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Pathogenicity and Toxicity of Entomopathogenic Nematodes Isolated from Egypt on Spodoptera littoralis and Agrotis ipsilon

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ABSTRACT

This study was managed to isolate and identify entomopathogenic nematodes from Egypt. In addition, the pathogenicity and toxicity of these isolated nematodes on Spodoptera littoralis and Agrotis ipsilon larvae were declared. Also, hemolymph protein patterns of uninfected and infected larvae of S. littoralis and/or A. ipsilon were screened. Results declare that EPNs were extracted from soil samples of Egyptian mango trees in Ismailia, Governorate, only 2% of soil samples were positive for the nematode Heterorhabditis which was confirmed by the morphological signs of dead Galleria larvae which turned dark-red colour and recorded the bioluminescence phenomena in the dark. Also, all tested Heterorhabditis strains induce a positive influence on larval mortality while a negative influence on pupation and adult emergence against both insects Spodoptera littoralis and A. ipsilon. After infection of S. littoralis 5th larval instar, LC₅₀ values were 33.51, 45.30, 73.24 and 39.11 IJs/Larvae, for Heterorhabditis bacteriophora HP88, Heterorhabditis indica AZ1, Heterorhabditis indica AZ2 and Heterorhabditis sp. HS, respectively. After infection of A. ipsilon 5th Larval instar, LC₅₀ values were 76.64, 82.58, 135.95 and 68.44 IJs/Larvae, at the previous arrangement for nematodes. From the previous results, the native isolates H. sp. HS was more pathogenic against tested insects than others. In addition, Spodoptera littoralis larvae were found to be more susceptible to all Heterorhabditis strains than A. ipsilon larvae. In addition, in both tested insects Spodoptera littoralis and A. ipsilon, larvae infected with the sub-lethal concentration LC_{50} showed a significant genetic variation in their protein pattern compared to control samples. The results declared that there is a protein band characteristic of all Heterorhabditis nematodes with M.W. 35KDa that may be defined as a toxic protein. From the above results, it can be concluded that the isolated strains of Heterorhabditis will be a promising agent in integrated pest management (IPM) Programs.

INTRODUCTION

The cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) belong to polyphagous insects and is one of the most agricultural insects dangerous in Egypt. It has seven generations per year so it attacks many important crops throughout the year and causes severe damage to various strategic crops and vegetables. The black

cutworm, Agrotis ipsilon (Hufnagel) (Lepidoptera: Noctuidae), is one of the most destructive species of soil insects and destroys more than 100 host plant species (Liu et al., 2015 and Hayat *et al.*, 2021). Black cutworm attacks a large number of field crops, in some cases, causing up to 100% damage at the seedling stage (Wang et al., 2021). Depending on their behaviour, larvae remain in the soil during the day and are therefore difficult to control (Bento et al., 2007). Thus, A. ipsilon is a serious pest not only in Egypt but also on many strategic economic crops around the world. Scientists are making great efforts to combat these pests by discovering co-friendly control agents. Egyptian fauna is populated by a large community of entomopathogenic nematodes (EPNs) from the families Steinernematidae and Heterorhabditidae. A symbiotic relationship occurs between Heterorhabditis nematode and its bacteria *Photorhabdus*, where the nematode relies on bacterial agents to quickly kill the insect host (Boemare, 2002; Ruisheng and Grewal, 2010; Vashisth et al., 2013). EPNs are considered one of the promising biological control strategies because they are safe for humans and the environment (Jagodič et al., 2019; Amutha et al., 2021; Devi et al., 2021). EPNs are distributed worldwide and include more than 16 Heterorhabditis species (Nguyen and Hunt, 2007). In Egypt, research on EPNs started in the 1970s when Dr. El-Kifl revealed that, Neoaplectana carpocapsae could act as a biological control agent on Spodoptera littoralis (El-Kifl, 1980). Shamseldean and Abd-Elgawad (1994) conducted a survey of Egyptian soils to isolate indigenous EPNs. The survey also declared that Heterorhabditidae were more abundant in the Egyptian fauna than Steinernematidae. EPN Varieties in Egypt include local and imported varieties. The imported ones do not give satisfactory results in many field experiments, perhaps because they are not adapted to the local environment. The use of local EPN may be more effective in controlling local insect pests because they are adapted to the local environment. Therefore, searching for effective local EPN strains from different parts of Egypt is a goal for researchers of bio-control agents. Consequently, the aim of this study was to extract local EPN isolates from Egyptian agricultural soils for later use in integrated pest management (IPM) programs, to evaluate the pathogenicity of EPN isolates on two cotton pests, S. littoralis and A. ipsilon and to reveal the changes in protein patterns of S. littoralis and/or A. ipsilon larvae due to EPNs infection.

MATERIALS AND METHODS

Insect's Cultures:

Three insect cultures were used in this study.

1-Greater Wax Moth, *Galleria mellonella:* Mass Production of *G. mellonella* was done in order to meet the requirement for isolation of entomopathogenic nematodes (Plate 1). A laboratory strain of the greater wax worm *Galleria mellonella* L. (Lepidoptera: Pyralidae) was obtained from the Plant Protection Research Institute, Agricultural Research Centre (ARC). The larvae were reared in 5-litre glass containers filled with a layer of artificial diet at the bottom and the container tightly covered with a muslin cloth. An artificial diet was prepared by the method of Metwally *et al.* (2012).



