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Identification of root-knot nematode species occurring on tomatoes in Kenya: use of isozyme phenotypes and PCR-RFLP

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Abstract. Root-knot nematodes (RKN) are serious pests of tomato production in Kenya. Accurate identification of the plant parasitic nematodes is important for their effective management. A study conducted to assess their prevalence and identify RKN occurring on tomatoes in Central Kenya showed infestation of the crop in all the three districts. Of the total sampled plants ($N = 900$), the RKN infestation level ranged from 28 to 62%. RKN disease severity ranged from 2.5 to 5.3 in all the locations. *Meloidogyne incognita*, *M. javanica* and *M. arenaria* were the only species found infesting tomatoes in these areas. Female nematodes sampled from the symptomatic root system that had root galls characteristic of RKN were analysed by isozyme phenotypes of esterase (EST) and malate dehydrogenase (MDH). EST phenotype was polymorphic and enabled identification of the three different species, while MDH was monomorphic. Polymerase chain reaction-restriction fragment length polymorphism with region between mitochondrial cytochrome oxidase subunit II and large subunit ribosomal RNA (mtDNA COII-LSUrRNA) using *Hin*fI showed that all isolates of *M. incognita* could be digested into three restriction fragments of about 1300, 400 and 100 bp, except for one species that showed an additional restriction site, giving four fragments of 900, 420, 380 and 100 bp. The 1800 bp PCR product of *M. javanica* was not digested by *Hin*fI. *Meloidogyne arenaria* (EST phenotype A1) PCR product was digested into two restriction fragments of about 1700 and 100 bp, while *M. arenaria* (EST phenotype A2) had two restriction fragments at 1100 and 100 bp.

Key words: *Meloidogyne* spp, tomatoes, isozyme profiles, molecular identification

Introduction

Tomato (*Lycopersicon esculentum* Mill [Solanaceae]) is one of the key vegetables produced by smallholder farmers usually for the domestic market in sub-Saharan Africa. About 54 million metric tonnes are produced worldwide and more than 10% of this in Africa (Wang *et al.*, 2006). Tomato is an integral part of an everyday diet for millions of people worldwide and has an important dietary value in respect of carotene, thiamine, niacin and vitamin C. Lycopene, a carotenoid, is a powerful

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antioxidant found in tomatoes and used in the management of cardiovascular diseases, urinary tract diseases and diabetes (Atessahin *et al.*, 2006).

Tomato is one of the most important local market vegetables in Kenya. The crop is mainly grown by small-scale farmers in most arable areas of the country. The main production areas in Central Province are Kirinyaga, Nyeri, Kiambu, Mwea and Gikambura. In 2004, an estimated 75,101 tonnes of tomato valued at over Kshs 1 billion (approximately $12 million) were produced in Central Province, surpassing all the other vegetables in value (KARI, 2005). The major tomato production constraints reported in Kenya include diseases (bacterial wilt, early and late blight, leaf curl, tomato spotted wilt virus, leaf spot and powdery mildew), insect pests and other arthropods (spider mites, thrips, whiteflies, African bollworm) and nematodes, leading to high economic losses (Varela *et al.*, 2003; KARI, 2005).

Root-knot nematodes (RKN) are one of the major pathogens of tomatoes worldwide and limit fruit production (Sikora and Fernandez, 2005; Mehdi, 2009). There are more than 90 described species in the genus *Meloidogyne* (Tylenchida: Heteroderidae), but the four most commonly occurring species are *Meloidogyne incognita, M. arenaria, M. javanica* and *M. hapla* (Hunt *et al.*, 2005). They have a wide host range and are hence difficult to control as they can survive and reproduce on other host crops, including weeds. RKN infection is characterized by galled roots, yield losses, collapse of plants and stunted growth (Jacquet *et al.*, 2005).

