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Research Article

Isolation and *in vitro* studies of indigenous fungi against root-knot nematode, *Meloidogyne incognita*

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ABSTRACT: The present study was carried out to evaluate the indigenous fungi from soil against the root knot nematode, *Meloidogyne incognita.* Eleven isolates were isolated and screened for their efficacy against nematode under *in vitro* condition for egg hatching and juvenile mortality. The results revealed that the isolate F6 showed the highest egg hatching inhibition (88.5%) followed by F11 and F7 compared to control after incubation for 72 h. The highest juvenile mortality of 73% was recorded in isolate F6 followed by F11 and F9 after 72 h. Isolate F6 was morphologically and molecularly identified as *Talaromyces pinophilus and* F9 and F11 were identified as *Aspergillus corrugates* and *Aspergillus nidulans.* Among the fungi, *T. pinophilus* was found to be highly effective against root knot nematode.

KEY WORDS: Indigenous fungi, culture filtrate, *in vitro*, *Meloidogyne incognita, Talaromyces pinophilus*

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INTRODUCTION

Plant-parasitic nematodes (PPNs) are considered as farmer's hidden enemy (Mahfouz and Askary, 2018). Among all the genera, root-knot nematodes (*Meloidogyne* spp.) are the most destructive plant parasitic nematodes (Hussain *et al*., 2011). Worldwide, root knot nematodes stand top among the ten most important genera of plant parasitic nematodes (Kayani *et al.,* 2013) with estimated annual yield loss up to 16.9% (Bhatti and Jain, 1977) and $100 billion monetary loss per year globally (Mukhtar *et al.,* 2014). Among the damaging genera of plant parasitic nematode, root-knot nematodes, *Meloidogyne* spp. causes severe loss to vegetables crops worldwide (Galvez *et al.* 2019). It also survives under a wide range of soil moisture and temperature conditions and reduces both quality and quantity of vegetable crops (Sasser, 1979). Pandey and Nayak (2018) witnessed that *M. incognita* is responsible for alteration of host metabolic processes which causes changes in the infected host by cellular, physiological and biochemical and it is difficult to control till now. Even though chemical methods are effective, nematicides are being banned due to the residual effects and toxicity to humans and environments. Due to these concerns, chemical usage is being discouraged and development of alternative, ecofriendly strategies of biocontrol agents and organic amendments for the management of root-knot nematode is gaining importance (Noling and Becker, 1994)**.** The antagonistic activity of biological control agents like fungi, bacteria, viruses (Stirling, 1991) has caused a vast uprising in research (Stirling, 2014). Hence, the new era of nematode management by antagonistic microorganisms is gaining momentum. The essence of a sustainable agriculture lies in management of biological diversity, productivity and vitality. Fungi are the most important biocontrol agents for regulating soil nematode population and utilization of fungi for nematode control is an interesting and developing research area. In the present study, it has been discussed about isolation of indigenous fungi and *in vitro* studies viz., egg hatching, juvenile mortality against root knot nematode.

MATERIALS AND METHODS

Isolation of root knot nematode, *Meloidogyne incognita*

The infested roots of ridge gourd which showed root gall symptoms were collected from Theethipalayam, Coimbatore for the isolation of root knot nematode, *M. incognita.* The infested roots were washed thoroughly in the running tap

water to remove the adhering soil particles and cut into small root bits for the observation of root galls under binocular stereozoom microscope and females of root knot nematode were collected by piercing the galled roots.

Morphological identification of *Meloidogyne incognita* through Posterior Cuticular Pattern (PCP)

Posterior Cuticular Pattern (PCP) was performed to confirm the species of root knot nematode which was collected from the infested field. The root bits were stained by using acid - fuchsin lactophenol followed by de-staining the roots with plain lactophenol for 24–72 hrs. The stained females were confirmed as *M. incognita* by posterior cuticular pattern revealing distinct high dorsal arch of smooth to wavy striae with no distinct lateral incisures (Eisenback *et al*., 1981).

