Journal of Applied Microbiology, 2024, 135, lxae111
https://doi.org/10.1093/jambio/lxae111
Advance access publication date: 1 May 2024
Research Article

JOURNAL OF APPLIED MICROBIOLOGY
OXFORD UNIVERSITY PRESS

Control of the plant-parasitic nematode *Meloidogyne incognita* in soil and on tomato roots by *Clonostachys rosea*

Tobias Stucky[1], Eliana Thyda Sy[1], Jakob Egger[1], Enis Mathlouthi[2], Jürgen Krauss[2], Lara De Gianni[1], Andrea Caroline Ruthes[3,*], Paul Dahlin[1,*]

[1]Entomology and Nematology, Plant Protection, Agroscope, Müller-Thurgau-Strasse 29, 8820 Wädenswil, Switzerland
[2]Vegetable-Production Extension, Plants and Plant Products, Agroscope, Müller-Thurgau-Strasse 29, 8820 Wädenswil, Switzerland
[3]Mycology, Plant Protection, Agroscope, Route de Duillier 60, 1260 Nyon, Switzerland

*Corresponding authors. Mycology, Plant Protection, Agroscope, Route de Duillier 60, 1260 Nyon, Switzerland, andreacaroline.ruthes@agroscope.admin.ch; Entomology and Nematology, Plant Protection, Agroscope, Müller-Thurgau-Strasse 29, 8820 Wädenswil, Switzerland, paul.dahlin@agroscope.admin.ch

Abstract

Aims: *Clonostachys rosea* is a well-known mycoparasite that has recently been investigated as a bio-based alternative to chemical nematicides for the control of plant-parasitic nematodes. In the search for a promising biocontrol agent, the ability of the *C. rosea* strain PHP1701 to control the southern root-knot nematode *Meloidogyne incognita* was tested.

Methods and Results: Control of *M. incognita in vitro* and in soil by *C. rosea* strain PHP1701 was significant and concentration dependent. Small pot greenhouse trials confirmed a significant reduction in tomato root galling compared to the untreated control. In a large greenhouse trial, the control effect was confirmed in early and mid-season. Tomato yield was higher when the strain PHP1701 was applied compared to the untreated *M. incognita*-infected control. However, the yield of non-*M. incognita*-infected tomato plants was not reached. A similar reduction in root galling was also observed in a field trial.

Conclusions: The results highlight the potential of this fungal strain as a promising biocontrol agent for root-knot nematode control in greenhouses, especially as part of an integrated pest management approach. We recommend the use of *C. rosea* strain PHP1701 for short-season crops and/or to reduce *M. incognita* populations on fallow land before planting the next crop.

Impact Statement

The impact of using *Clonostachys rosea* strain PHP1701 lies in its ability to function as an alternative control of the devastating plant-parasitic nematode *Meloidogyne incognita*. The results showed that, as an integrated approach, this fungal strain PHP1701 can assist growers in controlling *M. incognita* during and between crop cycles when applied directly to the soil or to tomato rootstocks, supporting a more sustainable approach to crop protection.

Keywords: Nematophagous fungi; Biocontrol; *Clonostachys rosea*; *Meloidogyne incognita*.

Introduction

Sustainable control of plant-parasitic nematodes is of great importance in order to meet the world's growing demand for food. Current estimates suggest that plant-parasitic nematodes are responsible for >30% of global crop yield losses (Sikora et al. 2023). In particular, the southern root-knot nematode *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949 is one of the most damaging plant-parasitic nematodes worldwide (Subedi et al. 2020).

The sedentary endoparasitic *Meloidogyne* species belong to a devastating group of nematodes that cause severe root galling in plants, hence the name root-knot nematodes. The obligate plant-parasitic life cycle of *M. incognita* begins with the second-stage juveniles (J2) infecting the host root by penetrating the root cell walls near the root tip with their stylet. The J2 then migrate between cells to the vascular cylinder of the plant. There, they establish a permanent feeding site by secreting cell wall degrading enzymes and induce the surrounding root cells to differentiate into a multinucleate giant cell on which the sedentary nematodes feed (Tian et al. 2015). Nematode infection and root galling lead to poor plant growth and reduced plant resistance to other biotic and abiotic stresses (Jones et al. 2013, Siddique and Grundler 2018). After the third nematode molt in the plant, only males become motile and leave the host. The sedentary females produce eggs, which are mainly deposited outside the root surface in a gelatinous matrix (Papadopoulou and Triantaphyllou 1982, Jones et al. 2013). Males are thought to play a minor role in reproduction, as females can reproduce asexually (Jones et al. 2013).

The main pillar of integrated nematode management in annual crops is the use of different crops implemented in a crop rotation system (Sikora et al. 2023). Nonetheless, nematicidal products have to be applied to control polyphagous nematodes.

With the phase-out of "older" chemical nematicides, which are harmful to human health and the environment, there is a growing global demand for bio-based alternatives to control plant-parasitic nematodes. In Switzerland, only one bio-based nematicide is commercially available without strict regulation. This biological nematicide is a nematode egg parasitic fungus,

Received 12 February 2024; **revised** 24 April 2024; **accepted** 30 April 2024

named *Purpureocillium lilacinum* strain 251 (formerly *Paecilomyces lilacinus* strain 251) (Sikora and Roberts 2018).

Soil-borne microorganisms such as fungi are promising plant-parasitic nematode antagonists that should be further investigated (Pires et al. 2022). In addition to the already known nematophagous fungal families with the ability to capture and/or parasitize nematodes or secrete metabolites that control nematodes (Pires et al. 2022), there are new fungal families that have been less studied as plant-parasitic nematode antagonists. For example, the saprophytic filamentous fungus *Clonostachys rosea* (Link) Schroers, Samuels, Seifert, and W. Gams 1999, which is used as an active ingredient in biological crop protection products against various plant pathogens such as fungi and insects, shows promise in the control of plant-parasitic nematodes (Dong et al. 2005, Zou et al. 2010, Iqbal et al. 2018b, Sun et al. 2020). To date, *C. rosea* has been reported to be active against the nematodes *Helicotylenchus* spp., *Heterodera* spp., *Pratylenchus* spp., *Trichodorus* spp. (Iqbal et al. 2018b), and *Meloidogyne* spp. (Migunova et al. 2018, Cristóbal-Alejo et al. 2021), as well as the model nematode *Caenorhabditis elegans* (Dong et al. 2005).

