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Utilization of Entomopathogenic Nematodes Combined with Plant Extracts and Plant Essential Oils against Grasshopper, *Heteracrir littoralis*

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ABSTRACT

The two tested nematode species locally isolated belong to family Heterorahabitidae, *Heterorhabditis indica* and *H. bacteriophora* were used. The susceptibility of 5th nymphal instar of the Acridid grasshopper, *Heteracrir littoralis* to different inoculum levels of the insect pathogenic nematodes combined with plant oils and/or plant extracts were recorded. The 5th nymphal instar of *H. littoralis* grasshopper was susceptible to the tested nematode species, either combined with plant oils or plant extracts, however, there were significant degrees of susceptibility between the control (untreated) treatment and all other tested concentrations differs according to the used of nematode species combined with either plant oils or plant extracts. Highest numbers of emerging nematodes coming from cadavers treated with camphor oil and the nematodes followed by garlic mixture and the mint oil mixture with the nematodes have given the less numbers of emerging nematodes, while the mint oil mixture with the nematodes have given the less numbers . Nematodes were affected by the presence of the plant extracts and it was evident that *H. bacteriophora* was more susceptible to plant extracts than *H. indica*.

KEYWORDS: Grasshopper, *Heteracrir littoralis*, entomopathogenic nematodes, *Heterorhabditis* spp., plant oils, plant extracts.

1- INTRODUCTION

Trials were carried out and developed for controlling different species of acridid pests especially grasshoppers all-round the world. Chemical insecticides are the main method applied against grasshoppers especially during outbreak. Appearance of many problems such as insect resistance to chemical insecticides and environmental pollution led to search for effective and safe alternatives to be used in grasshoppers control.

Entomopathogenic nematodes are lethal parasites of many insects with relatively short lifecycle (Gaugler, 2002). Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae are soil inhabiting insect pathogens that possess potential as biological control agents (Gaugler, 1981; Kaya, 1985a; Poinar, 1986; Gaugler and Kaya, 1990; Kaya and Gaugler, 1993).

No other biological control agent offers a comparable combination of attributes: broad host range, high virulence, inexpensive mass rearing, and safety (Poinar, 1990). Researchers have investigated the relationship between grasshoppers and bio control agents such as protozoa (Henry and Oma, 1981) and entomopathogenic nematodes (Abdel-Kawy, 1981).

Recently, most research experiments tend to use some isolates/strains of entomopathogenic nematodes , plant extracts and plant oils to manage insect pests. From time to time efforts to institute the control of certain insects by such measures have been made in various parts of the world. In the current years, the need to reappraise the possibilities of these biological control measures in the light of novel application techniques and recent knowledge concerning the effect of entomopathogenic nematodes, plant essential oils and plant extracts on insect population and their control.

Knowledge on the effect of entomopathogenic nematodes as bio control agents of grasshoppers in Egypt is still sporadic. Both *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* species are the main insect pathogenic nematodes developed as biological control agents used against economic insect pests with a wide host range.

The aim of this work is to investigate the fect and the possibility of using some strains of entomopathogenic nematodes of family Heterorhabditidae in controlling *Heteracris littoralis*, when combined with garlic plant oils *Allium sativum*, camphor, *Eucalyptus globulus* and Mint, *Mentha*

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piperita, or combined with organic extracts of *Euphorbia pulchrrima, Euphorbia cotimfabia, Dodonea* viscose and *Eucalyptus rosrata.*

2- MATERIALS AND METHODS

2-1- Mass rearing of entomopathogenic nematodes

The technique of Dutky *et al.* (1964) followed by a modified White trap (White, 1927) was adapted in the mass rearing of these nematodes. The last instar larvae of the greater wax moth, *Galleria mellonella* and the 5th instar nymphs of Grasshopper, *Heteracris littoralis* were used as host insects in the in vivo production of these nematodes.

2-2- Mixing EPN with plant oils

Three essential oils were applied, Garlic, *Allium sativum* (Family: Liliaceae), camphor, *Eucalyptus globulus* (Family: Myrataceae), and Mint, *Mentha piperita* (Family: Labiatae) were obtained from EL-Captain Company (CAPPHARM), elcaptain@elcaptain.co, Al-Obour City (Cairo), Egypt.

