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Morphological identification of root-knot nematode and fungi associated with guava decline

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Abstract
The survey results revealed that *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *psidii* were identified as the most prominent pathogens causing guava decline in Hisar, Jind and Bhiwani districts of Haryana. Morphometric observations were used for identification of the major root-knot nematode obtained from the survey. Posterior cuticular pattern of adult females and morphological characters of males and second stage juveniles were also used for species level identification. Morphological identification of fungus associated with guava decline was identified as *F. oxysporum* f. sp. *psidii*.

Keywords: *Fusarium oxysporum*, guava, *Meloidogyne incognita*, morphological, survey

Introduction
Guava (*Psidium guajava* L.) is one of the most important commercial fruit crops grown in India and is known as poor man's fruit. It is native to tropical America, particularly in the region between southern Mexico and northern South America. As per estimates of 2019, the worldwide production of guava was 59.73 MT. India is the world's largest producer of guava followed by Indonesia and Mexico [1]. In India, 4.469 million MT of guava is produced annually on an area of 3.10 lakh hectares (DAC&FW, Horticulture statistics section, 3rd advance estimate 2020-2021). With a production share of about 21.78% of India's total guava production, Uttar Pradesh has the maximum production followed by Madhya Pradesh, Bihar, Andhra Pradesh, Haryana, and Punjab [2]. Guava is well known for its antioxidant dietary fiber and polyphenols, which promote health. These bioactive compounds possess significant antimutagenic and antiviral properties, contributing to their ability to counteract genetic mutations and inhibit viral activity [3]. Guava production is declining due to a disease complex involving parasitic nematode *Meloidogyne* spp and fungus *Fusarium oxysporum* f.sp. *psidii* [4]. The complex infestation of *Meloidogyne* spp. and *F. oxysporum* f.sp. *psidii* causes the sudden death of plants and severe loss in terms of quality and quantity of fruits [5]. Managing guava decline requires isolation, identification, and pathogenicity testing of *F. oxysporum*. Isolation and early detection of *F. oxysporum* from guava roots are essential for understanding the guava wilt complex. It plays a crucial role in disease epidemiology, host-pathogen interaction, and integrated disease management strategies. Accurate identification of *Meloidogyne* spp. is essential for effective nematode management in guava orchards.

Materials and Methods
Preparation of slides for nematode identification
The killing, fixing, and clearing method was used to process the nematode suspension that was extracted from the samples to prepare permanent slides [6]. An equivalent volume of boiling fixative (8% formalin) was added to the vials to kill and fix the nematodes. Nematodes were killed and fixed for additional processing to prepare permanent slides after the lid was securely closed and left for a full day. After transferring nematodes to a cavity block that contained Seinhorst's solution I, the partially covered cavity blocks were kept in the oven for 12 hours at 40 ºC. The cavity block was then partially covered, filled with Seinhorst's solution II, and placed in the oven for 4 hours at 40 ºC.

Then the nematode was transferred from the hollow block to a sanitized glass slide, and a drop of glycerol was placed to make permanent slides. A circular coverslip was carefully fitted over the glycerol drop after placing the tiny pieces of wax.

Identification of root-knot nematode species

The root samples infected with RKNs were washed in running tap water. After being chopped into tiny pieces of two centimeters, infected roots were boiled in 0.1% acid fuchsin lactophenol for two to three minutes. To de-stain the roots, the roots were kept in plain lactophenol overnight after being well-cleaned under running water. Under a stereo binocular microscope, adult females were removed from the root galls, their posterior portion was cut and their internal organs were cleansed. The perineal pattern was put on a glass slide with a drop of lactophenol and a cover slip was placed on it and sealed with nail polish. The species confirmation was done based on the perineal pattern [7].

Isolation and identification of fungi

The associated fungi were isolated from infected guava plant parts and rhizospheric soil on potato dextrose agar medium (PDA). Pure culture was obtained from the single spore method and was maintained on PDA slants. The roots were cleaned and washed in water to remove soil particles and were surface sterilized by using 0.1% sodium hypochlorite for two to three minutes and then rinsed with sterile distilled water. The small bits of roots were transferred to Petri plates containing sterilized PDA medium, and they were then incubated for seven days at 26±1^{0}C. Colonies were examined under a compound microscope for identification of fungus. The fungus was identified based on mycological observations such as colony color, mycelial growth, and micro and macro conidia [8].

