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Population Dynamics of a Nematophagous Fungus *Lecanicillium muscarium*, and Root Knot Nematode, *Meloidogyne incognita* to Assess the Disease Pressure and its Management

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***Lecanicillum muscarium*, *Meloidogyne incognita*, Reproduction factor, Tomato, Nematode population.**

ABSTRACT

A series of experiments were conducted in a greenhouse to determine the population dynamics of the root knot nematode, *Meloidogyne incognita*, and the nematophagous fungus, *Lecanicillium muscarium*, on tomato. The nematode population densities were measured by determining the number of galls and egg masses, juveniles and eggs per root system, and reproduction factor. Four initial populations (Pi) (500, 1000, 1500, and 2000 eggs) mixed with antagonistic fungal conidia levels (1×10^3, 1×10^4, 1×10^5, 1×10^6) were used during the experiments. The roots were stained with Phloxine B, the egg-masses were quantified, the root systems were rated for galling, and the egg masses were measured on a 0 to 5 scale, where 0 = no gall or egg masses, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 galls or egg masses per root system. The nematode reproduction rate (Pf/Pi, where Pf = final nematode population / initial nematode population) decreased proportionately with the increased initial fungal conidial Pi. Foliage growth was directly related to fungal Pi and inversely to nematode Pi. Our results showed that the higher Pi of *L. muscarium* was associated with the lower Pf of *M. incognita*. Foliage growth increased with increased fungus inoculum.

INTRODUCTION

Plant parasitic nematodes constitute the most abundant and successful animal phylum (Boucher and Lambshed, 1994) and cause great economic losses to agricultural crops and forestry worldwide (Sasser and Freckman, 1987; Siddiqui and Mahmood, 1996; Li *et al*., 2007; Zasada *et al*., 2008; Anwar and McKenry, 2010; Renčo *et al*., 2012; Renčo, 2013). Among plant parasitic nematodes, the root knot nematodes (*Meloidogyne* spp.) are considered the most dominant and extensive plant pathogens, and they attack a wide range of host plants including field crops, vegetables, fruit trees and ornamentals (Regaieg *et al*., 2010). In total, 2000 host plant species are prone to nematode infection, which causes an approximate 5% loss worldwide (Hussey and Janssen, 2002). The damage to the global crop production due to root knot nematodes is estimated to be approximately US$ 80 billion annually (Rodriguez and Canullo, 1992; Li *et al*., 2007). Root knot nematodes are sedentary endoparasites, polyphagous and considered a silent threat to agriculture. *Meloidogyne* species such as *M. incognita* and *M. javanica* are considered the most limiting factors for vegetative growth as well as harvest yield. The nematode second stage juvenile penetrates into plant roots and after a cascade of changes leads to successful infection by generating giant cells that ultimately result in root galls (Sharon *et al*., 2001). The galls reduce plant nutrient absorption, causing the plant with impaired growth to become stunted and chlorotic (Ellis *et al*., 2008). Root knot nematodes are not easy to control as they have a wide range of hosts, very short generation times, high production rate and endoparasitic nature (Manzanilla-Lopez *et al*., 2004). Concerns exist for the ecological and human health hazards as well as the breakage of resistance in cultivars due to the emergence of new pathogen races, and the usage of notorious chemicals is being restricted (Zuckerman and Esnard, 1994). Scientists are struggling to find other ways to get rid of these lethal chemicals that are carcinogenic.

The use of beneficial or antagonistic microorganisms is an alternative strategy to reduce the consumption of chemical pesticides for minimizing the parasitic nematode population on a threshold level (Stirling, 1991; Berg *et al*., 2005). Several fungi have been identified to reduce the nematode densities in soil that exhibit a range of antagonistic activities including a production of compounds that have nematicidal properties or parasitization of nematodes through prey devices or traps (adhesive knobs or nets,

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constricted and non-constricted rings) (Barron, 1977; Bird and Herd, 1995; Kerry, 2000; Lopez-Llorca and Jansson, 2006; Zouhar *et al*., 2013). Various studies have reported the production of nematicidal compounds by the fungi that become active against plant parasitic nematodes when they come in contact with each other (Anke *et al*., 1995; Anke, 2010; Hallmann and Sikora, 1996; Anke and Sterner, 1997; Chen *et al*., 2000; Meyer *et al*., 2000, 2004). The natural association among soil dwelling plant parasitic nematodes and fungi together in the soil rhizosphere keeps the nematode population low through the production of naturally produced toxic compounds and metabolites by fungi (Siddiqui and Mehmood, 1996).

