African Journal of Biotechnology Vol. 11(100), pp. 16546-16556, 13 December, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2502
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Prevalence, incidence and molecular identification of root-knot nematodes of tomato in Pakistan

Ishrat Naz[1]*, Juan E. Palomares-Rius[2], Vivian Blok[2], Saifullah[1], Sardar Ali[3] and Musharraf Ahmed[1]

[1]Khyber Pakhtunkhwa Agricultural University, Peshawar, Pakistan.
[2]Cell and Molecular Sciences, James Hutton Institute, Invergowrie, Dundee, DD2 5DA, United Kingdom.
[3]Hazara University, Khyber Pakhtunkhwa, Haripur Campus, Haripur, Pakistan.

Accepted 16 October, 2012

Tomato is a widely grown vegetable in Pakistan. However, its production is severely constrained by root knot nematodes (RKNs). Accurate identification of RKNs is essential for an appropriate control program. The current study evaluated the prevalence, incidence and diversity of RKNs of tomato crops grown in the Khyber Pakhtunkhwa Province and their identification using molecular tools. A field survey, including 30 commercial tomato fields, was conducted in ten major tomato growing areas of Swat and Malakand divisions during spring 2010. The overall prevalence and incidence in the study area was 83.3 and 52.0%, respectively. Three species of RKNs, *Meloidogyne arenaria, M. incognita* and *M. javanica* were found alone or in mixed populations. Disease incidence ranged from 10% in Malakandher to 100% and 90 to 100% in Jabban and Malakand, respectively. The greatest galling index (GI) (5.0) and egg mass index (EMI) (5.0) was recorded in samples from Jabban, whereas the lowest GI and EMI were recorded in samples from Malakandher and Peshawar. The population density of RKNs was highest in roots (633.0 eggs and second-stage juveniles) and soil (533.0 eggs and second-stage juveniles) samples of Jabban. DNA amplification with rDNA (D2A-D3B) and (194 to 195) primers amplified 750 and 720 bp products for *M. arenaria, M. incognita* and *M. javanica*, respectively. Amplification with sequence characterized amplified regions (SCAR) primers produced characteristic products of 420 bp for *M. arenaria* (Far/Rar), 1200 bp for *M. incognita* (Finc/Rinc), and 670 bp for *M. javanica* (Fjav/Rjav). DNA amplification of mtDNA with C2F3/1108 primers yielded a 1700 bp size product for all three species of RKNs in comparison with 520 and 750 bp for *M. chitwoodi* and *enterolobii*, respectively, which were utilized as control. Sequencing the 28S rDNA product generated with the D2A-D3B primers did not differentiate among the three *Meloidogyne* spp. from the study area.

Key words: *Meloidogyne*, species identification, perineal pattern, sequence characterized amplified regions (SCAR) primers.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family. It is the most popular and widely consumed vegetable crop grown in outdoor fields, green houses and net houses of the world. Worldwide, tomato is the second most consumed vegetable after potato (FAOSTAT, 2009). It is a rich source of potassium, iron and vitamins A, B, C (Baloch, 1994). Globally, tomato yields are reduced by plant-parasitic nematodes. Root-knot nematodes (RKNs) (*Meloidogyne*) (Goldi, 1892) are among the main obligate pathogens of tomato plants all over the world (Jacquet, 2005; Perry and Moens, 2009). The most economically significant species of RKNs in the tropical and temperate regions are *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. incognita* (Kofoid and White, 1919) Chitwood, 1949, *M. javanica* (Treub, 1885) Chitwood, 1949, whereas *M. chitwoodi* Golden, O'Bannon, Santo and Finley, 1980, *M. fallax* Karssen,

*Corresponding author. E-mail: ishratro@hotmail.com. Tel: +923329322581.

1996, and *M. hapla* Chitwood, 1949, are more important in cooler regions. Although tomato is often (but inappropriately) considered a universal host for *Meloidogyne* spp. (Perry and Moens, 2009), tomato yield losses of 22 to 30% have been reported by *M. incognita* (Sasser and Carter, 1982). Losses up to 80% in heavily infested fields have also been recorded (Kaskalvalci, 2007).

In Pakistan, the yield/unit area of tomato is considerably lower (10 to 12 t/ha) than in many other tomato growing countries and much less than the world average (36 t/ha) (Anonymous, 2009). Conditions that promote disease from RKN are severe in Pakistan, which has tropical and subtropical regions with warm sandy soils, which are quite suitable for nematode development and reproduction (Khattak, 2008). Specifically in the Khyber Pakhtunkhwa (KPK) province, the disease is more severe due to favourable environmental conditions, sandy loam soils and the short life cycle (six to eight weeks), in which the RKN populations can gradually build-up in the presence of suitable hosts (Gul, 1988). Indeed, many farmers have given up growing tomatoes in these areas (Khattak, 2008). Extensive surveys of nematodes associated with cereals, vegetables, fruits, and other crops in the Sindh, Punjab, Balochistan and KPK provinces of Pakistan have been carried out and have reported the presence of four species of RKNs, *M. arenaria* (race 1 and 2)*, M. incognita* (races 1 and 2)*, M. javanica,* and *M. hapla* (Gul and Saeed, 1990). *M. graminicola* was also reported from rice fields in Sheikhpura by Munir and Bridge (2003).

Root-knot nematode control is far more complex than many other kinds of pathogens because these pathogens mostly attack underground parts of plants resulting in poor growth and less yield (Williamson and Hussey, 1996). Additionally, the majority of these pathogens are polyphagous and infect over 5,500 plant species, including most of the economically important crops of the world and many non-crop species (Trudgill and Blok, 2001; Perry and Moens, 2009). Successful control of RKNs depends on accurate and rapid nematode identification. Traditional techniques for species identification of RKNs have relied on morphological characters (Eisenback, 1985); however, morphological identification is difficult, requires extensive experience and usually, several species of RKNs are commonly found together. For this reason, molecular identification is an important complement to morphological identification in order to identify individuals accurately to the species level.

