

Discrimination among *Meloidogyne Incognita* Isolates by Cellulase Activity, Protein and Dna Fingerprinting

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Abstract: Biochemical and molecular markers were used for discrimination five *M. incognita* isolates i.e M₁, M₂, M₃, M₄ and M₅. It was found variations in cellulase activity, protein patterns and DNA fingerprint. Comparing cellulase activity among isolates showed the differences in specific activity and recovery. Isolate M₄ was 928.5 mg protein in a rather total activity 2432.2 μ and low specific activity 2.44 IU/mg. While isolate M₃ was 892.3 mg protein in a rather total activity 229.9 μ and high specific activity with 2.90 IU SDS-PAGE of protein analysis showed distinct protein contents, polypeptides and density of bands, whereas, 11, 12, 6, 8 and 7 polypeptides for M₁, M₂, M₃, M₄ and M₅ respectively. The polymorphic was 21.43%. RAPD-PCR was used to analysis DNA of isolates. Three arbitrary random primers were successfully (producing polymorphic band) to reveal genetic variation among 5 isolates of *M. incognita*. Based on these identify markers, the genetic distances among isolates were determined and their genetic relationships were estimated. RAPD fingerprint of isolates revealed polymorphic in 27, 29 and 24% by OPA₁₃, OPD₈ and OPE₂₀ primers respectively. As well as 1, 2, 4, 3 and 1 genetic markers for *M. incognita* isolates M₁, M₂, M₃, M₄ and M₅ isolates. The phylogenetic tree revealed that, the five isolates showed close similarity with the group. The different in enzyme activities were recorded in isolates. The developed RAPD profiles of *M. incognita* isolates were not typical. The frequency of genetic variability was detected in *M. incognita* isolates dependent, variations in cellulase activity.

Key words: *Meloidogyne* isolates; SDS-PAGE; cellulase enzyme, RAPD-PCR.

INTRODUCTION

The detection of pests and diseases is fundamental to crop management. Modern developments in molecular biology have exploited the use of polypeptide fractions, isozymes and DNA fingerprints for detection and represent an active area of research in plant nematology (Williamson, 1991). Some of the most widespread pests limiting world agricultural productivity are the root-knot nematodes of the genus *Meloidogyne*. In Egypt, species of this genus are generally regarded as pests that are economically important in greenhouse and the field. However, in tropical regions they attack almost every type of crop, causing considerable losses of yield or adversely affect the quality of the produce. The impact of the root-knot nematode on a crop and the relationship between the nematode and host differ between nematode species.

Successful non-chemical control of root-knot nematodes by such means as resistant cultivar introduction and crop rotation depends on the accurate and rapid nematode detection. However, it is not easy to identify *Meloidogyne* species, especially the second stage juveniles (J2), by their morphological and isozyme characteristics.

Recently, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and polymerase chain reaction (PCR) have been employed for species identification of *Meloidogyne* J2. PCR-restriction fragment length polymorphism (RFLP) method is successfully applied for the identification of a single *Meloidogyne* J2 (Orul, 1998 and Zijstra *et al.*, 1995), but the PCR-RFLP method requires digestion of amplified products. Contrarily, RAPD-PCR method does not require the digestion, and so is more simple and rapid than PCR-RFLP method. This method should be further improved as a practical technique for species identification of a single J2 of various *Meloidogyne* spp. (Cenis, 1993).

This study focused on the genetic variations among five *Meloidogyne incognita* isolated from different soils using SDS-PAGE, cellulase activity and RAPD-PCR.

MATERIALS AND METHODS

Nematoda Cultures:

Population of five *M. incognita* isolates (Nematode Lab. Plant Protection, NRC) were cultured on tomato *Lycopersicon esculentum* cv. Castle rock, pot cultures were maintained in a greenhouse (approximately 25°C,

average relative humidity 80%; day length of 16 h). Females were recovered using the method of Hussey (1971). Root systems were excised, cleaned of debris and cut into approximately 1-2 cm pieces and placed in 1 liter Erlenmeyer flask with 250 ml of a 50% solution of liquid pectinase concentrate and agitated on a shaker overnight at room temperature. Released females were collected, washed with distilled water and transferred into 2 ml Eppendorf tubes and stored at -20°C until required.

