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EFFECTS OF COLD HARDENING ON ACYL LIPIDS OF CITRUS TISSUE

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Abstract—Cold hardened and unhardened 8- or 16-month-old citrus plants were examined for differences in fatty acid (FA) content Unhardened leaves from 8-month-old Valencia scion budded on sour orange rootstock had 29% less FAs than leaves from seedling sour orange After cold hardening triacylglycerol (TAG) FAs increased 4-fold in Valencia on sour orange and 6-fold in sour orange seedling The percentage of FAs associated with TAGs for unhardened–hardened 16-month-old Valencia on sour orange tissues were upper leaves 7–20, lower leaves 6–17, bark 6–9, and roots 57–73% Cold hardening increased the amount of TAG FAs of 16-month-old Valencia on sour orange in upper leaves by 226% and in lower leaves by 173% Concentrations of linoleic acid increased by 479% in upper leaves and by 303% in lower leaves Quantities of linolenic acid in monogalactosyl diacylglycerols declined by 27% in upper leaves and by 20% in lower leaves

INTRODUCTION

Plants vary in their inherent ability to withstand freezing temperatures Temperature cold hardening improves a plant's ability to withstand freeze stress Temperature cold hardening consists of exposing plants to progressively cooler temperatures from 1 to 4 weeks Lipids are one of several plant constituents which respond to this hardening process in which changes are noted in phospholipids and components of biomembranes [1] Lipid involvement in the hardening mechanism remains unclear [2, 3]

Citrus cultivars vary in their ability to withstand freezing temperatures [4] The rootstock and the scion apparently interact in determining citrus resistance to low temperature [5] Analyses of leaf fatty acids (FAs) indicate some changes that occur during cold hardening of three 8-month-old citrus rootstocks with various chill resistances [6] Quantities of FAs and degree of unsaturation were found to be lowest in the least resistant and highest in leaves of the most cold resistant rated rootstock Cold hardening increased the amounts of FAs and degree of unsaturation in leaves of all three rootstocks The greatest increase did not occur in phospholipids but in the triacylglycerol (TAG) fraction Linoleic (18 2) accounted for the major increase in FAs

In our earlier study [6], leaves were from seedling plants in which the leaf lipid profiles were representative of the rootstock's germplasm resistance towards cold stress An objective in the present study was to determine whether leaf lipid profiles from a citrus scion budded to one of these rootstocks would be different from the rootstock's normal leaf lipid profiles In the previous citrus study [6], leaves were selected from the top five leaves of ten trees Another objective of the present study was to determine whether cold hardening also alters lipid profiles in lower leaves and other tissues The third objective was to determine the quantities of the five major FAs within nine lipid classes under normal and cold hardening temperatures The emphasis in future hardening studies can then be placed on analyses of specific lipids and FAs

RESULTS AND DISCUSSION

Data comparing FAs in tissues from seedling and grafted citrus trees are presented in Table 1 Hardening increased the total FA content in sour orange seedling (SO) by 19% with 18 2 increasing by 172% FAs in the neutral lipid fraction of SO seedling increased by 190% Although all five FAs increased in neutral lipid with hardening, the greatest increase (6 6-fold) was with 18 2 Triacylglycerols (TAGs) accounted for 31% of the FAs in neutral lipid of unhardened leaves and 75% in neutral lipid of hardened leaves Total FAs in TAGs increased 6-fold and 18 2 in TAGs 13-fold with hardening

Total lipid FAs in leaves of Valencia budded on sour orange (Val/SO) increased by only 6% from the hardening process Amounts of the five FAs in total lipid of Val/SO leaves were from 40 to 94% of those in SO seedling leaves Since all cultural practices were similar for the two sets of plants, these lower quantities must be characteristic of the scion leaf Differences in the concentrations of 16·0, 18 0 and 18 3 between control and hardened samples of Val/SO leaves were minimal whereas 18 1 decreased by 52% and 18 2 increased by 112% The greatest lipid changes under hardening occurred in the neutral lipid TAGs FA changes, however, were less with Val/SO than with SO seedling Within the TAG subfraction of Val/SO total FAs increased 4-fold, 18 2 6 4-fold, and 18 3 3 8-fold

