exotherms at colder temperatures (-41.95 C, s^2 = 0.180), but no exotherms at or near -35 C. This confirms that the exotherms observed at the warmer temperatures actually were bud exotherms and not those of the attached stem tissue. The sections of stem without buds produced unexpected high temperature exotherms (HTE) that had previously not been seen in DTA's of cold hardy 'Valiant' buds during midwinter. These exotherms occurred at a mean of -23.2 C and are possibly produced by unbound water released during wounding.

Estimation of the Inheritance of Cold Tolerance in Grapes

Parents that differed considerably in midwinter hardiness were chosen so that progeny produced might give a good estimate of the inheritance of cold hardiness in grapes. 'Valiant' has been reported to have fruited well and shown no winter damage following winters when temperatures were near -45 C (Peterson, et al., 1988). It is the product of a cross between V. riparia and 'Fredonia'. 'Fredonia' is the result of a V. labrusca x V. vinifera cross (Hedrick, 1908). 'French Colombard' and 'Zinfandel' are both V. vinifera with hardiness levels which are quite similar (Clore, et al., 1974). 'French Colombard' has been

estimated to have a mean midwinter bud hardiness of -20.9 C using DTA (Andrews, et al., 1984).

DTA data of 'Valiant' were collected on two dates in January and February 1989, near the beginning and end of the progeny bud sampling period in midwinter. This was done to determine if there were differences in hardiness on these two dates. An F-test showed that the variances on both dates were equal ($p=0.01$), which justified comparing the equality of the means. A t-test indicated that the means were not significantly different ($p=0.0005$) on these two dates. This would tend to validate the use of progeny data taken between and around those two dates. The concern here is illustrated by the differences shown between the plot of a DTA of 'Valiant' during midwinter hardiness (Fig. 3) and during deacclimation (Fig. 4). During midwinter, distinct exotherms for the bud, as a unit, and the stem tissue exist. This is lost during deacclimation. The period for testing midwinter hardiness is shortened and spring weather must be monitored and taken into consideration. The shift in grape DTA during deacclimation has been observed by other researchers, most recently by Hamman (1988).

Given that the DTA's were determining mean bud hardiness temperatures, the progeny were evaluated for bud hardiness. Pedigrees, as far as they are known, are presented for 'Valiant'x'Zinfandel' (Fig. 5) and for 'Valiant'x'French Colombard' (Fig. 6) progeny. The numbers under the cultivar names give the estimated mean midwinter

Fig 3. Plot of DTA of one 'Valiant' bud in midwinter. The two exotherms occurring at higher temperatures are associated with unbound water. The exotherm at around -35 C is associated with the bud and the exotherm at the lowest temperature is from the stem.

