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Application of Isoelectric Focusing to the Taxonomic Identification of *Meloidogyne* spp.[1]

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Abstract: *Meloidogyne incognita, M. arenaria, M. hapla,* and *M. javanica* were distinguishable from each other by isoelectric focusing (IEF) of nematode egg proteins. Proteins extracted from larvae and adults of *Hoplolaimus columbus* and from eggs of *Heterodera glycines* had distinctive profiles, also. Protein profiles from eggs, preparasitic larvae and egg-laying adults of *M. incognita* showed differences. It was necessary to compare samples run at the same time to ensure reliability.

Key words: root-knot nematodes, biochemical taxonomy, isoelectric focusing, *Meloidogyne* spp., protein profiles.

Although 45 species of *Meloidogyne* have been described, four species, *M. incognita* (Kofoid & White, 1919) Chitwood, 1949, *M. javanica* (Treub, 1885) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, and *M. hapla* Chitwood, 1949, are responsible for 90% or more of the damage to crops caused by this genus (8). The increasing development and use of cultivars resistant to species of *Meloidogyne* make accurate identification of this species necessary for effective control procedures to be developed and implemented.

Within the past 15 years, significant efforts have been applied to developing biochemical methods for identification of *Meloidogyne* spp. and other nematode genera. These methods have included disc-gel electrophoresis of soluble proteins (9,24,29), karyology (28), serology (13,18,20), and isozyme analysis (2,7,11,12).

The root-knot nematodes (*Meloidogyne* spp.) are among the most important pathogens affecting soybean (*Glycine max* L.) production in South Carolina. There are at least four species and six physiological races of *Meloidogyne* in South Carolina (S. A. Lewis, unpubl.). Methods currently used for taxonomic identification of *Meloidogyne* spp. are perineal patterns of adult females (3), morphometrics of second-stage juveniles and adults (25), differential host assay (26), and morphology of stylet and labial regions of larvae and males (8).

Biochemical techniques that are potentially useful for identification of *Meloidogyne* spp. and races (5,7,8,13,18,28) have used electrophoretic methods for separation of soluble proteins. Analysis of enzyme activity and protein profiles generated by these techniques have been useful for nematode identification (12,15,27).

Isoelectric focusing (IEF) is an electrophoretic technique which separates proteins in complex mixtures (30). The proteins migrate in a continuous pH gradient to their specific isoelectric points. We have investigated IEF as a taxonomic tool for the identification of *Meloidogyne* spp. and comparisons of *Heterodera glycines* Ichinohe, 1952 and *Hoplolaimus columbus* Sher, 1963.

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Materials and Methods

Culturing and isolation of nematode eggs: The roots of 2-week-old *Lycopersicon esculentum* Mill. cv. Rutgers were inoculated with a suspension of 5,000–10,000 nematode eggs (*Meloidogyne* spp.). After 40–45 days, root-knot nematode eggs were freed of root and soil debris by the centrifugal-flotation method of McClure et al. (17). Eggs were stored in water at 4 C until needed.

Eggs from cysts of *Heterodera glycines* were recovered in the same manner except cysts were first isolated from soil by sequential sieving, then broken in a Waring blender at high speed for 5 minutes. *Hoplolaimus columbus* larvae and adults were recovered from infested soybean roots in a mist chamber and kept at room temperature in an aerated flask until needed.

Protein extraction from nematode eggs: Approximately 1×10^5 *Meloidogyne* spp. eggs were pelleted in a 0.5-ml Eppendorf tube and resuspended in 0.1-ml extraction buffer (5% 2-mercaptoethanol, 8 M urea, 1% Triton X-100, 1 M Tris [pH 8.0], 1 M NaCl, and 3% ampholytes corresponding to the pH gradient in the electrofocusing gel). The egg suspension was homogenized on ice in a ground glass tissue grinder for 5 minutes. Insoluble material (egg shells, unbroken eggs, and nematodes) were pelleted at 5,000 *g* for 10 minutes at 4 C. The aqueous phase was recovered by passing a 20-gauge needle through the side of the tube and withdrawing the aqueous phase without disturbing the pellet or the lipid layer.

