Disc-electrophoretic Patterns of Enzymes and Soluble Proteins of *Ditylenchus dipsaci* and *D. triformis*[1]

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Abstract: Soluble protein, esterase and oxidative enzyme patterns of the Waynesville, North Carolina, (WNC) and Raleigh, North Carolina, (RNC) populations of *Ditylenchus dipsaci* were compared. Polyacrylamide gel electrophoretic patterns of soluble protein extracts of nematodes of the two populations differed. Esterase and catalase patterns, however, were identical. Peroxidatic activity of the catalase isoenzymes from nematodes of the two populations differed when catechol was used as a cosubstrate. Distinct differences were demonstrated in soluble protein and enzyme patterns between *D. dipsaci* and *D. triformis*. *Key Words: Ditylenchus dipsaci, Ditylenchus triformis,* Proteins, Enzymes.

The Waynesville, North Carolina (WNC) and Raleigh, North Carolina (RNC) populations of *Ditylenchus dipsaci* (Kühn) Filipjev induced different responses in the garden pea, *Pisum sativum* L. 'Wando' (1). WNC nematodes stimulated a hypersensitive (resistant) reaction whereas RNC nematodes caused tissue disruption and galling characteristic of the susceptible response. Hussey and Krusberg (5) reported higher peroxidase activity in WNC and RNC nematode-infected 'Wando' pea plants than in noninoculated plants. Differences in the oxidative enzyme patterns of diseased and noninoculated tissues were detected by polyacrylamide gel electrophoresis of extracts, but not between the WNC and RNC nematode-infected tissues.

Oxidative enzymes in plant-parasitic nematodes have not been investigated. These enzymes have, however, been studied extensively in bacteria and fungi. For example, protein and enzyme patterns of urediospores from two physiological races of *Puccinia graminis* var *tritici* differed only in their peroxidase and polyphenoloxidase enzymes (8). More bands with peroxidase and polyphenoloxidase activity were detected in extracts from urediospores of the less virulent physiological race 111 than from the more virulent race 21 by polyacrylamide gel electrophoresis. Slight differences in soluble protein patterns were also detected.

The purpose of this study was to compare soluble protein and enzyme patterns in the two populations of *Ditylenchus dipsaci* and in *D. triformis*, a fungal feeder.

MATERIALS AND METHODS

WNC and RNC populations of *Ditylenchus dipsaci* (Kühn) Filipjev were propagated on alfalfa callus (6). *Ditylenchus triformis* Hirschmann and Sasser was cultured on the fungus, *Pyrenochaeta terrestris* de Not., grown on potato-dextrose agar.

Nematodes were separated from 6- to 8-week-old cultures by the Baermann funnel technique. Following concentration, nematodes were washed eight times with distilled water in centrifuge tubes then suspended in cold 0.05 M potassium phosphate buffer at *p*H 7.0 and homogenized by passing twice through a cold French pressure cell. The homogenate was centrifuged at 20,000 *g* for 30 min at 4 C and the supernatant solution, used as the source of proteins, was stored at 4 C and analyzed within 48 hr. Protein determinations were made on trichloroacetic

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acid precipitate dissolved in 0.1 N NaOH by the method of Lowry *et al.* (7). Protein preparations had to be analyzed within 48 hr; if stored longer or frozen prior to use, indistinct bands (soluble protein patterns) were obtained.

