

The compatibility of single and combined applications of the entomopathogenic nematode, *Heterorhabditis indica* with imidacloprid against red palm weevil, *Rhynchophorus ferrugineus* (Oliv.)

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Abstract

The present study aimed to investigate the biological potential of the entomopathogenic nematode (*Heterorhabditis indica*) for the management of the red palm weevil, *Rhynchophorus ferrugineus* O. and to evaluate its possible synergies with an insecticide application. Entomopathogenic nematode (EPN) and imidacloprid were applied either individually or in combinations. The virulence of EPN and imidacloprid was tested for three larval stages and the adults of *R. ferrugineus*. Moreover, pupation rate, adult emergence and egg eclosion were determined at sub-lethal doses. Our results showed that the mortality rate in all three larval stages and adults of *R. ferrugineus* was highest in combined treatments in comparison to single treatments. However, when compared to fourth and sixth instar larvae, second instar larvae were most susceptible, while the mortality rate was increasing over time. The mean mortality rate in adults was lower than in the larval stages. Furthermore, egg eclosion, pupation and adult emergence were lowest in the combined applications of EPN and imidacloprid. This research could contribute to the effective management of *R. ferrugineus*, especially in areas where it has developed pesticide resistance.

Keywords: Nematode-insecticide, Date palm, Mortality, Development, RPW, Insect stage

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Introduction

Date palm (*Phoenix dactylifera* L.) is the most important crop worldwide, especially in Arab

countries where it has been used as a staple food (Gaugler 1988; Erskine et al., 2004). Date palm trees show a high diversity in texture, chemical composition, color, and shape, depending on the



environment, seasonal effects, genotypes and cultivation methods (Hadrami and Jameel, 2012). Many coleopteran pests attack date palm trees. Among them, the red palm weevil (RPW), *R. ferrugineus* (Olivier, 1970), has been identified as the most notorious pest (Wakil et al., 2015; Dembilio and Jaques, 2015).

This pest is posing a severe threat to the date palm production and it is also known as the coconut weevil, Asiatic palm weevil, hidden enemy, red stripe weevil, and AIDS of date palm due to the slow death of infected trees (Khamiss and Abdel, 2013). This pest has a complex nature, damaging trees that are younger than 20 years (Nirula, 1956; Abraham et al., 1998). The weevil larvae feed within the tree trunk, damage the vascular system by mining into the heart of the trees, which results in the collapse of the tree (Ju et al., 2011). Fully matured grubs pupate within the chewed up frass for 11-40 days. Under unfavorable conditions and without control measures, the date palm tree dies within 6-8 months (Kurian and Mathen, 1971; Avand, 1996; Rajamanickam et al., 1995).

To manage this severe pest, date palm growers have, for many decades used fumigants, as well as insecticides. However, chemical applications have become a significant challenge for palm growers due to the complex nature of RPW (Hussain et al., 2013). Moreover, insecticide applications are not only potentially harmful to the environment and human health but also promote insecticide resistance in RPW. (Abraham et al., 1998). Alternative biological control agents such as entomopathogenic nematodes and its combination with chemicals could provide a significant breakthrough for the management of RPW (Khan et al., 2012; Charnley and Collins, 2007; De Faria and Wraight, 2007).

Entomopathogenic nematodes (EPNs) have proven to be efficient and potent microbial control agents against a variety of pests, especially against insect pests of stored grain (Dembilio et al., 2010). However, so far, very few studies have investigated the combined effect of EPNs with pesticides under laboratory as well as under field conditions (Dembilio et al., 2010; Rovesti and Deseo, 1990; Portman et al., 2016; Yan et al., 2012). Many scientists working in this area of research have successfully used EPNs for RPW control. EPNs are most effective as compare to others microbial control agents, kill the insect by damaging their cuticle instead of ingestion and can quickly transfer from one insect to another (Atwa, 2018; Lacey and Georgis, 2012; Pervez et al., 2012;

Yadav and Lalramliana, 2012). EPNs are obligate parasites mainly from the family of Heterorhabditidae and Steinernematidae. They kill insects with the help of mutualistic bacteria that live in their intestines (Poinar, 1990). EPNs are considered safe and less dangerous in pesticide applications to non-target organisms, environment, and human health as well as can be integrated with each other's, often showing potent synergistic and additive interactions (Thurston et al., 1994).

