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Evaluation of nematicidal and plant growth promotion properties of some soil nematophagous fungi against root-knot nematode *Meloidogyne incognita*: *in vitro* and greenhouse studies

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The root-knot nematode *Meloidogyne incognita* infects tomato plants, significantly reducing tomato yield. Using pesticides to manage crop diseases impacts crop yield quality, environmental pollution, and people's health. In this study, natural enemies such as soil fungi are used in biological management methods to combat nematodes, offering a potential substitute for chemical nematicides. All fungal treatments dramatically reduced the number of hatching juveniles and increased mortality. Spore suspension (SS) of *Aspergillus terreus* and *A. niger* produced the fewest hatched juveniles and the most significant number of dead juveniles. The *A. niger* showed the highest phosphate solubilization (207.1 µg ml^{-1}), and the production of IAA was produced in the absence and presence of tryptophan in *A. terreus*. *Trichoderma longibrachiatum* gave the highest siderophore. The greenhouse experiment showed that *T. longibrachiatum*_SS produced the longest plants (36.3 cm) and the highest fresh weight (8.9 g). The female number decreased by 93.8% compared to nematode treatment when *T. longibrachiatum*, *A. niger*, and *A. terreus*_SS was used. In soil, *A. niger* and *A. terreus*_SS had the highest nematode reduction percentage (75%). The number of eggs was significantly reduced in *A. terreus*_SS (55, 82.1%). *A. niger* and *T. longibrachiatum*_SS significantly reduced the number of galls (94.1%) and eggs (94.7%) for both fungi. *A. niger*_SS enhanced the production of the pigment. At the same time, *T. longibrachiatum*_SS significantly increased the peroxidase and polyphenol oxidase.

Keywords: Tomato, nematophagous fungi, root-knot nematode, greenhouse, oxidative enzymes, growth promotion

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INTRODUCTION

The genus *Meloidogyne* contains several species that cause root knots. Tomatoes are infected by several species of root-knot nematodes, such as *Meloidogyne arenaria* (peanut root-knot nematode), *M. incognita* (Southern root-knot nematode), *M. hapla* (Northern root-knot nematode), *M. javanica* (Javanese root-knot nematode), and the recently discovered *M. enterolobii* (guava root-knot nematode). *M. incognita* is the root-knot nematode that is most common in North Carolina (Sasser et al. 1983; Jones et al. 2013; Eisenback and Triantaphyllou 2020). In agriculture, these nematodes can infect more than 3000 host plant species (Jung and Wyss 1999; Hussey and Janssen 2002; Abad et al. 2008). *Meloidogyne* spp., among the many nematodes, is to blame for a significant portion of the 100 billion dollars in losses annually attributed to nematode damage globally (Ralmi et al. 2016).

In agriculture, chemical, biological, physical, and cultural controls are frequently used with cultivars resistant to plant-parasitic nematodes. Nematicides are primarily used to combat root-knot nematodes. Nematicides are an efficient way to cope with nematodes, but their use is restricted because of their high costs, short-term effects, lack of availability, nematode resistance development, environmental and health risks, residual toxicity, and negative impacts on soil beneficial fauna and microflora in addition to crop phytotoxicity effects (Mukhtar et al. 2017). Biocontrol is the best alternative to chemical control of nematodes, whether used alone or as part of Integrated Pest Management (IPM), because it is innocuous and economically feasible. Most antagonistic fungi and bacteria are used in the biological control of plant-parasitic nematodes (Oka et al. 2000; Mukhtar et al. 2017).

It is known that many fungi can infect vermiform nematodes and their eggs. Compared to the number of fungi that attack the cyst nematode eggs, very few fungi have been isolated from the root-knot nematode. On tomato roots, black-coloured egg masses of *M. javanica* were found to contain a fungal parasite (Oka et al. 1997). In the egg mass or on any of the examined culture media, this fungus did not sporulate. It appeared to resemble the hyphomycete genus *Scytalidium* based on its hyphal characteristics. The hyphae of this *Scytalidium*-like fungus proliferated in the gelatinous matrix of the egg mass and pierced the eggshell with penetration pegs (Morgan-Jones et al. 1984). *In vitro*, parasitism of the

egg mass drastically decreased the hatch rate of *M. javanica* juveniles. Adding fungus to the soil did not stop young pests from entering the tomato roots. The nematode population in the fungus-treated soil was, however, smaller after one generation than in untreated soil (Oka et al. 1997; Medeiros et al. 2017). Using pesticides to manage crop diseases impacts crop yield quality, environmental pollution, and people's health. According to Saudi Vision 2030, which deals with food security and public health for the kingdom, natural enemies such as soil fungi are used in biological management methods to combat nematodes, offering a potential substitute for chemical nematicides.

Most soil fungi have a dual function of disease suppression and plant growth promotion. This study investigated how some soil fungi suppress nematode infection and enhance tomato growth parameters as growth-promoting fungi.

MATERIALS AND METHODS

Plant materials

Tomato seeds (cv. super strain B) were purchased from the local Jeddah market. The seeds were grown in pots to test their viability, which must be more than 90%.

