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Virulence of *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) Produced in vitro Against *Galleria mellonella* (Lepidoptera: Pyralidae).

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ABSTRACT

Entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema* are obligate and lethal insect parasites. In last decade they are widely used as biological control agents for pest insects of commercial crops. In this study, the pathogenicity of the Egyptian EPNs strain; *H. bacteriophora* (BA1) was compared with the exotic strain (Hb 1-3), both produced *in vitro*, against last instar larvae of the greater wax moth, *Galleria mellonella*. Two bioassay methods were used to determine the virulence of both strains. Two densities of *Galleria* were used for each strain per each concentration. It was found that the infectivity of nematode strains against *G. mellonella* larvae was concentration dependent; i.e. the mortality percentage increased as the infective juvenile concentrations increased. Individual treatment (one larva/cup) led to more efficacy than using 5/ cup at 25°C. The productivity of both nematode strains in *Galleria* was estimated. The total yield of IJs on the *Galleria* was varied between native and exotic strains with significant different of multiplication. The amounts of IJs were produced in each *Galleria* from several thousands to hundreds thousands. In general, it was found that larvae infected with indigenous nematodes were more productive than that infected with the exotic strain. The histopathological patterns revealed variable effects on *G. mellonella* larvae after infection with both heterorhabditid nematodes. Muscles suffered destruction in the fibrillate with some fragments and fat body tissues showed high vacuolation

Key words: *Heterorhabditis*; Infectivity; *Galleria;* Bioassay; Production; Histopathology.

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INTRODUCTION

Entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* and their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, have been used to control a wide range of agriculturally important insect pests [1]. The mechanism by which these nematodes are able to infect and reproduce in the insect host involves a mutual relationship between the nematode and their symbiotic bacteria [2], [3]. Once an IJ penetrates into the haemocoele the bacterial symbiont is released from the nematode gut, septicemia becomes established and insect death occurs within 48 h. Although IJs play an important role in insect death by vectoring the bacteria, but, in most cases the bacteria alone are sufficient to cause insect mortality when injected into the haemocoele [4].

Inside their insect hosts, EPNs experience both intra and interspecific competition. Intraspecific competition takes place among nematodes of the same species when the number of infective juveniles penetrating a host exceeds the amount of resources available. Interspecific competition occurs when different species compete for resources. In both cases, the individual nematodes compete with each other indirectly by consuming the same resource, which reduces their fitness and may result in the local extinction of one species inside the host [5]. The greater wax moth, *G. mellonella* is widely used in mass production of biological control agents including the EPNs. Therefore, a good and less expensive artificial diet is important for mass rearing of *G. mellonella* [6].

The present study dealt with comparative laboratory assays of the Egyptian strain of the EPNs *H. bacteriophora* BA1 and the exotic strain *H. bacteriophora* Hb1-3 produced in vitro against last instar larvae of *G. mellonella* using two bioassay methods. The production of IJs of both strains of nematodes in *Galleria* larvae was investigated. The histopathological pattern of infected larvae with both nematode strains was carried out.

MATERIALS AND METHODS

Galleria mellonella

The greater wax moth, *G. mellonella* larvae were obtained from infested hives and reared on artificial media developed by Metwally, *et al.* 2012[6].

Entomopathogenic nematode

The native Egyptian EPNs *Heterorabditis bacteriophora* (BA1) was isolated form a soil sample collected from Nubaryia district, El-Behera governorat, Egypt by Hussein and Abou El-Soud (2006)[7], while the foreign *H. bacteriophora* (Hb 1-3) was kindly provided by Dr. R-U Ehlers, Raisdorf, University of Kiel, Germany. Both strains were mass produced *in vitro* using Bedding's technique [8].

