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Original Research Paper

Exploration and evaluation of root-associated endophytes against *Meloidogyne incognita* infecting tomato

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ABSTRACT

The fungal endophytes play a key role in habitat adaptation of plants resulting in enhanced plant performance and withstand against biotic and abiotic stresses in the environment. The present study was conducted to isolate root-associated fungal endophytes from cultivated crops and evaluated for their efficacy against *Meloidogyne incognita* infecting tomato plants. Total 262 fungal root endophytes were isolated from 14 cultivated crops and classified and also grouped into 4 genera (*Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Trichoderma* sp.) based on morphological structure. The colonization rate of these isolates ranged from 0 to 100%; Shannon's index (H) is 0 to 1.37; Simpson (1-D) is 0 to 0.74 and Dominance (D) is 0.4 to 1. Antagonism evaluation of fungal endophytic isolates on *M. incognita* revealed that 5 isolates caused 100 per cent juveniles (J2) mortality, 14 isolates caused mortality in the range of 90-99 per cent, 12 isolates caused 75- 89 per cent mortality, and remaining 26 isolates caused <75 per cent J2 mortality. The isolates that caused 100 per cent J2s mortality were molecularly characterized based on ITS-1 and ITS-2 gene nucleotide sequences and identified as *Aspergillus flavipes*, *Penicillium murcianum*, *Fusarium oxysporum*, *Trichoderma asperellum* and *Trichoderma viride.* These fungal root endophytic isolates possessed plant growth promotion traits, effectively parasitized the eggs of *M. incognita* and significantly reduced the infection in tomato.

Keywords: Antagonism, endophytes, fungus, *Meloidogyne incognita*, plant growth promoting traits

INTRODUCTION

The Root-knot nematode (RKN) species are cosmopolitan in nature globally and they cause severe damages and yield losses to numerous crops (El Aimani et al., 2022). RKN have been found in association with fungal and bacterial pathogens which utilize nematodes penetration pathways in plants roots to reinforce plant disease complex (Leoni et al., 2020). To manage these complex diseases and to enhance the plant health, it is necessary to suppress the nematodes population. Use of chemical pesticides is the most rapid method to control nematode pest. However, less than 1% of pesticides applied for pest control reach their target pests and remaining chemicals reach non-target flora and fauna (Bernardes et al., 2015). As pesticides contaminations have serious environmental risks and impacts on human health, hence gradual withdrawal of chemical nematicides from the market (Xiang et al., 2018). This stimulates the search for alternatives like eco-friendly strategies that could be useful in sustainable crop production (Stirling, 2011). The fungal endophytic microbes that reside inside host plants is largely underestimated and distribution is governed by host and/or environment specific factors (Coleman Derr et al., 2016; Hardoim et al., 2015). These microbes enhances the plant growth promotion and suppress the pests and pathogens through different mechanisms (Yu et al., 2022; Busby et al., 2016; Fesel & Zuccaro et al., 2016).

Several fungal endophytic microbial communities have been reported that direct parasitize nematode eggs and larvae by hyphal loops and other means to trap nematodes and their eggs and indirectly suppress the nematode population by release of nematicidal compounds and determine soil suppressiveness (Ciancio et al., 2019; Topalovic et al., 2020; Kumar & Dara, 2021). As such, there is a growing interest in finding microorganisms that may co-exist in the soil

or plant tissues and can inhibit the growth and spread of nematodes (Kumar & Dara, 2021). Therefore, our hypothesis postulates that the application of fungal endophytic microbes can parasitize *Meloidogyne incognita* and efficiently suppress its infection in tomato plants. To investigate this, the present study was conducted to isolate root-associated fungal endophytes from cultivated crops. Subsequently, identified based on molecular characterisation and assessed of their diversity and also evaluated for nematode antagonism against *Meloidogyne incognita*.

