Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(2): 483-486

Received: 13-01-2020
Accepted: 15-02-2020

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Identification of root-knot nematode infecting pomegranate, *Punica granatum* L. in Haryana

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Abstract
Root-knot nematodes are the most plant parasitic nematodes causing serious damage in pomegranate. Accurate identification of *Meloidogyne* species is of great importance for effective management of root-knot nematodes. Morphological identification of *Meloidogyne* spp. has been long and extensively used. However, molecular diagnostics was reported as a replacement or a complement method. Examination of the perineal patterns of the female was typically identified the nematode species as *Meloidogyne javanica* which was dominant in both pomegranate infected orchard samples. For further confirmation of identification, a polymerase chain reaction (PCR)-based assay with species-specific sequence characterized amplified regions (SCAR) primer set was performed. The Fjav/Rjav primer efficiently amplified SCAR markers of 670 bp, which were previously reported for *Meloidogyne javanica*. These results support that SCAR markers are a powerful tool for rapid and effective detection and could be used as a complementary tool together with the morphological identification of root-knot nematodes.

Keywords: Root-knot nematodes, *Meloidogyne javanica*, pomegranate, perineal patterns, polymerase chain reaction, SCAR markers

Introduction
Pomegranate (*Punica granatum* L.) is one of the most important fruit crops of the tropical and subtropical regions of India. India is world's leading producer of pomegranate. In India, pomegranates are commercially cultivated in Maharashtra. They are planted on a smaller scale in states of Gujarat, Rajasthan, Karnataka, Andhra Pradesh, Tamil Nadu, Punjab, Haryana and Uttar Pradesh. The area under pomegranate is increasing steadily due to its high monitory returns and export value. Recently pomegranate growers are encountering problem of yellowing of leaves, stunting and less productivity. Such trees were found to be severely infested with root knot nematodes on assaying of soil and root samples (Poornima K, *et al*., 2015) [15]. Several workers (Ansari MA, Devi G, Patel AD and Senthilkumar T. *et al*) [4, 5, 14, 18] have studied the community analysis of plant parasitic nematodes associated with various horticultural crops in India, but there is very little information available on the economic damage and community structure of phytoparasitic nematodes in pomegranate. Accurate and careful identification of *Meloidogyne* species infecting crops is a core for efficient use of plant resistance and successful management of root-knot nematodes. Several methods were used to identify root-knot nematode species such as morphological characters and molecular techniques. The perineal pattern is often an unreliable character when used alone for making diagnostic conclusions but, when used as a complementary tool together with enzyme characterization or molecular analysis, is essential for checking the morphological consistency of the identification (Carneiro, *et al*., 2004) [3]. Therefore, molecular diagnostics of *Meloidogyne* species has been sought as a replacement. PCR-based detection methods have been developed and widely used for nematodes identification, including species specific or sequence characterized amplification region (SCAR) primers (Daramola, *et al*., 2015) [4]. SCAR markers are more advantageous than RAPD markers because they usually detect only a single locus and it is more specific. Moreover, their PCR amplification is less sensitive to reaction conditions and therefore they are reproducible Incidence of plant parasitic nematodes like root knot in pomegranate being an emerging problem, in the present study taking into consideration and an investigation was planned and conducted for the presence of root knot nematode on pomegranate and identification of the infecting root knot species in Haryana based on morphological identification and molecular analysis.

Materials and Methods

Identification of nematode isolates: The soil and root samples of root-knot nematodes infecting pomegranate were collected from two localities of Haryana, (Kalanaur and Lahli) cultivated with pomegranate orchards Plate. (1). Adult females were extracted to prepare perineal patterns Plate. (5).

Perineal patterns: Infected roots Plate. (2) were collected and washed. Perineal patterns were prepared according to a method described by (Eisenback JD, Khan MR, Nono-Womdim and Taylor. *et al*) [7, 10, 13, 19], the root tissues were teased apart with forceps and half spear to remove adult females. Females were transferred to a drop of tap water on a glass microscope slide. The cuticle of the female ruptured near the neck and gently pushed the body tissue out. After that the cuticle was placed in a drop of 45% lactic acid on a glass microscope slide for 30 minutes. The cuticle was cutting in half with blade and the perineal patterns trimmed to a square. The perineal patterns were transferred to a drop of glycerin on a clean glass microscope slide. The interior surface of the cuticle was placed on the glycerin drop against the glass slide. Cover slip was sealed and the slide has been labeled and examined under compound microscope.