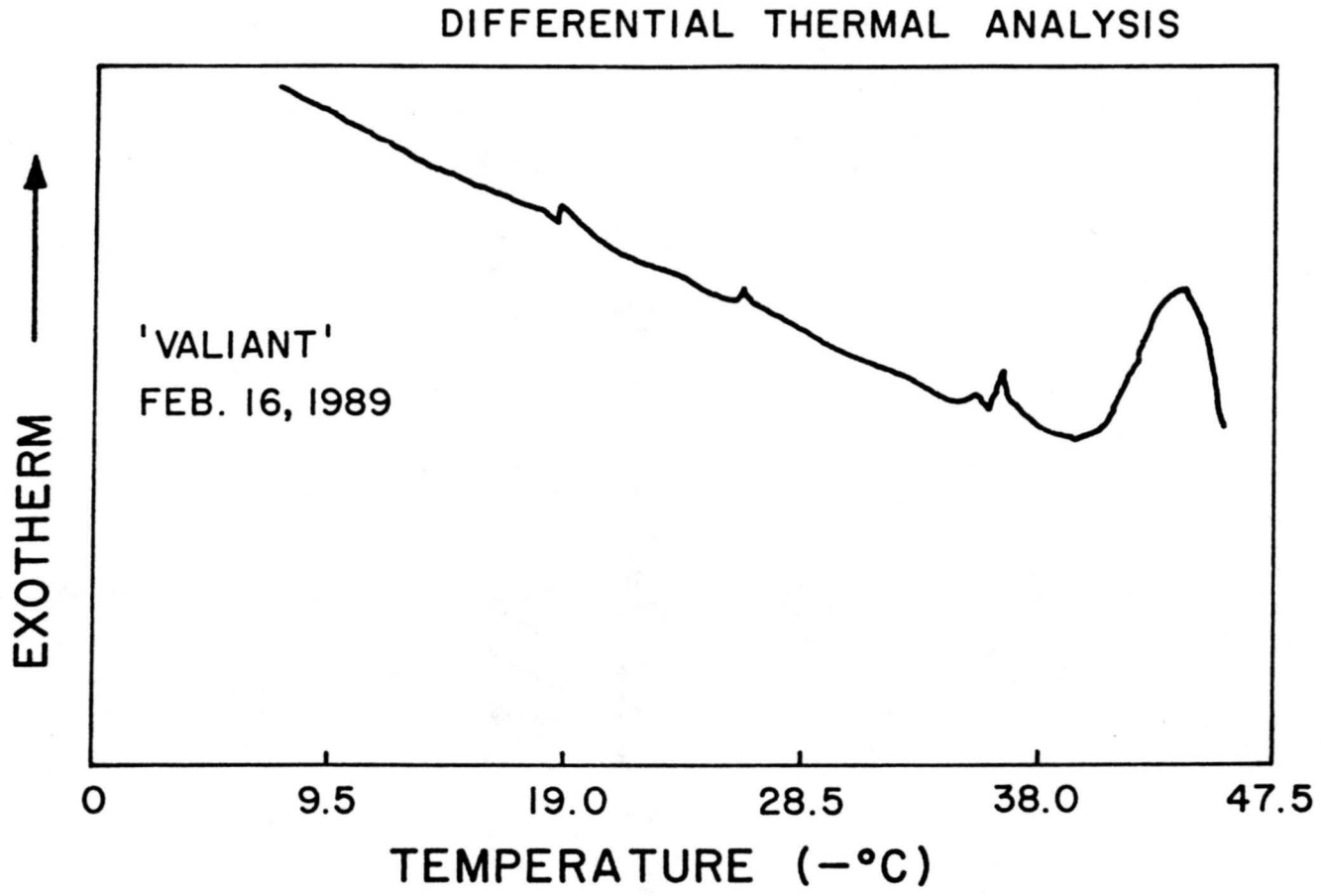
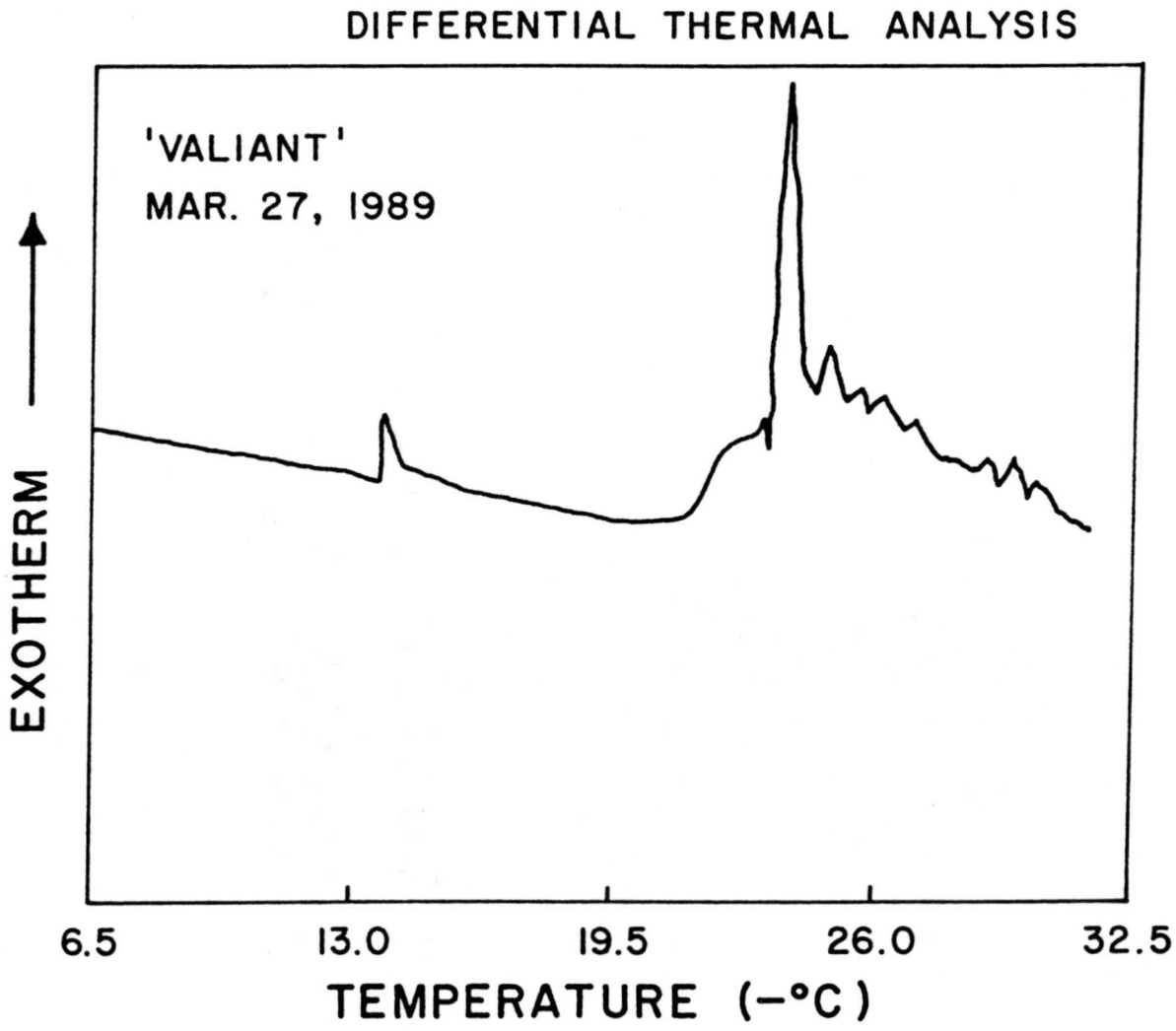

DIFFERENTIAL THERMAL ANALYSIS
'VALIANT'
FEB. 16, 1989
EXOTHERM
0
9.5
19.0
28.5
38.0
47.5
TEMPERATURE (−°C)

Fig. 4. Plot of DTA of one 'Valiant' bud during deacclimation. The high temperature exotherm is from unbound water, while the other peaks are from the bud exotherms superimposed on the stem exotherms.


DIFFERENTIAL THERMAL ANALYSIS
'VALIANT'
MAR. 27, 1989
EXOTHERM
6.5
13.0
19.5
26.0
32.5
TEMPERATURE (−°C)

'Fredonia'
(-32.78)

V. riparia
(-39.2)

'Valiant'
(-34.95)

'Zinfandel'
(-23.59)

Progeny
(-30.37)

Fig. 5. Pedigree of progeny resulting from 'Valiant' x 'Zinfandel' crosses. Numbers refer to mean bud cold hardiness (°C).

'Fredonia'
(-32.78)

V. riparia
(-39.2)

'Valiant'
(-34.95)

'French Colombard'
(-23.46)

Progeny
(-30.40)

Fig. 6. Pedigree of progeny resulting from 'Valiant' x 'French Colombard' crosses. Numbers refer to mean bud cold hardiness (°C).

bud hardiness temperatures, as measured by DTA in Fort Collins. An exception is the value given for V. riparia. This value is estimated from DTA data given by Pierquet and Stushnoff (1980). Data were plotted as normal distributions for the 'Valiant'x'Zinfandel' progeny and parents (Fig. 7) with progeny values tested for normality using a normal probability plot in Fig. 8 (Statgraphics, STSC, Inc., Rockville, MD). Each data point plotted represents the mean of eight bud DTA temperature measurements. The same holds true for 'Valiant'x'French Colombard' crosses illustrated in Fig. 9 (normal distribution) and Fig. 10 (normal probability plot). In both cases, the evidence presented suggests that it is reasonable to believe that the progeny mean cold hardiness values are normally distributed and lie between the parental types. This agrees with the report of Dorsey and Bushnell (1925) who stated that the crossing of parents of extreme hardiness would produce progeny of intermediate hardiness.

