AN ABSTRACT OF THE THESIS OF

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Title: Freeze Damage of Pinot Noir (Vitis vinifera L.) as Affected by Bud Development, INA Bacteria, and a Bacterial Inhibitor.

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Spring freeze decreased the yield of field grown grapevines by 70 %. The spring bud development of Pinot Noir was characterized into seven stages after the initial budbreak, 96 % of the buds attained full development in 44 days. Controlled freezing tests were used to determine bud hardiness. The T50 values for quiescent, swollen, budburst, first, second, and third flat leaf bud stages were, -14, -3, -2.2, -2, -1.7, and -1.1°C, respectively. The water content increased from 57 to 84 % from the quiescent to the swollen bud stage, thereafter little change occurred. A high correlation was found between hardiness, water content, and stage of bud development.

Genotype and culture age affected the ice nucleation activity of three Pseudomonas syringae strains. The cells attained stationary phase of growth after 60 hr in culture. The INA of PssB-15 occurred in the range of -2 to -4°C throughout the 7 days of evaluation. Pss2-3RNH INA was prevalent in 3 day old cultures only. Pss2-3 was a poor ice nucleator. The INA of bacterial

suspensions was directly proportional to concentrations ranging from 10^9 to 10^2cells/ml, which nucleated ice from -2.5 to -15.1°C. INA-bacteria at 10^8cells/ml incited freeze injury to grape leaf tissue specifically from -2 to -4°C.

Frost Gard, a commercial chemical promoted as a freeze protectant, did not show antifreeze action at concentrations of 0, 0.12, 0.25, 0.50, and 1 %. At these concentrations the water solutions froze at -11.6, -12.0, -11.8, -11.7, and -12.5°C respectively. Suggesting that the water ice nucleation temperature depended on the amount of impurities in the solutions. Frost Gard at 0.25, 0.50, and 1 % had a strong bactericide effect on a bacterial suspension of 8 x 10^9 cells/ml. The same concentrations also lowered the nucleation temperature to -4.5, -6.9, and -5.2°C compared to the control suspension at -2.5°. It was concluded that Frost Gard binds with the active site of nucleation, since it strongly interacted with an inorganic ice nucleator. Frost Gard at 0.25 % applied to grape leaf disks reduced the damaged area by 7 % only at - 2°C. Phytotoxicity due to Frost Gard was observed at rates above .25 %.

Grape cuttings were used to test the capacity of an antibiotic resistant strain of Pseudomonas syringae to translocate through the vascular tissue. The cuttings were placed in bacterial suspensions of ca. 10^2, 10^4, and 10^6 cells/ml for 24, 36, and 48 hr. Bacterial populations recovered were inversely proportional to the lenght of the cutting section, regardless of the time of exposure to the inoculum. It was determined that the liquids moved faster through the vascular tissue than the bacteria.

FREEZE DAMAGE OF PINOT NOIR (Vitis vinifera L.) AS AFFECTED BY BUD DEVELOPMENT, INA-BACTERIA, AND A BACTERIAL INHIBITOR.

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'My friend had a vineyard

on a fertile hillside,

he spaded it, cleared it of its stones

and planted with the choicest vines...'

Is. 5:2-3

Dedicated to Alfonso,

Felisa and Alfonsito.

Note: This thesis is presented in four papers written in the format of the Journal of the American Society for Horticultural Science.

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Chapter 1

INTRODUCTION.

Freezing injury is recognized as a major limiting factor in the production of horticultural crops (9). The danger of frost limits the suitability of areas for viticulture (19). Even for hardy grape cultivars, frost is a frequent form of damage. No viticultural area in the U.S. is completely free of spring freeze hazard (52).

Oregon viticulture is a new and expanding industry. The state vintage in 1986 accounted for 730,000 gal of wine. The area dedicated to the culture of grapes is estimated in 3,485 acres, from which at least 2,948 acres are located in the Willamette Valley. Pinot Noir is the leading variety, occupying 36 % of the acreage grown in western Oregon. The typical vineyard is represented by small areas of less than 20 acres (78). Oregon viticulture also has a spring freeze problem. It has been suggested the use of slopes, up to 10% with southern exposure, located a few hundred feet above the valley floor to avoid natural frost pockets (37). Nevertheless, despite good site selection the problem persists in certain regions and therefore, the frost control strategy used include microclimate modification. However, the cost and availability of a system to modify the vineyard environment is a matter of increasing concern, and under advective conditions any method is of limited value (48).

Expanding buds and shoots are usually the part of the vine most susceptible to freeze injury (8). Frosts kill the young primary developing buds and lead to yield reduction. Furthermore, later developing shoots originating from secondary buds are less fertile and have less available growing season than the primary shoots and consequently , the clusters born on these shoots seldom achieve commercial maturity standards (19).

One of the greatest difficulties in calculating the value a frost forecast, lies not only in the prediction of a given temperature, but also, in the lack of adequate data relating crop damage to various low temperature (89). Critical temperatures for Concord grapes have been estimated (86), although these were different than those observed to cause field kill (52), and this species is considered hardier than vinifera grapes. Also, budbreak dynamics are useful to understand the risk factor under Oregon conditions, as well as critical temperatures.

The role of ice nucleation-active bacteria in frost injury to plants has been studied extensively with contradictory results, particularly on woody species (5, 41, 64). Pseudomonas syringae, the main INA bacterium species, is reported to be a weak pathogen for grapevines (57). However, the effect of this bacteria is unknown on the freezing stress of young developing grape shoots. It has been assumed that INA-bacteria limit the supercooling capacity of grape buds, but no reports are available in the literature, and the matter needs to be studied. However, a selection procedure has to be used to choose an adequate INA-bacterial strain in order to carry out the proper evaluations on plant tissues.

Different methods of preventing frost injury incitated by epiphytic INA-bacteria have been explored; since a general description was reported (61), a wide variety of methods and chemicals have been tried (2, 29, 72, 87). However, little success has been attained on fruit trees.

There is a new chemical product on the market promoted for frost protection in viticulture. The producers claim that the product inhibits of INA-bacteria properties, with an antifreeze effect. Nevertheless, no information about its performance on vineyard protection is available in the literature at this point in time. Because of the increasing concern among grape growers and industry people, the effect of such chemical must be evaluated.

Suppression of the epiphytic populations of INA-bacteria has proved successful in reducing frost injury in tender annual plants; but in fruit tree species these attempts, including drastic surface treatments have failed, (10, 29, 41). The important question still to be answered is the presence of this bacteria or other nucleating agents on or in the plants (61). Different evidences indicate that other bacteria species can maintain internal populations which are able to move successfully through the grapevine vascular system (40, 100). In the search for possible internal populations of INA-bacteria and their potential role in freeze damage, a preliminary step is to determine whether these bacteria can be translocated through the vessels.

Chapter 2

LITERATURE REVIEW.

I. SPRING FREEZE HAZARD.

A. Plant Phenology.

Plants from the temperate zone have evolved different strategies to survive under the cold conditions of late fall, winter and early spring. Plants respond to various physiological mechanisms seasonally, and consequently they can adapt to low temperature environments. Understanding these mechanisms can imply many practical applications (Tanino, personal communication). Phenological data are key factors in the interpretation of such plant responses. Phenology is defined as the study of the relationship of the periodic events in the plant life cycle as related to climate (68). Phenological models have been used to time acceleration of pollinizer bloom (91), selection of specific genotypes (26, 39, 50), prediction of stages of development (34) and even in timing pest control applications (33).

Pinot Noir, the main cultivar grown in Oregon, has been susceptible to spring frost damage because of its comparatively early budbreak (36, 73). Determining budbreak is of major importance to predict frost risk. In Vitis vinifera L., xylem exudation starts 40 to 50 days before budbreak (76), while changes in its volumes and composition are preluded as well (75). However, different factors may have an influence, for example, the negative effect of previous overcropping on bud survival have been demonstrated in Carya illinoensis W.K. (109), and Vitis labruscana

B. (45). Early defoliations during the fall can lead to a more rapid deacclimation during the next spring, reducing bud survival in Prunus cerasus (46).

In Vitis labruscana B. the basal nodes are retained for fruiting, since their hardiness is of major interest irrespective of the progress of acclimation at the shoot tip (106). During the early fall acclimation the basal cane and primary bud tissues acclimated first, and there was an inverse relationship between hardiness and water content (105).

The role of tissue water content is considered to have a strong influence in the acclimation and deacclimation patterns of several species. In Cornus stolonifera M. water stress increased hardiness by decreasing tissue hydration (28), This was accompanied by decreases in proteins, RNAs and starch, as well as an increase of sugar (27). Also, in grape hybrids the carbohydrate content has been related to an antifreeze action (74). Nevertheless, in several Vitis species, summer drought enhanced winter injury by excessively stressing the vines and by delaying senescence once water became available in early fall (3).

It was suggested that the cell wall and/or plasmamembrane may have characteristics associated with a dehydrative resistance, which allows woody tissues to deep supercool (104). However, by spring the dehydrative resistance decreased with an accompanying increase in water content which reduced bud hardiness of Vaccinium australe S. Another important factor to consider is the water migration from a bud primordium to bud scales (21), which plays an important role in hardiness of flower buds of other plants. This factor was

confirmed in dormant buds of plum when Burke et al (23) found ice in the bud scales and axis, but not in the primordial region at -29.5°C.

B. Freeze Hazard.

Spring freeze damage of flowers and fruits is recognized as one of the most common types of freezing damage, despite the differential hardiness among various organs (101). The difference in hardiness between the same organs on different species may be a result of different deacclimation rates (98).

Hardiness is not a static entity but rather changes with time, temperature, day length, maturity, moisture content, nutrition and physiological age (98). Onset and rate of loss of cold tolerance are especially important when the tissues deacclimate at the end of the dormant season.

In grapevines, buds are usually the most susceptible tissue (8). The primary bud is the least hardy and most sensitive tissue to either cultural or environmental stresses (48); and its inherent difference with the secondary bud is responsible for differences in spring bud hardiness (93).

The yield reduction caused by a severe frost will depend on how many fruitful buds have started to grow at the time of the frost and on the fruiting habits of the cultivar (103). This observation was supported later, when genotypes were shown to be a major survival variable in cold stressed vines (67).

The air temperature of the preceding day was related to short term changes in cold resistance of Malus pumila cv.'Haralson' apple bark during the spring dehardening (47), This was confirmed later

on grape buds of 'Concord' (86). However, on developing shoots of the later species , prefreeze temperatures had no effect on their hardiness, because freeze resistance always decreased with the advance of phenological development (52).

It has been suggested that the northern distribution of many Angiosperm species and their low temperature exotherms (LTE) may be related in some way to wood morphology (38). The ice formation in Prunus persica L. appeared to be initiated within the woody tissue, spreading to the developing flowers and fruits; however, if the temperature drops very quickly, the opposite pattern may occur (11). On Prunus species (14), bark and xylem tissues exhibited contrasting freezing patterns and mechanisms of freezing resistance.

According to the thermodynamic components of this stress, it is considered that as the temperature decreases, the kinetic energy of all molecules is reduced. This allows potential energy to become increasingly effective. Water which is bonded in many patterns with plant components, also is more stable when bonded to itself, forming ice and releasing heat. If heat removal is slow, all of the liquid water associated with the plant components is in balance with ice. However, under non-equilibrium conditions the stress is of higher intensity and as a result death occurs (77).

Under natural (slow rate) freezing, plasma membrane permeability does not significantly limit water flux from plant cells to avoid intracellular ice formation. As stated previously, water efflux at slow freezing rates is controlled largely by the rate of heat removal from the cells (95). From new evidence, Gusta et al (44) found that storage of tissue at nonlethal subzero

temperatures for a time period had a reduction in the Low Temperature Exotherm (LTE), as well as a smaller sized LTE. It allowed the loss of cellular water to extracellular ice, with a concomitant increase in internal solute concentration, accompained by a further depression of the freezing point. This finding contrasts with the generally accepted hypothesis that freezing injury is due to limited plasma membrane water permeability causing the deadly intracellular ice formation.

Assuming that the release of hydrogen cyanide indicates membrane damage, it was concluded that during a fatal freeze-thaw cycle, membrane damage occurred during cell contraction and, therefore, was not dependent upon membrane area expansion during thawing (96).

The freezing process has been hypothesized as follows. During freezing a point is reached when all readily available water has been frozen extracellulary and only some water remains in the protoplasm. As the temperature continues to decrease this water is pulled away from protoplasmic constituents to the extracellular ice. This sets off a chain reaction of denaturation, additional protoplasmic water release, and death (101).

C. Frost Control.

Frost control has become an obligated cultural practice in critical situations where budbreak overlaps with the late spring frost period. However, it should be recognized that in most cases the problem has been created by an inadequate selection of an early budbreak cultivar planted in a risky area, which underlines the importance of a careful selection of site and genotype.

Nevertheless, even under an ideal combination of climate and agrosystem, the eventual occurrence of frosts, as part of the natural conditions, leads to the same and final conclusion: The necessity of crop protection.

The classical active methods of frost protection used in viticulture rely on modifying the vineyard microclimate during the frost. These methods have been widely described (18, 31, 80, 81, 89, 103), and can be summarized as follows: heating and use of wind machines, combined or alone; sprinkler or surface irrigation; and covering. Their common denominator is the enormous amount of energy and resources required. Considering the limited amount of conventional energy sources and their increasing cost, new approaches are needed. The knowledge of the low critical temperatures for different deciduous species at different stages of development represents a decisive managerial tool in the delicate decision making involved in frost control (86, 87). Nevertheless, it should also be considered that the produce value of some grape cultivars cannot absorve the cost of even limited frost control (48).

The second group of protection methods includes strategies such as late or double pruning to modify plant responses (103).

Recently, the problem has been focused on the biotic components of this energetic system. For example the importance of weed control as a part of a general program including cultural practices considered as classical when dealing with frosts (17, 51). Although, this recommendation is based upon the strict point of view of energy retention in the system, it should be considered the

dynamic relationship existing between its living parts.

II. BACTERIAL ICE NUCLEATION.

A. Ice Nucleation.

Water is considered to freeze at 0°C, however the homogeneous nucleation temperature of pure water can be as low as -38°C (70). This has been called supercooling and it occurs when the temperature drops below the freezing point. Supercooling is the lowest subfreezing temperature attained before ice is formed (49). Another characteristic of deep supercooled systems is that while their freezing point is near -40°C, they thaw near 0°C (20).

Many tree fruit species avoid freezing by deep supercooling. Water remains in a supercooled state in plants if there are no ice nuclei and/or if the temperature does not fall below the homogeneous nucleation point. External ice can readily nucleate plants via entry sites such as stomata, lenticels and wounds. It grows from one or more nucleation sites, and it is more rapid through the vascular tissue (24).

Extensive work has been done to elucidate the nature of the ice-nuclei. It has been shown that different ice nucleators occur in nature. The possibility of extraterrestrial, meteoritic origin has been considered based on climatological data showing rainfall peaks corresponding to meteorite showers. Inorganic soil particles are good ice nucleators at temperatures colder than -15°C. Clay particles have been found at the center of most snowflakes. A maritime source for ice nuclei has been reported as limited and poor. Recent data show no evidence that anthropogenic aerosols

contribute significantly to the global population of atmospheric ice nuclei. However, none of the sources discussed above are capable of producing high concentrations of nuclei active at temperatures warmer than -10°C., and they have a limited terrestrial distribution. The suggestion that materials of biological origin may be acting as atmospheric ice nuclei emerged when bacterial cells were found to become ice centers in a cloud chamber; they were very active, nucleating at temperatures close to -1°C. Their abundance in litter of plant origin depends on the climate. For example an analysis of freezing nucleus (FN) content active at -10°C per weight of litter in Poa spp from 3 different environments showed that litter from the tropical, mesothermal, and the humid microthermal areas contained an increasing gradient in concentration of bacterial nucleus. This clearly proves their abundance in the temperate zone, while in the tropical area their presence is very low (90).

It has been pointed out (64), that leaves of many plants have substantial numbers of two species of INA bacteria, Pseudomonas syringae and Erwinia herbicola. Frost injury to field grown corn leaves at -5°C was directly proportional to the logarithm of the INA bacterial population. These bacteria incited frost injury to tender plants, preventing supercooling below -2 to -5°C.

The later is considered less efficient as an ice nucleus than P syringae, which has been described as Gram negative, straight or curved rods, 0.5 to 1.0 by 1.5 to 4.0 (92), having up to 4 polar flagella and they are catalase positive and strict aerobes. The "Approved Lists of Bacterial Names" classified them as P. syringae

pv. syringae (Pss) and ice nucleation activity (INA) as well as pathogenecy tests are required for identification at the pathovar level, besides of the LOPAT tests (32). Although not all of the strains show the INA phenotype or it may be functional at different temperatures. Indirect immunofluorescence (IIF) and INA have been evaluated for detection and identification of two bacterial foliar pathogens of tomato transplants (54). The results showed that when both tests were used in combination they become a useful diagnostic tool for distinguishing P.s. pv. syringae (Pss) and P.s. pv. tomato.