MATERIALS AND METHODS

Isolation of root-associated endophytic fungi from selected crops

The root samples of 45 days old different crops (Table 1) were collected from research field at ICAR-Indian Agriculture Research Institute, New Delhi, India. Isolation of endophytic fungi was carried out under aseptic condition according to Dababat et al. (2008) protocol. After completing the surface sterilization of the roots, they were evenly plated on the surface of PDA media in petri dishes. To prevent bacterial contamination, the PDA media were supplemented with 150 mg/L streptomycin sulphate. Five replicates of the plates were then incubated for one week in a BOD incubator at 25°C. Following the incubation period, the fungi colonies were counted under a stereoscopic microscope at 10x magnification [Olympus Biological Microscope-Binocular LED (MX 21i)]. Distinct fungi colonies exhibiting morphological variations on the PDA media were carefully selected for nematicidal evaluation. To ensure purity, the chosen fungi isolates were sub-cultured and maintained in 15% glycerol vials, stored at -80°C for future use and preservation.

Nematode inoculum

The population of the test nematode was developed on brinjal plants and pure culture of *M. incognita* species was maintained in the Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, India and identified based on the perineal pattern structure of female nematode (Taylor et al., 1955).

Evaluation of endophytic fungi cell-free filtrates (CFF) on *M. incognita* juveniles (J2s) mortality

Egg masses of *M. incognita* were carefully collected from culture pots and incubated at 21°C in a BOD incubator for hatching. Freshly prepared potato dextrose broth (PDB) as inoculated with pure culture of endophytic fungi and incubated on a shaker at 120 rpm for 72 h, followed by centrifugation at 10,000 rpm for 10 min. The supernatants were filtered using Whatman No.1 paper, followed by nitrocellulose syringe filter (0.22 µm) to obtain cell-free filtrates (CFF). For the bioassay, 1 mL of the CFF was added to each well of pre-sterilized multiwell plates, and 100 freshly hatched second-stage juveniles (J2s) of *M. incognita* were added in 100 µl of water to each well. The plates were then incubated at 25°C, while sterile medium and sterile distilled water (SDW) were used as control treatments. After 24 h, the immobile J2s were counted under a stereoscopic zoom binocular microscope, and immobile J2s observed were transferred to distilled water for another 24 h to confirm mortality by touching of juveniles with pick, if it's moving then it's alive, if not its confirmed that dead. Each isolate was tested with five replicates.

Molecular characterization of rhizobacterial isolates

The 48 h fungus cultures were used for genomic DNA isolation by Zymo Research Crop quick DNA™ Fungal/Bacterial Miniprep kit (Cat. No. D6005) according to manufacturer's protocol. Sequencing of ITS-1 and ITS-2 regions were performed by using universal primers (58A1F – GCATCGATGAAGAA CGC, 58A2R- CTGCGTTCTTCATCGAT) as described by White et al. (1990). The nucleotide sequences obtained were aligned with the existing nucleotide database of NCBI GenBank and the reference sequences were retrieved for analysis of phylogenetic tree.

Evolutionary phylogenetic tree analysis by maximum likelihood method

The evolutionary history was inferred using the Maximum Likelihood method and the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree, inferred from 10,000 replicates (Felsenstein, 1985), was used to represent the evolutionary history of the analyzed taxa.

Evaluation of endophytic fungi isolates on *M. incognita* egg parasitism and egg hatch inhibition

The *in vitro* evaluation of nematode egg parasitism was conducted by placing surface-sterilized by 1% solution of freshly prepared antibacterial and antimycotic solution (ABAM) for 5 minutes and

placed *M. incognita* eggs on 1% water agar plates containing 0.1% streptocycline, which served to prevent bacterial contamination. On these plates, the test fungi, namely *Aspergillus flavipes*, *Penicillium murcianum*, *Fusarium oxysporum*, *Trichoderma asperellum*, and *T. viride*, had been cultured for 7 days at 28°C. After 7 days of co-incubation at the same temperature, the eggs were examined under a dissecting microscope. Eggs showing colonization by fungal hyphae were considered infected (Fig. 4). Control plates without any fungi were used as a reference for comparison. Each treatment was replicated five times, and each replicate consisted of four plates.

