ORIGINAL ARTICLE

Biocontrol efficacy of endophytic fungus, *Acremonium sclerotigenum*, against *Meloidogyne incognita* under in vitro and in vivo conditions

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Abstract

Biocontrol microorganisms are important tools for the control of root knot nematodes (*Meloidogyne* spp.). Endophytic fungi have shown great potential as biocontrol agents in such applications. We here isolated an endophytic fungus from tomato root galls infected with *M. incognita* and identified the isolate as *Acremonium sclerotigenum* based on morphology and the internal transcribed spacer sequence. The biocontrol potential of this fungus was evaluated both in vitro and in vivo. Specifically, in vitro analyses were conducted to determine the potential of *A. sclerotigenum* to increase *Meloidogyne incognita* juvenile (J2 stage) mortality and decrease *M. incognita* egg hatching rates. The results revealed that *A. sclerotigenum* culture filtrates caused high J2 mortality rates (up to 95.5%) and significantly inhibited egg hatching (by up to ~43%). Furthermore, eggs treated with the culture filtrate were disaggregated and could not develop into nematodes. An in vivo experiment showed that treatment of tomato plants with *A. sclerotigenum* suppressed root knot nematode populations and significantly reduced the galling index. Both *A. sclerotigenum* treatment and exposure to the nematicide abamectin had good control effects, with efficacy rates of 55.43% and 70.58%, respectively. In summary, the endophytic fungus *A. sclerotigenum* here showed excellent potential for biocontrol of *M. incognita*. Further studies should be conducted to identify the nematicidal compounds produced by this fungus and to establish the molecular mechanism of action associated with the observed biocontrol effects.

Keywords *Meloidogyne incognita* · *Acremonium sclerotigenum*, biocontrol

Abbreviations

DI   Disease index
GI   Galling index
PDA   Potato dextrose agar
PDB   Potato dextrose broth
RKN   Root knot nematode
RF   Reproduction factor
ITS   Internal transcribed spacer
qPCR Quantitative PCR

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Introduction

Members of the genus *Meloidogyne* are referred to as sedentary endoparasitic root knot nematodes (RKNs). These pests are economically important throughout the world due to the enormous financial losses they cause as a result of extensive crop damage (Abad et al. 2008). The RKN host range encompasses more than 3000 plant species, including nearly all cultivated crops, such as potatoes, carrots, and tomatoes (Przybylska et al., 2020). There are more than 100 species in the genus *Meloidogyne* (Tariq-Khan et al. 2017), the most important of which are *Meloidogyne incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, and *M. enterolobii* (Čepulytė et al. 2018; Collett et al. 2021; Rashidifard et al. 2018). *M. incognita* is a highly invasive species that has spread to at least 143 countries (Bebber et al. 2014).

Due to the heavy economic losses caused by RKNs, various measures have been tested and applied to control these species. However, RKNs are difficult to control due to their high multiplication rates, short life cycles, and broad

host range. The most common control methods are chemical nematicide application, crop rotation, and the use of resistant cultivars (Atia et al. 2020; Bhat et al. 2023). The latter use of resistant cultivars method is the most effective and economical approach to controlling nematodes. RKN-resistant cultivars of some crops, including tomato (Jacquet et al. 2005) and pepper (Fazari et al. 2012), are commercially available. Although breeders have undertaken research to identify and characterize RKN-resistant cultivars, such efforts have been hampered by a lack of resistant genetic resources. Chemical treatments are generally effective in combatting RKN, but their detrimental effects on the environment and human health, and instances of resistance developing among nematodes, have caused global restrictions on nematicidal chemicals. Although crop rotation is a common method of controlling RKN, their broad host range minimizes the efficacy of this approach as a nematode management method (Salem et al. 2019a).

Several other approaches have been tested to prevent RKN damage to plants, such as grafting (Salem et al. 2019b), application of organic amendments (Abdeldaym et al. 2014) or plant extracts (Ismail et al. 2020; Ahmad 2020), and biological control methods (Atia et al. 2016; Daneshkhah et al. 2013; Haque et al. 2018). Over the past few decades, there has been an increasing focus on the use of microorganisms as biocontrol agents (Atia et al. 2020). Fungal RKN antagonists can play crucial roles in nematode management; such species possess nematophagous or nematicidal activities (Bhat et al. 2023). These fungi are commonly classified into four types: endoparasites, nematode-trapping fungi, egg/female parasites, and toxin producers (Moosavi and Zare 2012; Anjum et al. 2019; Zhang et al. 2020). Nematophagous fungi grow slowly in soil, and a large fungal biomass is required for nematophagous activity in the field. Thus, these species do not have high control efficiency.

