Histochemical Localization and Nematoxicity of Terpenoid Aldehydes in Cotton

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Abstract: In healthy cotton, except for random occasional occurrence in cortical cells, terpenoid aldehydes (TA) are localized in the epidermis and, even there, are absent from the tip 2–4 cm of the root. Since constitutive TA do not occur in the endodermis and stele of the root, they cannot be effective agents against the development of the sedentary stage of the root-knot nematode, *Meloidogyne incognita*. Within 4 days after inoculation with the root-knot nematode, infection-induced TA accumulated in the endodermis and outer stele. These induced TA were thus localized where they could be effective against the sedentary stage of the nematode. Infection-induced TA accumulation was more rapid and occurred in more stele cells in a resistant cotton cultivar than in two susceptible cultivars.

TA extracts from cotton were inhibitory to nematode movement. All second-stage larvae exposed to 1,000 ppm TA for 3 h became rigid, made no movement, and appeared dead. Washing these larvae to remove the TA and incubating them for an additional 24 h did not change their appearance. Shorter exposure times or lower TA concentrations allowed some larvae to recover. Exposing larvae to 10 ppm of TA for 24 h had little effect on them. TA extracted from *G. arboreum*, a cotton that does not methylate TA, were slightly less inhibitory to the root-knot nematode than TA extracted from *G. hirsutum* which partially methylates TA. *Key Words:* Gossypol-like, resistance, gossypium, host-parasite interactions, toxicants, antibiotic compounds.

Gossypol and related terpenoids, potent toxicants produced by cotton (*Gossypium* sp.), are putatively involved in the resistance of the crop to insects (5, 6, 7, 11, 12) and fungi (1, 2, 5, 6, 15, 21). Their involvement in cotton resistance to the root-knot nematode, *Meloidogyne incognita*, has been reported by Veech and McClure (18). They suggested that infection-induced accumulation of terpenoid aldehydes (TA) might be an important mechanism of the resistance even though neither pre- nor postinfectional terpenoid concentrations in extracts of whole roots were related to resistance in a susceptible and a resistant cultivar. Their suggestions were tested further in five cotton varieties with different levels of resistance (19). The results confirmed that relative host resistance was not significantly correlated with the host's ability to produce TA in response to infection. That study also confirmed that neither pre- nor postinfectional TA concentrations in whole roots correlate with relative host resistance: the pre- or postinfection concentration in a resistant variety may be higher or lower than the pre- or postinfection concentration in a susceptible variety. Nevertheless, inoculated susceptible roots generally accumulated TA more slowly than their uninoculated controls, whereas inoculated resistant roots accumulated TA faster than their uninoculated controls.

Although the previous studies (18, 19)

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implicated TA in the resistance of cotton to the root-knot nematode, neither study took into consideration the histopathology of the host-parasite relationship or the toxicity of TA to nematodes.

Other studies (13) showed that except for occurrence in occasional cortical cells, the root cortex and stele do not contain TA. These compounds are localized in the epidermis through the length of the root, except the distal 2–4 cm of root tip, which is usually devoid of detectable concentrations. The area of the root surface devoid of constitutive TA corresponds to the preferred site of penetration by the root-knot nematode. Because the nematode penetrates, migrates, and becomes sedentary in root tissues devoid of constitutive TA, the preformed concentrations are of little consequence. Therefore, if TA are to have a role in the resistance of cotton to the root-knot nematode, new sites of TA accumulation must be formed that are anatomically situated so as to be effective against either the transitory or sedentary stage of the parasite. Additionally, because the effects of TA on nematodes have not been reported, it is necessary to demonstrate a toxic, inhibitory, or static effect to implicate these compounds in a resistance mechanism.

This paper reports the sites of histochemical localization of terpenoid aldehydes in root-knot-nematode-infected susceptible and resistant cottons and the results of the bioassay of these compounds on *M. incognita*.

MATERIALS AND METHODS

Histochemistry. The histochemical localization of constitutive and infection-induced TA was determined in cotton plants (*G. hirsutum*) that are resistant (cv Auburn 623 RNR), moderately susceptible (cv Deltapine 16), and susceptible (cv M8) to root-knot nematodes. Seeds of these cultivars were germinated at 30 C in ragdolls and inoculated 2 days later with 100 *M. incognita* second-stage larvae/seedling as described previously (9). The inoculated seedlings and controls were incubated at 28/18 C (day temp/night temp) until harvested. At harvest, fresh freehand sections were cut at the sites of inoculation and from comparable areas in uninoculated roots. The tissue sections were incubated in $HClO_3$ saturated with $SbCl_3$. The $SbCl_3$-$HClO_3$ reagent is a specific color indicator of TA (14). The stained tissue sections were wet-mounted in $SbCl_3$-$HClO_3$ on glass slides and observed under the microscope for the characteristic red antimony-terpenoid derivatives, and photographed.