Maintenance of pure culture of *M. incognita*

Root knot nematodes were multiplied and maintained as pure culture in tomato (PKM2 variety) under glass house condition in the Department of Nematology, TNAU, Coimbatore. After species confirmation, the egg masses were collected from the infested roots and kept in the beaker containing sterile distilled water and allowed for egg hatching for 4 days under room temperature. The earthen pots were filled with sterile pot mixture in the ratio 2:1:1of red soil: sand: FYM. Freshly hatched juveniles of *M. incognita* were inoculated near the root region of plants 25 days old seedling of tomato and ridge gourd. Egg masses were collected from this pure culture for the experiments.

Isolation of native antagonistic fungi from soil

Fungi were isolated by serial dilution and spread plate techniques in Potato Dextrose Agar media (PDA). Plates were incubated at 20-25˚C for 4 - 5 days and observed for the growth of fungi colonies. These colonies were isolated with the help of cork borer and subsequent sub culturing was done for maintaining the pure culture of isolated fungi. Fungal discs from pure cultures were inoculated into autoclaved potato dextrose broth for preparation of culture filtrate.

***In vitro* study of egg hatching against isolated fungi**

Two ml of 100% culture filtrate was poured in the petri dish (5 cm diameter) and egg masses were surface sterilized with 0.5% sodium hypochlorite and placed with one egg mass per petridish. Sterile distilled water with egg mass was served as control. Plates were incubated at room temperature (25 ± 2˚C). Observations were taken at an interval of 24 hrs upto 72 hrs. Experiment was carried out in Completely Randomized Design (CRD) with 3 replications.

***In vitro* study of juvenile mortality against isolated fungi**

Two ml of 100% culture filtrate was poured in the petri dish (5 cm diameter) and inoculated freshly hatched 100 second stage juveniles (J_2) into each petri dish. Sterile distilled water with freshly hatched 100 second stage juveniles served as control. Plates were incubated at room temperature (25 ± 2˚C). Observations were taken at an interval of 24 hrs, 48 hrs and 72 hrs. Experiment was carried out in Completely Randomized Design (CRD) with 3 replications.

Molecular identification of isolated native fungi

For identification of isolated fungi, total genomic DNA of 3 effective fungal isolates were extracted by using CTAB method. The genomic DNA of isolated fungi were amplified by using universal primer pair *viz.*, Internal Transcribed Spacer, ITS 1 and ITS 4 by Polymeric Chain Reaction (PCR) method. Forward primer ITS1 (5’- TCCGTAGGTGAACCTGCG G - 3’) and reverse primer (5’- TCCTCCGCTTATTGATATGC- 3’) (White *et al.,* 1990). PCR products were loaded in agarose gel electrophoresis for DNA confirmation. The gel was observed under an UV transilluminator and documented in Alpha imager TM1200 documentation and analysis system (Alpha Innotech Corporation, San Leandro, California).

Statistical analysis

The data were analysed using AGRES software and the significant means were separated by using Duncan’s Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The root knot nematode, *Meloidogyne* species was isolated from stained roots of ridge gourd which were morphologically verified by the presence of high dorsal arch with smooth to wavy striae (Fig. 1) and confirmed as *M. incognita.* Eisenback *et al*. (1981) explained the posterior cuticular pattern of *M. incognita* by the presence of a high, squarish dorsal arch which contains a distinct whorl in the tail terminal region and has smooth to wavy striae, sometimes zigzagged. The lateral field may be marked by breaks and forks in the striae and absence of distinct lateral lines.

The results of *in vitro* studies revealed that the isolates F6, F11and F7 showed the highest egg hatching inhibition at the rate of 88.5%, 87% and 84%, respectively with respect to control after incubation for 72 hours (Table 2). Singh and Mathur (2010) found that culture filtrates of *Acremonium strictum* was very effective against the nematode with regards to egg parasitism (53%), egg hatching inhibition (86%) and mortality (68%) compared to control.

