

ICE FORMATION AND THE AFFECTS OF COLD ACCLIMATION ON COLD
HARDINESS IN A SUBTROPICAL FRUIT SPECIES

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Philip Andrew Carter

Certificate of Approval:

Robert C. Ebel, Co-Chair
Associate Professor
Horticulture

Douglas A. Findley, Co-Chair
Assistant Professor
Horticulture

Jeff L. Sibley
Associate Professor
Horticulture

David G. Himelrick
Professor
Horticulture
Louisiana State University

George T. Flowers
Interim Dean
Graduate School

ICE FORMATION AND THE AFFECTS OF COLD ACCLIMATION ON COLD
HARDINESS IN A SUBTROPICAL FRUIT SPECIES

Philip Andrew Carter

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Philip Andrew Carter

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Signature of Author

Date of Graduation

VITA

Philip Andrew Carter, son of Douglas Ray and Kerry Magouirk Carter, was born on August 24, 1978, in Ohatchee, Alabama. He graduated as Salutatorian from Ohatchee High School in 1996. He received a Bachelor of Science in Agriculture Education in 2000 from Auburn University. He immediately entered graduate school in Horticulture at Auburn University. He worked briefly for the Randolph-Roanoke Career Technical Center, where he conducted Forestry and Horticulture classes. He married April Camile Dempsey on September 22, 2007, and is currently an organic vegetable grower.

THESIS ABSTRACT

ICE FORMATION AND THE AFFECTS OF COLD ACCLIMATION ON COLD
HARDINESS IN A SUBTROPICAL FRUIT SPECIES

Philip Andrew Carter

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Fruit production in the southeastern United States is highly vulnerable to freezes. Understanding the mechanism of damage in various fruit crops leads to methods for mitigation of damage through direct protection, cultural practices, and genetic modification to enhance cold hardiness. The current study was conducted to assess the pattern of ice formation in stems and/or leaf tissue of Satsuma mandarins (*Citrus unshiu* (Marc.) 'Owari') at various stages of acclimation. Plants, were subjected to subfreezing temperatures. Ice formation was measured in stems using differential thermal analysis. The first exotherm was bulk xylem water freezing, with secondary exotherms, when present, associated with tissue death. The temperature differential of the first exotherm varied with air temperature and acclimation treatment, indicating a differential rate and

amount of ice formation. Watersoaking and electrolyte leakage of leaves usually corresponded with the peak of the first stem exotherm, with the rate of electrolyte leakage being a function of acclimation and temperature treatment. For some treatments, leaf electrolyte leakage leveled off at 50%, which was attributed to a differential response of leaf tissues to treatments. Leaf death occurred when electrolyte leakage was greater than 50%.

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INDRODUCTION AND LITERATURE REVIEW

Introduction

Fruit production in the southeastern United States is highly vulnerable to freezes. Understanding the mechanism of damage in various fruit crops increases our comprehension of how to mitigate damage through direct protection, cultural practices, and genetic modification to enhance cold hardiness. The study presented here was conducted to assess the pattern of ice formation in stems and/or leaf tissue of Satsuma mandarins (*Citrus unshiu* (Marc.) 'Owari') at various stages of acclimation. Plants were subjected to subfreezing temperatures, with ice formation being measured using differential thermal analysis in stems, and by electrolyte leakage in leaves.

Citrus

Commercial citrus crops are cold tender evergreens (Webber et al., 1967) that are damaged by temperatures between -2.2°C to -13°C (Yelenosky, 1985; Yelenosky et al., 1981). In many parts of the world such as the southeastern U.S., citrus is grown in areas that are highly vulnerable to damaging freezes (Attaway, 1997). Damage occurs in citrus after ice forms (Yelenosky, 1985). Most studies conclude that ice forms exclusively in the apoplast because symplastic ice formation would be instantly lethal (Meryman, 1956;