Endophytic fungi, which colonize the plant tissue, lack the shortcomings of nematophagous fungi and provide benefits to the host plant, such as enhanced growth (Mehmood et al. 2019), improved stress tolerance (Bastias et al. 2017), or more robust defense responses (De Silva et al. 2019; Hu et al. 2023). Endophytic fungi that are capable of producing nematotoxic metabolites are the most promising as potential biocontrol agents. These fungi can effectively inhibit and suppress RKNs by reducing their penetration, delaying their development, or decreasing their reproductive capacities (Grabka et al. 2022). Many strains of the endophytic fungus *Fusarium oxysporum* can efficiently reduce *M. incognita* and *Radopholus similis* population densities and reproductive rates (Grabka et al. 2022; Kisaakye et al. 2022). Culture filtrate and purified Chaetoglobosin A (ChA) isolated from the endophyte *Chaetomium globosum* NK 102 both have nematicidal activity, increasing mortality and reducing J2

penetration into cucumber roots (Natori 1983). In tall fescue (*Festuca arundinacea*), application of *Neotyphodium coenophialum* enhances host nematode resistance (Choi et al. 2022).

Notably, *Acremonium* spp. have been shown to secrete nematicidal metabolites (Lozano et al. 2022), suppress nematode egg hatching, and reduce nematode populations (Tian et al. 2014). *Acremonium* spp. are also known to produce a large number of bioactive metabolites such as phenols (Lu et al. 2022), alkaloids (Januar and Molinski 2013), peptides (Chen et al. 2012), polyketides (Wicklow and Poling 2009), and terpenes (Suciati et al., 2013). Several studies have examined the effects of *Acremonium*-derived metabolites on insects or nematodes. Furthermore, some *Acremonium* species have been found to enhance host plant resistance to diseases caused by soil-borne pathogens (Bogner et al. 2016).

Endophytes and nematodes occupy a similar ecological niche, giving endophytes distinct advantages as biocontrol agents compared to nematophagous fungi (Tian et al. 2014; Chen et al. 2022). Indeed, endophytes are well-suited to competition with soil-borne pathogens (whether bacterial, fungal, or nematodal) to protect their niche (Schouten 2016). Previously, fungal endophytes with biocontrol potential have predominantly been isolated from healthy rather than from diseased plants. However, it is feasible to obtain endophytic fungi with latent biocontrol capacities from diseased tissues. In the present study, we therefore aimed to isolate and identify endophytic fungi from *M. incognita*-infected tomato roots and to evaluate the potential of these isolates to control RKNs.

Materials and methods

This research was carried out in 2022 at the Tianjin Academy of Agricultural Sciences, Institute of Plant Protection, Vegetable Disease Laboratory (39°6'14" N, 117°3'32" E). Infected tomato roots were obtained from the agricultural fields of a village in the North China Plain in which RKN disease was a serious problem in tomato production (Xiawuqi village, Wuqing district, Tianjin; 39°7'5" N, 116°46'43" E).

Isolation and identification of endophytic fungi

Endophytic fungi were isolated from the galls of tomatoes infected with RKN. Root galls were washed to remove soil particles, disinfected in 0.5% NaClO solution for 1 min, then washed three times with sterile water. Root knots were cut into pieces (0.5 × 0.5 cm), which were placed on potato dextrose agar (PDA) media containing 100 µg/mL ampicillin, then incubated at 25 °C for 7 d in the dark. Isolates were

purified using the single spore method (Constantinescu 1988) and stored at 4 °C.

To identify the isolated fungal species, Petri dishes containing root knots as described above were incubated for 7 d at 25 °C in the laboratory. Fungal cultures were then identified based on microscopic and macroscopic features as described by Wei (1979). Isolates were maintained on PDA plates for further experiments.

