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Detection, Diagnosis and Pathogenic Potential of *Meloidogyne incognita* on Passion Fruit from Mizoram, India

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Authors' Contributions
MRK conceived the project, identified the nematode species and supervised the study. SP, AS, PS and SL conducted laboratory processing, microscopic observation and pathogenicity test. GTM conducted biochemical and molecular characterizations under the supervision of MRK and SB. MRK wrote the article.

Key words
Passion fruit, *Meloidogyne incognita*, Identification, Pathogenicity, Mizoram.

ABSTRACT

Root knot nematode (*Meloidogyne incognita*) infects passion fruit (*Passiflora edulis*) in Mizoram, India and infected plants showed declined symptoms and poor fruit yield. Infested plants displayed stunting and yellowing and thick-root symptoms. The association of the root knot nematode species was identified based on observations on morphology of different life stages, beta-esterase phenotype and amplification of rDNA and partial sequence homology of ITS-1and ITS-2. Host differential tests confirmed the race 2 of *M. incognita* infecting passion fruit. Pathogenic relationship with the passion fruit was proved through inoculation studies and inoculated plants also produced almost similar above-ground symptoms and profuse root galling. The pathogenic potential of *M. incognita* on passion fruit was determined; the minimum 10 second stage juvenile (J2)/200 cc soil can cause reduction of plant growth parameters and highest reproduction (R=87.27).

INTRODUCTION

Phytonematodes are commonly associated with passion fruit (Sharma and Loof, 1972; Boesewinkel, 1977; Loof and Sharma, 1979; Milne, 1982). Among them, reniform and root-knot nematodes (*Meloidogyne* spp.) are considered potential threat to cause economic damage in passion fruit (Cohn and Duncan, 1990). Root knot nematode (*M. incognita*) shows variation in virulence to passion fruit (De Villeiers and Milne, 1973) and has been reported to cause of concern for profitable cultivation of passion fruit in Kenya (Ondieki, 1975). In South Africa, root knot nematodes particularly *M. javanica* have been considered as serious pest on yellow and purple passion fruit (Milne, 1982). However, in Fiji, passion fruit has been recommended as a suitable crop for rotation for the control of root knot nematodes as because some genotypes in yellow and purple passion fruit have been reported as resistant to root knot nematodes from Brazil (Klein *et al*.,1984; Costa *et al*., 1997). In India, purple passion fruit (*Passiflora edulis*) are mostly grown in the Nilgiris in the south India and in eastern parts of India. It has been recorded as a host of root knot nematode (Parvatha Reddy *et al*., 1980). However, infestation of root knot nematode on passion fruit has not been studied much from India. Recently, in Khanpui of Aizawl in Mizoram, passion fruit orchard showed decline of plant growth and poor yield of the crop. The preliminary studies confirmed association of root knot nematodes in the root system of declined plants. Therefore, the present study focused to identify nematode species and to establish the association of root knot nematode and formation of typical thick-root symptoms on passion fruit.

MATERIALS AND METHODS

Sample collection and processing of root and nematodes

Infested root samples obtained from orchard of passion fruit grown in Khanpui (GPS: 23°51'23.4468" N/92° 54'13.9140"E), Aizawl, Mizoram, India and

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processed in laboratory. Root samples stained with NaOCl-Acid fuchsin (Byrd *et al*., 1983) and a part of the root sample was cut into a small pieces for extraction of second stage juveniles (J2s) and males following modified Baermann's technique (Whitehead and Hemming, 1965). Egg mass was dissected with the help of a forceps from root sample, individual egg mass teased out on a glass slide with help of a needle under stereoscopic trinocular microscope (Carl Zeiss- Stemi 2000-C) at magnification of 32X to release eggs. Nematode specimens killed by hot-water bath, fixed in 3% formaldehyde and processed by Seinhorst method (Seinhorst, 1959). Specimens were then mounted in anhydrous glycerine on glass slides for morphological studies.

Morphological characterization

For morphological characterization, the most differential and supplementary characters for *Meloidogyne* as detailed by Jepson (1987) and Karssen (2002) were studied. The de-Man ratios and other values were determined from the measurements taken with the help of an ocular micrometer (Hopper, 1986). The species was identified using the techniques described by Taylor and Netscher (1974) and Hartmen and Sasser (1985). The variations in body shape, anterior end and perineal pattern of female; anterior and posterior end of male and juvenile were recorded for characterization of the species. Photomicrographs of males, females, perineal patterns and juveniles were taken by a Color Digital Camera (Cannon, 20IS) attached with a compound microscope (Zeiss-Axioskop 40, Germany).