Lecanicillium muscarium (ex. *Verticillium lecanii*) (Gams and Zare, 2001) is widely known as an entomopathogenic fungus (Cuthbertson *et al*., 2010) that also possesses mycoparasitic and nematicidal properties against food spoilage (Fenice *et al*., 1998a) and plant diseases (Ownley *et al*., 2010; Goettel *et al*., 2008). The fungus also has the capability to produce chitinolytic enzymes, including chitinases, glucanases and proteases (Fenice and Gooday, 2006; Fenice *et al*., 1998a, 2012) that have been shown to be more efficient than *Trichoderma harzianum* (Fenice *et al*., 1998b) against plant pathogens.

Lecanicillium spp. successfully parasitizes the eggs of *M. incognita* (Gan *et al*., 2007; Nguyen *et al*., 2007) and the females, cysts and eggs of *Heterodera glycines* under both lab and greenhouse conditions (Meyer and Meyer, 1996). Moreover, the studies claim that immature J2 are more susceptible to *Lecanicillium* spp. (Chen and Chen, 2003; Irving and Kerry, 1986; Kim and Riggs, 1991). To establish a successful infection to their hosts, *L. muscarium* conidia adhere to the host cuticle through mucilage and germinate, penetrate and produce blastospores inside the nematode eggs and J2. The fungus also produces various toxic secondary metabolites that induce resistance in plants in order to overcome other pathogens (Hirano *et al*., 2008).

In our previous studies we demonstrated that *L. muscarium* was able to reduce the population level of *Meloidogyne hapla* and *Heterodera Schachtii* under both lab and greenhouse conditions. The objective of the present study was to examine 1) whether the concentration of *L. muscarium* directly affects the nematode population and 2) which concentration best reduces the infestation of nematodes in the soil.

MATERIALS AND METHODS

A nematode culture was prepared on a susceptible tomato variety, and the eggs were extracted from the roots using 0.05% NaOCl. The extracted eggs were gently washed with tap water to remove NaOCl (Hussey and Barker, 1973). *M. incognita* was identified by morphological characteristics (Eisenback, 1985). The desired inoculum density was prepared by stirring the egg suspension in distilled water. The inoculum density was prepared as 100 eggs per ml of water. *Lecanicillium muscarium* previously isolated from the egg mass of *Meloidogyne incognita* in Turkey was cultured and processed for conidia production. Fungus was maintained on potato dextrose agar (PDA). Conidia were obtained in deionized water containing 0.03% Tween 80 and filtered through four layers of sterile cheesecloth to remove mycelium (Güçlü *et al*., 2010). Conidia were counted and standardized using a hemocytometer under a compound microscope.

The experiment was carried out with a susceptible variety of tomato i.e., Beril. The seeds of the tomato were surface sterilized in 0.05% NaOCl for 1 minute before nursery growth. After three weeks of being in the nursery, the plants were shifted to pots with a 5 x 6 cm dimension containing 500 cm^3 of sterilized sandy loam soil (62% sand, 18% silt, and 20% clay). The pots were placed in a completely randomized design with five replications on a bench in a green house. The pots were irrigated after two-day intervals throughout the period of the study. The daily temperature ranged between 25-28°C. After planting, fungus conidia with different densities (1×10^3, 1×10^4, 1×10^5, and 1×10^6) were pipetted onto each pot. One week later, nematode eggs of different concentration (500, 1000, 1500 and 2000 eggs) were inoculated near the root zone of the plant in each pot. The plants without fungus inoculum served as the control.

