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ATTEMPTS TO INCREASE TOLERANCE OF GRAPEFRUIT SEEDLINGS TO THE BURROWING NEMATODE (*RADOPHOLUS SIMILIS*) BY APPLICATION OF PHENOLICS*

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Abstract—In tests using burrowing nematode-susceptible grapefruit seedlings, host tolerance (as measured by feeder root and total plant weight) could be increased by exogenous applications of vanillic acid. Burrowing nematode infection reduced total phenolics in the roots of the nontreated seedling by 25 per cent, but total phenolics in the infected root were increased by treatments with hesperidin, ferulic acid and vanillic acid. Only with the latter treatment was total phenolics in the infected root maintained at a level comparable to the healthy control. Quantitative determinations were made in the feeder roots for 13 phenolics which constitute 70–80 per cent of the total phenolics. Treatments of infected seedlings especially manifested increases in: vanillic acid, salicylic acid, *p*-hydroxybenzoic acid, *o*-coumaric acid, and gentisic acid. The increase in total and specific phenolics resulting from the vanillic acid treatment could account for the sustained host development in spite of the presence of the pathogen, but that consideration should also be given to other phenolic-mediated effects. No direct relationship could be found between the total phenolic content of the feeder roots and the degree of host tolerance that was produced by the various phenolic treatments.

INTRODUCTION

ONE OF Florida's most destructive citrus diseases, 'spreading decline', is caused by the soil-borne, obligate, endoparasitic burrowing nematode (*Radopholus similis* (Cobb) Thorne). Ingress of the pathogen is through young feeder roots[1] which are subsequently destroyed. There is extensive browning and necrosis of the parasitized root tissues, and trees so affected subsequently sustain considerable physiological changes in all portions of the tree.[2–8] One of these modifications in the host is a significant reduction in both the free and bound phenolics in the roots and leaves of susceptible *Citrus* cultivars.[8] With tolerant cultivars, however, the net effect of *R. similis* infection is a 27–300 per cent increase (depending on the cultivar) in the bound phenolics, primarily in the roots.[8] In tolerant cultivars, there is also extensive browning and necrosis of the parasitized roots, but the nematode is slowly eliminated and seedlings recover within 6 months after infection. This interval is equal to the time of *R. similis* survival without food.[9,10] The disappearance of the nematode from these

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roots has been interpreted to indicate a post-infectional defense mechanism that possibly may be attributed to a change or accumulation of aromatic compounds in the host.

Essentially the same kinds and amounts of phenolics, with few exceptions, were previously found in both the healthy tolerant and healthy susceptible *Citrus* cultivars;[8,11] but after infection the phenolic composition in both plant groups was quantitatively changed.[8] Thus, the reduction of phenolics in the susceptible roots in contrast to an increase in specific phenolics in the tolerant roots following *R. similis* infection, prompted further investigations to determine the extent to which phenolics could be modified in the roots of a *R. similis*-susceptible citrus cultivar.

As part of a continuing study on the biochemical changes in *Citrus* infected with the burrowing nematode, the present investigation has been concerned with two objectives: (i) to establish if phenolics applied to the soil can be taken up by the plant so as to modify the individual and/or total phenolics in the root system of a *R. similis* susceptible cultivar and (ii) whether there is any relationship to the total phenolic content of the feeder roots and the observed host tolerance to *R. similis* infection. Phenolics selected for study are, with the exception of coumarin and hesperidin, normal constituents of grapefruit (*C. paradisi* Macf.) feeder roots and are the ones most involved with changes associated with nematode infection. The term host tolerance, as used in the text, indicates a sustained root and shoot development of the infected host.

RESULTS

Twin-Root Seedlings

Plant response. High rates of *o*-coumaric acid, ferulic acid, vanillic acid, gentisic acid, and hesperidin produced the best root systems in the nematode-infected portion of the twin-root seedlings (Table 1). These phenolics produced root systems that were essentially comparable to, or better than, the healthy controls. Only hesperidin (high rate) produced a total plant weight that was greater than the healthy controls. *R. similis* was not eliminated by any of the treatments and no significance can be assigned to the differences in the number of nematodes recovered per treatment.

Total phenolics. All phenolic treatments either maintained or increased the total phenolics in the parasitized roots, with the greatest increase (*ca.* 50 per cent) obtained with the caffeic acid treatment (Table 1). Total phenolics from the roots of the infected, non-treated seedlings were reduced approximately 25 per cent.

