EVALUATION OF CERTAIN ENTOMO-PATHOGENIC NEMATODES AS BIOCONTROL AGENTS FOR THE CITRUS NEMATODE, *TYLENCHULUS SEMIPENETRANS*.

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Abstract

A pot experiment was carried out to evaluated the ability of entomopathogenic nematodes (EPNS), *Steinernema* sp, *Steinernema carpocapsae*, *Heterorhabditis bacteriophora* (B20) and *Heterorhabditis bacteriophora* (HP88) as biological control agents of the citrus nematode *Tylenchulus semipenetrans*, plant-parasitic nematodes (PPNS) infecting sour-orange plants in the greenhouse. Entomopathogenic nematodes as added at three doses (2000, 4000, 8000 infective juveniles) to sour-orange plants pots one week before adding the inoculations of citrus nematode, *T. semipenetrans* (3000 juveniles/pot). The highest reduction of *T. semipenetrans* juveniles in the soil and egg-masses were recorded at the treatment of adding 8000 IJS of *H. bacteriophora* (HP88). *Steinernema* sp. This research may contribute in the novel direction of using EPNS as biological agents of PPNS.

Keywords: sour-orange plants, Entomopathogenic nematode, *Steinernema* sp.; *Steinernema carpocapsae*; *Heterorhabditis bacteriophora*, *Heterorhabditis bacteriophora* and *Tylenchulus semipenetrans*.

INTERDICTION

Entomopathogenic nematodes (EPN) in the family's Steinernematidae and Heterorhabditidae are found in many region throughout the world including Egypt (Shamseldean and Abd-Elgawad, 1994). They selectively infect many insects and a few other pests but do not adversely affect mammals or plants. Limited nematicide availability and high costs of nematicides development have created a need to discover alternative methods for plant-parasitic nematodes (PPN). Other studies have shown that EPN and their associated bacteria possibly may interfere with the infection and reproduction of some PPN (Grewal *et al.*, 1999). Some nematologists are interested in determining this interaction between EPN and PPN were first shown by Bird and Bird (1986), who showed that a reduction of the infection of *Meloidogyne*

javanica in tomato plants was caused by *Steinernema glaseri* (Steiner) in greenhouse pot tests. Additional research has documented PPN suppression by EPN (Ishibashi and Kondo, 1986; Lewis *et al.* 2001; Perez and Lewis 2004 and Shapiro-Ilan *et al.* 2006). Our objective in this study was to evaluate the effect of the EPN in the infection of the citrus nematode *Tylenchulus semipenetrans* (PPNS) infecting sour-orange plants in the greenhouse,in addition to this, the effect of Egyptian entomopathogenic nematode strains compared to foreign strains was conducted. The primary objective of this study was to evaluate whether entomopathogenic nematode applications would suppress the citrus nematode *Tylenchulus semipenetrans* infecting sour-orange seedlings. As a secondary objective, we evaluated to this nematode pest as well as suppression of root infection.

MATERIALS AND METHODS

Entomopathogenic nematode :

Two species of *Heterorhabditis bacteriophora* (B20) and *Steinernema* sp. from Applied Center for Entomonematodes, Faculty of Agriculture, Cairo University, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* (HP88) form (Biosys,Palto-Alto,California) all were tested for efficacy against *Tylenchulus semipenetrans* infected sour-orange plants under greenhouse conditions. The entomopathogenic nematodes population used in this research originated from Plant Protection Research Inst. Agricultural Research Centre (ARC) Dokki, Giza., Egypt, where greater wax moth *Galleria mellonella* was used as host insect to *in vivo* culture; to be used in this work as the biological agent.

Source of the Citrus Nematode, Inoculum:

Second stage juveniles of *T. semipenetrans* were obtained from a pure culture propagated on sour-orange, *Citrus aurantium* L. in the greenhouse of Nematology Research Unit, Agricultural Zoology Department, Faculty of Agriculture, Mansoura University, Egypt. Nematodes were extracted from soil by sieving and modified Baermann technique **(Goodey, 1957).** and inoculum level was determined according to the design of each experiment during the course of this investigation which was done in the greenhouse of the department mentioned above.

