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Water-stress-induced Cold Hardening of Young Citrus Trees[1]

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Abstract. **Water-stressed young, potted citrus seedlings and budded trees on different rootstocks were tested for freeze avoidance and tolerance. Water stress increased the supercooling of cold-sensitive citrus seedlings in freeze-avoidance tests and increased the cold hardiness of grapefruit and orange trees in freeze-tolerance tests. Water stress was most effective in reducing injury during freezes above –6.7°C. Temperatures below –6.7° resulted in complete kill regardless of –25 bars water stress in the leaves. Tissue analyses showed increased in proline accompanying increases in sugars during forced dehydration of citrus leaves.**

Cold-tender citrus trees cold harden readily when exposed to low temperatures. Temperatures between 15.6 and 4.4°C and photosynthetically active radiation (PAR) less than 600 μeinsteins/m² per sec induced cold hardening that enabled citrus trees to survive freezes that killed unhardened trees under controlled conditions (15). The importance of prefreeze hardening conditions is well documented in freeze damage surveys (19). Favorable cold-hardening temperatures do not always precede damaging freezes, and alternate ways to induce cold hardiness are needed to minimize citrus freeze losses. Alternatives include chemicals (10) and water stress (2, 10). Chemical cryoprotectants remain largely experimental in citriculture, and even less is known about water stress.

In a series of tests, the effects of water stress on citrus cold hardiness were examined and some of the concomitant biochemical changes were identified.

Materials and Methods

Trees. Tests were made on 8-month-old seedlings of very cold-tender-rated citron, *Citrus medica* L., and more cold-hardy, 18-month-old budded trees of 'Star Ruby' grapefruit, *C. paradisi* Macf., and 'Valencia' sweet orange, *C. sinensis* (L.) Osbeck, on three different rootstocks. These rootstocks ranged from the cold-hardy trifoliate orange, *Poncirus trifoliata* (L.) Raf., to the less cold-hardy Koethen sweet orange, and *C. miaray* Wester.

Citron and rootstock seedlings were from open-pollinated seed. Grapefruit and orange budwood were from single trees in a variety collection planting. Individual trees were grown in a mixture of 1 part sand:2 parts vermiculite:4 parts of sphagnum peat moss in 2.5-liter containers in a greenhouse under natural daylight. Maximum light in the greenhouse approximated 875 μeinsteins/m² per sec (PAR), and maximum temperature was 31°C day, and minimum temperature was 22° night. These temperatures do not induce cold hardening in citrus trees. Single-stem test trees were selected for uniform appearance and absence of visible active growth.

Water-stress and tissue analyses. Water stress was induced by withholding water from potted citrus plants for about 8 days. Stress was measured in the leaves immediately before freeze tests. In freeze avoidance tests limited to citron seedlings, water content in composite samples of 3 leaves per seedling averaged 1.3 g to 1.5 g of water per gram of ovendry wt for water-stressed seedlings and 3.1 to 3.3 g for nonstressed seedlings. Stressed seedlings showed pronounced wilt of the leaves relative to no wilt in nonstressed seedlings. When rewatered, nonfrozen seedlings fully recovered and showed no injury.

Rewatered, nonfrozen, water-stressed trees used in freeze-tolerance tests also recovered completely. In these tests, stress was measured with a Scholander pressure bomb (4). Data were averaged from 3 leaves per tree with a minimum of 5 single-tree replicates. Fully mature leaves were arbitrarily selected from between 15-45 cm of terminal shoot growth. Composite samples of 2 leaves per tree were analyzed and partitioned for measurements of sugars (17) and proline (13).