A number of molecular techniques have been developed for the identification of RKNs species (Blok and Powers, 2009). Among these, polymerase chain reaction (PCR)-based diagnostics have provided fast, accurate and sensitive tools for RKN identification (Niu et al., 2011). A molecular diagnostic key was developed by Adam et al. (2007) for identification of seven economically important root knot nematodes that are routinely encountered in many nematological research and diagnostic laboratories. The key uses several molecular diagnostic techniques for the identification of these seven common species. Additionally, with the increase of DNA sequencing, several nuclear DNA regions such as the partial 18S, ITS regions, and the D2 and D3 expansion segments of the 28S rDNA and mitochondrial DNA (mtDNA) have proved to be useful diagnostic targets for the identification of RKNs (Landa et al., 2008).

To our knowledge, RKNs in Pakistan have only been identified on the basis of morphology and there is no information available in literature about the molecular identification of these RKNs populations. For this reason, we conducted a survey of the major tomato growing areas in the Swat and Malakand divisions from the KPK province, with the following main objectives: (a) to determine the prevalence, incidence and species diversity of RKNs using perineal pattern and molecular tools; (b) to determine the suitability of molecular tools for identification of RKN populations from Pakistan; (c) to determine the molecular diversity between RKNs populations of the same species using sequence analysis of the D2-D3 expansion segments of 28S rDNA.

MATERIALS AND METHODS

Soil and root sampling

Tomato growing areas of Dargai and Swat of Malakand divisions in Khyber Pukhtunkhwa (KPK) Pakistan were surveyed during spring, 2010. Fields with tomato crop history were selected randomly in the localities (Santhosh et al., 2005). A total of 30 fields belonging to ten localities of Malakand and Peshawar divisions (KPK province) were surveyed, and ten plants per field (1/2 acre) were collected in a zigzag pattern. 100 g of feeder roots and 1 kg soil from the rhizosphere of each of the ten plants per field were collected from a depth of 20 cm. Samples were stored at 4°C until nematode extraction.

Nematode infestation of root samples

The roots were carefully washed in tap water, blotted dry and their gall ratings determined and recorded (Taylor and Sasser, 1978). Roots were visually assessed and scored for the galling index (GI) using a 0 to 5 galling scale, where 0 = no gall on roots, 1 = 1 to 2, 2 = 3 to 10, 3 = 11 to 30, 4 = 31 to 100, 5 = more than 100 galls per root. 20 g of the roots were stained in Phloxine B for 15 to 20 min according to Holbrook et al. (1983). The stained roots were rinsed in water and egg masses (EM) visually counted and scored using a 0 to 5 egg mass rating index; where 0 = no egg masses; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100 and 5 = > 100 EM/root system (Taylor and Sasser, 1978).

Extraction of nematodes from soil

The soil was thoroughly mixed and a 100 cm^3 sub-sample was used for nematode extraction, employing a modified sieving/Baermann funnel technique (Thistlethwayte, 1970). The

Table 1. Primers used for molecular identification of *Meloidogyne* species.

Primers code	Primer sequence 5′−3′	Specificity and source
D2A	ACAAGTACCGTGAGGGAAAGTTG	28S rDNA región, De Ley et al. (1999)
D3B	TCGGAAGGAACCAGCTACTA	
194	TTAACTTGCCAGATCGGACG	5S-18S rDNA region, Blok et al. (1997)
195	TCTAATGAGCCGTACGC	
Far	TCGGCGATAGAGGTAAATGAC	*M. arenaria*-specific SCAR, Zijlstra et al. (2000)
Rar	TCGGCGATAGACACTACAAACT	
Fjav	GGTGCGCGATTGAACTGAGC	*M. javanica*-specific SCAR, Zijlstra et al. (2000)
Rjav	CAGGCCCTTCAGTGGAACTATAC	
Finc	CTCTGCCCAATGAGCTGTCC	*M. incognita* specific SCAR, Zijlstra et al. (2000)
Rinc	CTCTGCCCTCACATTAGG	
C2F3	GGTCAATGTTCAGAAATTTGTGG	COII/lrRNA region of mtDNA, Hugall et al. (1994)
1108	TACCTTTGACCAATCACGCT	

recovered nematodes were enumerated using a stereo-binocular microscope (Olympus SZ 61) at 3.5× magnification and the density expressed as the number of nematodes in 100 cm^3 of soil.

Extraction of nematodes from roots

Galled roots of tomato plants were washed thoroughly in running tap water and cut into small segments (1 to 2 cm long) and agitated for 1 min in 1% NaOCl. The suspension was passed through 75 and 5 µm sieves, eggs and second-stage juveniles (J2) collected on the 5 µm sieve were washed several times with water, re-suspended, and their concentration determined by dilution counts (Hussey and Barker, 1973) using a stereo-binocular microscope (Olympus SZ 61) at 3.5× magnification. The frequency of occurrence (prevalence = number of fields with RKNs/total number of fields surveyed) and incidence of the disease (number of plants galled/total number of plants sampled) in each field were calculated. Identification of *Meloidogyne* species collected from Malakand and Peshawar divisions was carried out using the perineal pattern method as described by Eisenback et al. (1981).

Molecular identification of *Meloidogyne* spp.

DNA was extracted from individual J2 and single females using worm lysis buffer (WLB) [50 mM KCl, 10 mM Tris pH 8.0, 15 mM $MgCl_2$, 60 µg ml^{-1} proteinase K (Roche, UK), 0.45% Tween 20 (Sigma, UK)] (Castagnone-Sereno et al., 1995). An individual J2 was placed in 10 µl worm lysis buffer on a glass microscopic slide and cut into two pieces with a scalpel under a stereomicroscope (Nikon, UK). Using a micropipette, the cut nematode was then transferred to a 0.5 ml centrifuge tube, the slide was washed with another 10 µl of WLB and this was combined with the first 10 µl. The same procedure was applied to the females. Females were picked up with tweezers, rinsed in distilled water and squashed with a mini plastic pestle in 20 µl lysis buffer in a 1.5 ml tube. Squashed females were then transferred to a 0·5 ml PCR tube using a micropipette. The tubes were centrifuged at 13,500 rpm for 2 min (25°C), then placed at -80°C for 10 min, incubated at 65°C for 1 h, followed by 95°C for 10 min. The samples were then frozen at -20°C or used immediately for PCR. For identification of *Meloidogyne* specimens, one or a combination of the following primers was used. PCR DNA quality reaction was performed using D2A-D3B primers (De Ley et al., 1999) amplifying the D2 and D3 expansion region of the 28S rDNA nuclear gene. More species specific regions were used for species identification: 194/195 primers amplifying the intergenic spacer region between the 5S to 18S rDNA (Blok et al., 1997); species-specific primers (sequence characterized amplified region-SCAR primers) for diagnosis of *M. arenaria, M. incognita,* and *M. javanica* (Zijlstra et al., 2000) (Table 1); and C2F3/1108 primers amplifying the COII/lrRNA region of the mtDNA of RKNs (Hugall et al., 1994) (Table 1). All PCRs were performed as described for their specific sets of primers, except for the C2F3/1108 primers, in which an extension temperature of 60°C was used. All reactions were performed using PuReTaq Ready-to-Go™ PCR beads (GE Healthcare, UK) or *Taq* polymerase (Promega, UK) in a Gene-Amp Applied Biosystem 9700 thermocycler (Applied Biosystems, UK).

