Phenol Accumulation Related to Resistance in Tomato to Infection by Root-Knot and Lesion Nematodes

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Abstract: Host-parasite relationships of *Meloidogyne incognita acrita* and *Pratylenchus penetrans* were compared on three closely related cultivars of tomato: 'Nemared', resistant to root-knot nematodes; 'Hawaii 7153', moderately resistant; and 'B-5', susceptible. Root-knot nematode larvae induced typical galls on the roots of B-5; larvae that entered Nemared were walled-off by necrotic cells; both reactions occurred in Hawaii 7153 . Lesion nematodes caused surface lesions which were initially similar on all cultivars. Five weeks after infection, they penetrated into the stele of the B-5 roots, whereas in Nemared and Hawaii 7153, injury was confined to the cortex. Chlorogenic acid was identified as the major phenolic compound in healthy tomato roots. Nemared contained the highest concentration of the acid and B-5 the lowest. Histochemical tests showed that chlorogenic acid was concentrated in the endodermis. The localized accumulation of chlorogenic acid and its oxidized products in host root cells infected by nematodes was concluded to be an important mechanism of resistance. *Key Words: Lycopersicon esculentum*, histochemistry, polyphenol oxidase.

The occurrence and metabolism of phenolic substances in plants, in response to injury or invasion by pathogens, such as fungi, bacteria and viruses, have been studied extensively (6, 10). Oxidized compounds produced in plants after invasion by pathogens often show considerable biological activity and are a common mechanism of resistance to plant pathogens.

Root-knot nematodes stimulate giant-cell formation in vascular tissue of susceptible plants, but a necrotic reaction takes place in resistant plants. Nematode larvae surrounded by necrotic cells fail to develop normally and soon die (5). Lesion nematodes destroy parenchyma cells of the cortex. Several authors have noted that the endodermis browns extensively and is not penetrated, at least in early stages of infection (9, 11, 14, 15). Phenolic compounds have been associated with nematode injury, including browning of plant tissues (1), but the role of phenolic substances in both resistant and susceptible reactions is not well understood.

Our paper reports histological and histochemical effects of *Meloidogyne incognita*

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acrita and *Pratylenchus penetrans* on tomato cultivars differing in resistance to root-knot nematodes.

MATERIALS AND METHODS

The tomato (*Lycopersicon esculentum* Mill.) cultivars used in this study are genetically similar except that 'Nemared' is resistant to *M. incognita acrita*, 'Hawaii 7153' is partially resistant and 'B-5' is fully susceptible.

Meloidogyne incognita acrita (Kofoid and White) Chitwood, was cultured aseptically in tomato roots grown in nutrient agar in petri dishes. Larvae to be used for inoculum were obtained by picking egg masses from the surfaces of galls and hatching the eggs in sterile water. *Pratylenchus penetrans* (Cobb) was cultured aseptically in alfalfa callus tissue grown in nutrient agar containing 2,4-D (8).

A suspension of each nematode species was pipetted over the roots of 7-day-old tomato seedlings in separate nutrient agar plates. Inoculated seedlings and noninoculated controls were maintained at 20 C.

Observations of infected roots: Living roots showing lesions or galls were sectioned 30-μm thick in cross and longitudinal planes in a cryotome at −20 C. The sections were placed on a clean glass slide; a few drops of diazotized sulfanilic acid (DSA) (a general histochemical reagent for phenolic compounds) were added and any color changes observed (3). Attempts were made, without success, to quantify histochemical tests for phenols. Usually the reaction was confined to relatively few cells surrounded by unreactive tissue. A failure to stain was interpreted as an absence of phenols, and an intense stain was considered evidence for a relatively large concentration of phenols.

Paraffin sections of root lesions and galls were prepared following standard procedures described by Jensen (7, p. 78-82). Root samples were fixed in Navashin's solution for 24 hr, washed in running tap water for 2 hr and dehydrated through a tertiary butyl alcohol series. Dehydrated roots were embedded in Tissuemat®, sectioned at 10 μm and stained with safranin and fast green.

