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Effect of some Nematophagous Fungi on Reproduction of a Nematode Pest, *Heterodera schachtii,* and Growth of Sugar Beet

Manzoor Hussain*, Miloslav Zouhar and Pavel Ryšánek

Department of Plant Protection, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Prague, Czech Republic

ABSTRACT

Heterodera schachtii is a globally important and often marginalized pest of sugar beet, cabbage, broccoli and radish, among other crops. It is a cyst-forming nematode that affects plant growth and yield. We report on studies aimed to evaluate the effects of five nematophagous fungi on the population dynamics of this pest in sugar beets in laboratory and greenhouse trials. The fungi chosen were *Arthrobotrys oligospora*, *Dactylella oviparasitica*, *Clonostachys rosea*, *Stropharia rugosoannulata*, and *Lecanicillium muscarium.* In the laboratory experiment, *S. rugosoannulata* proved to be the most efficient biocontrol agent by parasitizing the maximum number of eggs, whereas *D. oviparasitica* appeared to be the least efficient after 72 hours. The greatest numbers of cysts and eggs were found to be colonized with *L. muscarium* during microscopic observations. In the greenhouse experiment, *L. muscarium* had significant effects in reducing the nematode population in soil compared to the other treatments. In regard to the growth parameters, root and shoot growth (cm) were enhanced after the application of *L. muscarium*, followed by *D. oviparasitica* and *S. rugosoannulata.* The reproductive rate (Rf = Pf/Pi) of nematodes was much higher in the non-treated plants than those that were treated. The root quality of the fungus-treated plants was significantly improved. All fungi conclusively proved to be effective against *H. schachtii* and need to be further investigated at the molecular level.

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Key words
***Heterodera schachtii*, Nematophagous fungi, Sugar beet, *Lecanicillium muscarium*, *Stropharia rugosoannulata*.**

INTRODUCTION

Various phytoparasitic nematodes pose a great threat to several vegetable and field crops worldwide, but sedentary endoparasitic nematodes from the genera *Meloidogyne*, *Globodera,* and *Heterodera* are the most important (Anwar and Mckenry, 2012; Singh and Kumar, 2015; Greco *et al*., 1993; Sasser and Freckman, 1987). The sugar beet cyst nematode, *Heterodera schachtii* Schmidt, is an alarming pest of sugar beet (*Beta vulgaris* L.) and many other plant species. Second-stage juveniles (J2), an infective stage of nematodes, penetrate into the roots of host plants, where they subsequently congregate and sustain specialized feeding structures called syncytia (Jones, 1981; Hussey, 1989; Wyss *et al*., 1992; Sijmons *et al*., 1994; Williamson and Hussey, 1996). Plants infested with nematodes incite or exacerbate the effects of other pathogens (pests) by worsening disease through decreased growth, wilted leaves, abnormal root development and the reduction of sugar contents. *Heterodera schachtii* is a main hindrance in the production of sugar beets in central Europe, where it is responsible for economic losses estimated to be 90 million Euros annually (Müller, 1989, 1999). Higher soil population levels of this nematode result in potential losses of sugar beet and sugar yields (Heijbroek *et al*., 2002; Heinrichs, 2011; Kenter *et al*., 2014; Hauer *et al*., 2016).

Management of the sugar beet nematode is challenging, as this nematode species has the ability to survive in a protective cyst for many years (Sharma, 1998). Researchers are struggling to manage cyst-forming nematode species using various alternative strategies such as the use of organic amendments (Renčo *et al*., 2007; Renčo and Kováčik, 2012, 2015), soil solarization, biofumigation, fallowing land, rotating crops with non-hosts or trap crops (Renčo *et al*., 2012; Renčo, 2013), and planting antagonistic plants. However, these strategies may be either very expensive for farmers or not effective because of the high cost-benefit ratio and pollution (Hussain *et al*., 2016).

