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Comparative Virulence Assessment of Different Nematophagous Fungi and Chemicals against Northern Root-Knot Nematodes, *Meloidogyne hapla,* on Carrots

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MH designed experiments, collected and analyzed the data and wrote the article. MM helped in collection of data. MZ and PR planned the study and proofread the article.

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ABSTRACT

Nematodes are considered to be the main pests in vegetables and crops. The problem is being increased due to lack of our farmer's knowledge, repetition of the same crop in field, and non-awareness of pesticides applications. Ten isolates from seven different fungi, *Arthrobotrys oligospora*, *Dactylella oviparasitica*, *Clonostachys rosea*, *Stropharia rugosoannulata*, *Lecanicillium muscarium*, *Trichoderma harzianum* and *Pleurotus ostreatus*, along with two chemicals, Vydate and Basamid (G), were evaluated against northern root-knot nematodes, *Meloidogyne hapla,* on carrots in a greenhouse. All fungi and chemicals proved to be efficient in reducing the infestation level of *Meloidogyne hapla* and providing better growth of carrots compared to their controls. Maximum reductions in nematode population were observed in the plants treated with *Lecanicillium muscarium* and both chemicals. *Lecanicillium muscarium* treatments alone or with nematodes had significant ($P = 0.01$) positive effects on plant shoot and root growth among all other treatments in the experiment. After *L. muscarium* and the chemicals (Vydate and Basamid), *Stropharia rugosoannulata* ranked second in reducing the nematode numbers of galls, egg masses, and second-stage juveniles (J2) and rate of nematode reproduction (Pf/Pi) and improving plant growth factors.

INTRODUCTION

Soil-borne diseases are chiefly caused by bacteria, fungi and nematodes and are considered to be the main hindrance in the economics of many major crops. The estimated annual yields are 30 to 35% less than they could be in the absence of pests (Zechendorf, 1995). The world pesticide market in 1987 was valued at US $20 000 million (Zechendorf, 1995), of which the nematicides market's share was estimated to be US $500 million (Nordmeyer, 1992). The economic loss caused by nematodes is estimated to be US $100 billion worldwide, of which the United States alone shares almost US $6 billion (Nordmeyer, 1992).

Synthetic pesticides such as bactericides, fungicides, and nematicides have been successfully used to manage soil-borne plant pathogens (Hussain *et al*., 2017a, b). Although these pesticides seem to be the most economical and effectual means of controlling plant pathogens, environmental, toxicological, and sociological concerns have drastically reduced the availability of these competent commercial pesticides, especially nematicides. These restrictions have forced scientists and growers to look for an integrated management system that makes use of other means of disease control. This approach involves a mixture of agrotechnical, biological, chemical and genetic (breeding) means of control and is termed integrated pest management (IPM).

IPM is associated with proper inspection, authentic identification and virtuous treatment of pests. This approach is environmentally safe and pest-specific with limited persistence. Therefore, biological pest management is considered an important part of IPM. Moreover, extensive use of these synthetic lethal chemicals and insect pests has led to resistance against them, further resulting in environmental pollution and adverse effects on human health and other beneficial organisms. The demand for limited chemical inputs in the agriculture sector has provided us momentum for the evolution of alternate measures (Khan *et al*., 2012).

Currently, more than 700 species of nematophagous fungi have been illustrated that mainly belong to classes such as Ascomycota, Basidiomycota, Zygomycota and Chytridiomycota. Recently, a few species from class

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Oomycota have also been addressed. Furthermore, based on modes of action, nematophagous fungi are classified into four groups: nematode trapping (formerly called predatory fungi), endoparasitic, egg and female parasitic, and toxin-producing fungi (Barron, 1977; Dackman *et al*., 1992; Jansson and Lopez-Llorca, 2001). The successful interactions between nematophagous fungi and their hosts include numerous steps of recognition (attraction phenomena and contact), production of adhesives and lytic enzymes, and differentiation of infectious structures (appressoria and trapping organs) of nematode digestion (Tunlid *et al*., 1992).