For preparation of proteins from *Meloidogyne* spp., approximately 1×10^4 larvae hatched from eggs (17) or 20–40 hand-picked adult females were used per extraction. About 1×10^4 *Hoplolaimus columbus* larvae or adults were homogenized in the same manner as *Meloidogyne* eggs.

Gel preparation: Gels contained 5% total polyacrylamide with 30:1 acrylamide/bisacrylamide ratio, 4 M urea, 0.25% Triton X-100, 3% ampholytes, and 0.25% TEMED, 0.03% ammonium persulfate. The gels were made on the Bio-Rad CTL casting system and allowed to polymerize 2 hours at room temperature.

Electrofocusing was performed according to the procedure outlined for use with the LKB Multiphor system (LKB Instruments, Inc.), except that 0.05 M NaOH and 0.05 M H_3PO_4 were used as the electrolytes for the cathode and anode, respectively.

Approximately 30 µl of supernatant was absorbed into two 0.5 × 1-cm filter paper strips (Whatman 3 mm) and applied to the surface of the gel 1 cm from the cathode. The power was applied in a stepwise fashion from a LKB 2117 power supply. The power was initially set at 5 watts, 400 volts for 1 hour, then the sample applicators removed. After an additional hour, the power supply was set at 600 volts for 2 hours, then 800 volts and 10 watts for 1 hour. The gel was removed from the cooling platform and immersed in 20% trichloroacetic acid for 12 hours, then soaked in 25% methanol–10% glacial acetic acid for 30 minutes or until the gel became transparent.

The gel was stained with 0.5% Coomassie brilliant blue R250 at room temperature for 1 hour, then destained with several changes of the methanol–acetic acid solution. Protein profiles were recorded with a Gelman ACD-18 densitometer (Gelman Instrument Co., Ann Arbor, MI) set at 595 nm with a slit width of 0.1 × 5 mm and scan length varied from 60 mm to 75 mm.

Results

Most resolvable *Meloidogyne* egg proteins had isoelectric points between pH 4–9. In numerous tests where egg proteins from the four *Meloidogyne* species were run side by side, differences in the protein profiles were observed. Figures 1, 2, and 3 show representative egg protein profiles of the four *Meloidogyne* spp. as represented by densitometer scans. Significant variations in protein profiles occurred when comparing profiles from different runs. We found that reliable comparisons were possible only when comparing profiles run on the same gel.

Two prominent protein bands in the profile of *M. arenaria* were not found in the profile of *M. incognita* when these were run on the same gel (Fig. 1). The profiles represented in Figs. 2 and 3 were run on the same gel. In Fig. 2, four protein bands distinguish *M. javanica* and *M. incognita*, two in each profile. Figure 3 compares *M.*

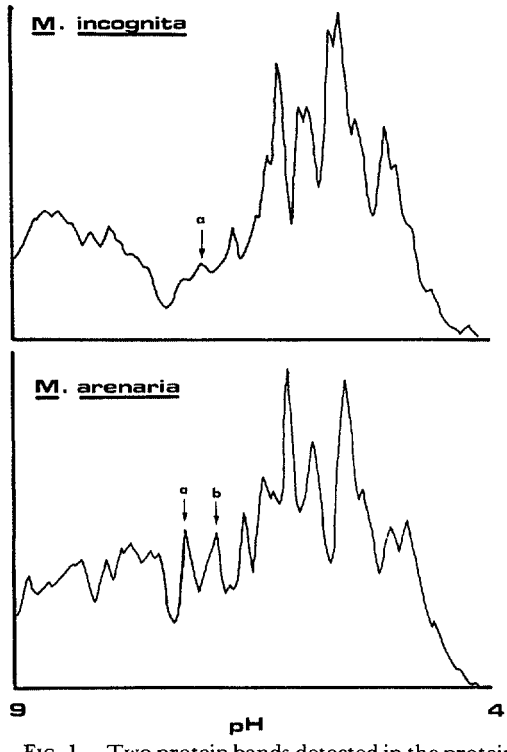

FIG. 1. Two protein bands detected in the protein profile of *M. arenaria* not found in the profile of *M. incognita* differentiate these two species.

