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INFLUENCE OF COLD TEMPERATURES ON LEAF LIPIDS OF *HIBISCUS ROSA-SINENSIS*

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Key Word Index—*Hibiscus rosa-sinensis*; Malvaceae; lipids; linoleic acid; cold hardening; cyclopropene fatty acids; freeze stress; triacylglycerol; polyprenols.

Abstract—Triaclyglycerols (TAG) accounted for 15% of the leaf acyl lipids in a *Hibiscus rosa-sinensis* plant that survived the January 1986 freeze in Florida, U.S.A. This high TAG level suggested that the plant had cold hardened. The TAG concentration in leaves on new shoots from this plant was greater than that in new leaves on new shoots from five plants frozen to the roots during the freeze. However, five months later, the amounts of TAG in the six *Hibiscus* plants were nearly the same. The minor differences in TAG levels, however, related to the survival rate of these six plants during subsequent freezes. In addition to linoleic acid, two cyclopropene fatty acids, malvalic and sterculic, were major constituents of the leaf TAG. *Hibiscus* plants placed in environmental chambers under control and cold-hardening regimes optimized for *Citrus* showed TAG concentrations of 7% in control and 20% in hardened plants. A survey of neutral lipids in *Hibiscus* and other plants showed that plastoquinone A (B) and α-tocopherol decreased and plastoquinone C increased under cold-hardening conditions. Polyprenols, a major component of *Hibiscus* leaves under normal conditions, declined greatly under cold-hardening regimes.

INTRODUCTION

Dramatic changes occur in the leaf lipids of *Citrus* when the plants are placed in a cold-hardening environment; triacylglycerol (TAG) species rich in linoleic acid (18:2) accumulate [1–4]. The increases are greater in cold-tolerant than in more cold-sensitive cultivars [1–4]. Since these TAG increases with cold hardening have not been reported for temperate plants, the question arose whether *Citrus* was unique or whether TAG synthesis might be a general reaction with subtropical plants placed in a cool environment. Following the 15 January 1985, freeze in central Florida a freeze survey of surviving plants revealed two shoots of a *Hibiscus* bush that had survived the freeze without any sign of damage. Seventeen other *Hibiscus* bushes in the same area of the yard were killed to the roots. Preliminary lipid analyses from the surviving *Hibiscus* showed TAG to be a major constituent of uninjured leaves. Thus, *H. rosa-sinensis* was selected as a model system for studying lipid changes in cold hardiness of subtropical plants. The objectives of this study were to (i) determine whether the TAG level in the hibiscus bush that survived the freeze was unusually high for this genus, (ii) determine whether new shoots from roots of this hardy plant produced leaves showing the same high amounts of TAG, (iii) determine whether TAG synthesis could be enhanced by placing *Hibiscus* plants in cold hardening environmental chambers optimized for cold hardening *Citrus*, and (iv) to observe other lipid changes that occur during cold hardening of subtropical plants.

RESULTS AND DISCUSSION

Studies in which lipid composition of leaves have been examined have generally ignored TAG as being a possible major constituent. In our previous cold-hardening studies [1–4], the TAG level in nonhardened citrus leaves ranged from 2 to 7%, while in hardened leaves the TAG/total lipid range was 11–40%. The mean TAG concentration for the *H. rosa-sinensis* plant that survived the 1985 freeze (HHl) was 15% (Table 1), which suggested that this plants survival might be associated with a high level of TAG in the leaves and that the plant was cold hardened. Three weeks prior to the January 1985 freeze, temperatures ranged from a night low of 14° to a day high of 28°F; temperatures not conducive to cold hardening of *Citrus* and, presumably, not to *Hibiscus*. Because HHl was the only survivor out of 18 equally healthy plants, we hypothesized that it was genetically different.

