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Virulence of Native EPN Strains and their Symbionts alone to Polyphagous Lepidopteran Pests vis a vis Model Insect *Galleria melonella* along with *in vivo* Production

Vinay K. Kalia[1]*, Garima Sharma[1] and Sudershan Ganguly[2]
[1]*Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi, 110012 India*
[2]*Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, 110012 India*

Abstract

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are obligate parasites of insects and can control pests due to the symbiotic bacteria that kill the insect host by septicemia and make the environment favourable for EPNs development and reproduction. In the present paper the virulence of three *Heterorhabditis* sp. strains and their respective symbiotic bacteria strains alone (*Photorhabdus luminescens* strains) was tested under laboratory conditions against larval stages of model insect host *Galleria mellonella* along with two important polyphagous pests *viz., Helicoverpa armigera* and *Spodoptera litura*. The results revealed that the virulence of the three strains of *Heterorhabditis* sp. tested, varied considerably in terms of both LC_{50} as well as LT_{50}. Feeding assays of symbiotic bacteria *Photorhabdus luminescens* showed that strain SG-*Ngp* was most effective against *S. litura* (LC_{50} = 4.06 x 10^5 cells/gm). It is worth mentioning that all the three strains showed lower LC_{50} against *S. litura* compared to *H. armigera* which concurrent with the results of IJ experiments. Among the three strains, H*ms1* was found to be most efficient IJ producer, via both *G. mellonella* and *H. armigera*. Although, when *H. armigera* was used as the host for this strain, the yield increased by 16%. Thus, this study provides an important insight on the native EPN strains with possible insecticide potential. Besides our studies suggest that not only EPN but also its associated symbiotic bacteria alone can be used for effective pest control.

Keywords: Entomopathogenic nematode; Growth inhibition; *Helicoverpa armigera; Spodoptera litura*; *Photorhabdus luminescens;* Virulence

Introduction

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are obligate parasites of insects and are used as biological control agents of economically important insect pests. Genera *Steinernema* and *Hetrorhabditis* possess a symbiotic association with pathogenic bacteria from the *Xenorhabdus* and *Photorhabdus* genera, respectively [1]. EPNs are ubiquitous, as they have been found in a wide range of ecologically diverse soil habitats including cultivated fields, woodlands, grasslands, deserts and ocean beaches, except Antarctica [2]. Being insect's natural enemy, having wide host range, host location searching / locating capability (particularly of some soil pest and stem borers) makes EPN a successful biocontrol agent [3]. Total 18 pesticide formulations, based on 12 different EPN species are commercially available worldwide as of now, whereas only 2 *Steinernema carpocapsae* formulations are available in India [4]. As potential natural enemies of insect pest, EPNs dominate its native habitat so there is the need of hour to investigate their entomopathogenic potential against major insect pests in India. Thus, the call for development of formulations using native EPNs has necessitates the search for new strains. Moreover, applying exotic EPNs may negatively affect native communities of EPNs and apparently dampen their rate of natural control [5]. Decrease in widespread EPN after application of exotic strain has been also reported with detrimental effects in long term [6]. Consequently, the isolation of native species of EPNs provides a valuable source for both biodiversity perspective and applicability prospect [7].

Infective juveniles (IJs), the only free-living stage of EPNs, enter the host insect through its natural apertures (oral cavity, anus and spiracles) or in some cases through the cuticle. Once inside the host insect, the nematodes and the multiplying bacteria in the hemocoel produce virulence factors resulting in insect death [8]. Developing nematodes feed on the bacteria by disintegrating host tissues, produce 1-3 generations and when the food resources are exhausted; nematodes emerge as IJs to seek new hosts [9]. During host invasion, the bacteria release several toxins and exoenzymes that play a role in insect death. The genome of *Photorhabdus luminescens* encodes a variety of virulence factors including toxins, hemolysins, adhesins, proteases and antibiotic-synthesis genes [10]. A number of insecticidal toxins from sp. have been reported *viz.,* Tc toxin complex [11], mcf1 which is apoptotic to insect and mammalian cell lines [12], PVC (Photorhabdus virulence cassettes) similar to bacteriocins in *P. asymbiotica* [13] and the PirAB (Photorhabdus insect related) toxins which are similar to insect's juvenile hormone esterase (JHE) of beetles [14].

The cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous insect pest distributed over the world [15]. It causes an estimated loss of US$927 million in chickpea and pigeon pea, over US$5 billion on over 200 crop species belonging to 45 families worldwide [16] and over $1 billion (USD) annually on different crops in India alone [17]. Globally, the *H. armigera* caterpillar consumes up $5 billion each year as control costs and production losses. China and India devote 50% of their insecticide applications to controlling it. Even

***Corresponding author:** Vinay K. Kalia, Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi, 110012 India, Tel: +919868548444; Fax: +9125842482; E-mail: vkalia_1998@yahoo.com; vkalia@iari.res.in

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more alarming is the rise of Bt-resistant populations of *H. armigera*, which have been identified in Pakistan, China, India and Australia [18].

Spodoptera litura (Fabr.), the 'tobacco cut worm' is a ubiquitous, polyphagous, lepidopterous pest that feeds on 112 cultivated crops all over the world [19]. Its larvae normally feed on the tender leaves causing serious damage to a variety of crops such as tomato, chilies, banana, castor, ground nut, soya bean, winged bean and cocoa [20].

The present study was undertaken as an extension to the biocontrol potential study of native EPN isolates collected from different parts of India and to investigate their efficacy against major insect pests. Eight isolates were collected from different parts of India [21]. The preliminary study of virulence of all the strains was carried out on *G. mellonella* and the three best strains *viz., Heterorhabditis* sp. strain H*mg3* (accession no. FJ751864), strain H*ms1* (accession no. HQ637414) and strain H*gj* (accession no. FJ744544) were shortlisted for further studies. This paper reports the virulence of above mentioned three strains of *Heterorhabditis* sp. as well as symbiotic bacteria alone (*Photorhabdus luminescens* strains) to three lepidopteran pests *viz., H. armigera, S. litura* and *G. Mellonella* along with their reproduction potential.

Materials and Methods

Test insects

H. armigera and *S. litura* were reared in the laboratory on a chickpea based semi-synthetic diet as described by Kalia et al., *Galleria mellonella* was cultured on semi-synthetic diet as per [22]. Rearing environment of 27 ± 2°C temperature with 60 ± 5% relative humidity (RH) was maintained throughout the experiment. All the adult moths were offered 10% honey solution fortified with multivitamins during their egg-laying period, in addition *G. mellonella* and *S. litura* were also provided with butter paper and blotting paper folded in a fan shape respectively for egg laying in the mating jars.

Test entomopathogenic nematodes

Three *Heterorhabditis* sp. strain H*mg3* (accession no. FJ751864), strain H*ms1* (accession no. HQ637414) and strain H*gj* (accession no. FJ744544) were obtained from Dr. S. Ganguly, Division of Nematology, IARI, New Delhi and maintained at the Insect Physiology and Molecular Biology laboratory. All strains of EPN were cultured through last instar *G. mellonella*. The emerging infective juveniles (IJ) were harvested using White traps [23] and stored at 10-15°C until further use within 2 weeks. The *Photorhabdus luminescens* strains namely SG-MG3 (accession no. JX221722), SG-NGP (accession no. JX240394) and SG-GJ were isolated from strains H*mg3*, H*ms1* and H*gj* respectively.

Bioassays with EPNs

The bioassays were performed on 12 well cavity sterile plates (2.5-cm-dia. x 2-cm-depths), where each well was lined at the bottom with Whatman No.1 filter paper. Hundred microliters of IJs suspensions were prepared in double distilled water (ddw) containing 10, 20, 50, 100 and 200 IJs of each isolates individually and incorporated onto the filter paper before releasing final instars larvae of *H. armigera, S. litura* and *G. mellonella*. In the control 100 µl ddw alone was introduced onto the filter paper. Ten replicates per concentration were used and each treatment was repeated thrice. Incubation was performed at a constant temperature of 27 ± 2°C and 60 ± 5% RH. Mortality was observed at 12 h interval for first 48 h followed by 24 h interval up to 168 h of IJs inoculation. Corrected mortality was calculated by [24]. Median lethal concentration (LC_{50}) was calculated at 36 and 48 h as 100% mortality in *G. mellonella* was attained by 48 h. Median lethal time (LT_{50}) was calculated at different concentrations to evaluate the most efficient dose with minimum time.

