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PHENOLIC CONTENT IN THE ROOTS AND LEAVES OF TOLERANT AND SUSCEPTIBLE CITRUS CULTIVARS ATTACKED BY *RADOPHOLUS SIMILIS**

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Abstract—The free and bound phenolics were quantitatively determined in the roots and leaves of both healthy and *Radopholus similis*-infected citrus cultivars, three of which were susceptible and three tolerant to the pathogen. Forty-one phenolics were detected, thirty-two of which were present in significant amounts. The same kinds of phenolics, with few exceptions, were isolated from the six cultivars whether healthy or infected and changes in the individual phenolics in the susceptible and tolerant groups were principally quantitative. In *R. similis*-infected plants, eleven of the bound phenolics in the roots of tolerant cultivars were appreciably increased while in the susceptible cultivars, the individual bound phenolics either remained unchanged or were lowered. The net effect of infection was a 27–300 per cent increase in the bound phenolics in the roots from tolerant cultivars while in the roots from the susceptible group, the bound phenolics were reduced 16–34 per cent. There was no consistent pattern of accumulation or reduction of the total free or individual free phenolics in infected roots from any of the cultivars. The total bound phenolics in the leaves from both the tolerant and susceptible cultivars were not significantly increased as a consequence of infection, although increases in specific individual bound phenolics were observed. After infection, the free phenolics in the leaves from the tolerant cultivars were increased from 2 to 7 per cent of the total phenolics, while in the susceptible cultivars, the free phenolics remained at the same level.

INTRODUCTION

SPREADING decline of citrus trees caused by *Radopholus similis* (Cobb) Thorne, the burrowing nematode, is one of Florida's most destructive citrus diseases. The injury to the rootlets by the obligate, endoparasitic nematode is both mechanical and physiological.[1,2] Trees so affected show a reduction and compaction of terminal twig growth and develop small lateral flushes with small leaves and thin petioles. The foliage and twig symptoms evolve slowly for several years, suggesting a gradual debilitation of the host possibly as a direct effect of the pathogen and/or indirectly, due in part, to a disproportionate accumulation of hesperidin (7-β-L-rhamnosyl-D-glucoside of 5,7,3′-trihydroxy-4′-methoxyflavanone), growth promotors and inhibitors in the terminals.[3]

Several citrus rootstocks tolerant to *R. similis* have been found.[4] When infected, these rootstocks show some initial reduction (*ca.* 20 per cent) in growth and sustain a population of *R. similis* for the first 6 months. If no additional source of nematodes is provided, the nematodes generally die within 6–9 months and the tree recovers.[5] This disappearance of the

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[1] E. P. DUCHARME, *Florida Hort. Soc.* **70**, 58 (1957).
[2] E. P. DUCHARME, *Phytopathology* **49**, 388 (1959).
[3] A. W. FELDMAN, R. W. HANKS, and R. J. COLLINS, *Phytopathology* **56**, 1312 (1966).
[4] H. W. FORD and W. A. FEDER, *Rio Grande Valley Hort. Soc.* **16**, 35 (1962).
[5] H. W. FORD and W. A. FEDER, University of Florida Circ. S-151 (1964).

nematodes was interpreted to indicate a post-infectional defense mechanism that is sometimes attributed to a change or accumulation of aromatic compounds in the host.[6–9] There have been many investigations on aromatic compounds in host tissue invaded by fungi, bacteria, and virus,[6, 9] but there appears to be no report on the quantification of phenols in the host where a nematode is the initial incitant. As part of a study on the biochemical changes in citrus trees infected with burrowing nematodes, the present investigation has been concerned with: (i) the isolation and quantification of the free and acid hydrolysable (bound) phenolics in the leaves and roots of healthy and *R. similis*-infected citrus three of which are susceptible and three tolerant to the pathogen and, (ii) ascertaining if significant differences in amounts and kinds of these constituents occur between healthy and infected seedlings of the susceptible and tolerant groups.

RESULTS

Plant weights were less in all infected seedlings of the six cultivars studied, particularly in the susceptible group. Plants from the tolerant group, in contrast to those from the susceptible group, appeared to recover. Although some necrosis of the tolerant roots were still evident, no *Radopholus similis* were found (Table 1).