Structures of two unusual fatty acids in HHl were determined to be a C_{18} (malvalic) and a C_{19} (sterculic) cyclopropene fatty acids (CPFA). CPFAs were first discovered in leaves of *Malva parviflora* [5]; however, in the Mervalus family, CPFA are generally concentrated in seeds. In *M. sylvestris* and *M. parviflora* leaves. CPFA were more prevalent in neutral than in polar lipids and occurred in larger amounts in winter than in summer [6, 7]. In none of the leaf studies, however, were CPFA determined to be specifically associated with TAG. CPFA accounted for 39% of TAG fatty acids in HHl (Table 1), whereas in the other lipids combined, the amount of CPFA was 0.4%

Chilling resistance of a plant has been correlated with the degree of fatty acid unsaturation; the postulation being that an increase in unsaturation will lower the temperature at which phase separation of the membrane lipid occurs. TAG lack the amphipathic physical properties common to membrane lipids. Nevertheless, we have postulated that in some presently unknown manner,

Table 1. Seasonal variation of TAG and component fatty acids in leaves of hardy *Hibiscus* (HH) and five nonhardy *H. rosa-sinensis* plants (NH)

Sample date H/NH	TAG (%)	Relative percentage of major fatty acids in TAG					
		16:0	18:1	18:2	18:3	Malvalic	Sterculic
24 Jan 85							
HH1	15	10	4	20	23	35	4
15 May 85							
HH2	37	2	11	64	9	10	4
NHH2	18±4	3±1	9±3	51±8	12±2	15±7	8±2
18 Jun 85							
HH2	34	3	8	27	7	40	13
NHH2	20±7	3±1	10±4	47±9	11±1	17±9	10±2
17 Jul 85							
HH2	20	4	6	50	18	17	4
NHH2	17±5	4±3	9±4	57±7	17±4	8±6	2±1
23 Aug 85							
HH2	13	5	5	15	11	51	11
NHH2	16±4	7±3	6±1	27±5	18±6	29±7	9±4
23 Sep 85							
HH2	13	7	6	20	15	40	8
NHH2	14±2	9±5	7±2	28±5	18±6	27±9	7±4
25 Dec 85							
HH2	12	9	4	21	13	45	4
NHH2	10±3	7±5	4±1	34±10	20±6	27±11	5±2
9 Jan 86							
HH2	13	9	3	22	24	37	4

the increased content of 18:2, as a component of TAG in citrus leaves, lowered the temperature at which freezing of the leaves occurred [1–4]. A suggestion has been made that TAG does not play a direct role in maintaining this fluidity; the postulation being that this lipid stores fatty acids produced by a low temperature-induced breakdown of MGDG and DGDG which may occur from photoinhibition [8]. This does not seem too probable since with both *Citrus* and *Hibiscus*, 18:2 is the major constituent of TAG whereas linolenic (18:3) is the major acid in the two glycolipids. 18:2 in citrus leaf TAG increased from 22 to 47% while palmitic (16:0), steartic (18:0) and oleic (18:1) decreased and linolenic (18:3) stayed the same when subjected to a hardening regime [1–4]. The melting point of a fatty acid is dependent not only on the degree of unsaturation, but also on chain length and spatial configuration. Zarins *et al.* [9] determined the decreasing order of melting points for CPFA to be sterculic > 18:1 > malvalic > 18:2. The effect of the fatty acids on the physical properties of TAG also depends on the combinations of these fatty acids in the TAG molecule [3]. Thus, the combined influence of 18:2 and malvalic in TAG of the hardy *Hibiscus*, HH1 (Table 1) should equal the influence of 18:2 in TAG of citrus for lowering the temperature at which freezing of the leaves would occur. This postulated correlation is even more realistic if we consider that HH1 survived a severe freeze. The citrus data were from cold-hardened plants.

