Novel approaches for sustainable crop yield and management of plant-parasitic nematodes

Edited by

Maria L. Inácio, Solveig Haukeland and Jorge M. S. Faria

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Novel approaches for sustainable crop yield and management of plant-parasitic nematodes

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Editorial: Novel approaches for sustainable crop yield and management of plant-parasitic nematodes

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Editorial on the Research Topic

Novel approaches for sustainable crop yield and management of plantparasitic nematodes

Plant-parasitic nematodes (PPNs) are a major concern in agriculture as they cause significant crop damage resulting in yield losses and economic losses for farmers (FAO, 2019). For the past 50 years, the control of PPNs has relied heavily on the use of synthetic nematicides and soil fumigants, which have been effective in rapidly controlling nematode populations. However, due to environmental and health concerns, many traditional nematicides have been banned or withdrawn from the market. To achieve sustainable PPN control, it is advisable to adopt control strategies that are safer and more selective. These strategies include the use of bionematicides, biocontrol agents, cultural methods, and plant resistance (Jones et al., 2013). Bionematicides include biochemical antagonists such as natural products or microbial antagonists, which can induce chemical and/or physical damage against PPNs. Biocontrol agents, such as beneficial nematodes, fungi, and bacteria, can then be used to control PPNs (Pires et al., 2022). Cultural methods, such as crop rotation and the use of resistant cultivars, can also play a significant role in PPN control. The use of resistant cultivars is another effective strategy, as it involves breeding plants with resistance genes that can withstand PPN infections (Djian-Caporalino et al., 2014).

The aim of this Research Topic on research for integrated management of plant parasitic nematodes is to explore and advance innovative strategies for effectively control of these pests in a sustainable manner. This includes improving identification methods, investigating the potential of biological control agents and developing functional genomics for targeted control. By addressing these research areas, scientists can contribute to the development of sustainable and effective management strategies for plant parasitic nematodes.

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The contributions include new updates in the identification and distribution of the impactful root-knot nematodes (RKNs) (Rusinque et al.), the use of alternative products to control different PPNs (Elsharkawy et al.; Pulavarty et al.; Kim et al.) and the investigation of genomic tools to confer disease resistance (Joshi et al.; Westerdahl et al.).

The accurate diagnostic of diseases caused by PPNs is the first step for preventing the spread of these pests, being essential for the sustainable management of cultural systems (Palomares-Rius et al., 2021). This is even more important because different nematode species can have different host ranges and pathogenicity. Rusingue el al. conducted extensive surveys across Portugal for the detection of RKNs, in close collaboration with phytosanitary authorities, confirming other reports on the global distribution of Meloidogyne sp. This study included crops of economic significance grown intensively, favouring the rapid build-up of nematode populations in the soil, with the detection of several species in a wide variety of hosts. The information gathered on the RKN species found in the country is crucial at a local level for farmers and technicians in adopting sustainable management practices, and in a broader context for decision-makers in establishing phytosanitary measures and monitoring programmes to prevent the introduction and spread of these pests of concern in Europe. This is particularly relevant for quarantine and regulatory purposes.

Elsharkawy et al., Pulavarty et al. and Kim et al. focused on the use of alternative products to control different PPNs, namely through volatiles, microbial fermentation products and bacterial filtrates. The study of terpenes as natural products for the control of nematodes has increased in recent years (Faria et al., 2023). Elsharkawy et al. obtained promising results using monoterpenes as plant-derived natural compounds with nematicidal activity against M. incognita under laboratory, greenhouse, and field conditions. Carvone, cuminaldehyde, cineole, and linalool were effective for the control of root-knot nematode of tomato. Pulavarty et al. tested microbial fermentation products which are organic based soil health products, against the golden potato cyst nematode (PCN), Globodera rostochiensis, a major threat to potato crop in many countries (Price et al., 2021). These formulations provided by Alltech displayed nematicidal properties against PCN, with no detrimental effects on other soil nematodes and on plant growth promotion. Burkholderia sp. are multifunctional plant growth-promoting rhizobacteria (PGPR) with some species known to have nematicidal activity (Meyer et al., 2000; Liu et al., 2022; Zhang et al., 2022). Kim et al. studied the nematicidal effect of Burkholderia sp. JB-2 strain in suppressing M. incognita on tomato, with promising results. The strain also effectively promoted the growth of tomato plants, in addition to upregulating the gene expression linked to plant defence and growth.

Targeting of major nematode parasitism genes *via* Host Delivered-RNAi (HD-RNAi) to confer silencing is one of the most effective new approaches to limit nematode infection. In the study of Joshi et al., through silencing of the nematode effector gene Mi-msp2 in *Arabidopsis* HD-RNAi lines, a decrease of *M. incognita* infection in CaMV35S:: Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi lines was observed. The tissue-specific HD-RNAi

suppression technique has also proven to be a useful tool for the production of transgenic crops. Westerdahl et al. performed field studies involving Easter lilies, a staple of the floral industry, engineered with a rice cystatin gene for migratory nematode resistance, namely to the root-lesion nematode *Pratylenchus penetrans*. The authors concluded that transformed lines although not fully resistant, when planted in the field demonstrated and maintained a degree of resistance to *P. penetrans* and displayed desirable growth and quality characteristics similar to non-transgenic lilies.

In conclusion, the adoption of safer and more selective PPN control strategies, such as bionematicides, biocontrol agents, cultural methods, and plant resistance is proving to be a sound alternative to the sole reliance on synthetic nematicides. Also, the access to DNA sequencing and to genome-editing tools, and the possibility to design new plant incorporated protectants are also paving the way for new possibilities for PPNs control. However, the integration of these control strategies is not always straightforward, and their effectiveness can vary depending on the specific nematode species and the local environment. The rhizosphere is a complex system that contains thousands of microbial species, and their interactions with PPNs are still not fully understood. Further research is needed to better understand the interactions between PPNs, microbial antagonists, and plant resistance mechanisms in order to develop more effective and sustainable control strategies.

Author contributions

MI: Conceptualization, Writing – original draft. JF: Writing – review & editing. SH: Writing – review & editing.

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Resistance induction and nematicidal activity of certain monoterpenes against tomato root-knot caused by Meloidogyne incognita

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This research was performed to evaluate the potential of carvone, cuminaldehyde, cineole, and linalool for the control of root-knot of tomato. The tested control agents were evaluated for their ability to stimulate systemic resistance to Meloidogyne incognita in tomato by monitoring the transcription levels of defense-related genes. Moreover, the ability of the tested agents to induce nematicidal activity concerning second-stage juveniles (J2) hatching and mortality was evaluated. Furthermore, the effect of the tested agents on certain tomato growth and yield parameters was assessed. The tested monoterpenes showed high nematicidal activity against M. incognita concerning J2 hatching inhibition and mortality. Carvone, cuminaldhyde, linalool, and cineole had LC50 values of 123.5, 172.2, 354.9, 466.4, and 952.3 μg/mL, respectively. Carvone was found to be the most efficient hatching inhibitor. The tested monoterpenes showed a high potential against root-knot under greenhouse and field conditions with respect to root-galling, egg masses, and the number of J2. Carvone was the most effective treatment. The growth and yield characters of treated tomato were significantly increased in monoterpenes treatments compared to untreated control. Treated tomato plants showed expression of defense-related genes (PR1 and PAL) 5-8 folds higher than the control. The results also showed

that cuminaldhyde, followed by carvone, linalool, and cineole, had the greatest levels of expression in tomato plants. Taken together, the selected monoterpenes could be used as alternatives to control the root-knot of tomato.

KEYWORDS

tomato, Meloidogyne incognita, resistance induction, control, monoterpenes

Introduction

Tomato (Solanum lycopersicum L) is an important crop worldwide. Tomato fruits are popular all over the world. Ripe tomato fruit is highly nutritious as a good source of vitamins and minerals (USDA, 2005). In addition, as a processed crop, it ranks first among vegetables (Kessel, 2003). It is consumed fresh and/or used to make pastries, mash, ketchup, and fruit drinks (Ogwulumba and Ogwulumba, 2018). Tomato is grown on various seasonal farms throughout the year in Egypt as one of the most important crops that can provide a higher income for small and large farmers than other crops (Malash et al., 2005; Abd-Elgawad, 2020).

Tomato is among the most susceptible crops to several biotic and abiotic stresses compared to other vegetables. Among the different biotic stresses, plant parasitic nematodes (PPN) are the most common and widespread pests that may cause major damage to tomato production (Abd-Elgawad, 2014, 2020; El-Shafeey et al., 2019). Plant parasitic nematodes are obligate parasites that feed on the plant. Therefore, they cause significant damage to a wide range of crops and lead to significant production losses of about \$78 billion worldwide annually (Caillaud et al., 2008). The intensive use of chemical pesticides to manage PPN has resulted in environmental and health issues, as well as nematicides resistance (Molinari, 2011; Abd-Elgawad, 2020). Hence, it has become very necessary to look for alternative strategies to control PPN. Among the possible alternative strategies are the use of plant extracts, secondary plant metabolites, and plant-based essential oils (Echeverrigaray et al., 2010; Kundu et al., 2021). In nature, essential oils play an important role in the protection of plants against microorganisms, insects, and nematodes. Monoterpenoids, are the most representative molecules in more than 90% of the essential oils extracted from plants (Bakkali et al., 2008). The activity of many plant compounds and metabolites, including essential oils (mainly monoterpenes), has been reported against PPN and may provide a potential alternative to currently used nematicides (Pérez et al., 2003; Onifade et al., 2008; Echeverrigaray et al., 2010; Kundu et al., 2016; Dutta et al., 2021; Keerthiraj et al., 2021).

Resistance in crops is an important strategy for the protection from PPN (Oka et al., 2000). Plant treatments

with several biotic or abiotic agents can simulate plants to resist PPN attack (Walters et al., 2005). The stimulated defense potential induced by particular environmental factors is known as induced resistance (Van Loon et al., 1998). This resistance is successful against fungi, bacteria, viruses, and PPN (Vallad and Goodman, 2004). Several low molecular weight molecules identified as phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), control the immune response in plants (Vallad and Goodman, 2004). The plant's detection of invading pathogen results in the production of signaling molecules such as phytohormones, SA, JA, and their derivatives. SA promotes resistance to biotrophic pathogens, while JA promotes resistance to necrotrophic pathogens. The induction of pathogenesis-related (PR) genes is believed to be an indicator of plant-induced defense responses against diseases attack. PR genes have been frequently used as marker genes for systemic acquired resistance in many plant species (Mitsuhara et al., 2008; Mazarei et al., 2011). In fact, only a few studies have examined the expression of the PR gene in response to root knot and cyst nematodes (Mazarei et al., 2011). Despite the fact that nematode diseases are thought to be managed, above-ground symptoms are frequently the result of below-ground infections that are difficult to detect by farmers. Nematode damage to most crops is almost always linked to the nematode's initial numbers in the soil (Lutuf et al., 2018). Genes that give host resistance to root knot nematodes (RKN, Meloidogyne sp.) have been reported by Williamson and Roberts (2009) in annual and perennial crops. PR gene expression, particularly PR-1 expression, was dramatically elevated in shoots of resistant infected plants (Vallad and Goodman, 2004). The expression of a phenylalanine ammonia-lyase (PAL) gene, which encodes PAL enzyme involved in the biosynthesis of the signal molecule, SA (Mauch-mani and Slusarenko, 1996). Induction of PAL activity is a reliable indicator of plant resistance expression (Mauch-mani and Slusarenko, 1996; Safaie-Farahani and Taghavi, 2017). Pathogen-infected plants have been demonstrated to produce PAL (Campos et al., 2003; Farahani et al., 2016). Plant resistance depends on PAL expression that is both more rapid and more persistent. Two non-host bacteria, Pseudomonas syringae pv. phaseolicola and P. syringae pv. glycinea, were inoculated into Arabidopsis plants,

and an increase in PAL protein accumulation was observed (Mishina and Zeier, 2007).

The current study evaluated the *in vitro* nematicidal activity of carvone, cuminaldehyde, cineole, and linalool against *Meloidogyne incognita*, as well as their ability to control the diseases caused by the RKN in tomato plants under greenhouse and field conditions. The effect of monoterpenes on root-galling, egg masses, and the number of J2 were evaluated. Additionally, the abilities of monoterpenes to stimulate defense relatedgenes (*PR1* and *PAL*) expression and certain growth and yield characters of tomato were investigated.

Materials and methods

Chemicals

Sigma Aldrich, United States, provided the monoterpenes (carvone, cineole, cuminaldehyde, and linalool) with a purity of 99%. Oxamyl with a trade name of Vydate 310 SL produced by DuPont Company Wilmington, United States, was used as a recommended nematicide for RKN control on tomato.

Isolation and identification of nematode

The southern root-knot nematode (M. incognita) was isolated from infected tomato and the adult females' perineal patterns, as well as the morphology of second-stage juveniles (J2), were used. The isolate was reared on tomato plants Cv super strain B under greenhouse conditions (temperature at 25°C with 14 h light/10 h dark photoperiod). The roots of tomato heavily infested with pure cultures of M. incognita were used for the extraction of eggs, according to Hussey and Barker (1973) and Ghahremani et al. (2019). RKN egg masses were placed in sterilized distilled water (SDW) containing a sodium hypochlorite solution (0.5%) and incubated for 72 h at 25 \pm 2°C for hatching. Every day, the newly hatched second-stage juveniles (J2) were collected and stored (Goodey, 1957). All experiments were carried out with J2 obtained within 72 h.

Laboratory experiments

Mortality

Four concentrations (125, 250, 500, and 1,000 μ g/mL) were prepared in 0.3% Tween 20 for each compound. There were four replicates of each concentration, each treatment comprising about 100 of *M. incognita* J2. The bioassay was performed in cavity watch glasses with four batches of 25 J2 individually in 2 ml of each test solution. Distilled water with

0.3% tween 20 was used as control. Treatments were incubated at $25 \pm 2^{\circ}$ C and nematode mortality was assessed after 72 h of exposure. According to the methods of Finney (1971) and Cheng et al. (2015), the lethal concentrations for half of the treated J2 (LC₅₀) for each treatment were determined.

Hatching

Nearly 100 eggs were selected from mature egg masses using sterile forceps from roots free of soil and were transferred to glass bottles with 2 mL of each concentration (125, 250, 500, and 1,000 μ g/ml) of carvone, cuminaldehyde, cineole, and linalool which were prepared in distilled water. Four replicates were made in each treatment. The hatched juveniles were counted under a stereo microscope (Commack, NY, United States) after incubation for 7 days at room temperature (25 \pm 2°C). The rate of hatching inhibition was calculated, and Probit analysis was used to measure the inhibition concentration for half of eggs hatching (IC50) values (Finney, 1971; Damascena et al., 2019).

Greenhouse experiment

The efficacy of the compounds on *M. incognita* reproduction under greenhouses was also evaluated at a concentration of 250 mg/kg soil at temperature ranged between 25 and 30°C. Oxamyl was used at a concentration of 8 mL/L as a reference nematicide. Forty-day-old seedlings were planted singly in a plastic container (25 cm in diameter) filled with 2 kg sandy clay soil (3:1, v: v, sand: clay) sterilized with steam. One week after planting and selecting the seedlings with the best growth of the roots, 100 mL of each compound solution was injected into the soil around the stem of the plant (soil drenched). After 2 days, each pot was inoculated with an initial inoculum level (500 juveniles in 100 mL per pot) from root-knot nematodes around the stem of the plant within a radius of 2 cm. After 2 days, each pot was inoculated with 500 J2 around the stem of each plant within a radius of 2 cm. After 10 days, plants were treated again with the compounds as mentioned above. There were four replicates for each treatment, including untreated controls. Two months after nematode inoculation, tomato plants were carefully uprooted and cleaned under running tap water.

Plant height and fresh and dry weight of the plant were recorded. In addition, the number of galls and measurements related to nematodes were recorded, including the gall index, egg masses, and the final population density (number of J2) (Barker, 1985). Egg masses number was determined according to Daykin and Hussey (1985) method by dipping the roots in a 0.015% Phloxine-B coloring solution for 20 min. The quantity of J2 per 250 cm³ soil was measured using serial sieves and an adapted Baermann's technique on a slide under a stereomicroscope (Goodey, 1957). Roots are indexed on a scale

as described by Barker (1985) from 0 to 5 scale (0; 0–10%, 1; 11–20%, 21–50%, 3; 51–80%, 4; 81–90%, and 5; 91–100%). The reduction percentage (R%) of nematode parameters were calculated according to the following equation:

ReductionPercentage (R%)

 $= \ ({\rm Population~of~control-Population~of~treatment})/$ ${\rm Population~of~control} \ \times \ 100$

Analysis of defense related genes expression

Tomato plants were treated with the selected monoterpenes and inoculated with M. incognita as described previously. RNA extraction was carried out at 2 days after nematode inoculation from tomato leaves using RNA Purification Kit (Thermo Scientific, Fermentas, #K0731). Complementary DNA (cDNA) was synthesized using Reverse Transcription Kits (Thermo Scientific, Fermentas, #EP0451). Quantitative RT-PCR (qRT-PCR) with SYBR Green was utilized to measure the expression of the target genes (PR1 and PAL), with LeUBI3 **Table 1** as an internal reference following the manufacturer protocol (Thermo Scientific, United States, # K0221). The $2^{-\Delta\Delta Ct}$ method was used to normalize the numbers of target genes' critical thresholds (Ct) with the numbers (Ct) of a housekeeping gene (LeUBI3) (Livak and Schmittgen, 2001).

Field experiment

This experiment was performed to evaluate the effectiveness of carvone, cineole, cuminaldehyde, and linalool under field conditions compared to oxamyl. Experiments were conducted in fields naturally infested with *M. incognita* in Baltim, Kafr Elsheikh Governorate. All treatments were arranged in a split plot design with four replications. Each treatment consisted of one row and each row was 1 meter wide. The general cultivation recommendations for tomato plants were applied during the 2019 and 2020 growing seasons. Untreated plants were used as a control. After 2 months of treatment, the tomato roots were uprooted and washed. Then the numbers of nematodes were calculated as previously described in addition to determining the tomato yield.

TABLE 1 Forward and reverse primers.

Gene Forward primer		Reverse primer Siz		Accession number	References
PR1	GCCAAGCTATAACTACGCTACCAAC	GCAAGAAATGAACCACCATCC	139	DQ159948	Safaie-Farahani and Taghavi, 2017
PAL	CTGGGGAAGCTTTTCAGAATC	TGCTGCAAGTTACAAATCCAGAG	150	AW035278	Safaie-Farahani and Taghavi, 2017
LeUBI3	TCCATCTCGTGCTCCGTCT	GAACCTTTCCAGTGTCATCAACC	144	X58253	Song et al., 2015

Statistical analysis

To determine LC_{50} and IC_{50} , mortality and inhibition rates were subjected to a probit analysis (Finney, 1971). If the 95 percent confidence limits did not overlap, the LC_{50} and IC_{50} values were considered significantly different. All experiments were repeated three times. Greenhouse and field data were statistically analyzed using ANOVA. Averages were compared by Fisher's LSD test. The analysis was performed using XLSTAT PRO (statistical analysis software, Addinsoft).

Results

Efficacy of monoterpenes on the second-stage juveniles and eggs hatchability

The efficiency of the selected compounds on the J2 of M. incognita was evaluated under laboratory conditions **Table 2**. The results indicated that the highest mortality in the number of J2 was recorded for carvone followed by the cuminaldhyde, linalool, and cineole, where the LC₅₀ values were 123.5, 172.2, 354.9, 466.4, and 952.3 μ g/mL, respectively.

The efficacy of the selected compounds on hatching inhibition of M. incognita eggs under laboratory conditions was presented in Table 3. The results showed that the carvone was the most effective compound on hatching inhibition, followed by cuminaldhyde, cineole, and linalool, where the IC₅₀ values were 88.2, 102.1, 480, 646.9, and 780.4 μ g/mL, respectively.

Effect of the selected monoterpenes on growth characteristics of tomato

The effect of the selected compounds on certain growth characteristics of tomato crop (plant height as well as fresh and dry weight of the plant) under greenhouse conditions compared to the recommended nematicide was presented in **Table 4**. The measured growth characters were improved in tomato plants treated with the selected monoterpenes compared to untreated control. The results showed that the measured growth characters (plant height as well as fresh and dry weight of the plant) were the highest in tomato plants treated with

TABLE 2 Effect of the used compounds on the mortality.

Treatment	$LC_{50} (\mu g/mL)$	95% Confidence	Slope \pm S.E	
		Lower	Upper	
Cineole	952.3a	763.1	1277.4	1.84 ± 0.24
Cuminaldehyde	172.2 c	145.1	199.9	2.35 ± 0.26
Linalool	354.9b	301.3	439.4	1.53 ± 0.18
Carvone	123.5 d	116.7	164.2	2.77 ± 0.27

Different letters mean 95 % confidence limits did not overlap and the LC_{50} values were significantly different.

TABLE 3 Effect of the used compounds on the hatching.

Treatment	$LC_{50} (\mu g/mL)$	95% Cor	nfidence Limits	Slope ± S.E
		Lower	Upper	
Cineole	646.9b	535.7	848.3	1.56 ± 0.19
Cuminaldehyde	102.1 c	81.9	120.1	3.23 ± 0.29
Linalool	780.4 a	609.1	1144.1	1.35 ± 0.18
Carvone	88.2 c	66.9	109.3	$2.43 \pm\ 0.28$

Different letters mean 95 percent confidence limits did not overlap and the LC_{50} values were significantly different.

TABLE 4 Effect of treatments on the plant height (cm), shoot fresh and dry weights (g).

Treatments	Plant height	Shoot v	veight
		Fresh weight	Dry weight
Control (healthy)	47.5 ± 1.25 ^a	178.15 ± 3.25 ^a	20.7 ± 0.12^{a}
Control (infested)	$27.4 \pm 0.75^{\rm e}$	$64.2 \pm 1.17^{\rm e}$	$7.2 \pm 0.11e$
Cineole	$41.1 \pm 1.10^{ m d}$	133.7 ± 2.65 d	$15.1\pm0.45^{\rm d}$
Cuminaldehyde	$44.8\pm1.12^{\mathrm{b}}$	165.9 \pm 1.85 $^{\mathrm{b}}$	18.3 ± 0.36^{b}
Linalool	$42.7\pm1.45^{\text{ c}}$	151.1 ± 1.36^{c}	$17.1\pm0.38^{\rm c}$
Carvone	$45.9 \pm 2.10^{\mathrm{b}}$	$167.2 \pm 2.47^{\mathrm{b}}$	$18.4\pm0.37~^{\rm b}$
Oxamyl	$43.3\pm0.95^{\text{c}}$	149.4 ± 1.10^{c}	$16.9\pm0.74^{\text{c}}$

Weight of tomato plants grown in soil infested with nematode under greenhouse conditions. Statistical comparisons were made among treatments within a single column. The different letters represent significant differences using Fisher's LSD test at $P \leq 0.05$. Each mean value came from four replicates.

carvone followed by the cuminaldhyde, linalool, nematicide, and cineole, respectively.

Efficiency of the selected monoterpenes on root knot nematodes of tomato under greenhouse conditions

The results in **Table 5** showed that the recommended nematicide and the carvone were the most effective treatments at the level of all the measurements taken (root-galling, egg masses, and the number of J2 per 250 cm³ soil), followed by cuminaldhyde, linalool, and cineole, respectively. **Figure 1**

shows a comparison between symptoms of RKN attack observed on tomato roots treated with carvone compared to control (untreated) one.

Efficiency of selected monoterpenes on root knot nematodes of tomato under field conditions

The results in **Table 6** showed that the recommended nematicide and the carvone were the most effective treatments with respect to all measurements taken (root-galling, the number of J2 in 250 cm³ soil, and yield of tomato as a ton per hectare), followed by cuminaldhyde, linalool and cineole, respectively.

TABLE 5 Effect of treatments on root galling, egg masses, and juveniles in tomato plants under greenhouse conditions.

Treatments	Root-galling		Egg-masses		Nematode population	
	Gall index	Reduction (%)	Number of egg-masses /Plant	Reduction (%)	No. of J2/250 cm ³ soil	Reduction (%)
Control healthy	0.0	-	0.0	-	0.0	-
Control infested	$4.8\pm0.13^{\text{a}}$	-	$147.7 \pm 2.58^{\mathrm{a}}$	-	1876 ± 3.10^{a}	-
Cineole	$3.0\pm0.10^{\text{b}}$	37.5 ± 1.10	$78.3\pm1.34^{\text{ b}}$	47.0 ± 0.84	$1229\pm2.65^{\mathrm{b}}$	34.5 ± 1.12
Cuminaldehyde	$1.5\pm0.07~^{\rm d}$	68.8 ± 1.34	$34.8\pm1.97^{\rm d}$	76.4 ± 1.25	$629\pm2.97~^{\rm d}$	66.5 ± 0.74
Linalool	2.0 ± 0.10 c	52.1 ± 1.17	$51.2\pm1.10^{\text{ c}}$	65.3 ± 0.77	$801 \pm\ 4.10^{\ c}$	57.3 ± 0.69
Carvone	$1.2\pm0.06^{\text{e}}$	75.0 ± 1.28	23.1 ± 1.11^{e}	84.4 ± 0.89	$513\pm2.74^{\text{ e}}$	72.7 ± 1.14
Oxamyl	$1.1\pm0.10^{\rm e}$	77.1 ± 1.55	$18.7\pm0.89^{\;\mathrm{f}}$	87.3 ± 1.10	$491\pm3.14^{\rm \;f}$	73.8 ± 1.34

Statistical comparisons were made among treatments within a single column. The different letters represent significant differences using Fisher's LSD test at $P \leq 0.05$. Each mean value came from four replicates.

Expression of defense-related genes

The effect of the compounds on the expression of defense genes (*PR1* and *PAL* gens) in the treated and inoculated tomato plants is investigated **Figure 2**. Higher transcription levels of *PR1* and *PAL* gens in treated tomato plants in comparison to untreated control was obtained. The findings also showed that the highest level of expression in tomato plants was cuminaldhyde followed by carvone, linalool, and cineole, respectively.

Discussion

Meloidogyne incognita causes root-knot, which is an important disease in different regions of the world (Onyeke and Akueshi, 2012; Devi and Kumari, 2014). M. incognita has a wide range of host plants, causing yield losses in severely infested fields (Onyeke and Akueshi, 2012; Mukhtar et al., 2014). In this study, under laboratory, greenhouse, and field conditions,

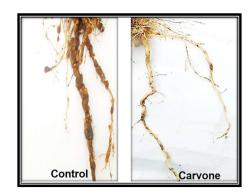


FIGURE 1

Symptoms on tomato roots treated with carvone and non-treated control plants.

the four monoterpenes (carvone, cuminaldehyde, cineole, and linalool) demonstrated nematicidal behavior against the RKN. Monoterpenes are the primary components of aromatic plant essential oils, and they are responsible for the majority of the biological activities of plant extracts (Sacchetti et al., 2005; Bakkali et al., 2008; Abdel Rasoul, 2013; Naz et al., 2013; Khan et al., 2020). Our results are in line with many investigation which reported that several essential oils and some of their major components have nematicidal activity against RKN (Abdel Rasoul, 2013; Naz et al., 2013; Lu et al., 2017; D'Addabbo et al., 2021). The most effective compounds against M. incognita in this study were carvone and cuminaldehyde, which is in line with the findings of Abdel Rasoul (2013). The high efficacy of carvone and cuminaldehyde against RKN may be due to the presence of a hydroxyl or carbonyl group in these two compounds. This indicates that the functional group is very important in their nematicide activity (Echeverrigaray et al., 2010).

Carvone, cominaldehyde, cineole, and linalool were evaluated in controlling *M. incognita* in tomato plants under greenhouse and field conditions. The results obtained in this study showed that they significantly reduced the numbers of galls, egg masses, and J2 in the root system compared to the infected control. These results were similar to those reported by other studies on essential oils (Echeverrigaray et al., 2010; Abdel Rasoul, 2013). The results of this study, however, differ from those of others in that the monoterpenes utilized were examined at all levels in the laboratory, greenhouse, and field. The ability of tomato plants to display induced resistance to RKN was also investigated by looking at the expression of defense genes in treated tomato plants.

Several studies have demonstrated the nematicidal activity of plant extracts in several plant species (Barbosa et al., 2010; Sivakumar and Gunasekaran, 2011; Zahradnikova and Petrikova, 2013). For example, the severity of *M. incognita* was significantly reduced in plants treated with neem extracts (Sivakumar and Gunasekaran, 2011). Pumpkin

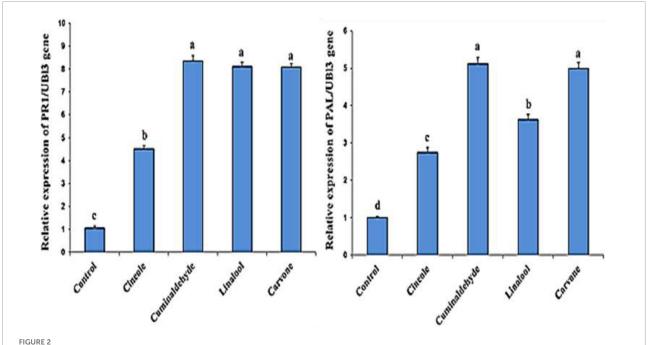
TABLE 6 Effect of treatments on root galling, nematode population and yield in tomato plants grown in soil infested with *M. incognita* under field conditions.

Treatments	Root-galling		Nematode pop	Yield (Ton/ Hectare)	
	Gall index	Reduction (%)	No. of J2/250 cm ³ soil	Reduction (%)	
Control infested	4.2 ± 0.16^{a}	-	2611 ± 1.45^{a}	-	15.3 ± 0.25 ^e
Cineole	$3.3 \pm 0.14^{\text{b}}$	21.4 ± 0.74	$1530\pm2.84^{\text{b}}$	41.4 ± 2.10	$25.7\pm0.23~^{\mathrm{d}}$
Cuminaldehyde	$\rm 1.9 \pm 0.10^{\rm d}$	54.8 ± 1.10	$1130\pm2.64^{\text{ d}}$	56.7 ± 1.57	$31.6\pm0.54^{\text{ b}}$
Linalool	$2.6 \pm\ 0.12^c$	38.1 ± 0.37	$1258\pm3.97^{\text{ c}}$	51.8 ± 1.97	$27.5\pm0.78~^{\mathrm{c}}$
Carvone	$1.8\pm0.10^{\text{ d}}$	57.1 ± 1.16	1102 ± 1.79^{d}	57.8 ± 1.35	33.7 ± 0.69 a
Oxamyl	$1.4 \pm\ 0.10^e$	66.7 ± 1.74	$1031\pm2.67^{\text{ e}}$	60.5 ± 1.68	$30.4\pm0.47^{\text{b}}$

Statistical comparisons were made among treatments within a single column.

Superscript of different letters represents significant differences using Fisher's LSD test at $P \le 0.05$.

Each mean value came from five replicates.



Effect of different treatments on the expression levels of *PR1* and *PAL* genes. Columns represent mean values. The control corresponds to inoculated and non-treated plants. Bars indicate standard errors. Different letters above columns indicate significant differences by Fisher's LSD test at significant values of $P \le 0.5$.

oil also showed nematicidal activity (Ayaz et al., 2015). Moreover, the treatment with watercress oil resulted in a significant decrease in symptoms caused by *M. hapla* nematode disease and a significant increase in the fruit yield (Zahradnikova and Petrikova, 2013).

A plant self-defense mechanism against infectious parasites can be initiated within plant root consisting of many biologically active secondary metabolites. There are a limited number of researches conducted to understand the effectiveness of induced resistance within the same species (Navyashree et al., 2021). Different studies have indicated the use of plant resistance inducers to alleviate these adversities, increase plant metabolic activity, and develop a defense mechanism against various

parasites. This kind of studies will also add value to our understanding of naturally occurring interactions between plant and RKN (Dutta et al., 2015).