As was mentioned previously, grape bud samples also contained subjacent stem tissue. This allowed for DTA of stem tissue associated with buds to take place at the same time as DTA of buds. The regression of mean bud cold hardiness on mean stem cold hardiness (Fig. 11) shows a weak relationship among all parents and progeny with a linear correlation coefficient (r) of 0.46. Layne (1983) also noted that the correlation of bud hardiness to wood hardiness of peaches and nectarines was usually low. This

Fig. 7. Normal distribution plot of mean bud hardiness temperatures of 'Valiant', 'Zinfandel' and their progeny as determined by DTA.

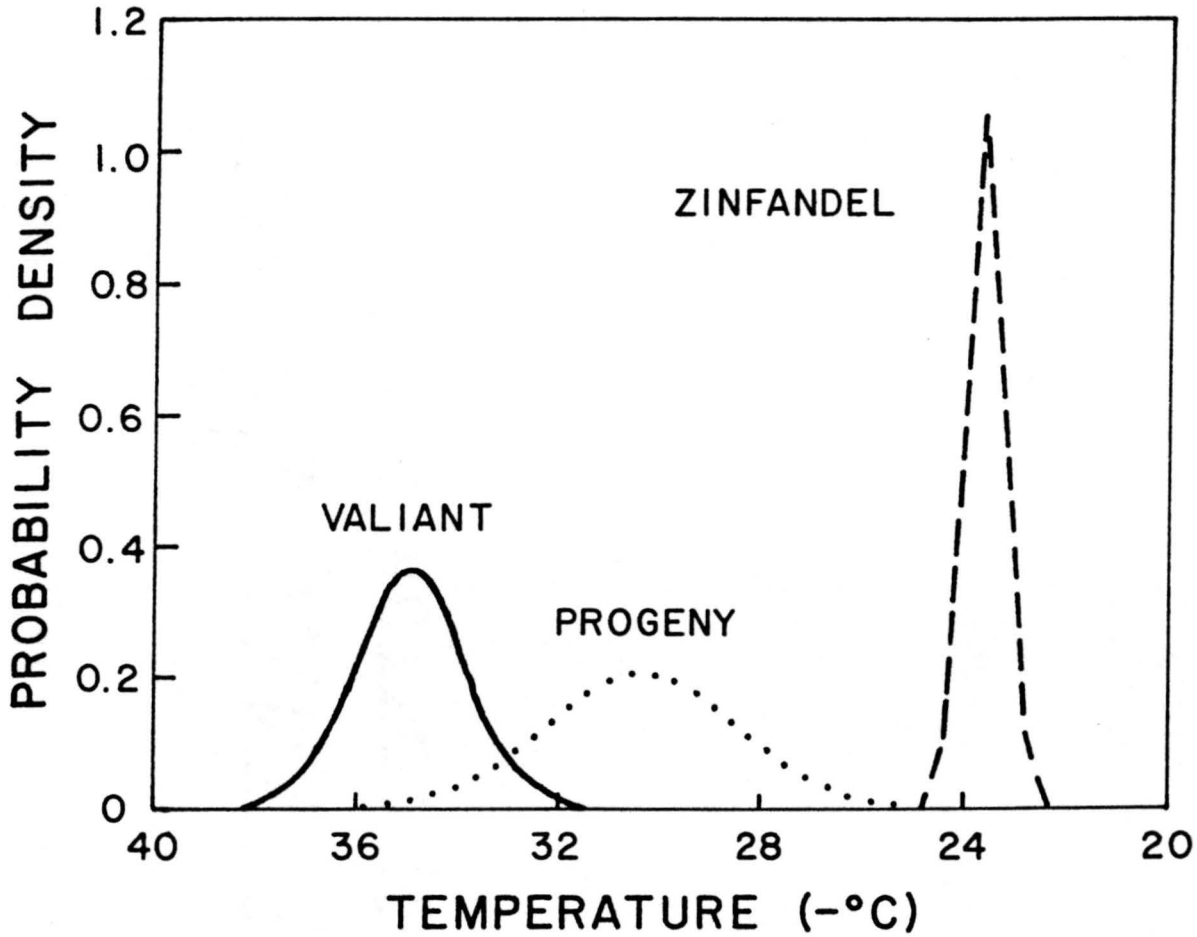

1.2
1.0
0.8
0.6
0.4
0.2
0
PROBABILITY DENSITY
ZINFANDEL
VALIANT
PROGENY
40
36
32
28
24
20
TEMPERATURE (-°C)

Fig. 8. Normal probability plot of 'Valiant' x 'Zinfandel' progeny mean bud hardiness temperatures as determined by DTA. These data indicate that the population of progeny are normally distributed.