Plate 1: Mass Production of Galleria mellonella.

2-Black Cut Worm, Agrotis ipsilon:

A.ipsilon was reared for evaluation of the pathogenicity of isolated entomopathogenic nematodes. A laboratory strain of *A. ipsilon* was obtained from the Cutworm Research Department, Plant Protection Research Institute, Agricultural Research Centre. *A. ipsilon* larvae were established and maintained in a growth chamber at 27 ± 2 °C, R.H 60%, and a photoperiod of 16:8 (L: D) (El-Shershaby, 2010) (Plate-2).



Plate 2: Mass Production of Agrotis ipsilon

3- Cotton Leafworm, Spodoptera littoralis:

For examination of the virulence of isolated entomopathogenic nematodes, mass rearing of *S. littoralis* was executed (Plate-3). *S. littoralis* was obtained from the Cotton leafworm Research Department, Plant Protection Research Institute, Agricultural Research Centre (ARC). This strain was reared under constant laboratory conditions of 27 ± 2 °C and 65 ± 5 % relative humidity (EL- Defrawy *et al.*, 1964).



Plate 3: Mass Production of Spodoptera littoralis

Searching for EPNs in Egyptian Soil.

To extract ENPs, fifty soil samples from several locations in Ismailia Governorate, Egypt. Soil samples were transported to the cotton leafworm research lab. at PPRI, ARC, Egypt. All details were written on each sample bag, i.e., location, soil type, date, etc., according to Vashisth *et al.*, (2013). Entomopathogenic nematodes were extracted from samples by insect baiting method (Bedding and Akhurst, 1975). The baiting agents used in this study were Galleria larvae. For each soil sample, a jar was filled with 250 g of moistened soil. Ten larvae were placed on the soil surface, covered the container, and converted. Keep the container at 25 ± 2 °C for 7 to 10 days. Dead larvae showing symptoms of EPNS infection were placed in white traps to collect the free-living stage of EPNs. **Nematodes Culture:**

To collect infective juveniles from cadavers, White traps were used (White, 1927) (Plate-4). Two Petri dishes were placed, one smaller than the other, the small one which served as a holding dish was placed in the middle of the storage dish (the large one), and twenty infected larvae were placed on the small Petri dishes that contained a layer of filter paper in the large Petri filled with sterile water. New IJs are released from cadavers approximately 10 to 14 days after infection.



Plate 4: White traps

Pathogenicity and Toxicity Test:

Toxicity Test of Entomopathogenic Nematodes:

To assess the pathogenicity and toxicity of four *Heterorhabditis* entomopathogenic nematodes, *viz.*, *Heterorhabditis bacteriophora* HP88, *Heterorhabditis indica* AZ1, *Heterorhabditis indica* AZ2 and *Heterorhabditis sp.* HS, different concentrations were prepared as follows: 25, 50, 100 and 200 IJ/larvae for each tested nematode. Freshly moulted 5th instar larvae were used in the experiments. Three replicates, containing 10 larvae/cub for *S. littoralis* or/ plastic ice cube for *A. ipsilon*, were set up for each infection with a nematode strain. These experiments were conducted to determine the larval mortality, pupation and adult emergence percentage.

Protein Bands Detection by Electrophoresis:

Hemolymph Samples Collection:

The hemolymph of normal and infected 5th larval instar with LC₅₀s were collected after 120h from infection by puncturing a pro-leg of tested insect and placing the drawn hemolymph in1.5-ml ice-cold microcentrifuge tubes that containing a few crystals of phenylthiourea. The hemolymph was centrifuged at 8000 rpm for 5min at 4°C.

Detection of Protein Bands:

Proteins were detected by Polyacrylamide gel electrophoresis (PAGE) according to the method of Davis, (1964). The concentration of the gel used in this study was 10% SDS-PAGE. The basic role of protein electrophoresis is the movement of the charged molecules towards an electrode with the opposite charge through a supporting buffer. After electrophoresis, the protein bands were showed by staining with Coomassi Blue R 250. Then, the gel was de-stained with the de-staining solution until the protein bands only appeared on the gel.

Calculations and Data Analysis:

The larval mortality percentages were corrected according to Abbott's formula (1925). Estimation of the LC_{50} according to Finney (1971).

The similarity index and genetic distance were determined according to (Nei and Li, 1979).

RESULTS

1-Source of EPNs in This Study.

The EPNs used in this study, the geographical location and the sources from which they were obtained are declared in Table (1). In this study, authors used an insect baiting method and *Galleria mellonella* (Linnaeus) was used as a baiting agent for extracting EPNs from the soil. Only one nematode sample was isolated from the soil of mango gardens in the Governorate of Ismailia, Egypt, out of 50 samples from sandy clay loam soil type; i.e., only one sample (2%) was positive for the appearance of *Heterorhabditis* nematodes which was confirmed by the morphological signs of the death of Galleria larvae with dark-red color and the bioluminescence phenomena in the dark as shown in Figure (1). Other entomopathogenic nematodes have been obtained from the Physiology department, PPRI, ARC, Dokki, Egypt.