Pss has been reported (83), as epiphytes on plants; they were the dominant pseudomonads on the leaves of certain fruit tree species. Although histological examination did not reveal their presence, they were found frequently associated with overwintering buds. This pathovar is also well known for its pathogenicity on members of the Rosaceae Family. However, it appears to be a weak pathogen for grapevines (57).

Not every cell of a given bacterial isolate with ice nucleation (IN) properties can serve as an ice nucleus at any given time and temperature. The ratio between ice nucleus and number of bacterial cells in a culture varies depending on the genotype and culture age. It was reported that the conditions to obtain the maximum IN expression were incubation temperature at 24°C and growth medium containing at least 1% glycerol (65). The distribution of the INA phenotype was determined within populations of Pss isolated from different woody species infected showed that 85 % of the isolates were INA at -5°C (15).

The presence of INA-bacteria in deciduous fruit tree orchards

in the Pacific Northwest was surveyed over a 3-year period (42). The authors found low populations or even the absence of INA-bacteria during the season when critical temperatures were common. These bacteria comprised over 10 % of the total bacterial population. Their populations fluctuated during the year with the highest levels developing on buds and flowers during cool, wet spring, followed by a drop during the summer and finally a gradual increase in the autumn, which agrees with other reports (60).

At least three major ecotypes of INA P. syringae were discerned in the Pacific Northwest (43). They were found as epiphytes from pome and stone fruit orchards. Half of the 82 isolated strains were pathogenic with different degree of virulence. However, nearly all strains induced hypersensitivity in tobacco and produced syringomycin. The in vivo frequency of ice nucleation was 6,000 cells per -5^{o}C/ice nucleus. Neither bacteriocin and phage typing demonstrated an appreciable phenotypic difference among the strains. All of them produced at least one bacteriocin and five phage sensitivity groups were identified. However, the phage typing differentiated Pacific Northwest INA strains from others reported in the literature.

B. Effect of INA-Bacteria on Frost Injury to Plants.

Lindow et al (62), and Anderson et al (5), have shown that INA-bacteria prevent supercooling in tender annual plants, initiating ice formation on corn in a range from -2 to -4^{o}C, while the controls supercooled to -8^{o}C. Ornamental plants with INA-bacteria were killed 2 to 4^{o}C warmer than the control treatments without bacteria; tomato and soybean plants without INA-bacteria

were able to supercool to -8°C, while INA-bacteria induced ice at -4 and -5°C respectively (6).

On tender annual plants it is clear that epiphytic INA-bacteria are incitants of frost injury. Their presence may become a real problem either when immature crops are exposed to early frosts during the autumn or seedlings during the spring, and the intensity of damage expected is directly proportional to the epiphytic population of such microorganisms. It has been demonstrated that grasses are sources of inoculum (16), and that these bacteria have limited migration toward woody plants (71).

On woody fruit tree species, the lack of the natural capacity of the plants organs to supercool has been reported. As an example, the temperature required to kill 50% of the buds at the full bloom stage was reported as -3.7°C for Red Delicious apples, Bartlett pears, -3.6° C; Bing cherries, -3.2° C; and Early Italian prunes, -4.5° C. Such limitation proved that some factor was preventing the buds to supercool (88).

Later, the capacity of supercooling was evaluated for apricots, peaches and sweet cherry blossoms (85). It was demonstrated that when the organs were detached from the woody tissue, they were able to supercool to -5 to -8°C whereas attached fruit supercooled only to -2 to -3.8°C. INA-Bacteria raised the nucleation temperature of detached fruit. The authors proposed that ice crystals appeared to form within the vascular system and then advance by progressive crystal growth into all the fruit on a single stem. They proposed that there were ice nucleating sites of non bacterial origin in the wood which were active at -2 to -3°C. This was supported later by an

experiment designed to study the ice nucleation within peach trees (13). It was concluded that the ice-nucleating agent associated with peach shoots was active at -2°C and it was: 1) present in both field and greenhouse grown plants; 2) associated with overwintering dormant tissue and throughout blossom development; 3) resistant to surface disinfestants; 4) resistant to bacterial nucleation inhibitors; and 5) inactivated by autoclaving. It was proposed that it might be a constitutive component of mature wood (10). However, a direct evidence for this intrinsic IN substance is lacking. It has not been identified, and it is not demonstrated if it is inducible or constitutive. If the later is true, it is possible to regulate its presence and modify plant freezing temperatures (9). Intrinsec leaf nucleators did not substantially limit supercooling in potato (53).

The effects of INA-bacteria on the freezing temperatures of Prunus flower buds at different stages of development were evaluated. The report concluded that a nonbacterial source of ice nucleation was present in the stem tissue, active at temperatures similar to INA-Bacteria, which limited supercooling in the floral organs (41). While the ice-nucleation temperature did not change with respect to bud development, the susceptibility of the floral organs to frost injury did change. However, it may be premature to assume the non-bacterial origin of the intrinsic ice nucleators, based only on the unsuccessful attempts to isolate the nucleator using conventional methods for surface epiphytic bacteria.

III. POSSIBILITIES OF IN PREVENTION.

The first situation involves tender annual plants with early or

late frosts, the second is related with perennial woody species and late spring frosts. The methods discussed have shown to be a possibility for the first condition (60). Their approach is the avoidance of frost injury by eliminating high populations of surface epiphytic bacteria or by preventing their growth, consequently eliminating the sites for ice nucleation. This is accomplished by, bactericides, antagonistic bacteria, or chemical inhibitors.

a) Bactericides. Significant frost control has been achieved experimentally with bactericides, although effective rate, timing and frequency remain to be determined. Because dead or dying cells also act as nuclei, the strategy should be prevention of bacterial build-up, rather than killing established populations.

b) Antagonistic bacteria. These bacteria are used to alter the composition of microorganisms on leaf surface to facilitate increased populations of non-ice nucleation active-bacteria on plants. Such is the case of the INA^- Pss strain (59, 66).

c) Chemical Ice nucleation inhibitors. These are chemicals that quickly inactivate the INA-bacteria without necessarily killing bacterial cells. They may offer a "day before" type of frost protection useful in areas where frost is infrequent, but a good predictor of meteorological conditions is also required. Generally speaking, several authors have reported contradictory results about their performance (4, 29, 40, 60).

In the second situation , the experimental results have not been encouraging, and the attempts to control frost by manipulating the INA-bacteria populations are considered premature (7, 13, 41)

However, several points should be taken in consideration.

a) Frost control should involve a multidisciplinary approach and every single component of the freezing stress must be taken into consideration to design an integrated strategy.

b) The natural ice-nucleators of non-biological origin nucleate ice at temperatures well below the supercooling capacity of flowers and fruits.

c) The nature of the proposed intrinsic nucleator should be defined.

d) Although the control of epiphytic populations has not proven totally effective by itself. It is possible that a reduction in inoculum diminishes the probabilities of ice initiation and consequently diminishes the possibility of damage, which is important when controlling frosts because if the threshold temperature is lowered, then it is possible to reduce costs.

Although, it is a general consensus that this approach is premature for frost control in deciduous fruit orchard. It is clear that much remains to be done and new possibilities can be expected.

IV. BACTERIAL VASCULAR TRANSLOCATION.

Although different evidences minimize and even exclude the action of INA-bacteria in the freezing injury of woody plant flowers, there are different experiences showing a positive translocation of bacteria through the vessels of grapevines. The detection of Agrobacterium tumefaciens in own rooted plants suggested the presence of an established internal population (25), and later a clear systemic spread of the Biovar 3 of the same microrganism was detected in the xylem of grapevines (40, 100).

Chapter 3

BUD DEVELOPMENT AND HARDINESS TO SPRING FREEZE OF PINOT NOIR GRAPEVINES.

ABSTRACT

The impact of frost damage on Pinot Noir buds was evaluated in a commercial vineyard. Plants growing on a hilltop were slightly affected, while those on the bottom showed a 70 % decrease in yield and a lower ripening level. A phenological study was carried out on 427 buds on vines growing in the field during the 1987 budbreak period. The bud development was characterized into seven stages. Budbreak was completed 44 days after it started, and only 4 % of the buds did not break. Overlapping populations of various bud stages were found at all times. Single node cuttings were forced in the greenhouse and used in controlled freezing tests to estimate the bud hardiness at different stages. The T50 values were; quiescent buds,-14^{o}C; swollen buds, -3.4^{o}C; budburst, -2.2^{o}C; and first, second, and third leaf stages were, -2^{o}C, -1.7^{o}C, -1.1^{o}C, respectively. It is proposed that the extent of freeze injury in the vineyard, depends on the proportion of buds at each stage, and their specific hardiness, as well. The bud water content at each stage was determined by dehydration. A rapid increase in water content occurred between quiescent and swollen buds, from 57 to 84 %. After that little changes occurred. A high correlation was found between hardiness levels, water content, and the time required for the buds to reach each stage of development.

INTRODUCTION

Spring freeze damage of developing buds and shoots is recognized as a major limiting factor in viticulture (1, 8). The extent of freeze damage on buds at different stages of development (SD) appears to be temperature dependent. Since budbreak is a dynamic process, it is important to determine the time of occurrence of the stage of bud development, as well as their killing points, to understand the risk factor. The temperature required to kill 50 % of the buds (T50) has been determined in Vitis labruscana B. cv. Concord (14). These results differ from the ones observed to cause field kill in other regions (8). Nevertheless, different genotypes differ in hardiness, and it is generally agreed that Concord is hardier than most vinifera grapes.

Dormant and deacclimating grape buds exhibit deep supercooling, even though they are the part of the vine most susceptible to freezing injury (1, 6). Artificial freezing techniques are preferred to estimate the killing temperatures, because of the unpredictability of frosts under field conditions (16, 21).

A detailed protocol has been standardized to carry out this kind of experiment (10). Viability of the organs is evaluated using different assay methods (11). In grapes several viability test have been evaluated (2), and the preferred methods are regrowth, tissue browning, and specific conductivity (15). Differential Thermal Analysis is recommended only for certain varieties (12, 17).

Environmental conditions before the occurrence of frost and water content of the tissues are two factors affecting the damage the vine hardiness. During dormancy, the temperature of the preceding day is closely related to changes in cold resistance (7, 13). However, after budbreak freeze resistance of grapevine buds is not affected by the preceding temperatures and always decreases with time (8).

Tissue water content of buds varies depending on the season. The greatest reduction in Concord buds occurs during the fall, and an inverse relationship was noted between hardiness and water content (18, 19). However, by mid winter specific hardiness differences could not be correlated with changes in water content (20). During the spring deacclimation period, the flower buds of Vaccinium australe increased in water content and decreased in bud hardiness (3). However, it has been questioned if this process affects only their ability to supercool (10).

The purposes of this study were to evaluate the budbreak phenology, the hardiness and water content of buds at several SD during budbreak, and to determine the relationship of SD, tissue water content and hardiness during budbreak.

MATERIALS AND METHODS.

Spring Freeze Damage Analysis

A preliminary analysis of frost damage was done on a commercial vineyard located near Rickreall, Oregon during the spring and fall of 1987. It is located on a South facing hillside

with a slope of approximately 6 degrees, with rows perpendicular to the slope. Bearing Chardonnay vines were pruned to two 14-bud canes. Four representative plants were evaluated along the slope, either at the bottom or at the top of the hillside.

Budbreak Dynamics Classification.

Fifteen year old 'Pinot Noir' vines head trained and cane pruned were growing in a clay loam soil at the Lewis-Brown Horticultural Farm near Corvallis. Two 15-node canes per plant were arched, and tied to a supporting wire. Fifteen plants were assigned to 3 replications. A total of 427 buds were monitored every other day according to the following phenological stages (5, 9, 13).

1. Quiescent Bud.
2. Swollen Bud.
3. Budburst.
4. First Flat Leaf.
5. Second Flat Leaf.
6. Third Flat Leaf.
7. Fourth Flat Leaf.

Critical Temperatures.

Four node cuttings of the basal 4th to 8th buds were obtained from plants growing in the field prior to budbreak, they were surface disinfested with Maneb, Omite, and Supracid ; and stored in polyethylene bags at $0^{o}C$ until forcing. Single-node cuttings were planted in a sterilized propagation medium of 1:1:1 mixture of soil, perlite, and sphagnum moss. The cuttings were placed in a greenhouse and watered daily.

The temperature levels used in the freezing test and the sample size used are summarized in Table 3.1. The freezing tests were done at the desired growth stages. The samples for the freezing study were prepared by aligning the buds on the cuttings

in the same direction and wrapping the basal ends in a wet cheese cloth. The cuttings were then wrapped in aluminum foil. The treatments were randomly assigned to the packages. The samples were placed in either the center of a Kelvinator ultra cold programmable freezer or in plastic bags submerged in an Endocal LT-50DD refrigerated circulating bath. The former was used for the quiescent bud stage , while the later was used for the other treatments. The sample temperature was allowed to equilibrate for 1 hour at $0^{o}C$, after which the temperature decreased at a rate of $1^{o}C$/hour. At $-1^{o}C$ the samples were inoculated with ice crystals to ensure proper nucleation, and at predetermined temperature intervals, samples were removed from the freezing unit and thawed at $4^{o}C$. They were placed in a flat with water in the greenhouse. Damage evaluation was made 1 week later by tissue browning observation.

The temperatures at 50 % of the buds was damaged (T50) were estimated by the Spearman-Karber method, as modified by Bittenbender and Howell (4).

Bud Water Content.

Ten representative buds of each stage were randomly selected, weighed individually, dried to constant weight, and reweighed for dry weight. The water content (WC) was estimated according to:

$$WC = 100 - 100dw/fw$$

where:

dw = dry weight (mg).

fw = fresh weight (mg).

The experimental design was a completely randomized with 7

stages as treatments and 10 buds as replications.

Stage of Development, Hardiness and Water Content.

Multiple regression analysis was used to determine the relationship between age and water content in the T50 of developing buds.

RESULTS AND DISCUSSION.

Spring freeze damage analysis.

The extent of freeze damage to grapevine bursting buds was greater at the bottom of the hillside (96 % compared to 6 % at the top). Table 3.2. Although temperature records were not available, it was assumed that the damage was caused by cooler temperatures occurring at the base of the hillside. Although, the primary buds were damaged the plants responded by pushing out secondary buds. These buds developed into full grown shoots. This variable was the same at both sites. However, the amount of clusters per shoot was considerably lower for those shoots originated from secondary buds, which carried a mean of only 0.9 clusters, compared with 2.5 on primary bud shoots growing on the top.

The amount of soluble solids (SS) on September 9 was 19.8 and 20.1^{o}Brix for the plants growing at the base and the top of the hillside, respectively, suggesting that the secondary bud clusters had less available ripening time (Table 3.3). This suggestion is confirmed by the higher acid content of top plant clusters. Assuming that most of the SS are sugars, the total SS produced per plant represents the total expression of the plant to elaborate sugars, which are a decisive final raw material for winemaking and

strongly determine the crop marketability. This preliminary evaluation agreed with previous reports suggesting that the crop originated from secondary buds was delayed in development and of a lower quality. It is important to note that during the fall of 1987, an unusually dry and warm period occurred which was extremely favorable for grape production. But under a normal season, this kind of secondary crop would have very few chances to attain commercial maturity standards.

Budbreak Dynamics.

The phenological development of the buds is shown in Table 3.4. Out of 427 buds, only 1.4% of the buds did not break, and 2.6 % did not develop normally. It can be inferred that western Oregon conditions favor sufficient chilling for Pinot Noir buds.

A total of 44 days had elapsed from the start of budbreak to the completion of all 7 stages of development for the 427 buds studied. The heat units accumulated between each sample period are shown in Table 3.5. The overlapping of various stages is found in Figure 3.1. Several stages were recorded during any given time, the most being 5 stages. The extent of freeze damage will depend on the proportion of buds at each stage and their corresponding critical temperature.

The mean date at which each stage occurred and the time interval between the means are shown in Table 3.6. The transition from quiescent (stage 1) to swollen buds (stage 2) required the longest interval. As the stages progressed, the time intervals became shorter when expressed as days, although not necessary so, when expressed as heat units. An exception was the interval to get

to the last stage peak, which is explained by the slow growth rate of late buds.

Critical Temperatures.

T50 of Pinot Noir buds are shown in Table 3.6. A rapid loss in hardiness occurred between the quiescent and swollen bud stages from -14°C to -3.4°C. More advanced stages had a T50 at slightly higher temperatures. These results suggest that during the quiescent phase, Pinot Noir buds maintain a level of hardiness similar to those reported for other varieties (Proebsting et al, 1980). The rapid lose in hardiness at the subsequent stages are likewise similar to those reported for other varieties; however, the T50 values were consistently higher than reported for Concord grapes (14). Therefore the extent of damage may be dependent on the proportion of buds at various stages, and the potential of a freezing temperature to cause a damage.

Bud Water Content.

Highly significant differences in WC were found among the 7 stages. The results are summarized in Table 3.7. Water content of quiescent buds was consistently lower (eg. 57 %) than the buds at the other bud stages (eg. above 84.4 %). The increment in bud WC that occurred during spring, coincides with a concomitant loss in cold hardiness. This agrees with previous reports of WC and hardiness of blueberry flower buds (3).

Stage of development, T50 and WC Relationship.