Table 1 shows percentages of TAG in total lipid and component fatty acids of these TAGs for the new growth from new shoots of the hardy habiscus (HH2) and from new shoots from five other *Hibiscus* plants that froze to the roots during the 1985 freeze, NHH2. During this study, the amount of lipid in the leaves of the six plants was not significantly different (unreported data). On May 15, the concentration of TAG in HH2 was twice the mean level in the other plants, NHH. One month later, only minor changes had occurred in the amounts of TAG in HH2 and NHH2. However, in July and continuing to the end of the year, TAG concentrations were the same in HH2 and NHH2; both declining to 13–15% observed for HH1 and for HH2 sampled 9 January 1986. If we consider that a high content of TAG in leaves is a marker for cold tolerance, then it is probable that during May–June HH2 could be identified as a cold-hardy type. Further evidence that leaf TAG synthesis under hardening regimes occurs mostly with nontemperate plants was that TAG was not found in leaves of *H. syricus* and in the leaves of *Petunia hybrida* under identical growing conditions to that of *H. rosa-sinensis*.

Discrete but minor trends were observed in the relative percentages of 16:0, 18:1, 18:3 and sterculic acids in TAGs of both HH2 and NHH2 during the May–December 1985 season. During this period, 16:0 and 18:3 increased while 18:1 and sterculic acids decreased. The rates of change, however, for all four acids in both sets of *Hibiscus* were less than 1.3% per month. Moreover, the rates of change for 18:2 and malvalic acids ranged from 2.2 to 5.2% per month with the rates of HH2 being twice that of NHH2. Plots of percentage × time for 18:2 and malvalic acids were very poorly correlated, e.g. $R = 0.62–0.65$; but, when these percentage per TAG values are converted to percentage per total lipid values, the biosynthesis of these acids in TAG is more apparent (Fig. 1). During May (and possibly also in April), 18:2 in TAG is extensively synthesized, then during June the two

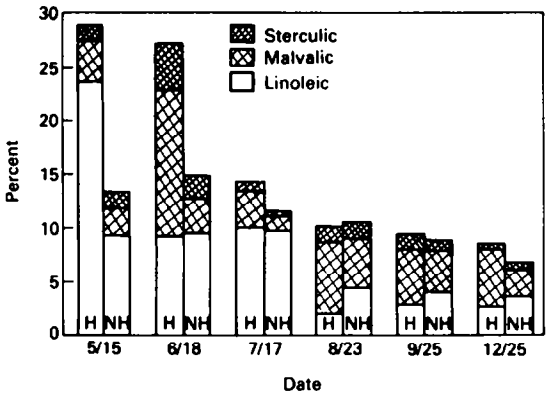

Fig. 1. Tag fatty acids in hardy (H) and nonhardy (NH) *Hibiscus* as percent of total leaf lipid.

CPFAs are synthesized and synthesis of 18:2 is reduced. During the remaining months, there is a decline in 18:2 but an increase in malvalic acid. These biosynthetic changes were not observed in NHH2 to the extent that they were observed in HH2 (Fig. 1). Thus, as observed with citrus [1–4], increases in polyunsaturates within TAG were greater for hardy than for cold-sensitive cultivars. Yano *et al.* [7] have shown the most likely biosynthetic pathway for malvalic acid is by α oxidation of sterculic, which is derived through the intermediate dihydrosterculic from 18:1. 18:2 also is derived from 18:1. Thus, from the data presented in this study it appears that, during May, 18:1 is desaturated to 18:2. In June, however, 18:1 is diverted towards the synthesis of CPFA.

Twenty-four hr prior to the 26 December 1985, freeze, leaves from six *Hibiscus* plants in the seasonal study were sampled in anticipation of the next-evening freeze. The 26 December 1985, freeze was not as severe as anticipated and only one of the six plants showed extensive damage when sampled 16 hr after the freeze. Nineteen days following the subsequent 1986 freeze, further extensive freeze damage was observed in all six plants. Various trends were observed in the intensity of damage with the TAG contents and component polyunsaturates in the leaves prior to the first freeze (Table 2). Plants A and B had the lowest amounts of TAG and greatest damage, while plants, D, E, and F had the highest TAG concentrations and the least freeze damage. High contents of 18:3 in plant E and malvalic acid in plants D and F accounted for total TAG polyunsaturate concentrations being higher than in the other, more damaged plants.