TABLE 1. COMPARISON OF THE AVERAGE FRESH WEIGHT OF THE HEALTHY AND *R. similis*-INFECTED CITRUS CULTIVARS AT TERMINATION OF EXPERIMENT

	Fresh weight (g)						
	Healthy seedlings			Infected seedlings			*R. similis* per g root
Cultivars	Roots	Shoots	Total	Roots	Shoots	Total	
Tolerant							
'Ridge Pineapple'	67	89	156	58	67	125	0
'Milam'	63	85	148	60	76	126	0
'Carrizo' citrange	58	66	124	56	62	118	0
Susceptible							
Sour orange	60	76	136	22	27	49	5
Grapefruit	65	83	148	36	70	106	10
Rough lemon	41	62	103	22	44	66	12

Forty-one phenolics were quantitatively determined but only thirty-two (twenty-two knowns and ten unknowns) are reported (Table 2). The remainder were generally in amounts of less than 1 μg/g fresh weight and included *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylacetic acid, syringic acid, cinnamic acid, 4-hydroxycoumarin, and four unknowns. In general, about 50–60 per cent of the total amount of phenolics present in the root and leaf tissue extracts could be removed from the chromatograms and analyzed. In 'Carrizo' citrange only 23 per cent of the total phenolics in the infected roots were recovered from the chromatogram and analyzed.

[6] G. L. FARKAS and Z. KIRALY, *Phytopathol. Z.* **44**, 105 (1962).
[7] R. C. HARE, *Botan. Rev.* **32**, 95 (1966).
[8] J. KUC, in *Perspectives of Biochemical Plant Pathology* (edited by S. RICH), *Conn. Agr. Exh. Sta. Bul.* **663**, 20 (1963).
[9] I. A. M. CRUICKSHANK and D. R. PERRIN, in *Biochemistry of Phenolic Compounds* Chapter 13 (edited by J. B. HARBORNE). Academic Press, New York (1964).

TABLE 2. AVERAGE AMOUNTS OF SOME OF THE BOUND PHENOLICS IN THE ROOTS AND LEAVES OF THREE TOLERANT AND THREE SUSCEPTIBLE CULTIVARS OF HEALTHY AND *R. similis*-INFECTED CITRUS SEEDLINGS

	ug/g Fresh weight							
	Tolerant cultivars*				Susceptible cultivars†			
	Healthy		Infected		Healthy		Infected	
Phenolics	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf
p-hydroxybenzoic acid	13	2	20	2	20	4	16	3
Vanillic acid	1	2	2	2	1	4	1	4
Salicylic acid	7	10	12	15	9	7	5	10
Gentisic acid	6	23	9	33	6	19	6	11
Homovanillic acid	2	13	4	14	2	10	2	12
o-coumaric acid	2	2	2	3	2	5	2	7
m-coumaric acid	1	4	3	5	2	3	1	5
p-coumaric acid	0	6	0	11	0	5	0	4
Ferulic acid	8	58	10	61	6	37	3	37
Caffeic acid	1	2	1	2	1	10	1	9
Sinapic acid	16	51	29	38	14	23	8	27
Isoferulic acid (C, F)‡	0	84	0	92	0	13	0	17
Umbelliferone	13	19	11	28	8	18	7	15
Scopoletin	54	16	62	24	54	24	41	19
Esculetin	2	0	2	0	1	0	1	0
Limettin	3	0	4	0	4	0	4	0
Bergaptol (E)	0	0	0	0	0	8	0	4
Naringenin (E)	0	0	0	0	0	44	0	30
Quercetin (A, C)	0	13	0	13	0	0	0	0
Hesperitin (A, B, C, D)	0	12	0	11	0	16	0	5
Phloretin (B, F)	0	2	0	7	0	29	0	30
Eriodictyol (D)	0	0	0	0	0	26	0	29
Unknowns								
1. t-28-5§	4	0	2	0	2	0	2	0
2. bv-50-50	2	0	4	0	2	0	2	0
3. t-85-27	2	0	5	0	2	0	2	0
4. ltb-13-70	0	11	0	12	0	17	0	17
5. t-35-0 (C, F)	0	10	0	16	0	26	0	0
6. q-27-75 (A, B, C, F)	13	16	5	15	0	17	0	20
7. t-40-78 (C, E)	19	27	22	36	0	14	0	16
8. bv-6-78 (A, B, C, F)	0	14	0	16	0	42	0	34
9. ltb-18-85 (B, F)	0	8	0	12	0	19	0	16
10. ltb-5-50 (B)	0	15	0	19	0	0	0	0

* Tolerant cultivars are 'Ridge Pineapple' (A), 'Milam' (B), and 'Carrizo' citrange (C).

† Susceptible cultivars are sour orange (D), grapefruit (E), and rough lemon (F).

‡ Letters indicate that the phenolic was found only in the cultivars designated (see footnotes * and †, above). Absence of letter indicates phenolic present in all cultivars.