The analysis of the relationship between hardiness and WC in Pinot Noir primary bud tissues during budbreak showed that there was a strong negative correlation of 0.9905 between the bud

hardiness and the other 2 variables (Figure 3.2). However, it is necessary to sample at closer intervals between quiescent and swollen bud stages, since the greatest changes in WC occurs during this phase. Although, the model indicates that WC may play a role in hardiness, it is very unlikely that the changes in hardiness are directly associated with the increase in bud WC. Nevertheless, a third factor is closely related with time, explaining the high significance of the model. The actual nature is not clarified. It can be hypothesized that during this period, nucleating agents can develop and translocate in the plant. Or, that it may indicate a slow release of a barrier to xylem water through the bud, which may impede the translocation of nucleating agents (Lombard, personal communication).

Table 3.1. Temperature levels used to estimate T50 of Pinot Noir freeze damaged buds at several stages of development

Stage of development [a]	Temperature levels (° C) [b]					
Quiescent bud	+4,	-4,	-8,	-12,	-16,	-20
Swollen bud	+4,	-2,	-4,	-6,	-8,	-10
Budburst	+4,	-1,	-2,	-3,	-4	
First flat leaf	+4,	-1,	-2,	-3,	-4	
Second flat leaf	+4,	-1,	-2,	-3,	-4	
Fourth flat leaf	+4,	0,	-1,	-2,	-3,	-4

a) Single node cuttings were collected from the field and stored at 0°C for 2 months. Later, they were forced to break in a 20°C greenhouse. The sample size for the first 3 stages was 4 buds, for the last 3 stages was 8 buds.

b) A controlled freezing technique was used. The temperature of the samples was equilibrated at 0°C for 1 hour, following a decreasing rate of 1°C/hr.

Table 3.2. Effect of site elevation on spring freeze damage to developing buds of Vitis vinifera cv. Chardonnay

Site *	Nodes per Plant		Frost ** Damage (%)	Shoots per Plant		Clusters per Shoot	
	Mean	SD	Mean	Mean	SD	Mean	SD
Bottom	28.5	4.9	92.1	21.5	3.8	0.9	0.2
Hilltop	26.3	3.4	6.8	22.0	3.2	2.5	0.5

* The slope was approximately 6%.

** No temperature records are available.

Table 3.3. Effect of Site on Yield Components of Vitis vinifera cv. Chardonnay after a Spring Frost.

Site	Yield per Plant (Kg)		Soluble Solids (^{o} Brix)		Acidity (g/l) *		Total Soluble per Plant (Kg)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D
Bottom	2.25	1.2	19.8	0.6	10.7	2.8	0.43	0.2
Hilltop	6.95	2.1	20.1	0.3	8.9	0.9	1.39	0.4

* Expressed as Tartaric Acid.

Table 3.4. Budbreak dynamics of Vitis vinifera L. cv. Pinot Noir in the Willamette Valley in 1987 a)

Date	Days	c) Cumm. Heat Units	Proportion of Buds at Various Stages of Development (%) b)						
			1	2	3	4	5	6	7
03/28	0	0	100.0	0	0	0	0	0	0
03/31	3	6	98.5	1.5	0	0	0	0	0
04/02	5	15	81.4	18.6	0	0	0	0	0
04/05	8	16	70.0	30.0	0	0	0	0	0
04/07	10	16	50.5	49.5	0	0	0	0	0
04/10	13	19	28.1	70.4	1.4	0	0	0	0
04/13	16	20	23.2	72.3	4.5	0	0	0	0
04/15	18	27	10.0	47.9	41.8	0	0	0	0
04/17	20	29	4.9	34.9	60.0	0	0	0	0
04/19	22	29	3.5	30.0	65.8	0.5	0	0	0
04/21	24	33	1.9	22.7	58.0	17.2	0	0	0
04/23	26	42	1.4	12.2	53.7	31.2	1.2	0	0
04/26	29	51	1.4	5.3	14.7	50.9	27.1	0.5	0
04/28	31	65	1.4	3.0	4.1	18.2	47.9	25.5	0.2
04/30	33	73	1.4	1.4	1.4	5.2	17.3	50.4	22.7
05/02	35	75	1.4	1.4	1.2	3.6	13.1	50.1	29.0
05/04	37	88	1.4	1.4	0.7	0.8	9.1	44.7	41.7
05/06	39	107	1.4	1.4	0.7	0.5	1.6	14.5	79.7
05/08	41	136	1.4	1.4	0.7	0.3	0	4.9	91.1
05/11	44	165	1.4	1.4	0 7	0.3	0	0	96.2

a) 427 buds were monitored every other day and classified according to their stage of development.

b) 1 Quiescent, 2 swollen, 3 burst, 4 first flat leaf, 5 second, 6 third, and 7 fourth flat leaf.

c) Sheltered temperature was recorded every 1/2 hr and heat units were estimated by ((Max+Min)/2)-10^{o}C.

Table 3.5. Mean Date and intervals at which peak populations of 7 stages of bud development of Vitis vinifera L. cv. Pinot Noir occurred in 1987.

Parameters	Stage of Development						
	1	2	3	4	5	6	7
Weighed Mean *	0	15.7	22.6	28.3	31.8	34.8	39.9
Actual Date	03/28	04/13	04/20	04/25	04/29	05/02	05/07
Interval (days) **	0	16	7	5	4	3	5
Cummulative Heat Units ***	0	20	30	44	70	75	121

* Estimated mean for the period when the stage occurred. The date before recording the first swollen bud is day 0.

** Time interval from the preceding stage.

*** Sheltered temperature recorded every 1/2 hr. Heat units estimated by ((Max+Min)/2) -10^{o}C

Table 3.6. Critical temperatures of Vitis vinifera L. cv Pinot Noir buds at 6 stages of development estimated from controlled freezing tests on single node cuttings.

	Stage of Bud Development					
				Flat Leaves		
	Quiescent	Swollen	Burst	First	Second	Fourth
T50 (°C)	-14.0	-3.4	-2.2	-2.0	-1.7	-1.2
S.D.	1.4	0.8	0.6	0.3	0.3	0.3

* T50 estimated by the Spearman-Karber method as modified by Bittenbender and Howell, 1974.

Table 3.7. Mean percentage of water content in developing Vitis vinifera cv Pinot Noir buds measured after dehydration at 70°C *

Stage of bud development **	1	2	3	4	5	6	7
Mean water content (%) ***	56.9a	84.4b	86.6bc	86.7bc	88.6c	85.5b	84.4b

* Estimated by WC= 100-100dry wt/fresh wt

** 1 quiescent bud, 2 swollen, 3 budburst, 4 first flat leaf, 5 second, 6 third, and 7 fourth flat leaves stages.

*** Mean separation at 0.01 level

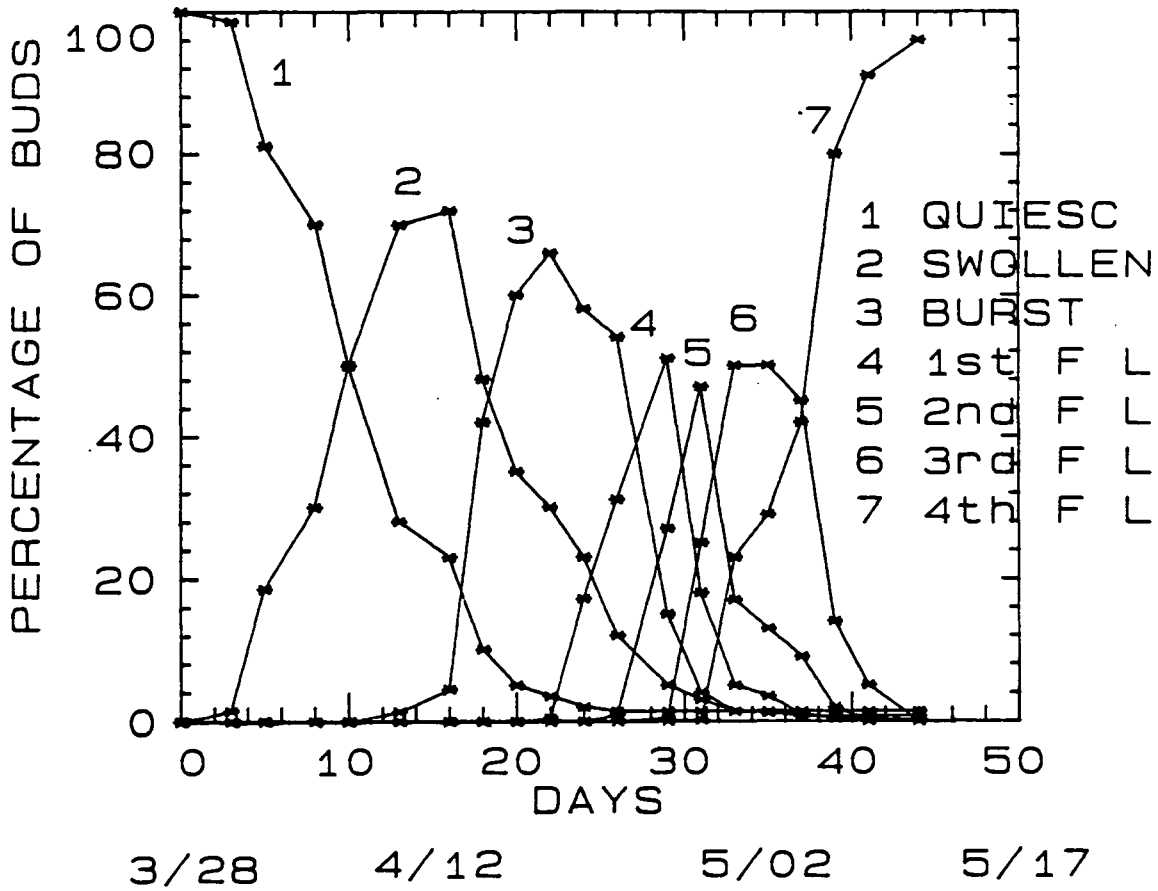
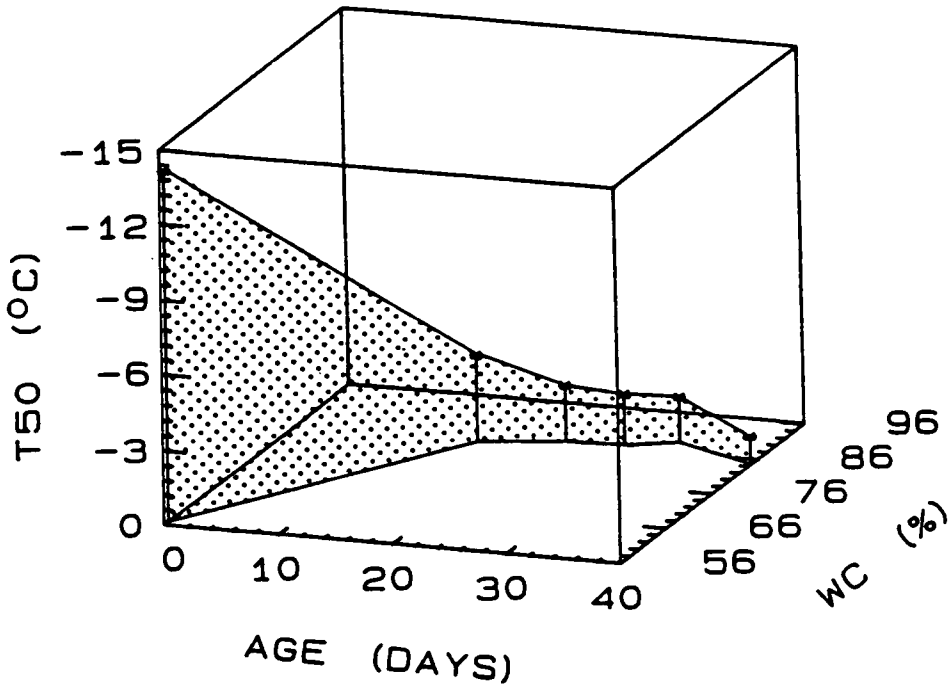


Figure 3.1 Budbreak dynamics of Vitis vinifera cv Pinot Noir at seven stages of development expressed as a percentage of the total population.



$$Y = -32 + 0.32(WC) + 0.09(AGE)$$

$$r^2 = 0.9905^{**}$$

Figure 3.2 Killing temperature of Vitis vinifera cv Pinot Noir at 6 stages of bud development as affected by water content and age.

REFERENCES

1. Andrews, P.K., C.R. Sandidge, and T.K. Toyama. 1984. Deep Supercooling of Dormant and Deacclimating Vitis Buds. Research Note. Am. J. Enol. Vitic. Vol. 35.(3): 175-177.

2. Ahmmedulla, M., and A. Kawakami. 1986. Evaluation of Laboratory Tests for Determining the Lethal Temperature of Vitis labruscana B. Concord Roots Exposed to Subzero Temperatures. Vitis 25, 142-145.

3. Bittenbender, H.C. and G.S. Howell. 1975. Interaction of Temperature and Moisture Content on Spring Deacclimation of Buds of Highbush Blueberry. Can. J. Plant Sci. 55:447-455.

4. Bittenbender, H.C., and G.S. Howell. 1974. Adaptation of the Spearman-Karber Method for Estimating the T50 of Cold Stressed Flower Buds. J. Amer. Soc. Hort. Sci. 99(2):187-190.

5. Flaherty, D.L., F.L. Jensen, A.N. Kasimatis, H. Kido, and W.J. Moller. 1982. Grape Pest Management. University of California. Publication 1981. pp. 12-15.

6. Howell, G.S., B.G. Stergios, and S.S. Stackhouse. 1978. Interrelation of productivity and Cold Hardiness of Concord Grapevines. Am. J. Enol. Vitic. 29(3):187.

7. Howell, G.S., and C.J. Weiser. 1970. Fluctuations in the Cold Resistance of Apple Twigs during Spring Dehardening. J. Am. Soc. Hort. Sci. 95:190-192.

8. Johnson, D.E., and G.S. Howell. 1981. Factors Influencing Critical Temperatures for Spring Freeze Damage to Developing Primary Shoots on Concord grapevines. A,m. J. Enol. Vitic. 32:144-143.

9. Jordan, T.D., R.M. Pool, T.J. Zabadal, and J.P. Tomkins. 1980. Cultural Practices for Commercial Vineyards. New York State College of Agriculture and Life Sciences. Cornell University. Miscellaneous Bulletin 111, pp. 22-24.

10. Levitt, J. 1980. Responses of Plants to Environmental Stresses. Second Edition. Vol. 1. Chilling, Freezing and High Temperature Stresses. Academic Press Inc. N.Y.

11. Palta, J.P., J. Levitt, E.J. Stadelmann. 1978. Plant Viability Assay. Cryobiology. 15:249-255.

12. Pierquet, P., and C. Stushnoff. 1980. Relationship of Low Temperature Exotherm to Cold Injury in Vitis riparia M. Am. J. Enol. Vitic. 31(1):1-6.

13. Proebsting, E.L., M. Ahmmedullah, and V.P. Brummund. 1980. Seasonal Changes in Low Temperature Resistance of Grape Buds. Am. J. Enol. Vitic. 31(4):329-336.

14. Proebsting, E.L., V.P. Brummund, and W.J. Clore. 1978. Critical Temperatures for Concord Grapes. Washington State University. Extension Leaflet EM 4330.

15. Stergios, B.G., and G.S. Howell. 1973. Evaluation of Viability Tests for Cold Stressed Plants. J. Am. Soc. Hort. Sci. 98(4):325-333.

16. Stushnoff, C. 1972. Breeding for Cold Hardiness. Hortscience. 7(1):10-12.

17. Wolf, T.K., and R.M. Pool. 1987. Factors affecting Exotherm Detection in DTA of Grapevine Dormant Buds. J. Amer. Soc. Hort. Sci. 112(3):412-415.

18. Wolpert, J.A., and G.S. Howell. 1986. Cold Acclimation of Concord Grapevines. III Relation between cold Hardiness, Tissue Water Content and Shoot Maturation. Vitis 25;151-159.

19. Wolpert, J.A., and G.S. Howell. 1985. Cold Acclimation of Concord Grapevines. II Natural Acclimation Pattern and Tissue Moisture decline in Cane and Primary Buds of Bearing Vines. Am. J. Enol. Vitic. 36(3):189-194.

20. Wolpert, J.A., and G.S. Howell. 1984. Effects of Cane Length and Dormant Season Pruning Date on Cold Hardiness and Water Content of Concord Bud and Cane Tissues. Am. J. Enol. Vitic. 35(4):237-241.

21. Young, R., and C.J. Hearn. 1972. Screening Citrus Hybrids for Cold Hardiness. Hortscience. 7(1):14-18.

Chapter 4

SELECTION AND EVALUATION OF A Pseudomonas syringae pv. syringae STRAIN AND ITS EFFECT ON THE FREEZE DAMAGE OF GRAPE LEAF TISSUE.