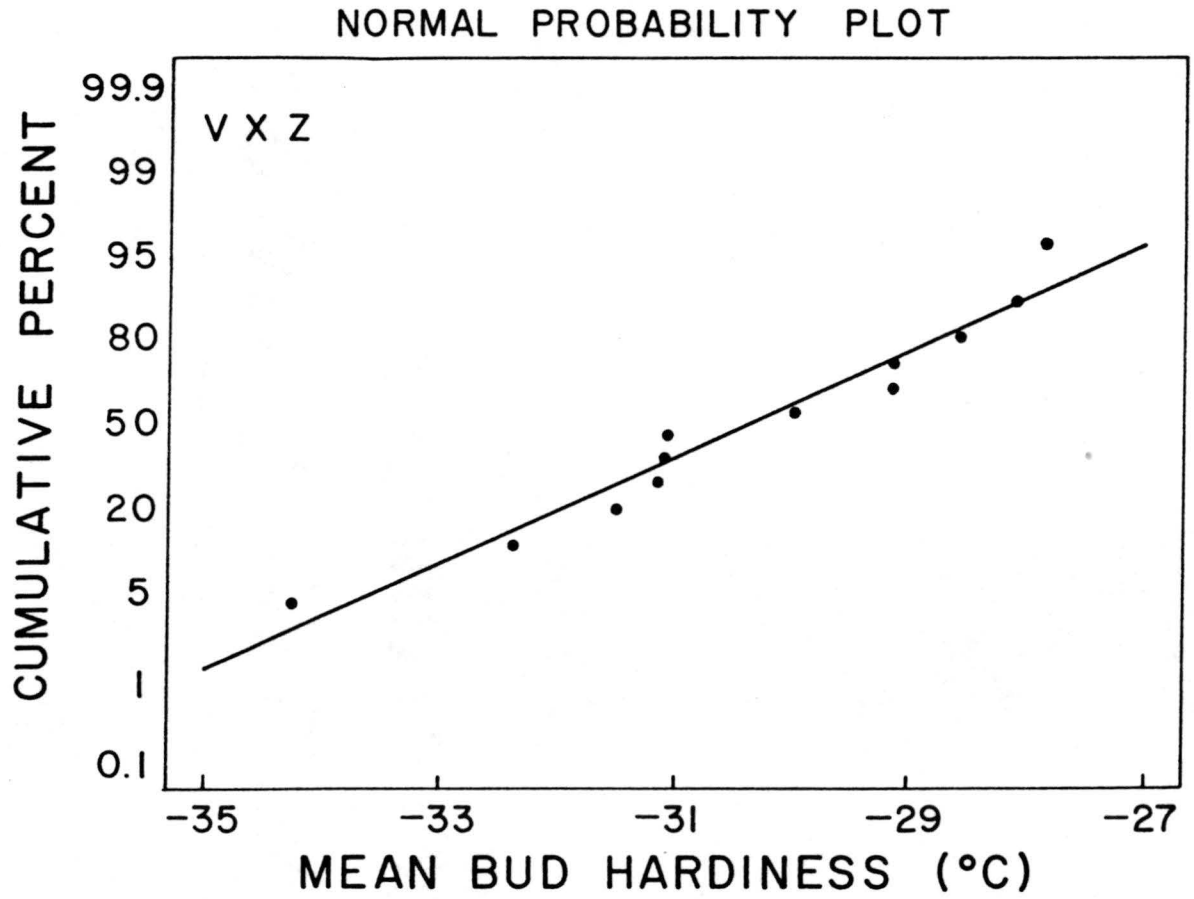

NORMAL PROBABILITY PLOT
V X Z
CUMULATIVE PERCENT
99.9
99
95
80
50
20
5
1
0.1
−35
−33
−31
−29
−27
MEAN BUD HARDINESS (°C)

Fig. 9. Normal distribution plot of mean bud hardiness temperatures of 'Valiant', 'French Colombard' and their progeny as determined by DTA.

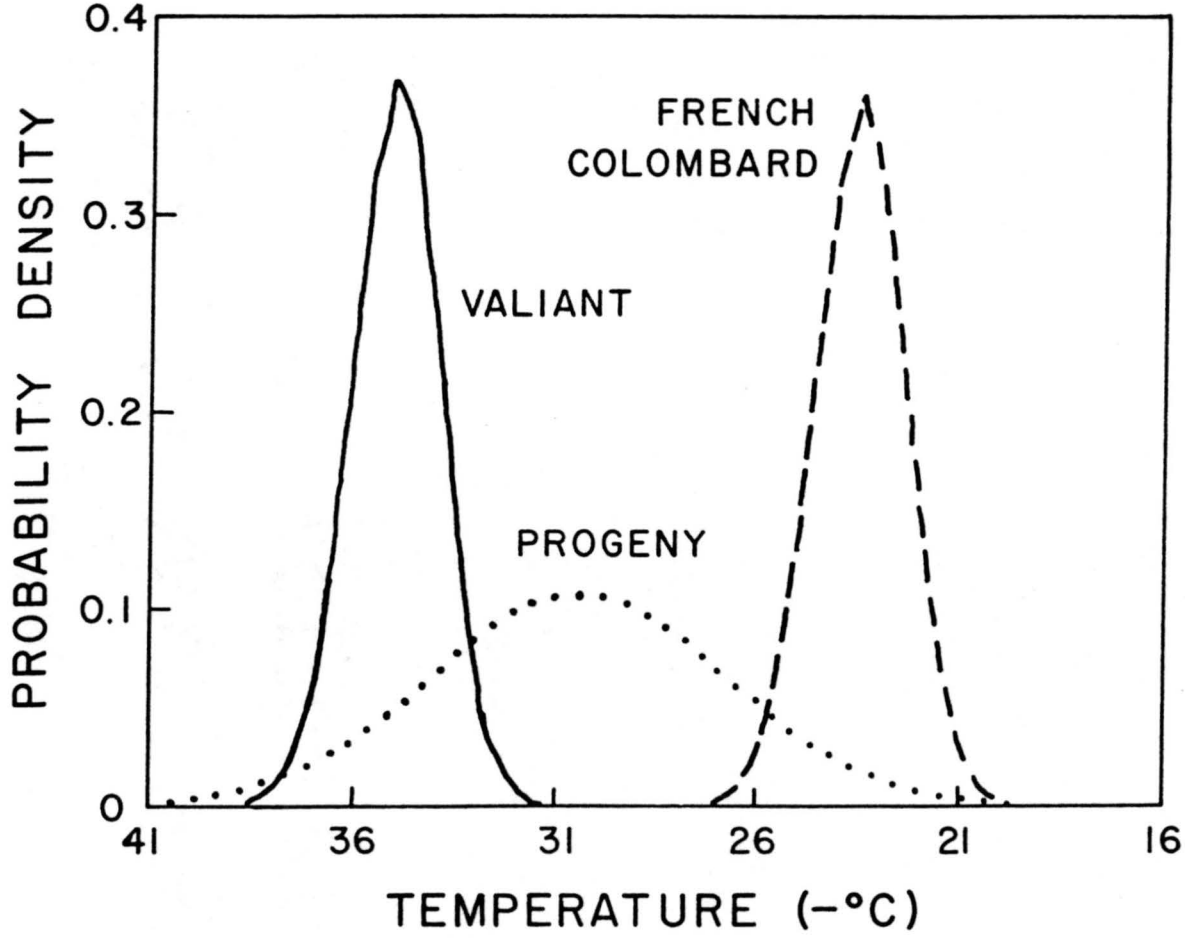

0.4
0.3
0.2
0.1
0
PROBABILITY DENSITY
FRENCH
COLOMBARD
VALIANT
PROGENY
41
36
31
26
21
16
TEMPERATURE (−°C)

Fig. 10. Normal probability plot of 'Valiant' x 'French Colombard' progeny mean bud hardiness temperatures as determined by DTA. These data indicate that the population of progeny are normally distributed.