Greenhouse experiment: In order to carry out this investigation either out of fifty four plastic pots 25 cm-d. filled with 3kg/pot steam-sterilized sandy loam soil (1:1,

V:V) separately. Each pot contained one sour-orange seedling. Respectively one week after planting of sour-orange 2years old were then inoculated with 3000 second stage juveniles of *T. semipenetrans* /plant one week after applying the tested treatments the other three sour-orange plants incoulum by plant parasitec nematode only without any treatment to serve as control (CK). Each treatment was replicated three times, pots were assigned to 13 groups as follows:

1- Three sour-orange seedlings inoculated with 2000 infective juveniles of *Steinernema* sp.
2- Three sour-orange seedlings inoculated with 4000 infective juveniles of *Steinernema* sp.
3- Three sour-orange seedlings inoculated with 8000 infective juveniles of *Steinernema* sp.
4- Three sour-orange seedlings inoculated with 2000 infective juveniles of *Steinernema carpocapsae.*
5- Three sour-orange seedlings inoculated with 4000 infective juveniles of *Steinernema carpocapsae.*
6- Three sour-orange seedlings inoculated with 8000 infective juveniles of *Steinernema carpocapsae.*
7- Three sour-orange seedlings inoculated with 2000 infective juveniles of *Heterorhabditis bacteriophora* (B20).
8- Three sour-orange seedlings inoculated with 4000 infective juveniles of *Heterorhabditis bacteriophora* (B20).
9- Three sour-orange seedlings inoculated with 8000 infective juveniles of *Heterorhabditis bacteriophora* (B20).
10- Three sour-orange seedlings inoculated with 2000 infective juveniles of *Heterorhabditis bacteriophora* (HP88)
11- Three sour-orange seedlings inoculated with 4000 infective juveniles of *Heterorhabditis bacteriophora* (HP88)
12- Three sour-orange seedlings inoculated with 8000 infective juveniles of *Heterorhabditis bacteriophora* (HP88)
13- Three sour-orange seedlings with no inoculum of entomopathogenic nematode as check treatment.

Pots were randomly arranged on a greenhouse bench at 18±4ºC. Plants were watered regularly and treated horticulturally as recommended. After 60 days from nematode juveniles inoculation, plants were harvested Infected plant roots were examined uprooted and washed with tap water and the number of egg masses after staining by lactic acid fuchsin **(Byrd *et al.* 1983)** were recorded. Number of *T. semipenetrans* in 3g soil/replicate/treatment was also determined by extracting through sieving and modified Baermann technique **(Goodey, 1957)** and recorded.

Data were statistically subjected to analysis by Duncan's multiple-range test. The least significant differences (LSD) between means were also calculated at 5% significant level.

RESULTS AND DISCUSSION

The obtained results in (Table, 1) revealed that the use of *Steinernema* sp. of 8000 Infective Juveniles (IJ)/pot recorded a 74% reduction in number of *Tylenchulus semipenetrans* Juveniles in the soil, while *Steinernema carpocapsae* caused 54% reduction of *T. semipenetrans* juveniles in the soil with the same concentration.

Treatment of 8000 IJs/pot *Heterorhabditis bacteriophora* (B20) and *H. bacteriophora* (HP88) recorded a 46 and 76% reduction in number of *T. semipenetrans* Juveniles in the soil, respectively. Data showed that, number of *T. semipenetrans* juveniles in the soil were decreased by increasing number of used *Steinernema* and *Heterorhabditis* IJs. The highest reduction percentage of *T. semipenetrans* juveniles (76%) was achieved by using 8000 IJs/pot *H. bacteriophora* (HP88).

Statistical analysis showed high significant differences between the doses(df=2,F=16114.8,P=0.0000) and four entomopathogenic nematodes (df=2,F=66541.5,P=0.0000) .Also, found significant differences between all treatments (df=8,F=2257.4,P=0.0000).

Table 1. Effect of Entomopathogenic nematode on Tylenchulus semipenetrans infecting sour-orange plant under greenhouse condition at (18 ± 5 °C).

Treatments		Average number of *T. semipenetrans* juveniles in soil	Reduction of *T. semipenetrans* juveniles	Reproduction of *T. semipenetrans* (RF)**	Efficacy % ***
Nematode type	Doses				
Steinernema sp.	2000	9860 Ae	51	2.3	60
	4000	7040 Be	65	1.4	75
	8000	5220 Ce	74	0.9	86
S. carpocapsae	2000	12380 Ac	38	3.2	43
	4000	11550 Bc	43	2.9	49
	8000	9200 Cc	54	2.0	62
H. bacteriophora (B20)	2000	13850 Ab	31	3.6	37
	4000	11300 Bb	44	2.8	50
	8000	10890 Cb	46	2.6	54
H. bacteriophora (HP88)	2000	13150 Ad	34	3.4	40
	4000	7100 Bd	65	1.4	75
	8000	4870 Cd	76	0.7	88
Control		20120 a		5.7	

Numbers followed by the same (capital letter of doses and small letter of treatment) within a row are not significantly to LSD test at 0.05 level of probability L.S.D.=56.73 (treatment) and 43.94 (doses).

