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PHYSIOLOGICAL and BIOCHEMICAL ASPECTS

Coumarin Accumulation in Citrus Tissue Infected with Exocortis Virus, as Influenced by Virus Titer, Strain, and Citron Clone

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Thin-layer chromatography (TLC) of extracts from midribs and young bark of citrus exocortis virus-infected (CEV) citron showed one to three blue to violet fluorescent bands (2, 8), that were not found in extracts of tissue from healthy plants. The principal fluorescent substance (R_f 0.40 to 0.45) consisted of scopoletin and umbelliferone, primarily as bound coumarins (about 90 per cent), plus small amounts of sinapic, ferulic, and coumaric acids (2, 7, 8). Even though the concentration of scopoletin and its ratio to umbelliferone (S/U ratio) in the principal fluorescent band varied with the kind of

tissue and the duration of infection, the ratio was usually higher in tissues from severely affected plants (2, 8).

This apparent association between severity of CEV symptoms and higher S/U ratios prompted us to investigate the relation of S/U ratio and total phenolics in the principal fluorescent zone to the virus content of infected citron tissues. Although the method for assaying CEV titer *in vitro* (9) is not precise, the technique would disclose large differences. Citron clones of different reactivity and different CEV isolates were used to test the relationship between phenolic content and virus titer.

MATERIALS AND METHODS

Citron plants. Four citron clones were used either as donor or receptor plants in the five experiments reported. Arizona 861, OES-4, and 60-7-26 clones react moderately to strongly to infection by common isolates of CEV (1). Leaf epinasty is more pronounced and consistent in Arizona 861 and 60-7-26 plants infected with severe isolates of CEV than in OES-4 plants. The fourth clone, 60-7-4, rarely exhibits leaf epinasty, even when infected with severe isolates of CEV, although slight browning may occur at the base of the petiole of older leaves.

All plants were cuttings rooted in a steam-sterilized potting mixture and maintained in a partially shaded, air-cooled, insect-controlled greenhouse.

Temperatures ranged from 21 to 31°C, and light intensity normally ranged from about 10^4 to 1.5×10^4 lux.

Virus isolates. Three CEV isolates, mechanically transmitted serially through at least three citron plants, and considered to be free of other citrus viruses, were used. Symptoms induced by these isolates had been consistent in previous experiments, and each isolate appeared to consist of only a single strain.

Isolate E-9 was originally obtained from a sweet orange tree budded on trifoliolate orange with bark scaling symptoms. This isolate causes severe leaf epinasty symptoms as well as marked stunting in Arizona 861, 60-7-26, and OES-4 citron plants.

Isolate FS-189 was originally obtained from a symptomless Persian lime tree budded on Rangpur lime. This isolate causes mild leaf epinasty symptoms and little stunting in Arizona 861, 60-7-26, and OES-4.

Isolate HT-67-4 was obtained from a Temple orange which had undergone several successive heat-therapy treatments. This isolate also causes mild symptoms in Arizona 861, 60-7-26, and OES-4 citrons similar to those induced by FS-189.

Preparation of inoculum. Healthy citron plants were inoculated with the virus isolates indicated. After these plants showed systemic symptoms, they were topped to force new growth of uniform age. Tissue for testing was harvested from several plants and combined.

Tissues from top, midleaf, and lower leaf midrib, young bark, old bark, young wood, feeder root tips, and from older feeder roots were assayed for infectivity as follows: Samples were triturated in a chilled mortar with cold (0.05 M) potassium phosphate buffer (pH 7.2) at a ratio of 1:9 (W/V) tissue to buffer. An aliquot of this homogenate was diluted 10-fold with buffer, and filtered through glass wool and a 1.2 μ Millipore filter in a Swinnex holder. A portion of the filtrate was further diluted 10- and 100-fold with buffer to yield 10⁻², 10⁻³, and 10⁻⁴ dilutions of original tissue. All inocula were stored in an ice bath prior to use. Time interval between trituration and inoculation was about 1.5 hours for all experiments.

