USE OF ISOENZYME PHENOTYPES TO CHARACTERISE THE MAJOR ROOT-KNOT NEMATODES (*Meloidogyne* spp.) PARASITISING INDIGENOUS LEAFY VEGETABLES IN KISII

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Abstract

Root knot nematodes belonging to the genus *Meloidogyne* are the most economically important group of plant parasitic nematodes. Their worldwide distribution, extensive host range and interaction with other plant pathogens in disease complexes rank them among the top plant parasitic pathogens affecting the worlds food supply. Yearly crop losses of 5% have been reported worldwide. The damage inflicted in certain regions of developing countries exceeds this level. To improve the identification of *Meloidogyne* populations , a rapid technique should be developed which is easy to apply. The objective of this study was to determine the occurrence, distribution and identity of root knot nematodes (RKN) affecting Indigenous leafy vegetables (ILVs) in Kisii District. A total of 200 samples of (*Solanum nigrum*, *Amaranthus hybridus* and *Cleome gynandra*) affected by RKN were collected from two divisions, Sameta and Kilgoris.

Previous studies on the diversity of *Meloidogyne* spp. have focused on morphology (perineal patterns, stylet structure, body length), and the response of the populations to differential host test. Morphological differences may be difficult to observe making identification of nematodes by these procedures difficult. Enzyme phenotype, using esterases (Est) and malate dehydrogenase (Mdh), can be used to characterize *Meloidogyne* spp. populations from different ILVs using a simplified technique for routine analyses. Soluble proteins from macerates of individual females were separated by vertical electrophoresis on 0.7 mm-thick-polyacrylamide gels (12 %). After electrophoresis gels were stained for enzymatic activity in a Petri dish at 37º C with different staining solution. The species identity of forty RKN affecting ILVs in Kisii was reviewed using isoenzyme analysis. *Meloidogyne javanica* had the phenotype J3 while *M. arenaria* had phenotype A2. Species specific phenotype I1 was 50%, J3 was detected in 25% of the samples from Kisii while phenotype A2 was found in 25 %. Mdh and Estarase phenotypes are species specific and are used for identification of RKN.

Key words: Polyacrylamide gel electrophoresis (PAGE), *Meloidogyne* spp.

Introduction

Indigenous leafy vegetables (ILVs) comprise a large number of species that are unrelated and whose leaves are used as food by many local communities in Africa and therefore play a crucial role in food security (Maundu, 1997; Mathenge, 1997). Indigenous leafy vegetables are those plants whose leaves or aerial parts have been integrated in a community's culture for use as food over a large span of time (Ogoye and Aagaard, 2003).

Root-knot nematodes (RKN), *Meloidogyne* spp., are among the most destructive plant-parasitic nematodes in Kenya. They belong to the order Tylenchida. These small round worms (300 mm and 2mm for vermiform juveniles and pyriform females, respectively) live in soils and are obligate and sedentary endoparasites of plant roots. They harbour a hollow, protrusible stylet, which they use to inject secretions into and withdraw nutrients from the infected root cells. These pathogens have co-evolved sophisticated interactions with their host (Abad *et al*, 2003). Identification of these nematodes is necessary to effectively implement nonchemical management strategies. The most common and agronomically important species are identified using a microscope to examine perineal patterns of females on differential hosts. Identification by these procedures is difficult, even for well-qualified taxonomists. Biochemical studies have demonstrated that major species of *Meloidogyne* can be differentiated by species-specific enzyme phenotypes using the isoesterases, (Est), and the malate dehydrogenases, (Mdh) (Dalmasso and Bergé, 1978; Esbenshade and Triantaphyllou, 1985a, 1990). Protein analyses have been demonstrated to be reliable, and esterase isoezyme patterns are probably the most widely accepted markers used to identify four major RKN species (Esbenshade and Triantaphyllou, 1985). More recently, DNA-based techniques for species identification such as restriction fragment length polymorphisms have been developed (Cenis *et al*., 1992). Polymerase chain reaction patterns are also used to distinguish the four common species of *Meloidogyne* (Cenis, 1993; Powers and Harris, 1993).