Recommended control measures for RKN include use of nematicides, soil solarization and crop rotation. However, use of these methods by small-scale growers in Kenya is greatly hampered due to high cost of using nematicides and diminishing size of land holdings. Plant resistance is one of the most environmentally safe and economically viable means for controlling RKN. Several cultivars of tomatoes such as Montelle, Sun6082, Pik Red, Celebrity and Beefmaster have been developed in an attempt to produce RKN-resistant cultivars (Milligan *et al.*, 1998; Jacquet *et al.*, 2005). Resistance mechanism in response to invasion by RKN involves the formation of necrotic cells at the infection site to prevent the juveniles from developing any further. However, a high level of genetic variability of RKN has led to the development of races and virulent populations which can reproduce even on plants carrying the resistance genes (Castagnone-Sereno, 2006). Successful use of tomato cultivars resistant to RKN in Kenya depends on the accurate identification of RKN species found associated with tomatoes.

In Kenya, identification of RKN has primarily relied upon morphological characteristics. This technique suffers major limitations. Morphological characterization relies on variability of the perineal patterns in the natural populations of the *Meloidogyne* spp. that may be indistinct (Baum *et al.*, 1994) but which has proved to be useful and remains a tool in diagnostics. It also relies on the variability of the stylet length, which values do overlap, making species identification difficult. Isozyme phenotype analysis used in RKN identification is more accurate and less subjective (Carneiro *et al.*, 2000). To complement isozyme phenotype technique, DNA markers have been useful for the identification and phylogenetic comparison of plant-parasitic nematodes (Curran, 1991; Jones *et al.*, 1997; Floyd *et al.*, 2002). The objective of the present study was to assess the prevalence and identify RKN infesting tomatoes using isozyme phenotypes and restriction fragment length polymorphism (PCR-RFLP).

Materials and methods

Study sites and sampling

Surveys were conducted in three major tomato-growing districts in Kenya that are hot spots for nematodes: Mwea (1°24.0′S; 37°25.0′E), Gikambura (1°16.6′S; 36°37.6′E) and Athi River (1°26.60′S; 36°58.6′E). Ten fields per district were selected and 30 young tomato plants (total of 900) were uprooted at random and inspected for occurrence of RKN symptoms, which were characterized by galled roots. Root samples were kept in polyethylene bags, brought to the laboratory and thoroughly examined for the presence of galls. Debris on the roots was washed off with distilled water and then immersed in an aqueous solution of phloxin B (0.15 g/l) for 15 min to stain egg masses. The number of root galls per root system was counted and prevalence determined on the following scale: 0 = 0, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100 and 5 = greater than 100 galls. The number of egg masses per root system was counted and prevalence determined on the following scale: 0 = 0, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100 and 5 = greater than 100 egg masses (Taylor and Sasser, 1978). RKN infestation load in each locality was calculated using the formula:

$$\text{Nematode prevalence} = (\text{total number of infested plants sampled}/\text{total number of plants sampled}) \times 100.$$

Maintenance of inoculum

The inoculum of selected field populations from each locality was maintained on RKN-susceptible tomato var. Money Maker in a greenhouse at

25 ± 3°C, 90 ± 2% relative humidity with a photoperiod of 12 h light:12 h dark by inoculating each seedling in a pot containing autoclaved soils with chopped infested roots collected from the field. To make pure cultures of field populations maintained on tomato in the greenhouse, a single egg mass inoculation was made (Mehdi, 2009). Single mature egg masses were inoculated in pots around the roots of a young tomato seedling for each population and the collection maintained separately. To maintain sufficient inoculum for further studies, sub-culturing was done. Each new seedling was inoculated with at least 15 egg masses obtained from a pure culture.

Identification of RKN using isozyme profiles

Sample preparation

Six females per plant were isolated under a dissecting microscope. After the isolation, they were rinsed in reagent-grade water and then transferred to an ice-cold 1.5 ml Eppendorf tube containing 60 µl extraction buffer (20% sucrose, 2% Triton X-100 and 0.01% Bromophenol Blue) (Esbenshade and Triantaphyllou, 1985) and squashed to release body contents. Samples were centrifuged at 12,000 *g* at 4°C for 1 min.

