Pakistan J. Zool., vol. 49(1), pp 215-220, 2017. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2017.49.1.215.220

Comparison between Biological and Chemical Management of Root Knot Nematode, *Meloidogyne hapla*

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ABSTRACT

These studies were employed to compare the potential of the nematophagous fungus *Lecanicillium muscarium* and the chemical nematicides Vydate and Basamid (G) with the fertilizer calcium cyanamide in the control of root knot nematode, *Meloidogyne hapla*, through soil application under greenhouse conditions. A significant (P=0.05) reduction in the number of galls and egg masses, eggs and juveniles (J2), and the reproduction factor (Rf=Pf/Pi) was observed in the plants treated with *L. muscarium* compared to the control. However, all chemical nematicides were also effective in reducing the nematode infestation significantly (P=0.05) in soil compared to the control, but there was no significant difference (P=0.05) among them. Plant growth was significantly (P=0.05) improved in the plants treated with *L. muscarium*.

Article Information
Received 04 August 2016
Revised 20 August 2016
Accepted 30 August 2016
Available online 18 December 2016

Authors' Contributions
MH designed experiments, collected data and wrote article, MZ and PR advised the study and proofread article.

Key words
***Lecanicillium muscarium*, Nematicides, *Meloidogyne hapla*, Plant growth, Reproduction factor**

INTRODUCTION

Plant parasitic nematodes are among the most notorious crop pests and are difficult to control due to their short and productive life cycle. Management of nematodes has been accomplished through plant resistance, crop rotation, cultural practices and chemical nematicides. Among known chemical treatments, two prevailing groups of low molecular weight soil fumigants and contact carbamates, or organophosphates, have the most conspicuous effects (Bakker, 1993; Whitehead, 1997). The development of new nematicides is the most challenging task of this era because several of the most economically important phytoparasitic nematode species stay in the soil in protective cysts or within plant roots. Since the targets of chemical nematicides mostly remain away from the actual site of application, adequate chemical nematode control may not be achieved more successfully (Chitwood, 2002). Furthermore, some nematodes produce special cysts that have cuticles impermeable to many organic molecules (Chitwood, 2002). Most nematicides are notoriously toxic or volatile with poor target specificity and are extremely hazardous to animals, humans, and the environment, causing problems such as groundwater contamination or ozone depletion (Thomas, 1996; Chitwood, 2002). Moreover, few chemical nematicides are available, and most of them have deficient broad spectrum efficacy to control soil borne disease. Currently, several research groups are struggling to develop chemical nematicides that control nematodes, although the chemicals have negative effects on plant health. In addition to chemicals, organic or green manure is also being used to mitigate the nematode population below the soil surface (Renčo *et al.*, 2007; Renčo and Kováčik, 2012, 2015). Certainly, the incorporation of organic residues enhances the physical and biological properties of soil by promoting an environment favorable to nematode antagonistic microorganisms (Stirling, 1991). Crops, mainly rapeseed or canola, neem and marigold, are used in crop rotation or manuring (Halbrendt, 1996; Brown and Morra, 1997). Additionally, alternate management strategies, including soil solarization, biofumigation, mycorrhization, fallowing land, crop rotation, and host plant resistance, are also taken into account (Renčo *et al.*, 2012), but these measures do not achieve the success rate growers expect. Based on the limitations of chemicals and other strategies, there is interest in controlling plant parasitic nematodes through nematophagous fungi, which belong to a group of organisms with the ability to infect and parasitize nematodes for nutrients and survival. Currently, more than 700 species of nematophagous fungi have been described. These fungi belong to the Ascomycota, Basidiomycota, Zygomycota and Chytridiomycota classes. Recently, a few species from class Oomycota have also been reported.

Furthermore, based on modes of action, the nematophagous fungi are categorized into four groups: nematode trapping (formerly called predatory fungi), endoparasitic, egg and female parasitic, and toxin producing fungi (Barron, 1977;

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0030-9923/2017/0001-0215 $ 9.00/0

Dackman *et al.*, 1992; Jansson and Lopez-Llorca, 2001). The interactions between nematophagous fungi and their hosts comprise various 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).

