ISOZYME PATTERNS OF EXOTIC *MELOIDOGYNE* SPP. POPULATIONS

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Summary. Isozyme patterns of esterase (EST), malate dehydrogenase (MDH) and superoxide dismutase (SOD) were used to determine the species of thirteen *Meloidogyne* isolates from populations originating from India (6), Venezuela (5), Cuba (1), and Egypt (1) and collected from different hosts. It was possible to characterize most of the populations tested as *M. incognita* and *M. javanica*, but only a part of them showed the typical species-specific EST phenotypes. Atypical EST phenotypes were also obtained, which made identification possible only by comparison of additional MDH and SOD phenotypes. By means of a combination of EST and MDH phenotypes, one population collected from guava in Venezuela and one population from tomato in Cuba were both identified as *M. mayaguensis*. Although SOD patterns did not clearly discriminate between *M. incognita* and *M. mayaguensis*, they showed the greatest polymorphism among populations, with a total of eight polymorphic bands detected.

Root-knot nematodes (RKN, *Meloidogyne* spp.) are widespread parasites of plants in tropical and sub-tropical countries, where they cause severe yield losses in a wide range of crops (Trudgill *et al*., 2000). The importance of RKN to tropical agriculture has been already outlined by the International *Meloidogyne* project (Sasser *et al*., 1983). Along with the most known and most widely distributed tropical species *M. incognita* (Kofoid *et* White) Chitw., *M. javanica* (Treub) Chitw. and *M. arenaria* (Neal) Chitw., other more recently described species, such as *M. hispanica* Hirschman*, M. mayaguensis* Rammah *et* Hirschmann*, M. konaensis* Eisenback, Bernard *et* Schmitt, *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos *et* Almeida, etc., have become common in surveys from warm climate areas of the world (Eisenback *et al*., 1995; Carneiro *et al*., 1996; Trudgill *et al*., 2000). For instance, *M. mayaguensis*, first described from Puerto Rico in 1988 (Rammah and Hirschmann, 1988), has been detected in Trinidad, Malawi, Senegal and on guava in Brazil (Trudgill *et al*., 2000; Carneiro *et al*., 2001). Moreover, most RKN populations from exotic countries have been found to be able to reproduce on resistant tomato when tested under controlled temperature conditions in glasshouses (Molinari and Caradonna, 2003).

The spread of these exotic *Meloidogyne* populations, because of their pathogenicity and virulence, is cause for concern and, therefore, the valuable tools for rapid identification used so far for the major species must be re-examined and widened in scope to include such exotic species. Although DNA-based diagnostic techniques have been successfully applied for RKN (Petersen and Vrain, 1999; Zijlstra *et al*., 2000), they remain of limited use for large surveys because the mere discrimination of species may be complex, time-consuming and expensive. Species-specific isozyme patterns revealed by polyacrylamide-gel electrophoresis have been proved to be excellent tools for the screening and identification of many populations of *Meloidogyne* spp. collected from different parts of the world (Esbenshade and Triantaphyllou, 1985; Carneiro *et al*., 2000; Molinari, 2001).

This paper reports the findings of a survey of *Meloidogyne* spp. populations collected from tropical and sub-tropical countries, and identified by means of the isozyme patterns of esterase (EST, E.C. 3.1.1.1), malate dehydrogenase (MDH, E.C.1.1.1.37) and superoxide dismutase (SOD, E.C.1.15.1.1) obtained by using automated and miniaturized electrophoresis equipment.

MATERIALS AND METHODS

Thirteen populations of *Meloidogyne* spp., originating from India (6), Venezuela (5), Cuba (1), and Egypt (1), were characterized (Table I). Nematodes were collected from their natural hosts and reared on susceptible tomato Roma VF in a greenhouse at 24-27 °C. Infested roots were rinsed, finely chopped and incubated in a solution of cellulytic and pectolytic enzymes (15% Pectinex® Ultra SP-L, 35% Celluclast®, Novo Nordisk Ferment Ltd, Denmark) at 32 °C whilst stirring overnight. Afterwards, the extracted females were collected on a 250 µm sieve; roots were saved and blended in a minimal volume of 0.9% NaCl. Again, the homogenate was filtered and the remaining females collected.

