

Original Research Article

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Characterization of Root Knot Nematode- Fungal Disease Complex of Pomegranate Isolated from Coimbatore District of Tamil Nadu, India

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ABSTRACT

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Root-knot nematode (*Meloidogyne* spp.) is the most important nematode with a wide range of host. Pomegranate cultivation has been challenged by a complex of nematode disease complex that involves both root knot nematode and a fungal organism. This study was aimed at identifying the root knot nematode, *Meloidogyne* species and the fungal organism that cause wilt disease in pomegranate. Based on the morphological means using posterior cuticular pattern and molecular characterization using internal transcribed spacer (TW81-AB28) region tools, the root knot nematode was identified as *M. incognita*. The fungal causal organism of pomegranate wilt was identified as *Fusarium* species by conidial and hyphal structures and confirmed by molecular means by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS1 and ITS4.

Introduction

Pomegranate (*Punica granatum*) is a high value horticultural crop, commercially cultivated in the states of Maharashtra, Karnataka, Gujarat, Andhra Pradesh, Tamil Nadu and Rajasthan. Wilt is one of the limiting factors in pomegranate cultivation in all the major growing regions of the country.

Pomegranate wilt was first reported in India from Nashik district in Maharashtra in 1978 and subsequently from Kaladgi and Kanamadi areas of Karnataka in 1988 and Cadapa, Andhra Pradesh in 2002. Somasekhara, (1999, 2006) attributed the disease to *Ceratocystis fimbriata* while Chavan and Dake, 2001 observed wilt in Maharashtra, India in 1998 and reported it to be caused by

Fusarium oxysporum. Root-knot nematodes *Meloidogyne incognita* has emerged as important problem in pomegranate in India, aggravating the incidence and loss caused by wilt disease, as nematodes pave way for the entry of disease causing organisms through the open wounds caused on the roots.

The root-knot nematode, *Meloidogyne incognita* (Kofoid and White, 1919; Chitwood, 1949) and *M. javanica* (Chitwood, 1949), are the economically important nematode species of pomegranate cultivars in the world (Singh *et al.*, 2019). Illangovan and Poornima, 2017 have reported root knot nematode incidence in pomegranate from Tamil Nadu. Root knot nematodes always increase the severity of *Fusarium* wilt (Atkinson's, 1982). The present study was used to identify the root knot nematode *M. incognita* and *Fusarium* disease complex in pomegranate field through morphological and molecular characterization.

Materials and Methods

Morphological characterization of Root Knot Nematode, *M. incognita*

Roots of pomegranate showing galls were stained with acid fuchsin. Fully developed females of *Meloidogyne* were dissected out from the stained roots for the preparation of perineal patterns. Ten adult females were selected randomly and used for species identification by posterior cuticular pattern (PCP) variations.

The variations in the perineal patterns were observed, photographed using Image Analyzing Camera fitted with a Trinocular compound microscope and compared with the descriptions of Jepson (1987), Karssen (2002), and Karssen and Moens (2006).

Molecular characterization of root knot nematode associated with pomegranate

Extraction of DNA from female root knot nematodes

DNA of root knot nematode, *M. incognita* was extracted from female root knot nematode by using worm lysis buffer [WLB; 50 Mm KCL, 10 Mm Tris Ph 8.2, 2.5 Mm MgCl₂ 20 µg/ml proteinase K, 0-45% Tween 20 and 0-0.1% gelatin] (Castagnone-Sereno *et al.*, 1995). Ten females were picked up and transferred to 1.5 ml centrifuge tube containing 25 µl worm lysis buffers. The female were crushed with needle or micropipette tips and centrifuged at 13,500 rpm for 2 min and placed at -80°C for 30 minutes. The samples were then stored at -20°C (Adam *et al.*, 2007).