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Table 1 Effect of cold hardening on major fatty acids (FAs) in leaves and bark of 8-month-old citrus plants

	μg FA/g fresh tissue											
	16 0		18 0		18 1		18 2		18 3		Total	
	UH*	H*	UH	H	UH	H	UH	H	UH	H	UH	H
Sour orange seedling												
Leaf												
Total lipid	1772	1669	188	206	507	565	1090	2961	4538	4217	8095	9618
Neutral lipid	598	763	11	52	73	299	200	1520	277	732	1159	3366
TAG	90	333	9	45	41	260	89	1235	125	648	354	2521
Bark												
Total lipid	1001	1421	80	104	522	634	1291	2664	1217	1758	4111	6581
Valencia on sour orange												
Leaf												
Total lipid	1389	1366	86	97	476	228	711	1505	3070	2864	5723	6060
Neutral lipid	369	415	7	19	45	56	90	443	116	311	627	1244
TAG	34	150	6	12	18	42	48	357	53	257	159	818
Bark												
Total lipid	1250	1104	73	73	811	341	1422	1771	1703	1468	5259	4757

*Unhardened and hardened tissue, respectively

FAs in SO seedling bark increased by 60% under hardening conditions Increases were observed in all five of the bark major FAs Amounts of 18 3 in bark were from 27% (unhardened) to 42% (hardened) of those in leaves from the same plant Whether citrus bark contains lower concentrations of 18 3 rich glycolipids than citrus leaves is still to be investigated Changes in bark FA concentrations with hardening were smaller for Val/SO than for SO seedling Under hardening temperatures, 18 2 in Val/SO bark increased by only 25% while in SO seedling bark 18 2 increased by 106% The amount of 18 1 in Val/SO bark hardened samples was 50% less than that of the unhardened sample Again this reduction must be characteristic of the scion since this decrease was not observed with SO seedling

To compare citrus tissues for lipid changes in response to hardening the contribution of TAGs to the total lipid was calculated for each tissue Leaves from 8-month-old SO seedling changed the most upon hardening The percentage of TAGs in SO seedling lipid increased from 4 to 26% or 22 percentage units while the unit increase for 8-month-old Val/SO leaves was 11 (calculated from data in Table 1) With 16-month-old Val/SO, the unit increases in TAGs with hardening were upper leaves 14, lower leaves 11, bark 3, and roots 16 (calculated from data in Table 2) Saturated FAs accounted for the TAG increase in roots in contrast with the polyunsaturated FA increases in the other citrus tissues (Table 2) The presence of TAGs in citrus epicuticular leaf wax was not established Bands with TLC R_f values similar to TAGs were esterified, however, their fatty acid methyl ester (FAME) profiles were more characteristic of free fatty acids, an established component of citrus leaf epicuticular wax [7, 8] The absence of a definite difference between unhardened and hardened FAME profiles for this area was evidence that the TAG synthesized in the citrus leaf with hardening was not deposited in the epicuticular wax

Percent polyunsaturated FA values for TAGs of hardened samples were greater than total lipid, sterol esters or other acyl lipids from the same tissue (Table 2) With only one exception (sterol esters, lower leaves), the relative percentage value of 18 2 increased upon hardening of the plant This occurred along with decreases in relative percentages of 18 1 in leaves and in bark

To determine whether TAGs were possibly synthesized at the expense of other lipids during the hardening process, 'balance sheets' were prepared for both upper (Table 3) and lower (Table 4) 16-month-old Val/SO leaves In upper leaves total FAs increased by *ca* 8% This consisted of an 84% increase in neutral lipid and a 13% decrease in glycolipids In the three neutral lipid subfractions, FAs associated with TAGs increased by 226% while acids in neutral lipid more polar than TAGs, increased by 114% and sterol ester FAs decreased by 38% Monogalactosyl diglycerides could account for all of the decline in glycolipid FAs Profiles of the four phospholipids were similar to those in our first study [6]