The term "nematophagous fungi" is defined as a group of organisms with the ability to infect and parasitize nematodes to obtain nutrients for survival. Based on their modes of action, nematophagous fungi are categorized into four groups: nematode-trapping (formerly called predatory fungi), endoparasitic, egg- and female-parasitic and toxin-producing fungi (Barron, 1997; Dackman *et al*., 1992; Jansson and Lopez-Llorca, 2001). The interactions between nematophagous fungi and their hosts include various steps of recognition (attraction phenomena, contact), the production of adhesives and lytic enzymes,

* Corresponding author: manzoor.sahi@gmail.com
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and the differentiation of infectious structures (appressoria and trapping organs) for nematode digestion (Tunlid *et al*., 1992). The nematode-trapping fungi *A. oligospora*, *S. rugosoannulata,* and *D. oviparasitica* produce adhesive networks, large spike cells called acanthocytes, and adhesive knobs or non-constricting rings, respectively (Jansson and Nordbring-hertz, 1979; López-LIorca *et al*., 2007; Zouhar *et al*., 2013). These traps are imperative to infect nematodes and attract nematodes to the fungi. The fungi *C. rosea* and *L. muscarium* have the ability to penetrate eggshells and nematodes directly or through enzymatic lysis, as nematode eggshells and bodies mostly consist of protein and chitin (Charnley, 1997; Butt, 2002; Clarke *et al*., 1967; Bird and Bird, 1991; López-LIorca *et al*., 2007).

The practical control of nematodes relies mainly on highly toxic nematicides, nematode-resistant varieties, crop rotation and the genetic modification of hosts. However, these control strategies are not effective enough due to their limitations, cost-benefit ratios, environmental pollution, residual toxicity and human health hazards (Thomason, 1987; Mukhtar *et al*., 2013; Hussain *et al*., 2016). Several interactions occur in the soil between both ubiquitous organisms, i.e., fungi and nematodes. The population levels of both of these taxa is higher in agricultural soil than in root-free soil (López-LIorca *et al*., 2007). This study aims to introduce potential antagonists to reduce the use of lethal chemicals to control the nematode population level in the soil and to thereby enhance the yield of cultivated sugar beets. The objective of this study was to compare the effectiveness of different promising antagonistic fungi on the population growth of *H. schachtii* infecting sugar beet and on plant growth.

MATERIALS AND METHODS

Nematode inoculum preparation

Nematode inoculum was prepared by inoculating seedlings of the susceptible sugar beet variety Alpaca with second-stage juveniles (J2) of *H. schachtii* ("Straškov isolate"*)* in a greenhouse. The soil used for this experiment was autoclaved in advance. The experiment was maintained in greenhouse pots for approximately four months, and the cysts were extracted from the infested soil using the Fenwick can flotation method (Fenwick, 1940). Mature brown cysts were separated and ruptured between slides in the presence of water to obtain J2 for inoculation.

Fungi cultures

The fungi used for the experiment were *Arthrobotrys oligospora*, *Dactylella oviparasitica*, *Clonostachys rosea*, *Stropharia rugosoannulata*, and *Lecanicillium muscarium*. *Lecanicillium muscarium* was isolated from the egg masses of root knot nematodes collected from twenty fields of tomato, eggplant, and carrot, whereas the other fungi (*Arthrobotrys oligospora*, *Dactylella oviparasitica*, and *Clonostachys rosea*) were isolated directly from the soil using the soil dilution method from the locations of Semice (N 50.16265, E 14.89643) and Litol (N 50.18404, E 14.83742) in Central Bohemia, Czech Republic. The *Stropharia rugosoannulata* isolate was obtained from a company that commercially produces mushrooms, the Mycelium Wolf. Uprooted roots with simultaneous infection by nematodes and fungi were observed. Black-colored egg masses were associated with fungal infection. These egg masses were inoculated axenically onto Petri plates containing potato dextrose agar (PDA) amended with streptomycin at 1 g/L after surface sterilization with 0.5% NaOCl for 2 min (Singh and Mathur, 2010). The Petri plates were incubated at 25 ± 2°C for ten days. The fungal colonies were isolated and purified by repetitive culturing and were later identified morphologically, which was confirmed using the molecular technique polymerase chain reaction (PCR) and sequencing in the laboratory. The fungal DNA was extracted by drying the mycelium in liquid nitrogen, which was then crushed in a Petri dish using the DNeasy Blood & Tissue Kit (Qiagen). The universal primer for the amplification of cistron rDNA was used. All amplicons were sequenced, and the sequences were compared by BLAST algorithms (data not shown). All the tested fungi were then grown in potato dextrose broth and kept under room temperature on an orbital shaker. After two weeks, the fungal mycelia were harvested, and culture filtrates were prepared in distilled water by W/V and were standardized. Each isolate was replicated seven times, and the experiment was repeated once to authenticate our findings.