Chemical analysis: Similar amounts, about 0.05 g, of lesion areas, root galls and healthy roots were dropped separately into 10 ml of boiling methanol. Each extract was concentrated to 1 ml per g fresh tissue in a rotary evaporator under reduced pressure at 48 C. The concentrated extracts were stored at −15 C.

Concentrated methanolic extracts were analyzed chromatographically on Whatman No. 1 paper (3). Solvent mixtures used were: 4:1:2 (v:v:v) and 4:1:5 (v:v:v) *n*-butanol/acetic acid/water (BAW); 2% acetic acid; and 3:30:10 (v:v:v) hydrochloric acid/acetic acid/water (HAW).

The following color reagents were used for detection and identification of compounds: diazotized sulfanilic acid (DSA) (3); ferric chloride reagent (12); ammoniacal silver nitrate (3); Arnow's reagent (2); anthrone reagent (3); and 0.3% ninhydrin in 95% ethanol (3).

Unidentified fluorescent spots, detected in unsprayed paper chromatograms illuminated with ultraviolet light, were cut out and eluted in methanol. Absorption spectra of eluates were measured in a Bausch & Lomb Spectronic 505 recording spectrophotometer.

Methanol extracts of equal weights (0.048 g) of the same age (2-week-old) root tissues from all three tomato varieties were analyzed for chlorogenic acid by the method of Ruckenbrod (3). Optical density at 324-326 nm was compared with a standard curve prepared with known chlorogenic acid.

Six days after inoculation with *P. penetrans*, equal weights of healthy roots and infected roots of each tomato cultivar were homogenized with 1 ml of phosphate buffer (0.02 M, pH 7), and centrifuged to remove debris.

The assay for polyphenol oxidase activity is based on the formation of a dark-colored polymeric compound from catechin. One hundred and fifty μl of extract was added to a cuvette containing 3 ml of 0.25% catechin solution. The formation of brown pigment was measured spectrophotometrically at 400 nm during the interval from 30-210 s after addition of extract (16).

RESULTS

Observations of infected roots: Galls appeared on the roots of the susceptible tomato, B-5, within 3-4 days after inoculation with *M. incognita acrita* larvae. Female nematodes matured after 2-3 weeks and egg mass formation and egg laying began 20-30 days after initial penetration. No galls formed on the resistant Nemared. Dark-brown necrotic flecks appeared on the roots where larvae had penetrated. Many larvae failed to penetrate and died outside the roots. In roots of the moderately resistant Hawaii 7153, both necrotic lesions and galls were formed. The galls

appeared similar to those formed on B-5, except that few egg masses were produced.

Necrotic surface lesions developed on the roots of all three tomato cultivars after invasion by *P. penetrans*.

Whole galls from B-5 roots were crushed slightly and stained with DSA reagent for detection of phenols. Little or no reaction occurred in galled tissues in any stage of development. The addition of DSA caused the necrotic flecks on the Nemared roots to become intensely brown. In all our studies, a positive response to DSA was obtained only in tissues which were already brown.

In cross and longitudinal sections of frozen living B-5 galls, no browning occurred and DSA reactions were negative (Fig. 1). Similar sections of Nemared lesions revealed brownish endodermal cells which reacted strongly to DSA. Necrotic lesions on Hawaii 7153 showed a similar positive reaction to DSA, whereas galls on the same roots did not react.

Root surface lesions on all three tomato cultivars were yellow-to-brown and color was greatly intensified by DSA. Cross sections of all three varieties of infected roots stained with DSA showed an overall brownish color, but the endodermis stained much more deeply than any other tissue indicating a relatively large concentration of phenolic substances in this layer (Fig. 2, 3).

Cross sections of galls on B-5 plants infected with *M. incognita acrita* showed typical giant cells with thick walls and dense cytoplasm, distortion of the vascular elements and proliferation of parenchyma.

In Nemared plants, the nematodes were found in the midst of necrotic cells which were deeply stained with safranin. Larvae did not develop and giant cells were never found (Fig. 4).