Females were then freed from plant debris by several

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steps of purification under a stereo microscope and suspended in 0.9% NaCl. Extracts for electrophoresis (10-20 µl) were usually prepared by homogenizing two females/µl of an extraction buffer containing 20% sucrose, 0.1 M Trizma-Base, 0.08 M boric acid, pH 8.4, 2.5 mM EDTA, 5 mg bromophenol blue and the protease inhibitors PMSF (1 mM), pepstatin (1 mM) and leupeptin (1 mM). The females were homogenized on ice using plastic, Eppendorf-shaped, miniature homogenizers (Biomedix, UK) with blunt bottoms and provided with small pestles that can be suspended within the "mortars"; this equipment is particularly suitable for homogenization in very small volumes. The homogenizers, with the pestles suspended, were centrifuged first at 2,432 *g* for 2 min to collect any drops of homogenate at the bottom. The pestles were then removed and the homogenates further centrifuged at 14,000 *g* for 15 min, at 4 °C in a bench centrifuge. Pellets were discarded and supernatants immediately used for electrophoresis or stored at -80 °C until use.

About 8 µg of female proteins were loaded in each sample and separated by native polyacrylamide gel electrophoresis using PhastSystem equipment (Pharmacia Biotech, USA), which permits pre-programming of the chosen separation method. Reproducibility of the runs is ensured by microprocessor control and by the use of pre-cast 0.45 mm thick gels whose separation zone is only 3.8 x 3.3 cm. Homogeneous gels (12.5% acrylamide) were used when gels were to be stained for esterase (EST, E.C.3.1.1.1) and malate dehydrogenase (MDH, E.C.1.1.1.37), whilst gradient gels (8-25% acrylamide) were used when superoxide dismutase (SOD, E.C.1.15.1.1) was to be visualized in order to have the finest separation of the very close bands obtained for this enzyme; such gradient gels were used to further differentiate esterase bands that appeared to have very similar relative mobility on homogenous gels. EST isozyme bands were stained according to Molinari (2001). SOD activity was stained using the technique described in Molinari *et al*. (1997), with slight modifications: gels were immersed in the staining solution (0.12 mM NBT) for 5 min at 37 °C, then rinsed with distilled water and incubated at room temperature in 0.1 M Na-K-phosphate buffer at pH 7.8 containing 15 mM TEMED and 0.26 mM riboflavin. Gels were maintained on a white light trans-illuminator until white bands over a dark background appeared. MDH bands were stained according to Esbenshade and Triantaphyllou (1985). Mini-gels were dried, directly scanned by means of a ScanJet II cx (Hewlett Packard), arranged as digital images and printed on photo quality paper. Species-specific esterase phenotypes were designated with the first capitalized letter of the appropriate species and a number indicating the number of the apparent bands; species polymorphism was indicated by a small letter (Esbenshade and Triantaphyllou, 1985, 1990). MDH and most of the SOD patterns were indicated as non-specific (N). Internal standards from pure populations of known species were added to the gels to ensure reliable identification. Protein content of the samples was determined according to Lowry *et al*. (1951).

RESULTS AND DISCUSSION

Five different bands of esterase activity were observed in the electrophoresis patterns of the thirteen *Meloidogyne* populations tested (Fig. 1). Two patterns, I1 and S1, made up of two close bands, identified, respectively, five and one populations as *M. incognita* (Table I, Fig. 1B). These results resembled those from other wider surveys reported in the literature; in a study of 291 RKN populations from all over the world, 105 out of 111 *M. incognita* populations showed only the I1 phenotype and the remaining six showed the S1 phenotype (Esbenshade and Triantaphyllou, 1985). Significantly, the S1 phenotype was not species-specific and also characterized four populations of *M. chitwoodi* and one population of *M. platani* Hirschmann. In our case, the identification as *M. incognita* of the population collected from coffee in Venezuela is due to the specific association of the S1 EST phenotype with the N1 MDH phenotype (Table I). Accordingly, *M. incognita* has already been reported on coffee from plantations of Brazil (Carneiro *et al*., 1996). Esterase is a monomeric enzyme in *Meloidogyne* spp. and patterns I1 and J2/J3, which are the most common of, respectively, *M. incognita* and *M. javanica*, have been considered to be expressed by two genetic *loci*, L1 and L2 (Navas *et al*., 2001). S1 and M2 phenotypes might be representative of a different uncommon *locus* (L3); in this case, the single bands involved would come from the expression of two homozygous alleles (*i.e.* S1, aa and M2, bb) (Fig. 1B).