The phytochemicals with potential nematicidal properties are also derived from different parts of plants and used against nematodes, but information related to their methods and application is still lacking (Akhtar and Mahmood, 1994; Renčo, 2013). Methyl bromide is extensively used by farmers in tunnel farming, but it has been implicated in the depletion of the ozone layer because it has high ozone depleting potential (Bell *et al.*, 1996; Ristaino and Thomas, 1998).

Registration of new chemicals is an immense hurdle for a prospective control of nematode diseases. Based on high performance during our previous studies, *L. muscarium* was selected and planned to investigate its comparative potential with chemical nematicides against economically important plant parasitic nematodes, *M. hapla*. Our main aim is to examine comparative potential of fungus, *L. muscarium* with hazardous chemicals against plant parasitic nematodes to avoid their high consumption in fields.

The European Community (EC) is more concerned with regulating the consumption of methyl bromide, as it depletes the ozone layer gradually. The EC, under regulation No. 2037/2000, covers the production, import, export, marketing and usage of methyl bromide. In 2005, the EC was able to reduce the so-called critical units (CUs) to 76 in 10 countries, while there were five CUs in two countries in 2008 and zero in 2009. Moreover, in 2005, 128 licensed fumigators were given permission to use methyl bromide in ten countries, whereas in 2007, there were 57 in five countries and zero in 2009 (European Commission, 2006; European Initiative, 2008). Keeping in mind the lethal activity of methyl bromide, different combinations of fumigants and non-fumigants were used as alternatives, such as metham-sodium, which is rapidly converted to methyl isothiocyanate while in contact with moist soil (Hague and Gowen, 1987). Nematode control by metham-sodium has been declared to be non-consistent or marginal (Haglund, 1999), and the ineffectiveness is attributed to its mode of action. The adoption of cultivars resistant to nematodes is a possible means of replacing lethal nematicides, but high soil temperature has led to a breakdown of this resistance (Netscher and Sikora, 1990; Tzortzakakis and Gowen, 1995; Giannakou and Karpouzas, 2003). The chemical nematicide Vydate (active ingredient, oxamyl) has the tendency to degrade rapidly in soil and become extremely toxic to nematodes while in contact. It also remains as residual accumulation in food and is poisonous to birds, humans, and animals when swallowed or in contact (Wagenet, 1985; Hayes and Laws, 1990; Meister, 1991; Witt, 1985). Basamid (active ingredient, dazomet) is a fumigant that is able to sterilize soil and kill the nematodes through gaseous degradation. It is used as an alternative to methyl bromide. The fertilizer calcium cyanamide is also used to kill nematodes (Cornforth, 1971; D'Addabbo and Sasanelli, 1998) and plays a role in enhancing plant growth.

Registration of new chemicals is a large hurdle for the prospective control of nematode diseases. Based on its high performance during our previous studies, *L. muscarium* was selected, and its potential effectiveness was compared with that of chemical nematicides against economically important plant parasitic nematodes, *M. hapla*. Our main aim is to examine the potential use of the fungus *L. muscarium* against plant parasitic nematodes compared with the effectiveness of hazardous chemicals to avoid their high consumption in fields.

MATERIALS AND METHODS

Nematode culture

Nematode galled roots of carrot plants were collected from the greenhouse of the Czech University of Life Sciences, Prague. A single egg-mass was used to establish a nematode population. The eggs were extracted from 70-day-old carrot roots using 0.05% sodium hypochlorite for 2 min (Hussey and Barker, 1973). The extracted eggs were gently washed with running water to remove excessive NaOCl. *Meloidgyne hapla* was identified based on morphological and morphometric characteristics (Eisenback, 1985). Extracted eggs were stored at room temperature (25˚C) to be used for experiments.

Fungal cultures

Lecanicillium muscarium previously isolated from the egg masses of *M. Hapla* were collected from the localities of Semice (N 50.16265, E 14.89643) and Litol (N 50.18404, E 14.83742) in Central Bohemia, Czech Republic. The tested fungus was cultured on Potato Dextrose Broth (PDB). Fungal mycelia were harvested from PDB media, weighed, and a standard solution (W/V) was prepared in distilled water. A 20 ml sample of the 30% (W/V) fungus solution was pipetted on top of the soil in each pot.

Nematicides applications

The chemicals Vydate (active ingredient, oxamyl), Basamid (G) (active ingredient, dazomet) and calcium cyanamide (fertilizer) were used at the rate of 4.85 g/L, 2 g/L and 5 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, two weeks for Basamid (G), and three weeks for calcium cyanamide to avoid phytotoxicity.