1966; Yelenosky, 1985, 1996), and there is considerable evidence that citrus plants can tolerate some intra-plant ice (Young and Peynado, 1967). Citrus plants dehydrate during acclimation to cold temperatures (Yelenosky, 1982; Young and Peynado, 1965), so that there is less probability for expanding apoplastic ice crystals to penetrate cells. Death of cold-hardened citrus is likely due to dehydration of the symplast. The mechanism of death for unhardened citrus plants, which are typically fully hydrated, is less clear. In this instance, expanding ice crystals may directly penetrate and kill cells (Young and Mann, 1974), which is more likely to occur for tightly packed cells such as palisade parenchyma of leaves, rather than loosely packed cells such as spongy mesophyll (Anderson et al., 1983). Tissues with loosely packed cells probably die due to symplastic dehydration.

The first water to freeze in plants is bulk water in xylem (Ketchie and Kammereck, 1987), and this is probably true for citrus considering the rapid pace at which ice propagates along stems (Yelenosky, 1975, 1991). As temperatures continue to decrease the water potential gradient between ice and symplastic water intensifies, further dehydrating the symplast. Cells would first lose turgor, but the next step that occurs in citrus is unclear. Some studies indicate that additional water is held by negative pressure potential caused by resistance of the cell wall to inward bending (Anderson et al., 1983). Other studies, however, indicate that osmotically active substances accumulate (Yelenosky, 1978, 1979, 1982) and lower the osmotic potential of cells (Cuilan et al., 2000) thus reducing the probability of plasmolysis and subsequent damage (Young and Mann, 1974). Regardless of the mechanism by which cells retain water, once the matric potential of ice is lower than the water potential of the cell, water is pulled from cells to form more apoplastic ice. Release of water by various tissues has been shown as multiple exotherms by differential thermal analysis for

several deciduous species (Ketchie and Kammereck, 1987; McLeester et al., 1969; Quamme et al., 1972; Rajashekar et al., 1982). No studies have been found demonstrating multiple exotherms for citrus stems, yet studies have demonstrated variation in cold hardiness of tissues with the cambium being the most sensitive followed by phloem and cortex, and xylem and pith (Nesbitt et al., 2002).

Citrus leaves present during winter months are necessary for fruit production the following growing season (Ebel et al., 2000). To maintain continuity in commercial production, research is needed to develop methods of preserving leaves during freezes. Natural freeze events are characterized by a gradual decline in temperature to some minimum followed by a gradual rise. Once ice is initiated in the plant, ice crystals grow until temperatures rise to a point that prevents additional ice formation. Thus, the minimum temperature that occurs during a freeze event is likely to be a less accurate predictor of the total amount of damage than is the length of time and the associated temperatures that occur during the freeze (Yelenosky, 1985).

Avoidance and tolerance are the two mechanisms by which plants may withstand freezing temperatures (Levitt, 1980). The effectiveness of these mechanisms varies in species and as plants acclimate to environmental conditions. Avoidance, or supercooling, is the ability to suppress ice formation below 0°C, and tolerance is the ability to withstand ice formation to some degree. Citrus plants have a limited ability to avoid ice formation, and are vulnerable to ice formation at temperatures of -2.2°C and below, but can survive ice formation under some plant acclimation and environmental conditions (Yelenosky, 1985). This study will focus on the amount of time citrus leaves can tolerate ice at various stages of acclimation.

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PATTERN OF EXOTHERM AND ELECTROLYTE LEAKAGE MEASURED AT
HIGH FREQUENCY OF SATSUMA MANDARIN EXPOSED TO SUBFREEZING
TEMPERATURES