Isozyme analysis

EST phenotype of root knot nematode population was compared with those of reference population of *M. javanica* from Kalyani, Nadia, following the protocol detailed by Esbenshade and Triantaphyllou (1985). Five young egg-laying females of each nematode population were dissected from roots, macerated in 20 ml of extraction buffer (20% sucrose, 2% Triton X-100 and 0.01% Bromophenol blue) and squashed to release body contents. Electrophoresis was done using mini-polyacrylamide gel (6%) at 4ºC with dual-gel electrophoresis unit (Double Helix, India). The gel electrophoresis was carried out as described by Karssen *et al*. (1995). The gel was stained at 37ºC with a staining solution containing 100 ml of 0.5 mM Tris solution, 100 mg fast blue RR salt (Sigma-Aldrich) and 40mg α-Napthyl acetate (Sigma-Aldrich) dissolved in ethanol. This was further rinsed with distilled water, and fixed for 5 min in a solution of 10% acetic acid, 10% glycerol and 80% distilled water.

Molecular characterization

Nematode DNA extraction

DNA extraction procedure adopted as detailed by Floyd *et al.* (2002). Individual female was transferred directly into 20µL of 0.25m NaOH in 0.2mL tubes, and preserved at room temperature for 3-16 h (Stanton et al., 1998). This lysate was then heated for 3 min at 95°C, 4µL of HCl and 10µL of 0.5mTris-HCl buffered at pH 8.0 added to neutralize the base; 5 µL of 2% Triton X-100 was also added, and finally the lysate was heated for a further 3 min at 95°C and stored at -20 °C.

PCR amplification and sequencing

The KAPA2G Robust PCR Kit (http://www.kapabiosystems.com/public/pdfs/kapa2g-robust-pcr-kits/KAPA2G_Robust_TDS.pdf) was used for PCR reaction using 2µL of each DNA lysate to a 25µL PCR reaction. The primer pair used was MIGF: 5'TTGATTACGTCCCTGCCCTTT3' and MIGR: 5'TTTCACTCGCCGTTACTAACG3' (Vrain *et al*., 1992) obtained from Xcelris Genomics Lab Limited. The reaction condition was as 94°C for 10 min; 38 cycles of (94°C for 30 sec; 55°C for 1 min; 72 °C for 1 min); 72 °C for 7 min. The amplified product after PCR reaction was observed on 1% agarose gel and size was determined against DNA lader of 100bp. Sequencing was performed for both strands from a commercial service (www.Xcelrisgenomics.com, 10A, Shakespeare Sarani, Kolkata). Sequence confirmation matched with the NCBI sequence database using BLAST search (https://www.google.co.in/#q=NCBI+blast).

Host differential test

Population of *M. incognita* were tested on North Carolina host differentials *viz*., tomato cv Rutgers tobacco cv NC-95, cotton cv Delta pine 61, peanut cv Florunner, and pepper cv California wonder (Taylor and Sasser, 1978) for identification of race. They were grown in earthen pot (10cm dia) filled autoclaved soil sandy soil (500cc) under net-house conditions. There were four pots for each host and individual pot contained only on plants. Each plant was inoculated with 10 ml suspension of 500 J2 around the seedlings of 20 day-old and plain water of same volume was given in the uninoculated control plants. Fifty-five day after inoculation, plants were carefully uprooted, washed in tap water to clear adhered soil particles and stained with NaOCl- Acid Fuchsin method (Byrd *et al*., 1983).

Pathogenicity test

Pathogenicity of root knot nematode population infecting passion fruit was tested under net-house conditions during July-Dec, 2014. Inoculum of *M. incognita* was