Leaves from *Hibiscus* plants held in environmental chambers at normal (NHH3) and cold-hardening temperatures (HH3) for four weeks showed no difference in their total lipid content. This contrasts with citrus where cold-hardening regimes increased the amount of total lipid from 6 to 67% [1–4]. Since total acyl lipid levels in this *Hibiscus* study were essentially the same in all plants, the GC analyses are presented in Table 3 as a balance sheet. Eleven major fatty acids from three lipid classes and some individual lipids are given as percentages of total fatty acids. In *Citrus*, the 6–67% increase in total fatty acids was largely due to a significant increase in 18:2 and a decrease in 18:1 [1–4]. In *Hibiscus*, individual acid differences between NHH3 and HH3 were less than those observed with *Citrus*. Of the five major acids, 16:0 decreased by 4% and 18:2 increased by 3%. Two minor acids, 3-*trans*-hexadecenoic (3*t*-16:1) and sterculic, increased 0.6 and 0.9%, respectively, while the hydroxy fatty acid decreased by 1.3%.

The nearly two fold increase of 3*t*-16:1 with hardening is interesting in light of recent studies linking this acid, found exclusively in phosphatidyl glycerol, with the cold sensitivity of plants [10–14]. To date, we have not determined in *Citrus* or *Hibiscus* the 16:0/16:0 and 16:0/3*t*-16:1 C-1,2 ratios. However, if chilling-resistant species tend to have low amounts of 3*t*-16:1, then our results in this study do not agree with the theory proposed in ref. [10, 11].

The cold-hardening regime increased the total fatty acids in phospholipids by 2.4% and in neutral lipids by 13.1%. These increases were at the expense of the two glycolipids, MGDG and DGDG. Within the phospholipids, the major change with hardening was an increase in 18:2 at the expense of 16:0. This was in agreement with the results obtained previously with *Citrus* [1, 2, 4]. Minor but definite decreases in the two CPFAs in phospholipid were observed in phosphatidyl choline. This finding adds to the knowledge that phosphatidyl choline is a precurser to TAG in many plants. Three quarters of

Table 2. Leaf lipid composition of six *Hibiscus* plants before* adjacent freezes† and accumulated leaf kill from these freezes

		Percentage of TAG fatty acids					
Hibiscus	TAG (%)	18:2	18:3	Malvalic	Sterculic	Total	Leaf kill (%)
A	6.5	3.19	1.17	.90	.30	5.56	85.0
B	8.2	3.17	1.61	2.45	.34	7.57	100.0
C	10.0	3.35	1.82	2.55	.42	8.14	78.0
D	12.2	2.54	1.58	5.44	.47	10.03	67.0
E	12.4	2.89	3.53	2.89	.21	9.52	43.0
F	12.8	3.21	1.75	5.68	1.08	11.72	67.0
Mean	10.4	3.06	1.91	3.32	.47	8.76	73.3
s.d.	2.6	.29	.83	1.87	.32	2.14	19.3

*Leaves sampled 25 December 1985.
†6 to 9 hr below 2° on 26 December 1985 and 9 January 1986, Winter Haven, Florida.

Table 3. Range of fatty acids in total lipid between nonhardened (NHH3) and hardened (HH3) *H. rosa-sinensis* leaves (relative percentage)