After 60 days, the plants were removed from the pots and washed in water carefully. The washed roots were blotted on paper, dried, and weighed.

Data collection

The data were calculated for plant growth (top fresh weight and root weight) and nematode reproduction parameters (number of galls and egg masses, number of eggs per root system, second-stage juveniles (J2) per 100 cc of soil, and reproduction factor).

After counting the galls, the whole root system was stained with a 0.005% "Phloxine B" (Holbrook *et al*., 1983) solution for 30 minutes to facilitate the counting of egg masses. The eggs were obtained from the roots using a 0.5% NaOCl solution passed through a sieve with a pore size of 74 and 25 mm (Hussey and Barker, 1973). The total number of eggs and juveniles were extracted from the soil of each individual plant from their respective pots following the Whitehead and Hemming Tray Method (Whitehead and Hemming, 1965).

The root system was rated for galling and egg mass presence on a 0 to 5 scale (Lamberti, 1971), where 0 = no gall or egg masses, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 galls or egg masses per root system.

Table I.- Effect of graded concentration of nematophagous fungus, *Lecanicillium muscarium* on different inoculum densities of root knot nematode, *M. incognita* with respect to nematode reproduction factors.

Conc. of *L. muscarium*	Inoculum densities of *M. incognita* 500	1000	1500	2000
Galls root system*				
0	25.00 e	53.00 d	74.80 b	104.00 a
1×10^3	13.80 g	20.00 f	27.00 e	58.00 c
1×10^4	8.60 j-l	11.00 h-j	11.60 gh	21.40 f
1×10^5	5.20m-o	7.40 k-m	9.00 i-k	11.20 hi
1×10^6	2.80 o	4.00 no	6.20 l-n	7.00 k-m
				LSD = 2.43
Egg masses root system				
0	21.20 e	45.80 c	64.80 b	87.20 a
1×10^3	9.60 hi	15.20 g	17.80 f	31.60 d
1×10^4	7.20 j-l	9.60 hi	10.40 h	14.00 g
1×10^5	3.80 mn	7.60 i-k	8.20 h-j	8.00 ij
1×10^6	2.00 no	4.20 mn	5.60 k-m	5.00 lm
				LSD =2.35
Eggs root system				
0	7764.0 e	15140.0 c	22600 b	32200 a
1×10^3	4135.0 g	5388.0 f	5680.0 f	9766.0 d
1×10^4	2080.0ij	2997.0 h	3027.0 h	4182.0 g
1×10^5	1330.0lm	2046.0 j	1886.0 jk	2463.6 i
1×10^6	734.00 n	1004.8 mn	1184.0lm	1524.0 kl
				LSD = 416.02
Eggs/g of root				
0	1494.2 d	2076.6 c	2455.6 b	2851.2 a
1×10^3	1038.6 e	912.20 f	785.80 g	981.20 e
1×10^4	711.80hi	720.20 h	564.80 k	604.00 jk
1×10^5	601.80jk	654.40 ij	550.40 k	403.20 lm
1×10^6	372.80m	463.80 l	395.80 m	362.20 m
				LSD = 63.95
J2 root system				
0	4726.0 e	9265.0 c	14595 b	17995 a
1×10^3	1431.0 j	1947.0 h	2839.0 f	4904.0 d
1×10^4	617.00 l	1071.0 k	1581.0 i	2218.0 g
1×10^5	361.00no	345.00 no	971.00 k	1078.0 k
1×10^6	192.00 p	305.00 op	468.00mn	518.00 lm
				LSD =145.25
J2 cc of soil				
0	945.00 e	1853.0 c	2919.0 b	3599.0 a
1×10^3	286.20 j	389.40 h	567.80 f	980.80 d
1×10^4	123.40 l	214.20 k	316.20 i	443.60 g
1×10^5	72.20 no	101.00 lm	194.20 k	215.60 k
1×10^6	39.20 p	61.00 op	93.60 mn	103.60 lm
				LSD =27.03
Pf/Pi**				
0	24.980 a	24.405 b	24.797 a	25.098 a
1×10^3	11.132 c	7.3350 d	5.6793 e	7.3350 d
1×10^4	5.3940 e	4.0680 f	3.0720 g	3.2000 g
1×10^5	3.3820 g	2.3910 h	1.9047 i	1.7708 i
1×10^6	1.8520 i	1.3098 j	1.1013 j	1.0210 j
			LSD =0.31	

Data are mean of ten replications; Means within a column followed by the same letter are not significantly different according to Least Significant Difference Test $P = 0.05$.

