ELECTROPHORETIC PATTERNS OF SOME *Meloidogyne* spp. FROM COSTA RICA[1] /

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Resumen

Se comparó las características de diez poblaciones de Meloidogyne *spp. de Costa Rica obtenidas mediante la técnica de electroforesis con gel de almidón. Se encontró que, con unas pocas excepciones, los patrones enzimáticos de deshidrogenasa de malato, isomerasa de fosfoglucosa, fumarasa, deshidrogenasa de* α *– glicerofosfato y deshidrogenasa de isocitrato podrían ser usados para diferenciar las especies de* Meloidogyne *estudiadas (*M. incognita, M. hapla, M. arenaria, M. exigua *y* M. salasi*). Se encontró diferencias intraespecíficas en los patrones de las cinco enzimas entre dos poblaciones de* M. hapla, *y en los patrones de las mismas, excepto deshidrogenasa de isocitrato, entre dos poblaciones de* M. exigua

Introduction

The variability of many morphological characters, and the presence within species of so-called physiological races, are two of the most important problems associated with the taxonomy of plant parasitic nematodes (1). These problems have prompted the search for other approaches not based entirely on anatomy and morphology, to aid in the identification and characterization of species and races of nematodes (10). Among these approaches, biochemical systematics in one which has provided new and helpful information about nematodes and their phylogenetic relationships, complementing and extending the information provided by classical morphologically-based taxonomy (9).

Biochemical systematics exploits the subtle molecular differences that underlie taxonomic variation (7). As suggested by Hussey (10), the ultimate goal of taxonomy should be the classification of the genotypes of the organisms. Practical methods for the analysis of the nucleotide sequence in genes are not currently available. Proteins, on the other hand, are an expression of the sequence of the nucleotides in a gene, and the analysis of these molecules may provide an approach for comparing the genotypes of nematodes.

Electrophoretic techniques allow the separation and identification of specific soluble enzymes and other nonenzymatic proteins. Many enzymatic proteins have different molecular forms, with identical or similar substrates, called isozymes (15).

Electrophoretic comparisons of the isozyme patterns obtained from individual specimens or mass homogenates of a population, can give an idea of the similarity between different populations (14).

The objective of this study was to use starch gel electrophoresis to differentiate several species of root-knot nematodes found in Costa Rica.

Materials and methods

Nematode Populations

Ten populations of *Meloidogyne* spp. from Costa Rica were studied. These were *M. salasi* (CR2), (13),

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M. arenaria race 2 (CR4), *M. exigua* (CR7,9), *M. hapla* (CR10,14), and *M. incognita* race 2 (CR3, 11, 12, 16). Most populations were increased on tomato, cv. Rutgers. *M. exigua* was increased on pepper, cv. California Wonder, and *M. salasi* was increased on the wild grass *Echinochloa colonum*. A mixture of soil and builders sand (3:1 v/v, pH 7.1, 1.5% O. M., 91.8% sand, 6.2% silt and 2.0% clay) treated with steam at 100°C for 24 hours was used to grow the plants. Each pot was fertilized twice a week during the first five weeks of growth with 100 ml of a 1% solution of Nutrisol (12–10–20). The pots were placed randomly on a greenhouse bench and kept separated from other populations by plastic dividers to avoid contamination.

Sample Preparation

Sixty days after inoculation, the root systems were cut into sections approximately 3 cm long and treated as described by Hussey (8), except for a few modifications. These modifications consisted of agitating the flasks containing chopped roots in 200 ml of Pectinol 59L at 150 oscillations per minute for 18-24 hours, the use of a 1.0 M sucrose solution for the centrifugation of the suspension of females, and the transferring of the females directly from the 60-mesh sieve to a beaker with a 1% NaCl solution. The females were collected free of debris, with a Pasteur pitette. Twenty females of each nematode population, except *M. exigua*, were placed in polyethylene microcentrifuge tubes (7 x 30 mm, 250 μl), the saline solution was removed and replaced with 10 μl of 0.1 M K_2 HPO_4 buffer with 0.8% of NaCl and 0.001 M $MgCl_2$ (11). *M. exigua* samples were comprised of 40 females. Samples were stored at – 85°C with no detectable loss of enzymatic activity noted in most samples stored up to eight months.

