Identification and Quantification of Phenolics in the Leaves and Roots of Healthy and Exocortis-Infected Citrus

A. W. FELDMAN and R. W. HANKS

SIGNIFICANT DIFFERENCES in the amount of amino acids in healthy and exocortis-infected citrus were observed only in the leaves of exocortis-infected trees (6) where the total amount of the free amino acids was reduced by approximately 50 per cent. One of the "healthy" trees used for these comparative analyses contained total amounts of leaf amino acids comparable to the amount in leaves of the exocortis-infected trees. This tree had been without visible symptoms for eight years and was considered a healthy tree. However, two years later, it was proven to be exocortis-infected by bark scaling and through further indexing on citron (*Citrus medica*, L. var. Corsican). From the amino acid investigation, it appeared that studies on the non-nitrogenous constituents might prove valuable as a means of diagnosing virus infection and thus possibly afford an additional clue to some of the biosynthetic pathways in exocortis-infected citrus.

The purpose of this investigation was to isolate and identify some of the free and bound phenolics in the leaves and roots of both healthy and exocortis-infected citrus and to determine whether significant differences in the amounts and kinds of these constituents occur between healthy and infected trees.

Materials and Methods

The trees used in these investigations were 3 field-grown healthy (designated H-1, H-2, and H-3) and 3 exocortis-infected (designated E-1, E-2, and E-3) trees of Valencia [*C. sinensis* (L.) Osb.] on rootstocks of *Poncirus trifoliata* (L.) Raf. (Table 1). The budwood for these trees was obtained from 6 different sources. All had been indexed and found to be

TABLE 1. GENERAL CHARACTERISTICS OF THE SOURCE TREES USED FOR PHENOLIC ANALYSES

Tree no.	Year budded	First visible exocortis symptoms on trifoliata[a]	Color test[b]	Condition and size of tree at sampling (1965)			
				Vigor	DAB[c]	DBB[d]	Ratio[e]
E-1	4/55	4/61	+ (1957)	Fair	44	70	1:2
E-2	4/55	4/62	+ (1963)	Fair	44	73	1:2
E-3	4/55	4/62	+ (1958)	Fair	42	61	2:3
H-1	9/55		? (1965)	Good	89	115	3:4
H-2	9/55		− (1965)	Good	76	105	3:4
H-3	9/55		? (1965)	Good	64	80	3:4

a. The E-1, E-2, and E-3 trees exhibited exocortis symptoms when budded with Rangpur lime (*C. limonia* Osbeck) in 1964. The H-1, H-2, and H-3 trees did not exhibit symptoms on Rangpur lime in 1964 or on Corsican citron (*C. medica* L.) in 1965.

b. Phloroglucinol-HCl color test for presence (+) or absence (−) of exocortis with year tested shown in parentheses.

c. Diameter of stem above bud in mm.

d. Diameter of root below bud in mm.

e. Ratio DAB/DBB.

free of tristeza, xyloporosis, and psorosis viruses. Permission to use these trees was kindly given by G. D. Bridges, Citrus Budwood Registration Office, Florida Department of Agriculture.

In September, 1965, samples of feeder roots and mature leaves, 25 g fresh weight each, were collected from each of the 6 trees and frozen immediately. Procedures for extracting the free and acid-hydrolyzable (bound) phenolics, using 2-dimensional paper chromatography and solvent systems, and methods for their identification have been published previously (7). For quantitative determination of the phenolics, the

individual components were separated on Whatman No. 1 paper, eluted, and analyzed by the Folin-Ciocalteu method (11) with ferulic acid used for a standard curve. The optical density of the phenolic-Folin mixture was determined at 660 mμ with a Spectronic-20 spectrophotometer equipped with a red sensitive phototube and an infrared filter. Only those phenolics that could be readily separated by paper chromatography were analyzed. Total phenolics were determined directly from the tissue extract rather than from chromatograms because of the inherent difficulty in recovering all the phenolic spots from the chromatogram. Phenolics present in amounts less than 0.3 μg are reported as trace (tr.). The reported data are based on at least two chromatograms for each free and each bound phenolic sample.