The objective of the current study was to investigate the potential of EPNs applications for the effective control of RPW, especially in combination with chemicals. Development, growth, and mortality of RPW were examined under laboratory conditions followed by treatments with the EPN, *Heterorhabditis indica*, or the insecticide alone and in combination. The obtained results could be helpful in decision making for the choice of suited treatments and application times during oncoming future research trials. This research will lead to successful and environmentally safe management control of RPW existence in date palm trees and their hosts.

Material and Methods

Insect collection

Adults of the red palm weevil, *R. ferrugineus*, were collected from different districts of Punjab, Pakistan from infested date palm trees. All stages of RPW were collected in separate boxes closed with rubber bands. Collected insects were fed with fresh pieces of a date palm during the collection.

Insect rearing

Collected insects were brought to the laboratory where they were supplied with clean and fresh food to increase their population. In the absence of a date palm, sugarcane was used as an alternative host. The insects were provided with optimal conditions; $27\pm 4^{\circ}\text{C}$ and 65% relative humidity. Both males and females were kept together to harvest eggs. Eggs hatched after 4 to 5 days and first instar larvae were collected. The diet was changed at regular intervals.

Nematode culture

We used nematodes from our laboratory culture reared on the wax moth *Galleria mellonella* L. Larvae killed by nematodes were placed on white traps for harvesting. Emerging IJ stages of nematodes were collected. Collected nematodes were transferred to Petri dishes,



wrapped with parafilm and stored at 10°C.

Pesticide

The insecticide used in our experiments was obtained from the company Bayer Crop Science, Pvt. Ltd. Pakistan. The chemical was labeled with all pieces of information, including active ingredients. The required formulation was produced following the instructions provided.

Bioassay tests

Against RPW larvae

Small plastic cups were used for our bioassay tests. For each treatment, 15 RPW larvae were inoculated with two different concentrations of nematodes (50IJ and 100IJ) and were placed in cups filled with sterilized sand. The cups were closed with cotton wools. A camel hairbrush was used to inoculate the nematodes. For the insecticide treatments, two different concentrations (1ppm and 2ppm) of imidacloprid were poured on filter papers and placed within cups filled with sterilized sand. Fifteen larvae were placed in each cup. A micropipette was used to apply the insecticide to the filter papers. Each treatment was replicated three times. Three bioassay tests were performed to check RPW larval mortality after 7, 14 and 21 days. Larvae that were showing slow movement or were unable to move were counted as dead. The environmental conditions were the same, as mentioned in the rearing section. The same method was applied to test for the combined effect of the nematodes with the insecticide.

Against RPW larvae development

In this trial, we tested the combined effect of the nematodes and the insecticide on the development of RPW and evaluated the egg eclosion, pupation and adult emergence of RPW. The nematode was applied to the RPW larvae with a camel hairbrush and placed in cups filled with sterilized sand. For the insecticide treatments, RPW larvae were dipped into the imidacloprid solution (1ppm and 2ppm) for ten seconds and placed in cups with sterilized sand. The diet provided to the test insects was also treated with an imidacloprid solution with different concentrations. Mortality data were collected until adult emergence. The same method was used to test for the combined effect of the nematode and the insecticide. The whole experiment was repeated three times.

Against RPW adults

In this trial, we measured the mortality of adult RPWs in response to nematode and insecticide application and their combination. Adults of RPW were treated with different nematode concentrations applied with a camel hairbrush. For the insecticide tests, sugarcane pieces (10 mm) were first dipped into two different imidacloprid concentrations for one minute and then offered to RPW adults. After 48 hours of feeding, the treated sugarcane pieces were replaced by new, untreated ones. In the combined treatments, sugarcane pieces treated with imidacloprid were offered to RPW adults for 48 hours and then after 24 hours of time intervals, the same adults were treated with the nematodes. Ten adults were used in each treatment and each treatment was replicated three times. Adult mortality was recorded after 7, 14 and 21 days.