Table 1: Geographical location and sources of EPNs used in this study.

Nematodes	Strain	Geographical Location	Source
Heterorhabditis sp.	HS	Ismailia, Egypt	Mango Soil
Heterorhabditis indica	AZ1	Suez, Egypt	Nectarines Soil
Heterorhabditis indica	AZ2	El-kasasein, Ismailia, Egypt	Mango Soil
Heterorhabditis bacterionhora	HD88	LISA	Commercial product
neterornaballis bacteriophora	111 00	USA	BioLogic, Inc., USA





Normal G. mellonella larvae

The infected G. mellonella larva showed a darkred color.



The bioluminescence phenomena of infected G. mellonella larvae in the dark.

Fig. 1: The morphological signs of death of infected *Galleria mellonella* with *Heterorhabditis* nematodes.

The Pathogenicity of Tested Nematodes Against Some Cotton Pests:

In the present study, pathogenicity and toxicity of four Heterorhabditis entomopathogenic nematodes, *viz.*, *Heterorhabditis bacteriophora* HP88, *Heterorhabditis indica* AZ1, *Heterorhabditis indica* AZ2 and *Heterorhabditis sp.* HS was evaluated against *S. littoralis* and *A. ipsilon* after infection of 5th instar larvae.

The Pathogenicity and Toxicity of Tested Nematodes Against S. littoralis:

To achieve this goal, four concentrations of each **Heterorhabditis** strain were prepared: 200, 100, 50 & 25 IJ/larvae. Use plastic cups filled with sterilized sand and moistened with 20% nematode suspension at different concentrations. Thirty newly moulted 5^{th} instar larvae were placed in these plastic cups for each concentration. Control larvae were allowed to be placed in the plastic cup filled with sterile sand moistened only with 20% water (v/w). The insecticidal activities of these *Heterorhabditis* and their effects on pupation % and adult emergence % were recorded as follows:

Pathogenicity and Toxicity of Heterorhabditis bacteriophora HP88 against S. littoralis:

After infection of 5th instar larvae of *S. littoralis* with four concentrations (200, 100, 50 & 25) IJs/larvae of the EPN *Heterorhabditis bacteriophora*, data of the nematode virulence were arranged in Figure (2). Depending on these data, the corrected mortality rate of infected larvae was found in a conc. dependent trend (100, 93.10, 65.52, 37.93% mortality, at 200,100, 50 & 25 IJs/larvae, respectively). At the highest concentration, pupae died completely while at the lowest concentration, only 50 % of larvae can pupate and only 30% can emerge as adults.

Pathogenicity and Toxicity of Heterorhabditis indica AZ1 against S. littoralis.

After placing the 5th instar larvae of *S. littoralis* in plastic cups filled with sterilized sand supplemented with four concentration levels of *Heterorhabditis indica* AZ1 (25, 50, 100 and 200 IJ/larva), results of the toxic activity were diagramed in Figure (3). According to these data, *H. indica* AZ1 exhibited acute toxicity on larvae at all concentrations (34.49, 55.18, 65.52 and 86.21%) at previous concentrations. *H. indica* AZ1 showed chronic toxicity against developing pupae with adult emergence decreased with increasing

concentrations (56.67, 26.67, 13.33 and 6.67% adult emergence, at 25, 50, 100 and 200 IJ/larvae, respectively, compared to 96.67% adult emergence of control.

Pathogenicity and Toxicity of Heterorhabditis indica AZ2 against S. littoralis.

After infecting newly moulted 5th instar larvae with *H. indica* AZ2 concentrations, the pathogenicity data were reported in Figure (4). Data showed that *H. indica* AZ2 exhibited moderate toxicity against larvae (75.87, 62.07, 37.93& 20.69 % larval mortality, at 200, 100, 50 & 25 IJ/Larvae respectively). The pupation rate was decreased with increasing concentration, and the most effective concentration was 200 IJ/Larvae as expressed by the lowest pupation rate (16.67 %). It was found that different concentrations of *H. indica* AZ2 clearly affected the emergence of adults. For example, the results showed that the adult emergence rate gradually decreased with increasing concentrations until reached 13.33 at a concentration of 200 IJ/Larvae.

Pathogenicity and Toxicity of Heterorhabditis sp. HS against S. littoralis.

Results recorded in Figure (5) illustrated that the larval mortality increased with the increase in the concentrations as showed finally in corrected percentages of 31.03, 62.07, 82.7and 96.56 % at the concentrations of 25, 50, 100 and 200 IJ/larvae, respectively. With regard to the pupation percentage, there is a great decline through successive concentrations as compared with 96.67 % in the control samples. The rate of adult emergence also decreased with increasing concentrations as shown in Figure (5).



Fig. (4): Pathogenicity of *H indica* AZ2 against 5th instar larvae of *S. littoralis*.



Fig. (3): Pathogenicity of *H. indica* AZ1 against 5th instar larvae of *S. littoralis*.



against 5th instar larvae of *S. littoralis*

LC50 Values of Heterorhabiditis Strains against S. littoralis Larvae.