			Glycolipids		Neutral lipids		
FA/NHH or HH	Total lipid	Phospholipids	MGDG	DGDG	TAG	SE ± PQ-B	Other
14:0							
NHH 3	.55	.07	.04	.01	.11	.23	.09
HH 3	.66	.07	.04	.01	.20	.26	.08
16:0							
NHH 3	19.55	13.85	.76	2.43	.59	.27	1.25
HH 3	15.75	11.48	.38	2.00	1.41	.31	.17
16:1							
NHH 3	.66	.07	.05	.01	.17	.11	.25
HH 3	.56	.07	.04	.01	.22	.13	.09
3t-16:1							
NHH 3	.85	.85					
HH 3	1.47	1.47					
18:0							
NHH 3	2.21	1.09	.15	.52	.17	.08	.20
NN 3	2.12	1.14	.07	.35	.32	.07	.17
18:1							
NHH 3	2.97	2.14	.23	.10	.27	.13	.10
HH 3	3.64	2.23	.08	.07	1.02	.14	.10
18:2							
NNH 3	21.02	6.89	7.02	5.09	1.56	.32	.14
HH 3	23.90	10.42	2.08	2.59	8.30	.35	.15
18:3							
NHH 3	44.43	7.15	22.16	13.43	.98	.31	.40
HH 3	45.33	7.92	19.47	12.93	4.68	.28	.05
Malvalic							
NHH 3	2.76	.22			2.54		
HH 3	2.39	.01			2.38		
Sterculic							
NHH 3	.76	.21			.55		
HH 3	1.62	.13			1.49		
Hydroxy							
NHH 3	4.24						4.24
HH 3	2.56						2.56
Total							
NHH 3	100.00	32.54	30.41	21.99	6.94	1.45	6.67
HH 3	100.00	34.94	22.16	17.96	20.00	1.54	3.40

the decrease in glycolipid acids was due to the decrease in 18:2 in both MGDG and DGDG. In *Citrus*, we found with temperature cold hardening that only MGDG decreased. This decrease was due almost entirely to 18:3. It is very probable that *Hibiscus* gives us a clearer picture as to the origin of 18:2 in TAG with cold hardening. As expected, the increase in neutral lipids of cold-hardened *Hibiscus* was confined almost entirely to TAG, increasing from 7% in NHH3 to 20% in HH3. Data suggest that in *Hibiscus*, TAG hardening increases the concentration of 10 of the 11 fatty acids with the major increases occurring with 18:2 and 18:3. In *Citrus*, 18:2 alone accounted for the majority of the increase in TAG.

Neutral lipids in various tissues were ovserved as fluorescent and absorbing bands on TLC plates sprayed with Rhodamine 6G (Table 4). TAG with a R_f of 0.45 was rated 5 in the original hardy *H. rosa-sinensis*, various cold-hardened *Citrus* which were reexamined, and in *H. rosa-sinensis* placed in the cold-hardening chamber for four weeks. Ratings of 4 for this lipid were given for hardy *H. rosa-sinensis* leaves stripped of their epicuticular wax prior to extraction, and also for *H. syricus* flower petals. Ratings of 1 or 2 were given to nonhardened samples, whereas TAG was not detected in a leaf lipid extract of *Petunia* plants that survived the January 1985 freeze, nor in the epicuticular wax of HH2 and the leaves of *H. syricus*. Inability to detect TAG in these three tissues is evidence that TAG is not a major leaf component of temperate plants, nor is it deposited to any appreciable extent in the outer wax layer of subtropical plants when they are cold hardened.

A fluorescent band at R_f 0.24 which differed between nonhardened and hardened hibiscus samples was shown to contain polyprenols. In seven TLC systems, its R_f values were identical with a ficaprenol standard and slightly different from a dolichol standard. When plates were sprayed with a molybdenum blue reagent and heated at 115°, farnesol, geraniol and ficaprenol standards all gave

Table 4. Survey of neutral lipids in *Hibiscus rosa-sinensis* and tissues of other plants

Plant	Tissue	Detection rating† of lipids‡					
		TAG	PP	Toc	PQA(B)	PQC	Ket
Citrus H*	Leaves	5	—	1	1	3	—
Hibiscus rosa-sinensis H 1	Leaves	5	2	1	1	3	2
Hibiscus rosa-sinensis H 2	Leaves	5	2	1	1	3	2
Hibiscus rosa-sinensis NH 2	Leaves	1	5	3	3	1	2
Hibiscus rosa-sinensis H 2	Dewaxed leaves	4	2	1	1	3	—
Hibiscus rosa-sinensis H 2	Wax of leaves	—	—	—	—	—	5
Hibiscus rosa-sinensis H 3	Leaves	5	1	—	—	3	2
Hibiscus rosa-sinensis NH 3	Leaves	1	5	3	3	1	2
Hibiscus syricus H	Leaves	—	3	1	1	—	—
Hibiscus syricus H	Flowers	4	2	—	—	—	—
Petunia H	Leaves	—	—	—	—	—	—

*H, Cold hardy or cold hardened; NH, nonhardened or control; 1, survival of freeze; 2, foliage on new shoots; 3, conditioned in chambers.