Abstract

The current study was conducted to relate ice formation to the pattern and rate of leaf and stem injury of Satsuma mandarins on trifoliolate orange rootstock. Potted trees were unacclimated, moderately acclimated or fully acclimated by exposing trees to 32/21°C, 15/7°C or 10/4°C, respectively. Freezing treatments consisted of decreasing air temperature at 2°C•h⁻¹ until ice formed as evidenced by exotherms determined using differential thermal analysis of stems. Air temperature was then decreased, held constant, or increased and held constant to simulate severe, moderate and mild freeze conditions, respectively. All treatment exhibited exotherms at -2 to -4°C, which were smaller with milder freezing treatments. Only the fully acclimated trees exhibited multiple exotherms. Leaf watersoaking, an indication of ice formation, occurred concurrently with stem exotherms except for fully acclimated trees where there was up to a 30-min delay that corresponded to the second exotherm. Electrolyte leakage of leaves began to increase near the peak of the stem exotherm, but increased more slowly with milder freezing temperature treatments. In some treatments, electrolyte leakage reached a plateau near 50% but leaves survived. Leaves died when whole-leaf electrolyte leakage exceeded

50%. These data are discussed within the framework of a proposed mechanism of injury of Satsuma mandarin leaves by subfreezing temperatures, especially multiple exotherms of fully acclimated trees, and the plateau of electrolyte leakage of leaves at the critical level for survival

Introduction

Commercial citrus crops are cold tender evergreens (Webber et al., 1967) that are injured by temperatures between -2.2°C to -13°C (Spiegel-Roy and Goldschmidt, 1996; Yelenosky, 1985; Yelenosky et al., 1981). In many parts of the world such as the southeastern U.S., citrus trees are grown in areas that are highly vulnerable to freezes that cause injury or tree death and significant economic losses (Attaway, 1997). There has been substantial research conducted to study the mechanism of injury (Spiegel-Roy and Goldschmidt, 1996; Yelenosky, 1985, 1996), however the mechanism is still not fully understood.

Citrus plants are injured by subfreezing temperatures only after ice forms within the tree (Spiegel-Roy and Goldschmidt, 1996; Yelenosky, 1985, 1996), which usually occurs between -2.2 to -6.7°C (Young, 1966). Ice can be initiated anywhere in the canopy, and propagates rapidly to the rest of the canopy (Lucas, 1954; Yelenosky, 1975, 1991). Most studies conclude that ice forms exclusively in the apoplast because symplastic ice would be instantly lethal (Meryman, 1956, 1966; Yelenosky, 1985, 1996), and there is considerable evidence that citrus plants can tolerate some intraplant ice (Young and Peynado, 1967). As ice forms, it expands by 9%, which can directly penetrate the

symplast and kill cells if the apoplast is saturated or nearly saturated with water (Young and Mann, 1974). Actively growing trees are typically unacclimated and are relatively hydrated (Yelenosky et al., 1984). During freezes, stem water freezes and exerts an outward pressure on bark until it cracks, causing direct injury to symplasts (Yelenosky et al., 1984). Citrus trees dehydrate as they acclimate to cold temperatures (Yelenosky, 1978, 1982; Young, 1970; Young and Peynado, 1965), and there is probably enough room in the apoplast for ice crystals to form without directly penetrating the symplast. However, as the temperature decreases further, the matric potential of ice drops below that of the osmotic potential of the symplast causing water to move toward the ice, which dehydrates the symplast. In cold hardy species, the symplast is highly elastic, thus dehydration is not lethal as the symplast is able to regain normal structure and functioning upon rehydration (Johnson-Flanagan and Singh, 1986; Ristic and Ashworth, 1994; Singh, 1979). Citrus symplast, however, is probably not as elastic as in deciduous species, and there is a level of dehydration that is lethal when the temperature drops below a critical minimum (Anderson, 1983; Young and Mann, 1974). Under some conditions, ice may penetrate into the symplast (Young and Mann, 1974) and propagate intercellularly (Rogers, 2000).

Studies that have related ice formation to injury did not use modern technology that allows data collection at high frequency. With the advent of computers, air temperature (T_{air}) in freeze chambers can be accurately controlled to within $\pm 0.2^{\circ}\text{C}$ (Nesbitt et al., 2002), and the latent heat of fusion from ice formation can be measured at high frequency using differential thermal analysis (DTA). Such data may increase our understanding of the specific mechanism by which intra-plant ice causes injury. This

study was conducted to measure electrolyte leakage (EL) of leaves as an indicator of injury and ice formation as measured by DTA at frequent intervals. Results are explained within the framework of our current theory of the mechanism of freeze injury of Satsuma mandarins.