Freeze tests. Water-stressed and non-water-stressed citrus trees were tested simultaneously in a walk-in, controlled-freeze room (18). Tests included freeze avoidance (supercooling of citron seedlings) and freeze tolerance (ice tolerance of budded trees) in freezes with ± 0.5°C temperature control, no light, and 50% ± 5% relative humidity. Citron seedlings were paired in 15 sets of 2; 1 water-stressed seedling was matched with 1 nonstressed seedling. One set was frozen in each of 3 freeze

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tests daily for 5 consecutive days. After plants were held for 1 hr at 1.1°, the temperature of the freeze room was cooled 5.5° per hr. The start of plant freezing (nucleation), or the end of supercooling was measured with 36-gauge, copper-constantan thermocouples taped firmly against the surface of the green stem 10 cm below maximum tree height. Exotherms were referenced with digital multimeters connected to variable strip-chart recorders as previously described (18). The beginning of exotherms coincided with water soaking in the leaves and stems.

Freeze tolerance was tested on groups of trees with at least 5 single-tree replicates at each temperature and duration. Freezes were of 2 general types. One type was a stepdown freeze with abrupt changes to progressively colder temperatures at preset time intervals. These freezes were manually controlled. Supercooled trees were maintained for 1 hr between –3° and –5°C in different studies before trees were misted with ice-cold water to induce uniform ice inoculation in the trees. Equal numbers of frozen trees were removed to room temperature (approx 25°) after each completed stepdown of progressively colder temperature.

Trees were held for 1 hr at 4.4°C and then cooled at a rate of 1.1° per hr to a minimum test temperature of –6.7°. After holding at –6.7° for 4 hr, trees were rewarmed at a rate of 1.1° to 4.4° and subsequently held at 25° for 3 hr before trees were returned to the greenhouse. Trees were rated during 5 weeks of observation for percentage of leaf kill and stem dieback. Percentages were converted to arc sine transformations in statistical analyses.

Results and Discussion

Freeze avoidance. Water stress increased supercooling in stems of citron seedlings. In 14 of 15 consecutive tests, the critical supercooling temperatures averaged 1.1°C colder in water-stressed than in nonstressed seedlings. This 1.1° difference is statistically significant at the 5% level in a comparison of the paired mean differences. Water-stressed seedlings froze between –6.4° and –9.5°, and nonstressed between –5.1° and –7.3°. The overlap is shown in Fig. 1, where the lower limits of supercooling in stressed seedlings coincide with the upper limits of supercooling in nonstressed seedlings. The linear associations of heat evolution at critical nucleating temperatures are similar to published correlations, and the maximum temperatures of exotherms in Fig. 1 may not reflect true freezing points (18). Also, the frequency distribution of the stressed seedlings throughout their range of supercooling is skewed to the right. This skewness reflects the increased depth of supercooling attributed to water stress. Water stress affects the capacity of plants to supercool (1). In plants other than citrus, increased supercooling is associated with maturation of tissues (9) and morphological features (6).

Supercooling increased in water-stressed citron seedlings probably because there is less free water readily available for freezing than in nonstressed seedlings, and thus require a higher energy of activation for ice crystal formation. Seemingly, water conditions are even more stable in leaves of grapefruit trees cold hardened with progressively colder temperature regimes than in seedlings in this study. Grapefruit trees placed in a chamber at –9.4°C showed 4° more supercooling in leaves of cold hardened than of unhardened trees (20). Some of the