Starch Gel Electrophoresis

Horizontal starch gel electrophoresis was performed on equipment modified from Bush and Huettel (3). The modification consisted of using a plexiglass U-shaped mold (15 cm long, 17.4 cm wide, 1 cm deep, with legs 1.5 cm long, 4 cm high and 17.4 cm wide for holding the starch gel. The bottom opening along each leg of the mold were taped shut and the mold was filled with starch gel solution. The tape along each leg was removed just prior to placing them into the electrode buffer (17). The starch solution was prepared by adding a mixture of 47.5 g of hydrolysed starch (Connaught Laboratories Limited, Ontario, Canada) and 21 g of electrostarch (Otto Hiller Co., Wisconsin, USA) to 450 ml of the appropriate buffer for the protein system under analysis. Three buffer systems were used for the analysis of the different isozymes. C-buffer system (2) was used for malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), and malic enzyme (ME). Ca-7 buffer system was used for α – glycerophosphate dehydrogenase (α–GPDH) and glucose–6–phosphate dehydrogenase (G–6–PDH), whereas CA–8 buffer system was used for hexokinase (HK) (17). Poulik and tris-ethylenediamine-tetracetic (EDTA)-borate buffer system was used for phosphoglucose isomerase (PGI) and fumerase (FUM) (3).

The nematode protein was prepared as described previously (3), except that the grinding buffer consisted of 0.01 M trisma base, 0.001 M EDTA free acid in 500 ml of deionized water, pH 7.0.

At 2.5 cm from the cathode end of the starch gel, two adjacent strips, 1 cm wide each, were cut with a metal spatula. The strip closer to the end was temporarily removed and the second strip was slid back into the space that was occupied by the first. The homogenate saturated wicks were blotted lightly and loaded on the vertical edge of the large portion of the gel, ca. 0.5 cm apart, starting from the lateral edge. The second strip of gel was then moved back to its original position, and the strip which had been temporarily removed was put into place. The gel was gently pressed back together, and placed inside a refrigerator at 5°. Each gel was covered with plastic food wrap, a glass plate was put on top and then a metal container with ice, to which NaCl had been added, was placed on top of the glass plate (3). The gels were electrophorized for 15 hours at 30 milliamps for the C-buffer system, 15 hours at 50 milliamps for the CA-7 buffer stystem, 6 hours at 30 milliamps for the CA-8 buffer system, and 6 hours at 50 milliamps for the Poulik and Tris-EDTA-borate buffer system.

After electrophoresis gels were removed from the trays and sliced horizontally, that is, parallel to the surface, into four slices, 2 mm thick. The sites of isozyme activity were determined by immersing the slabs in the appropriate enzyme reaction mixtures. The mixtures described by Steiner and Joslyn (17) were used for the detection of MDH, IDH, ME, α–GPDH, G–6–PDH, and HK. The reaction mixtures detailed by Bush and Huettel (3) were used for the determination of PGI and FUM activity.

The gel slabs were incubated in the dark at 37°C for 15-75 minutes depending upon the enzyme. Once stained, the slabs were removed from the reaction mixture, washed with running water and photographed. They were immersed in a fixing solution consisting of methanol, deionized water and glacial acetic acid (5:5:1, v/v) for 18 hours, washed again with running water, wrapped in plastic food wrap and stored in a refrigerator at 5°C.

Two preliminary tests were conducted for each system with a few samples, and after obtaining consistent results, four gels were run with samples from each population for each of the systems studied.

Results

All isozymes resolved migrated anodically on the gels. The bands of each enzyme were numbered consecutively beginning with the one nearest the origin.

