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# Efficacy of *Trichoderma reesei* for the management of root-knot nematode, *Meloidogyne incognita* infecting tomato under field conditions

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**Abstract**
Root-knot nematode, *Meloidogyne incognita* is important pest of agriculture and horticulture crops in India. Biological control has been developed as an eco-friendly treatment to control of plant parasitic nematodes. A Survey was conducted for isolation of antagonistic fungus from five districts of north eastern region of Tamil Nadu *viz.*, Tiruvannamalai, Cuddalour, Villupuram, Kallakurichi and Vellore districts. From the survey, 35 cultures were isolated among them *Trichoderma reesei* (TVM1) culture showed very effective against root knot nematode. *Trichoderma reesei* (TVM 1) strain showed high virulence against second stage juveniles ($J_2$) of *M. incognita* by morphology analysis as well as biomolecular assay. Besides, *Trichoderma reesei (*TVM 1) significantly inhibited egg hatching with the inhibition percentages of 80.20, 67.40, and 57.00% at 48, 72, and 96 h after the treatment, respectively. The results of pot culture experiment showed that the modification of *Trichoderma reesei* TVM 1 significantly decreased the number of root galls, $J_2$ and nematode egg masses and $J_2$ population density in soil and significantly increased the growth of tomato plants. In the field experiment, the efficacy of *Trichoderma reesei* TVM 1 showed more than 60 percent against root-knot nematode. *Trichoderma reesei* (TVM 1) could be used as a potential biological control agent against root-knot nematode, *M. incognita* under pot and field conditions.

**Keywords:** *Trichoderma reesei*, *M. incognita*, nematode management, plant growth promotion, tomato

## Introduction
Tomato (*Solanum lycopersicum* L.) is one of most important vegetable crop in the globe. In India contributes 9 percent share in world production and the Tamil Nadu state one of the main vegetables cultivation regions in among southern states. Major production area of Tamil Nadu is Coimbatore, Dharmapuri, Salem and Krishnagiri districts. Tomato is mostly affected by many biotic and abiotic factors. Among them, Root-knot nematode (RKN) is one of the most seriously damaging plant-parasitic nematode in the world threatening to the growth and production of more than 5500 plants species, including vegetable crops and weeds [Fan *et al*., 2020] [4]. The most frequently occurring, species of root-knot nematodes include *Meloidogyne incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, which were responsible for high economic losses to different crops. Root-knot nematodes (RKNs, *Meloidogyne* spp.) were sedentary, polyphagous, root endoparasites and among the most damaging agricultural and horticultural pests, attacking a wide range of crops (Mumpi Ering and Sobita Simon, 2018) [9]. Nematode larvae infect the plant root system and develop root-knot galls that exhaust the plant's nutrients and photosynthate. Plant yields were reduced due to nematode infection, while infection may be lethal in young plants (Bradshaw *et al*., 2016) [1]. Rapid development of high-value crops, such as tomato (*Solanum lycopersicum*), are severely damaged by *M. incognita* and has led to severe losses.

Currently, use of chemical and physical measures have limited for nematode management. Biological control occurs by reducing nematode infection and/or regulation of its populations through the activity of organisms that are antagonistic to them [Poveda *et al*., 2020] [13]. These organisms can interact with nematodes directly through antibiosis and competition for space or nutrients or interact indirectly with plant pathogenic nematodes by inducing resistance in the host plant [Poveda *et al*., 2020; Xiang *et al*., 2018] [13, 21]. Plant-growth-promoting fungi (PGPF) can act as efficient and ecofriendly nematode biocontrol, as well as biofertilizers for plant growth and yield improvement. Biological control with microbial antagonists has received a great deal of attention as a promising measure to control different plant diseases. Many antagonistic microorganisms including *Trichoderma* spp., *Streptomyces* spp.,

*Pseudomonas* spp., *Bacillus* spp. have been screened and widely exploited to control a wide range of plant pathogens [Fan *et al*., 2022]. In recent years, several fungal and bacterial antagonists have been identified and tested for the management of root-knot nematodes. The main criteria for successful deployment of these biocontrol agents in fields are their ability to suppress nematode populations and restrain their multiplication and enhance yields profitably in the presence of nematodes.