ABSTRACT

This study was carried out to select a strain with a high level of Ice Nucleation Activity (INA), to determine its growth pattern and to evaluate the effect of bacterial concentration gradients on the nucleation temperature of suspensions. A time course study demonstrated a strong interaction between genotype and culture age relative to Ice Nucleation Activity (INA). The INA of Pseudomonas syringae pv. syringae strain B15 (PssB-15) was between -2 to -4°C throughout the 7 days of evaluation. The INA of Pss2-3RNH was observed on 3 day old cultures only, and Pss 2-3 was a poor nucleator. The first 2 strains attained a stationary phase of growth after 60 hr in culture. The INA of Pss-B15 suspensions was directly proportional to its concentration. The mean nucleation point of the bidistilled water control and the bacterial suspension (log 9.31 cells/ml) was -17.2 and -2.5°C, respectively. Surface sterilized grape leaf disks (25 mm^2) sprayed with either bidistilled water or a Pss-B15 suspension of ca. 10^8 cells/ml were used to evaluate the ability to incite freeze injury. A highly significant effect of INA-bacteria to incite freeze injury was detected, especially in the range of -3 to -4°C.

INTRODUCTION

Spring weather in Western Oregon is characterized by high rainfall conditions (7). Vineyards are located mainly on hillsides and require the use of grass cover crops to prevent erosion (11). Grasses were recently found to be potential sources of Pseudomonas syringae pv syringae (Pss) inoculum (2). The ice nucleation (IN) phenotype was associated with 85 % of the isolates in a local survey (1). IN phenotype has been implicated as a predisposing factor for infection to occur (10). However, these microbes appear to be weak pathogens of grapevines (3). And, there is a possibility that temporary epiphytic populations may incite frost injury since vertical dispersions have been reported (8), and the inoculum is widely dispersed on the cover crops. This may explain field observations, where regardless of topographic conditions, there is greater susceptibility to freeze damage of buds on canes laying in the grass.

The objectives of this study were to select a bacterial strain with a strong expression of the ice nucleation-active (INA) phenotype, to determine its growth pattern, and to evaluate the effect of a concentration gradient on the freezing temperature of bacterial suspensions. The final objective was to evaluate the effect of INA-Bacteria on the freezing injury to grape leaf tissue.

MATERIALS AND METHODS

Several conditions were common to all the experiments, as shown below.

Culture media and bacterial suspensions.

To get a better expression of the INA characteristic, the strains were grown on King's B media with the glycerol content increased to 1 % (6). The incubation temperature was 27°C, while the cultures were acclimated at 4°C for 12 hr before the freezing tests. A Klett-Summerson Photoelectric Colorimeter was used to adjust the bacterial suspension concentrations by optical density.

Freezing Tests.

A programmable Endocal LT-50DD refrigerated circulating bath was used to lower the temperature of the samples, after equilibrating their temperatures at 0°C for an hour. The samples consisted of a 1 ml aliquot of the different suspensions contained in 4 ml disposable culture tubes. Previously, the tubes were screened for INA at -14°C, and sterilized before use.

EVALUATION OF THE INA-PHENOTYPE IN THREE Pss STRAINS.

The INA of bacterial PssB-15, Pss2-3, and Pss2-3RNH was evaluated at different ages from 2 to 7 day old cultures. The suspension concentrations were adjusted to 80 Klett units (KU), which yielded approximately 10^8 cells/ml. Serial dilutions were plated to verify the concentration.

A completely randomized design with a factorial arrangement of 3 genotypes and 6 was and 20 replications per treatment was used in this study.

GROWTH PATTERN DETERMINATION.

The best ice nucleator (PssB-15) was selected from the previous experiment, and its growth pattern was determined and compared with Pss2-3RNH as a reference control. Unwashed cells from

three day old cultures were suspended in sterile bidistilled water, and the optical density was adjusted to 70 KU (ca 10^6 cells/ml). Plates with solid media were spread evenly with 0.1 ml of that suspension and incubated. At each sampling time, the lawn of bacteria from 4 plates were individually harvested by double rinsing the dishes with known aliquots of sterile bidistilled water, and added to a side-arm flask. The volume was adjusted to 100 ml, the suspension shaken for 1 min, and the optical density determined as stated above. The sampling times were 0, 24, 36, 48, and 60 hr.

A regression analysis of the growth patterns was carried out and a non-linear model was selected.

EFFECT OF SUSPENSION CONCENTRATION ON THE NUCLEATION POINT.

The PssB-15 strain was cultured, incubated, and acclimated as before, a stock suspension was made with an original concentration of ca. 10^9 colony forming units (CFU)/ml. The stock suspension was serially diluted, nine concentrations were made and each was subjected to the freezing test described previously. One ml of suspension was placed in a 4 ml test tube and each dilution was replicated 10 times.

An analysis of variance was done, and a regression analysis was used to illustrate the response gradient.

EFFECT OF Pss ON THE FREEZING INJURY OF GRAPEVINE LEAF TISSUE.

Ten terminal leaves were collected from plants growing in the greenhouse. They were surface sterilized by immersion in ethanol 70 % for 2 sec and in 10 % chlorox for 5 minutes, plus 1 drop of Tween 20.

Twenty five sq mm leaf disks were cut from these leaves, randomly separated, and assigned to one of two Petri dishes. One was sprayed with a bacterial suspension of 10 CFU/ml; the other was sprayed with sterile bidistilled water. Two experiments were done with this material.

In the first study, the INA of the bacteria on the disk surfaces was determined. Five leaf disks of either, control or inoculated treatments were placed in each of 20 test tubes containing 1 ml of sterile bidistilled water. The temperature at which the water in the test tube frozen was recorded. The experimental design was completely randomized with 2 treatments and 10 replications, the nucleating point was considered as a response variable.

In the second study, inoculated and control disks were evaluated for frost injury at +4, -1, -2, -3, -4, and -5°C. Individual samples consisted of 5 leaf disks wrapped in a moist kimwipe, and arranged between 2 sheets of aluminum foil. The samples was placed on a floating aluminum pan in a programmable circulating bath. After the temperature of the samples was equilibrated, the bath temperature was reduced by 1°C/hr. At -1°C the treatments were inoculated with ice. At each test temperature a set of samples was removed and stored at +4°C until the next day. The samples were placed in 8 ml of bidistilled water in 10 ml flasks and evaluated for tissue damage by electric conductivity.

The experimental design was completely randomized, with a factorial arrangement involving the presence or absence of inoculum, as well as the 6 temperature levels mentioned before.

RESULTS AND DISCUSSION

INA-PHENOTYPE OF 3 BACTERIAL STRAINS.

A highly significant interaction was found between genotype and age of the 3 strains used in this study, their INA was different (Figure 4.1). INA of Pss-B15 was more constant and occurred at a higher temperature than the other 2 strains tested. Pss2-3RNH showed an intermediate INA among the 3 strains evaluated and for some unknown reason only the 3 day old cultures were able to nucleate at temperatures as warm as -3°C (this was confirmed three times); whereas, before and after that period the INA was lower. This temporal expression of a metabolic pathway is an interesting phenomenon reported previously in Bacillus subtilis (9), and it had been eventually observed in INA-bacteria strains (Lindow, personal communication). Pss2-3 was the poorest ice nucleator in this study; its freezing point was in the range of -8 to -10.5°C. This difference in behavior between wildtype and mutant strains can be explained as an unexpected change in certain traits during the selection process for the drug resistant mutant.

Optical density measures only the suspension turbidity, not the viability of the suspended cells. So, the viable concentration was estimated by the dilution plating method, and results are shown in Figure 4.2. The variations in populations were probably due to inaccurate dilutions. This variability probably occurred during the preparation of the suspensions. There was not a coincidence between higher populations and warmer freezing points. Instead, it is more probable that changes in INA are closely related with changes in cell metabolic activity as affected by age. Other evidence

supporting this statement is that not every single cell acts as an ice nucleus, and the probability of finding one INA cell is as low as 1 in 1,000 cells (4). Considering the size of the variation among concentrations it is more likely that the changes in INA are related to culture age. Apparently the cells stop growing during the first 7 days, but do not die.

In summary the INA phenotype differed greatly between genotypes Pss-B15 was a better ice nucleator for the purpose of this study, and there is evidence of temporary factors in Pss2-3RNH which affects the expression of the INA.

BACTERIAL GROWTH PATTERN.

The growth pattern of Pss-B15 and Pss2-3RNH are shown in the Figure 4.3. The non-linear model of the data was highly significant with very acceptable coefficients of determination. Essentially, both strains grew at the same rate and the estimation of their growth patterns were statistically the same. And, a common model was estimated with a coefficient of determination of 0.81, the cell density was 5.7 KU at time 0, and their growth rate was 0.18 KU/hr. After 60 hr in culture, the strains were at stationary phase with an optical density of 254 KU. Although the growth rate of PssB-15 was slower (0.21 vs 0.23 KU/hr), it could grow longer than Pss2-3RNH, because its growth ceased few hours after. It is thought important for cultures to be in stationary phase to aid cell survival when the bacteria are transported from an in vitro environment to the more hostile conditions on the leaf surface (Moore, personal communication).

EFFECT OF SUSPENSION CONCENTRATION ON THE NUCLEATION POINT.

The concentration of bacterial cells had a highly significant effect on the freezing point of the suspensions. It was clear that the higher the concentration, the warmer the temperature at which nucleation occurred, ranging from -2.5°C for the most concentrated treatment (log 9.31 cells/ml), to -17.2°C for the control containing only bidistilled water (Table 4.1). A highly significant exponential model between suspension concentration and ice nucleation temperature was calculated with a coefficient of correlation of 0.91 (Figure 4.4). According to the estimated parameters, the nucleation point rose exponentially as the concentration increased. The exponential rise was explained by the higher probability of finding an ice nucleus in the more concentrated treatments, while the opposite occurred at concentrations below log 3 cells/ml. It can be concluded that the nucleation point is directly proportional to the suspension, and similar results were also reported by others (5).

EFFECT OF Pss ON THE FREEZING INJURY TO GRAPEVINE LEAF TISSUE.

Leaf disks immersed in bidistilled water had a highly significant difference in the ice nucleation point of the water. Surface sterilized disks nucleated ice through the water at a mean temperature as low as -7.5°C, while those inoculated with INA-bacteria catalized ice formation at -3.1°C (Table 4.2).

A highly significant interaction was found between temperature and the presence of INA-bacteria in causing damage to leaf disks (Table 4.3). The biggest difference in damage was found at temperatures in the range of -3 and -4°C. It is apparent that above

that range, the amount of damage incited by INA-bacteria is not significant. However, at -5°C, the damage is the same regardless of the presence of external nucleators suggesting that the capacity of the leaf tissue to supercool was limited by other nucleators which were less active than the sprayed bacteria. However, no information about their nature is provided in this study. It can be concluded that epiphytic INA-bacteria on grape leaf tissue can induce freeze injury, with the strongest effect in the range of -3 to -4°C.

Table 4.1. Effect of Pseudomonas syringae pv. syringae Strain B-15 concentration on the nucleation temperature of suspensions.

Suspension Concentration (log cells/ml)	Mean Ice Nucleation Point (°C)
None *	-17.2 f
1.77	-15.1 e
2.83	-13.7 d
3.79	- 8.1 c
4.64	- 4.1 b
5.68	- 3.6 b
6.74	- 3.5 ab
7.86	- 3.0 ab
9.31	- 2.5 a

Standard Error = 0.55°C

* None means absence of bacteria

Table 4.2. Freezing temperature of water as affected by epiphytic INA-bacteria on Pinot Noir grape leaf disks

	Temperature (°C)
Inoculated	-3.1 a
Non-inoculated	-7.5 b

Mean separation at 0.01 level

Table 4.3. Effect of low temperatures in a controlled freezing test on injury to Pinot Noir grapevine leaf disks inoculated in vitro with INA-bacteria

	Leaf disk area damaged (%)				
Temperature (°C)	-1	-2	-3	-4	-5
Inoculated	5.24	59.4	86.4	88.4	94.7
Control	6.27	56.9	68.6	78.7	92.6

Significant at the 0.01 level

Standard error= 0.707 % damage.

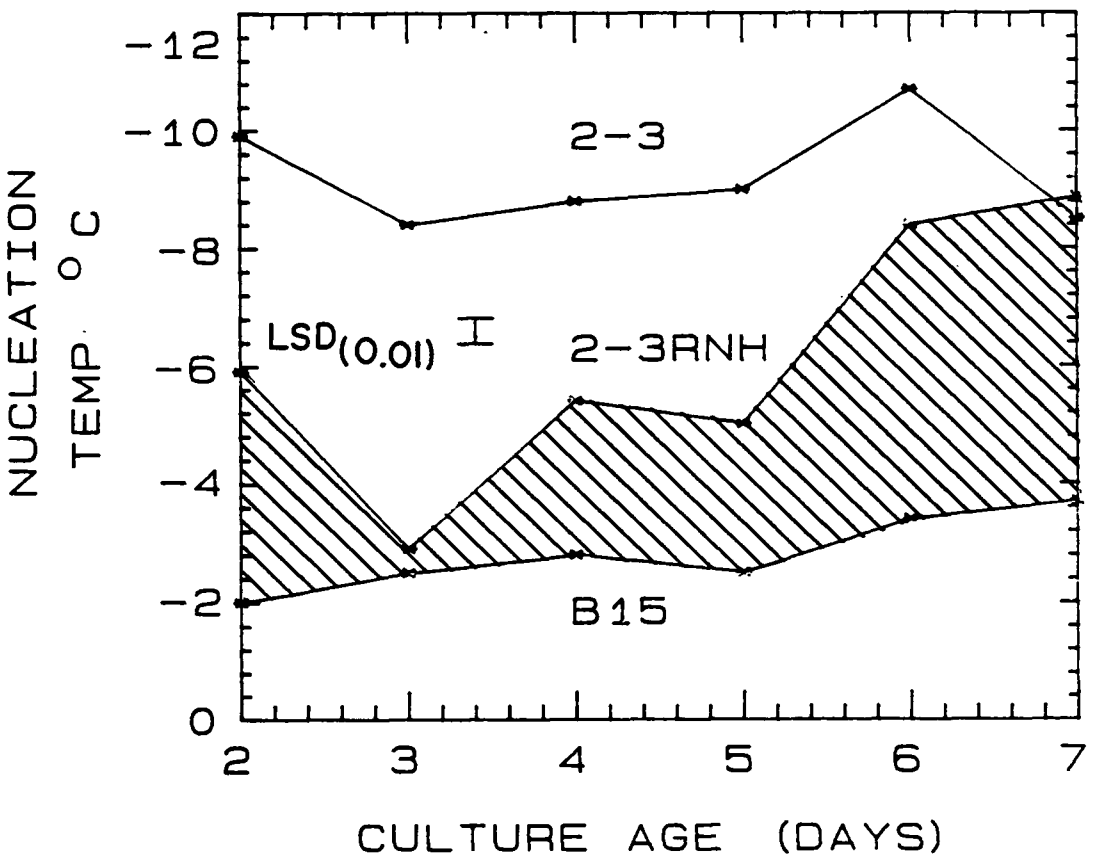


Figure 4.1. Effect of culture age on the ice nucleation-activity of three Pseudomonas syringae pv. syringae strains in vitro at constant optical density

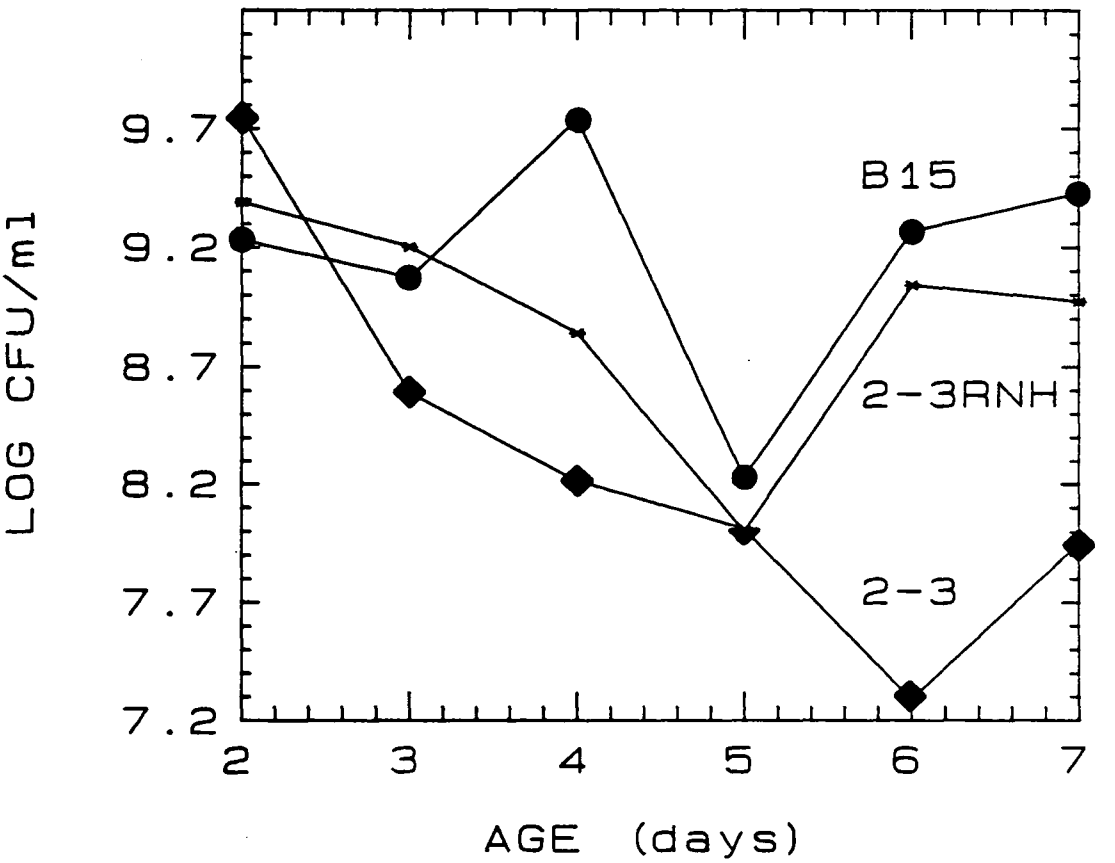


Figure 4.2. Effect of suspension concentration of three strains of Pseudomonas syringae pv. syringae determined by dilution plate assay. Colony forming units (CFU)

