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### Authors

Feldman, A. W.

Hanks, R. W.

Garnsey, S. M.

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## Localization and Detection of Coumarins in Exocortis-Virus-Infected Citron

A. W. FELDMAN, R. W. HANKS, and S. M. GARNSEY

OBJECTIVES of the investigation to be reported in this paper were: 1. to determine by thin-layer chromatography (TLC) the fluorescent band(s) that best characterize exocortis virus infection in citron, and 2. to ascertain the tissue(s) involved and the pattern of coumarin distribution in citron plants after infection. The investigation was prompted after a recent finding that a specific violet fluorescent material, gentisoyl glucose (5), is present in the albedo and bark extract of both stubborn- and greening-affected trees (8, 9), and by the more recent observation that TLC of tissue extracts of citron clones reveal 1-3 blue to violet fluorescent bands. These bands are primarily the coumarins, scopolin and skimmimin (Fig. 1), with moderate amounts of both free and unidentified bound forms of scopoletin and umbelliferone (1).

### *Methods*

PLANT MATERIAL.—The sources of plant material used were rooted greenhouse-propagated cuttings from Etrog citron clones 7-4, 7-7, OES-7, OES-10, 7-26, and Arizona 861. A standard isolate of exocortis virus, free of other known viruses, was introduced by mechanical inoculation as required (6). Plants of

clone 7-4 remained symptomless, whereas plants of all other clones developed typical exocortis symptoms. Graft-inoculated plants of OES-7 and OES-10 obtained from G. D. Bridges—State Department of Agriculture, Winter Haven, Florida—were used in several tests. These graft-inoculated plants, inoculated with several sources of exocortis virus, were still symptomless at time of analyses, about 7 months after inoculation.

Plant tissues analyzed were: 1. whole tip leaves (ca. 0.5 cm long) from new shoots; 2. midribs from expanding young leaves (ca. 2-3 cm long); 3. midribs from fully expanded young leaves; 4. midribs from mature leaves; 5. bark from young shoots; 6. bark from mature stems; 7. young root tips; 8. inactive feeder root tips; and, in some instances, 9. the woody stele from immature stems. Investigations were made on tissues 1-6 beginning 3 weeks after inoculation and continued for 3 months on all citron clones except 7-4. With the latter clone, analyses were made biweekly for 8 months. In 4 experiments, analyses were made on each leaf midrib from the top 10 leaves, exclusive of the very small tip leaves of new shoots, and on 8 cm strips

of young bark taken in sequence, beginning 3 cm below the stem tip. These experiments and analyses were conducted over a 3-year period and in different seasons.

**TISSUE EXTRACTION AND CHROMATOGRAPHY.**—All tissues from healthy and exocortis-infected plants were finely chopped and extracted at 4°C, for 72 hours, in 50 per cent ethanol at a ratio of 1:3 w:v. Fifty to 100 µl of tissue extract was spotted

drolyses and two-way paper chromatography (4) in which standard curves were prepared with either scopoletin or umbelliferone.

### Results

**TISSUES INVOLVED IN COUMARIN ACCUMULATION AND SEQUENCE IN DEVELOPMENT OF FLUORESCENT BANDS.**—Extracts from tip leaves, from midribs of expanding young leaves, from midribs of young ex-

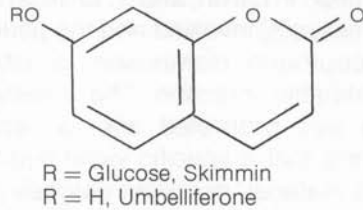
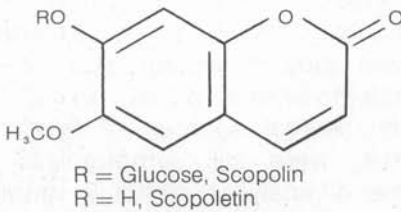


FIGURE 1. Principal coumarins in extracts of midribs and young bark from *Etrog citron* infected with exocortis virus.

on 20 x 20 cm TLC plates (Mallinckrodt 7G silica gel), which were developed in H<sub>2</sub>O-saturated n-butanol at 26°C (1). After development, chromatograms were air-dried and viewed under UV at 366 and 253 nm.