The use of microorganisms as bioagents is a less hazardous method for controlling plant pathogens. Almost 20 species of the genus Trichoderma act as bioagents against many soil-borne as well as foliar plant pathogens. *T. harzianum, T. koningii, T. viride, T. atroviride, T. pseudokoningii, T. longibrachiatum, T. hamatum, T. polysporum* and *T. reesei* are the most important species, which act as potential antagonists (Monaco *et al*. 1991) [8].

*Trichoderma* genus is one of the most frequently studied groups of fungi used as biological control agents. These species are often very fast growing, rapidly colonize substrates and effectively control different diseases by using a variety of mechanisms. Furthermore, *Trichoderma* showed many unique properties including the ability to colonize plant root, being easily culturable and propagated, improving plant growth and disease resistance, and improving nutrient utilization efficacy. Interestingly, *Trichoderma* spp. were investigated by many authors for their potentiality in controlling plant pathogenic nematodes; however, there is little information concerning the mechanisms of *Trichoderma*–nematode plant interactions (Nafady *et al*., 2022) [10]. In addition to that, some endophytes such as *Paecilomyces* and *Trichoderma* also trap and kill root knot nematode in the soil or root systems. These may act at different life stages of nematode (i.e. eggs, juveniles, or adults) (Yao *et al*., 2015; Schouten, 2016) [22, 15].

The present study revealed that, there are no reports about using *Trichoderma reesei* as a biological control agent against root knot nematode, *M. incognita*. The objective of this study was to isolate and identification of effective fungal strains against *M. Incognita* and to evaluate the biological control activity under *in vitro* and *in vivo* based on morphology and sequence analysis and their effects on the growth of tomato plants in pot and field experiments.

## Methods

### Nematode Inoculum

The experimental trial was laid out in the glasshouse at Agricultural College and Research Institute, Tamil Nadu Agricultural University, Vazhavachanur, Tiruvannamalai district, Tamil Nadu, India. The *Meloidogyne incognita* nematode population was isolated from infested tomato plants grown in field and maintained on susceptible host tomato plants in the glasshouse. For nematode inoculum preparation, the tomato root system was separated from the whole plant and washed with water to remove soil adhesion. Egg masses of *M. incognita* were recovered from galls of the infected tomato roots and stirred for 4 min in 0.5% NaOCl described by Hussey, R.S.; Barker, K.R (1973) [6] witth some modifications. Nematode egg suspensions were sieved through a 500 mesh sieve (25 µM) and collected in sterilized water. The second stage juveniles (J2) were obtained from the incubated eggs (after 3–5 days) using a modified Baermann funnel method described by Cesarz *et al*., (2019) [3] with some modifications. *M. incognita* inoculum was used for the pot experiment consisting of 3000±5 J2 dispensed in 50 mL of water.

### Isolation of fungal antagonist

Antagonistic fungal biocontrol agents were isolated from rhizosphere soil collected healthy plants of tomatoes in different locations in Tiruvannamalai and adjoining districts, Tamil Nadu, India. Isolation of fungi, the soil serial dilution plate method was described by Pradhan and Sukla, 2006 with some modifications. In brief, a 1 g of rhizosphere soil samples was added into 9 mL of sterilized water and then mixed to get soil suspension. The soil suspension was serially diluted to the appropriate concentration ($10^{-2}$ g/mL and$10^{-2}$ g/mL) and plated on Potato Dextrose Agar (PDA) plates. The plates were then incubated at 28 °C for 5 days. Individual fungal colonies were isolated and purified for further screening.