*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; 5, >100 galls or egg masses per root system (Lamberti, 1971).

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

The extracted eggs were rinsed thoroughly in tap water and then counted at 40X magnification. Nematode reproduction was assessed by calculating the nematode reproduction rate as P_f / P_i, where P_i = initial inoculum level, and P_f = final population at harvest.

Statistical analysis

The experiment was repeated once in a greenhouse. All the data from two experiments were subjected to factorial analysis using Statistica 10.4 software.

RESULTS

In our greenhouse trials approximately 60 days after nematode infestation, the initial population densities of nematophagous fungus, *L. muscarium* and *M. incognita* were regressed with the final population of *M. incognita* using the combined data from two consecutive experiments (Table I). The initial population densities of *L. muscarium* were significantly associated with the final population densities of *M. incognita* in terms of the second stage juveniles (J2) per root system, J2 per cm^3 of soil, gall and egg masses per root system, eggs per root system, eggs per gram of root, and reproduction factor. As the fungal population densities increased, the nematode population level decreased, which is clear evidence of fungal parasitic activities on nematodes. Maximum parasitism was noted at a higher concentration of fungus conidia (1×10^6).

The minimum number of eggs and J2 in the soil was recovered even at higher initial population densities of nematodes (Table I). Likewise, *L. muscarium* caused a significant increase in root shoot length and fresh and dry shoot weight compared to control plants (Table II). Top fresh weight (g) significantly improved with increased initial population densities of fungus (Table II), whereas fresh root weight decreased gradually with an increased fungus concentration.

The maximum top fresh weight was obtained when the fungal concentration was 1×10^6 compared to the control and other treatments in the experiment, while the minimum was recorded at concentration of 1×10^3. Similarly, the response of the roots in terms of induction of the root galls and egg production to *M. incognita* infection was directly proportionate to the Pi levels of the nematode and inversely proportional to *L. muscarium*. The minimum root weight was obtained at a higher level of fungal conidial suspension (Table I).

Moreover, *L. muscarium* showed no negative impact on plant growth. During our observations, the fresh root weight (g) and top fresh weight (g) gradually improved with increasing *L. muscarium* concentrations (Table II). The maximum plant growth parameters (root weight and top fresh weight) were obtained at the highest level of fungal conidia, whereas the minimum parameters were noted at a lower level. These studies demonstrate that there is a strong association of *L. muscarium* with the nematode population and plant growth.

Table II.- Effect of graded concentration of nematophagous fungus, *Lecanicillium muscarium* on different inoculum densities of root knot nematode, *M. incognita* with respect to plant growth parameters

Conc. of *L. muscarium*	Inoculum densities of *M. incognita*				
	0	**500**	**1000**	**1500**	**2000**
Fresh root weight (g)					
0	4.16 j	5.20 i	7.30 e	9.20 c	11.30 a
1×10^3	6.66 g	3.98 j	5.90 h	7.23 ef	9.96 b
1×10^4	7.62 d	2.92 l	4.17 j	5.37 i	6.99 f
1×10^5	9.20 c	2.21 m	3.13 l	3.47 k	6.16 h
1×10^6	11.28 a	1.97 n	2.17 m	2.99 l	4.24 j
					LSD =0.2715
Top fresh weight (g)					
0	171.60ij	132.60 o	97.40 q	72.40 r	52.20 s
1×10^3	204.40d	161.20 k	143.60 m	137.80 n	128.00 p
1×10^4	235.00c	174.80hi	162.60 k	155.80 l	146.80 m
1×10^5	246.40b	191.40 f	175.80 h	169.76 j	184.78 g
1×10^6	272.40a	203.80 d	199.24 e	185.80 g	204.20 d
					LSD =0.3181

Data are mean of ten replications; Means within a column followed by the same letter are not significantly different according to Least Significant Difference Test $P = 0.05$.