Cross and longitudinal paraffin sections of lesions caused by *P. penetrans* showed similar injury in all three tomato cultivars. Cells in the cortex were often badly damaged by several nematodes, resulting in a cavity. After 5-6 weeks (the later stages of infection), nematodes were found in the vascular tissues of B-5 (Fig. 5), but none was ever found in the vascular tissues of Nemared or Hawaii 7153.

Chemical analysis: Paper chromatography of methanol extracts from all three tomato cultivars revealed seven ultraviolet-fluorescing spots of the same size and location, regardless of whether the plants were healthy or infected with *M. incognita acrita*. An additional spot was observed, however, in extracts from all three cultivars infected with *P. penetrans* (Table 1).

By spraying chromatograms with a variety of detecting reagents, the major fluorescent spot, present in both healthy and lesion nematode-infected extracts at Rf = 0.58 was determined to be a phenolic compound. A weakly reacting spot (Rf = 0.46 in BAW), present only in extracts of infected tissue, was also identified as a phenolic compound (Table 1).

By chromatographic and spectrophotometric comparison with authentic phenolic compounds, the spot at Rf = 0.58 was identified as chlorogenic acid. Chlorogenic acid had been reported previously as the major phenol in the tomato plant by Spurr et al. (13). The additional spot (Rf = 0.46) in infected extracts remains unidentified.

Differences were found among chlorogenic acid concentrations in uninfected roots of the three tomato cultivars. Nemared roots contained 0.58 mg of chlorogenic acid per gram of root tissue; Hawaii 7153, 0.44 mg; and B-5, 0.38 mg.

Differences in amounts of chlorogenic acid were also demonstrated between extracts of healthy and infected roots within each cultivar. Roots infected by nematodes contained more chlorogenic acid than did healthy roots of the same cultivar; the amount was more nearly proportional to the severity of infection, although exact amounts were not determined.

Polyphenol oxidase activity in B-5 roots increased about 50% 6 days after infection with *P. penetrans*. Similar changes occurred in the other cultivars. As injury increased, so did polyphenol oxidase activity, although in heavily infected tissue, enzymatic activity was reduced as the cortex collapsed.

Plants infected with *M. incognita acrita* showed no varietal differences in polyphenol oxidase activity or levels.

DISCUSSION AND CONCLUSIONS

Susceptible B-5 tomato reacted characteristically to invasion by root-knot nematodes in that giant cells formed and the larvae developed to adults. Histochemical tests indicated little or no accumulation of phenolic compounds.

In contrast, roots of resistant Nemared became locally discolored and necrotic when invaded by root-knot nematode larvae. Histochemical tests indicated that large amounts of phenolic compounds accumulated