To determine the LC_{50} concentration of different oils on embryonated eggs and different life stages of the tested grasshopper, *H. littoralis,* as determined by Sharaby *et al.* (2012), five descending concentrations that permit the computation of LC_{50} were diluted on the basis of volume/volume (1.0, 0.5, 0.25, 0.13, 0.06%) from each plant oil were prepared by mixing known volume from the oil with 100ml of the insect diet that recorded by Sharaby *et al* (2010), during diet preparation, one drop of Triton X100 was added to obtain the desired concentration. The treated diet poured into plastic box and kept in refrigerator until needed. A piece of the treated diet was introduced into jars that contain nymphal instar as a food source for seven days then the remained diet replaced by untreated one, number of dead insects were counted each day after treatment till day 14 (end of the experiment) to calculate the LC_{50} and LC_{25} values. For each concentration, 25 individuals were tested in five replicates, 5 nymphs each. Controls were fed on untreated diet.

Forty insects were tested in 4 replicates, 10 nymphs each. Control individuals were fed on untreated original diet. The LC_{50} values were determined . After the determination of the LC_{50} and LC_{25} values of each oil, 3ml of nematode infective juveniles suspension that contain 1500 IJs/ml were concentrated with a 500 mesh stainless steel sieve to have a final concentration of 4500 IJs mixed with the LC_{50} and LC_{25} concentrations of the plant oils.

Three ml of the final concentration of the LC_{50} and LC_{25} of the plant oil mixed with the 4500 nematode IJs were evenly distributed with a pipette over a piece of tissue paper and placed inside a 250cc plastic cup. Four individuals of the 5th instar nymphs of the target grasshopper were placed in the plastic cup and covered with the lid. Clover leaves were added to each cup as a food source. Insects were examined every 24 hours for dead insects. Cadavers of dead insects were placed in White traps (White, 1927) and nematodes emerged from the cadavers were counted after 3 weeks.

2-3- Mixing EPN with plant extracts

To determine the LC_{50} concentration of different plant extracts on embryonated eggs and different life stages of the tested grasshopper as mentioned by Sharaby *et al*. (2011). Five descending concentrations that permit the computation of LC_{50} was diluted on the basis of weight/volume (25, 12.5, 6.25, 3.13 and 1.56%) each plant extract were prepared by mixing known weight from the extract with 100ml of grasshopper diet during diet preparation. Triton X 100 was added to reach the desired concentration. The modified diet poured into a plastic container and kept in the refrigerator until used. A piece of the modified diet was introduced into jars containing different insect nymphal instars as food source for five days, then the remnants of treated diet was replaced by the original untreated one.

Number of dead insects were counted each day after treatment until day 10 to calculate the LC_{50} and LC_{25} values for each concentration. Forty insects were tested in 4 replicates, 10 nymphs each. Controls were fed on untreated original diet. The LC_{50} values were determined. After the determination of the LC_{50} and LC_{25} values of each plant extract, 3ml of nematode infective juveniles suspension that contain 1500 IJs/ml were concentrated with a 500 mesh stainless steel sieve to have a final concentration of 4500 IJs mixed with the LC_{50} and LC_{25} concentrations of the plant extracts.

Three ml. of the final concentration of the LC_{50} and LC_{25} of the plant extracts mixed with the 4500 nematode IJs were evenly distributed with a pipette over a piece of tissue paper and placed inside a 250cc plastic cup. Four individuals of the 5th instar nymphs of the target grasshopper were placed inside the plastic cup and covered with the lid. Clover leaves were added to each cup as a food source. The cup was examined daily for dead insects. Cadavers of dead insects were placed on a White traps (White, 1927) and nematodes emerged from the cadavers were counted after 3 weeks.

Statistical analyses

Corrected mortality was carried out using Abbott's formula (Abbott, 1925). All data were subject to analysis of variances (ANOVA) through SPSS computer programme. The mean values were compared using Duncan's Multiple Range test (Duncan, 1965). The LC_{50} and LC_{25} values were calculated according to Finney's equation (Finney, 1971).

3- RESULTS AND DISCUSSION

3-1- Sources of used nematodes:

Native species of entomopathgenic nematodes were used. These species and/or isolates belong to the genus *Heterorhabditis* (Table 1). This table indicates the scientific and code name of these entomopathogenic nematodes as well as names of places from which they were isolated and/or obtained.