Plant-parasitic nematodes are considered silent threats and dangers to several crops and vegetables worldwide, but sedentary endoparasitic nematodes, including *Meloidogyne* spp., *Heterodera* spp., and *Globodera* spp., are the most hideous pathogens that can inhabit roots for most of their life's intervals (Hussey and Grundler,1998; Renčo *et al*., 2012). Considering their feeding behavior and life cycle, it is challenging and precarious to control them with nematicides and microbial antagonists while they become established into host tissues (Stirling, 1991; Renčo *et al*., 2011). *Meloidogyne hapla* has been exposed as a flagrant vegetable pest in the Czech Republic over the past few years (Nováková and Zouhar, 2009). Due to the wide prevalence of this pest, the losses have reached 50 to 90% of the total crop (Nováková and Zouhar, 2009). More specifically, production losses for carrots as well as sparsley grown in the sandy soils of the Elbe lowland in the Czech Republic have also been reported by Douda *et al*. (2010).

The objective of our study was to assess and compare the virulence potential of different fungi against *M. hapla* among themselves and between synthetic nematicides under greenhouse conditions.

MATERIALS AND METHODS

Nematodes culture

Nematode galled roots of carrot plants from our greenhouse were collected, egg masses were isolated, and a single egg mass was used to establish a nematode culture (Hussain *et al*., 2016). Eggs were extracted from 90-day-old galled carrot roots using 0.05% NaOCl. Extracted eggs were gently washed with tap water to remove NaOCl (Hussey and Barker, 1973). *Meloidogyne hapla* species were identified based on perineal patterns (Eisenback, 1985). One thousand fresh extracted eggs from roots were used for greenhouse experiments.

Fungi culture

All fungi previously identified in our laboratory were grown on potato dextrose agar (PDA) for two weeks and then transferred to 500 ml flasks containing potato dextrose broth amended with streptomycin at 1 g/L. The flasks were kept under room temperature on an orbital shaker for almost four weeks. The solution from flasks was collected by staining the mycelia with cheese cloth and used for experiments. Twenty milliliters of each fungus was inoculated in each pot. The fungal isolates used in this study were *Arthrobotrys oligospora*, *Dactylella oviparasitica*, *Clonostachys rosea* 156, *C. rosea* 224, *Stropharia rugosoannulata* 5083, *S. rugosoannulata* 5131, *S. rugosoannulata* 5133, *Lecanicillium muscarium*, *Trichoderma harzianum* and *Pleurotus ostreatus.*

Nematicide application

The chemicals Vydate (active ingredient, Oxamyl) and Basamid (G) (active ingredient, Dazomet) were used at rates of 4.85 g/L and 2 g/L, respectively. The chemicals were weighed using a sensitive balance and mixed with sterilized soil. Chemical mixed soil was transferred to pots and left for one day in the case of Vydate and two weeks for Basamid (G) to avoid phytotoxicity (Hussain *et al*., 2017a, b).

Greenhouse setting

This experiment was carried out in the greenhouse of Czech University of Life Sciences, Prague, Czech Republic. To investigate the effectiveness of fungi against nematodes, a susceptible variety of carrot "Darina" was used. One carrot seedling aged two weeks was used and one week later inoculated simultaneously with fungi and nematodes eggs. Similarly, one seedling was also planted in each pot treated with chemicals. A total of 20 ml from each fungus was pipetted on top of the soil in each pot. The control treatments contained plants without nematodes and fungi, with nematodes only, with chemicals only, and with fungi only. Moreover, chemicals and fungi were also tested against nematodes in soil in the absence of plants. A treatment with only nematodes in soil was also included in the experiment. The pots were placed in a completely randomized design (CRD) with seven replications on a bench in a greenhouse. The experiment was repeated once. The pots were irrigated at two-day intervals throughout the study period. The daily temperature ranged between 25°C and 28°C. The whole experiment lasted three months, while the experiments with treatments with only soil and nematodes, soil with fungi and nematodes, and soil with nematodes and chemicals lasted one month.

