Compatibility of three virulant Egyptian entomopathogenic nematod strains and two organophosphus insecticids in controlling Zeuzera pyrina L. in olive orchards

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Abstract
This investigation aimed to explore the efficacy of three Egyptian isolates and/or strains of entomopathogenic nematodes and/or two recommended insecticides, to control Zeuzera pyrina L. under laboratory and field conditions. Results revealed that in all tested EPNs, LC50s were lower in larval stages than pupae. All nematode strains were not significantly affected by any of the tested concentrations of diazinox or basudin, i.e., IJs survival was held over 75% and 66% when checked by 1500 and 4500ppm, respectively. Insecticides exposure period affected to some extent the nematode survival regardless of their concentration. Z. pyrina highest mortality (100%) was obtained from the mixture of basudin (half recommended rate) and LC90 of EGB20 strain. Data extracted from field results recommends that, all tested nematode strains at 1000 IJs/ml proved to be more effective against Z. pyrina in olive tree than diazinox and basudin. The EGB20 strain showed superior effect over EGB13 and EBN16, especially with injection methods and addition of evaporation retardant agent. The joint action of mixing nematode strains together with insecticides as a co-toxicity factor did not achieve its goal or fulfilled our expectations. Yet, combinations of EGB13 and EGB20 strains, proved to be slightly higher in their effectiveness than using insecticides or entomopathogenic nematodes separately and multiple co-applications of insecticides and nematodes were associated with a longer protection period of olive trees. The current study might be an important step towards establishing an efficient control strategy capable of minimizing Z. pyrina population below economic thresholds in olive farms.

Key-words: Entomopathogenic Nematodes, Basudin, Diazinox, Interaction, Leopard Moth, Olive

Introduction
The olive tree (Olea europaea L.) belongs to the family Oleaceae, is native to the Mediterranean region, tropical & central Asia and various parts of Africa (Syed, 2008). It plays different roles in lives of Mediterranean people, i.e. nutritional, social, cultural, economic and political. Yet, Z. pyrina L. (Lepidoptera: Cossidae) is considered as a serious pest of olive trees. The impact of this pest in North Africa has increased during the last few decades (Hegazi et.al., 2015). The larval stage of Z. pyrina are cryptic woodborers affecting a wide variety of trees and shrubs, comprising over 150 plant species from 20 genera (Kutinkova et al., 2006). Newly established olive orchards suffer a great damage, including the death of young trees, whereas, in nurseries, damage can be particularly extensive (Castellari, 1986). In Egypt, damage caused by Z. Pyrina led to uprooting of olive groves by growers. The damage caused by larval tunnels in structurally critical wood can be
extremely serious to a tree already bearing a fruit load; it causes ordinary branches to break under a medium load, whereas it may cause complete death of young trees with a heavy load as a result of damage to the major branches and trunk (Hegazi et al., 2015).

The potential disappointment of insecticides application including development of insect resistance, contamination of plant and animal products, and environmental pollution, served as a strong impetus for developing alternative measures to control insect pests. In this respect, entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae have been successfully used throughout the world for the control of important agricultural insect pests (Atwa, 2014). There are many advantages that make EPNs excellent biocontrol agents such as their broad host range, their ability to search actively for their hosts, kill them relatively quickly, in addition, they are economically mass produced, non-injurious to vertebrates, easily applied, compatible with most chemical insecticides, and environmentally safe (Garcia Del Pino and Palomo, 1996). All these attributes indicate that EPNs could be used in IPM programs involving the use of chemical insecticides. Therefore, this investigation was implemented to determine the combined effect of EPNs and/or insecticides on the leopard moth (Zeuzera pyrina).

Although results from many laboratory tests concerning the efficiency of EPNs have been promising in controlling insect pests, yet, field evaluation results have often been highly variable particularly against well-hidden insects of cryptic habitats such as soil and tunnel living insects (Atwa, 2018). Those pests are well protected with a high survival rate. Thus, these insect hosts are capable of producing new populations with large numbers that subsequently disperse or migrate or both to find more susceptible hosts where control measures are essential. Therefore, field trials were conducted to validate laboratory findings in the field. In addition, the use of naturally occurring nematodes in a particular area as biological control agents may also reduce the risk to non-target organisms when compared with exotic isolates (Blackshaw, 1988).