Chemical nematicide

Vydet® is the trade name of oxamyl-24 nematicide, a water-soluble liquid, and the active ingredient is Methyl N N-dimethyl-N ((methylcarbamyl)oxy)-1-thioxamidate (Figure 1), 24%, and the other ingredients are 76%. The recommended dose was calculated as 0.003 ml/pot. This nematicide was purchased from local markets in Jeddah, KSA.

Isolation of fungi from soil

On the Jeddah-Makkah Road, soil samples were gathered from seven distinct locations. Dilution plate and soil plate techniques were used to isolate fungi from the soil's subsurface layer, which is around 15 to 30 cm deep (Johnson and Curl 1972).

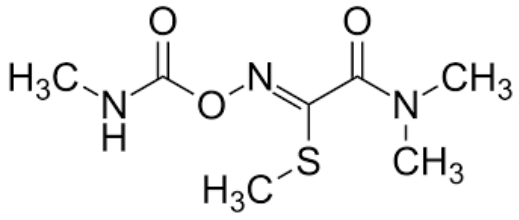

Figure 1. The structural formula of oxamyl nematicide

For each sample, six plates were used. As previously mentioned, isolation techniques were conducted using Potato Dextrose Agar (PDA) medium supplemented with 50 ppm of chloramphenicol and 1/15000 of Rose Bengal (Alzahrani et al. 2021).

Molecular identification of fungal isolates

Internal Transcript Spacer (ITS) was used to identify the most potent fungal isolate according to the manufacturer's instructions using the QIAamp DNA Mini Kit (Qiagen, France). The fragments containing ITS1 and ITS2 were amplified with primers ITS1, ITS2, ITS3, and ITS4 (ITS1: 5´-TCCGTAGGTGAACCTGCGG-3´; ITS2: 5´-GCTGCGTTCTTCAT CGATGC-3´; ITS3: 5´-GCATCGATGAAGAACGCAGC-3´; ITS4, 5´-TCCTCCGCTTAT TGATATGC-3´). The PCR products were sequenced (Macrogen, South Korea). The sequence was aligned using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted for an accession number.

Test the fungal isolates for plant growth-promoting (PGP) activities

The three fungal isolates were tested for PGP activities as follows:

Indole acetic acid (IAA) production: A submerged malt extract broth (2%) with or without 0.1% (w/v) L-tryptophan (pH 5.5) is used to produce IAA (Bose et al. 2013). Each conical flask containing 100 ml medium was inoculated with two cubes of fungal isolates (108 colony-forming units (CFU)/ml), then incubated on a rotary shaker at 28±2°C and 150 rpm for 10 days. After the incubation period, the cultures were centrifuged at 4000 rpm for 10 min, and one ml of supernatant was added to 2 ml of Salkowski's reagent and then incubated for 30 min at room temperature. The production of IAA was determined by spectrophotometric measurement at 530 nm as described by Gordon & Weber (Gordon and Weber 1951).

Siderophore production: The fungal isolates were checked for siderophore production using a universal CAS assay (Schwyn and Neilands 1987). Two fungal discs (108 colony-forming units (CFU)/ml) in malt extract broth for 10 days at 180 rpm. 100 µl of supernatant was mixed with 100 µl CAS reagent in 96 well plate. After 20 min, optical density was measured at 630 nm using a microplate reader (Tecan i-control) (Arora and Verma 2017). Siderophore was measured as percent siderophore unit (PSU), which was calculated according to the following formula (Payne 1993).

$$Percent\ Siderphore\ Unit\ (PSU) = \frac{(Ar - As)}{Ar} x100$$

Where A_r = absorbance of reference (CAS solution and uninoculated broth), and A_s = absorbance of the sample (CAS solution and cell-free supernatant of the sample).

Phosphate solubilization: Tri-calcium phosphate (TCP) was supplied per flask (w/v), and the fungal isolates' solubilization of TCP was quantitatively estimated in Pikovskaya liquid broth medium. After incubation for 10 days at 180 rpm, the supernatant was extracted by centrifugation for 10 min at 10,000 *g*(Zhang et al. 2018). Using the KH_2PO_4 standard curve, the phospho-molybdate blue colour method was used to quantify phosphate solubilization, which was then represented in ppm (Jackson 1967).

Extracellular hydrolytic enzyme production

To test the production of cellulase, xylanase, pectinase, and chitinase, the hydrolytic enzyme production was screened on basal medium (1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgSO_4{\cdot}7H_2O$, 1 ml trace element solution, and 1000 ml of water) supplemented with (1% w/v) various sole carbon sources (carboxy methyl cellulose (CMC), oats' pelt xylan, pectin of citrus peel, and colloidal chitin). Fresh fungal discs were injected into the center of a particular medium and cultured at 25±2°C for 10 days. Gram's iodine (2.0 g KI and 1.0 g I_2 in 300 ml of distilled water) was then added to the plates and left for five minutes. After that, plates were checked for a clear area surrounding the colony (Soares et al. 1999; Gohel et al. 2014; Meddeb-Mouelhi et al. 2014; Sharma and Salwan 2015). The following formula is used to compute the enzyme index (EI):