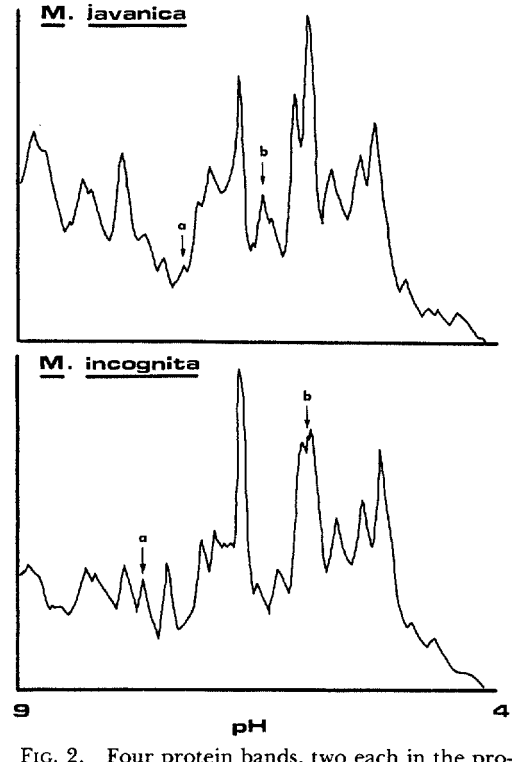

FIG. 2. Four protein bands, two each in the protein profiles of *M. javanica* and *M. incognita*, differentiate these two species.

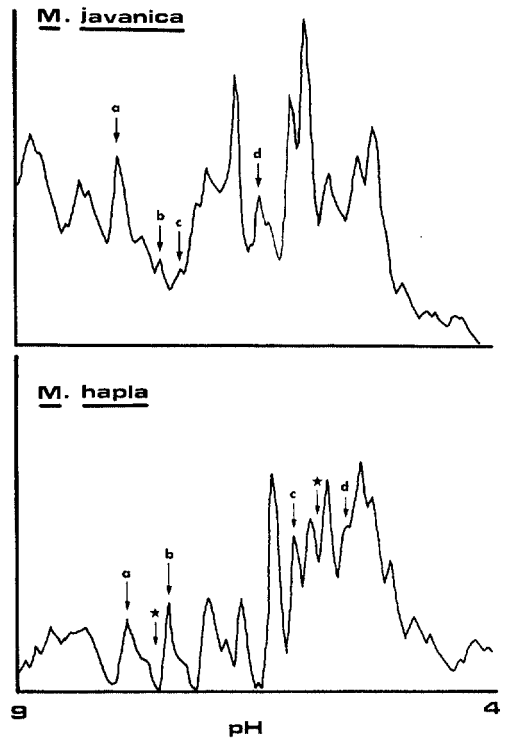

FIG. 3. Eight protein bands, four in the protein profile of *M. javanica* and four in the profile of *M. hapla*, distinguish these two species. The star indicates where protein bands diagnostic for *M. incognita* would be (Fig. 2).

javanica and *M. hapla*. Eight protein bands were distinctive.

Two South Carolina populations of *M. arenaria* that did not reproduce on peanut and that were pathogenic on *M. arenaria*-resistant *Glycine max* cv. Govan had identical protein profiles (Fig. 4). Subsequent differential host assay (26) also found the two populations to be identical (S. A. Lewis, unpubl.).