Each enzyme is discussed separately, and the results mentioned constitute an average of the four observations performed in each case. The intensity of the staining reaction on the gel surface was related to enzyme activity. The results obtained with all of the enzymes are illustrated in Figure 1.

Malate Dehydrogenase

M. salasi showed four bands, with MDH1 appearing weakly stained at 25 mm from the origin. MDH2 produced a heavily stained band starting 38 mm from the origin. MDH3 and MDH4 appeared as two weakly stained bands which started 46 and 61 mm from the origin, respectively. In population CR7 of *M. exigua* two weakly stained bands were located 10 and 25 mm from the origin, whereas CR9, the other *M. exigua* population, had one weakly stained band which started 19 mm from the origin. Population CR10 of *M. hapla* had a heavily stained band which started 24 mm from the origin; there was evidence of a weakly stained, narrow band located 34 mm from the origin. CR14, the other population of *M. hapla*, had three bands; MDH1 was heavily stained and started 54 mm from the origin. MDH2 and MDH3 were weakly stained, and started 64 and 75 mm from the origin, respectively. *M. arenaria* (CR4) and *M. incognita* (CR3, 11, 12, 16) had all three bands located at the same position. In all cases MDH1 was heavily stained and started 34 mm from the origin. MDH2 was weakly stained and started 34 mm from the origin. MDH3 was weakly stained and started 42 from the origin. With the exception of CR16, in which case the band stained heavily, all MDH bands were weakly stained.

Isocitrate Dehydrogenase

Population CR10 of *M. hapla* had two weakly stained bands which started 33 and 37 mm from the origin. CR14, the other *M. hapla* population, had three bands which started 44, 50 and 56 mm from the origin. The IDH3 band was heavily stained whereas IDH1 and IDH2 were weakly stained. *M.*