### Screening of fungal isolates against *M.* incognita *in vitro*

The J2s of *M. incognita* were used to detect the nematicidal activities of fungal isolates. The fermentation broth of fungal strains was prepared in Potato Dextrose Broth (PDB) medium in a shaker at 150 rpm for 5 days at 28 °C. Cell-free supernatant was obtained after centrifugation at 4500×g for 15 min and filtered using the filter. A 50 µL suspension containing 50 J2s was added into each petri dish with 950 µL of the prepared fungal cell-free supernatant. The J2s suspension mixed with PDB medium alone was used as control. Five dishes were used for each treatment. All the dishes were incubated at 25 °C for 72 h. The number of dead and alive J2s was examined using the stereoscopic microscope at 24 h, 48 h, and 72 h after treatment. A nematode that malformed, immobile or motionless even probed with a fine needle was deemed dead [Cayrol *et al*., 1989] [2]. The J2s mortality and corrected J2s mortality were calculated as following:

J2s mortality (%) = The number of dead J2s / total number of J2s x 100,

Corrected J2s mortality (%) = (J2s mortality in the treatment - J2s mortality in the control) / (100 - J2s mortality in the control) x 100

The strains that exhibited the strongest nematicidal activity against J2s of *M. incognita* were chosen for the future study. The effects of selected strains on J2s mortality of *M. incognita* were conducted as described above and repeated three times.

### Assay for Plant Growth-Promoting and Enzymatic Activities of *T. reesei*

The fungus *T. reesei* was grown on potato dextrose agar (PDA) at 28 °C for 7 days until sporulation. *T. reesei* spores were collected in sterile distilled water to form a homogenous spore suspension, and then a 10 mL spore suspension was used as starter inoculum for liquid PD medium. After 7 days, the suspension cultures, maintained in an incubator with agitation (150 rpm), were used to inoculate the tomato seedlings as bioagents [Yin *et al*., 2014] [23]. The spore suspension concentration was adjusted to 2 x$10^6$ spores/mL.

**Identification of strain TVM1**

To identify strain TVM1, the growth pattern and microscopic observation of the morphology of conidia and conidiophores were performed. Single spore of strain TVM1 was grown on the PDA plate at 25 °C for 5 days. Then, a 5-mm-diameter fungal plug was placed in the center of PDA plates (90 mm) at 25 °C in the dark for 7 days. The color, smell, growth, and shape of the colony and conidiophores branching pattern and conidia were examined [Kim *et al*., 2012] [7]. Afterwards, strain TVM1 was further identified through a phylogenetic analysis ITS region sequence. The phylogenetic tree of strain TVM1 based on the sequence of ITS region was constructed using the neighbor joining method in MEGA 4.0 software. The topology of the phylogenetic tree was evaluated using 1000 bootstrap re sampling replicates.

**Raising of Test Plants**

Tomato seeds (*Solanum lycopersicum* L.), variety PKM 1 was used in the glasshouse pot experiment. The seeds were soaked in 70% ethanol as surface sterilization for 2 min, and then the seeds were rinsed several times with sterile $H_2O$ and planted in plastic cell plug trays (4 x 4 cm) packed with vermiculite. The trays were kept under glass house conditions for 3 weeks and irrigated regularly.

**Efficacy of *Trichoderma reesei* under pot condition**

The pot experiment was laid out to evaluate the effects of T. reesei on tomato growth and root-knot nematode infection in the glasshouse using a completely randomized design (CRD). Tomato seedlings were planted in pots containing a 5 kg mixture of loam and sandy soil (1:1, w/w). Five tomato seedlings were planted in each pot and they were thinned to three per pot after 6 days of planting. Pots were irrigated regularly, and plants were grown under natural conditions. The experiment consisted of seven treatments, $T_1$-*Purpureocillium lilacinum* (Root dip + soil application 30 DAP), $T_2$-*Trichoderma viride* (Root dip+soil application 30 DAP), $T_3$- *P. Lilacinum* (root dip)+ *T. viride* (2 kg/ha)soil application 30 DAP), $T_4$- *T. reesei* (Root dip+soil application 30 DAP), $T_5$-*P. Lilacinum* (root dip)+ *T. reesei*(2 kg/ha), $T_6$-Carbofuran 3G @1kg a.i./ha, $T_7$-Untreated control (Healthy control). Each treatment was performed in five replicates. Plants were harvested 50 days after planting, comparable to the late vegetative stage of tomato growth.