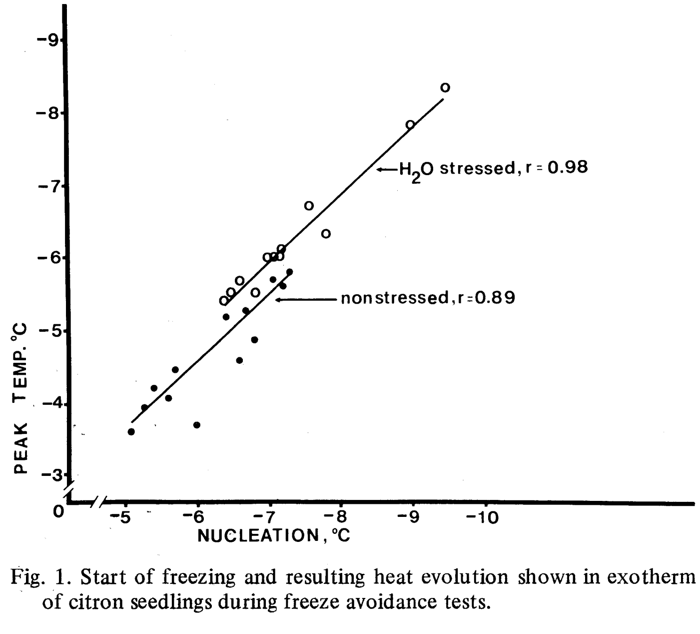

Fig. 1. Start of freezing and resulting heat evolution shown in exotherm of citron seedlings during freeze avoidance tests.

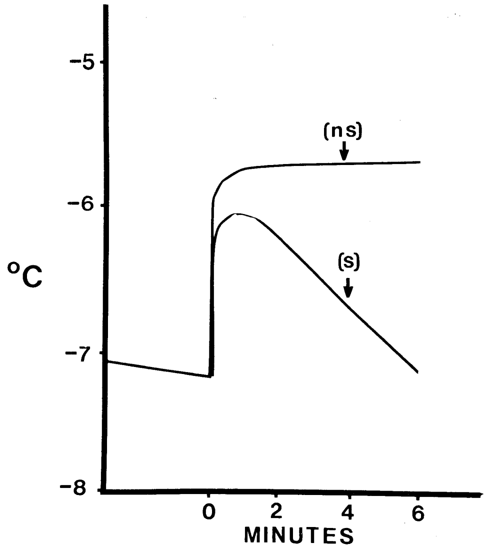

Fig. 2. Different exotherms associated with water-stressed (s) and nonstressed citron seedlings (ns) during freeze avoidance tests with a –5.5°C per-hr freezing rate.

Table 1. Effect of withholding water on freeze injury of 18-month-old 'Star Ruby' grapefruit trees on sweet orange rootstock during a freeze profile of 1.1°C per hour from 4.4°C.

°C	Watering treatment	Percent kill[z]	
		Leaves	Stem
–3.3	(supercooled trees inoculated with a mist of ice-cold water)	0	0
–4.4	Watered[y]	58	0
	Water withheld[x]	19*	0
–5.5	Watered	92	6
	Water withheld	68*	0*
–6.7	Watered	100	25
	Water withheld	100	0*
–7.8	Watered	100	100
	Water withheld	100	94
–8.9	Watered	100	100
	Water withheld	100	100

[z] Avg of 5 trees.

[y] Avg of –5 bars water stress in the leaves and no wilt.

[x] Avg of –25 bars water stress in the leaves and moderate wilt.

*Significantly different at the 5% level in comparison of the means of watered and water withheld at a given temperature.

Table 2. Effect of withholding water on freeze injury of 18-month-old 'Star Ruby' grapefruit trees on *C. miaray* rootstock after 2 hr at progressively colder temperatures.

°C	Watering treatment	Percent kill[z]	
		Leaves	Stem
–3.3	(supercooled trees inoculated with a mist of ice-cold water)	0	0
–3.3	Watered[y]	81	5
	Water withheld[x]	22*	0*
–4.4	Watered	90	60
	Water withheld	31*	0*
–5.5	Watered	100	100
	Water withheld	73*	0*
–6.7	Watered	100	100
	Water withheld	100	100

[z] Avg of 5 trees.

[y] Avg of –7 bars water stress in the leaves and no wilt.

[x] Avg of –25 bars water stress in the leaves and moderate wilt.

*Significantly different at the 5% level in comparison of the means of watered and water withheld at a given temperature.

most stable water conditions in plants result in deep supercooling of –40° and colder, and this largely determines the northern limits of plant distribution (5). Deep supercooling is also important in seed survival (8).