Results

Thirty-nine phenolics, of which 25 were identified, were isolated from the roots and leaves of both healthy and infected trees. All of the 25 identified phenolic compounds were present in both healthy and exocortis-infected trees, but some of these phenolics were present in significantly different amounts in healthy and infected trees. Nine of the 14 unidentified phenolics were analyzed, but only unknown-B was included in Table 3. There was a significant difference in the amount of "unknown-B" in healthy and exocortis-infected plant samples.

The amount of each phenolic as well as the total amount of phenolics were similar in the H-1 and H-3 trees, but the amounts were slightly lower in the H-2 tree. Of the exocortis-infected trees, the E-2 tree had slightly less total phenolics than did the E-1 and E-3 trees, though all three trees exhibited the same general phenolic pattern. To simplify the reporting of data, only the averages for the three healthy and for the three exocortis-infected trees are given. For brevity, samples from exocortis-infected trees are referred to in the text as either diseased leaves or diseased roots.

LEAVES.—Slight differences were found between the leaves from healthy and exocortis-infected trees in the total amount of both bound phenolics and free phenolics, but these differences were not considered significant (Table 2). The differences were primarily in the individual phenolics of the bound group. Major differences were found in p-hydroxybenzoic acid and vanillic acid which occurred in large amounts in the healthy leaves, and in sinapic acid which was increased in diseased leaves (Table 3). The amount of increase of p-hydroxybenzoic acid and vanillic acid in healthy leaves is essentially equal to the increase in the

amount of sinapic acid in diseased leaves (Table 3); thus, these increased amounts offset each other without affecting the total amounts of phenolics found in healthy and diseased leaves. In the free phenolic group, salicylic acid and especially unknown-B were evident in larger amounts in the healthy leaves.

ROOTS.—Total free phenolics, determined directly from the tissue extract, showed approximately a threefold increase in diseased roots as compared with healthy roots (Table 2), even though there appeared to be no significant differences in the amounts of the individual phenolics as measured from the chromatogram (Table 3). Total bound phenolics

TABLE 2. TOTAL BOUND AND FREE PHENOLICS (μG/G FR. WT.) IN PLANT EXTRACT FROM LEAVES AND ROOTS OF HEALTHY AND EXOCORTIS-INFECTED CITRUS. DATA ARE AVERAGE OF 3 EACH OF HEALTHY AND EXOCORTIS TREES

	Leaf			Root			Total of root and leaf phenolics
	Bound	Free	Ratio[a]	Bound	Free	Ratio	
Healthy	687	100	7:1	247	7	35:1	1041
Exocortis	747	83	9:1	244	23	10:1	1097

a. Ratio: bound/free.

were virtually the same in the roots of both tree groups (Table 2), but vanillic acid, gentisic acid, sinapic acid, and scopoletin, from the bound group, were especially increased in the diseased roots (Table 3).

Discussion

Changes in phenolic constituents have been observed in plants subjected to stress, be it virus (5, 8), fungus (10, 12), insect (1), or injurious chemicals (15). The accumulation of the coumarins, umbelliferone, and scopoletin (4, 13) is especially noticeable in affected plants and suggests a common response in some hosts. Attempts to ascertain virus infection in *Solanum tuberosum* L. by differences in scopoletin content were successful only when analyses were made just prior to flowering (14). Attempts to use the phenolic, leuco anthocyanin, as a basis for a color test to detect the swollen shoot virus in cacao (*Theobroma cacao* L.) led to anomalous results since age of leaf and variety of cacao, which affect the total tannin content, also affect the leuco anthocyanin content (9). Therefore, leaf age, as it influences the phenolic pattern in exocortis-infected citrus, may be important and is being investigated.