Cellulase Activity Assay:

Cellulase activity is determined by the hydrolysis of 200 µl 1% CMC in 100 mM sodium acetate buffer containing 100 mM NaCl (pH 5.2) at 50°C for 10 min. Dinitrosalicylic acid (0.5 ml) was added to stop the reaction by boiling in a water bath for 5 min and quick cooling to room temperature. The absorbance at 540 nm was measured (as standard assay). One unit of cellulase activity is defined as the amount of enzyme that yields 1 µmol glucose in 1 min at 50°C (Li *et al.*, 2005).

Determination of Protein:

Protein content was determined by lowry's method according to Lowry, *et al.* (1951) using bovine serum albumin as a standard.

SDS-polyacrylamide Gel Electrophoresis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) under reducing conditions in discontinuous electrode buffer system. Protein samples were denatured and reduced completely before electrophoresis by mixing the samples with an equal volume of 2x sample buffer (Lucius *et al.*, 1987) and heating the mixture at 95°C in a water bath for 5 min. Treated samples were centrifuged at 12,000 rpm for 10 min and chilled on ice before use. Each lane was charged with 25 µg protein of supernatant. Electrophoresis was carried out at room temperature at a constant current at 25 mA for 1 hr. Followed by 30 mA for 4 hrs. At end of the run the gel was stained with coomassie blue, destained in the stain solvent and photographed.

Extraction of t-DNA:

Miniprep method was used for t-DNA extraction from nematoda. This is the slightly modified method of Cenis (1993) for extracting a large quantity of DNA from many adult females. Fifty adult females were manually picked from roots of each host plant and washed in a sterile plastic dish (9 cm diameter) containing sterile water. They were ruptured with a pestle on a slide glass, and 250 µl of DNA extraction buffer (200 mM Tris-HCl; pH 8.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) was added and transferred to a 0.5 ml microcentrifuge tube then added 75 µl of 3 mM potassium acetate pH 5.2. The lysate was kept at -20°C for 10 min. After centrifugation, the supernatant was transferred to a new micro centrifuge tube. Nucleic acids were precipitated with an equal volume of isopropanol at room temperature for 30 min and pelleted by centrifugation at 15,000 rpm for 15 min. The pellet was washed with 70% ethanol, dried and dissolved in TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). The concentration of the template DNA was adjusted to about 1 ng/µl.

Amplification of DNA:

Ten arbitrary primers were used for RAPD-PCR amplification. The amplification was performed in 25 µl of reaction buffer (10 mM Tris HCl pH 8.3; 50 mM KCl, 1.5 mM MgCl₂) with 100 mM of each dNTP, 0.1 µl Taq DNA polymerase, and 5 µl crude DNA extract in the case of single extraction. The PCR thermal program was 35 cycles of 94°C for 1 min 36°C for 1 min and 72°C for 1 min. These amplified products were stored at -20°C until used.

Conformation of PCR Amplification:

The electrophoresis of the amplified DNA was performed through 1.5% agarose gel at a constant voltage of 50 V in 0.5 x TBE buffer (50 mM Tris, 50 mM boric acid and 1 mM EDTA pH 8.5) at room temperature. The gels were stained with ethidium bromide solution (0.5 µg/ml) and were photographed on a UV transilluminator.

RESULTS AND DISCUSSION

Genetic variability among 5 nematode isolates was detected by SDS-PAGE, cellulase activity and DNA fingerprint by RAPD-PCR.

Protein Patterns:

Cellulose hydrolytic activity was assayed of each isolate. The specific activities of 2.58; 2.55; 2.90; 2.44 and 2.76 1 μ/mg protein for *M. incognita* isolates M₁, M₂, M₃, M₄ and M₅ respectively (Table, 1).

The obtained results recorded in Table (2) and Fig. (1) showing the number and density of protein fractions. Whereas 10 bands for (isolate (M₁), 12 bands, isolate (M₂) and 6 bands (M₃, and 7 bands for M₄ and M₅). The polymorphism between nematoda isolates, 5 unique bands (36%), 6 monomorphic bands (43%) and 3 polymorphic bands with (21%). The proteins patterns divide the two groups. Weak similarity between M₁ and M₂ (group 1) and complete similarity between M₃, M₄ and M₅ (group 2).

Table 1: Protein content and cellulase activity of nematoda isolates.