Statistical analysis

The mortality data were analyzed using Abbot's Formula. To test for significant differences among treatments, larval and adult mortality was analyzed with an Analysis of Variance (ANOVA). Corrected mortality was calculated using Abbot's formula. The differences among means were calculated using Tukey's HSD test at a 5 % level. Excel sheet was used for descriptive analysis.

Results

Efficacy of EPN and insecticide against *R. ferrugineus* larvae

The mortality rate under different concentrations of EPN and imidacloprid was observed. In the first week of exposure, imidacloprid showed the highest mortality rate against *R. ferrugineus*, which was significantly lower in the nematode treatment. The mortality of *R. ferrugineus* was evaluated in three different larval stages (second, fourth and sixth). The mortality rate in the combined treatment was gradually decreasing, with a maximum of 59.36 ± 1.97 , in the second instar larvae and with 49.52 ± 1.57 and 45.39 ± 2.07 in the fourth and sixth instars, respectively. Our results show that the combined efficacies of nematodes and insecticide directly depend on the larval stage. The same treatment was applied to all stages; the developmental stage of the RPW was positively correlated with its ability to cope with the insecticide –nematode treatment (Table 1).



Table-1. Mean mortality of red palm weevil, *R. ferrugineus* larvae treated with two different concentrations of *H. Indica* and Imidacloprid under different time intervals

Treatment	Week 1			Week 2			Week 3		
	2 nd instar	4 th instar	6 th instar	2 nd instar	4 th instar	6 th instar	2 nd instar	4 th instar	6 th instar
Hi1 (50IJ)	11.32±1.56e	8.20±1.47g	5.97±2.06f	17.35±1.94g	11.95±1.43e	8.87±1.92e	25.72±1.83e	15.82±1.17f	13.38±2.20f
Hi2 (100IJ)	16.40±1.89e	14.97±1.43f	11.95±1.8ef	23.28±1.55g	20.21±1.83de	16.45±1.91e	31.53±1.87de	24.07±1.63e	21.07±1.68f
I1 (1ppm)	26.19±2.24d	23.28±1.08e	19.52±1.71de	35.28±1.33f	28.46±1.68d	27.72±1.92d	40.58±1.84d	32.21±1.99d	30.79±1.88e
I2 (2 ppm)	38.25±2.03c	32.27±1.38d	27.03±1.50cd	43.54±1.37e	38.41±1.73c	37.46±2.01c	50.31±2.41c	42.12±0.95c	40.53±1.77d
I1 × Hi1	47.35±1.46b	36.08±1.03cd	33.06±1.68bc	52.65±1.84d	47.40±1.64b	45.76±2.02bc	61.58±2.37b	53.38±1.26b	48.04±1.42cd
I1 × Hi2	51.90±1.60ab	39.15±1.17bc	38.30±1.80ab	60.85±1.96c	54.07±1.70b	49.47±2.87b	66.88±2.67b	58.57±1.57b	53.23±2.21c
I2 × Hi1	54.13±2.14ab	42.72±1.63b	41.27±1.47a	71.38±1.95b	64.76±2.96a	60.11±2.40a	78.15±2.30a	74.39±1.91a	62.45±2.47b
I2 × Hi2	59.36±1.97a	49.52±1.57a	45.39±2.07a	80.53±2.28a	72.17±2.06a	66.67±2.08a	87.25±1.72a	78.94±2.10a	72.69±1.99a

Means showing different letters in each column are significantly different at a 5% level ($P < 0.05$, Tucky HSD test). **Hi**: *Heterorhabditis indica*; **I**: Imidacloprid; **IJ**: Infective Juvenile

Nematodes usually have a slow mode of action to kill herbivores insects; however, when they are combined with insecticides, they become more productive and fastest to kill.