Clonostachys rosea, first described by Bainier (1907) and formerly known as *Gliocladium roseum*, relies on multiple mechanisms to control plant-parasitic nematodes. However, preliminary control mechanisms employ cell wall degrading enzymes (mainly chitinases, glucanases, and proteases) and secondary metabolites (Dong et al. 2005, Chatterton and Punja 2009, Fatema et al. 2018, Iqbal et al. 2018a, 2020). For example, an extracellular serine protease in *C. rosea* was found to degrade the cuticle of the free-living nematode *Panagrellus redivivus* (Li et al. 2006), and deletion of a gene encoding a subtilisin-like protease from *C. rosea* reduced its virulence against nematodes (Zou et al. 2010).

Studies by Dong et al. (2005) showed that a class of secondary metabolites belonging to the verticillin-type epipolysulfanyldioxopiperazine from *C. rosea* strain 1A had nematicidal activity against the model nematode *C. elegans* and the nematode *P. redivivus*. However, nematode mortality in *C. rosea* culture filtrates against *Heterodera glycines* and *Pratylenchus penetrans* varied widely between strains (Iqbal et al. 2020), suggesting that *C. rosea* lacks host specificity and that nematode control can vary widely between selected strains. It has also been reported that some strains of *C. rosea* were shown to have a parasitic lifestyle against *Helicotylenchus* sp., *Heterodera* sp., *Paratylenchus* sp., *Pratylenchus* sp., and *Trichodorus* sp. (Iqbal et al. 2018b, 2019).

Therefore, in this study, we selected the promising *C. rosea* strain PHP1701 from an *in vitro* test for further testing, in soil, in small pot experiments, and in large greenhouse conditions, for its ability to control the root-knot nematode *M. incognita*.

Materials and methods

Meloidogyne incognita rearing and collection of second-stage juveniles

Three-week-old tomato (*Solanum lycopersicum*) cv. Oskar plants were used to propagate a *Mi*-virulent *M. incognita* population described by Hallmann and Kiewnick (2018) under greenhouse conditions (25°C/19°C, 60% humidity, 15/9 h light/dark cycle). Heavily galled root systems were used to extract J2 using a mist chamber or eggs by cutting roots into 1 cm pieces and shaking vigorously for 3 min in 1% NaOCl water solution, and collecting the eggs in a 20 μm mesh sieve. Nematode densities (eggs or J2 ml $^{-1}$) were counted under an inverted light microscope (Zeiss, at 5× magnification) using a counting chamber, and nematode suspensions were kept refrigerated at 4°C until further use. Nematode suspensions were adjusted accordingly for each experimental setup. Periodically, total DNA was extracted from *M. incognita* for barcoding analyses to ensure the identity of *M. incognita* according to Kiewnick et al. (2013).

Clonostachys rosea isolate and culture preparation

The *C. rosea* strains tested were of two different origins. A Swiss strain of *C. rosea*, designated F20, was isolated from individually selected free-living nematodes observed to be infected by fungi during diagnostic analysis. The selected fungus-infected nematode was placed on a Petri dish (100 × 15 mm) containing potato dextrose agar (PDA) medium supplemented with antibiotics (ampicillin and erythromycin) (Oxoid) at 22°C in the dark. The fungal isolate was then propagated on new PDA plates for maintenance and identified as *C. rosea* by the amplification of the internal transcribed spacer (ITS) region of its ribosomal DNA using the primers ITS1f (Gardes and Bruns 1993) and ITS4 (White et al. 1990).

Clonostachys rosea strain PHP1701 was obtained as a formulated powder from Andermatt Biocontrol Suisse (Grossdietwil, Switzerland). For *in vitro* analysis, *C. rosea* strain PHP1701 was grown on PDA plates as described for the Swiss *C. rosea* strain.

In vitro comparison of two *C. rosea* strains against *M. incognita* second-stage juveniles

To compare the efficacy of the Swiss *C. rosea* strain F20 with *C. rosea* strain PHP1701 in controlling root-knot nematodes, the selected *C. rosea* were tested *in vitro* against *M. incognita* J2 using six-well plates. In each well, 1% water–agar medium was added just enough to cover the bottom of the plate. Conidia were suspended in MiliQ water from the PDA plates and counted under a light microscope using an improved Neubauer counting chamber. The conidium concentration was adjusted to 1×10^7 conidia ml^{-1} and individual plates were inoculated with either strain F20 or PHP1701 (1×10^7 conidia ml^{-1}). One plate was used as a control (no fungi). After 7 days, surface-sterilized nematodes (EPPO Standard Diagnostics, PM 7/148) were added (150 J2 per well) and J2 viability was scored under an inverted light microscope (Zeiss, 10× magnification) as active, inhibited noninfected, and inhibited fungus infected after 1, 3, and 6 days ($n = 6$).

Testing of *C. rosea strain* PHP1701 against *M. incognita* under soil conditions

As *C. rosea* strain PHP1701 against *M. incognita* showed most promising results *in vitro*, its biocontrol activity under different application concentrations was tested in pots filled with 100 ml of steamed soil: sand mixture (1:3) inoculated with 150 J2 (as referred to in the "*Meloidogyne incognita* rearing and collection of J2" section). Three days after J2 inoculation, a dilution of the recommended concentration per plant of 0.2, 0.02 (one- tenth of the recommended concentration per plant), 0.01, or 0.005 g of *C. rosea* pot was applied as a 15 ml solution ($n = 6$). For the negative control, 25 ml of water was used. Pots were covered with a plastic foil and kept moist at

23°C with 60% humidity for 14 days in the dark. J2 was extracted from the entire 100 ml of soil from each treatment, using the Oostenbrink dish method described by Hallmann and Subbotin (2018). Nematodes were counted under an inverted light microscope (Zeiss, 5× magnification) in a counting chamber.

Test of *C. rosea* strain PHP1701 against *M. incognita* in a small greenhouse trial

To test the efficacy of *C. rosea* strain PHP1701 in a small greenhouse trial, 40 pots filled with 1 l of steamed soil:silver sand (1:3, v/v) were inoculated with 6000 J2/pot and allowed to settle for 3 days before applying *C. rosea* PHP1701 at the recommended concentration of 0.2 g/pot. Subsequently, 3-week-old tomato plants cv. Moneymaker were planted in the pots as indicator plants.

The treated and untreated control pots were arranged in a randomized block design and maintained at 25°C/19°C, 60% humidity, and a 15 h/9 h light/dark cycle in the greenhouse. After 4 and 9 weeks ($n = 10$; replicates), tomato roots were washed free of soil and roots were scored according to Zeck (1971), where 0 represented no root galling and 10 represented dead galled roots caused by root-knot nematodes.