TA Bioassay. TA extracted from a *G. hirsutum* cultivar according to previously described procedures (3) were concentrated in ethyl acetate. The extract, containing hemigossypol, methoxyhemigossypol, gossypol, methoxygossypol, dimethoxygossypol, and several unidentified TA, was stored at −10 C. To prepare solutions for bioassay, 1.0 ml of the concentrated TA solution was dissolved in 25 ml of 0.1 M KOH containing 0.1% Tween 20. This solution was titrated with HCl to pH 6.8–7.0, rotary-evaporated to remove the ethyl acetate, and centrifuged at 5,00z × *g* to remove any precipitate. The total TA concentration (expressed as gossypol equivalents) was determined spectrophotometrically from the extinction coefficient of the phloroglucinol derivatives as described previously (18). Concentrations of 1,000, 500, 250, 125, 50, and 10 ppm TA were prepared by dilution with 0.1 M KOH, adjusted to pH 6.8–7.0. Control solutions were 0.1 M KOH, containing 0.1% Tween 20, adjusted to pH 6.8–7.0 in distilled water. Ten replicate 0.5-ml samples of each test concentration and each control solution were placed in 1-dram screw-cap vials to which ca. 1,000 larvae in 0.01 ml distilled water were added. The number of inactive larvae, expressed as a percentage of the controls, was determined after various exposure times. The larvae were then removed from the TA test solutions by centrifugation, washed with, and resuspended in, 0.1 M KOH solution at pH 6.8–7.0 for 24 h, and checked for recovery. Also determined after this recovery period was the number of inactive larvae, expressed as a percentage of the controls.

A concentrated solution of TA in ethyl acetate was also prepared from a cultivar of *G. arboreum* which is reported to lack the gene for methylation of TA (5). This extract was bioassayed because I (19) reported earlier that methylated terpenoids held greatest promise for involvement in a mechanism of resistance to root-knot nematodes.

Bioassays of these solutions were prepared and evaluated as described above.

As a standard for comparison with toxicity tests on other animals, pure gossypol acetate solutions were bioassayed as described above. Used in addition to the standard controls was a 1,000-ppm acetic acid solution adjusted to pH 6.8–7.0 with KOH.

The standard deviation (S_x) for each treatment is given. Where the percentages of inactive larvae were between 10 and 90% for comparable treatments with *G. hirsutum* and *G. arboreum* TA, the differences were analyzed by the *t*-test.