$$EI = \frac{Diameter\ of\ hydrolysis\ (cm)}{Diameter\ of\ the\ colony\ (cm)}$$

Preparation of *M. incognita* inoculum

Infected tomato plants had their root galls removed, and soil particles were carefully cleaned with tap water. 500 ml of 0.5% sodium hypochlorite (NaOCl) solution was used to sterilize plant roots (2-3 cm long) (Hussey and Barker 1973). After being vigorously shaken for two minutes, the solution was filtered through 60 and 500-mesh sieves to separate the massive number of *M. incognita* eggs inside egg masses found on plant roots. After thoroughly cleaning the 500 mesh eggs with sterile water to remove any remaining NaOCl, the eggs were placed in a Petri plate and incubated for two days at 25±2°C. All of the *M. incognita* eggs that hatched in the water and the active juveniles (J2) that emerged during that time were gathered for *in vitro* and greenhouse experiments (Coyne et al. 2007; El-Ashry et al. 2022).

Fungal inoculum preparation

The culture filtrate was tested for nematode mortality. The mortality assay protocol involved preparing the fungal spore suspension (SS) at a concentration of 10%, mycelia homogenate (MH), and fungal cell-free culture (CC) (80%) using distilled water. The concentration of the fungal spores grown on potato dextrose broth (PDB) (Lab M) was adjusted to be approximately 108 colony-forming units (CFU)/ml. A hemocytometer was used to adjust the fungal spore concentration.

Effect of different fungal isolates on *M. incognita* J2s mortality

In Petri dishes with 10 mL of each concentration and 2 mL of a nematode-water solution, about 200 J2s of the RKN *M. incognita* were inserted. Plates were incubated in the laboratory at 25°C. At 6, 12, 24, and 48 h after exposure to the fungal suspension, a 1 ml portion of each plate was visually counted for both live and dead larvae using a Hawksley counting slide to estimate the percentage mortality rate (El-Ashry et al. 2022). The following equation was employed to calculate the percentage mortality rate.

$$Egg\ hatching\ inhibition\ (\%) = Control - \frac{Treatment}{Control}\ X\ 100$$

Greenhouse experiment

The greenhouse experiment was carried out to compare the antagonistic properties of the four fungal isolates to the commercial nematicide oxamyl against the root-knot nematode (M. incognita) infecting tomatoes. Twenty-day-old tomato seedlings of the cv. super strain B was planted in 75 plastic pots (20 cm in diameter), each containing 2.0 kg of sterilized clay: sandy soil (1:1, w/w).

Each fungal isolate was subjected to five replicates of the three treatments (filtrate, spore suspension, and mycelial suspension), oxamyl, nematode, and plant alone, using the abovementioned concentrations. The negative control (without nematodes) and a positive control (a tomato injected with nematodes) were also used.

Five days after transplanting, second-stage juveniles of *M. incognita* at a rate of 1400 J2s/pot were introduced, and three days after nematode infection, oxamyl and fungal treatments were administered. Under ideal environmental circumstances (12–14 hours of day length, 28–30°C temperature, and 65%

humidity), the greenhouse experiment was run for 55 days.

Plant and nematode parameters

After nematode infestation for 55 days, plants were harvested. Measurements were made of the shoot fresh and dry weight, root fresh weight, and plant length associated with tomato growth. Plants were delicately uprooted. Tap water was used to wash the roots gently to remove soil particles. Then, roots were stained with acid fuchsin in lactic acid and counted under binocular imaging for nematode phases (females, developmental stages, egg masses, and galls) (Bybd Jr et al. 1983). A modified Baermann approach was used to remove nematode juveniles from the soil, and they were subsequently counted using a counting slide and a stereoscopic microscope (Hooper et al. 2005; Hallmann and Subbotin 2018). The Galls Index (GI), nematode reproduction factor (Rf), and egg masses index (EI) were measured (Taylor and Sasser 1978).

The percent increase in plant length and fresh and dry weights was calculated using the following equation:

$$Increase\ (\%) = \frac{Treatment - Nematode}{Nematode} \times 100$$

The reproduction factor of the nematode was calculated according to the following equations:

$$Rf = \frac{Pf}{Pi}$$

Rf is the reproduction factor, Pf is the final population, and Pi is the initial population.

$$Pf = SNP + NDS + Female\ number + (\frac{No.of\ eggs}{Egg\ mass}\ x\ No.egg\ mass)$$

SNP is the soil nematode population, and NDS is the number of developmental stages.

Measurement of some resistance-related enzymes

Fresh tomato leaves were used to assay the enzymes. By detecting the oxidation of pyrogallol to purpurogallin in the presence of H_2O_2 at 425 nm, peroxidase activity (PO), polyphenol oxidase (PPO) activity at 400 nm, and catalase activity were determined using a UV spectrophotometer (Allam 1972).

Determination of photosynthetic pigments of tomato leaf

Dimethyl sulfoxide (DMSO) was used for the extraction of chlorophyll from fresh tomato leaves (Hiscox and Israelstam 1979; Hiscox and Israelstam 1980). The absorbance of both blank and sample was measured at 470, 645, and 663 nm. The method of Arnon (1949) was followed to determine photosynthetic pigments.