**Effect of *Trichoderma reesei* against root knot nematode under field condition**

The field experiment was carried out in a field and severely infested with *M. incognita*, located in Agricultural College and Reseasrch Institute, Tiruvannamalai, Tamil Nadu in two growing seasons in 2020 and 2021. The four week-old tomato seedlings without infestation with *M.incognita* were transplanted into the field. The 196 mL sterile distilled water was added into 4 mL fermentation broth (106 spores/mL) of strain TVM1 or 4 mL PDB culture medium to obtain the desired concentrations and poured into the planting hole during plant transplantation. The experimental plots were 6m long, 5.5 m wide and separated by 0.5 m (6 plant rows) and contained 21 transplanted seedlings per row. A randomized complete block design was adopted in this experiment, and each treatment consisted of three replications. The fields were irrigated and fertilized followed as per the schedule. Fifteen plants and rhizosphere soil samples were randomly selected and collected from each treatment, 30 days after transplantation. The population at harvest stage from root and soil samples, root knot index and yield kg per plant were observed as per the procedure.

**Statistical analysis**

Data were statistically analyzed using SPSS software 20.0. Duncan's one-way analysis of variance was used to determine the significant differences.

**Results**

**Identification of Trichoderma isolates from Rhizosphere regions**

In total, 35 fungi isolates were obtained from rhizosphere soil of different crops and screened for the potential against *M. incognita in vitro* under *in vitro* condition. Among these isolates, TVM 1 (NCBI Accession No. OP895726) showed the strongest nematicidal activity against second stage juveniles (J2s) of *M. incognita*. Moreover, strain TVM1 showed significant antagonistic activity towards pathogens of Fusarium wilt in tomato under *in vitro*.

**Identification of *Trichoderma reesei* (TVM1)**

To identify strain TVM1, morphological observation was performed. The isolate TVM1 was grown rapidly on PDA medium forming a white, 60-mm-diameter colony at 25 °C under dark for 2 days. Then, the colony changed to greyish green or dark green and formed a wide conidial zone at the edge of the colony at 7 days (Fig. 1). The conidiophores were erect showing a long axis of the structure and fertile to the top. The cylindrical or spindle-shaped bottle stems were solitary born on main branch and spirally arranged on top of lateral branches. The conidia were yellowish green to dark green, globose with smooth walls. No distinct coconut-like odor was detected. Based on the above microscopic observations, isolate TVM1 was tentatively identified as

***Trichoderma***

To further identify isolate TVM1, a phylogenetic analysis of its 5.8 S-ITS sequences was performed. Sequence data were edited using BioEdit (ver. 7.0) software to derive a consensus sequence for each virus and aligned with a multiple alignment program ClustalX, (ver. 1.81) (Vaucheret, 2006). Conserved domains were identified using the blastp search tool. Phylogenic trees were constructed using the neighbor-joining method calculated with MEGA, ver. 3.1 (Rodriguez-Negretel *et al*., 2008). The phylogenetic tree showed that the small sub unit ITS sequence (GenBank accession number: OP895726) of isolate TVM1 was clustered with *T. reesei* (Fig. 2). These results demonstrated that isolate TVM 1 was identified as *T. reesei*.

***In vitro* study on nematode penetration**

Culture filtrate of *Trichoderma reesei* was tested against root knot nematode. The results showed that the culture filtrate of *Trichoderma reesei* TVM 1 isolate had an inhibitory effect on nematode penetration. The same observation was reflected in the number of galls per plant as well as number of eggmasses per root system (Table 1).