In-vitro study: *Effect of fungi on egg parasitism*

One milliliter of the filtrate from each fungus was poured onto the center of a 60 × 15 mm Petri dish (each dish had sixteen 10 × 10 mm square grid cells, which were marked numerically in one direction and alphabetically in the other) containing PDA medium. The plates were then uniformly spread with 100 eggs of *H. schachtii* and incubated at 25 ± 2°C for 3 days. Seven replicates were produced for each fungus, whereas the eggs without fungi were kept as controls for comparison. The effects of the fungi were evaluated after 24, 48, and 72 h, and the percent of parasitized eggs was measured by staining the eggs with cotton blue and counting them under a stereo-binocular microscope. Infected eggs with hyphae or disintegrated contents were declared as infected (Khan *et al*., 2006; Singh and Mathur, 2010). Eggs containing live J2 and that

had hatched J2 were counted as viable. The experiment was repeated once.

Greenhouse study

This experiment was carried out in a greenhouse at the Czech University of Life Sciences (CULS), Prague, Czech Republic, to investigate the effectiveness of fungi against nematodes on a susceptible variety of sugar beet, Alpaca. The soil was sterilized by autoclaving at 121°C for 30 min at 15 kPa. Pots of 500 cm^3 were filled with sterilized soil taken from the fields at CULS, and three seeds of sugar beet were sown per pot. The pots were irrigated to ensure that the soil did not become dry. The inoculation of fungi and nematodes was conducted at the two-leaf stage. Mycelia from all five fungi were harvested from the PDB media, weighed, and a standard solution (W/V) was prepared in distilled water. Twenty milliliters of 30% (W/V) solution for each fungus was pipetted onto the top of the soil in each pot. Two days after the fungal inoculation, 2000 freshly hatched J2 were introduced to each pot, whereas only J2 were applied to the control plants. The pots were placed in a completely randomized design (CRD) with seven replicates on a greenhouse bench. The pots were irrigated at two-day intervals throughout the growth period. The daily temperatures ranged between 25° and 28°C. The experiment was repeated once.

Data collection

After four months of growth, the plants were carefully removed from the pots, and their roots were cut from the shoots. The roots were gently washed, blotted dry, and weighed, and the length was also recorded. The number of cysts in the roots and soil was recorded. The total number of eggs and J2 constituted the total nematode population. The potential of nematophagous fungi was evaluated in terms of the nematode reproduction factor (Rf), where Rf is the final nematode population at harvest (Pf) divided by the initial nematode population (Pi). The number of eggs per cyst and the colonization of eggs and cysts by fungi were also measured under a high-resolution microscope. To assess the effects of the fungi, the percent increases and decreases in growth parameters compared to the controls were calculated (Irshad *et al*., 2012; Hussain *et al*., 2016) as follows:

$$\% \text{ reduction or increase } = \frac{\text{Un-inoculated - Inoculated}}{\text{Un-inoculated}} \text{ x100}$$

Statistical analysis

All experiments were repeated twice. All data from the experiments were subjected to analysis of variance (ANOVA). The means were compared by t-tests at $P = 0.05$ using the software Statistica 8.1.