A toxicity test of *Heterorhbditis* strains was performed on 5th larval instars of *Spodoptera littoralis* at different concentrations of the tested strains. Deaths were recorded after 120 hours of infection. Results in Table (2) declared that, the LC₅₀ values of *H. bacteriophora* HP88, *H. indica* AZ1, *H. indica* AZ2 and *H. sp.* HS were 33.51, 45.30, 73.24

and 39.11 IJ/Larvae i.e. *H. bacteriophora* HP88 was the most effective followed by *H. sp.* HS, *H. indica* AZ1 and *H. indica* AZ2 in descending order.

Heterorhabiditis strains	LC ₅₀ IJ/Larvae	Lower limit	Upper limit	Slop
HP88	33.51	29.29	38.32	3.08 ± 0.099
AZ1	45.30	36.45	56.22	1.55 ± 0.042
AZ2	73.24	61.52	87.20	1.7 ± 0.042
HS	39.11	33.65	45.42	2.48 ± 0.063

Table 2: Toxicity of *Heterorhabiditis* strains against *S. littoralis* 5th instar larvae.

Pathogenicity and Toxicity of H. bacteriophora HP88 against A. ipsilon.

The delayed effect of *Heterorhabditis bacteriophora* HP88 against 5th larval instars of *A. ipsilon* was seen in **Figure (6)**. Data clearly showed that the larval mortality increased as the concentration increased. The total corrected larval mortalities reach 70% at the highest concentrations. In addition, results indicated that the percentage of pupation and adult emergence resulted from the larvae treated as 5th larval instar with different concentrations of *H. bacteriophora* HP88 were highly reduced as compared with untreated one.

Pathogenicity and Toxicity of Heterorhabditis indica AZ1 against A. ipsilon.

It is clear from the results shown in **Figure** (7) that there is an effect resulting from *Heterorhabditis indica* AZ1 on the larval mortality rate, as stated in the corrected percentages of 20.00, 40.00, 60.00, and 66.67 % at the ascending concentration. The percentage of pupation was decreased, recording 73.33, 53.33, 40.00, and 33.33%. In addition, the adult emergence rate was 66.67, 50.00, 36.67, and 26.67 % compared to 96.67% in the control.

Pathogenicity and Toxicity of Heterorhabditis indica AZ2 against Agrotis ipsilon.

The results declared in Figure (8), illustrate that the corrected larval mortality rate has a positive relationship with *Heterorhabditis indica* AZ2 concentrations, which reaches 13.33, 26.67, 46.67, and 56.67 at the concentrations of 25, 50, 100 and 200 IJ/larvae, respectively. On the other hand, an inverse relationship between *H. indica* AZ2 concentrations and the pupation rate, (83.33, 70, 53.33, and 40 % at concentrations of 25, 50, 100, and 200 IJ/larvae), respectively, compared to 100% in the control group. Regarding adult emergence, there was a decrease in the percentage of adult emergence by 80.00, 70.00, 46.67, and 33.33 %, respectively, at the previous concentrations.

Pathogenicity and toxicity of Heterorhabditis sp. HS against Agrotis ipsilon.

Data presented in Figure (9), reveals that there is an effect on larval mortality that varies by corrected percentages of 23.33, 40.00, 63.67 and 76.67 at concentrations of 25, 50, 100 and 200 IJ/larvae, respectively. In addition, the pupation percentage decreased to 73.33, 60.00, 36.67, and 16.67 compared to 100 % in the control group. The rate of adult emergence decreased with increasing concentrations, as indicated by 63.33, 53.33, 33.33 and 10.00%, respectively, at ascending successive concentrations as shown in Figure (9).



LC50 Values of Heterorhabditis Strains Toward A. ipsilon Larvae.

Results of the present investigation indicated that, the LC₅₀ values of *H.* bacteriophora HP88, *H. indica* AZ1, *H. indica* AZ2 and *H. sp.* HS varies according to strain shown in Table (3). The susceptibility of the 5th larval instars of *A. ipsilon* was applied at different concentrations of tested nematodes. Deaths were listed after 120 hours of infection. LC_{50s} ranged from 61.62 to 95.36 IJ/Larvae of *H. bacteriophora* HP88, 66.91 to 101.97 IJ/Larvae of *H. indica* AZ1, 104.77 to 176.84 IJ/Larvae of *H. indica* AZ2 and 57.11 to 82.00 IJ/Larvae of *H. sp.* HS i.e. *H. sp.* HS was the most effective followed by *H. bacteriophora* HP88, *H. indica* AZ2.

Heterorhabiditis strains	LC ₅₀ IJ/larvae	Lower limit	Upper limit	Slop
HP88	76.64	61.62	95.36	1.35 ± 0.039
AZ1	82.58	66.91	101.97	1.42 ± 0.04
AZ2	135.95	104.77	176.84	1.43 ± 0.043
HS	68.44	57.11	82.00	1.65 ± 0.042

Table 3: Toxicity of *Heterorhabditis* strains toward A. ipsilon larvae.