To genetically confirm the identities of the fungal isolates, selected fungi were cultured on PDA media for 7 d at 25 °C, then mycelia were transferred to 1.5-mL centrifuge tubes. Fungal DNA was extracted using a fungal genome extraction kit (B511375; Sangon Biotech, Shanghai, China). The nuclear ribosomal internal transcribed spacer (ITS) sequence of each fungus was amplified via PCR with the IST1/IST4 primer pair (Van Nguyen et al., 2007). Each 50-μL reaction contained 100 ng genomic DNA, 0.5 μM each of the ITS1 and ITS4 primers, and 2× EsTaq Master Mix (Dye) (CW0690H, CWBIO, Jiangsu, China). The thermocycling program consisted of denaturation for 4 min at 95 °C; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s; and a final elongation step at 72 °C for 10 min. ITS amplicons were then sequenced by Azenta Life Sciences. The NCBI database was searched using the resulting sequences as queries to identify similar sequences. The ITS sequences of several species in the genus *Acremonium* were downloaded from GenBank. These sequences were aligned and a phylogenetic tree was constructed in MEGA7.0 (Mega Limited, Auckland, New Zealand) using the neighbor-joining algorithm with 1000 bootstrap replicates.

Nematode culture preparation

A pure culture of the RKN *M. incognita* has been maintained on pepper (*Capsicum annuum* L. cv. 'Qiemen') plants in the greenhouse at the Tianjin Academy of Agricultural Sciences. For this study, two months after inoculation, galled pepper roots were hand-picked and washed to remove soil particles. Egg masses were collected and sterilized in 0.5% NaClO solution for 1 min, then rinsed twice in sterile distilled water. The final concentration of the egg suspension was adjusted to ~1000 eggs/mL. To obtain active *M. incognita* juveniles at the J2 stage (J2s), eggs were incubated at 25 °C in a Petri dish containing sterilized water to promote hatching. The resulting nematode suspension contained ~1000 J2s/mL.

In vitro nematicidal bioassays

Nematicidal bioassays were conducted in vitro to screen 81 fungal isolates for activity against *M. incognita*. Each isolate was grown on PDA for 5 d, then agar blocks of 5 mm in diameter were removed from the plate and placed in 500-mL flasks containing 200 mL of potato dextrose broth (PDB). After incubation at 25 °C for 5 d with 120 rpm shaking, cultures were centrifuged at 1000 × g for 5 min. The supernatant was passed through a 0.22-μm micropore filter (Pall Life Sciences, Ann Arbor, MI, USA) to remove the conidia and mycelia. Undiluted culture filtrate (100% concentration) was diluted in sterile deionized water to generate 25%, 50%, and 75% culture filtrate solutions. Each well of a sterile 24-microwell plate (Costar® 3526, Corning Incorporated, China) was filled with 100 μL of *M. incognita* J2 suspension (~1000 J2s/mL) and 1 mL of fungal culture filtrate (25%, 50%, 75%, or 100%) or PDB as a control. Microwell plates were then incubated at 25 °C in the dark for 24, 48, and 72 h. Plates were examined under a stereomicroscope at 10× magnification to count the number of dead J2s at each concentration of culture filtrate. Individuals were classified as dead based on their shape and the absence of mobility as described by Duong et al. (2021). The percentage of dead nematodes was calculated to estimate the toxicity of each concentration of fungal filtrate. This experiment was repeated five times independently. Based on the results of this screening, one promising fungal endophyte (*A. sclerotigenum*) was selected for further testing.

In vitro egg hatching activity assay

Egg hatching bioassays were conducted in vitro following the protocol described above for the nematicidal assays, with the substitution of eggs for J2s. The numbers of hatched nematodes were counted at 3, 4, and 5 d of treatment, then the hatching inhibition rate for each treatment was calculated at each timepoint. The experiment was repeated five times independently.