Fig. 1. Production of prey device, acanthocytes by *S. rugosoannulata* and parasitism of J2.

RESULTS

In vitro *study*

In the laboratory experiment, *S. rugosoannulata* extensively parasitized *H. schachtii* eggs, followed by *L. muscarium and C. rosea*, whereas the other two fungi had moderate impacts. The effects of the *S. rugosoannulata* spores can be seen in Figure 1, showing that the acanthocytes sheared the nematodes while they were moving. *Lecanicillium muscarium* and *D. oviparasitica* grew more quickly on the media than the other fungi. A mat of mycelium was observed in the eggs during the examination under the microscope. Hyphae of *L. muscarium* and *D. oviparasitica* completely occupied the eggs and vacuolated after the consumption of the egg contents. The contents of the eggs were completely putrefied and decayed. The fungus *A. oligospora* quickly parasitized the eggs at 24 h or 48 h, but it later became less effective compared to the others (Table I). No eggs were found dead or parasitized in the controls. The lowest percentage (5%) of eggs hatched into J2 in the case of *S. rugosoannulata*, followed by *L. muscarium* (7%), *D. oviparasitica* (11%), *C. rosea* (13%) and *A. oligospora* (15%) compared to the controls. None of the fungi produced resting spores in the media. Cleared zones were found in the case of *L. muscarium* on media containing colloidal chitin, indicating that this fungus produced some active enzymes in culture.

Greenhouse study

The effectiveness of the five nematophagous fungi on *H. schachtii* reproduction and the growth of sugar beets was evident. A significant reduction ($P = 0.05$) in

the nematode population occurred in the fungus-treated plants compared deviation of fourteen replications; Means in each column with different letters differ significantly according to T. Test at $P = 0.05$ to that in the controls. A few cysts were recovered from the plants treated with *L. muscarium*. The reductions observed for the other fungi were also significant ($P = 0.05$) but less pronounced than those of *L. muscarium* (Table II). A significant reduction in the Pf/Pi occurred in all fungi treatments. The soil of the control plants had a significantly higher number of cysts (Table II) compared to the fungus-treated plants. The smallest number of eggs was recovered from the cysts collected from the pots treated with *L. muscarium* compared to the other treatments, and more than 95% of the cysts, containing 92% of the eggs, were found to be colonized with fungus and were not viable (Table III).

The lowest colonization rate of eggs and cysts by fungi occurred in plants treated with *C. rosea* (Table III). A significant reduction in eggs and J2 occurred in soil treated with *L. muscarium* followed by *S. rugosoannulata, D. oviparasitica*, *A. oligospora* and *C. rosea* compared to the controls. The lowest number of J2 per root system was recovered from *L. muscarium*-treated plants compared to

Table I.- Efficacy of fungi on viability of eggs of *Heterodera schachtii.*

Test Fungi	Percent egg parasitism at three time intervals		
	24h	48h	72h
A. oligospora,	25.4±1.17 a	42.8±0.92 a	65.8±1.02 c
D. oviparasitica	14.4±1.54 cd	27±1.41 b	63.2±1.36 cd
S. rugosoannulata	26.2±2.01a	46.2±2.65 a	83.8±3.34 a
C. rosea	11.8±0.86 c-e	32.2±5.20 b	66±2.28 c
L. muscarium	21.8±2.15 b	32.4±2.68 b	75.2±1.74 b
Control	00	00	00

*After revival in water; Data are presented as mean ± standard.

Table II.- Influence of nematophagous fungi on reproduction of *Heterodera schachtii,* 120-days after inoculation with an initial population density of 2000 J2 per plant.