Results and Discussion

Morphological characterization of *M. incognita* population

Morphological characterization is a fundamental approach to identifying and differentiating RKN populations. In the present study, five nematode specimens of each stage (male, female, and juveniles) from each district (Jind, Hisar, and Bhiwani) were isolated from infected guava roots and surrounding rhizosphere soil. The extracted juveniles, males, and adult females were examined under a compound microscope for key morphological characters. Morphological characterization primarily involves the examination of key diagnostic features such as perineal patterns of adult females, morphometric traits of second stage juveniles (J2s), and stylet structure. The identification was based on the length of the males, tail morphology, and perineal pattern analysis of adult females, which included observations on the shape and arrangement of striae, dorsal arch configuration, etc. Second stage juveniles (J2s) were characterized by body length, stylet length, tail shape, and hyaline tail terminus. Morphometric measurements were recorded and compared with standard diagnostic keys for *M. incognita* identification.

Female: The variations among the posterior cuticular patterns were observed, all the population showed a high dorsal arch, wavy striae, and distinct dorsal lines (Figure 1). Females were pear-shaped, without posterior terminal protuberance. These measured 498-650 µm in length and 350-640 µm in width (Table 1). The neck protruded anteriorly, with a length of 107-250 µm. The maximum body diameter (MBD) ranged from 238-520 µm. The stylet was 10.7-13.1 µm long with a conus that varied from straight to curved dorsally. The stylet knobs were rounded or slopping posteriorly having 1.5-3.1 µm length and 2.8-4.9 µm width with excretory pores located posterior to the stylet knobs. The perineal pattern was rounded to oval-shaped having a high dorsal arch with vulva slit length of 15-23.7 µm. These morphometric parameters provide valuable taxonomic insights for species delimitation and phylogenetic studies.

Male: The morphological characterization of vermiform males revealed distinct diagnostic features crucial for taxonomic identification (Figure 2). The vermiform males had 1100-1380 µm body length (Table 2). The head cap was high with a large labial annulus. The lip width and length were 4.1-5.8 and 8.5-11.5 µm, respectively. The stylet was robust, measuring 15.4-19.4 µm in length with pointed conus that was smaller than the shaft and knobs. The dorsal esophageal gland opening (DEGO) was positioned at 3.5-7.4 µm. The spicules were slightly curved with a length and width of 15-36 µm and 2.8-4.5 µm, respectively. The tail was short and bluntly rounded with a length of 8.7-18.6 µm. The observed morphological characteristics aligned with previously described species within the genus, reinforcing their significance in nematode systematics and biodiversity studies.

Second stage juveniles (J2s): The J2s had a vermiform, slender body that tapered at both ends. The body length varied from 320-412 µm (Table 3). The labial region was weakly sclerotized and continuous to the body with lip length and width of 3.5-5.7 µm and 2.2-4.2 µm, respectively. The stylet was weakly sclerotized with small and oval knobs slopping backward. The length of the stylet ranged from 9-12.2 µm having knob length and width of 1.3-2.3 µm and 2.2-4.2 µm, respectively. The metacorpus was oval with a heavily sclerotized valve, playing a crucial role in regulating esophageal pumping and food passage. The pharyngeal gland exhibited variability in length and overlapped the intestine ventrally. The tail was conoid, measuring 35-64 µm, gradually tapered towards the terminus, serving as an important taxonomic characteristic (Figure 3).