Materials and Methods

Study sites and Sampling

Sampling was done in two sites in Kisii district (Sameta and Kilgoris). From each selected zone; 10 plants of each vegetable i.e. *S. nigrum*, *A. hybridus* and *C. gynandra* infected by RKNs were randomly selected and uprooted. The rhizosphere soil up to a depth of about 30cm was collected. To raise pure cultures for the isoenzyme analysis, single females with egg masses were isolated from the infected roots under a stereomicroscope (Spider plant, African spinach and Black nightshade) and inoculated on tomato seedlings *Lycopersicum esculentum*, variety money maker. The seedlings were maintained under greenhouse conditions at 20-28^{0}C for 55 days. Plants were then harvested and females used for isoenzyme characterization.

White females from each tomato seedling were isolated from root tissues under a stereomicroscope. These females were rinsed in reagent grade water and transferred to an ice bath containing 60 µl extraction buffer (20% sucrose, 2% Trinton X-100 and 0.01% Bromophenol blue (Esbenshade and Triantaphyllon, 1985b).The nematodes were macerated to release body contents. Polyacrylamide gel electrophoresis (PAGE) was performed using the Phastsystem (Amershan Pharmacia) on 12% straight gels and native buffer strips. The system was cooled to a stand by temperature of 5^0 C. Individual females were macerated and the resulting solution drawn into a well sample applicator. Samples were loaded on two Phast system devices. *Meloidogyne javanica* females were used as a standard in each gel for reference.

After electrophoresis gel was stained for enzymatic activity in a Petri dish at 37 ºC with different staining solutions. Malate dehydrogenase staining solution contained 0.05 g β-NAD, 0.03 g Nitro Blue Tetrazolium, 0.02 g Phenazine Methosulfate, 50.0 ml 0.5 M Tris pH 7.1 and 7.5 ml stock (10.6g Na2CO3 + 1.34 g L-malic acid in 100 ml water) dissolved in 70 ml of reagent-grade water. For EST activity we used staining solution contained 100 ml 0.1 M Phosphate buffer pH 7.3, 0.06g Fast Blue RR salt, 0.03 g EDTA and 0.04 g α-Naphthyl acetate dissolved in 2 ml acetone (Karsen *et al*., 1995). Incubation for MDH lasted 5 minutes, after that gel was washed twice with distilled water and stained for EST activity for 30 minutes. When isozyme phenotypes patterns were clearly visible the enzymatic reaction was stopped by rinsing gels with distilled water and fixed for 5 minutes in a solution of 10% acetic acid, 10% glycerol and 80% distilled water. Photographs of gels were taken with a digital camera for further processing.

Results

Forty specimens of *Meloidogyne* from the two selected areas in Kisii Kenya subjected to isozyme analysis yielded positive results. Malate dehydrogenase and Esterase activities were investigated for the 40 Kisii populations of *Meloidogyne*. Four esterase phenotypes were recognized on the basis of single bands or combinations of several bands. A distinct phenotype was associated with most specimens of each major species: *M .arenaria*, *M. javan*ica and *M. incognita*. Due to such associations a letter was designated suggesting the particular nematode species followed by a number indicating the number of major bands of enzymatic activity was used. These are the phenotype nematode species association that were recognized J3 for *M .javanica*,A1 and A2 for *M. arenaria* race 1 and *M. arenaria* race 2 respectively and I1 *M. incognita.*

One Mdh phenotypes (i.e. N1) and four Est phenotypes (i.e. I2, J3, A1 and A2) were detected (Plate 1-5). Based on the established relationship of Mdh–Est profiles and species of *Meloidogyne*, three species were identified from the populations, of which 20 were *M. incognita* (Mdh–Est phenotype N1–I2), 10 were *M. javanica* (N1–J3), 10 were *M. arenaria* (N1–A2).