Selection of appropriate EPNs as biological control agents includes laboratory bioassays to identify virulent strains and evaluate efficacy under simulated field conditions (Jansson et al., 1993). Nematodes field release strategy may be risky and may result in poor efficacy if environmental conditions are not favorable and/or nematode quality is poor. Similarly, a possible reason for poor control or rapid resurgence of insect populations by insecticide applications could be attributed to the increase in insect tolerance and/or the suppression of natural enemies (Shorey, 1961; Georgmou, 1963). Accordingly, reducing the use of insecticides in pest management through mixing low compatible concentrations with virulent EPNs might maximize the pest management and minimize environmental hazards (Sammour and Saleh, 1996). However, different factors such as application techniques, using of evaporation retardant agents, and proper time of application, play different roles in co-applications of nematodes and chemical insecticides which reflect on field efficacy against noxious insect pests. Mixed application of S. carpocapsae with certain insecticides to the soil has provided more effective insect control than separate applications of each (Hatsukade, 1987; Hatsukade et al., 1988; Ishibashi et al., 1987).

In addition, Ishibashi (1993) reported that soil applications of carbamate and organophosphate insecticides with S. carpocapsae (All strain) increased field efficacy of the nematodes against three species of lepidopterous larvae. The range of carbofuran doses used in these studies (0.01 to 50 μg/ml) was below that used by other researchers who reported adverse effects consequent to treating IJs with the carbamates, oxamyl or methomyl (Gaugler and Campbell, 1991; Hara and Kaya, 1983; Ishibashi, 1993; Ishibashi and Takii, 1993). Some reports indicated that compatible combinations were sharply effective against Z. pyrina larvae infesting apple and olive trees. Othman (1994) reduced Z. pyrina larval population significantly by injecting the galleries with phenthoate, cidal, vydate and inserting the suspension of N. carpocapsae into the galleries entrance holes by using a soaking cotton-wool. He found also that the larval galleries can be left open after treatment with the chemical insecticides but in case of the nematode suspensions, plugging the galleries is preferable. According to Abd-Elgawad (2019) more innovative thinking, systematic experiments, and well-designed field trials examining new options and showing EPNs worth are desperately needed, consequently, this study may assist in establishing an efficient control strategy capable of minimizing population densities of the leopard moth
(Zeuzera pyrina) below economic thresholds and optimizes EPNs capabilities to be more active in the biological control strategies of insect pests.

Material and methods

Laboratory experiments

Z. pyrina collection

Last instar larvae and pupae of Z. pyrina were collected to evaluate the virulence of the selected nematode isolates. Insects were collected from apple and olive fields in New Nubaria city - Beheira Governorate; which is a coastal Egyptian governorate, located in the northern part of the country, west of the Nile Delta (between 30° 36' 36" N and 30° 25' 48" E). Captured insect stages were directly placed individually in 30 ml plastic cups with screw plastic lids and transferred immediately to the laboratory.

Nematode Strains

Three Egyptian nematode strains isolated and identified by Atwa (2003), i.e. *H. bacteriophora* (EGB13), *H. indica* (EBN16) and *Steinernema* sp. (EGB20) were in vivo reared, in great numbers, on the last instar larvae of *G. mellonella* according to the technique of Dutky et al. (1964) and IJs were harvested from nematode traps as described by White (1927) at 25 ± 1°C. A stock suspension of IJs in sterilized distilled water at a concentration of 1000 IJs/ml was stored at 10°C until needed. All nematodes were used within 14 days post-harvest and a new stock was produced every 2 weeks.

Insecticides

Two widely recommended used insecticides from the organophosphates group were tested against the three EPNs strains (EGB13; EBN16 and EGB20), i.e. the Diazinox (60%EC) provided by the "Nippon Kayaku Co. Ltd, Japan" and Basudin (60% EC) provided by "the Syngenta Agrochemical, Switzerland", according to their commercial formulation. Stock solution and dilutions of these insecticides were freshly prepared in distilled water based on their formulation. Concentrations employed in this investigation were determined as half, equal or one and half recommended field rate which was 3000ppm, for both insecticides.

Virulence Bioassays

The virulence of the Egyptian strains; *H. bacteriophora* (EGB13), *H. indica* (EBN16) and *Steinernema* sp. (EGB20) were assessed against the late instar larvae and pupae of *Z. pyrina*. Comparative nematode infectivity was measured as LC50s (concentration of IJs required to kill 50% of treated insect stages) and LT50s (time required to kill 50% of treated insect stages). The capture insect stages were washed with sterile distilled water and exposed to the nematode suspensions on the day of collection. The LC50s were determined by placing individual insect larvae/pupae in 30ml plastic cups with screw plastic lids and transferred immediately to the laboratory.

Insecticides–Nematodes–Insect Interactions

After detecting the most virulent nematode strains from the previous bioassay tests, the study extended to another stage, where we determined the insecticides’ toxicity to these strains, in addition to assessing the ability of the insecticides - treated IJs to cause host mortality. Moreover, a successive series of toxicological tests were maintained to identify the insecticidal role in this complex.