§ Indicates color of u.v. fluorescence, R_f in benzene–acetic acid–water, and the R_f in sodium formate–formic acid–water.

t=tan; bv=blue violet; ltb=light blue; q=quench.

In spite of a 3-fold increase in bound phenolics in the roots from 'Carrizo' citrange on infection (see Table 3), the individual phenolics analyzed from the chromatograms were essentially in the same amounts as those in the infected roots from the other two tolerant cultivars. A considerable amount of unidentified Folin reactive material was present in tissue extract of infected 'Carrizo' citrange roots, substances that were not detected on the chromatogram by either the u.v. or the diazo spray. A chromatogram of the extract of infected 'Carrizo' citrange roots sprayed with 1:1 Folin reagent–water produced a multitude of diffused blue areas so that "new" spots were difficult to detect.

TABLE 3. TOTAL BOUND AND FREE PHENOLICS IN THE ROOTS AND LEAVES OF THE THREE TOLERANT AND THE THREE SUSCEPTIBLE CULTIVARS OF HEALTHY (H) AND *R. similis*-INFECTED (I) CITRUS SEEDLINGS

	μg/g Fresh weight							
	Bound[1] phenolics				Free[1] phenolics			
	Roots		Leaves		Roots		Leaves	
Cultivars	H	I	H	I	H	I	H	I
Tolerant								
'Ridge Pineapple'	326	416	524	560	5	8	27	72
'Milam'	260	380	508	588	8	5	21	27
'Carrizo' citrange	220	632	608	616	8	38	3	34
Average	270	476	547	588	7	17	17	44
Susceptible								
Sour orange	200	132	404	428	30	8	2	2
Grapefruit	368	292	492	472	8	8	5	6
Rough lemon	296	228	520	508	2	8	30	20
Average	288	217	472	469	13	8	12	9

[1] Phenolic analyses were made directly from the processed tissue samples.

The same kinds of phenolics, with few exceptions, were isolated from the six cultivars whether healthy or infected. The exceptions were, for the most part, those phenolics specific to a cultivar. Because of similarity of phenolic patterns within each of the tolerant and susceptible plant groups, the phenolics are listed as an average of the three cultivars in each group (Table 2).

Roots

The total amounts of bound phenolics from the roots of the healthy tolerant and healthy susceptible cultivars were essentially the same (Table 3). In *R. similis*-infected plants, eleven of the bound phenolics in the roots from the tolerant clones were considered significantly increased (more than 15 per cent) while in the susceptible clones, the individual bound phenolics either remained unchanged or were lowered (Table 2). Those lowered were salicylic acid, *p*-hydroxybenzoic acid, ferulic acid, sinapic acid, and scopoletin. With the exception of *p*-hydroxybenzoic acid and scopoletin, these phenolics were present in the infected roots from the susceptible clones in relatively small amounts (Table 2). However, in the roots from the tolerant clones, the same phenolics, along with several others, were increased so that moderate amounts of each were present after infection (Table 2). The net effect of infection was a 27–300 per cent increase in the bound phenolics in the roots from tolerant

cultivars while in the susceptible group, the bound phenolics were reduced 16–34 per cent (Table 3).

Free phenolics in the roots were present in amounts that constituted less than 4 per cent of the total phenolics found in the roots. Chromatograms of the free phenolics generally showed from ten to eighteen fluorescent areas including trace amounts of umbelliferone, scopoletin, *o*-coumaric acid, vanillic acid, vanillin, salicylic acid, and syringic acid. There was no consistent pattern of accumulation or reduction of the total free (Table 3) or individual free phenolics in infected roots from any of the cultivars. Also, no free phenolic compounds were eliminated nor were any new free phenolic compounds developed as a consequence of infection.

Leaves

Leaves from the healthy and infected tolerant cultivars had considerably larger amounts of bound ferulic acid, sinapic acid, and isoferulic acid than did the leaves from the susceptible cultivars (Table 2). After nematode infection, leaves from the tolerant cultivars had more gentisic acid, *p*-coumaric acid, umbelliferone, scopoletin, phloretin, and unknowns 5, 7, 9, and 10 while in the susceptible cultivars, there was more homovanillic acid, sinapic acid, isoferulic acid, and unknown 6 than in the corresponding healthy cultivars (Table 2). Increases in salicylic acid and *o*-and *m*-coumaric acid were common to both plant groups. In spite of the increase in specific individual bound phenolics, the total phenolics in the leaves from both the tolerant and susceptible cultivars were, with the possible exception of 'Milam,' not significantly increased as a consequence of *R. similis* infection (Table 3).