Materials and Methods

Plant material and culture

Satsuma mandarin [*Citrus unshiu* (Marc.) 'Owari'] budded on trifoliolate orange seedlings [*Poncirus trifoliata* (L.) Raf. 'Rubidoux'] with a single, staked shoot that was 31 cm to 85 cm in height were used in this experiment. Trees were grown in 3.7 L pots, in 1 pine bark: 1 sand substrate amended with 4.45 kg•m⁻³ of dolomitic lime, 8.9 kg•m⁻³ of N-P-K (18-6-12, Polyon 12-14 month slow-release fertilizer, Pursell Technologies, Inc, Sylacauga, AL) and 0.89 kg•m⁻³ of micronutrients (Micromax, The Scotts Co., Marysville, OH). Trees were grown outdoors in ambient conditions in Auburn, AL, until acclimation treatments were initiated. Trees were irrigated daily with overtree sprinkler irrigation and fertilized weekly with Hoagland's solution (Hoagland and Arnon, 1938).

Acclimation treatments

Acclimation treatments were conducted in an environmental growth chamber (Model E15, Conviron, Winnipeg, Canada) with florescent lighting (320 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at midplant height) and ambient relative humidity. The trees were irrigated daily and not

fertilized during acclimation. There were three acclimation treatments. Trees were unacclimated, moderately acclimated or fully acclimated to cold temperatures. Acclimation treatments were chosen based on the relationship between temperature, growth and acclimation. Citrus tree growth ceases below 12°C, they cold-acclimate below 10°C and are fully cold-hardened when exposed to 10°C or less for 3 weeks (Bain, 1949; Cooper et al., 1954; Yelenosky et al., 1984; Yelenosky, 1985; Young, 1961; Young and Peynado, 1962).

Trees in the unacclimated treatment were held at air temperatures (T_{air}) of 32/21°C and 12/12 h of light/dark photoperiod for 3 weeks, after which they were actively growing. Trees in the moderately acclimated treatment were exposed to 26/16°C for 7 d, 20/11°C for 7 d, and 15/7°C for 11 d, after which the plants had ceased growing. For each decrease in temperature, photoperiod was decreased 30 minutes to 10.5/13.5 h of light/dark by the end of the moderate acclimation treatment. The decreases in photoperiod were conducted to approximate natural decreases in daylength for the Gulf Coast region of the U.S. where Satsuma mandarins are grown commercially. Trees in the fully acclimated treatment were exposed to temperatures that promoted maximum cold hardiness, T_{air} of 15/7°C for 3 weeks followed by 10/4°C for 4 weeks (Yelenosky et al., 1984). Photoperiod was decreased similarly as for the moderately acclimated trees but decreased further for the fully acclimated trees and was 10/14 h by the last 4 weeks of the acclimation treatment.

Freezing treatments

Freezing treatments were conducted after each acclimation treatment using a computer-controlled, chest freezer (Model FFC25C4AWD, Electrolux Home Products, Inc., Cleveland, OH) modified as described previously (Nesbitt et al., 2002). The freezer was further modified by fitting a Plexiglas lid with a small access door below the freezer lid, which reduced the change in T_{air} within the freezer headspace as leaves were removed for sampling. Tree pots were fitted into styrofoam wells and potting media surfaces were covered with two layers of 1.6 cm thick, styrofoam insulation (R3, Dow, Midland, MI) and a layer of 6 mm insulating bubble wrap (R9.8, Reflectix, Inc., Markleville, IN) to prevent potting the media from freezing (Nesbitt et al., 2002). The freezer was programmed to drop T_{air} linearly at $2^{\circ}\text{C}\cdot\text{hr}^{-1}$ to 4°C and held at that temperature for 1 hour. The freezer was then programmed to decrease or increase T_{air} at $2^{\circ}\text{C}\cdot\text{hr}^{-1}$ or hold it constant, depending on the specific freezing treatment. The first freezing treatment was conducted to determine the temperature of exotherms, and consisted of a continuous drop to -12°C (Fig. 1; U1, M1 and F1). The second freezing treatment consisted of a decrease in temperature to about 0.5°C to 1°C below the peak of the exotherm of the -12°C freezing treatment. T_{air} in the third freezing treatment was dropped to the same temperature as the second freezing treatment, but then increased to and held constant at -2.0°C or -3.5°C .