In vivo experiments under greenhouse conditions

Seeds of the susceptible *Solanum lycopersicum* variety 'Jinpeng No. 1' (produced by Xi'an Jinpeng Seedlings Co., LTD) were obtained from a market. Tomato seeds were sown in a seedbed containing sterilized soil mix (1:1:1 clay:sand:loam [v/v/v]) and grown at 25 °C under a 16/8 h light/dark photoperiod. When tomato seedlings reached the trifolate stage, a suspension of 5-d-old *A. sclerotigenum* conidia was prepared, containing 10^6 conidia as measured with a hemocytometer under an optical microscope. After the seedlings were gently removed from the soil, the roots were drenched in the conidia suspension for 10 s, then replanted in 140×125×100 mm plastic pots. A 1% microcapsule suspension of the nematicide abamectin (Jiangsu Yancheng Shuangning Agrochemical Co. LTD, China) at a dosage of 5.52 kg/hm^2 was used as the positive control, and

sterilized water was used as the negative control. After 7 d, each pot was inoculated with 1000 fresh *M. incognita* J2s at three locations surrounding the stem. Each treatment group contained 10 seedlings. After 2 months, roots were harvested from the soil and infected lateral roots were examined to evaluate the control effects of the selected fungal isolate (*A. sclerotigenum*) and abamectin. Control effects were determined based on the galling index (GI) scores and the reproduction factor (Rf) values. GI scores were assessed for each plant using a scale from 0 to 4 as follows: 0, no galls on root; 1, 1–25% of the root area covered in galls; 2, 26–50% of the root area covered in galls; 3, 51–75% of the root area covered in galls; and 4, 75–100% of the root area covered in galls (Ma et al. 2017). Disease index (DI) values were calculated with the following formula:

$$DI(\%) = \frac{\sum(\text{selected GI value} \times \text{number of plants with the selected GI value})}{4 \times \text{total number of plants}} \times 100$$

The control efficacy was calculated as follows:

$$\text{Control efficacy}(\%) = \frac{\text{DI in control group} - \text{DI in treatment group}}{\text{DI in control group}} \times 100$$

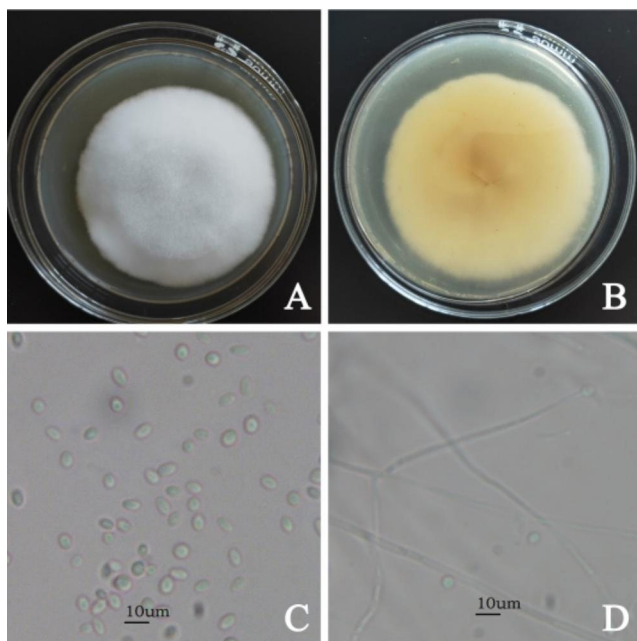Rf was calculated as described by Naz et al. (2021):

$$Rf = Pf/Pi$$

Fig. 1 Colony and conidia morphology of the selected endophytic fungus after 7 d of growth at 25 °C on potato dextrose agar. (**A, B**) Colony morphology as viewed from the (**A**) upper and (**B**) lower side of the plate. (**C, D**) Conidia as viewed at 100× magnification under a light microscope. Scale bar = 10 μm

Where Pf and Pi are the final and initial nematode populations, respectively. This experiment was repeated three times independently.

Statistical analysis

Data were analyzed using SPSS v21.0 (IBM, Chicago, IL, USA). Analysis of variance (ANOVA) was conducted to analyze differences in the mean mortality rate, egg hatching rate, RKN population, GI, and DI between treatment groups. Where ANOVA revealed significant differences, a post-hoc Tukey's honestly significant difference (HSD) test was applied. Significant differences were called at $p \leq 0.05$.

Results

Isolation and identification of endophytic fungi

A total of 81 endophytic fungi were obtained in this study, and all strains were screened against *M. incognita* in laboratory. Base on the results of this screening, on promising fungal endophyte (*A. sclerotigenum*) was selected for further testing, which showed over 90% mortality rates of after exposure for 72 h.

After the selected fungal isolate was grown, morphology was assessed to establish its identity (Fig. 1). When grown on solid medium, colonies were white on the top surface and light yellow on the lower surface. Conidia were hyaline and ellipsoidal and were 4.0–5.1 μm × 1.1–1.9 μm in size.