Lower leaves of Val/SO had higher concentrations of FAs than upper leaves The differences were for unhardened samples 25% and for hardened samples 13% The greater amount of FAs in lower leaves was due mainly to 18 3 (Table 4) 18 2 was less in hardened lower leaves than the corresponding upper leaves In contrast with the upper leaves, total FAs from all three lower leaf lipid fractions were influenced by the hardening process Within the neutral lipid fraction, hardening increased TAG FAs by 62%, due primarily to 18 2 and 18 3 The FA decrease in glycolipids was due to 18 3 in monogalactosyl diglyceride Of the four phospholipids, phosphatidylcholine showed the greatest unhardened–hardened difference in total fatty acids (22%) Hardening increased the 18 2 content of all lipids with the exception of sterol esters, as observed with upper leaves

FAs of total lipid, the three lipid fractions and eight out of the nine subfractions all increased in unsaturation with hardening The increases ranged from 3 percentage units for monogalactosyl diglycerides in lower leaves to 27 for TAGs in upper leaves Hardening had more influence on

Table 2 Effect of cold hardening on major fatty acids (FAs) in tissues from 16-month-old Valencia on sour orange

	% Of total FAs		% Polyunsaturates*		Rel % FAs†					
					18 1		18 2		18 3	
Tissue/lipid	UH‡	H‡	UH	H	UH	H	UH	H	UH	H
Upper leaves										
Total lipid			66 7	72 9	7 4	4 5	19 7	32 6	47 0	40 3
TAG	6 8	20 4	51 1	78 2	14 2	6 9	30 0	53 3	21 1	24 9
SE	8 8	5 1	63 9	77 0	9 9	5 6	35 9	50 0	28 0	27 0
OL	84 4	74 5	68 3	71 4	6 6	3 8	17 2	26 0	51 1	45 4
Lower leaves										
Total lipid			68 7	74 2	6 6	4 1	16 5	23 5	52 2	50 7
TAG	6 1	16 9	64 9	77 6	16 6	7 6	24 0	35 3	40 9	42 3
SE	4 6	5 1	73 9	53 4	12 9	6 9	33 6	31 1	40 6	22 3
OL	89 3	78 0	68 7	74 8	5 5	3 2	15 1	20 4	53 6	54 4
Bark										
Total lipid			58 2	69 3	17 6	8 5	30 3	43 0	27 9	26 3
TAG	5 9	9 3	68 8	74 5	15 1	7 6	41 1	55 0	27 7	19 5
SE	1 5	0 8	55 1	60 5	28 2	17 9	32 7	43 4	22 4	17 1
OL	92 6	89 9	53 5	63 9	17 3	9 0	26 2	36 3	27 3	27 6
Roots										
Total lipid			36 5	39 6	32 2	32 4	25 9	31 0	10 6	8 6
TAG	56 6	72 7	44 8	40 5	31 7	32 3	27 4	30 4	17 4	10 1
SE	1 2	0 8	38 3	39 3	42 5	31 5	29 4	32 0	8 9	7 3
OL	42 2	26 5	33 9	38 4	33 2	31 8	27 1	31 1	6 8	7 3
Leaf wax										
TAG?	n d	n d	4 0	6 3	43 1	37 0	4 0	6 3	—§	—§

*(18 2+18 3)/(16+18+18 1+18 2+18 3)×100
†Relative per cent of the five major fatty acids
‡Unhardened and hardened tissues, respectively
§—if present—less than 0 1 rel %
SE = sterol ester, OL = other lipids, TAG = triacylglycerol, n d = not determined

Table 3 Effect of cold hardening on major fatty acids (FAs) in upper leaves of 16-month-old Valencia/sour orange