One of the primary impacts of changes in the gene expression of plants is to elicit resistance to a large variety of pathogens and parasites including nematodes (Druzhinina et al., 2011; Cameron et al., 2013). In this study, we analyzed changes in the expression of defensive genes after treating tomato plants (soil drench) with control measures. The defensive genes analyzed in this study were highly expressed in tomato plants treated with the tested inducers. *PR1* and *PAL* genes were overexpressed after treatment with the selected compounds. This is in line with Sharaf et al. (2016) results, who reported that

the expression of defense-related genes elevated in nematodeinfected tomato plants and treated with inducers.

Plant extracts and plant residues, alone or in addition to physical measures, have been proven promising effects in weeds, microbial, and insect control, and certain commercial natural oils are currently accessible for organic agriculture purposes (Dayan et al., 2009). Essential oils and organic amendments derived from essential oil-rich plants have been used to successfully protect plants against phytonematodes (Pérez et al., 2003; Onifade, 2007). For the mode of action of essential oils, it has been found that some essential oils have genotoxic activity in Drosophila melanogaster (Karpouhtsis et al., 1998; Enan, 2001; Lazutka et al., 2001), to activate octopamine receptors (Karpouhtsis et al., 1998; Enan, 2001), and interfering with GABA receptors for insects (Priestley et al., 2003). As lipophiles, essential oils and terpenoids interfere with the cytoplasmic membrane of yeasts, destroying the structure of carbohydrates, fatty acids, and phospholipids, causing mitochondrial membrane depolarization and leakage of radicals, cytochrome C, calcium ions, and proteins (Bakkali et al., 2008). The presence of phenols, aldehydes, and alcohols in essential oils has been related to their in vitro cytotoxic activity (Bruni et al., 2004; Onifade et al., 2008).

The results showed that the plants infected with nematodes and non-treated with selected compounds displayed a delay in growth as plant height decreased, and the fresh and dry root weights were significantly reduced due to nematode infection, which is in line with Radwan et al. (2009) and Elsayed and Edrees (2014). The significant increase in growth characteristics is the primary criterion for judging the incidence of induced systemic resistance (ISR) in nematode-infected plants treated with inducible control agents (Sharaf et al., 2016). The results in this study clearly indicated a significant improvement in the growth characteristics and productivity of tomato plants treated with the selected monoterpenes. This improvement may result in the ability of the treatments to reduce nematode infection on the roots. This is because healthy or low-infected roots have the ability to transport water and nutrients from the soil through the xylem, which is reflected in the growth of tomato plants (Bakr and Hewedy, 2018).

Conclusion

The present results showed that the used monoterpenes as plant-derived natural compounds possess strong nematicidal activity against *M. incognita* under laboratory, greenhouse, and field conditions. Antihatching and/or anti-juveniles action against *M. incognita* was revealed in most tested monoterpenoids. For the management of *M. incognita*, carvone proved to be the most effective natural nematicide, as shown by its ability to reduce egg hatching and the number of J2. The growth and yield characters of tomato treated with the tested

compounds increased compared to untreated control. Since phytochemicals have high nematicidal ability, the current study suggests that they may be used as alternatives in an integrated disease management program against *M. incognita*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AD and ME: conceptualization, formal analysis, investigation, data curation, writing – original draft preparation, and supervision. ME: methodology and software. AD, AA, SB, MS, MK, and ME: validation. SB, AA, AA-A, MK, and ME: resources. AD, AA, SB, and ME: writing – review and editing. SB, AA, AA-A, and ME: visualization and funding acquisition. AD, AA-A, and ME: project administration. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sustainable management of the potato cyst nematode, Globodera rostochiensis, with two microbial fermentation products

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Potato cyst nematodes (PCN) cause an overall 9% yield loss of total potato production worldwide. Research on sustainable management of PCN is still under progress. Two microbial fermentation products (MFPs) from Alltech, a proprietary blend formulated with a bacterial fermentation media and a Cu component (MFP5075), and a microbial based product (MFP3048), were evaluated against the PCN Globodera rostochiensis. In laboratory tests, effectiveness of the MFPs was recorded in terms of PCN juveniles (J2) hatching from cysts, J2 mortality and their attraction toward potato roots using pluronic gel. Greenhouse trials were conducted to study the effect of the products on PCN infestation in potato plants and a pilot scale experiment was conducted to study the impact of these MFPs on nematode biodiversity in garden soil. All treatments were performed within a concentration range of 0, 0.5, 1, and 2% (v/v) MFP5075 and 2, 6, 10, and 20 g/10 ml (w/v) MFP3048. The attraction assay, juvenile hatching and the PCN infestation in plants results were compared with those in an untreated control and a commercial nematicide (Nemguard $^{\mathrm{TM}}$) treatment. After 24 h of treatment with 0.5 and 1% MFP5075, a 13-fold and 43-fold reduction, respectively, relative to J2 survival was recorded compared to that of untreated control. However, no J2 survived at 2% and above concentration of the MFP5075 treatment. Treatment with MFP3048 was effective in causing mortality of J2 only after 48-h. In the attraction assay, a 20-fold and 8-fold reduction in number of J2 attracted toward potato roots was observed, when treated with MFP5075, compared to the untreated and the NemguardTM treatment, respectively. Subsequently, 30-35 PCN cysts were treated with both products dissolved in potato root diffusate and the results were recorded in terms of number of J2 hatched in each treatment after 10 days. No J2 hatched in the MFP5075 treatment, whereas mean numbers (\pm SE) of 243 \pm 11.5, 30 \pm 2.5, and 1.3 \pm 0.6 J2 were noted in the untreated control, MFP3048, and the NemguardTM treatment,

respectively. The treatment with the MFPs compromised the integrity of the unhatched J2, which looked granular, whereas the internal organs of the unhatched J2 could be clearly identified in the untreated control. In plant infestation studies, treatment with MFP3048 and MFP5075 caused 90.6 and 84.9 percent reduction in PCN infestation, respectively, in terms of cysts developed on roots compared to untreated control. Overall, results indicate that the MFPs could potentially provide a promising alternative for sustainable PCN management.

KEYWORDS

potato cyst nematodes, Globodera rostochiensis, MFP5075, MFP3048, Alltech

Introduction

Potatoes are one of the most important non-grain food crops in the world with an annual production of approximately 314,140,107 tons/year. Potatoes can be grown in varied climatic conditions and take less time and input to grow. They are considered to be an essential food security crop by United Nations due to their nutritional value, high food energy and complex carbohydrate content per unit of land (Wijesinha-Bettoni and Mouillé, 2019). China is the top potato producer in the world with 90,321,442 tonnes produced per year, followed by India (48,529,000 tonnes yearly), Ukraine, Russian Federation, United States, Bangladesh, Germany and France.1 With an increasing world population and urbanization, there is a global increase in potato consumption and therefore there is a high demand for its production (Price et al., 2021). Furthermore, there is a huge demand from the fast-food industry for processed potatoes (e.g., crisps, starch, and chips) due to huge economic returns (Abong and Kabira, 2013). Various recent reports highlight the importance of global potato production and limitations faced by growers due to increasing demands (Mburu et al., 2020; Gartner et al., 2021; Price et al., 2021; Pulavarty et al., 2021b).

Over the last 50 years, in terms of yield development, potatoes (46.2%) lag behind other crops such as corn (158.8% increase), wheat (135.5%), rice (109.6%), and soy (94.6%) (Redcliffe and Australia, 2021). This could be attributed to various pests infesting the potato crops globally, improper cropping practices and substandard tuber quality (Kaguongo et al., 2014). Some of the important diseases, disorders or defects associated with potato crop are early blight, late blight, root knot nematodes, potato cysts nematodes, fusarium rot, ring rot, pink rot, fusarium wilt, verticillium wilt, blackspot, blackheart, and leafroll (Olivier et al., 1998). Globodera spp., Meloidogyne spp., Pratylenchus spp. and Trichodorus spp. are reported to be some

of the major nematode pests infecting potato plants (Nicol et al., 2011).

Potato cyst nematodes (PCN; Globodera rostochiensis and Globodera pallida) are such economically important pathogens causing huge crop losses in potato crop worldwide (De Boer et al., 1996; Aires et al., 2009; Niere and Unger, 2012; Eche et al., 2018; Mburu et al., 2020; Price et al., 2021; Ochola et al., 2022). Jones et al. (2013) have reported PCN as one of the top ten plant parasitic nematodes (PPN) based on their economic and scientific importance. They are known to cause enormous crop losses and various researchers have reported decline in potato yields in various parts of the world, such as in Kenya (Mwangi et al., 2015; Mburu et al., 2020), Rwanda (Niragire et al., 2019), Uganda (Cortada et al., 2020), North America (Dandurand et al., 2019), United Kingdom (Gartner et al., 2021), India (Chandel et al., 2020), South Korea (Kwon et al., 2018), China (Zhang et al., 2021), Iran (Hajihassani et al., 2013), Columbia (Vallejo et al., 2021), and Switzerland (Ruthes and Dahlin, 2022). Due to their wide spread and prolonged survival in soil (upto 20 years) and resistance to extreme temperatures it is tough to permanently eradicate PCN (Chandel et al., 2020).

Applications of synthetic chemical nematicides are expensive and have known to cause various environmental issues and a long term impact on human health (Douda et al., 2021). Many chemical nematicides have been therefore banned in the recent past (Pulavarty et al., 2021b). The biofumigation approach has been practiced in the past few years to manage PCN, however, the efficacy of the fumigant deteriorates over time due to various environmental factors such as temperature, humidity and soil characteristics (Hajihassani et al., 2018).

Some research groups have reported the application of extracts from Brassicaceae plants (Aires et al., 2009; Lord et al., 2011; Ngala et al., 2015), but, the application of such extracts have been reported to cause root decay and eventually lead to rotting of the plant biomass resulting in unpleasant odors in the fields (Ngala et al., 2015). In most cases, with the application of various

¹ https://atlasbig.com/en-ie/countries-by-potato-production

products there is a lack of information on the effect of those products on other beneficial soil microbes and soil biodiversity. A thorough investigation on application of any product on beneficial nematodes, fungi, bacteria, or soil biodiversity as a whole is essential for a sustainable PCN management.

Recently, a most successful wrap-and-plant technology was reported by a research group in East Africa, which uses banana-matrix as a seed wrap to control the *G. rostochiensis* infestation (Ochola et al., 2022). This approach has proven very successful to sustainably manage PCN and other crop pests while increasing the potato yields significantly. However, large scale application of this technology needs to be validated and confirmed on larger scale production. Despite much international collaborative research and significant efforts, PCNs still continue to manifest vast damage to potato crops universally.

The current study aims to assess the potential of two microbial fermentation products, a proprietary blend formulated with a bacterial fermentation media and a Cu component (MFP5075), and a microbial based product (MFP3048), against the PCN *G. rostochiensis. In vitro* laboratory experiments were conducted to study the effect of MFPs on juvenile (J2) mortality, J2 hatching from the cysts, granulation of unhatched eggs and attraction of J2 toward potato roots. Subsequently, plant trials were conducted in greenhouse conditions to study the efficacy of the MFPs in controlling *G. rostochiensis* infestation in potato plants. The effect of MFPs on PCN, in terms of laboratory bioassays and greenhouse trials were compared with those of the untreated control and a commercial organic nematicide (NemguardTM) treatment.

The nematode biodiversity in soil after application of the MFPs was compared with that of biodiversity in the untreated garden soil and treatment with two commercial nematicides, NemguardTM (organic), and VydateTM (synthetic).

Materials and methods

Sourcing of potato cyst nematodes cysts and hatching of *Globodera* rostochiensis juveniles (J2)

Globodera rostochiensis cysts were received as a kind offer from Dr. Colin C. Fleming, Grassland and Plant Science Branch, Agri-Food and Biosciences Institute, Belfast, United Kingdom. The cysts were incubated at 4°C to break their dormancy for 6 months. After 6 months, a hatching assay was performed by incubating the cysts in potato root diffusate (PRD) to check their viability. Subsequent experiments were all performed only after ensuring the viability of the cysts.

Collection of potato root diffusate

Potato rood diffusate was collected following protocol described by Turner et al. (2009) with the following modifications: tubers of susceptible variety, Désirée, were grown in a one liter soil pot, and the plants were allowed to grow until they reached an approximate height of 15 cm and roots were seen growing out from the base of the pots. These plants were then used for PRD collection. On the day of collection, the plants were not watered. One hundred milliliters of distilled water was gradually added per pot until the soil was saturated and a pale brown flow through liquid leached out from the bottom of the pot. A plastic saucer was placed underneath to collect the initial flow through liquid. The collected liquid was added back into the pot and was let drain for a second time. This process was repeated two more times. The liquid flow through that was collected in the saucer was poured into an amber colored glass bottle. A 1 month old potato plant was then kept at 25°C in the same glass bottle with the roots submerged in the flow through liquid for 24 h. Subsequently, the PRD was obtained by filtering the flow through liquid through a 90 µm sieve to remove the soil particles. The diffusates were then stored at 4°C until use.

Bioassay: Experimental design and microbial fermentation products treatment to determine lethal concentrations and doses

Both products were tested initially on beneficial entomopathogenic nematodes (EPN; Pulavarty et al., 2020) and results indicated that 7% MFP5075 was considered as LC50 concentration and no mortality was noted when treated with MFP3048 (Pulavarty et al., 2020). These findings also revealed that at 4% MFP5075 and below there was no mortality caused to EPN (Pulavarty et al., 2020), therefore, all subsequent treatments were performed below 4% to ensure no harm is caused to EPN in the soil. The products were also reported to reduce *Meloidogyne javanica* infestation in tomato plants when treated with 1–3% MFP5075 and also when combined with 3 g MFP3048 (Pulavarty et al., 2021a).

Globodera rostochiensis cysts were hydrated in distilled water for 24 h. After 24 h, the cysts were incubated in PRD for 1 week. First batch of J2 obtained after 1 week of incubation were discarded and the cysts were placed into new petri dishes containing fresh PRD for another few days to collect J2 that were used for the bioassays.

All the treatment studies were conducted in 96-well plates with 10 replicates per treatment concentration. Freshly cultured J2, no older than 2 days post-cyst emergence, were used for the study. In each well, 200 μ L of PRD containing approximately 20–30 J2 and various product (MFP5075 and MFP3048 Alltech

ACS, Dunboyne, Co. Meath, Ireland) dilutions were added to obtain the required concentration. Potato root diffusate (200 μ l) containing J2 without any product was considered as control. The concentration range for MFP5075 was 0–2% and for MFP3048 was 0–20 g dissolved in 10 ml of water. MFP5075 was directly pipetted in to 200 μ l PRD in 96-well plate to obtain 0.5, 1, and 2% v/v, respectively. MFP3048, a powdered product, was prepared by adding 2, 6, 10, and 20 g in 10 ml w/v of distilled water in a falcon tube. These tubes containing MFP3048 product were kept in the incubator shaker for 24 h before use. After 24 h, 100 μ l supernatant from each of the falcon tubes containing 2, 6, 10, and 20 g in 10 ml was added into each well to obtain an concentration range of 1, 3, 5, and 10 g per 10 ml, respectively.

After 24 h incubation at 20–22°C, survival and mortality were calculated by counting the motile and immotile J2 using an Olympus Stereo Microscope (SZX7). The mortality of immotile J2 was ensured by gently touching them with a needle. Another set of plates with the same concentration range was analyzed after 48 h incubation. It was observed that in the MFP5075 treatment, the mortality rate was similar to that of 24 h incubation. Therefore, data recorded after 24 h incubation were considered for statistical analysis for the MFP5075 treatment. However, in the MFP3048 treatment, J2 mortality occurred only after 48 h treatment, therefore, data recorded after 48 h incubation was considered for statistical analysis. All these bioassays were repeated thrice to ensure the reproducibility of the results.

Attraction assays using 20% (w/v) pluronic gel

Fifty milliliters of a 20% (w/v) pluronic gel PF-127 (Sigma, St. Louis, MO, United States) solution was prepared in distilled water (Sasaki-Crawley et al., 2012) and the dissolved gel was stored at 4°C until use. Attraction assays were performed on glass slides using a protocol reported by Pulavarty et al. (2021a). Approximately 40-50 J2 were applied onto each slide containing pluronic gel solution (300 µl) in 10 mM sodium phosphate and PRD (200 µl). A 1-month-old potato root tip, 1 cm in length, was placed on the center of the glass slide containing the gel-nematode mixture. Ten microliters of MFP5075 (1%) was added to three of the glass slides, ten microliters of MFP3048 (20 g/10 ml) was also added to another set of three slides. Three slides containing the gel-nematode mixture without any products was regarded as the negative control (untreated). Three more slides with the gel-nematode mixture treated with 10 microliters of NemguardTM (20 Kg/hectare; which corresponds to 13.3 mg/L) was considered a positive control for the experiment.

The potato root tip on each slide was covered with a transparent cover slip to prevent evaporation and drying of liquid and to clearly visualize movement of J2 toward potato roots. Individual slides were all incubated at room temperature, and the movement of J2 in each of the slides was closely monitored after every 10 min, upto 60 min, using an Olympus Stereo Microscope (SZX7). After 60 min of treatment, the mean number of J2 attracted toward the potato root in each slide was noted and the data recorded was used for statistical analysis. All the treatments including positive and negative controls were repeated nine times over a span of 1 week with three replicates each time (total of 27 replicates/treatment) to ensure the reproducibility of the results. The mean values obtained were used for statistical analysis.

Globodera rostochiensis juvenile hatching assay from the cysts

Globodera rostochiensis cysts after incubation at 4° C for a period of 6 months were used for this assay. Approximately, 30–35 cysts were placed in individual petridishes (30 mm \times 15 mm) for treatment with MFP5075 (2%), MFP3048 (20 g/10 ml), and NemguardTM (13.3 mg/L) dissolved in PRD. The Petri dish containing only PCN cysts and PRD without any product were considered negative controls. Mean number of PCN J2 hatched in each petri dish was manually recorded after every 7 days upto a period of 4 weeks. After each week, all the J2 were removed in each petri dish and were replaced with fresh PRD containing product's dilutions to continue the assay. The mean number of J2 recorded after 4 weeks of treatment was used for statistical analysis.

After 4 weeks of treatment with various products, some cysts were carefully broken open to study the granulation of unhatched eggs in the treated and untreated conditions (Feist et al., 2020). Individual cysts were removed from the treatment solutions, washed with ddH2O and transferred on to a glass slide containing a few drops of ddH2O. Cysts were carefully broken open with scalpel and tweezers without causing any damage to the eggs. Juvenile integrity within each unhatched egg was observed under the microscope. The total number of unhatched and granular eggs were enumerated in the treated and control groups. The unhatched eggs were considered as "granular," where the mouth part and tail of the enclosed J2 was not distinguishable and appeared dark. The percentage of granular unhatched eggs in each cyst was calculated as: (Number of granular unhatched eggs)/(Total number of unhatched eggs) × 100. Data was represented as the percentage of unhatched eggs that appear granular per cyst in each treatment and control group.

Some of the cysts, after 4-weeks of treatment with MFP3048, MFP5075, and NemguardTM, were transferred back into individual wells of the 24 -well plates, containing normal PRD without any products to record the J2 hatching in absence of the products. After 10 days, number of J2 in each well were manually counted under the stereoscope.

Effect of microbial based products on Globodera rostochiensis infestation in potato plants

New potato Solanum tuberosum Désirée tubers were collected from Teagasc, Oak Park, Carlow and exposed to cold spells at 4°C with high humidity for 2 months. Subsequently, the tubers were shifted to a dark room with 20-22°C and high humidity to encourage their germination. Within a period of 2 weeks sprouting was observed on the tubers. Immediately the small sprouted eye regions were gently excised from the tuber using a melon scoop and were potted on to small plastic pots containing thoroughly dried and sterilized sand: soil mixture 90:10. These potato tubers were maintained in an environmentally controlled glasshouse conditions at 32 \pm 2°C, 70 \pm 10% relative humidity (RH), and natural 14 h day/10 h night cycle, until the seedlings attained a height of 10-15 cm. Then, the nematode bioassays were performed to study the effect of the MFPs on PCN infestation in potato

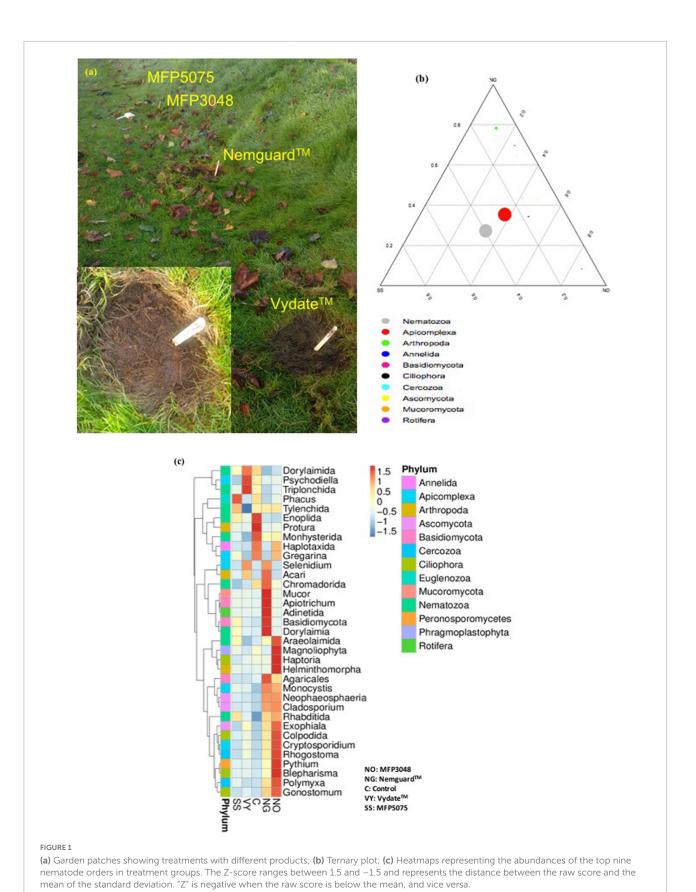
Prophylatic treatment: For this study, the individual 1 month old potato seedlings were first treated with MFP5075 (2%), MFP3048 (20 g/10 ml), and NemguardTM (13.3 mg/L) dissolved in water. After treatment for a period of 2 weeks, each seedling was infected with approximately 500-600 freshly hatched G. rostochiensis J2. Plants with only infection and no treatment were considered as inoculated untreated control. After infection, the potato seedlings were maintained in an environmentally controlled plant growth room at 32 \pm 2°C, 70 \pm 10% relative humidity (RH), and natural 14 h day/10 h night cycle. Treatments, including control pots, were set up in triplicate and were allowed to grow for a period of 60 days. After the treatment duration of 60 days, the potato plants were harvested to record the various growth parameters in terms of shoot height (SH; cm), root length (RL; cm), fresh weight (FW; g), dry weight (DW; g), and number of leaves (NL). SH and RL were measured using a meter ruler, FW and DW were recorded using laboratory weighing scales, NL was enumerated by visual counting. The number of the new cysts or females produced in each root system was used as a measure to study the impact of the MFPs on PCN infestation. For this, the potato roots were carefully observed to manually count the number of females developed on each root system. The results were compared with those of the inoculated untreated control plants and with those of commercial nematicide (NemguardTM) treatment. All experiments were repeated three more times over a period of 6 months to ensure the reproducibility of the results.

Garden soil treatment with microbial fermentation products, deoxyribonucleic acid extraction, and sequencing

A pilot scale experiment was conducted to study the impact of the MFPs on nematode biodiversity in untreated garden soil. For this study a random plot within the Kilkenny Road campus of SETU was chosen. In that, an individual garden patch as shown in Figure 1a of approximately, $45~\mathrm{cm} \times 45~\mathrm{cm}$ size, was treated with the following: MFP5075 (2%), MFP3048 (20 g/10 ml), Nemguard TM (20 Kg/hectare), and VydateTM (55 Kg/hectare) dissolved in water. A patch without any treatment was considered as untreated negative control. Treatment with NemguardTM, a commercial organic nematicide and VydateTM, a commercial synthetic chemical nematicide were considered as positive controls (Figure 1a). The individual products were mixed uniformly in the soil upto a depth of 20 cm. After 15 days, soil samples (approximately 25 g) were freshly collected from each patch. A total of 30 samples were obtained (5 treatments X 6 replicate samples per plot) and sieved through a 2 mm mesh. An individual soil sub-sample was added to individual centrifuge tubes containing 25-ml of deionized water and centrifuged for 10 min at 3500 rpm. The supernatant was discarded and the residue was dried overnight at room temperature (Karpinska et al., 2021). Subsequently, from the 0.25 g of soil sub-samples per treated patch, total soil deoxyribonucleic acid (DNA) was extracted using the Qiagen DNeasy[®] PowerSoil[®] Pro kit, as per the manufacturer instructions. Before outsourcing to Novogen Co., Ltd., the total DNA was quantified using both InvitrogenTM Qubit 4 Fluorometer and NanoDropTM and its quality and integrity was measured by performing agarose gel electrophoresis.

The nematode18S V4 rRNA region was sequenced using the MN18F (5'CGCGAATRGCTCATTACAACAGC 3') and 22R (5'GCCTGCTGCCTTCCTTGGA 3') primer pairs, on an Illumina paired-end platform (Bhadury et al., 2006; Karpinska et al., 2021). The data obtained from the sequencing company was studied and analyzed to understand the effect of the MFPs on nematode biodiversity in comparison to the untreated control soil and that treated with commercial nematicides.

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (Magoè and Salzberg, 2011), which was designed to merge paired-end reads when at least some of the reads overlap with the read generated from the opposite end of the same DNA fragment; the splicing sequences were called raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al., 2013) according to the Qiime (V1.7.0) quality control process.



The tags were compared with the reference database (SILVA138 database²) using UCHIME algorithm (UCHIME Algorithm³) (Edgar et al., 2011) to detect chimera sequences⁴ and the chimera sequences were removed (Haas et al., 2011) leaving the effective tags.

Sequences analysis were performed by Uparse software (Uparsev7.0.1090⁵) (Edgar, 2013) using all the effective tags. Sequences with \geq 97% similarity were assigned to the same OTUs and representative sequences for each OTU were screened for further annotation. For each representative sequence Qiime (Version 1.7.0⁶) (Altschul et al., 1990) in Mothur method was performed against the SSUrRNA database of SILVA138 database⁷ (Wang et al., 2007) for species annotation at each taxonomic rank (Threshold: 0.8 \sim 1) (Quast et al., 2013) (kingdom, phylum, class, order, family, genus, species).

To obtain the phylogenetic relationship of all OTUs, the MUSCLE (Edgar, 2004) (Version 3.8.318) compared multiple sequences rapidly.

Operational taxonomic units abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on this output normalized data.

Statistical analysis

All the experiments were statistically designed and analyzed. The experiments were arranged in a completely randomized factorial design (CRD). The results recorded from the MFP treatments in terms of PCN J2 survival, hatching assays, pluronic gel attraction assays, granulation of unhatched eggs and the plant-PCN trials were subjected to analysis of variance (ANOVA), using IBM-SPSS, version 23. Three sets of experimental plant trials in prophylactic treatments were performed over a span of 6 months to confirm reproducibility of results. Each trial had three replications per concentration including the untreated controls. Analysis of Variance was conducted for each experimental trial separately and the results from each trial were subjected to factorial ANOVA, to determine if the data was similar. Due to no significant difference among the trials, results presented in this study were the mean values of all the trials combined. Treatment means were separated using least significant differences (LSDs) obtained from analysis of variance (ANOVA) using IBM SPSS version 23.

- 2 http://www.arbsilva.de/
- 3 http://www.drive5.com/usearch/manual/uchime_algo.html
- 4 https://drive5.com/usearch/manual/chimeras.html
- 5 http://drive5.com/uparse/
- 6 http://qiime.org/scripts/assign_taxonomy.html
- 7 http://www.arb-silva.de/
- 8 http://www.drive5.com/muscle/

Results

Bioassay: Experimental design and microbial fermentation products treatment to determine lethal concentrations and doses

Treatment of *G. rostochiensis* J2 with MFP5075 had a very strong and significant impact ($p \le 0.05$). Preliminary experiments were conducted at concentrations ranging from 0 to 10% MFP5075. After 24 h of treatment in the 96-well plates, the J2 at all the dilutions except for untreated control did not survive. Subsequently, concentration range was reduced to 0–2%. In this concentration range, after 24 h of treatment in 96-well plates at 0.5 and 1% a 13-and 43-fold reduction in survival was recorded compared to untreated control. However, at 2%, the number of juveniles (J2) which survived dropped to zero (**Figure 2A**). Therefore, 2% MFP5075 could be considered as the lethal concentration for *G. rostochiensis* J2.

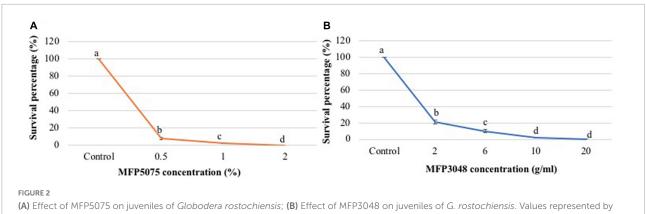
In the case of MFP3048, concentrations used were in the range of 0–20 g/10 ml (w/v) dissolved in water. After 24 h treatment in 96-well plates, in all the replicates, J2 were viable up to 10 g of product. Subsequently, the treatment duration was increased to 48 h with the same concentration range. After 48 h of treatment, percent survival at 2, 6, 10, and 20 g/10 ml dropped to 21.3, 10.2, 2.2, and 0.3%, respectively (**Figure 2B**). Therefore, 20 g/10 ml (w/v) MFP3048 could be considered as the lethal dose for *G. rostochiensis* J2.

Attraction assays using 20% (w/v) pluronic gel

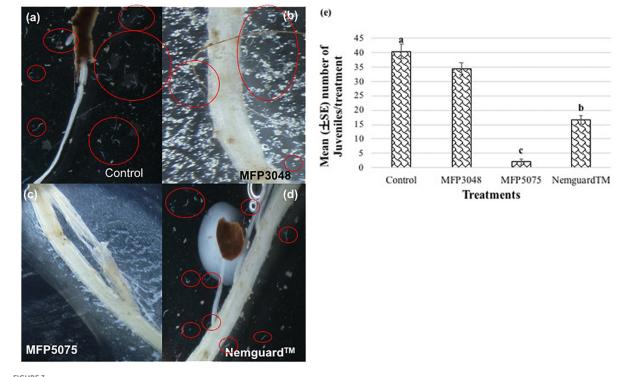
The MFP5075 treatment had a significant effect on PCN J2 in their attraction toward the host roots (**Figure 3**). There were only 1–2 J2 found attracted to the roots with this treatment ($p \leq 0.05$). A 20- and 8-fold reduction in number of J2 attracted toward potato roots was observed with MFP5075 (2 \pm 0.1) treatment compared to that of untreated control (40.3 \pm 2.5) and NemguardTM (16.7 \pm 1.5) treatment, respectively. In the case of treatment with MFP3048, an average of 34.3 \pm 2.1 J2 were found attracted to the potato roots, which was not significantly different from the untreated control. All the individuals and clusters of nematodes are indicated using red circles in **Figure 3**.

Globodera rostochiensis J2 hatching assay from cysts

Approximately 30–35 cysts were treated with MFP5075 (2%), MFP3048 (20 g/10 ml), and Nemguard TM (13.3 mg/L)



similar letters are not significantly different from each other ($p \le 0.05$).