Data collected

Ice formation was determined in tree stems for all freezing treatments by differential thermal analysis (Ketchie and Kammereck, 1987). Two 32-gauge copper-constantan thermocouples (Omega Wire Inc., Camden, NY) were attached at approximately 10 cm and 35 cm above the graft union of each plant using parafilm. Thermocouple leads were connected to a computer and temperature data were collected and stored every 2 s (PC64 data logging software, Lawson Labs, Inc., Kalispell, MT).

Electrolyte leakage (EL) of leaves was determined before freezing and every 30 minutes during each freezing run. A single mature leaf was randomly selected and removed from each tree at each sampling time. EL was determined according to Nesbitt et al. (2002). EL due to freeze damage was expressed as a percent using the following equation proposed by Flint et al. (1967) where $EL = [(EL_{\text{frozen}} - EL_{\text{boiled}}) / (EL_{\text{prefrozen}} - EL_{\text{boiled}})] \times 100$.

After each freezing run, trees were removed from the freezer and placed in a greenhouse under ambient conditions and cultured to promote growth. After 2-4 weeks, stem dieback was determined by measuring the length of dead tissue from the tip of the main central leader and was expressed on a percent dieback basis by measuring the length of the entire stem from the tip to the graft union.

Statistical analysis

Six trees were used as replications for each freezing treatment, except the first freezing run for fully acclimated trees where four trees were used.

Stem exotherms for each thermocouple were smoothed by taking the average of readings every 800 s before and after each data point. Smoothing reduced fluctuations caused by the on/off cycling of the freezer heaters that resulted in a $\pm 0.2^{\circ}\text{C}$ variation about the mean T_{air} (Nesbitt et al., 2002), and during opening of the lid to remove samples, which increased air temperature an average of 2°C .

Differences in dieback among freezing treatments within each acclimation treatment were determined using the GLM procedure of SAS. Means were separated using Duncan's Multiple Range Test ($P \leq 0.05$).

Results and Discussion

Ice formation

A major exotherm occurred for all treatments between 1 to 3 hrs after the start of each freezing treatment (Fig. 1, U2, M2, and F2). Exotherms occurred when T_{air} was between -2.0 to -4.0°C , which lies in the range that typically causes ice to form in citrus trees (Yelenosky, 1985). The highest temperature differentials of exotherms varied from 0.3 to 0.7°C and exhibited a rapid increase followed by a gradual decline. In general, exotherms were smaller with milder freezing treatments within each acclimation

treatment, indicating less water freezing. In most treatments, stem differential temperature eventually dropped to the baseline (stem differential temperature of 0°C).

Trees in most treatments exhibited only one exotherm, however, there appeared to be up to three exotherms for fully acclimated trees, with the peak of the second exotherm occurring at 4 h and the third at or after 6 h when data collection was terminated (Fig. 1, C2). Multiple exotherms have been reported for deciduous crops, which have been related to differential cold hardiness among tissues in deciduous species (Ketchie and Kammereck, 1987; McLeester et al., 1969; Quamme et al., 1972; Rajashekar et al., 1982). Only one report indicated multiple exotherms occurred for citrus leaves (Jackson and Gerber, 1963). Citrus stems vary in cold hardiness (Nesbitt et al., 2002), but no reports have related exact tissues to specific exotherms.

