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FLAVONOID CHANGES IN DEVELOPING LEMONS GROWN *IN VIVO* AND *IN VITRO*

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Key Word Index—*Citrus limon*; Rutaceae; lemon; tissue culture; flavonoids; diosmin; eriocitrin; hesperidin; rutin.

Abstract—Concentrations and HPLC profiles of the major flavonoids of lemon juice vesicles (*Citrus limon* cv. Eureka) were determined for tree-grown fruit 2–55 mm diameter and in 25–30 mm diameter fruit halves cultured *in vitro* for up to four months. In tree-grown fruit, the total amount of hesperidin per lemon accumulates rapidly from fruit set to its maximum value at the 25–30 mm diameter stage; and thereafter the concentration continually decreases as the fruit increases in diameter. At the 25–30 mm stage, the total amount of eriocitrin begins to increase rapidly and continues until the fruit reaches full size. The amount of diosmin per fruit increases gradually throughout the development of the lemon. Flavonoids of juice vesicles from *in vitro* cultured fruit halves show a pattern of development similar to fruit grown on the tree. In contrast, flavonoid patterns from callus derived from juice vesicles were quite dissimilar to those of developing fruit.

INTRODUCTION

The major flavonoids of citrus, identified in the late 1950's and 1960's by Horowitz and Gentili [1–6], were studied with respect to their taste characteristics [7]. Numerous quantitative studies on citrus flavonoids have been conducted since then [8–11], and their role in the quality of processed citrus juices has been extensively covered [12, 13]. The flavonoid compounds in citrus, such as hesperidin and naringin, greatly influence the quality of both the fresh fruit and processed products. Hesperidin is a significant component of the cloud in lemon and orange juice [14], and naringin plays a major role in the bitterness of grapefruit [15].

The study of flavonoid synthesis in young citrus has been rather limited. Maier and Metzler [16] and Albach *et al.* [17] demonstrated the rapid accumulation of naringin in very young grapefruit, and this correlated with high enzyme activities of the fruit [18]. Much of the general information on the biosynthesis of flavonoids was derived from studies using parsley suspension cultures [19, 20]. Rapidly growing citrus callus tissue can be successfully obtained from a variety of explant types [21–24]. However, citrus callus tissue apparently has an altered flavonoid pathway, since it has been reported that naringin was not produced in grapefruit callus [25]. Isolated lemon juice vesicle clusters and fruit parts containing vesicles of lemon have been established and maintained in culture [26, 27]. However, those cultures also produced substantial callus growths on the vesicle surfaces.

In this work we cultured small lemon fruit halves [28]. This technique has been valuable in the morphological study of lemon juice vesicles, since callusing from vesicles was minimal (to none) and the proportion of normal appearing vesicles in culture frequently exceed 90% of the vesicle population. The value of this cultured juice vesicle system for studying the biosynthesis, metabolism and regulation of the lemon flavonoids would be greatly enhanced if it could be shown that the flavonoid metabolism of the cultured vesicles is similar to that of tree-grown fruit. This is a report of a comparative study of the patterns of the major flavonoids during growth of tree-grown and cultured lemon juice vesicles.

RESULTS AND DISCUSSION

HPLC patterns

The chromatograms in the present work, which are from extracts of mostly young fruit, reveal that there are many more presumptive flavonoid peaks occurring than have been reported in the literature [1–4, 6]. The chromatograms in Fig. 1 illustrate the differences in the UV absorbing compounds between young and mature lemons. Identification of the major flavonoids was accomplished by comparing their spectral properties and retention times with known standards. They are eriocitrin (ERC), hesperidin (HSP), rutin (RTN), and diosmin (DSM). The unknown compounds represented by peaks UK1, UK2, and UK3 have spectra characteristic of the flavanones (UV absorption maxima: 286, 284 and 286 nm, respectively) [29]. The compounds represented by peaks UK4, UK5 and UK6 have spectra characteristic of flavones. Many minor peaks have been observed in chromatograms of extracts of lemons at various stages of fruit development. Spectra of the compounds represented by most of these peaks were typical of the coumarins and substituted cinnamic acids.

The accumulation of the major flavonoids as a function of fruit growth is illustrated in Fig. 2 on a per-fruit basis. It can be seen that the hesperidin level builds up rapidly in the very small fruit then tapers off in lemons from *ca* 25 mm diameter to full size. This appears to be analogous to the build-up of naringin in small grapefruit [15–17].