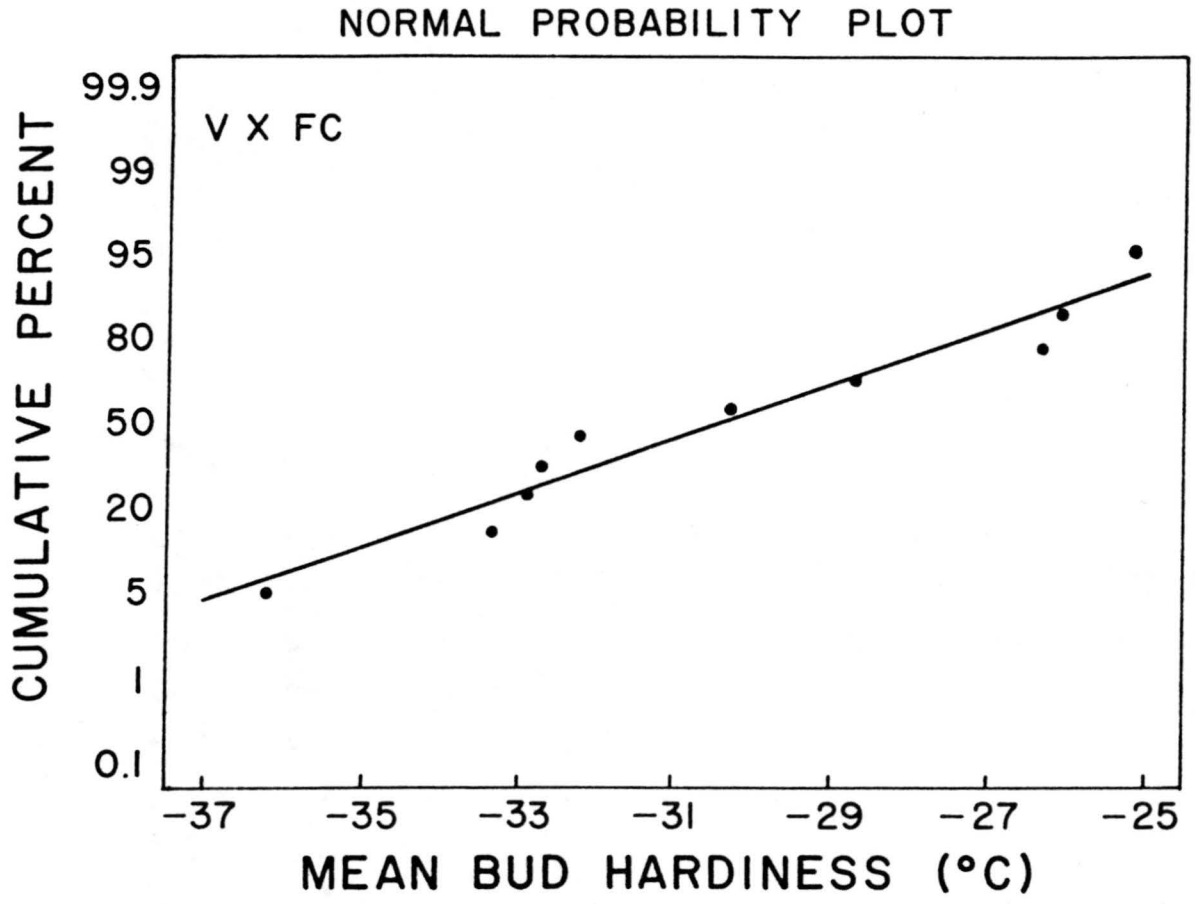
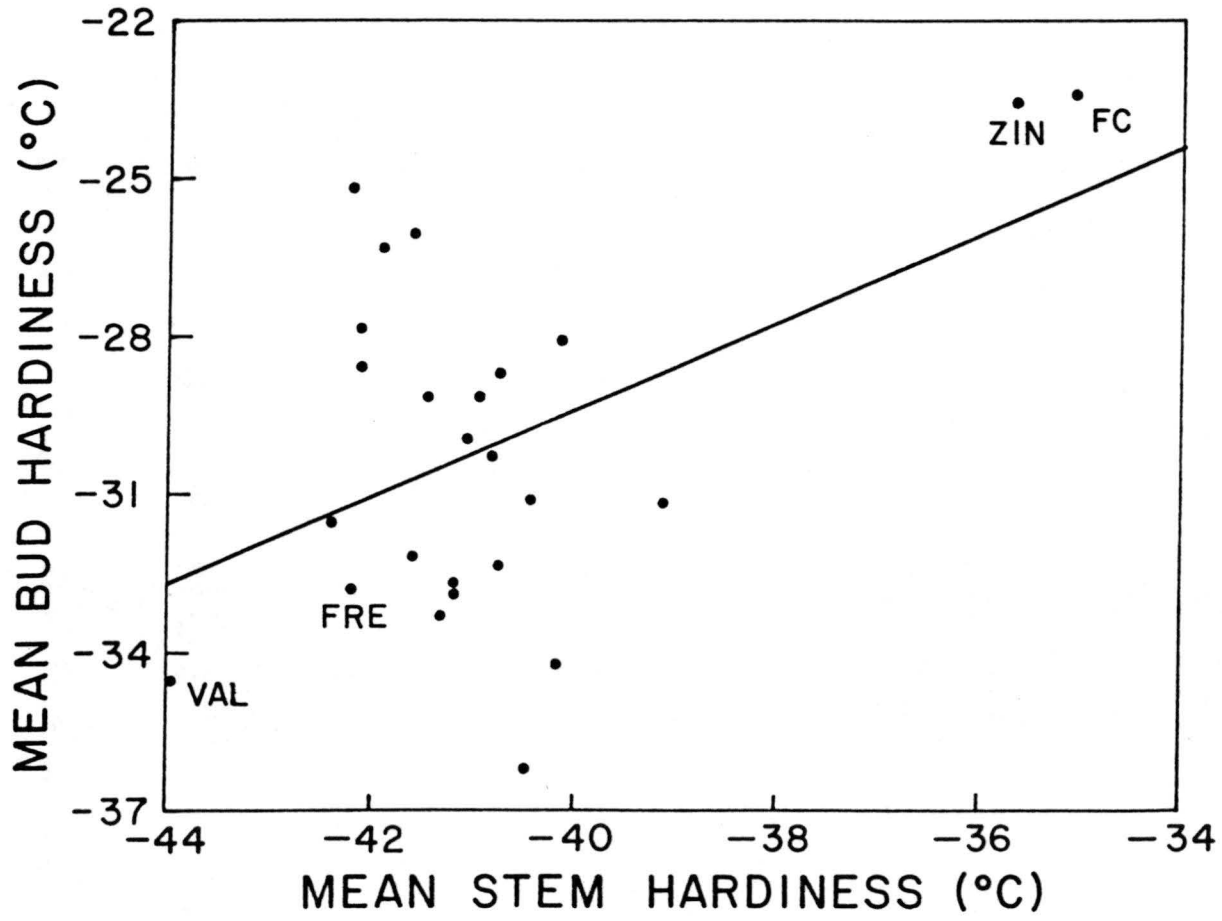