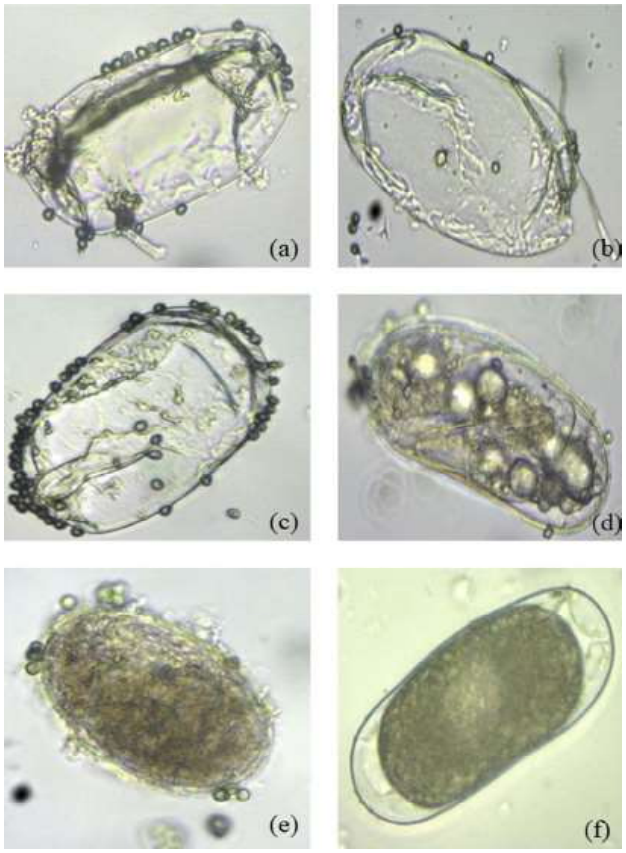

Fig. 4 : Microscopic observation of *Meloidogyne incognita* eggs parasitism by root endophytic fungal isolates

(a) *Trichoderma asperellum*, (b) *Trichoderma viride*, (c) *Aspergillus flavipes*, (d) *Penicillium murcianum*, (e) *Fusarium oxysporum*, (f) untreated egg (control)

Effect of endophytic fungi isolates CFF on *M. incognita* egg hatch inhibition

For egg hatch inhibition study, fresh egg masses of *M. incognita* were meticulously hand-picked from the host plant, washed with sterile distilled water, and then treated with a 1% solution of freshly prepared antibacterial and antimycotic solution (ABAM) for 5 minutes. The egg masses underwent three rinses with sterile distilled water to ensure complete removal of the solution. Next, five egg masses were carefully placed in 55 mm pre-sterilized petri dishes, with each dish containing 2 mL of cell-free filtrates (CFF) of the selected fungi (*A. flavipes*, *P. murcianum*, *F. oxysporum*, *T. asperellum* and *T. viride*). The plates were then incubated at 25°C, with sterile medium and sterile distilled water being used as control treatments for comparison. To assess the effect of the fungal CFF on hatching, the hatched juveniles (J2s) were collected and counted at specific intervals of 2, 4, 6, 8, 10, 12, and 15 days after the initial inoculation, employing a stereoscopic binocular microscope. For each treatment, five replicates were maintained to ensure statistical validity. After 16 days, any unhatched eggs were separated by immersing the egg mass in a 1% sodium hypochlorite (NaCOl) solution for 0.5 minutes. Following the separation, the unhatched eggs were counted, and the percentage of J2 hatch was calculated based on the total number of eggs present.

Bio-efficacy of endophytic fungal isolates against *M. incognita* infection in tomato

Three weeks old tomato cv. Pusa Ruby seedlings were transplanted in 10 inch plastic pots that were filled with 2 kg soil mixed with 3% each fungal culture (*A. flavipes*, *P. murcianum*, *F. oxysporum*, *T. asperellum* and *T. viride*) at ~10^8cfu mL^{-1}. The *M. incognita* was inoculated at 2 J2s/cc soil after seven days of transplanting. The nematicide, Fluopyram 400SC (Velum prime (VP), Bayer crop science) was taken as an additional treatment for chemical control and applied at 0.56 µL/kg soil after mixing in 500 mL sterilised water for uniform spread. The treatments were, T_1: Water + Mi, T_2: *A. flavipes* + Mi, T_3: *P. murcianum* +Mi, T_4: *F. oxysporum* + Mi, T_5: *T. asperellum* + Mi, T_6: *T. viride* + Mi and T_7: VP+ Mi. Pots were kept inside the polyhouse in winter season (December to February) and temperature inside the polyhouse was 12-18° C. Six replications of the seven treatments were maintained in a polyhouse for 75 days. The plants were uprooted and plant growth parameters and number of root galls per root, number of J2s per cc soil, number of egg masses per root and number of eggs per masses and nematode reproduction factor were recorded.