Bioassay

First method

Infection of *G. mellonella* larvae was carried out in plastic cups (100 cc) half-filled with moistened sterile sandy soil and covered with plastic lids. The cups were treated with each of the two *H. bacteriophora* strains using 5 concentrations; 5,10,20,40 and 80 IJs/cup. Five larvae were used/cup for each replicate/ concentration. The cups were inspected daily one week post-treatment and percentage mortality recorded. The dead larvae (cadavers) were dissected to insure that they were nematode infected. Control treatment was carried out using distilled water.

Second method

Infection took place in similar cups lined with filter paper treated with the nematode suspensions at the same concentrations. Only one larva was used for each cup (25 replicates/ concentrations).

Production of entomopathogenic nematode

Five *Galleria* larvae were used for each concentration. Last instar larvae were confined, individually, in plastic cups (100 cc capacity) lined with filter paper and covered with plastic lids. Infection took place using five concentrations of 1,2,4,8 and 16 IJs/cup for each of the two strains of *H. bacteriophora*. IJs were harvested daily using White traps according to White, 1927. The whole number of IJs produced /larvae were estimated. All experiments were carried out in a conditioned laboratory at 25 ±2 °C and 50 – 60 % R.H.

Histological Investigation

Full– grown larvae were confined, individually, in plastic cups (100 cc capacity) lined with filter paper and covered with plastic lids. The filter paper was treated with the nematode suspension at five Concs., i.e., 5,10,20,40 and 80 IJs/cup. Histological studies were carried out in larvae after 4 and 6 days of treatment. Larvae were fixed in hot Bouin's solution for 4 hours and dehydrated through series of ethanol and embedded in paraffin wax 58 – 60 °C M.P. for half an hour. Longitudinal sections were cut at 5µ thickness by a microtome and mounted on glass slides. The sections were photographed under microscope using 40 X magnification [9].

Statistical analysis

Mortality rates were corrected according to Abbott's formula [10] and toxicity lines and LC_{50} values were calculated according to Finney [11]. The mass production of the two strains were statistically analyzed by ANOVA and mean values were separated by the least significant difference (L.S.D.) procedure [12]. T-test between the average production of the two strains at different concentrations or at each one was calculated.

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RESULTS AND DISCUSSION

Bioassays

Data presented in Tables (1) and (2) showed mortality percentages among *G. mellonella* larvae one week post-treatment by *H. bacteriophora* (Hb 1-3 and BA1) at different concentrations. These data showed that the percentage mortality increased as the concentration of IJs increased. Percentage mortalities by strain (Hb 1-3) reached 28,52,68,80 and 100 %, for density of 5 larvae / cup (first bioassay method) and increased to 32,56,72,88 and 100 %, for density of single larva / cup(second bioassay method), at concentrations of 5,10,20,40 and 80 IJs / cup, respectively,. The LC_{50} of the foreign strain of *H. bacteriophora* (Hb 1-3) to *G. mellonella* larvae in the two densities were 10.546 and 9.071 IJs /cup, respectively. As for strain (BA1), mortalities' percentages were 32, 56, 68, 76 and 96 %, at the same concentrations, respectively at the density of 5 larvae/ cup. These percentages were 36, 60, 76, 84 and 100 %, at the same concentrations, respectively at the density of one larvae/cup. The LC_{50} values for the indigenous nematode (BA1) were 9.544 and 8.288 IJs/cup, respectively (Figs. 1and 2). The slopes of probit-regression mortality lines were 1.564, 1.463 for Hb 1-3 and 1.283, 1.637 for BA1, at the two methods of treatment, respectively. El-Bishry *et al*. [13] used three doses 10, 20 and 40 IJs/larva and three nematode isolates *H. bacteriophora* against *G. mellonella* (S1, TWF and N1). The effect of nematode dose on penetration rate was only evident in case of TWF, which caused complete mortality to *G. mellonella* larvae at the three doses, while with the other two isolates, mortality was dose-dependent.