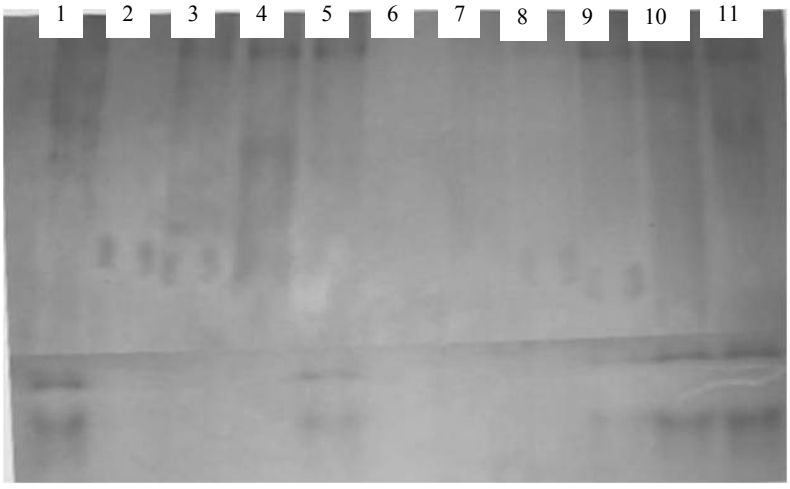

Plate 1: Malate dehydrogenase (Mdh) and Esterase (Est) Phenotypes of representative populations of M. javanica M. arenaria and M. incognita from Sameta division of Kisii.

Lanes 1 Mdh-Esterase phenotype (N1-J3); 3 and 4 (N1-I2), 5, 9 and 10(N1-A2) Lane 11: (N1-J3);
M. javanica *M. incognita* *M. arenaria*

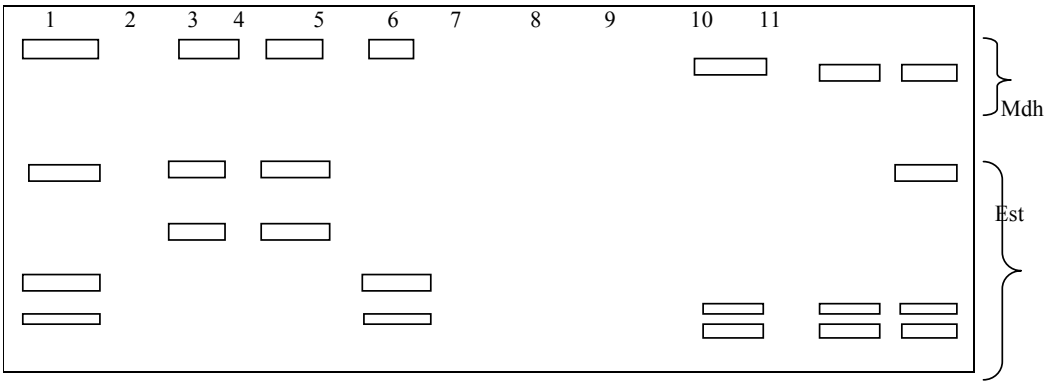

Fig. 1: A schematic representation of the gel image shown in Plate 1 Lanes 1Mdh-Esterase phenotype (N1-J3); 3 and 4 (N1-I2), 7,9 and 10: (N1-A2); 11: (N1-J3)

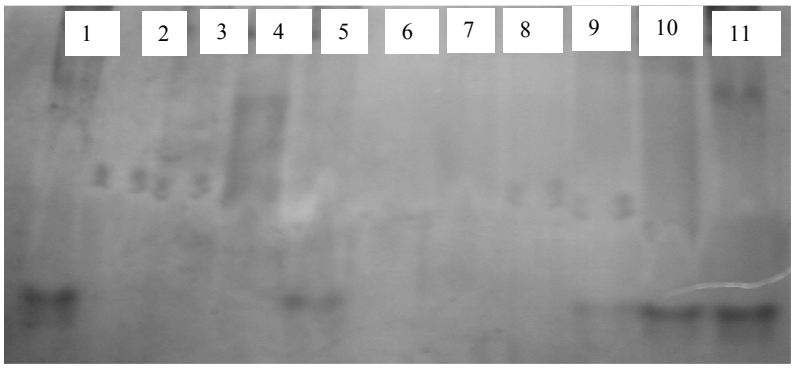

Plate 2. Malate dehydrogenase (Mdh) and Esterase (Est) Phenotypes of representative populations of M .javanica , M. arenaria and M. incognita.