DNA amplification by Polymerase Chain Reaction

PCR amplification using DNA extracted from root knot females with ITS primers were carried out using a total volume of 25 µl reaction mixture, which contained 2.0 µl of DNA, 1.0 µl of each 10-µM primer (Forward TW 81 GTTTCCGTAGGTGAACCTGC and Reverse AB28-ATATGCTTAAGTTCAG CGGGT, Joyce *et al.*, 1994), 2.5 µl of 10X buffer, 2.5 µl 200 Mm of each dNTP and 2 units of Taq polymerase enzyme and made up to 25 µl (Adam *et al.*, 2007). The PCR amplification conditions used for each primer sets were initial denaturation at 94 °C for 2 min followed by 45 cycles at 94 °C for 20 s, 62° C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 7 min. Amplified PCR products were separated on an agarose gel (1 % w/v) in 1X TAE buffer at 65 V for 150 min. The DNA profile was visualized in a gel documentation unit. The gel was documented with the help of GELSTAN 4X™ documentation unit. They were then

eluted and further sequencing carried out at Agri genome Biotech Pvt Ltd, Kerala.

Sequence of ITS rDNA gene

The PCR products were sequenced at Agri genome Biotech Pvt Ltd, Kerala. The sequence of the ITS-rDNA region of root nematode female, *M. incognita* was compared with pair wise sequences available in GenBank through Nucleotide BLAST (Basic Local Alignment Search Tool). The similarity index was calculated and used for cluster analysis and construction of phylogenetic tree based on maximum neighbour joining method.

Isolation and characterization of fungal pathogen associated with disease complex

The fungal pathogen *Fusarium* sp. were isolated from the roots of wilt infested pomegranate plants (*Punica granatum* var. Bhagwa) collected from pomegranate growing regions of Tamil Nadu. Infested root samples were sterilized by dipping in 10% (w/v) sodium hypochloride solution for 3-5 min and washed thrice with sterile water. The root was cut with a sterile blade and four pieces of diseased vascular tissue (5×5 mm) placed on the surface of Potato Dextrose Agar (PDA) media. Streptomycin sulphate was added to the PDA to minimize chances of bacterial contamination. Plates were incubated at 28±2°C for five days. After five days of inoculation, fungal growth was observed and the fungi were purified by single hyphal tip technique (Tutte, 1969). The fungi were identified following sporulation and pure culture stored at 4°C on PDA slants (Fig. 2a) and (Fig. 2b).

Morphological characterization

For morphological identification, single spore isolates were grown for 10-15 days on PDA

medium. *Fusarium* culture was multiplied by 10-15 days old PDA cultures. Microscopic features of conidia, conidiophores and chlamydo spores were also determined based on Summeral *et al.*, (2003).

Isolation of fungal DNA

Fungus was grown in potato dextrose broth for seven days at room temperature for mat formation. The mycelial mat was dried on a country filter paper, ground using liquid nitrogen and transferred to 1.5 ml eppendorf tube (100-200 mg). Added 100 µl of CTAB buffer, mixed and incubated @ 65°C for 10 min/ water bath. Half of the contents were transferred to a new tube. Added 750 µl of chloroform: Isoamylalcohol 24:1) and mixed well by inverting the tubes and centrifuged @ 10,000 rpm 10 min. Transferred 300 µl of the supernatant for incubation. Added 150 µl of 5M NaCl₂ into 600 µl of ice cold isopropanol, mixed well and incubated @ -20°C overnight. This was further centrifuged @ 13,000 rpm for 10 min. The supernatant was discarded and the pellets air dried. Washed it with 75 % ethanol twice and resuspended the DNA pellets in 50 µl Tris EDTA buffer or double sterilised distilled water. The RNase (1mg/ml) was added and incubated at 37°C for 1 hour and stored at -20°C.µl

Molecular characterization of using ITS primer

Molecular identification of *Fusarium* cultures were carried out based on conserved ribosomal internal transcribed spacer (ITS) region. The amplification of the ITS regions using universal primer pairs ITS1 (50-TCCGTA GGTGAACCTGCGG-30) and ITS4 (50 TCCTCCGCTTATT GATATGC-30) (White, 1990). Amplification was performed on a Thermal Cycler (Applied Biosystems 9700) with 25 µl reaction mixture containing 2.5 µl of 10X buffer (10 mM Tris–