MDH patterns were the least polymorphic with only two observed bands: *M. mayaguensis* showed the pattern N1a, characterized by a band migrating faster than the widespread N1 band and slower than the H1 band, which specifically characterizes *M. hapla* phenotypes (Fig. 2). However, detection of the sole N1a pattern is not sufficient to identify *M. mayaguensis*: it must be associated with the M2 EST phenotype. Although this phenotype has been designated as M, indicating a specificity for *M. mayaguensis*, Esbenshade and Triantaphyllou (1985) differently identified populations characterized by the present M2 EST phenotype (or VS1-S1(a) as named by them) because they did not show the N1a MDH phenotype: one population (VS1-S1a/H1) from NC, USA was ascribed to *M. carolinensis*, and one other (VS1-S1/N3c) from Puerto Rico was left unidentified. Therefore, the association of the M2 with the N1a pattern was a strong clue for the identification of one population from fields of guava in Venezuela and one other from tomato in Cuba as *M. mayaguensis* (Table I). Certainly, the accurate identification of such populations as *M. mayaguensis* was supported by considering all three isozyme patterns, as a previous screening carried out us-

Table I. Origins, hosts, species identification and enzyme phenotypes of *Meloidogyne* populations.

Acronym	Species	Country, Region, Locality	Crops	Enzyme phenotypes		
				EST	MDH	SOD
IN1	*M. javanica*	India, Pallipalem	Peanut, *Arachis hypogaea*	J3	N1	J2
IN2	*M. javanica*	India, Tiripurati	Peanut, *Arachis hypogaea*	J3	N1	J2
IN3	*M. javanica*	India, Patancheru (BP9)	Peanut, *Arachis hypogaea*	J3	N1	J2
IN4	*M. javanica*	India, Makila	Peanut, *Arachis hypogaea*	J3	N1	J2
IN5	*M. incognita*	India, Kalimala	Peanut, *Arachis hypogaea*	I1	N1	N3
IN6	*M. incognita*	India, Patancheru (RP14)	Peanut, *Arachis hypogaea*	I1	N1	N3
Ven0	*M. incognita*	Venezuela, Maracay, Aragua State	Coffee, *Coffea arabica*	S1	N1	N3a
Ven1	*M. incognita*	Venezuela, Sabana Grande, Lara State	Tomato, *Lycopersicon esculentum*	I1	N1	N3
Ven2	*M. incognita*	Venezuela, Barinas	Sweet potato, *Ipomea batatas*	I1	N1	N3
Ven3	*M. incognita*	Venezuela, Barinas	Cassava, *Manihot esculenta*	I1	N1	N3
Ven4	*M. mayaguensis*	Venezuela, Mara Municipality, Zulia State	Guava, *Psidium guajava*	M2	N1a	N3b
Cu0	*M. mayaguensis*	Cuba, Zona Franca	Tomato, *Lycopersicon esculentum*	M2	N1a	N4
Eg	*M. javanica*	Egypt, Noubaria	Grapes, *Vitis vinifera*	J3a	N1	J3

ing only the EST isozyme patterns was not sufficient to identify such populations to species (Molinari, 2001). On the other hand, a Martinique population collected from tomato and RKN infesting guava in Brazil have already been reported as *M. mayaguensis* (Carneiro *et al.,* 2000, 2001). Furthermore, *M. mayaguensis* has recently been distinguished from the major species of tropical RKN by mitochondrial DNA (Blok *et al.,* 2002). Population Ven4 from Zulia State was preliminarily identified as *M. incognita* race 1 by Crozzoli and Casassa (1998) using inoculum from infested guava on differential plants, and was found to be less aggressive than populations Ven1 and Ven2 on rice in Venezuela (Greco *et al*., 2000). The presence of *M. mayaguensis* has also been recorded on coffee plants from the eastern region of Cuba since 1995 (Rodriguez *et al*., 1995) and in a large survey of the island it was confirmed that various