Test Fungi	Cysts / 250g of soil	Egg / 250g of soil	J2 / root system	Eggs/cyst	J2/250g of soil	Pf/Pi*	Colonized cysts (%)	Colonized eggs (%)
L. muscarium	5.6±1.1ac	221±13.9a	1006.2±5.4a	127.0±12.5a	211.4±4.0a	1.43±0.17a	95.8±1.9a	92.4±3.9a
A. oligospora	8.2±0.8bc	265.4±9.7b	1130.4±4.4b	154.6±3.6b	240±7.9b	2.07±0.16b	90.8±2.4b	86±4.7bc
D. oviparasitica	9.2±1.1b	268±7.8b	1172.0±15.2c	173.4±3.4c	258.6±13.9c	2.43±0.20c	88.6±5.8b	84±3.5b
S. rugosoannulata	7±1.6c	280.4±3.4c	1243.4±11.5d	190.4±9.4d	231.4±18.4b	2.19±0.38bc	91.8±2.4b	90.8±1.8ac
C. rosea	9.4±1.1b	300.4±8.6d	1291.2±12.7e	189.8±6.5d	317.4±21.9d	2.75±0.20d	71.6±2.4c	73±1.6d
Control**	13.2±1.3d	502.2±9.0e	2144.0±9.6f	295.8±19.8e	455.4±4.2e	5.43±0.36e	00±00d	00±00e

Data are presented as mean ± standard deviation of fourteen replications. Values within a column followed by the same letter are not significantly different according to T.Test (two tail, equal variances) at $P = 0.05$.

*RF, Reproduction factor whereas "Pf" is final nematode population density divided by initial nematode population density (Pi).

**With nematode only.

Table III.- Influence of nematophagous fungi on plant growth of sugar beet, 120-days after inoculation of *Heterodera schachtii* with an initial population density of 2000 J2 per plant.

Test Fungi	Fresh shoot wt. (g)	Fresh root wt. (g)	Fresh root length (cm)	Fresh shoot length (cm)	% increase length		% increase weight	
					root	shoot	root	shoot
A. oligospora	16.85± 0.97c	19.28±2.1 e	7.28± 1.2cd	26.71± 1.3b	6±1.3c	36±3.4b	114±8.7c	87±11.2b
D. oviparasitica	24.42± 2.1b	31.42±2.5 b	10.00± 1.6ab	27.85± 1.9b	45±2.3b	42±3.9b	249±12.8b	171±13.2b
S. rugosoannulata	22.71± 1.9b	23.28± 1.9d	8.00± 0.9b-d	21.00± 1.4c	16±1.9c	7±1.2c	158±13.9c	152±17.9b
C. rosea	17.42± 1.2c	26.85± 1.8c	9.14± 0.8bc	27.14± 1.2b	33±2.3b	38±3.8b	198±14.3c	93±21.3b
L. muscarium	33.14± 1.2a	44.28± 3.5a	12.14±0.95 a	42.57± 2.3a	77±4.5a	117±7.9a	392±19.8a	268±24.5a
Control (nematodes)	9.00± 1.3d	9.00± 1.23f	6.85± 0.87d	19.57± 1.86d	-	-	-	-

Data are mean of fourteen replications; Means within a column followed by the same letter are not significantly different according to TTest (two tail, equal variances) at $P = 0.05$.