Final chlorophyll contents were calculated by using the equations:

$$Chlorophyll\ a = \frac{12.7\ (OD\ 663) - 2.69\ (OD\ 645)V}{1000x\ W}$$

$$Chlorophyll\ b = \frac{22.9\ (OD\ 645) - 4.68\ (OD\ 663)V}{1000x\ W}$$

$$Total\ chlorophyll = Chlorophyll\ a + Chlorophyll\ b$$

The total carotenoids were calculated using the following equation Wellburn (1994).

$$Total\ carotenoids = \frac{\left(\frac{(1000\ (OD\ 470) - 1.29Ca - 53.78Cb)}{220}\right)V}{1000x\ W}$$

Where OD is the optical density (nm), V is the final volume (ml), and W is the fresh weight of the sample (g).

Statistical analysis

All data represented in this paper were the mean of four replicates. Standard deviations (SD) were evaluated. The least significant difference (LSD) of the measured data was considered as follows: non-significant when $P>0.05$ and significant when $P<0.05$ (Calinski et al. 1981).

RESULTS AND DISCUSSION

The biggest dangers to crop productivity worldwide are plant pathogenic nematodes such as root-knot and cyst nematodes (Barker and Koenning 1998; Abad et al. 2008). Researchers looking for efficient management methods are interested in nematode infections since they present a serious challenge to many farmers. The most researched nematode-antagonistic species are nematophagous fungi, which effectively regulate the number of parasitic nematodes on their host. These fungi are common in soil and saprophytes, endophytes, and rhizosphere pathogens (Freitas Soares et al. 2023). Researchers have been investigating using PGPF as a substitute for chemical nematodes to treat nematode infestations (Attia et al. 2021).

The three fungi were identified by aligning the sequence of the four soil fungi with blast analysis at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).*A. niger, T. longibrachiatum,* and *A. terreus* had accession numbers LC577101, LC577103, and LC577104. A maximum-likelihood phylogenetic tree for the four fungal isolates was constructed using a

fast tree with slow NNI and MLACC=3 (Price et al., 2010). The four fungal isolates gave high similarities with *A. terreus* (MH065614), *A. niger* (KU847851), and *T. longibrachiatum* (KY764849) in the NCBI GenBank database (Price et al. 2010; Alzahrani et al. 2021). After 24, 48, and 72 hours, the number of larvae hatched from the egg masses and the number of dead larvae were noted. Compared to the untreated check, the data in Table 1 demonstrated that all fungal treatments dramatically reduced the number of hatching juveniles and increased mortality. Spore suspension (SS) of *A. terreus* and *A. niger* produced the fewest hatched juveniles and the most significant number of dead juveniles, followed by cell-free culture (CC) of the same two fungi. However, the highest number of hatched juveniles and no mortality were noted in the untreated check.

The CC of all fungi tested (*Trichoderma harzianum, T. viride, Pochonia chlamydosporia, Purpureocillium lilacinum,* and *Pseudomonas fluorescens*) considerably raised the percentage of juvenile mortality and decreased the rate of hatched juveniles. With the lowest percentage of hatched juveniles and mortality on *M. javanica* eggs and juveniles, *T. viride* was the most successful therapy among the studied fungi (Choudhary et al. 2023). In comparison to the controls, *Lecanicilliummus carium* was shown to be very successful in infecting eggs (79.6%), decreasing egg hatching (6.9%), and causing J2 mortality (78%), followed by *L. psalliotae* and *Trichoderma hamatum*. Conversely, *Fusarium solani* showed minimal infection of J2 and nematode eggs (Hussain et al. 2017).

The three fungal isolates were tested for PGP traits to detect whether they were used as biological control agents. *A. niger* showed the highest phosphate solubilization (207.1 µg ml^{-1}), while *A. terreus* and *T. longibrachiatum* are almost the same. The production of IAA was considerable in the absence and presence of tryptophan in the culture medium for *A. terreus*, and there was no IAA production in the other two fungal isolates in the absence of tryptophan. The highest producible isolate for siderophore was *T. longibrachiatum,* and the lowest was *A. terreus* (Table 2). The ability of the PGPF to solubilize phosphates and produce IAA, siderophores, cellulase, chitinase, and other chemicals can either directly or indirectly enhance the growth and development of a host plant in addition to fostering disease resistance (Zhang et al. 2018; Muslim et al. 2019).

Figure 2 shows that the efficiency of producing hydrolytic enzymes was calculated as enzyme index (EI); the highest EI was xylanase, followed by cellulase for *A. terreus*, while the lowest was chitinase for *A. niger*. Xylanase, pectinase, and chitinase were not detected in *T. longibrachiatum*.

From a biological perspective, enzymes are essential to the fungus-parasite interaction process. According to the molecular makeup of these several possible catalytic targets, nematophagous fungus-produced enzymes, including lipases, chitinases, and proteases, make up a robust toolkit in the predatory mechanism. Enzyme usage is a green technique that could be the global standard for sustainable pest management in the future (Freitas Soares et al. 2023).

Hydrolytic enzymes, organic acids, and low molecular weight natural compounds are secreted by filamentous fungi, which have many uses, including the solubilization of P (Cairns et al. 2021), as well as the solubilization of calcium and iron phosphates (Vera et al. 2002). Therefore, they have much promise for creating biofertilizers, which are crucial for sustainable agriculture (Alves et al. 2021) since they increase soil fertility and encourage plant growth (Xiao et al. 2009).

