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Parasitic Activity of the Root Knot Nematode *Meloidogyne incognita* in Okra in a Naturally Infested Field and its Suppression by *Lecanicillium muscarium*

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MH designed the study, collected the data and wrote the manuscript. MZ and PR supervised the study and proofread manuscript.

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***Meloidogyne incognita*, *Lecanicillium muscarium*, Reproduction factor, Infestation, Plant parasitic nematodes, Biological control.**

ABSTRACT

The experiment was conducted in a grower's field naturally infested with *Meloidogyne incognita* in Layyah District. Six okra genotypes, including 19,224; 19,235; Pusa Swami; Ikra-1; Ikra-2; and Sabzperi China Red, were selected from our previous study according to their galling index and level of susceptibility. These genotypes were used as test genotypes for the interaction between *M. incognita* and the entomopathogenic fungus, *Lecanicillium muscarium*. The initial inoculum was measured before okra was seeded in the field. At harvest, the numbers of root galls, egg masses, and eggs per root system, the root weight, the final population of the second stage juveniles, and the reproduction rate were detected at the highest levels in untreated soil, whereas low numbers were encountered in soils initially treated with the fungus, *L. muscarium*. *Meloidogyne incognita* and other plant parasitic nematodes multiplied significantly more in untreated soil than in treated soil, which clearly indicated that *L. muscarium* effectively reduced the nematode infestation level in the soil.

INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench) is a biannual crop with two growing seasons, one starting in early March and one in late July (Hussain *et al*., 2016a). The okra vegetable is consumed in the Indo-Pak sub-continent because of its rich nutritional value, even though it originates from the tropics of Afro-Asian countries. Globally, it suffers from the sessile endoparasitic and polyphagous root knot nematode, *Meloidogyne* spp. (Goswami *et al*., 2008). The most predominant species in Pakistan is *M. incognita,* which causes yield losses and impairs the root systems of plants by producing root galls that can be seen with the naked eye (Anwar *et al*., 2007; Anwar and McKenry, 2010; Hussain *et al*., 2015). Most of the grower's fields in Pakistan are naturally infested with root knot nematodes, especially *M. incognita*, due to conventional cropping systems and cultivation of the same vegetable hosts in the fields where the nematodes are already present. Due to a lack of awareness and crop rotation, a huge nematode build-up is being created by farmers not educated about the nematodes. The population densities of plant parasitic nematodes attained through crop rotation varies considerably with the year, location, pathogen-associated weed hosts and the nature and length of the rotation (Clayton *et al*., 1994). Rotation is considered a primary tactic for reducing the nematode population, and 2-4 years of rotation are generally required to obtain the best results. Non-host plantings, particularly grasses and monocotyledon crops such as sorghum or corn, are also effective in mitigating the effects of nematodes, but farmers are reluctant to use these crops due to the length of the growing period. The most successful component of crop rotation is the availability of resistant or tolerant genotypes that could also be used to limit the magnitude of the nematodes. Unfortunately, due to a wide range of hosts, especially in the case of *Meloidogyne* spp. the option for alternate crops is not fruitful.

The estimated overall losses of vegetables caused by *Meloidogyne* spp. are 5-43% (Sasser, 1979; Gautam *et al*., 2014). The production of okra is not sufficient to meet the demand of the people in Pakistan due to the intervention of several plant pathogens, including bacteria, fungi, viruses, nematodes and abiotic factors, but root knot nematodes have proven a considerable threat to crop yields (Sasser and Freckman, 1987; Kinlock and Sprenkel, 1994; Baird *et al*., 1996; Kamran *et al*., 2013; Hussain *et al*., 2016a). Several studies have reported that the root knot nematode contributes to yield losses in okra of up to 27% (Anwar and McKenry, 2012; Sikora and Fernandez, 2005).

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Table I.- Interaction of *Meloidogyne incognita (Mi)* with *Lecanicillium muscarium* on roots of 6 okra genotypes in natural *Mi*- infested field (March season).