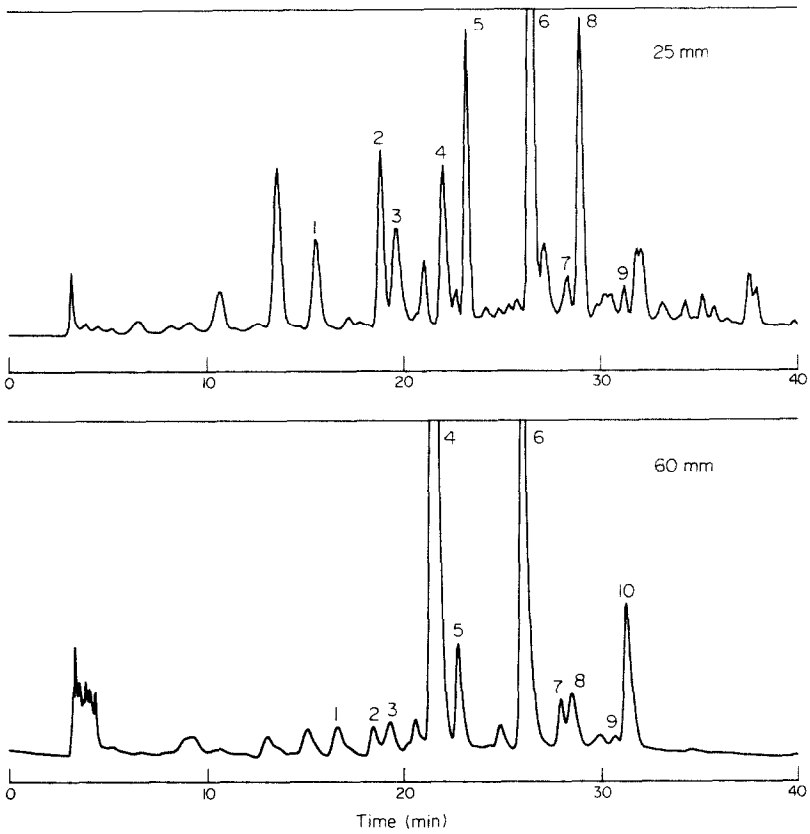

Fig. 1. Chromatographic comparison of the flavonoid patterns of extracts of lemon juice vesicles of 25 and 60 mm tree-grown lemons. The chromatogram of the 60 mm lemon required *ca* 10 times the amount of extract to give peak sizes comparable to the 25 mm fruit. Eluent monitored at 285 nm. Identifications: 1–3, unknown flavanones; 4, eriocitrin; 5, 7, 9 unknown flavones; 6, hesperidin; 8, rutin; 10, diosmin.

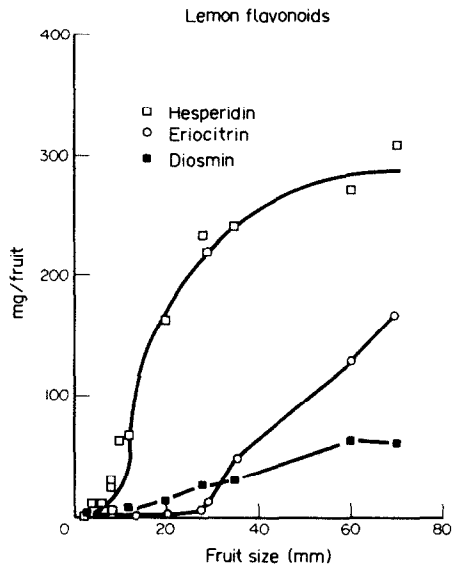

Fig. 2. Increase in the total eriocitrin, hesperidin, and diosmin content per lemon (mg/fruit; *in vivo*) as a function of fruit size.

Eriocitrin starts to increase significantly after the hesperidin is near its maximum or at the 25–30 mm diameter size. It continues to increase as the fruit grows to full size. The total amount of diosmin increased gradually throughout the development of the lemon. However, the concentrations of these compounds (mg flavonoid/g fresh tissue) are actually decreasing due partly to growth and the increase in water content of the juice vesicles (Table 1). The moisture content of lemon juice vesicles increased from 68.1 to 90.0% as the fruit grew from 14 to 56 mm in diameter. The flavonoid concentrations are also dropping with growth on a dry weight basis, which could be explained by an increase in citric acid as the vesicle grows. A net increase in total flavonoids with growth and a corresponding drop in concentration suggest that the rate of accumulation of the flavonoids in lemon juice vesicles is highest in the small fruit and tapers off (but does not cease) as the fruit grows. It also should be noted in Table 1 that as a percentage of total flavonoids eriocitrin increases while hesperidin decreases with fruit growth.