Table 1 : Details on the crops, isolates, colonization rate and diversity indices of fungal root endophytes

Crop habitat	Fungal species	No. of isolates	Colonization rate (%)	Shannon's index (H)	Simpson (1-D)	Dominance (D)
Cowpea	*Aspergillus* sp.	5	50	1.093	0.6627	0.3373
	Penicillium sp.	4	40			
	Fusarium sp.	5	50			
	Trichoderma sp.	4	40			
Soybean	*Aspergillus* sp.	5	50	1.371	0.7422	0.2578
	Penicillium sp.	3	30			
	Fusarium sp.	4	40			
	Trichoderma sp.	4	40			
Mungbean	*Aspergillus* sp.	4	40	1.352	0.7337	0.2663
	Penicillium sp.	3	30			
	Fusarium sp.	2	20			
	Trichoderma sp.	4	40			
Urdbean	*Aspergillus* sp.	3	30	1.371	0.7422	0.2578
	Penicillium sp.	4	40			
	Fusarium sp.	5	50			
	Trichoderma sp.	4	40			
Pigeonpea	*Aspergillus* sp.	3	30	1.355	0.7336	0.2664
	Penicillium sp.	4	40			
	Fusarium sp.	6	60			
	Trichoderma sp.	4	40			
Clusterbean	*Aspergillus* sp.	4	40	1.371	0.7422	0.2578
	Penicillium sp.	5	50			
	Fusarium sp.	3	30			
	Trichoderma sp.	4	40			
Rice	*Aspergillus* sp.	5	50	1.334	0.7245	0.2755
	Penicillium sp.	4	40			
	Fusarium sp.	3	30			
	Trichoderma sp.	2	20			
Maize	*Aspergillus* sp.	5	50	1.334	0.7245	0.2755
	Penicillium sp.	4	40			
	Fusarium sp.	2	20			
	Trichoderma sp.	3	30			
Bajra	*Aspergillus* sp.	4	40	1.352	0.7337	0.2663
	Penicillium sp.	4	40			
	Fusarium sp.	2	20			
	Trichoderma sp.	3	30			
Cucumber	*Aspergillus* sp.	8	80	1.375	0.7444	0.2556
	Penicillium sp.	7	70			
	Fusarium sp.	9	90			
	Trichoderma sp.	6	60			
Pumpkin	*Aspergillus* sp.	9	90	1.383	0.7485	0.2515
	Penicillium sp.	8	80			
	Fusarium sp.	10	100			
	Trichoderma sp.	9	90			
Tomato	*Aspergillus* sp.	10	100	1.363	0.7392	0.2608
	Penicillium sp.	10	100			
	Fusarium sp.	9	90			
	Trichoderma sp.	6	60			
Okra	*Aspergillus* sp.	3	30	0	0	1
	Penicillium sp.	2	20			
	Fusarium sp.	2	20			
	Trichoderma sp.	1	10			
Banana	*Aspergillus* sp.	6	60	0.9743	0.5938	0.4063
	Penicillium sp.	2	20			
	Fusarium sp.	8	80			
	Trichoderma sp.	5	50			

Statistical analysis

The numerical data of different experiments were square-root transformation before analysis. Only the back-transformed data were reported after the transformed data were subjected to an analysis of variance (ANOVA) using PROC GLM SAS (version 9.3; SAS institute 2011; Cary, NC, USA). Utilizing Tukey's significance test results at the 5% level of significance, relevant means were compared.