Infectivity assay. Assay plants were inoculated by cutting the stem of each receptor plant 10 times with a razor blade freshly contaminated with inoculum. Four or five plants were used for each tissue dilution. Cuts were made in two series of five cuts on opposite sides of the stem, which was wrapped with self-adhesive tape following inoculation. Plants were cut back to force

new growth, which was thinned to a single sprout. Plants were observed weekly for the presence of diagnostic symptoms (1). They were cut back at least twice during the six months after inoculation, to force growth. Data were recorded on both infectivity titer and on the infectivity index. The latter was a weighted index and was calculated by the following formula (10):

$$\frac{\text{No. plants with exocortis symptoms} \times \log \text{ of the reciprocal of dilution used for inoculation}}{\text{Total no. plants inoculated}}$$

Healthy plants were included to detect possible contamination.

Chromatography. Five ml of the cold, tissue-phosphate-buffer homogenate (0.5 gm tissue, fresh weight) were saturated with NaCl, and 5 ml of *n*-propanol were then added for partition. The mixture was shaken periodically while maintained at 3°C for 96 hours. The *n*-propanol (top layer) was removed with a pipet and stored in a vial at 3°C for chromatography and analyses.

Fifty to 100 μ l of tissue extract (*n*-propanol) were spotted on TLC plates (Merck, silica gel-G), which were developed in water-saturated *n*-butanol, air dried, and viewed under ultraviolet light at 360 nm (7, 8).

Determination of total phenolics. The principal fluorescent zone at R_f 0.40 to 0.45 was removed from the TLC plate and eluted in ethylacetate-acetic acid-H₂O (5:1:1, V/V), dried *in vacuo* at 40°C, and taken up in 3 ml of 50 per cent ethanol (5) for spectrophotofluorometric (SPF) analyses (Aminco-Bowman). A commercial source (K & K Laboratories) of scopoletin, similarly prepared, was used as a standard (8). Data are expressed as μ g phenolics per gm tissue, fresh weight.

Determination of scopoletin and umbelliferone. An aliquot of tissue extract (*n*-propanol) equivalent to 100 mg of tissue (fresh weight) was streaked onto three TLC plates (20 \times 20 cm) and developed as above. The principal fluores-

TABLE 1
 SCOPOLETIN/UMBELLIFERONE (S/U) RATIOS, TOTAL PHENOLICS, SYMPTOM SEVERITY, AND INFECTIVITY INDEX FROM
 YOUNG BARK AND LEAF MIDRIB TISSUE FROM FOUR CLONES OF CITRON INFECTED WITH THREE ISOLATES OF CEV
 (Data from five experiments)

Citron clones and tissue	Virus isolate											
	E-9				FS-189				HT-67-4			
	Total phenolics*	S/U ratio	Inf. index†	Symptom severity‡	Total phenolics*	S/U ratio	Inf. index†	Symptom severity‡	Total phenolics*	S/U ratio	Inf. index†	Symptom severity‡
	($\mu\text{g/gm}$)				($\mu\text{g/gm}$)				($\mu\text{g/gm}$)			
Arizona 861:												
Midrib§.....	16.8	11.1	1.5	++++	0.8	0.7	0.3	+	1.8	2.0	0.3	+
Young bark.....	10.7	10.1	1.7	++++	6.6	0.8	0.6	+	1.5	1.9	0.1	+
OES-4:												
Midrib.....	8.1	5.6	1.6	++	5.6	0.3	0.4	+	2.5	1.8	0.2	+
Young bark.....	0.7	0.5	0.7	++	5.9	1.0	0.1	+	0	0	0.1	+
60-7-26:												
Midrib.....	12.0	2.3	2.0	+++
Young bark.....	4.8	2.8	2.0	+++
60-7-4:												
Midrib.....	Trace	0	0.9	±
Young bark.....	0	0	0.9	±

* Total phenolics, including coumarins, in the principal fluorescence at R_f 0.40–0.45. No coumarins were found in extracts of tissues from healthy citron.

† No. plants with exocortis symptoms \times log of the reciprocal of dilution used for inoculation \div total no. of plants inoculated.

‡ A relative designation based on the over-all response to a given virus isolate. +++ = severe; ± = showing only very mild leaf symptoms.

§ Midribs from both expanding top and newly expanded mid-leaves.

cent zone was eluted in ethylacetate-acetic acid-water and dried *in vacuo*. The residue was acidified with 5 ml 2N HCl, hydrolyzed in a boiling water bath, and partitioned into diethyl ether to recover coumarins, which were then separated by two-way paper chromatography (3). After recovery from the paper

by means of 50 per cent ethanol, the amount of each coumarin was determined by SPF. Commercial sources of scopoletin and umbelliferone (K & K Laboratories) similarly prepared were used as standards. Scopoletin/umbelliferone (S/U) ratio was determined from amounts of each coumarin present.