Table 1: Morphological characteristics of females of *Meloidogyne incognita* infecting guava orchards of Hisar, Jind, and Bhiwani districts

Measurements (µm)	(n=5)		
	Hisar Population	Jind Population	Bhiwani Population
Body length (L)	597.0±65.0 (508-609)	598.0±35 (500-601)	610.0±137.0 (498-650)
Body width	450.0±60.3 (322-560)	480.0±74.2 (400-606)	508.0±102.9 (350-640)
Neck length	169.0±34.0 (108-235)	192.0±40.0 (107-240)	212.0±22.5 (192-250)
Maximum body diameter (MBD)	470.0±52.0 (349-520)	349.0±80.0 (238-492)	464.0±55.0 (352-510)
a=L/MBD	1.2±0.2 (1.3-16)	1.6±0.37 (1.4-2.0)	1.2±0.2 (1.1-1.6)

Lip length	1.7±0.1 (1.3-2.2)	2.4±0.2 (1.7-2.5)	2.8±0.2 (2.6-3)
Lip width	3.8±0.6 (3.4-4.8)	4.1±0.7 (3.4-4.8)	4.6±1.1 (3.8-6.3)
Stylet length	11.2±0.5 (10.7-12)	12.1±1.2 (11.2-13.1)	12.4±0.1 (12.3-12.6)
Stylet knob length	1.9±0.3 (1.5-2.1)	2.5±0.4 (1.9-3)	2.3±0.5 (1.8-3.1)
Stylet knob width	3.8±0.3 (2.8-3.4)	4.5±0.5 (3.8-5.3)	4.2±0.6 (3.4-4.9)
DEGO	4.2±0.9 (3.8-4.6)	3.4±1.2 (3.2-4.2)	4.6±0.8 (3.6-5.3)
Distance from anterior end to excretory pore	18.4±0.5 (17.5-18.5)	17.8±0.8 (17.2-18.5)	20.4±5.0 (15.6-24)
Distance from anterior end to end of metacorpus	72±5.9 (71-79)	74±5.6 (72-78)	72±5.7 (70-75)
Vulva slit length	18.3±1.7 (15-19.7)	22.6±1.3 (20.8-23.7)	18.8±1.1 (17.4-20.4)
Vulva- anus distance	17.8±2.0 (15.4-18.5)	19.7±0.6 (17.5-19.9)	18.2±0.7 (17.7-19.4)

All measurements are in micrometers and Mean± SE (range)

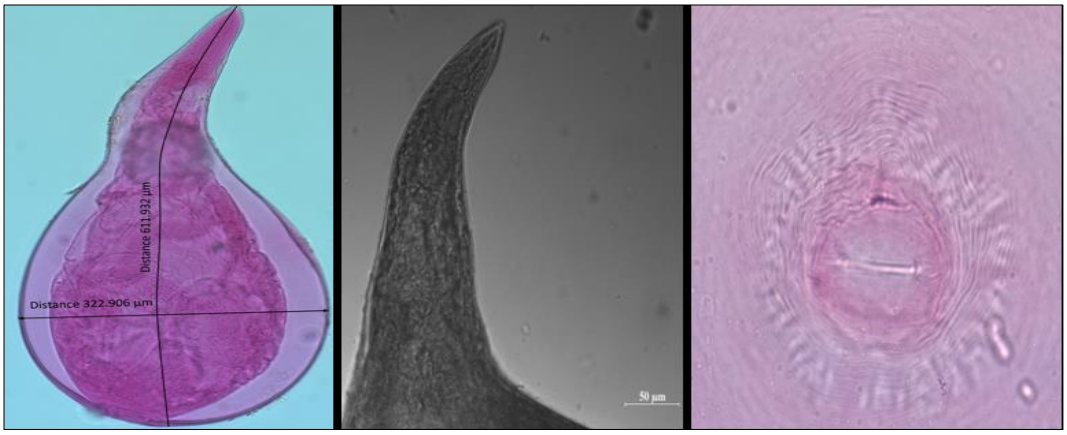

Fig 1: Morphological characteristics of *Meloidogyne incognita* females

Table 2: Morphological characteristics of males of *Meloidogyne incognita* infecting guava orchards of Hisar, Jind, and Bhiwani districts