Production of entomopathogenic nematode

Table (3) illustrated the average production at different concentrations of *Heterorhabditis* Hb 1-3 and BA1 strains. Strain BA1 was higher in productivity than Hb 1-3 strain at the five tested concentrations, 1,2,4,8 and 16 IJs/cup. A single full-grown larva of *G. mellonella* treated with *H. bacteriophora* (Hb 1-3) produced 15000 (12180-16800) IJs/larva, at the lowest concentration, one IJs/ larva. The production rate increased to 93090 (82200-103700) IJs/larva at the highest concentration 16/larvae. Treatment of *G. mellonella* with BA1 strain produced

Table (1) Percentage mortality by different concentration and comparative toxicity of *Heterorhabditis bacteriophora* strain HB 1-3 and BA1 one week post-treatment against full grown larvae of *Galleria mellonella* using 5 larvae / cup.

Concentrations (IJs/cup)	% Mortality	LC_{50}	Slope	Concentrations (IJs/cup)	% Mortality	LC_{50}	Slope
Hb 1-3				*BA1*			
0.00	0.00	10.546	1. 564	0.00	0.00	9.544	1.283
5	28			5	32		
10	52			10	56		
20	68			20	68		
40	80			40	76		
80	100			80	96		

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Table (2) Percentage mortality by different concentration and comparative toxicity of *Heterorhabditis bacteriophora* strains HB 1-3 and BA1 one week post-treatment against the full grown larvae of *Galleria mellonella* using a single larva/ cup.

Concentrations (IJs/cup)	% Mortality	LC_{50}	Slope	Concentrations (IJs/cup)	% Mortality	LC_{50}	Slope
Hb 1-3				*BA1*			
0.00	0.00	9.071	1.463	0.00	0.00	8.288	1.637
5	32			5	36		
10	56			10	60		
20	72			20	76		
40	80			40	84		
80	100			80	100		

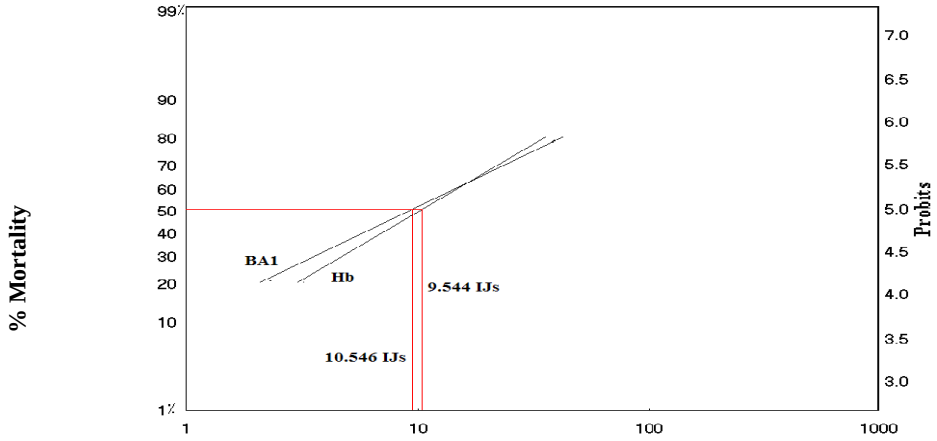

Fig. (1) Probit-Regression mortality lines showing response of full grown larvae of *Galleria mellonella* treated with different concentrations of *Heterorhabditis bacteriophora* (strains Hb 1-3 and BA1) using 5 larvae/ cup