When citrus trees freeze, ice is typically initiated at a single or few locations and spreads rapidly throughout the entire canopy (Yelenosky, 1991). The time exotherms traveled between the two thermocouples on a tree was <1 min. Water soaking, an indicator of ice formation in leaves was observed to occur within 30 min after the exotherm peak of the stems (Young and Peynado, 1967).

Leaf electrolyte leakage

For trees exposed to the -12°C freezing treatment, EL began to increase near the peak of the single exotherm for unacclimated (Fig. 1, U3) and moderately acclimated

(Fig. 1, M3) trees, whereas EL of the fully acclimated trees (Fig. 1, F3) increased with onset of the second exotherm. EL increased with time for all treatments, but increased more slowly for milder freezing treatments. For some treatments, the stem differential temperature dropped back to 0°C, yet EL still increased. For example, unacclimated trees exposed to -4°C had the stem differential temperature drop to 0°C by 4 h (Fig. 1, U2), but EL continued to increase from 50% to 70% from 4 to 6 h (Fig. 1, U3). It is likely that additional water froze, but the heat released probably dissipated to such an extent that what reached the thermocouple was below its detection limit. Moderately acclimated trees exposed to -4.5°C (Fig. 1, M2) and fully acclimated trees exposed to -6°C (Fig. 1, C2) had EL level off near 50%, which may indicate differential response of various tissues. Since these leaves survived, the tissue critical to survival was not killed.

Leaf watersoaking was observed to develop simultaneously with the increase in EL for all treatments. Watersoaking developed near the petiole first along with small patches scattered throughout the lamina that grew and coalesced. The entire leaves of unacclimated trees were completely watersoaked for all freezing treatments. Leaves of fully acclimated trees were not completely watersoaked. The amount of watersoaking tended to be less with increased acclimation and milder freezing treatments.

Stem dieback and bark cracking

The entire stem and all leaves were killed when T_{air} was decreased to -12°C for all acclimation treatments, and -6°C for unacclimated trees (Table 1). Dieback for the -4.0°C

freezing treatment of unacclimated trees was intermediate to the -2.0 and -4.0°C freezing treatments. Whole-leaf survival related closely to stem survival. In all treatments where there was stem dieback and leaf necrosis, whole-leaf EL increased to over 50%. Bark cracking was evident only on unacclimated trees and was observed to be less with milder freezing treatments.

The results of this study fit within the framework of our current theory of citrus tree injury by subfreezing temperatures. Ice spreads rapidly throughout the entire canopy. Bulk water froze in the stem followed by an increase in EL, which was very slow in some freezing treatments, supporting the contention that water freezes in the apoplast. If water within the leaf symplast froze during this exotherm, the symplast would have been killed instantly causing EL to rise above 50%, the level that is lethal (Levitt, 1956). Although tissue water content was not measured, unacclimated trees had larger exotherms, more watersoaking, and more bark cracking indicating a higher water content than would be found in acclimated trees. For unacclimated trees, it is clear that at least with bark cracking, the expansion of ice caused direct injury to the trees, although this does not preclude symplastic dehydration. For fully acclimated trees, the lack of complete watersoaking of leaves indicated that there was still sufficient space in the apoplast for ice to form, and thus direct penetration of cells probably did not occur. The presence of multiple exotherms for fully acclimated trees indicates a differential response of different tissues in the stems, but the tissues that correspond to each exotherm needs to be determined. The plateau in EL near 50% for the -4.5°C freezing treatment of the moderately acclimated trees and the -6°C of the fully acclimated trees probably indicates differential tissue response within leaves, and that the tissues critical to survival were not

killed. Additional research needs to be conducted to determine which leaf tissues are critical to survival. This study demonstrated that under some acclimation treatments and subfreezing temperatures, Satsuma mandarins exhibit multiple exotherms in stems and a plateau in leaf EL, which likely indicates differential response among tissues of these two organs.