RESULTS

Data are from five experiments conducted from 1968 to 1972. Tissues from top, midleaf, and lower leaf midrib, young bark, old bark, young wood, feeder root tips, and from older feeder roots were analyzed in the initial trials with Arizona 861 infected with CEV isolate E-9 (table 1). The principal TLC band indicative of CEV infection (R_f 0.40 to 0.45) occurred most strongly in extracts from leaf midribs, young wood, and young bark; it was faintly detectable in the old bark, and was absent in young and old feeder roots.

Second and third bands at R_f 's 0.47 to 0.59 and 0.68 to 0.75, respectively, were sometimes evident, depending on the duration of infection and on the severity of symptoms. The three fluorescent bands were not detected in tissue extracts from healthy citron. Variations in R_f values and color of fluorescence in each band were considered due to concentration of the coumarin(s) in the band and to interference and accumulation of other phenolics at the same R_f .

In initial trials with Arizona 861 infected with isolate E-9, the infectivity index was not directly correlated with higher phenolic and S/U values found in tissue from young wood, young bark, and midribs (table 1). Total phenolics and S/U ratios in midrib tissue also tended to decrease with leaf age, but the infectivity index was essentially similar for the three sources of midrib tissue (table 1). Although no fluorescent spots were observed on TLC with extracts from root tissues, a low virus titer of

CEV was recovered from young and older feeder roots (table 1). Subsequent experiments on host and virus isolate effects were therefore confined to tissue extracts from midribs of young, expanding leaves and from young bark, since these tissues tended to exhibit a better correlation between the infectivity index and the phenolic constituents in the principal fluorescent zone.

Host effect on S/U ratios, total phenolics, and infectivity index. The S/U ratios and total phenolics from leaf midrib and young bark tissue differed considerably among the four citron clones infected with CEV isolate E-9. Total phenolics were consistently higher in the midrib tissue and were generally related to symptom severity (table 2). A high S/U ratio and high levels of phenolics were obtained in the midrib and young bark tissue of Arizona 861. This clone showed strong symptoms and also had a high infectivity index. Citron clone 60-7-4 exhibited very mild symptoms, had a moderate infectivity index, and showed no coumarins or phenolics (table 2).

The S/U ratio and total phenolics for citron clones OES-4 and 60-7-26 were generally at a moderate level. Symptoms in clone OES-4 were moderate, and in clone 60-7-26, moderate to strong. Both midrib and young bark tissue from the latter clone had more phenolics than did OES-4. The infectivity index for both midrib tissue and bark tissue of 60-7-26 was also higher than those from comparable tissues of OES-4.

TABLE 2
TOTAL PHENOLICS, SCOPOLETIN/UMBELLIFERONE (S/U) RATIO, AND VIRUS TITER
OBTAINED FROM EIGHT SAMPLING SITES OF CITRON CLONE ARIZONA 861
INFECTED WITH EXOCORTIS VIRUS ISOLATE E-9

Sampling site	Total phenolics* ($\mu\text{g}/\text{gm}$)	S/U ratio	Infectivity titer at dilutions of:			Infectivity† index
			10^{-2}	10^{-3}	10^{-4}	
Midrib						
Top leaf.....	10.0	5.8	7/9	5/9	0/9	1.1
Midleaf.....	5.8	4.1	8/9	3/9	2/9	1.2
Lower leaf.....	3.8	2.9	4/4	2/4	0/4	1.2
Young bark.....	8.4	6.1	9/9	5/9	2/9	1.5
Old bark.....	Trace	0	4/4	1/4	0/4	0.9
Young wood.....	13.5	6.3	3/5	1/5	0/5	0.6
Tips						
feeder root.....	0	0	1/5	0/5	0/5	0.1
Older						
feeder root.....	0	0	2/5	1/5	0/5	0.5

* Total phenolics, including coumarins, in the principal fluorescence at R_f 0.40–0.45. No coumarins were found in the extracts of tissues from healthy citron.

