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Accumulation of Free Proline in Citrus Leaves during Cold Hardening of Young Trees in Controlled Temperature Regimes

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ABSTRACT

Free proline increased in leaves of orange (*Citrus sinensis* [L.] Osb. cv. Valencia) and grapefruit (*Citrus paradisi* Macfad. cv. Star Ruby) trees on a wide range of citrus rootstocks during cold hardening. Increases in sugars accompanied proline accumulation. During cold hardening, the rate of proline accumulation was greater in old than in young leaves. In leaves of grapefruit trees kept in the dark during cold hardening, neither proline nor sugars increased and the degree of cold hardiness was less than in trees exposed to light. Like sugar accumulations, proline accumulation does not reflect specific degrees of cold hardiness in citrus cultivars.

Accumulation of free proline is frequently observed in plants subjected to environmental stresses and is sometimes associated with increased cold hardiness in plants (2, 6, 8). Possible roles suggested for free proline in plant cold hardiness range from protection of cellular membranes (5) to regulation of enzymes (10).

Proline is one of the most abundant amino acids in citrus tissues. It is an important soluble nitrogen store in citrus leaves (12) and is one of four amino acids that peak in accumulation during water stress, generally at the wilting range (3). Accumulation of free proline is one of the features of water stress-induced cold hardening of citrus trees (16). During temperature-induced cold hardening, proline was one of three amino acids that increased in leaves of citrus trees withstanding −6.7 C without injury (15). In this work we report the accumulation of proline in citrus leaves. Accumulation was measured in young trees on different rootstocks during temperature-induced cold hardening, during different visible stages of tree growth, and in relation to light exposure and sugar accumulation.

MATERIALS AND METHODS

Trees. Citrus trees were 1-year-old sweet orange (*Citrus sinensis* [L.] Osb., cvs. Valencia and Koethen) and grapefruit (*C. paradisi* Macfad., cv. Star Ruby) budded on 1.5-year-old rootstocks: rough lemon, *C. limon* Burm. f.; sour orange, *C. aurantium* L.; large flower trifoliate, *Poncirus trifoliata* (L.) Raf.; *C. volkameriana* Wester; *C. macrophylla* Wester; Swingle citrumelo, *C. paradisi* × *P. trifoliata*; Carrizo and Troyer citranges, *C. sinensis* × *P. trifoliata*; and a hybrid of Rangpur lime, *C. reticulata* var. *austera* hybrid × Troyer citrange. Buds were from single trees and rootstocks were grown from open-pollinated seed.

Individual trees were grown in a mixture of sand-Vermiculite-sphagnum peat moss (1:2:4, v/v) in 3-liter containers under natural daylight in a greenhouse. Maximum light in the greenhouse measured 875 $\mu E/m^2 \cdot s$ PAR on a Lamda L1-185 meter[1] (Lambda Inst., Inc., Lincoln, Nebr.). Air temperatures during daylight ranged from 24 to 32 C and RH ranged from 40 to 70%. During the nights, air temperatures ranged from 21 to 24 C and RH ranged from 70 to 90%. Single-stem trees were maintained under routine greenhouse procedures and test trees were selected for uniformity.

Cold Hardening and Freeze Tests. Cold hardening and freeze tests were in controlled environment facilities previously described (14). Temperature regimes included unhardened trees directly from the greenhouse and trees cold hardened by progressively colder temperatures. Temperatures were controlled ± 0.5 C during alternating 12 h of abrupt light and dark. Light was a mixture of 86% cool-white fluorescent and 14% incandescent. Illumination at the top of the trees averaged 375 $\mu E/m^2 \cdot s$. Individual hardening treatments are given in tables.

Freeze tolerance was tested in the dark with 50 ± 5% RH. Ambient air temperatures were either automatically cooled and warmed 1.1 C/h with predetermined minimum and duration, or they were manually controlled with abrupt 1 C change between progressively colder freeze levels. Standard freeze programs started with 2 h at 4.4 C and ended at 4.4 C. In other freezes, trees were abruptly removed to 25 C after constant durations at different freeze levels. Differences in supercooling were avoided during the warmer freezes with ice-cold water mists to start freezing in trees at −2.2 C. After all freezes, trees were kept at 25 C for 3 h and then returned to the greenhouse for 5 weeks of injury observations. Trees were rated for percentage of leaves killed and dieback of the main stem.