Performance of *C. rosea* strain PHP1701 against *M. incognita* under a large greenhouse setting

In the greenhouse, eighty 15 l pots were filled with heat-sterilized field soil and arranged in four rows, to test the performance of the biocontrol agent on a large scale. Nematode-positive pots were inoculated with a *M. incognita* suspension containing 5000 J2/egg (52% eggs and 48% J2). Nematode-free control pots and those inoculated with *M. incognita* were paired as untreated and treated with 0.2 g *C. rosea* strain PHP1701/plant. Treatments with *C. rosea* strain PHP1701 were repeated every month or every second month with 0.2 g/plant. Each treatment was replicated eight times ($n = 8$). Three days after nematode inoculation, the first treatments were applied directly to tomato rootstocks 'Ubari' [containing Mi resistance and grafted with tomato variety 'Cristal' (The Rootstock Company)] and plants were planted the next day. The plants were watered and fertilized as needed with soluble NPK fertilizer (Kristalon Red Acid, Yara, UK) using a drip irrigation system. Red tomato fruits were harvested, counted, and weighed for yield estimation. At the beginning, middle, and end of the growing season, root galling by *M. incognita* was indexed on a scale of 0–10 according to Zeck (1971) by harvesting one row at the beginning and middle of the season and two rows at the end of the season.

On-farm trial to evaluate the performance of *C. rosea* PHP1701 against *Meloidogyne* sp.

To evaluate the efficacy of the biocontrol agent in the field, a small on-farm trial was conducted as a block of four 50 m rows, divided in half, and used as *C. rosea* PHP1701 treated or untreated blocks. At planting in late March, the grower applied 0.2 g *C. rosea* PHP1701/plant to half of the planted area and repeated the applications on a monthly basis. Root galling was indexed according to Zeck (1971) on 2 May and 5 July 2023 ($n = 10$).

Antibiosis assay

To test whether the biocontrol activity was due to direct parasitism or due to the indirect effect of secondary metabolites produced by *C. rosea*, strain PHP1701 was cultured in Erlenmeyer flasks (100 ml) containing liquid (50 ml) potato dextrose broth) inoculated with 1×10^6 conidia ml^{-1} (as described in the "*In vitro* comparison of two *C. rosea* strains against *M. incognita* second-stage juveniles" section) and incubated at 25°C on a rotating shaker (120 rpm) for 18 days under dark conditions. The fungal biomass was separated from the broth by filtration through a milk cloth. The culture filtrate was further filtered through a 0.22 µm polyethersulfone (PES) filter (Sartolab® BT Vacuum Filtration) to remove residual conidia.

The antibiosis effect of strain PHP1701 was evaluated *in vitro* on the developmental stage of *M. incognita* eggs (Cabianca et al. 2022) and J2 motility (Oldani et al. 2023). A nematode suspension in water (700 ml) containing 150 eggs or 100 J2 was added to 24-well plates, followed by the addition of the culture filtrate and water to achieve dilutions of 0.5%, 1.0%, 5.0%, 10%, or 25% of the culture filtrate, up to a final volume of 1.5 ml per well. Water was used as a control (1.5 ml), each treatment was replicated five times ($n = 5$), and plates were incubated at 20°C in the dark. Egg development (egg, pretzel, and J2) and J2 motility were evaluated under an inverted light microscope (Zeiss, 10× magnification) after 1, 3, 6, and 9 days of incubation or after 1, 2, 3, and 7 days of incubation, respectively. To confirm whether or not immotile J2 could induce root galling, the *in vitro* assay was followed by an *in planta* bio assay. After the seventh day of J2 incubation with different concentrations of PHP1701 culture filtrate, treatment suspensions were collected from their respective wells and applied to pregerminated cucumber seedlings (*Cucumis sativus*, cv. Landgurken, Bigler Samen), sown in 10 ml pots containing potting soil (10 ml). The cucumber plants were grown for 21 days in a climate chamber (at 24°C, and 60% relative humidity). The root systems were then graded according to a 10-point root gall scale as described by Zeck (1971) (0: no galls, up to 10: completely galled roots).

Data analysis

Root gall index data were $\log_{10}(x + 1)$ transformed. Experiments were analyzed by one-way ANOVA with *post-hoc* Tukey's honestly significant difference test ($P \leq 0.05$).

Results

In vitro comparison of two *C. rosea* strains against *M. incognit a* second-stage juveniles

The *in vitro* comparison of the Swiss *C. rosea* strain F20 with the *C. rosea* strain PHP1701 from Andermatt Biocontrol Suisse showed that the strain PHP1701 had the strongest nematicidal effect on J2 after 3 and 6 days (Fig. 1). After 3 days, 22.0% of *M. incognita* J2 exposed to *C. rosea* strain PHP1701 were significantly inhibited + immotile (free), compared to only 11.2% inhibited + immotile (free) when exposed to *C. rosea* strain F20 and 8.7% in the control. After 6 days, the natural decline of nematode motility in the control reached 12.7%, while both *C. rosea* strains significantly affected J2 with 40.3% of inhibited + immotile (free) and 6.3% of inhibited + immotile (infected) J2 under strain PHP1701

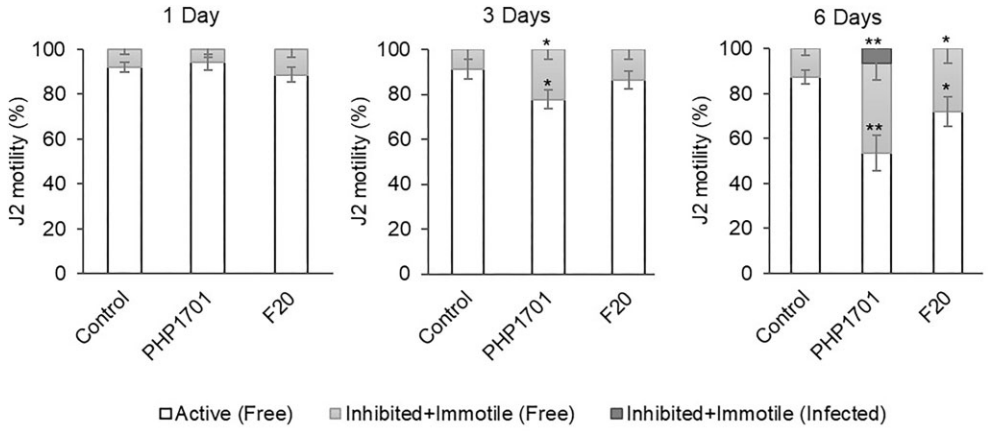

Figure 1. *In vitro* nematicidal activity of two *C. rosea* strains against the root-knot nematode *M. incognita* after 1, 3, and 6 days. Error bars represent standard deviations of replicates ($n = 6$). ∗Significant differences in % relative to the control, calculated by one-way ANOVA with *post-hoc* Tukey's Honest Significant Difference (HSD) test, $P < 0.05$.