Enzymes in the protein preparations were separated by disc electrophoresis on polyacrylamide gels by the method of Ornstein (10) and Davis (2). A fraction of the protein preparation (approximately 0.15 mg of protein) in a 10% sucrose solution was applied directly on top of the spacer gel. Electrophoresis was conducted anionically at 4 C with 5 ma per column. Following electrophoresis the gels were incubated in freshly prepared substrate solutions to define the locations of specific enzymes. Electrophoretic mobility (E_f) values presented in the text were determined directly from the gel columns by comparing the migrating distance of the protein band with that of the bromophenol blue marker dye. Controls consisted of heating gels in test tubes in boiling water for 10 min or incubating gels in a solution minus the substrate. Nonspecific esterases were detected by incubating the gels in the dark at 25 C in a solution of 49 ml of 0.1 M potassium phosphate buffer at *p*H 7.0; 1 ml of 1% α-naphthylpropionate, α-naphthylacetate, or α-naphthylbutyrate in acetone; and 25 mg of Fast Blue RR until bands appeared (9). Procedures for the oxidative enzyme tests (dihydroxyphenylalanine (DOPA)-oxidase, peroxidase, catalase) were previously described (5) excepting that pyrogallol was the cosubstrate in the test for peroxidase. The terms DOPA-oxidatic, peroxidatic, and catalatic are used to indicate activities demonstrated in the gel and not to indicate specific enzymes since certain oxidative enzymes seem to possess more than one type of activity. Commercial horseradish peroxidase and mushroom tyrosinase (Nutritional Biochemicals Corporation, Cleveland, Ohio) were used as standards.

Average pore size in the separating gel was varied by changing the concentration of acrylamide (5, 6, 7, 8.5%) to determine whether the enzymes could be resolved into additional components. Soluble protein patterns were obtained by covering gels for at least 1 hr with a solution of 0.5% Amido Schwarz in 7% acetic acid. Gels were stored in 7% acetic acid following electrophoretic destaining.

Peroxidatic cosubstrate specificity of oxidative isoenzymes in the protein preparations of WNC and RNC nematodes were determined as follows. The gels were incubated for 30 min in 2×10^{-2} M aqueous solutions of the following phenols and aromatic amines: catechol, gallic acid, guaiacol, hydroquinone, benzidine dihydrochloride, *p*-phenylenediamine, *p*-anisidine, and chlorogenic acid. Specificity was visually estimated and rated from 1 to 4 by relative intensities of the developed bands 15 min after gels were post-incubated in 0.3% hydrogen peroxide.

Results

Twenty-one to 22 soluble proteins were consistently separated by disc electrophoresis of which five to six appeared to be major proteins based on intensity of staining with Amido Schwarz. Preparations from RNC nematodes contained a major protein band not detected in the preparations from WNC nematodes. A schematic diagram of the soluble protein profiles of RNC and WNC nematodes is presented in Fig. 1. An attempt to depict the variation in the intensity of staining of the proteins is represented by the differences in the widths of the individual bands. An arrow indicates the protein band present in RNC but absent in WNC nematodes.