Table 1: SCAR primers used for molecular identification of *Meloidogyne javanica*

Primer name	Fragment size (bp)	Sequence (5'-3')
Fjav/Rjav	670	F: GGTGCGCGATTGAACTGAGC R: CAGGCCCTTCAGTGGAACTATAC

DNA Extraction and PCR assay: DNA extraction from nematode juveniles was performed following cetyl trimethyl ammonium bromide (CTAB) method (Mondino EA. *et al* and Sambrook J. *et al*) [11, 17] with slight modifications. In order to confirm morphological identification of nematode species, species-specific SCAR primer set selected from previous studies as marker specific for *Meloidogyne javanica*, namely Fjav/Rjav (Zijlstra C, *et al.*, 2000) [20] was used in the PCR (Table 1). PCR amplifications were performed in 25µl reaction mixtures, each containing 2.0µl of genomic DNA, 1X PCR buffer 2.5 µl, 50 mM $MgCl_2$ 0.75 µl, 10 mM dNTP mix 0.5 µl, 10 µM of forward primer 0.5 µl, 10 µM of reverse primer 0.5 µl and Platinum^{TM}Taq DNA-polymerase 0.1 µl. Amplifications were performed in a using the following PCR profile: initial denaturation at 94 °C for 4 min, followed by 45 cycles each consisting of 30 sec at 94 °C, 30 sec at 60 °C for Fjav/Rjav, followed by 90 sec at 72 °C, with a final extension at 72 °C for 10 min.

Electrophoresis: PCR products were separated using horizontal gel electrophoresis unit on 1% agarose gel stained with ethidium bromide in 1 X TBE buffer. A 1000 bp DNA ladder was used to estimate the size of each amplified DNA fragment. The gel was run for approximately 1 hours using constant voltage of around 80 V and then visualized and photographed under UV light using a gel documentation system.

Results and Discussion

Examination of the perineal patterns of the females, hand picked up from infected pomegranate roots exhibited features typical to *Meloidogyne javanica* Plate. (3). This species was dominant in both pomegranate samples collected from Kalanaur and Lahli. The important diagnostic characters of perineal patterns of the *Meloidogyne javanica*, were summarized as low and rounded dorsal arch, contain lateral ridges that divide the pattern into dorsal and ventral regions or striae. Striae were coarse and smooth to slightly wavy and tail terminus often with distinct whorl. This species *Meloidogyne javanica* was differed from the three other common species (*Meloidogyne arenaria, Meloidogyne hapla* and *Meloidogyne incognita*) by containing its pattern on lateral field as described by (Eisenback JD, *et al*., 1981) [8]. A combination of identification methods was used to separate root-knot nematode species. Results from the infected areas indicated that, the presence of *Meloidogyne javanica* which is not surprise as it is mentioned as the most common *Meloidogyne* species in tropical regions like Haryana (India) where, annual temperatures are between 17-32˚C. *Meloidogyne javanica* was dominated at the present studied samples.