NORMAL PROBABILITY PLOT
V X FC
CUMULATIVE PERCENT
99.9
99
95
80
50
20
5
1
0.1
−37
−35
−33
−31
−29
−27
−25
MEAN BUD HARDINESS (°C)

Fig. 11. Linear regression of midwinter mean bud cold hardiness on mean stem cold hardiness of parents and their progeny. The parents are noted as 'Valiant' (VAL), 'Zinfandel' (ZIN) and 'French Colombard' (FC). The seed parent of 'Valiant', 'Fredonia', is denoted as FRE.


MEAN BUD HARDINESS (°C)
-22
-25
-28
-31
-34
-37
-44
-42
-40
-38
-36
-34
MEAN STEM HARDINESS (°C)
ZIN
FC
FRE
VAL

is in contrast to the relationship of the parents, which when analyzed separately, along with 'Fredonia' (the seed parent of 'Valiant') had a linear correlation coefficient of r=0.99. All of the mean stem hardiness temperatures of the progeny lie between the mid-parent value for this characteristic and the cold hardy parent, 'Valiant'. This implies that there may be numerous dominant genes involved with this trait or that there may be some cytoplasmic inheritance at work, since the seed parent in all cases is 'Valiant', the cold hardy parent. Reciprocal crosses were not made, so that speculation on this point is not justified. Limin and Fowler (1984) concluded that the cytoplasm had little direct effect on cold hardiness of rye or triticale, although Sasahara, et al. (1982) reported the opposite in rice.

Bud DTA data from the parents and progeny of both mating pairs were statistically analyzed as random effects factors. Three hypotheses were raised in terms of the relationship of the parents and progeny. First of all, the average of the true means of the parents (mid-parent) is equal to the true mean of all possible progeny of these two parents (p=0.05). In the case of 'Valiant'x'Zinfandel', the hypothesis was rejected. However, with 'Valiant'x'French Colombard', the data were such that the null hypothesis could not be rejected. All of the progeny were found to be more cold hardy than their V. vinifera parents and were clustered near the mid-parent values. The means were

slightly more negative in both cases (see Figs. 7 and 9). The mean progeny cold hardiness values of the 'Valiant'x'French Colombard' crosses were more variable and the null hypothesis could not be rejected. The progeny mean of 'Valiant'x'Zinfandel' crosses was significantly more negative than the mid-parent value according to these data.

The second hypothesis stated that the true mean bud hardiness of the progeny was equal to the true mean bud hardiness of the V. vinifera parent. In both cases of 'Valiant'x'Zinfandel' and 'Valiant'x'French Colombard', this null hypothesis was rejected at $p=0.0005$. The same was true for the third hypothesis that the true mean bud hardiness of the progeny is equal to the true mean bud hardiness of 'Valiant'. These results are, of course, not surprising, given that the progeny data are all near the mid-parent values and the bud cold hardiness variances are fairly low (typically around 1.00).

Field observations were made in June, 1989, to determine whether injury or death had occurred to any of the plants during the winter in the vineyard. During the time of this study, an unexpected 'test winter' occurred, which allowed for spring confirmation of some of the DTA data. The single representatives of each V. vinifera parent were killed to the ground. This was expected, since an extended eight day cold period occurred in early February, with an extreme minimum temperature of -28.3 C reported at the campus weather station. Both V. vinifera parents had bud

hardiness levels near -23 C according to DTA measurements. All of the progeny reported here and 'Valiant' survived. Approximately 77% of the progeny and 'Valiant' eventually flowered. One individual produced no exotherms and was dead to the ground in the spring. It had been sampled several times during the winter, but no exotherms were observed. This was in contrast to previously reported results that some dead tissues have produced exotherms (McLeester, et al., 1969). The exact time of death or by what means (freezing, insects or disease) was not determined.

The bud hardiness data of both the parents and progeny were also used to estimate the inheritance of grape bud cold hardiness in the broad sense. The two sets of parents plus their progeny and the parents only were used separately in two independent estimates of heritability. A method of Cain and Anderson (1980) was used for computation. The formula calculates broad sense heritability in the following manner:

$$h^2 = (\delta_{BP}^2 + \delta_{WP}^2 - \delta_{WC}^2) / (\delta_{BP}^2 + \delta_{WP}^2),$$

where δ_{BP}^2 is the variance between progeny, δ_{WP}^2 is the variance within progeny, and δ_{WC}^2 is the variance within parental clones. The within parental clones variation was used to estimate environmental variation. Using this method, heritability of midwinter bud cold hardiness in progeny of a 'Valiant'x'Zinfandel' cross is equal to:

$$(27.701 + 1.167 - 0.8188) / (27.701 + 1.167) = 0.972.$$

In a 'Valiant'x'French Colombard' cross, the heritability is:

(103.719 + 1.292 - 1.1328) (103.719 + 1.292) = 0.989.

These numbers represent the degree to which the phenotype, in this case midwinter bud cold hardiness, is determined by the genotype. One, or unity, would be a perfect relationship.

Cain and Anderson (1980) also estimated broad sense heritability using parental data only. The formula used was:

$$h^2 = (\delta_{BC}^2 - \delta_{WC}^2) / \delta_{BC}^2 ,$$

where δ_{BC}^2 is the between parental clone variance and δ_{WC}^2 is the within parental clone variance. In this instance, 'Valiant' x 'Zinfandel' gave a heritability close to that of the previous estimate, only slightly higher. The value derived came to :

$$h^2 = (805.306 - 0.8188) / 805.306 = 0.9999.$$

The result was virtually the same with 'Valiant'x'French Colombard', with:

$$h^2 = (830.507 - 1.1328) / 830.507 = 0.9986.$$

All of the estimated heritability values are very close to unity, which is not unexpected given that the progeny are clustered near the mid-parent values, the parents are extreme in their hardiness and the variances within progeny and parents are quite low.