The reaction products were resolved by electrophoresis on 1.0% agarose gels in 1× Tris borate EDTA (TBE), stained with Sybr® safe DNA staining dye (Invitrogen, USA) and visualized with UV light (Uvitech-Cambridge, UK). D2 and D3 expansion regions of the 28S rDNA PCR products of individual nematodes from selected nematode populations (W2, M3, J3, J4, F2, T1, H1 and R2), belonging to the main tomato growing areas (Heroshah, Swat, Skhakot, Peshawar, Malakand, Jabban and Thana), were sequenced at the James Hutton Institute (JHI) sequencing facilities using an ABI PRISM-3100 genetic analyzer (Applied Biosystems, UK). The positive control populations used were *M. arenaria, M. chitwoodi, M. enterolobii* (syn. *M. mayaguensis*), *M. fallax* and *M. incognita* from the JHI collection, and *M. javanica* was provided by Dr. Castillo (Insituto de Agricultura Sostenible, Cordoba, Spain).

RESULTS

Prevalence, incidence and population density of nematodes

The 30 tomato fields belonging to ten localities of Swat

and Malakand division were surveyed during spring 2010, and 300 roots (ten samples per field) and 150 (five cores per field) soil samples were collected, out of which 241 (80.3%) root and 131 (87.3%) soil samples were infested with RKNs. The overall prevalence of RKNs in Swat and Malakand division was 83.3%. *M. incognita* and *javanica* were found co-infesting 10 (33.3%) of the fields. Three (10.0%) of the fields were infested by *M. javanica* and *arenaria* together; whereas three (10.0%) fields were concomitantly infested by *M. javanica*, *incognita* and *arenaria*. *M. incognita* and *javanica* were recovered from 4 (13.3%) and 6 (20.0%) fields, respectively, whereas *M. arenaria* was recovered alone from one (3.3%) sampling site.

There was significant variability in the prevalence and incidence of *Meloidogyne* spp. (Table 2) between localities. Disease prevalence was 100% in all localities except of Thana and Swat localities, where the prevalence was 66.6%. Field infestation ranged from 0 to 100, with an average of 52.0 % in the studied areas. Maximum incidence (100%) was recorded in all the fields of Jabban followed by Malakand (90 to 100%). Disease incidence ranged from 0.0 to 80% in all the fields of Swat, 60 to 80% in Dargai and 40 to 80% in Wartair followed by 40 to 70% in Skhakot. The lowest disease incidence was in Malakander (10.0%) followed by Peshawar (0.0 to 20.0%) (Table 2). The greatest galling index (GI) and egg mass index (EMI) were obtained from Jabban, in which the incidence was also the greatest (Table 1), whereas minimal GI and EMI were recorded in samples collected from fields of Malakandher and Peshawar. RKN populations per 100 cm^3 of soil and 20 g of roots varied among the sampling sites (Table 3).

Tomato cultivars, Riogrande and Raja were mostly grown in these areas. However, there was no difference in the response of these two cultivars in terms of their susceptibility to RKN species (Table 2). RKN populations in the soil ranged from 0 to 670, with an average of 259.5 RKNs in 100 cm^3 of soil and 0 to 633, with a mean of 196.2 RKN J2s + eggs in 20 g of roots. The root and rhizosphere soil analysis also revealed the presence of six other endo- and ecto- parasitic nematode genera namely *Criconema*, *Helicotylenchus*, *Hoplolaimus*, *Longidorus, Pratylenchus* and *Xiphinema.* However, root and soil populations of these nematodes were very low and not reaching threshold plant damage levels (Table 3). *Praylenchus* spp. was the only endoparasitic nematode found in the roots, ranging from 0 to 6 individuals in 20 g of roots and from 0 to 11 individuals in 100 cm^3 soil (Table 3).

Perineal pattern identification

Microscopic examination of the perineal pattern morphology of adult females from the different fields revealed the presence of three different species, *M. incognita*, *javanica* and *arenaria.*

Molecular identification

Ribosomal DNA amplification (D2A/D3B and 194/195 primers)

DNA obtained from individual J2s/females belonging to tomato fields collected from ten different localities of KPK province of Pakistan was used. The D2-D3 extension fragment of 28S rDNA was used in order to check the DNA quality and prevent false negatives in the species-specific PCRs. All nematodes tested showed the correct band size (750 bp), except for L1 and L2 samples. The sizes of the amplified PCR products with 194/195 primers were all approximately 720 bp, with a single amplicon, reflecting no variation in size among *M. javanica*, *incognita* and *arenaria* species. Nematodes L1 and L2 did not yield any amplification product with 194/195 primer set (Table 4).