Haemolymph Protein Pattern of Uninfected and Infected Larvae of Some Cotton Pests by Entomopathogenic Nematodes:

Fraction Protein Pattern of Uninfected and Infected *S. littoralis* Larval Haemolymph by Entomopathogenic Nematodes (EPNs):

SDS- PAGE declared that the hemolymph proteins of control and infected samples were separated into 19 different bands according to their relative frequency (Rf), and molecular weight (M.W.) values and visualized by using COBB stain. Results given in Table (4) and illustrated in Figure (10) showed that the total number of bands in control, H. bacteriophora HP88, H.indica AZ1, H. indica AZ2, and H. sp. HS were 9, 16, 13, 9 and 15, respectively. In addition, the band no. 2, 3, 4, 9, 12, 14 and 15 with (M. W) 237, 197, 165, 101, 66, 50 and 42 K.Da respectively, were common for control, H. bacteriophora HP88, H.indica AZ1, H.indica AZ2 and H. sp. HS samples. On the other hand, the band's no. (7 and 17 with Mol. w. 127 and 34 K.Da.) were characteristic for control only while the band no. 1 with (M.W) 255 K.Da. characteristic for H. bacteriophora HP88 only. Band no. 5, 16 and 19 with (M. W) 145, 35 and 10 K.Da respectively, detected only in H. bacteriophora HP88, H.indica AZ1 and H. sp. HS. While the band no. (6 and 18 with M.W 135 and 32 K.Da.) shared bands between bacteriophora HP88 and H. sp. HS. In spite of band no. 10 with M.w.84 K. Da shared between H.indica AZ1 and H.indica AZ2. Data of SDS protein pattern in Table (4) and Fig. (10) revealed that the fifth band in control had the highest concentration with a percentage amounting to 34.19. While, in H. bacteriophora HP88 the eighth band had the highest amount with a percentage amount of 22.56. In H. indica AZ1 the highest density band was the fifth band with a percentage amount of 24.90. The fourth band had the highest density in *H. indica* AZ2 with a percentage amount of 36.15. On the other hand, the seventh band had the highest amount with a percentage amount of 22.42 in H. sp. HS.



Fig 10: Electrophoretic fraction protein pattern of *S. littoralis* larval haemolymph as control and infected samples by EPNs.

Pand Mal		0	Control	H. bacteriophora HP88		H. indica AZ1		H. indica AZ2		<i>H. sp.</i> HS		
no.	no. Rf. W.	Wol.	+ or -	% amount	+ or -	% amount	+ or -	% amount	+ or -	% amount	+ or -	% amount
1	0.01	255	-		+	4.27	-		-		-	
2	0.04	237	+	8.71	+	7.82	+	8.27	+	9.49	+	10.28
3	0.10	197	+	12.74	+	13.69	+	15.32	+	16.41	+	12.16
4	0.15	165	+	7.49	+	5.60	+	5.23	+	6.73	+	6.19
5	0.20	145	-		+	5.17	+	6.92	-		+	4.67
6	0.23	135	-		+	4.05	-		-		+	3.47
7	0.25	127	+	8.35	-		-		-		-	
8	0.26	125	-		+	4.08	-		-		+	4.64
9	0.33	101	+	34.19	+	22.56	+	24.90	+	36.15	+	22.42
10	0.39	84	-		-		+	4.47	+	6.35	-	
11	0.44	74	-		+	4.47	+	5.53	+	6.61	+	5.01`
12	0.47	66	+	9.56	+	5.37	+	5.26	+	6.71	+	5.39
13	0.54	55	-		+	4.42	+	5.45	-		+	4.91
14	0.57	50	+	6.21	+	4.00	+	5.41	+	5.49	+	5.24
15	0.62	42	+	8.13	+	5.09	+	5.92	+	6.07	+	4.31
16	0.68	35	-		+	3.07	+	3.80	-		+	4.89
17	0.69	34	+	4.63	-		-		-		-	
18	0.72	32	-		+	3.35	-		-		+	3.36
19	0.97	10	-		+	3.02	+	3.50	-		+	3.06

Table 4: Molecular weight (M.W.) and % amount of *S. littoralis* larval haemolymph fractionation protein pattern as control and infected samples by EPNs.

Results in Table (5), declared that, the similarity index (S. I) between the control sample and *H. bacteriophora* HP88, *H. indica* AZ1, *H.indica* AZ2, and *H. sp.* HS samples were 0.56, 0.48, 0.78 and 0.58 respectively. The highest S.I were recorded between *H. bacteriophora* HP88 and *H. sp.* HS (0.97).

Table 5: Similarity index and genetic distance between uninfected and infected samples of larval hemolymph protein of *S. littoralis*

	S. I										
	Sample	Control	H. bacteriophora HP88	H. indica AZ1	H. indica AZ2	H. sp. HS					
G. d	Control	-	0.56	0.48	0.78	0.58					
	H. bacteriophora HP88	0.44	-	0.83	0.64	0.97					
	H. indica AZ1	0.52	0.17	-	0.81	0.86					
	H. indica AZ2	0.22	0.36	0.19	-	0.67					
	H. sp. HS	0.42	0.03	0.14	0.33	-					

Fraction Protein Pattern of Uninfected and Infected A. *ipsilon* Larval Hemolymph by Entomopathogenic Nematodes (EPNs):

There were many clear differences between the five larval haemolymph proteins when re-fractionated under denaturing conditions; Table (6) and Fig (11). Fifteen bands were separated from control and infected samples. The total number of bands in control and infected samples *H. bacteriophora* HP88, *H. indica* AZ1, *H.indica* AZ2 and *H. sp.* HS were 7, 8, 10, 8 and 9, respectively. As shown in Table (6), band no. (2 with M.W. 199 K.Da.), (5 with M.W. 114 K.Da.) and (7 with M.W. 70 K.Da.) were common for control, *H. bacteriophora* HP88, *H. indica* AZ2 and *H. sp.* HS samples. While band no.