Leaf Pubescence as a Selection Criterion for Cold Hardiness

It was previously observed that cold hardy grapes tend to have glabrous abaxial leaf surfaces, while less hardy types were more hirsute (Yeager, 1936). This observation was critically evaluated by looking at leaf surfaces with a dissecting microscope. Eighteen grape cultivars were studied in September, 1986, to establish the validity of this assertion. Nine of the cultivars were reported to be cold hardy and were thus expected to have glabrous lower leaf surfaces, while the rest were less hardy V. vinifera cultivars. The V. vinifera cultivars studied were 'French Colombard', 'Chenin Blanc', 'Cabernet Sauvignon', 'Zinfandel', 'Pinot Noir', 'Semillon', 'Riesling', 'Petite Syrah', and 'Merlot'. The hardier types were 'Valiant', 'Beta', 'Lakemont', 'Fredonia', 'Himrod', 'Swenson's Red', 'Concord', 'Rougeon' and 'Aurore'. All of the V. vinifera cultivars were hairy to some extent, which was expected. However, three of the more cold tolerant cultivars, 'Fredonia', 'Lakemont', and 'Concord' had long filamentous hairs, with 'Concord' having very dense hairs. This failed to support the premise that the presence of leaf hairs indicated lack of hardiness.

In September, 1988, the progeny of the crosses being investigated herein were studied for abaxial leaf pubescence. Most were seen to be smooth, although some had sparse hairs along veins, margins and between veins. These

varied in shape and length but were not as dense as seen in the viniferas. This tended to support the original premise, but closer scrutiny at higher magnification revealed that some leaves which appeared glabrous at lower magnifications were quite hirsute, with close thick mats of tiny hairs. The problem was a lack of a standard by which to quantify the presence of leaf pubescence. It could be said, however, that a grape plant showing obvious and copious amounts of abaxial pubescence is probably not very cold hardy, but that is not a surety. Selection for cold hardiness should not be based on this procedure alone.

Embryo Rescue of Grape

Of the 'Valiant' grape seeds that were originally planted, only three percent germinated. This was similar to the poor performance that had been reported by a number of researchers and prompted efforts to improve germination (Cain, et al., 1983; Einset and Pratt, 1975; Ellis, et al., 1983; Manivel and Weaver, 1974; Olmo, 1936; Ottenwaelter, et al., 1974; Pal, et al., 1976; and Selim, et al., 1981). The technique for rescuing grape embryos described earlier was quite successful, with no contamination of the 32 embryos or medium. All but one developed, enlarged and spread their cotyledons. The exceptional embryo developed a large mass of callus only. However, it was evident that some of the problems of seed germination are not necessarily

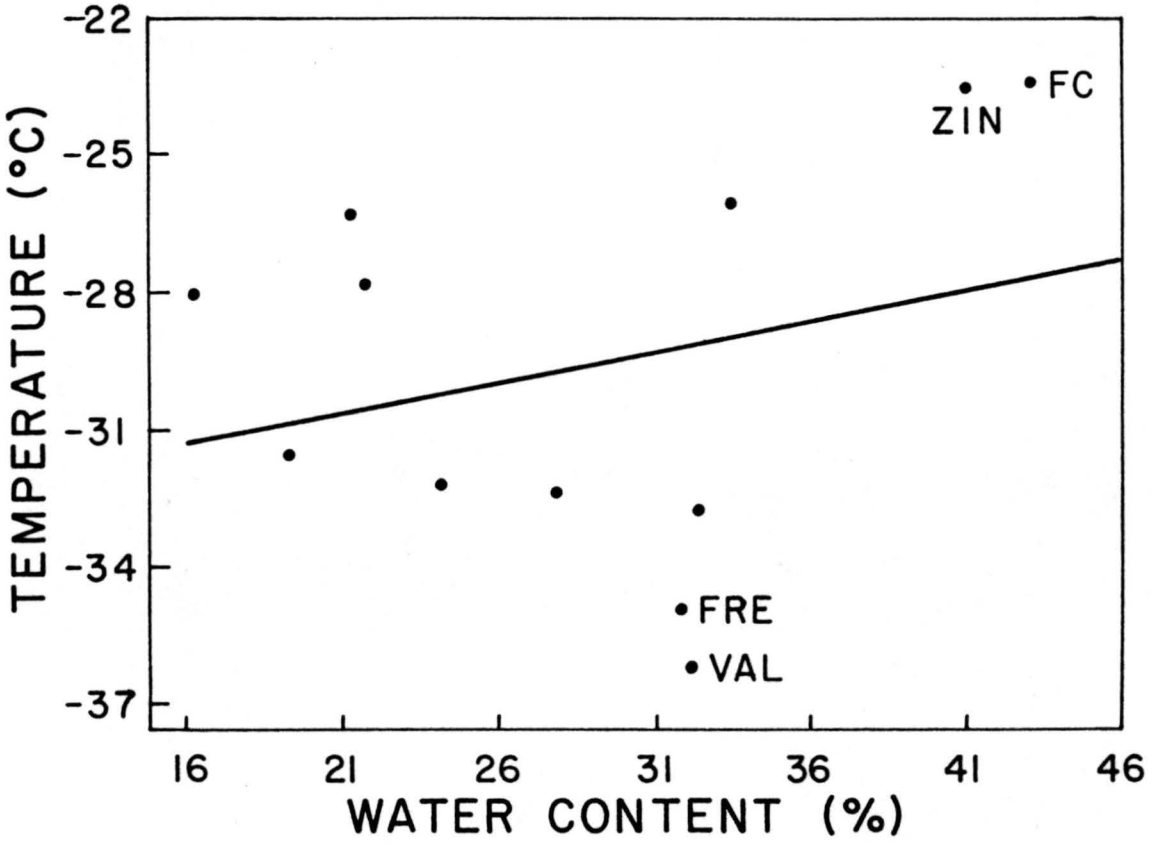
insurmountable nor are they always the result of inviability.

Midwinter Water Content of Grape Buds and its Relationship to Cold Hardiness

After the moisture contents of the parents and 'Fredonia' had been determined, regression analysis was performed to determine its relationship to midwinter cold hardiness. There was a very strong linear relationship ($r = 0.984$, with a slope of 1.02) which indicated that cold hardy buds have a lower water content in midwinter than do the lesser hardy buds. This is in agreement with the previously reported work of Graham and Mullin (1976a) who noted a strong linear relationship of water in the floret of azalea and the average exotherm temperature. When the progeny data were determined later and analyzed with the parents, the relationship was changed dramatically (Fig. 12). The linear regression correlation coefficient (r) became 0.262, and the slope became a small (0.133) positive value. These data are completely different from the observations made previously by other researchers (Gusta, et al., 1983; Kaurin, et al., 1984; Pierquet and Stushnoff, 1980; Quamme, 1983; and Wolpert and Howell, 1985a; 1985b), and with my data of the parent types.