(Supplementary Fig. S1) and 27.9% of inhibited + immotile (free) J2 under isolate F20.

Testing of *C. rosea* strain PHP1701 against *M. incognita* under soil conditions

Clonostachys rosea strain PHP1701 was further evaluated for its ability to control *M. incognita* J2 in soil (Fig. 2). Of the 150 J2 applied to the soil, 72.7 ± 9.4 J2 were re-extracted after 14 days from the control treatment. Significantly fewer nematodes, 29.8 ± 4.6 J2, were re-extracted from 100 ml of soil treated with 0.02 g *C. rosea* strain PHP1701. However, when the soil was treated with 0.01 or 0.005 g *C. rosea* PHP1701, fewer nematodes, 40.3 ± 7.0 or 49.0 ± 13.6 J2, were re-extracted from 100 ml of soil after 14 days, but not significantly different compared to the control.

Test of *C. rosea* strain PHP1701 against *M. incognita* in a small greenhouse trial

In the small pot experiment with *M. incognita* infecting tomato plants, *C. rosea* PHP1701 showed a significant reduction in root galling after 4 and 9 weeks compared to the untreated control (Fig. 3). The root system grown in soil treated with *C. rosea* PHP1701 also exhibited a higher root weight than the untreated control plants (Supplementary Fig. S2).

Performance of *C. rosea* strain PHP1701 against *M. incognita* under a large greenhouse setting

In the large greenhouse trial over a 12-week period, reduced root galling caused by *M. incognita* could be observed in early and mid-season as a result of *C. rosea* PHP1701 application (Table 1). However, only at mid-season, the monthly application significantly reduced root galling compared to the control plants. At the end of the season, with a difference of ±0.2, no significant differences in root galling were observed between treated and control plants. With a root galling index of 6.8 for the monthly *C. rosea* PHP1701 application, 7.0 for the control roots, and 7.2 for the bimonthly application, the majority of the roots were severely affected by *M. incognita* infection.

Tomato yield showed the greatest difference between *M. incognita*-infected and *M. incognita*-uninfected plants, with the lowest yield in the infected (positive) control and the highest yield in the uninfected (negative) control (Table 2).

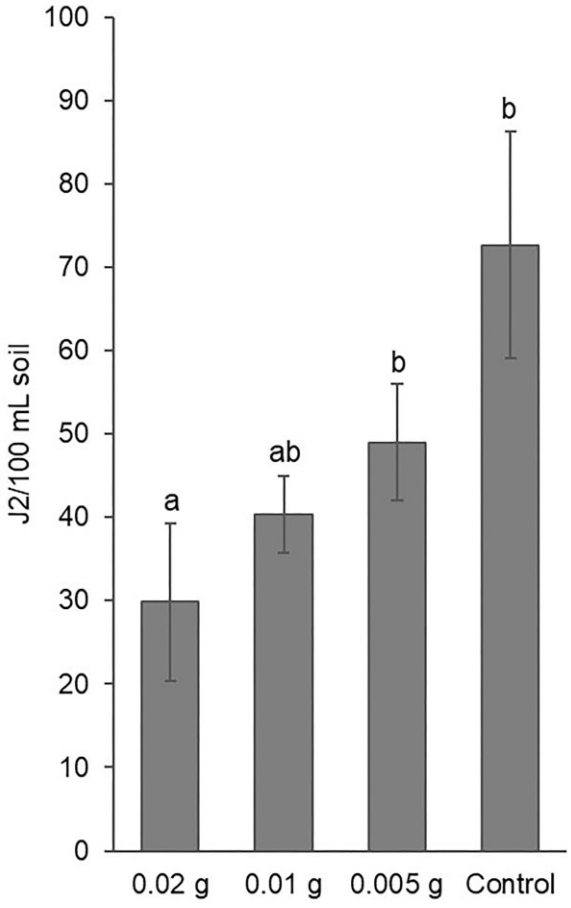

Figure 2. Concentration-dependent control of *M. incognita* by *C. rosea* PHP1701 over 14 days in soil. Error bars represent standard deviations of replicates ($n = 6$). Statistical significance was calculated using one-way ANOVA with the *post-hoc* Tukey–Kramer HSD test, $P < 0.05$. Means followed by the same letter are not significantly different.

Infected plants treated with *C. rosea* PHP1701 reached 92.41% of the yield potential when applied monthly or 97.07% of the yield potential when applied every second month compared to the yield of the negative control plants. The negative control plants had the highest average fruit

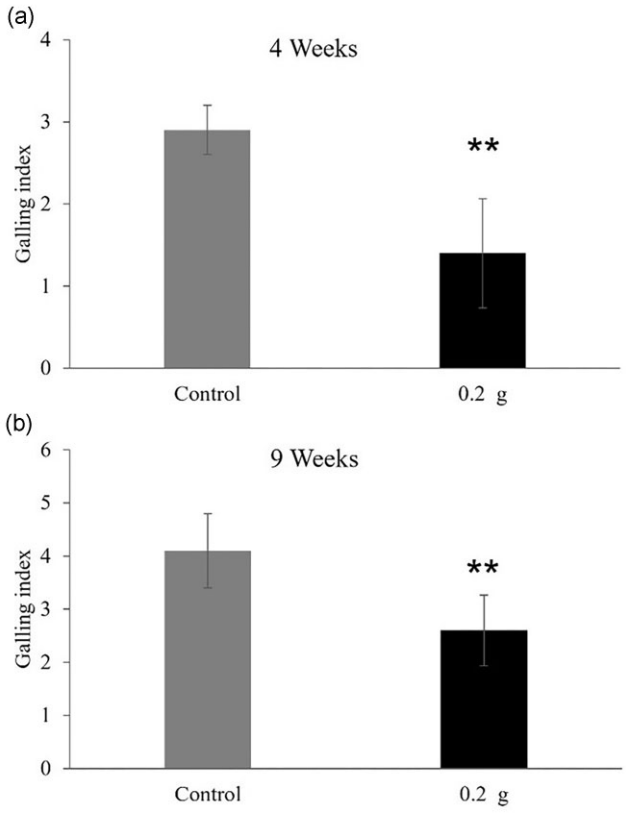

Figure 3. Protection of tomato cv. Moneymaker against the root-knot nematode *M. incognita* by *C. rosea* PHP1701 after 4 (a) and 9 (b) weeks of exposure. Error bars represent standard deviations of replicates ($n = 10$). Significant differences compared to control, calculated by one-way ANOVA with the *post-hoc* Tukey's HSD test, $*P < 0.05$ and $**P < 0.01$.

weight of 118.65 g, followed by the plants treated with *C. rosea* PHP1701 every second month, 111.89 g. While the plants treated with *C. rosea* PHP1701 but not infected with *M. incognita* had an average fruit weight of 111.48 g, the infected plants treated monthly had an average fruit weight of 108.89 g, and the infected untreated (positive) control plants had the lowest fruit weight, 108.00 g (Table 2).