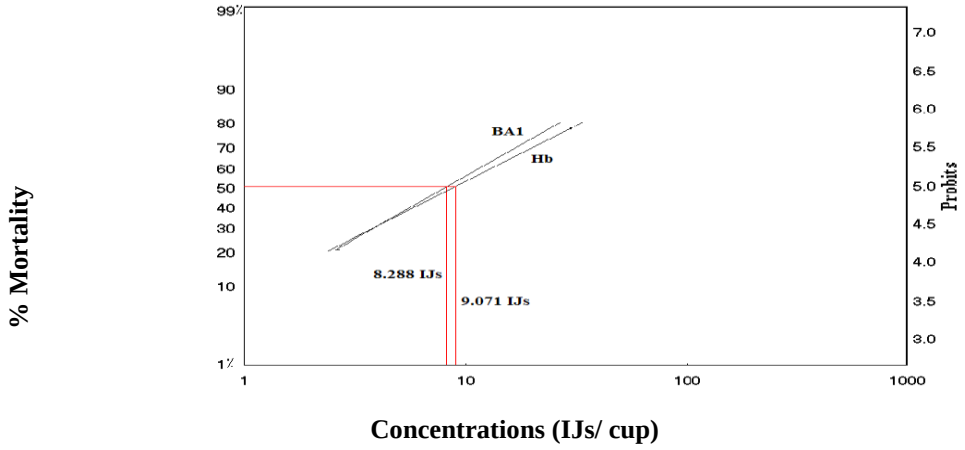

Fig. (2) Probit-Regression mortality lines showing response of full grown larvae of *Galleria mellonella* treated with different concentrations of *Heterorhabditis bacteriophora* (strains Hb 1-3 and BA1) using single larva/ cup.

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Table (3) Production of *Heterorhabditis bacteriophora* (strains HB 1-3 and BA1) in full grown *Galleria mellonella* larvae.

Concentrations (IJs / Larva)	Replicates	*H. bacteriophora*	
		Hb 1-3	BA1
1	R1	16800.00	39200.00
	R2	12180.00	25510.00
	R3	15270.00	33200.00
	R4	16550.00	29500.00
	R5	14200.00	35620.00
Average±S.E.		**15000±845.834**	**32606±2376.188**
2	R1	29700.00	47250.00
	R2	22670.00	55200.00
	R3	39000.00	41000.00
	R4	36900.00	49500.00
	R5	29250.00	56900.00
Average±S.E.		**31504±2929.951**	**49970±2858.916**
4	R1	44100.00	63600.00
	R2	56500.00	75400.00
	R3	53000.00	66750.00
	R4	49600.00	79060.00
	R5	58500.00	70200.00
Average±S.E.		**52340±2560.268**	**71002±2808.876**
8	R1	67150.00	82500.00
	R2	66000.00	97060.00
	R3	65000.00	93200.00
	R4	77350.00	86500.00
	R5	72500.00	99500.00
Average±S.E.		**69600±2330.255**	**91752±3189.809**
16	R1	87500.00	116800.00
	R2	82200.00	168000.00
	R3	103700.00	160000.00
	R4	99500.00	152500.00
	R5	92550.00	136900.00
Average±S.E.		**93090±3897.477**	**146840±9094.696**

32606 (25510 -39200) and 146840 (116800 - 168000) IJs/ larva at concentrations of 1 and 16 IJs/ larvae, respectively.

Table (4) showed the statistical analysis of the average production for the two strains in full grown *G. mellonella* larvae. Production of Hb 1-3 and BA1strains in larvae differed significantly between the different concentrations (F= 129.7842, df=4, P=<0.001, L.S.D.= 7967.627 at 5%) and (F= 85.330, df=4, P=<0.0001, L.S.D.= 14122.73 at 5%), respectively. Insignificant difference was found between the production averages at different treated

concentrations for the two strains (P< 0.001). Highly significant difference of production was found between the two strains for each concentration (P < 0.001).

Table (4) Statistical analysis of the average production of *Heterorhabditis bacteriophora* strains in full grown larvae *Galleria mellonella*.

Concentrations (IJs / Larvae)	Average Production of *H. bacteriophora* strains			
	Hb 1-3	BA1	T-test (1)	T-test (2)
1	15000 **e**	32606 **e**	**N. S.**	**H. S.**
2	31504 **d**	49970 **d**		**H. S.**
4	52340 **c**	71002 **c**		**H. S.**
8	69600 **b**	91752 **b**		**H. S.**
16	93090 **a**	146840 **a**		**H. S.**
L.S.D. (0.05)	**7967.627**	**14122.73**		

T-test (1) = between the different concentrations of the two strains.