After the second and third weeks of the exposure, the mortality rate was much higher than in the first week. It can also be an indication that time is an essential factor for the efficacy of nematode applications.

Efficacy of EPN and insecticide against *R. ferrugineus* adults

To test our hypothesis, the effectiveness of the nematodes and their combinations with the insecticide were also tested against adult RPW (Table 2).

Table-2. Mean mortality of *R. ferrugineus* adults treated with two different concentrations of *H. Indica* and Imidacloprid under different time intervals

Treatments	Week 1	Week 2	Week 3
Hi1 (50IJ)	5.55±2.94e	11.23±1.99e	21.36±2.47g
Hi2 (100IJ)	8.89±2.60e	13.58±1.61e	28.27±2.02fg
I1 (1ppm)	14.57±2.39de	21.60±1.09d	33.70±2.32ef
I2 (2 ppm)	20.25±2.23cd	32.27±1.74cd	41.85±2.23de
I1 × Hi1	27.16±2.20bc	36.08±2.04c	47.78±1.74cd
I1 × Hi2	31.73±1.84ab	39.15±1.78b	54.44±1.9bc
I2 × Hi1	33.83±2.60ab	42.72±2.47b	61.24±2.43b
I2 × Hi2	41.97±1.78a	49.52±1.25a	73.58±1.73a

Means showing different letters in each column are significantly different at a 5% level ($P < 0.05$, Tucky HSD test). **Hi**: *Heterorhabditis indica*; **I**: Imidacloprid; **IJ**: Infective Juvenile

The adults were treated in the same way as the larvae with regard to the application of the insecticide and the nematodes. Our results showed that the mortality rate in *R. ferrugineus* adults was lower than in the larval stages. Under individual treatments of nematode and insecticide,

the *R. ferrugineus* showed lowest adults mortality (8.89±2.60 and 20.25±2.23), which was recorded higher (16.40±1.89 and 38.25±2.03) in larval stages.

The mortality rate for the insecticide treatment was higher than for the nematode treatment. The mortality rate was gradually increasing with increasing concentration being highest in the combined treatment. In the combined treatment, the maximum mortality was 41.97±1.78 after the first week of exposure and it steadily increased to 49.52±1.25 and 73.58±1.73 after the second and third weeks, respectively. Overall, the mortality was lowest in the adults in comparison to larval stages.

Efficacy of EPN and insecticide against *R. ferrugineus* development

The results for the impact of the different treatments on egg eclosion, pupation rate and adult emergence are summarized in Table 3.

For these trials, we used the same concentrations and treatments as for the larvae and adults in the previous section. Second, fourth, and sixth instar larvae were selected to test the impact of nematodes and their combinations with the insecticide on pupation, egg eclosion, and adult emergence. Based on our findings, pupation rate, egg eclosion and adult emergence were highest in the nematode applications than in the insecticide. The development of the larvae was decreasing with increasing nematode and insecticide concentration. In the combined treatments, the developmental rate of *R. ferrugineus* was being reduced in comparison to the single treatments. Under the combination of treatments, the second instar larvae showed lowest pupation rate, egg eclosion, and adult emergence (4.44±1.76, 00.00±0.00 and 00.00±0.00), respectively. However, in the fourth instar larvae, pupation rate and adult emergence were slightly increased but egg eclosion was continuously observed lowest.



Table-3. Adult emergence, pupation and egg eclosion of *R. ferrugineus* larvae treated with two different concentrations of *H. Indica* and Imidacloprid under different insect stages.