Significantly higher juvenile mortality was found in isolates F6, F11and F9 as 73%, 63% and 54%, respectively compared to control after 72 hrs of incubation (Table 3). Similarly, on citrus seedlings, the culture filtrate of *Talaromyces cyanescens* inhibited the motility of second stage juveniles, egg hatching and nematode reproduction (Lucas *et al.,* 2009).

Morphological changes in the eggs of *M. incognita* was observed under light microscope which indicated the disintegration of eggs treated with isolated fungal culture filtrate compared with control healthy eggs in untreated control (Fig. 2). Sikandar *et al.* (2020) revealed that *in vitro* evaluation of *Penicillium chrysogenum* Snef1216 against *M. Incognita* recorded the highest ovicidal activities as only 5.20% eggs hatched at 100% conc. with compared to 86.8% hatching of *M. incognita* eggs in distilled water (Control). Egg hatching inhibition of *M. incognita* increased with increasing concentration and exposure time.

Morphological changes in second stage juveniles of *M. incognita* showed the dead juveniles with deformation like bulging of internal content, disintegration of cuticle (Fig. 3) after 72 hrs of incubation. Studies of Hamza *et al.* (2017) revealed that *Talaromyces assiutensis* killed all *M. javanica* juveniles during *in vitro* predation tests.

The effective fungal isolates were molecularly identified by using universal primer ITS1 and ITS4 in PCR which revealed that the isolates were amplified with amplicon size of approximately 650 bp with respect to 18SrRNA region (Fig. 4). By resolving the PCR product on 1.2 % agarose, they were purified and sequenced at Barcode Biosciences, Bangalore, India. The sequence was analysed in National Centre for Biotechnology Information (NCBI) – BLAST (Basic Local Alignment Search). Analysis results of the isolate F6 unveiled that it had nucleotide sequence identity 98.46% homology with *Talaromyces pinophilus* and the sequence deposited in the genbank with the accession number MZ930495. Phylogenetic relationship (Fig. 5) was analysed with MEGA11 software to construct the neighbour joining tree with boot strap method of 1000 replications. F9 and F11 were identified as *Aspergillus corrugates, Aspergillus* nidulans, respectively. Since *Aspergillus* spp., are found to be human pathogen further studies was carried out only with *Talaromyces pinophilus*. Zaki and Maqbool (1991) found that *Pasteuria penetrans*, *Paecilomyces lilacinus, Talaromyces flavus,* and *Bacillus subtilis* reduced gall index and individual application were more effective than combinations of these biocontrol agents.

The isolated fungus F6 was confirmed as *Talaromyces pinophilus* by colony growth, growth texture and micromorphological characteristics which exhibited dull green with white or yellow mycelia and greyish orange in reverse (Fig. 6) with biverticillate conidiophore with globose shape (3µm) conidia (Fig. 7) and confirmed the characters (Abdel and Elyousr, 2018).

Table 1. Soil collection at different regions of Coimbatore district

Isolates	Location	Crop	Latitude	Longitude	Crop stage
F1	Theethipalayam I	Ridge gourd	10.9321° N	76.8767° E	Harvesting stage
F2	Theethipalayam II	Ridge gourd	10.9456° N	76.8847° E	Flowering stage
F3	Theethipalayam III	Bottle gourd	10.9550° N	76.8829° E	Harvesting stage
F4	Madampatti	Bitter gourd	10.9731° N	76.8573° E	Flowering stage
F5	Karadimaadai	Bitter gourd	10.9399° N	76.8579° E	Harvesting stage
F6,F7	Deenampalayam	Ridge gourd	11.0090° N	76.8538° E	Harvesting stage
F8,F9	Kinathukadavu	Ridge gourd	10.8224° N	77.0161° E	Vegetative stage
F10	Samiyandipudur	Bitter gourd	10.6663° N	76.9215° E	Harvesting stage
F11	Andipalayam	Ridge gourd	11.0925° N	77.3123° E	Vegetative stage