The profiles from protein extracted from eggs, preparasitic larvae, and egg-laying females of *M. incognita* are compared in Figs. 5 and 6. *M. incognita* egg and preparasitic larvae extracts differed in one protein band from the profile of egg extracts and two bands in the profile of preparasitic larvae. Two bands distinguished eggs and egg-laying female extracts (Fig. 6). Protein profiles from *H. columbus* and *H. glycines* obtained from nematodes and eggs, respectively, showed few similarities in their protein profiles (Fig. 7).

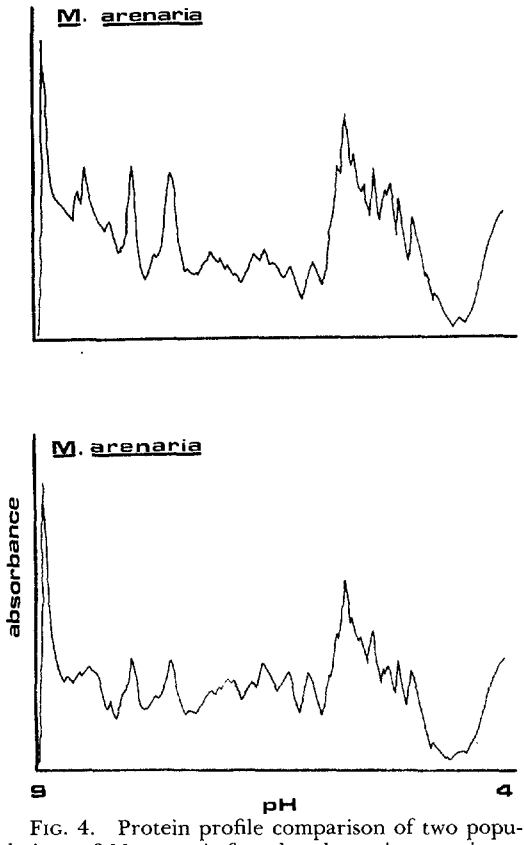

FIG. 4. Protein profile comparison of two populations of *M. arenaria* found pathogenic on resistant Govan soybean. Except for some quantitative differences, these populations were found to be identical.

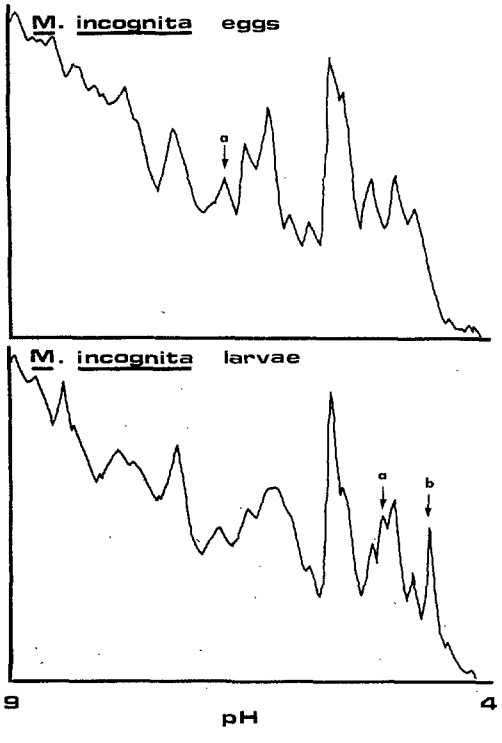

FIG. 5. Protein profiles of extracts from *M. incognita* eggs and preparasitic larvae showing three protein bands that separate these two developmental stages.