From Fig. 2 it is apparent that the flavonoids are rapidly changing in lemons at the 25–30 mm diameter stage. We chose this size fruit for comparing the *in vitro* system with tree-grown lemons. In Table 2 one can see the

Table 1. Average composition of the major flavonoids in lemon juice vesicles as a function of fruit growth (diameter) for tree-grown fruit

Fruit diameter (mm)		Flavonoids									
		UK1	UK2	UK3	ERC	UK4	HSP	UK5	RTN	UK6	DSM
20	F.W.	0.74	1.20	1.33	1.32	1.34	43.8	2.13	1.89	0.47	4.84
	D.W.	2.46	4.01	4.41	4.38	4.45	146	7.08	6.28	1.58	16.13
	%	1.3	2.0	2.2	2.2	2.3	74.2	3.6	3.2	0.8	8.2
25	F.W.	0.45	0.95	0.69	0.37	0.84	27.0	0.84	1.46	0.39	4.84
	D.W.	1.60	3.46	2.44	1.42	3.12	99.7	3.15	5.30	1.41	17.35
	%	1.2	2.5	1.8	1.0	2.2	71.8	2.3	3.8	1.0	12.5
30	F.W.	0.12	0.23	0.14	0.41	0.49	4.4	0.23	0.22	0.15	0.88
	D.W.	0.50	0.91	0.46	1.64	1.97	17.5	0.91	0.89	0.59	3.54
	%	1.7	3.2	1.6	5.7	6.8	60.6	3.2	3.1	2.0	12.2
37	F.W.	0.05	0.13	0.03	0.28	0.34	3.1	0.16	0.05	0.06	0.62
	D.W.	0.26	0.65	0.16	1.4	1.71	11.6	0.79	0.26	0.31	3.10
	%	1.3	3.2	0.8	6.9	8.5	57.3	3.9	1.3	1.5	15.3
40	F.W.	0.05	0.08	0.02	0.44	0.22	2.2	0.13	0.05	0.04	0.42
	D.W.	0.21	0.44	0.10	2.46	1.25	12.2	0.54	0.26	0.22	2.32
	%	1.0	2.2	0.5	12.3	6.2	61.0	2.7	1.3	1.1	11.6
45	F.W.	0.00	0.05	0.00	0.44	0.16	1.3	0.07	0.05	0.02	0.28
	D.W.	0.08	0.33	0.00	2.95	1.07	8.5	0.5	0.42	0.12	1.87
	%	0.5	2.1	0.00	18.6	6.7	53.7	3.2	2.6	0.7	11.8
50	F.W.	0.02	0.04	0.01	0.52	0.18	1.0	0.07	0.07	0.01	0.31
	D.W.	0.14	0.33	0.08	4.03	1.37	7.44	0.51	0.53	0.08	2.41
	%	0.9	2.0	0.5	23.8	8.1	44.0	3.0	3.1	0.5	14.2

UK1-UK3, unknowns with flavanone spectra; ERC eriocitrin; UK4-UK6, unknowns with flavone spectra; HSP, hesperidin; RTN, rutin; DSM, diosmin.

F.W.: fresh weight mg/g tissue.

D.W.: dry weight mg/g tissue.

Individual compound as a percentage of the 10 major compounds.

Table 2. Changes in the major flavonoids of lemon juice vesicles of 25 mm diameter lemon halves grown *in vitro* over a four week period

Time (weeks)		Flavonoids									
		UK1	UK2	UK3	ERC	UK4	HSP	UK5	RTN	UK6	DSM
0	F.W.	0.77	0.76	1.10	0.26	0.94	17.18	0.79	1.04	0.30	3.90
	%	2.8	2.8	4.1	1.0	3.5	63.5	2.9	3.8	1.1	14.4
1	F.W.	0.27	0.50	0.44	1.26	1.15	11.66	0.58	0.90	0.19	2.94
	%	1.4	2.5	2.2	6.3	5.8	58.6	2.9	4.5	1.0	14.8
2	F.W.	0.14	0.46	0.26	1.31	1.18	9.26	0.44	0.78	0.19	2.40
	%	0.9	2.8	1.6	8.0	7.2	56.4	2.7	4.7	1.2	14.6
4	F.W.	0	0.24	0	3.12	0.91	6.66	0.39	0.65	0.10	1.89
	%	0	1.7	0	22.3	6.5	47.7	2.8	4.7	0.7	13.5

F.W.: fresh weight mg/g tissue.