RESULTS

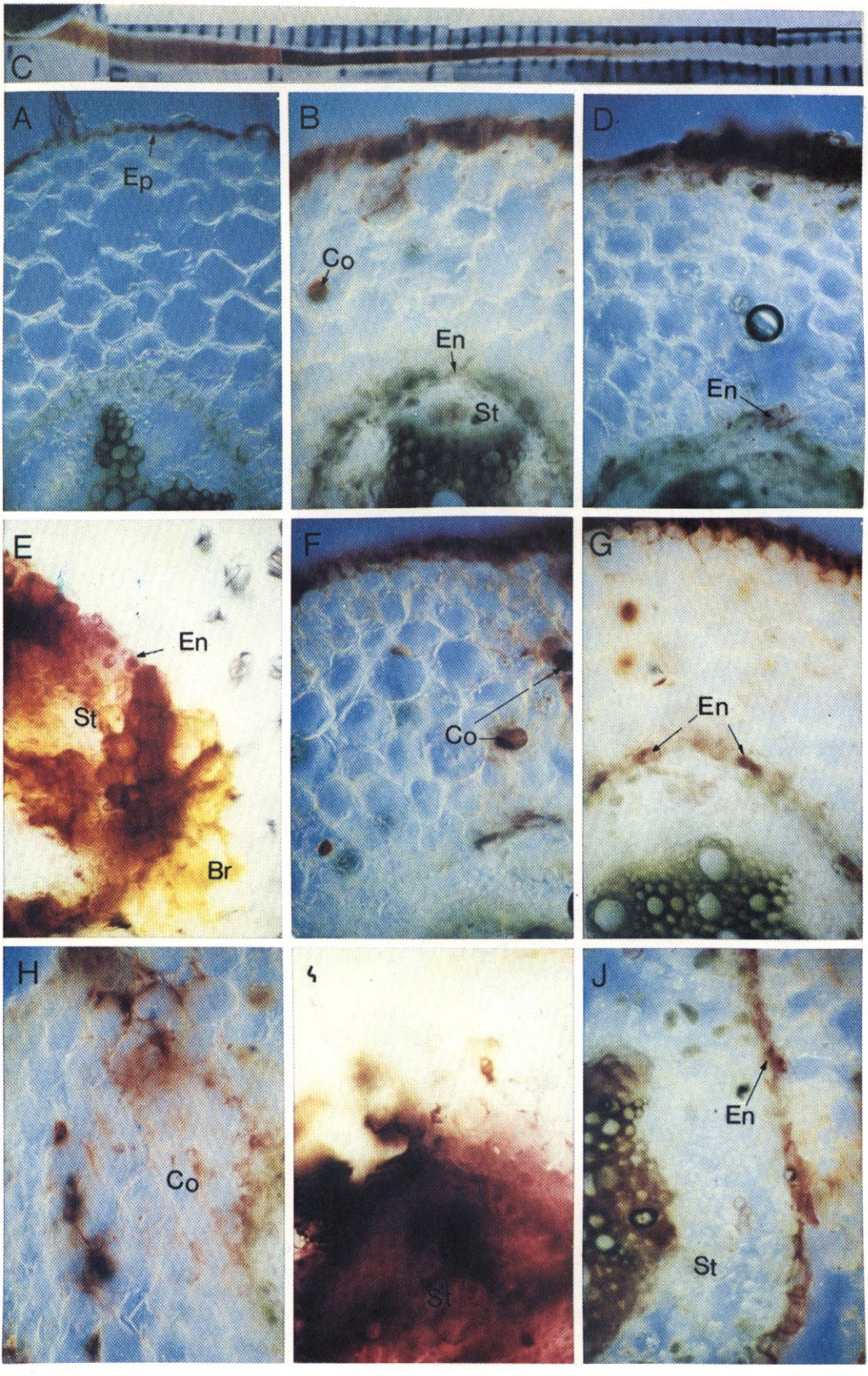
Histochemistry. In healthy cotton roots, TA demonstrated by the deposition of the red antimony-terpenoid precipitate are normally localized in the epidermis (Fig. 1A) and, occasionally, cortical cells (Fig. 1B). TA accumulation was first detectable about 2 cm from the root tip (Fig. 1C). It is apparent from these three photographs that TA in healthy roots are not localized in cells which the root-knot nematode is likely to encounter either on penetration of the root or during migration to a feeding site. Because root-knot-nematode-susceptible and -resistant cottons have identical sites of localization of constitutive TA, these sites are probably not involved in the resistance response mechanism.

Upon infection by the root-knot nematode, new sites of TA accumulation are formed. The accumulation often begins in one or two cells of the endodermis (Fig. 1D) and spreads to many cells in the endodermis and vascular cylinder (Fig. 1E). The resistant (Fig. 1D), moderately susceptible (Fig. 1F) and susceptible (Fig. 1G) cottons have all been found to respond in this way. However, the infection-induced response seems to be stronger and occurs earlier in the resistant cotton. The susceptible (Fig. 1G) and moderately susceptible (Fig. 1F) cottons showed about the same extent of infection-induced TA accumulation respectively at 12 and 14 days after inoculation whereas the resistant cotton (Fig. 1D) did so at 4 days after inoculation. TA accumulation in the cortex, but not the stele, was often greater in the susceptible cotton (Fig. 1H) than in the resistant cottons. By 11 days after inoculation, TA accumulation in the resistant cotton (Fig. 1I) was very intense throughout the entire vascular cylinder, whereas even at 14 days after inoculation, the moderately susceptible cotton (Fig. 1J) contained TA in only the endodermis and cortex.

Bioassay. TA extracted from cotton were inhibitory to the larvae of *M. incognita.* Within 20 min of exposure to 500 ppm TA, all larvae became straight and rigid (Fig. 2A) and displayed no muscular activity. In contrast, at least 90% of the larvae in the control solution were active and easily identified by their sinuous shape (Fig. 2B) and body movements.