*Reduction % = $\frac{\text{N. control} - \text{N. treatment}}{\text{N. control}} \times 100$

** Reproduction (RF) = $\frac{\text{Number after treatment - Number before treatment}}{\text{Number after treatment}}$

*** Efficacy % = $\frac{\text{RF of control} - \text{RF treatment}}{\text{RF of control}} \times 100$

Data in Table 2 show that using *Steinernema* sp. and *Steinernema carpocapsae* at a rate of 8000 IJs/pot recorded 83% and 71% reduction of egg-masses, respectively. Results also have showed that *H. bacteriophora* (HP88) in 8000 IJs/pot was the best used treatment rising the percentage reduction of egg-masses to 96%. Entomopathogenic nematode, *Steinernema* and *Heterorhabditis* were present around the roots of sour-orange seedlings and produced toxic agents to plant-parasitic nematodes causing a boundary area of protection around plant roots against the infection by infective juveniles of *T. semipenetrans*.

Statistical analysis showed high significant differences among tested doses (df=2,F=2145.9,P=0.0000) and the four entomopathogenic nematodes (df=2,F=13363.5,P=0.0000).Also, significant differences between all treatments (df=8,F=315.4,P=0.0000) were found.

Table 2. Effect of Entomopathogenic nematode on egg-masses number of *Tylenchulus semipenetrans* infecting sour-orange plant under greenhouse condition at (18 ± 5 °C).

Treatments		Number of egg-masses in root	Reduction of egg-masses %
Nematode type	Doses		
Steinernema sp.	2000	262 Ac	66
	4000	254 Bc	67
	8000	136 Cc	83
S. carpocapsae	2000	422 Ab	42
	4000	365 Bb	51
	8000	233 Cb	71
H. bacteriophora (B20)	2000	210 Ad	72
	4000	188 Bd	73
	8000	133 Cd	81
H. bacteriophora (HP88)	2000	351 Ac	55
	4000	276 Bc	62
	8000	26 Cc	96
Control		762 a	

Numbers followed by the same (capital letter of doses and small letter of treatment) within a row are not significantly to LSD test at 0.05 level of probability L.S.D.=6,05 (treatment) and 4.68 (doses).

Entomopathogenic nematodes are obligated parasites of soil-living insects. Nearly 80 species have been described from two genera. They are used successfully as biological control agents in several cropping systems to reduce populations of soil insects. Application at this rate impact insects, plant-parasitic nematodes and free-living nematodes in various way. The interaction between plant-parasitic nematodes (PPN) and entomopathogenic nematode (EPN) is especially unexpected because these nematodes do not compete for common resources nor do they interact directly in any way. The reduction of plant-parasitic nematodes is attributed at least partially to compounds produced by symbiotic bacteria associated with (EPN). These bacteria are produced in large quantities during an (EPN) infection and cadavers of insects with ongoing infections were repellents to plant-parasitic nematodes, and the cell-free extract of the bacteria in culture to be toxic to most nematodes other than their symbiotic partners.

All the above mentioned entomopathogenic nematode species reduced number plant parasitic nematode in the soil and egg-masses of *Tylenchulus semipenetrans.* This may be attributed to competition at the root surface which may affect plant parasitic nematodes behavior or entomopathogenic nematodes crowded along the roots of plants force plant parasitic away. Suppression of plant-parasitic nematode populations has been demonstrated in a number of greenhouse (Ishibashi and Choi, 1991; Ishibashi and Kondo, 1987) and field studies (Grewal *et al.*, 1997; Smilety *et al.*, 1992) in different cropping systems. It is also possible that experimental differences between greenhouse and field conditions significantly affected the suppressive action of *S. feltiae* on plant-parasitic nematodes.

Earlier research demonstrating suppression of plant-parasitic nematodes after the application of entomopathogenic nematodes was primarily conducted with root-knot nematodes (Bird and Bird, 1986; Ishibashi and Choi, 1991). Suppression of other plant-parasitic nematodes has yielded inconsistent results. Alternatively, *P. penetrans* number were not affected by entomopathogenic nematode application, but not at 3, 5, or 8 weeks in the second year.