The data in Table 3 revealed that the use of spore suspension of *T. longibrachiatum* gave the highest plant length (36.3 cm), followed by *A. niger* (36.0 cm) and *A. terreus* (34.8 cm). The same pattern in fresh weight of the tomato plant where the SS of *T. longibrachiatum* gave the highest plant fresh weight (8.9 g) followed by *A. terreus* (8.2 g) and *A. niger* (8.1 g) and in case of the dry weight of shoot system, the best fungal isolate was *A. niger* (1.3 g) followed by *A. terreus* (1.2 g) and *T. longibrachiatum* (1.0 g).

In the greenhouse experiment, the fungal spore suspension enhanced tomato growth to resist the invasion of the juveniles (J2) of *M. incognita* in all fungal isolates' tests. This appeared in plant length using different fungal treatments, which is almost the same as using different fungal treatments, nearly the same as using nematicide oxamyl.

In greenhouse experiments, *T. harzianum* formulated in a peat-bran decreased root galling and increased the shoot fresh weight of tomatoes cultivated in soil infested with nematodes (Spiegel and Chet 1998; Schubert et al. 2008). When used, *T. harzianum* and *T. viride* significantly increased tomato shoot weight and decreased tomato root weight (Mukhtar et al. 2017; Mukhtar 2018). The outcomes showed how

Table 1. Effect of different fungal treatments on mortality percentage and hatchability of *Meloidogyne incognita* eggs

Fungal isolate	Fungal treatment	% of mortality			% of hatched eggs	% of unhatched eggs
		24 h	48 h	72 h	7 days	
Control		0	0	3	88	12
A. terreus	CC	24	55	73	30	70
	SS	27	40	70	27	73
	MH	30	60	81	43	57
A. niger	CC	30	60	100	30	70
	SS	33	65	100	27	73
	MH	24	59	100	32	68
T. longibrachiatum	CC	22	40	60	50	50
	SS	27	40	56	53	47
	MH	20	35	50	48	52

Note: each value is the mean of five replicates. CC: Cell-free Culture filtrate, SS: Spore suspension, MH: Mycelial homogenate.

Table 2. Some plant growth-promoting traits of the three fungal isolates

Fungal isolate	P-Solubilization (µg ml^{-1})	IAA (µg ml^{-1})		Siderophore production (%)
		With Tryptophan	Without Tryptophan	
A. terreus	61.4±1.24^{b}	13.91±1.18^{a}	34.54±0.86a	22.34±1.53^{b}
A. niger	207.1±5.51^{a}	0.64±0.64^{b}	0.00	92.12±2.19^{a}
T. longibrachiatum	62.6±5.57^{b}	0.03±0.03^{b}	0.00	95.26±0.37^{a}

Data are average values of three replicates, (±) standard error of mean, values within the same vertical area with the same letter are not significantly different at 5% probability level by Duncan's Multiple Range test.

Table 3. Impact of four soil fungi on growth parameters of tomato infected with *Meloidogyne incognita* under greenhouse conditions

Treatments		Length (cm)				Fresh weight (g)				Dry weight (g)	
		Shoot	Root	Total	Inc. (%)	Shoot	Root	Total	Inc. (%)	Shoot	Inc. (%)
A. niger	CC	13.3**	15.0*†	28.3	6.4	6.2**††	1.0**	7.2	28.6	0.6**†	-14.3
	SS	19.0	17.0**	36.0	35.3	6.6**††	1.5**†	8.1	44.6	1.3**††	85.7
	MH	15.0**††	12.0††	27.0	1.5	4.5	1.3**	5.8	3.6	0.5**††	-70.3
T. longibrachiatum	CC	18.5*	16.3**	34.8	30.8	4.9	1.8**††	6.7	19.6	0.6**†	-14.3
	SS	19.3	17.0**	36.3	36.5	6.4**††	2.5	8.9	58.9	1.0**††	42.8
	MH	15.0**††	12.3††	27.3	2.6	4.8	1.9*††	6.7	19.6	0.8†	14.3
A. terreus	CC	19.3	15.5*†	34.8	30.8	5.0	1.8*††	6.8	21.4	0.8†	14.3
	SS	21.0†	13.8*†	34.8	30.8	5.7*†	2.5	8.2	37.5	1.2**††	71.4
	MH	17.0*	14.5*†	31.5	18.5	5.7*†	2.0	7.7	46.4	0.9*††	28.6
Oxamyl		16.5	20.3	36.8	38.3	5.2	2.6	7.8	39.3	1.1	57.1
Non-infested control		20.0	20.0	40.0	50.4	4.7	2.5	7.2	28.6	0.8	14.3
Infested control		17.3	9.3	26.6	-	4.8	0.8	5.6	-	0.7	-
	1%	3.8	6.8	-	-	1.4	0.8	-	-	0.2	-
LSD											
	5%	1.5	4.3	-	-	0.9	0.6	-	-	0.1	-

Note: Each value is the mean of five replicates. CC: cell-free culture, SS: Spore suspension, MH: Mycelial homogenate. *, significant at $P < 0.05$ relative to non-infested control; **, significant at $P < 0.01$ relative to non-infested control; †, significant at $P < 0.05$ relative to infested control; ††, significant at $P < 0.01$ relative to infested control. LSD, Least Significant Difference.

