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Characterization of Anionic Peroxidases in Tomato Isolines Infected by *Meloidogyne incognita*[1]

G. Zacheo, C. Orlando, and T. Bleve-Zacheo[2]

Abstract: Changes in peroxidase activity during nematode infection were studied using root extracts of tomato near-isogenic lines differing in resistance to *Meloidogyne incognita*. Total peroxidase activity increased slightly in crude extracts of four susceptible isolines but doubled in two resistant lines, Monita and Motaci. Nematode infection enhanced levels of both *p*-phenylenediamine-pyrocatechol oxidase and syringaldazine oxidase 7 days after inoculation, especially in resistant lines. This elevated peroxidase activity in resistant isolines was caused by an increase in anionic peroxidase activity. These enzymes, which likely are involved in lignification, were isolated and purified from tomato isolines by ammonium sulfate precipitation, high performance ion-exchange chromatography, and gel electrophoresis. The purified anionic peroxidase extracts contained an electrophoretic band with R_f 0.51 that was present in extracts of infected but not uninfected roots.

Key words: enzyme, isoline, isoperoxidase, *Lycopersicon esculentum*, *Meloidogyne incognita*, nematode, peroxidase, resistance.

Multiple forms of peroxidase (E.C.1.11.1.7, donor: hydrogen peroxide oxidoreductase) exist in many plant species (6,27). Their universal distribution and the availability of many colorimetric assays have made them convenient enzymic markers in genetic, physiological, and pathological studies (10,21,31,32).

Although peroxidase is a highly catalytic enzyme, it has very little specificity and exists in a multitude of isoforms (6). These characteristics impede understanding the actual function of peroxidase in plants and plant pathogenesis. Because of the many reports of enhanced peroxidase activity during the interaction of plants with potential pathogens (15,25,29,33,34), the enzyme has been concluded to be possibly important in plant defense mechanisms (13,32). In many cases, the biosynthetic pathways leading to disease resistance involve lignification of injured host cells. For example, resistance to the fungus *Verticillium albo-atrum* in one tomato line is correlated with its ability to coat the xylem vessels more rapidly after inoculation than a susceptible isogenic line (24). The coating material appears to consist of suberin and lignin (24,29) and presumably assists in the defense of the plant by forming a barrier that mechanically excludes the pathogen. Histochemical and biochemical investigations (8,30) indicate that isoperoxidases participate in lignin biosynthesis. Specifically, peroxidases catalyze the last steps of lignin biosynthesis, i.e., the polymerization of phenolic compounds and the formation of cross-links between extensin, lignin, and feruloylated polysaccharides (4,10). Anionic peroxidases (syringaldazine oxidases) were reportedly involved in polymerization of substituted cinnamyl alcohols to lignin (12,17). Thus, peroxidases are certainly important in the reinforcement of the cell wall, and there appears to exist more than a casual relationship among pathogen infection, peroxidases, and resistance.

To understand better the function of peroxidases during infection by the root-knot nematode *Meloidogyne incognita* (11, 20,22), we undertook a comparative study of isoperoxidase isolated from near-isogenic tomato lines of the Moneymaker type with or without the *Mi* gene (16), which is a single dominant gene in tomato cultivars that confers resistance against all common *Meloidogyne* species except *M. hapla* (28).

Materials and Methods

Seeds of several near-isogenic tomato lines of the Moneymaker type, susceptible

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[2] Istituto di Nematologia Agraria, CNR, Via Amendola 165A, 70126 Bari, Italy.

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(Moneymaker, Monalbo, Moboglan, and Momor) and resistant (Monita and Motaci) to *M. incognita* (16), were germinated in sterilized quartz sand. Each uniformly germinated 10-day-old seedling was transferred into 3-cm-d clay pots containing quartz sand and divided into two groups—one uninfected and used as the control, and the other immediately inoculated with active second-stage juveniles (J2) of *M. incognita* race 2 (80 juveniles per seedling). For determination of penetration and nematode growth 40 days after inoculation, roots were stained with lactophenol–acid fuchsin (19).