HCl, pH 8.8); 2.5 mM MgCl₂; 2 mM each of dNTP; 25 pmol ml⁻¹ primer (each of ITS-1 and ITS-4); 1U of Taq DNA Polymerase; 60–100 ng genomic DNA. The amplification cycle consisted of an initial denaturation at 95 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 8 min. Amplified PCR products were separated on an agarose gel (1.5% w/v) in 1X TAE buffer at 65 V for 150 min. They were then eluted and further sequencing carried out at Agri genome Biotech Pvt Ltd. Kerala.

ITS data analysis

The ITS nucleotide sequence for isolate were then compared to those in the public domain databases NCBI (National Centre for Biotechnology information; www.ncbi.nih.gov) using Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). Alignment of ITS DNA sequences was done using CLUSTAL W programme.

Results and Discussion

Morphological characterization of Root Knot Nematode, *M. incognita*

The Posterior Cuticular Patterns of *M. incognita* were characterized by the presence of a high, squarish dorsal arch contains a distinct whorl in the tail terminal area. The striae were smooth to wavy, sometimes zigzagged on dorsal and lateral side. Lateral field were not clear. The pattern confirmed its similarity with the original description of *M. incognita* by Tesařová *et al.*, (2003).

Molecular characterization of Root Knot Nematode, *M. incognita*

The isolate of root knot nematode, *M. incognita* were characterized through PCR using ITS rRNA gene. The amplicon size of

approximately 730 bp was amplified corresponding to ITS rRNA region (Fig. 3a). The PCR product were resolved on 1.2% agarose gel, purified and sequenced at by Agri genome Biotech Pvt Ltd, Kerala, India. The sequence analysis of isolate through BLAST search revealed that they had nucleotide sequence identity of 81.49% with their existing isolate available in NCBI databases. The result confirmed the species of root knot nematode as *M. incognita* and was submitted in NCBI GenBank with accession numbers of TNAUP1-MW111209. Thus it is summarised that the root knot nematode was identified as *M. incognita* based on morphological and molecular tools.

Phylogenetic analysis of ITS region of *Meloidogyne* sp.

The neighbour joining tree was constructed with the closely related organism of *M. incognita* using MEGA 7 software. The phylogenetic analyses based on the ITS region sequences of *M. incognita* and the cluster was formed. The *M. incognita* formed in the single cluster of *M. incognita* (MW111209) (Fig. 3b).

Morphological characterization of *Fusarium* sp.

The pathogen was isolated from the root and stem bits of infected roots using Potato Dextrose Agar (PDA) medium by half plate technique. The pathogen produced a dense, milky white to light pink coloured fluffy mycelial growth on petridish. The pathogen when observed microscopically by observing their two types of conidia *viz.*, micro and macro conidia. Micro conidia were small, oval shaped, hyaline and single or bicelled. Macro conidia were sickle shaped hyaline and multicelled with three to five septum. Abundant chlamydospores were observed terminally and as intercalary (Fig. 1). The

microconidia were hyaline, single celled consisting a septae and slightly curved $16.2 - 17.4 \times 3.6 - 3.8 \mu\text{m}$. Macro conidia were sickle shaped with single hyaline with three septa measuring $35.0 - 36.5 \times 3.5 - 3.7 \mu\text{m}$. The Chlamydospores were intercalary or terminal in position that were produced singly and sometimes in pairs measuring $11.70 - 18.65 \times 10.94 \mu\text{m}$. The above features matched with those of *Fusarium* spp.

Molecular identification and sequence analysis of ITS region of *Fusarium* sp.