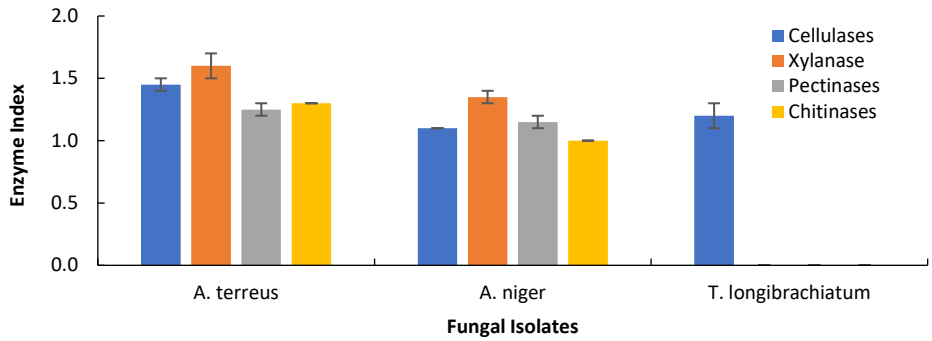

Figure 2. Enzymatic activity indexes of the three fungal isolates.

effective the therapies may be at reducing the negative impacts of RKN-infected plant output. On the other hand, even in the presence of nematode stress, *Alternaria photistica*, *A. niger*, and *Penicillium buchwaldii* were successful in fostering plant growth (Kandil et al. 2024).

According to our data (Table 4), compared to nematode treatment, the number of females significantly decreased (93.8%) when *T. longibrachiatum*, *A. niger*, and *A. terreus* SS was used. In soil, *A. niger* and *A. terreus SS* had the highest nematode reduction percentage (75%). Compared to nematode treatment, the number of eggs was significantly reduced, with the most significant reduction occurring in *A. terreus* (55, 82.1%). The maximum decrease in total nematode populations was obtained in both *A. niger* and *A. terreus* SS (92.6%). *A. niger*, *T. longibrachiatum*, and *A. terreus* SS significantly reduced the number of galls (94.1%, 94.1%, and 91.2, respectively) and eggs (94.7%) for all three fungi (Table 5). The data in Table 5 showed that *A. niger, T. longibrachiatum,* and *A. terreus* have the same effect on the nematodes, whether galls or eggs. The spore suspension treatment of *A. niger, T. longibrachiatum,* and *A. terreus* significantly reduced the number of galls (94.1%, 94.1%, and 91.2, respectively) and eggs (94.7%) for all three fungi. The mycelial treatment of *A. terreus* also resulted in a high reduction in gall numbers (91.2%) and eggs (94.7%). The rhizospheric and endophytic fungi and bacteria are the best biological control agents because they may either directly or indirectly protect plants from nematode parasitism. These microbes likely affect the plant by inducing resistance or impeding the nematode's ability to recognize its host (Poveda et al. 2020).

It is reported that tomato seeds inoculated with *T. harzianum* significantly decreased the nematode *M. javanica* disease level, influencing their reproduction, establishment, and development (the number of egg masses per plant and the number of eggs in each mass, as well as the number of galls per plant) under greenhouse conditions (Sahebani and Hadavi 2008; Naserinasab et al. 2011; Abootorabi and Naraghi 2017; Singh et al. 2020). They also observed a significant decline in egg hatching, demonstrating the considerable potential of this type of *Trichoderma* as a biocontrol agent against this plant parasite. Thus, it was discovered that *T. harzianum* root colonization hindered the local nematode's performance at many parasitism stages, including invasion, galling, and even reproduction in tomatoes (Martínez-Medina, Fernandez, et al. 2017; Martínez-Medina, Appels, et al. 2017). Significant drops were seen in the number of reproductive factors, eggs per egg mass, egg masses, and galls of *M. incognita* due to both antagonistic fungi (*T. harzianum* and *T. viride*) (Mukhtar et al. 2017; Mukhtar 2018).

Trichoderma virens uses salicylic acid (SA) and jasmonic acid (JA) to make tomatoes more resistant to *F. oxysporum* f.sp. *lycopersici* (Jogaiah et al. 2018). Numerous studies have shown that these two hormones control how vulnerable and resistant plants are to nematodes (Martínez-Medina, Fernandez, et al. 2017; Martínez-Medina, Appels, et al. 2017). *Trichoderma* upregulates the expression of JA-dependent defenses in response to *M. incognita* inhibition of JA-related defenses in the roots during the second phase. This counteracts *M. incognita* suppression of defenses and stops the nematodes' growth and reproduction. To strengthen defenses against the invasion of new juveniles, the fungus, perhaps by recognizing eggs, encourages the activation of SA-dependent defenses once parasitism is established(Martínez-Medina, Fernandez, et al. 2017). Fungal strains isolated from plants' rhizosphere effectively combated the northern RKN (*M. hapla*) (Nekoval et al. 2024).