However, for all yield data, including average weight (g)/harvest, average tomato fruit/harvest, total fruit weight/plant, average tomato fruit/plant, and average fruit weight, no statistically significant differences were observed between and among *C. rosea* PHP1701 treated and untreated control plants.

On-farm trial to evaluate the performance of *C. rosea* PHP1701 against *Meloidogyne* spp.

Application of *C. rosea* PHP1701 in the greenhouse on-farm trials showed a significant reduction in root galling compared to the untreated control, at both evaluation dates (Fig. 4).

Early root galling was assessed at the beginning of the trial and the differences were highly significant. In July, the root galling of *C. rosea* PHP1701 treated plants showed a high standard deviation but still resulted in significantly lower root galling index compared to the control plants.

Antibiosis assay

Clonostachys rosea PHP1701 culture filtrate was shown to affect *M. incognita* egg development (Supplementary Fig. S3A-D). A decrease in the percentage of eggs and a relative increase in J2 were observed in the control after the sixth day of incubation compared to the different treatments (Supplementary Fig. 3C). After 9 days of incubation, at the concentration of 25% of culture filtrate, most of the eggs developed to the pretzel stage (J1; 55.2% ± 0.58%), and only 19.3% ± 1.15% developed to J2, while in the control 46.2% ± 1.15% of the initial eggs developed to the J2 stage (Supplementary Fig. 3D).

When evaluating the effect of *C. rosea* PHP1701 culture filtrate on J2 motility, a statistically significant increase in affected and immotile J2 and a consequent decrease in normal motile J2 was observed, as early as the first day of incubation (Supplementary Fig. 3E). This trend was also observed on the second and third day of incubation (Supplementary Fig. 3F and G). However, after day 7, the amount of affected J2 (56.6% ± 0.52% and 61.0% ± 1.15%) is significantly higher than normal (34.7% ± 1.73% and 30.0% ± 1.73%) or immotile (8.7% ± 1.15% and 9.0% ± 0.89%) J2 at 10% and 25% culture filtrate treatments (Supplementary Fig. 3H).

To verify whether the culture filtrate treatment could affect *M. incognita* J2 infectivity capacity, cucumber seedlings were grown for 21 days after application of treated J2. Only the 25% culture filtrate treatment showed to have a significant impact on the capacity of *M. incognita* J2 to induce root gall formation (Supplementary Fig. 4). While the control plants had a gall index of 7.0 ± 0.00, plants treated with 25% PHP1701 culture filtrate had a gall index of 5.5 ± 0.42.

Discussion

Our study showed that the *C. rosea* strain PHP1701 has promising application potential for agronomic root-knot nematode control in small and large greenhouse trials. As the Swiss *C. rosea* strain F20 appeared to be less effective *in vitro*, we refrained from further investigations. Nonetheless, the search for more potent strains should continue as different *C. rosea* strains were able to control nematodes in various crops and countries, including Czech Republic (Hussain et al. 2017 and 2018), Iraq (Lafta and Kasim 2019), Japan (Toju and Tanaka 2019), Russia (Migunova et al. 2018), South Africa (Pambuka 2014), and Sweden (Iqbal 2019).

Based on the results of the *in vitro* assay, we concluded that the inhibited + immotile (free) J2 were affected by nematicidal compounds secreted by the *C. rosea* strains rather than directly parasitizing the nematodes as seen later in the experiment, after 6 days, shown as inhibited + immotile (infected), confirming that the two *C. rosea* strains tested, F20 and PHP1701, do not appear to have the same nematicidal activity/intensity *in vitro*. The differences in nematicidal activity were also previously observed by Iqbal et al. (2020), who tested a large number of culture filtrates of 53 *C. rosea* strains against the root lesion nematode *Pratylenchus penetrans*.

The significant control effect on *M. incognita* J2 *in vitro* was replicated in soil, meaning that *C. rosea* strain PHP1701 can be used to reduce the *M. incognita* J2 population in soil. Control of plant-parasitic nematodes in soil by *C. rosea* has also been previously described for *M. incognita* in greenhouse tomato plants (Cristóbal-Alejo et al. 2021), *Trichodorus* sp. in wheat, and *Paratylenchus* sp. in carrot (Iqbal et al. 2018b).

Table 1. Temporal evaluation of the fungus *C. rosea* as *M. incognita* control in greenhouse tomato production.

Treatment		*M. incognita* infected			Nematode free control	
C. rosea application		Every month	Every second month	Positive control	Every month	Negative control
Time points of root gall rating	Early	2.00 ± 0.70	1.75 ± 0.40	2.50 ± 0.50	0	0
	Mid	3.25 ± 0.80∗	4.00 ± 0.70	4.75 ± 0.40	0	0
	Late	6.80 ± 0.60	7.25 ± 0.70	7.00 ± 0.80	0	0

Values are means of eight replicates. Significant differences are indicated by an asterisk, calculated by one-way ANOVA with the *post-hoc* Tukey's HSD test, $P < 0.05$.

Table 2. Tomato yield of plants infected (positive control) and not infected (negative control) with the root-knot nematode *M. incognita* and of plants treated monthly and bimonthly and untreated with *C. rosea* PHP1701 over time from 14 June to 21 August 2023.

Treatments	Average weight g/harvest	Average tomato fruit/harvest	Total fruit weight g/plant	Average tomato fruit/plant	Average fruit weight (g)	Yield potential %
Negative control	851.50 ± 384.42	7.21 ± 3.20	9168.29 ± 740.29	77.75 ± 4.41	118.65 ± 9.84	100.00
Negative Control + *C. rosea*	810.44 ± 417.52	7.20 ± 3.47	8609.96 ± 1629.51	77.13 ± 13.23	111.48 ± 7.84	93.91
Every month	774.11 ± 378.32	7.08 ± 3.33	8472.25 ± 1271.64	77.88 ± 11.06	108.89 ± 7.02	92.41
Every second month	803.01 ± 354.67	7.10 ± 2.98	8900.00 ± 1262.53	79.75 ± 9.79	111.89 ± 12.20	97.07
Positive control	771.28 ± 315.11	7.08 ± 2.84	8345.25 ± 1173.73	77.63 ± 11.61	108.00 ± 8.34	91.02

Values are means of eight replicates. One-way ANOVA with the *post-hoc* Tukey's HSD test, $P < 0.05$. Yield potential relative to the nematode-free control plants expressed in percentage (%).