PCR amplification of sequence characterized amplified region (SCAR) primers

The SCAR primer pairs (Table 1) were used for diagnosis of *M. incognita*, *javanica* and *arenaria*. PCR with specific SCAR primers Fjav/Rjav produced a 670 bp product for nematodes M1, M2, M3, M4, J1, J3, F2, T1, T2, T3, W1, W2, W3, R1, R3, Q1, Q2, Q3, H2 and E1(*M. javanica*) (Table 4 and Figure 1A). Out of 31 nematodes, only six nematodes (L3, J2, J4, F1, F3 and F4) were identified as *M. incognita* and produced a 1200 bp product with the species-using the Finc/Rinc primer pair (Figure 1A) while three nematodes (R2, H1 and E2) produced a SCAR product of 420 bp with specific primer pair Far/Rar for *M. arenaria* (Table 4 and Figure 1A). The results for two nematodes from Dargai (L1 and L2) gave equivocal results with the SCAR primers. The SCAR primers identified *M. javanica* with the highest frequency (64.5%) followed by *M. incognita* (19.3%) and *M. arenaria* (9.7%). *M. javanica* was present in all localities tested except for field D. *M. incognita* was present in localities Dargai and Swat and finally, *M. arenaria* was present in localities Jabban, Thana and Wartair. Two fields presented mixtures of *M. javanica* and *arenaria.* Positive control samples for *M. incognita*, *arenaria* and *javanica* amplified the expected products for each species.

PCR amplification of the COII/LrRNA of mtDNA region

Figure 1B illustrates the sizes of amplified products of the 31 different nematodes belonging to 11 populations. All produced one major product of approximately 1700 bp corresponding to *M. incognita*, *javanica* and *arenaria*. *M.*

Table 2. Incidence, galling index (GI), egg mass index (EMI), densities and perineal pattern identifications of RKNs in soil and roots collected from tomato growing areas of Khyber Pakhtunkhwa Province (KPK) in Pakistan.

Locality	Cultivar	Field	Incidence*	GI	EMI	RKN nematodes in 100 cm^3 soil	RKN nematodes in 20 g root	*Meloidogyne spp*
Dargai	Riogrande	D1	80.0 (10)	5.0	4.0	435	327	*M. incognita*
	Riogrande	D2	60.0 (10)	5.0	4.5	335	289	*M. javanica* + *M. incognita*
	Riogrande	D3	70.0 (10)	4.0	3.5	314	312	*M. javanica*
Heroshah	Riogrande	H1	60.0 (10)	3.5	3.0	321	189	*M. javanica* + *M. incognita*
	Riogrande	H2	50.0 (10)	3.5	4.0	269	201	*M. javanica* + *M. incognita*
	Riogrande	H3	40.0 (10)	3.0	3.0	118	179	*M. incognita*
Swat	Riogrande	S1	70.0 (10)	4.0	4.0	209	69	*M. incognita*
	Riogrande	S2	0.0 (10)	0.0	0.0	0.0	0.0	NIL
	Riogrande	S3	80.0 (10)	4.0	3.0	295	114	*M. arenaria* + *M. javanica*
Skhakot	Raja	SK1	70.0 (10)	4.0	2.0	201	256	*M. javanica* + *M. incognita*
	Riogrande	SK2	60.0 (10)	3.5	2.0	199	121	*M. javanica* + *M. incognita*
	Riogrande	SK3	40.0 (10)	3.0	3.0	285	165	*M. javanica*
Peshawar	Raja	P1	0.0 (10)	0.0	0.0	0.0	0.0	NIL
	Riogrande	P2	20.0 (10)	2.0	1.0	58	38	*M. javanica* + *M. incognita*
	Riogrande	P3	10.0 (10)	3.0	2.0	32	31	*M. javanica*
Malakand	Riogrande	M1	100.0 (10)	4.5	5.0	481	512	*M. incognita* + *M. javanica*
	Riogrande	M2	100.0 (10)	5.0	4.0	670	431	*M. incognita* + *M. javanica*
	Raja	M3	90.0 (10)	5.0	3.5	458	267	*M. incognita* + *M. javanica*
Jabban	Riogrande	J1	100.0 (10)	5.0	5.0	533	633	*M. incognita* + *M. javanica* + *M. arenaria*
	Riogrande	J2	100.0 (10)	5.0	5.0	670	319	*M. incognita* + *M. javanica*[b] + *M. arenaria*
	Riogrande	J3	100.0 (10)	5.0	5.0	486	230	*M. incognita* + *M. javanica* + *M. arenaria*
Malakander	Raja	MK1	10.0 (10)	2.0	3.0	56	19	*M. javanica*
	Riogrande	MK2	10. 0 (10)	2.0	2.0	45.0	21.0	*M. javanica*
	Raja	MK3	10.0 (10)	2.0	2.0	83.0	78.0	*M. incognita*
Thana	Riogrande	T1	30.0 (10)	3.0	4.0	185	211	*M. arenaria*
	Riogrande	T2	0.0 (10)	0.0	0.0	0.0	0.0	NIL
	Riogrande	T3	30.0 (10)	2.5	3.0	223	165	*M. javanica*
Wartair	Riogrande	W1	50.0 (10)	4.0	4.0	290	315	*M. javanica* + *M. arenaria*
	Riogrande	W2	80.0 (10)	4.5	3.5	331	215	*M. javanica* + *M. arenaria*
	Riogrande	W3	40.0 (10)	3.5	4.0	203	180	*M. incognita* + *M. javanica*

*Number of plants in brackets.

Table 3. Population of plant-parasitic nematodes other than root-knot nematodes identified at genus level in root and soil samples collected from 30 fields from tomato growing areas of Khyber Pakhtunkhwa Province in Pakistan.

Locality	Field	Nematodes in 20 root	Nematodes in 100 cm^3 soil					
		Pratylenchus	*Xiphinema*	*Pratylenchus*	*Hoplolaimus*	*Longidorus*	*Helicotylenchus*	*Criconema*
Dargai	D1	2	3	5	2	1	2	2
	D2	3	2	3	3	1	1	1
	D3	4	1	5	0	2	0	0
Heroshah	H1	2	1	2	1	0	2	1
	H2	1	2	4	1	0	1	0
	H3	3	4	3	0	1	1	1
Swat	S1	2	3	2	2	2	1	2
	S2	4	2	3	0	2	0	3
	S3	3	3	3	2	3	0	0
Skhakot	SK1	2	3	4	4	1	1	1
	SK2	4	4	8	1	2	0	0
	SK3	1	3	6	2	1	3	1
Peshawar	P1	2	2	2	0	4	3	0
	P2	3	1	3	2	0	0	0
	P3	1	0	1	1	1	1	0
Malakand	M1	3	0	5	2	2	2	2
	M2	5	2	6	3	3	3	1
	M3	5	6	9	2	1	3	2
Jabban	J1	4	2	7	2	3	1	1
	J2	5	3	8	3	4	2	0
	J3	6	2	11	1	3	1	2
Malakander	MK1	1	2	3	2	2	2	1
	MK2	1	4	2	0	1	0	0
	MK3	0	1	0	0	0	0	0
Thana	T1	2	2	3	0	2	2	1
	T2	5	1	6	1	1	1	0
	T3	6	1	3	1	3	3	0
Wartair	W1	2	1	2	2	3	1	1
	W2	0	2	3	1	3	0	0
	W3	2	2	1	1	2	2	1

Table 4. Molecular identification of root knot nematodes collected from tomato growing areas of Khyber Pakhtunkhwa Province (KPK) in Pakistan based on size of band depending of primer combination.