Table 2. Effect of isolated fungal culture filtrate on egg hatching of *M. incognita* at 2ml concentration

Treatments	No. of juveniles hatched out*		
	24 hrs	48 hrs	72 hrs
F1	39^{ef} (6.13)	44.67^{f} (6.74)	57.33^{de} (7.57)
F2	9.7^{b} (3.10)	26.33^{cd} (5.12)	29.67^{c} (5.43)
F3	33^{de} (5.74)	41.67^{f} (6.45)	47.67^{d} (6.90)
F4	9.7^{b} (3.06)	69.33^{g} (8.32)	73.33^{f} (8.55)
F5	26.7^{d} (5.12)	39.33^{ef} (6.26)	64^{ef} (7.99)
F6	5.0^{a} (2.16)	12.67^{a} (3.55)	19.33^{a} (4.39)
F7	3.7^{a} (1.86)	12.33^{a} (3.49)	27.67^{bc} (5.25)
F8	17.7^{c} (4.18)	64.67^{g} (8.03)	104^{g} (10.19)
F9	11.3^{bc} (3.35)	19.33^{b} (4.39)	96.33^{g} (9.81)
F10	15.0^{bc} (3.86)	32.67^{de} (5.69)	35c (5.90)
F11	17.3^{c} (4.14)	24^{bc} (4.89)	22^{ab} (4.67)
SDW	45.3^{f} (6.71)	125^{i} (11.17)	168^{i} (12.95)
PDB	35.3^{def} (5.94)	103.33^{h} (10.16)	147^{h} (12.12)
SeD	0.43	0.34	0.36
CV	12.48	6.39	5.60
CD(P = 0.01)	1.20	0.94	0.99

*Mean of three replications; Figures in parenthesis are square root transformed values. In a column, means followed by common alphabet are not significantly differ from each other at 1% level by Duncan's Multiple Range Test (DMRT).

Table 3. Effect of isolated fungal culture filtrate on juveniles' mortality of *M. incognita* at 2ml concentration

Treatments	No. of dead juveniles*		
	24 hrs	48 hrs	72 hrs
F1	14.33^{e} (3.84)	21.33^{cd} (4.66)	22.33^{f} (4.78)
F2	16.67^{de} (4.11)	18^{d} (4.29)	22.33^{f} (4.77)
F3	8^{g} (2.91)	10^{e} (3.22)	22.67^{f} (4.80)
F4	33.33^{b} (5.81)	44.67^{b} (6.72)	53.67^{c} (7.36)
F5	10^{fg} (3.20)	23.33^{c} (4.88)	27.33^{de} (5.27)
F6	40.67^{a} (6.41)	61^{a} (7.84)	73.33^{a} (8.59)
F7	13.67^{ef} (3.76)	23.33^{c} (4.88)	24^{ef} (4.94)
F8	6.67^{fg} (2.67)	22^{cd} (4.74)	27^{de} (5.24)
F9	37^{b} (6.12)	42.67^{b} (6.57)	54^{c} (7.38)
F10	23.33^{c} (4.87)	24.33^{c} (4.97)	30^{d} (5.52)
F11	20^{cd} (4.52)	48^{b} (6.96)	63.33^{b} (7.98)
SDW	0^{h} (0.71)	0^{f} (0.71)	0^{g} (0.71)
PDB	0^{h} (0.71)	0^{f} (0.71)	0^{g} (0.71)
SeD	0.29	0.25	0.20
CV	9.24	6.46	4.51
CD(P = 0.01)	0.80	0.69	0.54

*Mean of three replications; Figures in parenthesis are square root transformed values. In a column, means followed by common alphabet are not significantly differ from each other at 1% level by Duncan's Multiple Range Test (DMRT).