DISCUSSION

We posit that the growth and yield of vegetables might be enhanced by incorporating population densities of *L. muscarium* or in combination with other nematophagous fungi. The soil with higher population densities of *L. muscarium* had a lower final nematode population, ultimately resulting in positive effects on plant growth factors (root shoot lengths and shoot weight) (Yang *et al*., 2012). The number of galls, egg masses, J2 population, and eggs were significantly decreased (P=0.05) with increasing *L. muscarium* conidia (Table I). This decrease in the final nematode population was due to a higher mass of conidia with a successful antagonistic reaction towards *M. incognita* eggs as well as J2 (Zhang *et al*., 2008). Previously, during in-vitro observations, *L. muscarium* produced higher densities of conidia on potato dextrose agar (PDA) media under room temperature (data not published) since conidia are the main propagule of infection. Spore attachment, germination and enzyme activity are important factors for fungal virulence (Kim *et al*., 2014). *L. muscarium* was believed to produce mucilage matrix, which help fungus conidia stick to nematode eggs and J2 to successfully penetrate germination of fine hyphae (Veenhuis *et al*., 1985). Additionally, plant roots also provide a biological environment with facilitation of nutrients to nematode and fungi (Curl and Truelove, 1986) and niches for their survival. The aggressiveness of *L. muscarium* could be correlated to a higher production of chitinolytic enzymes (generally called chitinases) in a wide range of temperatures (5-30°C) with an optimum temperature of 25°C. (Fenice *et al*., 1996, 1997, 2012; Khan *et al*., 2004) The cuticle of nematode eggs and J2 are mainly comprised of proteins and chitin (Blaxster and Robertson, 1998). *L. muscarium* is more powerful and efficient than *Trichoderma harzianum*, which is commercialized as a strong mycoparasitic and entomopathogenic agent exhibit scarce activity even in cold environments (Malathrakis and Kritsotaki, 1992; Fenice *et al*., 1998a), low or higher moisture (Jackson *et al*., 1985; Kope *et al*., 2008) and broad humidity levels (Kope *et al*., 2008). Furthermore, studies showed that *L. muscarium* also plays a vital role in activating the plant defensive system by triggering induced resistance (Hirano *et al*., 2008). This could be another reason to build the plant confidence level against pathogens. A gradual increase of fungal propagules enhanced plant growth by limiting nematode reproduction factors, which might be due to endophytic colonization of the roots by the fungus, as examined under microscope. Our study validates the results of Trifonova *et al*. (2009). These authors isolated three fungi viz *Fusarium oxysporum*, *Gliocladium roseum* and *Verticillium chlamydosporium* from infected *M. incognita* females in southern Bulgaria and found that these fungi decreased the number of eggs from 7.6% to 23.5%. Similarly, Dababat and Sikora (2007) investigated *Fusarium oxysporum* and found that it inhibited juvenile penetration, gall formation and egg mass production in tomato plants and that a double dose of the fungus had more precise results comparatively. According to Kiewnik and Sikora (2006), nematophagous fungi *Paecilomyces lilacinus* decreased the number of egg masses by 74%, the root galling by 66% and the final population of nematodes in the roots by 71% compared to the control (inoculated treatment).

CONCLUSION

The above discussion reports that *Meloidogyne* spp. could be managed by increasing the biocontrol concentration in the soil. By this means we could spare the environment and underground water from notorious chemicals that cause pollution and carcinogenic effects. Scientists must try to find alternative management strategies so that farmers may easily benefit from these strategies. Our findings show that the magnitude of the nematode reproduction rate, development of root galls, production of egg masses, eggs and J2 population are inversely related to levels of the fungus “Pi” . Our results also revealed that *L. muscarium* could be a potential biocontrol agent against plant parasitic nematodes by protecting the roots from extensive colonization.

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Conflict of interest statement

We declare that we have no conflict of interest.

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