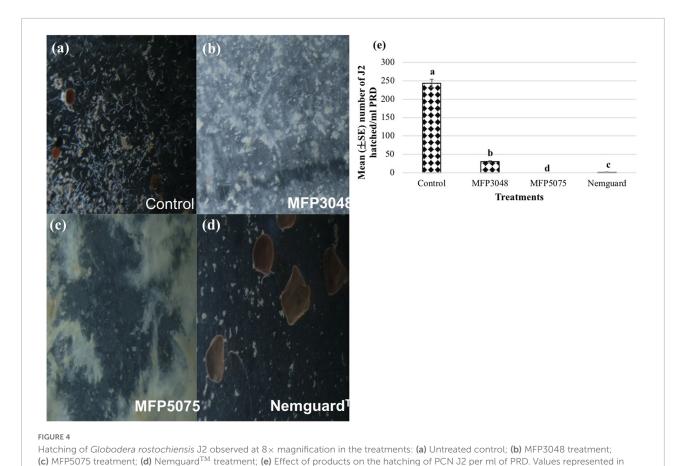
Attraction of Globodera rostochiensis J2 toward potato roots observed at 8x magnification: (a) Control; (b) MFP3048 treatment; (c) MFP5075 treatment; (d) Nemguard TM treatment; (e) Effect of products on the attraction of PCN J2. Values represented in graph by the same letters are not significantly different from each other ($p \le 0.05$). Individual and cluster of nematodes are indicated using red circles.

dissolved in PRD. After 4 weeks of treatment (243.3 \pm 11.5), (30 \pm 2.6), (0 \pm 0), and (1.3 \pm 0.6) J2 hatched in untreated control, MFP3048, MFP5075, and NemguardTM treatments, respectively (Figure 4).

Approximately 10-15 cysts per treatment were broken open carefully after 4 weeks. Efforts were made to identify the stylet, esophagus and the tail regions of the unhatched juveniles within the cysts. Most of the eggs in the untreated control were found as empty shells. However, many eggs were found granular in

the MFP3048 treated cysts when compared to those treated with MFP5075, NemguardTM treatment, and in the untreated control (Figure 5). Treatment with MFP5075 caused granulation in 47.7% of the unhatched eggs which was higher than that of the eggs that were found granular when treated with NemguardTM treatment (39.3%). However, the number of granular eggs was highest in the MFP3048 (59.3%) treatment (Figure 5).

Upon transferring the individually treated cysts into normal PRD without products, no hatching in the MFP3048 treated



cysts was observed. However, approximately 3-4 and 60-70 J2/ml PRD were found hatched in the MFP5075 and the NemguardTM treated cysts, respectively.

graph by the same letters are not significantly different from each other (p < 0.05)

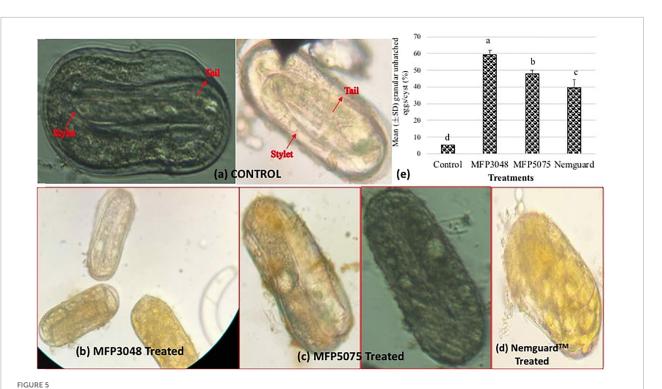
Effect of microbial based products on *Globodera rostochiensis* infestation in potato plants

Globodera rostochiensis J2 completely killed all the inoculated untreated potato plants in all the trials. The inoculated plants were all found completely dried up, wilted and dead within 1 month of infection. Treatment with the MFP products and Nemguard $^{\rm TM}$ had a very significant impact on the growth of the potato plants (Table 1). Potato plants that were infested with PCN J2 and treated with 1% MFP5075 performed very well regarding all the growth parameters. The MFP5075 (24.7 \pm 5.0) treatment enhanced the root growth by 1.6–2.1-fold when compared to Nemguard $^{\rm TM}$ (15.2 \pm 2.75) and the MFP3048 (11.5 \pm 2.6) treatments. Only root length was measured and recorded in the inoculated control plants, whereas all other growth parameters were not recorded as

the plants were all completely dried up and dead. A 2.68-fold increase in root length of MFP5075 treated plants was noted when compared to that of untreated inoculated control plants.

MFP5075 treatment also had a significant effect on FW, DW, and SH. The SH in the plants treated with MFPs was 1.2-fold greater compared to that of the Nemguard TM treatment. However, MFP5075 had the largest impact on plants in terms of both FW (10.0 \pm 2.9 g) and DW (2.1 \pm 0.1 g) compared to other treatments. The increase in FW was 1.8- and 1.5-fold for MFP 5075 compared to MFP3048 (5.7 \pm 1.6 g) and Nemguard TM (6.6 \pm 0.7 g) treatments. The increase in DW was 2.6- and 2.3-fold compared to the MFP3048 (0.8 \pm 0.1 g) and Nemguard TM (0.9 \pm 0 g) treatments.

The most interesting observation was made in relation to the number of females/cysts developed per root system (Table 1 and Figure 6); the cysts developed on each root system are indicated using red arrows in Figure 6. The plants treated with both MFPs had less PCN infestation with least number of cysts noted in the MFP3048 (3.3 \pm 0.2) treatment when compared to MFP5075 (5.3 \pm 1.5), Nemguard TM (23.7 \pm 1.5), and the untreated control (35 \pm 5) (Table 1). The infection was reduced by 10.6- and 6.6-fold with MFP3048 and MFP5075 treatments compared



treated; (c) MFP5075 treated; (d) NemguardTM treated; (e) Percentage of granular unhatched eggs/cyst in various treatments. Data shown are mean (\pm SE) percentage of unhatched eggs that appeared granular, per cyst. Values represented in graph by the same letters are not significantly different from each other ($\rho \leq 0.05$).

Treatment with products cause granulation in the unhatched eggs. Picture of the unhatched eggs from (a) Untreated control; (b) MFP3048

TABLE 1 Effect of various treatments on: number of leaves (NL), shoot height (SH), root height (RH), fresh weight (FW), and dry weight (DW) of treated, inoculated, and untreated potato plants.

Treatments	Mean (±SE) Number of leaves (NL)	Mean (±SE) Shoot height (SH) (cm)	Mean (±SE) Root length (RL) (cm)	Mean (±SE) Fresh weight (FW) (g)	Mean (±SE) Dry weight (DW) (g)	Mean (±SE) Number of cysts/plant
Inoculated control	0 ± 0 c	0 ± 0 c	$9.2\pm0.7~\mathrm{c}$	$0\pm0~\mathrm{c}$	0 ± 0 c	$35\pm5~\text{a}$
10 g MFP3048	$19.7\pm1.2~\text{a}$	$41.3\pm4.2~a$	$11.5\pm2.6\text{b}$	$5.7\pm1.6\mathrm{b}$	$0.8\pm0.1b$	$3.3\pm0.6~\mathrm{c}$
1% MFP5075	$22\pm6.6a$	$41.7 \pm 5.5~\text{a}$	$24.7 \pm 5.0 \; a$	$10.0\pm2.9~a$	$2.1\pm0.1~a$	$5.3\pm1.5~\text{c}$
Nemguard TM	$21.7\pm3.5~a$	$34.7 \pm 9.8 b$	$15.2\pm2.75\mathrm{b}$	$6.6\pm0.7~\mathrm{b}$	$0.9\pm0b$	$23.7\pm1.5~\text{b}$

Values represented in the table by the same letters are not significantly different from each other ($p \le 0.05$).

to untreated control plants, respectively. This reduction was 7.2- and 4.5-fold when compared to the treatment with the commercial nematicide Nemguard $^{\rm TM}$ treatment.

Garden soil treatment with microbial fermentation products, deoxyribonucleic acid extraction, and sequencing

Garden soil patches were treated with MFP5075, MFP3048, Nemguard TM , Vydate TM , and there was an untreated control. A dominant phylum detected along with Nematoda was Apicomplexa. A few other minor

organisms belonging to the phyla Arthropoda, Annelida, Basidiomycota, Ciliophora, Cercozoa, Ascomycota, Rotifera, and Mucoromycota were also detected, however, they were not in the interest of our study.

The ternary plot is shown in **Figure 1b**. In the plot the three vertices are represented by the three samples or groups. Circles represent dominant taxa and the size of circles is proportional to the relative abundance of the phyla. The sample or group to which the circle is closer, is represented in higher abundance in the respective phylum. The plot clearly displayed that the phylum Nematoda was dominant in soil samples treated with MFP5075 followed by the soil samples treated with MFP3048. Members of the phylum Nematoda were least abundant in samples treated with the commercial nematicide NemguardTM.

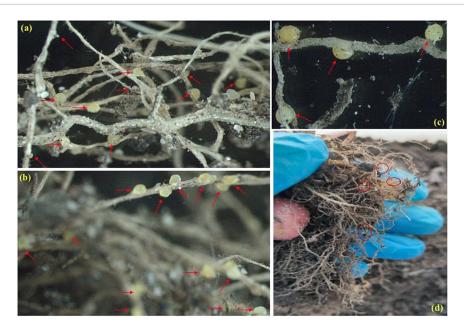


FIGURE 6 Images of cysts developed on potato roots ($2-10 \times$ magnification) in: (a) Untreated control; (b) NemguardTM treated; (c) MFP5075 treated; and (d) MFP3048 treated. Red arrows in the pictures indicate the cysts or females developed on each root system.

Heatmaps representing the abundances of the top nine nematode orders in treatment groups are shown in Figure 1c. The identified nine dominant nematode order were Dorylaimida, Triplonchida, Tylenchida, Enoplida, Monhysterida, Chromadorida, Dorylaimia, Araeolaimida, and Rhabditida. Among them Dorylaimida, Triplonchida, and Dorylaimia mostly contain free-living terrestrial and freshwater species. The orders Monhysterida, Enoplida and Araeolaimida contain marine free living nematodes; Tylenchida are mostly parasites of insects or plants and Rhabditida is an order of free-living, zooparasitic, and phytoparasitic microbivorous nematodes living in soil. From the heat map it is evident that, the nematodes belonging to the order Dorylaimida and Triplonchida were abundant in the untreated control, VydateTM and MFP5075 treated samples whereas, they were least abundant in case of MFP3048 and NemguardTM treated soils. A significantly different observation was made regarding the order Tylenchida, which were highly abundant in all the treatments except in the soils treated with the chemical nematicide VydateTM. Similarly, the VydateTM treatment completely reduced the abundance of the nematodes belonging to the orders Monhysterida, Chromadorida, Dorylaimia, Araeolaimida, and Rhabditida. The heat map gives an overall indication, that out of the nine nematode orders identified, seven were abundant in the soils treated with MFP5075 and six of them were found abundant in the soils treated with MFP3048 and NemguardTM. The lowest nematode abundance was recorded in the soils treated with the chemical nematicide

VydateTM, which had only three out of the nine orders identified.

Discussion

Although there are many reports on applications of various products to control PCN infestation, most of them still remain incomplete due to certain limitations. Some of the nematicides that are currently in use are synthetic based and therefore highly toxic for the environment (Feist et al., 2020).

The bioassays clearly indicated the nematicidal potential of the products by causing 100% mortality at very low concentrations. Similar observations were noted when the root-knot nematode, M. javanica was treated with MFP5075 (Pulavarty et al., 2020, 2021a). Application of pluronic gels facilitates easy observation of juvenile movement toward host roots (Wang et al., 2009; Sasaki-Crawley et al., 2012; Pulavarty et al., 2021a). The in vitro bioassays that are reported here on PCN juvenile mortality, J2 hatching from the cysts and attraction towards host roots, give a clear indication that the product MFP5075 has an impact on the activity of the PCN. It has the potential to cause mortality directly to J2 as 2% of its concentration caused 100% mortality within 24 h. Similarly, the attraction assays also revealed the potential of the product as it completely blocked the movement of J2 toward the host roots by killing the J2. Similar effect was noticed while treating M. javanica J2 with the same product (Pulavarty et al., 2021a). This observation indicates that the application of MFP5075 to

the PCN infected soil, it could potentially supress the infection by causing death of the juveniles that are available in the soil and also cease the hatching process. Potentially, if a product can cease the hatching process then it has the ability to interfere with the life cycle of the nematode and could stop the release of the next generation of the PCN (Zakaria et al., 2013). Therefore, in the course of time the nematodes could be completely eradicated from the soils. However, in such cases, the shelf-life of the applied products must be thoroughly investigated so as to ensure its prolonged effect on nematodes.

The observation on egg viability or juvenile integrity within the eggshells, suggests the in ovo necrotic potential of the MFPs, especially, MFP3048, as it caused the highest number of granular unhatched eggs. This gives an indication that onset of favorable conditions might not promote the hatching process as the product possibly is causing morphological necrosis within the egg itself. This again indicates that this product interferes with the PCN life cycle by irreversibly inhibiting the hatching process. This loss of J2 integrity clearly depicts the necrosis and suggests a potential mode of action of MFP3048. This could possibly explain the 8.1-fold reduction in J2 hatching, when cysts were treated with MFP3048 when compared to that of untreated control. The in ovo exposure to MFP3048 caused an impairment in J2 hatching even after transferring the cysts to normal root diffusates. MFP5075 treatment also caused granulation in the G. rostochiensis eggs but this was lower than that caused by the MFP3048 treatment, with only a few J2 observed to hatch when the cysts were put back into normal PRD. However, the Nemguard $^{\mathrm{TM}}$ treatment caused least granulation in the unhatched eggs, therefore the cysts when treated with this nematicide resulted in the hatching of juveniles following the onset of normal conditions. Similar observations were reported in G. pallida, when its eggs were treated with aldicarb, as there was no evidence of irreversible inhibition of the hatching process (Feist et al., 2020). Conversely, treatment with Fluopyram and Abamectin was reported to cause heavy granulation within the eggs of G. pallida and therefore reduced the J2 hatching process due to morphological disruption of eggs (Feist et al., 2020). All these nematicides are synthetic agrochemicals and have been reported to be highly toxic to birds, mammals, and aquatic organisms (European Food Safety Authority, 2013).

Prophylactic treatment with the MFPs clearly showed potential of plant growth promotion. The potato plants treated with MFP5075 had the highest biomass accumulation compared to the plants in other treatments. These observations were in agreement with previous findings that were reported on tomato plants (Pulavarty et al., 2021a). MFP3048 also contributed toward growth of potato plants compared to those in the untreated control but had less biomass accumulation compared to that in the MFP5075 treatment. However, nematode suppression potential was more evident in the MFP3048 treatment (Table 1), as the roots treated with this product

had the least number of females or cysts developed on them compared to any other treatment. The reason for this could be directly related to the granulation that was caused in eggs treated with this product. Treatment of plants with this product might have increased the number of granular eggs thus reducing J2 hatching therefore interrupting the lifecycle of PCN and ultimately reducing the cyst formation on the roots. The untreated potato plants were all dead with highest number of cysts or females developed in their roots, whereas, the MFP treated plants not only had higher biomass accumulation but also were found to have the least number of cysts or females developed on their roots compared to the plants in the untreated control and the commercial nematicide treatment (NemguardTM). This could be due to the presence of essential micronutrient, bacterial fermentation extracts and Cu component within these products that are contributing toward plant growth promotion (Pulavarty et al., 2020). Aires et al. (2009) reported G. rostochiensis suppression following treatment with extracts of Brassicacea plants. The study showed that maximum nematode suppression was noted when the plants that were treated with extracts of watercress, cauliflower and Brassica rapa (Aires et al., 2009). However, the effect of these extracts on other soil nematodes, EPN and on potato plant growth have not been reported.

Prior to large scale field application, a pilot scale trial needs to be performed to study the nematode abundance in the soils after application of any products (Abawi and Widmer, 2000; Briar et al., 2007; Oka, 2010). This kind of analysis would provide an indication on what could possibly happen to non-target beneficial nematodes in the soil. This would certainly help to prevent causing any long term damage to natural soil ecology (Abawi and Widmer, 2000; Pulavarty et al., 2021b). Therefore, in this study with Alltech MFPs we tried to fill the knowledge gaps by studying their impact on EPN (Pulavarty et al., 2020), other soil nematodes and on plant growth promotion were assessed in a small pilot scale study.

Our results in relation to nematode abundance in soil indicated that the application of the MFPs had a very similar effect to that in the untreated control and the Nemguard TM treatment. These observations when combined with that of the previous results reported in terms of the MFPs effect on the beneficial EPN (Pulavarty et al., 2020), indicate that the MFP formulations could be safe for the environment. However, these results are very preliminary and further repetition and large-scale field trials are essential to validate the reported results on nematode biodiversity.

Compiling all the findings, it could be concluded that under the conditions of this study the MFPs, which are organic based soil health products, displayed nematicidal properties against *G. rostochiensis*. These products reduced PPN infestations in the host plants and displayed a positive impact on the beneficial

EPN (Pulavarty et al., 2020, 2021a). Application of these products could potentially improve the potato yield and help toward enhancing the global potato production. Therefore, subsequent large scale field applications should be performed to further confirm the findings of this work and potentially contribute toward the sustainable management of PCN.

Data availability statement

The data presented in this study are deposited in the NCBI repository, accession numbers SAMN30418779, SAMN30418780, SAMN30418781, SAMN30418782, SAMN30418783, SAMN30418784, SAMN30418785, SAMN30418786, SAMN30418787, and SAMN30418788.

Author contributions

AP: planning, execution, statistical analysis, and manuscript drafting. AS: performing experiments and helping AP. DS: performing nematode biodiversity studies. JM: bioinformatic analysis of nematode biodiversity studies. KH: manuscript editing, mentoring, and funding the research. TK-D: manuscript editing, mentoring, and through supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author KH was employed by Alltech Biosciences, Ireland.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Gall-specific promoter, an alternative to the constitutive *CaMV35S* promoter, drives host-derived RNA interference targeting *Mi-msp2* gene to confer effective nematode resistance

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One of the major obligate plant parasites causing massive economic crop losses belongs to the class of root-knot nematodes (RKNs). Targeting of major nematode parasitism genes via Host Delivered-RNAi (HD-RNAi) to confer silencing is established as one of the most effective approaches to curb nematode infection. Utilizing nematode-responsive root-specific (NRRS) promoters to design a dsRNA molecule targeting approach to hamper nematode parasitism. Here, a previously validated peroxidase gall specific promoter, pAt2g18140, from Arabidopsis was employed to express the dsRNA construct of the nematode effector gene Mi-msp2 from Meloidogyne incognita. Arabidopsis RNAi lines of CaMV35S::Mi-msp2-RNAi and pAt2q18140::Mi-msp2-RNAi were compared with control plants to assess the decrease in plant nematode infection. When subjected to infection, the maximum reductions in the numbers of galls, females and egg masses in the CaMV35S::Mi-msp2-RNAi lines were 61%, 66% and 95%, respectively, whereas for the pAt2g18140::Mi-msp2-RNAi lines, they were 63%, 68% and 100%, respectively. The reduction in transcript level ranged from 79%-82% for CaMV35S::Mi-msp2-RNAi and 72%-79% for the pAt2g18140::Mi-msp2-RNAi lines. Additionally, a reduction in female size and a subsequent reduction in next-generation fecundity demonstrate the efficacy and potential of the gall Joshi et al. 10.3389/fpls.2022.1007322

specific promoter pAt2g18140 for utilization in the development of HD-RNAi constructs against RKN, as an excellent alternative to the CaMV35S promoter.

KEYWORDS

HD-RNAi, *Meloidogyne incognita*, pAt2g18140, gall specific promoter, *Mi-msp2*, effector gene

1 Highlights

- Reduced nematode infection in RNAi lines *CaMV35S:: Mi-msp2-RNAi* and *pAt2g18140::Mi-msp2-RNAi*.
- Reduced fecundity and size of females in RNAi lines for both promoters.
- The gall-specific promoter is very effective in gene silencing via HD-RNAi.

2 Introduction

Plant parasitic nematodes (PPNs) have been identified as one of the major plant pest groups causing large crop losses annually (Chitwood, 2003). The major groups among the PPNs are root knot nematodes (RKN- Meloidogyne spp.) and cyst nematodes (Heterodera spp. and Globodera spp.). Among the RKNs, Meloidogyne incognita (southern root knot nematode) is a sedentary obligate parasite with a dynamic geographical distribution (Abad et al., 2008). With annual crop losses of USD 173 billion and a wide host range of as many as 2000 crop plant species, RKN is one of the primary parasites threatening agriculture (Elling, 2013). The major feature that enables M. incognita to successfully parasitize host plants for a longer duration is the formation of permanent nematode feeding sites (NFSs) comprising giant cells. Infective juveniles (J2s) penetrate the host plant roots upon receiving a suitable stimulus in the soil and then migrate to the cortex region to develop giant cells (Jones and Payne, 1978).

The functional mechanisms for the establishment, construction and later maintenance of the NFS are strategically controlled by a group of nematode parasitism genes known as effector genes (Gheysen and Mitchum, 2011). The effector proteins encoded by the effector genes have been reported to play a vital role in nematode parasitism. 16D10, calreticulin and MSP18 nematode effector proteins are functionally involved in disrupting the host plant basal immune system and rendering the host plant prone to infection (Huang et al., 2006; Jaouannet et al., 2012; Jaouannet et al., 2013; Grossi-de-Sa et al., 2019). Effector protein 8D05

influences water transport to nematode feeding sites, and protein Mj-FAR-1 is involved in modifying cell wall-related plant gene functions for developing feeding sites (Iberkleid et al., 2013; Xue et al., 2013). However, the effector protein Mi-MSP40 is actively involved in suppressing the effector triggered immunity (ETI) signalling-associated cell death response in the host (Niu et al., 2016).

The availability of plant genome sequences has made it feasible to characterize the functional aspects of their genes, leading to immense progress in plant genome research. One of the most applicable tools for functional genomics studies is reverse genetics. In the field of reverse genetics, *in vivo* dsRNA delivery *via* Host Delivered-RNAi (HD-RNAi) technology has proven to be an effective method for gene silencing (Timmons et al., 1998). For the purpose of combating several PPNs, many effector genes have been targeted by HD-RNAi and successfully silenced. Silencing of various effector genes has directly affected the functionality of nematode parasitism to different degrees, as reported in the cases for different effectors genes termed as *Meloidogyne* secretory proteins of *M. incognita* (*Mi-msp*) (Joshi et al., 2022).

The majority of promoters used in gene silencing via RNAi are expressed constitutively, such as CaMV35S and pUbi1 (Sindhu et al., 2009). Lacking restrictive spatial expression, the constitutive promoters drive the dsRNA expression in RNAibased gene construct throughout the plant tissues/organs in transgenic plants. By silencing the target gene and its homologues in all of the plant tissues, the constitutive promoter poses the risk of causing off-target pleiotropic phenotypes thus altering the desired results (Peremarti et al., 2010). One of the major risks in such cases is embryo lethality caused by silencing of homologous sequences. Selective functionality of the common transgenic promoter, CaMV35S, for expression of nematode RNAi constructs in NFS is questionable (Goddijn et al., 1993; Bertioli et al., 1999). The CaMV35S driven gusA and gfp have been reported to be downregulated at NFS of RKN and cyst nematodes in Arabidopsis thaliana (Urwin et al., 1997). Thus, due to a many off target effects across homologous sequences in different tissues/organs it is difficult to assess the reason for phenotypic variations in transgenic plants compared to the control ones. On the other hand employing regulated promoters proves as a better option

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for expression of RNAi constructs these regulated promoters are stage specific, expressed in certain selective tissues/organs, and can be physically/chemically induced by specific external or internal stimuli. The use of stress-inducible promoters is highly desirable due to a potential decrease in off target effects (Yamaguchi-Shinozaki and Shinozaki, 2001). Previously a range of NRRS promoters have been successfully identified and employed to combat PPN infection to varying degrees (Opperman et al., 1994; Thurau et al., 2003; Sukno et al., 2006; Kumar et al., 2016). The A. thaliana promoter MDK4-20 and the Zea mays promoter ZmRCP-1 are expressed specifically in the root tip making them suitable to control the expression of the antinematode defence genes and dsRNA molecules targeting PPN genes (Lilley et al., 2011; Onyango et al., 2016). Therefore, the study of RNAi constructs under the control of NRRS promoters will provide insights into the molecular functionality of the nematode responsive promoters as well as the mechanisms of their effector genes.

The gall-specific promoter pAt2g18140, which drives the expression of endogenous peroxidase in A. thaliana, has been previously reported as responsive to infection by M. incognita. (Kakrana et al., 2017). A substantial reduction in the gene expression of the effector gene Mi-msp2 was reported, which led to a decrease in parasitization by M. incognita. Also, there was evident reduction in infection in phenotype in of the Mi-msp2 RNAi lines in comparison with the control plants (Joshi et al., 2019). The adult M. incognita females feeding on the Mi-msp2 RNAi lines presented an up to 85% reduction in gene transcript levels (Joshi et al., 2019). On the other hand, a previously reported gall specific promoter, At2g18140, was found to express specifically at the sight of M. incognita infection in the transgenic lines (Kakrana et al., 2017). In light of these promising results in this study the RNAi construct was prepared expressing siRNA of the effector gene Mi-msp2 under the control of the gall specific promoter At2g18140. A comparative study was performed to assess the reduction in M. incognita infestation to evaluate the efficacy levels of effector gene Mi-msp2 silencing in the Arabidopsis HD-RNAi lines driven by the CaMV35S and At2g18140 promoters. Five independent events of each CaMV35S::Mi-msp2-RNAi Arabidopsis line and pAt2g18140::Mi-msp2-RNAi Arabidopsis line were evaluated along with control Arabidopsis (Col-1) plants.

3 Material and methods

3.1 Procurement and maintenance of the *M. incognita* culture on tomato plants (PUSA Ruby)

The Division of Nematology, Indian Agricultural Research Institute (IARI), New Delhi, provided the pure culture of RKN *M. incognita* (Kofoid & White) Chitwood race 1, which was maintained on Pusa Ruby tomato (*Solanum lycopersicum L.*)

plants at ambient culture room conditions at NIPB-IARI. The identification of M. incognita was performed from an isolated single egg mass from a pure culture (Zijlstra et al., 2000; Joshi et al., 2019). Hatched J2s obtained from pure cultured egg masses were used for multiplication and culture maintenance. The host plant, PUSA Ruby tomato seedlings, was grown and irrigated with Hoagland solution at regular intervals (Joshi et al., 2019). Twenty-five-day-old plants were subjected to M. incognita juvenile (500) infection, and the infected roots were harvested and analysed for gall development after 6 to 7 weeks of infection. The plants were harvested after the developed egg masses were visible; the latter were then hand-picked with forceps. The isolated egg masses were allowed to hatch immersed in M9 medium at 28°C over a wire gauge covered with tissue paper on Petri plates (the modified Baermann funnel technique) (Baermann, 1917; Hooper, 1986). The released J2s were collected for the maintenance of a continuous culture of *M*. incognita and for transgenic infection analyses.

3.2 Preparation of RNAi constructs pAt2g18140::Mi-msp2-RNAi and CaMV35S::Mi-msp2-RNAi

3.2.1 Cloning of sense, antisense and promoter fragments in the RNAi plasmid pHANNIBAL

Selective primers for the sense and antisense sequences of the Mimsp2 gene and the gall specific promoter pAt2g18140 were synthesized with flanking sequences for suitable restriction enzymes using Primer3 plus (Supplementary Table S1). The gall specific promoter of the At2g18140 gene (1572 bp) was amplified from DNA isolated from Arabidopsis (Col-0) plants using genespecific primers and the Mi-msp2 gene fragments (sense and antisense- 590 bp) were amplified from M. incognita DNA with gene-specific primers (Supplementary Table S1). Primers for cloning in pHANNIBAL (Wesley et al., 2001) were designed from the Mimsp2 gene plus restriction sites for EcoRI and KpnI for the sense fragment and HindIII and XbaI for the antisense fragment. For the promoter gene At2g18140, primers with flanking sequences for the enzymes MluI and XhoI were designed suitably for cloning in pHANNIBAL (Wesley et al., 2001) (Figure 1A). The amplicons were purified via agarose gel electrophoresis, cloned into the pGEMT easy vector (Promega Corporation, Wisconsin-Madison, USA) and confirmed by Sanger sequencing. For cloning, the gene fragments were digested with suitable restriction enzymes from pGEMT plasmids and confirmed by sequencing. The digested fragments were further purified and ligated within the digested RNAi vector pHANNIBAL (Figure 1A). The ligated vector was transformed into E. coli (DH5a), and confirmation of the positive clones was performed by a colony PCR protocol (Supplementary Table S1). The positive clones were further selected for cloning the RNAi cassette into the binary vector pCAMBIA 1302 for transformation into A. thaliana (Col-0) plants.

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3.2.1.1 RNAi cassette transfer from pHANNIBAL to the binary vector pCAMBIA 1302

The RNAi cassette from transformed pHANNIBAL was restricted with SacI and PstI and cloned into the digested binary vector pCAMBIA 1302 via ligation. The ligated vector was transformed into E. coli (DH5α) (Figure 1B). A colony PCR protocol was used to confirm transformed positive clones. The RNAi cassette bearing the pCAMBIA 1302 plasmid was isolated and transformed into the Agrobacterium tumefaciens GV3101 strain by the standard freeze-thaw method (Clough and Bent 1998). A. tumefaciens was grown on selective YEP medium (50 mg/L kanamycin, 50 mg/L gentamycin and 25 mg/L rifampicin) for the selection of recombinant transformants. The transformed A. tumefaciens GV1301 was confirmed to contain the insert by colony PCR. Wild-type Arabidopsis thaliana was transformed with positive A. tumefaciens clones via Agrobacterium-mediated transformation via floral dip method (Clough and Bent 1998). The seeds harvested from the T₁ plants were screened on selective MS media (15 mg/L hygromycin), and the selected transgenic pAt2g18140::Mi-msp2-RNAi plants were confirmed by PCR (Figure S2). The T₁ transgenic pAt2g18140::Mi-msp2-RNAi plants were propagated up to the T3 homozygous generation (Figure S1).

3.2.2 Transformation of *Arabidopsis thaliana* (Col-1) *with the CaMV35S::Mi-msp2-RNAi* vector pYSB

The CaMV35S::Mi-msp2-RNAi construct was prepared using Gateway technology (Invitrogen Corpration, Waltham, Massachusets, USA) in the RNAi plant expression vector pYSB (generated by IIT Kanpur). The recombinant pYSB RNAi

construct was transferred into Agrobacterium tumefaciens strain GV3101 and introduced into Arabidopsis thaliana (Col-1) via Agrobacterium-mediated transformation (Clough and Bent 1998). T₃ homozygous CaMV35S::Mi-msp2-RNAi transgenic lines were used for this study (Joshi et al., 2019).

