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Morphological and Molecular Identification of Root-knot Nematode, *Meloidogyne incognita* Infecting Pomegranate (*Punica granatum* L.) in Jodhpur, Rajasthan, India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) pose a major threat to pomegranate (*Punica granatum* L.) cultivation in India, leading to significant yield losses. In this study, galled roots of pomegranate were collected from orchards in Jodhpur, Rajasthan and nematode infestation was confirmed through root staining. The perineal pattern of females displayed the typical characteristics of the *Meloidogyne incognita*. To further confirm the species, DNA was extracted and a polymerase chain

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reaction (PCR) assay using species-specific SCAR (sequence-characterized amplified region) primers was conducted. The *M. incognita*- species specific primers, MincF1/MincR1, yielded the expected 150 bp product, verifying the presence of *M. incognita*. This study marks the first molecular confirmation of *M. incognita* infecting pomegranate orchards in this region, demonstrating the efficacy of SCAR markers in complementing traditional morphological identification as well.

Keywords: Root-knot nematodes; Meloidogyne incognita; pomegranate; perineal patterns; polymerase chain reaction; SCAR markers.

1. INTRODUCTION

Pomegranate (*Punica granatum* L.) is a highly adaptable crop, thriving in a wide range of climatic conditions, including Mediterranean, subtropical and tropical regions. Its versatility is reflected in global production, which reaches approximately 6.3 million metric tons (MT) from 556 thousand hectares of cultivated land. India is the leading producer, with an output of 3,186 thousand MT, followed by China, Iran, Turkey, the USA, Afghanistan and Spain (Sarkhosh et al., 2021). In India, pomegranate is commercially cultivated year-round in states such as Maharashtra, Gujarat, Karnataka, Andhra Pradesh, Tamil Nadu, Madhya Pradesh and Rajasthan, with availability from January to December. Despite its resilience, pomegranate faces significant challenges from non-insect pests, with root-knot nematodes emerging as a major threat to sustainable production. These nematodes, particularly prevalent in arid climates and sandy soils, can cause yield losses ranging from 30% to 40% and reduce fruit quality (Singh et al., 2019; Singh et al., 2021; Khan et al., 2014). Root-knot nematodes spread via water, soil, farm equipment and infested planting materials, often going undetected in substrate mixtures used during seedling preparation. Their feeding activity disrupts root function, causing gall formation, impairing nutrient, water uptake, leading to symptoms like yellowing, tip drying, and stunted growth. Additionally, affected plants are more susceptible to secondary infections by fungi and bacteria, as well as nutritional deficiencies, further exacerbating yield losses (Sikora et al., 2018). Several methods were used to identify root-knot nematode species, including morphology and molecular techniques. The perineal pattern alone is often unreliable but, when combined with enzyme or molecular analysis, helps confirm its identification (Carneiro et al., 2004). Molecular methods, like PCR-based detection using species-specific SCAR primers, are now widely used for identifying nematodes (Daramola et al., 2015). We conducted a survey in a pomegranate orchard located in Jodhpur, Rajasthan, and observed wilting symptoms in some of the pomegranate plants. Upon examining the roots of these plants, we noticed the presence of galls (Fig. 1C). In this study, we confirmed the presence of *Meloidogyne incognita* based on the perineal pattern and species-specific SCAR marker analysis.

2. MATERIALS AND METHODS

2.1 Sample Collection and Nematode Identification

Soil and root samples were collected from pomegranate orchards in Jodhpur, Rajasthan, to study root-knot nematodes (RKN) infecting pomegranate (Fig. 1). Adult females were extracted from the roots using a needle and scalpel under a binocular microscope. Their perineal patterns were prepared by cutting in 45% lactic acid and mounted in glycerin (Hooper, 1986) and light microphotographs of perineal pattern captured in stereobinocular compound microscope (1023270192). Morphological identification was conducted based on the methods described by Jepson (1987) and Karssen (2002) (Fig. 2).

2.2 DNA Extraction and PCR Amplification

DNA was extracted from a single female nematode using a worm lysis buffer (0.2M NaCl, 0.2M Tris pH 8.0, 1% β-mercaptoethanol, 800 µg/ml proteinase K), following the protocol described by Castagnone-Sereno et al. (1995). The lysates were stored at –20°C for further molecular analysis. DNA was examined using species-specific primers designed for the common root-knot nematodes, including *M. incognita, M. javanica, M. arenaria* and *M. hapla* (Devran et al., 2018; long et al., 2006; Dong et al., 2001; Zijlstra et al., 2000). PCR reactions

were performed in a Thermal Cycler following the protocol described by Devran et al. (2018).

2.3 Electrophoresis

PCR products were separated using horizontal gel electrophoresis on a 1.2% agarose gel stained with ethidium bromide in 1X TBE buffer. A 100 bp DNA ladder (MBT049) was used as a size reference to estimate the length of the amplified DNA fragments. The gel was run for about 40-minute at a constant voltage of 90 V. Afterward, the bands were visualized and photographed under UV light using a gel documentation system (AlphaImager, Alpha Innotech USA).