Biochemical measurements were made on seedlings 7 days after inoculation. The roots of the tomato seedlings at this stage developed a great number of short lateral roots, creating a suitable system to obtain uniform nematode invasion. Segments (ca. 2-cm-long, 30 g total) of root tissues from 300 seedlings per treatment were homogenized in 10 mM N-(2-hydroxyethyl)piperazine-N′-3-propanesulfonic acid (EPPS) buffer, pH 7.8, containing 1% insoluble polyvinylpyrrolidone, in a Potter homogenizer cooled in ice. The resultant slurry was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 20 minutes. Solid ammonium sulfate was slowly added to the supernatant for 1 hour to create a 1.37 M solution, which was stirred for an additional hour. The suspension was centrifuged and the supernatant was adjusted to 3.71 M with ammonium sulfate by the same procedure. The suspension was then centrifuged and the precipitate was dissolved in 50 mM potassium phosphate buffer (PPB), pH 7.0, and dialyzed against the same buffer. The protein solution was centrifuged and concentrated with an Amicon (Danvers, MA) Centricon PM 10 membrane unit (23).

The samples were partially purified by mixing for 90 minutes in an equal volume of pre-swollen DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden), equilibrating in 50 mM PPB (pH 7.0), and then filtering through a Millipore (Bedford, MA) apparatus equipped with a 0.22-μm filter. The recovered resin was mixed for 90 minutes with PPB containing 0.5 M NaCl and then filtered similarly. Pigments in the protein extracts partially remained attached to the resin. The aqueous filtrates from these two steps were mixed and dialyzed against PPB three times. The resultant solutions were concentrated with the Centricon apparatus and then used as crude extract for assay of peroxidase activity and separation of peroxidase isoenzymes by polyacrylamide gel electrophoresis (PAGE) or by high performance liquid chromatography (HPLC).

The crude peroxidase extracts were purified by ion exchange HPLC through a Pharmacia CM-Sepharose CL-6B column (2.5 × 30 cm) equilibrated with 50 mM Na-K phosphate buffer (pH 6.0). The anionic proteins that did not bind to the column were recovered in the eluate. Flow rate was 1 ml/minute, 1 ml fractions were collected, and a 50-μl aliquot was used to assay for syringaldazine oxidase activity. The detector was set at 280 and 407 nm, and fractions with a Reinheitzahl (RZ) value (A_{407}/A_{280}) ≥ 0.8 were pooled and concentrated with a Centricon apparatus. The anionic proteins were applied to a DEAE-Sepharose column (2.5 × 20 cm) and equilibrated with pH 6.0 PPB. Bound isoperoxidases were eluted with a 0–0.5 M linear NaCl gradient in PPB and concentrated and desalted with the Centricon apparatus. Peroxidase activity was assayed spectrophotometrically with two different electron donors: syringaldazine (Syr), which has a special affinity for peroxidases involved in cell wall lignification, and *p*-phenylenediamine-pyrocatechol (PPD-PC), which is oxidized by a number of isoperoxidases present in all cellular tissues (12). Syr oxidase was assayed in 50 mM PPB (pH 6.0) containing 25 μM Syr and 250 μM H_2O_2, and PPD-PC oxidase was measured in 0.1 M Tris-HCl (pH 7.6) containing 0.17 mM PPD, 4.5 mM PC, and 250 μM H_2O_2. The increase in absorbance was recorded at 530 and 575 nm (12). For determination of temperature sensitivity, identical assays were performed with en-

zyme solutions heated to 70 C for 10 minutes and then rapidly cooled by immersion in an ice-water bath. Sensitivity to cyanide, an inhibitor of peroxidase (26), was investigated in 1 mM potassium cyanide. Protein concentration was measured with Bradford's (2) protein–dye binding assay.