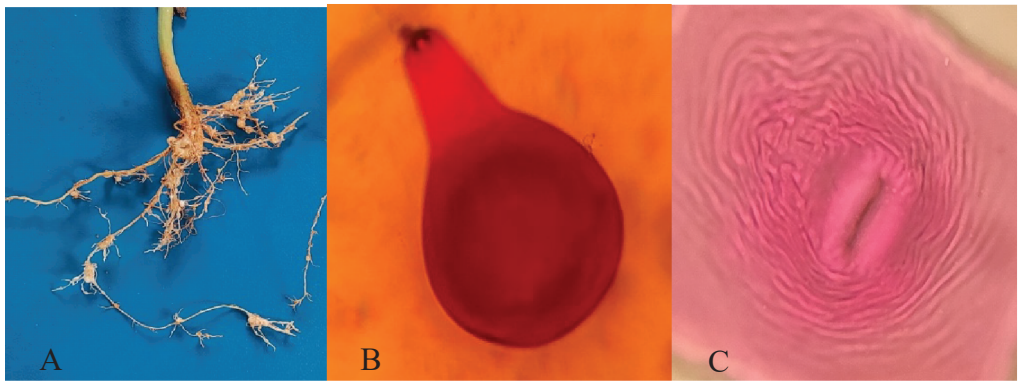


Fig. 1. A. Morphological identification of root knot nematode, *Meloidogyne incognita* infected root sample. **B.** Adult female (10X). **C.** Perineal pattern of *M. incognita* (40X).