The effect of entomopathogenic nematodes on plant-parasitic nematodes may be partially explained in context of the mechanism(s) proposed for plant-parasitic nematode suppression. First, competition at the root surface may affect plant-parasitic nematode behavior and subsequent population densities (Bird and Bird, 1986) Ishibashi and Choi, (1991) determined that the nearly half inculated *S.*

carpocapsae moved to the area of the root tips and remained there for some time. The repellence of plant-parasitic nematodes from root tips may explain significant delays in the root penetration by plant-parasitic nematodes associated with entomopathogenic nematode treatments. In addition, stilbene derivatives and ammonia produced by bacteria show selective nematicidal activity (Hu *et al.*, 1995) .The positive movement towards CO2 is the basis of investigations on the use of entomopathogenic nematodes as a prophylactic soil treatment to coat the surface of plant roots and prevent invasion by plant-endoparasitic nematodes (e.g. Ishibashi and Choi, 1991).The second proposed mechanism concerns the build-up of natural nematode antagonists in soil as a result of massive increases in numbers of nematodes (Ishibashi and Choi, 1991; Ishibashi and Kondo, 1987). The third proposed for entomopathogenic nematodes associated symbiotic bacteria (*Xenorhabdus* and *Photorhabdus* spp) may be directly toxic or have detrimental behavioral effects on plant-parasitic nematodes (Grewal *et al.*, 1999; Hu *et al.*, 1999).

In (2014 Nour El-Deen *et al.,*) experiment the nematotoxic activities of the cell-free conditioned media (CFCM) of entomopathogenic bacterium species *Photorhabdus* luminescens (strain: TT01), *Xenorhabdus budapestensis* (strain: AF 2013 or EMA) and *X. szentirmaii* (strain: EMC), isolated from entomopathogenic nematodes *H. bacteriophora* and *S. bicornutum* and *S. rarum*, respectively. The applied doses were 1, 2, 5, 10, 20, 40, 60 and 80 V/V %, respectively. The test organism was *Meloidogyne incognita* (J 2 s) and the rate of mortalities were determined at the 6 th , 12 th , 24 th and 48 th hrs following exposure. Data revealed that the rate of larval mortality proved dose/dependent. Only the highest dose of TT01 CFCM resulted in 100% mortality rate after 48 hrs of exposure time. On the other hand, lower concentrations of EMA and EMC CFCM resulted in the same results. 6 hrs following application of 10 %, CFCM resulted in significantly the highest larval mortality rate 59.4%; in comparison with EMA (48 %) and TT01 (40%).

(Kella *et al.,* 2011) tested the ability of entomopathogenic nematodes, *S. carpocapsae* and *H. bacteriophora* (B20) as biological control agents against *M. incognita* infecting tomato plants in the greenhouse. Entomopathogenic nematodes were added at three inoculum levels 1000, 2000 and 4000 IJs/pot. The results reported that the use of both entomopathogenic nematodes (EPN) effective in the biological control programs of *M. incognita* and other plant parasitic nematodes.

The symbiotic bacteria associated with Steinernematids, *Xenorhabatdus* spp., produce metabolites that are toxic to nematodes. These metabolites include indole, which produced by *Photorhabdus* in culture. Indole was associated with *M. incognita* paralysis, but was not produced in *G. mellonella* cadavers (Hu *et al.*, 1999). A similar mechanism may explain the results (LaMondia and Cowles, 2002) with *P. penetrans* and *S. feltiae.* Results obtained by Kella and Eman, 2007 have also indicated the toxic effect of Stalpene and indole on plant-parasitic nematodes. The allelochemicals produced by *Xenorhabatdus* spp. As the cause of antagonism to *M. incognita.* In this study *T. semipenetrans* suppression using *Heterorhabditis* was more effective than using *Steinernema.* We found that pre-infestation applications of EPN suppress *T. semipenetrans* on greenhouse sour-orange. Other results by Hu *et al.*, (1999) prove that alellelopathic substances produced by live or dead IJs may be toxic and/or repellent to PPN, thus reducing their population density. EPN-associated bacteria, *Xenorhabdus* spp. or *Photorhabdus* spp., produce endotoxins composed of lipopolysacarides that are toxic and could kill or affect in another way the evaluated stages (Dunphy and Webster, 1988).

Jagdale *et al.*, (2002) stated that live and dead *S. carpocapsae* IJ reduced PPN populations 15 and 30 days after the application by more than 50%. They also suggested a chemical disturbance instead of a physical one.