Genotypes	Root galls	Gall index[1]	Egg masses	Egg mass index[1]	Eggs per root system	Root weight (gm)	Eggs per gram of root	Final J2 population in soil (100 cm^3)	Rate of reproduction[2]
Pusa Swami	103a	5	106a	5	24671a	21.9a	1124a	602a	55.8a
Ikra-1	75c	4	73c	4	22384c	19.8c	1122a	542b	50.9b
Ikra-2	77b	4	75b	4	22985b	20.8b	1105b	445d	49.9c
Sabzperi China Red	68e	4	65d	4	19885e	19.6c	1009d	540c	46e
19224	70d	4	73c	4	21982d	19.9c	1098c	600a	50.9b
19235	72d	4	73c	4	19838f	19.9c	998e	601a	46.9d
Pusa Swami$^{\Psi}$	26f	3	21e	3	2113g	17d	124i	116e	4.36f
Ikra-1$^{\Psi}$	25f	3	19f	3	2097g	17d	123i	116e	4.34f
Ikra-2$^{\Psi}$	21g	3	19f	3	2017h	15e	124i	115e	4.26fg
Sabzperi China Red$^{\Psi}$	18h	3	15g	3	1901j	14f	135g	98f	4.0fgh
19224$^{\Psi}$	14.6i	3	13h	3	1850k	14f	133h	96g	3.7h
19235$^{\Psi}$	13.9i	3	14gh	3	1935i	13g	149f	94g	3.85gh

[1]Gall and egg mass indices, 0-5 scale; where 0 is no galls or egg masses, 1 is 1-2 galls or egg masses, 2 is 3-10 galls or egg masses, 3 is 11-30 galls or egg masses, 4 is 31-100 galls or egg masses and 5 is > 100 galls or egg masses per root system (Quesenberry *et al*., 1989). [2] Rate of reproduction = Pf/Pi (Final Population / Initial Population*). * Mean initial population = 55 J2/100 cm^3 of soil. [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. $^{\Psi}$ is *Lecanicillium muscarium* treated varieties.

Many strategies, such as the application of chemicals or fumigation; crop rotation; the use of antagonistic plants, trap crops, and resistant genotypes; the application of organic manure; and the use of biocontrol, are practiced to mitigate nematode infestations in soil, but all practices have limitations with respect to the yield, environment, duration of crops, and encounter with useful microbes.

Nematophagous fungi have been studied extensively for the control of soil-borne plant parasitic nematodes because the use of nematicides, soil sterilants, and fumigants has ecological and toxicological threats (Jatala, 1986; Santos *et al*., 1992; Zouhar *et al*., 2013). Biologically suppressive soil has a mitigating influence on soil-borne pathogens, including bacteria, viruses, fungi and nematodes, and affects plant vigor and yield despite the virulence of the pathogen, a conducive environment, and susceptible hosts (Hornby, 1990; Becker *et al*., 2013; Renčo, 2013). Nematophagous fungi have developed exclusive abilities to parasitize nematodes in various ways. For example, *L. muscarium* is a parasitic fungus of eggs; it uses appressoria, specialized penetration pegs, or lateral mycelial branches to penetrate the egg shell (López-Llorca *et al*., 2007). Moreover, the fungus has the ability to penetrate the egg shell and the nematode directly via hyphae or through enzymatic actions because the nematode egg shells (Charnley, 1997; Hussain *et al*., 2017d) and body mostly consist of protein and chitin (Clarke *et al*., 1967; López-LIorca *et al*., 2007). The objective of this study was to evaluate the nematode population pressure and its multiplication and to measure the effectiveness of our previously studied entomopathogenic fungus, *L. muscarium,* against root knot nematodes in farmer's fields.

MATERIALS AND METHODS

Based on its crop and soil history, the experimental field, which had soil comprising 90% sand, 8% silt, 2% clay and 2.35% organic matter, was selected and marked for the experiment. The pH of soil was measured as 6.5. Initially, 75 soil samples from 15-23 cm depth were taken from the field and transported to the laboratory for analysis. The initial data on the presence of root knot nematodes, as well other plant parasitic nematodes, were recorded by using sieving and sucrose centrifugation technique. At harvest, five soil samples of 200 g from each plant root zone were taken and used to record the final nematode population. In total, 75 samples were taken from each plot, and the average nematode population was recorded as initial inoculum. In selected areas, the six susceptible okra genotypes (Table I) were sown on ridges in a plot of size 25m^2 each and allowed to grow for 2 months. The rows in plots without fungus application were considered the control. The inoculum of *L. muscarium* was prepared in the lab on a specialized substrate after pellets from grain