The advantages of water stress in freeze avoidance did not extend to the freeze tolerance of supercooled citron seedlings in this study. All seedlings were severely injured within 30 min after ice formed at critical supercooling temperatures. Seedling injury included total leaf kill and as much as 80% stem kill within 10 minutes after ice formed in the stems, regardless of water stress. The rapid and extensive injury is attributed mostly to increased intracellular freezing caused by non-equilibrium freezing, which intensifies with increased supercooling (12). Because of differences in water content, exotherms were smaller with water-stressed than nonstressed seedlings (11). The exotherms of nonstressed seedlings had considerably broader plateaus than those of stressed seedlings (Fig. 2). These features indicate that less ice formed in water-stressed than in nonstressed seedlings, but in citron seedlings injury was similar in both cases.

Freeze tolerance. Water stress consistently increased the freeze tolerance of young citrus trees on different rootstocks in tests on more than 150 trees. Water-stressed trees showed pronounced leaf wilt and averaged –25 bars to –26 bars water stress in the leaves. Nonstressed trees showed no wilt and averaged –4 to –7 bars of water stress in the leaves. The increased cold hardening due to water stress did not extend to freezes below –6.7°C. Trees cooled below –6.7° were virtually completely killed regardless of differences in water stress.

Water-stressed 'Star Ruby' grapefruit trees on sweet orange rootstock were cold hardy to temperatures as low as –5.5°C in the leaves and –6.7° in the stems (Table 1). One hour after the supercooled grapefruit trees were frozen at –3.3°, and temperature lowered to –4.4°, trees showed an average leaf kill of 19% compared to 58% for nonstressed trees. Stressed trees continued to show less injury at –5.5°, where nonstressed trees began to sustain stem kill. Stems of water-stressed trees were not injured until temperatures were colder than –6.7°.

Holding trees for 2 hr at –6.7°C resulted in complete kill of 'Star Ruby' trees on *C. miaray* rootstock in a stepdown type of freeze regime (Table 2). Increased cold hardiness due to water stress was evident at temperatures above –6.7° and of the same 2-hr duration. Water-stress-induced increases in freeze tolerance were most evident in the stems of water-stressed trees. Differences in stem kill were maximum at –5.5°; stem kill was absent in stressed trees but was complete in nonstressed trees. These observations were similar to previous studies of sweet orange seedlings that were cold-hardened with low temperature regimes instead of water stress prior to stepdown freezing tests (16, 20).

Both 'Star Ruby' grapefruit and 'Valencia' orange trees on trifoliate orange rootstock increased in cold hardiness with increased water stress (Table 3). However, neither selection showed uninjured stems after 4 hr at –5°C. On nonstressed trees, leaf kill was complete, whereas on water-stressed trees, leaf survival was 16% for grapefruit and 31% for orange. Stem kill was less than 15% for stressed trees compared to 40% and 70%, respectively, for nonstressed orange and grapefruit trees. Total sugars and proline concentrations increased in the leaves of water-stressed trees. Both fractions are associated with increases of cold hardiness in citrus trees (14).

Increased freeze tolerance due to water stress was also effective in freezes more severe than –5°C for 4 hr. After 4 hr at –6.7°, water-stressed trees showed 75% leaf kill and only 3% stem kill compared to almost total kill of nonstressed trees (Table 4). This equaled the increase in freeze tolerance of trees cold-hardened with low temperatures in this test. Combining water stress and low-temperature cold-hardening treatments was not more effective in hardening trees than the individual treatments. Water-stress-induced cold hardening and temperature-induced cold hardening had similar end results in tree survival although the physiological routes differed.

Table 3. Effect of withholding water on freeze injury of 18-month-old 'Star Ruby' grapefruit and 'Valencia' orange trees on trifoliate orange rootstock after 4 hr of –5°C.