PCR was performed to identify the *Fusarium* sp. using ITS1 and ITS4 region. The results revealed that the isolate were amplified with amplicon size of approximately 560 bp corresponding to 18S-28S rDNA region (Fig. 4a). The PCR products were resolved on 1.2 % agarose, purified and sequenced at Agri

genome Biotech Pvt Ltd, Kerala, India. The sequence analysis of *Fusarium oxysporum* isolate revealed that they had nucleotide identity 100 % with existing *F. oxysporum* isolate from NCBI database. The isolate TNAUFP1-MW092746 was identified as *F. oxysporum* and the isolate was submitted in NCBI GenBank.

Phylogenetic analysis of 18S-28S rDNA region of *Fusarium* sp.

The neighbour joining tree was constructed with the closely related organism of *Fusarium* sp. collected from NCBI database. The phylogenetic analysed based on the 18S-28S sDNA sequence of *Fusarium oxysporum* revealed that cluster were formed. The clusters formed with five isolates of *F. oxysporum* (MW092746) were formed in cluster (Fig. 4b).



A - Micro conidia B - Chlamydospores

Fig.1 Morphology of *Fusarium* sp.

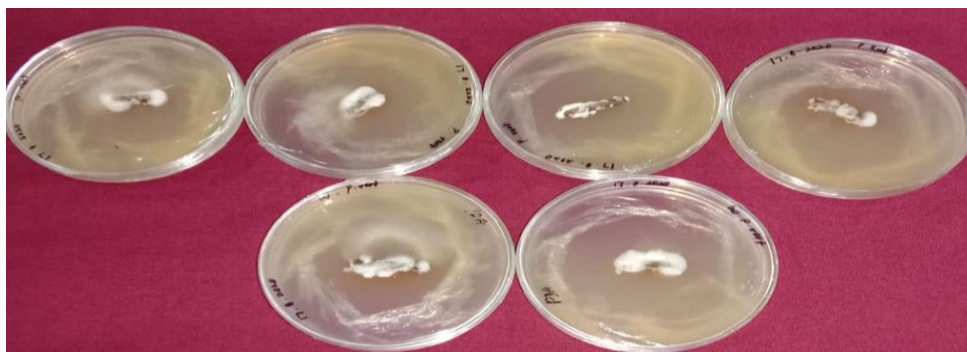


Fig.2a Isolation of *Fusarium* sp. from root bits



Fig.2b Growth of *Fusarium oxysporum*

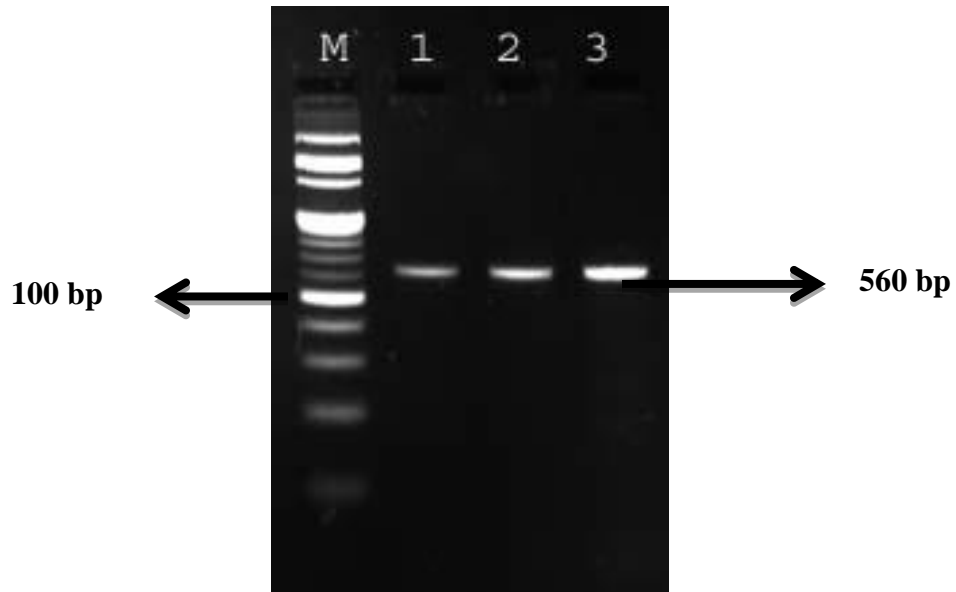


Fig.3a PCR amplification of 18S rDNA region of *F. oxysporum*

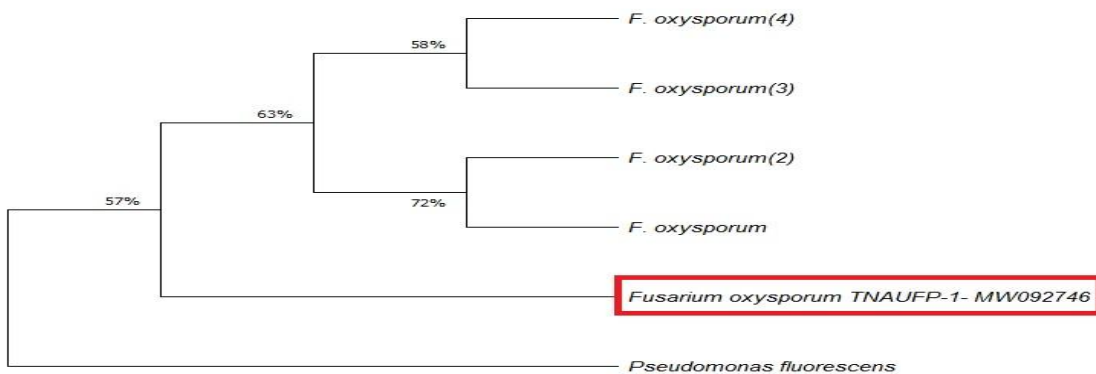


Fig.3b Phylogenetic analysis of *F. oxysporum*

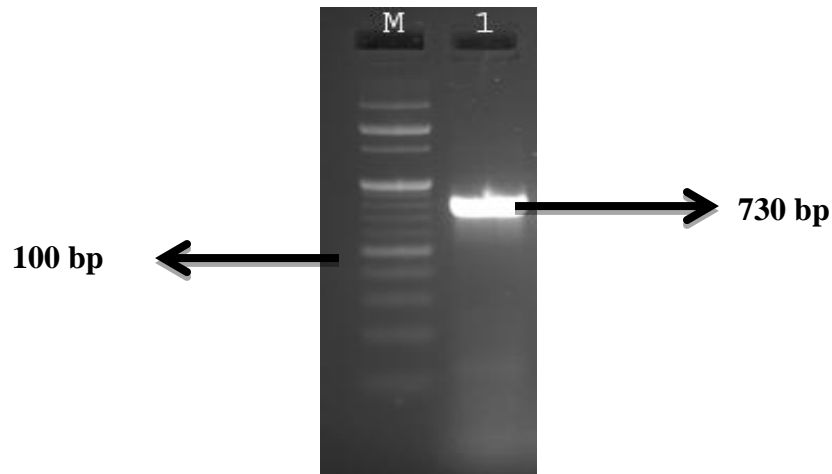


Fig.4a PCR amplification of ITS rDNA region of *M. incognita*

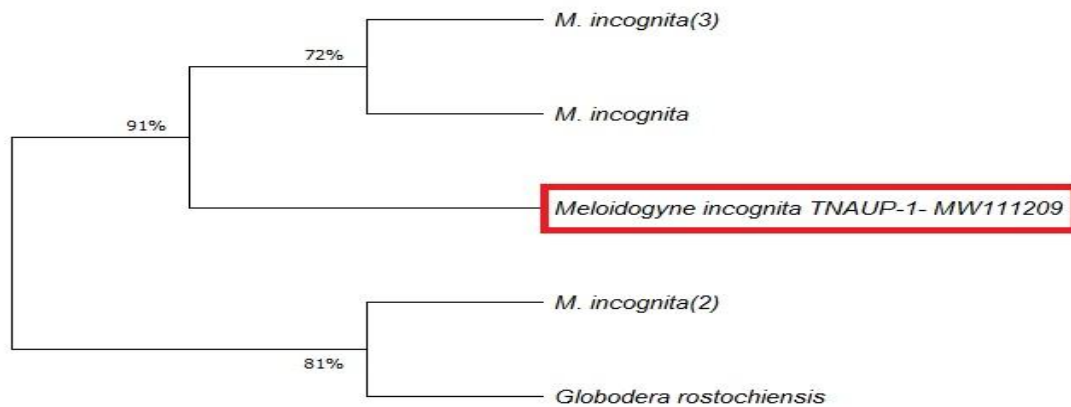


Fig.4b Phylogenetic analysis of *M. incognita*