Samples (6 μg protein) were analyzed with native PAGE (14) on 10–13% linear gradient polyacrylamide gels. Electrophoresis was conducted for 1 hour at 40 mA and 900 V at 7 C. Isoperoxidases were stained with 1 mM diaminobenzidine (DAB) in 50 mM sodium acetate buffer (pH 5.0) and 250 μM H_2O_2, or with 0.6 mg/ml 4-chloro-1-naphthol (NA) and 0.16% H_2O_2 (15). For Syr oxidase and PPD-PC oxidase, the gels were stained in the same solutions utilized for the spectrophotometric analyses.

The molecular weights of the separated isoenzymes were determined with SDS-PAGE (14) on 10–13% linear gradient by comparison to the following standards: bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and cytochrome *c* (12,400). Proteins (50 μg) were denaturated by boiling for 3 minutes in 1% SDS–4% mercaptoethanol. Gels were stained with 0.2%. Coomassie blue and destained in aqueous 7% acetic acid–5% methanol. Molecular weight and R_f values were obtained from densitometric scanning of the gels. All reported values are the means of three different experiments with triplicate analyses of each extract.

Results

Forty days after inoculation, the two resistant isolines contained fewer females and J4 of *M. incognita* and fewer root galls than the susceptible lines (Table 1). All the isolines tested had different degrees of resistance, as indicated by the numbers of juveniles that entered roots and reached the J4 stage. More females were produced in the susceptible isolines than in the two containing the *Mi* gene, Monita and Motaci (Table 1).

Table 1. Numbers of galls and fourth-stage juveniles and females of *Meloidogyne incognita* in roots of near-isogenic lines of *Lycopersicon esculentum* 40 days after inoculation.

Isoline†	Galls	J4	Females
Moneymaker	75 ± 8.0	50 ± 9.0	52 ± 5.0
Monalbo *Ve*	232 ± 26	100 ± 11	159 ± 21
Moboglan *Ve, pyl*	80 ± 10	56 ± 3.0	58 ± 3.0
Momor *Ve, Tm-2²*	104 ± 13	72 ± 11	63 ± 4.0
Monita *Mi*	22 ± 1.0	4 ± 1.0	24 ± 1.0
Motaci *Tm-1, Mi*	17 ± 2.0	2 ± 1.0	21 ± 2.0

Data are the means of three different experiments (12 replicates for each) ± standard error.

† Lines have genetic resistance as follows: *Ve* = *Verticillium; pyl* = *Pyrenochaeta lycopersici; Tm-2²* = tobacco mosaic virus strain 2^2; *Mi* = *Meloidogyne* spp.; *Tm-1* = tobacco mosaic virus strain 1.

After partial purification on DEAE-Sepharose resin to remove the highly colored, presumably phenolic contaminants, tomato root extracts contained various amounts of peroxidase activity (Table 2). Seven days after inoculation, the average total peroxidase activity was higher in the resistant than in the two susceptible lines. Compared to levels in uninoculated controls, levels of both PPD-PC oxidase and Syr oxidase doubled in Monita and Motaci, the two lines carrying the *Mi* gene. The susceptible lines showed a moderate increase following nematode infection (Table 2).

The isoperoxidases from crude extracts separated under native PAGE revealed specific isoenzymes characteristic for each tomato line (Fig. 1). Except for Moboglan and Moneymaker, all isolines yielded patterns containing at least three groups of isoenzymes: one group of bands with R_f between 0.30 and 0.40, and two faster migrating groups with R_f of ca. 0.5 and 0.6. Moboglan contained a band with R_f 0.68 not present in the other lines. The group of isoenzymes at R_f 0.50 and 0.51 represented 70% of the densitometrically determined peroxidase present in the gel. For each tomato line, substantial qualitative or quantitative differences in isoenzyme patterns were not apparent between crude extracts from infected plants and those from uninfected controls.

