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

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 *Meloidogyne incognita*. To further confirm the species, DNA was extractedand a polymerase chain reaction (PCR) assay using species-specific SCAR (sequence-characterized amplified region) primers was conducted. The *M. incognita*-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 methods.

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

Introduction

Pomegranate (*Punica granatum* L.) is a highly adaptable crop, thriving in a wide range of climatic conditions, including Mediterranean, subtropicaland 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 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.

Materials and Methods

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 (Figure 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 patterncapturedin stereobinocularcompound microscope (1023270192). Morphological identification was conducted based on the methods described by Jepson (1987) and Karssen (2002) (Fig. 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).

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.1 The 25µl reaction composition for PCR amplification

Table 2: Species-specific primersused 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		

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).

Result 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).

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.

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

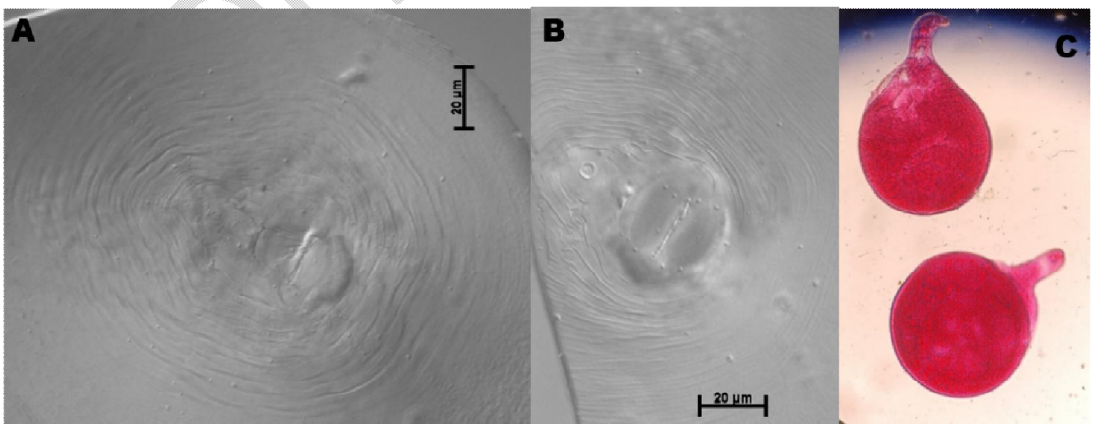

Fig. 2.A&**B** Perennial pattern of *M. incognita***C**, *M. incognita* females dissected from the infested pomegranate roots

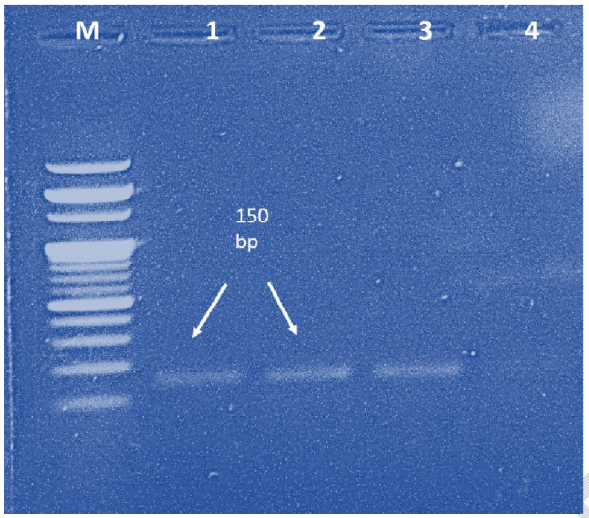

Fig. 3. Amplified DNA of using *Meloidogyne incognita*-specific primers: MincF1/MincR1 primer set, M: HIMEDIA 100 bp DNA Ladder (MBT049); 1-2: Samples; 3: *M. incognita* (positive control); 4: Water.