Statistical analysis

Data from each experiment were subjected to analysis of variance (ANOVA). Means were partitioned by the least

significant difference (LSD) at $P = 0.01$ using the Statistica 8.1 software package.

RESULTS

The aim of the study was to compare the effectiveness of some potential fungi and commercially available nematicides. The effects of all fungi and nematicides were obvious in the reduction of gall and egg mass indices, J2 population, egg production per root system and rate of nematode reproduction, as indicated in Table I. If we divide all fungi into groups according to their effects, *Lecanicillium muscarium* ranked first with minimum number of galls (21 galls) and egg masses (18 eggmasses); *Stropharia rugosoannulata* isolates 5131, 5133, and 5083 second; and *Trichoderma harzianum* third, while the rest fungi (*Clonostachys rosea, Arthrobotrys oligospora* and *Pleurotus ostreatus*) ranked fourth. Moreover, the effects of *L. muscarium* in comparison to nematicide and Basamid (G) were not significantly ($P = 0.01$) different. Both were able to reduce the nematode galls, egg masses, egg production per root system, J2 population and nematode reproduction rate over the control. In addition, *L. muscarium* also had positive effects on the growth of plants regarding root shoot lengths and weights (Table I). The plants treated with other fungi also had better results on plant growth factors compared to the control treatments, while the minimum plant growth factors were observed in the plants treated with chemicals (Table I). The plants treated with fungi alone exhibited better growth than those treated with nematodes and chemicals. The maximum fresh root shoot lengths and weights were observed in the plants when only *L. muscarium* was inoculated. Overall, the chemicals and *L. muscarium* were aggressive against nematodes in soil, but the chemicals had some negative effects on plant growth, which can easily be seen in Table I. Moreover, no nematodes were recovered from the soil treated with only nematodes, with nematodes and fungi or with nematodes and chemicals in the absence of plants (Table I). The results from both experiments were quite similar except for a few treatments.

DISCUSSION

The parasitic activities of different fungi against plant-parasitic nematodes have been substantially studied by scientists around the globe as the whole world is interested in looking for alternative measures to manage soil-borne diseases, especially nematode and fungal diseases, instead of drastic chemicals (Cayrol *et al*.,1989; Saifullah, 1996; Zaki, 1999; Nicola *et al*., 2014; Hussain *et al*., 2017c, d). Although these synthetic chemicals improve yield and production by controlling insect pests, they also exert negative effects on the environment and life on earth (Hussain *et al*., 2017c). Therefore, alternative strategies are being introduced by researchers to combat this problem. Based on parasitic performance, all tested fungi were grouped into four different categories. In the first category, *L. muscarium* ranked first, as it tremendously reduced the number of galls, egg masses, J2 population, egg production per root system and rate of nematode reproduction compared to their respective controls. The enormous effects of *L. muscarium* could be due to its multiple ways of action. The activity of *L. muscarium* could be correlated with the production of its very sharp and fine hyphae to puncture the cuticle of nematode eggs mechanically; also, enzymes, specifically chitinases, help in the maceration of egg shells and rupturing of J2s (Zhang *et al*., 2008). Moreover, fungi have been proven to stimulate induced resistance in plants (Hirano *et al*., 2008). In comparison to chemicals, *L. muscarium* had somewhat similar effects, which strongly suggested that *L. muscarium* could be a better candidate than lethal chemicals for controlling nematodes (Hussain *et al*., 2017d). In addition, *L. muscarium*-treated plants have greater root shoot weights and lengths than those of plants treated with other fungi (Hussain *et al*., 2018) and chemicals (Hussain *et al*., 2017b, e), which also led us to study it further. It has also been documented that *L. muscarium* works well at a wide range of temperatures (5-30°C), with an optimum temperature of 25°C (Fenice *et al*., 1996, 1997; Hussain *et al*., 2017). The second category comprised isolates from the fungus *Stropharia rugosoannulata*. This fungus also produced remarkable effects in reducing the nematode infestation level in soil (Hussain *et al*., 2017c). Microorganisms produce toxic metabolites, such as antibiotics, to prevent other microorganisms from competing for nutrients and space in ecological niches. Similarly, toxin-producing basidiomycetous fungi such as *Stropharia rugosoannulata* also have the ability to attack nematodes through their hydrolytic enzymes and metabolites (Dong *et al*., 2006; Stadler *et al*., 2006). The pedantic modes of action of these compounds against nematodes are still unknown. The nematicidal mode of *Stropharia rugosoannulata* has been reported by Luo *et al*. 2006, 2007. It produces special nematode-attacking devices: three-dimensional acanthocytes (Fig. 1) resemble a very sharp sword that could damage the nematode cuticle, leading to the leakage of inner materials of nematodes. Many studies have shown that the main virulence factors of this fungus are mechanical force and toxin production (Luo *et al*., 2006). The third category consists of *Trichoderma harzianum*, which has also been used as a biocontrol agent not only against plant parasitic nematodes but also against