3.3 Seed storage and sterilization for plating on MS media

The seeds of A. thaliana (Col-1) and five independent events of each CaMV35S::Mi-msp2-RNAi line and pAt2g18140::Mimsp2-RNAi line were taken (approximately 100-150 seeds each) for sterilization and plating. The seeds were placed in a microfuge tube and surface sterilized by the standard two-step protocol (Joshi et al., 2019). The sterilized seeds were suspended in a 0.1% agarose solution. These seeds were then kept at 4°C for 72 hours for vernalization and plated on selective MS media (pH 5.8) (HiMedia) with the antibiotic hygromycin (15 mg/ml) for pAt2g18140::Mi-msp2-RNAi lines and kanamycin (50 mg/L) for the CaMV35S::Mi-msp2-RNAi lines. Within 12-15 days, the resistant plants grew with bright green and healthy leaves and a well-developed root system after plating and they were allowed to grow until the 6-leaf stage. The plants were grown under a 16h light/8-h dark photoperiod at 21°C. After 14 days, the seedlings (ten plants per plate) were transferred to half concentration MS media with CleriGel (HiMedia) in round Petri dishes (9 cm diameter). The plants were kept slightly angled at 45° for unidirectional root proliferation for 7 days until secondary roots appeared. The A. thaliana (Col-1), pAt2g18140::Mi-msp2-RNAi lines and CaMV35S::Mi-msp2-

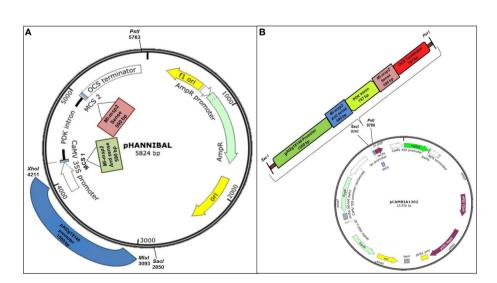


FIGURE 1 Constructs used in the study. (A) Cloning of the Mi-msp2 gene and promoter pAt2g18140 in the RNAi vector pHANNIBAL. (B) Insertion of the RNAi cassette from pHANNIBAL into the binary vector pCAMBIA 1302 expressing the dsRNA construct for pAt2g18140::Mi-msp2-RNAi.

RNAi lines showed no differences in their morphological features, such as root and shoot growth patterns, life cycle or flowering responses. Furthermore, no phenotypic distinctions were observed among the different RNAi lines.

3.4 Nematode J2 sterilization, infection of the plants and analyses of the infected plants

M. incognita J2s obtained after hatching at 28°C were suspended in M9 buffer and sterilized by the standard protocol using nematode sterilization buffer (Kakrana et al., 2017). After secondary root growth, 21-day-old plants were infected with approximately 300 M. incognita J2s per plant. Thirty-five days post infection (dpi), the plants were carefully harvested from the MS media (CleriGel), and the fresh weight of the root mass was recorded. The roots were visualized under a light microscope to count the numbers of galls, females and egg masses per gram of root fresh weight. The egg masses were picked with forceps, and the females were extracted after dissecting the roots under a light microscope. A mean of 10-15 biological replicates was taken for the counted numbers of galls, females and egg masses per gram of root fresh weight per RNAi line. The data obtained from the control plants versus the pAt2g18140::Mi-msp2-RNAi and CaMV35S::Mi-msp2-RNAi RNAi lines were compared to assess the gene-silencing efficiencies.

3.5 Reproductive capacity and fecundity of M. incognita in the A. thaliana (Col-1), pAt2g18140::Mi-msp2-RNAi lines and the CaMV35S::Mi-msp2-RNAi lines

To estimate the level of susceptibility of the CaMV35S::Mimsp2-RNAi and pAt2g18140::Mi-msp2-RNAi line plants, the M. incognita egg masses were isolated, and the number of eggs in each single egg mass was recorded. Five egg masses each were isolated from the five independent CaMV35S::Mi-msp2-RNAi lines (CaMV35S::Mi-msp2-RNAi E1, CaMV35S::Mi-msp2-RNAi E2, CaMV35S::Mi-msp2-RNAi E3, CaMV35S::Mi-msp2-RNAi E4 and CaMV35S::Mi-msp2-RNAi E5) and from the five independent pAt2g18140::Mi-msp2-RNAi lines (pAt2g18140::Mi-msp2-RNAi E1, pAt2g18140::Mi-msp2-RNAi E2, pAt2g18140::Mi-msp2-RNAi E3, pAt2g18140::Mi-msp2-RNAi E4 and pAt2g18140::Mi-msp2-RNAi E5). The isolated egg masses were kept for hatching at 28° C, and the number of J2s that hatched was recorded. An average of the total number of eggs was calculated for CaMV35S::Mi-msp2, pAt2g18140::Mi-msp2-RNAi and the control plants from the respective isolated egg masses. A comparative evaluation of the averaged values of the egg masses was made for the CaMV35S::Mimsp2-RNAi lines, pAt2g18140::Mi-msp2-RNAi lines and the control plants. The susceptibility of the control plants versus the pAt2g18140::Mi-msp2-RNAi and CaMV35S::Mi-msp2-RNAi lines after Mi-msp2 gene silencing was assessed by Oostenbrink's reproduction factor (Oostenbrink, 1966; Joshi et al., 2019). Phenotypic effects on the infected roots were acquired at a suitable magnification with a NIKON® microscope. The extracted females were also captured to compare the sizes (lengths and widths) of 20 nematode females (NIS element D-Nikon Instruments Inc., New York, USA). The average values were taken for further analyses.

3.6 Comparative gene expression analysis in RNAi females after gene silencing

The adult feeding females were carefully excised from control plants and five events of each pAt2g18140::Mi-msp2-RNAi and CaMV35S::Mi-msp2-RNAi lines. The excised females (200-250 per sample) were flash-frozen in liquid nitrogen for total RNA isolation (PureLink RNA Mini Kit-Thermo Fisher Scientific, Wilmington, USA). Around 250-300 ng/µl RNA was isolated from each sample. For expression analysis by qRT-PCR (Applied Biosystems StepOne PlusTM Real-Time PCR (USA, Massachusetts), cDNA was synthesized from the total RNA (SuperScript III First-Strand Synthesis System-Thermo Fisher Scientific, Wilmington, USA). RT primers designed for the Mi-msp2 cDNA sequence were used for the expression analysis (http://frodo.wi.mit.edu/primer3/) (Supplementary Table S1). Quantitative real-time PCR was performed to analyse the gene expression of the Mi-msp2 gene in females feeding on each of the five RNAi lines of CaMV35S::Mimsp2-RNAi and pAt2g18140::Mi-msp2-RNAi, each expressing dsRNA specific for the Mi-msp2 gene. The females feeding on control plants were also isolated and analysed for Mi-msp2 gene expression. The $\Delta\Delta$ Ct values of the obtained gene expression of the effector gene Mi-msp2 were compared for females feeding on the control plants, and five events for each pAt2g18140::Mi-msp2-RNAi and CaMV35S::Mi-msp2-RNAi line were evaluated using nematode actin reference gene (Accession no. Mi-actin-Minc06769) (Livak and Schmittgen, 2001). For each event three biological and three technical replicates were taken. For the assessment of significance, statistical analysis using one-way ANOVA and Tukey's test were performed.

4 Results

4.1 Phenotypic analyses of control and transgenic lines before and after *M. incognita* infection

All of the transgenic lines of both RNAi constructs (*CaMV35S::Mi-msp2-RNAi*) and p*At2g18140::Mi-msp2-RNAi*) were plated on selective MS media and observed for phenotypic differences in plant growth. Twelve-day-old seedlings of

transgenic lines were found to have similar growth patterns to that of the control plants (wild-type *Col-0*). There were no phenotypic differences observed among the different RNAi lines.

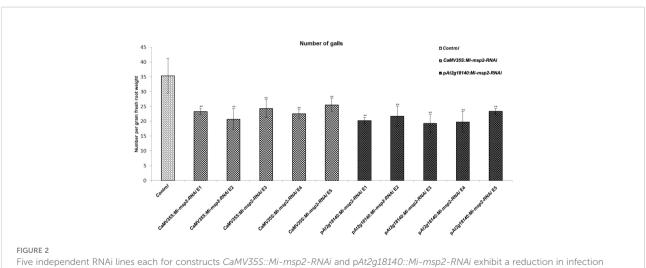
4.2 *M. incognita* infection assay on *Mi-msp2 HD-RNAi* lines of *Arabidopsis thaliana*

Five independent homozygous T₃ transgenic events (E1, E2, E3, E4 and E5) of both promoter-driven Mi-msp2 RNAi lines, namely, CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi, were selected for M. incognita infection analyses. The intensity of infection of the RNAi lines (of both promoters) was compared to the control plants (wild-type Col-0). The recorded numbers of galls, females and egg masses per gram fresh weight of root were compared (Figures 2-4). All of the transgenic events of the Mi-msp2 RNAi lines (CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi) depicted a significant reduction in M. incognita infection compared to the control plants, indicating successful gene silencing. For the CaMV35S::Mimsp2-RNAi lines, the percent reduction in the numbers of galls was recorded as 56%, 61%, 54%, 57% and 52% for CaMV35S::Mi-msp2-RNAi E1, CaMV35S::Mi-msp2-RNAi E2, CaMV35S::Mi-msp2-RNAi E3, CaMV35S::Mi-msp2-RNAi E4 and CaMV35S::Mi-msp2-RNAi E5, respectively (Figure 2). The reduction in the number of females was 60%, 64%, 61%, 59% and 66% for CaMV35S::Mi-msp2-RNAi E1, CaMV35S::Mi-msp2-RNAi E2, CaMV35S::Mi-msp2-RNAi E3, CaMV35S::Mi-msp2-RNAi E4 and CaMV35S::Mi-msp2-RNAi E5, respectively (Figure 3). The reduction in the number of egg masses was the maximum recorded as 83%, 80%, 87%, 75% and 95% for CaMV35S::Mi-msp2-RNAi E1, CaMV35S::Mi-msp2-RNAi E2,

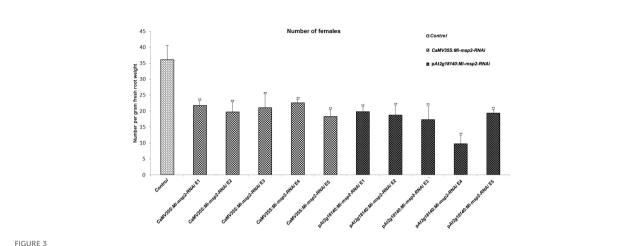
CaMV35S::Mi-msp2-RNAi E3, CaMV35S::Mi-msp2-RNAi E4 and CaMV35S::Mi-msp2-RNAi E5, respectively (Figure 4). In the pAt2g18140::Mi-msp2-RNAi transgenic lines, the number of galls were reduced by 62%, 59%, 63%, 63% and 56% for pAt2g18140::Mi-msp2-RNAi E1, pAt2g18140::Mi-msp2-RNAi E2, pAt2g18140::Mi-msp2-RNAi E3, pAt2g18140::Mi-msp2-RNAi E4 and pAt2g18140::Mi-msp2-RNAi E5, respectively (Figure 2). The reduction in the number of females was 64%, 66%, 68%, 67% and 64% for pAt2g18140::Mi-msp2-RNAi E1, pAt2g18140::Mi-msp2-RNAi E2, pAt2g18140::Mi-msp2-RNAi E3, pAt2g18140::Mi-msp2-RNAi E4 and pAt2g18140::Mi-msp2-RNAi E5, respectively (Figure 3). Similarly, the reduction in the number of egg masses was the maximum recorded as 86%, 73%, 89%, 100% and 75% for pAt2g18140::Mi-msp2-RNAi E1, pAt2g18140::Mi-msp2-RNAi E2, pAt2g18140::Mi-msp2-RNAi E3, pAt2g18140::Mi-msp2-RNAi E4 and pAt2g18140::Mi-msp2-RNAi E5, respectively (Figure 4). A significant decline in infection with constitutive promoters and the gall specific promoter-driven RNAi lines for the secretory gene Mi-msp2 reveals the potential applicability of the gall specific promoter pAt2g18140 in nematode control (Figure 5).

4.3 Comparative analyses of morphometry of adult nematode females feeding on infected transgenic Arabidopsis RNAi lines CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi and control plants

For the assessment of any differences in the growth and development of adult female nematodes, the sizes of the females isolated from the dsRNA-expressing transgenic lines (*CaMV35S*::



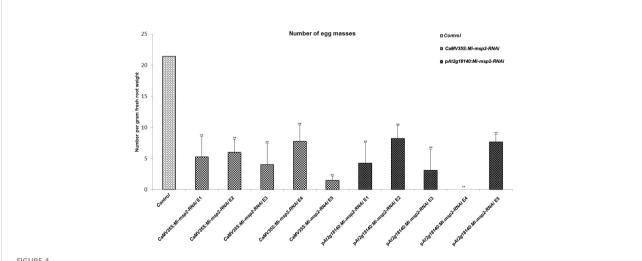
Five independent RNAi lines each for constructs CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi exhibit a reduction in infection represented by a reduction in the number of galls compared to the control line evaluated for M. incognita infection. Each bar represents the mean \pm SE. Double asterisks indicate statistically significant differences by one-way ANOVA and Tukey's test ($p \le 0.01$).



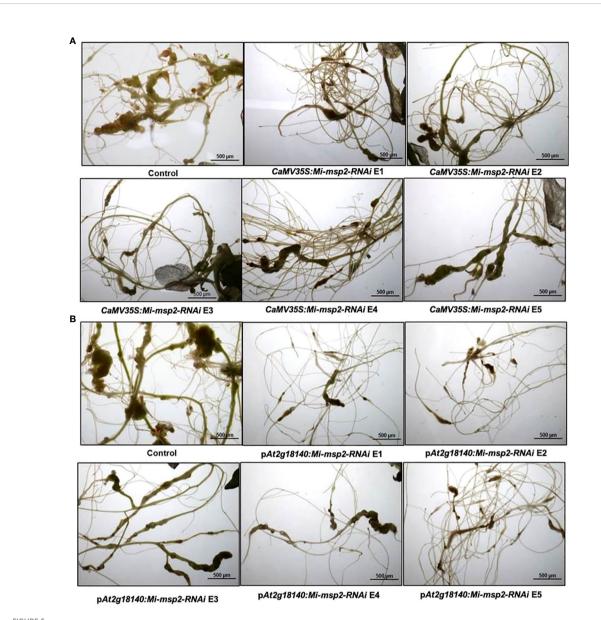
Five independent RNAi lines each for constructs CaMV35S:Mi-msp2 and pAt2g18140::Mi-msp2-RNAi exhibit a reduction in infection represented by a reduction in the number of females isolated from infected roots compared to the control line evaluated for M. Incognita infection. Each bar represents the mean \pm SE. Double asterisks indicate statistically significant differences by one-way ANOVA and Tukey's test ($p \le 0.01$).

Mi-msp2-RNAi and p*At2g18140::Mi-msp2-RNAi*) and control plants were compared. Morphometric analyses were conducted to assess the effect of silencing on the growth patterns by measuring the average surface area (μm²) and the average width (μm) of the feeding females. Twenty females were selected from each of the five RNAi lines of *CaMV35S::Mi-msp2-RNAi* and p*At2g18140::Mi-msp2-RNAi* and also from the control plants. Their lengths and diameters were documented and averaged. A visible reduction in size along with slight deformities in shape were evident in the cases of females feeding on transgenic RNAi lines compared to those feeding on control plants (Figure 6C). The reduction in the average

diameter for females isolated from the *CaMV35S::Mi-msp2-RNAi* and p*At2g18140::Mi-msp2-RNAi* lines was 32% in both cases (Figure 6A). The reduction in the average area for females isolated from the *CaMV35S::Mi-msp2-RNAi* lines was recorded as 55% and for the p*At2g18140::Mi-msp2-RNAi* lines as 56% compared to the females isolated from the control plants (Figure 6B). In previous studies the reduction in female width and area for *Mi-msp2* RNAi lines was reported to be 32.2% and 54.8% respectively (Joshi et al., 2019). These results indicate that the *Mi-msp2* gene driven by both promoters affected the development of feeding *M. incognita* females.



Five independent RNAi lines each for constructs CaMV35S:Mi-msp2 and pAt2g18140::Mi-msp2-RNAi showing a reduction in infection represented by a reduction in the number of egg masses produced by the females in infected roots compared to the control line evaluated for M. incognita infection. Each bar represents the mean \pm SE. Double asterisks indicate statistically significant differences by one-way ANOVA and Tukey's test (p \le 0.01).



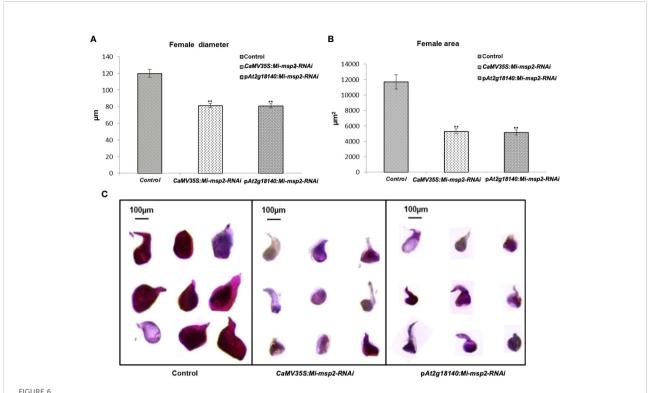
Representative images of *M. incognita*-infected *Arabidopsis thaliana* roots of control and RNAi plants. **(A)** Roots of a control plant compared with *CaMV355::Mi-msp2-RNAi* RNAi lines (*CaMV355::Mi-msp2-RNAi* E1, *CaMV355::Mi-msp2-RNAi* E2, *CaMV355::Mi-msp2-RNAi* E3, *CaMV355::Mi-msp2-RNAi* E4, *CaMV355::Mi-msp2-RNAi* E4, *CaMV355::Mi-msp2-RNAi* E4, *CaMV355::Mi-msp2-RNAi* E3, *CaMV355::Mi-msp2-RNAi* E4, *CaMV355::M*

4.4 Fecundity of *M. incognita* females feeding on *CaMV35S::Mi-msp2-RNAi* line and *pAt2g18140::Mi-msp2-RNAi* line plants and control plants

The reduction in the number of eggs was recorded as 80% for *CaMV35S::Mi-msp2-RNAi* and 77% for p*At2g18140:: Mi-msp2-RNAi* (Figure 7C). Thus, silencing of the gene *Mi-*

msp2 driven by the promoters CaMV35S and pAt2g18140 successfully hampered the fecundity of M. incognita.

Another method to estimate nematode fecundity was the reproduction factor, which was calculated by measuring the number of juveniles hatched from the cumulative egg masses isolated from five independent RNAi lines of both CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi. For the egg masses excised from the five independent RNAi lines of CaMV35S::Mi-



Morphological size comparison of female nematodes isolated from the control and RNAi plant lines CaMV355::Mi-msp2-RNAi and Dat2g18140::Mi-msp2-RNAi (cumulative females from all five lines of each of the RNAi constructs). (A) Average female width (μ m), (B) average female area (μ m²). Each bar represents the mean \pm SE. Double asterisks indicate statistically significant differences according to one-way ANOVA and Tukey's test ($p \le 0.01$). Representative images of adult females isolated from (C) Control, CaMV355::Mi-msp2-RNAi and Dat2g18140::Mi-msp2-RNAi RNAi lines.

msp2, the reduction in the reproduction factor was 0.145–0.749 compared to 10.2 for the control plant juveniles (Figure 7A). The reproduction factor for the five independent RNAi lines of pAt2g18140::Mi-msp2-RNAi was decreased to 0–0.896 compared to 10.2 for the control plant juveniles (Figure 7B). Comparable results were obtained in previous reports for the effector gene Mi-msp2 RNAi lines (Joshi et al., 2019).

4.5 Gene expression analysis for *Mi-msp2* in females feeding on *CaMV35S:: Mi-msp2-RNAi* and *pAt2g18140::Mi-msp2-RNAi* lines

The expression of the *Mi-msp2* gene was observed to be downregulated relative to the nematode *actin* gene in each RNAi line. The reduction in gene expression for the *CaMV35S::Mi-msp2-RNAi* RNAi lines was 82%, 81%, 79%, 78% and 80% for E1, E2, E3, E4 and E5, respectively, compared to the control (Figure 8). The reduction in gene expression for the *pAt2g18140::Mi-msp2-RNAi* lines was 79%, 77%, 76%, 72% and 74% for E1, E2, E3, E4 and E5, respectively, compared to the control (Figure 8). Thus, from the reduced transcript levels, we can conclude that dsRNA for the effector gene *Mi-msp2* was

efficiently expressed by the RNAi constructs under the control of both promoters CaMV35S and p*At2g18140* and that HD-RNAi-based silencing was successful for the targeted gene.

5 Discussion

The NRRS promoters are responsible for triggering gene expression under the external stimulus provided by the pathogen at the time of nematode infection. The expression of the *Arabidopsis* promoter p*At2g18140* is highly restricted to the RKN galls present in the infected plant roots where the sedentary nematodes are at their feeding sites among the giant cells (Kakrana et al., 2017). When subjected to *M. incognita* infection, expression from this promoter was found to be spatiotemporally localized and it was not expressed in any other plant tissue or organ in the transgenic plants. This promoter is specifically expressed in the roots of infected plants as visualized by GUS expression (Kakrana et al., 2017).

The *At2g18140* gene encodes a plant peroxidase protein that has a plausible function in host-plant parasitic nematode interactions (Vercauteren et al., 2001; Jammes et al., 2005; Severino et al., 2012). Previously, plant peroxidases were found

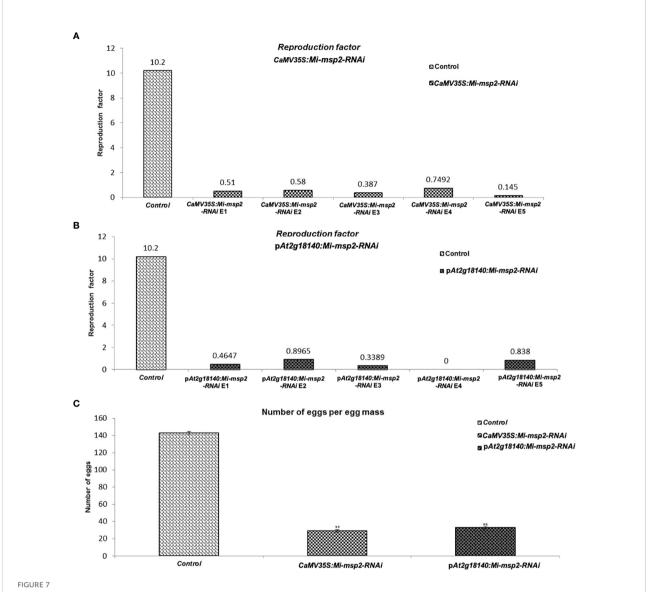
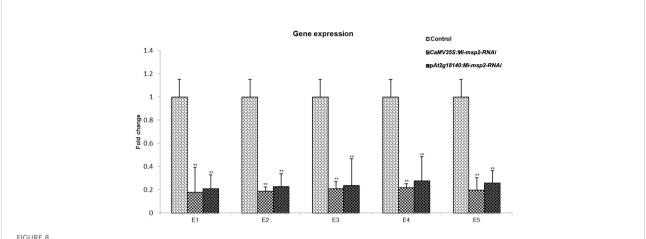


FIGURE 7 Fecundity of *M. incognita.* (A) Reproductive factors for the control and CaMV35S::Mi-msp2. (B) Reproductive factors for the control and pAt2g18140::Mi-msp2-RNAi. (C) The average number of eggs per egg mass isolated from the control and CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi (cumulative egg masses from five lines of each RNAi construct). Each bar represents the mean \pm SE. Double asterisks indicate statistically significant differences according to one-way ANOVA and Tukey's test ($p \le 0.01$).

to assist in host plant parasitization in *Coffea canephora* sp., hence confirming the conserved roles of peroxidases in response to PPNs (Severino et al., 2012). Peroxidases are functional in the hypodermis and enable scavenging of host-derived reactive oxygen species, thereby protecting the external cell membranes from oxidation.

As one of the most widespread plant parasites, PPNs are a constant threat to many crops. Able to infect almost all of the vascular plants, they pose a threat to agriculture worldwide (Sato et al., 2019). The available genomic data and molecular information for PPNs can enable the development of methodologies to curb nematode infestation. RNAi technology

has provided a promising way to curb nematode infection by silencing targeted nematode genes (Fire et al., 1998). Indigenous to *M. incognita*, the *Mi-msp* effector genes, a class of key genes involved in nematode parasitism, have served as targets for effective resistance *via* HD-RNAi. Silencing of various effector genes has directly affected the functionality of nematode parasitism to different degrees, as reported in the cases for effectors *Misp12*, *Mimsp40*, *Mimsp18*, *Mimsp20*, *Mi-msp-1*, *Mimsp2*, *Mi-msp3*, *Mi-msp5* and *Mi-msp24* (Xie et al., 2016; Niu et al., 2016; Shivakumara et al., 2017; Chaudhary et al., 2019; Joshi et al., 2019; Joshi et al., 2020). The fusion constructs for the effector genes *Mi-msp1*, *Mi-msp16*, *Mi-msp20* and *Mi-cpl*, *Mi-icl*,



QRT-PCR-based expression analysis of *M. incognita* females isolated from infected roots of the control plants and five independent lines of each RNAi construct CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi. Each bar represents the mean \pm SE. Double asterisks indicate statistically significant differences according to one-way ANOVA based on F and P values (p \leq 0.01).

Mi-sf have been successfully transformed in crop plants like eggplant and tomato to impart gene silencing *via* HD-RNAi (Hada et al., 2021; Lisei-de-Sá et al., 2021).

The expression of RNAi constructs under the control of their constitutive promoters serves the purpose of gene silencing, but a controlled targeting approach using the NRRS promoter will be more specific while effectively deterring PPN infection (Goddijn et al., 1993; Kakrana et al., 2017). Over the past few decades, various NRRS promoters have been found to be effectively activated in response to PPN infection, such as TobRB7, LEMMI9, Hahsp17.7G4, AtCel-1, AtWRKY23, ZmRCP-1, pAt1g74770 and pAt2g18140 (Opperman et al., 1994; Escobar et al., 1999; Escobar et al., 2003; Sukno et al., 2006; Grunewald et al., 2008; Onyango et al., 2016; Kakrana et al., 2017). These NRRS promoters can be employed to drive RNAi constructs for specific nematode target genes to dissuade PPN infection (Sukno et al., 2006; Kakrana et al., 2017). TobRB7 was used to drive dsRNA against the MjTis11 gene of M. javanica in tobacco, which resulted in a nonsignificant reduction in the number of galls. The probable reason for this could be the weak promoter nature of TobRB7. A significant reduction of 32% was observed in dsRNA expressing RNAi lines from the M. incognita splicing gene under the control of the NRRS promoter pAt1g74770 (Kakrana et al., 2017).

The class *Mi-msp* of nematode effector genes has been observed to impart promising results to curb PPN infection when employed *via* HD-RNAi technology (Niu et al., 2016; Joshi et al., 2019; Joshi et al., 2020). The effector gene *Mi-msp2* originates in the subventral oesophageal glands of *M. incognita* and is maximally expressed in the earlier stages of the lifecycle (Joshi et al., 2019). Recently the HD-RNAi based silencing of the genes involved in chitin biosynthesis has been demonstrated in

tobacco lines. The reduction in M. incognita infection has been reported in terms of number of eggs and root knots of up to 90% (Mani et al., 2020). This suggests its involvement in penetration and migration in host plants. The Mi-msp2 gene has shown a promising reduction in M. incognita infection in Arabidopsis transgenic RNAi lines. The reduction in infection in terms of the number of galls for the constitutive promoter RNAi line CaMV35S::Mi-msp2-RNAi was similar to that of the gall specific promoter RNAi line pAt2g18140::Mi-msp2-RNAi. In a similar manner, all of the lines of both constructs showed a reduction in the number of females, which was comparable for the RNAi lines driven by both promoters. The development of J2s feeding on the CaMV35S::Mi-msp2-RNAi and pAt2g18140:: Mi-msp2-RNAi lines was hampered, as demonstrated by the reduction in the diameter and area in the extracted fully developed adult females from all of the RNAi lines compared to the control plants. There was a reduction in both reproduction factor and the number of eggs per egg mass in CaMV35S::Mi-msp2-RNAi and pAt2g18140::Mi-msp2-RNAi RNAi lines with a collective reduction in fecundity of the next generation of M. incognita. The females feeding on the RNAi lines also presented reduced production of Mi-msp2 transcripts compared to females feeding on control plants.

6 Conclusion

Expressing defence-related pathogen genes under the control of constitutive promoters has been observed to cause the development of disease symptoms even in the absence of respective pathogens, hampering the growth of transgenic plants, resulting in sterile transgenic plants, and causing inefficient

expression of the genes of interest and their expression in unrelated tissues (FitzGerald et al., 2004; Venter, 2007). Naturally occurring constitutive promoters might underperform and have downregulated expression at the desired site, as seen for the promoter Act2 (of A. thaliana) in seed coats and the actin promoter (of rice) in xylem tissue (McElroy et al., 1990; An et al., 1996; Biłas et al., 2016). To overcome these problems while providing nematode resistance, NRRS promoters can serve as a tool for quantitatively, temporally and spatially controlling the expression of a dsRNA construct transferred via HD-RNAi. Here, we constructed a promoter and an effector gene RNAi system that allows for the activation of effective silencing of the gene of interest only after the host is attacked by the nematode. The peroxidase promoter pAt2g18140 can be applied for producing localized dsRNA constructs of various PPN parasitism genes at the time of infection in several host plants. Our results show that in a controlled manner, suppression by RNAi could be achieved by selecting a suitable selective promoter instead of a constitutive promoter. The tissue-specific HD-RNAi suppression technique has also proven to be a useful tool for the production of transgenic crop plant materials, providing insights into fundamental studies of biotic stress/disease resistance. Temporal and spatial gene control as an alternative approach via inducible promoters is desirable as a new system for cases with external stimulus-specific responses. Applying specifically inducible silencing systems can overcome lethality problems and aid in revealing information about gene function in specific knockout studies. We have developed a nematode-inducible system offering rigorous, noninvasive, stimuli-controlled gene expression in plants, a technology that can be further expanded to various crop systems. This system can be applied to generate a series of effective silencing constructs via a gene pyramiding technique, clubbing together different RNAi constructs expressed by the gall specific promoter pAt2g18140.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

PJ conceived the idea. PJ, AC, RB, and AS designed the experiment. IJ, AK, and DK conducted the experiments. IJ performed the data analyses. IJ and DK prepared the Figures

and/or tables. IJ wrote the manuscript. PJ, AC, RB, and AS edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1007322/full#supplementary-material

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Field test of Easter lilies transformed with a rice cystatin gene for root lesion nematode resistance

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Easter lilies, Lilium longiflorum cv. Nellie White are a staple of the floral industry. In the U.S. most of the Easter lilies are grown in Oregon and California along the coast where there is a micro climate that is favorable to growth of lilies. The main pest when growing lilies in the field is Pratylenchus penetrans, the root lesion nematode. Easter lilies are one of the most expensive crops to produce because of the cost of chemicals used to control P. penetrans and other pathogens that infect the lilies. Our previous study had shown that transgenic Easter lilies containing a rice cystatin gene (Oc-IAD86 that has a deleted Asp86) were resistant to P. penetrans in vitro. This study examined growth characteristics of five independently transformed lines of the cystatin Easter lilies compared to nontransformed Nellie White for three seasons in the field in Brookings, Oregon. Liles grown in three soil chemical treatments 1) preplant fumigation, 2) preplant fumigation plus at plant organophosphate, and 3) at plant organophosphate were compared to those grown in nontreated soil. Growth characteristics evaluated included: time of shoot emergence, survival of plants, size of plants, visual ratings of plant health, basal roots and stem roots, weight of foliage and roots, and number and size of bulblets that developed on stems. Nematodes were counted following their extraction from the roots. While not totally resistant, when planted in the field, transformed lines demonstrated and maintained a degree of resistance to lesion nematode over two growing seasons and displayed desirable growth and quality characteristics similar to non-transformed lilies.