Trees	Watering treatment	Leaves			Percent kill[z]	
		Water stress (bars)	Total sugars (mg/g O.D. wt)	Proline	Leaves	Stem
Star Ruby	Watered	– 6	56	5	100	70
	Water withheld	–25*	66*	11*	84	13*
Valencia	Watered	– 4	38	13	100	40
	Water withheld	–26*	47*	16*	69*	7*

[z] Avg of 7 trees inoculated at –4.4°C with a mist of ice-cold water during cooling of 1.1° per hr from 4.4°.

*Significantly different at the 5% level in comparison of the means of watered and water withheld within citrus cultivars.

Table 4. Effect of withholding water on freeze injury of 18-month-old 'Star Ruby' grapefruit trees on trifoliate orange rootstock after 4 hours of –6.7°C.

Treatment	Leaves			Percent kill[z]	
	Water stress (bars)	Total sugars (mg/g O.D. wt)	Proline	Leaves	Stem
Greenhouse, watered	– 6	54 a[x]	9a	100 a	84 a
Greenhouse, water withheld	–35	62 b	12 b	75 ab	3 b
Cold hardened[y], watered	– 9	97 c	10 a	87 ab	1 b
Cold hardened, water withheld	–30	118 d	14 c	68 b	2 b

[z] Avg of 10 trees inoculated at –5.5°C with a mist of ice-cold water during a cooling of 1.1° per hr from 4.4°.

[y] In controlled-environment room with 21.1°C, 12-hr days (450 μeinsteins/m^2 per sec PAR) and 10° nights for 1 week followed by 2 weeks of 15.6° days and 4.4° nights.

[x] Mean separation within columns by Duncan's multiple range test, 5% level.

Apparently a key factor is decreased tissue hydration in both cases. Other researchers have expressed similar opinions in studies on buds of fruit trees (7), wood of *Cornus* (2), and leaves of *Brassica* (3). In the present study, there was a partially additive effect of water stress on low-temperature cold-hardening treatments on increases of sugars and proline in the leaves of citrus, but these increases did not alter the freeze tolerance of citrus trees. Within practical limitations, the dryness of trees is yet another factor of many that are associated with the cold hardiness of citrus trees.

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Effect of Ethylene, Maturity, and Attachment to the Parent Plant on Production of Volatile Compounds by Muskmelons[1]

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Additional index words. Cucumis melo, cantaloupe, 'Honey Dew'

Abstract. **Gas samples from the central cavities of 3 cultivars of muskmelon fruits (*Cucumis melo* L.) were resolved by gas chromatography into at least 12 organic volatile fractions. Harvesting of 'Top-Mark' and 'PMR-45' cantaloupe up to 12 days before normal abscission did not reduce the final concentration of volatiles attained. Production of volatiles by cantaloupe was inhibited while still attached to the parent plants, but no evidence for inhibition by the parent plant was obtained in 'Honey Dew', nor was the final concentration of volatiles reduced by early harvest. High concentrations of exogenous ethylene applied to harvested melons accelerated the onset of production of the organic volatiles but did not significantly affect their ultimate concentrations. Exogenous ethylene altered the relative amounts of at least 2 fractions. The production of volatiles is closely coordinated with the other aspects of ripening, but the control mechanisms appear to be different.**

Although much effort has been devoted to the identification of the volatile compounds responsible for the characteristic flavors of many fruits and vegetables, relatively little attention has been given to the factors affecting their production. Maturity has a clear effect on the quantity and variety of volatiles produced by peaches (2, 9); not only do immature peaches produce less, but some volatiles found in mature peaches are not produced in detectable quantities by peaches harvested before maturity, even after several days of subsequent incubation. Exposure to gamma radiation suppresses volatile production, and this suppression coincides with a retardation of ripening (13).

Many volatile compounds produced by muskmelon have recently been identified; most are alcohols and fatty acid esters (5, 6, 7, 16). Although some work elucidating the biosynthesis of these compounds has been published (17), and although Paillard (10) has reported the synthesis by apple tissue of several esters from the corresponding alcohol and acid

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