Some of the phenolics or their metabolites were absorbed and were translocated in grapefruit roots so that the net effect was an increase in the total phenolics and a sustained root development in the parasitized root system. The lower root weights observed with *p*-hydroxybenzoic acid, caffeic acid, coumarin, and *p*-hydroxyphenylacetic acid treatments, even though the total phenolics were increased, were due to the effects of the nematode and/or toxicity from these phenolics. The experimental design, limited by the number of acceptable twin-root seedlings, precluded the separation of these two factors.

Single-Root Seedlings

Plant response. The weights of all phenolic-treated nematode-infected seedlings were considerably reduced after 8 months except in those seedlings treated with vanillic acid (Table 2). The infected plants from this treatment maintained growth at a level comparable

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TABLE 1. INFLUENCE OF PHENOLICS APPLIED AS SOIL TREATMENTS TO TWIN-ROOT GRAPEFRUIT SEEDLINGS INFECTED WITH *R. similis**

Treatments§	Rate (mg)‖	Fresh weight (g)		Total phenolics§ in infected root (μg/g fr. wt.)
		Feeder roots from infected root system	Total plant	
Control (non-infected)	—	24‡	157‡	300
Control (infected)	—	16	122	228
p-OH Benzoic acid	2·5	14	103	—
	5	16	122	396
o-Coumaric acid	2·5	21†	136†	—
	5	27‡	147‡	312
Ferulic acid	2·5	13	128	—
	5	24‡	143†	296
Caffeic acid	2·5	18	129	—
	5	14	123	440
Sinapic acid	2·5	13	123	340
Vanillic acid	2·5	19	134	—
	5	23‡	149‡	380
Coumarin	2·5	19	124	—
	5	15	127	388
Cinnamic acid	2·5	21†	142†	—
	5	13	126	368
Salicylic acid	2·5	17	123	—
	5	20	148‡	336
p-OH Phenylacetic acid	2·5	14	124	—
	5	13	129	340
Gentisic acid	2·5	20	145‡	—
	5	22†	156‡	336
Hesperidin	2·5	18	150‡	—
	5	24‡	173‡	358
Naringin	2·5	21‡	149‡	—
	5	18	159‡	320

* Phenolics were applied semiweekly for 4 months to one of the twin-root systems. That portion of the root system not receiving the phenolic treatment was inoculated with *R. similis* 30 days after treatment began.

†,‡ Differences compared to infected control are significant at 5 and 1 % levels, respectively.

§ Phenolic analyses were made directly from the processed tissue samples.

‖ All treatments except non-infected control were carried out on seedlings with one of the twin-root systems infected. Number of *R. similis* per g feeder root ranged from 3 to 20. No significance could be assigned to the differences in numbers of nematodes recovered per treatment. Data taken at termination of 3 month's infection.

to those of the treated and non-treated healthy controls. Response of the infected seedlings treated with ferulic acid was also comparable to the healthy controls, but when compared with the corresponding healthy, ferulic acid-treated seedlings, feeder root and total plant weights were 29 and 18 per cent less, respectively. All phenolic treatments except vanillic acid and *o*-coumaric acid increased the weight of the non-infected seedlings. As in the tests with the twin-root, grapefruit seedlings, *R. similis* was not eliminated by any of the treatments.

TABLE 2. PLANT RESPONSE AND TOTAL PHENOLIC CONTENT IN THE FEEDER ROOTS OF BOTH HEALTHY AND INFECTED SINGLE-ROOT GRAPEFRUIT SEEDLINGS THAT RECEIVED SOIL APPLICATIONS OF PHENOLICS

Treatment	Rate‡ (mg)	Fresh weight (g) feeder roots		Per cent reduction	Fresh weight (g) total plant		Per cent reduction	Total phenolics (μg/g fr. wt.)‖	
		H	I§		H	I		H	I
None (control)	—	34†	19	44	122†	95	22	464	351
Hesperidin	5	53†	21	60	160†	99	38	401	360
	10	49†	24	51	142†	97	32	328	404
Ferulic acid	5	45†	32	29	146†	119	18	360	376
	10	42†	21	50	136†	101	26	340	388
Vanillic acid	5	32	30	6	126	115	9	404	468
	10	31	25	19	131*	110	16	428	452
o-Coumaric acid	5	47†	26	45	145†	103	29	436	404
	10	34*	25	26	127*	109	14	468	452

*,† Difference between healthy (H) and infected (I) is significant at 5 and 1% levels, respectively.
‡ Phenolics were applied weekly for 9 months. *R. simils* added to root zone 30 days after treatments began. Data taken at termination of 8 month's infection.
§ *R. similis* recovered from roots of all infected seedlings at termination of experiment.
‖ Phenolic analyses were made directly from the processed tissue samples.