Native PAGE electrophoresis and enzyme staining

Electrophoresis was done on native polyacrylamide gels (8–25%) at 4°C as described by Karssen (2002). *Meloidogyne javanica* (laboratory reared) obtained from the botanical farm at Kenyatta University in Nairobi, Kenya, was used as a reference standard. After electrophoresis, gels were stained for enzymatic activity in a Petri dish at 37°C. Malate dehydrogenase (MDH) activity was measured using a staining solution containing 0.05 g β-NAD, 0.03 g nitroblue tetrazolium, 0.02 g phenazine methosulphate, 50 ml of 0.5 M Tris pH 7.1 and 7.5 ml stock (10.6 g Na_2CO_3 + 1.34 g L-malic acid in 100 ml H_2O) dissolved in 70 ml reagent grade water. Incubation of MDH lasted for 5 min. Following this, the gel was washed twice with distilled water and further stained for esterase (EST) activity for 30 min. EST was measured using a staining solution containing 100 ml of 0.1 M phosphate buffer pH 7.3, 0.06 g fast blue RR salt, 0.03 g EDTA and 3 ml of 0.1 g α- and 0.1 g β-naphthyl acetate dissolved in 10 ml acetone (Karssen, 2002). The reaction was terminated at 30 min. This was achieved by rinsing the gel with distilled water and fixing for 5 min in a solution of 10% acetic acid, 10% glycerol and 80% distilled water. This experiment was conducted four times.

Identification of RKN using PCR-RFLP

DNA extraction and purification

Six female nematodes were handpicked from the infected root systems under a dissecting microscope and second-stage juveniles (J_2) were allowed to hatch in 2% sucrose at 25°C. Three J_2 were used for the subsequent analysis. The respective J_2 were frozen in a pre-cooled mortar and ground to fine powder in liquid nitrogen. A 50-µl lysis buffer (1–10 mg/ml proteinase K, 100 mM Tris–HCl pH 8.0, 5 mM EDTA, 200 mM NaCl and 1% w/v of SDS buffer) was added to the fine powder, mixed gently and incubated at 37°C overnight. The liberated DNA was extracted against an equal volume of phenol saturated with TE (100 mM Tris–Cl pH 8.0 and 1 mM EDTA pH 8.0). The aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube using a wide-bore pipette and mixed with an equal volume of chloroform isoamyl alcohol (24:1). DNA was then precipitated from the final aqueous phase by addition of an equal amount of ice-cold 95% ethanol with an incubation period of 5 h at −20°C. DNA was pelleted by centrifugation at 12,000 *g* for 10 min, washed with 500 µl of 70% ethanol, air-dried and re-suspended in 20 µl of 10 mM Tris and 1 mM EDTA at pH 7.5. DNA was quantified using spectrophotometer at 280/260 nm.

Mitochondrial DNA COII PCR amplification

Primers C2F3 (5′-GGTCAATGTTCAGAAATTT-GTGG-3′) (Powers and Harris, 1993) and MRH106 (5′-AATTTTCTAAAGACTTTTCTTAGT-3′) (Stanton *et al.*, 1997) were used to amplify the region between (MtDNA COII-LSUrRNA) genes. Amplifications were performed using 25-µl volumes containing 12 ng DNA, 200 µM dNTPs (MBI Fermentas®), 0.4 µM primers, 1 U *Taq* DNA polymerase (MBI Fermentas®) and 1 × *Taq* DNA polymerase reaction buffer. The temperature profile for all reactions was 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 2 min, with a final extension at 72°C for 10 min. Confirmation of the amplified DNA fragments was conducted on 1% agarose gel in 1 × TAE buffer at 70 V for 1.5 h.

Restriction digestion of mitochondrial DNA COII PCR products

The region between mtDNA COII-LSUrRNA PCR products was digested using *Hin*fI (Fermentas®). The restriction digestion with *Hin*f1 was carried out on 1 µl buffer, 1 µl (10 U) of *Hin*f1, 4 µl water and 4 µl of PCR product. Digestion was performed for 4 h at 37°C. Restricted products (5 µl) were separated on a 2% agarose gel in 1 × TAE at 70 V for 1.5 h.

Table 1. Prevalence and distribution of root-knot nematodes on tomatoes in three districts in Kenya

Location	Total no. of plants infected	Disease prevalence (%)	GI (average)	EMI (average)
Athi River	188	62.6	5.3	4.6
Mwea	143	47.6	3.9	4.4
Gikambura	86	28.6	2.5	2.9

GI, gall index; EMI, egg mass index on Taylor and Sassers' scale.