TABLE 2. *p*-Phenylenediamine-pyrocatechol (PPD-PC) oxidase and syringaldazine (Syr) oxidase activity from roots of uninfected and *Meloidogyne incognita*-infected near-isogenic lines of *Lycopericon esculentum* with or without the *Mi* gene. Values represent the recovered activity of peroxidase extracted from roots 7 days after inoculation and partially purified with DEAE-Sepharose.

	PPD-PC oxidase‡		Syr oxidase‡	
Isoline†	Uninfected	Infected	Uninfected	Infected
Moneymaker	4.15 ± 0.2	5.35 ± 0.5	4.81 ± 0.1	5.45 ± 0.1
Monalbo *Ve*	3.35 ± 0.1	5.00 ± 0.4	5.65 ± 0.3	6.35 ± 0.2
Moboglan *Ve, pyl*	4.25 ± 0.3	4.60 ± 0.2	7.80 ± 1.0	7.65 ± 0.8
Momor *Ve, Tm-2²*	4.38 ± 0.3	5.24 ± 0.2	7.70 ± 0.5	9.70 ± 1.0
Monita *Mi*	4.45 ± 0.1	8.32 ± 0.1	5.06 ± 0.2	9.84 ± 0.7
Motaci *Tm-1, Mi*	4.40 ± 0.2	8.78 ± 0.8	5.00 ± 0.1	9.22 ± 0.4

Data are the means of three experiments with triplicate analyses of each extract.

† Genetic resistance as follows: *Ve* = *Verticillium; pyl* = *Pyrenochaeta lycopersici; Tm-2²* = tobacco mosaic virus strain 2²; *Mi* = *Meloidogyne* spp.; *Tm-1* = tobacco mosaic virus strain 1.

‡ Activity is expressed as increase in absorbance per minute per mg of protein (± standard error) in spectrophotometric assays.

Subsequent HPLC purification of the crude extracts through a cation-exchange resin resulted in two fractions (anionic and cationic) which, regardless of the sample, contained 80% of the peroxidase activity loaded on the column. The anionic peroxidases were recovered between 90 and 105 minutes in the flow-through fraction, whereas the cationic peroxidases were eluted at 0.5 M NaCl. Most (ca. 90%) of the peroxidase activity recovered from the CM-Sepharose column represented anionic peroxidases (Fig. 2).

The anionic peroxidases were purified further with DEAE-Sepharose chromatography, in which peroxidase activity was initially bound but eluted immediately after the NaCl concentration reached 0.5 M (Fig. 3). Absorbance at 407 nm indicated that most of the hemoproteins eluted in

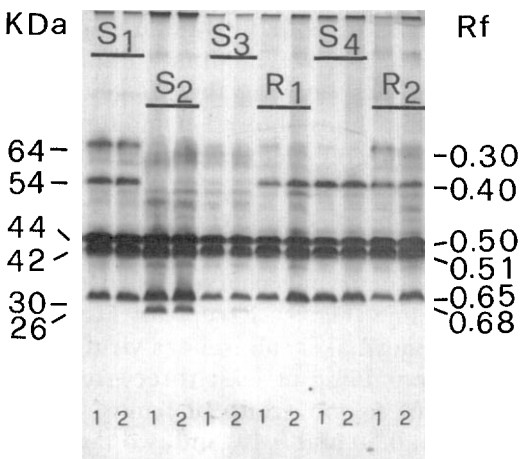

FIG. 1. Polyacrylamide gel electrophoresis (PAGE) of native peroxidases from crude extracts of roots of six different isolines of *Lycopersicon esculentum,* susceptible (S) or resistant (R) to *Meloidogyne incognita.* Root samples were taken 7 days after nematode inoculation. Peroxidases were extracted in potassium phosphate buffer and partially purified on DEAE-Sepharose CL-6B. Lanes 1 and 2 in each pair represent extracts from uninfected and infected roots, respectively. Peroxidases were visualized by staining the gels with diaminobenzidine. S_1 = Momor, S_2 = Moboglan, S_3 = Moneymaker, R_1 = Monita, S_4 = Monalbo, and R_2 = Motaci.