RESULTS AND DISCUSSION

Total of 262 fungal root endophytes were isolated from 14 cultivated crops of root tissue and presented in Table 1. Isolates were observed and evaluated for macroscopic characteristics (colour, texture, and growth rate) and microscopic characteristics (size and shape of the conidia). Based on morphology and conidial structure, isolates were classified and grouped into 4 genera and identified as *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Trichoderma* sp. The colonization rate of these isolates irrespective of the crop, ranged from 0 to 100%. The species diversity of isolated fungal root endophytes was calculated based on Shannon's index (H), Simpson (1-D) and Dominance (D). Shannon index (H) in the range of 0 to 1.37, Simpson index was 0 to 0.74 and Dominance index (D) was 1 to 0.4 (Table 1). Cell-free filtrates of endophytic fungi on *M. incognita* juveniles (J2s) mortality exhibited that J2s mortality, 5 isolates caused 100 per cent J2 mortality, 14 isolates caused mortality in the range of 90-99 per cent, 12 isolates caused 75- 89 per cent mortality and remaining 26 isolates caused < 75 per cent J2 mortality (Fig. 1).

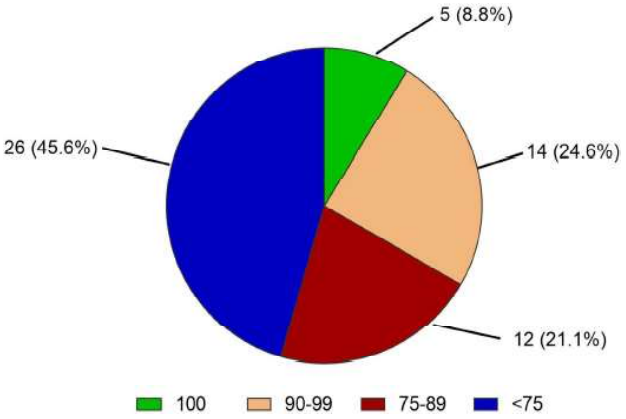

Fig 1 : Percentage fungal endophytes isolates causing mortality in *Meloidogyne incognita* juveniles

Molecular characterization

Potential 5 isolates of endophytic fungi were selected (Fig. 1) based on causing 100% mortality of *M. incognita* J2s and The ITS-1 and ITS-2 gene nucleotide sequenced and were identified *as A. flavipes*, *P. murcianum*, *F. oxysporum*, *T. asperellum*, *T. viride,* respectively and their corresponding GenBank accessions numbers are OR267516, OR267517, OR267518, OR267519, OR267520, respectively (Table 2). The phylogenetic tree was found to possess substantial genetic homogeneity between isolates and database of NCBI reference sequences (Fig. 2). The isolates of the experiment have formed five distinct clades in the phylogenetic tree (Fig. 2).

Egg parasitism and hatch inhibition

Under a dissecting microscope, the nematode eggs wrapped with fungal hyphae were clearly distinguishable from the uninfected ones (Fig. 4).

Table 2 : Fungal root endophytes isolates and their identity based on ITS 1 and ITS 2 gene sequence similarity

Isolates	Fungal endophytes	Similarity (%)	GenBank Accession No.
F2	*Aspergillus flavipes*	100	OR267516
F4	*Penicillium murcianum*	100	OR267517
F6	*Fusarium oxysporum*	100	OR267518
F10	*Trichoderma asperellum*	100	OR267519
F15	*Trichoderma viride*	99	OR267520

Homology and phylogenetic identity of the root-associated fungal endophytes isolates were obtained by comparing the ITS1 and ITS2 gene sequence similarity with related isolates available at the NCBI database