Table I. Influence of nematophagous fungi to *Meloidogyne hapla* reproduction and plant growth parameters of carrot in greenhouse, 90-days after inoculation with an initial population density (Pi) of 1000 eggs per plant.

Treatments	No. of galls	No. of egg masses	No. of eggs per root system	J2 per 100 cm^3 of soil	Nematode reproduction rate*	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
Trichoderma harzianum +nematodes	33.5d	31.2c	2255f	2120cd	6.4d	26ab	24.2bcde	15.2abcde	10.4abc
Lecanicillium muscarium+ nematodes	21f	18.5f	1260g	829f	2.6e	28.8a	28ab	17.2abc	11.7a
Stropharia rugosoannulata. 5131+ nematodes	30.2de	27.7d	3346de	2011de	6.6d	26.4ab	26.1abc	16abcd	11ab
Stropharia rugosoannulata .5133+ nematodes	29.7e	25.4de	3300de	1979de	6.5d	25.8ab	25.8abcd	15.4abcde	10.5abc
Stropharia rugosoannulata .5083+ nematodes	29e	24e	3187e	1888e	6.3d	25abc	25abcde	15.1abcde	10.1bcd
Clonostachys rosea. 156+ nematodes	40.7bc	37b	3757bc	2328b	7.6bc	25.5ab	22.8cdef	14.8bcde	9.7bcdef
Clonostachys rosea. 224+ nematodes	43.2b	39.1b	3883b	2398b	7.9b	25.2ab	22cdefg	14.5bcde	9.4cdefg
Pleurotus ostreatus+ nematodes	42.4bc	38.5b	3714bc	2398b	7.8bc	24.7bc	21.7cdefgh	14.2bcdef	9.8bcde
Arthrobotrys oligospora 5/10+ nematodes	39.7bc	36b	3589bcd	2326b	7.5bc	24bc	21.1efghi	13.8cdefg	9.5bcdefg
Dactylella oviparasitica+ nematodes	38.8c	36b	3522cd	2276bc	7.4c	22.7bc	19.4fghij	11.8efgh	9.2cdefgh
Nematodes+ plants+soil	78.8a	70a	5707a	3756a	12.2a	15.8efg	17.1hij	5.7k	4.7m
Plants+ *Lecanicillium muscarium*	0h	0h	0j	0h	0h	22.8bc	29.5a	19.1a	10.4abc
Plants + *Trichoderma harzianum*	0h	0h	0j	0h	0h	18.7de	26.2abc	18.2ab	9.5bcdefg
Plants+ *Stropharia rugosoannulata*. 5131	0h	0h	0j	0h	0h	18def	24.4bcde	17.4abc	8.8defghij
Plants+ *Stropharia rugosoannulata* .5133	0h	0h	0j	0h	0h	17efg	21.4defghi	16.7abcd	8.4efghijk
Plants+ *Stropharia rugosoannulata* .5083	0h	0h	0j	0h	0h	15.4efg	21efghij	16.4abcd	8.2fghijk
Plants+ *Clonostachys rosea. 156*	0h	0h	0j	0h	0h	15.1efg	20.7efghij	15.7abcde	8.1ghijk
Plants+ *Clonostachys rosea*. 224	0h	0h	0j	0h	0h	14.8efg	19.2fghij	15.1abcde	7.8hijk
Plants+ *Pleurotus ostreatus*	0h	0h	0j	0h	0h	14.2fg	18.8fghij	14.5bcde	7.7ijk
Plants+ *Arthrobotrys oligospora* 5/10	0h	0h	0j	0h	0h	13.8gh	17.4ghij	14.1cdef	7.4jkl
Plants+ *Dactylella oviparasitica*	0h	0h	0j	0h	0h	13.4gh	16.8ij	13defg	7.1kl
Only plants +soil	0h	0h	0j	0h	0h	10hi	21.7cdefgh	8.2hijk	6.1lm
Nematodes+ soil	0h	0h	0j	0h	0h	0j	0k	0l	0n
Nematode +fungi**	0h	0h	0j	0h	0h	0j	0k	0l	0n
Nematode+ Vydate	0h	0h	0j	0h	0h	0j	0k	0l	0n
Nematode+ Basamid(G)	0h	0h	0j	0h	0h	0j	0k	0l	0n
Plants+ only Vydate	0h	0h	0j	0h	0h	7.1i	17.4ghij	6.4ijk	5.2m
Plants+ only Basamid (G)	0h	0h	0j	0h	0h	6.8i	16.4j	6.2jk	4.9m
Plants +nematodes+ Vydate	13g	8g	477i	564g	1.6g	26ab	19.4fghij	10.4fghi	9.1cdefghi
Plants+ nematodes+ Basamid (G)	18f	17f	854h	709fg	2.2f	21.2cd	18.5fghij	9.8ghij	9.2cdefgh