Measurements (µm)	**(n=5)**		
	Hisar Population	**Jind Population**	**Bhiwani Population**
Body length (L)	1174.0±125.0 (1120-1410)	1143.0±134.0 (1116-1380)	1113.0±142.0 (1100-1220)
Lip width	5.3±0.4 (4.8-5.8)	4.9±0.5 (4.1-5.8)	4.1±0.7 (3.7-5.6)
Lip length	9.7±0.6 (8.5-10.3)	9.6±1.1 (8.7-11.5)	9.2±0.4 (8.7-9.7)
Stylet length	17.8±0.8 (16.6-19.4)	16.9±1.2 (15.4-18.2)	18.1±0.8 (16.5-18.7)
DEGO	5.9±1.7 (4.8-7.4)	4.8±0.9 (3.5-5.9)	5.4±1.8 (4.7-7.2)
Distance from anterior end to end of metacorpus	82.0±9.8 (66-95)	71.0±12.0 (58-92)	84.0±3.1 (79-87)
Distance from anterior end to pharyngo-intestinal junction	110.0±10.3 (87-125)	104.0±5.1 (102-114)	109.0±0.9 (107-111)
Distance from anterior end to excretory pore	128.0±14.4 (108-142)	147.0±11.6 (138-165)	136.0±13.6 (108-140)
Maximum body diameter (MBD)	25.8±1.6 (23.4-26.7)	28.1±4.4 (22.5-35.8)	24.5±2.1 (22.4-26.7)
Anal body diameter (ABD)	21.5±3.6 (17.1-26)	19.3±1.1 (17-20.3)	18.8±1.2 (18-20.5)
Tail length	13.2±2.4 (8.7-18.6)	10.6±1.3 (10.5-13.7)	11.4±1.2 (10.5-13.5)
Spicule length	24.2±5.8 (16.9-36)	25.8±4.6 (20.9-34)	25.6±6.9 (15-30)
Spicule width	3.4±0.3 (3.2-3.9)	3.8±0.5 (3.1-4.5)	3.6±0.5 (2.8-4.4)
a=L/MBD	45.3±7.9 (37-60)	40.5±8.2 (36-62)	45.4±7.9 (38-61)
b=L/Distance from anterior end to pharyngo-intestinal junction	10.4±1.9 (9.6-13.7)	10.9±1.8 (9.4-13.6)	10.2±1.9 (9.7-13.7)
c=L/tail length	88.9±10.6 (66-95)	107.8±22.9 (62-145)	97.6±9.9 (65-102)
c'=tail length/ABD	0.61±0.1 (0.5-0.6)	0.54±0.2 (0.4-0.7)	0.60±0.1 (0.4-0.7)

All measurements are in micrometers and Mean± SE (range)

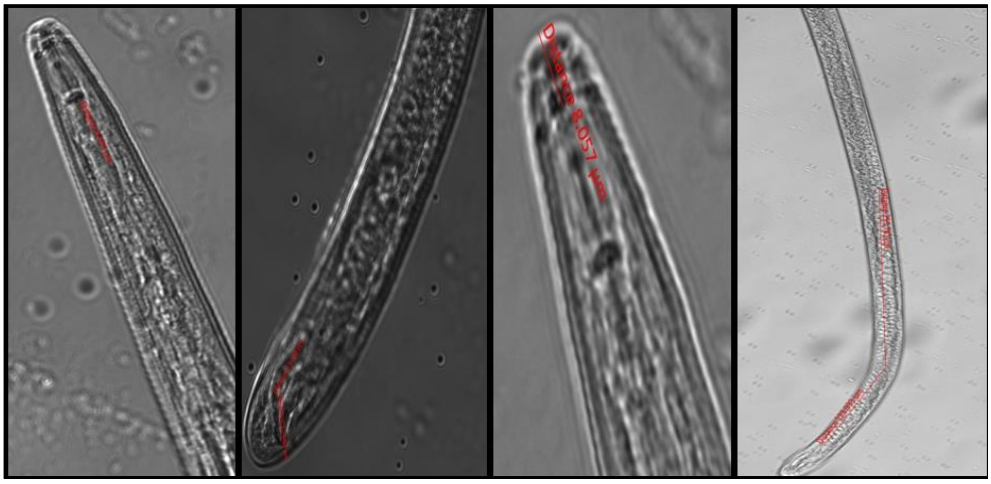

Fig 2: Morphological characteristics of *Meloidogyne incognita* males

Table 3: Morphological characteristics of juveniles of *Meloidogyne incognita* infecting guava orchards of Hisar, Jind, and Bhiwani districts