**DETERMINATION OF TOTAL PHENOLS.**—Fluorescent zones were removed from the TLC plates and eluted in ethylacetate-acetic acid-H<sub>2</sub>O (5:1:1, v:v), dried in vacuo at 40°C, and taken up in 3 ml of 50 per cent ethanol for analysis by spectrophotofluorometry (SPF) (Aminco-Bowman spectrophotofluorometer) with scopoletin as a standard.

**COUMARIN DETERMINATION.**—The quantity of the specific coumarins was determined by SPF after hy-

panded leaves, from young bark, and from immature wood of all exocortis-infected citron clones exhibited 1–3 violet to blue fluorescent bands on the chromatogram. These fluorescent bands were not—with the occasional exception of tip leaves—present on chromatograms from comparable tissue extracts of healthy citron (Table 1). The violet to blue fluorescent bands were located at R<sub>f</sub> 0.40–0.45, 0.47–0.59, and 0.68–0.75. When only 1 fluorescent band was present, it was located at R<sub>f</sub> 0.47–0.59. The variation in the range of the R<sub>f</sub> and the color of the fluorescence of each band was due to the concentration of the coumarin(s) in the band and to interference and accumulation of

other phenolics at the same Rf. The Rf for a given fluorescence was always higher in extracts obtained during spring and summer when tissues were most succulent.

Coumarin(s) associated with the fluorescent band at Rf 0.47–0.59 was always produced first, often prior to visible symptoms, and generally 4–6 weeks after inoculation, depending upon seasonal conditions. This fluorescent band

0.45 in the extract from the midrib of fully expanded young leaves and occasionally in the young bark. This band contained other bound derivatives of both scopoletin and umbelliferone, including small amounts of scopolin and skimmin. Depending on seasonal conditions, a third fluorescent band (Rf 0.68–0.75) often appeared with the second fluorescent band, or 1–2 weeks later.

TABLE 1. BOUND FORMS OF SCOPOLETIN AND UMBELLIFERONE IN THE PRINCIPAL BLUE TO VIOLET FLUORESCENT ZONE (RF 0.47–0.59) FROM THIN-LAYER CHROMATOGRAMS OF TISSUE FROM EXOCORTIS-INFECTED CITRON<sup>a</sup> (AVERAGE OF 3 TRIALS)

Tissue <sup>b</sup>	Total phenolics at Rf 0.47–0.59 µg/g tissue (fr. wt.)	Bound scopoletin at Rf 0.47–0.59 µg/g tissue (fr. wt.)	Bound umbelliferone at Rf 0.47–0.59 µg/g tissue (fr. wt.)	Ratio: <sup>c</sup> scopoletin/ umbelliferone
Whole tip leaves <sup>d</sup>	0.95	0.34	0.12	3
Midribs from expanding top leaves <sup>d</sup>	1.65	0.68	0.28	2.4
Midribs from fully expanded young leaves	6.9	5.4	1.3	4.2
Young stem bark	12.4	8.9	1.4	6.4
Immature stem wood	11.3	5.1	0.9	5.7
Mature stem bark	Tr. <sup>e</sup>			

a. Plants of citron clone 7-26, sampled 2–3 months after inoculation when symptoms first appeared.

b. Fluorescent zone at Rf 0.47–0.59 was not observed in the tissue extract of midribs from mature leaves, young root tips, and old feeder-root tips or in any other tissue extracts from healthy plants with the occasional exception of whole tip leaves.

c. Concentration and ratio of bound scopoletin to bound umbelliferone will vary according to season, clone, and duration of infection.

d. The fluorescent band at Rf 0.47–0.59 from these tissue extracts often was diffuse and not so well defined.

e. Less than 0.1 µg/g.

was found in extracts obtained either from the midribs of expanding young leaves, midribs of fully expanded young leaves, young bark, or any 2 of these tissues or in all of them. The concentration and ratio of bound scopoletin to bound umbelliferone in the fluorescent band at Rf 0.47–0.59 varied according to the tissue, duration of infection (Table 1), season, and clone (1). When symptoms first appeared (leaf epinasty), a second fluorescent band was often observed at Rf 0.40–

The fluorescent band at Rf 0.47–0.59 was found in all the citron clones studied, whether mechanically or bud inoculated, and we consider it the principal fluorescent band associated with exocortis virus infection in citron. This fluorescent band, however, appeared slowly in the symptomless clone no. 7-4 since it was first detected 6 months after inoculation in the extract of the midribs from fully expanded young leaves and 2 weeks later in the extract from young bark.