[1]Gall and egg mass indices: 0-5 scale; where 0: no galls or egg masses; 1: 1-2 galls or egg masses; 2: 3-10 galls or egg masses; 3: 11-30 galls or egg masses; 4: 31-100 galls or egg masses and 5: > 100 galls or egg masses per root system (Quesenberry *et al*., 1989); [2]*Rate of reproduction: Pf/Pi (Final Population / Initial Population*); [3]Means with in a column sharing the same letter are not significantly different from each other at *P: 0.01* according to Least significant difference; [4]**The data were merged from all treatments with fungi and nematodes.

soil-borne, foliar, and postharvest phytopathogenic fungal pathogens (Chet, 1987; 1990). Moreover, it has also been proven that *Trichoderma* promotes plant growth (Inbar *et al*.,1994) and has the ability to colonize root surfaces and the cortex (Kleifeld and Chet, 1992; Yedidia, 1999), which provides a protecting shield against second-stage juveniles (J2). Reduction of egg production has also been reported by Windham *et al*. (Windham *et al*., 1989), which strengthens our studies.

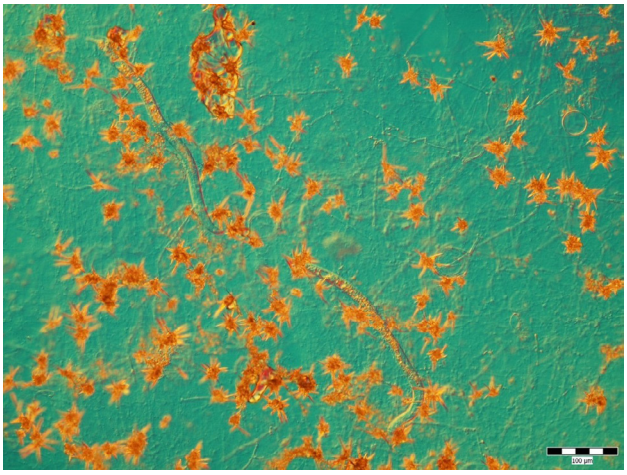

Fig. 1. Nematode juveniles captured by specialized three-dimensional attacking devices, acanthocytes produced by *Stropharia rugosoannulata*