The ITS region of the isolated fungus was then sequenced (GenBank OP926024). In a phylogenetic analysis of related ITS sequences, the selected fungal isolate was in the same clade as an *A. sclerotigenum* strain, with which the selected isolate had 100% sequence similarity (Fig. 2). Thus, based on morphological characteristics and the ITS sequence, the isolated fungus was identified as *A. sclerotigenum*.

Effects of *A. sclerotigenum* on *M. incognita* J2 mortality

All four *A. sclerotigenum* culture filtrate concentrations (25%, 50%, 75%, and 100%) showed significant ($p < 0.05$) nematicidal effects against *M. incognita* J2s after 24, 48, and 72 h of exposure (Fig. 3). The highest concentration of culture filtrate (100%) killed 95.5% of *M. incognita* J2s after exposure for 72 h. This was significantly higher than the mortality rate in the control group (3.0%), which was treated with PDB.
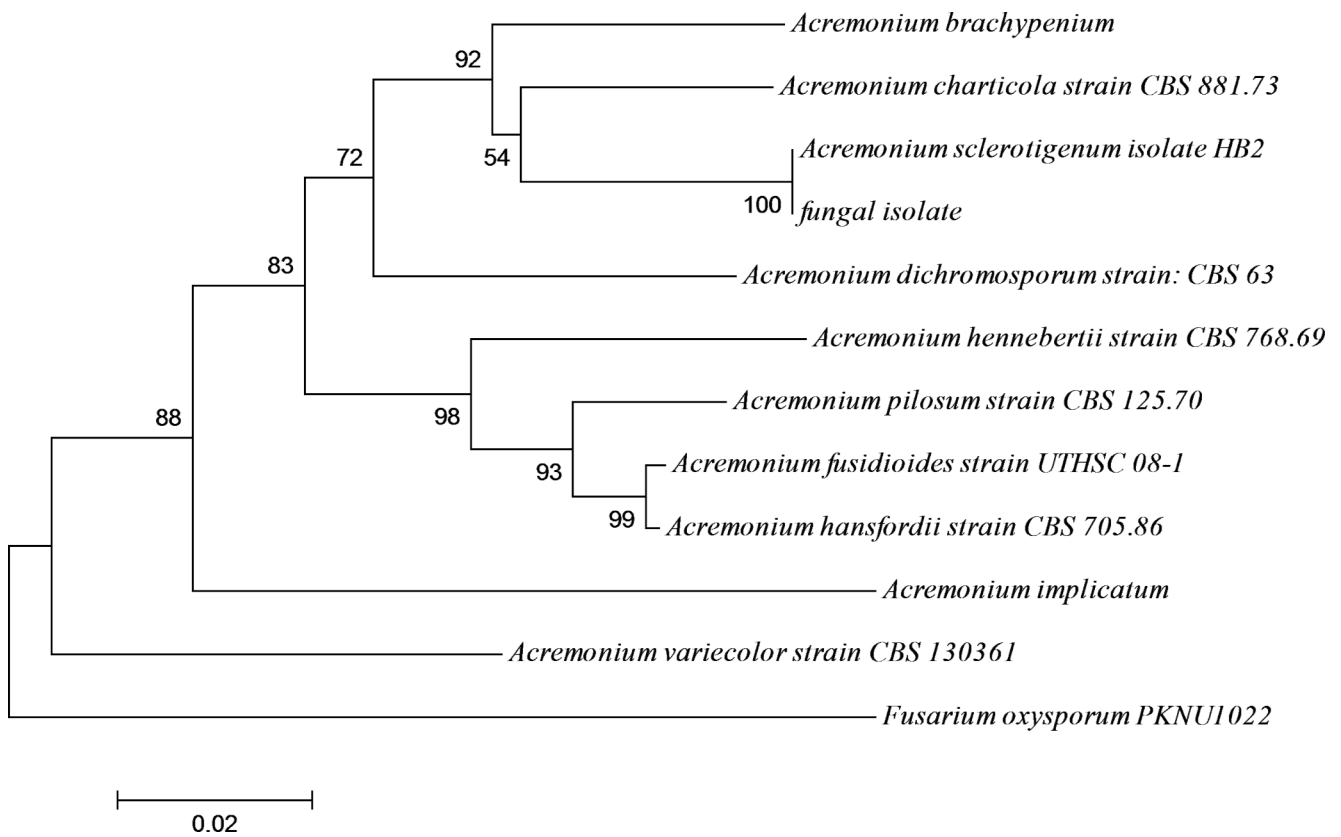
Fig. 2 Phylogenetic tree showing relationships between the nuclear ribosomal internal transcribed spacer (ITS) regions of the isolated fungus (OP926024) and other *Acremonium* species and strains, with *Fusarium oxysporum* serving as the outgroup. The tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. Bootstrap support values are shown at each node
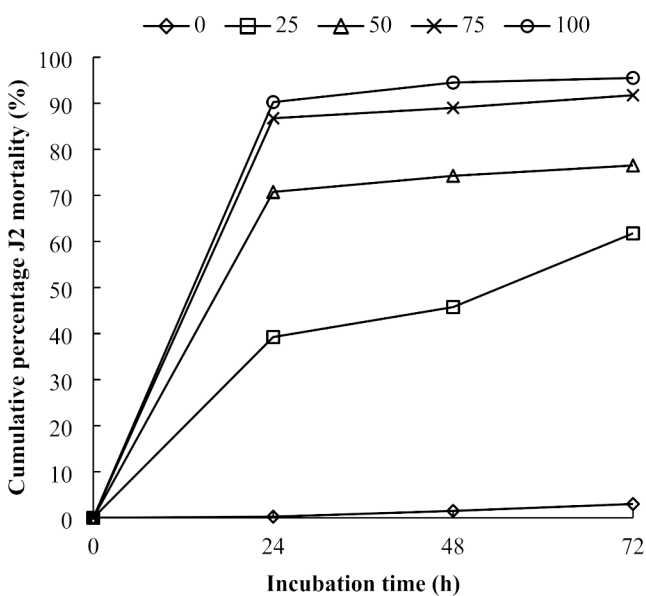
Effects of *A. sclerotigenum* on *M. incognita* egg hatching rates