Fig. 1. A) Isozyme patterns of esterase (EST) of *Meloidogyne javanica* from Makila, India (J3), and *M. javanica* from Egypt (J3a), *M. incognita* from Barinas, Venezuela (I1) and from Maracay, Venezuela (S1), *M. mayaguensis* from guava, Venezuela (M2). B) Phenotypes of esterase (EST) observed in 13 exotic populations of *Meloidogyne* spp. (L1-L3: *Locus*1-*Locus* 3).

species of RKN damage coffee, guava, soybean and various field and vegetable crops (Fernández Díaz-Silveira and Ortega Herrera, 1998). These two described populations of *M. mayaguensis* from Venezuela (Molinari, personal communication) and Cuba (Molinari and Caradonna, 2003) have been found to be virulent to *Mi*-bearing resistant tomato plants. The findings of the present study confirm that *M. mayaguensis* may be a widespread pest of major crops over the whole tropical and sub-tropical American area.

SOD patterns showed the greatest polymorphism, with eight polymorphic bands. The presence of a large number of bands might be considered the result of at least three different loci (L1, L2, L3) at genomic level. Because of its dimeric structural configuration, it is likely that SOD also forms inter-loci bands. Significantly, as many as six different patterns over thirteen populations were detected (Fig. 3). J2/J3 patterns may be explained by the presence of an infrequent locus (L4). N3a and N3 show quantitative differences in the activity of the 0.77 Rm band because of diverse chromosomal content of the sampled populations, but both patterns might be considered as variants of the same genetic profile. Fast-migrating bands (Rm>0.69) were common to all samples, whilst slow-migrating bands were apparent only in some patterns (Fig. 3B). *Meloidogyne incognita* (N3, N3a) and *M. javanica* (J2, J3) phenotypes were easily distinguishable, whilst phenotypes N3b and N4 both characterized *M. mayaguensis*. However, SOD pattern analysis was the only means of detecting intra-specific variability that would

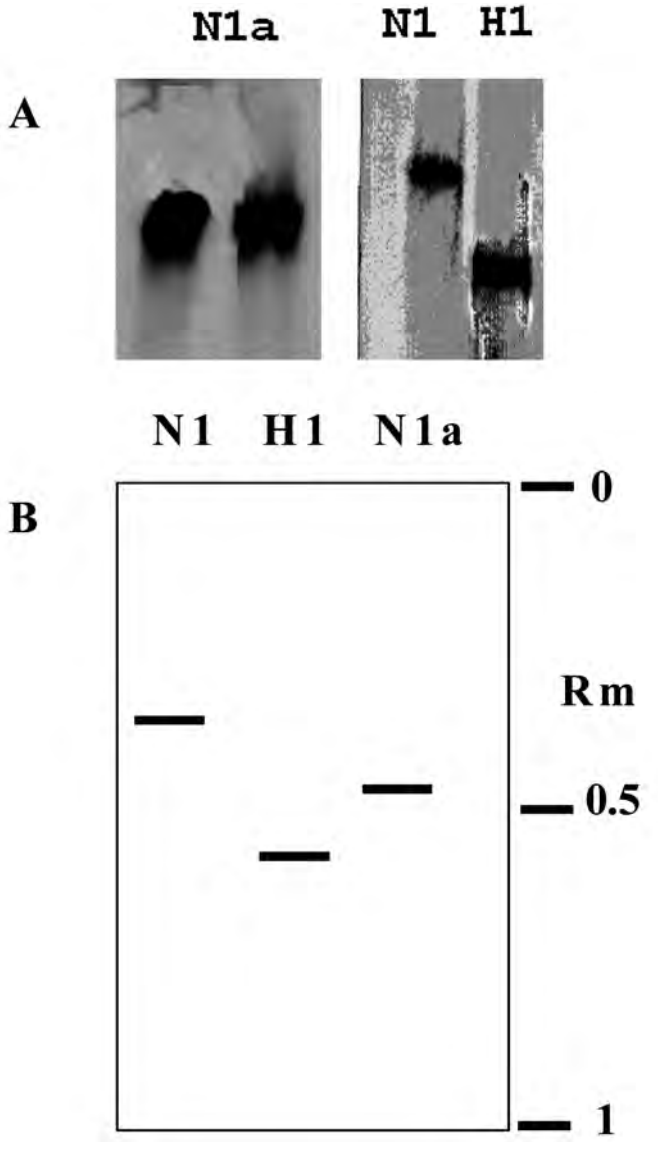

Fig. 2. A) Isozyme patterns of malate dehydrogenase (MDH) of *M. mayaguensis* from Venezuela and Cuba (N1a), *M. incognita* from Maracay, Venezuela (N1) and *M. hapla*, standard population (H1). B) Phenotypes of malate dehydrogenase (MDH) of the 13 populations tested plus one standard population of *M. hapla*.