Measurements (µm)	(n=5)		
	Hisar Population	**Jind Population**	**Bhiwani Population**
Body length (L)	377.0±20.9 (335-410)	419.0±9.0 (320-400)	383.0±12.6 (365-412)
Body width	14.2±2.4 (14.1-16.8)	15.3±1.5 (13.5-17.8)	15.1±2.0 (13.2-17.9)
Lip length	4.1±0.3 (3.5-4.7)	4.6±0.6 (4.1-5.6)	4.7±0.5 (4.1-5.7)
Lip width	3.2±0.4 (2.6-3.7)	2.9±0.4 (2.2-3.7)	3.4±0.4 (2.3-4.2)
Stylet length	10.9±0.7 (9.1-12.1)	10.4±0.7 (9-11.7)	10.7±0.8 (9.2-12.2)
Stylet knob length	1.6±0.4 (1.4-2.3)	1.4±0.5 (1.3-2.2)	1.9±0.2 (1.6-1.9)
Stylet knob width	3.4±0.6 (2.3-4.2)	2.8±0.7 (2.2-3.8)	3.1±0.7 (2.3-4.2)
DEGO	2.8±0.3 (2.2-3.2)	3.2±0.4 (2.4-3.6)	3.1±0.5 (2-4.5)
Maximum body diameter (MBD)	12.8±1.1 (11.4-14.8)	11.9±0.9 (10.7-13.7)	12.6±2.2 (10.2-17.5)
Anal body diameter (ABD)	10.2±2.1 (6.1-14.3)	9.8±0.8 (7.5-10.5)	10.1±2.1 (6.4-13.8)
Tail length	48.0±6.9 (35-57)	47.0±7.8 (34-64)	47.0±6.9 (35-56)
Distance from anterior end to pharyngo-intestinal junction	65.0±7.4 (63-70)	63.0±7.5 (61-82)	67.0±7.3 (59-80)
a=L/MBD	29.4±4.9 (20.5-40.2)	35.2±1.6 (25-37)	30.3±4.8 (20.6-38)
b=L/Distance from anterior end to pharyngo-intestinal junction	5.8±0.5 (4.2-6.5)	6.65±0.4 (4.7-7.1)	5.71±0.6 (4.1-6.5)
c=L/tail length	7.85±1.3 (6.5-11.3)	8.91±1.1 (5.8-10.8)	8.14±1.5 (5.9-10.6)
c'=tail length/ABD	4.70±0.7 (3.7-5.8)	4.79±0.5 (4.1-6)	4.65±0.7 (3.5-5.9)

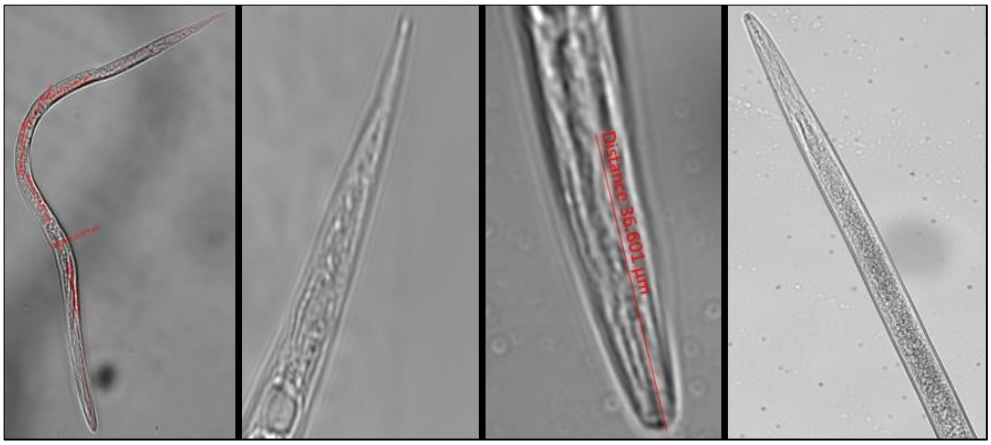

Fig 3: Morphological characteristics of *Meloidogyne incognita* second stage juveniles