	μg FA/g fresh leaf											
	16 0		18 0		18 1		18 2		18 3		Total	
	UH*	H*	UH	H	UH	H	UH	H	UH	H	UH	H
Total lipid	1037	1002	119	92	331	218	883	1579	2101	1946	4471	4837
Neutral lipid	280	368	15	22	105	105	262	736	197	347	859	1578
TAG	101	136	4	11	43	68	91	527	64	246	303	988
N(p)	78	182	9	8	23	23	29	96	22	35	161	344
SE	101	50	2	3	39	14	142	113	111	66	395	246
Glycolipid	372	273	62	39	70	38	146	235	1667	1429	2317	2014
MGDG	72	33	7	3	25	10	88	136	1099	767	1291	949
DGDG	300	240	55	36	45	28	58	99	568	662	1026	1065
Phospholipid	385	361	42	31	156	75	475	608	237	170	1295	1245
PC	152	129	23	15	57	28	217	269	111	80	560	521
PE	80	88	8	7	25	10	172	226	64	43	349	374
PI	84	89	4	4	9	6	55	81	45	35	197	215
PG	69	55	7	5	65	31	31	32	17	12	189	135

*Unhardened and hardened leaves, respectively
N(p) = neutral lipid more polar than TAG, SE = sterol ester, MGDG = monogalactosyl diacylglycerol, DGDG = digalactosyl diacylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PG = phosphatidylglycerol

Table 4 Effect of cold hardening on major fatty acids (FAs) in lower leaves of 16-month-old Valencia/sour orange

	μg FA/g fresh leaf											
	16 0		18 0		18 1		18 2		18 3		Total	
	UH*	H*	UH	H	UH	H	UH	H	UH	H	UH	H
Total lipid	1285	1106	99	77	368	225	923	1282	2918	2768	5593	5458
Neutral lipid	337	441	22	27	106	102	203	459	284	508	952	1537
TG	55	126	8	11	56	71	81	326	139	391	339	925
N(p)	252	209	10	11	16	12	35	47	40	55	353	334
SE	30	106	4	5	34	19	87	86	105	62	260	278
Glycolipid	478	300	51	25	86	49	181	246	2165	1904	2961	2524
MGDG	100	50	7	3	30	14	109	152	1535	1200	1781	1419
DGDG	378	250	44	22	56	35	72	94	630	704	1180	1105
Phospholipid	470	365	26	25	176	74	539	577	469	356	1680	1397
PC	205	147	14	13	92	27	292	299	273	195	876	681
PE	106	83	5	5	27	9	172	180	114	79	424	356
PI	96	81	2	1	9	2	55	60	64	55	226	199
PG	63	54	5	6	48	36	20	38	18	27	154	161

*Unhardened and hardened leaves, respectively
For abbreviations consult Table 3

the upper than on the lower leaves Upper leaves are younger and thus generally would be more biologically active

In this study potted citrus plants kept under normal greenhouse conditions were the source of unhardened or 'control' lipid samples During the course of this study the question arose whether humidity and light differences between greenhouse and environmental chambers may account for the lipid differences presented in this study A recent study, however, minimizes the possible effects of these two parameters FA profiles of Val/SO plants kept in chambers at 30/21 1° and at 15 6/4 4° showed differences (unpublished data) comparable to those for greenhouse 15 6/4 4° (Table 1) Thus, as has been observed in other plants [9–11], cool conditions increase the quantities of unsaturated TAGs in vegetative tissues The present data substantiate the earlier results [6] that 18 2 increases in all lipids Furthermore, these increases occur primarily in upper leaves but also in other tissues 18 3 was preferentially present in lower leaves When the leaves were cold hardened this acid decreased as a component of monogalactosyl diglyceride in both upper and lower leaves Leaves from Val/SO had lower FA concentrations as well as a lower degree of unsaturation compared to leaves of the sour orange rootstock The grafted plants also showed less change in FA profiles when cold hardened Cold hardening increased the amounts of TAGs in citrus tissue The major change was an increase in 18 2