Table 1 shows the percentage of larvae that became inactive after exposure to various TA concentrations for various periods, and the percentage that remained inactive after a 24-h recovery period in the control solution. Also shown are the effects of TA

FIG. 1. The histochemical localization of terpenoid aldehydes (TA), characterized by the red antimony-terpenoid precipitate, in cotton (*Gossypium hirsutum*) roots before and after infection by *Meloidogyne incognita.* The cotton cultivars were root-knot resistant (cv Auburn 623 RNR), moderately susceptible (cv Deltapine 16), and susceptible (cv M8). A & B) Uninoculated roots showing the localization of constitutive TA (red precipitate) in the epidermis (Ep) and random cortical cells (Co). Note the absence of constitutive TA in the endodermis (En) and stele (St). C) A complete illustration of an intact cotton tap root showing the absence of constitutive TA from the distal 2 cm of the root and then the gradual accumulation of TA toward the hypocotyl. D) Infection-induced TA accumulation in one or two endodermal (En) cells of resistant cotton 4 days after inoculation. E) Infection-induced TA in many endodermal (En) and stele (St) cells of resistant cotton 8 days after inoculation. Also note the early stage of tissue browning (Br). F) TA in the cortex (Co) of moderately susceptible cottons 14 days after inoculation; it is impossible to distinguish constitutive from induced terpenoids in this tissue. Slight TA accumulation is in the endodermis (En). G) Infection-induced TA in the endodermis (En) of susceptible cotton 12 days after inoculation. H) TA accumulation in the cortex (Co) of moderately susceptible cotton 8 days after inoculation; most of the accumulation was probably infection-induced. I) Extensive infection-induced TA accumulation in the stele (St) of resistant cotton 11 days after inoculation. J) Infection-induced TA accumulation in the endodermis (En) but not in the stele (St) of moderately susceptible cotton 14 days after inoculation.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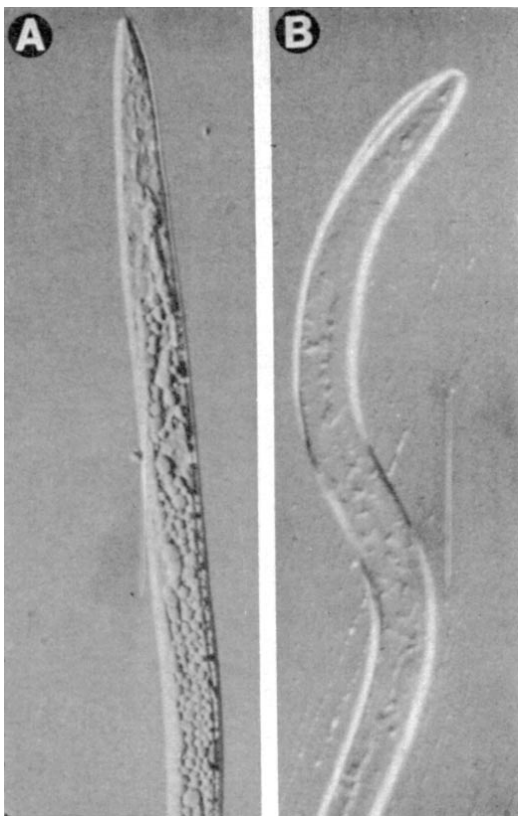

FIG. 2. Second-stage larvae of *Meloidogyne incognita*. A) A larva exposed to cotton terpenoids showing the inactive pencil shape. B) An active larva in the characteristic sinuous shape.

extracted from both *G. hirsutum* and *G. arboreum*.

After a 24-h exposure to various concentrations of TA from *G. hirsutum*, only 9% of the larvae were inactive in 10 ppm, 71% in 50 ppm, and 100% in 125 ppm or greater. Almost none of these last larvae recovered after being washed free of the TA. Shorter exposures to TA, however, were reversible in some cases, and most of the affected larvae regained muscle activity during the 24-h recovery period. Larvae that regained body movement were probably in a metabolic state analogous to anesthesis. All larvae exposed to 1,000 ppm TA were inactive after a 1-h exposure; about 25% regained activity during a 24-h recovery period. Similarly, all larvae were inactive after a 3-h exposure to 1,000 ppm terpenoid, but none of these larvae regained activity. Clearly, the number of larvae reversibly affected decreased with exposure time and/or terpenoid concentration.