produced on the passion fruit plants raised in cemented pot (25cm dia). Six week-old seedlings of passion fruit were singly transplanted into 15cm dia plastic pots containing 1000cc sterilized soil, mixed with decomposed farm yard manure. Two weeks after transplantation, each seedling was inoculated with a series of 0, 10, 100, 500, 1000, 5000 and 10000 freshly hatched second stage (J2) of *M. incognita* (Pi) in 10ml of water by pouring around each plant. Each treatment was replicated six times and arranged on cemented platform in randomized block design. General care and maintenance of plants was taken to provide adequate growing conditions for the crop. After six months, individual plant along with their roots gently uprooted and washed in tap water. The root systems of each plant were gently cut from the stem and observable parameters *viz*., shoot length, fresh shoot weight, root length, fresh root weight, galling index (on a 1-5 scale), egg mass index. Root galling severity was assessed on a 1-5 scale (1 = no galls/ egg masses, 2 = 1-10 galls/ egg masses; 3 = 11-30 galls/ egg masses; 4= 31-70 galls/ egg masses and 5 = 70-above galls/ egg masses per root system). Final J2 and male populations were obtained from root system and soil; soil nematodes extracted from soil by modified Cobb's sieving method (Whitehead and Hemming, 1965) and root population (mature and immature females) from the root system stained by NaOCl- Acid Fuchsin method (Byrd *et al*. 1983). The final population (Pf) included root population and soil population. The reproduction factor (R) = Pf/Pi, where Pf represents final and Pi initial population of nematode and pathogenicity was determined according to Vovlas *et al*. (2008).

Statistical analysis

All data were analyzed statistically using SPSS, and mean, standard error (SE) and coefficient of variation (CV) was calculated based on multiple numbers of specimens or samples. Significance of treatments was conducted using Duncan's Multiple Range Test (DMRT) at P≥0.05.

RESULTS

A population of root knot nematode infesting passion fruit in Khanpui of Aizawl district in Mizoram was identified based on studies of morphology and morphometrics of mature female, perineal pattern, male, eggs and second stage juvenile (Fig. 1). Measurements of different life stages of passion fruit population are given below:

Measurements

Female (n=20): L, 629µ±51.60 (510-770); BW, 415µ±27.43 (350-460); a, 2.25±0.17 (2.11-2.63); stylet, 15.94µ±0.76 (15.20-18.05); stylet cone, 9.14µ±0.55 (8.55-10.45); stylet shaft, 5.13µ±0.33 (4.75-5.70); DGO, 3.94µ±0.44 (3.33-4.75); length median bulb, 38.33µ±2.62 (33.25-42.75); width median bulb, 34.39µ±2.72 (29.45-42.75); head-SE pore, 34.91µ±7.78 (26.60-47.50).

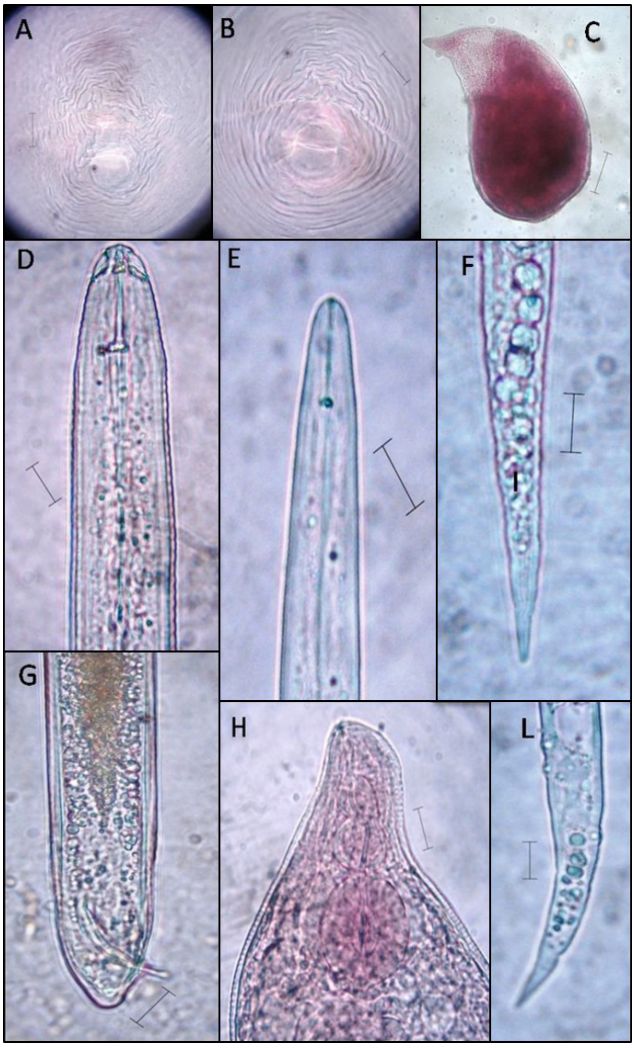

Fig. 1. Morphology of *Meloidogyne incognita* from Mizoram infecting passion fruits: A and B, perineal pattern of mature female; C, mature female; D, male anterior end; E, J2 anterior end; FL, J2 posterior end; G, male posterior end; H, female anterior end. Bar length: AB&H= 20, C=200, DEF&G=10, L=5 microns

Perineal patterns (n=20): length vulva-slit, 23.28µ±1.29 (20.90-25.65); inter-phasmid, 24.94µ±2.52 (20.90-29.45); vulva-anus, 17.03µ±1.99 (15.20-20.90); vulval slit-tail terminus, 26.84µ±2.58 (22.80-32.30).