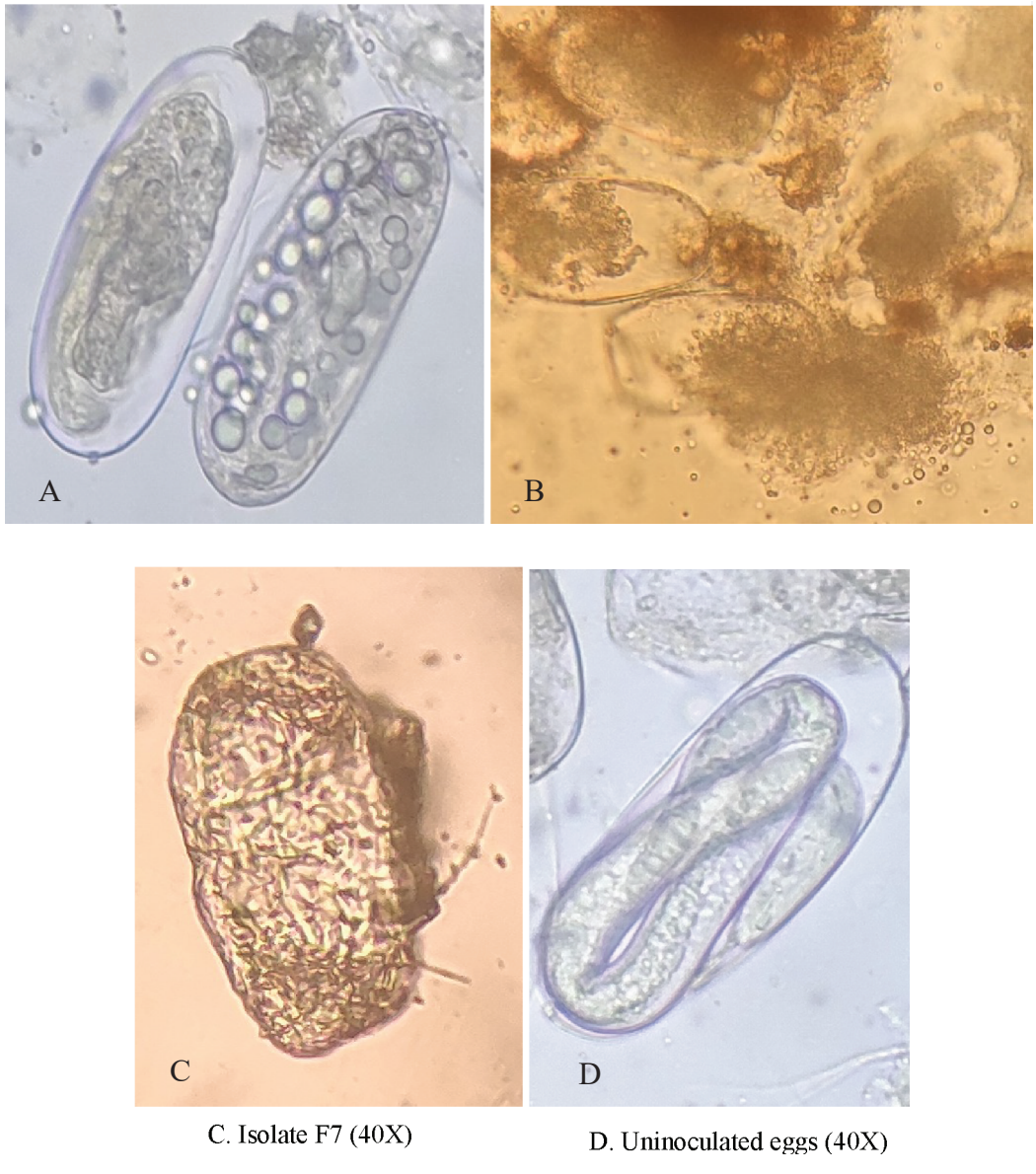
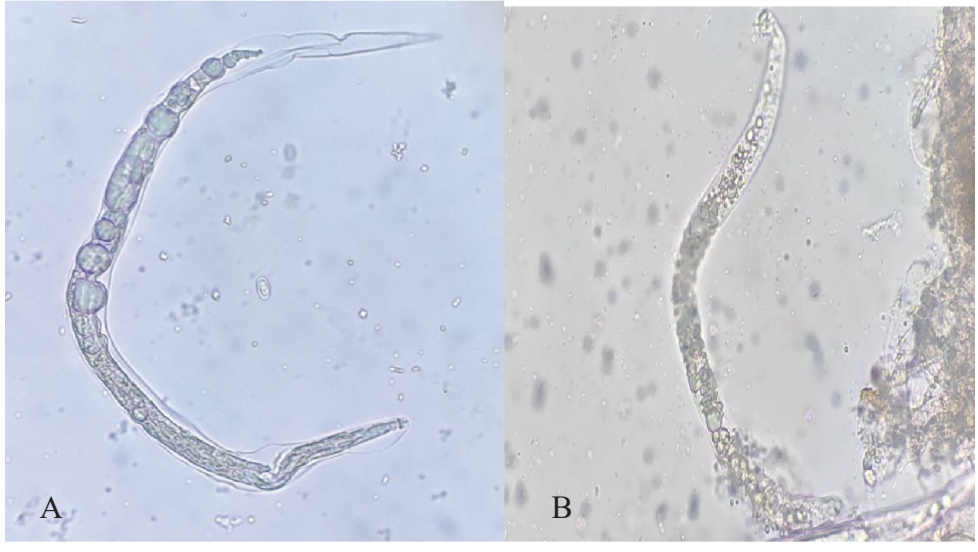


Fig. 2. *In vitro* study of isolated fungi against *M. incognita* eggs at 40X
A. Bulging of eggs. B. Sporulated eggs C. Hyphal growth on eggs D. Juvenile inside the egg.