Nematoda	Protein content (mg)	Total activity (U)	Specific activity (1 u/mg)
<i>M. incognita</i> (M ₁)	980.5	2521.1	2.62
<i>M. incognita</i> (M ₂)	925.9	2621.5	2.83
<i>M. incognita</i> (M ₃)	892.3	2299.4	2.57
<i>M. incognita</i> (M ₄)	928.5	2432.2	2.62
<i>M. incognita</i> (M ₅)	859.5	2375.3	2.75

In recent year, owing to the unique characteristics of animal cellulases, a number of laboratories have paid great attention to finding animal sources for cellulases for example, root-knot nematode *M. incognita* (Rosso, et al., 1999), crayfish (Byrne et al., 1999); blue mussel (Xu, et al., 2001) and Mollusca and *Ampullaria crossean* (Wong, et al., 2003 and Yan-Hong et al., 2005). We have reported a multi functional cellulose (EGX) from the gastric juice of *A. crossean*. The cellulolytic and hemicellulolytic enzymes in Mollusca, *A. crossean*, have not been investigated systemically. It is worth while, identifying new enzymes from this species.

Cellulases are responsible for the hydrolysis of the b-1,4-glucosidic bands in cellulases. Ordinarily it was accepted that effective biological hydrolysis of cellulose to glucose required synergistic collaboration of three different kinds of enzymes endo-b-1,4-gluconase (EC 3.2.1.4 EG) which randomly cleaves internal linkages in cellulose chains, cellobryhdrolase (EC 3.2.1.91, CBH) which specifically cleaves cellobiosyl units from non-reducing ends of cellulose chains and b-D-glucosidase (EC. 3.2.1.21) which cleave glucosyl units from celooligo-saccharides (Perez et al., 2002 and Li et al., 2005).

The obtained data of SDS-protein fingerprint showed that 5 isolates of *M. incognita* were differed in number and density of protein patterns the analysis of nematoda isolates species and strains for somaclonal variation has yet to be established traditional methods based on protein fractions and isozymes analysis have been used to determine genetic variabilities and identify parental hybrids (Sharma, 2003, Swelam, 2005 and Misra et al., 1993).

Table 2: Protein fractions and content of Nematoda isolates by SDS-PAGE.

Isolates	M ₁		M ₂		M ₃		M ₄		M ₅		Polymorphism
M.W.	M.W	Density	M.W	Density	M.W	Density	M.W	Density	M.W	Density	
107.5	-	-	107.5	+++	-	-	-	-	-	-	Unique
97.4	-	-	97.4	++	-	-	-	-	-	-	Unique
85.7	85.7	++	85.7	+++	85.7	+	85.7	+	85.7	+	Monomorphic
81.5	81.5	++	81.5	++	-	-	-	-	-	-	Polymorphic
75.2	75.2	++	75.2	+++	75.2	+	75.2	+	75.2	+	Monomorphic
58.1	58.1	++++	58.1	++++	58.1	+++	58.1	+++	58.1	+++	Monomorphic
39.2	39.2	+++	39.1	+++	39.1	++	39.2	++	39.2	++	Monomorphic
32.5	32.5	++	32.5	++	-	-	32.5	+	32.5	+	Polymorphic
30.1	-	-	30.1	++	-	-	-	-	-	-	Unique
29.0	29.0	+	-	-	-	-	-	-	-	-	Unique
14.3	14.3	+	-	-	-	-	-	-	-	-	Unique
13.0	13.0	++	13.0	++	13.0	++	13.0	++	13.0	++	Monomorphic
12.0	12.0	+++	12.0	+++	12.0	++	12.0	++	12.0	++	Monomorphic
Number of polypeptides	10		11		6		7		7		

MW : Molecular weight of protein markers (KDa)

Density of band++++ Very strong+ Weak

+++ Strong- Absent band ++ Moderate

RAPD pattern of five *Meloidogyne* isolates: Five *Meloidogyne* isolates examine were successfully identified using the primer OPA₁₃, OPD₈ and OPE₂₀. The reproducible RAPD patterns were obtained by using the template DNA extracted with the mini prep method from the adult female.

The miniprep extraction method produced the more clearly intense bands and fewer minor ones. The reproducible RAPD pattern was successfully obtained by using ¼ volume of the template DNA extracted with 20µl of the lysis buffer from single individuals of adult female. The RAPD patterns of the adult female were practically identical. The yield of DNA was determined spectrophotometrically as 10 µg/0.05 tissues. The purity of DNA genome as indicated by A₂₆₀/A₂₈₀ ratio was 1.8.