Male (n=4) : L, 1830±177.76 (1600-2020); BW, 39.90±3.00 (36.10-42.75); a, 45.88±3.22 (42.11-48.78); stylet, 22.56±0.91 (21.85-23.75); stylet cone,

11.88±0.55(11.40-12.35); stylet shaft, 7.72±0.60 (7.13-8.55); length knobs, 2.97±0.24(2.85-3.33); width knobs, 4.87±0.24 (4.75-5.23); DGO, 2.38±0.39 (1.90-2.85); head-metacorpus, 95.71±8.19 (85.50-105.45); b'', 19.11±0.38 (18.71-19.61); head-SE pore, 160.08±12.52 (142.50-171.95); spicule, 30.64±0.91(29.45-31.35).

Second stage Juvenile (n=20): L, 410.50µ±21.51 (365.00-455.50); BW, 13.35µ±0.55 (12.35-14.25); a, 30.80±1.98 (27.98-34.21); stylet, 10.45µ±0.34 (9.98-10.93); DGO, 3.21µ±0.21(2.85-3.33); length median bulb, 12.16µ±0.85 (10.45-13.30); width median bulb, 8.41µ±0.22 (8.08-8.55); head-metacorpus, 55.39µ±2.60 (50.35-59.85); b'', 7.42±0.48 (6.68-8.54); head-SE pore, 82.13µ±3.86 (1.25-86.45); head-esophageal gland, 135.52µ±9.06 (114.00-150.10); b', 3.04±0.26 (2.70-3.55); tail length, 49.50µ±2.20 (46.55-53.20); c, 8.30±0.46 (7.50-9.58); hyaline tail terminus length, 11.97µ±1.09 (10.45-13.30); width anal body, 9.33µ±0.52 (8.55-10.45); c', 5.31±0.28 (4.90-6.11); rectum inflated (n=15).

Egg (n=20): length, 97.50µ±3.44 (95-105); width, 43.50µ±2.35 (40-45); ratio, 2.25±0.17 (2.11-2.63).

Morphological and morphometric variations in the population infecting passion fruit

Female

Measurement of body length (629µ), body width (415µ), stylet cone (9.14µ), stylet shaft (5.13µ), length (38.33µ) and width (34.39µ) of median bulb did not show much variation (CV<10). Stylet length (15.94µ) showed least variation (CV<5); DGO (3.94µ) and a -value (2.25) moderate variation (CV<20); and head to SE pore distance (34.91µ) high variation (CV>20).

Perineal pattern

Striae fine to thicken wavy and zigzagged (Plate 10). Length of vulval slit (20.90 to 25.65µ) and tail terminus to vulva-slit (22.80 to 32.30µ) showed low variation (CV<10); and inter- phasmid (24.94µ) and vulva to anus distance (17.03µ) moderate variation (CV<20).

Male

Body length (1.60 to 2.02mm) and width (36.10 to 42.75µ); stylet shaft (7.72 µ), knobs length (2.97µ), head to metacorpus distance (95.71µ), head to SE pore distance (160.08µ) and a-value (45.88) showed low variability (CV<10); stylet length (22.56µ), stylet cone (11.88µ), knobs width (4.87µ), spicule length (30.64µ) and b'' – value (19.11) showed least variability (CV<5); and DGO (1.90 to 2.85µ) moderate variability(CV<20).

Second stage juvenile

The mean body length and width of J2 were 410.50mm and 13.35µ, respectively. Measurements of stylet length (10.45µ), width of median bulb (8.41µ), head to median bulb (55.39µ), head to SE pore (82.13µ) and tail length (49.50µ) showed least variation (CV<5). Most of the other parameters such as DGO (2.85 to 3.33µ), length of median bulb (10.45 to 13.30µ), head to esophageal gland end (114 to 150.10µ), hyaline tail length (10.45 to 13.30µ), abw (8.55 to 10.45µ) and ratio values (a, b'', b', c and c',) did not show much variation (CV<10). Inflated rectum was found in 15 J2 out of 20 specimens.