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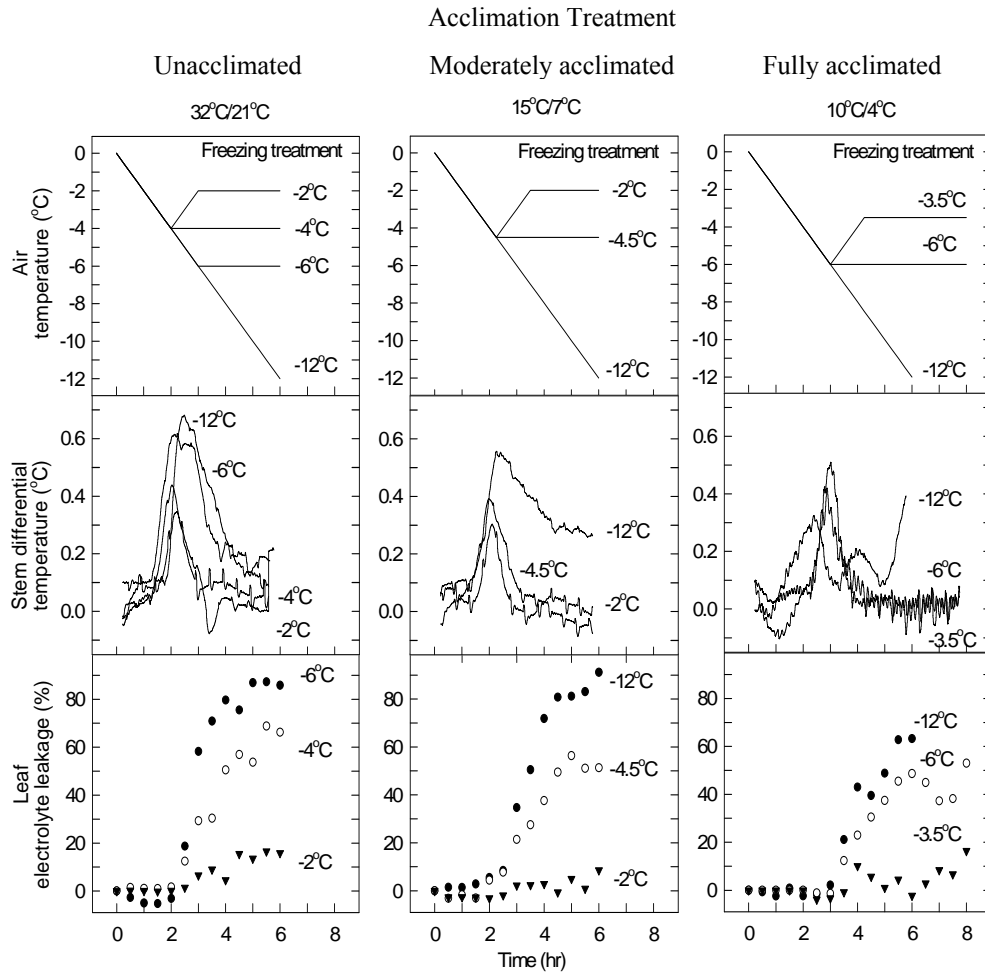
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Table 1. Percent dieback of Satsuma mandarin shoots at different cold hardiness levels induced by three acclimation treatments and exposed to various freezing temperatures.

<u>Day/night acclimation temperature</u>					
32/21°C		15/7°C		10/4°C	
Freezing treatment (°C)	Shoot dieback (%)	Freezing treatment (°C)	Shoot dieback (%)	Freezing treatment (°C)	Shoot dieback (%)
-12.0	100 a ^z	-12.0	100 a	-12.0	100 a
-6.0	100 a	-4.5	5 b	-6.0	0 b
-4.0	38 b	-2.0	0 b	-3.5	0 b
-2.0	0 c	---	---	---	---

^zLetters within columns indicate significant differences using Duncan's Multiple Range Test ($P \leq 0.05$).

Figure 1. Air temperatures for each freezing treatment (U1, M1, and F1) and the effect on stem ice formation measured by differential thermal analysis (U2, M2, and F2), and leaf electrolyte leakage (U3, M3, and F3). Each differential thermal analysis curve and electrolyte leakage data point is a mean from six trees.