straw were ground, as shown in Figure 1, and mixed well into the soil up to 23cm deep before seeding. In total, 1 kg of substrate including the fungal mycelium (CFU: 1×10^6/g of substrate) grown on grain straw (Fig. 1) was mixed uniformly and applied to each plot. A randomized complete block design was used. Each plot layout comprised a plant-to-plant distance of 30cm and row-to-row distance of 60cm with fifteen plants in total. Each treatment was replicated five times. Plants were allowed to grow for 60 days. The soil nematode population was assessed before the transplanting and at harvest to determine the rate of reproduction. The plants were irrigated with tube well water and fertilized with NPK. The first study was done in early March, whereas the same experiment was repeated in July in the same year (2017).

Fig. 1. Preparation steps of *L. muscarium* inoculum on grain straw substrate in Lab (A, pellets from grain straw; B, autoclaved substrate inside box; C, growth of mycelium on substrate).

Parameters recorded

Sixty days after inoculation, the plants were uprooted with a spade. The roots were washed free of soil and blotted with paper to damp dry. Data were recorded on the following parameters: the number of galls, gall index, egg masses, egg mass index, eggs per plant, eggs per gram of root, and reproduction factor. Roots were weighed separately, in grams, on a balance.

The following 0-5 scale was used for gall and egg mass indices: 0 = no galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 ≥ 100 galls or egg masses per root system (Quesenberry *et al*., 1989). The root systems of the plants were stained with 0.005% Phloxine B (Holbrook *et al*., 1983) solution for 30 min to facilitate the counting of egg masses. Eggs were collected from the roots using 0.5% NaOCl solution, which was passed through sieves with pore sizes of 74 and 25 µm (Hussey and Barker, 1973). The extracted eggs were rinsed thoroughly in tap water and then counted at 40X magnification. Nematode reproduction was assessed by the following calculation: the nematode reproduction factor (Rf) = P_f / P_i, where P_i = the initial inoculum level that was measured before seeds were sown in the field, and P_f = the final population of the nematodes in the soil at harvest. The numbers of all other plant parasitic nematodes were also measured in the soil before and at harvest.

Data analysis

The data were subjected to analysis of variance and a least significant difference test at the 0.01% level of significance to compare the differences among the treatment means by using Statistica 10.4 software.

RESULTS

Experiment 1 (March season)

All okra genotypes in the control treatments could be classified into two groups according to the number of galls and egg masses per root system. The cultivar Pusa Swami was the best host of all the genotypes, with the highest root gall and egg mass indices of 5. The second group comprised five genotypes, including Ikra-1, Ikra-2, Sabzperi China Red, 19224, and 19235, which exhibited a high root galling and egg mass indices of 4. Overall, all genotypes were documented as highly susceptible based on their high potential for nematode multiplication. In treated soil, the gall and egg mass indices were reduced from 5 to 3, with fewer galls and egg masses per root system. Likewise, the root weight, eggs per gram of root, final population of second-stage juveniles (J2) in the soil and the rate of reproduction were reduced significantly (P = 0.01) in the fungus-treated soil compared to all control treatments of the genotypes (Table I). In the untreated cv. Pusa Swami, the final rate of reproduction was 55.8, whereas in the treated cv. Pusa Swami, it was 4.36. Similar reductions were observed in the remaining genotypes, as shown in Table I.

In addition to *M. incognita*, other *Meloidogyne* spp., such as *M. javanica, M. arenaria,* and *M. hapla*, were also reduced in the treated soil. The relative frequency of occurrence and the mean density decreased for all *Meloidogyne* spp. and other plant parasitic nematodes (Table III).

Table II.- Interaction of *Meloidogyne incognita (Mi)* with *Lecanicillium muscarium* on roots of 6 okra genotypes in natural *Mi*- infested field (July season).