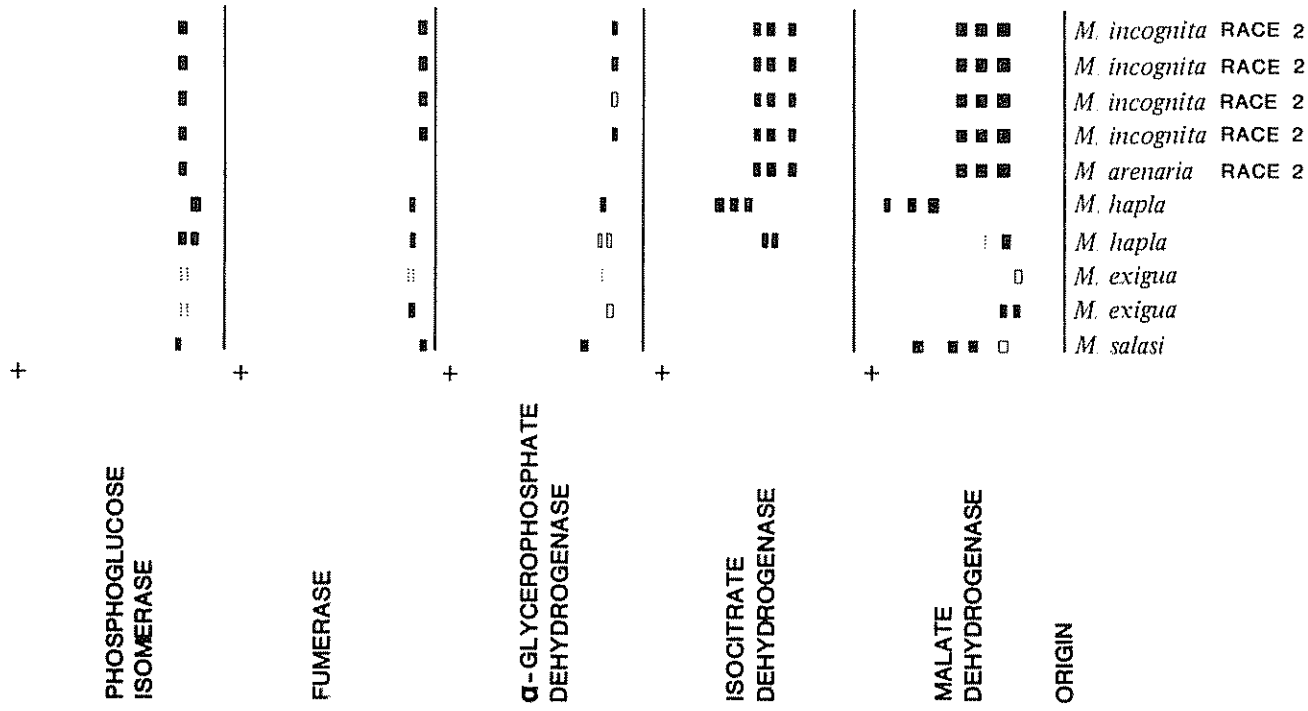

Fig. 1 Diagramatic sketch of comparative electrophoretic patterns of some *Meloidogyne* spp. from Costa Rica. Left to right: *M. salasi* (CR2); *M. exigua* (CR7); *M. exigua* (CR9); *M. hapla* (CR10); *M. hapla* (CR14); *M. arenaria* (CR4); *M. incognita* (CR3); *M. incognita* (CR11); *M. incognita* (CR12) and *M. incognita* (CR16).

arenaria CR4) and *M. incognita* (CR3, 11, 12, 16) all had three bands located at the same positions. IDH1, IDH2 and IDH3 started 25, 34 and 40 mm from the origin, respectively. With the exception of the bands of *M. arenaria*, and IDH3 of the *M. incognita* population CR12, which were heavily stained, the other bands stained relatively weakly.

Activity of this enzyme was not detected in homogenates of *M. salasi* and *M. exigua*.

∝–glycerophosphate Dehydrogenases

In *M. salasi* there was one heavily stained band which started 24 mm from the origin. Population CR7 of *M. exigua* had one weakly stained band which started 13 mm from the origin. Population CR9 of *M. exigua* showed only one weakly stained, narrow band located 18 mm from the origin. Population CR10 of *M. hapla* had two weakly stained bands, which started 14 and 18 mm from the origin. CR14, the other *M. hapla* population, had one heavily stained band which started 17 mm from the origin. All populations of *M. incognita* had one band which started 11.5 mm from the origin. In CR3 and 16 the band was heavily stained, whereas it was weakly stained in the other populations. Activity of this enzyme was not detected in homogenates of *M. arenaria*.

Fumerase

One heavily stained band, starting 4 mm from the origin, was detected in *M. salasi*. In *M. exigua* no activity of the enzyme was detected on two occasions, but when the number of females per samples was increased to 100, population CR7 showed one weakly stained band which started 9 mm from the origin. CR9 also had the same as described for CR7, except that it was very weakly stained. Population CR10 of *M. hapla* had one weakly stained band which started 8 mm from the origin. The other *M. hapla* population (CR14) had one heavily stained band which started 9 mm from the origin. All populations of *M. incognita* had one band, heavily stained, which started 4 mm from the origin.

The activity of this enzyme could not be detected on homogenates of *M. arenaria*.

Phosphoglucose Isomerase

M. salasi had one heavily stained band which started 18 mm from the origin. *M. exigua* had, in 3 out of 4 gels, one very weakly stained band which started 16 mm from the origin. Population CR10 of *M. hapla* had two heavily stained bands which started 11 mm and 16 mm from the origin, whereas population CR14 of this same species had only one heavily stained band, which started 10 mm from the origin. *M. arenaria* and all populations of *M. incognita* had one heavily stained band which started 16 mm from the origin.

No activity was detected for G–6–PDH and HK, and no discrete bands were observed for ME, although some smearing was evident on all gels indicating enzyme activity.