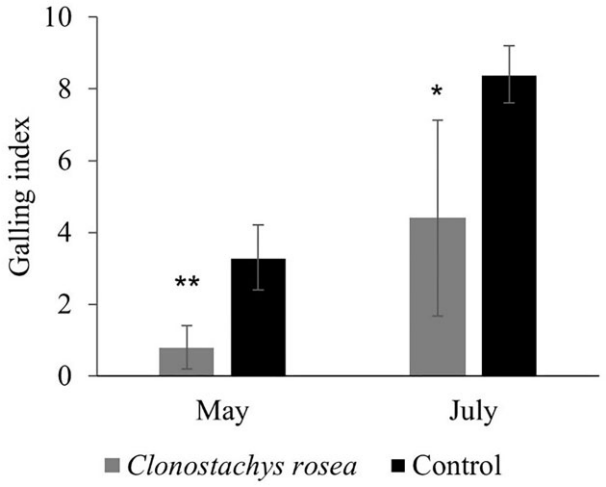

Figure 4. Tomato root galling in early (May) and mid-season (July) during an on-farm trial using *C. rosea* PHP1701 to control *Meloidogyne* spp. ($n = 10$). One-way ANOVA with the *post-hoc* Tukey's HSD test, $*P < 0.05$ and $**P < 0.01$.

Although there are other *C. rosea* strains active against *M. incognita* in soil [strain ACM908 (Wang et al. 2011)], our results support previous findings that the selected *C. rosea* strain PHP1701 can control *M. incognita* in a concentration-dependent manner. This finding is of great importance as this antagonist is not crop dependent and can be applied weeks before planting.

In the large greenhouse trial and in the on-farm trial, we can see that the effect of *C. rosea* PHP1701 decreases over time. As biological control is a population density-dependent process, where it is expected that the biological control products will keep the nematode population below an economic threshold (Abd-Elgawad 2021), often, as in our trial, the *M. incognita* populations increase during the middle and end of the season. There are several reasons why the biocontrol agent effectiveness declines over time. In particular, biological and environmental factors have a major impact on their success. Soil moisture, soil temperature and texture, pH, salinity, and pre-existing microorganisms can have a great impact on the nematode biocontrol agent (Abd-Elgawad 2021). However, each organism has its own biocontrol limitations. For example, the nematode egg parasitic fungus, *Verticillium chlamydosporium*, decreases in efficacy as the nematode population increases (De Leij et al. 1992). The reduction in efficacy is attributed to the ability of *V. chlamydosporium* to reach only a limited number of nematode eggs. Similar studies with the egg parasitic fungus *P. lilacinum* found that only 50% of nematode eggs were parasitized (Carneiro and Cayrol 1991). Egg development studies on *Meloidogyne* spp. using *C. rosea* culture filtrate in Petri dishes showed a maximum reduction in egg hatching of ~60% (Lafta and Kasim 2019). Based on the previous investigations and the population development in the greenhouse trials, we assume that *C. rosea* PHP1701 controls *M. incognita* with a similar intensity to that of *P. lilacinum* strain 251, as similar experiments in the same greenhouse resulted in a similar control with a "population plateau" of a galling index of 6.94–8.00 at the end of the season (Dahlin et al. 2019).

Antibiosis, rather than parasitism, has previously been hypothesized as the mode of action due to the secretion of nematicidal compounds (Iqbal et al. 2018b). We decided to additionally test the antibiosis potential of *C. rosea* PHP1701. Culture filtrate from *C. rosea* PHP1701 was shown to affect *M. incognita* egg development by delaying or inhibiting the maturation of J1 into J2, and also affected *M. incognita* J2 motility and consequently its ability to induce root gall formation. Given that *C. rosea* PHP1701 was shown to be capable of infecting *M. incognita* J2 in the initial *in vitro* assay, we cannot confirm whether nematodes were directly killed or nematode mortality was inhibited and fungal hyphae subsequently infected the inhibited nematodes. However, similar *in vitro* studies have shown that the *C. rosea* secretome has nematicidal properties and does not require live

hyphae to parasitize the nematodes for control (Iqbal et al. 2020).

Since *C. rosea* strain PHP1701 showed a great capacity to decrease the nematode population in the soil as described above, *C. rosea* PHP1701 could potentially be used by farmers suffering from a high *M. incognita* pressure after a long period of tomato cultivation or other long-standing crops to give the new crop a better start in a reduced *M. incognita* infected soil.

Regarding regular application, further studies are needed to evaluate different crops and their yields. In our experiment, the bimonthly application resulted in a higher yield than the monthly application, despite the fact that the monthly application resulted in better root-knot nematode control, which is puzzling. In addition, the nematode-infected control had the lowest yield and the non-*M. incognita*-infected plants had the highest yield. However, since the nematode population reached similar root gall levels at the end of the season and the yield differences were not significant, the cost of application must be considered. We also observed no increase in tomato yield in the uninfected tomato plants inoculated with *C. rosea*. This was despite the increase in root weight in the small pot experiment and previous studies describing plant growth benefits such as increased leaf area and girth in oil palm seedlings (Goh et al. 2020).

Further research is needed to evaluate whether an initial application will have the best economic effect on *M. incognita* population compared to a monthly application, as no growth-promoting effects over a tomato season have yet been described for this *C. rosea* strain. In particular, crops such as salad, which have a shorter growing season, may more strongly benefit from a pre-plant application of *C. rosea* PHP1701. In particular, a pre-plant application on black fallow could be beneficial for the next planted crop, as the results showed a significant downregulation of the soil *M. incognita* population. In addition, longer standing crops such as tomato plants may benefit from the use of *C. rosea* in the control of multiple plant pathogens, as recent publications have shown that *C. rosea* can induce *Botrytis cinerea* resistance in tomato plants (Li et al. 2023). However, whether *C. rosea* PHP1701 also has this ability to control *B. cinerea* needs to be evaluated.

Although *C. rosea* PHP1701 successfully controls *M. incognita*, little is known about its ecology and plant interactions with the rhizobiome. Based on the stronger control in the field trial, we can only speculate that *C. rosea* PHP1701 may have had a more favorable environment compared to the large pot trial, which needs to be evaluated in further studies.

It is important to consider that *C. rosea* PHP1701 should be used in an integrated pest management program, as *M. incognita* control alone can currently not match the nematicidal efficacy of chemical nematicides as a single application, despite its valuable contribution to a more sustainable crop production.