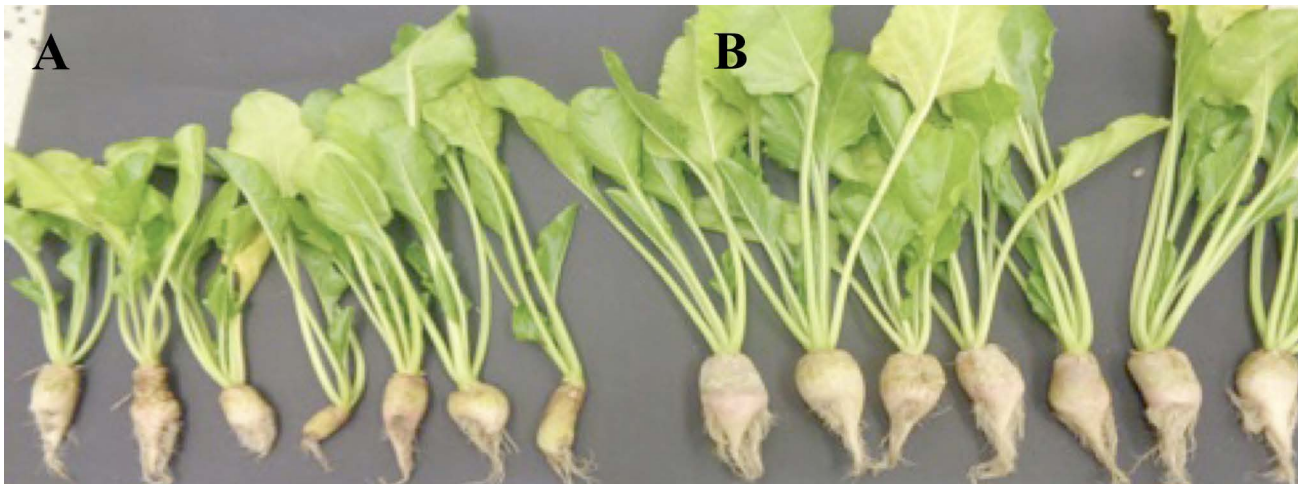

Fig. 2. Comparison of the growth between sugar beet plants treated with (A) Heterodera schachtii alone (control) and (B) together with nematophagous fungus, *Lecanicillium muscarium* and *Heterodera schahctii*.

all other treatments. Overall, the performance of all fungus-treated plants was excellent compared to their respective control plants. In regard to the plant growth parameters, all fungi played a significant role in improving plant growth. *Lecanicillium muscarium* showed a remarkable impact on increasing the root and shoot lengths by 77% and 117% and the weights by 392% and 268%, respectively, compared to the controls (Table III). *Arthrobotrys oligospora* also increased the plant root and shoot lengths and growth significantly but was the least significant compared to the other treatments and the controls (Table III, Fig. 2). Moreover, all fungi were observed to be more efficient in reducing the final population of nematodes and improving the plant growth factors.

DISCUSSION

The efficiency of five fungi against *H. schachtii* was evaluated in sugar beet. All fungi were declared as nematophagous fungi with the ability to control nematode population pressure in soil as well as in plant roots. Overall, the parasitic reaction of all fungi to *H. schachtii* was significant ($P = 0.05$), but some of the species proved to be more efficient in reducing the infestation level and improving plant vigor. During the *in vitro* examination, *S. rugosoannulata* was able to infect more eggs than the other fungal treatments, which could be due to its high production of traps, acanthocytes (Luo *et al*., 2006; Zouhar *et al*., 2013) and enzymes (Dong *et al*., 2006; Stadler *et al*., 2006; Yang *et al*., 2007) in media. The microscopic observations showed that the acanthocytes resemble sharp swords that might puncture the shell of nematode eggs and the cuticle of J2, resulting in the leakage of inner contents and desiccation. The infection of nematode eggs by fungi appeared to depend on mechanical force, which could be an important virulence factor for the parasitism of eggs and nematode juveniles (Luo *et al*., 2006). *Lecanicillium muscarium* ranked second, excellently infecting nematode eggs as well. Its effectiveness could be correlated to its high levels of conidia production as well as enzymatic action (Zhang *et al*., 2008). *Lecanicillium muscarium* was observed to produce more conidia in the media than all the other fungi, and conidia are the main propagules of this fungus. It also produces a mucilage matrix, which helps it to stick to eggs and J2 to facilitate its germination and penetration into the host (Veenhuis *et al*., 1985). An interesting observation was noted in the case of *A. oligospora*, which parasitizes eggs relatively more during 24 h and 48 h but less after 72 h. This potentially moderating effect might be associated with a short-term increase in the fungal populations (Bird and Herd, 1995). Overall, all fungi were shown to be a potential parasites of *H. schachtii* eggs in the laboratory experiments.