Treatment with *A. sclerotigenum* culture filtrate markedly inhibited *M. incognita* egg hatching after 5 d (Fig. 4). In the control group, 88.75% of the eggs hatched, whereas only 50.75% of eggs in the group treated with 100% culture filtrate hatched. Notably, the egg hatching inhibition rate increased along with the culture filtrate concentration and incubation time. Furthermore, eggs in the control group were smooth and intact and could hatch into nematodes (Fig. 5C). However, treated eggs tended to disaggregate and could not develop into nematodes (Fig. 5A, B).

Effects of *A. sclerotigenum* treatment on *M. incognita* in vivo

Data obtained from greenhouse-grown tomato plants infected with *M. incognita* indicated that treatment with *A. sclerotigenum* suppressed RKN infection, significantly reducing the GI values of treated plants ($p < 0.05$) (Fig. 6). Compared with the control treatment, both the *A. sclerotigenum* and the abamectin treatments had good control effects;
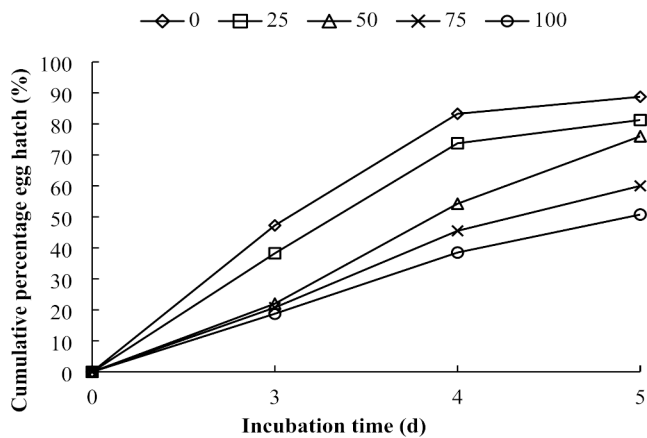

Fig. 3 Effects of treatment with *Acremonium sclerotigenum* culture filtrate on J2-stage *Meloidogyne incognita* mortality rates over time