Activities for the three oxidative enzyme

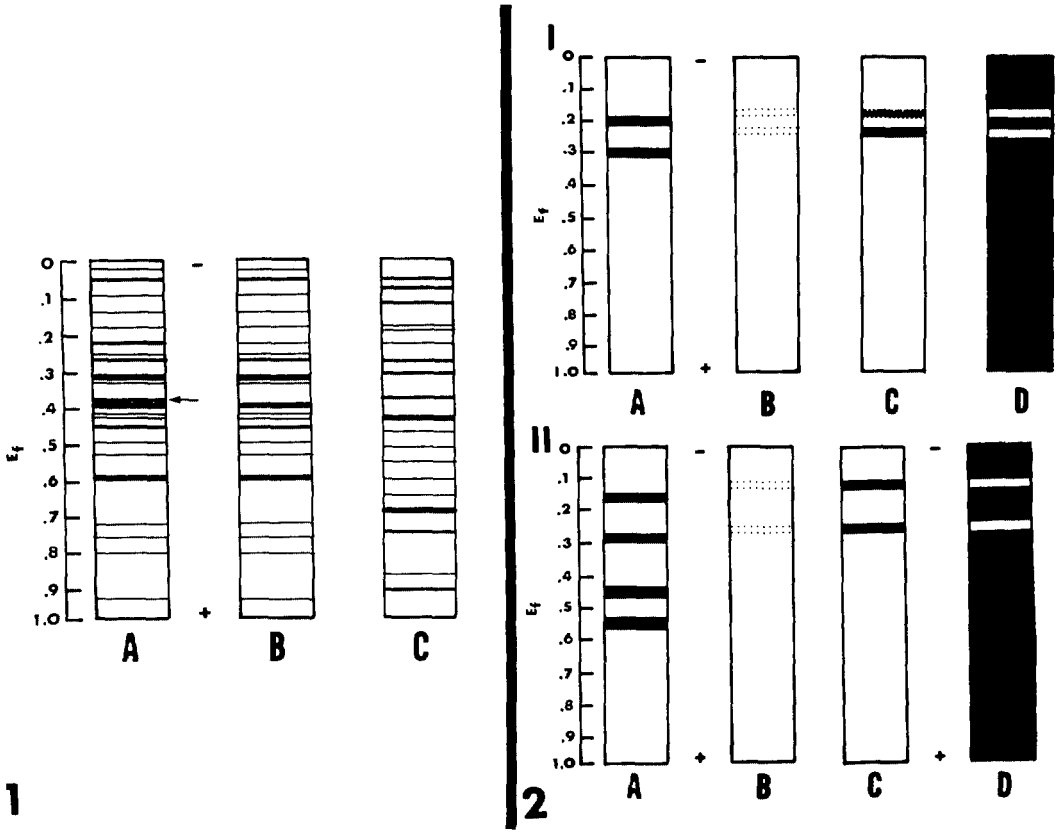

FIG. 1–2. Electrophoretic patterns of proteins from *Ditylenchus dipsaci* and *Ditylenchus triformis*. **1.** Schematic diagram of soluble protein profiles from nematodes of the Raleigh, North Carolina (A) and Waynesville, North Carolina (B) populations of *Ditylenchus dispsaci* and *D. triformis* (C) Arrow indicates the protein band present in RNC but absent in WNC nematodes. **2.** Zymograms obtained from protein preparations of WNC and RNC nematodes of *D. dipsaci* (I) and *D. triformis* (II). Zymograms show sites of esterase (A), DOPA-oxidatic (B), peroxidatic (C), and catalatic (D) activities.

tests occurred at the same sites in the gels (Fig. 2). Two bands with peroxidatic activity were localized in the gels with pyrogallol as the cosubstrate. The E_f values for the two components were 0.16 and 0.23. With pyrogallol cosubstrate the isoenzyme with the greater mobility (E_f 0.23) had higher activity (more intense cosubstrate reaction) than the slower migrating component (Fig. 3). Identical isoenzyme reactions were observed for both WNC and RNC nematodes.

Weak DOPA-oxidatic activity was also demonstrated at sites where peroxidatic activity occurred. An inhibitor of DOPA-oxidase was used to determine if a mixture of enzymes occurred at these sites. The gels were incubated in a 100 μg/ml buffered solution of phenylthiourea (PTU), a known DOPA-oxidase inhibitor, before incubation in the substrate solution. An equal concentration of PTU was also added to the substrate solution. PTU had no effect on the nematode enzyme activity, but markedly

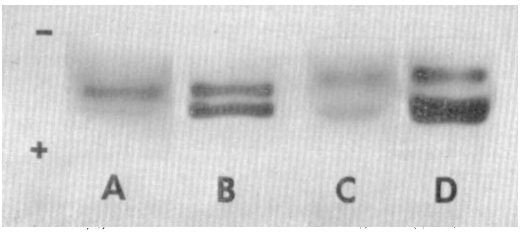

FIG. 3. Localization of sites of peroxidatic activity in extracts of *Ditylenchus dipsaci* in polyacrylamide gels with catechol (A, C) and pyrogallol (B, D) as cosubstrates. Bands were developed following electrophoretic separation of extracts of RNC nematodes (A, B) in 8.5% gels and WNC nematodes (C, D) in 7% gels.

inhibited the DOPA-oxidatic activity of a commercial preparation of mushroom tyrosinase. After development of the DOPA-oxidatic activities, the gels were incubated 15 min in 0.3% hydrogen peroxide, which resulted in intensification of the bands. This intensification was not observed when a commercial preparation of mushroom tyrosinase was used. Peroxidase isoenzymes separated by electrophoresis from a commercial preparation of horseradish peroxidase also possessed weak DOPA-oxidatic activity similar to that in the nematode preparations. Addition of hydrogen peroxide enhanced the intensity of staining of these bands.