Results

Prevalence and severity of RKN in tomato fields

Tomato plants in all the three districts were found infested with RKN, but the severity of infestation varied. Highest prevalence per plant (62.6%) was found in Athi River, closely followed by Mwea (47.6%) and Gikambura (28.6%). The disease severity on tomato was estimated on the basis of average gall and egg mass indices. Both gall and egg mass indices (averages) ranged from 2.5 to 5.3. The greatest egg mass and gall indices were found in Athi River, the area in which the prevalence of RKN was also greatest (Table 1). *Meloidogyne incognita* was found in all the three districts and accounted for 59.1% of all the samples analysed, closely followed by *M. javanica* (35.7%). *Meloidogyne arenaria* (EST phenotypes A1 and A2) occurred only in Athi River and accounted for 5.2% of the total samples.

RKN identification by isozyme profiles

On the basis of isozyme profiles ($N = 900$), three species of RKN (*M. incognita, M. javanica* and *M. arenaria*) were identified in the three districts. Five EST phenotypes were detected in this study. *Meloidogyne javanica* produced three bands at EST loci 1, 2 and 3, while *M. incognita* occurred in two EST phenotypes; *M. incognita* (EST phenotype I1) with one strong band at EST locus one and *M. incognita* (EST phenotype I2) with a strong band at locus one and an allele at the same band. Two EST phenotypes of *M. arenaria* were detected in this study: *M. arenaria* (EST phenotype A1), which had two strong bands at second and third EST locus, and *M. arenaria* (EST phenotype A2), which had a strong band at the second locus (Fig. 1). One MDH phenotype designated N1 was observed for all the species (*M. javanica, M. incognita* and *M. arenaria*) identified in this study (Fig. 2).

RKN identification by mitochondrial DNA (mtDNA) COII PCR-RFLP

Using mtDNA COII-LSUrRNA primers (C2F3 and MRH106), an amplification product was obtained from purified DNA fragments of two different sizes from the same samples used for isozyme identification. *Meloidogyne incognita, M. javanica* and *M. arenaria* (EST phenotype A1) had a PCR product of approximately 1800 bp, while *M. arenaria* (EST phenotype A2) produced smaller products of approximately 1200 bp (Fig. 3). When the mtDNA COII-LSUrRNA PCR products were digested using restriction enzyme *Hin*fI, different restriction profiles on the 1800 bp fragment of *M. incognita, M. javanica* and *M. arenaria* (EST phenotype A1) were observed. *Meloidogyne incognita* produced three fragments of approximately 1300, 400 and 100 bp, except one specimen that had an additional restriction site in the 1300 bp fragment producing four fragments of approximately 900, 420, 380 and 100 bp (Fig. 4, lane 4). Two fragments were generated in *M. arenaria* (EST phenotype A1) of 1700 and 100 bp, while *M. arenaria* (EST phenotype A2) 1200 bp PCR products was cleaved into two fragments of 1100 and 100 bp. The 1800 bp PCR product of *M. javanica* had no restriction sites with *Hin*fI (Fig. 4).

Discussion

RKN were found infesting tomatoes in all the three districts with a high prevalence. The occurrence of *M. incognita, M. javanica* and *M. arenaria* from 900 samples collected clearly demonstrated the widespread distribution of these species. The high

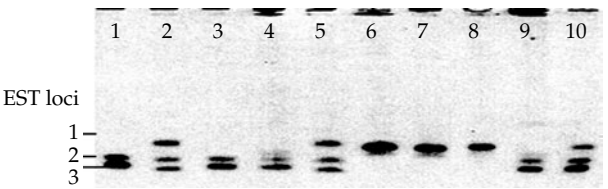

Fig. 1. Esterase (EST) phenotypes of representative specimens of *Meloidogyne incognita, M. javanica* and *M. arenaria* from the three locations. Lane 1: *M. arenaria* (EST phenotype A2); 2: *M. javanica* (J3); 3: *M. arenaria* (EST phenotype A2); 4: *M. arenaria* (EST phenotype A2); 5: *M. javanica* EST phenotype (J3); 6: *M. incognita* (EST phenotype I1); 7: *M. incognita* (EST phenotype I1); 8: *M. incognita* (EST phenotype I1); 9: *M. arenaria* (EST phenotype A2); 10: Standard (*M. javanica*).

