Pattern of Exotherm and Electrolyte Leakage Measured at High Frequency of Satsuma Mandarin Exposed to Subfreezing Temperatures

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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 water soaking, an indication of ice formation, occurred concurrently with stem exotherms except for fully acclimated trees where there was up to a 30-min delay and which corresponded with 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.

Commercial citrus crops are cold tender evergreens (Webber et al., 1967) injured by temperatures between -2.2 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 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 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 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 exerting an outward pressure on bark until it cracks, which causes 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 temperature decreases further, the matric potential of ice drops below that of the osmotic potential of the symplast causing water to move towards 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, are probably not as elastic as deciduous species, and there is a level of dehydration that is

lethal when 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 ± 0.2 °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 understanding of the specific mechanism by which intraplant 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

Tree material and culture. Satsuma mandarin [*Citrus unshiu* (Marc.) 'Owari'] budded on trifoliolate orange seedlings [*Poncirus trifoliata* (L.) Raf. 'Rubidoux'] with a single, staked shoot and that were 31 to 85 cm high were used in this experiment. Trees were grown in 3.7-L pots in a 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 to 14 month slow-release fertilizer, Pursell Technologies, Inc, Sylacauga, Ala.) and 0.89 kg·m⁻³ of micronutrients (Micromax, The Scotts Co., Marysville, Ohio). Trees were grown outdoors in ambient conditions at Auburn, Ala., 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 fluorescent lighting (320 μ mol·m⁻²·s⁻¹ at midtree 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 growth ceases below 12 °C, 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, 1965).

Trees in the unacclimated treatment were held at 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 trees had ceased growing. For each decrease in temperature, photoperiod was decreased by 30 min to 10.5/13.5 h of light/dark by the end of the moderate acclimation treatment.

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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 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 FFF25C4AWD; Electrolux Home Products, Inc., Cleveland, Ohio) modified as described previously (Nesbitt et al., 2002). The freezer was further modified by fitting a Plexi-glass 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, Mich.) and a layer of 6 mm insulating bubble wrap (R9.8, Reflectix, Inc., Markleville, Ind.) to prevent potting media from freezing (Nesbitt et al., 2002). The freezer was programmed to drop T_{air} linearly at 2 °C·h⁻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 °C·h⁻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 to 1 °C below the peak of the exotherm of the -12 °C freezing treatment, and then held constant at that temperature. 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 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 were attached at about 10 and 35 cm above the graft union of each tree and wrapped in parafilm. Thermocouple leads were connected to a computer and temperature collected and stored every 2 s (PC64 datalogging software; Lawson Labs, Kalispell, Mont.).

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

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 to 4 weeks, stem dieback was determined by measuring the length of dead

tissue from the tip back of the main central leader and 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 ± 0.2 °C variation about the mean T_{air} (Nesbitt et al., 2002), and during opening of the lid to remove samples, which increased T_{air} 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 h 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 (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 baseline (stem differential temperature of 0 °C).

Trees of 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 stem tissues vary in cold hardiness (Nesbitt et al., 2002), but no reports have related tissues to specific exotherms.

Fig. 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). Above each column of graphs is the acclimation treatment, which was 32/21 °C, 15/7 °C or 10/4 °C for the unacclimated (U), moderately acclimated (M) or fully acclimated (F) trees, respectively. Each differential thermal analysis curve and electrolyte leakage data point is a mean from six trees.

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

Day/night acclimation temp					
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 ²	-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	0c	---	---	---	---

²Letters within columns indicate significant differences using Duncan's multiple range test ($P \leq 0.05$).

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 (Young and Peynado, 1967), was observed to occur within 30 min of each exotherm. Based on these data, we conclude that ice began to form in leaves within 30 min after the exotherm peak of the stems.

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 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 dissipated such 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 water soaking was observed to develop simultaneously with the increase in EL for all treatments. Water soaking 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 water soaked for all freezing treatments. Leaves of fully acclimated trees were not completely water soaked. The amount of water soaking 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 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), also during the exotherm. Although tissue water

content was not measured, unacclimated trees had larger exotherms, more water soaking, and more bark cracking indicating higher water content than 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 water soaking of leaves indicates 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 what leaf tissue is 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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