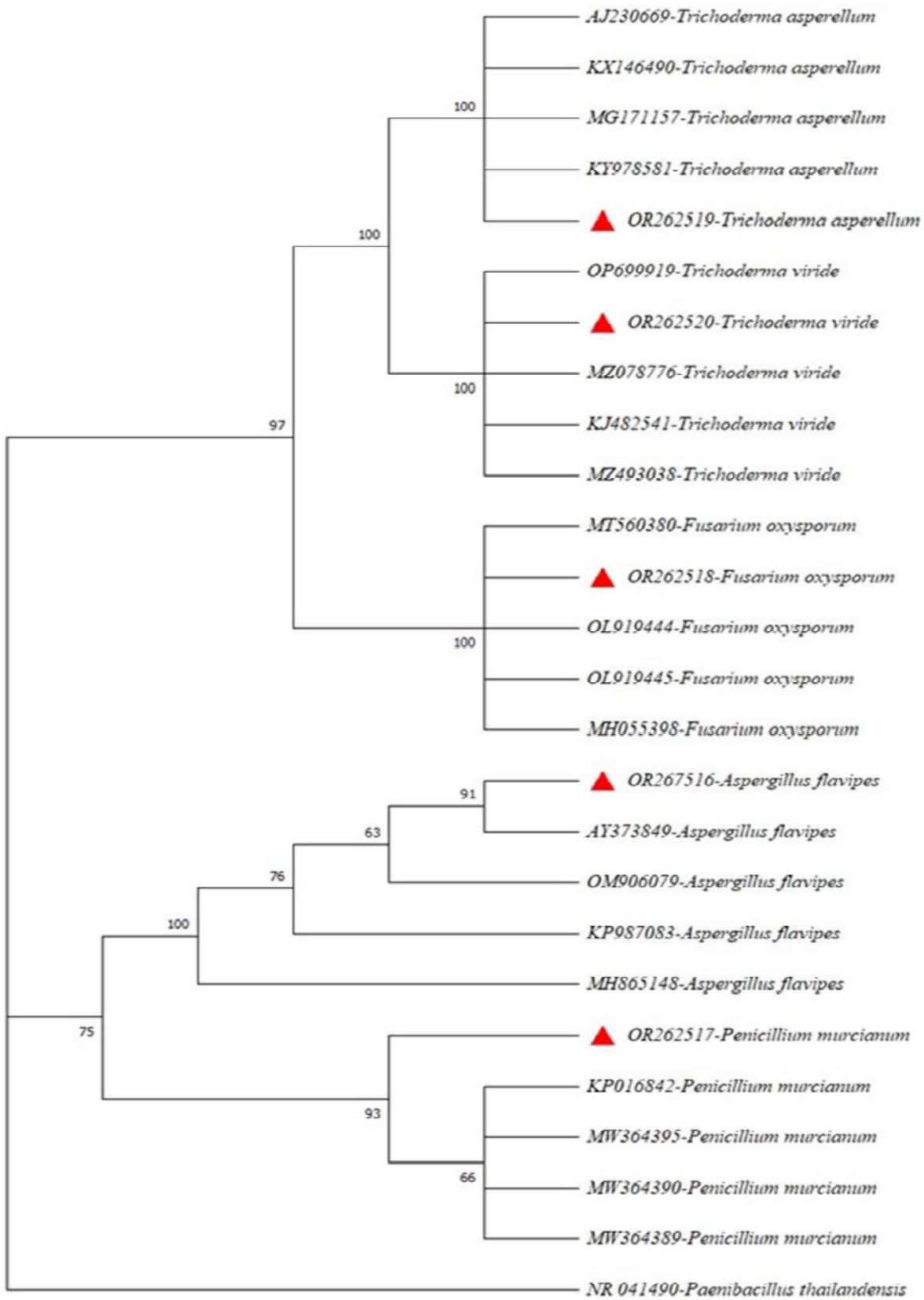

Fig. 2 : Phylogenetic tree relationship of identified fungal endophytes isolates with selected reference strains available in the NCBI depository

Nematode eggs that exhibited contents disintegration and evident penetration and parasitism by the inoculated fungal hyphae were considered as parasitized. All five fungal species demonstrated equal potential in infecting approximately 60-90% of the eggs (Fig. 4). Following fungal parasitism, the nematode eggs were rendered nonviable, leading to a complete halt in the hatching process. When the fungal CFF was applied to the egg masses, it significantly inhibited egg hatching with hatch inhibition range from 83. 28% to 100% compared to the control after 15 days of incubation (Fig. 3). In the control treatment, hatching continued up to 15 days. However, among the different treatments, *F. oxysporum* and *T. viride* caused the most substantial hatch inhibition (88.70% to 100%), followed by *T. asperellum* (86.44% to 100%), *A. flavipes* (83.62% to 100%), and *P. murcianum* (83.28% to 100%) as compared to the control (Fig. 3). The findings demonstrate the potent nematode egg hatching inhibition capability of the studied fungal isolates.