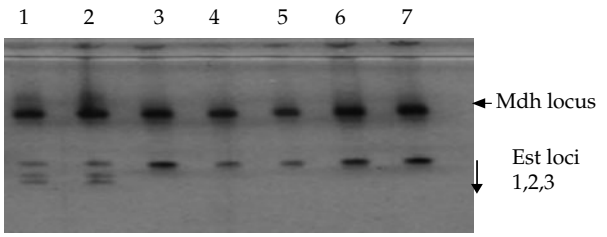

Fig. 2. Malate dehydrogenase (MDH) and esterase (EST) phenotypes of the representative populations of *Meloidogyne incognita* and *M. javanica* in tomato production systems (Athi River samples). Lane 1: *M. javanica* A13 (MDH-EST phenotype) (N1-J3); 2: *M. javanica* A14 (N1-J3); 3: *M. incognita* A5 (NI-I1); 4: *M. incognita* A6 (N1-I1); 5: *M. incognita* A10 (N1-I1); 6: *M. incognita* A16 (I1); 7: *M. incognita* A18 (N1-I1).

prevalence of these nematodes shows the significance of RKN as a threat to tomato production. These three species were associated with yield loss of tomato ranging from 24 to 38% in Brazil (Sikora and Fernandez, 2005). *Meloidogyne incognita* was the most common and widely spread species in all tomato production areas compared with *M. javanica* and *M. arenaria,* which showed that this species is significant and a damaging tomato pest. *Meloidogyne incognita* is reported to be widely distributed in the vegetable-growing regions of Pakistan (Anwar *et al.*, 2007; Shahid *et al.*, 2007). Soil type is a primary factor that may influence the damage potential of *Meloidogyne* spp (Siddiqui and Mahmood, 1998; Desaeger and Rao, 2000). In Athi River, sandy-loamy soils are mostly found while in Gikambura and Mwea, black cotton soils are predominant (Thomas, 1997). The sandy-loamy soils in Athi River correlate with high magnitude of damage by nematodes due to easier movement and reproduction rate of nematodes.

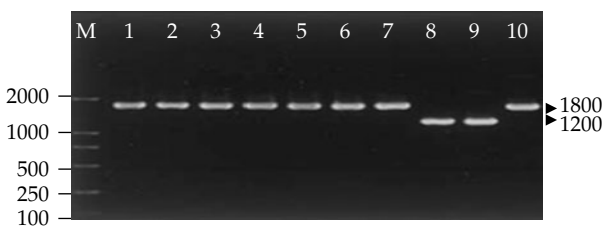

Fig. 3. PCR products amplified using mtDNA COII-LSUrRNA primers from representative specimens of *Meloidogyne incognita*, *M. javanica* and *M. arenaria.* Lane 1: *M. incognita* (EST phenotype I1); 2: *M. incognita* (EST phenotype I1); 3: *M. incognita* (EST phenotype 12); 4: *M. arenaria* (EST phenotype A2); 5: *M. arenaria* (EST phenotype A1); 6: *M. javanica* (EST phenotype J3); 7: *M. javanica* (EST phenotype J3); 8: *M. arenaria* (EST phenotype A2); 9: *M. arenaria* (EST phenotype A2); 10: *M. javanica* (EST phenotype J3); M: 2000 bp ladder.