TA from *G. arboreum* also inhibited the movement of *M. incognita* larvae. Wherever valid comparisons could be made (cases in which both test solutions effected between 10 and 90% inhibition), *t*-test analysis indicated that TA from *G. hirsutum* were more effective than TA from *G. arboreum*. Exposure to 250 ppm TA for 5 h, followed by a 24-h recovery period, yielded 88% and 49% inactive larvae for *G. hirsutum* and *G. arboreum* TA, respectively. The *t*-value −7.08** was highly significant.

Table 2 shows the results of exposing *M. incognita* larvae to purified gossypol. Gossypol was less effective than the natural mixtures of TA in inhibiting larval movement. After a 24-h recovery period, all larvae exposed to 1,000 ppm for 1 h were active; 74% of the larvae similarly treated with the mixture of terpenoids extracted from *G. hirsutum* were inactive. Exposure to 250 ppm gossypol for 1 h resulted in only 19% of the larvae being inactive; 91% of the larvae similarly treated with the mixture of TA extracted from *G. hirsutum* were inactive.

DISCUSSION

The major function of TA in cotton seems to be to protect the plant from various adversities. In 1905, Quaintance and Brues (17) showed that the cotton bollworm would rather feed on upland cotton (*G. hirsutum*) than on Egyptian cotton (*G. barbadense*). From current knowledge, it is likely that this preference was mediated by the terpenoid complement of the respective plants. Cook (10) suggested that "oil glands" had a protective value to the cotton plant, and Withers and Carruth (20) identified the pigment in the glands as the terpenoid gossypol and associated it with the toxicity of cottonseed to swine. More recently numerous additional terpenoids and TA from cotton have been identified (2, 3, 4, 5, 6) and their toxicity to a broad range of organisms demonstrated. Bottger *et al.* (7) were the first to demonstrate the toxicity to insects of gossypol (actually the entire complement of terpenoids).

This report is the first observation of the toxicity of terpenoids from cotton to a nematode. Although larvae exposed to low TA concentrations (< 50 ppm) for short periods (< 3 h) did recover muscle activity,

TABLE 1. The mean percentage of the *Meloidogyne incognita* larvae that were inactivated by exposure to terpenoid aldehydes extracted from *Gossypium hirsutum* (a cotton that can methylate terpenoids) and *G. arboreum* (a cotton that lacks the gene for methylating terpenoids). The standard deviation (S_x) for each ten replicate treatments is given. The *t*-value is given where valid comparison between the cotton species can be made, i.e., where the treatment affected 10–90% of the larvae.

Terpenoid concn. (ppm)	Exposure time (h)	A[1] B[1]	Inactive larvae in TA solutions From *G. hirsutum* (%)	(S_x)	From *G. arboreum* (%)	(S_x)	*t*
1000	1	A	100	0.0	99	0.5	
		B	74	4.6	66	4.7	−2.35*
	3	A	100	0.0	—	—	—
		B	100	0.0	—	—	—
	5	A	100	0.0	100	0.0	
		B	100	0.0	100	0.0	
	24	A	100	0.0	100	0.0	
		B	100	0.0	100	0.0	
500	1	A	100	0.0	100	0.0	
		B	5	2.0	13	4.8	
	3	A	100	0.0	—	—	—
		B	51	11.5	—	—	—
	5	A	100	0.0	100	—	—
		A	100	0.0	100	0.0	
	24	B	100	0.0	100	0.0	
250	1	A	91	6.3	100	0.0	
		B	2	3.7	9	5.8	
	3	A	95	5.4	—	—	—
		B	0	2.2	—	—	—
	5	A	100	0.0	100	0.0	
		B	88	2.7	49	13.0	−7.08**
	24	A	100	0.0	100	0.0	
		B	98	1.5	100	0.0	
125	1	A	73	7.0	98	1.1	
		B	4	0.9	6	1.7	
	3	A	92	4.4	—	—	—
		B	1	4.3	—	—	—
	5	A	100	0.0	100	0.0	
		B	12	3.2	7	3.6	
	24	A	100	0.0	100	0.0	
		B	83	4.6	74	7.4	−4.08*
50	24	A	71	7.7	—	—	
10	24	A	9	4.4	—	—	

[1]A % larvae inactive at the end of the exposure time.
B % larvae inactive after a 24-h recovery period following the treatment.

the inability of larvae treated with higher concentrations (> 125 ppm) to regain muscle activity is a strong argument supporting a toxic effect of TA. At low concentrations, the terpenoids, like many toxicants applied at sublethal concentrations, appear to act like anesthetics. Because the effects of TA on the transitory phase of the nematode have not been implicated in the resistance response, it is not important that nematodes recover from ephemeral exposure to low concentrations. However, it is important to the sedentary phase of the nematode that constant exposure to 125 ppm TA or greater inhibits muscle activity irreversibly. Once a larva establishes a feeding site and begins to enlarge, its ability to become transitory again is substantially reduced.