Egg

Egg size was 97.50 µ × 43.50µ and length/width ratio varied from 2.11 to 2.63.

Biochemical and molecular characterisation

Isoenzyme patterns of esterase (Est) phenotype was analyzed and partial sequence of rDNA were obtained for further confirmation of species. Enzyme electrophoresis results showed the characteristic Est phenotypes of 'I1' (single major band) of *M. incognita* (Fig. 2A). Amplification and sequencing of the partial rDNA gene was accomplished with primers: one primer (F: 5'TTGATTACGTCCCTGCCCTTT3'), located in the 3' portion of 18S, the small ribosomal subunit gene, approximately 182bp from its junction with ITS1, the first internally transcribed spacer (110bp) and the second primer (R: 5'TTTCACTCGCCGTTACTAACG3'), is located in the 5' portion of 28S, the large ribosomal subunit gene approximately 80bp from the junction with ITS2, the second internally transcribed spacer (101bp). Between both spacers is the 5.8S ribosomal gene that is of 259bp in length. A DNA fragment of 712bp was obtained and the sequence was submitted to NCBI GenBank database (Accession No. KP179225, Ghule *et al.*, 2014) and compared with those in GenBank. A BLAST search results indicated the sequence was identical to the sequences of *M. incognita.* On the basis of these results, the root-knot nematodes isolated from passion fruit in Mizoram, India was confirmed as *M. incognita*.

Race identification

Inoculation of *M. incognita* population infecting passion fruit was evaluated on NC host differentials and results (Table I) revealed that the population was unable to infect and reproduce on cotton cv Delta Pine 61 and peanut cv Florunner. However, it was capable of infecting and multiplying on tomato cv Rutgers, Tobacco cv NC 95 and Pepper cv California wonder. Therefore, the population of *M. incognita* was identified as race 2.

Table I.- Infection and reproduction of *Meloidogyne incognita* population on differential hosts.

Tomato cv Rutgers		Tobacco cv NC 95		Peanut cv Florunner		Cotton cv Delta pine 61		Pepper cv California wonder	
Gall index	Egg mass	Gall index	Egg mass	Gall index	Egg mass	Gall index	Egg mass	Gall index	Egg mass
2.86 (1-3)	5.17 (1-8)	2.17 (2-3)	4.67 (1-12)	1.00 (1-1)	0	1.00 (1-1)	0	3.25 (2-4)	18.67 (7-50)

Figures in parentheses are range value; Gall Index on 1-5 scale (1, no gall; 2, 1-10; 3, 11-30; 4, 31-100 and 5, >100 galls in a root system).

Table II.- Effects of different inoculums level of *M. incognita* on the growth of passion fruits and nematode reproduction [Mean value of six (range)].

Inoculum (Pi)	Plant growth parameters				Gall index (1-5)	Egg mass index (1-5)	Final nema. population (Pf)	Reproduction factor (R=Pf/Pi)
	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g.)				
0	55.00b (49-61)*	18.63b (16.71-20.55)	31.5b (29-34)	16.71ac (14.6-18.82)	1.00b (1-1)	1.00dc (1-1)	0	0
10	53.33b (44-65)	11.25a (10.16-2.82)	21.00a (19-23)	11.25c (5.49-14.66)	4.00a (3-5)	2.33cb (1-3)	436b (258-615)	87.27b (51.6-123)
100	43.33a (39-46)	11.39a (7.2-14.86)	21.33a (11-33)	10.26ac (4.15-13.58)	4.33a (4-5)	3.33b (3-4)	973b (941-1014)	19.47a (19.32-20.28)
500	40.67a (24-51)	7.32a (4.75-9.69)	12.00a (9-14)	6.19b (3.58-7.83)	4.67a (4-5)	4.00a (3-5)	1395b (414-2690)	5.58a (1.65-10.76)
1000	38.00a (25-45)	9.35a (4.55-14.75)	18.67a (11-30)	7.55b (2.85-11.04)	5.00a (5-5)	3.67b (3-4)	1037b (546-1954)	2.08a (1.09-3.90)
5000	36.00a (31-42)	8.73a (6.78-10.41)	15.50a (14-16.5)	6.14b (4.5-8.2)	5.00a (5-5)	3.67b (3-4)	2182ab (1838-2843)	0.87a (0.73-1.13)
10000	30.17a (29-31)	8.50a (7.8-9.09)	17.67a (15.5-19.5)	15.21ac (13.69-16.72)	5.00a (5-5)	5.00a (5-5)	3467a (2253-5851)	0.69a (0.45-1.17)

*Figures in parentheses are range value; Means in the same row followed by the same letter are not significantly different at P=0.05 by DMRT; gall and eggmass indices (1, no gall/eggmass; 2, 1-10; 3, 11-30; 4, 31-100 and 5, >100 galls/eggmasses in a root system).