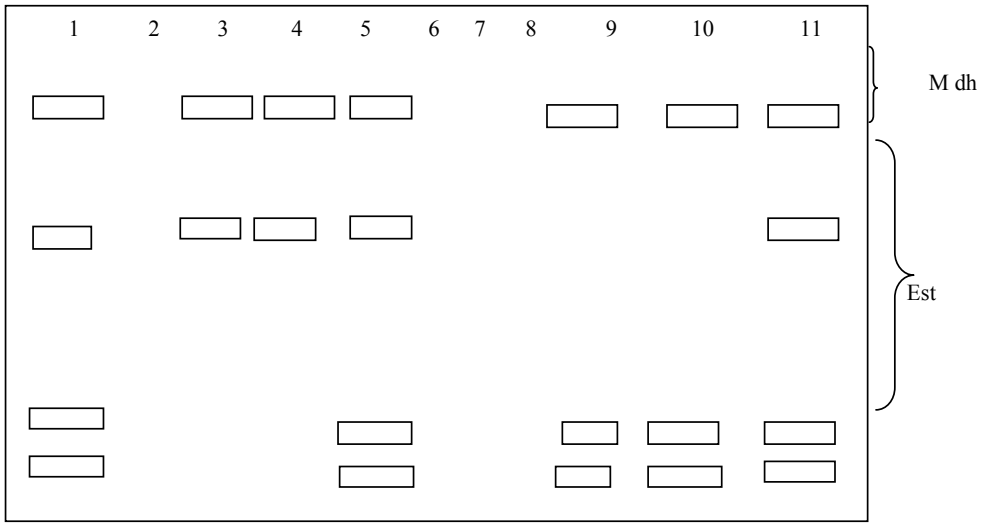

Fig. 2: A schematic representation of the gel image shown in Plate 2 Lanes 1, 5 and 11Mdh-Esterase phenotype (N1-J3); 3 and 4(N1-I1), 9 and 10: (N1-A2); 11: (N1-J3)

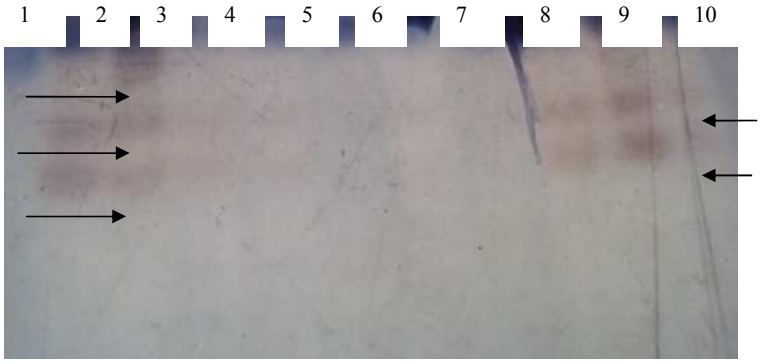

Plate 3. Malate dehydrogenase (Mdh) and Esterase (Est) phenotypes of representative populations of M. javanica , M. arenaria from Kisii.