The high reduction percentage in the gall number means the fungus affects the J2 (larvae) stage, and the infection rate decreases. In contrast, the reduction in the percentage of egg numbers means that the fungus affects females. From our results, the spore suspension of *A. niger*, *T. longibrachiatum*, and *A. terreus* significantly affects the J2 stage and females.

From the results, we selected plants treated with spore suspension of *A. niger, T. longibrachiatum,* and *A. terreus* for the analysis of plant pigment production and oxidative enzymes.

The data represented in Figure 3 showed that the spore suspension of *A. niger* enhanced the production of plant pigments (chlorophyll and carotenoids) (2.88 mg g^{-1} total chlorophyll and 0.77 mg g^{-1} carotenoids), followed by *T. longibrachiatum* (2.81 mg g^{-1} total chlorophyll and 0.74 mg g^{-1} carotenoids). The difference in total chlorophyll between the treatments of *A. niger* and *T. longibrachiatum* was in the content of chlorophyll a (1.93 and 1.86 mg g^{-1}, respectively), while the content of chlorophyll b was the same (0.95 mg g^{-1}).

Table 4. Development and reproduction of *M. incognita* as influenced by the addition of the four soil fungi under greenhouse conditions

Treatments		Nematode population			No. Eggs/egg mass	Total nematode population	Rf*
		Root		Soil			
		DS	Females				
A. niger	CC	1.0††	9.0**	718**††	100**††	1328	0.95
	SS	1.0††	2.0**	527**	61.0**†	591	0.49
	MH	1.0††	10.0**†	633**†	129**††	1547	1.11
T. longibrachiatum	CC	1.0†	10.0**†	799**††	192**††	2346	1.68
	SS	0.0††	2.0**	731**††	76.0**††	809	0.58
	MH	2.0†	10.0**†	1011**††	151**††	2080	1.49
A. terreus	CC	1.0††	9.0**	735**††	90.0**††	1375	0.98
	SS	1.0††	2.0**	527**	55.0**	591	0.49
	MH	1.0††	2.0**	676**††	61.0**†	734	0.52
Oxamyl		5.0	1.0	335	20.0	361	0.26
Nematode		0.0	32	2120	308	8004	5.72
1%		3.1	13.4	300.0	50.0	-	-
LSD							
5%		2.0	9.0	200.0	40.0	-	-

Notes: Nematode = 1400 eggs and juveniles (J_2) of *M. incognita*; DS = Developmental stages; each value is the mean of five replicates; Rf = Reproduction factor. CC: cell-free culture, SS: Spore suspension, MH: Mycelial homogenate. *, significant at $P < 0.05$ relative to nematode treatment; **, significant at $P < 0.01$ relative to nematode treatment; †, significant at $P < 0.05$ relative to oxamyl; ††, significant at $P < 0.01$ relative to oxamyl. LSD, Least Significant Difference.

Table 5. Reduction percentages in root galling and number of egg masses of *Meloidogyne incognita* in tomato plants as influenced by the addition of the four soil fungi under greenhouse conditions

Treatments		No. of galls	Reduction (%)	RGI	No. of egg masses	Reduction (%)	EI
A. niger	CC	9.0**†	73.5	2.0	6.0**	68.4	2.0
	SS	2.0**	94.1	1.0	1.0**	94.7	1.0
	MH	10.0**†	70.6	2.0	7.0**	63.2	2.0
T. longibrachiatum	CC	11.0**††	67.6	3.0	8.0**	57.9	2.0
	SS	2.0**	94.1	1.0	1.0**	94.7	1.0
	MH	10.0**†	70.6	2.0	7.0**	63.2	2.0
A. terreus	CC	10.0**†	70.6	2.0	7.0**	63.2	2.0
	SS	3.0**	91.2	2.0	1.0**	94.7	1.0
	MH	3.0**	91.2	2.0	1.0**	94.7	1.0
Oxamyl		1.0	97.1	1.0	1.0	94.7	1.0
Infested control		34.0	-	4.0	19.0	-	3.0
1%		10.0	-	-	10.0	-	-
LSD							
5%		7.2	-	-	7.2	-	-

Note: initial nematode concentration = 1400 eggs and juveniles (J_2) of *M. incognita*.RGI: Root gall index or EI: Egg-masses index was determined according to the scale given by Taylor & Sasser (1978) as follows: 0= no galls or egg masses, 1= 1-2 galls, or egg masses; 2= 3-10; 3= 11-30;4= 31-100; and 5= more than 100 galls or egg masses. CC: cell-free culture, SS: Spore suspension, and MH: Mycelial homogenate.
*, significant at $P < 0.05$ relative to nematode treatment; **, significant at $P < 0.01$ relative to nematode treatment; †, significant at $P < 0.05$ relative to oxamyl; ††, significant at $P < 0.01$ relative to oxamyl.