Survival of Insecticide-Treated IJs

About 1000 IJs/ml of EGB13, EBN16 and EGB20 were used in this experiment. Three serial dilutions of each insecticide were made, viz., 1500, 3000 and 4500 ppm for diazinox and basudin. Each insecticide dilution was added individually to Petri dishes (60 X 15mm) in 5- ml aliquots. Five ml of each nematode strain suspension was added in as well to obtain the desired mixture. Untreated controls were employed using distilled water in place of the
insecticide solution. The Petri dishes were sealed with parafilm to avoid evaporation. Each treatment was replicated three times and incubated at 25 ± 2°C. Nematode survival was determined after 48 and 96 hour of exposure to insecticides by taking a sample of 1 ml of the mixed suspension from the Petri dishes. Nematodes that responded to prodding by movement were considered alive; those that did not move were recorded as dead (Hara and Kaya, 1983). Counts were repeated three times for each concentration at each time interval. The data were analyzed using the analysis of variance (ANOVA) and Least Significant Differences (LSD) tests.

**Infectivity of Insecticides–Treated IJs**

The ability of the two insecticide–treated nematode strains to cause host mortality was tested at 25 ± 2°C against last instar larvae of *Z. pyrina*. The nematode inocula of each strain were applied at concentrations equivalent to LC90 for each larva to compare nematicidal potency. These LC90 values were constructed on the log–concentration scale based on previous experiments. A single larva of *Z. pyrina* was placed on a 5.5 cm diameter filter paper in a 60 x 15mm petri dish containing 3 ml of nematode–insecticide suspension and was exposed to combinations of LC90 of each IJs strain plus 1500 ppm (half recommended dose) of diazinox and basudin. Insect mortality was recorded after 72 hours from initial exposure to nematode–insecticide suspension, all dead insects were dissected to verify that they harbored nematodes. Nematode concentrations which reach the LC90 and insecticide solutions were tested in parallel with the above treatments. Ten replicates were used per treatment.

**Field Evaluation**

Field trials were designed based on the data extracted from the laboratory assays. Reasonably, the incompatible nematode–insecticide combinations were impractical for field work. Experiments were conducted before sunset at 6:00 PM. Temperatures and relative humidity ranged from 14.8–32.4°C and 28–91%, respectively. In all tests, water was used as the control solutions in non-treated trees.

The experiments were carried out in an olive tree orchard (5-7 year-old) located in Abbas El-Akkad village, El-Nubaria region. Trees in the orchard, naturally infested with *Z. pyrina*, were used in this investigation. The Farm was divided in plots; each contained a minimum of ten trees. All experimental units were treated during May 2019.

**Effect of Application Techniques on Z. pyrina Infestation**

The nematodes suspension of each of the Egyptian strains mixed with one of the chemical insecticides (diazinox or basudin) were delivered into the active tunnels of the destructive larval stages of *Z. Pyrina* through two application techniques, i.e. injecting and spraying.

The recommended concentration (3000 ppm) of diazinox 60% E.C. and basudin 60% E.C. to control *Z. pyrina* under field conditions were used, whereas, half the recommended concentration (1500ppm) was utilized only in the compatible insecticide-nematode combination treatments. The tested evaporation retardant agent superfilm (tricitin polymers, Baico Company, USA) was added at 1m/ liter of either nematode suspensions and/or insecticide solutions.

Injecting technique was carried out by applying the liquid inside the galleries using a 30 cm long flexible plastic tube (2 mm in diameter) fixed in a 60-cc plastic syringe. On the other hand, the spraying technique was achieved by using a 1-liter portable backpack sprayer with a diaphragm pump. The spraying liquid was always directed towards the opening of the tunnels and overall directed to the infested areas. Each application method included 12 treatments: i.e. nematode suspension, insecticide emulsion and mixtures of nematode species and insecticides. In the control experiments all treatments, liquid materials were applied with or without evaporation retardant agent (superfilm). Five replicates (infested trees) per treatment were used. At time of application, active galleries (5-15 live larvae/tree) were tagged on the selected trees. Frasses were removed from the entry of the holes and from the ground surface around the tree trunk at the time of application and two days later. Seven days post applications all marked galleries were examined individually. Fresh frasses’ explosions at the opening of the active tunnels pointed to live larvae. Dead larvae inside inactive galleries were determined by

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breaking the small branches or using a flexible hooked wire for larval extraction. Percentage of insect mortality was calculated using Henderson and Tilton formula (1955).