3. RESULTS AND DISCUSSION

The cuticular markings pattern high squarish dorsal arch with smooth to wavy striations and no distinct lateral lines in the perineal region of mature females confirmed that only *Meloidogyne incognita* was present in the samples collected from Jodhpur, in both the Mridula and Sinduri varieties. The overall morphology of this population appears to be similar to *M. incognita* (Eisenback and Triantaphyllou, 1991; Whitehead, 1968). *M. incognita*-specific and MincF1/MincR1 primer set (Devran et al., 2018) primers only produced an expected approximately 150 bp products, but other primers failed to amplify any products (Fig. 3).

Table 1. The 25µl reaction composition for PCR amplification

GoTaq Green Master mix	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Crude extracted genomic DNA	2 µl
Nucleus free water	8.5 µl

Table 2. Species-specific primers used in the study

	Target species	Code	Sequence (5’ 3’)	Amplicon size (bp)	Reference
1	*M. incognita*	MincF1	AAAAACACGCGATAACAAAAA	150	Devran et al. (2018)
		MincR1	ATTCAAAACTTGGGGGAAAAA		
2	*M.enterolobii*	Me F	AACTTTTGTGAAAGTGCCGCTG	236	Long et al. (2006)
		Me R	TCAGTTCAGGCAGGATCAACC		
3	*M. javanica*	Fjav	GGTGCGCGATTGAACTGAGC	670	Zijlstra et al. (2000)
		Rjav	CAGGCCCTTCAGTGGAACTATAC		
4	*M.hapla*	MhaF1	GGCTGAGCATAGTAGATGATGTT	1500	Dong et al. (2001)
		MhaR1	ACCCATTAAAGAGGAGTTTTGC		

Fig. 1. A, wilted pomegranate plant, B & C Galls caused by *Meloidogyne incognita* on the roots of pomegranate (*Punica granatum* L.), arrow indicating on galls

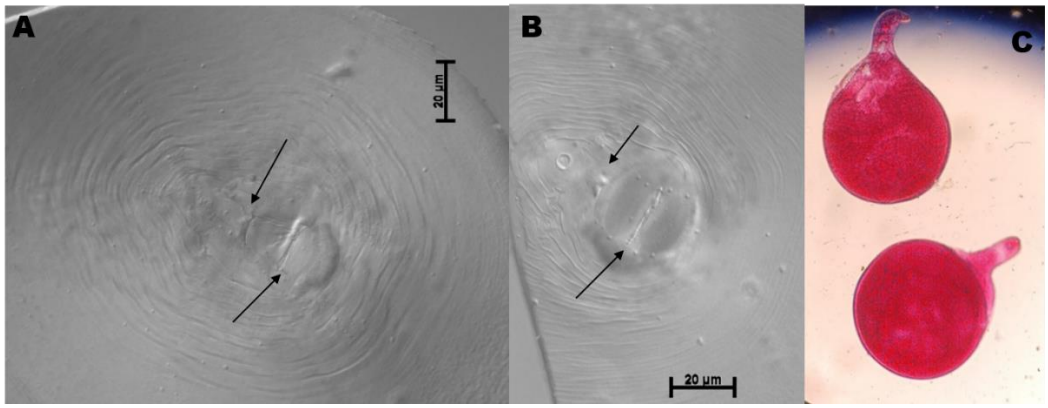

Fig. 2. A & B Perennial pattern of *M. incognita,* arrows pointing anal opening and vulval slit C, *M. incognita* females dissected from the infested pomegranate roots

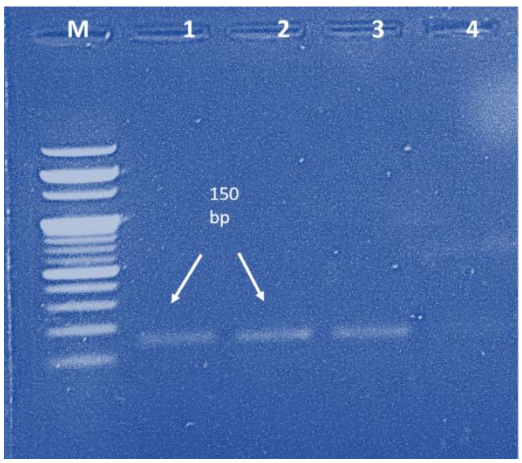

Fig. 3. Amplified DNA of using *Meloidogyne incognita*-specific primers: MincF1/MincR1 primer set, M: HIMEDIA 100 bp DNA Ladder (MBT049); 1-2: Samples; 3: *M. incognita* (positive control); 4: Water

4. CONCLUSION

In conclusion, the results from both the perineal pattern examination and molecular analysis were consistent, indicating that molecular identification using SCAR markers can serve as a valuable complementary tool along with morphological identification for root-knot nematodes. These findings hold significant value for horticulturists and can be applied to manage the damage caused by *M. incognita* in pomegranate orchards.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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