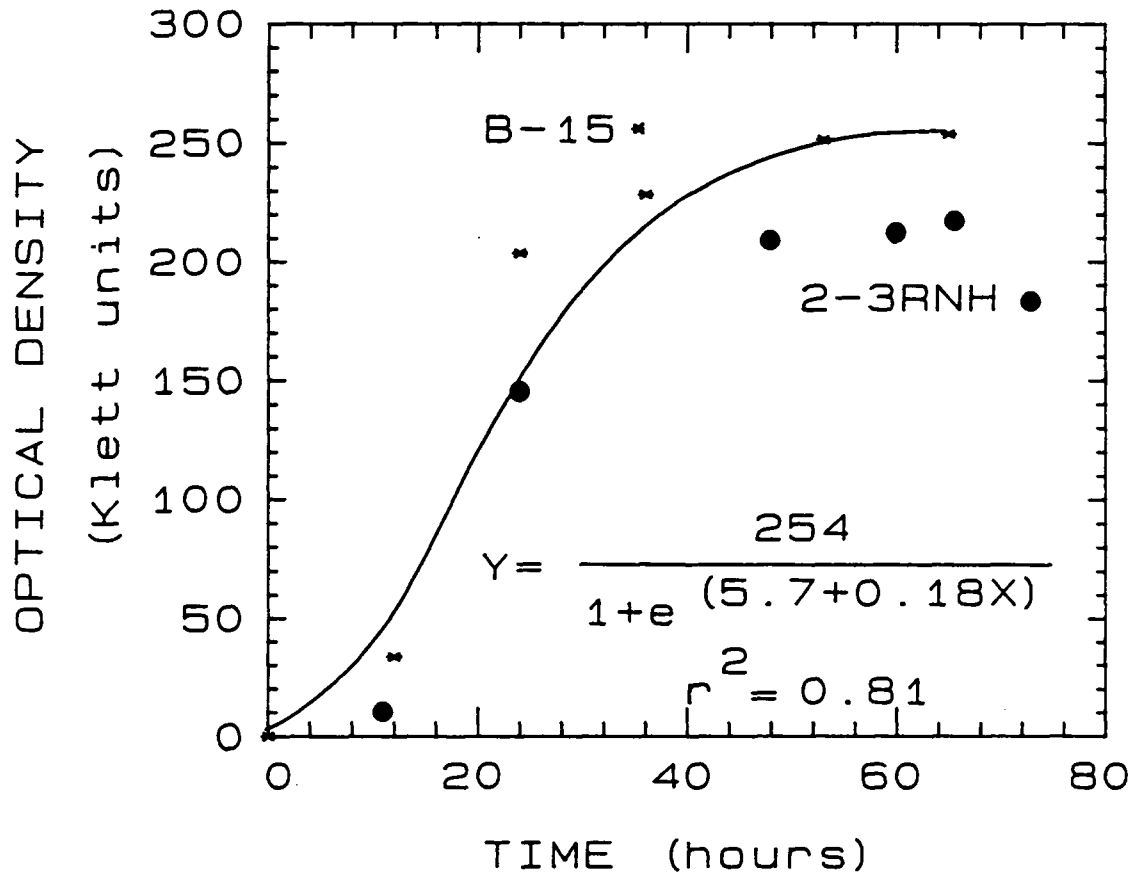


Figure 4.3. Growth pattern of two Pseudomonas syringae pv. syringae strains.

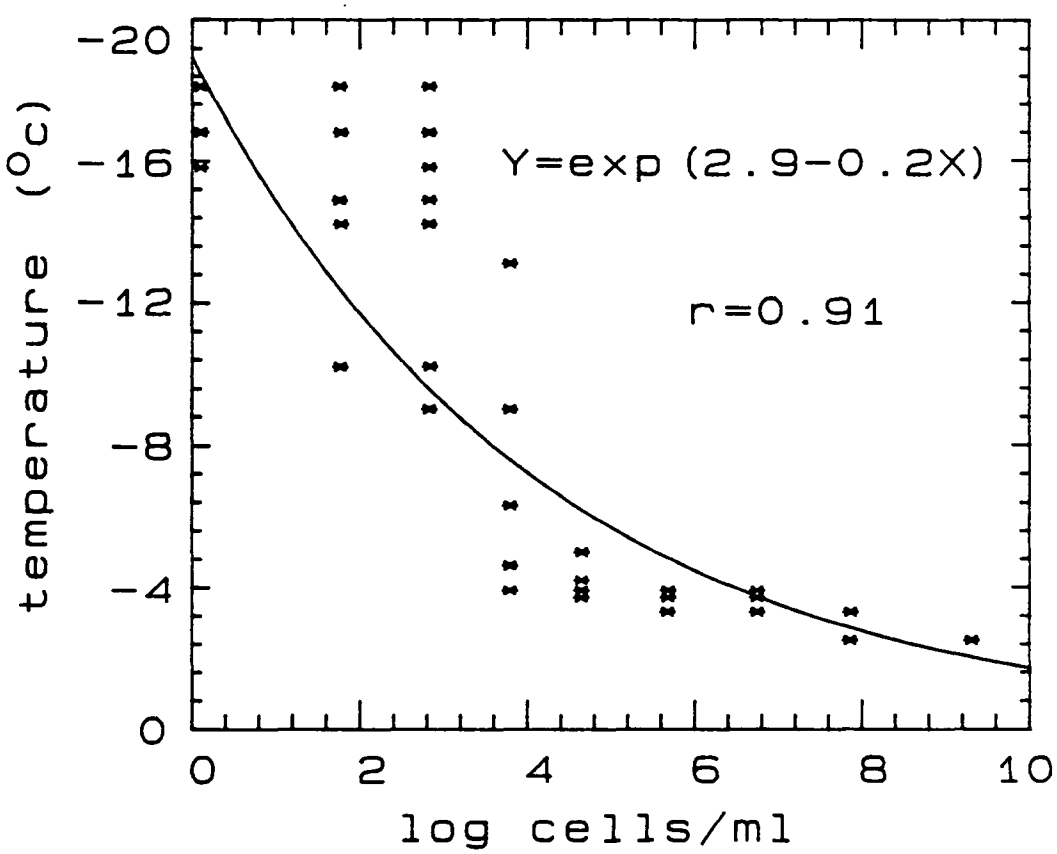


Figure 4.4 Effect of suspension concentration on the ice nucleation activity of Pseudomonas syringae pv. syringae strain B-15.

REFERENCES

1. Baca, S., M. Canfield, and L.W. Moore. 1987. Variability in Ice Nucleation Strains of Pseudomonas syringae Isolated form Diseased Woody Plants in Pacific NW nurseries. Plant Disease 71(5):412-415.

2. Baca, S. and L.W. Moore. 1987. Variations in Pseudomonas syringae Isolated from Grass Species Occurring in Woody Plant Nurseries in the PNW. Plant Disease Vol 71(8):724-727.

3. Klinger, A.E., N.J. Palleroni, and R.E. Poutis. 1976. Isolation of Pseudomonas syringae from lesions on Vitis vinifera. Phytopath. 86:107-16.

4. Lindow, S.E. 1983. The Role of Bacterial Ice Nucleation in Frost Injury to Plants. Ann. Rev. Phytopathology 21:363-384.

5. Lindow, S.E., D.C. Arny, and C.D. Upper. 1982. Bacterial Ice Nucleation A Factor in Frost Injury to Plants. Plant Physiol. 70:1084-1089.

6. Lindow, S.E., S.S. Hirano, W.R. Barchet, and D.C. Arny. 1982. Relationship between Ice Nucleation Frequency of Bacteria and Frost Injury. Plant Physiol. 70:1090-1093.

7. Lombard, P.B., B.T. Watson, D.A. Heaterbell, and R. Garren. 1986. Production, Maturity and Composition of Winegrape Varieties in Western Oregon. Agricultural Experiment Station. O.S.U. Corvallis, Or. Circular of Information 701 pp 12.

8. Malvick, D.K. 1987. Survival and Dispersal of Pseudomonas syringae in a Maple Nurseries and Pear Orchards. M.S. Thesis. O.S.U. Corvallis, Or. pp 74.

9. Stubbs, J.J., R.E. Feeney, J.C. Lewis, I.C. Feustel, H.D. Lightbody, and J.A. Garibaldi. 1947. Subtilin Production in Submerged culture. Archives of Biochemistry. Vol 14 (3):427-435.

10. Sule, S., and E. Seemuller. 1987. The Roll of Ice formation in the infection of Sour Cherry leaves by Pseudomonas syringae pv syringae. The American Phytopathological Soc. 77(2):173-176.

11. Wilson, W.D. 1985. Response of 'Pinot Noir' grapevines to a Perennial Ryegrass Cover Crop and Irrigation in the Willamette Valley. M.S. Thesis. O.S.U. Corvallis, Or. pp 2.

Chapter 5

EVALUATION OF A COMMERCIAL ANTIFREEZE CHEMICAL.

ABSTRACT.

Frost Gard is a commercial chemical advertised for frost control in vineyards. The objective of this study was to evaluate the potential ability of Frost Gard to act as an antifreeze, cryoprotectant and inhibitor of INA in Pseudomonas syringae by measuring the nucleating temperature of water solutions and bacterial and inorganic ice nucleator suspensions and by freeze damage to grape leaf tissue. Frost Gard at 0.1 to 1 % was ineffective as a cryoprotectant and had little influence on the freezing point of several water types with various impurities. Instead, Frost Gard acted as a bactericide. At 0.5 % reduced the nucleation temperature of the cell suspension from -2.5 to -6.7^{o}C, while heat treated bacterial suspension froze at -14^{o}C. Frost Gard at 0.5 % was also effective in binding with an inorganic ice nucleator, Thermica (0.3 mg/ml), reducing its nucleation point from -2.8 to -11.1^{o}C. Grape leaf disks grown under simulated field conditions were damaged severely at -2 to -4^{o}C. Frost Gard at 0.25 % reduced the damaged area from 66 to 59 %, but only at -2^{o}C. The greatest increase in damage occurred in the range of -2 to -3^{o}C.

INTRODUCTION.

Besides the traditional systems several methods for frost control have been evaluated. Growth regulators have been applied to either directly increase plant hardiness (5), or indirectly to delay budbreak and avoid frosts (1, 10). Also, antitranspirants and cryoprotectants have been used unsuccessfully (7, 9).

Frost injury has been related to the presence of epiphytic populations of ice nucleation-active (INA) bacteria, and various methods to reduce frost damage by controlling the INA-bacteria had been tested on several crops. Spring populations of epiphytic INA bacteria have been reduced with antibiotic sprays (8). However, even dead cells retain the ice nucleation properties (2). Antagonistic INA$^-$bacteria are aggressive competitors and can suppress the presence of INA-bacteria. Its use, however, was not effective in controlling frost injury to pear blossom (4). Results have shown that bacterial inhibitors inactivate the INA-bacteria without necessarily killing the cells. In tender annual plants the above methods have been used successfully to reduce freeze damage by eliminating or preventing the growth of epiphytic INA-microbes (8).

Attempts to control frost damage in woody plants by suppressing the effect of epiphytic INA-bacteria have failed (4, 6). A general consensus that frost control strategies designed to reduce the population of INA-bacteria within deciduous orchards may be premature (3, 6).

Various chemicals on the market are being advertised for increasing frost protection in plants. This has aroused the

interest of growers about their performance. Frost Gard is claimed to be a plant antifreeze chemical and an inhibitor of the active ice nucleation site of INA-bacteria. Although its formulation is not available, its label confirm the presence of certain cryoprotectant agents in the solution, as well as heavy metals and nitrogen.

The purposes of this study were to determine whether Frost Gard was a cryoprotectant, antifreeze, and/or competitor of the ice nucleation site of INA-bacteria, and to evaluate its protective action on grape leaf tissue.

MATERIALS AND METHODS.

The following conditions were common for the different experiments. The evaluations of the freezing events of the various treatments were done under controlled freezing tests in a controlled glycol bath. The treatments consisted in 1 ml of either chemical solutions or bacterial suspensions, contained in ten-4 ml sterilized Difco disposable test tubes, previously screened for INA at -14°C. The sample temperatures were equilibrated at 0°C for 1 hr before dropping the temperature at a rate of temperature drop was 0.5°C/hr.

Frost Gard on INA of water solutions.

This study tested the antifreeze and cryoprotectant characteristics of Frost Gard. Since it was assumed that the presence of ice nucleators varies depending on water purity, Frost Gard at 0, 0.125, 0.25, 0.50, and 1.0% concentration was applied to 4 water quality classes (tap, sterile tap, bidistilled, and

sterile bidistilled), and a fifth treatment of sterile bidistilled water inoculated with ice crystals at -0.5°C, to test the cryoprotectant nature of Frost Gard. The experimental design was a completely randomized with 10 replications, with a factorial arrangement involving the 5 Frost Gard concentration and the 4 water classes mentioned above. The temperatures at which the samples were frozen (nucleation temperature) was considered as the response variable.

Frost Gard on bacterial suspensions.

Pseudomonas syringae pv. syringae strain B15 were grown onto King's B plates for 60 hr at 27°C. They were then subjected to an acclimation treatment of 4°C for 12 hr. The suspension concentration of the bacteria was adjusted to a final density of $80x10^8$ colony forming units/ml. The concentration was determined turbidimetrically and confirmed by a standard dilution plate assay. The stock suspension was divided equally and the following treatments applied 2 hr before the freezing test.

a) Control,

b) 0.25 % Frost Gard

c) 0.50 % Frost Gard

d) 1.00 % Frost Gard

e) An aliquot of the stock suspension was autoclaved at 110°C for 20 minutes,

f) Stocked suspension exposed to chloroform fumes for 5 hr. Its concentration was adjusted to the same optical density than the stock suspension.

The nucleation points of individual replications were recorded

for each treatment, and their bacterial populations were estimated before and after the freezing test. The experimental design was completely randomized with 20 replications.

Frost Gard and an inorganic ice-nucleator.

Thermica, a fine, inert clay which nucleates ice at warm temperatures was used to differentiate the antifreeze from the biotic effect of Frost Gard. A factorial experiment with 5 levels of Thermica (0, 0.3, 0.6, 1.2 and 2.4 mg/ml) and 4 levels of Frost Gard (0, 0.25, 0.50 and 1%). The experimental design was a completely randomized with 10 replications. The nucleation temperature of individual samples was recorded.

Frost Gard on grape leaf tissue.

Single node grape cuttings were forced in the greenhouse in early July. The cuttings were exposed to high humidity to simulate spring conditions at the Willamette Valley. Care was taken to prevent the mist from wetting the cuttings directly. The maximum temperatures in the greenhouse were 20°C and the minimum 12°C. The cuttings were sprayed either with a Pss-B15 suspension of ca 10^8 CFU/ml or sterile bidistilled water. The bacterial suspension was applied twice, one week after the first application. Frost Gard (0, 0.25, 0.50, and 1 %) was applied 36 hours before the freezing test. The bacterial population was checked after the Frost Gard application and before the freezing test.

Disks (5 mm diameter) from the terminal leaf of each cutting were sampled, pooled and randomized for each Frost Gard treatment. Ten disks per treatment were placed on a moist Kimwipe between two aluminum foils and placed on a floating aluminum pan in a low

temperature glycol bath. The temperature was equilibrated at 0°C for 1 hour and then dropped to -2, -3, and -4 at a rate of 1°C/hr After the freezing test, the samples were stored at 4°C for about 12 hr. The disks were then placed on moist filter paper in petri dishes and kept for 72 hr in an incubation chamber at 25°C, with a 12 hr photoperiod. The damage was evaluated visually by the tissue browning method. The injury to disks was scored according to the percentage of damaged area. The experimental design was a completely randomized with 10 replications. A factorial arrangement was used to evaluate the interaction between inoculum, Frost Gard and temperature.

RESULTS AND DISCUSSION.

Frost Gard on the INA of water solutions.