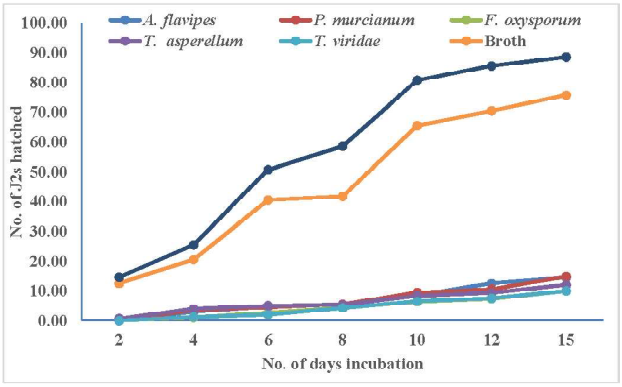

Fig. 3 : Effect of fungal endophytes isolates on egg hatch inhibition of *M. incognita*

Effect of fungal endophytes on plant growth parameters and suppression of nematode infection in tomato cv. Pusa Ruby

The soil application of fungal root endophyte isolates resulted in a significant enhancement of plant growth parameters and effectively suppressed *M. incognita* infection in tomato plants. The average plant height ranged from 171 to 186 cm and total biomass from 230 to 280 g when compared to the control treatment (Fig. 5).

Notably, *F. oxysporum* treatments exhibited the maximum enhancement in all these growth parameters. However, the chemical nematicide fluopyram 400SC

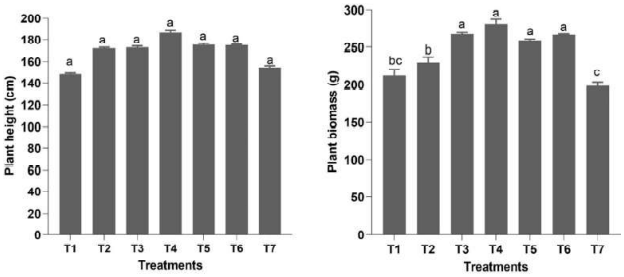

Fig. 5 : Treatment effects of fungal endophytes on plant growth parameters in tomato

The data are mean ± standard error (SE)
Different letters on each column indicate statistically significant difference between treatments at $P < 0.05$ using Tukey's HSD test

did not show any increase in plant growth parameters compared to the control treatments. At 75 days after nematode inoculation, an average of 257 galls per plant was observed in the control treatment. However, in the presence of *A. flavipes* (44.6), *P. murcianum* (47.3), *F. oxysporum* (36), *T. asperellum* (47.6), and *T. viride* (42.3), significantly less number of galls were recorded (P<0.05). The effect of *F. oxysporum* was particularly noteworthy, as it showed the most substantial reduction in gall formation among all treatments. This trend was also evident in the number of egg masses per plant, eggs per egg mass, and the reproduction factor of the nematode in all the treatments. Comparing the treatment effect of the fungal endophyte isolates with fluopyram 400SC, it was found that the fungal treatments were at for effective in reducing gall formation, average number of egg masses per plant, eggs per egg mass, and the nematode reproduction factor (Table 3).

The diversity of fungal endophytes is influenced by the soil edaphic factors and host plant (Arnold, 2007), present study encompassed a total of 262 isolates, which were classified into five distinct species, namely *A. flavipes*, *P. murcianum*, *F. oxysporum*, *T. asperellum*, *T. viride* (Table 1 & 2). These findings are in alignment with previous research, which has also demonstrated the presence of endophytic fungal isolates belonging to the *Trichoderma* spp. group in various plant species. Further, *Trichoderma* spp. endophytes have been identified in the roots and corms of banana (*Musa* sp.), French bean (*Phaseolus vulgaris*), tomato (*Solanum lycopersicum*), maize (*Zea mays*), and other crops (Natsiopoulos et al., 2024). Notably, the presence of these fungal endophytes has been associated with antagonistic activity against