**Efficacy of *Trichoderma reesei (TVM1)* isolate against root-knot nematode (*Meloidogyne incognita*) as root dip and soil treatment under pot conditions.**

The result of the experiment revealed that, maximum root length (15.88 cm) was noted in *Trichodema reesei* ($T_4$)

inoculated pot followed by ($T_1$) *Purpureocillium lilacinum* (15.21 cm) inoculated pots. Minimum (8.12 cm) root length was recorded in inoculated control. Whereas shoot length maximum shoot length (45.40 cm) was noted in *Trichodema reesei* ($T_4$) inoculated pot followed by ($T_2$) *Trichoderma viride* (43.55 cm) inoculated pots when compared to control (30.11 cm) shoot length was recorded. (Table 1). Fresh weight of shoot was also influenced by *Trichoderma reesei* at (52.31gm/kg) and minimum fresh shoot weight (28.10 gm) were recorded in inoculated control. Minimum number of (25.41 galls/plant) was recorded in *Trichoderma reesei* treated pots followed by the combination of *Purpureocillium lilacinum* + *Trichoderma reesei* (30.31 galls/ plant) was recorded. For egg masses minimum number of (25.31egg masses/ gall) was recorded in *Trichoderma reesei* treated pots followed by the combination of *Purpureocillium lilacinum* + *Trichoderma reesei* (30.22 egg masses/ gall) was recorded (Table 1). The nematode population at harvest also recorded, *Trichoderma reesei* minimum population of 210.66 cc in soil and 141.21 cc in root when compared to inoculated control of 365.20 in soil and 1415.20 in root respectively. For yield performance, maximum of *Trichoderma reesei* (1.2 kg per plant) was recorded and minimum of 0.75 kg per plant was recorded in untreated control (Table 2).

**Biocontrol of root-knot nematode in the field experiment**

The field experiments showed similar results to the glasshouse pot experiments. The results indicated that *T. reesei* TVM1 is a biological control agent for effectively controlling root knot nematode under field conditions. Population at harvest stage both soil and root were calculated as per the procedure, root knot index and yield character also observed in the field experiments. The population at harvest stage in soil 210.66 cc was observed in soil and 141.21 cc was observed in root system when compared to control (365.20 cc in soil and 1415.20 cc in root). (Table 3)

**Fig 1:** The colony changed to greyish green or dark green and formed a wide conidial zone at the edge of the colony at 7 days