KEYWORDS

Pratylenchus penetrans, cysteine protease, Lilium longiflorum, nematode management, pesticide use

1 Introduction

Pratylenchus penetrans, the root lesion nematode, is a major pest that ranks third for the economic damage that it causes to crops worldwide (Davis and MacGuidwin, 2000). It is a particular problem in the Pacific Northwest where it infects Easter lilies (Westerdahl et al., 2003; Westerdahl et al., 2020). The wholesale value of Easter lilies is \$24 million, and their cultivation impacts 890 hectares of land, 320 U.S. greenhouse growers and countless retailers (USDA Floriculture Crops 2015 Summary). The main pest threatening lily production is P. penetrans which can reduce size and quality of the plant by feeding on its roots. There are no known cultivated lily species resistant to P. penetrans. Currently soil fumigation and other pesticide applications directed against this nematode pest costs growers \$3,840/0.41 hectare.

Effective nematode management requires a combination of clean planting stock and clean soil, as well as an understanding of the biology of the pests involved. Lily growers are very interested in alternatives to pesticides because of their effect on human health, production costs, and their anticipated removal from the market. In the Easter lily cropping system, severe pest pressure resulting from both nematode infested soil and infected planting stock results in growers using a dual nematicide application consisting of a dual preplant fumigant treatment followed by an organophosphate at planting (Westerdahl et al., 2003; Westerdahl et al., 2020).

Cystatins are proteinase inhibitors that interfere with digestion of protein in various nematode species resulting in nematodes that have delayed development. The rice cystatin (oryzacystatin), Oc-IΔD86, has a deleted Asp86. Hairy roots of tomato transformed with this gene were found to have resistance to Globodera pallida, the potato cyst nematode as the female nematodes were smaller and had decreased fecundity (Urwin et al., 1995). This variant cystatin Oc-IDD86 was compared to the intact rice cystatin lacking the amino acid deletion, and tomato roots with Oc-IDD86 were more effective for G. pallida resistance (Urwin et al., 1995). Effective resistance to *G. pallida* in potato plants transformed with Oc-IΔD86 was demonstrated in a field study (Urwin et al., 2001). Rice plants transformed with Oc-IDD86 showed a 55% reduction in egg production by Meloidogyne incognita, root-knot nematodes after growing rice plants 42 days in nematode-infested soil in pots (Vain et al., 1998). Transgenic Arabidopsis plants containing Oc-IΔD86 were resistant to the migratory nematode Rotylenchulus reniformis (the reniform nematode) and to two sedentary nematode species, Heterodera schachtii (beet-cyst nematode) and M. incognita (rootknot nematode) in a greenhouse study (Urwin et al., 1997; Urwin et al., 2000). Resistance against Radopholus similis was achieved in transgenic Cavendish banana plants expressing Oc-IΔD86 when grown in the greenhouse (Atkinson et al., 2004).

Cystatins other than Oc-IAD86 have been shown to affect migratory nematodes. Alfalfa plants containing either the rice oryzacystatin I or II genes showed resistance to *P. penetrans* when plants were growing in sterile conditions in a growth chamber (Samac and Smigocki, 2003). Sweetpotato plants transformed with the oryzacystatin-I gene showed resistance in the field to *Ditylenchus destructor* (stem nematodes) (Gao et al.,

2011). Plantains (*Musa* spp.) were transformed with either maize cystatin which is a cysteine proteinase and/or a synthetic peptide that interferes with chemoreception of the nematode (Roderick et al., 2012). The highest level of resistance against *R. similis* was in plantains with the maize cystatin (84% resistance), followed by dual defense genes (70% resistance), and then the synthetic peptide (66%) in screen house trials. Select lines of the transgenic plantains were grown in the field in Uganda where the main nematode pests are *R. similis* and *Helicotylenchus multicinctus* (Tripathi et al., 2015). The highest resistance was found in transgenic plantains with the synthetic peptide or dual defense genes, and several transgenic plant lines have been selected for further evaluation of plant vigor, yield, root necrosis and death, and toppling of the plants.

Field grown Easter lily bulbs are sold to greenhouse operations nationwide for forcing to produce flowering plants at Easter. Bulbs are grown for two to four years before they are large enough for sale. Typically, land is prepared in May, fumigated in July, bulblets are planted from August through October, and bulbs are harvested the following August through October (Roberts et al., 1985). Planting stock can be from immature bulbs, individual scales from a bulb, or bulblets that develop on the underground portion of the stem intermingled with stem roots. Roots grow both on the below ground portion of the stem and from the base of the bulb. Bulbs not reaching marketable size are replanted for an additional year.

Over a period of more than 40 years of trials, rating and measurement systems have evolved for the different parts of an Easter lily plant (Figure 1A). From top to bottom, the parts of the plant utilized in ratings and measurements are the foliage, belowground stems that emerge from the bulb or bulblet that was planted, stem roots that grow on the belowground stems, bulblets that develop amongst the stem roots, the bulb or bulblet that was planted, and basal roots growing from the base of the bulb or bulblet.

In three field trials, growth characteristics of *Lilium longiflorum* cv Nellie White plants that had been propagated *in vitro* (nontransformed, NTr), and five lines (CYS 11, CYS 25, CYS 55, CYS 59, CYS 71) of Easter lilies transformed with the Oc-IΔD86 gene were planted and evaluated in three soil chemical treatments 1) preplant fumigation, 2) preplant fumigation plus at plant or post plant organophosphate, and 3) organophosphate alone; and compared to those grown in nontreated soil to see if they could provide an improvement over or replace the standard soil treatments.

2 Materials and methods

Trials were conducted at the Easter Lily Research Foundation (ELRF) Station in Brookings, OR, in a field managed to provide a uniform population of *P. penetrans* by rotating lilies with clover. Tractor drawn implements were used for land preparation and bed formation and was done with great precision to ensure the integrity of the individual plots. Because several years of pasture rotation are practiced between crops, the initial soil population of *P. penetrans* at planting of trials is typically at a level that is not detectable by

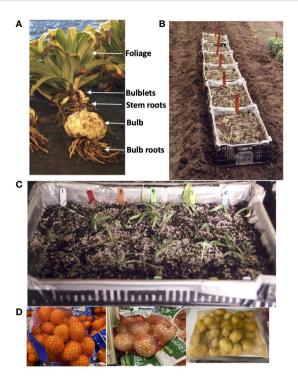


FIGURE 1

(A) View of a mature Easter lily plant indicating location of the parts analyzed in the trials: foliage (visually rated), bulblets (counted and weighed), stem roots (visually rated), bulb (weighed), and bulb roots (visually rated). (B) View of perforated plastic crates used in Trial 1. To be certain that all plantlets could be recovered, we modified a standard technique used in the greenhouse of planting in perforated plastic crates and then planted the crates in the field. The crates were placed in flat bottomed furrows in the field either in nontreated soil or in soil that had been previously fumigated. Soil from the same treatment was later filled in around the crates. Photo is of the six crates placed in untreated soil. Half of the crates were later treated with an organophosphate. (C) View of a single plastic crate with three plants of each lily type planted in a row identified by colored stakes: NTrNW (white), CYS 11 (red), CYS 25 (orange), CYS 55 (green), CYS 59 (yellow) and CYS 71 (blue). (D) Mesh bags of the type used to hold plants and bulbs for individual replicates prior to dipping in a preplant fungicide bath and then planting in the field.

standard soil extraction techniques. However, the levels present combined with low levels of nematodes in planting stock are sufficient to cause significant damage at harvest (Westerdahl et al., 2003).

2.1 Trial 1: May 2014 to September 2014

Lilium longiflorum 'Nellie White' (NW) were transformed with the rice cystatin gene Oc-IΔD86 under control of the CaMV 35S promoter as previously described (Vieira et al., 2015). The lilies were developed from NW field planting stock obtained from Dahlstorm and Watt Bulb Farms in Smith River, CA. Transgenic lilies growing *in vitro* were shipped to the Easter Lily Research Foundation in Brookings, Oregon (Curry County) under an APHIS interstate transport permit 436422. The field release permit was 14-056-103n.

We expected to receive plantlets in the fall of 2013 to plant for the 2013 to 2014 growing cycle. but the plants were not available until May of 2014. Because the ability of the laboratory raised plantlets to survive under field conditions had never been tested, the decision was made to proceed with Trial 1 to determine if the plantlets could survive when removed from tissue culture and planted in the field. To be certain that all plantlets could be recovered, we modified a standard technique used in the greenhouse of planting in perforated plastic crates (Figure 1C) and then planted the crates in the field (Figure 1B). Once planted, the crates were placed in flat bottomed furrows in the field and surrounded with soil. The plants not planted in the field were grown in plastic boxes in the greenhouse until fall when they were recovered and planted in the field for Trial 2.

Plantlets were received on 7 May and were removed from culture tubes and washed free of agar on 8 to 9 May. The largest 36 plantlets of each of the five independently transformed plant lines (CYS 11, CYS 25, CYS 55, CYS 59, and CYS 71) and nontransformed (NTr) plantlets were planted into 12 perforated plastic crates (55.9 X 35.6 X 15.2 cm) filled to 10.2 cm deep with soil taken from either a nontreated (NT) area, or with soil from a preplant fumigated area (PP) (1,3-dichlororpropene [Telone II, Dow AgroSciences, Indianapolis, IN] at 374.2 l ha-1 plus metamsodium [Amvac, Los Angeles, CA] at 702 l ha-) that had been treated on 20 July 2013. Each crate contained 18 plantlets in 6 rows (Figure 1C). Each row consisted of 3 plantlets from one plant line marked with colored stakes, and the rows within each crate were randomized. Plantlets were planted in the crates on 9 May. From 9 to 22 May, the crates were kept indoors under 24-hour lights. On 23 May, the crates were placed in a furrow in the field (Figure 1B) and later surrounded with soil. Crates were placed in the same field soil

treatment as the soil in the crate. An additional crate was placed upside down to create a protective cover. On 27 May, half of the crates were treated with an organophosphate (fosthiazate 10G [Nemathorin, Syngenta International AG, Basel, Switzerland] at 4.5 kgha-1). Thus, 16 plantlets of each line were exposed to four different soil treatments in a randomized complete block design. On 25 July 2014, the foliage quality was visually rated. All visual ratings in all trials were conducted subjectively on a scale from 1-10 with 10 being the best by comparison with other plants in the same trial by an observer with more than 30 years of experience at conducting visual ratings of Easter lilies.

Prior to harvest, on 13 September 2014, the plants were again rated visually using the same scale. The number of surviving plants was determined. Plant weight and root weight were determined, and lesion nematodes were counted after removal from roots using a modified Baermann funnel technique (Ayoub, 1977). A plastic mesh screen was suspended in a cup of water, roots were placed on the screen, submerged in water, and covered with a plastic lid. Nematodes were allowed to emerge for 48 hours at which time, those that emerged into the water were counted using a stereoscopic microscope.

The harvested plants were destroyed. On 9 May, the remaining smaller plantlets to be used in Trial 2 were planted in plastic crates filled with Pro-mix HP potting mix and grown in the greenhouse until 4 November 2014.

2.2 Trial 2: November 2014 to September 2015

This field release was covered under the APHIS permit 15-036-102n. The plants that had been growing in the greenhouse from 9 May to 4 November 2014 were harvested and the number were counted and visually sorted into large, medium and small size categories for each line. The total number available for each line were: 96 non-transformed NW, 60 CYS 71, 36 CYS 11, 108 CYS 55, 84 CYS 59, and 72 CYS 25.

These were divided among 72 plastic mesh bags similar to those used for marketing produce (Figure 1D) from which they would be planted in the field to become 3 replicates in each of 4 soil treatments per line (3 replicates X 4 soil treatments X 6 lines = 72). The four soil treatments were: 1) NT (nontreated), 2) PP (fumigation on July 25, 2014 as in the previous trial), 3) AP (an at planting treatment of phorate [Thimet, Amvac, Los Angeles, CA] at 24.4 kgha-1 plus Ethoprop [Mocap, Amvac, Los Angeles, CA] at 20.9 lha-1, and 4) PP/AP (treatments 2 and 3 combined).

Prior to planting, bags were dipped for one hour at 12°C in a freshly made fungicide solution of 0.72 kg a.i. pentachloronitrobenzene (Terraclor 400, PCNB, 40% pentachloronitrobenzene, Uniroyal Chemical Company, Middlebury, CT), 0.95 kg a.i. tetramethylthiuram disulfide (42-S Thiram, 42% tetramethylthiuram disulfide, Gustafson, Plano, TX), 0.11 kg a.i. Thiophante-methyl (Systec FL 46.2%, Regal Chemical Company, Alpharetta, GA) and 0.81 kg a.i. carboxin (Vitavax-34, Gustafson, Plano, TX) per 379 liters of water and planted 9 November, 2014, within 24 hours of treatment.

Plants were hand planted and harvested. Plots were 1.5 m long with 1.2 m between plots (Figure 2A). The numbers of plants per plot ranged from 3 to 9 depending on the line. The trial area was separated from other lilies by at least one row. Emergence was determined on 10 February, 16 February, and 27 March 2015. Visual ratings were conducted mid-season on 3 June and on 18 September 2015. Plants were hand dug, washed, and graded on 21 September 2015. If fewer than five plants were harvested from a replicate, all plants were evaluated. If five or more plants were harvested, the largest, the smallest, and three randomly selected intermediate plants were evaluated. Data collected at harvest included survival, bulb weight, visual rating of basal roots and stem roots (1 to 10 with 10 being the highest score), stem weight, and number and weight of bulblets that grew on the stems. Basal roots were removed from the bulbs, weighed, and then placed in a modified Baermann apparatus (Ayoub, 1977) for nematode extraction for two days at which time the number of nematodes were counted. Bulbs from this trial were saved for planting the subsequent year (Figures 2B, C).

The entire yield of each replicate harvested (with the exception of CYS 11) was placed into a separate plastic mesh bag from which to be planted in Trial 3. CYS 11 bulbs were not saved due to poor growth and health in Trials 1 and 2.

2.3 Trial 3: October 2015 to September 2016

This field release was done under the APHIS permit 16-033-103n. The individual mesh bags from Trial 2 were fungicide dipped on 14 October 2015 as in the previous trial. The bulbs were planted 15 October in a different location at the ELRF Research Station using the same plot design and soil treatments as the previous year. Plots were 1.5 m long with 1.2 m between plots. PP fumigation had been conducted 16 July 2015 and the AP treatment was applied at planting. A visual rating was conducted on 1 June 2016. The trial was harvested on 21 September 2016 (Figure 2D). Plants were harvested by shovel and hand-washed. If fewer than 5 plants were harvested from a replicate, all plants were evaluated. If 5 or more plants were harvested, the largest, the smallest, and 3 randomly selected intermediate plants were evaluated. Survival was determined, and the circumference of the bulbs was measured using calipers. Circumference was converted to grams using a previously developed regression formula: grams = 7.542417 × centimeters - 62.54368 (Westerdahl et al., 2003). Basal and stem roots were visually rated as before. Basal roots were removed and weighed followed by nematode extraction as before. Bulblets were removed from the stems, counted, and weighed. Following bulblet removal, the stems were rated visually and weighed.

Data was analyzed by Analysis of Variance (ANOVA) ($P \le 0.05$) followed by Fisher's Protected Least Significant Difference Test to facilitate comparison of 1) all treatments with each other and in particular with NT/NTr NW, and 2) treatments within each of the four soil treatment groups (PP, PP/AP, AP, and NT)

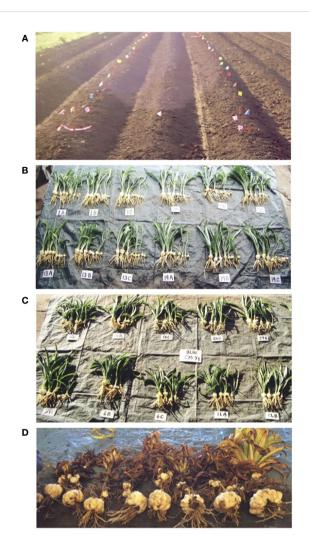


FIGURE 2

(A) View of field plot following planting of Trial 2. The location of each treatment was marked with a colored ribbon. (B) View of three replicates (a–c) of four treatments harvested from the Non-transformed Easter lily treatments in Trial 2 ready to be planted in Trial 3. Treatment 1 - Nontransformed Nellie White (NTrNW) planted in preplant fumigated soil followed by an at plant organophosphate (PP/AP), Treatment 7 - Nontransformed Nellie White (NTrNW) planted in preplant fumigated soil (PP), Treatment 13 - Nontransformed Nellie White (NTrNW) planted in nontreated soil followed by an organophoshate application (AP), Treatment 19 - Nontransformed Nellie White (NTrNW) planted in nontreated soil (NT). (C) View of three replicates (a–c) of four treatments of transformed lily CYS 71 harvested in Trial 2 ready to be planted in Trial 3. Treatment 18 - CYS 71 that had been planted in nontreated soil (NT), Treatment 6 - CYS 71 that had been planted in soil treated preplant with a fumigant followed by an at plant treatment with an organophosphate (PP/AP), Treatment 12 - CYS 71 planted in soil treated preplant with a fumigant followed by an at plant treatment with an organophosphate (PP/AP), Treatment 12 - CYS 71 planted in soil treated preplant with a fumigant (PP). (D) A representative replicate of transgenic lilies harvested from Trial 3 that had been planted with bulbs harvested from Trial 2 that are pictured in (C).

compared to NTr NW in that same group (JMP Pro 16, SAS Institute, Cary NC).

3 Results and discussion

Data has been summarized and statistically analyzed to facilitate comparisons of all treatments with each other, and comparison of treatments within a soil treatment group (Tables 1–6). Results are summarized here for 1) all treatments compared to nontreated (NT) non-transformed (NTr) Nellie White (NW) and 2) comparison within the four soil treatment groups (PP, PP/AP, AP, NT) for transformed lines compared to NTrNW.

The transformed lines were not totally resistant to lesion nematode, but significant nematode reductions were documented with and without chemical soil treatments (Table 1). This was most evident in Trial 1 where 19 of 20 transformed lines grown in various soil treatments had lower levels of lesion nematode than NT NTrNW ($P \leq 0.05$) and in Trial 3 where nematode levels in all transformed lines were lower than corresponding NTrNW for PP/AP, PP, and AP soil treatments ($P \leq 0.05$). Determining degree of nematode control is confounded by root growth (Table 4) and health (Table 5). Roots that have been damaged by nematode or fungal infestation may not be able to support nematode levels as high as can healthy roots. For example, in Trial 3, the relatively small size of root systems in NT NTrNW (Table 4) compared to PP/

TABLE 1 Densities of lesion nematode (*Pratylenchus penetrans*) per gram of roots at harvest in three field trials conducted in naturally infested soil at the Easter Lily Research Foundation Research Station in Brookings, OR, USA.

Soil Treatment	Lily	Tria	al 1	Tria	al 2	Tri	al 3
PP/AP	NTrNW	0.09	b,x	0.04	с,у	4.43	bcd,x
PP/AP	CYS 11	0.00	b,y	0.73	bc,x	not	tested
PP/AP	CYS 25	0.00	b,y	0.19	с,ху	0.74	efg,yz
PP/AP	CYS 55	0.04	b,xy	0.17	c,xy	0.42	g,z
PP/AP	CYS 59	0.00	b,y	0.05	vc,y	2.50	bcdefg,y
PP/AP	CYS 71	0.00	b,y	0.05	с,у	0.75	efg,yz
PP	NTrNW	0.52	a,x	0.02	c,x	5.45	ab,x
PP	CYS 11	0.00	b,x	0.80	bc,x	not	tested
PP	CYS 25	0.00	b,x	0.05	c,x	1.43	defg,y
PP	CYS 55	0.00	b,x	0.29	c,x	2.14	cdefg,y
PP	CYS 59	0.00	b,x	0.07	c,x	1.28	defg,y
PP	CYS 71	0.00	b,x	0.59	c,x	0.68	fg,y
AP	NTrNW	0.14	ab,x	1.33	abc,xy	8.54	ab,x
AP	CYS 11	0.00	b,x	5.13	a,x	not	tested
AP	CYS 25	0.00	b,x	1.03	bc,y	1.25	defg,y
AP	CYS 55	0.22	ab,x	1.93	abc,xy	5.11	bc,xy
AP	CYS 59	0.00	b,x	2.51	abc,xy	1.49	defg,y
AP	CYS 71	0.00	b,x	1.59	abc,xy	3.70	bcdefg,y
NT	NTrNW	0.49	a,x	0.49	c,x	0.45	g,x
NT	CYS 11	0.00	b,y	4.62	abc,x	not tested	
NT	CYS 25	0.00	b,y	0.46	c,x	0.82	efg,x
NT	CYS 55	0.00	b,y	1.74	abc,x	4.03	bcde,x
NT	CYS 59	0.00	b,y	5.16	a,x	0.43	g,x
NT	CYS 71	0.00	b,y	1.82	abc,x	3.78	bcdef,x

Each figure is the mean of three replicates. Means followed by the same letter are not significantly different at $P \le 0.05$ either comparing all treatments in a trial (ab), or within a soil treatment group (xy). Trials 1 and 2 were planted with tissue cultured plantlets. Each replicate in Trial 3 was planted with surviving bulbs from the same treatment replicate in Trial 2. Trials tested the effects of four soil treatments: preplant fumigant (PP), after planting or at plant organophosphate (AP), a combination of preplant fumigation followed by after planting or at plant organophosphate (PP/AP), or nontreated (NT) soil that were planted to six different lines of Easter lily: non-transformed Nellie White variety (NTrNW) and five transformed lines of Nellie White (CYS 11 CYS 25, CYS 59, CYS 71) except that CYS 11 was not tested in Trial 3.

AP NTrNW could be related to lower levels of lesion nematode in NT NTrNW than in PP/AP NTrNW.

When planted in Trial 2, the plantlets were not infested. There was potential for the roots to become infested during the trial and for this infestation to be carried over into Trial 3. This is a normal progression in the culture of Easter lilies. Bulblets planted commercially are potentially infested with low levels of lesion nematode and are planted into nematode infested soil.

Comparing all treatments in Trial 1, lesion nematode was numerically lower than NT NTrNW for all PP/AP; all PP except NTrNW; for all AP except NTrNW and CYS 55 and for NT CYS 71 (Table 1). Comparing all treatments to NT NTrNW, at $P \leq 0.05$, lesion nematode was lower for all PP/AP; all PP except NTrNW; all AP except NTrNW and CYS 55; and for all NT. Within soil treatment groups at $P \leq 0.05$ lesion nematode was lower than NTrNW for all PP/AP except CYS 55; and all NT.

Comparing all treatments in Trial 2, numerically lesion nematode was lower than NT NTrNW for all PP/AP except CYS 11; PP NTrNW, CYS 25, CYS 55, and CYS 59; and NT CYS 25. Comparing all treatments to NT NTrNW, at $P \leq 0.05$, lesion nematode was higher in AP CYS 11; and NT CYS 11 and CYS 59. Within soil treatment groups at $P \leq 0.05$ lesion nematode was greater than NTrNW in PP/AP CYS 11

Comparing all treatments in Trial 3, numerically, only PP/AP CYS 55 and NT CYS 59 were lower than NT NTrNW. Numerically within soil treatment groups, lesion nematode was lower than NTrNW for all PP/AP, PP, and AP treatments. The same is true at $P \leq 0.05$ except for AP CYS 55.

The size and appearance of bulbs is the primary criterion of marketability of bulbs to greenhouses for forcing. Seasonal weather patterns greatly affect quality and size of bulbs even in the absence of nematode pests. For example, NW bulbs produced one year can

TABLE 2 Size of transformed and non-transformed plants (Trial 1) and bulbs at harvest (Trials 2 and 3) in field trials conducted at the Easter Lily Research Foundation Research Station in Brookings, OR, USA in soil naturally infested with lesion nematode (*Pratylenchus penetrans*).

Cail tuantum out	1:1.	Trial 1		Trial 2		Trial 3	
Soil treatment	Lily	Weight	(grams)	Weight (grams)		Weight	(grams)
PP/AP	NTrNW	18.54	abc,x	47.41	ab,xy	105.73	abc,xy
PP/AP	CYS 11	4.03	c,x	26.77	cdefgh,z	not to	ested
PP/AP	CYS 25	21.09	abc,x	33.57	bcdef,yz	82.10	cdefg,z
PP/AP	CYS 55	19.76	abc,x	51.79	a,xy	114.03	a,xy
PP/AP	CYS 59	19.90	abc,x	43.06	abc,xyz	88.36	bcdefg,yz
PP/AP	CYS 71	15.48	abc,x	54.45	a,x	99.34	abcde,xyz
PP	NTrNW	22.42	abc,x	32.96	bcdef,x	94.23	abcdefg,xy
PP	CYS 11	33.05	ab,x	10.93	h,y	not tested	
PP	CYS 25	19.18	abc,x	23.54	efgh,xy	70.28	g,y
PP	CYS 55	15.04	abc,x	32.09	bcdefgh,x	99.02	abcde,x
PP	CYS 59	36.90	a,x	26.44	defgh,xy	96.15	abcdef,xy
PP	CYS 71	12.71	abc,x	30.01	cdefg,x	75.39	efg,xy
AP	NTrNW	14.52	abc,x	39.96	abcde,x	113.07	ab,x
AP	CYS 11	1.84	c,z	10.74	h,z	not tested	
AP	CYS 25	10.18	bc,xyz	29.54	cdefg,xy	96.47	abcdef,x
AP	CYS 55	5.03	c,xyz	28.27	cdefg,xy	102.53	abcd,x
AP	CYS 59	12.18	abc,xy	20.85	fgh,yz	100.87	abcd,x
AP	CYS 71	3.85	c,yz	27.64	cdefg,xy	102.21	abcd,x
NT	NTrNW	10.74	bc,xy	19.71	fgh,y	80.82	defg,x
NT	CYS 11	2.27	с,у	29.62	cdefg,xy	not tested	
NT	CYS 25	12.54	abc,x	15.61	gh,y	73.67	fg,x
NT	CYS 55	7.47	с,ху	26.61	cdefgh,xy	91.68	abcdefg,x
NT	CYS 59	4.66	с,ху	40.94	abcd,x	83.12	cdefg,x
NT	CYS 71	2.01	с,у	23.80	efgh,xy	101.00	abcd,x

Each figure is the mean of three replicates. Data was analyzed by Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference Test. Means followed by the same letter are not significantly different at $P \le 0.05$ either comparing all treatments in a trial (ab), or within a soil treatment group (xy). Trials 1 and 2 were planted with tissue cultured plantlets. Each replicate in Trial 3 was planted with surviving bulbs from the same treatment replicate in Trial 2. Trials tested the effects of four soil treatments: preplant fumigant (PP), after planting or at plant organophosphate (AP), a combination of preplant fumigation followed by after planting or at plant organophosphate (PP/AP), or nontreated (NT) soil that were planted to six different lines of Easter lily: non-transformed Nellie White variety (NTrNW) and five transformed lines of Nellie White (CYS 11 CYS 25, CYS 59, CYS 71) except that CYS 11 was not tested in Trial 3.

be more than double the size of those produced in another year (Roberts et al., 1985).

Trials conducted at the ELRF Station rotate through four different fields. Therefore, in addition to weather variation, there is additional variability in soil characteristics and nematode population levels. Even the standard products utilized by growers have shown year-to-year variability working better in some years than others (L.J. Riddle, pers. comm.). The PP and AP chemical treatments used in these trials have been developed over many years of research and always provide superior growth compared to nontreated soil. However, there are years when the PP or AP treatments alone provide better growth than the combined PP/AP treatment. Comparing the size of NW bulbs, in all three trials, the PP/AP, PP, and AP soil treatments all resulted in better growth than

NT. PP provided the best growth in Trial 1, PP/AP in Trial 2, and AP in Trial 3 (Table 2).

Easter lily bulbs are typically calipered, boxed and sold based on circumference with larger bulbs being sold at a higher price. Bulbs were weighed in Trials 1 and 2 because they were too small to caliper for circumference measurements. For comparison with Trials 1 and 2, the circumference data for Trial 3 was converted to grams using a previously developed regression formula: grams = $7.542417 \times \text{centimeters} - 62.54368$ (Westerdahl et al., 2003).

Even though Trial 1 was in the ground for less than half a normal growing season, many of the transformed lines were numerically larger at harvest than NT NTrNW and were equivalent to NTrNW within soil treatment groups ($P \le 0.05$) (Table 2). In trial 2, with the exception of CYS 11, most transformed lines were numerically larger

TABLE 3 Size of largest and smallest bulbs at harvest in field trials conducted at the Easter Lily Research Foundation Research Station in Brookings, OR, USA in soil naturally infested with lesion nematode (*Pratylenchus penetrans*).

C 11			Largest B		Smalles	t Bulb				
Soil treatment	Lily	Trial 2 (g	rams)	Trial 3 (g	Trial 3 (grams)		ırams)	Trial 3	Trial 3 (grams)	
PP/AP	NTrNW	99.11	bcde,xy	143.40	bcde,y	12.10	bcde,xy	39.63	abcd,xy	
PP/AP	CYS 11	35.81	hijk,z	not tes	ted	17.73	abcd,x	not t	ested	
PP/AP	CYS 25	94.04	bcde,yz	130.63	cde,y	2.78	e,y	31.65	abcd,xy	
PP/AP	CYS 55	156.00	a,x	168.95	ab,x	7.40	cde,y	49.21	abc,x	
PP/AP	CYS 59	105.19	bcde,xy	137.02	bcde,y	10.28	bcde,xy	8.50	d,y	
PP/AP	CYS 71	114.60	abc,xy	159.37	abc,x	19.40	abcd,x	34.84	abcd,xy	
PP	NTrNW	121.86	abc,x	156.17	abcd,x	5.42	de,x	46.02	abc,x	
PP	CYS 11	20.65	jk,z	not tested		3.08	e,x	not t	ested	
PP	CYS 25	67.20	defghi,yz	146.59	bcde,xy	4.57	e,x	26.86	bcd,x	
PP	CYS 55	83.93	bcdef,xy	146.59	bcde,xy	6.36	de,x	36.44	abcd,x	
PP	CYS 59	81.50	bcdefg,xy	143.40	bcde,xy	3.06	e,x	46.02	abc,x	
PP	CYS 71	47.54	fghijk,yz	132.23	cde,y	8.50	cde,x	30.05	abcd,x	
AP	NTrNW	83.20	bcdefg,x	162.56	abc,xy	8.01	cde,x	52.40	abc,x	
AP	CYS 11	13.60	k,y	not tes	ted	7.87	cde,x	not t	ested	
AP	CYS 25	94.92	bcde,x	183.31	a,x	3.73	e,x	23.67	cd,x	
AP	CYS 55	63.31	defghij,x	140.21	bcde,y	8.04	cde,x	49.21	abc,x	
AP	CYS 59	63.56	defghij,x	141.81	bcde,y	1.86	e,x	58.79	ab,x	
AP	CYS 71	55.04	efghijk,xy	154.58	abcd,xy	13.08	bcde,x	52.40	abc,x	
NT	NTrNW	44.32	fghijk,xy	119.45	ef,x	4.09	e,y	52.40	abc,x	
NT	CYS 11	32.96	ijk,y	not tes	ted	26.27	a,x	not t	ested	
NT	CYS 25	38.44	ghijk,y	97.10	f,x	2.68	e,y	50.81	abc,x	
NT	CYS 55	80.46	bcdefgh,x	137.02	bcde,x	5.71	de,y	33.25	abcd,x	
NT	CYS 59	72.20	cdefghi,xy	141.81	bcde,x	20.87	ab,xy	25.26	cd,x	
NT	CYS 71	44.55	fghijk,xy	125.84	def,x	8.16	cde,xy	60.39	a,x	

Each figure is the mean of three replicates. Data was analyzed by Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference Test. Means followed by the same letter are not significantly different at $P \le 0.05$ either comparing all treatments in a trial (ab), or within a soil treatment group (xy). Trial 2 was planted with tissue cultured plantlets. Each replicate in Trial 3 was planted with surviving bulbs from the same treatment replicate in Trial 2. Trials tested the effects of four soil treatments: preplant fumigant (PP), after planting or at plant organophosphate (AP), a combination of preplant fumigation followed by after planting or at plant organophosphate (PP/AP), or nontreated (NT) soil that were planted to six different lines of Easter lily: non-transformed Nellie White variety (NTrNW) and five transformed lines of Nellie White (CYS 11 CYS 25, CYS 55, CYS 59, CYS 71) except that CYS 11 was not tested in Trial 3.

than NT NTrNW and those in the PP/AP soil treatment groups CYS 55. CYS 59, and CYS 71 were significantly larger ($P \le 0.05$). Because of poor vigor, CYS 11 was eliminated from consideration after Trial 2. In Trial 3, growth of the transformed lines was essentially equivalent to that of NTrNW demonstrating that transformed lilies retain their vigor for at least two years. Looking at the largest bulb that developed in Trial 2, with the exception of CYS 11, compared to NT NTrNW, the largest bulbs developed in the PP/AP soil treatment group ($P \le 0.05$) (Table 3). In Trial 3, growth of the transformed lines was for the most part equivalent to that of NTrNW. In Trials 2 and 3, growth of the smallest bulbs harvested was typically equivalent to NTrNW (Table 3).