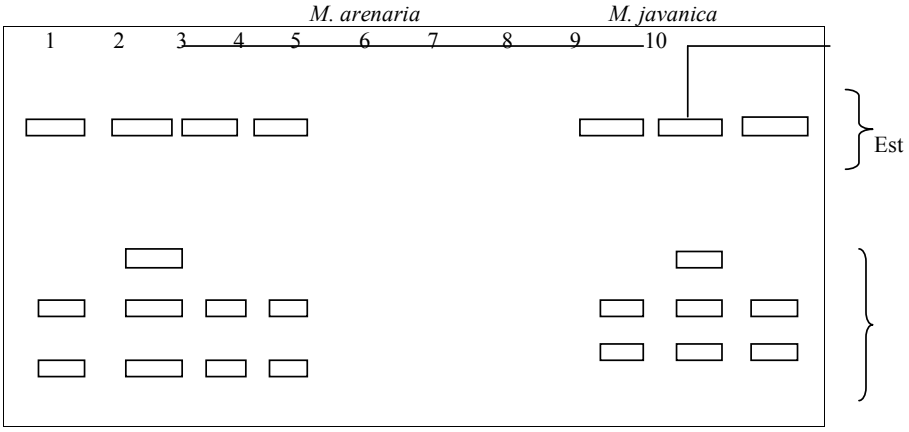

Fig.3: A Schematic representation of the gel image as shown in Plate 3 Lanes 2 and 9 M. javanica Lanes 3, 4, 8 and 10 M .arenaria

Plate 4:. Gel photo showing Malate dehydrogenase (Mdh)and Estarase (Est) phenotypes of the representative specimens of M. arenaria and M.javanica. Lanes 1, 9, and 10 is (N1-A2).Lanes 3 to 7 is N1-J3).

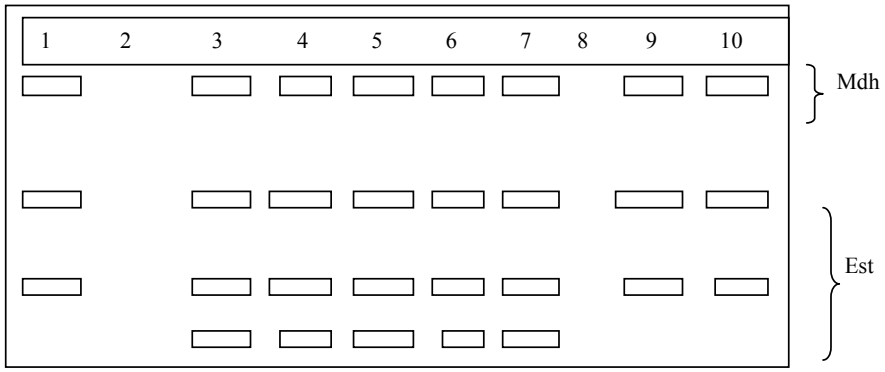

Fig. 4: A schematic representation of the gel image shown in Plate 4 showing Meloidogyne javanica and Meloidogyne arenaria

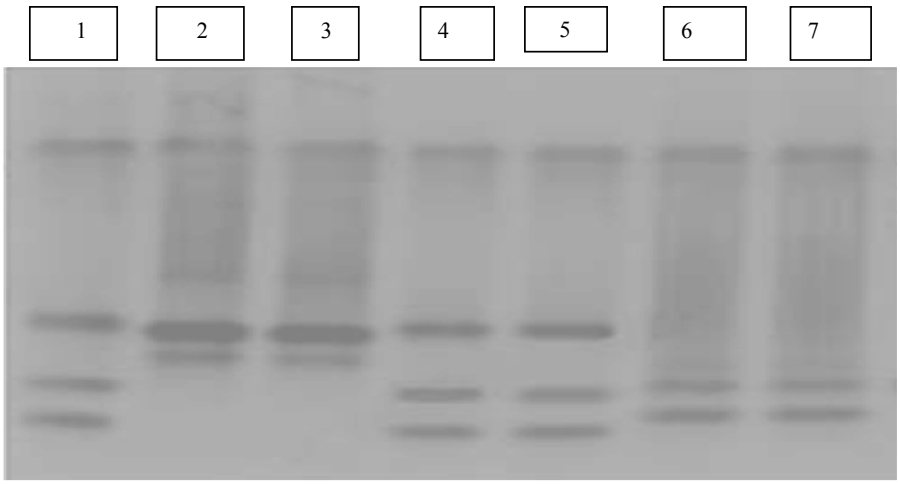

Plate 5 .Malate dehydrogenase (Mdh) and Esterase (Est) Phenotypes of representative populations of M. incognita, M. javanica and M. arenaria from Kisii. Lanes 1(Mdh-Est phenotype N1-J3); 2: (N1-I2); 3: (N1-I2); 4&5 (N1-J3); 6&7 (N1-A2)