Tissue Analyses. Tissue analyses were made on composite samples of three arbitrarily selected leaves per tree with a minimum of five single-tree replicates. Samples dried in a forced-air oven at 90 C for 25 h were micromilled, and 2- to 3-g subsamples were Soxhlet-extracted in 76% ethanol (v/v) and partitioned with 100 to 200 mesh ion exchange resins, Dowex 1-X-8 and 50-W-X4 for determination of proline and sugars. Proline was determined according to Troll and Lindsley (13) with modifications by Singh *et al.* (9). Total sugar and sucrose were determined according to procedures previously reported (17). Concentrations reported are means of three determinations per each of two subsamples.

Cold Hardening in Darkness. Dark treatments were limited to controlled temperature regimes. Single trees were covered with aluminum foil and randomized with uncovered trees during cold-hardening regimes. These covers did not significantly change leaf temperatures measured with 36-gauge, copper-constantan thermocouples taped to the leaves.

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

RESULTS AND DISCUSSION

Free proline accumulated in leaves of all orange and grapefruit trees tested during cold hardening. Proline concentrations before cold hardening and rates of accumulation during cold hardening were different in leaves of trees on different rootstocks. In general, proline increases relative to initial concentrations were greater in leaves of Valencia orange trees on citrus rootstocks rated cold-hardy than on those rated cold-tender. This association was not evident for leaves of Star Ruby grapefruit trees. Levels of proline found in this study are probably less significant than relative comparisons, since oven-dried procedures may not have stopped enzyme action and CH_2O transformation soon enough.

In one phase of this study, the smallest increase in free proline during cold hardening, 127% based on dry weight, was found in leaves of Valencia orange trees on cold-tender rough lemon rootstock (Table I). These trees were also the most injured after −6.7 C for 4 h. Less wood injury and greater increases in proline were evident in the leaves of trees on rootstocks more cold-hardy than rough lemon. However, proline increases did not correlate well with relative cold hardiness ratings of rootstocks (18). Trifoliate orange rootstock ranges from moderately cold-hardy to very cold-hardy. In this study, proline increases in leaves of Valencia orange trees on trifoliate rootstocks averaged 157% (next lowest to cold-tender rough lemon) in contrast to 483% for cold-hardy sour orange. Proline increased 247% in leaves of orange trees on moderately hardy to hardy Swingle. The greatest increase of 557% was found in leaves of orange trees on moderately hardy to hardy Carrizo citrange.

In another phase of this study, Valencia orange trees on rough

Table I. *Proline Accumulation in Leaves of Valencia Orange Trees on Different Rootstocks After Cold Hardening*

Rootstock	Freeze Injury[a]		Cold Hardened	Proline	Proline Increase After Hardening
	Leaf kill	Stem kill			
	%			mg/g dry weight	%
Rough lemon$_T$[b]	92	39*	Yes[c]	10.2*	127
	100	100	No[d]	4.5	
Carrizo citrange$_{I-H}$	82*	4*	Yes	9.2*	557
	100	100	No	1.4	
Swingle citrumelo$_{I-H}$	72*	9*	Yes	13.2*	247
	100	100	No	3.8	
Sour orange$_H$	90	3*	Yes	17.5*	483
	100	100	No	3.0	
Trifoliate orange$_{I-VH}$	93	4*	Yes	8.5*	157
	100	100	No	3.3	

[a] After −6.7 C for 4 h.

[b] Relative cold hardiness: T, tender; I, moderately hardy; H, hardy; VH, very hardy (18).

[c] Two weeks each successively of 21.1 C days and 10 C nights and 15.6 C days and 4.4 C nights.

[d] Greenhouse controls.

* Means significantly different from those of unhardened trees at the 5% level.

Table III. *Effect of Cold Hardening on Sugars, Proline, and Water Content in Leaves of Valencia Orange Trees on Rangpur × Troyer Rootstock at Different Stages of Growth*

Visible Stage of Growth	Age of Leaves	Cold Hardened	Total Sugars	Sucrose	Proline	H_2O
	weeks		mg/g dry weight			g/g dry weight
Active	<2	Yes[a]	170*	63*	8.5*	2.5*
		No[b]	62	30	5.3	3.5
	>8	Yes	92*	58*	18.2*	1.8*
		No	43	35	3.0	2.1
			no new growth			
Quiescent	<2	Yes	95*	52*	20.5*	2.0*
	>8	No	48	37	6.5	2.4

[a] Five weeks of 15.6 C days and 4.4 C nights.

[b] Greenhouse controls.

* Significantly different from unhardened trees at the 5% level in comparison of the means.