In Trial 1, numerically, over all treatments plant weight was greater than NT NTrNW for all PP/AP except CYS 11; all PP; AP

NTrNW and CYS 59; and NT CYS 25. Comparing all treatments to NT NTrNW, at $P \le 0.05$, plant weight was greater for PP CYS 59. Within soil treatment groups at $P \le 0.05$ plant weight was equivalent to NTrNW with the exception of AP CYS 11.

In Trial 2, numerically, over all treatments. average bulb weight was greater than NT NTrNW for all PP/AP; all PP except CYS 11; all AP except CYS 11; and all NT except CYS 25. Over all treatments compared to NT NTrNW, at $P \leq 0.05$ average bulb weight was greater for PP/AP NTrNW, CYS 55, CYS 59 and CYS 71; AP NTrNW; and NT CYS 59. Within soil treatment groups at $P \leq 0.05$ average bulb weight was less than NTrNW for PP/AP CYS 11; PP CYS 11; AP CYS 11 and CYS 59; and greater for NT CYS 59.

In Trial 3, numerically, over all treatments, bulb circumference was greater than NT NTrNW for all treatments except PP CYS 25 and CYS $\,$

TABLE 4 Weight of basal roots (grams) per plant at harvest in field trials conducted at the Easter Lily Research Foundation Research Station in Brookings, OR, USA in soil naturally infested with lesion nematode (*Pratylenchus penetrans*).

Soil treatment	Lily	Trial 1		Trial 2		Trial 3	
PP/AP	NTrNW	5.25	ab,x	16.36	bcde,w	10.40	a,x
PP/AP	CYS 11	1.75	bcde,x	6.45	ghi,x	not t	ested
PP/AP	CYS 25	4.65	abcd,x	19.94	abc,w	7.87	abcde,x
PP/AP	CYS 55	4.43	abcde,x	13.08	cdefg,wx	9.33	abc,x
PP/AP	CYS 59	4.79	abc,x	20.09	abc,w	8.13	abcd,x
PP/AP	CYS 71	2.77	abcde,x	15.96	bcde,w	7.73	abcde,x
PP	NTrNW	4.10	abcde,x	6.66	fghi,yz	8.13	abcd,x
PP	CYS 11	1.82	bcde,x	3.91	hi,z	not t	ested
PP	CYS 25	5.33	ab,x	21.54	ab,w	6.67	bcdefg,x
PP	CYS 55	3.47	abcde,x	14.54	bcdef,wxy	6.27	cdefg,x
PP	CYS 59	6.63	a,x	17.16	bcd,wx	7.33	abcdef,x
PP	CYS 71	3.10	abcde,x	8.55	efghi,xyz	4.13	fg,x
AP	NTrNW	5.26	ab,x	4.39	hi,z	9.20	abc,x
AP	CYS 11	0.44	e,z	1.22	i,z	not t	ested
AP	CYS 25	2.88	abcde,xyz	18.04	bcd,w	9.96	ab,x
AP	CYS 55	1.89	bcde,yz	12.42	cdefg,x	7.47	abcdef,x
AP	CYS 59	3.99	abcde,xyz	11.17	defgh,x	8.96	abcd,x
AP	CYS 71	0.75	de,z	2.01	i,z	4.40	efg,y
NT	NTrNW	3.20	abcde,xy	3.59	hi,z	6.67	bcdefg,x
NT	CYS 11	1.18	cde,xy	5.22	ghi,z	not tested	
NT	CYS 25	3.70	abcde,x	16.62	bcd,x	5.49	defg,x
NT	CYS 55	1.96	bcde,xy	12.41	cdefg,xy	5.60	defg,x
NT	CYS 59	2.54	bcde,xy	26.91	a,w	6.62	bcdefg,x
NT	CYS 71	0.46	е,у	2.73	i,y	3.50	g,x

Each figure is the mean of three replicates. Data was analyzed by Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference Test. Means followed by the same letter are not significantly different at $P \le 0.05$ either comparing all treatments in a trial (ab), or within a soil treatment group (xy). Trials 1 and 2 were planted with tissue cultured plantlets. Each replicate in Trial 3 was planted with surviving bulbs from the same treatment replicate in Trial 2. Trials tested the effects of four soil treatments: preplant fumigant (PP), after planting or at plant organophosphate (AP), a combination of preplant fumigation followed by after planting or at plant organophosphate (PP/AP), or nontreated (NT) soil that were planted to six different lines of Easter lily: non-transformed Nellie White variety (NTrNW) and five transformed lines of Nellie White (CYS 11 CYS 25, CYS 55, CYS 59, CYS 71) except that CYS 11 was not tested in Trial 3. Visual ratings in all trials were conducted subjectively on a scale from 1-10 with 10 being the best by comparison with other plants in the same trial by an observer with more than 30 years of experience at conducting visual ratings of Easter lilies.

71. Over all treatments compared to NT NTrNW, at $P \le 0.05$ bulb circumference was greater for PP/AP NTrNW and CYS 55; and AP NTrNW. Within soil treatments groups, there were no differences at $P \le 0.05$ except that PP/AP CYS 25 bulbs were smaller than NTrNW.

In Trial 2, numerically, over all treatments the weight of the largest bulb was greater than NT NTrNW for all PP/AP except CYS 11; all PP except CYS 11; all AP except CYS 11; and NT CYS 11, and CYS 25 (Table 3). Over all treatments compared to NT NTrNW, at $P \leq 0.05$ the weight of the largest bulb was greater for all PP/AP except CYS 11; PP NTrNW; and AP CYS 25. Within soil treatment groups at $P \leq 0.05$ the weight of the largest bulb was less than NTrNW in that group for PP/AP CYS 11; PP CYS 11, CYS 25 and CYS71; and for AP CYS 11.

In Trial 3, numerically, over all treatments the weight of the largest bulb was larger than NT NTrNW for all treatments except NT CYS 25. Over all treatments compared to NT NTrNW, at $P \le 0.05$ the weight of the largest bulb was larger for PP/AP CYS 55 and CYS 71; PP CYS 71; and for AP NTrNW, CYS 25 and CYS 75. Within soil treatment groups at $P \le 0.05$ the weight of the largest bulb was larger than NTrNW for PP/AP CYS 55 and CYS 71; and for PP CYS 71.

In Trial 2, numerically, over all treatments the weight of the smallest bulb was greater than NT NTrNW for all PP/AP except CYS 25; all PP except CYS 11 and CYS 59; all AP except CYS 25 and CYS 59; and all NT except CYS 25. Over all treatments compared to NT NTrNW, at $P \le 0.05$ the weight of the smallest bulb was greater for PP/AP CYS 11 and CYS 71; and for NT CYS 11 and CYS 59.

TABLE 5 Basal root visual rating at harvest in field trials conducted at the Easter Lily Research Foundation Research Station in Brookings, OR, USA in soil naturally infested with lesion nematode (*Pratylenchus penetrans*).

Soil treatment	Lily	Tri	al 1	Tr	ial 2	Tri	al 3
PP/AP	NTrNW	7.67	a,x	8.16	a,x	9.67	a,x
PP/AP	CYS 11	4.67	a,x	8.17	a,x	not tested	
PP/AP	CYS 25	8.00	a,x	7.46	ab,x	8.67	abc,xy
PP/AP	CYS 55	4.67	a,x	7.96	a,x	7.67	abcd,xy
PP/AP	CYS 59	6.33	a,x	7.59	ab,x	8.33	abc,xy
PP/AP	CYS 71	4.67	a,x	7.09	abc,x	6.67	cd,y
PP	NTrNW	7.00	a,x	6.61	abcd,x	9.67	a,x
PP	CYS 11	5.33	a,x	5.39	bcdefg,x	not	tested
PP	CYS 25	8.33	a,x	5.80	abcdef,x	8.00	abcd,xy
PP	CYS 55	9.00	a,x	6.29	abcde,x	7.67	abcd,yz
PP	CYS 59	7.33	a,x	6.64	abcd,x	8.33	abc,xy
PP	CYS 71	4.67	a,x	6.85	abcd,x	6.00	d,z
AP	NTrNW	7.33	a,x	4.89	cdefgh,x	9.67	a,x
AP	CYS 11	4.33	a,x	2.33	i,y	not t	tested
AP	CYS 25	7.33	a,x	4.93	cdefgh,x	9.00	ab,xy
AP	CYS 55	5.00	a,x	4.12	efghi,xy	8.67	abc,xy
AP	CYS 59	7.67	a,x	2.68	hi,xy	9.00	ab,xy
AP	CYS 71	4.67	a,x	3.73	fghi,xy	7.33	bcd,y
NT	NTrNW	8.00	a,x	4.51	defghi,x	8.33	abc,x
NT	CYS 11	4.67	a,x	3.67	fghi,x	not tested	
NT	CYS 25	6.33	a,x	4.42	defghi,x	8.00	abcd,x
NT	CYS 55	7.33	a,x	4.47	defghi,x	7.67	abcd,x
NT	CYS 59	5.67	a,x	3.69	fghi,x	7.33	bcd,x
NT	CYS 71	4.00	a,x	2.98	ghi,x	7.00	bcd,x

Each figure is the mean of three replicates. Data was analyzed by Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference Test. Means followed by the same letter are not significantly different at $P \le 0.05$ either comparing all treatments in a trial (ab), or within a soil treatment group (xy). Trials 1 and 2 were planted with tissue cultured plantlets. Each replicate in Trial 3 was planted with surviving bulbs from the same treatment replicate in Trial 2. Trials tested the effects of four soil treatments: preplant fumigant (PP), after planting or at plant organophosphate (AP), a combination of preplant fumigation followed by after planting or at plant organophosphate (PP/AP), or nontreated (NT) soil that were planted to six different lines of Easter lily: non-transformed Nellie White variety (NTrNW) and five transformed lines of Nellie White (CYS 11 CYS 25, CYS 59, CYS 71) except that CYS 11 was not tested in Trial 3.

Within soil treatment groups at $P \le 0.05$ the weight of the smallest bulb was greater than NTrNW for NT CYS 11.

In Trial 3, numerically, over all treatments the weight of the smallest bulb was greater than NT NTrNW for AP CYS 59; and NT CYS 71. Over all treatments compared to NT NTrNW, at $P \leq 0.05$ the weight of the smallest bulb was less for PP/AP CYS 59. There were no significant differences within soil treatment groups.

The size and apparent health of a bulbs basal root system is also an important factor in marketability of bulbs. For the most part, root systems of transformed bulbs were of similar size to NTrNW (Table 4). In Trial 2, in several instances CYS 25, CYS 55, and CYS 59 had larger root systems than NTrNW ($P \le 0.05$). Visually, in the three trials, there were few significant differences in basal root ratings with the exception that in Trial 2, all PP/AP ratings were better than NT NTrNW ($P \le 0.05$) (Table 5).

In Trial 1, numerically, over all treatments there was a greater root weight than NT NTrNW for PP/AP NTrNW, CYS 25, CYS 55, and CYS 59; PP NTrNW, CYS 25, CYS 55, and CYS 59; AP NTrNW, and CYS 59; and NT CYS 25. Over all treatments compared to NT NTrNW, there were no significant differences ($P \le 0.05$). Within soil treatment groups root weight was lower for AP CYS 11, CYS 55, and CYS 71 compared to NTrNW.

In Trial 2, numerically, over all treatments, root weight was greater than NT NTrNW for all PP/AP, all PP, all AP except CYS 11, and CYS 71; and for NT all except CYS 71. Over all treatments, root weight was greater than NT NTrNW for all PP/AP except CYS 11; PP CYS 25, CYS 55, and CYS 59; AP CYS 25, and CYS 55; and for NT CYS 25, CYS 55, and CYS 59 ($P \le 0.05$). Within soil treatment groups root weight was greater than NTrNW for PP CYS 25, and CYS 59; AP CYS 25, and CYS 59; and for NT CYS

TABLE 6 Percent survival of Easter lily plants at harvest in field trials conducted at the Easter Lily Research Foundation Research Station in Brookings, OR, USA in soil naturally infested with lesion nematode (*Pratylenchus penetrans*).

Soil Treatment	Lily	Tr	ial 1	Tı	rial 2	Tria	l 3
PP/AP	NTrNW	88.89	ab,xy	91.67	a,x	87.50	abc,x
PP/AP	CYS 11	46.67	b,y	55.56	bc,y	not te	sted
PP/AP	CYS 25	88.89	ab,xy	88.89	a,x	100.00	a,x
PP/AP	CYS 55	88.89	ab,xy	85.19	ab,x	100.00	a,x
PP/AP	CYS 59	100.00	a,x	85.71	a,x	100.00	a,x
PP/AP	CYS 71	66.67	ab,xy	86.67	a,x	100.00	a,x
PP	NTrNW	77.78	ab,x	87.50	a,xy	95.83	a,x
PP	CYS 11	66.67	ab,x	100.00	a,x	not te	sted
PP	CYS 25	100.00	a,x	72.22	abc,y	88.89	abc,x
PP	CYS 55	77.78	ab,x	81.48	ab,xy	88.89	abc,x
PP	CYS 59	77.78	ab,x	85.71	a,xy	100.00	a,x
PP	CYS 71	66.67	ab,x	93.33	a,xy	100.00	a,x
AP	NTrNW	88.89	ab,x	83.33	ab,x	95.83	a,x
AP	CYS 11	55.56	ab,x	55.56	bc,x	not te	sted
AP	CYS 25	77.78	ab,x	83.33	ab,x	83.33	abcd,x
AP	CYS 55	44.44	b,x	74.08	abc,x	81.48	abcd,x
AP	CYS 59	66.67	ab,x	80.95	ab,x	57.14	cd,x
AP	CYS 71	66.67	ab,x	80.00	ab,x	80.00	abcd,x
NT	NTrNW	77.78	ab,x	83.33	ab,x	83.33	abcd,x
NT	CYS 11	44.44	b,x	44.44	C,X	not te	sted
NT	CYS 25	77.78	ab,x	72.22	abc,x	61.11	cd,x
NT	CYS 55	55.56	ab,x	74.08	abc,x	92.59	ab,x
NT	CYS 59	66.67	ab,x	47.62	c,x	66.67	bcd,x
NT	CYS 71	66.67	ab,x	73.33	abc,x	60.00	cd,x

Each figure is the mean of three replicates. Data was analyzed by Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference Test. Means followed by the same letter are not significantly different at $P \le 0.05$ either comparing all treatments in a trial (ab), or within a soil treatment group (xy). Trials 1 and 2 were planted with tissue cultured plantlets. Each replicate in Trial 3 was planted with surviving bulbs from the same treatment replicate in Trial 2. Trials tested the effects of four soil treatments: preplant fumigant (PP), after planting or at plant organophosphate (AP), a combination of preplant fumigation followed by after planting or at plant organophosphate (PP/AP), or nontreated (NT) soil that were planted to six different lines of Easter lily: non-transformed Nellie White variety (NTrNW) and five transformed lines of Nellie White (CYS 11 CYS 25, CYS 59, CYS 71) except that CYS 11 was not tested in Trial 3.

25, CYS 55 and CYS 59 (P \leq 0.05). PP/AP CYS 11 had poorer root growth than NTrNW ($P \leq$ 0.05).

In Trial 3, numerically, over all treatments, root weight was greater than NT NTrNW for all PP/AP; PP NTrNW and CYS 59; and all AP except CYS. 71. Over all treatments, at P \leq 0.05 only PP/AP NTrNW was better than NT NTrNW. Within soil treatment groups at P \leq 0.05 AP CYS 71 had a lower root weight than AP NTrNW.

In Trial 1, numerically, over all treatments, PP CYS 25 and CYS 55 had a higher basal root visual score than NT NTrNW (Table 5). Statistically, there were no differences over all or within soil group treatments ($P \le 0.05$).

In Trial 2, numerically, over all treatments, the basal root visual score was greater than NT NTrNW for all PP/AP; all PP all bulbs; and AP NTrNW, and CYS 25. At $P \leq 0.05$, overall treatments, all PP/AP were visually better than NT NTrNW. Within soil treatment groups, AP CYS 11 was not as good as NTrNW ($P \leq 0.05$).

In Trial 3, the visual rating of basal roots showed that numerically over all treatments, PP/AP NTrNW, and CYS 25; PP NTrNW; AP NTrNW, CYS 25, CYS 55, and CYS 59 looked better than NT NTrNW. Over all treatments, PP CYS 71 had a lower visual basal root rating than NT NTrNW ($P \leq 0.05$). Within soil treatment groups, PP/AP CYS 71, PP CYS 55 and CYS 71; and AP CYS 71 received a lower score than NTrNW in that same group ($P \leq 0.05$).

Survival in Trial 1 ranged from 44.44 to 100 percent with lowest survival occurring in CYS 11 and CYS 55, but no statistically significant differences were delineated ($P \le 0.05$) (Table 6). Survival was again variable in Trial 2, but only significantly lower for CYS 11 and CYS 59 ($P \le 0.05$). There were no significant differences in survival in Trial 3 ($P \le 0.05$). Statistically significant differences due to variability between replicates and economically significant differences due to crop loss are not always equivalent.

Because a variable number of bulbs were planted in Trials 2 and 3, survival was analyzed on a percent basis. In Trial 1, numerically, over all treatments, survival was greater than NT NTrNW for all PP/AP except CYS 11; for PP CYS 25; and for AP NTrNW. There were no significant differences either over all or within soil treatment groups. In Trial 2, numerically, over all treatments, survival was greater than NT NTrNW for all PP/AP except CYS 11; and all PP except CYS 25 and CYS 55. Over all treatments compared to NT NTrNW, at $P \leq 0.05$ survival was lower for NT CYS 11 and CYS 59. Within soil treatment groups at $P \leq 0.05$ survival was lower than NTrNW for PP/AP CYS 11. In Trial 3, numerically, over all treatments, survival was greater than NT NTrNW for all PP/AP; all PP; AP NTrNW, and for NT CYS 55. There were no significant differences either over all or within soil treatment groups.

Time of emergence of shoots above ground was monitored on three dates in Trial 2 (Supplementary Material 1.1). Statistically, there were no differences in emergence either overall or within soil treatment groups ($P \le 0.05$). Weather conditions prevented obtaining emergence data for Trial 3.

Plant growth and health was rated visually in all three trials (Supplementary Material 1.2). When rated mid-season this can provide an early indication of how plants will perform at harvest. Poor visual ratings for CYS 11 in both Trial 1 and Trial 2 (which were only significant in the AP soil group treatments) as well as there being very few surviving bulbs at harvest contributed to its not being included in Trial 3.

The weights of stems and stem root scores were highly variable (Supplementary Material 1.3). Stem weight and stem root visual scores are not available for Trial 1 because plants were not in the ground long enough for these characteristics to develop. Largest stem weights were associated with PP/AP soil treatment in Trial 2 and with AP soil treatment in Trial 3.

The number and size of bulblets that develop on stems belowground is important because they are often utilized in subsequent plantings (Supplementary Material 1.4). Trial 1 conducted May-September 2014 was planted from the tissue cultured plantlets received from USDA. This was too short a period of time to produce bulblets. Plantlets not used for Trial 1 were raised in the greenhouse until used for Trial 2. The plants in Trial 2 were in the field for a full growing season, developed bulbs at the base of the plant that were to plant Trial 3, and produced bulblets on the underground stems. Both number and weight of bulblets generally increased from Trial 2 to Trial 3 correlated with the greater size of bulbs. The largest number and weight of bulblets in Trial 2 were associated with the PP/AP soil treatment.

This study confirmed what others have found when comparing the resistance of plants engineered for disease and pest resistance in the field and *in vitro*. Very often plants that showed disease resistance in the greenhouse or *in vitro* were not resistant in the field. Environmental conditions differ, and the field is a much harsher environment with temperature, water, drought, salt stress, etc. A recent study found that environmental stress was the major cause of transcriptomic and proteomic changes in both GM and non-GM plants (Batista et al., 2017). The microbe environment is also complex in the field. We identified several fungi including *Fusarium oxysporum*, *F. tricinctum*, and *Rhizoctonia* sp. AG-I from necrotic roots of lilies and demonstrated *in vitro* that root lesion infection proceeds more quickly in the presence of the fungal isolates (Lakshman et al., 2017).

There have only been two field studies involving plants engineered for migratory nematode resistance, and both studies found that transgenic plants were more resistant to the infecting migratory nematodes than non-transformed plants (Gao et al., 2011; Tripathi et al., 2015). Rice plants transformed with *oryzacystatin*-1 (OC1) showed resistance to stem nematodes (*D. destructor*) in the field (Gao et al., 2011). Plantains transformed with a synthetic peptide that interferes with chemoreception or dual defense genes (a maize cystatin combined with synthetic peptide) showed resistance in the field trial in Uganda (Tripathi et al., 2015).

Development of the transgenic Easter lilies involved tissue culture techniques, and this may have affected growth characteristics of the transgenic lilies. Bulb scales of lily plants micropropagated in vitro were cultured four months on MS medium containing either 1 mg/L picloram (CYS 55 and CYS 59) or 2 mg/L dicamba (CYS 25) and mannitol as an osmoticum prior to bombardment with the gene gun. CYS 71 was regenerated from bombarded suspension cells of Easter lily that had been on 0.5 mg/L picloram for one year. Following bombardment, bulb scales were cultured on selection medium containing both picloram and phosphinothricin for approximately one year to select for regenerated plantlets that were putatively transformed. Somaclonal variation has been reported to occur when plants are regenerated from callus in vitro (Larkin and Scowcroft, 1981; Lee and Phillips, 1987; Phillips et al., 1994; Park et al., 2009). Also, some in vitro conditions such as growing plants on osmoticum prior to gene gun bombardment have been shown to cause cytogenetic abnormalities in transgenic barley plants (Choi et al., 2001). Fonseca et al. (2014) found proteomic differences between transgenic and non-transgenic plants that were thought to be caused by in vitro culture.

In our field study micropropagated, non-transformed lilies were compared to transformed lilies. It may have been informative to include lilies transformed with a vector only (lacking the cystatin gene) as an additional control so that all lilies with or without the cystatin gene had been through tissue culture. Nonetheless, the lilies with the cystatin gene had shown resistance to *P. penetrans* as compared to micropropagated lilies *in vitro* (Vieira et al., 2015). Results from our field trials demonstrate the importance of conducting field trials to determine effective resistance to nematodes.

While not totally resistant, when planted in the field, transformed lines demonstrated and maintained a degree of resistance to lesion nematode over two growing seasons and displayed desirable growth and quality characteristics similar to non-transformed lilies. They also reacted similarly to non-transformed lilies to standard soil chemical treatments. In spite of the variability that naturally occurs

in field trials conducted over multiple years, our results were fairly consistent. With further development, transformed lilies could help producers meet the overall goal of reducing pesticide use.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1134224/full#supplementary-material

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Assessment of nematicidal and plant growth-promoting effects of *Burkholderia* sp. JB-2 in root-knot nematode-infested soil

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Root-knot nematodes (RKN), Meloidogyne spp., are plant-parasitic nematodes that are responsible for considerable economic losses worldwide, because of the damage they cause to numerous plant species and the inadequate biological agents available to combat them. Therefore, developing novel and eco-friendly nematicides is necessary. In the present study, Burkholderia sp. JB-2, isolated from RKN-infested rhizosphere soil in South Korea, was evaluated to determine its nematicidal and plant growth-promoting effects under in vitro and in vivo conditions. Cell-free filtrates of the JB-2 strain showed high levels of nematicidal activity against second-stage juveniles (J2) of M. incognita, with 87.5% mortality following two days of treatment. In addition, the assessment of the activity against other six plant parasitic nematodes (M. javanica, M. hapla, M. arenaria, Ditylenchus destructor, Aphelenchoides subtenuis, and Heterodera trifolii) showed that the cell-free filtrates have a broad nematicidal spectrum. The three defense-responsive (MiMIF-2, MiDaf16-like1, and MiSkn1-like1) genes were activated, while Mi-cm-3 was downregulated when treated with cell-free filtrates of JB-2 cultures on J2. The greenhouse experiments suggested that the cell-free filtrates of the JB-2 strain efficiently controlled the nematode population in soil and egg mass formations of M. incognita in tomato (Solanum lycopersicum L., cv. Rutgers). An improvement in the host plant growth was observed, in which the shoot length and fresh weights of shoots and roots increased. The treatment with 10% of JB-2 cell-free filtrates significantly upregulated the expression levels of plant defenses (SIPR1, SIPR5, and SIPAL) and growth-promoting (ACO1, Exp18, and SIIAA1) genes compared with the corresponding parameters of the control group. Therefore, JB-2 could be a promising candidate for the sustainable management of RKN.

KEYWORDS

root-knot nematode, nematicidal activity, biological control, *Burkholderia*, plant growth, *Solanum lycopersicum*

1 Introduction

Root-knot nematodes (RKN), Meloidogyne spp., are one of the most economically damaging genera of plant-parasitic nematodes, with an estimated economic loss over 100 billion USD annually (Wang et al., 2021). They are obligate endoparasites, which can infect over 3,000 plant species worldwide by invading root cells (Abad et al., 2003; Anwar and McKenry, 2010; Mitiku, 2018). The RKN J2 migrates through intercellular spaces, becomes sedentary, and enters vascular cylinders. Multinucleated feeding cells are then formed, which transform into enlarged giant cells with special nutrient-feeding structures by the end of the sedentary life cycle. It destroys the structure of host roots by depriving it of nutrients, resulting in stunted growth and production. In turn, the host gets susceptible to biotic and abiotic stresses (Ralmi et al., 2016; Liu et al., 2022). Here, the inhabitation and parasitism of RKN can be difficult to control, as its symptoms can be easily confused with other plant problems hence, identifying and controlling the occurrence of RKN in plants remain an ongoing challenge (Gillet et al., 2017; Subedi et al., 2020).

Generally, managing RKN involves the application of synthetic nematicides, botanical essential oils, biological control, hostdelivered RNAi approach and resistant cultivars among others (Collange et al., 2011; Joshi et al., 2020; Subedi et al., 2020). Specifically, synthetic nematicides such as organophosphates, carbamates and fumigants have been extensively used for their efficiency; however, they are currently being restricted, owing to their negative environmental impacts such as non-target toxicity and agricultural ecosystem disturbance, as well as human health safety concerns (Forghani and Hajihassani, 2020). In the past decades, studies have been conducted to develop novel ecofriendly nematicides and address the negative impacts of the synthetic ones. Recently, biological control agents based on microorganisms have been used as more environment-friendly alternatives and are considered a sustainable nematode control strategy (Abd-Elgawad and Askary, 2018). Fungi, such as Purpureocillium lilacinum (e.g. BIOACT® by Bayer Crop Science), Trichoderma spp. (e.g. Trifesol® by Biocultivos Agricultura Sostenible) and Pochonia chlamydosporia (e.g. KlamiC® by BiotorLabs) has been widely accepted and utilized by farmers and other stakeholders for controlling nematode populations in soil (Moliszewska et al., 2022). Certain bacterial species, including Bacillus spp. (e.g. Aveo®EG by Valent BioSciences) and Pasteuria spp. (e.g. ClarivaTM by Syngenta International AG) are also known to be safe and cost-effective and are well-received to control a wide range of nematode species (Roth et al., 2020; Machado, 2022).

The rhizosphere environment harbors diverse bacteria that affect the soil ecosystem, promote plant growth, improve the plant defense system and exert direct antagonistic effects on plant pathogens. Particularly, certain strains of rhizosphere bacteria such as *Bacillus*, *Pseudomonas*, *Pasteuria*, *Serratia* and *Burkholderia* have been reported to play a crucial role in controlling RKN through their nematicidal and plant growth-promoting properties (Collange et al., 2011; Mhatre et al., 2019; Ahmad et al., 2021). They have been

reported to effectively suppress RKN by directly paralyzing, killing and inhibiting J2 hatching through their toxic metabolite and enzyme production capabilities, in addition to having indirect suppressing abilities through the induction of systemic plant resistance (Siddiqui and Mahmood, 1999; Tian et al., 2007; Lamovšek et al., 2013). However, despite continuous research and notable success in laboratory studies of bacterial nematicides, their mechanisms in field conditions remain poorly understood; hence, extensive research, particularly on novel nematicidal strains with high field adaptability and activity in the rhizosphere, remains necessary and crucial for the development of sustainable RKN control strategies.

In the present study, the nematicidal strain *Burkholderia* sp. JB-2 was isolated from RKN-infested rhizosphere soil in South Korea, in which its ability to control RKN was assessed under laboratory conditions. Furthermore, changes in the relative expression levels of seven *M. incognita* J2 genes were studied. Potted tomato (*Solanum lycopersicum* L. cv. Rutgers; susceptible control) (Kokalis-Burelle et al., 2013) in soil infested with *M. incognita* under greenhouse conditions was also monitored to assess whether the JB-2 can control and promote plant growth.

2 Materials and methods

2.1 Nematodes

Meloidogyne incognita was collected and identified based on the method described by Saeki et al. (2003) from roots of the oriental melon (*Cucumis melo* L. var. *makuwa*) in a commercial greenhouse at Yesan-ri, Seongju-gun, Republic of Korea, which were then grown on tomato (*Solanum lycopersicum* L., cv. Rutgers, Seedway, Hall, NY) 28 ± 2 °C, under greenhouse conditions.

The egg masses were obtained from infected *S. lycopersicum* using a 0.5% NaOCl solution according to Hussey and Barker (1973) and incubated at 28°C for 24 h in distilled water. *Meloidogyne incognita* J2 were obtained using a modified Baermann funnel (Viglierchio and Schmitt, 1983) and used for *in vitro* and *in vivo* experiments. Other pure cultured-nematodes such as *M. javanica, M. hapla, M. arenaria, Ditylenchus destructor, Aphelenchoides subtenuis* and *Heterodera trifolii* were obtained from the Nematode Research Center, Life and Industry Convergence Research Institute, Pusan National University, Miryang-si, Republic of Korea for the activity spectrum analysis.

2.2 Isolation and identification of bacterial strains

Rhizosphere soil samples were collected from a *M. incognita*-infested commercial greenhouse in Seongju-gun, Republic of Korea (35°55'32.2" N, 128°17'13.8" E). Thereafter, one gram of the collected soil samples were diluted with phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.144% Na₂HPO₄, 0.024% KH₂PO₄, pH 7.4). The soil suspension was subsequently spread onto Reasoner's

2A agar medium (MBcell, Seoul, Republic of Korea) and incubated at 30°C for two days. The bacterial colonies were isolated according to color and morphological properties and stored at –70°C in R2A broth with 25% sterilized glycerol for further analysis. A total of 28 bacterial isolates from rhizosphere soil samples were evaluated for nematicidal activity against *M. incognita* J2.