Code[a]	Locality	Field	194/195 (rRNA) primer	Fjav/Rjav (SCAR) primer	Finc/Rinc (SCAR) primer	Far/Rar(SCAR) primer	C2F3/1108(mtDNA) primer	Molecular Identification
L1	Dargai	D1	-	-	-	-	-	Unknown
L2	Dargai	D1	-	-	-	-	-	Unknown
L3	Dargai	D1	720 bp	-	1200 bp	-	1700 bp	*Meloidogyne incognita*
M1	Heroshah	H1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
M2	Heroshah	H2	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
M3	Heroshah	H2	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*[b]
M4	Heroshah	H2	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*

Table 4. Continued.

J1	Swat	S3	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
J2	Swat	S1	720 bp	-	1200 bp	-	1700 bp	*Meloidogyne incognita*
J3	Swat	S3	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*[b]
J4	Swat	S1	720 bp	-	1200 bp	-	1700 bp	*Meloidogyne incognita*[b]
F1	Skhakot	SK1	720 bp	-	1200 bp	-	1700 bp	*Meloidogyne incognita*
F2	Skhakot	SK2	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*[b]
F3	Skhakot	SK1	720 bp	-	1200 bp	-	1700 bp	*Meloidogyne incognita*
F4	Skhakot	SK1	720 bp	-	1200 bp	-	1700 bp	*Meloidogyne incognita*
T1	Peshawar	P2	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*[b]
T2	Peshawar	P2	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
T3	Peshawar	P3	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
W1	Malakand	M1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
W2	Malakand	M1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*[b]
W3	Malakand	M1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
R1	Jabban	J1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
R2	Jabban	J1	720 bp	-	-	420 bp	1700 bp	*Meloidogyne arenaria*[b]
R3	Jabban	J1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
Q1	Malakander	MK1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
Q2	Malakander	MK1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
Q3	Malakander	MK1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
H1	Thana	T1	720 bp	-	-	420 bp	1700 bp	*Meloidogyne arenaria*[b]
H2	Thana	T3	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
E1	Wartair	W1	720 bp	670 bp	-	-	1700 bp	*Meloidogyne javanica*
E2	Wartair	W1	720 bp	-	-	420 bp	1700 bp	*Meloidogyne arenaria*

[a]Individual nematodes (juveniles or females); [b]individual nematodes with D2 and D3 expansion region of the 28S rDNA nuclear gene sequenced.

chitwoodi and *fallax* produced 520 bp products, whereas *M. enterolobii* produced a 750 bp product (Figure 1B). The nematodes L1 and L2 did not yield any amplification.

DNA sequencing of D2-D3 expansion segments of 28S rDNA

Nematodes W2, M3, J3, F2, and T1 were identified as *M. javanica*; J4 (*M. incognita*) and H1 and R2 (*M. arenaria*) were selected for sequencing (Table 4). Intraspecific variability ranged from three nucleotides (0.4% differences) (between J3 and T1) to 27 nucleotides (4.2% differences) (between W2, M3 and F2) for *M. javanica* (636 bp alignment) while for *M. arenaria*, no differences were found (H1 and R2). *M. javanica* nematodes (T1, W2, M3, J3 and F2) showed the highest similarity with *M. hispanica* (98%) (EU443606.1) and *M. thailandica* (97%)(EU364890.1). *M. arenaria* (R2 and H1) showed the highest similarity with *M. incognita* (99%) (AF435794.1), *M. paranaensis* (99%) (AF435799.1) and *M. thailandica* (97%) (EU364890.1) whereas *M. incognita* (J4) showed similarity with *M. hispanica* (99%) (EU443606.1) and *M. thailandica* (97%) (EU364890.1). In general, sequencing the 28S rDNA with D2A/D3B primers did not differentiate the three tropical *Meloidogyne* species. New sequences obtained in this study were deposited in GenBank, with accession numbers JQ317912-19 for T1, W2, M3, J3, F2, J4, R2, and H1, respectively.

DISCUSSION

In the present study, the occurrence of *M. incognita, javanica* and *arenaria* alone, or in mixed populations from samples collected from 30 tomato production fields, clearly demonstrate the widespread occurrence of these species in KPK. These RKNs are considered the most common species in Pakistan (Gul and Saeed, 1990). Amongst them, *M. incognita* has been ranked first by these authors with respect to host range and geographical distribution (Taylor et al., 1982). However, in the present survey, *M. javanica* was found to be more widely distributed than *M. incognita*. These differences to our results may be ascribed to different soil characteristics and the climatic conditions of the studied areas. Particularly, these areas are characterized by sandy soil, with sands comprising more than 70%, whereas in most parts of the KPK, the soil is about 50% of sand (Gul, 1988).