This data suggesting that some of entomopathogenic nematodes can suppress plant-parasitic nematode species. Genefally, to achieve the second part out of a two-fold goal, *i.e.* control of insect and nematode pests, necessitates optimal application tactics to maximize field effectiveness of EPN, *e.g.* delivery of the dauer stage juveniles near the plant roots for effective phytonematode control (Abd-Elgawad and Aboul-Eid, 2002; Abd-Elgawad *et al.,* 2008 and Kella *et al.,* 2008). In this respect, Lewis and Grewal (2005) wondered what a possible nematode control product based on EPN would look like. One possibility is to market EPN product in the same way as they are currently marketed for insect control. Another possibility is to develop a nematode management product based on the bacteria (or metabolites) alone. However, the broad-spectrum activity of the bacteria (*Xenorhabdus* spp. for Steinernematidae and *Photorhabdus* spp. for Heterorhabditidae) would warrant studies of how these applications would impact non-target species. Eventually, further experiments that account for and standardize the above-mentioned factor, responsible for disparity in EPN efficacy against PPN, are needed. On the other hand

,*Steinernema* sp and *Heterorhbditis bacteriophora* gave reduction of number *T. semipenetrans* juveniles and egg-masses of treated plants than untreated plants.

CONCLUSION

Our results agree with those reported previously to use EPN in the control of PPN. We specifically conclude that entomopathogenic nematodes show promise for control *Tylenchulus semipenetrans*. On the other, showed the effect of Egyptian strains ,*Steinernema* sp. and *Heterorhbditis bacteriophora* (B20) on *T. semipenetrans* gave reduction of number PPN and egg masses than foreign strains. Steinernema carpocapsae and *Heterorhbditis bacteriophora* (HP88) .This result compatible with Abd-Elgawad, and Aboul-Eid(2002) using four Egyptian isolates of entomopathogenic nematodes give some hopping or promising results in this respect (Azazy,1996 and 2001; EL-Deeb *et al.*, 2004)Egyptian isolates are becoming established as biological control agents of some plant parasitic nematodes.

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تقييم النيماتودا الممرضة للحشرات فى المكافحة البيولوجية لنيماتودا الموالح المتطفلة على نباتات النارنج *Tylenchulus semipenetrans.*

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- معهد بحوث وقاية النباتات- مركز البحوث الزراعية- الدقى – جيزة.

و تهدف هذه الدراسة لمعرفة كفاءة النيماتودا الممرضة للحشرات *Steinernema sp.*; *Steinernema carpocapsae*, *Heterorhabditis bacteriophora* (B20) and *Heterorhabditis bacteriophora* (HP88) فى مكافحة نيماتودا الموالح *Tylenchulus semipenetrans* المتطفلة على شتلات النارنج تحت ظروف الصوبة الزجاجية، تم استخدام الطور المعدى من النيماتودا الممرضة بتركيزات ٢٠٠٠، ٤٠٠٠، ٨٠٠٠ طور معدى /اصيص . وبعد اسبوع تم عمل عدوى بنيماتودا *T. semipenetrans* بمعدل ٣٠٠٠ فرد /أصيص.

أوضحت النتائج أن معاملة شتلات النارنج بالنيماتودا الممرضة للحشرات سواء *Steinernema* أو *Heterorhabditis* أدى الى خفض تعداد يرقات نيماتودا الموالح مقارنة بالكنترول فى حالة استخدام التركيز ٨٠٠٠ طور معدى من نيماتودا *Steinernema*، وقد بلغ معدل التكاثر ٠,٧-٢,٦ فى حالة استخدام التركيز ٨٠٠٠ طور معدى من النيماتودا *Heterorhabditis bacteriophora* (HP88) و *H. bacteriophora* (B20) على الترتيب. حيث أكدت الدراسة أنه كلما زاد مستوى العدوى بالنيماتودا الممرضة كلما زادت نسبة الانخفاض فى عدد كتل البيض لنيماتودا الموالح *T. semipenetrans* حيث كانت *Steinernema* sp. اعلى خفضا من النيماتودا *S. carpocapsae* فى عدد كتل البيض ٨٢% ،٧١% على الترتيب عند نفس التركيز. اما بالنسبة لنيماتودا *Heterorhabditis bacteriophora* (HP88) كانت أعلى كفاءة من النيماتودا *H. bacteriophora* (B20) فى خفض تعداد كتل البيض بنسبة ٩٦% ، ٨٢% على الترتيب عند نفس التركيز.ومن التحليل الاحصائى إن السلالات المحلية كانت أفضل من السلالات المستوردة فى تقليل أعداد و كتل البيض للنيماتودا للمرضة للحشرات. وتشير الدراسة إلى أن تواجد الاطوار المعدية من النيماتودا الممرضة للحشرات حول جذور شتلات النارنج وافرازها لمواد سامة من البكتريا المصاحبة لها يشتت نيماتودا الموالح *T. semipenetrans* ويحد من تطفلها على الجذور.