Genomic DNA was extracted using a standard phenol-chloroform extraction method (Wilson, 2001) and a partial 16S rRNA gene was amplified using a polymerase chain reaction (PCR) to identify the molecular characteristics of the bacterial isolates, in which the universal primers 27F (5'-AGAGTTTGATC MTGGCTCA-3') and 1492R (5'-TACGGYTACCTTGTTACG ACTT-3') were used (Weisburg et al., 1991). The sequencing of the purified products was performed at Macrogen Inc. (Seoul, Republic of Korea). The sequence of the 16S rRNA gene was then evaluated against the type strains available in the EzBioCloud database (ChunLab Inc., Seoul, Republic of Korea) to identify closely related species. Molecular phylogeny of 16S rRNA was inferred using the neighbor-joining method in MEGA X software (Kumar et al., 2018).

2.3 *In vitro* nematicidal activities of cell-free filtrates

Bacterial isolates were fermented in a 500 mL baffled Erlenmeyer flask containing 100 mL of Luria-Bertani (LB) broth (BD Difco, Franklin Lakes, NJ, United States) on a shaking incubator (200 rpm) at 28°C for 48 h. Following fermentation (Approximately equivalent to 3.0×10^8 colony-forming unit/mL), supernatants were separated by centrifugation at 10,000 rpm for 15 min at 4°C, and subsequently filtered using a 0.22 µm pore filter (Millipore, Burlington, MA, USA). An aliquot containing 50 fresh hatched-J2 in $90 \,\mu L$ sterilized water was transferred to each well of a 96-well plate (SPL Life Sciences Co. Ltd., Gyeonggi-do, Korea) and treated with 10 µL of cell-free filtrates at a final concentration of 10% (v/v). LB broth was used as the negative control. Abamectin (1 μg/mL, Supelco, Bellefonate, PA, USA) and 2,000-fold diluted Sunchungtan 150 EC (150 µg/mL of fosthiazate, Farm Hannong Co., Seoul, Korea) was used as the positive control. The 96-well plates were incubated at 28°C for 48 h. Following incubation, the J2, in each treatment, were observed using a stereo microscope (Olympus SZ61, Olympus Corporation, Tokyo, Japan). They were considered dead when they exhibited a straight form and immobility following stimulations using a fine needle. The relative mortality rate was calculated based on the Abott (1925) formula: [(mortality rate on the treatment-mortality rate on the negative control)/(1-mortality rate on the negative control)]. All experiments were performed in triplicate wells and repeated three times under similar conditions.

2.4 Total RNA isolation and cDNA synthesis

Following 24 h of treatment, total RNA was extracted from J2 using TRIzol reagent solution (Ambion, Carlsbad, CA, USA), which

was then subjected to purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The complementary DNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and was used as a template.

2.5 Gene expression analysis

The relative expression levels of six M. incognita genes (response to oxidative stress, MiMIF-2, MiDaf16-like1, and MiSkn1-like1; nematode development, Mi-Cpl-1 and Mi-SER-1; nematode parasitism, Mi-cm-3) which were treated with 10% culture filtrates of JB-2 and investigated by real-time quantitative PCR. The expression levels were analyzed using SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany) and genespecific primers (Supplementary Table S1) on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The 18S rRNA and MiActin were used as the reference genes. The relative transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Beaubois et al., 2007). All experiments were performed in triplicate.

2.6 Reactive oxygen species assay

The fluorescent probe, 2,7-dichlorodihydrofluoresceindiacetate (H2DCF-DA, Sigma-Aldrich, St Louis, MO, USA), was used to detect intracellular reactive oxygen species (ROS) levels in M. incognita (Yoon et al., 2018; Maleita et al., 2022). The J2 of M. incognita were collected in sterilized water and 50 nematodes were transferred, per well, into 96-well plates. The 10% cell-free filtrates, 50 mM H₂O₂ (Daejung Chemicals & Metals Co., Ltd, Gyeonggi-do, Republic of Korea; as positive control), LB broth (negative control), 1 μg/mL abamectin and 2,000-fold diluted sunchungtan 150EC (150 µg/mL of fosthiazate) were poured in each well, respectively, and were subsequently incubated for 24 h at 25°C. Thereafter, the nematodes were washed thrice with sterilized water and transferred to the 96-well plate. The H₂DCF-DA was then added to each well at a final concentration of 50 µM. The fluorescence was measured using a fluorescence spectrophotometer (Wallac Victor 3 1420 multilabel counter, Perkin-Elmer, Wellesley, USA) by recording the fluorescence intensity at \(\lambda \text{x} \) 485 nm and \(\lambda \text{em} \) 535 nm, at 60 min intervals for 120 min at 20°C. The data were collected from the 60 min time point. Assays were performed in triplicate wells and repeated three times under similar conditions. The nematodes were immobilized on a glass slide using 10 mM sodium azide. Images were captured using a fluorescence stereo microscope (Olympus SZX16, Tokyo, Japan) equipped with a TUCSEN Dhyana 400 DC digital camera (Olympus).

2.7 Greenhouse experiment

The experiment was performed in a controlled greenhouse located in the Nematode Research Center, Life and Industry Convergence Research Institute, Pusan National University,

Miryang-si, Republic of Korea, under the following conditions: the temperature range was $25 \pm 3^{\circ}$ C and relative humidity was 70%, with a 12 h light/12 h dark cycle. Pots with a diameter and depth of 12 cm and 10 cm, respectively, were filled with 500 g of autoclave-sterilized soil (121°C for 1 hour) obtained from a commercial greenhouse in Seongju-gun, Republic of Korea (35°55'32.2" N, 128°17'13.8" E) and inoculated with *M. incognita* (1 J2/g of soil) in 1 mL of sterilized water. Cell-free filtrates of JB-2 were similarly prepared as described above and were used for *in vivo* assays. Following 24 h of inoculation, the experiment involved four treatments: (1) cell-free filtrates of JB-2 (10%, 1%, and 0.1%), (2) 1.8% abamectin (Sun Moon Green Science Co. Ltd., Seoul, Korea) as a positive control, (3) 2,000-fold diluted sunchungtan 150EC (150 µg/mL of fosthiazate, Farm Hannong Co) as a positive control, and (4) LB broth as a negative control.

The experiments were divided into three groups. The first group was an evaluation of the effects of four treatments on nematodes in 500 g of soil from each replicate pot, one week after treatment. The number of nematodes was determined based on the method described by Coolen (1979) under a stereo microscope (Olympus SZ61). The second and third groups consisted of S. lycopersicum at the two-leaf stage, which were transplanted into pots (one for each pot) one week after treatment. The nematode population density in S. lycopersicum roots was determined by extracting J2 from 1 g of each S. lycopersicum roots at 7 days post-transplant. Meanwhile, the latter involved assessing the 45-day post-transplant plant growth parameters such as the shoot length of the plant and fresh weight of the roots and shoots, expression levels of plant defense genes, and nematode parameters such as the number of egg masses on each tomato roots. The number of egg masses was determined using phloxine B staining (Southey, 1986).

Finally, grounded root samples (0.5 g) were frozen in liquid nitrogen. The expression levels of the plant defense (salicylic acid response, *SIPR1* and *SIPR5*; salicylic acid biosynthesis, *SIPAL*) and growth-promoting (*ACO1*, *Exp18*, and *SIIAA1*) genes, were determined using gene-specific primers. The *Ubi3* and *SIActin* genes were used as the reference genes (Supplementary Table S2). All experiments were performed in triplicate.

2.8 Statistical analyses

One-way ANOVA was performed using SPSS software (version 24, SPSS, Inc., Chicago, IL, United States). The mean values were compared using Scheffé's method and p values <0.05 were considered statistically significant.

3 Results

3.1 Isolation and identification of bacterial strain with nematicidal activity

Among the isolated strains, cell-free filtrates of the JB-2 strain showed high levels of nematicidal activity against J2, with a mortality rate of 87.46% (Figure 1). Meanwhile, the mortality rates of J2 in the positive controls of the 150 μ g/mL fosthiazate and 1 μ g/mL abamectin were 97.56% and 98.43%, respectively. Hence, the JB-2 strain exhibited a high nematicidal activity against J2 of *M. incognita* and was selected for the further studies.

The assessment of the activity spectrum using the bioassay showed broad-spectrum activities of the seven plant parasitic nematodes used *M. incognita*, *M. javanica*, *M. hapla*, *M. arenaria*, *D. destructor*, *A. subtenuis* and *H. trifolii* with a mortality rate of 87.46%, 84.36%, 83.19%, 82.83%, 81.33%, 79.28% and 74.05%, respectively (Table 1).

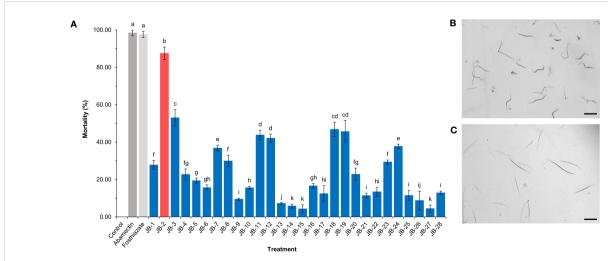


FIGURE 1 Isolation and screening of *Burkholderia* sp. JB-2 with nematicidal activity against the second-stage juveniles of *Meloidogyne incognita*. The mortality rate of the 50 fresh hatched-J2 of *M. incognita* after 48 h treated with 10% cell-free filtrates of isolated bacteria (A). Abamectin (1 μ g/mL) and 2,000-fold diluted Sunchungtan 150EC (150 μ g/mL of fosthiazate) used as the positive controls, a LB broth used as the negative control. Morphological observation of the active nematodes (B) and the dead nematodes with straight form and immobility (C) post-stimulation using a fine needle. The experiment was performed in triplicate under the same conditions. Different letters above the error bars indicate significant differences by Scheffé's test (P < 0.05). Scale bar: 100 μ m.

TABLE 1 Assessments of the mortality rate (%) of cell-free filtrates of Burkholderia sp. JB-2 against the second-stage juveniles of seven plant parasitic nematodes.

Diant paracitic nomatodo	Mortality rate (%)						
Plant parasitic nematode	Control	Fosthiazate (150 μg/mL)	JB-2 (10% cell-free filtrates)				
Meloidogyne incognita	$0.00 \pm 0.00^{\circ}$	97.56 ± 1.85 ^a	87.46 ± 1.09 ^b				
Meloidogyne javanica	0.00 ± 0.00^{c}	96.02 ± 0.73 ^a	84.36 ± 0.55 ^b				
Meloidogyne hapla	0.00 ± 0.00^{c}	97.63 ± 1.00 ^a	83.19 ± 0.74 ^b				
Meloidogyne arenaria	0.00 ± 0.00°	96.19 ± 0.96^{a}	82.83 ± 0.66 ^b				
Ditylenchus destructor	0.00 ± 0.00^{c}	88.41 ± 3.29 ^a	81.33 ± 1.28 ^b				
Aphelenchoides subtenuis	$0.00 \pm 0.00^{\circ}$	90.11 ± 0.32 ^a	79.28 ± 0.67 ^b				
Heterodera trifolii	0.00 ± 0.00°	95.08 ± 0.77 ^a	74.05 ± 4.29 ^b				

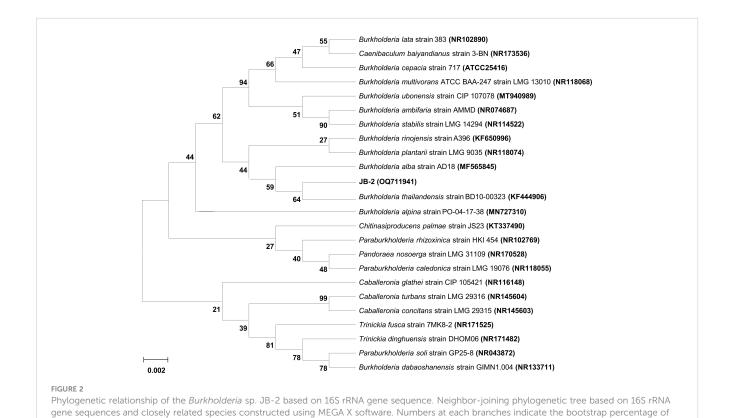
Within a row, values with different letters indicate significant differences by Scheffe's test at P < 0.05. Data are presented as Mean \pm SD (n = 3).

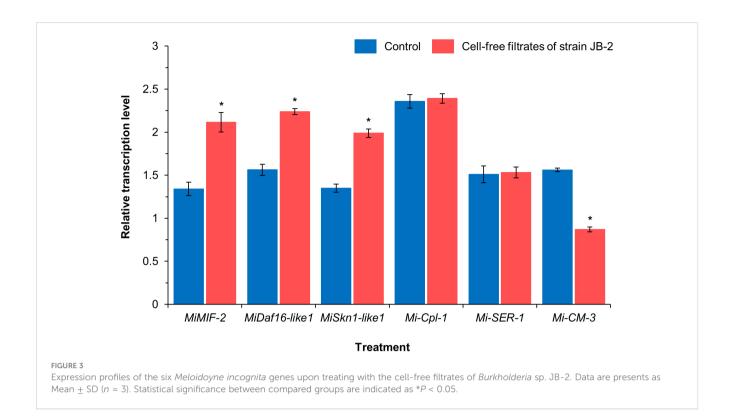
The phylogenetic profiling of the JB-2 strain based on comparison between the nucleotide sequence of its partial 16S rRNA gene with that of the type strains available in the EzBioCloud database showed that JB-2 strain was most closely related to *B. thailandensis* BD10-00323 (GenBank accession number KF444906), in which 99.04% of the 16S rRNA nucleotide sequence showed similarities (Figure 2). The 16S rRNA nucleotide sequence was deposited in GenBank under accession number OQ711941. With this, the JB-2 strain was deposited in the Korean Collection for Type Cultures under code number KCTC14976BP.

1,000 replications

3.2 Changes in the response properties of *M. incognita* upon JB-2 treatment

Based on the relative transcription levels of the six M. incognita genes using real-time quantitative PCR, the expression profiles of the four genes were significantly different between the control and JB-2 treatment groups (Figure 3), in which the expression levels of the former, including MiMIF-2 (P < 0.005), MiDaf16-like1 (P < 0.005), and MiSkn1-like1 (P < 0.005), and defense-responsive genes from oxidative stress were higher by approximately 1.4 folds than that of the control. The relative expression of Mi-cm-3, which is a





negative regulator of the salicylic acid pathway, was also reduced by 1.8-fold in treated nematodes compared with that of the control. Meanwhile, no significant differences were found between the expression levels of Mi-Cpl-1 and Mi-SER-1 between the control and JB-2 treatments (P > 0.05).

The measurement of the intracellular ROS levels in J2 using $\rm H_2DCF\text{-}DA$ (Figure 4) showed that, in the JB 2 treatment, there were numerous giant vacuole formations and high levels of DCF fluorescence, with a value of 11,445 \pm 934 rfu, which was similar to that of the pattern observed in the $\rm H_2O_2\text{-}treated$ group. Meanwhile, the LB broth, abamectin and fosthiazate-treated groups exhibited less vacuole formation and lower levels of DCF fluorescence, with 740 \pm 34, 2176 \pm 210 and 1232 \pm 934 rfu, respectively. These results indicate that the treatment with the JB-2 cell-free filtrate results in an increase in ROS accumulation in *M. incognita*, which may consequently cause oxidative stress and potential apoptosis in nematodes.

3.3 Effect of JB-2 strain on *M. incognita* under greenhouse conditions

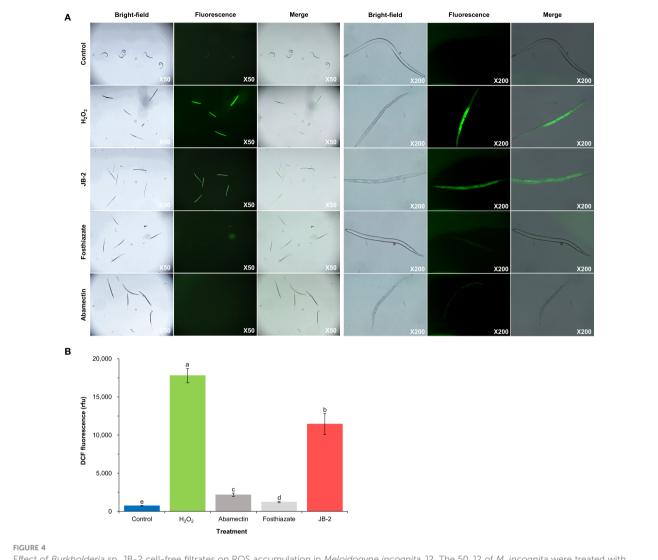
Based on the week-long treatment observation of the population of M. incognita in the tested soil and S. lycopersicum roots (Figure 5), the nematode population in the soil treated with JB-2 cell-free filtrates was found to be significantly reduced (P < 0.005) by up to 75.9% compared to of the control. Meanwhile, no statistically significant differences were found between JB-2 and the positive control (fosthiazate and abamectin) treatment (P > 0.05) (Figure 5A). The number of nematodes per gram of S. lycopersicum root also significantly decreased in a concentration-dependent

condition compared to the control when treated with cell-free filtrates of JB-2 (Figures 5B, C).

Based on the number of egg masses on each *S. lycopersicum* root following 45 days of transplanting (Figure 6), it was found that the number of egg masses decreased in varying degrees in all treatments. Specifically, the number of egg masses (6.8 \pm 3.4 egg masses/g) in the 10% cell-free filtrates of the JB-2 strain was reduced. Meanwhile, the number of egg masses in abamectin (6.5 \pm 1.9 egg masses/g), including the other concentrations was also reduced and similar to that of the positive control. Hence, this suggests that the reduction was dependent on the concentration. The treatment of foasthiazate showed the highest reduction in egg masses with 2.1 \pm 0.4 egg masses/g of root.

3.4 Effects of JB-2 strain on *S. lycopersicum* infested with *M. incognita*

The application of 10% cell-free filtrates of the JB-2 strain was found to significantly affect all plant growth parameters following 45 days of treatment. Specifically, it was found that it had significant effect on the growth of *S. lycopersicum* compared to that of the positive control (abamactin and fosthiazate) (Figure 7), in which shoot length growth reached 48.5 \pm 1.41 cm. Other treatments did not have significant differences (P > 0.05), except for the fosthiazate treatment which had a shoot length growth that reached 43.4 \pm 3.1 cm. In addition, the fresh weight of the shoots also increased by approximately 2.4-fold and 2.6-fold compared to that of the control and abamectin treatments, respectively. However, no statistical differences were found in the fresh weight of the roots among all the groups tested.

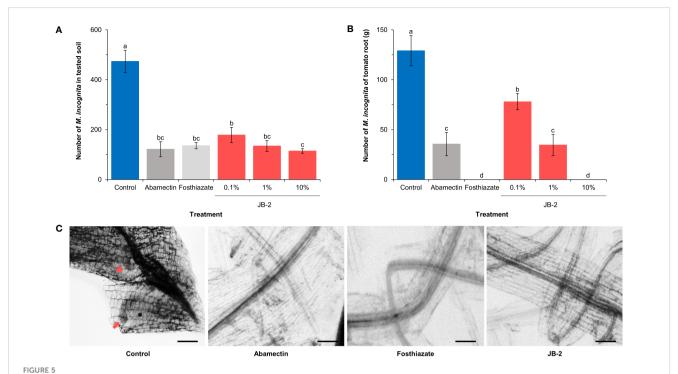


Effect of *Burkholderia* sp. JB-2 cell-free filtrates on ROS accumulation in *Meloidogyne incognita* J2. The 50 J2 of *M. incognita* were treated with 10% cell-free filtrates of JB-2 and were subsequently incubated for 24h at 25°C. 50 mM H_2O_2 was used as the positive control and LB broth was used as the negative control. Quantitative analysis of vacuolization and ROS accumulation in *M. incognita* using a fluorescence probe, $H_2DCF-DA$ (A). Left-hand panels are bright-field images; right-hand panels are fluorescent images of the nematodes captured by the fluorescence stero microscope equipped with a TUCSEN Dhyana 400 DC digital camera. Qualitative analysis of ROS accumulation in *M. incognita* J2 using a fluorescence spectrophotometer by recording the fluorescence intensity at λ ex 485 nm and λ em 535 nm (B). The experiment was performed in triplicate under the same conditions. Different letters above the error bars indicate significant differences by Scheffé's test (*P* < 0.05).

Based on the analysis of the expression levels of salicylic acid response (SIPR1 and SIPR5), salicylic acid biosynthesis (SIPAL) and growth-promoting (ACO1, SIIAA, and Exp18) genes using real-time quantitative PCR (Figure 8), to determine the molecular effect of cell-free filtrates of the JB-2 strain in plants, the expression patterns of defense-related genes (SIPR1, SIPR5, and SIPAL) were significantly upregulated following treatment with 10% JB-2, whereas, no significant differences were found between the control and the positive control treatments (P > 0.05) (Figures 8A-C). Compared to the control, it showed more than 3-fold increases of both SIPR1 and SIPR5 expression and three plant growth-related genes (ACO1, SIIAA, and Exp18) exhibited enhanced expression levels in 10% of the JB-2 treatments by approximately 11.2-fold, 6.0-fold, and 6.4-fold, respectively (Figures 8D-F). Similarly, treatments with positive controls (abamectin and fosthiazate) were significantly upregulated (P < 0.05).

4 Discussion

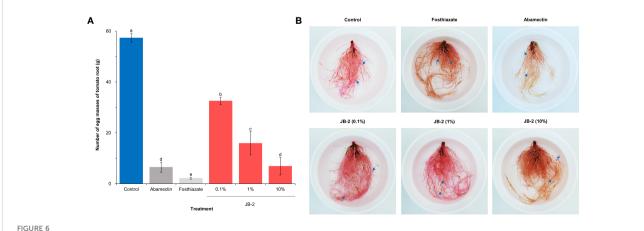
Considering the undesirable effects of synthetic nematicides to the environment and human health, eco-friendly biological nematicides have been extensively developed over the last several decades in which biological control agents, such as numerous microorganisms, have been identified as effective alternatives that can act as nematicidal and promote plant growth (Siddiqui and Mahmood, 1999; Forghani and Hajihassani, 2020). Specifically, rhizospheric bacteria have been considered as promising alternatives, owing to their natural abundance in soils and beneficial effects to the host plant, in which they can stably colonize the rhizosphere, stimulate plant growth and development and offer host plants the essential nutrients through its dynamic interaction with the surrounding soil environment (Gray and Smith, 2005; Groover et al., 2020; Khairy et al., 2021).



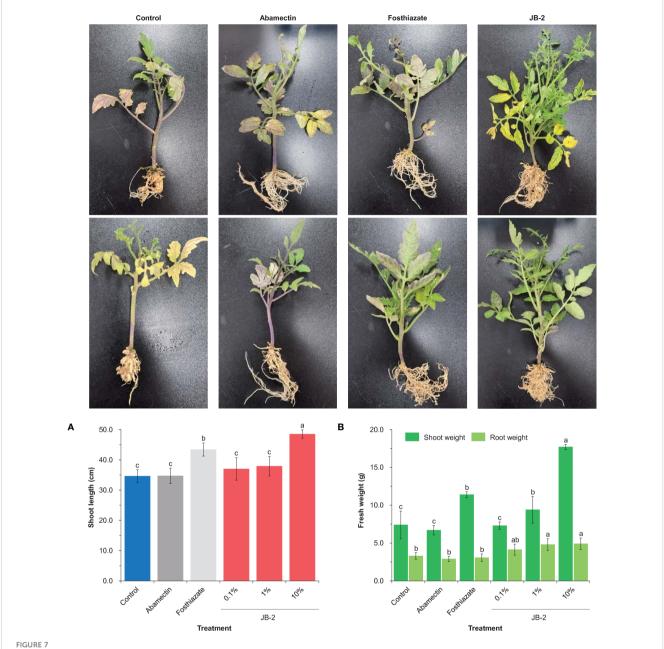
Effect of *Burkholderia* sp. JB-2 cell-free filtrates on *Meloidogyne incognita* in the pot experiment (n=5). **(A)** Number of nematodes in the 500 g of soil tested. **(B)** Number of nematodes per gram of *Solanum lycopersicum* roots. **(C)** Images of *S. lycopersicum* roots at 7-day post-transplant. The arrows indicate live *M. incognita*. The experiment was performed in triplicate under the same conditions. Different letters above the error bars indicate significant differences by Scheffé's test (P < 0.05). Scale bars: 200 μ m.

Recently, *Burkholderia* species, which are gram-negative proteobacteria, widely distributed in various terrestrial and aquatic environments, have gained increasing attention as one of the most beneficial biological nematicides to control RKN, owing to their bioremediation properties, particularly of xenobiotic compounds and plant growth promotion (Coenye and Vandamme, 2003; Mahenthiralingam et al., 2005). In the present study, the 10% cell-free filtrates of *Burkholeria* sp. JB-2 had an 87%

mortality rate against J2 of *M. incognita*, which also included a broad nematicidal spectrum. This indicates that the strain may possess nematicidal metabolites secretion properties; therefore, it can be considered as a suitable candidate for biological control of RKN. The results of the present study were consistent with those of previous studies, in which the nematicidal capabilities were also reported based on the effective control of *M. incognita* in the culture filtrates of *B. arboris* J211 from tobacco rhizosphere soils (Zhang



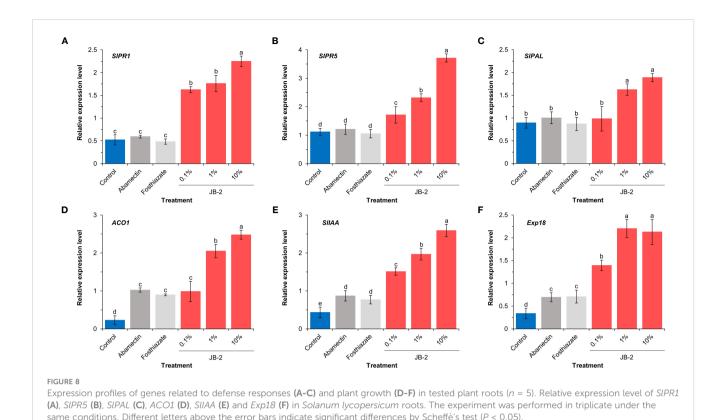
Effects of *Burkholderia* sp. JB-2 cell-free filtrates on the number of egg masses of *Meloidogyne incognita* per plant in the pot experiment (n = 5). (A) Number of egg masses on each *Solanum lycopersicum* root following 45 days of transplanting in the 500 g of the soil tested. (B) Root symptoms of *S. lycopersicum*. The arrows indicate egg masses of *M. incognita*. The experiment was performed in triplicate under the same conditions. Different letters above the error bars indicate significant differences by Scheffé's test (P < 0.05).



Effects of Burkholderia sp. JB-2 cell-free filtrates on the growth of Solanum lycopersicum in the pot experiment (n = 5). Shoot length of S. lycopersicum (A) and fresh weight of S. lycopersicum roots and shoots (B). The experiment was performed in triplicate under the same conditions. Different letters above the error bars indicate significant differences by Scheffé's test (P < 0.05).

et al., 2022). Similarly, it was also found that it stimulated plant growth. Meanwhile, the *B. vietnamiensis* B418, obtained from the barley soil and belonging to the *B. cepacia* complex, also exhibited nematicidal efficacy, with a 71.15% mortality rate against RKN in *Citrullus lanatus* cv. Jingxin (watermelon), in which it was found to simultaneously modulate the rhizosphere microbial community (Liu et al., 2022); additionally, the *B. rinojensis* A396 strain from the soils in Japan had already been commercialized as a broadspectrum bionematicide by Marrone Bio Innovations and has been registered under the product name Majestene® (Cordova-Kreylos et al., 2013; Arthurs and Dara, 2019).

Understanding the nematicidal mechanism in controlling RKN is crucial, as they are also affected by diverse biotic and abiotic factors, in which these mechanisms largely influence their activity and stability in various environmental conditions. In turn, more effective and long-lasting RKN management can be developed to aid in identifying suitable integrated pest management strategies. However, most *Burkholderia* species have limited nematicidal mechanisms, as they are more generally associated with host defenses such as systemic acquired resistance. Additionally, its other mechanisms are yet to be identified. Macrophage migration inhibitory factor (MIF)-like proteins are multifunctional proteins that mainly regulate innate and adaptive



immune responses (Leyton-Jaimes et al., 2018), for which previous research demonstrated that the MIF-like protein MiMIF-2 protected M. incognita against oxidative stress by modulating host immunity (Zhao et al., 2020). Similarly, MiDaf16-like1 and MiSkn1-like1 were also found to have modulating effects in response to oxidative stress, thereby activating insulin/insulin-like signaling pathways (Basso et al., 2020). In the present study, three defense responsive genes (MiMIF-2, MiDaf16-like1, and MiSkn1-like1) from oxidative stress were upregulated upon treatment with cell-free filtrates of JB-2 on M. incognita J2 for 24 h, indicating the activation of the defensive responses to oxidative stress. Here, the oxidative stress caused by the excessive accumulation of ROS is extensively affected by metabolic processes, leading to cell death as damage was induced to cell components (Berlett and Stadtman, 1997). The significant differences found in the fluorescence intensity and vacuole formation between the control and treatment groups indicate that there was excessive generation of ROS in nematodes, as induced by cell-free filtrates of JB-2. These results suggest that JB-2 has a direct nematicidal mechanism by inducing ROS accumulation and internal damage to M. incognita. This result was also consistent with those of Gao et al. (2016), in which the Bacillus cereus strain S2 was found to induce ROS accumulation in the intestinal tract and destroy the genital areas of nematodes by producing sphingosine. Three nematicidal volatiles, including dimethyl disulfide, methyl isovalerate and 2-undevanone from the B. atrophaeus strain GBSC56, also showed strong nematicidal activity, causing excessive ROS production in M. incognita (Ayaz et al., 2021). The present study is the first to demonstrate one of the prospective mechanisms of the direct nematicidal action of Burkholderia sp. against M. incognita. However, the exact nematicidal metabolites produced by the JB-2 strain remain unascertained. Consequently, further studies focusing on the identification of chemical properties, including the characteristics of ROS damages and its mechanisms, are necessary to validate the results. Chorismate mutase, encoded by Mi-cm-3, is an enzyme that plays an important role in the successful parasitism of M. incognita in its early parasitic stages by regulating the plant salicylic acid pathway (Wang et al., 2018). In the present study, Mi-cm-3 was downregulated upon treatment with cell-free filtrates of JB-2 on M. incognita J2. However, the expression levels of Mi-Cpl-1, which encodes the cathepsin L-type cysteine protease, and Mi-SER-1, which encodes the chymotrypsin-like serine protease, did not have significant differences between the control and JB-2 treatment, despite being implicated in nematode parasitism and development (Shindo and Van der Hoorn, 2008; Antonino de Souza Júnior et al., 2013).

The results of the pot experiment indicated that despite the removal of the cells, the cell-free filtrates of JB-2 can still control *M. incognita*, in which plant growth-promoting effects were observed under greenhouse conditions. This also indicates that cells are not required for their activity; instead, it is inferred that secreted metabolites from JB-2 may be responsible for inducing the direct nematicidal effects and changing the soil microbiome. According to Liu et al. (2022), *B. vietnamiensis* B418 significantly suppresses RKN by modulating the rhizosphere microbial community, based on the changes in the composition of the soil bacterial community. Similarly, previous studies have also indicated that the changes in soil microbial communities are key factors that affect RKN colonization

(Cao et al., 2022; Lu et al., 2023). It is recommended that the soil microbiome, including its microbial communities, is further studied using metatranscriptomic, metabolomic and proteomic studies to understand and verify the results of the present study.