Dargai, Malakand, Heroshah and Jabban soils in particular, have a large amount of sand and gravel and are most suitable for the RKNs development. Soil type has been found as a primary edaphic factor that may influence the damage potential of *Meloidogyne* species

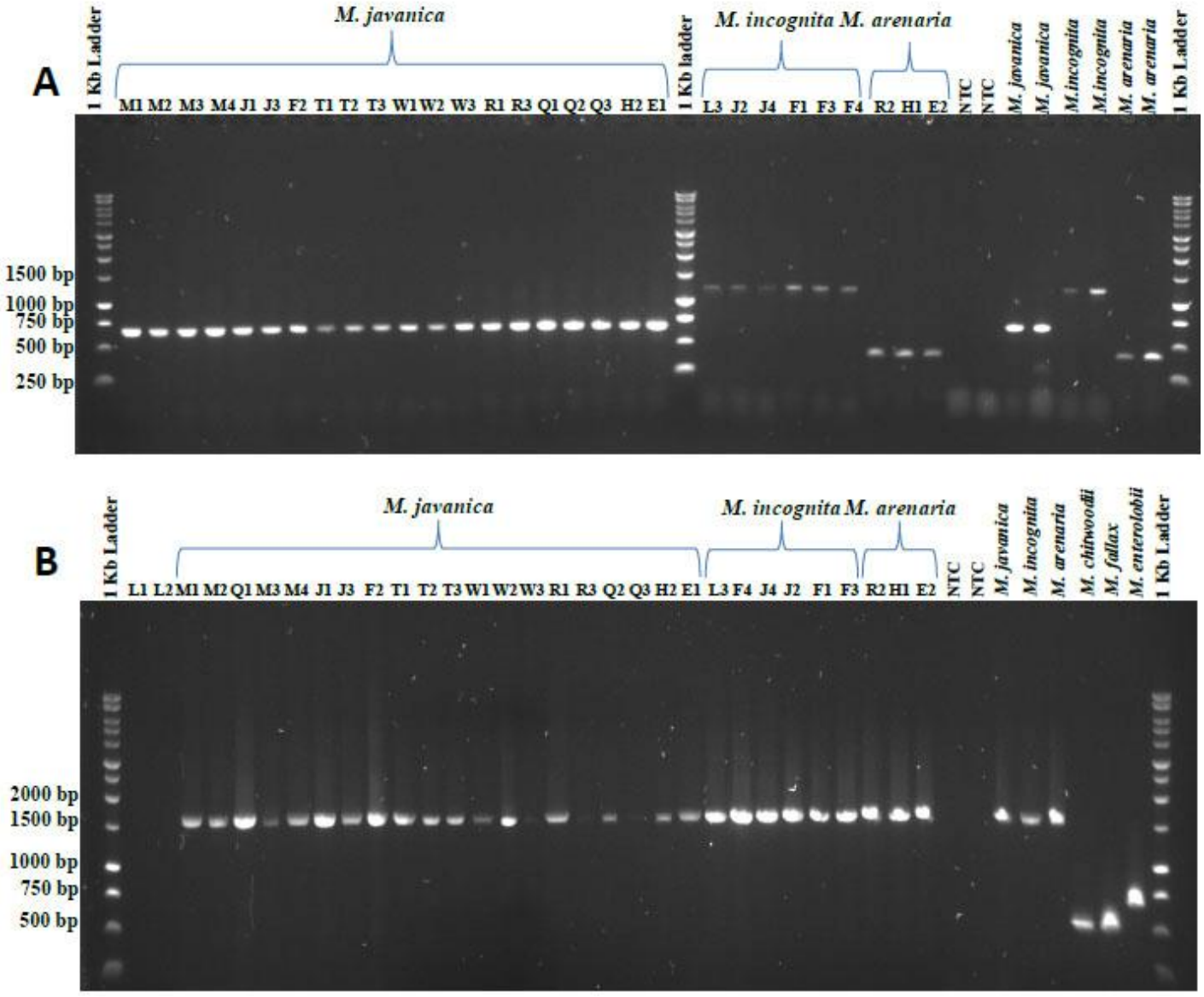

Figure 1. A) Amplification products of PCR reactions using Fjav/Rjav (*M. javanica*), Finc/Rinc (*M. incognita*) and Far/Rar (*M. arenaria*) specific SCAR primers with DNA extracted from single females from 11 RKN nematodes (Table 4) and the three positive control nematodes (*M. javanica, incognita and arenaria*), NTC (no template control-water) and 1 Kb ladder (Promega, UK). B) Amplified PCR products of COII/lrRNA region of mtDNA (*Meloidogyne* spp) with DNA extracted from single females from 11 RKN populations/isolate (Table 4) and positive control nematodes (*M. javanica, incognita, arenaria, chitwoodi, fallax and enterolobii*), NTC (no template control-water) and 1 kb ladder (Promega, UK).

(Jain, 1992). Soil type influences nematode movement while searching for hosts, penetration of roots, reproduction, and buildup of population densities in fields (Prot and Van Gundy, 1981). The high prevalence and incidence of these nematodes suggests their Importance as a potential threat to tomato in KPK. The major tomato growing areas particularly the Dargai and Jabban are surrounded by the Malakand hills, which protect the winter tomato crops from frost, and tomatoes are grown successfully. One of the reasons for high incidence of RKNs in these areas is monoculturing of tomato, and the information obtained from growers indicate that most of the fields observed had been under vegetable cultivation for several years (Anwar et al., 2007). Another factor for higher nematode density and severe incidence might be the use of susceptible rotation crops like okra and egg plants by the growers and the use of susceptible tomato cultivars in the rotations. These findings agree with others who have reported that these nematodes are widely distributed in vegetable growing regions of Pakistan

(Khan et al., 2006).

The fields with minimum infestation of RKNs, located at Peshawar and Malakandher might be due to silt loam and clay soil types (Ogbuji, 2004) and the climate in surrounding areas is tropical, dry and very hot. Clay soil has unfavourable pore size and aeration which probably resulted in poor nematode multiplication and movement (Young and Heatherly, 1990). Persistence of dry conditions and climatic factors may affect the survival of RKNs (Taylor et al., 1982). In this study, several other plant-parasitic nematodes were collected and identified. Among them, *Pratylenchus* spp. was found to be most widely distributed. Other important groups of nematodes isolated from these areas were *Xiphinema* spp., *Helicotylenchus* spp., *Hoplolaimus* spp., and *Criconema* spp. However, the population density of these nematodes in tomato fields was quite low, showing that the major nematological problem in these areas are related to RKNs. Kamran et al. (2010) observed *Pratylenchus* spp., *Xiphinema* and *Helicotylechus* spp. in tomato roots and soil infested with RKNs in Punjab province. Most of these nematodes have been reported to be associated with other crops (Khan and Shaukat, 2005).