Conclusion

This study showed that *C. rosea* strain PHP1701 can be used to control *M. incognita* under greenhouse conditions. We report a strong nematicidal property in soil in the presence and absence of plants. However, the nematicidal effect is concentration dependent and limited to the duration of culture. We hypothesize that with increasing *M. incognita* population, the biology of the fungi cannot control enough nematodes, so there is a tipping point when the fungi reach their limit. In addition, *C. rosea* PHP1701 showed an increase in root biomass in small pot experiments, but no increase in yield was measured in the larger experiment.

Finally, we recommend the use of *C. rosea* strain PHP1701 for short-season crops and to reduce *M. incognita* populations without a host crop, such as on fallow land before planting the next crop in an integrated pest management program. Overall, *C. rosea* strain PHP1701 is a promising new biocontrol agent.

Acknowledgements

The authors would like to acknowledge Andermatt Biocontrol Suisse for providing us with fresh *Clonostachys rosea* strain PHP1701. Marco Eigenmann and Miro Nützi are acknowledged for their help with greenhouse experiments and tomato harvesting and Patrik Kehrli for proofreading the manuscript and for his insightful comments.

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest: None declared.

Funding

This research has not received funding from any source.

Author contributions

Tobias Stucky (Data curation, Investigation, Methodology), Eliana Thyda Sy (Data curation, Investigation, Methodology), Jakob Egger (Investigation, Methodology), Enis Mathlouthi (Investigation, Methodology), Jürgen Krauss (Conceptualization, Investigation, Methodology, Supervision), Lara De Gianni (Investigation, Methodology), Andrea Caroline Ruthes (Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing), and Paul Dahlin (Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing)

Data availability

The data underlying this article are available in the article and in its online supplementary material.

References

Abd-Elgawad MMM. Optimizing safe approaches to manage plant-parasitic nematodes. *Plants* 2021;**10**:1911. https://doi.org/10.3390/plants10091911.

Bainier G. *Gliocladium roseum* sp. nov. et *Cephalosporium acremonium* (Corda). *Bull Soc Mycol Fr* 1907;**23**:111–4.

Cabianca A, Ruthes AC, Pawlowski K *et al*. Tomato sterol 22-desaturase gene *CYP710A11*: its roles in *Meloidogyne incognita* infection and plant stigmasterol alteration. *Int J Mol Sci* 2022;**23**:15111. https://doi.org/10.3390/ijms232315111.

Carneiro RMDG, Cayrol JC. Relationship between inoculum density of the nematophagous fungus *Paecilomyces lilacinus* and control of *Meloidogyne arenaria* on tomato. *Rev Nematol* 1991;**14**:629–34.

Chatterton S, Punja ZK. Chitinase and β-1,3-glucanase enzyme production by the mycoparasite *Clonostachys rosea* f. *catenulata* against fungal plant pathogens. *Can J Microbiol* 2009;**55**:356–67. https://doi.org/10.1139/W08-156.

Chitwood BG. Root-knot nematodes. Part 1. A revision of the genus *Meloidogyne* Goeldi, 1887. *Proc Helminthol Soc Wash* 1949;**16**:90–114.

Cristóbal-Alejo J, Diaz-Braga A, Herrera-Parra E *et al*. *Clonostachys rosea* selected by nematicidal screening and its efficacy against *Meloidogyne incognita* in a greenhouse. *Biocontrol Sci Technol* 2021;**31**:1283–97. https://doi.org/10.1080/09583157.2021.1944607.

Dahlin P, Eder R, Consoli E *et al*. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. *Crop Prot* 2019;**124**:104874. https://doi.org/10.1016/j.cropro.2019.104874.

De Leij FAAM, Dennehy JA, Kerry BR.. The effect of temperature and nematode species on interactions between the nematophagous fungus *Verticillium chlamydosporium* and root-knot nematodes (*Meloidogyne* spp.). *Nematologica* 1992;**38**:65–79.

Dong JY, He HP, Shen YM *et al*. Nematicidal epipolysulfanyldioxopiperazines from *Gliocladium roseum*. *J Nat Prod* 2005;**68**:1510–3. https://doi.org/10.1021/np0502241.

EPPO Standard Diagnostics. PM 7/148 (1) Guidelines for the management of nematode collections used for the production and maintenance of reference material. *EPPO Bull* 2021;**51**:351–679. https://doi.org/10.1111/epp.12798.

Fatema U, Broberg A, Jensen DF *et al*. Functional analysis of polyketide synthase genes in the biocontrol fungus *Clonostachys rosea*. *Sci Rep* 2018;**8**:15009. https://doi.org/10.1038/s41598-018-33391-1.

Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol Ecol* 1993;**2**:113–8. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x.

Goh YK, Marzuki NF, Tuan Pa TNF *et al*. Biocontrol and plant-growth-promoting traits of *Talaromyces apiculatus* and *Clonostachys rosea* consortium against *Ganoderma* basal stem rot disease of oil palm. *Microorganisms* 2020;**8**:1138. https://doi.org/10.3390/microorganisms8081138.

Hallmann J, Kiewnick S. Virulence of *Meloidogyne incognita* populations and *Meloidogyne enterolobii* on resistant cucurbitaceous and solanaceous plant genotypes. *J Plant Dis Prot* 2018;**125**:415–24. https://doi.org/10.1007/s41348-018-0165-5.

Hallmann J, Subbotin SA. Methods for extraction, processing and detection of plant and soil nematodes. In: Sikora RA, Coyne D, Hallmann J, *et al*. *Plant-Parasitic Nematodes in Subtropical and Tropical Agriculture*(3rd edn). Wallingford: CAB International, 2018, 87–119. https://doi.org/10.1079/9781786391247.0087.

Hussain M, Zouhar M, Ryšánek P. Potential of some nematophagous fungi against *Meloidogyne hapla* infection in Czech Republic. *Pakistan J Zool* 2017;**49**:35–43. https://doi.org/10.17582/journal.pjz/2017.49.1.35.43.

Hussain M, Zouhar M, Ryšánek P. Attraction of root-knot nematodes, *Meloidogyne incognita*, to living mycelium of nematophagous fungi. *Pakistan J Zool* 2018;**50**:2073–8. https://doi.org/10.17582/journal.pjz/2018.50.6.2073.2078.

Iqbal M. Biological control of plant-parasitic nematodes by the fungus *Clonostachys rosea*. *Acta Uni Agric Suec* 2019;**2019**:72.