Differences in the total amounts of phenolic compounds in healthy and exocortis-infected trees are of no significance, but differences in the

TABLE 3. AMOUNTS (μG/G FR. WT.) OF BOUND AND FREE PHENOLIC IN THE LEAVES AND ROOTS OF HEALTHY AND EXOCORTIS-INFECTED CITRUS

	Leaf				Root			
	Bound		Free		Bound		Free	
Phenolics	Healthy	Exocortis	Healthy	Exocortis	Healthy	Exocortis	Healthy	Exocortis
Hydroxybenzoics								
p-hydroxybenzoic	12.2	5.2			3.4	4.4		
m-hydroxybenzoic	+	+						
Vanillic acid	12.7	9.7			4.7	9.1		
Homovanillic acid	+	+						
Vanillin							0.5	1.1
Salicylic acid	tr.	tr.	2.7	1.5	2.9	3.0	0.7	0.7
p-hydroxyphenylpyruvic					+	+		
p-hydroxyphenylacetic	+	+			+	+		
Gentisic acid	49.9	53.1	1.0	1.0	5.4	10.5		
Syringic acid					tr.	tr.		
Syringealdehyde					tr.	tr.		
Cinnamic Acids								
o-coumaric	2.5	2.0			3.0	4.2	0.5	0.8
m-coumaric	+	+			+	+		
p-coumaric	2.9	2.6						
Ferulic	97.6	102.4			18.4	19.2		
Caffeic	1.0	1.0						
Sinapic	49.6	60.5	1.0	1.0	41.9	46.7	0.3	0.3
Cinnamic					tr.	tr.		
Coumarins								
Umbelliferone	2.0	2.6			19.1	19.3	0.5	0.7
Scopoletin	31.1	27.2	1.0	0.7	41.2	53.7	0.5	0.8
Esculetin					tr.	tr.		
Limettin							0.5	0.8
4-hydroxy coumarin	tr.	tr.						
Flavonoids								
Quercetin	7.0	10.9					0.5	0.8
Hesperitin	33.0	34.5						
Unknown-B[a]			13.4	8.9				

a. Unknown B has a blue fluorescence under UV and has an Rf of 0.4 in benzene:acetic acid:water (125:72:3) (v/v/v) and an Rf of 0.65 in sodium formate: formic acid:water (10:1:200) (w/v/v).

Tr. = less than 0.3 μg.

\+ = present but unable to separate for analyses.

amounts of certain phenolic constituents in healthy and diseased trees tend to indicate a specific phenolic pattern associated with the disease. For example, 93 per cent more vanillic acid and 94 per cent more gentisic acid occurred in exocortis-infected roots than in healthy roots, whereas, scopoletin was increased 30 per cent in diseased roots (Table 3). Sinapic acid was increased in diseased leaves and roots by 21 per cent and 11 per cent, respectively, although the latter increase is not significant.

The total amount of free phenolics was three times greater in exocortis-infected roots than in healthy roots (Table 2). However, a difference of this magnitude could occur without modifying the total phenolics because the free phenolics constitute such a small part of the total.

Thirty-nine phenolic compounds were separated, but these constitute only about half of the total amount of phenolics present in the tissue extract. The other phenolics are "missing" in separation and identification procedures because they are probably phenolic compounds that neither fluoresce under ultraviolet light nor react with diazo-detecting sprays when present on paper chromatograms. Some may be present in such small amounts as to escape detection.

The increase in scopoletin in diseased roots may be sufficient to account partially for the stunting effect of the virus on citrus. This particular coumarin is a competitive inhibitor of indole-3-acetic acid, and at concentrations of 50 ppm it depressed root growth of peas (*Pisum sativum* L.) (2). Scopoletin at 25 ppm inhibited growth of 7 cm grapefruit seedlings planted in vermiculite and the growth of grapefruit seedling root tips in petri dishes (unpublished data).

Sinapic acid is utilized as a lignin precursor (3), and the sinapic acid increase may be sufficient to account for the hardened condition associated with the disease syndrome.

Citrus was found to respond to exocortis virus infection by a considerable increase in several phenolic compounds. The increase in vanillic acid and gentisic acid seems adequate to serve as a means of diagnosing exocortis virus infection in the roots of *P. trifoliata* grafted with Valencia. Measuring the increase (threefold) of free phenolics in diseased roots compared with that in healthy roots may be more satisfactory for the same purpose. However, determination of the total free phenolics is much simpler than quantitative determinations of individual phenolic compounds.