Table 3 : Effect of fungal endophytes on *Meloidogyne incognita* in tomato under pot conditions

Treatment	No. of galls/ plant root system	No. of EM/plant root system	No. of eggs/EM	FNP in 100 CC Soil oil	RF
T_1: Water	257.0 + 4.8^{a}	160.3 + 5.9^{a}	262.5 + 4.8^{a}	406.7±3.1^{a}	2.03
T_2: *Aspergillus flavipes*	44.6 + 1.6^{bc}	37.5 + 1.1^{b}	149.1 + 4.3^{cd}	138.2±2.2^{bc}	0.69
T_3: *Penicillium murcianum*	47.3 + 3.3^{bc}	34.5 +1.0^{b}	170.3 + 3.4^{bc}	145.2±1.5^{bc}	0.73
T_4: *Fusarium oxysporum*	36.0 + 1.4^{c}	39.6 + 2.0^{b}	144.1 + 8.3^{d}	137.3±1.9^{c}	0.69
T_5: *Trichoderma asperellum*	47.6 + 1.6^{b}	44.3 + 1.3^{b}	179.2+ 6.5^{b}	148.5±3.3^{b}	0.74
T_6: *Trichoderma viride*	42.3 + 1.7^{bc}	38.6 + 2.4^{b}	160.8 + 8.1^{bcd}	144.7±2.8^{bc}	0.72
T_7: Fluopyram 400SC	45.0 + 1.5^{bc}	40.3 + 1.2^{b}	137.1 + 3.3^{d}	142.0±1.2^{bc}	0.71
df	6, 35	6, 35	6, 35	6, 35	
F value	587.59	268.88	43.24	1109.87	
Pr (>*F*)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	

The data are mean ± standard error (SE). Different letters on each column indicate statistically significant difference between treatments at $P < 0.05$ using Tukey's HSD test

specific pests and pathogens affecting these plants. This suggests that the endophytic fungal community, particularly those of the *Trichoderma* genus, could play a vital role in protecting plants from harmful pests and diseases, thereby contributing to the overall health and productivity of the crops. Present results confirmed that, dominant endophytes belong to *Aspergillus* spp., followed by *Fusarium* spp., and *Trichoderma* spp. and identified genera of fungal endophytic microbes were most commonly found in plants grown under a tropical and sub-tropical environment, hence these results were in parallel with the findings of Zida et al. (2014) who observed *T. asperellum* as dominant fungal endophytic species, followed by *T. harzianum*, and *Fusarium* spp. CFF of fungal endophytic isolates exhibited a remarkable effect on the mortality of *M. incognita* juveniles. Parasitism activity of these fungal isolates on the nematode eggs was so effective that it rendered the eggs nonviable, preventing them from hatching into juveniles. It is also reported that *T. harzianum* and *T. asperellum* strains parasitize *M. javanica* eggs and inhibit egg hatching of juveniles (Devindrappa et al., 2018). However, *Trichoderma* species produces several lytic enzymes like proteases, lipases, and glucanases, hence these enzymes were inhibits the nematode egg hatching (Tyskiewicz et al., 2022). Fungal endophytes have been reported to express strong antagonistic activity against target pathogens while establishing mutualistic endophytic associations with their host plants. As a result, they can modulate the host plant's physiological and biochemical processes, leading to the induction of the host's defence mechanisms against target pests and pathogens. This intricate interplay between the endophytes and their host plants can act as a natural defense system, providing sustainable protection against nematode infestations and promoting healthier plant growth (Khare et al., 2018; Kiarie et al., 2020; Li et al., 2021).

CONCLUSION

The root-associated fungal endophytic isolates *viz*., *A. flavipes*, *P. murcianum*, *F. oxysporum*, *T. asperellum* and *T. viride* have exhibited nematicidal activity and also effectively reduced *M. incognita* infection and promoted plant growth in tomato plants. These potential isolates can be utilised in nematode pest management practices moreover it offer sustainable and eco-friendly alternatives to traditional agrochemicals for plant protection.

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