**Effect of Successive Applications on Z. pyrina Infestation**

In this experiment, the two application methods were compared in relation to applications’ date. Each of the injection and spraying techniques included the following treatments: EGB13 (1000 IJs) + Diazinox (1500 ppm), EGB20 (1000 IJs) + Diazinox (1500 ppm), EGB13 (1000 IJs) + Basudin (1500 ppm) and EGB20 (1000 IJs) + Basudin (1500 ppm). Infested olive trees were treated on 20 March, 10 April, 30 May and 15 October 2019 (as this is the pest’s second peak). The previously mention methodology and application techniques concerning preparation, implementation and results recording of nematode-pesticides combinations was followed. Experimental units consisted of individual olive trees, five replicates per treatment and four treatments per test. All treatments were applied just before sunset.

**Data Analysis**

The data were normalized using an arcsine transformation. The significance of differences between the means was determined using analysis of variance (ANOVA). Comparisons were made by using Tukey’s multiple range test. The LC50s and LT50s were estimated using the log - concentration scale. Percentage of insect mortality was calculated using Henderson and Tilton formula (1955). Considering the joint action of the combination of each insecticide with each nematode strains against the infestation of Z. pyrina, the co-toxicity factor (C.F.) was calculated to differentiate the final effect of the combination, if it is additive, synergistic or antagonistic according to the equation of Sun and Johnson (1960).

\[
C. F. = \frac{\text{Observed } \% \text{ mortality } - \text{ expected } \% \text{ mortality}}{\text{Expected mortality } \%} \times 100
\]

A positive factor of (+20) or more means potentiation, a negative factor of (-20) or more means antagonism, and intermediate values (i.e. between – 20 and +20) is considered as additive effect.

**Results and Discussion**

### Laboratory Experiments

**Nematode Virulence vs. Z. pyrina larval and pupa stages**

Glazer et al. (1996) explained that insect hosts’ rapid mortality due to nematodes infestation indicates high nematode virulence which was attributed to the combination of faster penetration rate and rapid propagation of the symbiotic bacterium. In our assessment on the EPNs virulence we found that the LC50s and LT50s of all tested nematode species and/or strains varied under laboratory conditions according to the insect stage and the used EPN, as presented in Figure (1a). Among all the tested strains, *H. bacteriophora* (EGB13) recorded the lowest LC50s (10.6 and 15.8 for both larvae and pupa, respectively) and LT50s (24hrs for both stages), followed by *Steinernema* sp. which took the same time to cause death of larvae and pupa but needed more concentration as the LC50s were 12.5 and 16.9, respectively. The least effectiveness and longest time were recorded by *H. indica* as presented in Figure 1a and b. Other researchers tested the differences in pathogenicity of both *Heterorhabditis* and *Steinernema* against Z. pyrina, i.e. Saleh et al. (1994) indicated *H. bacteriophora* achieved better control than *S. glaseri* against Z. pyrina larvae. On the contrary, Abdel-Kawy et al. (1988) stated that *S. carpocapsae* has more potential against this pest than *H. bacteriophora*. Similar results were obtained by Elham et al (2015) who stated that *S. carpocapsae* was more virulent than *H. bacteriophora* against Z. pyrina larvae under laboratory conditions. Considering the findings of those studies compared to our experiments, it could be suggested that the variation in data might have resulted from the origins of the tested EPN stains or the used technique.
Insecticides–Nematodes–pest Interactions
Survival of Insecticide-Treated IJs

The three tested nematode strains showed different survival ability in relation to the used insecticides concentration and exposure time. However, all used nematode strains were affected to some extent by the tested concentrations of diazinon and basudin. This finding is in accordance with Gordon et al. (1996) and Rovesti and Desko (1990) who highlighted that organophosphates compounds are toxic against Steinernematids and Heterorhabditids. Basudin was significantly more toxic to all nematode strains than diazinon as presented in Table 1. On the other hand, the IJs survival of EPNs strains decreased in the higher concentration, i.e. 4500 ppm, than the recommended and the half-recommended concentrations in both insecticides. After forty-eight hours exposure to the recommended concentration of diazinon (3000 ppm), about 92.7% of H. bacteriophora IJs survived, whereas IJs survival percentage recorded only 82.5% in case of H. indica. Steinernema sp. showed a survival percentage very similar to that of H. bacteriophora (95.9%). This was the case also with the other diazinon concentrations as both H. bacteriophora and Steinernema sp. IJs survived better than those of H. indica. Several researchers confirmed our findings, i.e. Laznik et al. (2012), Radová (2010) and Yan et al. (2012) who stated that infective juveniles of different species of Steinernema and Heterorhabditis were evaluated against different agro-chemicals, each species exhibited varying degree of mortality.