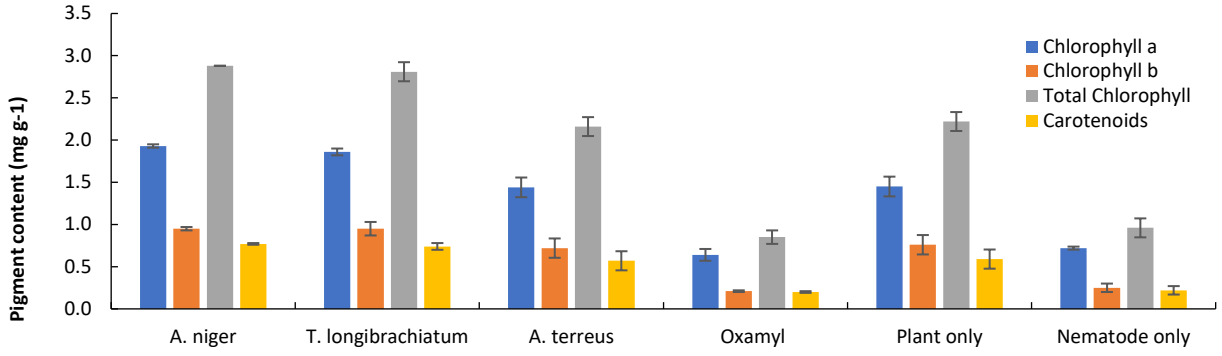

Figure 3. Tomato pigments (chlorophyll and carotenoids) production as a response to different treatments under greenhouse experiments. Error bars are ±SE.

The photosynthetic process depends on chlorophyll pigments because they absorb light and promote the conversion of carbon dioxide and water into carbohydrates (Fang and Agarwal 2024). The PGPF mitigated the adverse effects of RKN-infected plants on pigment content. The RKN-infected plants performed better and became more resilient because of the combined impacts of PGPF, which included enhanced photosynthetic capacity, higher nutritional availability, regulation of antioxidant defenses, and potential direct nematocidal or antagonistic actions (Hashem et al. 2023).

It was evident from Table 6 that the nematicide oxamyl significantly increased overall oxidative enzyme production. In the presence of oxamyl, the peroxidase enzyme was at its most significant level (1.571 U). The spore suspension of *T. longibrachiatum*, followed by A. terreus (0.527 U and 0.526 U, respectively), was one-third. The same behaviour was observed in the polyphenol oxidase enzyme. However, there was no discernible difference between oxamyl and the spore suspension of *T. longibrachiatum* (0.527 U and 0526 U, respectively), followed by *A. terreus* (0.451 U). In the catalase enzyme, the spore suspension of *A. terreus* produced the most catalase (0.916 U), approximately twice as much as the oxamyl (0.537 U). Although the oxamyl treatment gave the highest production value of proline (2.422 mg), the *A. niger* spore suspension was (1.46 mg) followed by *A. terreus* (1.266 mg).

Peroxidase has been connected to pathogen resistance mechanisms. Peroxidase enzyme produces active oxygen species and thickens cell walls to defend against pathogen attack (Hiraga et al. 2001; Anterola and Lewis 2002). Peroxidases are essential to produce reactive oxygen species (ROS) linked to the hypersensitive reaction (HR) in the incompatible plant-nematode interaction (Melillo et al. 2006). It has been suggested that the genus *Meloidogyne*-specific resistance to the CM334 may be due to the accumulation of phenolic chemicals (Pegard et al. 2005). A significant rise in chlorogenic acid was linked to CM334's resistance to nematodes that cause root knots. The browning and resistance of the CM334 chili pepper and the Nemared tomato cv to *M. incognita* are assumed to be caused by the buildup of this acid (Pegard et al. 2005).

Accordingly, the enzymatic activity of peroxidases or polyphenol oxidases may cause the oxidation of phenolic compounds (Gómez-Vásquez et al. 2004).

Table 6. Some oxidative enzymes and proline secreted by the tomato cv. super strain B as a response to nematode infection and its control by spore suspension of soil fungi

Treatments	Peroxidase (U ml^{-1})	Polyphenol oxidase (U ml^{-1})	Catalase (U ml^{-1})	Proline (mg ml)
A. niger	0.565±0.030	0.105±0.002	0.576±0.024	1.46
T. longibrachiatum	0.580±0.027	0.526±0.002	0.025±0.002	0.964
A. terreus	0.573±0.027	0.451±0.001	0.916±0.028	1.266
Oxamyl	1.571±0.028	0.527±0.003	0.537±0.003	2.422
Tomato	1.683±0.097	0.289±0.002	0.409±0.001	2.392
Nematode	1.091±0.102	0.517±0.005	0.605±0.005	0.686

All data are the mean of three replicates (±SD)

CONCLUSION

The advantage of using fungi as control agents for nematode infection is not only for their effects on all nematode stages but also for their role in plant growth promotion, which was clear in the increase in chlorophyll and carotenoids production and oxidative enzymes over the nematicide oxamyl and in the plant only. The most effective biocontrol agent was *A. niger* and *A. terreus* SS, which suppressed the root-knot nematode *M. incognita* and enhanced the growth parameters of tomato cv super strain B.

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AUTHORS' CONTRIBUTIONS

Nuha Alhazmi, Dina Ibrahim, Tarek Moussa: Research concept and design, writing manuscript draft. Nuha Alhazmi, Dina Ibrahim: Experimental work and writing the article. Tarek Moussa: Critical revision of the article. Dina Ibrahim, Tarek Moussa: Data analysis and interpretation.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

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