The reproducibility of RAPD analysis is known to be highly influenced by experimental conditions. It is therefore essential to optimize the PCR condition to obtain reproducible and interpretable results before going to routine analysis. The PCR condition for RAPD analysis were optimized by investigating each factor individually. This include genomic DNA quality and concentration primer annealing and extension temperature as well as denaturation time and temperature. The optimized conditions were detailed in materials and method section. It was found that, quality of DNA genomic extracted was a good template PCR amplification. However, treatments of DNA with RNase gave sharp and clear amplification products compared with untreated DNA Castiglione *et al.* (1994) also reported similar observations.

After optimization of the reach conditions, polymorphism among the five *Meloidogyne incognita* isolates was detected using different random primers. RAPD analysis gave the best results amplification, and expressed as average number of bands and density per primer. Twelve random primers screened (operon random primer) were survey. For the reproducibility of RAPD patterns, two independent experiments were performed for each primer. Repetition of the experiments using different DNA samples conforming the stability and reproducibility of the results. Of the ten random primers that were screened in RAPD analysis for their ability to produce sufficient amplification products, five primers were selected in the first primer screening. In the second screening three primers namely OPA₁₃, OPD₈ and OPE₂₀ were more stable and reproducible and gave sufficient polymorphism among 5 *M. incognita* isolates. The PCR amplification patterns obtained by other primers did not distinguish variations between 5 *Meloidogyne* isolates. Therefore were focused our efforts on these primers. The distribution of the polymorphism bands were generated using three selected random primers, among different isolates of *M. incognita* are summarized in Table 3 and 4 and Fig. 3.

The results revealed the polymorphism among 5 *M. incognita* isolates where as TAF, MAF, PAF and unique were 52, 20, 27, 5 with (OPA₁₃); 46, 15, 29, 4 with OPD₈; 45, 15, 26, 4 bands with OPE₂₀ respectively. As with as the results revealed that by using the primer OPA₁₃, OPA₁₃, OPD₈ polymorphic with 52, 63 and 58%, respectively among 5 *Meloidogyne incognita* as average 57.34%. In addition the genetic marker for 5 isolates, one band for each isolate with molecular weight 1383, 740, 655, 703 and 191p for M₁, M₂, M₃, M₄ and M₅ isolate respectively. Two unique bands 1230 and 255 for M₂ and M₄ isolates respectively and four unique bands with 1383, 413, 255 bp for M₃ and 1053 bp for M₄ (Table 3 & 4).

Table 3: DNA fingerprint of five *Meloidogyne incognita* isolates using RAPD-PCR.

Primers	OPA ₁₃					OPD ₈					OPE ₂₀					Polymorphism
	M ₁	M ₂	M ₃	M ₄	M ₅	M ₁	M ₂	M ₃	M ₄	M ₅	M ₁	M ₂	M ₃	M ₄	M ₅	
1627	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	polymorphic
1383	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	polymorphic
1230	-	-	-	-	-	-	+	-	-	-	+++	+++	-	+	-	polymorphic
1125	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	polymorphic
1053	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	polymorphic
025	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	polymorphic
851	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	polymorphic
740	-	-	+	-	-	-	-	+	+	-	+++	+++	-	+	-	polymorphic
655	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	polymorphic
541	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-	polymorphic
460	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	monomorphic
413	+	+	+	+	-	-	-	-	+	+	-	-	+	-	-	polymorphic
355	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	monomorphic
307	-	-	-	+	-	-	+	+	-	-	-	-	-	+	+	polymorphic
274	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	monomorphic
255	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	polymorphic
235	+	+	-	-	+	+	+	+	-	-	-	-	-	-	+	polymorphic
207	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	polymorphic
191	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	polymorphic
178	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	polymorphic
156	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	polymorphic
102	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	polymorphic
Total DNA markers	14	13	10	8	7	7	10	10	10	9	10	9	8	11	7	

Table 4: Genetic markers and polymorphism of five *Meloidogyne incognita* isolates using RAPD-PCR.