Individual compound as a percentage of the 10 major compounds.

concentration changes in the major flavonoids of lemon juice vesicles grown *in vitro* from 25 mm diameter fruit halves. This data can be compared with the concentration changes of flavonoids of tree-grown lemons in Table 1. With the exception of eriocitrin, it is apparent that lemons grown in the two systems follow a similar trend with respect to the flavonoids, that is, a decrease in the concentrations. However, the flavonoid concentrations of the *in vitro* lemons were higher than those of the tree-grown fruit. This is the stage (Fig. 2) in tree-grown lemons where eriocitrin begins to accumulate rapidly. The rapid accumulation of eriocitrin also occurs in lemons grown *in vitro*. It appears to be more pronounced than in the tree-grown fruit, and it counteracts the dilution effects of vesicle growth.

Direct comparison of the two systems is rather difficult because the fruit in both the *in vitro* and the *in vivo* systems is changing with time and size. In both cases the percentage of eriocitrin increases while that of hesperidin decreases. After one to two weeks in culture, the eriocitrin and hesperidin percentages looked more like those of the 37 mm tree-grown lemons (Table 1). The flavonoid percentages of *in vitro* fruit after four weeks growth resembled that of 45–50 mm tree-grown lemons (Table 1). However, the 45 mm lemon was *ca* 10 mm larger than the original 25 mm diameter lemon would have been after an additional four weeks on the tree. The increased fruit diameter from 25 to 45 mm represents *ca* nine weeks of normal growth. This would suggest that fruit grown in the *in vitro* system develops at a faster rate than it does on the tree.

A separate experiment (Table 3) confirmed the buildup of eriocitrin *in vitro* along with the decrease of the other flavonoids. In addition, this experiment illustrates the critical effect of explant size. In this case both explant sizes showed increases in eriocitrin, but the 25 mm fruit had a larger gain than the 30 mm diameter fruit. The increased time in culture from 48 to 120 days resulted in additional growth of the vesicles as reflected in the decrease in concentration of the flavonoids (except for eriocitrin). The percentage of eriocitrin increased while its concentration remained approximately constant. Hesperidin, on the other hand, decreased both in percentage and concentration.

Culture characteristics

After 60 days in culture, a visual examination of the externally visible juice vesicles from 25 mm (165 days old) and 30 mm (180 days old) diameter fruit showed that they produced callus appendages on vesicles at rates of 12.1% and 0.0%, respectively. Any occurrence of callus on the surface of the juice vesicle was recorded as abnormal. Normal juice vesicle development in culture is described as the continued growth of the juice visicles as intact structures in a mode of development paralleling that found within the tree-grown fruit. When cultured fruit halves were sliced open longitudinally it was found that the internal vesicles were living, healthy in appearance, and elongating normally. These vesicles were totally devoid of callus. The flavedo and most portions of the albedo of 25 mm diameter fruit died during culture. When larger fruits were used, e.g. fruits over 30 mm in diameter, all visible and internal vesicles remained normal and living; also, the flavedo remained green for over 4 months in culture. The albedo retained its characteristic white colour and appeared healthy during culture. After four to six months in culture the flavedo in some halves turned yellow-green or yellow corresponding to the changes occurring in tree-grown fruit.

Originally, the juice vesicles in 25 mm diameter fruit were green, about 2 mm in length × 1 mm in width, and

Table 3. Changes in the flavonoids of lemon juice vesicles grown *in vitro* from lemon halves as a function of fruit explant size and time in culture

		Flavonoids						
Treatments		ERC	UK4	HSP	UK5	RTN	UK6	DSM
0 days:								
25 mm	F.W.	0.41	0.85	26.0	0.88	1.23	0.35	3.77
	%	1.2	2.5	77.5	2.6	3.7	1.0	11.2
30 mm	F.W.	0.41	0.49	4.4	0.23	0.22	0.15	0.88
	%	6.0	7.2	64.9	3.4	3.2	0.7	13.0
48 days								
25 mm	F.W.	3.79	0.61	7.28	0.65	0.81	0.44	2.28
	%	23.9	3.8	45.9	4.1	5.1	2.8	14.4
30 mm	F.W.	2.51	0.45	6.05	0.47	0.65	0.23	1.78
	%	20.7	3.7	49.8	3.9	5.4	1.9	14.7
120 days:								
25 mm	F.W.	3.68	0.68	4.28	0.27	0.46	0.17	1.24
	%	34.1	6.3	39.7	2.5	4.3	1.6	11.5
30 mm	F.W.	2.10	0.57	3.87	0.23	0.41	0.12	1.01
	%	25.3	6.9	46.6	2.8	4.9	1.4	12.2

F.W.: Fresh weight mg/g tissue.
Individual compound as a percentage of the 10 major compounds.

were at the early elliptical-conical stage of development. After 30 days in culture the vesicles were the same width but had grown 3–4 mm in length. Vesicles remained green with a characteristic elliptical structure. Following 60–120 days in culture vesicles turned a whitish colour and had enlarged to as long as 10–15 mm.