Summary

Thirty-nine phenolics, free and bound, of which 25 were identified, were isolated from the roots and leaves of healthy and exocortis-infected Valencia on *P. trifoliata* rootstock. The phenolic composition of healthy and exocortis-infected trees was essentially similar, but differences were found in amounts of certain individual phenolics.

Major differences were observed in the bound group of phenolics from healthy and diseased leaves; p-hydroxybenzoic acid and vanillic acid occurred in larger amounts in the leaves of healthy trees, whereas sinapic acid was increased in those from exocortis-infected trees.

In the roots of exocortis-infected trees, the total free phenolics were increased approximately threefold, whereas in the bound phenolics vanillic acid, gentisic acid, sinapic acid, and scopoletin were especially increased.

This paper is the Florida Agricultural Experiment Stations' Journal Series No. 2423.

Literature Cited

1. Akazawa, T., Uritani, I., and Kubota, H. 1960. Isolation of ipomeamarone and 2 coumarin derivatives from sweet potato roots injured by the weevil *Cylas formicarius elegantalus*. Arch. Biochem. Biophs. 88: 150-156.
2. Andreae, W. A. 1952. Effect of scopoletin on indoleacetic acid metabolism. Nature 170: 83-84.
3. Brown, S. A. 1961. Biosynthesis of plant phenols, p. 9-47. *In* Symposium on Biochemistry of Plant Phenolic Substances. Colorado State Univ. Press, Fort Collins.
4. Brown, S. A. 1964. Biosynthesis of coumarins - V. Pathways of umbelliferone formation in *Hydrangea macrophylla*. Phytochem. 3: 469-476.
5. Durbin, R. D., Castillo, B. S., and King, T. H. 1960. The occurrence of chlorogenic acid in strawberry plants affected with crinkle and yellows viruses. Plant Disease Reptr. 44: 536-537.
6. Feldman, A. W., and Hanks, R. W. 1965. Quantitative determination of the free amino acids and amides in roots and leaves of healthy and exocortis-infected *Citrus sinensis* Osbeck on *Poncirus trifoliata* Raf., p. 285-290. *In* W. C. Price [ed.], Proc. 3d Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
7. Feldman, A. W., and Hanks, R. W. 1965. Phenolic compounds in roots and leaves of four citrus cultivars. Nature 207: 985-986.
8. Geisman, T. A. 1956. The flavonoid constituents of normal and virus-infected peach and cherry leaves. Arch. Biochem. Biophys. 60: 21-26.
9. Holden, M. 1957. An investigation of polyphenolic compounds of the cacao leaf in connection with a chemical method for detecting virus infection. J. Sci. Food Agr. 8: 553-561.
10. Hughes, J. C., and Swain, T. 1960. Scopolin production in potato tubers infected with *Phytophthora infestans*. Phytopathology 50: 398-400.
11. Keith, R. W., LeTourneau, D., and Mahlum, D. 1958. Quantitative paper-chromatographic determination of phenols. J. Chromatog. 1: 534-536.

12. MINAMIKAWA, T., AKAZAWA, T., and URITANI, I. 1962. Isolation of esculetin from sweet potato roots with blackrot. Nature 195: 726.
13. MINAMIKAWA, T., AKAZAWA, T., and URITANI, I. 1963. Analytical study of umbelliferone and scopoletin synthesis in sweet potato roots infected by *Ceratocystis fimbriata*. Plant Phys. 38: 493-497.
14. REPPEL, L. 1959. The relations between scopoletin content and virus infections in leaves and tubers of *Solanum tubersum* L. Planta Med. 7: 206-218.
15. URITANI, I., URITANI, M., and YAMADA, H. 1960. Similar metabolic alterations induced in sweet potato by poisonous chemicals and by *Ceratostomella fimbriata*. Phytopathology 50: 30-34.