Table (1): Scientific, code names and geographic distribution of native *Heterorhabditis* spp. used in this study

Nematode species	Isolate designation	Source
1- ***Heterorhabditis bacteriophora***	EKB20*	El-Sheikh Mobarak Village, Balteam, Kafr El-Sheikh, Egypt.
2- ***Heterorhabditis indica***	EGB4**	Alrigha, Badrshain Center, Giza, Egypt.

*E = Egypt, KB = Kafr El-Sheikh, Balteam.
**E = Egypt, G = Giza, B = Badrshain.

3.2. Nematode and plant oils

Nematodes (4500 IJs) were mixed with the most three effective plant oils plant oils, garlic, *Allium sativum* (Family: Liliaceae), camphor, *Eucalyptus globulus* (Family: Myrataceae) and mint, *Mentha piperita* (Family: Labiatae) Sharaby *et al*. (2012). The mixtures were applied against the tested 5th instar nymphs of the grasshoppers, numbers of emerging nematodes from the cadavers treated with the LC_{25} of the plant oils and nematode mixtures have varied greatly. When the LC_{25} of the Garlic oil, and the nematode *Heterorhabditis bacteriophora* (EKB20) was applied, high numbers of infective nematode juveniles were produced (40447 IJs). Meanwhile, when the LC_{50} of the same plant oil was mixed with the nematodes and applied, less nematode numbers have emerged (29967 IJs). The same trend was recorded in the other two plant oils, camphor, *Eucalyptus globulus* and mint, *Mentha piperita*. With the highest numbers of emerging nematodes coming from cadavers treated with camphor oil and the nematodes followed by garlic mixture and the mint oil mixture with the nematodes have given the less numbers of emerging nematodes (Table 2).

In contrast, when the LC_{25} mixture of the garlic oil and the nematode species, *Heterorhabditis indica* (EGB4) was applied, more or less the same numbers of nematodes (40933 IJs) have emerged from the insect cadavers in comparison with *H. bacteriophora* (EKB20). The same trend was observed in the other two plant oils, camphor, *Eucalyptus globulus* and mint, *Mentha piperita*. With the highest numbers of emerging nematodes coming from cadavers treated with camphor oil and nematode mixtures followed by garlic mixture while the mint oil mixture with the nematodes have given the less numbers of emerging nematodes (Table 3).

Laboratory investigation was carried out to study the susceptibility of certain life stages of the desert locust, *Schistocerca gregaria* Forsk. to an Egyptian strain of the entomopathogenic nematode *Heterorhabditis bacteriophora* (TWF). The obtained results indicated that the tested 1st, 2nd and 5th nymphal instars as well as the adult insects were very susceptible to infection when subjected to moistened sand contaminated with nematode juveniles. Complete mortality was recorded within 72 hours. Nematode development and recovery were evident in dead insects. In cages provided with oviposition sites contaminated with nematode juveniles, mortality was higher in females (100.00%) than in males (83.33%). In a T-tube choice assay, a 62.09% of nematode juveniles could locate, find and infect different developmental stages of the desert locust (Badawy, 2000).

Mixing plant extracts and plant oils with entomopathogenic nematodes accomplished in the present study is a novel idea to increase the efficacy of both biological agents to be used in a synergy instead of using them singly (Tables 2 & 3). Recently, Negrisoli *et al*. (2010) demonstrated that entomopathogenic nematodes including *Heterorhabditis indica*, *Steinernema carpocapsae* and *Steinernema glaseri* were found to be compatible with many insecticides including chlorpyrifos, deltamethrin, lufenuron, deltramethrin + triazophos, diflubenzuron, gamacyhalothrin, lambda cyhalothrin, spinosad, cypermethrin, triflumuron, and permethrin in the laboratory.

Table (2): Mean number of emerging nematode juveniles from insect cadavers of the 5th nymphal stage of *Heteracris littoralis* treated with different plant oils exposed to an Egyptian isolate of *Heterorhabditis bacteriophora* (EKB20) at 4 IJs/ml. of nematode suspension mixed with plant oils

Type & potency of plant oils	Mean number of emerging infective juveniles/insect at nematode concentration of (4 IJs/ml) mixed with different plant extracts
	Mean ± Std. Error
Garlic (*Allium sativum*) (LC_{20})	40446.67±887.47b
Camphor (*Eucalyptus globulus*) (LC_{20})	66700.00±3094.08b
Mint (*Mintha pipreta*) (LC_{20})	35926.6±1809.80b
Control	476866.67±60227.02a
F-value	50.791**
Garlic (*Allium sativum*) (LC_{50})	29966.67±1441.29b
Camphor (*Eucalyptus globulus*) (LC_{50})	55260.00±2612.69b
Mint (*Mintha pipreta*) (LC_{50})	29066.67±2196.79b
Control	476866.67±60227.02a
F-value	53.037**