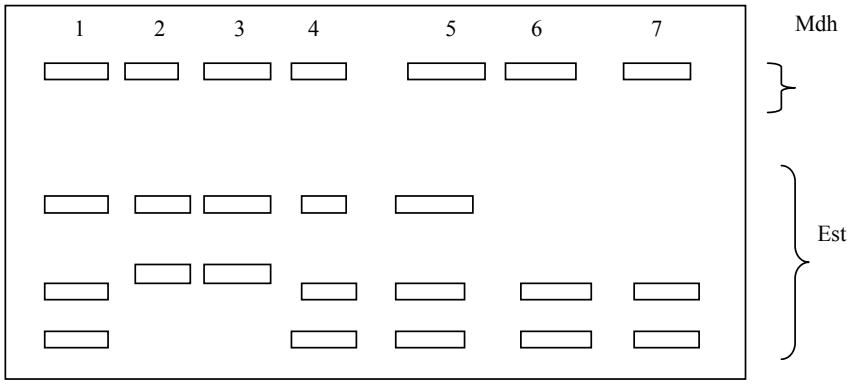

Fig. 5: A schematic representation of the gel image shown in Plate 5.Lanes as described in Plate 5.

Discussion

The present study included populations of *Meloidogyne spp*. originating from indigenous leafy vegetables in Kisii. The results provide species identification and an outline of the diversity of root-knot nematodes parasitizing indigenous leafy vegetables using enzyme phenotypes and molecular polymorphism. Isozyme phenotypes have been shown to be a valuable tool for precise identification of the major *Meloidogyne* species (Carneiro *et al*., 2000). Esterase phenotypes are among the isozymes that are highly polymorphic and are being used for the differentiation of the various species (Triantaphyllou, 1985; Fargette, 1987; Carneiro *et al.,* 2000).

With the electrophoretic procedure used herein, esterase phenotypes are species specific and are a good tool for identifying root-knot nematodes species from indigenous leafy vegetables .i.e., *M. incognita* (Est I1, I2), *M .arenari*a (Est A2) and *M. javanica* (Est J3). These phenotypes were previously reported by Carneiro *et al* (2000)

Although some intraspecific variability was observed in the populations of *M. incognita* using esterase enzymes phenotype analysis, it was observed that intraspecific variation in the enzymatic level was low. This observation is supported by the fact that enzymes are produced via the expression of genes which are often highly conserved between closely related taxa and which represent only a minor fraction of the total genome. Non-coding regions are more abundant and subjected to extensive evolutionary changes due to the absence of, or low selection pressure (Mclain *et al.,* 1987).Conversely, the perineal pattern can be used as a complementary tool to enzyme characterization and is important for checking the morphological consistency of the identification. Features of the male are essential for assisting in the diagnosis of some species.

Conclusion

The use isoenzyme profiles, Malate dehydrogenase and Esterase enzymes for the identification of root knot nematodes (RKNs) species has been demonstrated in this study. The limitation of these profiles is that they are only performed with single females and not the second-stage juveniles (J2). Since the female stage is unavailable in the soil samples the labour and space to establish and maintain in culture from single egg masses is discouraging. Second-stage juveniles are more readily available from the soil samples and can also be obtained by hatching the eggs or releasing juveniles from the eggs by physical pressure.

Recommendation

Further studies should be undertaken on other varieties of indigenous leafy vegetables in Kenya in order to understand the diversity of the species in these regions.

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