The PCR assay for the two nematode popoulation with the specific SCAR primer Fjav/Rjav clearly produced a specific DNA fragment of 670 bp (Plate. 4), which was expected for *Meloidogyne javanica* as reported by (Zijlstra C, *et al*., 2000) [20]. Similar findings were reported by (Devran Z, *et al*., 2009) [6] in Turkey and (Naz I, *et al*., 2012) [12] in Pakistan**.** Identification by morphological characters and host plant response is time consuming and needs extensive labor. Isozymes analysis can be carried out only on female individuals and can be affected by environmental factors (Esbenshade PR, *et al*., 1990) [9]. Unlike, molecular techniques based on DNA can be used in every stage of the nematode's life cycle, and they are rapid, and reliable (Devran Z, *et al*., 2009) [6]. For molecular identification of species, the characteristic sequence of genomic DNA of different species should differ to allow the delineation of species, but at the same time, no/minor variation within the species should exist (Devran Z. *et al.*, 2009) [6]. PCR-based markers have been used especially to allow a clear and rapid species diagnosis when external morphological characters are not fully discriminated. SCAR markers have been commonly used in genomic analysis and widely used for molecular identification of root-knot nematodes to confirm morphological identifications as well as to identify unknown isolates (Akyazi F. *et al*, Devran Z.*et al*, Naz I. *et al*, Randig O. *et al* and Ziljstra C. *et al*) [1, 6, 12, 16, 20]. Species-specific SCAR primers amplify the DNA fragment(s) belonging to only one species and are desirable to accurately identify nematode species Moreover, SCAR markers can be amplified from DNA from egg masses, second stage juveniles and females and was successfully applied using DNA extracts from infested plant material. Therefore, the method has potential to be optimized for routine practical diagnostic tests facilitating the control of these economically important pest organisms (Ziljstra C, *et al*., 2000) [20]. In conclusion, it is clear that, results from perineal pattern examination and molecular analysis were consistent with each other, suggesting that molecular identification using SCAR markers could be used as a complementary tool together with the morphological identification of root-knot nematodes.

Plate 1: Wilted Pomegranate plant

Plate 2: Galls on root produced by *Meloidogyne javanica*

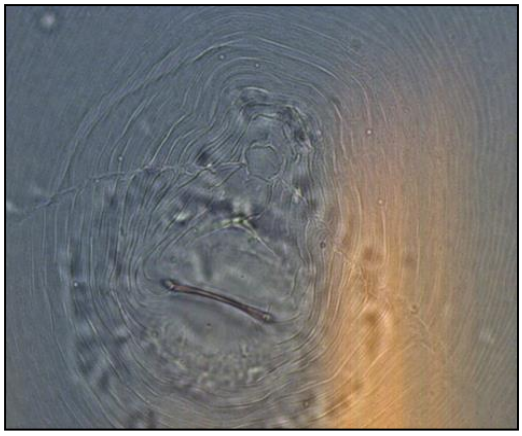

Plate 3: Perineal pattern of *Meloidogyne javanica*

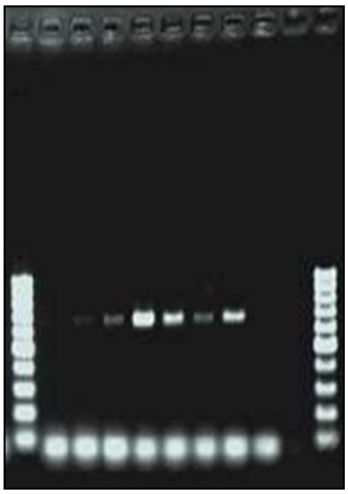

Plate 4: Amplification at 670bp using Fjav/Rjav SCAR primer of *Meloidogyne javanica* 1000bp DNA ladder

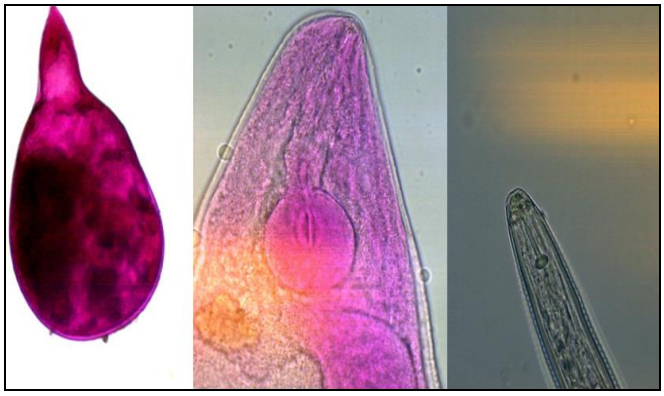

Plate 5: (A) Matured female of *Meloidogyne javanica* (B) Anterior region of female (C) Anterior region of juvenile

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