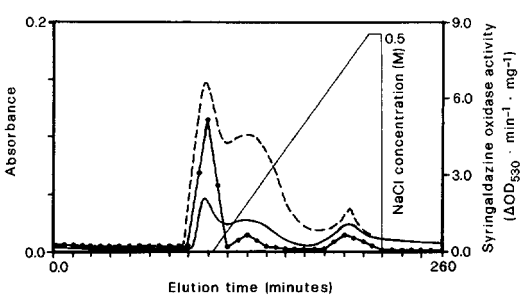

FIG. 2. High performance liquid ion-exchange chromatogram on a CM-Sepharose CL-6B column (2.5 × 30 cm) of crude peroxidase from roots of *Lycopersicon esculentum* Monita infected with *Meloidogyne incognita.* Anionic peroxidases were eluted in 50 mM potassium phosphate buffer (PPB). A gradient from 0.25 to 0.5 M NaCl in 50 mM PPB was applied to elute the cationic peroxidase; 1-ml fractions were collected, and syringaldazine oxidase (•) activity in 50-µl aliquots was expressed as increase in OD_{530} per minute per mg protein. Absorbance at 280 and 407 nm is represented by the dashed and smooth solid lines, respectively.

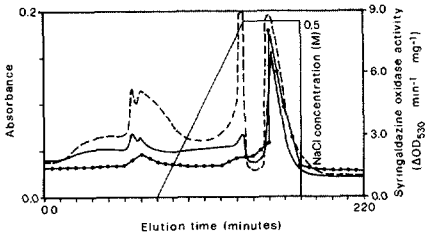

FIG. 3. High performance liquid chromatogram of eluate between 80 and 130 minutes from Figure 2, subsequently applied to a DEAE-Sepharose CL-6B column (2.5 × 20 cm). Proteins were eluted with 50 mM pH 6.0 potassium phosphate buffer, the peroxidases were eluted with a NaCl gradient, and the syringaldazine oxidase activity in 50-μl aliquots was expressed as increase in OD_{530} per minute per mg protein. Legends for lines as in Figure 2.

one peak that coincided with peroxidase activity measured with syringaldazine as the electron donor (Fig. 3).

After the DEAE-Sepharose purification, the specific activity of anionic peroxidase was ca. 3–6 fold greater than that of the crude extract. Anionic peroxidase activity increased either as Syr oxidase or as PPD-PC oxidase, 7 days after nematode inoculation (Table 3). By this time, the anionic peroxidase activity increased slightly in the susceptible isolines but approximately doubled in resistant isolines.

The anionic peroxidases extracted from uninfected and infected plants exhibited differential heat sensitivity. After 10 minutes at 70 C, only 40% and 50% of the initial activity in the extracts from uninfected roots of either Moboglan or Monita remained with PPD-PC and Syr as substrates, respectively. The resistance to heat treatment of peroxidase from infected Moboglan roots was the same as that from uninfected roots (i.e., 40% and 50%), whereas in the extract from infected Monita roots, 70% of the PPD-PC and Syr oxidase remained active.

Inactivation of the anionic peroxidases by cyanide was ca. 90% in all the samples and thus indicated that the enzymes may be hemoproteins.

The PAGE profile of anionic peroxidase revealed the presence of more than one enzyme form (Fig. 4). Indeed, all isolines contained a diaminobenzidine-stained protein band (R_f 0.50) with high peroxidase activity; a second band (R_f 0.51) was present in uninfected or infected extracts from roots of the two susceptible isolines (Moboglan and Moneymaker). This isoenzyme was absent in uninfected resistant isolines (Monita and Motaci), but it was induced in these two isolines by nematode infection. Although the isoforms at R_f 0.50 and 0.51 accounted for most of the anionic peroxidase activity, some fainter bands were obvious (Fig. 4).