The free phenolics in the leaves from all healthy cultivars constituted approximately 2 per cent of the total leaf phenolics. After infection, the free phenolics in the leaves from the tolerant cultivars were increased to 7 per cent of the total, while in the susceptible cultivars, the free phenolics still constituted 2 per cent of the total phenolics. Considerable increases in free phenolics in the leaves were noted from both 'Ridge Pineapple' and 'Carrizo' citrange as a consequence of infection. The latter showed an elevenfold increase (Table 3). Chromatograms of the free phenolics usually had from ten to fourteen fluorescent areas which included trace amounts of scopoletin, sinapic acid, ferulic acid, salicylic acid, and cinnamic acid. Leaves from 'Carrizo' citrange not only had larger amounts of all the individual free phenolics, especially scopoletin, ferulic acid, and *o*- and *p*-coumaric acid, but also contained several additional small unidentified fluorescent materials.

DISCUSSION

Essentially the same kinds and amounts of phenolics, with few exceptions, were found in both the healthy tolerant and healthy susceptible cultivars. After infection, the phenolic composition in both plant groups were quantitatively changed, especially the bound phenolics in the tolerant roots. Sinapic acid was considerably reduced in the leaves from the infected tolerant cultivars while salicylic acid, gentisic acid, *p*-coumaric acid, umbelliferone, and scopoletin were increased. The accumulation of the coumarins, umbelliferone and scopoletin is especially noticeable in affected plants and appears to indicate a response common in a number of species.[10–14]

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The free phenolics in the tolerant cultivars, though representing only a small portion of the total phenolic content, were increased only in the leaves from the infected seedlings so that there appeared to be some mobilization of these in the tolerant hosts. Free phenolics are less soluble, and, hence, less likely to be translocated than bound phenolics, yet the free phenolics were concentrated at a considerable distance from, rather than at, the infection site where these substances generally tend to accumulate.[14–16] This pattern of accumulation, also observed for amino acids,[17] proteins,[18, 19] hesperidin, and growth inhibitors[3] at sites far removed from the area of *Radopholus similis* infection, appears to be characteristic of this disease and suggests a systemic response to infection by *R. similis*.

The net effect of parasitism in the tolerant roots is an increase in bound phenolics of approximately 30 per cent for 'Ridge Pineapple' and 'Milam' and 300 per cent for 'Carrizo' citrange. Considered on the basis of total root weight of these tolerant seedlings (Table 1), this represents 2·4, 2·3, and 3·5 mg of phenolic substances, respectively, as compared to 0·29, 1·1, and 0·50 mg in the infected roots from sour orange, grapefruit, and rough lemon (Table 1). The question then is whether the increase in these post-infectionally formed phenolics can be considered as one of the causes of the observed tolerance to *R. similis*. A 1–3 mg increase in phenolic substances which was found in the tolerant roots, could conceivably be responsible for the slow starvation of a given population of the nematode once the pathogen had entered the root and initiated the physiological processes associated with feeding. If these phenolics are mobilized to the site of injury (feeding area) as has been observed with other host-pathogen interactions,[14–16] it is conceivable that sufficient phenolic substances (or possibly a specific, as yet, unidentified material) could accumulate to inhibit the extracellular lytic enzymes[20–22] or other vital physiological processes of the pathogen. Direct proof, however, will require further investigation.

Without food, *R. similis* can survive 4 to 6 months,[23] the latter period has generally been observed to be the minimum time required for the disappearance of the nematode from the roots of these tolerant cultivars. Since *R. similis* is an obligate parasite, it has not been possible to evaluate *in vitro* the effect of either the individual phenolics or the phenolics in the tissue extract on the feeding activity of this parasite.

Although not established with the present data, the phenolics in the roots from these susceptible cultivars may have been mobilized at the infection site but were readily inactivated through condensation and coagulation with the amino acids and proteins that accumulate in the areas of infection in the susceptible citrus roots.[17–19] The rather extensive necrosis in the infected susceptible roots also suggests the involvement of phenolics even though these condensation products were incapable of producing a physical barrier so often associated in resistant hosts with pathogen inactivation or restriction.[8, 24]

The specific pattern of response to stress initiated by the nematode appears to be manifested in different ways depending on whether the host is either susceptible or tolerant. Three main

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[21] D. E. HATHWAY and J. W. T. SEAKINS, *Biochem. J.* **70**, 158 (1958).
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differences noted were: (i) an increase in bound phenolics in the roots and an increase in the free phenolics in the leaves from the tolerant cultivars, (ii) a decrease in bound phenolics in the roots from the susceptible cultivars, and (iii) quantitative changes in the individual phenolics of both plant groups. In the latter case, a number of these phenolics have been identified as cofactors for indolyl-3-acetic acid oxidase where their function appears to depend on a critical proportion of each phenolic constituent[25, 26] so that further consideration should be given to their role, if any, in the growth manifestations characteristic of this disease.