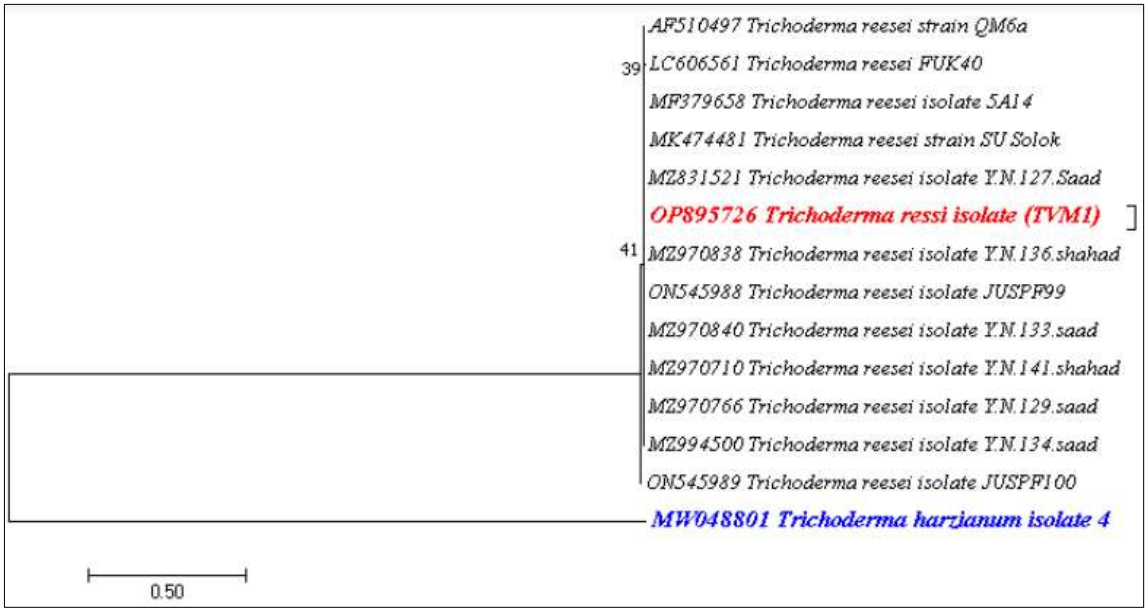


**Fig 2:** Phylogenetic tree showed similary with other sequences

**Table 2:** Assessment of morphometric characters, status of galls and egg masses in tomato var. (PKM1) treated with various bioagents against root knot nematodes, *M. incognita* under pot culture condition

| Treatments | Root length(cm) | Shoot length (cm) | Shoot weight (g) | No of galls /root system | No of egg masses /gall |
|---|---|---|---|---|---|
| T1- *Purpureocillium lilacinum* (Root dip + soil application 30 DAP) | 15.21 | 42.75 | 33.43 | 39.43 | 35.25 |
| T2-*T.viride* (Root dip +soil application 30 DAP) | 13.22 | 43.55 | 42.83 | 51.30 | 41.72 |
| T3-*P. lilacinum* (root dip)+*T. viride* (2 kg/ha)soil application 30 DAP) | 12.41 | 35.21 | 34.21 | 41.10 | 41.91 |
| T4- *T. reesei*(Root dip +soil application 30 DAP) | 15.88 | 45.40 | 52.31 | 25.41 | 25.31 |
| T5-*P. lilacinum* (root dip)+*T. reesei*(2 kg/ha) | 09.21 | 40.20 | 34.40 | 30.31 | 30.22 |
| T6-Carbofuran 3G @1kg a.i./ha | 11.52 | 35.19 | 32.20 | 54.91 | 41.22 |
| T7-Untreated control | 08.12 | 30.11 | 28.10 | 85.61 | 62.32 |
| CD (p=0.05) | 3.90 | 12.69 | 12.25 | 15.37 | 12.48 |
| SED | 1.79 | 5.82 | 5.62 | 7.05 | 5.73 |

**Table 2:** Assessment of population and yield in tomato var. (PKM1) treated with various bioagents against root knot nematodes, *M. incognita* under pot culture condition at farm of AC&RI, VVNR, Tiruvannamalai

| Treatments | Population at harvest (soil 200 cc) | Population at harvest (root 5 g) | Root knot index % | Yield kg/plant |
|---|---|---|---|---|
| $T_1$- *Purpureocillium lilacinum* (Root dip + soil application 30 DAP) | 612.18 | 511.22 | 3.0 | 1.1 |
| $T_2$-*T.viride* (Root dip + soil application 30 DAP) | 401.21 | 414.12 | 3.0 | 1 |
| $T_3$-*P. lilacinum* (root dip)+*T. viride* (2 kg/ha)soil application 30 DAP) | 274.16 | 165.32 | 2.0 | 1 |
| $T_4$- *T.reesei* (Root dip +soil application 30 DAP) | 210.66 | 141.21 | 1.0 | **1.2** |
| $T_5$- *P. lilacinum* (root dip)+*T. reesei* (2 kg/ha) | 631.44 | 602.72 | 2.0 | 1 |
| $T_6$-Carbofuran 3G @1kg a.i./ha | 265.20 | 201.22 | 4.0 | 1.1 |
| $T_7$-Untreated control | 365.20 | 1415.20 | 5.0 | 0.75 |
| CD (p=0.05) | 139.57 | 202.38 | 0.92 | 0.32 |
| SEd | 64.05 | 92.88 | 0.42 | 0.14 |