Treatments	2 nd Instar	—	—	4 th Instar	—	—	6 th instar	—	—
	Pupation (%)	Adult emergence (%)	Egg eclosion (%)	Pupation (%)	Adult emergence (%)	Egg eclosion (%)	Pupation (%)	Adult emergence (%)	Egg eclosion (%)
Hi1 (50IJ)	63.58±1.86a	54.44±1.98a	45.31±2.08a	71.48±1.67a	62.35±2.00a	54.44±1.98a	73.83±1.82a	64.69±1.92a	59.14±2.23a
Hi2 (100IJ)	55.58±1.98a	45.43±1.55b	33.95±2.14b	65.80±1.90ab	54.32±2.34b	45.18±2.49b	63.46±2.15b	61.36±1.43a	49.75±2.66b
I1 (1ppm)	42.09±1.17b	35.06±2.17c	20.25±2.23c	56.67±2.53b	41.97±1.78c	36.29±1.52c	59.01±2.58bc	51.11±1.45b	41.97±1.78bc
I2 (2 ppm)	33.83±2.61b	24.94±1.63d	12.35±2.21d	41.97±1.78c	34.07±1.55d	30.49±2.10c	52.22±1.74c	45.30±2.67b	33.83±2.61cd
I1 × Hi1	22.59±2.12c	15.56±2.94e	00.00±0.00e	32.84±2.04cd	22.72±1.41e	21.11±1.11d	41.85±2.23d	32.72±2.51c	25.56±1.76de
I1 × Hi2	14.57±2.39cd	4.32±2.51f	00.00±0.00e	24.81±2.83de	15.80±1.66ef	14.44±1.76de	31.73±1.84e	21.36±2.48d	17.78±1.47ef
I2 × Hi1	11.11±2.61de	00.00±0.00f	00.00±0.00e	16.67±2.36ef	12.22±1.47fg	7.78±1.46e	23.46±2.27ef	15.56±1.76de	12.22±1.47f
I2 × Hi2	4.44±1.76e	00.00±0.00f	00.00±0.00e	11.11±2.61f	6.67±1.67g	00.00±0.00f	14.57±2.39f	7.78±1.47e	3.33±1.67g
<i>df</i>	9	9	9	9	9	9	9	9	9
<i>P</i>	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Means showing different letters in each column are significantly different at a 5% level ($P < 0.05$, Tucky HSD test). **Hi**: *Heterorhabditis indica*; **I**: Imidacloprid; **IJ**: Infective Juvenile

For the sixth instar, all three parameters were slightly increased, showing that time was a significant and most crucial factor for the control of red palm weevil, *R. ferrugineus* in all treatments. The second instar stage was the most susceptible in comparison to the fourth and sixth instar stages. Nematode and insecticide concentrations and their compatibility were also significant parameters for the mortality and development of *R. ferrugineus*.

Discussion

Due to multiple and excessive applications of pesticides for the control of herbivores communities, some pests have become able to resist these control measures due to changes in their internal mechanisms such as increasing their immunity levels (Dermauw et al., 2018; Delnat et al., 2019). Researchers are challenged to find effective control strategies that should not only be safe to the environment but should also effectively kill insect pests populations without causing resistance effects (Alvarez et al., 2019; Ali et al., 2019; Allan, 2018).

Due to global warming and climate change, researchers are struggling to develop biological control strategies for the most severe insect pests to reduce the use of chemicals (Bakera et al., 2020). EPNs mainly belong to the families heterorhabditidae and Steinernematidae are the most important and safe biological control agents due to their specificity, infectivity and their non-target effects. Many researchers have combined EPNs with insecticides to improve their effectiveness, which has proven successful (Garriga et al., 2019; Yuksel et al., 2019). The red palm weevil, *R. ferrugineus*, is the most

important and noxious pest of palm trees, affecting palm production worldwide (Yan et al., 2019). The management of this pest is broadly based on insecticides applications (Mashal and Obeidat, 2019).