Fig. 4 Effects of treatment with *Acremonium sclerotigenum* culture filtrate on *Meloidogyne incognita* egg hatching rates over time
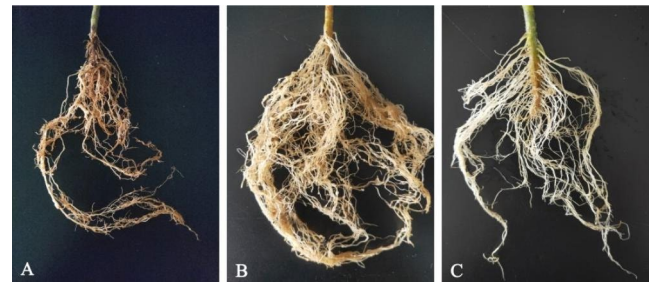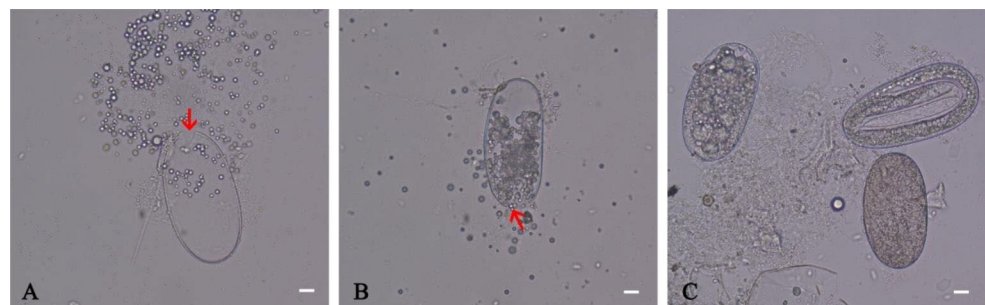

Fig. 6 Images showing representative roots of tomato plants infected with *Meloidogyne incognita*. Roots were collected from plants treated with (**A**) sterile water (control), (**B**) *Acremonium sclerotigenum*, and (**C**) the nematicide abamectin

the preventive efficacies of the two treatments were 55.43% and 70.58%, respectively (Table 1). The Rf of *M. incognita* was highest in the control group (3.06), lower in samples treated with the biocontrol agent *A. sclerotigenum* (1.57), and lowest in samples treated with the nematicide abamectin (1.33) (Table 1).

Table 1 Effects of *Acremonium sclerotigenum* treatment on *Meloidogyne incognita* infecting tomato plants

Treatment	Disease index (DI)	Control effect (%)	Rf (Pf/Pi)
A. sclerotigenum	15.71 ± 2.56 [b]	55.43 ± 3.27 [b]	1.57 [b]
Abamectin	10.48 ± 3.08 [c]	70.58 ± 5.77 [a]	1.33 [b]
Control	35.28 ± 5.16 [a]	–	3.06 [a]

Lowercase letters within each column indicate statistical significance groups at $p < 0.05$ (analysis of variance and post-hoc Tukey's test). Rf, reproduction factor; Pf, final nematode population; Pi, initial nematode population. The control effects were calculated with respect to the control group, and a control effect could therefore not be calculated for the control group itself

Discussion

Numerous publications have emphasized the negative impacts of chemical nematicide application on the ecological environment and human health (e.g., Hardoim et al. 2015; Mishra et al. 2021). It is therefore important to identify putative nematicidal biocontrol agents. Studying these fungi will enable researchers to identify potential biocontrol agents and understand their interactions with pathogenic nematodes, ultimately minimizing the necessity of chemical nematicide use.

We here studied application of the endophytic fungus *A. sclerotigenum* (in the form of culture filtrates or conidia suspensions) for RNK management. In vitro bioassays revealed strong nematicidal activity of the culture filtrate. In a test of varying culture filtrate concentrations, the highest concentration of culture filtrate (100%) caused 95.5% *M. incognita* J2 morality after 72 h. Moreover, treatment with *A.*

sclerotigenum culture filtrate markedly inhibited *M. incognita* egg hatching; the hatching inhibition rate increased along with the culture filtrate concentration and the incubation time. Fungal nematicidal activity can usually be attributed to either the production of nematicidal metabolites or to fungal parasitism. In this case, the use of culture filtrate precluded the possibility of parasitism. Consistent with the hypothesis that a nematicidal metabolite was involved, *M. incognita* responses to treatment with *A. sclerotigenum* culture filtrate were concentration-dependent. Similar effects have been reported previously. For example, undiluted *Trichoderma harzianum* culture filtrate causes higher *M. incognita* hatching inhibition rates than diluted cultures (Babu et al. 2021). Culture filtrates of some non-pathogenic endophytic *F. oxysporum* strains contain specialized metabolites that are highly toxic to *M. incognita* (Constantin et