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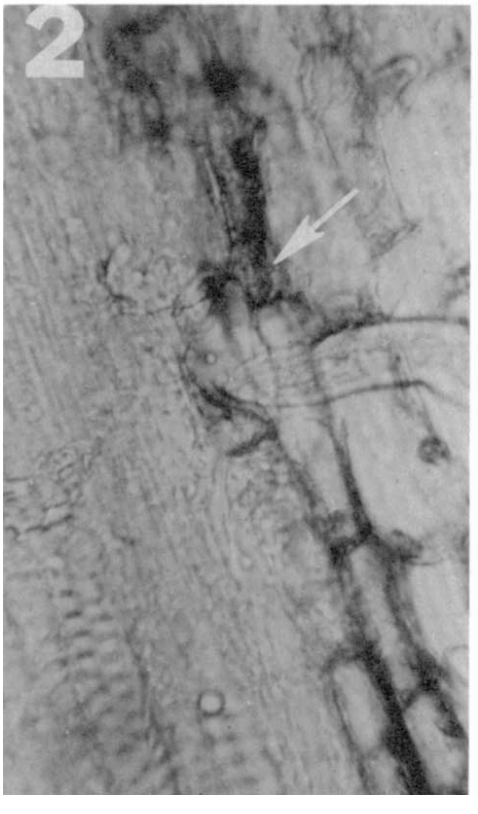
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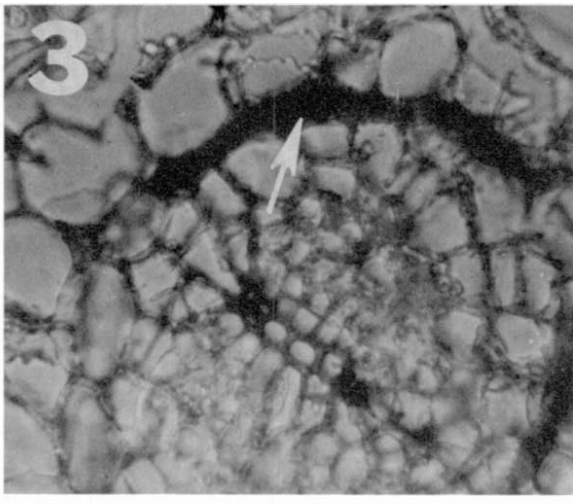
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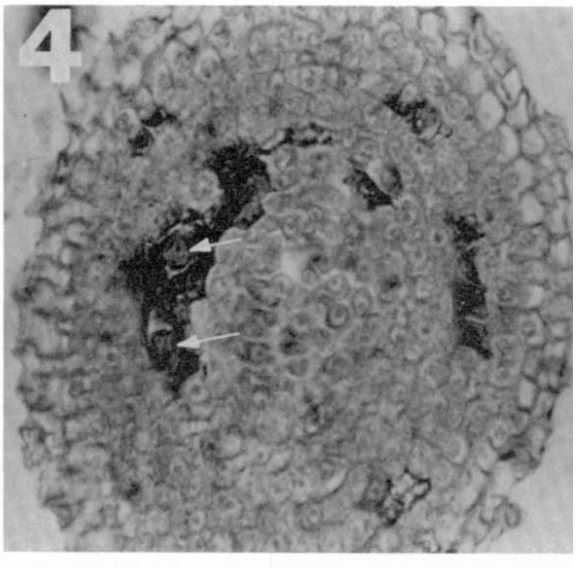
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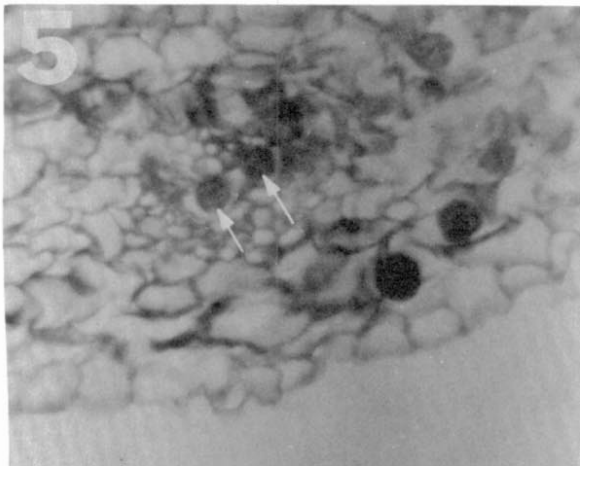
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in injured tissues. Larvae died within the necrotic areas and giant cells did not develop. If gall formation is considered a response to the presence of the nematodes, it is apparent that either Nemared tomato reacts differently to this stimulus than does B-5 or else the stimulus is never received, either because it is inactivated or never produced. The large numbers of dead larvae that never penetrated Nemared roots, point to some sort of inhibition.

In early stages of infection, all three tomato cultivars reacted similarly to *P. penetrans*. Injury in the resistant Nemared was confined to the cortex and nematodes did not pass through the endodermis, although the endodermis in the vicinity of lesions turned brown and contained large concentrations of phenolic compounds. Five weeks after inoculation, *P. penetrans* penetrated the endodermis of B-5 and destroyed vascular cells, whereas the endodermis of Nemared was not penetrated and vascular cells appeared normal.