Catalase activity in the gels was indirectly detected by incorporating soluble starch into gels prior to electrophoresis to provide the necessary component for staining with iodine. Starch reacting with iodine released by hydrogen peroxide oxidation of potassium iodide stained the gel dark blue. Areas where catalase had decomposed the hydrogen peroxide remained unstained. Two sites of catalatic activity similar in both nematode populations coincided with the sites of DOPA-oxidatic and peroxidatic activities (Fig. 2). Changing the pore size of gels revealed no additional bands of enzymatic activity (Fig. 3).

TABLE 1. Peroxidatic cosubstrate specificity of of two oxidative isoenzymes in preparations from nematodes of the Raleigh, North Carolina (RNC) and Waynesville, North Carolina (WNC) population of *Ditylenchus dipsaci*.

Cosubstrate (2×10^{-2} M)	Specificity[a]			
	RNC		WNC	
	I[b]	II[b]	I[b]	II[b]
Catechol	3	2–1	3	3
Gallic Acid	4	4	4	4
Hydroquinone	4	4	4	4
p-Phenylenediamine	1	1	1	1
Benzidine-HCl	–[c]	–	–	–
Guaiacol	–	–	–	–
p-Anisidine	–	–	–	–
Chlorogenic Acid	2	2	2	2
Pyrogallol	3	4	3	4

[a] Reactivity was rated 1 (low) to 4 (high) by visual observation of the intensity of disc electrophoresis bands following 30 min incubation in aqueous solutions of the cosubstrates and 15 min post-incubation in 0.3% H_2O_2.
[b] Isoenzymes separated from protein preparations by electrophoresis; I—slower migrating isoenzyme, II—faster migrating isoenzyme.
[c] No detectable activity.

Peroxidatic cosubstrate specificity of the isoenzymes was determined by using aqueous solutions of nine different phenols and aromatic amines as cosubstrates. Activity was observed with six of the nine cosubstrates, but the degree of activity varied (Table 1). Isoenzymes from neither population of *D. dipsaci* utilized benzidine-HCl, guaiacol, nor *p*-anisidine, common cosubstrates for peroxidase isoenzymes, at concentrations of substrate and cosubstrates used in the experiments. A commercial preparation of horseradish peroxidase utilized these three cosubstrates. Activity of the isoenzymes differed when catechol was used as a cosubstrate. The faster migrating (E_f 0.23) isoenzyme from RNC nematodes was less reactive with catechol than was the faster migrating (E_f 0.23) isoenzyme from WNC nematodes (Fig. 3).

Soluble protein and enzyme patterns from *D. triformis* were distinctly different from those of *D. dipsaci* (Fig. 1 and 2). Multiple forms of the enzymes were also present in

protein preparations from *D. triformis.* Four bands of non-specific esterase activity were localized in the gels. The two slower migrating (E_f 0.16 and 0.29) components had either a higher specific activity with α-naphthylacetate or were present in higher concentrations that the two faster migrating (E_f 0.45 and 0.55) components as the localization reaction was more intense with the two components (E_f 0.16 and 0.29) near the cathodic end of the gel. The second site of activity (E_f 0.29), from the cathodic end of the gel, appears as a broad band.

For the three oxidative enzyme tests activities occurred at the same sites in the gels. Although similar results were obtained for the tests with *D. dipsaci*, the electrophoretic mobilities of the two isoenzymes from *D. triformis* were distinctly different from those of *D. dipsaci.* The E_f values for isoenzymes in preparations of *D. triformis* were 0.10 and 0.25 (Fig. 2), with the former sometimes appearing as a broad band.