1 with Mol. W. 255 K.Da. shared between control and *H. bacteriophora* HP88. Band no. 14 shared between control and *H. sp.* HS. While the band no. 3 appeared in all samples except the *H. bacteriophora* HP88 sample. It is clear that bands no. 11 and 15 with M.W. 35 and 10 K.Da. were characteristic bands for all entomopathogenic nematodes infected samples. On the other hand, band no. 6 with M.W. 93 K. Da. shared band between *H. indica* AZ1 and *H.indica* AZ2. Also, band no. 8 with M.W. 54 K.Da. shared band between *H. bacteriophora* HP88 and *H. sp.* HS. While, the band no. (10 with M.W. 43 K.Da.), (12 with M.W. 34 K.Da.) and (13 with M.W. 32 K.Da.) were characteristic only for *H. indica* AZ2, control and *H. indica* AZ1 samples, respectively. Results recorded in Table (6) and Fig. (11) declare that the second band had the highest density in the control sample with a percentage amount to 43.63. While, in *H. bacteriophora* HP88, the highest density band was the fourth band with a percentage amount of 33.03. On the other hand, the first band had the highest densities in *H. indica* AZ1, *H. indica* AZ2 and *H. sp.* HS with percentage amounts of 37.78, 47.28 and 30.13, respectively.





Fig. 11: Electrophoretic fraction protein pattern of *A. ipsilon* larval haemolymph as control and infected samples by EPNs.

Table	6:	Molecular	weight	(M.W.)	and	%	amount	of	А.	ipsilon	larval	haemolymph
	fi	ractionation	protein	pattern a	s cor	ntro	l and infe	ecte	ed sa	amples b	y EPN	s.

Band no Rf.		Rf. Mol.		ontrol	<i>bacte</i> H	H. riophora IP88	Н.	<i>indica</i> AZ1	H.	indica AZ2	H.	sp. HS
110.		vv .	+	%	+ or	%	+	%	+	%	+	%
			or -	amount	-	amount	or -	amount	or -	amount	or -	amount
1	0.01	255	+	13.23	+	13.86	-		-		-	
2	0.1	199	+	43.63	+	30.41	+	37.78	+	47.28	+	30.13
3	0.17	167	+	6.80	-		+	3.18	+	5.04	+	4.78
4	0.27	132	-		+	2.92	+	4.04	-		+	4.06
5	0.30	114	+	4.35	+	33.03	+	4.28	+	4.52	+	4.51
6	0.36	93	-		-		+	5.28	+	4.58	-	
7	0.45	70	+	16.81	+	17.59	+	14.70	+	17.61	+	14.67
8	0.54	54	-		+	14.93	-		-		+	20.21
9	0.59	46	-		-	-	+	12.68	-		-	
10	0.62	43	-		-		-		+	8.30	-	
11	0.67	35	-		+	9.50	+	8.68	+	10.07	+	10.72
12	0.68	34	+	9.12	-		-		-		-	
13	0.70	32	-		-		+	5.41	-		-	
14	0.74	29	+	7.87	-		-		-		+	5.92
15	0.98	10	-		+	4.68	+	3.98	+	2.60	+	5.00

The similarity index (S.I.) and genetic distance (G.d) were declared between the control and other infected samples (Table 7). The similarity index between (*H. bacteriophora* HP88, *H. indica* AZ1, *H.indica* AZ2, and *H. sp.* HS infected samples) and their control was (0.53, 0.47, 0.53 and 0.62), respectively. *H. bacteriophora* HP88 and *H. sp.* HS-infected samples were more related to each other with S.I. equal 0.82.

	S. I										
G.d.	Sample	Control	H. bacteriophora HP88	H. indica AZ1	H. indica AZ2	<i>H. sp</i> . HS					
	Control	-	0.53	0.47	0.53	0.62					
	H. bacteriophora HP88	0.47	-	0.71	0.63	0.82					
	H. indica AZ1	0.53	0.29	-	0.78	0.74					
	H. indica AZ2	0.47	0.37	0.22	-	0.71					
	<i>H. sp.</i> HS	0.38	0.18	0.26	0.29	-					

Table 7: Similarity index and genetic distance between uninfected and infected samples of larval haemolymph protein of *S. littoralis*