Pathogenicity and disease symptoms

Plants inoculated with nematodes displayed distinctive difference in growth parameters (shoot length, fresh shoot weight, root length, fresh root weight), galling index, egg mass index and final population in comparison to uninoculated plants (Table II). Inoculated plant showed stunted growth and yellowing symptoms (Fig. 2B, C); these symptoms were prominent among different levels of inoculated and uninoculated conditions. Similar symptoms were also noticed in the naturally infested passion fruit plants; infected root appeared uneven thickness, slight swelling with scattered root galling in small roots (Fig. 2D). The reproduction of root-knot nematode was significantly reduced with the increase in the inoculum levels; the highest reproduction (R=87.27) was achieved at the minimum inoculum level (10 J2/1000 cm^3 soil) and the lowest (R=0.69) at the maximum inoculum level (10000 J2/1000 cm^3 soil). Further, the rate of nematode multiplication showed a declining trend with the increase in the initial inoculum level. The minimum number of *M. incognita* at which passion fruit plant suffers and become pathogen for the crop was 10 J2/1000 cm^3 soil.

DISCUSSION

Infestation of root knot nematode under natural conditions is responsible for decline and poor yield of purple passion fruit (*Passiflora edulis*). This has been recorded in the Champhai district of Mizoram, India. The present study conclusively confirmed the identity of the root knot nematode species as *M. incognita* (Kofoid and White) Chitwood, 1949. The morphological observations

Fig. 2. A, Esterase phenotypes of *Meloidogyne javanica* (MJ) as standard (J3) and *M. incognita* (Mi) from Mizoram on passion fruit (I1); symptoms induced B and C, Inoculated plants and root system; D, naturally infested root symptoms.

mostly agrees with the typical characteristics of *M. incognita* (Kofoid and White) Chitwood, 1949 with minor deviations in the measurements and perineal pattern morphology. Biochemical molecular studies helped to confirm the specific identity; Est phenotypes of 'I1' (single major band) was typical for *M. incognita* (Eisenback *et al*., 1981), and amplification of rDNA and and partial sequence (ITS-I and ITS-2) showed sequence homology with that of *M. incognita* (JQ405212, KJ739708 *etc.*). The host differential test (Taylor and Sasser, 1978) based on infectivity and reproduction of *M. incognita* (Khan *et al*., 2014) on tomato cv Rutgers, tobacco cv NC 95, peanut cv Florunner, cotton cv Delta pine 61, pepper cv California wonder revealed the population infecting passion fruit as race 2.

Nematode population isolated from naturally infested passion fruit, the nematode infected plant showed stunted growth and yellowing of leaves both natural and inoculated conditions. Infected old root showed root galling those are of typical in nature; root swells at different parts of root system and appeared thick as thick-root symptom. In the inoculated root, the nematode species induced a typical root galls with production of large eggmasses on root surface. Further, this study revealed pathogenic potential of the nematode species; a threshold limit of 10J2/1000 cc soil could be damaging at seedling stage of passion fruit. The infection of *M. incognita* either in nursery or in main-field conditions lead to poor growth and stand of uneconomic fruit yielding plants. Interestingly, the association of root knot nematode in passion fruit have been reported earlier (Sharma and Loof, 1972; Boesewinkel, 1977; Loof and Sharma, 1979; Milne, 1982) and predicted its damage concern and management for the crop (Cohn and Duncan, 1990) from Kenya (Ondieki, 1975), south Africa (Milne, 1982) and from Brazil (Klein *et al*., 1984; Costa *et al*., 1997). However, pathogenic potential of root knot nematodes have not been previously determined. The present study demonstrated the effect of inoculum levels on the plant growth parameters, symptom production and nematode reproduction for better understanding of dimension of crop damage due to the infestation of *M. incognita* on passion fruit. In fact, management strategies of root knot nematodes involve cultural practices, growing resistant cultivar, application of bioagents and chemical nematicides. Before, adoption of root knot nematode management practices, species identification and nature and extent of damages due to nematode infection are essential information for the extension workers and crop growers.

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Conflict of interest statement

We declare that we have no conflict of interest.

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