TABLE 3. CHANGES IN THE AMOUNTS OF THE PRINCIPAL PHENOLICS IN THE FEEDER ROOTS OF BOTH HEALTHY (H) AND INFECTED (I) SINGLE-ROOT GRAPEFRUIT SEEDLINGS BY SOIL APPLICATION OF PHENOLICS.*

Treatment	Rate† (mg)	Phenolics analyzed (μg/g fr. wt.)													
		Sinapic acid		Ferulic acid		*o*-Coumaric acid		Vanillic acid		Isoferulic acid		Salicylic acid		Naringenin	
		H	I	H	I	H	I	H	I	H	I	H	I	H	I
Control	—	28	20	16	11	17	8	18	10	20	14	22	15	11	5
Hesperidin	5	28	22	17	10	18	17	22	18	23	18	25	17	14	9
Hesperidin	10	22	29	17	18	26	12	14	20	17	20	23	26	7	7
Ferulic acid	5	32	29	17	14	14	20	17	18	18	18	23	23	12	10
Ferulic acid	10	44	34	18	14	12	12	17	18	17	18	31	31	10	9
Vanillic acid	5	30	34	14	17	14	26	28	39	25	17	23	28	9	7
Vanillic acid	10	29	28	17	16	19	32	47	35	22	17	25	32	10	7
o-Coumaric acid	5	31	20	14	12	31	25	32	22	20	20	25	22	10	10
o-Coumaric acid	10	44	34	14	17	50	34	26	15	29	15	26	20	9	7

Treatment	Rate† (mg)	Phenolics analyzed (μg/g fr. wt.)											
		Scopoletin		Umbelliferone		*p*-OH Benzoic acid		*p*-OH Phenylacetic acid		Gentisic acid		Esculetin	
		H	I	H	I	H	I	H	I	H	I	H	I
Control	—	75	56	35	20	41	59	17	15	18	16	0	0
Hesperidin	5	78	66	39	24	44	71	20	15	17	7	0	0
Hesperidin	10	76	77	24	24	33	82	20	20	9	22	0	0
Ferulic acid	5	78	81	25	27	38	60	20	18	14	12	0	0
Ferulic acid	10	73	73	33	26	39	60	12	18	14	22	0	0
Vanillic acid	5	72	80	23	30	60	69	17	18	15	22	0	0
Vanillic acid	10	71	70	25	28	50	74	17	22	14	18	0	0
o-Coumaric acid	5	82	76	30	24	38	69	15	20	12	9	9	0
o-Coumaric acid	10	74	68	31	22	38	62	22	17	14	18	20	12

* Data taken at termination of 8 months' infection.

† Phenolics applied weekly for 9 months.

Total phenolics. Total phenolics in the non-treated roots were reduced 25 per cent by *R. similis* infection (Table 2). Phenolics were moderately increased in the infected root by high rates of both hesperidin and ferulic acid and by the low rate of vanillic acid (Table 2). Only vanillic acid (both rates) and the high rate of *o*-coumaric acid maintained total phenolics in the infected root at a level comparable to those of the healthy control. Prolonged treatment (9 months) of healthy seedlings with phenolics tended to reduce the total phenolic content of the roots. The exception to this was with the high rate of *o*-coumaric acid.

Individual phenolics. Most of the 13 phenolics selected for analyses were generally increased in both the healthy and infected seedlings by the various phenolic treatments (Table 3). An unidentified Folin reactive material(s) was present in the roots from the healthy non-treated control since the 13 phenolics that were analyzed accounted for only 66 per cent of the total phenolics in these roots as compared to 75–85 per cent for all other root samples. Treatment of infected seedlings especially manifested increases in: *o*-coumaric acid, vanillic acid, salicylic acid, *p*-hydroxybenzoic acid, and gentisic acid. No one treatment except the low rate of vanillic acid resulted in a substantial increase in most of the individual phenolics. In comparing treatments, infected versus healthy, the general trend was a reduction in the amount of the individual phenolics in the non-treated diseased root, in which specific phenolics were considerably modified in amount by treatment alone or by treatment plus infection. For example: in the healthy roots, sinapic acid, vanillic acid, salicylic acid, *p*-hydroxybenzoic acid, scopoletin and umbelliferone constituted approximately 70 per cent of the phenolics. With the exception of the *o*-coumaric acid treatment (high rate), this percentage was changed only slightly by treatment and/or infection. All treatments considerably increased *p*-hydroxybenzoic acid only in the infected roots while salicylic acid was sustained at a high level in both the healthy and the infected roots. Applications of vanillic acid and *o*-coumaric acid, particularly the higher rates, produced a threefold increase in the healthy root and a fourfold increase of each respective phenolic in the infected root. Esculetin was not found in any roots except those treated with *o*-coumaric acid. In general, the low rate of ferulic acid caused less deviation in the overall phenolic pattern between the healthy and the similarly treated and infected seedlings, than did any of the other treatments.