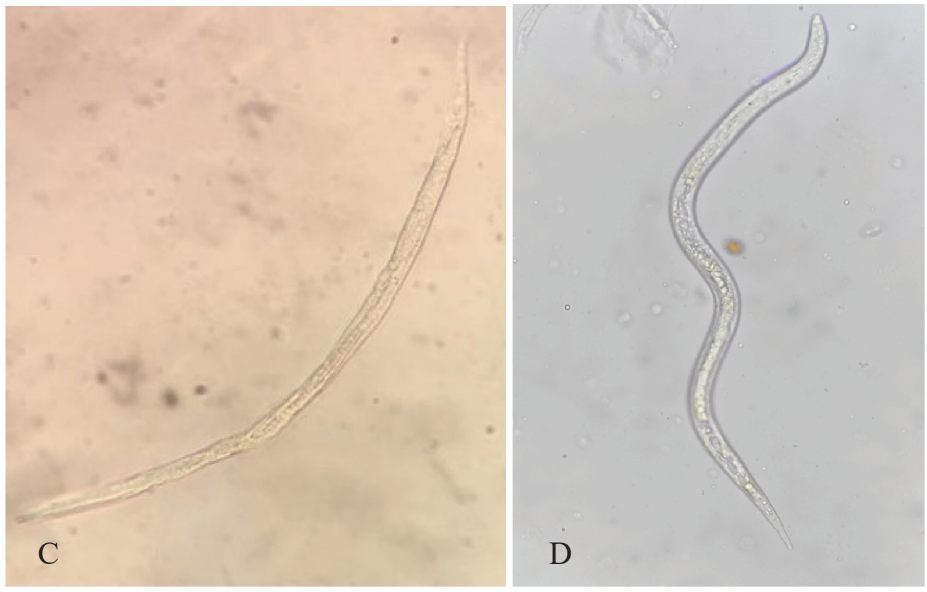


Fig. 3. *In vitro* study of isolated fungi against second stage juveniles of *M. incognita* (40X) A. Bulging of juveniles B. Disintegration of juveniles C. Dead juveniles D. Healthy juveniles.

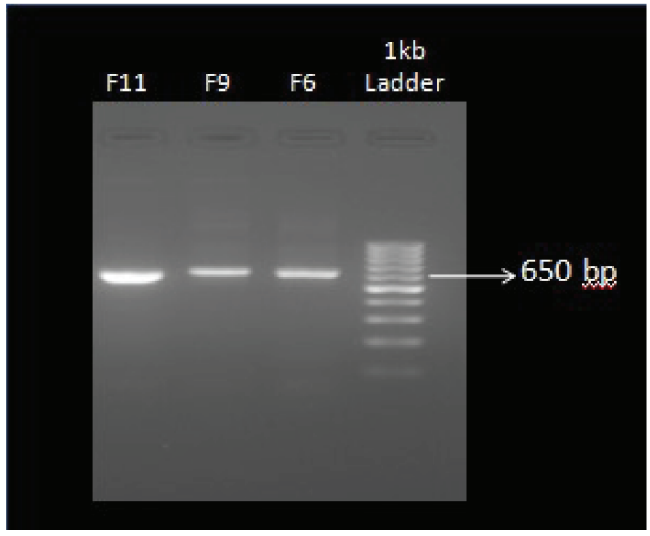


Fig. 4. PCR amplification of isolated fungi F6 with 1kb ladder.

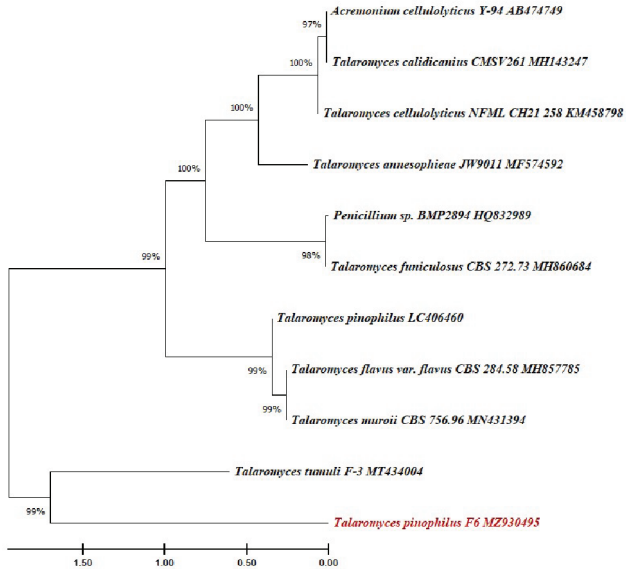


Fig. 5. Phylogenetic relationship of *Talaromyces pinophilus* F6 (MZ930495).