A survey was undertaken by Sudheer *et al.*, (2007) in major pomegranate (cultivars Ganesh, Mridula and Bhagva) growing areas in Anantapur district of Andhra Pradesh, India during kharif 2006 to assess the nematode problems. The intensity of root-knot nematode damage increased with an increase in age of the plant. In general, the more than five-year-old plants were severely affected by root-knot nematode. The highest juvenile population (370/200 g soil) was recorded in Madakasira mandal. Huge egg masses were observed inside as well as outside the galls.

In the present study, wilted pomegranate plants showed typical wilt symptoms *viz.*,

stunting, yellowing of leaves, defoliation, reduced fruit size due to improper nutrient uptake and premature leaf and fruit shedding. Similar symptomology with predominance of root knot nematode in wilt affected plants were documented by Poornima *et al.*, (2015) in pomegranate; Sharma *et al.*, (2010) in pomegranate; Ansari and Ahmed (2000) in guava; Senthilkumar and Rajendran (2005) in grapes; Patel *et al.*, (2007) in agricultural crops; Devi (2007) in pineapple and Srinivasan *et al.*, (2011) in banana. Suresh *et al.*, (2018) noticed yellowing of the leaves with inter-venial chlorosis, general drooping of the leaves, complete wilting of plants with almost dried leaves and small dried black

fruits hanging on the branch in guava plants infested with *Meloidogyne enterolobii*. Ilangovan and Poornima (2017) reported that the perineal pattern of all the root knot nematode females showed the typical characters of high squarish dorsal arch containing distinct whorl in the tail terminus, smooth/wavy striae, forks in the striae at the lateral sides. Hence the nematode was identified as *M. incognita* based on the conventional method using PCP and head regions of juveniles.