EXPERIMENTAL

Plant Propagation

Six citrus cultivars were used in this investigation. The three susceptible to *Radolpholus similis* were: *Citrus aurantium* L. (sour orange), *C. paradisi* Macf. var. 'Duncan' (grapefruit), and *C. limon* Osbeck (Florida rough lemon). The three tolerant to *R. similis* were: *C. sinensis* Osbeck var. 'Ridge Pineapple' (sweet orange), 'Milam,' a citrus hybrid of unknown parentage (probably a hybrid between rough lemon and sour orange), and 'Carrizo' citrange (*C. sinensis* Osbeck navel × *Poncirus trifoliata* Raf.).

One-year-old seedlings were arranged in a greenhouse into groups of twenty-four plants per cultivar, each having comparable size and weight. Twelve plants of each cultivar were then inoculated with approximately 100 female *R. similis* by pouring a 50 ml H_2O suspension of the nematodes into the root zone of each plant. To ensure a sustained nematode population, plants were reinoculated twice, each at 6-month intervals. The remaining twelve plants of each cultivar served as non-inoculated controls. All plants were fertilized monthly with a soluble commercial preparation of 20-20-20 analysis containing micronutrients and supplemented with $MgSO_4$. Seven months after the last inoculation, six plants of each of the healthy and infected cultivars were harvested prior to 10 a.m. on each of three successive days. Shoots and roots were weighed, portions of each root system were used to determine the number of *R. similis*,[27] and feeder roots and mature leaves were removed and immediately processed for extraction of the phenolics.

Extraction of Phenolics

One hundred g tissue were comminuted in a blendor with 300 ml hot 95 per cent ethanol, refluxed for 2 hr, and filtered hot through a sintered glass funnel. The solvent was removed under reduced pressure and the residue was triturated in 50 ml hot water for subsequent free[28] and acid hydrolysable[29] (bound) phenolic extraction.

Chromatography and Quantitative Determinations

Two-dimensional ascending chromatography was employed using Whatman No. 1 paper 22 cm.[2] Chromatograms, in quadruplicate, were spotted with the equivalent of either 250 or 500 mg of plant material. The first direction solvent system was benzene-acetic acid-water (125:72:3) v/v/v, equilibrated at least 5 hr at 17° prior to use. After drying overnight at 26°, the second direction was developed in sodium formate-formic acid-water (10:1:200) w/v/v, at 26°. Good resolution and excellent R_f reproducibility were obtained with these solvents at these temperatures. Details on the identification of the individual phenolics that were analyzed have been published elsewhere.[10, 30] Prior to analyses, the position of the individual phenolic on the chromatogram was determined by marking its fluorescent area under u.v. light. Phenolics that could not be located in this manner were compared to a diazo-sprayed chromatogram of the same tissue extract which then served as a template for marking the position of the phenolic. Only those phenolics that showed good resolution on the chromatogram and were present in amounts of at least 1 μg were removed for analyses. Total bound and free phenolics were determined directly from the tissue extract rather than from the chromatogram because of the inherent difficulty in recovering all the phenolic spots from the chromatogram.

Phenolic areas to be analyzed were cut from the chromatogram and eluted with 7 ml H_2O at 90° for 5 min. After removal of the paper, 0·1 ml Folin Ciocalteau reagent [31] and 0·2 ml 20% Na_2CO_3 were added and the

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solution heated for exactly 3 min at 92°, cooled for 30 min, and the absorptivity (A) determined at 660 nm. Paper blanks of comparable size to some of the phenolic spots were always included to determine background color. The concentration of the identified phenolic was then ascertained by comparison with suitable standards. Curves prepared with ferulic acid served as a standard for determining the concentration of the unknowns as well as the total bound and free phenolics in the tissue extracts. Because most of the phenolics in the free extract were present in amounts less than 1 μg/g tissue, it was not possible to analyze the individual phenolics present on the chromatogram. Data on phenolics, averaged from at least three chromatograms per tissue extract, are presented as μg/g tissue, fresh weight basis. Mature leaves and feeder roots from both healthy and infected seedlings contain 63–66 per cent moisture.