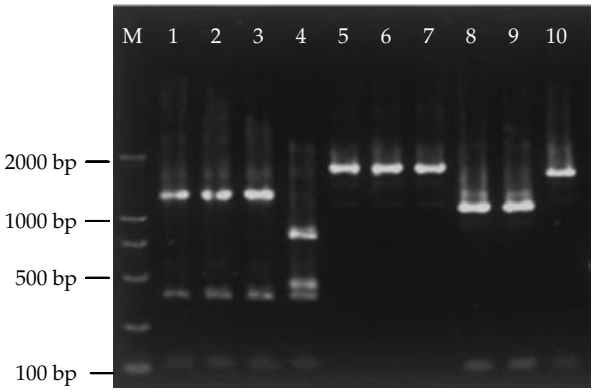

Fig. 4. *Hin*fI restriction patterns of the PCR products amplified using mtDNA COII primers from the representative specimens of *Meloidogyne incognita*, *M. arenaria* and *M. javanica.* Lane 1: *M. incognita* (EST phenotype Mwea); 2: *M. incognita* (EST phenotype I1); 3: *M. incognita* (EST phenotype I2); 4: *M. incognita* (EST phenotype I1); 5: undigested PCR product (*M. incognita* EST phenotype I2); 6*: M. javanica* (EST phenotype J3); 7: *M. javanica* (EST phenotype J3); 8: *M. arenaria* (EST phenotype A2); 9: *M. arenaria* (EST phenotype A2); 10*: M. arenaria* (EST phenotype A1); M: 2000 bp ladder.

Morphological characters have been the basis for nematode identification for many years in East Africa (Nono-Womdim *et al.*, 2002). Species identification based on differences in morphological characters requires a lot of skill and is often inconclusive at species level (Hartman and Sasser, 1985). Isozyme analysis used in the current study was a relatively fast and accurate method to identify species of the genus *Meloidogyne* as earlier reported by Esbenshade and Triantaphyllou (1990). Five EST phenotypes detected in this study were species-specific, which is in agreement with a previous study (Karssen, 2002). These were recognized on the basis of a single band or a combination of several bands. As a result of such associations, these phenotypes were designated a letter suggesting a particular nematode species, followed by a number indicating the number of major bands of enzymatic activity. The following phenotype nematode species associations were recognized: I1 and I2 for *M. incognita*, A1 and A2 for *M. arenaria* and the phenotype J3 for *M. javanica* (Ibrahim *et al.*, 1995). Differences in activities of the isozymes were indicated by the colour intensity of the bands of the different alleles from the same locus. The three species (*M. javanica*, *M. incognita* and *M. arenaria*) found in the three districts had one MDH phenotype designated as N1 that reconfirms their identity. MDH was found to be polymorphic with *M. hapla* species (Ibrahim *et al.*, 1995); however, this species was not found in the current study. A limitation of isozyme analysis is that it can

only be done on fresh, healthy, young females (Esbenshade and Triantaphyllou, 1985).

Genomic DNA-based techniques do not rely on the expressed products of the genome and are independent of environmental influence or developmental stage of an organism. In our analysis, all isolates of *M. incognita*, *M. javanica*, *M. arenaria* (EST phenotype A1) produced 1800 bp fragment, which is different from the isolates of *M. arenaria* (EST phenotype A2) that produced a 1200 bp fragment. Hence *M. arenaria* (EST phenotype A2) could be distinguished on the basis of the size of the PCR amplicon alone. The PCR-RFLP results of the current study agree with those of Powers and Harris (1993), which indicate different size classes of amplification products in reactions with five *Meloidogyne* species using mitochondrial DNA. The different restriction profiles of RKN isolates studied corroborate earlier studies (Castagnone-Sereno *et al.*, 1994; Blok *et al.*, 2002) except for the variant *M. incognita* (Fig. 4, lane 4), which showed an additional *Hin*fI restriction site on the amplification product. The size variation in this *M. incognita* is as a result of intraspecific variation in the cytochrome oxidase subunit II region for this species. The absence of the *Hin*fI restriction site for *M. javanica* in this study is a result similar to what was reported for the same species by Hugall *et al.* (1994).

The J_2 stage of plant-parasitic RKN has been difficult to identify because of their small size and lack of distinguishing morphological features. Identification of this stage is important, since they include the infective and overwintering stage of RKN species. Whereas the isozymes used were successful in identifying the enlarged female stages, they lack the sensitivity to detect the relatively smaller vermiform and this was resolved by the use of PCR-RFLP. This study confirms the identity of the prominent and prevalent species of RKN in three tomato-growing districts in Kenya.

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