** Highly Significant.

Means followed by the same letter within a single column are not significantly different (P> 0.05) according to Duncan Multiple Range test

Table (3): Mean number of emerging nematode juveniles from insect cadavers of the 5th nymphal stage of *Heteracris littoralis* treated with different plant oils exposed to an Egyptian isolate of *Heterorhabditis indica* (EGB4) at 4 IJs/ml. of nematode suspension mixed with plant oils

Type & potency of plant oils	Mean number of emerging infective juveniles/insect at nematode concentration of (4 IJs/ml) mixed with different plant extracts
	Mean ± Std. Error
Garlic (*Allium sativum*) (LC_{20})	40933.33±1106.12b
Camphor (*Eucalyptus globulus*) (LC_{20})	68306.67±3561.55b
Mint (*Mintha pipreta*) (LC_{20})	37006.67±1540.02b
Control	280566.67±36958.79a
F-value	39.440**
Garlic (*Allium sativum*) (LC_{50})	39266.67±1425.04 b
Camphor (*Eucalyptus globulus*) (LC_{50})	61166.67±3780.75 b
Mint (*Mintha pipreta*) (LC_{50})	33460.00±2878.64 b
Control	280566.67±36958.80 a
F-value	40.440**

** Highly Significant.

Means followed by the same letter within a single column are not significantly different (P> 0.05) according to Duncan Mutiple Range test.

3.3. Nematode and plant extracts

When nematodes (4500 IJs) were mixed with plant extracts and the mixture was applied against the tested 5th instar nymphs of the grasshoppers, numbers of emerging nematodes from the cadavers treated with the LC_{25} of the plant extracts and nematode mixtures have varied greatly. When the LC_{50} of the plant extract of *Dodonaea viscosa* and the nematode *Heterorhabditis bacteriophora* (EKB20) mixture was applied few nematodes were produced (1000 IJs). Meanwhile, when the LC_{50} of the *Schinus terebinthifolius* was mixed with the nematodes and applied, higher nematode numbers have emerged (2100 IJs). In contrast, no infective juveniles have emerged from insect cadavers treated with the LC_{50} of both green and reddish poinsettia (*Euphorbia pulcherrima* and *Euphorbia cotimfabia*) mixed with the nematodes and applied to the grasshoppers (Table 4).

In contrast, when the LC_{50} of the plant extract of *Dodonaea viscosa* and the nematode *Heterorhabditis indica* (EGB4) mixture was applied with higher numbers of nematodes produced (97850 IJs). While the LC_{50} of the *Schinus terebinthifolius* when mixed with the nematode *Heterorhabditis indica* (EGB4) and applied, less nematode numbers have emerged (41600 IJs). In contrast, no infective juveniles have emerged from insect cadavers when treated with the LC_{50} of the green poinsettia (*Euphorbia pulcherrima*). While the reddish poinsettia (*Euphorbia cotimfabia*) when mixed with the nematodes and applied to the grasshoppers, 21650 IJs have emerged from the cadavers of the treated insects (Table, 5). When the LC_{25} of all plant extracts were mixed with the nematode *Heterorhabditis indica* (EGB4) and applied to the 5th instar grasshopper nymphs. Infective nematode juveniles have emerged from the insect cadavers and their numbers have varied greatly in different mixtures of both plant extracts and nematodes used. The highest numbers of emerging nematode IJs was evident with the use of a mixture between *H. indica* nematode suspension and the LC_{25} of *Schinus terebinthifolius* (105,107 IJs) (Table 5).

Data extracted from tables (4 & 5) have shown number of infective juveniles (IJs) emerged from insect cadavers that killed by exposure to a mixture of plant extracts and nematode infective juveniles. Nematodes were affected by the presence of the plant extracts and it was evident that *H. bacteriophora* was more susceptible to plant extracts than *H. indica*.