Morphological characterization of *Fusarium oxysporum*

For the identification of *F. oxysporum*, macroscopic and microscopic characteristics were studied. Variations were observed in the macroconidial shapes and sizes, apical and basal cell morphology, and cultural appearances (Table 4). Macroconidia were single and sickle shaped with length and width of size from 23.75-35.43 and 3.730-4.534 µm respectively. They were predominantly falcate to nearly straight, usually 3-septate, though occasionally 4-5 septate. These macroconidia were thin-walled, pointed at both ends, and in some cases, slightly curved. The microconidia were hyaline to colorless, with shapes varying from cylindrical to oval (Figure 4). Chlamydospores appeared thick-walled and were found singly or in pairs. The cultural appearance of the

isolate was sparse to abundant cottony mycelium with colony color and pigmentations ranging from pale violet to peach-violet. Based on morphological characteristics, the isolate was compared with the description provided by Booth (1971) and was confirmed as *F. oxysporum*.

Table 4: Morphological characterization of *F. oxysporum* associated with guava decline

Macroscopic characteristics	
Colony colour	Pale violet, dark violet to peach-violet.
Pigmentation	Peach violet color
Microscopic characteristics	
Apical cell morphology	Tapered and curved
Microconidia	Cylindrical (colorless)
Length of macroconidia	23.75-35.43
Width of macroconidia	3.730-4.534
Macroconidia septation	3-5 (3 most common)