Chlorogenic acid was identified as the major phenolic compound in tomato roots, both before and after infection by both nematodes. An additional phenolic compound, which appeared in tomato roots infected with *P. penetrans*, remains unidentified. However, a spot with the same *Rf* values was found when 1-day-old solutions of chlorogenic acid were chromatographed; the unknown spot has been tentatively identified as an oxidation product of chlorogenic acid. Failure to detect the additional phenolic compound in Nemared infected with root-knot nematodes perhaps may have occurred because the lesions on Nemared were so small.

Uninfected Nemared roots contained the highest concentration of free chlorogenic acid of the three cultivars, and, since this compound is concentrated in a relatively small number of cells, as shown by histochemical tests, varietal differences would be quite large. Infection by lesion nematodes, resulting in a relatively large amount of cell damage caused a further over-all increase in total phenols and polyphenol oxidase activity which was quite pronounced in the vicinity of injured cells.

We propose that the invasion of tomato roots by nematodes results in accumulation of chlorogenic acid which is subsequently oxidized by the action of host or nematode polyphenol oxidase, resulting in the formation of the brown-colored melanins in injured areas. Such compounds might inhibit nematode activity and prevent root-knot nematode larvae from penetrating the endodermis into tissues suitable for giant cell production. Failure to penetrate the endodermis has relatively little effect on lesion nematodes which are cortical parasites. Varietal differences in amounts of free chlorogenic acid in the uninfected roots might explain differences in resistance to injury by nematodes, particularly since the free chlorogenic acid concentration and polyphenol

TABLE 1. *Rf* values of ultraviolet-fluorescent compounds isolated by paper chromatography from three cultivars of tomato roots ('Nemared', 'Hawaii 7153', 'B-5'), both healthy and infected with *Pratylenchus penetrans*.

Spots[a]	*Rf* BAW[b] (4:1:2)	*Rf* BAW[b] (4:1:5)	*Rf* 2% acetic acid	*Rf* HAW[c] (3:30:10)
1	0.58	0.63	0.82	0.69
1′	0.46	0.53	0.75	
2	0.42	0.45	0.66	0.79
3	0.35	0.31		
4	0.22	0.24		
5	0.16	0.17		
6	0.09	0.14		
7	0.04	0.10		
8	0.58	0.63	0.82	0.68

[a]Spots 1 to 7 were present in both healthy and infected tissue extracts of all varieties. Spot 1′ was present only in infected tissue extracts. Spot 8 was known chlorogenic acid.
[b]BAW = 4:1:2 (v:v:v) or 4:1:5 (v:v:v) *n*-butanol/acetic acid/water.
[c]HAW = 3:30:10 (v:v:v) hydrochloric acid/acetic acid/water.

FIG. 1-5. Photomicrographs of sectioned tomato roots showing local accumulation of polyphenols stimulated by nematode infection. DSA reagent (diazotized sulfanilic acid, specific for polyphenols) had been added to fresh frozen sections in Fig. 1-3. **1.** 'B-5' tomato cultivar showing a susceptible reaction to *Meloidogyne incognita acrita*. The larval head (arrow) is surrounded by enlarged cells and there is no response to added DSA (× 300). **2.** Longitudinal section of 'B-5' showing the head of a *Pratylenchus penetrans* adult in the area of the endodermis which resulted in the local accumulation of polyphenols (arrow) (× 300). **3.** 'Nemared' tomato cultivar infected with *P. penetrans* showing polyphenols concentrated in the endodermis (arrow) (× 300). **4.** 'Nemared' embedded in paraffin and stained with safranin and fast green. *M. incognita acrita* larvae (arrows), circular in cross section, are surrounded by deeply stained necrotic cells (× 100). **5.** 'B-5' root prepared as in Fig. 4. Lesion nematodes (arrows), after 5 weeks, can be seen within the stele (× 150).

oxidase activity were increased by infection. The reports of Chang (4) that lesion nematodes were repelled and their respiration was significantly reduced by the oxidation products of chlorogenic acid, strongly support this hypothesis.

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