Experimental protocol

The experiment was installed in a greenhouse under controlled conditions with a temperature range of 25-27˚C. Two-week-old seedlings were transplanted to plastic pots (volume 500 cm^3) with one seedling per pot. A 20 ml sample of the 30% (W/V) cultured fungus filtrate of was pipetted on top of the soil around the root zone. The experiment pots were placed on the greenhouse bench in a completely randomized design. Two treatments of nematicides, one treatment of fertilizer, and one treatment of fungi were used, each replicated five times, and the experiment was repeated once. Freshly hatched eggs were introduced at a rate of 2000 eggs per pot. The plants treated only with J2 served as the control. Experimental plants were allowed to grow for 70 days.

Data collection

After 70 days, the carrot plants were carefully uprooted from the pots, and their roots were clipped from the shoots. The roots were gently washed and blotted dry. Fresh root shoot weight and length were noted. The number of galls was counted, root systems were stained by Phloxin B, and egg masses were counted under a stereomicroscope at 40X magnification. The total number of eggs and nematodes in the soil and root system were counted and comprised the total nematode population. The reproduction factor was calculated by dividing the final total population by 2000, Pi.

Data analysis

Data from all experiments were pooled and subjected to ANOVA tests. Means were partitioned by the T-Test using the Statistica 8.1 software package.

RESULTS

The purpose of the study was to compare the potential of *L. muscarium* to the effectiveness of commercially available chemical nematicides and fertilizer. The effects of nematicides and fertilizer were more pronounced in the reduction of gall and egg mass indices, J2, and egg production per root system when compared to *L. muscarium* (Table I) and the control. While all chemical treatments were able to reduce gall and egg mass indices, J2 population, and egg production, necrotic lesions were observed during treatment with dazomet. *L. muscarium* not only produced excellent effects on controlling the nematode population but also had remarkable effects on plant growth, which are shown in Table II. However, the suppression of the nematode population when using fungus was less pronounced than when using nematicides. The subduing effects on nematode population densities were statistically identical among nematicides. Although all nematicides reduced nematode infestation rates in soil, none of them proved to be aggressive. In regards to plant growth parameters, maximum fresh root shoot weight and length were improved with *L. muscarium* compared to all other treatments. Fresh shoot weight (g) obtained from *L. muscarium* was significantly higher among all treatments, while the fresh shoot weight from nematicides and fertilizer was not significantly (P= 0.05) different from that of the control (Table II).

Table I.- Comparative effects of nematophagous fungi, *L. muscraium* and nematicides on *Meloidogyne hapla* reproduction on carrot in terms of root galling, egg mass and reproduction factor in the greenhouse, 70-days after inoculation with an initial population density (Pi) of 2000 eggs per plant.

Fungi/Treatments	Galls/ root system	Egg masses/ root system	Egg production/ root system	J2 production/ 100cc of soil	Reproduction factor ** (Pf/Pi)
L. muscarium	10 ± 1.2 a	7.8 ± 0.8 a	2260 ± 233 a	744 ± 12 a	2.99 ± 0.11 a
Vydate	5.4 ± 1.1 b	4.2 ± 0.8 b	1550 ± 79 b	179 ± 17 b	1.22 ± 0.06 b
Basamid (G)	5 ± 1.2 b	3.8 ± 1.3 b	1350 ± 146 c	175 ± 13 b	1.11 ± 0.06 c
Calcium cyanamide	4.6 ± 0.5 b	3.4 ± 1.1 b	1341 ± 173 c	168 ± 15 b	1.09 ± 0.11 bc
Control	35.6 ± 1.8 c	30.8 ± 0.8 c	9090 ± 317 d	2016 ± 51 c	9.58 ± 0.10 d

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

*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).

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

Table II.- Comparative effects of nematophagous fungi, *L. muscraium* and nematicides on plant growth parameters 70-days after inoculation with an initial population density (Pi) of 2000 eggs per plant.