Moreover, it was also noticed that increasing exposure period (from 48 to 96 hrs.) increased IJs mortality as presented in Table 1, although percentages of survival differed from one strain to the other. Basudin insecticide recommended concentration showed almost the same trend of diazinon, as the survival percentages of H. bacteriophora IJs decreased from 89 to 81%, H. indica from 86 to 71% and Steinernema sp. survival also decreased from 93 to 74%, on the two registration dates, respectively. These results were confirmed by the finding of Kumar et al. (2015) who tested the effect of increasing exposure time of nine insecticides (some of which are from the organophosphorus group) on the survival of IJs of both Steinernematids and Heterorhabditids and stated that increasing exposure periods caused higher mortality rates among IJs of both nematodes under laboratory conditions.

Obtained results indicate that both insecticides had a negative lethal effect on nematodes, yet a contrast concept should be borne in mind for beyond higher concentrations. The action of these organophosphates (basudin and diazinon) on nematode species was reviewed in literature. Zimmerman and Chranshow (1990) noted that diazinon (basudin) was not toxic to N. carpocapsae and N. bibionis during 48 hours of exposure whereas H. bacteriophora (Hp-88) was significantly affected at the end of 48 hours exposure to 400 ppm diazinon. The findings of Sammour and Saleh (1996) are in accordance with our results as they found that diazinon was safe to the two Egyptian nematodes Heterorhabditis sp. and Steinernema sp. while basudin was safe to Steinernema sp. and harmful to Heterorhabditis sp. after 3 days continuous exposure. In the current study we found the same; 48hrs exposure to the recommended concentration of basudin caused only 7% mortality in Steinernema sp. IJs whereas 11 and 14% mortality occurred in case of both Heterorhabditis strains, respectively, as shown in Table 1. Clearly, such comparative lethal effect varied with regard to nematode type, employed insecticide rate and time exposure as they explained.

Table 1. Effect of diazinon and basudin different concentrations on EPNs strains survival within 48 and 96 hrs of exposure.

| Nematode strain | Exposure period (hr.) | Corrected IJs survival % | Diazinon 60%EC | Basudin 60% EC |
|-----------------|-----------------------|--------------------------|---------------|---------------|
| Heterorhabditis bacteriophora (EGB13) | 48 | 95.7 | 95.0 | 78.0 | 92.0 | 89.0 | 72.0 |
| | 96 | 82.5 | 82.8 | 69.0 | 83.0 | 81.0 | 65.0 |
| H. indica (EBN16) | 48 | 89.2 | 87.3 | 76.7 | 90.5 | 86.0 | 73.0 |
| | 96 | 76.4 | 71.6 | 62.0 | 79.7 | 71.0 | 63.0 |
| Steinernema sp. (EGB20) | 48 | 95.9 | 89.5 | 79.0 | 92.0 | 93.0 | 68.0 |
| | 96 | 78.5 | 75.5 | 68.0 | 75.0 | 74.0 | 66.0 |

*Recommended field rate.
It could be concluded that diazinox and basudin have low toxicity to tested nematode strains at their practical recommended concentrations, therefore, these combinations might be suitable for co-applications against Z. pyrina, taking into consideration the usage of the recommended concentrations and IJs exposure period to the insecticides. This concept has been strengthened by Prakasa et al. (1975) Das and Divakar (1987) and Ishibashi and Takii (1993) who reported “no concern about problems associated with the application of nematodes with insecticides at their practical recommended concentrations”.

**Infectivity of Insecticides Treated- IJs**

As stressed earlier in Table 1, the effectiveness of diazinox and basudin on the IJs were poorly – toxic compiling which eliminated only about 4 – 25% of nematode IJs. Diazinox and basudin half recommended concentration (1500ppm) gave low control percentages as mortality of Z. pyrina larvae recorded 10 and 15 %, respectively after 48hrs of treatment. On the other hand, the combination of insecticides and nematode LC90 caused mortality percentages ranged from 90 to100% which is considered a high control level. However, these mortalities were either equal or in significantly greater than those caused by a single application of nematodes alone (LC90). In this line, there were no significant differences in infectivity between heterorhabditid and steinernematid strains as presented in Table 2. Nevertheless, the highest Z. pyrina mortality value (100%) was obtained from the mixture of basudin and EGB20 strain (LC90) whereas mixed application of diazinox and EBN16 strain did not improve insect control than that caused by nematode alone (90% insect mortality). Possibly, enhancing nictating behavior of EGB20 by basudin may support their highest infectivity as explained in other instances by Ishibashi (1993); Ishibashi and Takii (1993) and Gordon et al. (1996).