Plant roots provide a biological and chemical environment in the rhizosphere (Lynch, 1982; Persmark and Jansson, 1997). Several interactions occur among microbes near root zones due to the nutrients provided by root exudates and fragments. Among the microorganisms, fungi and nematodes are more prevalent in the rhizosphere than in root-free soil (Curl and Truelove, 1986). Moreover, nematophagous fungi are generally found in agricultural soils (Gary, 1988; Dackman *et al*., 1992; López-LIorca *et al*., 2007). They can live saprophytically in soil but prefer the parasitic form if appropriate hosts are available. The five fungi showed excellent results in the greenhouse studies, but *L. muscarium* had the most conspicuous effects on reducing nematode population growth and enhancing

plant growth compared to all other treatments. This might be due to the fast growth of this fungus, with its higher levels of its primary propagules, conidia, and secondary metabolites. These studies showed that *L. muscarium* also triggers the defensive system of plants when it infects the roots (Hirano *et al*., 2008). The maximum fresh plant growth was observed in *L. muscarium*-treated plants compared to that of the controls and the other treatments. The advantageous effects of this fungus over the others could be caused by multiple parasitic actions over a wide range of temperatures (5-30°C), with an optimum at 25°C (Fenice *et al*., 1996, 1997). The facultatively saprophytic fungus *C. rosea* significantly (P = 0.05) reduced the nematode Pf/Pi but ranked third in improving plant growth. Some studies have demonstrated that extracellular serine proteases isolated from *C. rosea* might be involved in the facilitation of egg and nematode cuticle penetration (Zhao *et al*., 2005; Li *et al*., 2006), as the cuticles of the eggs and nematodes consist mostly of protein (Clarke *et al*., 1967; Sugimoto *et al*., 2003). The nematode predacious fungi *A. oligospora*, *D. oviparasitica,* and *S. rugosoannulata* have to produce organs of capture (three-dimensional networks of hyphal loops produced by anastomosis, adhesive knobs or non-constricting rings, large spike cells, acanthocytes) (Fig. 1) to prey upon nematodes (Nensen *et al*., 1986; López-LIorca *et al*., 2007). These fungi played a role in suppressing the population in the soil, but they were not very effective in increasing plant vigor compared to the other species. The successful attachment and infection of nematodes by *A. oligospora* is also believed to be highly dependent on the adhesion capacity of the fungus (Nordbring-Hertz and Mattiasson, 1979). In our opinion, lower infection might have occurred due to less adhesive forces and a lower production of enzymes and prey devices. We suspect that this might have occurred due to the environmental conditions and the morphology of the plant roots. *Dactylella oviparasitica* and *A. oligospora* were appeared to be body guards of the plant roots, colonizing and protecting them from pathogen infection (Persson and Jansson, 1999). Furthermore, chemotropic growth of fungi occurred toward roots (Bordallo *et al*., 2002), and trap devices were triggered in response to the exudates released from the roots (Persmark and Nordbring-Hertz, 1997).

There is also evidence that nematodes are also attracted to fungi (Jasson and Norbring- Hertz, 1979; Jasson, 1982). Some studies have indicated that the endophytic colonization of roots by *L. muscarium* elicits systemic resistance in plants, but information is still lacking for other fungi (Hirano *et al*., 2008).

CONCLUSION

Heterodera schachtii densities were negatively correlated to the presence of nematophagous fungi. The ratio of the fungal population to nematodes in the rhizosphere showed that there was a strong antagonistic interaction involved. Nematodes may be controlled more effectively if multiform fungi with multiple actions are applied. The colonization of roots by nematophagous fungi provided protection against nematodes to plants. Furthermore, the nutrients provided for the saprophytic growth of fungi and nematodes in the rhizosphere should be taken into account and need further study.

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Conflict of interest statement

We declare that we have no conflict of interest.

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