Fungi/ Treatments	Fresh shoot wt. (g)	Fresh root wt. (g)	Fresh root length (cm)	Fresh shoot length (cm)	% increase of shoot wt.	% increase of root wt.	% increase of root length	% increase of shoot length
L. muscarium	18.3 ± 0.7 a	47.4 ± 1.1 a	10.58 ± 0.9 a	47.6 ± 1.1 a	77.6	404	120	77.6
Vydate	16.2 ± 0.8 b	44.6 ± 1.5 b	9.38 ± 0.9 ab	40.2 ± 2.6 b	572	374	95.4	50
Basamid (G)	15.6 ± 1.1 b	43.4 ± 1.8 bc	9.14 ± 0.8 b	38.6 ± 1.5 b	51.4	361	90.4	44
Calcium cyanamide	15 ± 1.4 b	40.6 ± 2.3 c	8.94 ± 0.9 b	37.2 ± 1.4 b	45.6	331.9	86.2	38.8
Control	10.3 ± 0.8 b	9.4 ± 1.1 d	4.8 ± 0.5 c	26.8 ± 0.8 c	-	-	-	-

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

DISCUSSION

Due to an increasing usage of chemicals in growers' fields, our health and environment are at risk. These chemicals improved yield by controlling insect pests but also damaged life on earth. Therefore, alternative strategies are of increasing concern in this modern era. All the nematicides had a remarkable impact on the reduction of nematode infestation, but Vydate was significantly able to kill nematodes the most effectively and promoted improved growth of plant shoot and root. This result might be due to the mode of action of Vydate, which is a broad spectrum systemic insecticide and nematicide (Mcgarvey *et al.*, 1984). Oxamyl, the active ingredient of the nematicide Vydate, has been known to be effective in controlling the invasion of roots by juvenile nematodes (Radewald, 1970). Vydate has the capacity to translocate within roots and to the upper parts of plants (Radewald, 1970; Atilano and Van Gundy, 1979; Potter and Marks, 1976). It also provides protection against other pathogens, which has been proven by its presence in plant shoots (Harvey *et al.*, 1978). Nematicides containing carbamate, even at low concentrations, affect the movement and orientation of nematodes toward host roots rather than killing them (Wright, 1981). However, nematicide concentration, dispersal, and depth of application is relatively important to killing a nematode population. Dazomet was the second most efficient treatment, significantly reducing nematode infestation levels in soil compared to other nematicides and an untreated control. Dazomet is a successful multi-fumigant that is degraded into dithiocarbamic acid, which in turn decomposes into methyl isothiocyanate, formaldehyde, hydrogen sulfide and methylamine (Roberts and Hutson, 1999). These byproducts enable a further reduction in nematode density and gall and egg mass indices. Calcium cyanamide, while effective in reducing the number of galls and egg masses with a reduced nematode multiplication rate, was comparatively less efficient.

Our study validates the results from Dickson (1998) and Giannakou and Karpouzas (2003). Our results suggest that no nematicide except oxamyl could efficiently diffuse within roots and kill nematodes living within galls. This is probably one of the reasons why oxamyl has been the most efficient nematicide for the last several years. However, *L. muscarium* had a large impact, reducing the nematode reproduction rate up to 70% that of the control, which is a great success for a biocontrol agent. *Lecanicillium muscarium* also effectively diminished juvenile penetration, which resulted in a reduced number of galls and egg masses. Furthermore, the size of galls was also reduced with a minimum number of egg masses, which indicated nematode static effects (McGarvey *et al.*, 1984). The efficacy of *L. muscarium* may correlate with temperature, producing high levels of infection propagules, conidia, and enzymes across a wide range of temperatures (5-30°C), with an optimal temperature of 25°C (Fenice *et al.*, 1996, 1997). There is also evidence that nematodes are attracted to fungi (Jasson and Norbring- Hertz, 1979; Jasson, 1982). Studies have also demonstrated the endophytic colonization of *L. muscarium* by roots eliciting systemic resistance (Hirano *et al.*, 2008).

CONCLUSION

Although chemical nematicides, fertilizer and *L. muscarium* had excellent effects on reducing nematode populations, the actual mechanism is still not fully understood and needs to be further examined. Furthermore, metabolites of chemical nematicides that have biological activity against nematodes and the formulation of *L. muscarium* for field application must also be identified.

ACKNOWLEDGEMENTS

Authors are grateful to Czech University of Life Sciences, Prague for provision of funds for this piece of research under CIGA project number PROJ201500056.

Conflict of interest statement

We declare that we have no conflict of interest.

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