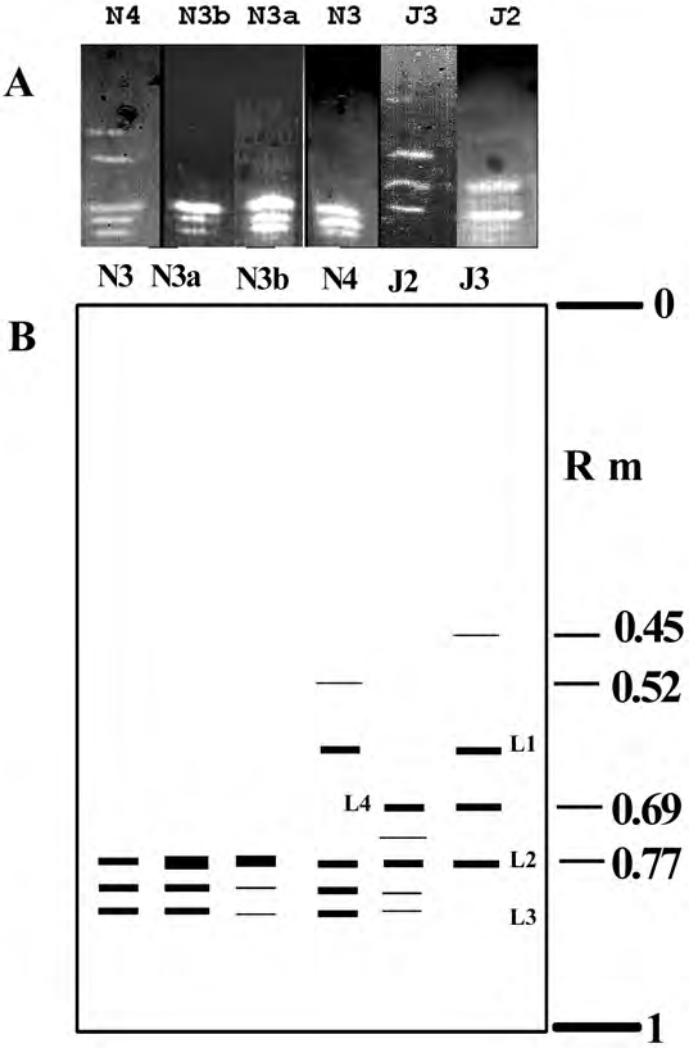

Fig. 3. A) Isozyme patterns of superoxide dismutase (SOD) of *M. mayaguensis* from Cuba (N4) and Venezuela (N3b), *M. incognita* from coffee and cassava, Venezuela (N3a and N3, respectively), *M. javanica* from Egypt (J3) and from Makila, India (J2). B) Phenotypes of superoxide dismutase (SOD) observed in 13 exotic populations of *Meloidogyne* spp. (L1-L4: *Locus*1- *Locus* 4).

permit the discernment of *M. javanica* Indian populations from the Egyptian one and the *M. mayaguensis* populations from different locations in the Caribbean area. However, the *M. javanica* population growing on grapes in Egypt was found to show an atypical *M. javanica* EST phenotype (J3a), slightly different from the standard pattern (J3) that characterizes *M. javanica* (Table I). SOD phenotypes have been reported to be sufficient to discriminate the four major *Meloidogyne* spp. (Molinari, 2004a), although, with specific regard to root-knot nematodes, they seem to have a more valuable application in assessing the intra-specific variability of different populations (Navas *et al*., 2001; Molinari, 2004b).

Thus, the data shown in this paper confirm that the geographic widening of *Meloidogyne* population surveys should be supported by an increase in the number of enzymes used in order to maintain the accuracy and reliability of species identification made by electrophoresis methods.

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