Chavan and Dake (2001) reported pomegranate wilt due to *Fusarium oxysporum* from the Ahmednagar and Solapur districts of Maharashtra, India. Wilt surveys of major pomegranate areas in India carried out Sharma *et al.*, 2010 during 2005-09 revealed disease prevalence in the states of Maharashtra (49.2%), Karnataka (61.11%) and Andhra Pradesh (8.69%). Wilt was prevalent on all important cultivars of all ages from 2-20 years. They observed *C. fimbriata* was observed to be the main cause of pomegranate vascular wilt apart from *Fusarium* sp. A dry root rot disease of pomegranate re-ported from Maharashtra, India was found to be caused by *Fusarium solani* (Kore and Mitkar 1993).). Isolations from the root and collar stem portion of the wilt-infected plants occasionally revealed association of *Fusarium* spp. (*F. solani* and *F. oxysporum*) and the pathogen was found to cause root rot and wilt, particularly in young plants (Anonymous, 2008). The entire tree eventually dies off within few weeks to display complete wilting. Wilt symptoms can at times spontaneously appear causing immediate senescing of the entire plants' foliage at once. Infected plants reveal dried foliage and fruits attached to the branches for many months (Sharma *et al.*, 2010). The xylem area becomes dark reddish-brown to purple deep-brown or black staining. The fungus can spread between adjacent trees and

at times randomly across different locations in the orchard. The disease can be managed efficiently by integrated management practices involving sanitation, cultural methods, chemical control and use of resistant cultivars (Sharma *et al.*, 2010).

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