†Relative fluorescence-absorbance intensities of neutral lipids with Rhodamine GG-UV in 20–50 mg crude lipids on TLC plates rates (—), not detected-5, very strong.

‡TAG, triacylglycerol; PP, polyprenols; Toc, α-tocopherol; PQA(B), Plastoquinone A or B; PQC, plastoquinone C; Ket, ketone in epicuticular wax.

a red-violet colour, whereas dolichol, amyrin, lanosterol and sitosterol standards, all gave gray colours. The first set of standards are all isoprene alcohols in which every isoprene unit is unsaturated, whereas in the second set of standards, the first isoprene unit is saturated or the hydroxyl moiety is on the sterol ring. Thus, this red-violet colour with Molybdenum Blue reagent observed with the R_f 0.24 compound is evidence that the compound has a double bond in the first isoprene unit. HPLC analysis of the R_f 0.24 lipid from NHH2 showed the lipid to be a mixture of polyprenols nearly identical to that observed in the ficaprenol standard. The number of 5-carbon units and relative percentages were: 8(1.5%), 9 (6%), 10 (17%), 11 (45%), 12 (24%) and 13 (2%). Minor amounts of homologs having R_fs slightly greater than these polyprenols may be homologues of dolichol (α-saturated) or the all *trans* homologue, solanesol. Polyprenols participate in the biosynthesis of asparagine-linked glycoproteins in yeasts, plants, and animals [15]. They are generally found in young plants during germination [16] or in seeds [17]. In this study, polyprenols appear to have an inverse relationship with the concentration of TAG (Table 4). Ratings of (−), 1 and 2 were given to hardy *H. rosa-sinensis*, cold-hardened *Citrus*, *Petunia*, chamber-hardened *H. rosa-sinensis*, dewaxed HH2 and *H. syricus* flowers, while ratings of 3 to 5 were observed for leaves of *H. syricus* and nonhardened *H. rosa-sinensis*. The presence of polyprenols in portions of a plant where chloroplasts are not present, such as flower petals, raises a question as to the role of these compounds in mature plants, especially in relation to cold hardening of the plant. In the chamber-hardening experiment, we observed glycolipids to decrease with hardening. It is possible that polyprenols are the carriers of sugars from these glycolipids to the membrane proteins.

Plastoquinones are generally classified into three groups, e.g. PQ-A, B, C. PQ-A, 2,3-dimethyl-5-prenyl benzoquinone with a 45 carbon prenyl side chain, was observed under UV with Rhodamine 6G as an absorbing band. The compound gave the typical plastoquinone blue colour when the plate was sprayed with reduced Methylene Blue reagent [8]. In the hexane–ethyl ether TLC system, this compound had an R_f of 0.68 which was not always resolved from carotene, sterol esters and hydrocarbons centered at R_f 0.75. Also in this TLC system, PQ-B would not be resolved from PQ-A. PQ-Bs are PQ-C with a hydroxyl moiety on a prenyl group esterified with $C_{16}C_{18}$ fatty acids. Its nonpolar nature would most likely prevent PQ-B from being resolved from PQ-A using our weakly polar TLC system [18]. To date the band at R_f 0.68 has not been characterized as PQ-A or PQ-B. Plastoquinone C can be considered to be unesterified PQ-B. The PQ-C band at R_f 0.38, like PQ-A, absorbed light with Rhodamine 6G and turned blue with reduced Methylene Blue. The concentration of this compound had an inverse relationship with that of PQ-A.