Thus, in the longer term experiment with single-root seedlings, treatments with phenolics modified the total and individual phenolic content of the feeder roots of healthy and infected seedlings, but no treatment increased the total phenolic content in the infected root beyond the level observed in the healthy roots (Tables 2 and 3). No direct relationship was evident between the total phenolic content of the feeder roots and the level of induced host tolerance. *R. similis* was not eliminated from the host by any of the treatments.

DISCUSSION

Attempts to assess *R. similis* induced physiological responses on a susceptible *Citrus* cultivar should take into account that significant changes in the host physiology are slow and usually are not evident until 2–3 months after infection with subsequent changes becoming more pronounced during the following 6–15 months. These physiological modifications are both local (at infection sites)[1,3,4,7,8] and systemic with lesser, though somewhat similar, changes at areas far removed from the site of pathogen establishment.[2,5–8] Plus the fact that *R. similis* is an obligate parasite, precludes testing *in vitro* of host metabolites or other substances.

Accumulation of phenolics and the induction of host tolerance has been demonstrated for a number of host–pathogen combinations.[12–19] Infusions of phenols,[12,13,19] phenolic mixtures,[19] and phenylalanine[20,21] have been shown to increase the resistance of the host to a given pathogen. This increase in resistance was directly attributed to increases in one or more phenolics and was not necessarily due to the elaboration of compounds or mixtures of compounds that were unique to the resistant cultivar.[19] All these investigations, however, have been relatively short-term trials, usually involving a span of from 30 min to several weeks.

Twin and single root grapefruit seedlings exhibited similar response to phenolic treatments, but with differences noted in the amount of the total phenolics. All treatments increased total phenolics in the infected roots over those of the infected controls, except in the twin-root seedlings where the total phenolics were sustained at a level that was greater than those in the healthy controls. Prolonged treatments (9 months) tended to reduce total phenolic content of healthy seedlings while considerably increasing total plant weight. From the latter effect it appeared that some phenolics could serve as metabolically functional carbon sources, particularly those with glycosidic residues. Extended treatment with some phenolics appeared to either suppress synthesis of aromatic compounds and/or accelerate their translocation to and their accumulation in, other portions of the plant. This latter effect was recently observed in hesperidin-induced dormancy experiments in *C. sinensis* Osbeck.[22] In twin-root seedlings, the increase in bound phenolics in the infected root may be a normal consequence of short-term treatment plus infection,[13,23] or may be due to the continued absorption and translocation of the phenolics from the treated healthy root to the infected portion of the same root system.

In a susceptible grapefruit seedling, the compatible host–*R. similis* reaction involves a mobilization of phenolics, but the rate of synthesis is probably insufficient to sustain those phenolics inactivated by condensation at the infection site. Thus, one effect of parasitism in the compatible host is a reduction in total phenolics in the feeder roots. In tolerant citrus seedlings, the host–*R. similis* relationship appears to be an incompatible reaction which also involves accelerated phenolic synthesis and accumulation of phenolics at the infection site. In addition, there is possibly a rapid tissue collapse so that the pathogen is restricted. In contrast to the response of the compatible host, then, the effect of *R. similis* infection in the incompatible host is a moderate to large increase in a number of the individual as well as total phenolics in the infected root and a subsequent elimination of the pathogen in *ca.* 6 months.

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Thus, in a *R. similis*-compatible citrus cultivar, vanillic acid treatments induced physiological modifications in the host with the consequence that there was less host–parasite compatibility so that essentially normal plant development was maintained despite the pathogen.

On the basis of total weight of feeder roots, single-root seedlings treated with vanillic acid had approximately 13 mg of phenolic substances in the non-infected root and 14 mg in the infected root as compared to 15·7 mg in the healthy controls and 3·5 mg in the infected controls. These phenolic changes could account for the improved host tolerance and would imply a continuous elaboration of phenolic substances and that these substances were translocated to the infection site as has been observed in other host–pathogen interactions.[12,16,23] Necrosis and browning of the roots were extensive indicating these phenolics were subsequently condensed with the accumulated amino acids and proteins[3,4] conceivably producing a physical barrier that partially restricted or inactivated the pathogen.[24,25]