| Insecticides (1500 ppm) | Nematodes strains nematode/larva (LC90) | Control |
|------------------------|----------------------------------------|---------|
|                        | H. bacteriophora LC90= 72 IJs/L         |         |
| Diazinox               | 95 b                                   | 10 a    |
| Basudin                | 95 b                                   | 15 a    |
|                        | H. indica LC90= 95 IJs/L                |         |
| Diazinox               | 90 b                                   |         |
| Basudin                | 95 b                                   |         |
|                        | Steinernema sp. LC90= 85 IJs/L          |         |
| Diazinox               | 95 b                                   |         |
| Basudin                | 100 b                                  |         |

On feet of results, the effectiveness of tested insecticide–nematode combinations on larvae could be summed up into two levels. The first is high – toxic level, i.e. diazinox– EBN16; which recorded 90% mortality equal to nematode LC90 treatments and the second; is extremely high–toxic level in the other remainder combinations which exercised 95 – 100% insect mortality. Several authors discussed the importance of using combinations of insecticides and EPNs in controlling pests. Hara and Kaya (1983) suggested that the combination between insecticides and nematodes should result in additive or supplemental mortality to the pest species or reduce the amount of chemical insecticides required for insect control. Further, Kumar et al. (2015) mentioned that the use of EPNs in conjunction with insecticides not only reduces the cost of application/pesticide dosage but also helps to achieve higher percentage mortality of target pests within short time. In these respects, Zhang et al. (1994) and Gordon et al. (1996) reported minimal effects of a variety of organophosphates on nematode infectivity.

The discussion on insecticides’ role in nematode infectivity would evolve physiological points of view. Pristarko (1967) reported that the addition of small amount of certain insecticides causes physiological weakening of the insect organism and reducing its resistance to EPNs. Poinar (1986) mentioned that the epithelial cells of insect, where nematodes usually penetrate, are structurally and physiologically weakened by using insecticides due to increase or decrease in their “pH values”. The increase or decrease in the esterase titer which play an important role in the resistance mechanism in insects, may be governed by the type and dose of the insecticide used and insect instar. On the other hand, Ahmed (1982) stated that insecticides treatment may affect the activity of cholinesterase enzyme that is responsible for the protein synthesis due to the dysfunction of the endocrine hormone which controls the release of this enzyme. Thus, nematode-insecticide
combination is able to overcome physiological defense system in insects. Another concept was proposed by Forshler et al. (1987) in the search of pesticide effect on nematode activity and infectivity, they claimed that reduction in nematode activity after exposure to chemicals is not accompanied with concomitant reductions in infectivity but nematode exposed to chemicals became quiescent and then they gain their activity again after being removed from contact with chemicals. The reason for the slow death state may be due to rates of penetration, metabolism and detoxification of the chemical by the nematode. Other investigators (Saleh and Sammour, 1995) proposed that the potential joint action of insecticide – nematode was probably due to the easy and fast reaching of insecticides to their target site in the insect. This may be enhanced through pathways induced by the nematodes and their associated bacteria besides the separate action of both insecticides and nematodes.

**Field Evaluation**

**Effect of Application Techniques on Z. pyrina Infestation**

The efficacy of the tested insecticides, EPNs and their combination against Z. pyrina in the field are presented in Table 3. The percentage of infestation reduction was computed by Henderson and Tilton equation (1955). All tested nematode strains at 1000IJs/ml proved to be more effective than both diazinon and basudin at their recommended concentration (3000ppm). These finding was manifested in the two applied treatments’ methods, with or without evaporation retardant agent super film (tricitin polymers).

**Table 3. Effect of EPNs strains and/or insecticides on the population reduction of Z. pyrina infesting olive trees by using different application techniques.**