Alpha tocopherol was detected as a Rhodamine 6G absorbing band at R_f 0.32 giving an instant pink colour when sprayed with $FeCl_3$-dipyridyl. The intensity of the compound paralleled that of PQ-A (B) and, to a lesser extent, that of polyprenols, suggesting that all three compounds are changed to other compounds when the plant is subjected to cold hardening regimes. Krol and Huner [19], however, have observed PQ-A and α tocopherol in wheat to be in higher concentrations when grown at cold-hardening temperatures than when grown at normal temperatures.

A Rhodamine 6G fluorescent band at R_f 0.57, when subjected to transesterification with NaOH–BCl_3, failed to produce FAMEs. The band was very strong in epicuticular wax, but was not detected in the lipid extract of leaves from which the wax had been stripped. The amount of this compound did not change under the cold-hardening regime. These data along with the R_f of the lipid suggest that it is an epicuticular wax ketone(s).

Under low temperature conditions, *H. rosa-sinensis* behaves in many ways like *Citrus*; however, in a number of ways it is unique. TAGs are present in higher amounts in nonhardened *Hibiscus* leaves than in nonhardened *Citrus* leaves. Fatty acid composition of *Hibiscus* TAG is more complex than for *Citrus* TAG, with up to 62% of the acids having a cyclopropene ring. The reporting for the first

time of CPFA present almost exclusively in TAG of leaf lipids raises a number of questions as to what purpose these CPFAs might have in plants and specifically, what role they have in protecting the plant from cold damage. When subjected to a cold-hardening regime idealized for *Citrus*, *Hibiscus* likewise increased its leaf TAG content. Instead of increasing TAG at hardly any expense to other *Citrus* lipid species, *Hibiscus* increased its TAG at the expense of its two glycolipids. Polyprenols were not observed in previous *Citrus* studies. Minor amounts of polyprenols may have been overlooked, however, since the probable importance of this lipid in cold hardening was not known at that time. In this study, polyprenols showed an inverse relationship with TAG and PQ-C and a direct relationship with PQ-A (B), α tocopherol, MGDG and DGDG.

A recent study [20] states that evidence is overwhelming that polyunsaturated acids are not critical in maintaining the fluidity of the lipid biolayer for life processes of the biological membrane in plants. However, the probability that neutral lipids such as TAG and polyprenols are involved in fluidity is now suspect. Evidence presented in our present study and in our previous studies [1–4] with *Citrus* suggest that TAG may play a significant role in protecting subtropical evergreen plants from stresses such as cold.

EXPERIMENTAL

Plants and temperature acclimation. Twenty *H. rosa-sinesis* plants were planted in July 1983 in Winter Haven, Florida. All plants were killed to the roots during the 24 December 1983 freeze. Eighteen plants regenerated from the roots following the freeze and 17 of these again killed to the roots during a January 23, 1985, freeze. However, 2 of 10 shoots survived on one *Hibiscus*. The 10 leaves on these shoots were mature, bright green and displayed no signs of freeze damage. Four of these leaves, selected for uniformity, were taken for a composite sample of the hardy *Hibiscus*, HH1. This plant and the roots of the other 17 plants were kept mulched and watered. By 15 May 1985, new shoots had reemerged from HH1 and 15 of the other *Hibiscus* plants; leaves appeared to be fully mature. Three leaf samples were taken from 5 of the rated nonhardy plants (designated NHH2) and from new shoots of the hardy *Hibiscus* (designated HH2) on 15 May, 18 June, 17 July, 23 August, 23 September and 25 December 1985. Leaves from the hardy *Hibiscus* plant were also sampled on 9 January 1986, 2 weeks after the 26 December 1985, freeze and just prior to the 9 January 1986, freeze. A 20-leaf composite sample was obtained from flowering *Petunia* × *hybrida* plants which withstood the 23 January 1985 freeze (designated NP). On 23 August 1985, 10-leaf and 5-flower (minus calyx) composite samples were obtained from a *H. syricus* plant adjacent to the *H. rosa-sinensis* plants (designated HsL and HsF). For the controlled hardening study, two *H. rosa-sinensis*, var. Single Scarlet from a local nursery were selected for their uniformity in size, health and foliage. One plant was placed in the control and the other in the cold-hardening chamber, along with *Citrus* and avocado plants and subjected to an optimum *Citrus* cold-hardening regime [1] for 4 weeks. Ten-leaf composite samples were designated NHH3 for control and HH3 for cold-hardened samples. On 11 May 1985, 10 leaves of HH2 were individually dipped in $CHCl_3$ for 1 min to remove epicuticular wax, the composite leaves then extracted; leaf extract = HH2-EW, wax extract = HH2W.