† No. plants with exocortis symptoms \times log of the reciprocal of dilution used for inoculation \div total no. of plants inoculated.

Effect of different virus isolates. Leaf midrib and young bark tissues from Arizona 861 and OES-4 citrons infected with E-9 (severe), FS-189 (mild), and HT-67-4 (mild) isolates of CEV were also evaluated to determine the effect of CEV isolate on virus and phenolic content. In Arizona 861 citron, S/U ratios, virus titer, and symptom severity were markedly lower in tissues infected with mild isolates than in tissues infected with the severe isolate. The same was generally true for total phenolics

except for the rather high value for FS-189-infected bark tissue (table 1). In OES-4 citron a similar correlation for midrib tissue was observed, although total phenolics for FS-189 tissue were high. A less clear pattern was observed with OES-4 bark tissue, especially in regard to total phenolics (table 1); values for E-9-infected tissue were quite low, and the total phenolics value for FS-189-infected tissue was extremely high in relation to the other values recorded.

DISCUSSION

Phenolic substances in CEV-infected citron, which form the principal fluorescent band on TLC, accumulate mainly in the more succulent tissues of the stem and leaf midrib. These phenolic substances, primarily coumarins, occur in highest concentration in the midrib tissues. They correlate positively with symptom severity and to a lesser extent with the virus titer in infected citron plants.

Several reasons may account for the

inconsistent correlation between the infectivity index and the total phenolic content, especially in midrib and young bark samples: (1) When a systemic assay of virus titer is used, only the end result of infection (total host reaction) is measured, and the dilution range and number of plants used provide only a broad estimate of titer. (2) The phenolic assay measures more precisely a specific physiological change associated with the response of a given tissue. (3) While the

extraction method for phenolics recovers about 80 to 90 per cent of the phenolic constituents from the tissue (3, 6), no information is available on the efficiency of virus extraction from citron tissue or the number of virions in the tissue extract that would be required to initiate infection. (4) Coumarins in a given tissue are randomly distributed (8), so that larger tissue samples or a composite of several types of tissue (midrib and young bark) would be needed to provide a better general "fit" for the relationship between infectivity index and phenolic accumulation. In spite of these inadequacies, extracts of midrib tissue, especially from citron clones Arizona 861 and OES-4, showed infectivity indices for each of the three virus isolates that were quite comparable, although not on an absolute basis, with the quantity of the phenolic recovered from the principal fluorescent band on TLC.

The effect of virus isolate on observed S/U ratio in midrib and young bark tissue of a given clone seems related to susceptibility of host tissue, and may be related to the degree to which a particular virus isolate is established in the cit-

ron host. S/U ratios greater than 1 occur in other citrus-pathogen combinations, and appear to be directly related to the degree of susceptibility of the tissue (3, 4, 6, 8). In host-parasite interactions in which the pathogen multiplies freely, there is either less reduction or a greater accumulation of scopoletin as compared with greater loss or less accumulation in the amount of umbelliferone. These higher S/U ratios are especially apparent with Arizona 861 infected with the severe isolate E-9, while S/U ratios of less than unity are evident with the milder FS-189 isolate.

Data from these experiments lend general support to the relationship among symptoms, phenolics, and virus content of reactive citron tissue. In these instances, a high virus concentration is associated with severe symptoms and with production of substantial amounts of phenolics. These relationships are perhaps less consistent in the less reactive virus-citron combinations. Presence of mild isolates of CEV in reactive citron indicators or of strong isolates of CEV in nonreactive indicators would not be reliably detected by our chromatographic procedures.

CONCLUSIONS

The principal fluorescent band on thin-layer chromatograms (R_f 0.40 to 0.45) of extracts from CEV-infected citron tissues was analyzed for coumarins and total phenolic substances. Tissues from eight sites were measured for the scopoletin-to-umbelliferone (S/U) ratios, total phenolics, and infectivity index. Young leaf midrib and young bark tissues had the highest virus and phenolic titer. Highest concentrations of

phenolic materials and virus occurred in midrib tissues of highly reactive citron plants infected with a severe isolate of CEV. Plants of the same clone infected with mild isolates, and nonreactive plants infected with the severe isolate had lower concentrations of phenolics and virus. The S/U ratio varied with the susceptibility of the host tissue. Higher ratios were produced with the severe isolate than with the mild ones.

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