Genotypes	Root galls	Gall index[1]	Egg masses	Egg mass index[1]	Eggs per root system	Root weight (gm)	Eggs per gram of root	Final J2 population in soil (100 cm^3)	Rate of reproduction[2]
Pusa Swami	150a	5	160a	5	38,001a	25a	1525a	1116d	65.3a
Ikra-1	149a	5	158b	5	32895d	25a	1342e	1382a	62.2b
Ikra-2	136b	5	139c	5	32996c	23.6b	1404c	1282b	62b
Sabzperi China Red	130c	5	129d	5	32490e	23bc	1407c	1040e	60.6c
19224	130c	5	110f	5	31896f	22.9c	1391d	1205c	57.8d
19235	127d	5	119e	5	33315b	22.7c	1467b	1302b	61.8b
Pusa Swami$^{\Psi}$	26e	3	20h	3	2144g	16.93d	126h	117f	4.41e
Ikra-1$^{\Psi}$	26e	3	22g	3	2099h	16.89de	125h	118f	4.35e
Ikra-2$^{\Psi}$	22f	3	19h	3	2050i	16.33e	125h	115f	4.37e
Sabzperi China Red$^{\Psi}$	19g	3	16i	3	1937j	13.86g	134g	101f	3.85e
19224$^{\Psi}$	16h	3	19h	3	1850k	14.5f	135g	100f	3.89e
19235$^{\Psi}$	16h	3	19h	3	1962j	13.3g	149f	97f	3.84e

For explanation of symbols used in this table, see Table I.

Table III.- Occurrence frequency and density of other nematodes species before and after experiment (March and July season).

Nematode species	March season (n=70*)						July season (n=70*)					
	Before experiment		After harvest without fungus application		After harvest with fungus application		Before experiment		After harvest without fungus application		After harvest with fungus application	
	RF	MD	RF	MD	RF	MD	RF	MD	RF	MD	RF	MD
M. javanica	60	2334	63	2980	31	988	55	2290	59	2790	29	790
M. arenaria	20	1669	31	2340	17	544	13	702	15	799	13	459
M. hapla	65	1569	63	2120	29	1003	30	799	25	804	23	876
Pratylenchus penetrans	40	1703	35	1802	19	955	38	1608	29	1708	23	899
Radopholus similis	20	966	25	1123	21	988	23	740	32	1002	24	590
Aphelenchus avenae	35	1233	34	1522	21	844	29	1156	36	1699	28	790
Helicotylenchus dihystera	12	988	14	1144	10	955	16	1877	20	1980	34	803
Xiphinema spp.	29	870	32	1230	22	988	60	1840	68	1990	45	1766
Hoplolaimus columbus	13	970	15	1240	7	455	23	1230	34	1980	40	399
Free living nematodes	68	3599	69	4580	70	2110	67	4567	70	5670	68	3450

*Number of soil samples collected. RF-Relative frequency of occurrence (percentage of samples in which species were present). MD, mean population recovered from samples taken.

Experiment 2 (July season)

In repeated studies, all okra genotypes in the control treatments could be classified in one group based on their gall and egg mass indices. All genotypes including Pusa Swami, Ikra-1, Ikra-2, Sabzperi China Red, 19224, and 19235 exhibited the maximum number of gall and egg mass indices (5), which proved that they are good nematode hosts. The number of eggs per root system, root weight, eggs per gram of root, final J2 population in 100 cm^3 of soil and final rate of reproduction were reduced significantly in all treated genotypes, as shown in Table II.

In the control treatments, cv. Pusa Swami showed a significantly higher rate of reproduction (65.3), with the maximum final population of soil nematodes, 1116 J2/100 cm^3 of field soil at harvest, whereas the initial inoculum before sowing was recorded as 75 J2/100 cm^3 of soil. In treated soil, the rate of reproduction was recorded as 4.41, with 117 J2/ 100 cm^3, which is quite low (Table II).

A comparison of the means indicated that cv. Pusa Swami had the significantly highest root weight (25 g) and eggs per gram of root system (38,001) when the soil was not treated with fungus, but the root weight was lower

at 16.93 g of root weight and 2144 number of eggs per root system in the fungus-treated soil. The same trend was observed in all genotypes (Table II). Overall, the reaction of the nematodes to all genotypes was more severe in the July than in the March season (Tables I, II). Compared to that of other plant parasitic nematodes, the relative frequency of the studied nematodes and their mean difference was lower in the treated soil than in the control soil (Table III). The relative frequency of all nematode species was reduced in the soil treated with the fungus. Overall, in both seasons, the numbers of all nematodes were reduced at harvest in the treated soil, as shown in Table III. The number of *Meloidogyne hapla* was greater in the soil in the March season than in July (Table III).