| Treatments (nematodes* and/or insecticides) | Insect population reduction % and co-toxicity factor |
|--------------------------------------------|-----------------------------------------------------|
|                                            | Injection methods                                    |
|                                            | Without Super film | With Super film | Without Super film | With Super film |
|                                            | % of population reduction | C. F. | % of population reduction | C. F. | % of population reduction | C. F. | % of population reduction | C. F. |
| EGB13 (1000 IJs)                          | 82.1 | - | 87.3 | - | 52.5 | - | 56.5 | - |
| EBN16 (1000 IJs)                          | 75.3 | - | 81.2 | - | 48.2 | - | 51.1 | - |
| EGB20 (1000 IJs)                          | 89.3 | - | 94.3 | - | 51.1 | - | 57.8 | - |
| Diazinon (1500 ppm)                       | 26.9 | - | 30.8 | - | 16.7 | - | 17.8 | - |
| Bassudin (1500 ppm)                       | 26.8 | - | 30.7 | - | 16.7 | - | 17.8 | - |
| Diazinon (3000 ppm)                       | 52.8 | - | 60.1 | - | 33.2 | - | 38.2 | - |
| Bassudin (3000 ppm)                       | 54.5 | - | 61.3 | - | 34.5 | - | 37.1 | - |
| EGB13 (1000 IJs) + Diazinon (1500 ppm)    | 90.3 | -17 | 95.2 | -19 | 62.3 | -10 | 69.2 | -5 |
| EBN16 (1000 IJs) + Diazinon (1500 ppm)    | 79.2 | -22 | 83.1 | -25 | 50.5 | -22 | 54.1 | -22 |
| EGB20 (1000 IJs) + Diazinon (1500 ppm)    | 94.1 | -19 | 91.2 | -22 | 53.2 | -22 | 69.1 | -5 |
| EGB13 (1000 IJs) + Basudin (1500 ppm)     | 91.3 | -17 | 81.3 | -26 | 67.2 | -4 | 72.1 | -4 |
| EBN16 (1000 IJs) + Basudin (1500 ppm)     | 82.5 | -20 | 85.3 | -24 | 61.1 | -7 | 65.1 | -7 |
| EGB20 (1000 IJs) + Basudin (1500 ppm)     | 84.0 | -28 | 87.3 | -30 | 63.2 | -8 | 74.2 | -3 |

*EGB13=H. bacteriophora, EBN16=H. indica and EGB20=Steinernema sp.

** The reduction population was calculated by Henderson and Tilton formula.

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Comparing the efficacy of nematode strains against *Z. pyrina*; data showed the superiority of EGB20 strain over both EGB13 and EBN16, especially with the injection methods. As presented in Table 3 the strain EGB20 caused a reduction percentage of 94.3% and 89.3% with and without the addition of super film, respectively, with a difference in reduction% that exceeded both EGB13 and EGB16 by about 7 and 13% when the super film was added and 7 and 14% without the super film addition. In general, data indicated that the efficacy of tested nematodes was more pronounced in the injection method than that of the spraying, especially when the super film technique was followed. This result is in accordance with El Ashry et al. (2018) who declared that combined EPNs with insecticides is more effective as injection technique than spray method. Apparently, the galleries caused by the insect larvae protected the injected IJs from the unfavorable conditions such as desiccation and ultraviolet. In contrast, spraying nematodes on the smooth surface of the infested trees bark cause a great loss of nematode inoculum by dripping and desiccation (Abdel-Kawy et al., 1988). On the other hand, in the spraying technique the addition of the super film succeeded in prolonging nematode survival which led to improving insect control to some extent as presented in Table 3. This finding was confirmed by several authors who tested variable materials and their effect of nematode survival, i.e. Welch and Briand (1961), Webster and Bronshill (1968) and Bedding, (1976). In addition, Grewal (2002) and Navon et al. (2002) mentioned that the addition of various polymers enhanced nematode survival and efficacy on foliage. Moreover, Massinon and Lebeau (2012) reported that adding surfactants to the spraying tank may help to prevent spray droplets from bouncing or rolling from the plant, by altering the surface tension of droplets and potentially increasing the deposition of EPNs on the plant.

Data in Table 3 indicated that the co-toxicity factor values ranged between -4 to -19. It could be concluded here that the combinations between the chemical insecticide at half recommended concentration and entomopathogenic nematodes did not achieve the required aim or fulfill our expectations. This may be due to the high efficacy of nematode virulence, when used alone. Yet, combinations, especially those of EGB13 and EGB20 strains, proved to be slightly higher in their effectiveness than using insecticides or entomopathogenic nematodes separately. In this context, Ishibashi and Kondo (1987) reported that the IJs of nematodes can be chemically activated with low concentrations of certain insecticides. Similarly, Sammour and Saleh (1996) demonstrated that combined applications of insecticides with an unidentified species of nematodes resulted in synergistic or additive effects on *Z. pyrina* larvae in the field. Consequently, we could summarize that using nematodes under field conditions needs the addition of certain compounds to maintain their activity for a long time provided that no detrimental effect occurs for the nematodes. In the present study, diazinon and basudin (half recommended concentration) could definitely play that role.