Primers	Polymorphism				Genetic markers of isolates						
	TAF	MAF ₁	PAF	Unique	Molecular weight (bp)	M ₁	M ₂	M ₃	M ₄	M ₅	
OPA ₁₃	52	20	27(52%)	5	1383	+					
					740			+			
					655		+				
					703					+	
					191						
OPD ₈	46	15	29(63%)	2	1230	-	+				
					255					+	
OPE ₂₀	45	15	26(58%)	4	1383				+		
					1053					+	
					413					+	
					255					+	
Total genetic markers	143	50	82	11		1	2	4	3	1	
% of polymorphism		34.97	57.34	7.69							

ATF = Total amplified fragments.
 MAF = Monomorphic amplified fragments or common amplified fragments
 PAF = Polymorphic amplified fragments or specific amplified fragments.
 No. of polymorphic bands
 % of poly morphic = $\frac{\text{No. of polymorphic bands}}{\text{Total amplified bands}} \times 100$

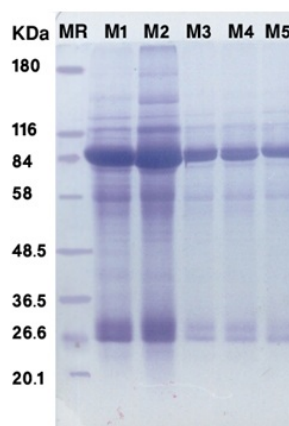


Fig. 1: SDS-PAGE 14% of protein fractions extracted from 5 *M. incognita* isolates (M₁, M₂, M₃, M₄, M₅). M = marker protein.

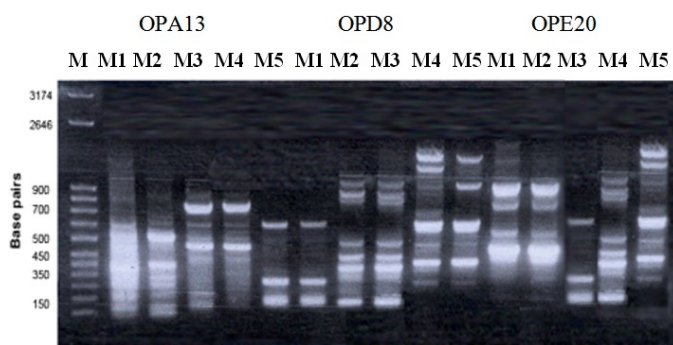


Fig. 2: Agarose gel electrophoresis (1%) stained with ethidium bromide showing RAPD-PCR products amplified of total DNA of *M. incognita* isolates:
 Lane M : DNA molecular weight marker.
 Lane M₁ to M₅. *M. incognita* isolates.

The results of the present study gave preliminary informative DNA based markers for 5 nematoda isolates. Also, optimization of physical experimental conditions of PCR amplification are a prerequisite for the performance of RAPD analysis. This increases the reproducibility and efficiency of RAPD as a molecular marker technique. However, three random primer gave reproducible and very stable results peculiar to the same specie from different accessions. The other primers sometimes did not give the exact fingerprints for 5 nematoda isolates. Accordingly, it may be suggested to use bulked DNA samples of different species to eliminate intraspecific variations.

It is concluded the distinct RAPD fingerprints among the different isolates and strains were obtained when suitable primers were used and PCR conditions were optimized. During the past years, numerous publications demonstrated the utility of RAPD markers for the analysis of the genetic diversity among isolates or strains within bacteria, fungi actinomycetes, nematoda and plants populations Orul (1999), Swalim, 2005; Nahid Aiat, 2006 and Girgis *et al.*, 2008). In the absence of DNA based markers, only the use of protein fingerprint and isozymes for distinguishing detection of somaclonal variations has been reported (Misra *et al.* 1993).

The molecular mechanism underlying somaclonal variations have been attributed to chromosome breakage, single base changes, changes in copy number of repeated sequences and alteration in DNA methylation patterns (Munthali *et al.*, 1996). The polymorphism in the amplification products may be either from changes in the sequence of the primer binding site (e.g. point mutations) or changes which after the size or prevent the successful amplification of the target DNA (e.g. insertion, deletions, inversions) as suggested by Roni *et al.* (1995).

It could be concluded that, protein fingerprint, isozymes and DNA fingerprint can be successfully used to detect somaclonal variations among 5 *M. incognita* isolates. Numerous researches proved that the sensitivity of protein and DNA fingerprint and isozyme analysis was sufficient enough to detect genetic change in many of bacteria, fungi, actinomycetes and nematoda (Perrent and Broughton, 1998; Sharma, 2003; Swelim, 2005; Nahid Aiat, 2007 and Seham Shash, 2009).

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