DISCUSSION

Bio-control agents are environmentally friendly and effective means of controlling pests through the use of natural enemies. This method relies on predation, parasitism, or other natural agents. One of these agents can infect and kill insects within 48h such as entomopathogenic nematodes (EPN). It is important to note that the infective juveniles (IJs) are the only free-living stage in the EPN life cycle that live freely in moist soils and move in search of their insect hosts. Once in contact with the insect host, they enter its body through natural openings of the alimentary or respiratory canals or directly through the cuticle (Dowds and Peters, 2002; Ciche and Ensign, 2003; Gulcu et al., 2017) and release their symbiotic bacteria (Photorhabdus) into the host to kill it. Therefore, great efforts are being made worldwide to isolate new strains of EPNs as a safe agent for controlling economic pests. In the present study, only one nematode sample was isolated from the soil of mango gardens in the Governorate of Ismailia, Egypt, out of 50 soil samples. This sample was positive for the nematode Heterorhabditis, which was confirmed by the morphological signs of Galleria dead larvae which were dark red in colour and exhibited the bioluminescence phenomenon in the dark. These results are consistent with those of Kaya and Gaugler (1993) who reported that the colour of carcasses of insects infected with Heterorhabditits turns red while those infected with Steinernema turn yellow. Boemare Heterorhabditits/Photorhabdus (2002)noted that to differentiate from Steinernema/Xenorhabdus; Photorhabdus is positive for bioluminescence and red pigment production, whereas Xenorhabdus is negative. Aliyu et al. (2015) showed that out of fifty soil samples, nematodes were extracted from only three larvae. Larvae infected with Steinernematidae nematodes turned yellowish brown, but larvae infected by Heterorhabditis nematodes turned brick red. Abdel-Razek et al. (2018) extracted only one Heterorhabditis isolate coded as IB from Sharkia governorates (Belbies). Abd El Azim and Khashaba (2021) found that only one of the fifteen sandy loam soil samples contained Heterorhabditis nematodes which were confirmed by the morphological indicators of dead Galleria larvae. Nouh (2021) isolated two strains of Heterorhabditis, (TAN5) and (PGN6) which were identified by Galleria death markers.

Another target of the current study is to show the pathogenicity and toxicity of native Heterorhabditis isolates (one isolate isolated in this study, two isolates have been obtained from (PPRI) and the last one as a reference HP88) against S. littoralis and A. ipsilon. Our results exhibited that, all tested Heterorhabditis strains induce a positive influence on larval mortality while negative influence on pupation and adult emergence percentage against both insects Spodoptera littoralis and A. ipsilon, Also, the native isolates *Heterorhabditis sp.* HS was more pathogenic against tested insects than others. In addition, Spodoptera littoralis larvae were found to be more susceptible to all *Heterorhbditis* strains than A. ipsilon larvae. These results are in harmony with the findings of many authors who recorded the pathogenicity of Heterorhabditis nematode against S. littoralis such as Reyad (2001) who recorded 100% mortality to larval instars of Spodoptera littoralis when infected with 40 IJs/ml of H. bacteriophora. Ahmed et al. (2014) observed that the mortality percentage of S. littoralis larvae increased with the increase of the concentrations of H. *bacteriophora*. Yağcı *et al.* (2022) showed a high mortality rate of the 6th larval instar of S. littoralis by H. bacteriophora (90.47%), at the highest concentration of 1000 IJ/ml. Abd El Azim (2022) tested H. taysearae isolate against S. littoralis. At 150 IJs/larvae, a 100% mortality rate was recorded, followed by 90% at 120 IJs/larvae and 60% at 60 IJs/larvae, 72 h post-treatment. Other authors tested the pathogenicity of Heterorhabditis nematodes on A. ipsilon like Shoeb et al. (2006) who tested the pathogenicity of H. bacteriophora against the 4th instar larvae of A. ipsilon, H. bacteriophora caused 49, 53 and 73% mortality rates, at 25, 50, and 100 IJs/ larva, respectively, 48 h post-infection. Many reported results of the increasing mortality rate of A. ipsilon larvae with increasing time interval of exposure to H. bacteriophora (Hassan et al. 2016) and (Yuksel and Canhilal 2018). Also, many authors studied the toxicity of Heterorhabditis nematodes against both insects S. littoralis and A. ipsilon such as Hassan et al. (2020) and Nouh (2021). In the current study, authors suggested that the variation in pathogenicity among Heterorhabditis strains due to the variation in virulence of infective juvenile IJs to penetrate through the host insect, to their ability to overcome the host insect immune system (Hassan et. al., 2016), to the types of protein toxins their symbiotic *Photorhabdus* bacteria secrete into the host and their secondary metabolites that also secrete. This suggestion was confirmed by Duchaud et al. (2003) who stated that Photorhabrus TT01 genome has two paralogs, plu4092 and plu4436, which encode proteins similar to juvenile hormone esterases (JHEs) of the insect Leptinotarsa decemlineata. Brown et al. (2006) mentioned that Photorhabdus strains possess txp40 gene that produces ubiquitous insect-killing proteins and is effective against the insect larvae belonging to the order Diptera and Lepidoptera. Zamora-Lagos et al. (2018) declared that the genome of Photorhabdus laumondii (TT01 strain) encodes for a wide range of metabolic compounds including lipases, toxins, adhesins, proteases, hemolysins and a variety of antibiotic substances. In the current study, the SDS protein pattern revealed several protein bands characteristic only for *Heterorhabditis* nematodes. These bands may be characteristic of nematodes or/and their associated bacteria or/and their toxin protein secretion. Authors remarked on a characteristic band with M.W. 35 KDa found in all tested Heterorhabditis strains. This band with M.W 35 KDa was isolated and identified as a Galtox toxin protein by Ahuja (2021). Another protein band with M.W 10 KDa were also, recorded in Heterorhabditis strains. This band may be a fragment cleaved from the TcA toxin protein during toxin processing. Many studies on TcdA1, an insecticidal toxic protein, have reported toxin processing by proteolytic cleavage (Liu et al., 2003); Proteolytic cleavage of the binding component TcA induced by P. luminescens protease PrtA1 or by collagenase largely increased the toxicity of the Tc toxin. Moreover, the binding of TcA to target cells was largely increased after cleavage. Ost et al. (2019) indicated that the Tc toxin is activated by proteolytic processing of the TcA component, resulting in increased receptor binding. in all reports, toxin processing appears to occur at the amino and carboxyl termini of TcdA1.