Mace (15) has shown that it takes between 24 and 48 h for resistant cotton stele cells devoid of constitutive TA to form induced TA in response to infection by *Verticillium dahliae*. If a similar or slightly

TABLE 2. The mean percentage of *Meloidogyne incognita* larvae that are inactivated by exposure to gossypol. The standard deviation (S_x) for each ten replicate treatment is given.

Gossypol acetate concn. (ppm)	Exposure time (h)	A[1] B[1]	Inactive larvae (%)	S_x
1000	1	A	100	0
		B	0	0.7
	5	A	100	0
		B	85	4.6
	24	A	100	0
		B	100	0
500	1	A	99	1.3
		B	1	0.9
	5	A	99	0.5
		B	33	0.9
	24	A	99	0.5
		B	85	2.6
250	1	A	19	3.0
		B	1	1.8
	5	A	98	2.7
		B	7	5.2
	24	A	100	0
		B	76	3.0
125	1	A	1	1.5
		B	0	0
	5	A	84	0.5
		B	6	5.9
	24	A	96	0.5
		B	23	2.6

[1]A % of larvae inactive at end of exposure period. B % of larvae inactive after a 24-h recovery period, following the treatment.

longer delay occurs for cells irritated by nematode feeding, the timing would be ideal for a resistance response. A very rapid response in the endodermis and stele might cause the larva that induced the response to abandon that feeding site and seek another. While that might eventually be fatal to the nematode, the effect on the plant could be undesirable. On the other hand, a very long delay in the synthesis of induced TA might allow the nematode to escape the effect by maturing before a toxic concentration was attained.

Lukefahr *et al.* (12) reported that gossypol had greater antibiotic activity than other terpenoids against the tobacco bud worm. My finding that gossypol was less inhibitory to the root-knot nematode than the combined terpenoids extracted from the cotton plant seems to support their suggestion that a synergistic effect between terpenoids may be involved in resistance mechanisms. I have suggested (19) that the methylated TA were the most likely TA to be involved in a mechanism of resistance to the root-knot nematode. The toxicology data presented here lend support to that hypothesis, but, because the nonmethylated TA also have high activity, the hypothesis should be amended to include both methylated and nonmethylated TA.

Because constitutive TA are not anatomically situated so as to be effective against the root-knot nematode, the hypothesis that TA are involved in a resistance mechanism was contingent upon the induction by infection of new sites of TA accumulation. The histochemical data presented here show that infection-induced TA do accumulate at sites where they will be effective against the sedentary nematode. Resistant and susceptible hosts responded to infection by synthesizing and accumulating TA at the feeding sites of the nematodes. Infection-induced TA, however, were detected earlier and accumulated in more cells in the resistant host than in the susceptible hosts. This situation is similar to that observed by Bell (1) and Mace (15) for cottons susceptible and resistant to Verticillium wilt. The difficulty of distinguishing susceptible and resistant responses histochemically is compounded by an inherent bias in the procedure. In tissue sections, nematode feeding sites that accumulate TA and stain red are much more evident than sites that do not accumulate TA and remain colorless.

In cotton the two major constitutive antibiotic metabolites are the terpenoids and the flavanols; both have never been found in the same cell (6). The constitutive flavan-3-ols (catechin and gallocatechin) are found in, among other cells, the endodermis (14). Upon infection by the root-knot nematode, TA synthesis also is induced in the endodermis. Thus, there may be a possible synergistic effect between flavanols and TA in cotton's resistance to the root-knot nematode.

Brodie *et al.* (8) and Minton (16) reported that root-knot nematodes penetrated susceptible and resistant cotton plants with equal facility. And both observed restricted larval development in resistant hosts. Minton (16) attributed resistance "to con-

ditions within the roots that prevented or delayed larval development. . . ." The data presented in this paper explain, in part, how larval development is restricted.

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