Lipid extraction and fractionation. Leaves and flower petals of *H. rosa-sinensis* and related plants were extracted for lipids as in previous studies [1–4] with the following modifications. A homogenizer replaced the blender for all extns of samples. In order to prevent any degradation of the sensitive cyclopropene ring, enzymes were not deactivated with boiling MeOH as in previous *Citrus* studies. Portions of lipid extracts were separated into TAG and 'other lipid' by TLC on 0.25 mm silica gel G plates with hexane–Et_2O (4:1). In the environmental chamber study, two TLC solvent systems were used (i) neutral lipids were sepd into sterol esters-plastoquinone B, TAG and other neutral lipid (lipid above polar lipid at origin and below TAG) with hexane–Et_2O (4:1) (ii) lipids were sepd into neutral lipids, MGDG, DGDG and phospholipids with Me_2CO. In a survey study, the hexane–Et_2O system was used. Polyprenols, α tocopherol and plastoquinones were eluted from the silica gel with $CHCl_3$ in preliminary identification studies. For determination of CPFA and other related structures, the methods of refs [21–23] were modified using 0.25 mm silica gel G TLC plates. For detection of lipids on TLC plates, Rhodamine 6G-UV light was used in recovery studies while phosphomolybdic acid, reduced methylene blue and $FeCl_3$–dipyridyl sprays, as well as Rhodamine 6G-UV, were used in the neutral lipid survey.

Lipid analyses. Portions of total lipid, lipid fractions and individual lipids were determined as fatty acid Me esters (FAME) prepared by methanolysis with NaOH–BCl_3–MeOH [1–3] after preliminary analyses of FAMEs by this method showed only slight destruction of the cyclopropene ring when compared to FAMEs prepared with NaOMe as catalyst. Oil from *Sterculia foetida* nuts was extd and used for stds of malvalic and sterculic acids. FAMEs were prepd from bands scraped from TLC plates without prior removal of silica gel. FAMEs were analysed by FID-GC using both packed glass and wide-bore columns. Packed columns. were 1.83 × 2 mm packed with 10% SP-1000 or with 5% SP-2100 [24]. Conditions were oven temp. SP-1000 190°, SP-2100 200°, inj and det both at 250° and He flow of 30 ml/min for both columns. Glass packed columns (1.83 × 2 mm) used were 3% SP-1000 at 180° or 5% SP-2100 at 200° with inj and det at 250° and He flow of 12.5 ml/min. In addition to the above four packed columns, normal, hydroxy and cyclopropene FAMEs were analysed on 30 m × 0.53 mm DB-WAX, 1.0 μm and DB-1, 1.5 μm Megabore FSOT columns. Temp programs were DB-Wax 175°/7 min, 1°/min to 205°, hold 10 min; DB-1 180°/17 min, 3°/min to 200°, hold 7 min. For both wide-bore columns, flow was 8.5 ml/min (He) with inj and det at 250°. Polyprenols were analysed by HPLC using 150 mm octadecyl column, solvent *iso*PrOH–MeOH (3.7), flow 1.0 ml/min, UV detector at 210 nm. HPLC standards were dolichol, ficaprenol and solanesol.

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