Iqbal M, Broberg M, Haarith D *et al*. Natural variation of root lesion nematode antagonism in the biocontrol fungus *Clonostachys rosea* and identification of biocontrol factors through genome-wide association mapping. *Evol Appl* 2020;**13**:2264–83. https://doi.org/10.1111/eva.13001.

Iqbal M, Dubey M, Broberg A *et al*. Deletion of the non-ribosomal peptide synthetase gene nps1 in the fungus *Clonostachys rosea* attenuates antagonism and biocontrol of plant pathogenic *Fusarium* and nematodes. *Phytopathology*® 2019;**109**:1698–709. https://doi.org/10.1094/PHYTO-02-19-0042-R.

Iqbal M, Dubey M, Gudmundsson M *et al*. Comparative evolutionary histories of fungal proteases reveal gene gains in the mycoparasitic and nematode-parasitic fungus *Clonostachys rosea*. *BMC Evol Biol* 2018a;**18**:171. https://doi.org/10.1186/s12862-018-1291-1.

Iqbal M, Dubey M, McEwan K *et al*. Evaluation of *Clonostachys rosea* for control of plant-parasitic nematodes in soil and in roots of carrot and wheat. *Phytopathology*® 2018b; **108**:52–59. https://doi.org/10.1094/PHYTO-03-17-0091-R.

Jones JT, Haegeman A, Danchin EG *et al*. Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol Plant Pathol* 2013;**14**:946–61. https://doi.org/10.1111/mpp.12057.

Kiewnick S, Wolf S, Willareth M *et al*. Identification of the tropical root-knot nematode species *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using a multiplex PCR assay. *Nematol* 2013;**15**:891–4. https://doi.org/10.1163/15685411-00002751.

Kofoid CA, White AW. A new nematode infection of man. *JAMA* 1919;**72**:567–9. https://doi.org/10.1001/jama.1919.02610080033010.

Lafta AA, Kasim AA. Effect of nematode-trapping fungi, *Trichoderma harzianum* and *Pseudomonas fluorescens* in controlling *Meloidogyne* spp. *Plant Arch* 2019;**19**:1163–8.

Li F, Ghanizadeh H, Song W *et al*. Combined use of *Trichoderma harzianum* and *Clonostachys rosea* to manage *Botrytis cinerea* infection in tomato plants. *Eur J Plant Pathol* 2023;**167**:1–14. https://doi.org/10.1007/s10658-023-02732-w.

Li J, Yang JK, Huang XW *et al*. Purification and characterization of an extracellular serine protease from *Clonostachys rosea* and its potential as a pathogenic factor. *Process Biochem* 2006;**41**:925–9. https://doi.org/10.1016/j.procbio.2005.10.006.

Migunova V, Sasanelli N, Kurakov A. Effect of microscopic fungi on larval mortality of the root-knot nematodes *Meloidogyne incognita* and *Meloidogyne javanica*. *IOBC-WPRS Bull* 2018;**133**:27–31.

Oldani E, Cabianca A, Dahlin P *et al*. Biogas digestate as potential source for nematicides. *Environ Technol Innov* 2023;**29**: 103025 https://doi.org/10.1016/j.eti.2023.103025.

Pambuka GT Biological control of root knot nematodes (*Meloidogyne* spp.) using bacterial and fungal antagonists. Master's thesis, 2014. College of Agriculture, Engineering and Science (CAES), University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Papadopoulou J, Triantaphyllou AC. Sex differentiation in *Meloidogyne incognita* and anatomical evidence of sex reversal. *J Nematol* 1982;**14**:549–66.

Pires D, Vicente CS, Menéndez E *et al*. The fight against plant-parasitic nematodes: current status of bacterial and fungal biocontrol agents. *Pathogens* 2022;**11**:1178. https://doi.org/10.3390/pathogens11101178.

Schroers HJ, Samuels GJ, Seifert KA *et al*. Classification of the mycoparasite *Gliocladium roseum* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. *Mycologia* 1999;**91**:365–85. https://doi.org/10.1080/00275514.1999.12061028.

Siddique S, Grundler FM. Parasitic nematodes manipulate plant development to establish feeding sites. *Curr Opin Microbiol* 2018;**46**:102–8. https://doi.org/10.1016/j.mib.2018.09.004.

Sikora RA, Helder J, Molendijk LP *et al*. Integrated nematode management in a world in transition: constraints, policy, processes, and technologies for the future. *Annu Rev Phytopathol* 2023;**61**:209–30. https://doi.org/10.1146/annurev-phyto-021622-113058.

Sikora RA, Roberts PA Management practices: an overview of integrated nematode management technologie. In: Sikora RA, Coyne D, Hallmann J, *et al*. (eds), *Plant-Parasitic Nematodes in Subtropical and Tropical Agriculture*(3rd edn). Wallingford: CABI, 2018, 795–839.

Subedi S, Thapa B, Shrestha J. Root-knot nematode (*Meloidogyne incognita*) and its management: a review. *J Agric Nat Res* 2020;**3**:21–31. https://doi.org/10.3126/janr.v3i2.32298.

Sun Z-B, Li S-D, Ren Q *et al*. Biology and applications of *Clonostachys rosea*. *J Appl Microbiol* 2020;**129**:486–95. https://doi.org/10.1111/jam.14625.

Tian BY, Cao Y, Zhang KQ. Metagenomic insights into communities, functions of endophytes and their associates with infection by root-knot nematode, *Meloidogyne incognita*, in tomato roots. *Sci Rep* 2015;**5**:17087. https://doi.org/10.1038/srep17087.

Toju H, Tanaka Y. Consortia of anti-nematode fungi and bacteria in the rhizosphere of soybean plants attacked by root-knot

nematodes. *R Soc Open Sci* 2019;**6**:181693. https://doi.org/10.1098/rsos.181693.

Wang L, Xu Y, Li S *et al*. Effects of metabolites of *Gliocladium roseum* on egg hatch and juvenile mortality of *Meloidogyne incognita*. *Soybean Sci* 2011;**30**:818–22.

White TJ, Bruns TD, Lees S *et al*. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, *et al*. (eds), *PCR Protocols: A Guide to Methods and Applications*. San Diego, CA: Academic Press, 1990, 315–22.

Zeck WM. Rating scheme for field evaluation of root-knot nematode infestations. *Pflanzenschutz-Nachr Bayer* 1971;**24**:141–4.

Zou CG, Tao N, Liu WJ *et al*. Regulation of subtilisin-like protease prC expression by nematode cuticle in the nematophagous fungus *Clonostachys rosea*. *Environ Microbiol* 2010;**12**:3243–52. https://doi.org/10.1111/j.1462-2920.2010.02296.x.

Received 12 February 2024; **revised** 24 April 2024; **accepted** 30 April 2024