Trichoderma involves different biocontrol mechanisms, such as antibiosis, competition, mycoparasitism and enzymatic hydrolysis (Elad, 1995; Sivan and Chet, 1992). Enzymes such as chitinases, glucanases, and proteases play a vital role in parasitism (Haran *et al*., 1996). A mechanism of induced resistance has recently been investigated by scientists, and evidence for defense responses induced by *T. harzianum* has been presented (Yedidia *et al*., 1999). In addition to competition, other mechanisms may potentially be involved in the nematode biocontrol process. The information related to the activity of this fungus is still very limited and needs to be investigated further for the development of improved biocontrol methods. During *in vitro* studies, Saifullah and Thomas (1996) observed the direct interaction of the fungus in potato cyst nematode, *Globodera rostochiensis*, in which the fungus successfully penetrated cysts and eggs to kill larvae inside. Furthermore, *Trichoderma* can provide better control in soil than in roots (Sharon *et al*., 2001), and the processes of anti-nematodes could be suggested: the metabolites produced by fungus in soil and direct parasitism.

The fourth category contained *Clonostachys rosea, Arthrobotrys oligospora* and *Pleurotus ostreatus*. *Clonostachys rosea* is associated with parasitic fungi, *Arthrobotrys oligospora* line up with trap fungi, while *Pleurotus ostreatus* is grouped into saprophytic fungi. In our study, less activity of *C. rosea* compared to other fungi might be correlated with its feeding behavior. *C. rosea* is a parasitic fungus, and this category cannot produce trap devices for J2s and eggs of nematodes. Egg shells could be a barrier for this fungus (Khan *et al*., 2004), but to establish an effective parasitic relationship between nematodes and parasitic fungi, the appressoria of *C. rosea* and *D. oviparasitica* must be affixed to the surface of nematode eggs or J2s (Jansson and Lopez-Llorca, 2001). The involvement of mucilaginous material during attachment of appressoria to the surface of eggshell was observed to serve as an adhesive to facilitate eggshell penetration by the fungus (Lopez-Llorca and Claugher, 1990; Stirling and Mankau, 1979). In addition, several extracellular enzymes such as serine proteases have been reported in nematophagous fungi that assist with successful penetration into nematodes. For example, two pathogenic proteases (PII and VCP1) were identified from *A. oligospora* by Tunlid *et al*., 1994 and Aoz1 was identified by Zhao *et al*., 2004. Some more proteases, Mlx, PrC, and Ds1, were identified in *L. psalliotae* (Yang *et al*., 2005), *C. rosea* (Li *et al*., 2006), and *Dactylella shizishanna* (Wang *et al*., 2006), respectively. All of these proteases were involved in nematode parasitism in assisting the hydrolytic activity and binding of the enzymes to the cuticle surface of nematodes and insects (St. Leger *et al*., 1986; Wang *et al*., 2006).

Additionally, no nematodes were retrieved from the pots to which only nematodes were applied, which suggested that nematodes died due to starvation and that nematodes are obligate in nature and always need host to proliferate and reproduce well in soil (Agrios, 2005). In the pots in which nematodes and chemicals were applied together, no nematodes were retrieved at all, possibly due to either the effectivity of chemicals or the absence of a host for nematodes to reproduce and to avoid starvation. Similar results were found for fungi. The plants treated with only fungi seemed to be healthy and grew well compared with those treated with chemicals. Based on our data, we concluded that *L. muscarium* and *S. rugosoannulata* could be potential candidates for replacing chemicals and improving soil health and overall production.

CONCLUSION

Based on our data, it is concluded that *L. muscarium*

and *S. rugosoannulata* are potential candidates for the management of nematodes and could be included as integrated pest management (IPM) in replacement to lethal chemicals.

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Statement of conflict of interest

The authors declares that there is no conflict of interests

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