There are several reasons why the progeny data may be anomalous. The first has to do with the fact that the

Fig. 12. Linear regression of midwinter mean bud cold hardiness on mean stem water content of parents and their progeny. The parents are noted as 'Valiant' (VAL), 'Zinfandel' (ZIN) and 'French Colombard' (FC). The seed parent of 'Valiant', 'Fredonia', is denoted as FRE.


-22
-25
-28
-31
-34
-37
TEMPERATURE (°C)
16
21
26
31
36
41
46
WATER CONTENT (%)
FC
ZIN
FRE
VAL

progeny samples had been in cold storage (-24 C) for one month. Although they were individually covered with plastic wrap, this may have been insufficient to prevent desiccation. The difference also may be due to the smaller average size of the sample buds which came from smaller and less mature vines. The number of buds per sample also varied. The parental types had 10 buds per cane while the progeny sample size ranged from four to ten, the result of limited sampling material in the vineyard. Another possible explanation of this disparity in progeny data is transgressive segregation. These data may actually represent the true moisture contents of the buds. There is insufficient data to make a sound, convincing conclusion at this time.

Conclusions

The results presented here lead to some interesting inferences. The use of DTA for selecting genotypes in improving the cold hardiness of grapes appears to be a valid and reliable procedure. The efficacy of this method for use with other plant species that employ supercooling as their means to avoid freezing is implied, but needs verification. The procedure is fairly rapid and gives the mean hardiness level, without a mathematical derivation to approximate the mean. Initial monetary investment may be prohibitive in some cases, but the multiple use of some of the equipment, especially the computer, printer and analog-to-digital

converter, may justify acquisition of any of these components.

It was also demonstrated that the broad sense heritability of midwinter cold hardiness is quite high. The expressed phenotypes of the progeny of the crosses made were associated very strongly with genotype. This was not surprising, even though this is the first reported study to quantify the relationship. What is needed in the future is that the crosses that were not used in this study, which are currently in the Colorado State University vineyard, at Fort Collins, be analyzed by DTA for cold hardiness. These data may then be used to give an estimate of cold hardiness in the narrow sense, when used in conjunction with data from parents and progeny used in this study. This information would be quite valuable in future grape breeding ventures when attempting to improve cold hardiness. Backcrosses have already been made with the progeny obtained from these experiments and any hardiness data obtained from these will further the effort started here. Some attempt should also be made to ascertain the importance of cytoplasmic effects by making reciprocal crosses of 'Zinfandel'x'Valiant' and 'French Colombard'x'Valiant'.

Leaf pubescence was shown to be an unreliable indicator of cold hardiness, although in most cases cold hardy grapes have leaves with glabrous abaxial surfaces. It would be interesting to know if there are any genetic linkages involved here. Quantification of the degree of pubescence

is a problem and one might look at reflectance as a possible measurement technique.

Embryo rescue techniques employed in this study have shown that low grape seed germination may not necessarily be due to inviability of the embryos. This technique may be used to obtain a greater number of grape seedlings from this and other breeding programs, besides those of seedless grapes for which the process was developed.

It is hoped that the disparity between water content data obtained in this study will be either explained or refuted by future studies using the same plant materials, but under more controlled circumstances. It may seem unlikely that transgressive segregation is taking place, but it is important that this be investigated to determine the mechanism underlying the process of decreasing water content to produce improved cold hardiness.

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APPENDIX

```
5 DIM Z(10)
10 OPEN "COM:88N2D" FOR INPUT AS 1
20 PN=192
30 CLS
40 PRINT "       DTA PROGRAM RUNNING"
70 FOR N=16 TO 25
80 CN=N
90 CCN=N-15
100 FOR I=1 TO 3:GOSUB 5000:NEXT
150 LPRINT USING "#####"; Z(CNN);
200 NEXT N
205 LPRINT CHR$(10)
210 GOTO 70
5000 OUT PN,CN
5010 GOSUB 5200
5020 OUT PN,161
5030 GOSUB 5200
5040 HB=CH
5050 IF(HB AND 128)<>0 THEN 5020
5060 OUT PN,145
5070 GOSUB 5200
5080 LB=CH
5095 HM-HB AND 15
5100 Z(CCN)=LB+256*HM
5105 IF (HB AND 16)=0 THEN Z(CCN)=-(Z(CCN))
5110 RETURN
5200 FOR DE=1 TO 1:NEXT
5210 CH=INP(PN)
5220 RETURN
```

Fig. 13. BASIC program used to control the analog-to-digital converter and to drive the printer.

$$T = A_1 + (A_2 * X) - (A_3 * X^2) + (A_4 * X^3) - (A_5 * X^4) + (A_6 * X^5) - (A_7 * X^6) + (A_8 * X^7), \text{ where:}$$

$A_1 = 0.10086091$

$A_{A2} = 25727.94369$

$A_3 = 767345.8295$

$A_4 = 78025595.81$

$A_5 = 9247486589$

$A_6 = 6.97688\ E11$

$A_7 = 2.66192\ E13$

$A_8 = 3.94078\ E14$

Fig. 14. Algorithm used to convert millivolt readings from thermocouple (X) into temperatures (T).

Table 4. Observed grape leaf abaxial surfaces.

Cultivar	Observed abaxial pubescence
Aurore	smooth
Beta	smooth
Cabernet Sauvignon	many medium length filamentous hairs (cylindrical)
Chenin Blanc	many medium length filamentous hairs (cylindrical)
Concord	very dense filamentous hairs
Fredonia	few long filamentous hairs
French Colombard	many long filamentous hairs
Himrod	smooth
Lakemont	few long filamentous hairs
Merlot	many clumps of long hairs wound into balls
Petite Syrah	few clamps of short filamentous hairs
Pinot Noir	sparse clumps of very long filamentous hairs
Riesling	many long filamentous hairs
Rougeon	smooth
Semillon	sparse clumps of very long filamentous hairs
Swenson Red	smooth
Valiant	smooth
Zinfandel	numerous short flat, ribbon-like hairs