At the amino termini, a 10 KDa fragment is cleaved.

Declarations:

Ethical Approval: Ethical Approval is not applicable to this study.

Competing interests: The authors declare that, there were no conflicts of interests

Authors Contributions: All authors listed have made significant contributions to the conception and design of the study, carefully reviewed the manuscript, confirmed the accuracy and reliability of the data and their interpretation, and approved its submission.

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ARABIC SUMMARY

القدرة المرضية و السمية للنيماتودا الممرضة للحشرات المعزولة من مصر على الاسبودبترا ليتورالس والاجروتس ابسيلون (Spodoptera littoralis and Agrotis ipsilon)

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لقد اجريت هذه الدر اسة لعزل سلالات جديدة من النيماتودا الممرضة للحشر ات من التربة المصرية وللتعرف على اى عائلة تنتمى هذه العزلات وكذلك لتقييم القدرة المرضية والسمية لهذه العزلات تجاه يرقات دودة ورق القطن والدودة القارضة و لفحص أنماط البروتين لدم كل من اليرقات المصابة وغير المصابة من الحشرات المستخدمة في الدراسة ولكي يتم عزل النيماتودا الممرضة للحشرات من التربة استخدمت طريقة إغراء الحشرات وتم استخدام دودة الشمع الكبري كعامل إغراء لاستخراج النيماتودا من التربة ولكي يتم التعرف على عائلة النيماتودا تم تسجيل العلامات المور فولوجية لموت يرقات دودة الشمّع الكبري و لتقييم التأثير السام لهذه العز لات، تم إعداد أربعة تركيز أت لكل منها وهي 25 و 50 و 100 و200 طور معدى لكل يرقة وتم وضع مجموعات من اليرقات حديثة الانسلاخ من الطور اليرقى الخامس في اكواب بلاستيكية مملوءة بالرمل المُعقم والمبللة بمحلول معلق من النيماتودا بنسبة 20٪ من تركيز ات مختلفة ثم تم تسجيل التاثير السام للنيماتودا المعزولة وتأثير اتها على نسبة التعذر ونسبة ظهور البالغين. وقد تم الكشف عن أنماط البر وتين لكل من البر قات المصابة وغير المصابة لدودة ودة ورق القطن و الدودة القارضة وذلك بأستخدام خاصية التفريد الكهربائي لهلام البولي أكريلاميد. وقد اظهرت النتائج أن العينات الإيجابية المعز ولة من تربة أشجار المانجو المصرية بمحافظة الإسماعيلية، تمثل فقط 2% من اجمالي عدد عينات التربة المجمعة وقد اوضحت النتائج ان النيماتودا المعزولة تنتمي الى عائلة ال Heterorhabditis والتي تم تأكيدها من خلال العلامات المور فولوجية لموت يرقات دودة الشمع الكبري حيث ظهرت اليرقات باللون الأحمر الداكن كما سجلت ظاهرة التلألؤ الحيوي في الظلام. لليرقات المصابة بالنيماتودا وقد اظهرت النتائج ان جميع سلالات Heterorhabditis المختبرة لها تأثير إيجابي على معدل وفيات يرقات دودة ورق القطن والدودة القارضة بينما كان لها تأثير سلبي على نسبة التعذر وخروج البالغين وقد اوضحت النتائج ان بعد إصابة الطور اليرقي الخامس لدودة ورق القطن كانت قيم التركيز المميت للنصف هي 33.51 و45.30 و73.24 و39.11 طور معدى لكل يرقة لكل من H. bacteriophora HP88 و H. indica AZ1 و H. sp. HS و H. indica AZ2 على التوالي بينما كانت قيم التركيز المميت للنصف هي 76.64 و 82.58 و 135.95 و 68.44 طور معدى لكل يرقة، وفقًا للترتيب السابق للنيماتودا وذلك بالنسبة للطور اليرقي الخامس للدودة القارضة ومما سبق يتضح ان من بين العز لات المصرية كانت العزلة H. sp. HS أكثر ضراوة ضد الحشرات المختبر ة من غير ها. كما وجد أن يرقات دودة ورق القطن أكثر عرضة للإصابة بجميع سلالات Heterorhbditis عن يرقات الدودة القارضة. وقد لوحظ تباين ملحوظ للانماط البروتينية بين دم العينة الضابطة والعينات المصابة بالتركيز المميت للنصف للنيماتودا المعزولة. ومن ضمن هذه الانماط نمط ذو وزن جزئي 35 كياو دالتون قد تم تعريفة كبروتين سام للحشرات ومن النتائج السابقة يمكن القول ان السلالات المعزولة سوف تلعب دور هام في برامج المكافحة المتكاملة للافات