The Meloidogyne species show wide morphological variations among and within species, making their identification difficult. The perineal pattern technique has been used to characterize *Meloidogyne* spp. (Chitwood, 1949), however, it has the problem of only being useful when females are present in the crop, and sometimes more than one species of RKNs are found together in the same plant root or soil. For these reasons, alternative fast and accurate identification methods for root-knot nematodes are needed for management and breeding (Powers and Harris, 1993). Molecular information can be used as a complement to morphological data in the identification process, particularly for *Meloidogyne* species (Moens et al., 2009). To our knowledge, this is the first report of molecular identification of RKNs in Pakistan.

M. incognita, *javanica* and *arenaria* from KPK, Pakistan, were successful identified using species-specific SCAR primers (Zjisltra et al., 2000). The other primers combinations used in this study [194 to 195) (rDNA) and C2F3/1108 (mtDNA)] and the sequencing of D2-D3 expansion segment of 28S rDNA clearly separated these species from others but not between themselves, as reported in other studies (Adam et al., 2007). For these reasons, species-specific primers are very well suited for the sampled area in Pakistan. These primers successfully amplified their expected fragment from single individual stages of RKNs from only 1/30 of the DNA extract. Similar results were found by Adam et al. (2007). Only two nematodes from Dargai locality failed to amplify the corresponding size of D2-D3 expansion segment of 28S rDNA and they failed in the posterior PCR reactions, showing the importance to use control PCR in order to prevent false negatives. Probably, the DNA of these two nematodes was degraded during the sampling or extraction procedures.

In general, the present results from perennial pattern examination and molecular identification of RKNs were consistent with each other. We obtained a higher proportion of molecular identification of *M. javanica* from our PCR results than of the other species of RKN, which clearly demonstrates the prevalence of this species in the study area. At present, only a few perineal pattern identifications were not coincident with molecular identification in some of the studied fields. For this reason, using molecular identification will complement the morphological identification and several individual nematodes; one per PCR reaction must be included in the analysis from each sample.

The mtDNA is an excellent source for genetic markers for population genetics and species identification (Blok and Powers, 2009). Amplification of the COII/lrRNA region of mtDNA with C2F3/1108 primers have successfully discriminated important species of RKNs (Orui, 1998). In the case of *M. arenaria*, Han et al. (2004) showed that the size of the amplified PCR product in the COII/lrRNA region was 1.1 kb in the USA isolates and 1.7 kb in the Korean and Japanese isolates, whereas the Chinese isolates revealed both sizes of the PCR products, indicating that the Chinese isolates have both genotypes. The size variability of PCR products among *M. arenaria* isolates from these countries was suggestive of intraspecific variation. Our results with *M. arenaria* mtDNA are similar to those reported by Han et al. (2004) and are in close agreement with those obtained by Powers et al. (2005). The reduction in extension temperature to 60°C dramatically improved the PCR performance in this A + T rich region.

Sequence analysis of the PCR products generated with the D2A and D3B primers showed the highest similarity between *M. incognita, javanica* and *arenaria*. De Ley et al. (1999) also studied ITS1, 5.8S, and ITS2 rDNA sequences from several *Meloidogyne* spp., including *M. hispanica,* and verified that a group of species (*M. hispanica, incognita, javanica, morocciensis*, and both races of *M. arenaria*) have nearly identical ITS region sequences. Diagnostic resolution of D2-D3 expansion segments of 28S rDNA is insufficient to discriminate between some of the most closely related, problematic and economically damaging species.

Although good phylogenetic resolution can be obtained for more distantly related species; relationships within species groups cannot always be resolved, and it may be more appropriate to analyze these with multiple loci and/or with longer sequence stretches.

In our study, the intraspecific variability for *M. javanica* ranged from 0.4 to 4.2%. The relatively high differences obtained are difficult to explain for the conserved region studied, in which similar morphological species have been reported to have differences in nucleotides around 4% or less (Landa et al., 2008).

ACKNOWLEDGEMENTS

The authors highly appreciate the Higher Education Commission of Pakistan for providing the necessary funds for this study.

REFERENCES

Adam MA, Phillips MMS, Block VC (2007). Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). Plant Pathol. 56:190-197.

Anonymous (2009). Agricultural Statistics of Pakistan, 2008-2009. Government of Pakistan. Ministry of Food, Agriculture and Livestock Division (Economic wing), Islamabad.

Anwar SA, Zia A, Hussain M, Kamran M (2007). Host suitability of selected plants to *Meloidogyne incognita* in the Punjab, Pakistan. Int. J. Nematol 17:144-150.

Baloch AF (1994). Vegetable crops. In: M. N. Malik (ed.) Horticulture. National Book Foundation Islamabad. pp.508.

Blok VC, Phillips MS, Fargette M (1997). Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major tropical root knot nematodes. J. Nematol. 29:16-22.

Blok VC, Powers TO (2009). Biochemical and Molecular identification. In: Root-knot Nematodes (eds.) Perry RN, Moens M, Starr JL. Wallingford/UK, CABI Publishing, pp. 98-112.

Castagnone-Sereno P, Esparrago G, Abad P, Leroy F, Bongiovanni M (1995). Satellite DNA as target for PCR-detection of the plant parasitic nematode *Meloidogyne hapla.* Curr Genet. 28:566-570.

Chitwood BG (1949). Root knot nematodes, Part I. A revision of the genus *Meloidogyne Goeldi*, 1887. Proc. Helminth. Soc. Wash. 16:90-104.

De Ley P, Félix MA, Frisse LM, Nadler SA, Sternberg PW, Thomas WK (1999). Molecular and morphological characterization of two reproductive species with mirror-image anatomy (Nematoda:Cephalobidae). Nematol. 1:591-612.

Eisenback JD (1985). Diagnostic characters useful in the identification of the four most common species of root knot nematodes (*Meloidogyne* spp.). In Sasser JN, Carter CC, (eds). An advanced treatise on *Meloidogyne,* vol. 1, Biology and control. Raleigh/North Carolina State University Graphics, pp.95-112.

Eisenback JD, Hirschmann H (1981). Identification of *Meloidogyne* species on the basis of head and stylet morphology. J. Nematol. 12:23-32.

FAOSTAT 2009. FAOSTAT On-line. Pakistan: United Nations Food and Agriculture Organization. http://faostat.fao.org/default.aspx.

