Morphological and molecular identification of root-knot nematodes infecting pomegranate in Assiut Governorate, Egypt

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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 isolates as M. javanica which was dominant in all five pomegranate samples. For further confirmation of identification, a polymerase chain reaction (PCR)-based assay with two species-specific sequence characterized amplified regions (SCAR) primer sets was performed. The Fjav/Rjav and MJ-F/MJ-R primers efficiently amplified SCAR markers of 670 and 517 bp, respectively, which were previously reported for M. 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.

Key words: Root-knot nematodes, Meloidogyne javanica, pomegranate, perineal patterns, polymerase chain reaction, SCAR markers.
Introduction

The pomegranate (Punica granatum L.) is an ancient fruit belongs to the family Punicaceae which includes one genus and two species. It is a widely grown horticulture crop in many tropical and subtropical countries. In Egypt, pomegranate considered one of the most important fruit trees cultivated in warm regions such as Assiut Governorate where the climate is characterized by long hot summer and low air humidity. The total area devoted for pomegranate was 26851 feddans and fruiting area was 9746 feddans producing about 89035 tons with the average of 9.136 tons/fedden. Assiut Governorate is considered as first cultivation of pomegranate that’s where the biggest area cultivated in Egypt (Ministry of Agriculture and Land Reclamation, 2012). Most of the pomegranate orchards found infested with root-knot nematodes (Khan and Shaukat, 2010; Khan et al., 2005). Root-knot nematodes are sedentary obligate endoparasitic nematodes, which common in Egypt and worldwide and cause severe damage especially in light soils that cause major economic damage to crops (Khan et al., 2008). 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 (Mwesige et al., 2016). Several methods were used to identify root-knot nematode species such as morphological characters (Eisenback & Triantaphyllou, 1991), host plant response (Hartman & Sasser, 1985), isozyme analyses (Esbenshade & Triantaphyllou, 1990) and molecular techniques (Daramola et al., 2015; Hassan et al., 2013; Powers et al., 2005; Powers et al., 1997; Powers & Harris, 1993). 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). Therefore, molecular diagnostics of Meloidogyne species has been sought as a replacement or complement for these procedures (Powers, 1992; Hyman, 1990; Burrows, 1990). Over the past years, different molecular analyses and PCR-based detection methods have been developed and widely used for nematodes identification, including amplified fragment length polymorphism (AFLP) (Hyman & Whipple, 1996; Hyman, 1990), random amplified polymorphic DNA (RAPD) (Naz et al., 2013; Carneiro et al. 2004), restriction fragment length polymorphism (RFLP) (Tomaszewski et al., 1994) and species-specific or sequence characterized amplification region (SCAR) primers (Daramola et al., 2015; Akyazi & felek, 2013; Naz et al., 2012;Devran & Sogut, 2009). RAPD technique is a simple molecular marker and easy to develop, but lack of reproducibility makes it less reliable for authentication. Therefore, to improve the reliability of RAPDs, (Paran & Michelmore, 1993) developed SCAR technique. SCARs are based on sequencing the polymorphic fragment derived from RAPD primers and designing longer primers that will specifically bind to this fragment. 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 (Idrees & Irshad, 2014). The present study was carried out in order to identify five root-knot nematode isolates collected from five localities cultivated with pomegranate in Assiut Governorate based on morphological identification and molecular analysis.

Materials and methods

Identification of nematode isolates:
Five isolates of root-knot nematodes were collected from five localities of Assiut Governorate, Egypt (El-Badary, Sedfa, Sahel-Selim, El-Fath and Manfalout Counties) cultivated with pomegranate orchards. Adult females were extracted to prepare perineal patterns.

Perineal patterns: Infected roots prepared from pure culture collected and washed. Perineal patterns were prepared according to a method described by Taylor and Netscher, 1974; Nano-Womdim et al., 2002; Eisenback, 2010 and Khan, 2014, the root tissues were teased apart with forceps and half spear to remove adult females. Females 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 placed in a drop of 45% lactic acid on a glass microscope slide. The interior surface of the cuticle must be placed against the glass. Cover slip placed on the glycerin drop. Cover slip sealed and the slide has been labeled and examined under research microscope.