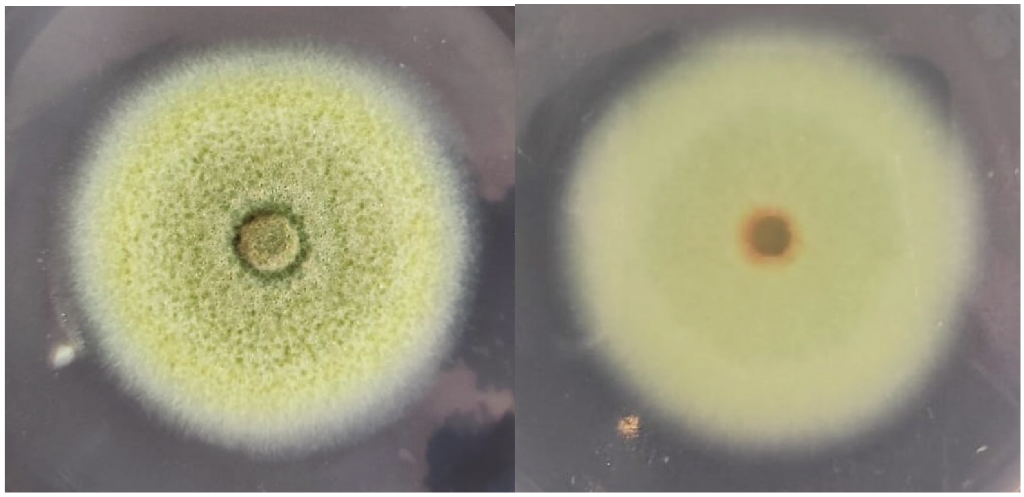


Fig. 6. Colony growth on PDA plates. A. dull green with white or yellow mycelia and B. greyish orange in reverse.

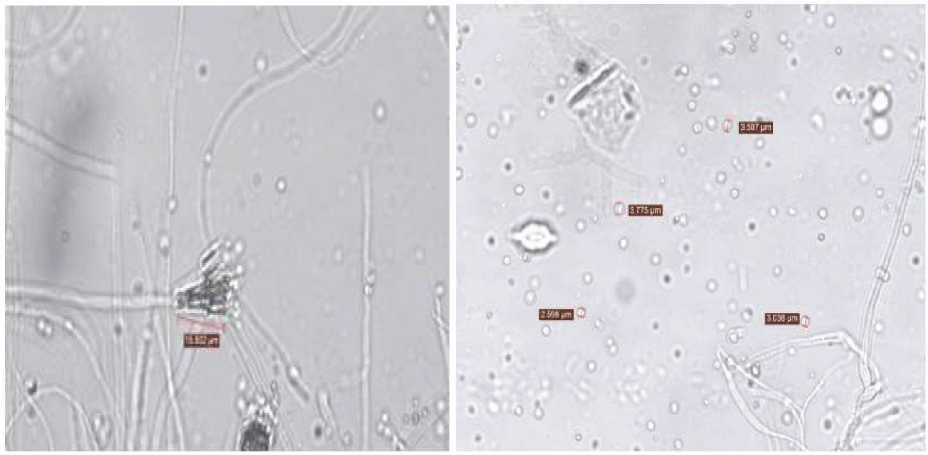


Fig. 7. Morphological description of Isolate 6 at 40x maginification
A. Biverticillate conidiophores B. Globose conidia.

CONCLUSION

The present study suggests that the indigenous fungi *Talaromyces pinophilus* has the ability to suppress *M. incognita* showing the highest egg inhibition and juvenile mortality percentage. Further explorations on mechanisms involved and identification of antinemic metabolic compounds needs to be focused for its use as an efficient biocontrol agent against plant parasitic nematode.

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