Gul A (1988). Studies on root knot nematodes (*Meloidogyne* spp.) in the North West frontier province of Pakistan with special reference to the association of *M. javanica* (Treub) Chitwood with peach (*Prunus persica* L Batsch). Karachi, Pakistan, University of Karachi, PhD thesis. pp.250.

Gul A, Saeed M (1990). A survey of root-knot nematode (*Meloidogyne* spp.) in North West frontier province (NWFP) of Pakistan. Sarhad J. Agric. 6:495-502.

Han HR, Cho MR, Jeon HY, Kim CK, Yan HI (2004). PCR-RFLP identification of three major *Meloidogyne* species in Korea. J. Asian Pasific Entomol. 7:171-175.

Holbrook, CC, Knaufft DA, Dickson SW (1983). A technique for screening peanut for resistance to *Meloidogyne arenaria*. Plant Dis. 67:957-958.

Hugall A, Moritz C, Stanton J, Wolstenholme DR (1994). Low, but strongly structured mitochondrial DNA diversity in root-knot nematodes (*Meloidogyne*). Genet. 136:903-912.

Hussey RS, Barker RR (1973). A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Dis. Rep. 57:1025-1028.

Jacquet M, Bongiovanni M, Martinez M, Verschave P, Wajnberg E, Castagnone-Sereno P (2005). Variation in resistance to the root-knot nematode *Meloidogyne incognita* in tomato genotypes bearing the *Mi* gene. Plant Pathol. 54:93-99.

Jain RK (1992). Nematode pests of vegetable crops. In: Bhatti, DS, Walia, RK (eds) Nematode pests of crops. CBS Publishers & Distributors, Delhi, India, pp.77-79.

Kamran M, Anwar SA, Javed M, Khan SA, Sahi GH (2010). Incidence of root knot nematode on tomato in Sarghoda, Punjab. Pakistan J Nematol. 28:253-262.

Kaskalvalci G (2007). Effect of soil solarization and organic amendment treatments for controlling *Meloidogyne incognita* in tomato cultivars in Western Antolia. Turk. J. Agric. For. 31: 159-167.

Khan A, Shaukat SS (2005). A survey of nematodes in pomegranates in lower Sindh. Sarhad J. Agric. 21:699-702.

Khan HU, Mukhtar R, Ahmad R, Iqbal MA (2006). Studies on the distribution and control of *Meloidogyne* root-knot nematodes in Faisalabad and Lahore divisions, Pakistan. Pakistan J. Nematol. 24:57-64.

Khattak B (2008). Biological management of root knot nematode *Meloidogyne javanica* (Treub) with *Trichoderma harzianum* Rifai in tomato. Peshawar, Khyber Pakhtunkhwa Agricultural University, Pakistan, PhD Thesis (Nematology). pp.157.

Landa BB, Palomares Rius JE, Vovlas N, Carneiro RMDG, Maleita CMN, Abrantes IM de O, Castillo P (2008). Molecular characterization of *Meloidogyne hispanica* (Nematoda, Meloidogynidae) by phylogenetic analysis of genes within the rDNA in *Meloidogyne* spp. Plant Dis. 92:1104-1110.

Moens M, Perry R, Starr JL (2009). *Meloidogyne species,* a diverse group of novel and important plant parasites. In: Perry RN, Moens M, Starr JL (eds) Root Knot Nematodes. Wallingford/UK, CABI Publishing, CAB International. pp.1-13.

Munir A, Bridge J (2003). Rice root-knot nematode *Meloidogyne graminicola* Golden and Birchfield, 1965 from rice in Pakistan. Pakistan J. Nematol. 21:133-135.

Niu JH, Jian H, Guo QX, Chen, CL, Wang XY, Liu Q, Guo YD (2011). Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii*. Plant Pathol. DOI: 10.1111/j.1365-3059.2011.02562.

Ogbuji RO. (2004). Soil depth distribution of the root-knot nematode (*Meloidogyne incognita*) from two farmlands in a humid tropical environment. Geogr. J. 5:79-80.

Orui Y (1998). Identification of Japanese species of genus *Meloidogyne* (Nematoda: Meloidogynidae) by PCR-RFLP analysis. Appl. Entomol. Zool. 33:43-51.

Powers TO, Harris TS (1993). A polymerase chain reaction method for identification of five major *Meloidogyne* species. J. Nematol. 25:1-6.

Powers TO, Mullin PG, Harris, TS, Sutton LA, Higgins RS (2005). Incorporating molecular identification of *Meloidogyne spp*. into a large scale regional nematodes survey. J. Nematol. 37:226-235.

Prot JC, Gundy DD (1981). Effect of soil texture and clay component on migration of *Meloidogyne incognita* second stage juveniles. J. Nematol. 12:213-217.

Santhosh JE, Beena B, Ramana KV (2005). Tropical soil microflora of species-based cropping systems as potential antagonists of root-knot nematodes. J. Invertebr. Pathol. 88:218-225.

Sasser JN, Carter CC (1982). Overview of International Meloidogyne project, Rational, goals, implementation and progress todate. Proceeding of Conference on root knot nematodes Meloidogyne spp. (Region 111). Brasillia Brazil. pp.3-13.

Taylor AL, Sasser JN (1978). Biology, Identification and Control of Root-knot Nematodes (*Meloidogyne* species). North Carolina State University, USAID, NC Graphics, Raleigh.

Taylor AL, Sasser JN, Nelson LA (1982). Relationship of climate and soil characteristics to geographical distribution of *Meloidogyne* species in agricultural soils. North Carolina State University, USAID, NC Graphics, Raleigh.

Thistlethwayte B (1970). Reproduction of *Pratylenchus penetrans* (Nematoda: Tylenchida). J. Nematol. 2:101-105.

Trudgill DL, Blok VC (2001). Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging root pathogens. Annu. Rev. Phytopathol. 39:53-77.

Williamson VM, Hussey RS (1996). Nematode pathogenesis and resistance in plants. Plant Cell. 8:1735-1745.
Young LD, Heatherly LG (1990). *Heterodera glycines* investigation and reproduction of soybean grown in clay and silt loam soils. J. Nematol. 22:618-619.
Zijlstra C (2000). Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: A powerful way of enabling reliable identification of populations or individuals that share common traits. Eur. J. Plant Pathol. 106:283-290.