Table IV. *Sugar and Proline Accumulation in Leaves of Star Ruby Grapefruit Trees on Carrizo Citrange Rootstock During Cold Hardening With and Without Light*

Exposed to Cold-hardening Temperatures	Sugars		Proline	Freeze Injury[a]	
	Total	Sucrose		Leaf Kill	Stem Kill
	mg/g dry weight			%	
No[b]	54	37	10.5	67	18
Yes, with light[c]	118*	71*	16.1*	4*	0*
Yes, without light[d]	26	16	10.5	100	12

[a] After −5 C for 4 h.

[b] Greenhouse controls.

[c] One week of 21.1 C days and 10 C nights immediately followed by 2 weeks of 15.6 C days and 4.4 C nights; trees uncovered.

[d] As above; but trees covered with aluminum foil.

* Significantly different from covered trees at the 5% level in comparison of means.

Table II. *Relative Changes in Levels of Total Sugars and Proline in Leaves of Citrus Trees on Different Rootstocks After Cold Hardening*

Rootstock	Cold Hardened	Valencia Orange			Star Ruby Grapefruit		
		Total sugars	Proline	Proline increase after hardening	Total sugars	Proline	Proline increase after hardening
		mg/g dry weight		%	mg/g dry weight		%
Rough lemon$_T$[a]	Yes[b]	83.6*	8.2*	173	108.0*	8.0*	1500
	No[c]	40.1	3.0		49.0	0.5	
C. macrophylla$_T$	Yes	50.8*	15.8*	177	120.0*	9.4*	276
	No	36.6	5.7		52.1	2.5	
C. volkameriana$_T$	Yes	81.5*	11.6*	346	106.0*	8.0*	264
	No	29.6	2.6		46.9	2.2	
Swingle citrumello$_{I-H}$	Yes	95.2*	11.0*	479	95.3*	4.4*	450
	No	55.9	1.9		47.7	0.8	
Troyer$_{I-H}$	Yes	56.2*	10.9*	419	86.5*	4.0*	233
	No	32.1	2.1		45.5	1.2	

[a] Relative cold hardiness: T, tender; I, moderately hardy; H, hardy (18).

[b] Two weeks each successively of 21.1 C days and 10 C nights and 15.6 C days and 4.4 C nights.

[c] Greenhouse controls.

* Means significantly different from those of unhardened trees at the 5% level.

lemon rootstock again showed the lowest relative proline increase in leaves (173%) (Table II). Cold-sensitive Macrophylla was next at 177%, followed by 346% for the slightly less cold-tender Volkameriana rootstock. The even more cold-hardy Troyer averaged 419%, and the highest was Swingle citrumelo with a 479% increase in free proline in leaves of Valencia orange during cold hardening. For Star Ruby grapefruit, the highest relative proline increase was in leaves of trees on cold-sensitive rough lemon, with no apparent association between rootstock cold hardiness and proline accumulation.

Stewart (12) reported proline concentrations may approach 2.5% but are usually 0.5% of the dry weight of Valencia orange leaves, and young leaves have more proline than old leaves. We found more proline in young leaves before cold hardening; but, the reverse after cold hardening. Proline concentrations increased 6-fold in older leaves and final concentrations exceeded twice the amounts found in the younger leaves on the same tree after 5 weeks of 15.6 C days and 4.4 C nights (Table III).

Two characteristic features of citrus cold hardening that favor proline accumulation are ample reserves of sugars and decreased water content in the leaves. Sugars are likely precursors for increased synthesis of free proline (11). Photosynthesis is implicated in cold hardening of citrus trees (14), and in this study, proline did not accumulate in leaves of grapefruit trees that were not exposed to light during cold-hardening regimes (Table IV). These trees also showed less sugar in the leaves and less freeze tolerance than the leaves of trees in the light. That proline increases are much lower in the dark than in the light is reported for leaves of winter rape (10), wheat (4, 10), barley (4), and cabbage (7). The other feature, water stress, induces proline accumulation in many plants (1, 9, 11) as well as in citrus (3, 16).

Free proline accumulation is considered a characteristic feature of citrus cold hardening and, seemingly, is influenced by rootstock-scion combinations. The increase in free proline levels in cold-hardened citrus trees appears to occur with accompanying effects on tissue water balance. Data do not differentiate cold-induced accumulation of proline from water stress-induced accumulation which has been done for barley (4). Like sucrose, neither the rates of proline accumulation nor the final concentrations of proline correlate well enough with cold hardening to be used as indexes of degrees of cold hardiness in citrus cultivars. However, cold protection provided by solute accumulation is increased intracellular viscosity and decreased cellular dehydration during the freezing process.

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