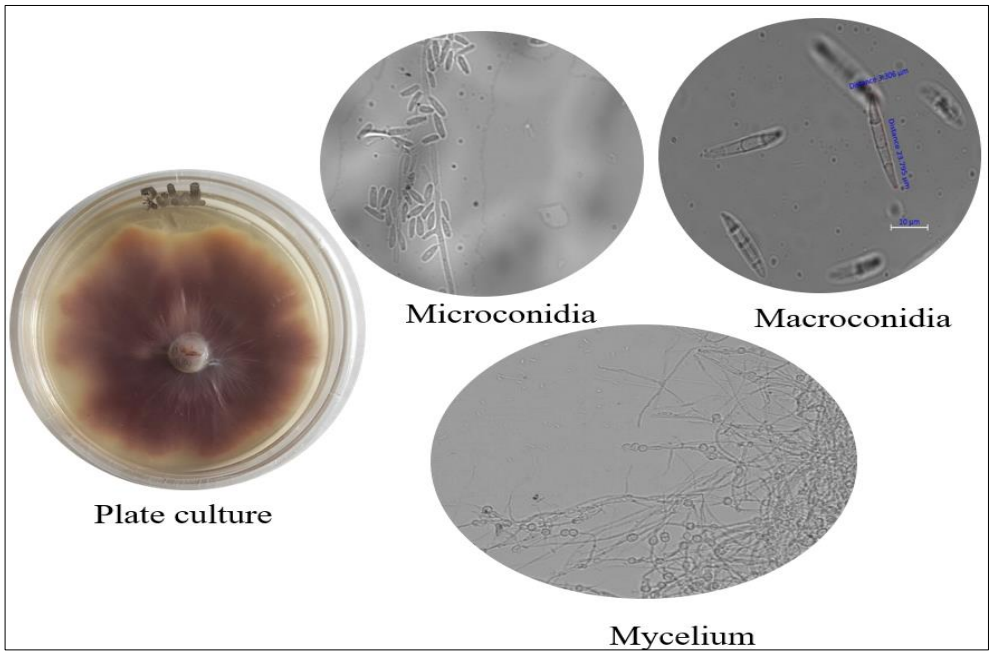

Fig 4: Cultural and morphological characteristics of *Fusarium oxysporum*

One of the most important diagnostic characteristics for identifying *Meloidogyne* species is the perineal pattern of mature females [9]. The current study uses morphological traits to confirm that *M. incognita* was found in guava orchards in Jind, Hisar, and Bhiwani districts. Perineal pattern analysis continues to be one of the most reliable diagnostic methods, confirming that populations of *M. incognita* have morphological characteristics that are well-preserved across several sites [10]. Our results showed wavy striae and a high dorsal arch, which are characteristics of *M. incognita* [11]. The identification was further supported by the lack of lateral lines, which is a characteristic that distinguishes *Meloidogyne* species [10]. The measurements of female body size, such as length (498-650 µm) and width (350-640 µm), were in agreement with earlier findings of Handoo *et al*. 2012 [12]. The species identity is further supported by the stylet length (10.7-13.1 µm) and structure, especially the posteriorly sloping knobs [13].

The morphological characteristics of vermiform males play an essential role in taxonomic classification. In this study, the body length (1100-1380 µm) and robust stylet (15.4-19.4 µm) were within the range described by Eisenback *et al*. 1981 [13]. The high head cap and large labial annulus observed in our populations were typical of *M. incognita* males. The slightly curved spicules, ranging from 15-36 µm in length, match previous descriptions and reinforce the taxonomic consistency of our findings [10]. The J2s are crucial for diagnosing *Meloidogyne* spp. due to their uniform morphology across populations. The juvenile body length (320-412 µm) observed in this study falls within the typical range reported for *M. incognita* [7]. The weakly sclerotized labial region, stylet length (9-12.2 µm), and conoid tail (35-64 µm), particularly tail length and esophageal structures, are the morphological traits that have been widely used in nematode taxonomy and remain reliable markers for species differentiation [13].

Morphometric parameters found in our study are similar to those found in previous investigations of *M. incognita* from various geographic locations [12]. According to Perry *et al*. 2009[14], environmental variables and host-induced phenotypic plasticity may be the cause of measurement variations, such as slight variations in stylet and tail length. The morphometric parameters align with classical descriptions, supporting their continued use as diagnostic tools in nematology. For nematode taxonomy, morphological characterization is still a popular method, especially in areas where molecular diagnostics are not easily available. The current study offers important insights into the variety and distribution of *M. incognita* by providing a thorough morphometric characterization of populations isolated from major growing

guava orchards in Jind, Hisar, and Bhiwani districts of Haryana.

The morphological characterization of *F. oxysporum* f.sp. *psidii* associated with guava decline showed notable differences in microscopic and cultural traits. The color of the colony varied from pale violet to peach violet, with matching pigmentation, as described by Booth 1971[14]. Mostly falcate to virtually straight, the macroconidia were usually 3-septate but sometimes 4-5-septate, which is consistent with observations by Leslie & Summerell 2006[15]. The chlamydospores were discovered alone or in pairs, the microconidia were hyaline and cylindrical to oval in shape. These observations confirm the morphological characteristics of *F. oxysporum*, as also noted in other studies on different formae species. The identification and characterization of *F. oxysporum* f.sp. *psidii* provided crucial insights into its role in guava decline. This forma specialis is a known vascular wilt pathogen, and its morphological confirmation suggests its significant impact on guava production. Future studies focused on pathogenicity tests, host range determination, and management strategies to mitigate the economic losses caused by this pathogen.

Conclusion

In the present study, the morphological parameters of juveniles, males, and adult females were examined. Morphometric measurements were compared with standard diagnostic keys, and the root-knot nematode was identified as *M. incognita*. The morphometric parameters of *M. incognita*, including body length, body width, stylet length, dorsal gland opening, head end to excretory pore distance, tail length, and body ratios observed in the present study, closely align with the original description. For the identification of *F. oxysporum*, macroscopic and microscopic characteristics were studied. Variations were observed in the macroconidial shapes and sizes, apical and basal cell morphology, and cultural appearances. Based on morphological characteristics, the isolate was compared with the standard description and was confirmed as *F. oxysporum*.

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