Meanwhile, the results from the relative transcription-level analysis of the six S. lycopersicum genes showed that treatment with JB-2 cellfree filtrates activated the defense mechanisms of the plants. Salicyclic acid (SA) and jasmonic acid are inferred to be the major signaling molecules that regulated plant defense responses (Shoresh et al., 2005; Chen et al., 2010), in which these were induced by several endogenous phytohormones, such as SA, SIPR1, SIPR5 and SIPAL which served as marker genes in the pathogen resistance (Seo et al., 2008; Li et al., 2015). In the present study, the relative expression levels of SIPR1, SIPR5 and SIPAL in the S. lycopersicum roots had a significant increase when treated with JB-2 cell-free filtrates, consistent with the results of a previous study, which indicated that certain bacteria promoted the expression of these genes (Ayaz et al., 2021; Tian et al., 2022). ACO1 was also suggested to be responsible for the final step in the ethylene biosynthesis pathway (Houben and Van de Poel, 2019), while Exp18 played a crucial role in the initiation of leaf primordium (Reinhardt et al., 1998); furthermore, SllAA, which is involved in auxin synthesis pathway genes (Nebenführ et al., 2000), was upregulated in the 10% JB-2 treatment.

5 Conclusion

The JB-2 strain from the RKN-infested rhizosphere soil in South Korea displayed high levels of nematicidal activity, in which the operational mechanism involved the induction of the accumulation of excessive ROS and internal damage of the J2 of M. incognita. Under greenhouse conditions, the JB-2 strain also showed suppression of M. incognita population by reducing the number of egg masses. The strain also effectively promoted the growth of S. lycopersicum based on plant height, fresh weight of root and shoot, in addition to upregulating the gene expression related to plant defense and growth. However, further studies are required to determine the major factors that affect nematicidal activities, host plant and soil microbiome interactions to aid in the identification of sustainable management of RKN (e.g. host plant and nematode species, verification of mechanisms at the molecular level, related hormone and metabolites, field conditions and soil microbial communities). The findings of the present study suggest that the JB-2 strain can be considered a potential alternative to nematicides with multi-functional benefits. Therefore, these findings provide further understanding of the multiple interactions that occur among rhizospheric bacteria, RKN and plants.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

J-HK, C-HL and K-HS participated in acquiring the data, the study design, drafted the manuscript, and revised the final manuscript. B-ML carried out the all of the laboratory experiments. M-KK participated in the data analyses. D-JP and H-YP participated in the protocol design and the statistical analyses. I-SC supplied the nematodes and conducted the pot trial. All the authors read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1216031/full#supplementary-material

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Root-knot nematode assessment: species identification, distribution, and new host records in Portugal

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Considered one of the most devastating plant parasitic nematodes worldwide, Meloidogyne spp. (commonly known as the root-knot nematodes (RKNs)) are obligate sedentary endoparasites that establish in the roots, causing hyperplasia and hypertrophy of surrounding cells, triggering the formation of galls. These galls will affect root development and physiology, leading to substantial yield losses. During 2017–2022, an extensive survey of *Meloidogyne* species was undertaken in Portugal (mainland and islands). A total of 1,071 samples were collected by the National Plant Protection Organization (DGAV) and private farmers from different regions of the country and were analysed at the Laboratory of Nematology (NemalNIAV). Samples in which the presence of Meloidogyne sp. was detected were used to perform bioassays to obtain females and juveniles for further studies. Since the accurate identification of RKNs is an important aspect of crop management, morphological and biochemical characterisation was performed. The most common morphological features were observed, showing consistency with previous descriptions of the genus. The biochemical identification using the esterase (EST) phenotype revealed the phenotypes of Meloidogyne arenaria, M enterolobi, M. hispanica, M. hapla, M. incognita, M javanica, and M. luci. Meloidogyne incognita and M. javanica were found to be the most prevalent species in the different regions followed by M. arenaria and M. hapla. This is the first distribution report performed in Portugal on RKNs, contributing to the development of management strategies and to updated information on the status of these pests in Europe.

KEYWORDS

esterase, horticulture, Meloidogyne, management, frequency

1 Introduction

Agriculture is the practice of cultivating natural resources to sustain human life and generate profits. In the EU, agricultural production is a big business, contributing EUR 217 billion towards the EU's overall gross domestic product (GDP) in 2022, and it is expected to grow due to the increase in global trade caused by the growing population. European countries contribute to the total output value of the EU's agricultural industry, being more than half (56.9%) coming from France, Germany, Italy, and Spain. Portugal contributes less than 5% (Eurostat, 2023).

In territorial, social, and economic terms, agriculture in Portugal has great importance for the whole country but particularly to rural areas concerning sustainable development. It is also well positioned in the European and world markets due to its climate, biodiversity, innovation, and ability to present differentiated and safe products. According to data from Instituto Nacional de Estatística's economic accounts, it appears that in 2018, vegetable and horticultural products represented 17% of the national agricultural production, of which fresh vegetables represent 50% of production (GPP, 2020; INE, 2022). According to PORDATA, agriculture and forestry have an essential role in preserving the environment and landscapes in Portugal; together, they cover 75.3% of the land. Moreover, approximately 26.2% of the agricultural area corresponds to arable land, which is divided into perennial crops (21.7%), pastures (51.7%), and family farming (0.4%).

Portugal is divided into seven regions (North, Metropolitan area-Lisbon, Centre, Alentejo, Algarve, Madeira, and Azores), which have different regional specialisations because of the considerable diversity of natural and economic-social conditions (Avillez, 2015; Freire and Lains, 2017). The regions of Alentejo and Azores have the highest significance in the national agriculture production, representing 8.6% and 6.8% of GDP, respectively (Marques, 2015; GPP, 2020).

Plant parasitic nematodes (PPNs) are regarded as one of the most important soil-borne pests, accounting for USD 175 billion per year in yield losses worldwide (Bernard et al, 2017). One of the oldest and most economically important PPNs are the root-knot nematodes (RKNs), *Meloidogyne* spp., which are considered serious pests for agricultural production, causing annual losses of USD 157 billion globally (Abad et al., 2008). This genus comprises more than 100 species (Subbotin et al, 2021); species *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949, and *Meloidogyne javanica* (Trub, 1885) Chitwood, 1949, are known as the most important due to their widespread distribution and broad host range (Jones et al., 2013). In Portugal, so far, only 10 species have been reported (Table 1).

Root-knot nematodes infect at the elongation zone and then move to the root tips to invade the vascular cylinder and form a feeding site, called a giant cell. At the same time, the neighbouring cells start to divide to form the typical gall or root-knot, affecting the development of the root system and causing significant yield losses (Jena and Rao, 1973; Norton and Niblack, 1991; Kyndt et al., 2014).

Most RKN species have high plasticity, enabling their establishment in different geographical areas and colonisation of

TABLE 1 Species of *Meloidogyne* sp. detected in Portugal.

Species	Author(s)
Meloidogyne arenaria (Neal, 1889)	Pais and Abrantes, 1989
Meloidogyne chitwoodi (Golden et al., 1980)	Da Conceição et al., 2009
Meloidogyne enterolobii (Yang and Eisenback, 1983)	Santos et al., 2019
Meloidogyne hapla (Chitwood, 1949)	Pais and Abrantes, 1989; Abrantes et al., 2008
Meloidogyne hispanica (Hirschmann, 1986)	Pais and Abrantes, 1989; Abrantes et al., 2008
Meloidogyne incognita (Kofoid and White, 1919) Chitwood, 1949	Pais and Abrantes, 1989; Abrantes et al., 2008
Meloidogyne javanica (Trub, 1885) Chitwood, 1949	Pais and Abrantes, 1989
Meloidogyne luci Carneiro et al, 2000	Maleita et al., 2018; Rusinque et al., 2021
Meloidogyne lusitanica (Abrantes and de A. Santos, 1991)	Abrantes and de A. Santos, 1991
Meloidogyne naasi (Franklin, 1965)	Viera dos Santos et al., 2020

different hosts. Moreover, projections by the intergovernmental panel for climate change indicate that the elevated temperature and moisture may result in an increasing rate of infection, development, and reproduction, causing shifts in their abundance and geographic distribution (Mbow et al., 2019).

Considering the impact that the RKNs have on agricultural production, species identification is essential to define sustainable management strategies. Morphological RKN identification is a valuable tool with low cost and accuracy depending on the number of characteristics and specimens evaluated. Furthermore, the biochemical electrophoretic analysis of non-specific esterase (EST), along with several molecular methods, such as internal transcribed spacer–polymerase chain reaction–restriction fragment length polymorphism (ITS-PCR-RFLP), sequence characterized amplified region (SCAR) markers, real-time PCR, and loop-mediated isothermal amplification (LAMP), have proved to be useful in the differentiation of economically important species of *Meloidogyne*.

Currently, in Portugal, there is a lack of detailed information on the root-knot nematode geographical distribution and species occurrence. Therefore, this study aimed to assess the presence and incidence of the RKNs in Portugal, thus contributing to knowledge about the wide dissemination of these nematodes and designing and implementing effective management practices.

2 Materials and methods

2.1 Sampling

From 2017 to 2022, soil and root samples were collected by inspectors of the National Plant Protection Organization (DGAV-Portugal) and by private farmers from the different regions of

Portugal (Figure 1; Table 2). Samples with a volume of 1,500 mL of soil/ha were collected from the rhizosphere at approximately 15–20-cm depth for horticultural crops and 90 cm for trees. At least 100 subsamples/ha were harvested in a rectangular mesh, not less than 5 m wide and no more than 20 m long between sampling points, covering the entire field. Samples were stored in polyethylene bags and individually coded. Geographical locations at district and county levels as well as the crops installed in these fields were accessed only after the result analysis.

Carrot, potato, and tomato were the main crops surveyed; however, samples from other crops such as broccoli, cabbage, chard, courgette, cucumber, orange tree, spinach, strawberry, and sweet potato were included.

2.2 Nematode extraction

Nematodes were extracted from a 500-mL subsample using the Oostenbrink dish technique according to protocol PM 7/119 (1) (Standard Protocol PM 7/119 (1), 2013). The suspensions were observed under a stereomicroscope (Nikon SMZ1500, Tokyo, Japan), and suspected specimens of *Meloidogyne* sp. were observed using a bright-field light microscope (Olympus BX-51, Hamburg, Germany) for confirmation. Roots were also examined for gall presence.

2.3 Morphological characterisation

Morphological characterisation was performed using secondstage juveniles (J2) and males individually placed in a drop of water on a glass slide and gently heat killed. Nematodes were observed using a bright-field light microscope (Olympus BX-51, Hamburg, Germany) and photographed with a digital camera (Leica MC190 HD, Wetzlar, Germany). The features observed for characterisation were stylet, excretory pore, tail, hyaline tail terminus, and spicule. Additionally, perineal patterns of mature females were cut in 45% lactic acid and permanently mounted in glycerin (Hartman and Sasser, 1985).

2.4 Biochemical characterisation

Bioassays were carried out by planting tomato plants cv. Oxheart in the remaining soil or inoculating them using egg masses and maintaining them in a quarantine greenhouse for 2 months to obtain material for further studies. From infected tomato roots, young egg-laying females were handpicked and transferred to micro-haematocrit capillary tubes (one female per tube) with 5 μL of extraction buffer (20% sucrose v/v and 1% Triton X-100 v/v). Maceration of the females was performed with a pestle, frozen, and stored at $-20^{\circ} C$ until use. After centrifugation, the protein extracts

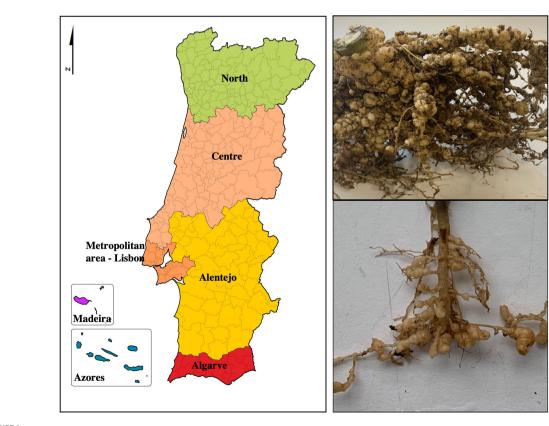


FIGURE 1
Regions of Portugal and symptoms of root-knot nematodes (*Meloidogyne* sp.) in (host) root.

|--|

Region	2017	2018	2019	2020	2021	2022	Total/region
North	32	33	17	11	15	23	130
Centre	40	42	59	122	70	27	360
Metropolitan area-Lisbon	5	32	12	21	9	23	102
Alentejo	14	28	78	70	55	49	293
Algarve	7	10	21	14	11	12	77
Azores Island	_	3	18	15	49	20	105
Madeira Island	_	_	_	3	_	1	4
Total per year	98	148	205	256	209	155	1,071

were separated by polyacrylamide gel electrophoresis (PAGE) on thin-slab 7% separating polyacrylamide gels in a Mini-Protean II (BioRad Laboratories, Hercules, CA, USA) according to Esbenshade (1985) and Pais et al. (1986). The gels were stained for EST activity with the substrate α -naphthyl acetate. Protein extracts of *Meloidogyne javanica* (Treub, 1885) isolate were included in the gel as a reference.

2.5 Statistical analysis

To evaluate the frequency and abundance of the different species of Meloidogyne in Portugal, multiple proportion tests were performed using the software R (https://www.r-project.org). For one of the tests, only samples identified to species level were used. The hypothesis tests were performed with a significance level $\alpha=0.05$.

Meloidogyne sp.-positive detections maps were made using the ArcMap 10.6 software (ESRI, USA): CAOP2017_PORTUGAL and CAOP2017_DISTRITOS shapefiles (DGT, 2017) for continental detections, CAOP2019_Madeira shapefiles (DGT, 2019a) for Madeira Island detections, and CAOP2019_Açores (Grupo Oriental), CAOP2019_Açores (Grupo Central), and CAOP2019_Açores (Grupo Occidental) shapefiles for Azores Island detections (DGT, 2019b; DGT, 2019c; DGT, 2019d).

3 Results and discussion

During the period 2017–2022, a total of 1,071 samples were collected and analysed from the seven regions of Portugal. Root-knot nematodes were detected in 243 samples distributed along the country (mainland and islands) and corresponding to 22.7% of the total (Figure 2). Among the positive detections, Azores Island contributed with 37% (90 samples), followed by the region of Alentejo at 22.7% (55 samples) and the Centre at 18.5% (45 samples) (Table 3).

The statistical analysis confirmed that the abundance of *Meloidogyne* is not equal in all regions, and comparison tests at a significance level of 5% showed that the region of Azores has a significantly higher abundance than the rest of the regions.

Morphological characterisation of second-stage juveniles recovered from soil was performed on 10 specimens. Nematodes were vermiform, slender, and annulated. The head region was slightly set off from the body. The stylet was delicate, narrow, and sharply pointed, with small knobs. The excretory pore was distinct. The tail was conoid with a hyaline terminus distinctive in most species. Males were vermiform, bluntly rounded posteriorly and with an anterior end narrowing. The head region was smooth, not set off from the body. The stylet was robust, with a straight cone, pointed and widen gradually to the posterior end. Knobs were rounded merging gradually into the shaft. The tail was short and round. Spicules were long and curved (Figure 3), agreeing with previous descriptions from Eisenback (1985) and Jepson (1987). Some specimens presented vesicle-like structures around the lumen of the juvenile metacorpus characteristic of Meloidogyne naasi and Meloidogyne sasseri. Therefore, to determine the identity of these specimens, as stated by Karssen (1996), morphometrics of secondstage juveniles (body, tail, and hyaline tail terminus length) were carried out, confirming the presence of M. naasi.

Females were elongated, ovoid, or pear-shaped. The perineal pattern comprised the vulva-anus area, tail terminus, phasmids, lateral lines, and surronding cuticule striae (Karssen et al., 2013). Some variability was observed among the perineal patterns. The shape was ovoid to rounded in the species *Meloidogyne arenaria*, *M. incognita*, *M. javanica*, and *M. enterolobii* while oval to squarish in species *M. luci* and *M. hispanica*. Distinctive lateral lines were present in *M. javanica* and *M. arenaria* whereas absent or weakly demarcated in *M. incognita*, *M. luci*, and *M. enterolobii* (Figure 4).

Enzyme phenotype analyses allowed us to identify some of the species present within the positive samples. Despite many attempts, it was only possible to reach the species identification of 51% of the positive samples (123). For the remaining 49%, we can only confirm the presence of individuals of the genus *Meloidogyne*. Seven different phenotypes were identified corresponding to the following species of root-knot nematodes: *Meloidogyne arenaria*–A2 phenotype, *M. enterolobii*–En5 phenotype, *M. hapla*–H1 phenotype, *M. hispanica*–Hi3 phenotype, *M. incognita*–I2 phenotype, *M. javanica*–J3 phenotype, and *M. luci*–L3 phenotype (Figure 5).

The host status of crops of economic importance and the host range of *Meloidogyne* spp. are issues of major concern in integrated nematode management recommendations. Currently, in Portugal,



TABLE 3 Positive and negative detections of *Meloidogyne* in the seven Portuguese regions between 2017 and 2022 (absolute values and %).

Region	Positive de	tections	Negative detections		
	Number of samples	%*	Number of samples	%*	
North	21	8.6	109	13.1	
Centre	45	18.5	315	38	
Metropolitan area Lisbon	13	5.4	89	10.8	
Alentejo	55	22.7	238	28.8	
Algarve	16	6.6	61	7.4	
Azores Island	90	37	15	1.8	
Madeira Island	3	1.2	1	0.1	
Total	243	22.7	828	77.3	

 $^{^{\}star}$ Corresponds to the percentage out of the total of positive/negative samples.

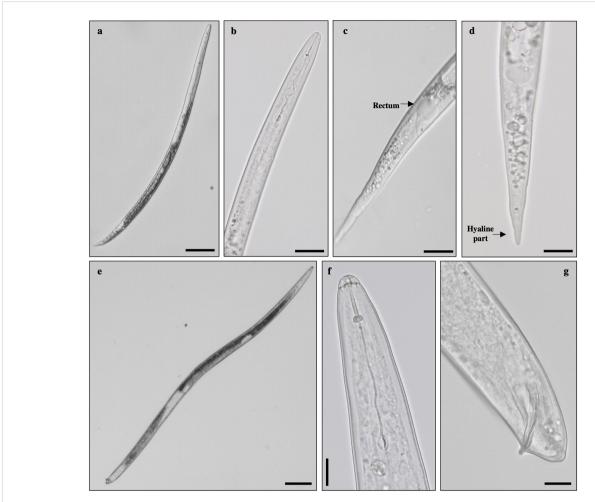


FIGURE 3
Morphological traits observed in specimens of *Meloidogyne* sp. Second-stage juvenile: (A) whole specimen, (B) anterior region, (C) tail region—rectum, (D) tail region—hyaline part. Male: (E) whole specimen, (F) head region, and (G) spicule. Bar = 20 µm.

this information is very incomplete. In this survey, RKN species were found parasitising 22 different plant hosts, mostly horticultural crops in open fields and also grasses, ornamentals, and fruit trees (Table 4). So far, many of these crops have not been reported in Portugal as hosts of *Meloidogyne*. Therefore, to our knowledge, this is the first report of species of *Meloidogyne* parasitising aubergine, broccoli, carrot, chard, courgette, orange tree, okra, pepper, and strawberry in Portugal's mainland.

The statistical analysis regarding the different species of RKNs showed that M. incognita and M. javanica presence was significantly different (p-value $\leq 2e-16$) from the rest of the species, indicating that the frequency of occurrence in fields across the country is high. The less frequent species found in the fields were M. enterolobii and M. enterolobii and enterolo

From the total samples identified to species level, *M. incognita* is the predominant species in the country (mainland and islands), as it was found in 71 samples (57.8%) and six of the seven regions surveyed. Following in prevalence, *M. javanica* was identified in 20 samples (16.2%) and present in five regions. *Meloidogyne arenaria* and *M. hapla* were detected in 10 samples (8.1%) and 12 samples (9.7%), respectively, and were present in four regions. Species of the

least frequent occurrence "minor species" such as *M. enterolobii*, *M. hispanica*, *M. luci*, and *M. naasi*, were also detected, corresponding to 8.2% of the total. The region with the highest diversity of species is the centre region (seven species) followed by the Azores Island (five species), Alentejo, Algarve (four species), and North (three species) (Figures 6A–D).

Plant parasitic nematodes represent a risk to agricultural production worldwide. Once a field is infested, it is difficult to eradicate them. Instead, the goal is to keep nematode densities low and reduce crop damage. RKNs are among the most widely distributed pests causing economically important damage to a great range of crops. Due to their importance and given the current concerns regarding climate change and food security, the main challenge is to find management strategies that can be efficient and sustainable to control them since the current practices are not enough; therefore, species identification is essential.

Although traditionally morphology is used for RKN identification, currently, it represents a challenge due to the variability between individuals, the indistinctive differences among them, and the increase in the number of species (Eisenback, 1985; Hirschmann and Volume, 1985; KarssenVan

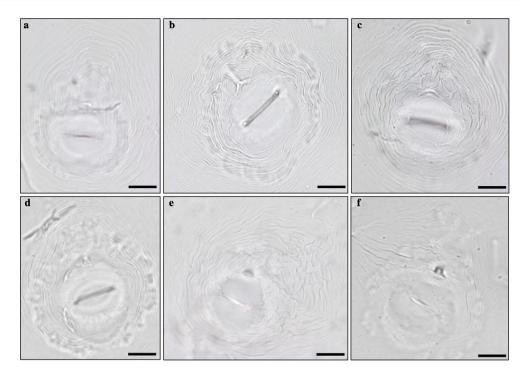


FIGURE 4
Perineal patterns observed in specimens of *Meloidogyne* sp.: **(A)** *Meloidogyne arenaria*, **(B)** *Meloidogyne incognita*, **(C)** *Meloidogyne javanica*, **(D)** *Meloidogyne enterolobii*, **(E)** *Meloidogyne luci*, and **(F)** *Meloidogyne hispanica*. Bar = 20 µm.

Aelst, 2001). Hence, it is of primary importance to have specialised and well-trained researchers to minimise the level of inaccuracy.

Furthermore, the effectiveness of the non-specific EST phenotype as the more stable and quicker method to identify *Meloidogyne* spp. has been demonstrated in many studies, showing to be highly polymorphic and able to detect different EST phenotypes of a single female (Esbenshade and Triantaphyllou, 1985; Carneiro et al., 2000). Nonetheless, its main disadvantage is

that requires females in a specific developmental stage (Hunt and Handoo, 2009).

Among the tropical species found in this study, *M. incognita*, *M. arenaria*, and *M. javanica* are probably the most widely distributed and economically important species of plant parasitic nematodes, so much that in some areas of the world, galls on roots are considered normal. The species *M. luci* is included in the European and Mediterranean Plant Protection (EPPO) alert list and *M.*

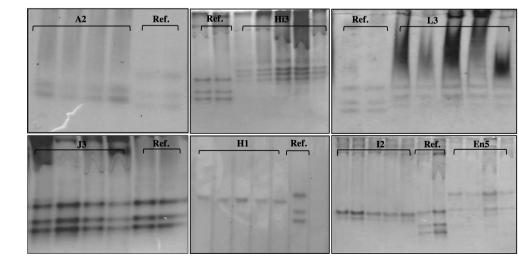
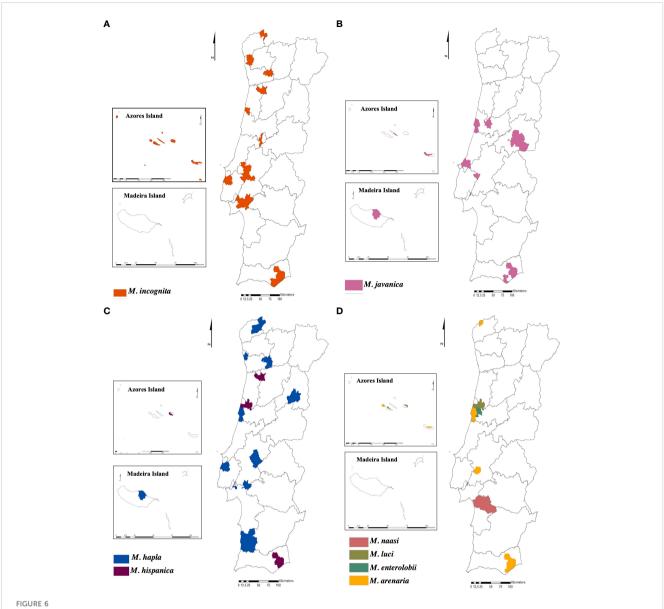


FIGURE 5
Esterase phenotypes of protein homogenates from one egg-laying female of Meloidogyne species: Meloidogyne arenaria (A2), Meloidogyne hispanica (Hi3), Meloidogyne luci (L3), Meloidogyne javanica (J3), Meloidogyne hapla (H1), Meloidogyne incognita (I2), Meloidogyne enterolobii (En5), and reference (J3)

TABLE 4 Crops and regions associated with each species of *Meloidogyne* sp. identified in Portugal.

Species identified	Region	Host plant	Cultivation system	
Meloidogyne arenaria	Azores Island	Green beans (Phaseolus vulgaris)	Greenhouse	
(10 samples = 8.1%)	Alentejo	Aubergine (Solanum melongena)	Open field	
	Azores Island	Cabbage (Brassica oleracea)		
	Centre	Cabbage tree (Cordyline australis)		
	Algarve	Orange tree (Citrus sinensis)		
	North, Algarve, and Azores Island	Potato (Solanum tuberosum)		
Meloidogyne incognita	Centre and Azores Island	Broccoli (Brassica oleracea cv. italica)	Greenhouse	
(71 samples = 57.8%)	Alentejo and Azores Island	Courgette (Cucurbita pepo)		
	Metropolitan area-Lisbon and Azores Island	Cucumber (Cucumis sativus)		
	Azores Island	Pea (Pisum sativum)		
	Alentejo and Azores Island	Pepper (Capsicum annuum)		
	Metropolitan area-Lisbon	Carrot (Daucus carota subsp. sativus)	Open field	
	Metropolitan area-Lisbon and Azores Island	Chard (Beta vulgaris subsp. vulgaris)		
	Azores Island	Leek (Allium porrum)		
	Azores Island	Onion (Allium cepa)		
	Alentejo	Okra (Abelmoschus esculentus)		
	North, Centre, and Azores Island	Potato (S. tuberosum)		
	North, Alentejo, Algarve, and Azores Island	Tomato (Solanum lycopersicum)	Greenhouse and open fie	
Meloidogyne javanica (20 samples = 16.2%)	Azores Island	Spinach	Greenhouse	
	Centre	Carrot (D. carota subsp. sativus)	Open field	
	Centre Centre	Cabbage tree (C. australis)		
		Grapevine (Vitis vinifera)		
	Alentejo	Okra (A. esculentus)		
	Alentejo and Azores Island	Potato (S. tuberosum)		
	Alentejo and Algarve	Tomato (S. lycopersicum)		
Meloidogyne hapla (12 samples = 9.7%)	Centre	Cabbage tree (C. australis)	Open field	
	Alentejo	Eucalyptus (Eucalyptus globulus)		
	Metropolitan area-Lisbon and Madeira Island	Grapevine (V. vinifera)		
	Alentejo	Pepper (C. annuum)		
	North	Potato (S. tuberosum)		
	Centre	Strawberry (Fragaria × ananassa)		
	North	Tomato (S. lycopersicum)		
Meloidogyne enterolobii (1 sample = 0.8%)	Centre	Cabbage tree (C. australis)	Open field	
Meloidogyne luci (4 samples = 3.3%)	Centre and Azores Island	Potato (S. tuberosum)	Open field	
Meloidogyne hispanica (4 samples = 3.3%)	Centre, Algarve and Azores Island	Potato (S. tuberosum)	Open field	
Meloidogyne naasi (1 sample = 0.8%)	Metropolitan area-Lisbon	Turfgrass	Open field	

Total of samples identified to species level (123 = 51%).



Distribution of the species of Meloidogyne sp. in Portugal (mainland and islands). (A) Meloidogyne incognita. (B) Meloidogyne javanica. (C) Meloidogyne hapla and Meloidogyne hispanica. (D) Meloidogyne naasi, Meloidogyne luci, Meloidogyne enterolobii, and Meloidogyne arenaria.

enterolobii in the A2 List of pests recommended for regulation as quarantine pests (EPPO, 2017a). Their habitats in general terms are the tropical and subtropical regions; however, they have also been found in temperate zones overwintering in mild winters. Meanwhile, *M. hapla* occurred mainly in temperate regions, being able to survive in temperatures below 0°C, though there is no evidence of its inability to survive in hot temperatures. Based on the above, the presence of these species in Portugal in a wide variety of hosts and climates is not an unusual event; on the contrary, it is an expected fact since the temperature increase is contributing to the geographical expansion not only of these major species but also of species of minor or restricted occurrence.

Management of RKNs is difficult due to the complexity of the soil environment (Norton and Schmitt, 1978). Biological, cultural, and chemical methods are some of the strategies that have reduced

the risk of damage by many nematode species (Hague and Gowen, 1987; Heald, 1987; Kerry, 1987; Halbrendt and La Mondia, 2004; Starr and Roberts, 2004). However, all these techniques have associated challenges (Abd-Elgawad, 2022a). Synthetic nematicides were a commonly used strategy; nevertheless, some active substances have been strictly regulated or banned from the market owing to adverse environmental and health impacts, reducing the number of alternatives for control.

Cultural methods also appear to control to some degree RKNs; however, the extensive host range that includes nearly every horticultural, fruit, and ornamental crop poses severe constraints. Similarly, many bacterial and fungal agents as well as chemical compounds have been described for *Meloidogyne* spp. as a potential strategy to be included in integrated pest management programs, among which some have not yet been tested in the field and others

have not provided consistent results (Faria et al., 2022; Pires et al., 2022). Resistant cultivars have also shown some efficacy on RKN control, but some species are able to overcome that resistance and the cultivars are not always commercially available (Abd-Elgawad, 2022b). A combination of microbial strategies using both bacterial and fungal agents with other cultural control practices or host resistance poses an alternative that can be used as a multidisciplinary approach to improve the management strategies for RKNs.

Finally, extensive surveys had not been performed in Portugal, and so, the results presented here confirm other reports on the widespread distribution of Meloidogyne, its high frequency of occurrence, and its potential as a problem for agricultural production. This assessment included crops of economic importance that are grown, intensively favouring the survival and rapid build-up of nematode populations in the soil. This fact and the ability of RKNs to be transmitted by soil, agricultural machinery, infected plants, and running water explain the presence of a high number of species in a wide diversity of hosts. The information here presented regarding the species of Meloidogyne found in the country will help farmers and technicians in the development and establishment of efficient and sustainable practices and policymakers in the provision of phytosanitary measures and monitoring programmes to prevent the introduction and spread of these pests of concern in Europe.

4 Conclusion

This study shows the high occurrence and frequency of RKNs in Portugal, confirming the widespread distribution of these nematodes. Moreover, the detection of a great variety of species of *Meloidogyne* in different regions around the country evidenced that there is a northward movement of pests caused by trade activity and climate changes. Due to this fact, the identification of *Meloidogyne* species is of great importance for the development of appropriate management practices for its control.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

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Conceptualisation: LR. Research and data analysis: LR, MC, MI, and FN. Writing—original draft preparation: LR. Writing—review and editing: LR, MC, CS, FN, and MI. Resources: MI. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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