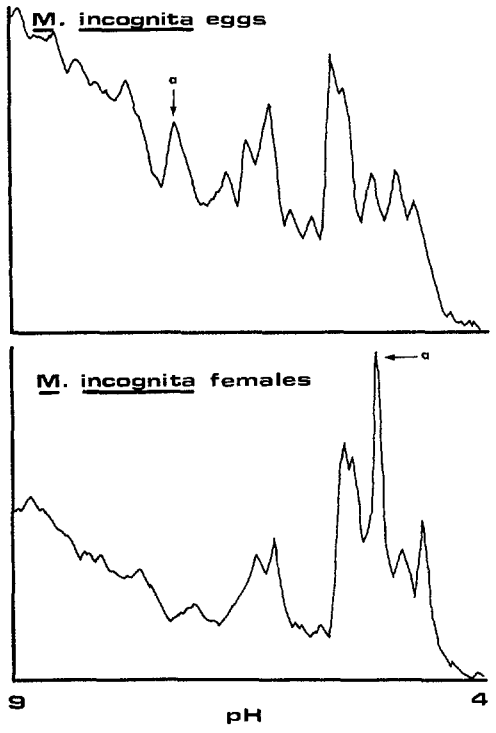

FIG. 6. Protein profiles of extracts from *M. incognita* eggs and females show two proteins differentiating these two developmental stages.

DISCUSSION

The use of electrophoretic data for taxonomic purposes has several advantages over morphological taxonomic methods (1) and can thus be used to supplement and to complement the other methods. Electrophoretic data reduce subjective determinations. New protein bands or changes in mobilities can be measured; these are often more objective than averages of morphological measurements. Electrophoretic data may be more precise for distinguishing genetic differences among organisms because each protein band is given the same weight. Problems associated with grading the importance of certain morphological characters are eliminated.

Root-knot nematode eggs have certain advantages for studying nematode pro-

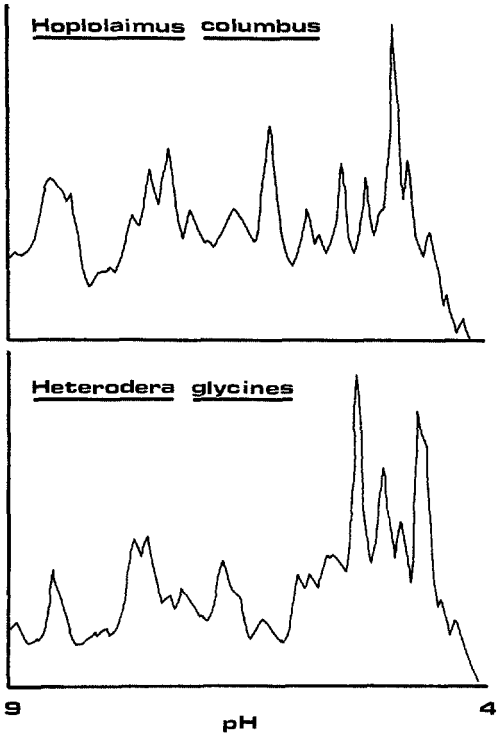

FIG. 7. Protein profiles of *Hoplolaimus columbus* (extracts from larvae and adults) and *Heterodera glycines* (egg extracts). Note lack of similarity.

teins. They can be obtained in relatively large numbers free from host debris. Eggs extracted from a single infected plant represent all egg-laying females in that plant and therefore help eliminate differences due to individual variation of adults in genetic makeup or life cycle. Eggs can be stored up to several months with no discernible changes in protein profiles.

Other researchers have used adult females as the source of protein (5,6,12–14) but have encountered several problems. Host proteins often contaminated preparations from feeding adults (12,14,15,21, 22). This problem was eliminated by using eggs and preparasitic larvae as the source of protein. Differences in isozyme and protein profiles have been detected between the developmental stages of *Meloidogyne* spp. and other genera (4,7,10,16,19,23) as were differences in protein profiles between eggs, larvae, and egg-laying adults (Figs. 5–7). Problems encountered with IEF analysis of nematode egg proteins necessitate running test samples on the same gel with the knowns. Quantitative differences between the same bands in different profiles are also a problem. In this study, distinctions made between nematode species were by the presence or absence of protein bands, and quantitative differences were not considered. The data presented in this study show that IEF of protein extracted from eggs are useful in making taxonomic determinations among species of *Meloidogyne*.

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