EXPERIMENTAL

Trees and hardening conditions Trees prior to hardening were grown in pots in a greenhouse under conditions previously described [6, 12] Trees used were 8-month-old seedling sour orange (*Citrus aurantium* L) and 8- as well as 16-month-old Valencia (*C sinensis* [L] Osbeck) budded to sour orange rootstock The trees were selectively matched for their similarity in health and size Unhardened trees were left in the greenhouse and cold hardened trees in controlled environment facilities for 4 weeks [6, 12] Temp regimes for cold hardened trees were 2 weeks of 21 1° days and 10° nights followed by 2 weeks of 15 6° days and 4 4° nights

Lipid extraction and fractionation Leaves from the top and bottom halves of each of six (three unhardened, three hardened) 16-month-old Valencia on sour orange trees were collected, washed, blotted dry and dewaxed with $CHCl_3$ [13] The main leaf vein was removed Roots and bark were removed from the plants Tissues were cut into small pieces and duplicate samples prepared (6 g) Enzymes were deactivated by boiling the sample in MeOH for 10 min In the scion–rootstock study all of the leaves from each of the four 8-month-old plants (two unhardened, two hardened) were removed Leaves from each plant were pooled and triplicate samples (15 g) taken In the scion–rootstock study, bark from above the graft was taken for samples Leaves in the scion–rootstock study were not dewaxed or deveined All tissue samples after enzyme deactivation were stored in MeOH at −60° until extracted within 2 weeks Leaf, bark and root samples were extracted for lipids as described previously for leaves [6] Wax extracts from upper and lower leaves were pooled, filtered, dried, concd and a band corresponding in R_f to TAGs fractionated by TLC [14] Portions (10%) of leaf, bark and root samples were separated into sterol ester, TAG and other lipid (OL) fractions on 0 25 mm silica gel TLC plates with hexane–Et_2O (9 1) OLs included all lipid components chromatographing from the origin to the TAG band Leaf samples (80%) were separated into neutral, glycolipid and phospholipids by CC [6] These three lipid fractions were further separated into their component lipids by TLC [6] Acyl lipid bands were located under UV after spraying with Rhodamine 6G On the neutral lipid plate the entire area from origin to TAGs was labeled N(p), neutral lipid more polar than TAGs In *ca* 40% of the samples the transferase activity of phospholipase D was not completely deactivated by boiling MeOH Phosphatidyl methanol (PM), formed from

phosphatidylcholine by this enzyme, eluted with the glycolipids from the silica gel column The PM was isolated by TLC as a narrow band between monogalactosyl diglyceride and digalactosyl diglyceride Analyses of FAMEs from PM were combined with phosphatidylcholine FAME analyses

Lipid analyses Aliquots of total lipid, lipid fractions and individual lipids were determined as FAMEs prepared by methanolysis with NaOH–BCl_3–MeOH [6] with methyl heptadecanoate as int standard FAMEs were prepared from bands scraped from TLC plates without prior removal of silica gel FAMEs were analysed by FID-GC using glass columns packed with 3% SP-1000 on GasChrom Q [6] In these studies total FAs were considered to include only the five major C_{16}–C_{18} fatty acids, 3-*trans*-hexadecenoate present in phosphatidylglycerol at 10–15% and C_{12}–C_{15} acids were excluded from FAME calculations Two FAMEs of undetermined structures and methyl palmitoleate, major components of sterol ester and N(p), were not always resolved from methyl palmitate under the GC conditions They are reported as 16 0 in Tables 1–4 Values in tables are the mean of duplicate analyses of from two to six samples The coefficient of variation (CV) determined for several mean ranges (MR) of values in Tables 1, 3 and 4 were as follows MR 1–50 μg/g, CV 10–35%, MR 51–300 μg/g, CV 7–10%, MR above 300 μg/g, CV 3–7%

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