When explants of juice vesicles smaller than 5 × 5 mm were cultured callus formed within 30 days. Once callus started to grow it obliterated normal visicle growth by submerging the vesicles under unorganized growth. After 60 to 120 days in culture the occurrence of normal vesicles was rare from such cultures. Anatomically and morphologically callus derived from vesicles was dissimilar to vesicles cultured in fruit halves. Furthermore, the HPLC patterns of extracts of callus and callus-free juice vesicles were completely different.

EXPERIMENTAL

Flavonoid analysis. Ca 200 mg of lemon tissue (from freshly picked fruit or from culture) was weighed and placed into a 1.5 ml plastic centrifuge tube. The tissue was finely chopped in 1 ml MeOH with a spatula. The mixture was centrifuged, and the supernatent decanted into a 5 ml volumetric flask. The extraction was repeated × 4 each with *ca* 1 ml of DMSO. The combined extract was diluted to 5 ml with H_2O and filtered (0.2 micron filter) prior to HPLC. The chromatographic system consisted of dual pumps, an automatic sampler, a C18 reverse phase column (25 × 0.4 cm), and a diode array detector. The detector was set to measure spectra from 220 to 400 nm and monitor the eluent at 285 nm. Concentrations of the compounds were calculated from integration peak areas of the sample and corresponding standards. At a flow of 1.0 ml/min the gradient elution schedule consisted of an initial 2 min of 80% 0.01 M H_3PO_4 and 20% MeOH followed by a linear gradient to 100% MeOH in 55 min.

Tissue culture. Fruits, 25–30 mm diameter, of *Citrus limon* (L.) Burm. f. cv. Eureka (lemon) were obtained from 6-year-old trees grown at the University of California, Riverside. The culturing technique was that of Tisserat *et al.* [28]. Fruits were surface sterilized in a 2.63% NaClO soln (containing 2 drops of Tween-20 emulsifier per 100 ml soln) for 30 min and then rinsed × 3 with sterile dist. H_2O. The extreme stylar and stem ends were then severed. Fruits were bisected equatorially and planted on the surface of nutrient agar medium. To induce callus from vesicles, explants of juice vesicles attached to 5 × 5 mm squares of albedo (*ca* 1 mm thick) were cultured.

The basal nutrient medium contained the following constituents, in mg/l: KNO_3, 500; $Ca(NO_3)_2 \cdot 3H_2O$, 500; $MgSO_4 \cdot 7H_2O$, 150; $MnSO_4 \cdot H_2O$, 5; $CuSO_4 \cdot 5H_2O$, 1; $ZnSO_4 \cdot 7H_2O$, 2; $MgCl_2 \cdot 6H_2O$, 100; KI, 0.5; $CaCl_2 \cdot 2H_2O$, 150; $CoCl_2 \cdot 6H_2O$, 100; H_3BO_3, 2.5; $Na_2MoO_4 \cdot 2H_2O$, 0.25; KH_2PO_4, 50; EDTA, 3.724; $FeSO_4 \cdot 7H_2O$, 2.784; 1-naphthal eneacetic acid, 0.1; sucrose, 30 000; thiamine-hydrochloride, 0.5; meso-inositol, 120; and agar, 8000. The pH was adjusted to 5.7 ±0.1 with 0.1 M HCl or NaOH before the addition of agar.

Five to ten fruits were originally planted in each medium type tested. All cultures were incubated in a temperature-controlled environmental chamber at 26 ± 1°. Growth tests were conducted under a 16-hr daily exposure to 2.2 W/m² using cool white fluorescent lamps. The number of vesicles producing callus appendages were recorded as percentages. Status of vesicles was determined using a dissecting microscope at 6–50 × magnification by viewing exposed vesicles. Fruit explants were measured before and after culture for fr. wt and for length and width of vesicles to determine % change occurring *in vitro*. Experiments were repeated at least twice.

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