There was no interaction between the various classes of water and Frost Gard (Table 5.1). Regardless of the Frost Gard concentration, mean nucleation temperatures of different water types varied from -13.9 to -16.9°C (Table 5.1). There was a highly significant difference between sterile bidistilled water and the other classes of water, suggesting that its impurities were less, and consequently the chances of nucleation were reduced. The addition of ice crystals to the sterile bidistilled water provoked an immediate nucleation in every Frost Gard concentration, at 0.5°C. This suggests that Frost Gard is not a cryoprotectant.

Frost Gard at the 1 % concentration was able to depress the nucleation point of the 4 water classes by about 1°C (Table 5.2). None of the other concentrations had any effect on the nucleation

point of the water classes. These studies suggest that Frost Gard does not act as an antifreeze and cryoprotectant chemical.

Frost Gard on bacterial suspensions.

The mean nucleation temperature of bacterial suspensions was strongly affected by several treatments (Figure 5.1). The control suspension nucleated at -2.3°C. When the cells where killed, different responses were observed depending on the treatment. The chloroform fumes killed the cells efficiently, but the INA-z protein, which is responsible for the INA phenotype, remained active, and nucleated at a mean temperature of -3.5°C. But autoclaving the suspensions which killed, also denatured the proteins and eliminated the INA. This treatment nucleated at -13.6°C. Frost Gard at 0.25, 0.5, and 1 % concentrations acted in an intermediate range, nucleating at -4.5, -6.9, -5.2°C, respectively. The 0.5 % level was more efficient, not only in terms of the mean nucleation point , but also in the cooler temperature range at which nucleation occurred. Therefore it is probable that competition for binding sites could become a factor affecting Frost Gard performance.

Table 5.3 shows the bacterial populations estimated after applying the treatments and after the freezing test. The freezing stress reduced the amount of viable cells in the control by 34 %, from $80x10^8$ to $53x10^8$CFU/ml. Chloroform fumes, autoclaving and the 3 Frost Gard concentrations killed the cells.

Frost Gard on an inorganic ice nucleator.

The results show a highly significant interaction between Frost Gard and Thermica (Table 5.4) its response surface is illustrated in

Figure 5.2. Sterile bidistilled water supercooled to -15°C in the absence of both, Frost Gard and Thermica. When the former was added, little changes occurred in the nucleation point and no real antifreeze action was observed. On the other hand, Thermica at every concentration actively nucleated at temperatures in the range of -2.3 to -3.5°C. However, when both products interacted, a dramatic change in the nucleation activity was observed. Nucleation temperatures decreased markedly when Frost Gard was added at any level, reaching a plateau ranging from -6.7 to -11.1°C. Again, it is apparent that Frost Gard at 0.5 % was the more effective concentration. The results suggest that Frost Gard was not acting as an osmoticum in reducing the INA of Thermica; but rather binding to the active site of nucleation. The competition of Frost Gard components to bind with the active sites explains why either under biological or non-biological conditions the mid concentration of 0.5 % offered better protection (Gusta, personal communication).

Frost Gard on grape leaf tissue.

The conditions obtained inside the greenhouse resembled those of spring, with 25°C and 80 % RH at daytime, and 15°C with 100 % RH during the night. The amount of inoculum recovered before the freezing test expressed as total fluorescent Pseudomonads averaged 584 and 20 CFU/gram fresh weight for inoculated and non inoculated plants, respectively.

The results from the freezing test indicate an interaction, between inoculation, Frost Gard concentration and temperature (Table 5.5) shows the individual and overall mean damage of the three factors involved and their respective levels. It is expressed

as percentage of disk area damaged.

In general, bacterial inoculation slightly increased the damage from 61 to 64 %. Some factors can be involved like a limited nucleation activity of the established bacteria and/or low population levels. Also, there may be inferred the presence of other kind of nucleators limiting the supercooling capacity of the tissue (3).

Temperature levels produced significant changes in leaf damage. Neither +4 nor -4°C were different in the damage level, damage did not occur in the controls. While, at -4°C the damage was nearly total. However, an intermediate level in damage was observed at -2 to -3°C with a significant increase in susceptibility occurring at -3°C. A damage of 59 % was attained with Frost Gard at 0.25 % at all temperatures. Although this represented the best protection, it has limited use for practical reasons, since there was 66 % damage in the control. These support the conclusion that Frost Gard at 0.25 % can reduce the leaf damage at -2°C. However, it was ineffective at lower temperatures regardless of INA-bacteria level.

Table 5.1. Mean ice nucleation temperature of different types of water containing Frost Gard at 0, .1, .2, .5, and 1 %.

Type of water	Nucleation temperature (^{o}C)
Tap	-13.96 b
Sterile tap	-14.00 b
Bidistilled	-14.42 b
Sterile bidistilled	-16.98 c
Sterile bidistilled + ice	-00.50 a

Mean separation at 0.01 level

Table 5.2. Effect of five concentrations of Frost Gard on the freezing temperature of tap, sterile tap, bidistilled and sterile bidistilled water.

Frost Gard (%)	Temperature (^{o}C)
0	-11.64 a
0.125	-12.06 ab
0.250	-11.84 a
0.500	-11.76 a
1.000	-12.56 b

Mean separation at 0.05 level

Table 5.3. Effect of heat, chloroform, and Frost Gard on viable cells of bacterial suspensions before and after freezing. *

Treatment	Bacterial population (CFU/ml) Before freezing	After freezing
Control	$80x10^8$	$53x10^8$
Chloroform fumes	0	0
Autoclaving	0	0
Frost Gard 0.2%	0	0
Frost Gard 0.5%	0	0
Frost Gard 1.0%	0	0

* Determined by dilution plate assay. Zero values were obtained by direct plating of the suspension.

Table 5.4. Effect of Frost Gard and Thermica at several concentrations on the ice nucleation temperature (^{o}C) of bidistilled water.

Thermica	Frost Gard (%)			
(mg/ml)	0	0.25	0.50	1.0
0	-15.00 g	-15.50 g	-13.80 g	-15.20 g
0.3	- 2.75 a	-10.40 ef	-11.10 f	- 9.70 def
0.6	- 3.95 a	-10.10 def	- 9.50 cdef	- 9.20 cdef
1.2	- 2.35 a	- 9.10 cde	-10.10 def	- 7.60 bc
2.4	- 2.45 a	- 6.70 b	- 8.50 bcde	- 8.40 bcd

Mean separation by Student's t at 0.01 level

Table 5.5. Percentage of disk area damaged as affected by the interaction of inoculum, low temperature, and Frost Gard concentration.

Mean disk area damaged (%)											
	Inoculated					Non-inoculated					Overall Frost Gard mean
Temp.	+4	-2	-3	-4	mean	+4	-2	-3	-4	mean	
Frost Gard (%)											
0	0	66	98	100	66	0	72	95	100	67	66.4
0.25	0	45	95	100	60	0	37	97	100	58	59.2
0.50	0	58	100	100	64	0	40	100	97	59	61.9
1.00	0	74	96	100	67	0	45	99	98	60	63.9
Means	0	61	97	100		0	48	98	99		
Inoc. avg.					64.5					61.1	
Temp.		+4		-2		-3		-4			
avg.		0		54.6		97.5		99.2			

$LSD_{(0.01)} = 1.00$

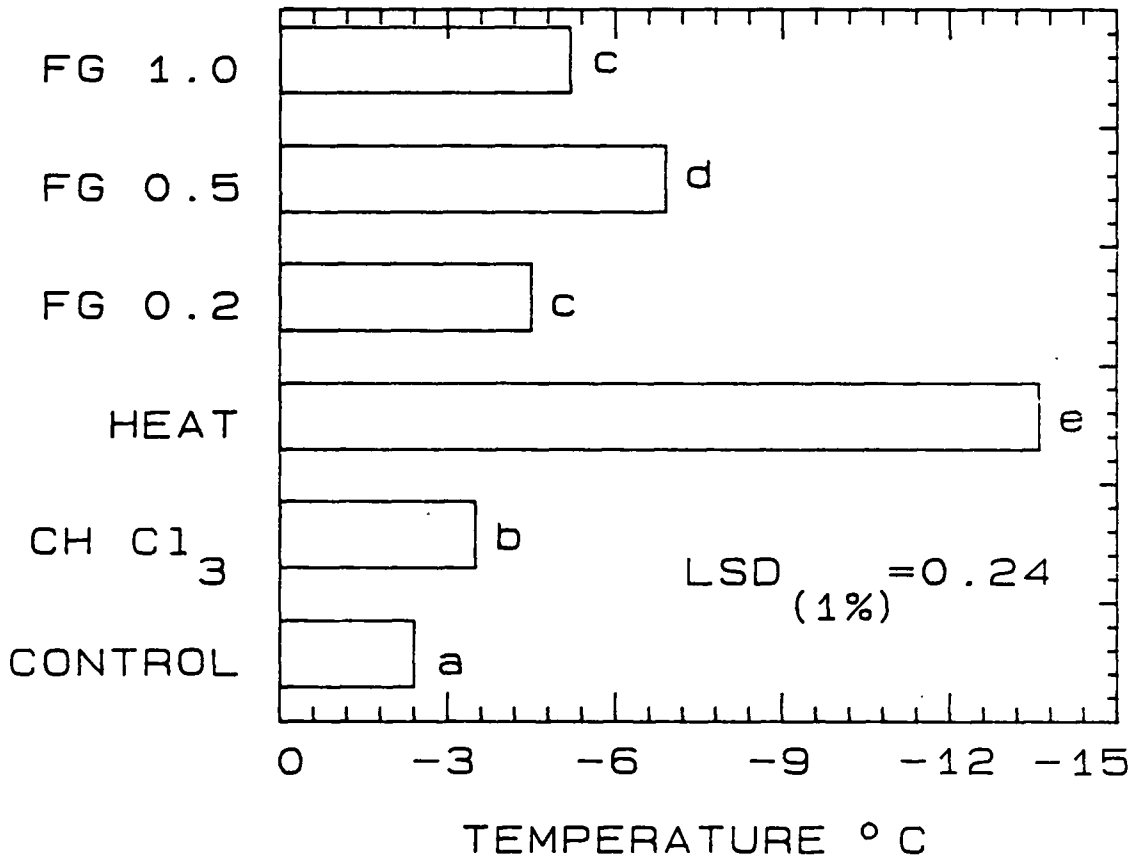


Figure 5.1. Effect of Frost Gard (FG), heat and chloroform ($CH\ Cl_3$) on the ice nucleation activity of Pseudomonas syringae pv syringae strain B-15.

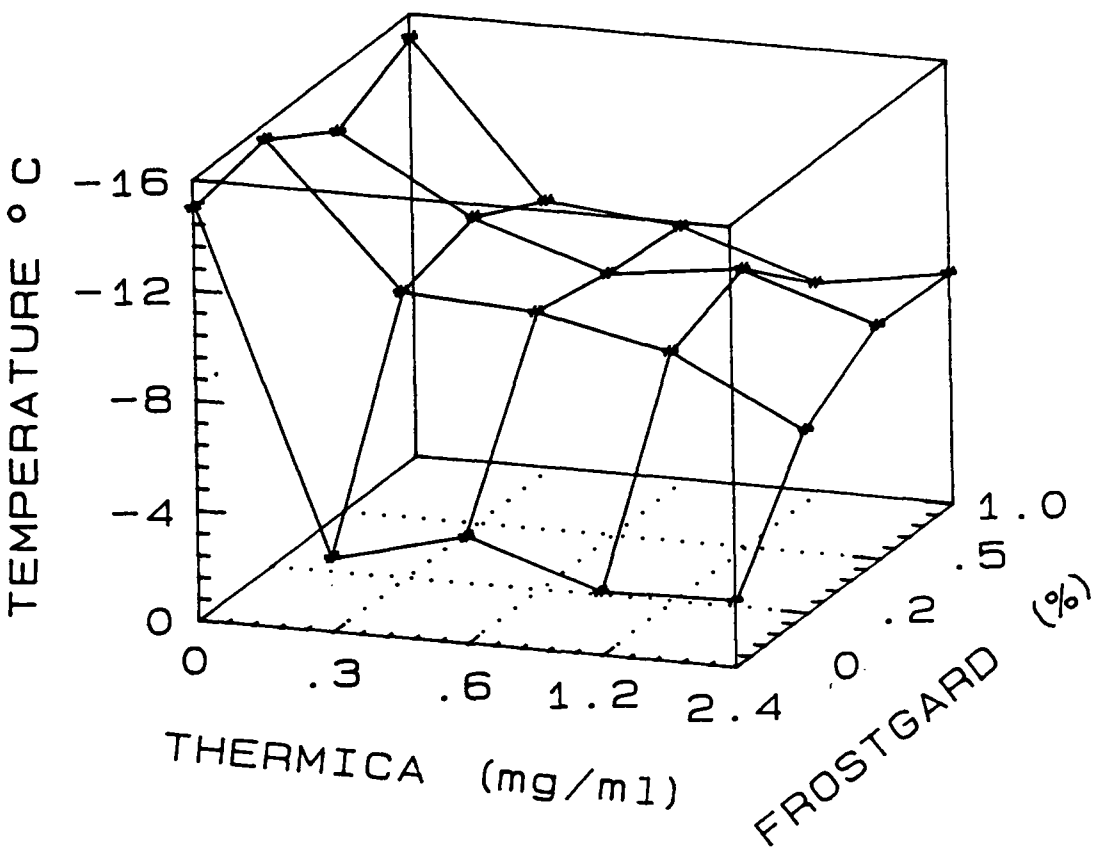


Figure 5.2. Interaction of several concentrations of Frost Gard and Thermica suspensions on the ice nucleation temperature of bidistilled water.

REFERENCES

1. Ahmmedullah, M., A. Kawakami, C.R. Sandidge, and R.L. Wample. 1986. Effect of PP333 on the Vegetative Growth, Yield, Quality, and Winter Hardiness of Buds of Concord Grapes. Hortscience 21(2):273-274.

2. Ashworth, E.N. 1986. Freezing Injury in Horticultural Crops. Research Opportunities. Hortscience 21(6):1325-1328.

3. Ashworth, E.N., and G.A. Davis. 1984. Ice Nucleation within Peach Trees. J. Amer. Soc. Hort. Sci. 109(2):198-201.

4. Cody, Y.S., D.C. Cross, E.L. Proebsting, and R.A. Spotts. 1986. Suppression of Ice Nucleation-active Pseudomonas syringae by Antagonistic Bacteria in Fruit Tree Orchards and Evaluation of Frost Control. Phytopathology 77:1036-1044.

5. Dalzel, J., and D.K. Lawrence. 1984. Biochemical and Biological Effects of Kaurene Oxidase Inhibitors, such as Paclobutrazol. British Plant Growth Regulators Group. Monograph II. pp 43-57.

6. Gross, D.C., E.L. Proebsting, and P.K. Andrews. 1984. The Effects of Ice Nucleation-active Bacteria on Temperatures of Ice Nucleation and Injury of Prunus Flower Buds at various Stages of Development. J. Amer. Soc. Hort. Sci. 109(3):375-380.

7. Hummel, R.L. 1987. The Role of Supercooling and Ice-nucleation Active Bacteria in Freeze Protection Washington State Nurserymen's Association. January Issue. pp 4-5.

8. Lindow, S.E. 1983. Methods of Preventing Frost Injury Caused by Epiphytic Ice Nucleation-active Bacteria. Plant Disease. Vol 67 (3):327-333.

9. Matta, F.B., S. Little, and R.H. Mullenax. 1987. Effects of Two Chemicals on Peach Fruit Survival Following Late Spring Frosts. Miss. Ag. and For. Exp. Sta. Research Report. Vol.12 No. 18.

10. Proebsting, E.L., and H.H. Mills. 1985. Cold Resistance in Peach, Apricot, and Cherry as Influenced by Soil Applied Paclobutrazol. Hortscience 20 (1):88-90.

Chapter 6

VASCULAR TRANSLOCATION OF *Pseudomonas syringae* pv.*syringae* IN *Vitis vinifera*. L.

ABSTRACT

An antibiotic resistance strain of *Pseudomonas syringae* pv. *syringae* was translocated through the vascular tissue of grapevine cuttings. Cuttings were placed in suspensions of ca. 10^2, 10^4, and 10^6 cells/ml for 24, 48, and 48 hr at room temperature. Sections of the cutting (4 cm) were removed at 9, 20, and 30 cm above the proximal end, cut in small pieces, and suspended in sterile water. The suspension was diluted serially and aliquots plated onto Kings B medium containing rifampicin and nalidixic acid. The populations of bacteria recovered were inversely proportional to the length of the cutting section regardless of the inoculation period. Cuttings were also placed in a dye solution to monitor its vascular movement through the xylem. The dye solution moved faster through the xylem than the bacterial suspensions.

INTRODUCTION

The biogenic origin of a large proportion of ice nuclei has been demonstrated; they are more abundant on plant litter of the temperate zone (12). Certain epiphytic bacteria act as ice-nuclei and are reported to incite frost injury to tender annual plants (10). Ice nucleation-active (INA) bacteria raise the freezing point, overriding the supercooling capacity of plants (1). Different methods to control the epiphytic population of this microorganism have been reported (8). However, attempts to achieve a good level of frost control by suppression of the INA-bacteria on leaf surfaces of deciduous fruit species have failed (3, 6) . The accepted conclusion suggests the presence of an internal ice nucleator of non-bacterial origin which nucleates ice at the same temperature as the bacteria do (2). But a basic question seems to remain unanswered. Are the bacteria located in or on the plant ? (9). Evidence from several sources shows that different bacteria are able to move through the vascular tissue of Vitis and Rubus (4, 7, 11). It has been reported also that INA-bacteria were found in the root xylem of alfalfa, increasing the freezing damage (5). The purpose of this work was to test the hypothesis that INA-bacteria can be translocated through the vascular tissue of grapevines.