DISCUSSION

Soluble protein patterns of nematode extracts of the WNC and RNC populations of *D. dipsaci* differed. One major protein was unique to RNC nematodes. The function of the protein was not determined although its high concentration suggests that it may be a fundamental protein. Identification of this protein would be valuable in determining specific differences between the WNC and RNC nematodes.

Serological characterization might be used to further identify the proteins separated by electrophoresis. Gibbins and Gradison (4) tested gel diffusion and immuno-electrophoresis in agar as means of separating biological races of *D. dipsaci.* Inconsistent differences were obtained when antiserum to a race isolated from red clover was tested against antigens prepared from several races isolated from different hosts. Combining the separating (sieving) ability of polyacrylamide gel with immuno-electrophoresis should improve the resolution and differences should be detectable.

This study utilized mixed stages of nematodes (larval and adult) and therefore, the protein band unique to the RNC nematodes may have been associated with individuals at a particular stage of development. Dickson (3) compared several enzymes from three stages (egg, larval, adult female) of *Meloidogyne incognita.* Five of seven enzyme patterns for adult and larval nematodes were similar with differences occurring in esterase and malate dehydrogenase patterns. Other differences were noted between enzyme patterns of eggs and adult females, and eggs and larvae. Soluble protein patterns of eggs, larvae and adults were not compared although differences would be expected. Slight differences in enzyme patterns might occur between larvae and adults of *D. dipsaci*, as a result of undeveloped reproductive organs in the larvae.

Esterase and oxidative enzyme patterns from WNC and RNC nematodes were similar. The two populations of *D. dipsaci* could not be distinguished on the basis of non-specific esterase patterns. DOPA-oxidatic, peroxidatic, and catalatic activities occurred at the same sites in gels, suggesting the present of multiple enzymes. Failure of varying concentrations of polyacrylamide gel to further resolve the bands, lack of phenylthiourea inhibition of the DOPA-oxidatic activity, and positive starch-iodine tests indicate that two catalase isoenzymes are responsible for the varied activity. The absence of detectable peroxidatic activity at these sites when guaiacol, benzidine-HCl, and *p*-anisidine were used as cosubstrates further demonstrates that these isoenzymes are catalases. Rudolph and Stahmann (11) reached similar conclusions with an enzyme from the bacterium, *Pseudomonas pha-*

seolicola, based upon the above specificity pattern. They found similar substrate specificities with two commercial preparations of catalase.

The significance of the difference in the peroxidatic cosubstrate specificity of the catalase isoenzymes from WNC and RNC nematodes is still uncertain. Catalase activities of the WNC and RNC populations of *D. dipsaci* were not compared in the present study. Secretion of these isoenzymes by the nematodes would have to be demonstrated before it could be suggested catalases play a role in inducing the hypersensitive response in pea tissues *via* oxidizing phenols to quinones which could inhibit cellular enzymes. Rudolph and Stahmann (11), however, suggested that pathogen catalases may be important for virulence of pathogens in some host-parasite combinations. They proposed that catalases may repress peroxidase activity important in host defense reactions by competing for hydrogen peroxide.

The two species of *Ditylenchus* studied can be distinguished by the differences in their soluble protein and enzyme patterns. *D. triformis* contained two esterase enzymes not detected in *D. dipsaci*. Dickson (3) has shown that four species of *Meloidogyne* had similar soluble protein patterns but differences in non-specific esterase patterns. These differences were considered valuable for distinguishing among these species. In addition, malate and α-glycerophosphate dehydrogenase patterns differed slightly.

Catalase reactions may be important in initiating the resistant response induced in 'Wando' pea by WNC nematodes. The occurrences, however, of two catalase isoenzymes in *D. triformis*, possibly indicates that these enzymes may only be important in metabolism of the nematode. Additional experiments to determine whether these enzymes are secreted by the nematodes should aid in elucidating the relation of nematode catalases to host resistance mechanisms.

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