T-test (2) = between each concentrations of the two strains. Values having same letters in a column are not significantly different.

El-Assal *et al.* [14] found that two Egyptian strains (TWF and Ar-4) of *H. bacteriophora* were more virulent to *G. mellonella* larva than the foreign strain (HP88) of the same nematode. Abd El- Rahman and Hussein [15] studied the effect of the initial infection densities (15, 100 and 1000 IJs) on the quality of the produced IJs of two nematode species, *H. bacteriophora* (H.b). The number of the initial penetrated IJs was increased by increasing in infection density of the two species. For (H.b), a highly significant increase in the number of penetrating IJs (penetration efficiency) in each larva at the three levels of infection (15, 100 and 1000) was found with the increased infection density (4.56, 48.82 and 398.33), respectively. Soliman [16] reported that an Egyptian strain of *H. bacteriophora* was more virulent that the foreign strain against the peach fruit fly, *Bacteroccra zonata.* Also its production rate was higher than the foreign nematode.

Histopathological investigation

Histopathological patterns revealed the activity of the EPNs *H. bacteriophora* (Hb 1-3 and BA1) inside *G. mellonella* larvae, after four and six days of inoculation. The two nematode strains affected muscles and fat tissues of the treated larvae. Muscle fibers suffered destruction with some fragmentation, complete destruction and disintegration in tissues was noticed. Also, fat tissues showed high vaculation. No haemocytic encapsulation is involved against nematode invasion (Figs.3 and 4). Tanada and Kaya [17] reported that depletion of fat bodies might result from catabolism stimulation of the fat body protein which could be utilized by the nematode

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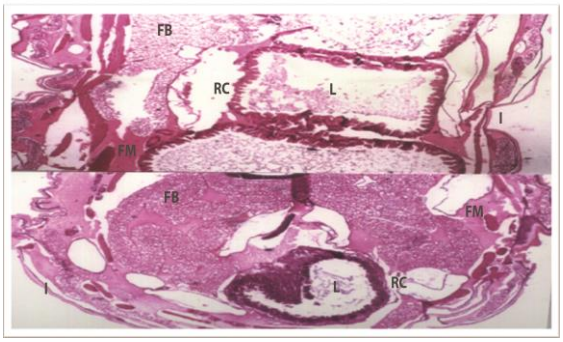

Fig.(3) Part of longitudinal section of full-grown *Galleria mellonella* larva uninoculated (control). X 40 FB= Fat body, FM=Fiber muscle, L=Lumen, RC=Regenerative cells, I=Integument.

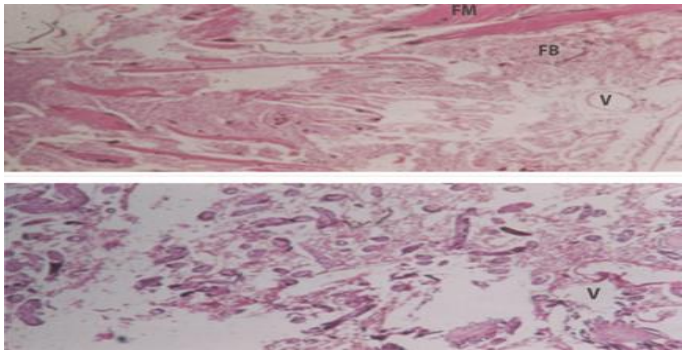

Fig. (4) Longitudinal section of *Galleria mellonella* larvae after 4 and 6 days from inoculation with *Heterorhabditis bacteriophora* (strain BA1) by concentrations 80 IJs/cup. X 40 V=Vacule,

FB= Fat body, FM=Fiber muscle.
for its growth. Soliman [18] documented similar results in *Ceratitis capitata* larvae infected with *H. bacteriophora* and *S. carpocapsae*.

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