MATERIALS AND METHODS

The plant materials used were 4 internode cuttings approximately 40 cm long of grapevine cv. Pinot Noir. The samples were either obtained directly from the field or collected

previously and stored in a cold room at 0°C.

Bacterial Strain.

Pss 2-3 RNH was used as inoculum. It is a spontaneous mutant of P. syringae pv. syringae strain Pss2-3 resistant to the antibiotics rifampicin and nalidixic Acid.

Inoculation technique.

Bacterial suspensions were prepared containing ca. 10^6, 10^4 and 10^2 colony forming units (CFU)/ml. The cuttings were prepared for inoculation by first immersing them in tap water and then removing 1 cm of the proximal end. The basal 5 cm of each cutting was placed in a beaker containing 100 ml of the bacterial suspension. The beaker and cuttings were kept in a 20°C greenhouse for either 24, 36 and 48 hours.

Isolation Technique.

Tissue and sap extraction methods were used to isolate the bacteria from the inoculated tissues. The basal 10 cm sections were eliminated to avoid possible surface contamination of the cuttings. This left 3 internodes per cutting from which the basal, middle and apical internodes were cut into 3-sections of about 10 cm long and prepared as follows.

Tissue Extraction Method.

An internode section of approximately 5 cm long was cut from each internode section and the bark was peeled off. Later a sample of the central part of each internode was cut out, sectioned, weighed and placed in a test tube. Ten ml of sterile water were added and the tube was shaken for 10 seconds. The samples were allowed to stand for 1 hour and shaken again. Serial dilutions were

prepared, and 0.1 ml was spread and cultured on King's B medium containing 0.1g/l of chlorothanilin (Bravo), 100 ppm of rifampicin and 150 ppm of nalidixic acid. The inoculated plates were allowed to incubate for at least 48 hours at 27°C. Before counting the colony forming units (CFU).

Sap extraction method.

A pressure chamber (4) was used to extract the sap from the internode sections prepared as described above. The range of pressure required had been determined previously, and rubber stoppers of different diameters were prepared for different cutting diameter. An aluminum foil was arranged in the bottom of the chamber to hold a culture tube of a sterile solution of azosulfamide (5%). The internode sections and the rubber stoppers were dipped in 70% ethanol and flamed. Small sections of both ends of the cuttings were eliminated after they were in the stopper and placed in the lid of the chamber. The bottom of the cuttings was inserted into the azosulfamide solution and the lid was clamped down. Pressure was applied until the dye solution appeared at the apical end of the cuttings, 0.1 ml was collected with a micropipete and it was diluted in 0.9 ml of sterile water. Later 0.1 ml was spread using the same procedure described for the other method.

Experiment 1.

A dye solution was used to follow the movement of the cells through the xylem. The bacterial population was determined by the tissue extraction method. The cuttings were inoculated for 24 hr with a bacterial suspension of 10^6CFU/ml. Cutting sections from the basal, middle, and apical internodes were assigned as treatments.

The experimental design used was a randomized block. Blocking was done according to 3 different conditions; i) cuttings inoculated with the bacterial suspension, ii) cuttings as in i) but including 100 ml of 5 % azosulfamide (dye), as an indicator of the capillary movement of liquids, and iii) cuttings in bidistilled water only. The variables evaluated were internode length, sample fresh weight and number of CFU per gram of fresh weight.

Experiment 2.

Three suspension concentrations of inoculum were used to determine whether the bacteria were translocated through the xylem. The experimental design was a randomized block with 5 replications. An interaction was evaluated through a factorial arrangement between inoculation periods at 3 levels; 24, 36, and 48 hr and suspension concentration at 3 levels 10^6, 10^4, and 10^2. The variable evaluated was the CFU/ml/g.

Experiment 3.

The sap extraction method was used to confirm the results from the former experiment. The data from this method were analyzed using the same design and conditions mentioned above.

RESULTS AND DISCUSSION

Experiment 1.

Results presented in Table 6.1 suggest that the difference between the mean length of the internode section from the basal cut was highly significant to test the translocation of the cells. The mean length was 10 cm. The same table shows no difference between the mean sample weight of the 3 cutting sections, which demonstrate

homogeneity of the samples.

The amount of inoculum recovered is presented in Table 6.2. The basal section from cuttings that had been immersed for 24 hr in the bacterial suspensions showed a similar amount of inoculum, regardless of the presence of dye solution. The amount of bacteria recovered from the cuttings was reduced drastically as the length of the section increased. Many fewer bacteria were isolated from the middle segment of the cuttings, and no bacteria was detected in the apical portions. The bacteria were completely absent in the cuttings immersed in distilled water. It was apparent that the dyed liquid moved faster than the bacteria. For example, the dye was seen at the top of the cutting in 24 hr, but no bacteria were isolated from the apical sections. It should be pointed out that in both cases the dye was observed at the top of the cuttings, indicating that capillary movement transports liquid throughout the xylem at a faster rate than bacteria, while the amount of bacteria inside start to increase from the base to the apice.

Experiment 2.

A significant interaction demonstrated that the amount of inoculum recovered was dependent on the height of the section above the base of the cutting and the inoculation period. Blocking by suspension concentration was highly efficient, with total amounts of recovery directly proportional to the concentration used. The results are summarized in Table 6.3, the amount of inoculum recovered at different combinations of time and section height shows a clear gradient which was inversely proportional to the height section sampled. Only a few colonies were found in the

apical section. Since, the size of the populations recovered at each section level did not show a constant pattern, it is apparent that differentials between intake, storing, and survival rates may be involved.

Experiment 3.

The results determined a positive interaction of sample height, and time of exposure to the bacteria, which was significant at the 5% level with a standard deviation of 22,510 CFU/cm/ml. Treatment means are showed in Table 6.4. Basal sections had higher amounts of inoculum at any given time and a clear gradient was observed in the medium and apical sections. These results confirm the usefulness of this method, reported on the translocation of Agrobacterium (4, 11).

The two methods used to isolate the bacteria from the inoculated tissues showed the same tendency, although the amount of inoculum recovered varied. It should be pointed out that a strong variability between plant material was observed for both techniques which probably influenced the amount of inoculum recovered. The population of bacteria recovered by sap extraction was smaller than by the other method. However, this method was more efficient to detect the populations in the apical sections. It should be noted also that this technique is more risky in terms of possible sample contamination. The concentration of bacteria in the basal section was initially higher, but as the populations start to build up in the upper sections, these numbers start to decrease maybe because of migration. The concentration of 10^6 CFU/ml may produce a clumping of bacterial cells, avoiding a more efficient

translocation. While 10^4CFU/ml showed a more stable amount of inoculum. In several cases a peak at 36 hr was observed followed by a decline maybe by cell death.

Table 6.1. Internode mean length of Pinot Noir grapevine cuttings and mean weight of fresh tissue sampled for xylem Pss2-3RNH strain

	Cutting Section			
	Basal	Middle	Apical	$LSD_{.05}$
Mean height (cm)	9.3 a	20.0 b	30.5 c	7.01
Mean weight (g)	2.6 a	2.3 a	2.1 c	1.05

Table 6.2. Amount of Pss2-3RNH recovered from Pinot Noir cuttings by tissue extraction after 24 hours of basal inoculation

	Population (x 10^6 CFU/gfw/ml)		
Blocks	Cutting section		
	Basal	Middle	Apical
Susp.(3x10^6CFU/ml)	87.2	0	0
Susp. + Dye Sol.	101.8	4	0
Distilled water	0	0	0

Table 6.3. Amount of Pss2-3RNH recovered (CFU/g/ml) by tissue extraction from 3 different heights of Pinot Noir grape cuttings and 3 inoculation periods

Population recovered (CFU/gfw/ml)			
	Cutting Section		
Time	Basal	Medium	Apical
24 hr	3 455,750	335,600	0
36 hr	6 174,700	165,300	0
48 hr	4 986,050	171,880	1,020
STD. ERROR	58,794 CFU/g/ml		

Table 6.4. Amount of Pss2-3RNH recovered (CFU/cm/ml) by sap extraction at 3 different heights of Pinot Noir grape cuttings and 3 inoculation periods

Population recovered (CFU/g/ml)			
	Cutting Section		
Time	Basal	Medium	Apical
24 hr	205,600	600	0
36 hr	164,850	160,520	210
48 hr	22,840	20,380	100
$LSD_{0.05}$	6,750 CFU/g/ml.		

REFERENCES

1. Anderson, J.A., D.W. Buchanan, and P.L. Ingam. 1981. The Role of Pseudomonas syringae pv. syringae, an Ice Nucleation Active Bacteria, in Frost Damage to Tender Annual Plants. Proc. Fla. State Hort. Soc. 94:72-74.

2. Ashworth, E.N., J.A. Anderson, G.A. Davis. 1985. Properties of Ice Nuclei Associated with Peach Trees. J. Amer. Soc. Hort. Sci. 110(2):287-291.

3. Cody, Y.S., D.C. Gross, E.L. Proebsting, and R.A. Spotts. 1986. Suppression of Ice-Nucleation Active Pseudomonas syringae by Antagonistic Bacteria in Fruit Tree and Evaluation of Frost Control. Phytopathology 77:1036-1044.

4. Goodman, R.N., D. Butrov, and F. Tarbah. 1987. The occurrence of Agrobacterium tumefaciens in Grapevine Propagating Material and a Simplified Indexing System. Am. J. Enol. Vitic., Vol. 38 (3):189-193.

5. Gagne, S., C. Richard, and H. Antoun. 1987. Isolation of Ice Nucleation Active Bacteria from Xylem of Alfalfa Roots and their Effect on Cold Resistance. Abstract. Third International Conference on Biological Ice Nucleation. Newport Or.

6. Hummel, R.L. 1987. The Role of Supercooling and Ice-Nucleation Active Bacteria in Freeze Protection. Washington State Nurserymen's Association Monthly Issue. January. pp. 4-5.

7. Kerr, A., and P.G. Brisbane. 1983. Agrobacterium. In: Plant Bacterial Diseases. P.C. Fahy and J. Persley (ed). Academic Press Australia. P. 28.

8. Lindow, S.E. 1983. Methods of Preventing Frost Injury Caused by Epiphytic Ice Nucleating Active Bacteria. Plant Disease Vol. 67 (3): 327-33.

9. Lindow, S.E. 1983. Role of Bacterial Ice-Nucleation in Frost Injury to Tender Plants. Ann. Rev. Phytopathology 21:363-84.

10. Lindow, S.E., et al. 1978. The Role of Bacterial Ice Nuclei in Frost Injury to Sensitive Plants. In: Plant Cold Hardiness and Freezing Stress. U.S.A. Academic Press. pp.249-63.

11. Tarbah, F., and R.N. Goodman. 1987. Systemic Spread of Agrobacterium tumefaciens biovar 3 in the Vascular System of Grapes. Phytopathology 77:915-920.

12. Schnell, R.C., and G. Vali. 1975. Biogenic Ice Nuclei : Part 1. Terrestrial and Marine Sources. J. Atmospheric Sciences. 33 (9):1551-61.

BIBLIOGRAPHY

1. Ahmmedullah, M., and A. Kawakami. 1986. Evaluation of Laboratory Tests for Determining the Lethal Temperature of Vitis labruscana B. Concord Roots Exposed to Subzero Temperatures. Vitis 25, 142-150.

2. Ahmmedullah, M., A. Kawakami, C.R. Sandidge, and R.L. Wample. 1986. Effect of Paclobutrazol on the Vegetative Growth, Yield Quality, and Winter Hardiness of Buds of Concord Grape. Hortscience 21(2):273-274.

3. Andersen, P.C., and M.B. Odneal. 1985. Effect of Soil Profile Modification and Irrigation on Senescence and Bud Hardiness of Young Grapes. Hortscience 20(4):749-751.

4. Anderson, J. A. and E. N. Ashworth. 1985. Ice Nucleation in Tomato Plants. J. Amer. Soc. Hort. Sci. 110(2):291-296.

5. Anderson, J.A., D.W. Buchanan and P.L. Ingram. 1981. The Role of Pseudomonas syringae, an Ice Nucleation Active Bacteria, in Frost Damage of Tender Annual Plants. Proc. Fla. State Hort. Soc. 94: 72-4.

6. Anderson, J.A., D.W. Buchanan, R.E. Stall, and C.B. Hall. 1982. Frost Injury of Tender Plants Increased by Pseudomonas syringae Van Hall. J. Amer. Soc. Hort. Sci. 107(1):123-125.

7. Andrews, P.K., E.D. Proebsting, and D.C. Gross. 1986. Ice Nucleation and Supercooling in Freeze - sensitive Peach and Sweet Cherry Tissues. J. Amer. Soc. Hort. Sci. 111(2):232-236.

8. Andrews, P.K., C.R. Sandidge, and T.K. Toyama. 1984. Deep Supercooling of Dormant and Deacclimating Vitis Buds. Research Note. Am. J. Enol. Vitic., Vol.35. No.3, pp 175-177.

9. Ashworth, E. N. 1986. Freezing Injury in Horticultural Crops - Research Opportunities. Hortscience. 21(6):1325-1328.

10. Ashworth, E. N., J. A. Anderson and G. A. Davis. 1985. Properties of Ice Nuclei Associated with Peach Trees. J. Amer. Soc. Hort. Sci. 110(2):287-291.

11. Ashworth, E. N., J. A. Anderson, G. A. Davis and G. Lightner. 1985. Ice Formation in Prunus persica under Field Conditions. J. Amer. Soc. Hort. Sci. 110(3):322-324.

12. Ashworth, E.N. and G.A. Davis. 1986. Ice Formation in Woody Plants under Field Conditions. Hortscience 21(5): 1233-1234.

13. Ashworth, E.N., and G.A. Davis. 1984. Ice Nucleation Within Peach Trees. J. Amer. Soc. Hort. Sci. 109(2):198 -201.

14. Ashworth, E. N., D. J. Rowse and L. A. Billmyer. 1983. The Freezing of Water in Woody Tissues of Apricot an Peach and the Relationship to Freezing Injury. J. Amer. Soc. Hort. Sci. 108(2):299-303.

15. Baca, S., M. Canfield, and L.W. Moore. 1987. Variability in Ice Nucleation Strains of Pseudomonas syringae isolated from Diseased Woody Plants in Pacific Northwest Nurseries. Plant Disease 71(5):412-415.

16. Baca, S., and L.W. Moore. 1987. Variations in Pseudomonas syringae Isolated from Grass Species Occurring in Woody Plant Nurseries in the PNW. Plant Disease Vol 71(8):724-727.

17. Balerdi, C. F. 1972. Weed Control in Young Vineyards. Amer. J. Enol. Vitic. 23(2):58-60.

18. Ballard, J.K., and E.D. Proebsting. 1978. Frost and Frost Control in Washington Orchards. W.S.U. Ext. Bull. 634.

19. Becker,N. 1984. Site Selection for Viticulture in Cooler Climates using Local Climatic Information. International Symposium on Cool Climate Viticulture and Enology. Eugene, Or. Proceedings. p 27.

20. Becwar,M., and M.J. Burke. 1982. Winter Hardiness Limitations and Physiography of Woody Timberline Flora. In: Plant Cold Hardiness and Freezing Stress. Academic Press Inc. pp.307-323.

21. Bittenbender H.C., and G.S. Howell. 1975. Interaction of Temperature and Moisture Content on Spring Deacclimation of Buds of Highbush Blueberry. Can. J. Plant Sci. 55:447-455.

22. Bittenbender, H.C., and G.S. Howell. 1974. Adaptation of the Spearman-Karber Method for Estimating the T50 of Cold Stressed Flower Buds. J. Amer. Soc. Hort. Sci. 99(2):180-190.

23. Burke, M.J., M.F. George, and R. Bryant. 1986. Water in Plant Tissues and Frost Hardiness. Handout from Hort 503.

24. Burke, M.J., L.V. Gusta, H.A. Quamme, C.J. Weiser, and P.H. Li. 1976. Freezing and Injury in Plants. Ann. Rev. Plant Physiol. 27: 507-528.

25. Burr, T.J., B.H. Katz, and A.L. Bishop. 1987. Ecology of Agrobacterium Biovars in Vineyard and Nonvineyard Soils and Detection in Association with Grape Roots from Nursery Storages. Plant Disease 71:617-670.

26. Byrne, D.H. 1986. Mechanisms of Spring Freeze Injury Avoidance in Peach. Hortscience 21(5):1235-1236.

27. Chen, P. and P. Li. 1976. Induction of Frost Hardiness in Stem Cortical Tissue of Cornus stolonifera Michx. by Water Stress. Plant Physiol 59:240-243.