Heterogenicity of isoperoxidase affinity for different substrates was apparent in DEAE-Sepharose-purified extracts from

TABLE 3. *p*-Phenylenediamine-pyrocatechol (PPD-PC) oxidase and syringaldazine (Syr) oxidase activity from roots of uninfected and *Meloidogyne incognita*-infected near-isogenic lines of *Lycopersicon esculentum* with or without the *Mi* gene. Values represent the recovered activity of peroxidase extracted from roots 7 days after inoculation and purified with CM- and DEAE-Sepharose ion-exchange chromatography.

	PPD-PC oxidase‡		Syr-oxidase‡	
Isoline†	Uninfected	Infected	Uninfected	Infected
Moneymaker	12.1 ± 0.6	16.2 ± 0.8	11.6 ± 0.9	18.0 ± 0.9
Monalbo *Ve*	17.5 ± 2.1	24.2 ± 2.6	18.0 ± 2.1	26.1 ± 2.1
Moboglan *Ve, pyl*	19.4 ± 1.4	18.9 ± 1.3	22.0 ± 3.0	30.1 ± 3.0
Momor *Ve, Tm-2²*	14.4 ± 2.0	16.8 ± 0.9	16.5 ± 1.8	17.0 ± 1.4
Monita *Mi*	22.2 ± 1.4	31.2 ± 1.9	23.3 ± 0.8	37.2 ± 1.9
Motaci *Tm-1, Mi*	20.2 ± 1.3	49.0 ± 3.0	29.8 ± 1.7	58.1 ± 2.7

Data are the means of three experiments with triplicate analyses of each extract.

† Genetic resistance as follows: *Ve* = *Verticillium; pyl* = *Pyrenochaeta lycopersici; Tm-2²* = tobacco mosaic virus strain 2²; *Mi* = *Meloidogyne* spp.; *Tm-1* = tobacco mosaic virus strain 1.

‡ Activity is expressed as increase in absorbance per minute per mg of protein (± standard error) in spectrophotometric assays.

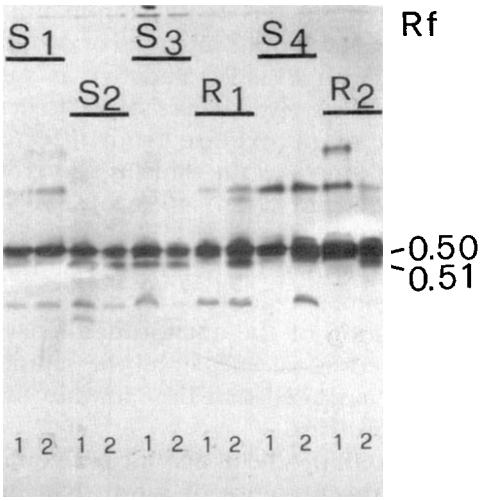

FIG. 4. PAGE of anionic isoperoxidase from extracts of tomato lines purified by HPLC on CM- and DEAE-Sepharose columns, as described in Figures 2 and 3. Peroxidases were stained with diaminobenzidine. Lanes 1 and 2 in each pair represent extracts from uninfected and *Meloidogyne incognita*-infected roots, respectively. S_1 = Momor, S_2 = Moboglan, S_3 = Moneymaker, R_1 = Monita, S_4 = Monalbo, and R_2 = Motaci.