Fig. 5 Effects of treatment with *Acremonium sclerotigenum* culture filtrate on *Meloidogyne incognita* eggs. (**A, B**) Eggs treated with undiluted *A. sclerotigenum* culture filtrate for 5 d. (**C**) Eggs treated with the control solution (potato dextrose broth) for 5 d. Scale bar = 10 μm

al. 2019). *C. globosum* is known to secrete many types of specialized metabolites with anti-nematode activity (Khan et al. 2019). A study conducted with endophytes isolated from banana roots showed that endophyte treatment caused 100% inhibition of *M. incognita* egg hatching and resulted in a 81.33–96.33% J2 mortality rate after treatment for 72 h (Ganeshan et al. 2021). The endophytic fungus *Acremonium implicatum* was previously obtained from the root galls of tomato plants infected with *M. incognita*; this fungus inhibited *M. incognita* egg hatching and showed excellent nematicidal effects against J2s (Tian et al. 2014). The filamentous fungus *Acremonium strictum*, which markedly reduces nematode populations and improves tomato plant health, was isolated from *M. incognita* egg masses (Oro et al. 2021). *A. strictum* also parasitizes *M. incognita* eggs, inhibiting hatching (Bhat et al. 2023).

In the present study, in vivo experiments revealed that *A. sclerotigenum* significantly suppressed RKN infection and drastically reduced the Rf of nematodes associated with treated compared to untreated plants. Similarly, the endophyte *C. globosum* significantly inhibits *M. incognita* J2 penetration and female reproduction, ultimately reducing galling in cotton plants (Zhou et al. 2016). Treatment with the endophytic fungus *Fusarium oxysurum* has also been shown to reduce the *M. incognita* populations associated with tomato, squash, and melon (Ganeshan et al. 2021). Endophytic *F. oxysporum* treatment reduces female *M. incognita* reproduction and J2 penetration of tomato roots (Bogner et al. 2016).

The benefits of endophytic fungal associations with crops are numerous. However, a key issue with the use of endophytes as biological control agents is the difficulty of detecting and quantifying them within the host plant to assess persistence. Quantitative PCR (qPCR) can be used to quantify microorganism abundance. In a study of tomato roots, qPCR was applied to measure colonization by *Pochonia chlamydosporia*. This experiment revealed that colonization decreased over time (Escudero and Lopez-Llorca 2012). Another study found that *P. chlamydosporia* 123 persisted endophytically in banana roots up to 75 d after a single inoculation, and that repeated application of the fungus lengthened the duration of root colonization (Mingot-Ureta et al. 2020). Such results demonstrate that endophytic fungi can persist in the roots over long durations. However, further optimization is required to increase fungal abundance in the roots, which decreased as the plants developed.

We here applied the endophytic fungus *A. sclerotigenum* to control RKNs, not only colonizing host plant tissue, but also producing a desirable control efficiency rate. These findings validated the strategy of screening infected plant tissues to identify organisms with putative biocontrol effects. However, the nematicidal metabolites produced by *A. sclerotigenum*, and the molecular genetic mechanism underlying their biosynthesis, remain unknown. Further analyses of this system should be conducted to delineate the relationships between endophytes, nematodes, and plants at the molecular level, specifically addressing the nematicidal effects of specialized metabolites produced by the fungi.

Conclusion

A. sclerotigenum culture filtrates were here shown to significantly increase *M. incognita* mortality and to decrease egg hatching rates. Treatment with culture filtrate also caused *M. incognita* eggs to disaggregate and fail to develop into nematodes. Importantly, treatment of tomato plants with *A. sclerotigenum* suppressed the RKN population and significantly reduced the GI to levels comparable to those of plants treated with the nematicide abamectin. Our study clearly showed that the endophytic fungus *A. sclerotigenum* isolated from tomato plants has great potential as a biocontrol agent against RKNs, advancing sustainable agricultural methods and promoting food security.

Data Availability Data analyzed for this study are included in the manuscript.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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