28. Chen,P., P.H. Li, and C.J. Weiser. 1975. Induction of Frost Hardiness In Red -Osier Dogwood Stems by Water Stress. Hortscience 10(4):372-374.

29. Cody, Y.S., D.C. Gross, E.L. Proebsting, and R. Spotts. 1986. Suppression of Ice-Nucleation Active Pseudomonas syringae by Antagonistic Bacteria in Fruit Tree Orchards and Evaluation of Frost Control. Phytopathology 77:1036-1044.

30. Dalzel, J., and D. Lawrence. 1984. Biochemical and Biological Effects of Kaurene Oxidase Inhibitors such as Paclobutrazol. British Plant Regulators Group. Monograph II. pp. 43-57.

31. Davies, F.S., L.W. Rippetoe, and L.K. Jackson. 1987. Intermittent Microsprinkler Irrigation and Tree Wraps for Freeze Protection of Young Hamlin Orange Trees. Hortscience 22(2):206-208.

32. Fahy, P.C., and A. Lloyd. 1983. The Fluorescent Pseudomonads. In: Plant Bacterial Diseases. P.C. Fahy, and G.J. Persley, (ed.) Academic Press Australia. pp. 141 - 143.

33. Flaherty, D.L., F.L. Jensen, A.N. Kasimatis, H. Kido, and W. Moller. 1982. Grape Pest Management. University of California. Publication 1981. pp 12-15.

34. Fuchigami, L.H., C.J. Weiser, K. Kobayachi, R. Timmis, and L.V. Gusta. 1982. A Degree Growth Stage Model and Cold Acclimation in Temperate Woody Plants. pp 93-116. In P. Li et al (ed.) Plant Cold Hardiness and Freezing Stress. Academic Press N.Y.

35. Gagne, S., C. Richard, and H. Antoun. 1987. Isolation of Ice Nucleation-Active Bacteria from Xylem of Alfalfa Roots and their Effect on Cold Resistance. Abstract. Third International Conference on Biological Ice Nucleation. Newport, Or.

36. Galet, P. 1979. A Practical Ampelography. Cornell University Press. New York. pp 82-83.

37. Garren, R. 1980. Soils and Site Selection for Grapes. Oregon Horticultural Society. Proceedings. p 213.

38. George, M.F., M.J. Burke, H.M. Pellet, and A.G. Johnson. 1974. Low Temperature Exotherms and Woody Plant Distribution. Hortscience Vol.9 (6): 519-521.

39. Gianfagna, T.J., and S. Mehlenbacher. 1985. Importance of Heat Requirements for Budbreak and Time of Flowering in Apple. Hortscience 20(5):909-911.

40. Goodman, R., D. Butrov, and F. Tarbah. 1987. The Occurrence of Agrobacterium tumefaciens in Grapevine Propagating Material and a Simplified Indexing System. Am. J. Enol. Vitic., Vol. 38(3): 189-193.

41. Gross, D.C., E.L. Proebsting and P. Andrews. 1984. The Effects of Ice Nucleation-Active Bacteria Temperatures of Ice Nucleation and Freeze Injury of Prunus Flower Buds at Various Stages of Development. J. Amer. Soc. Hort. Sci. 109(3):375-380.

42. Gross, D. C., Y. S. Cody, E. L. Proebsting, G. K. Radamaker and R. Spots. 1983. Distribution, Population Dynamics, and Characteristics of Ice Nucleation-Active Bacteria in Deciduous Fruit Tree Orchards. Applied and Environmental Microbiology. 46(6):1370-1379.

43. Gross, D.C. Y.S. Cody, E.L. Proebsting, G.K. Radamaker, and R. Spots. 1984. Ecotypes and Pathogenicity of Ice Nucleation Active Pseudomonas syringae Isolated from Deciduous Fruit Tree Orchards. Phytopatology Vol 74(2):241-248.

44. Gusta, L.V., N. Tyler, and T.H. Chen. 1983. Deep Supercooling in Woody Taxa Growing North of the -40°C Isotherm. Plant Physiol. 72: 122-128.

45. Howell, G.S., B.G. Stergios, and S.S. Stackhouse. 1978. Interrelation of Productivity and Cold Hardiness of Concord Grapevines. Am. J. Enol. Vitic., Vol 29, No. 3 pp. 187-190.

46. Howell, G.S., and S.S. Stackhouse. 1973. The Effect of Defoliation Time on Acclimation and Dehardening in Tart Cherry (Prunus cerasus L.). J. Am. Soc. Hort. Sci. 98:132-136.

47. Howell, G.S., and C.J. Weiser. 1970. Fluctuations in the Cold Resistance of Apple Twigs during Spring Dehardening. J. Am. Soc. Hort. Sci. 95:190-192.

48. Howell, G.S., and J.A. Wolpert. 1978. Nodes per Cane, Primary Bud Phenology, and Spring Freeze Damage to Concord Grapevines. A Preliminary Note. Am. J. Enol. Vitic., Vol.29(4):229-232.

49. Hummel, R.L. 1987. The Role of Supercooling and Ice Nucleation Active Bacteria in Freeze Protection. Washington State Nurserymen's Association Monthly Issue. January pp. 4-5.

50. Iezzoni, A.F., and R.L. Hamilton. 1985. Differences in Spring Floral Bud Development among Sour Cherry Cultivars. Hortscience 20(5):915-916.

51. Johns, R. 1986. Successful Frost Control in Vineyards. The Australian Grape Grower and Winemaker. Sept. Issue. pp. 14-16.

52. Johnson, D.E., and G.S. Howell. 1981. Factors Influencing Critical Temperatures for Spring Freeze Damage to Developing Primary Shoots on Concord Grapevines. Am. J. Enol. Vitic. 32: 144-149.

53. Johnson, L.K. 1986. Characteristics of Ice Nucleation in Potato Leaves. M.S. Thesis. Oregon State University. Corvallis, Or.

54. Jones, J.B., R.D. Gitaitis, and S.M. McCarter. 1983. Evaluation of Immunofluorescence and Ice Nucleation Activity as Rapid Test for Identifying Foliar Diseases of Tomato Transplants Incitated by Fluorescent Pseudomonads. Plant Disease 67:684 - 687.

55. Jordan, T.D., R.M. Pool, T.J. Zabadal, and J.P. Tomkins. 1980. Cultural Practices for Commercial Vineyards. New York State College of Agriculture and Life Sciences, Cornell University. Miscellaneous Bulletin 111, pp. 22-24.

56. Kerr, A., and P.G. Brisbane. 1983. Agrobacterium. In: Plant Bacterial Diseases. P.C. Fahy and J. Persley (ed). Academic Press Australia. P.28.

57. Klinger, A.E., N.J. Palleroni, and R.E. Pontis. 1976. Isolation of Pseudomonas syringae From Lesions on Vitis vinifera. Phytopathology 86(2):107 -116.

58. Levitt, J. 1980. Responses of Plants to Environmental Stresses. Second Edition. Vol. 1 Chilling, Freezing and High Temperature Stress. Academic Press Inc. N.Y.

59. Lindemann, J., and T.V. Suslow. 1987. Dispersal Survival and Efficacy of INA^- Deletion Mutant Strains of Pseudomonas in a Field Test for Frost Protection of Strawberry. Abstract. Third International Conference on Biological Ice Nucleation. Newport Or.

60. Lindow, S.E. 1983. Methods of Preventing Frost Injury Cause by Epiphytic Ice Nucleation Active Bacteria. Plant Disease Vol.67 (3):327-333.

61. Lindow, S.E. 1983. The Role of Bacterial Ice-Nucleation in Frost Injury to Tender Plants. Ann. Rev. Phytopathology 21:363-84.

62. Lindow, S.E., D.C. Arny, and C.D. Upper. 1978. Distribution of Ice Nucleation-Active Bacteria on Plants in Nature. Applied and Environmental Microbiology. Vol. 36 (6):831-838.

63. Lindow, S.E., D.C. Arny, and C.D. Upper. 1982. Bacterial Ice Nucleation: A Factor in Frost Injury to Plants. Plant Physiol. 70:1084-1089.

64. Lindow, S.E., D.C. Arny, C.D. Upper, and W. Barchet. 1978. The Role of Bacterial Ice Nuclei in Frost Injury to Sensitive Plants. In: Plant Cold Hardiness and Freezing Stress. U.S.A. Academic Press. pp.249-263.

65. Lindow, S.E., S.S. Hirano, W.R. Barchet, D.C. Arny, and C.D. Upper. 1982. Relationship between Ice Nucleation Frequency of Bacteria and Frost Injury. Plant Physiol. 70:1069-1093.

66. Lindow, S.E., N. Panapoulos, G. Andersen, C. Pierce, and G. Lim. 1987. Biological Control of Frost Injury to Potato with Ice⁻ Deletion Mutants Of Pseudomonas syringae Constructed in vitro. Abstract. Third International Conference on Biological Ice Nucleation. Newport Or.

67. Loeffler, G. 1984. 1983-1984 Freeze Damage to Grapes. Washington State Grape Society. Proceedings. pp 21-22.

68. Lombard, P.B., and E.A. Richardson. 1979. Physical Principles Involved in Controlling Phenological Developement. In: Modification of the Aerial Environment of Plants, (B. Barfield and J.F. Gerber, Editors). St. Joseph, Mi. pp 429-440.

69. Lombard, P.L., B.T. Watson, D.A. Heatherbell, and R. Garren. 1986. Production, Maturity, and Winegrape Varieties in Western Oregon. Agricultural Experiment Station. O.S.U. Circular of Information 701.

70. MacKenzie, A.P. 1977. Non-equilibrium Freezing Behavior of Aqueous Systems. Phil. Trans. R. Soc. Lond. B. 278:167-189.

71. Malvick, D. 1987. Survival and Dispersal of Pseudomonas syringae in a Maple Nursery and Pear Orchard. M.S. Thesis. Oregon State University. Corvallis, Oregon. p 74.

72. Matta, F.B., S. Little, and R.H. Mullenax. 1987. Effects of Two Chemicals on Peach Fruit Survival Following Late Spring Frosts. Mississippi Agricultural & Forestry Experiment Station. Research Report. Vol. 12, No. 18.

73. McIntire, G.N., L.A. Lider, and N.L. Ferrere. 1982. The Chronological Classification of Grapevine Phenology. Am. J. Enol. Vitic., Vol. 32, No. 2, pp 80-85.

74. Meiering, A. G., J. H. Paroschy, R. L. Peterson, G. Hostetter and A. Neff. 1980. Mechanical Freezing Injury in Grapevine Trunks. Am. J. Enol. Vitic. 31(1):81-89.

75. Ohkawa, M. 1982. Budbreak and Organic Acid Content in Xylem Exudate in Greenhouse Grown Vitis vinifera L. cv. Muscat of Alexandria. J. Japan. Soc. Hort. Sci. 51(2): 152-158.

76. Ohkawa, M. 1981. Budbreak and Xylem Exudation in Greenhouse Grown Vitis vinifera L. cv. Muscat of Alexandria. J. Japan. Soc. Hort. Sci. 50(1):10-14.

77. Olien, C.R. 1973. Thermodynamic Components of Freezing Stress. J. Theor. Biol. 39:201 - 210.

78. Oregon Wineline. 1987. OSU Report. Oregon Wine Advisory Board. Fall Issue.

79. Palta, J. P., J. Levitt and E. J. Stadelmann. 1978. Plant Viability Assay. Cryobiology. 15:249-255.

80. Parsons, L. 1987. Fight Frost with Microsprinklers. Western Fruit Grower. Vol. 107(2):6 - 7.

81. Perry, K. 1986. Frostpro, a Model of Overhead Irrigation Rates for Frost/Freeze Protection of Apple Orchards. Hortscience 21(4): 1060-1061.

82. Pierquet, P. and C. Stushnoff. 1980. Relationship of Low Temperature Exotherms to Cold Injury in Vitis riparia Michx. Am. J. Enol. Vitic. 31(1):1-6.

83. Preece, T.F., and C.H. Dikinson. 1971. Ecology of Leaf Surface Microorganisms. Academic Press Inc. pp.120 - 121.

84. Proebsting, E. L., M. Ahmmedullah and V. P. Brummund. 1980. Seasonal Changes in Low Temperature Resistance of Grape Buds. Am. J. Enol. Vitic. 31(4):329-336.

85. Proebsting, E.L., P. Andrews, and D. Gross. 1982. Supercooling Young Developing Fruit and Floral Buds in Deciduous Orchards. Hortscience 17(1):67-68.

86. Proebsting, E.L., V. Brummund, and W.J. Clore. 1978. Critical Temperatures for Concord Grapes. Washington State University. Extension Leaflet EM 4330.

87. Proebsting, E.L., and H.H. Mills. 1985. Cold Resistance in Peach, Apricot, and Cherry as Influenced by Soil Applied Paclobutrazol. Hortscience 20(1):88-90.

88. Proebsting, E.L., and H.H. Mills. 1978. Low Temperature Resistance of Developing Flower Buds of 6 Deciduous Fruit Species. J. Amer. Soc. Hort. Sci. 103(2):192-198.

89. Rosenberg, N.J., B.L. Blad, and S. Verma. 1983. Microclimate: The Biological Environment. Second Edition. John Wiley & Sons. New York. pp 368-369.

90. Schnell, R.C., and G. Vali. 1976. Biogenic Ice Nuclei: Part 1. Terrestrial and Marine Sources. J. Atmospheric Sciences. 33 (8):1554 -1564.

91. Seeley S.D., E.J. Seeley, and A. Richardson. 1987. Use of a Phenological Model to Time Acceleration of Pollinizer Bloom. Hortscience 22(1):51-52.

92. Shaad, N.W. 1980. Lab Guide for Identification of Plant Pathogenic Bacteria. The American Phytopathological Soc. St. Paul Mn.

93. Stergios, B.G., and G.S. Howell. 1977. Effect of Site on Cold Acclimation and Deacclimation of Concord Grapevines. Am. J. Enol. Vitic. Vol.28 (1):43-48.

94. Stergios, B.G., and G.S. Howell. 1973. Evaluation of Viability Tests for Cold Stressed Plants. J. Amer. Soc. Hort. Sci. 98(4): 325-33.

95. Stout, D.G. 1979. Plant Plasma Membrane Water Permeability and Slow Freezing Injury. Plant, Cell, and Environment (2):273-275.

96. Stout, D.G., W. Majak, and M. Reaney. 1980. In vivo Detection of Membrane Injury at Freezing Temperature. Plant Physiol. (66) 74-77.

97. Stubbs, J.J., R.E. Feeney, J.C. Lewis, I.C. Feustal, H.D. Lightbody, and J.A. Garibaldi. 1947. Subtilin Production in Submerged Culture. Archives of Biochemistry Vol 14(3):427-435.

98. Stushnoff, C. 1972. Breeding for Cold Hardiness. Hortscience Vol.7 (1):10-13.

99. Sule, S. and E. Seemuller. 1987. The Role of Ice Formation in the Infection of Sour Cherry Leaves by Pseudomonas syringae pv. syringae. Phytopathology 77:173-177.

100. Tarbah, F., and R.N. Goodman. 1987. Systemic Spread of Agrobacterium tumefaciens biovar 3 in the Vascular System of Grapes. Phytopathology 77:915-920.

101. Weiser, C.J. 1970. Cold Resistance and Injury in Woody Plants. Science 169 pp.1296 - 1278.

102. Wilson, W.D. 1985. Response of 'Pinot Noir' Grapevines to a Perennial Ryegrass Cover Crop and Irrigation in the Willamette Valley. MS Thesis. O.S.U. Corvallis, Or. p 2.

103. Winkler, A.J., J.A. Cook, W.M. Kliewer, and L.A. Lider. 1974. General Viticulture. Second Edition U.C. Press, Berkeley Ca. pp 205-233.

104. Wisniewski, M., and E.N. Ashworth. 1986. Seasonal Variation in Deep Supercooling and Dehydrative Resistance. Hortscience 21(3):503-505.

105. Wolf, T. W. and R. M. Pool. 1987. Factors Affecting Exotherm Detection in DTA of Grapevine Dormant Buds. J. Amer. Soc. Hort. Sci. 112(3):520-525.

106. Wolpert, J. A. and G. S. Howell. 1986. Cold Acclimation of Concord Grapevines III. Relationship between Cold Hardiness, Tissue Water Content, and Shoot Maturation. Vitis. 25:151-159.

107. Wolpert, J.A., and G.S. Howell. 1985. Cold Acclimation of Concord Grapevines. II. Natural Acclimation Pattern and Tissue Moisture Decline in Cane and Primary Buds of Bearing Vines. Am. J. Enol. Vitic. 36:189-194.

108. Wolpert, J. A. and G.S. Howell. 1984. Effects of Cane Length and Dormant Season Pruning Date on Cold Hardiness and Water Content of Concord Bud and Cane Tissues. Am. J. Enol. Vitic. 35(4):237-241.

109. Wood, B.W. 1986. Cold Injury Susceptibility of Pecan as Influenced by Cultivar, Carbohydrates, and Crop Load. Hortscience 21(2):825-826.

110. Young, R. and C.J. Hearn. 1972. Screening Citrus Hybrids for Cold Hardiness. Hortscience, Vol. 7(1):14-18.