Except when large accumulations of a specific phenolic have been directly implicated in toxicity to a given parasite, the role of the individual phenolics in the hosts defense mechanism is little understood. In some instances, no specific phenolic was implicated and host tolerance was attributed to a general accumulation of phenolic substances in the invaded tissue.[16–19] With vanillic acid treatment (low rate) of grapefruit seedlings, certain phenolics, particularly *p*-hydroxybenzoic acid, sinapic acid, salicylic acid, vanillic acid, gentisic acid, ferulic acid, scopoletin, umbelliferone and *o*-coumaric acid were increased. Most of these phenolics were also increased in the *R. similis*-infected tolerant citrus roots,[8] but unlike the roots from these tolerant hosts, total phenolics in the susceptible grapefruit roots were not increased above the amount of those phenolics found in the healthy roots. If certain phenolics did participate individually or collectively in maintaining good host development in spite of no large accumulation of total phenolics, then additional phenolic mediated changes might also be considered to account for the induced host tolerance with the vanillic acid treatment. These are: (i) that even with the increase in individual phenolics, the balance or ratio of each phenolic constituent in the root was maintained at a level capable of accelerating the necessary enzyme-mediated[26–31] defense mechanism and (ii) that vanillic acid *per se* may selectively neutralize phytotoxins elaborated by the host and/or pathogen.

Thus, in a compatible host–parasite combination such as grapefruit–*R. similis*, treatment with specific phenolics, while not eliminating the pathogen, can modify the individual and total phenolic content in the root. However, there does not appear to be any direct relationship between the total phenolic content of the feeder root and the level of host tolerance produced with phenolic treatments. We believe that the increase in total and specific individual phenolics from the vanillic acid treatment may account for some suppression of *R. similis* activity but that consideration should also be given to other phenolic-mediated effects.

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EXPERIMENTAL

Plant Propagation

Citrus seedlings with two types of root systems, all involving the *R. similis* susceptible grapefruit (*C. paradisi* 'Duncan'), were used in these investigations. For short-term experiments (3 months infection), twin-root plants[32] were used. Longer term infection trials (8 months) used single-root seedlings. All seedlings were maintained in a greenhouse as previously reported.[8] Plants selected for study had comparable size and weight.

Twin-Root Seedlings

Tap root from 2-yr-old seedlings was split lengthwise from the tip to the base of the stem. Each half-root system was then planted in a 1·4 l. paired-container of steamed Lakeland sand (organic-free subsoil). Seedlings were allowed to callus and become established for 6–8 months before use. Each of the 13 phenolics was tested at 2·5 and 5 mg per plant (except sinapic acid) and was applied twice a week for 17 weeks as an aqueous suspension in 50 ml H_2O (Table 1). The suspension was poured into the root zone of one of the twin roots through 3 or 4 holes punched *ca.* 6 cm into the soil. Controls were similarly treated using 50 ml H_2O. 5 plants were used for each treatment. The portion of the root system not receiving treatment was inoculated with 300 mature female *R. similis* 30 days after treatments were begun and phenolic applications were continued for an additional 3 months. The number of *R. similis* per g feeder root were determined at the termination.[33]

Single-Root Seedlings

2-year-old seedlings, selected for uniform root systems, were planted in 1·4 l. containers of steamed Lakeland sand (subsoil). After the seedlings were re-established (6 months), 10 plants per replicate were treated weekly with 5 and 10 mg rates of four phenolics (Table 2) that showed the best host response in the twin-root experiment. Seedlings were inoculated with 300 *R. similis*, as above, 30 days after treatments were initiated, and treatments continued for an additional 8 months. Seedlings similarly treated, but not inoculated, served as controls.

Extraction of Phenolics

100 g feeder roots, harvested before 10:00 a.m. from a given treatment were thoroughly washed in cold running H_2O, and each sample was divided into two 50 g lots for subsequent acid hydrolysable (bound) phenolic extraction.[8]

Chromatography and Quantitative Determinations

Two-dimensional ascending chromatography was employed using Whatman No. 1 paper 22 cm^2. Chromatograms in duplicate were spotted with the equivalent of 250 mg of root material. The first direction solvent system was benzene–acetic acid–water (125:72:3, by vol.), 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) at 26°. Identification of phenolics, and analyses for total and individual phenolics by the Folin Ciocalteau method have been published elsewhere.[8,11] Total phenolics were determined directly from the tissue extract rather than from the chromatogram because of the inherent difficulty in recovering all of the phenolic spots from the chromatogram. Analyses were made for 13 phenolics which constitute *ca.* 70–80 per cent of the total phenolics normally present in the feeder roots of *Citrus*. Data are an average of four determinations per tissue sample and are presented as μg/g tissue, fresh weight basis. Feeder roots from both healthy and infected seedlings contain 63–66 per cent moisture.

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