DNA Extraction PCR assay: DNA extraction from nematode isolates was performed following cetyltrimethylammonium bromide (CTAB) method (Mondino et al., 2015; Sambrook et al., 1989) with some modifications. In order to confirm morphological identification of nematode isolates, two species-specific SCAR primer sets selected from previous studies as markers specific for *M. Javanica*, namely Fjav/Rjav (Zijlstra et al., 2000) and MJ-F/MJ-R (Meng et al., 2004) were used in the PCR (Table 1). PCR amplifications were performed in 25μl reaction mixtures, each containing 5-10 ng of genomic DNA, 1X PCR buffer, 1.5 mM MgCl₂, 200 μM of each dNTP, 0.8 μM of each primer, and 1U Taq DNA-polymerase. Amplifications were performed in a SensoQuest Lab Cycler (SensoQuest GmbH, Göttingen, Germany) 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 and 55°C for MJ-F/MJ-R primers, 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 1.5% agarose gel stained with ethidium bromide in 0.5 X TBE buffer. A 100 bp DNA ladder was used to estimate the size of each
amplified DNA fragment. The gel was run for approximately 2-3 hours using constant voltage of around 80 V and then visualized and photographed under UV light using a gel documentation system. The specific band with the expected size was then detected for each SCAR marker separately.

Table 1: SCAR primers used for molecular identification of M. javanica.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Fragment size (bp)</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>Fjav/Rjav</td>
<td>670</td>
<td>F: GGTGCAGGGATTGAACTGAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CAGGCCCTTCATGGAACTATAC</td>
</tr>
<tr>
<td>MJ-F/MJ-R</td>
<td>517</td>
<td>F: ACGCTAGATTTCCAGGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTACCAGAAGGCAGCCATGC</td>
</tr>
</tbody>
</table>

Results and Discussion

Examination of the perineal patterns of the females which were hand picked up from infected pomegranate roots exhibited features typical to *M. javanica*. This species was dominant in all five pomegranate samples collected from Sedfa, El-Fath, Sahel-Selim, El-Badary and Manfalout counties Fig. (1). The important diagnostic characters of perineal patterns of the *M. javanica*, 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 *M. javanica* was differed from the three other common species (*M. arenaria*, *M. hapla* and *M. incognita*) by containing its pattern on lateral field as described by (Eisenback et al., 1981). A combination of identification methods was used to separate root-knot nematode species. Results from the sampled areas indicated that, the presence of *M. javanica* which is not surprise as it is mentioned as the most common *Meloidogyne* species in tropical and subtropical regions (Moens *et al.*, 2009; Taylor & Sasser, 1978) like Egypt where, annual temperatures are between 17-32˚C. *M. javanica* was dominated at the presently studied samples. A similar finding was reported by Nano-Womdin *et al.* (2002) in Tanzania on tomato plants. This result is in disagreement with Eisenback *et al.* (1981) where, *M. incognita* was the most prevalent among all *Meloidogyne* species in the studied areas in the international *Meloidogyne* project.

Figure 1: Perineal pattern of *M. javanica.*
The PCR assay for the five nematode isolates with the specific SCAR primer Fjav/Rjav clearly produced a specific DNA fragment of 670 bp (Fig. 2A), which was expected for *M. javanica* as reported by Zijlstra et al. (2000). A similar finding was reported by Devran and Sogut (2009) in Turkey and Naz et al. (2012) in Pakistan. Consistently, the *M. javanica*-specific primers MJ-F/MJ-R generated a SCAR product of 517 bp with all the five nematode isolates (Fig. 2B), which was identical to those previously reported for *M. javanica* (Meng et al., 2004). In accordance Song et al. (2017) have extracted DNA from a single J2 hatched from egg masses and used the MJ-F/MJ-R primers to further confirm species identification. They found that PCR products produced a fragment length of 517 bp (GenBank accession no. KX646189), which was identical to those previously reported for *M. javanica*.

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 & Triantaphyllou, 1990). 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 & Sogut, 2009). 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 & Sogut, 2009; Blok & Powers, 2009). 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 & felek, 2013; Naz et al., 2012; Jones et al., 2009; Devran & Sogut, 2009; Randig et al., 2002; Zijlstra et al., 2000). 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 (Zijlstra et al., 2000). In conclusion, it is clear that, results from perennial 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.

References


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