**Table 3:** Assessment of population and yield in tomato var. (PKM1) treated with various bioagents against root knot nematodes, *M. incognita* Under field conditions

| Treatments | Population at harvest (soil 200 cc) | Population at harvest (root 5 g) | Root knot index % | Yield kg/plant |
|---|---|---|---|---|
| T1-*T.viride* (Root dip + soil application 30 DAP) | 401.21 | 414.12 | 3.0 | 1 |
| T2- *T. reesei* (Root dip + soil application 30 DAP) | 210.66 | 141.21 | 1.0 | 1.2 |
| T3-Carbofuran 3G @1kg a.i./ha | 265.20 | 201.22 | 4.0 | 1.1 |
| T4-Untreated control | 365.20 | 1415.20 | 5.0 | 0.75 |
| CD (p=0.05) | 139.57 | 202.38 | 0.92 | 0.32 |
| SEd | 64.05 | 92.88 | 0.42 | 0.14 |

## Discussion

The results of the present study revealed that the native isolate of Trichoderma has an inhibitory effect on nematodes under pot and field conditions. In recent years, *Trichoderma* spp. has been used as biocontrol agents to control many plant pathogens as well as nematodes. Similar results indicated all the growth parameters increased as there was an increase in concentration of *Trichoderma viride* with increased in root-knot infested soil (Sonkar *et al*., 2018) [17]. Root-knot nematodes (*Meloidogyne* sp.) are sedentary endoparasites and are among the most destructive pests of agricultural crops. They are worldwide in distribution having a very wide host range. *Trichoderma* isolates have been used successfully to control the damage caused by soil-borne pathogens in greenhouses and under opened-field conditions (Papavizas, 1985) [11]. *Trichoderma* species also have been shown to have activity toward root-knot nematodes (Windham *et al*., 1989; Sharon *et al*., 2001) [20, 16]. Ering and Simon, (2018) revealed that the experiment has shown *Trichoderma* isolates can reduce the number of *Meloidogyne incognita* juveniles' counts, as the counts were much lower in the treated plots than the control plots. After 90 DAT the tomato plant was uprooted and the number of root gall/root system was observed. Pocurull *et al*. (2020) [12] revealed that, *Trichoderma asperellum* T34 reduced both nematode infectivity and reproduction ($P< 0.05$) by 71 and 54%, respectively. Meanwhile, *T. harzianum* T22 suppressed nematode reproduction by 48%, but did not affect nematode infectivity ($P< 0.05$). In addition, the biocontrol efficacy of another species *T. longibrachiatum* in controlling *M. incognita* was indicated from 30 to 78% for gall index on cucumber [Zhang *et al*., 2015] [24]. Nafady *et al*., (2022) [10] revealed that the damage of RKN can reduce over 50%, which suggested that *T. citrinoviride* Snef1910 has more biocontrol efficiency and potential application to control root knot nematode M. incognita on tomato. Interestingly, Zhang *et al*. [2017] [27] reported that the hydrolytic enzyme activity of Trichoderma species may explain the direct mechanism of *Trichoderma*–nematode interaction through the first step of Trichoderma parasitism on nematodes by hydrolyzing the nematode eggs and the second-stage juveniles. So, for disturbing nematode eggs and juveniles and consequently controlling the plant pathogenic nematodes, a combination of lytic enzymes including proteases, chitinases, and lipases are required [Tariq Javeed *et al*., 2021] [19].

## Conclusions

In conclusion, this study showed that *T. reesei TVM1* was screened from 35 fungi isolates and efficiently controlled root knot nematode caused by *M. incognita*, which played control

efficacy of more than 50% and increased egg hatching inhibition percentages and reduced root galls, egg masses and J2s on tomato. Moreover, *T. reesei TVM1* showed the plant growth promotion of shoot and root length of tomato. This is the first report on the use of a *T. reesei TVM1* as a potential biological control source to control root knot nematode caused by *M. incognita*. This study provides a new biological control agent and potentially practical strategies for sustainable management of root knot nematode.

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