Moboglan and Monita (Fig. 5). More bands were stained with PPD-PC than with the other chromogens; however, isoperoxidases exhibited the highest affinity towards DAB. In contrast, spectrophotometric analysis showed that syringaldazine had a lower affinity than PPD-PC for the isoperoxidase separated by gel electrophoresis. Relatively low activity of peroxidase was revealed by NA. The isoenzyme group with R_f 0.50 exhibited high affinity for all the chromogens used, whereas the isoperoxidase group with R_f 0.51 exhibited the highest rate of oxidation for DAB. Based on densitometrically calculated migration relative to the molecular weight standards, the isoenzymes were 64,000 (R_f 0.30), 54,000 (R_f 0.40), 44,000 (R_f 0.50), 42,000 (R_f 0.51), 30,000 (R_f 0.65), and 26,000 (R_f 0.68) (Fig. 1).

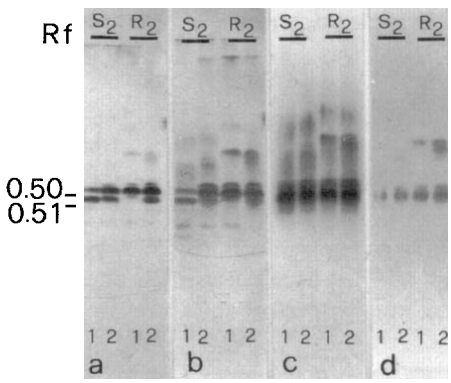

FIG. 5. PAGE of native anionic peroxidases extracted from roots of two tomato isolines, Moboglan (S_2) and Monita (R_2), and purified HPLC on CM- and DEAE-Sepharose columns. Peroxidases were stained with the following chromogens: a) diaminobenzidine, (b) chloronaphthol, c) *p*-phenylenediamine-pyrocatechol, d) syringaldazine. Lanes 1 and 2 contain peroxidases from uninfected and *Meloidogyne incognita*-infected roots, respectively.

DISCUSSION

Our experiments focused on the expression of peroxidase isoenzymes of tomato near-isogenic lines 7 days after nematode infection. An anionic group of isoperoxidases was identified in the two resistant isolines containing the *Mi* gene. In response to *M. incognita* infection, these two lines showed a specific increase in a peroxidase isoenzyme at R_f 0.51 that did not increase in the susceptible isolines. When infected, both resistant isolines displayed the same subset of isoenzymes. Because this type of response was not found in the susceptible lines, the enzyme at R_f 0.51 may be one of several responsible for the increase in total peroxidase activity. Isoperoxidase accumulation may be part of a general plant defense mechanism, presumably by catalyzing the final step of lignin biosynthesis (7,12,18). Upon nematode infection, the isoperoxidases that rapidly accumulate in the resistant isolines could function differently from those with the same R_f (0.51) in the susceptible lines.

Our results concerning the anionic peroxidase agree with those of Imberty et al. (12), who found that the fast migrating anionic isoperoxidase had particularly high affinity for syringaldazine. Histochemical analysis with syringaldazine showed intense staining on the walls of the cells surrounding cyst nematode feeding sites in resistant pea roots (1) and *M. incognita* feeding sites in resistant tomato roots (un-

publ.). The use of syringaldazine as a specific substrate for isoperoxidases involved in lignification (3,8) allows the levels of anionic peroxidase and lignification to be correlated (17). The *M. incognita*-induced isoperoxidases in tomato also resemble those in other systems with respect to fast migration during electrophoresis, resistance to heat treatment, molecular weight, inhibition by cyanide, and absorbance at 407 nm (3,6,12). The production of such isoenzymes in various resistant plants challenged with different pathogens indicates that peroxidases are certainly important in mechanical reinforcement of plant cell walls (7,9,15).

This study constitutes the first step of purification and identification of anionic peroxidase from tomato roots infected by root-knot nematodes. Further studies are necessary, however, to demonstrate the time course of appearance of this enzyme during infection of resistant tomato roots and to determine its localization within the roots. Although present evidence implicating this enzyme in resistance is indirect, further support for this hypothesis will come from further characterization of anionic isoperoxidases in tomato roots and the elucidation of their actual function, if any. The availability of purified anionic tomato peroxidase should help in the isolation of the gene coding for these enzymes.

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