**Effect of Successive Applications on *Z. pyrina* Infestations**

In the previous field trial, it appeared that co-application of EGB13 or EGB20 nematode strains with half the recommended concentrations of diazinon or basudin displayed a good role for reducing *Z. pyrina* infestations. This notion stimulated further investigation. In the present field-trial, a periodic inoculated release strategy has been extensively applied four times throughout the year (Table 4). Reduction percentages of *Z. pyrina* larvae within the same application technique resulted in statistically equal control levels within the four treatments/periods, where the mean reduction percentages in case of EGB13/EGB20/ diazinon in injection application ranged between 88.7 and 89.5%, whereas, EGB13/EGB20/Basudin recorded about 90% reduction percentages. However, spraying resulted in much lower reduction percentages that did not exceed 66% in all cases, as shown in Table 4.
Table 4. Effect of successive use of insecticides (1500ppm) - nematodes* (1000IJs/ml) combinations on reduction percentages in *Z. pyrina* population during 2019.

| Treatments + super film | Reduction Percentages in insect population and co-toxicity factor | Mean Population reduction % |
|-------------------------|---------------------------------------------------------------|-----------------------------|
|                         | March, 20 | April, 10 | May, 30 | October, 15 |               |
| Injection               |           |           |         |             |               |
| EGB13 + Diazinox        | 90.9      | -19       | 90.0    | -17         | 92.2          | -17           | 81.6          | -17           | 88.7          |
| EGB20 + Diazinox        | 91.5      | -18       | 92.0    | -16         | 93.0          | -14           | 82.6          | -17           | 89.8          |
| EGB13 + Basudin         | 89.4      | -10       | 90.5    | -11         | 91.7          | -11           | 91.0          | -11           | 90.65         |
| EGB20 + Basudin         | 89.0      | -8        | 90.9    | -9          | 92.2          | -8            | 88.1          | -7            | 90.05         |
| Spraying                |           |           |         |             |               |
| EGB13 + Diazinox        | 59.6      | -24       | 61.0    | -26         | 64.4          | -25           | 82.1          | -23           | 66.775        |
| EGB20 + Diazinox        | 58.2      | -21       | 60.9    | -24         | 62.9          | -21           | 61.1          | -21           | 60.775        |
| EGB13 + Basudin         | 62.2      | -23       | 65.9    | -30         | 67.0          | -28           | 65.8          | -29           | 65.225        |
| EGB20 + Basudin         | 61.0      | -22       | 61.9    | -23         | 68.1          | -21           | 65.1          | -23           | 64.025        |
| Min.-Max. Temperature (°C) | 5-18 | 15-28 | 14.5-29.8 | 12.8-25.9 |               |
| Min.- Max. Relative Humidity | 32.5-71.9 | 33.4-63.4 | 31.4-61.4 | 26.2-56.2 |               |

*EGB13=Heterorhabditis bacteriophora, EBN16=Heterorhabditis indica and EGB20= Steinernema sp.*

**The reduction percentages were calculated by Henderson and Tiltone formul a

These findings could be attributed to the favorable weather conditions of relatively low temperatures that reached in some cases 5-6°C) and high relative humidity that recorded 71.9 %in some days of the present experimentation. Adults of *Z. pyrina* that emerged from infested olive trees in the neighborhood fields cause re-infestation in the new olive trees. Consequently, it could be concluded that the long term of multiple co-applications of insecticides + nematodes was associated with efficient insect control and longer protection to the treated olive trees. This concept was supported by many authors who reported that starting a protective insecticidal spraying program (four sprays of diazinon 60% EC or basudin 60% EC) against *Z. pyrina* earlier in April or May is more efficient than late dates (El–Sherif et al., 1985; Ismail et al., 1990; Othman et al., 1993). Also, Abdel–Kaway et al. (1988) indicated that the nematode *N. carpopuspsae* applications in October gave a higher percentage mortality of *Z. pyrina* than the spraying application in March and April. They attributed these findings to the favorable weather conditions of low temperature and higher relative humidity prevailed during October. Abdel–Kaway et al. (1992) recommended that controlling *Z. pyrina* larvae and *S. littoralis* by *H. bacteriophora* could be done in the Summer time by the injection method since spraying in that time is entirely ineffective.

**Conclusion**

This study emphasizes that EPNs could play a complimentary or even superior role with chemical insecticides in controlling *Z. pyrina* pest in olive orchards. We strongly advise that the safe and effective combinations used in this study are to be included in IPM programs of this pest in Egypt. Excellent control could be achieved provided that suitable nematode strains, insecticide concentrations and repetition of nematode application are taken into account.
consideration. Needless to mention that farmers’ awareness concerning the important of this agent, its safety and capabilities will defiantly achieve sustainability in the pest control. Undoubtedly, extensions workers and instructors should be involved in this process.

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