The number of IJs emerging from *H. littoralis* nymphs and adults varied significantly among the tested nematode species. *H. indica* reproduced better than *H. bacteriophora*. The number of emerging juveniles varied greatly among the tested nematode isolates. Such variations have been attributed to differences in the proportion of infective juveniles retaining bacteria as well as the number of bacteria within the infective juveniles of each nematode strains and/or species. Whereas, growth of the nematodes inside the cadavers is contributed to the symbiotic bacteria which produce antibiotics preventing the growth of competing microorganisms and support the nematode growth and reproduction as the main food source for the nematodes (Akhurst, 1983).

Saleh and El-Kifl (1994) have also reported that, *H. bacteriophora* produced more IJs in the larvae of *Ostrinia nubilalis* than did *S. carpocapsae*. Shamseldean *et al*. (1994) when exposed certain insect pests to different *heterorhabditid* nematode isolates and observed differences in the reproductive potential among the tested nematodes, and Koppenhofer and Kaya (1995) who reported that, increasing densities of *S. glaseri* IJs in the soil affected reproduction of the nematode in *G. mellonella* larvae and the number of progeny produced per host cadaver decreased at the higher densities of IJs used in the infection process.

Table (4): Mean number of emerging nematode juveniles from insect cadavers of the 5th nymphal stage of *Heteracris littoralis* treated with plant extracts mixed with 4500 IJs of an Egyptian isolate of *Heterorhabditis bacteriophora* (EKB20).

Type & potency of plant extract	Mean number of emerging infective juveniles/insect at nematode concentration of (4500 IJs) mixed with different plant extracts
Dodonaea viscose **(LC_{50} = 13.32 mg./ 100 ml. insect diet)**	**1000.00±316.23^{c}**
Dodonaea viscose **(LC_{25} = 0.20 mg./100 ml. insect diet)**	**77460.71±22213.48^{b}**
Schinus terebinthifolius **(LC_{50} = 23.18 mg./100 ml. insect diet)**	**2100.00±428.04^{c}**
Schinus terebinthifolius **(LC_{25} = 0.38 mg./100 ml. insect diet)**	**34392.86±2290.65^{bc}**
Green Poinsettia (*Euphorbia pulcherrima*) (LC_{50} = 3.61 mg./100 ml. insect diet)	**0.00±0.00^{c}**
Green Poinsettia (*Euphorbia pulcherrima*) (LC_{25} = 0.41 mg./100 ml. insect diet)	**28750.00±5032.04^{bc}**
Reddish Poinsettia (*Euphorbia cotimfabia*) (LC_{50} = 3.75 mg./100 ml. insect diet)	**0.00±0.00^{c}**
Reddish Poinsettia (*Euphorbia cotimfabia*) (LC_{25} = 1.20 mg./100 ml. insect diet)	**31750.00±3223.52^{bc}**
Control	**476866.67±60227.02^{a}**
F-value	**51.105****

**Highly significant.

Means followed by the same letter within a single block are not significantly different (P> 0.05) according to Duncan Mutiple Range test.

Table (5): Mean number of emerging nematode juveniles from insect cadavers of the 5th nymphal stage of *Heteracris littoralis* treated with different plant extracts exposed to an Egyptian isolate of *Heterorhabditis* indica (EGB4) at 4 IJs/ml. of nematode concentrations mixed with plant extracts

Type & potency of plant extract	Mean number of emerging infective juveniles/insect at nematode concentration of (4 IJs/ml) mixed with different plant extracts
	Mean ± S.E.
Dodonaea viscosa **(LC_{50})**	**97850.00±46116.67b**
Dodonaea viscosa **(LC_{25})**	**50656.67±7152.47bcd**
Schinus terebinthifolius **(LC_{50})**	**41600.00±5198.15bcd**
Schinus terebinthifolius **(LC_{25})**	**105107.14±14313.81b**
Green Poinsettia (*Euphorbia pulcherrima*) (LC_{50})	**0.00±0.00d**
Green Poinsettia (*Euphorbia pulcherrima*) (LC_{25})	**58250.00±8500.32bcd**
Red Poinsettia (*Euphorbia cotimfabia*) (LC_{50})	**21650.00±11832.05cd**
Red Poinsettia (*Euphorbia cotimfabia*) (LC_{25})	**78600.53±7573.59bc**
Control	**280566.67±36958.80a**
F-value	**14.925****

** Highly Significant

Means followed by the same letter within a single column are not significantly different (P> 0.05) according to Duncan Mutiple Range test.

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