CONCLUSIONS

Plant injury occurs after ice formation (Yelenosky, 1985). Tissue damage may be inflicted by direct or indirect methods. Direct damage occurs due to the expansion of water due to freezing. Ice formation in the apoplast may cause direct damage in tightly packed cells (Young and Mann, 1974), or by symplastic freezing, which is considered instantly lethal (Meryman, 1956, 1966). Indirect damage occurs through symplastic dehydration. There are two mechanisms by which plants may withstand freezing temperatures, avoidance and tolerance (Levitt, 1980). The effectiveness of these mechanisms varies in species and as plants acclimate to environmental conditions. Avoidance, or supercooling, is the ability to suppress ice formation below 0°C, and tolerance is the ability to withstand ice formation to some degree, with the belief that individual tissues have temperature dependent water potential thresholds (Nesbitt et al., 2002).

It was observed in this study that a citrus species employed avoidance and tolerance mechanisms. The degree to which these mechanisms were effective varied by level of acclimation. These findings were expected, but this study generated many unexpected findings. The most unexpected finding was the discovery of multiple exotherms in citrus stems. Multiple exotherms have been demonstrated for many species (Ketchie and Kammereck, 1987; McLeester et al., 1969; Quamme et al., 1972; Rajashekar et al., 1982), and in citrus leaves under unnaturally rapid temperature

decrease (Jackson and Gerber, 1963), but no report was found demonstrating that they occurred in citrus stems or leaves at naturally occurring decreases in temperature.

Freezing occurred for all treatments between -2°C to -6°C , a range associated with ice formation in citrus (Yelenosky, 1985). Thermocouples on individual plants showed no differences in the time of exotherm appearance. These results are similar to those of previous research that demonstrated that ice can form anywhere within citrus stems, and propagates rapidly along the stem (Yelenosky, 1991). Intensity of exotherms varied with temperature and acclimation treatments, with the milder freezing treatments and increased acclimation levels having less ice formation. Citrus trees dehydrate during acclimation, which affects the amount of available water within the tissues (Yelenosky, 1982; Young and Peynado, 1965). Intensity could also vary due to a lack of multiple exotherms in the mild freezing treatments. This indicates that the temperature stress in these treatments was insufficient to cause symplastic dehydration, decreasing the amount of water available to freeze. Shoot death corresponded well to the presence of the multiple exotherms in acclimated treatments, another indication that certain tissue can tolerate ice formation with varying degrees of hardiness (Nesbitt et al., 2002).

Ice formation can be observed through watersoaking in leaves (Young and Peynado, 1967), which developed simultaneously with the first stem exotherm for plants from mild acclimation treatments. Plants acclimated at $10^{\circ}\text{C}/4^{\circ}\text{C}$ displayed a slow rate of ice formation in leaves, with ice developing along the petiole first, then small scattered patches of ice appeared throughout the lamina, grew and coalesced. The lack of watersoaking in leaves does not necessarily indicate a lack of ice formation, merely

insufficient amounts of ice to display visual watersoaking. Increases in electrolyte leakage (EL) corresponded with ice formation. As expected, the amount of EL was dependent upon acclimation treatment (Nesbit et al., 2002), but also upon freezing treatment. This implies that the duration of freezing temperatures, not only ice formation, is critical for the survival of citrus leaves.

Areas of Future Interest

It is clear that a great deal of work is still needed in our attempt to understand cold hardiness in citrus trees. A critical area in need of study is the association of exotherms with specific tissue damage. This step would lead to the determination of which tissue is critical for survival, and the development of protection strategies related to acclimation. Cultural practices and genetic modification directed at increased tissue hardiness could greatly reduce losses associated with freeze injury. Another area of interest is the affect of environmental factors on acclimation, and cultural practices that would enhance the rate of acclimation or stabilize plants in an acclimated condition. Ultimately, the future of fruit production depends on the dedication of researchers to push forward in the investigation of these and other problems facing the fruit production industry.

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