Discussion

The starch gel electrophoretic analyses demonstrated certain enzymes could be used to differentiate among most of the species of *Meloidogyne* used in this study. Thus isozyme patterns provide and additional criterion for the taxonomic classification of these nematodes and confirms other electrophoretic studies with root-knot nematodes (4, 5, 11, 12).

The MDH, IDH, ∝–GPDH, FUM and PGI patterns revealed intraspecific differences in *M. exigua* and *M. hapla*. Each population of these two species were collected from different localities in Costa Rica, and also different hosts in the case of *M. hapla*. Population CR7 of *M. exigua* reproduced on tomato to a limited extent, whereas CR9 reproduced well on this host. Some morphological differences between males of both populations were observed with the scanning electron microscope (R. López, unpublished data). Regarding the two populations of *M. hapla*, certain morphological differences in several characters of the second-stage juveniles were noted also (R. López, unpublished data). It seems likely that the populations of both *M. exigua* and *M. hapla* differed in their genetic composition. Therefore it is not surprising to have found differences in their enzyme patterns. Moreover, these electrophoretic differences could be considered as additional evidence that these populations are physiologically different. Other enzyme analyses should provide more confidence in their identification by enzyme profiles.

The comparison of four populations of *M. incognita* race 2 showed that no differences existed in any of the enzymes among these populations. Some slight differences were found in the intensity of the staining reactions of certain isozymes patterns, but they did not seem to be important enough to be considered diagnostic for a specific host race of *M. incognita*.

Several factors may be responsible for the failure to detect enzymatic activity of G–6–PDH and HK, and for obtaining only smears in the ME gels. The method of culturing, stage of development, physio-

logical state of the nematodes, protein extraction procedures, storage conditions of the protein extract and the method of protein analysis are factors that can induce variability in electrophoretic analyses of nematode proteins. These factors may influence the number of proteins or isozymes that can be detected, their electrophoretic mobility or both (11). Some or all of these factors, or the interaction of some of them could be responsible for the unsatisfactory results. A similar explanation could be given regarding the failures to detect activity of IDH in homogenates of *M. salasi* and *M. exigua*, and of α-GPDH and FUM in homogenates of *M. arenaria*. In any case, it seems desirable to investigate these aspects, not only with the same populations used in this study, but with others of the same species, to determine whether the unsatisfactory results are due to the specific population or to the methodology.

Those enzymes which did not appear consistently, or which appeared only after increasing the number of females in the samples, i.e., PGI and FUM in homogenates of *M. exigua*, may have concentrations that were too low to be suitable for starch gel electrophoresis. It is also possible that freezing affected the activity of these enzymes.

While tomato was used as the host for most populations, pepper was the host for *M. exigua* and *E. colonum* was the host for *M. salasi*. Evidence indicated that enzyme profiles may be influenced by the plant on which the root-knot nematodes are increased (11, 12, 16). Dickson *et al.* (5), however reported identical enzyme patterns for *M. javanica* isolated from different hosts.

When possible it is desirable to propagate all of the nematode populations on a common host to avoid the possibility of their influence on enzyme profiles. The use of life stages other than the females, such as the egg or the freshly hatched second-stage juvenile, which are free from plant protein, might prove to be a more reliable subject for electrophoretic analyses as was found for some round cyst nematodes (6).

Starch gel electrophoresis is a promising approach for the study of several specific enzymes in root-knot nematodes, and could be used, in addition to those based on morphology, responses of differential plants, cytology and mode of reproduction, for the taxonomic classification of this important group of plant pathogens.

Summary

Ten populations of *Meloidogyne* spp. from Costa Rica were compared by means of starch gel electrophoresis. With a few exceptions, malate dehydrogenase, phosphoglucose isomerase, fumerase, α-glycerophosphate dehydrogenase and isocitrate dehydrogenase isozyme patterns could be used to differentiate the species of *Meloidogyne* that were investigated. Intraspecific differences were noted in patterns of the five enzymes between two populations of *M. hapla* and in the patterns of all enzymes except isocitrate dehydrogenase between two populations of *M. exigua*.

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