Twenty-eight plants of citron clones OES-7 and OES-10, which remained symptomless 7 months after bud inoculation with tissue suspected of carrying exocortis virus, were examined chromatographically for the fluorescent band at Rf 0.47–0.59. The band was found in 11 plants. These 11 and 4 others eventually showed visible symptoms.

**PATTERN OF COUMARIN ACCUMULATION IN INDIVIDUAL LEAVES AND YOUNG BARK SEGMENTS.**—Derivatives of scopoletin and umbelliferone, primarily scopolin and skimmin, are always present in the principal blue-violet fluorescent band at Rf 0.47–0.59. These coumarins are readily found in the extract of midribs from leaves that show even very early leaf epinasty symptoms of exocortis virus infection. In some plants, only 1 leaf showed early symptoms; but coumarins were found in the extract of the adjacent young bark or in the young bark 5–8 cm above or below the node of the symptom-bearing leaf. These coumarins may also be found in the extract of midribs from symptomless leaves 2–6 leaves above or below the leaf that exhibits slight symptoms. When 3–4 leaves on 1 shoot exhibit slight leaf curl symptoms, coumarins may readily be found in extracts of midribs from all fully expanded young leaves, with and without symptoms, and in the young bark. In some instances, the distribution of coumarins was not uniform. Coumarins could be found in the midribs and young bark immediately above or below

the leaves that exhibited slight leaf curl symptoms, and also in the midribs of 1 or 2 fully expanded young leaves approximately 6–10 cm away but not in the midribs of the intervening leaves.

### *Discussion*

The accumulation of the bound and free coumarins in certain tissues of Etrog citron following infection by exocortis virus offers the possibility of using these phenolics as a presumptive aid for detection of exocortis virus. Though bound forms of scopoletin and umbelliferone are normal constituents of citrus (2, 3, 4) as well as of other plants, particularly those subjected to stress (3, 4, 7), they do not appear to be normal constituents of the citron tissues used in our tests. The extraction method outlined here apparently removes the extracellular or the more readily diffusible coumarins. The detection of exocortis-specific phenolics by TLC and UV is relatively simple, but careful selection of tissue is necessary to obtain a sharp fluorescent pattern at Rf 0.47–0.59. Separately prepared extracts of midribs from fully expanded young leaves, and from young bark in an area 5–10 cm long and beginning 8 cm below the shoot tip, consistently yielded the best fluorescent pattern, with little or no interference at the principal Rf (0.47–0.59). Comparable tissue extracts from healthy citron should be included as a basis for comparison.

Mild strains of exocortis virus may

not produce symptoms in citron until 1 or more years after inoculation, but coumarins do accumulate and can be detected in the young bark and midribs from fully expanded young leaves prior to appearance of symptoms. Vigorous growth of the host facilitates early detection of coumarins, and test plants should receive good care.

A variety of stress conditions can cause increased coumarin concentrations in plants. The specificity of coumarin formation as a result of exocortis infection should be re-examined whenever a stress factor that could cause coumarin accumulation is added. The effects of citrus yellow vein, stubborn, and seedling-yellows viruses were not investigated in this study and would require study in regions where they would be encountered. Citron plants grown in metal containers may develop root necrosis caused by the metal oxides of the container. Extracts of young bark or midribs from young expanded leaves from plants injured in this fashion have shown the principal fluorescence at Rf 0.47-0.59. Plastic liners for cans help to eliminate metal oxide-induced root necrosis.

The random accumulation of

coumarins in midribs and young bark of citron may be caused by erratic virus multiplication, by irregular or localized interference with normal host metabolism, or by both. A similar type of random distribution of gentisoyl glucose in the bark of greening-affected citrus was also noted by Schwarz (personal communication). In trifoliolate orange rootstock infected with exocortis virus, the concentration of coumarins in the root bark is highest in late spring and early summer. Coumarins are absent, or in very low concentration, in fall and winter (1). This absence is in contrast to the accumulation of gentisoyl glucose in citrus affected by the viruses that cause greening, dieback, and stem pitting where the highest concentration of gentisoyl glucose is found during late fall or early winter (5, 8, 9).

Data from this investigation and others (1, 3, 5, 8, 9) support the possibility of using specific host metabolic accumulations to ascertain the presence of a specific virus infection in citrus.

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