DISCUSSION

The root knot species, *M. incognita, M. javanica, M. hapla* and *M. arenaria,* were identified based on perineal pattern morphology using approximately 10 adult females. Based on the perineal pattern information, the field was declared as infested with *M. incognita.* The soils of Layyah generally comprise sand and sandy loam, which are conducive for the penetration, development, and reproduction of the root knot nematode (Ogbuji, 2004) and are therefore favorable to the development of root knot nematodes. Our previous study clearly demonstrated that *M. incognita* is distributed widely across the Layyah vegetable production areas (Hussain *et al*., 2015). This distribution also agrees with the observations of Anwar *et al*. (2007), who reported that root knot nematodes are a damaging pest in vegetables in the Punjab. The juvenile of the root knot nematode moves intercellularly after penetrating the root, migrating down the plant cortex toward the root tip (Williamson and Hussey, 1996). The primary symptom of root-knot nematode infection is the formation of typical root galls on the roots of susceptible host plants. The J2 develops into adult females, which lay eggs. The number of eggs laid by a female depends on the host status to a particular nematode, nematode species or population involved and other environmental factors (Anwar *et al*., 2002). The host status of a plant to nematodes may be estimated by the number of galls produced by the root system (Bélair and Benoit, 1996) or the final nematode population at harvest per gram of root (Gast *et al*., 1984; Jordaan *et al*., 1988). The host status may also be determined according to the magnitude of the reproduction factor (Rf = Pf/Pi = the final population of nematodes divided by their initial population), which is frequently used and is the most accurate measure of the nematode-host relation (Anwar *et al*., 2002).

Genotypes can also be evaluated for root knot nematode resistance based on the degree of root galling, egg mass number or total number of eggs collected from the root system (Hussey and Boerma, 1981). The plant response of okra genotypes to *M. incognita* infection assessed by the production of root galling, root galls and egg mass indices, and eggs per root system and per gram of root, as well as the rate of reproduction, varied considerably among the genotypes. This variation in genotypes to nematode infection is natural, as the genotypes vary in their genetic make-up (Ammti *et al*., 1985) and in the level of resistance mechanisms possessed by a particular cultivar/line (Anwar and McKenry, 2002).

The behavior of okra genotypes toward nematode populations in the soil differed little between March and July. The plants sown in July exhibited more galls, egg masses, eggs per root system, eggs per gram of root, and J2 population in 100 cm^3 and a higher final reproduction factor than those sown in March. The differences in the nematode-related parameters could be due to temperature (32-35°C in March-April and 33-42°C in July-August). *Meloidogyne* spp. favor higher temperatures, with greater reproduction, as described by Santo and O'Bannon (1981).

All okra genotypes were susceptible to the nematodes, but cv. Pusa Swami was designated the best host to the nematode, showing heavy root galling, higher gall and egg mass indices, more eggs per root system as well as per gram of root, and a higher reproduction factor (Hussain *et al*., 2016a, b). This finding suggests that cv. Pusa Swami lacks resistance genes to halt the penetration, arrest the development and suppress the reproduction of *M. incognita.* This susceptible cultivar attracted J2, allowing them to penetrate in the roots of cv. Pusa Swami (Fenoll *et al*., 1997; Castagnone-Sereno, 2002). The finding that the damage caused by nematodes to plants was directly proportional to the population densities of the nematodes in the soil and to their reproduction potential in the plant agree with Barker and Olthuf (1976) and Ahmad and Khan (1991).

Most entomopathogenic fungi such as *L. muscarium* produce hydrolytic enzymes that enable them to penetrate the cuticle of nematode eggs and J2 (Gabriel, 1968; Latgé, 1974; Leopold and Samsinokova, 1970; Shinya *et al*., 2008). Furthermore, the efficient antagonistic relation between fungi and nematodes has positive effects on plant growth compared to the controls (Hussain *et al*., 2017a-f). *Lecanicillium muscarium* also effectively diminished juvenile penetration, which resulted in a decreased number of galls and egg masses (Hussain *et al*., 2018). Furthermore, the size of the galls was smaller with fewer egg masses, which evidenced the static effects of the nematode (McGarvey *et al*., 1984, Hussain *et al*., 2017a, b).

CONCLUSION

Based on the above discussion, we conclude that *Meloidogyne* spp. is a threat to vegetable crops, including okra. The fungus *Lecanicillium muscarium* effectively controlled the nematode infestation in soil even at very high temperatures. Therefore, we recommend applying this bio-agent in fields before planting vegetables or other crops.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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