Under these circumstances, our research aimed to evaluate the efficacies of EPN and insecticide applications - singly and in combinations - against larval and adult stages of RPW. Besides this, we also assessed their effects on the development of *R. ferrugineus*. Our results showed that larval mortality was gradually increasing with increasing concentration and time. In single treatments, imidacloprid treated larvae showed the highest mortality rate in all larval stages, which was lower in the EPN. Maximum mortality was found in the combination treatments of EPN and imidacloprid, which was increased from lower to highest at first, the second and third weeks of exposures. Patil et al. (2017) showed that second instar *Leucopholis lepidophora* grubs were more susceptible to infections with nematodes when compared to subsequent insect stages. Wu et al. (2017) concluded that the combination of nematodes and thiamethoxam significantly increases the mortality of *Bradysia odoriphaga* larvae in comparison to single applications of nematodes and thiamethoxam. They showed that the combination of EPNs with insecticides was most effective (90 %) for the control of insect pests for up to six weeks. Koppenhofer et al. (2000) stated that the combination of EPNs and imidacloprid has proven fruitful for the control of white grubs showing high compatibility and a low environmental impact. Nematodes applications with insecticides present less hazardous effects on the environment and biotic



communities. Is our research answerable to a very significant hypothesis that how nematodes applications with combinations of insecticides could improve control strategies used to manage *R. ferrugineus*? Based on our results, time and insect stage appeared the most significant that could affect nematodes efficacies and their combinations with insecticides.

In our second experiment, we tested the effectiveness of EPN and imidacloprid against the adults of *R. ferrugineus*. Our results showed that adult mortality was lower than the mortality of the larval stages; however, it was gradually increasing with increasing concentration and time. In the single treatments, the mortality rate was observed lowest than in the combined treatments. Mbata et al. (2018) explain that nematodes are most virulent to adult maize weevils and caused the highest mortality, which was observed similar in our research against RPW. Rodriguez et al. (2007) showed that *Tribolium castaneum* larvae, which were treated with *Steinernema riobrave*, had higher mortality than adult beetles. Which confirms our hypothesis that *R. ferrugineus* increase their immunity levels during their growth and when they reached maturity, they develop effective internal mechanisms to cope with control applications. As compared to *R. ferrugineus* larval stages, time and concentration were also observed most significant factors against the mortality of adults.

We also evaluated the impact of the nematodes and the insecticide on the development of *R. ferrugineus*. Our results showed that pupation rate, adult emergence, and egg eclosion were higher in the nematode treatment than in the insecticide treatment. As compared to individual treatments, pupation rate, adult emergence and egg eclosion were lowest in the combined treatment. The second instar stage showed the lowest pupation rate, adult emergence and egg eclosion in comparison to the fourth and sixth instar. Egg eclosion appeared the most susceptible when compared to pupation rate and adult emergence. Hubner et al. (2017) concluded that the larvae of spotted wing drosophila, *Drosophila suzukii* when was treated with nematodes showed less adult appearance. Wakil et al. (2017) showed that egg eclosion, pupation rate, and adult emergence was decreased when *R. ferrugineus* larvae were treated with entomopathogenic fungi and nematodes alone and this effect was even higher in the combined treatment. There is a lack of information regarding the effectiveness of combined applications of nematodes and insecticides on adult mortality and the development of *R. ferrugineus*. Our research helps to fill this gap with accurate and significant results. Further investigations are needed that

would explain the molecular mechanisms involved in the synergies of nematodes and insecticide applications.

Conclusion

Biological control is the only way to suppress pest populations without or with minimum applications of chemicals. Entomopathogenic nematodes (EPNs), preferably in combination with insecticides, could provide effective management of pest species, especially the red palm weevil, *R. ferrugineus*. In response to these challenges, the current research was conducted to test the effectiveness of EPN and insecticide alone and in combination. The results showed that the mortality of *R. ferrugineus* larvae and adults was increasing with increasing concentrations. In individual treatments, the *R. ferrugineus* showed less mortality, which was recorded higher in insecticide exposure. However, in nematode-insecticide combinations, *R. ferrugineus* showed a higher mortality rate when compared to single treatments. We also evaluated the effect of EPN and their combinations with insecticide on the development of *R. ferrugineus*. Our results showed that pupation rate, adult emergence and egg eclosion was lower in the combined treatments as compared to the single treatments. In conclusion to our research, EPNs combinations with pesticides could provide effective biological control of *R. ferrugineus* with the advantage of being less hazardous to the environment and human health.

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