Estimation of EPN production

Ten dead larvae from the bioassay experiment (under 2.3), were selected randomly and rinsed with ddw. The larvae were then transferred aseptically on to the White trap and each concentration had a single white trap with 10 replicates. The IJs were collected during a period of 30 days starting from the date of first harvest. The IJs suspension was collected in a 50 ml culture bottle and a constant volume of 30 ml was maintained for all the suspensions. For counting the IJs, three samples were collected from each suspension and a 25 µl aliquot was withdrawn from each one and transferred to a 7 cm nematode counting dish to be counted separately. Final nematode concentration per ml was calculated by multiplying the average of the three 25 µl counts.

Isolation and bio efficacy of symbiotic bacteria

Last instar larvae of *H. armigera* were exposed individually in sterile plates (5 cm diam. x 2 cm depth) lined with Whatman No.1 filter paper to 100 IJs of *Heterorhabditis* strains suspended in 100 µl ddw. After 24 h of exposure, fresh haemolymph was collected by making a lesion in a proleg, in pre-cooled centrifuge tubes. Ten µl of this haemolymph was streaked on NBTA plates (Nutrient agar 7; Bromothymol blue 0.025; triphenyl-2,3,4-tetrazolium chloride 0.04 $g.l^{-1}$) and was incubated at 28°C for 24 h. Pure colonies were purified by sub culturing thrice on NBTA plates and subsequently inoculated in 250 ml Luria broth. The inoculated broth was incubated at 28°C and 200 rpm for 24 h. Culture broth was centrifuged at 8,000 rpm for 15 min. at 4°C. Bacterial cell counts were made using 'Neubaur Haemocytometer' (Germany) and the number of bacterial cells/ml calculated. A stock of suspension of 1 x 10^9 $cell.ml^{-1}$ was prepared.

Each *P. luminescens* strain was tested against neonates of *H. armigera* and *S. litura* by diet incorporation method using 100 µl suspensions maintaining the concentrations of 10, 1 × 10^2, 1 × 10^4, 1 × 10^6 and 1 × 10^8 bacterial cells/gm of diet as per [22]. The control was incorporated with 100 µl ddw only. Each test was replicated thrice with 10 neonates per replicate. The mortality observations were recorded at every 24 h till 7 days.

Growth inhibition of *H. armigera* larvae after *P. luminescens* treatment

On 7th day after treatment ten larvae from the above experiment were randomly selected from each set of concentration (treatment) and control. The percentage growth inhibition was calculated with the formula: Growth inhibition (%) = $(C_w\text{-}T_w)/C_w) \times 100$, where C_w is the average weight of 10 larvae in control and T_w is the average weight of 10 larvae in treatment.

Statistical analysis

The insect mortality data for median lethal concentration (LC_{50}) and median lethal time (LT_{50}) were analysed using maximum likelihood program for probit analysis [25]. The LC_{50} and LT_{50} were considered significantly different only in case of non-overlapping fiducial limits at 95% confidence level. The IJ production analysis was performed by one way analysis of variance (ANOVA) (SAS 9.2, SAS Institute Inc., Cary, NC, USA). Correlation between the parameters was determined by regression analysis.

Results

Bioassays with *Heterorhabditis* strains

Perusal of data in (Table 1) revealed that H*gj* strain was observed to be most virulent against *G. mellonella* with lowest LC_{50} at 36 h (13.02 IJs/larva) followed by *S. litura* (LC_{50} = 23.49 IJs/larva) and *H. armigera* (LC_{50} = 115.24 IJs/larva). However, all the three strains were found to be at par against *G. mellonella* at 36 h with LC_{50} values ranging from 13.02 to 14.58 IJs/larva and overlapping fiducial limits exhibiting no significant difference among them. By 48 h strains H*ms1* and H*gj* demonstrated 100% mortality, in contrast LC_{50} for H*mg3* was 7.59 IJs/larva only. On contrary, *H. armigera* was observed to be least susceptible to H*ms1* with highest LC_{50} (138.56 IJs/larva) followed by H*gj* (LC_{50} = 55.52 IJs/larva) and H*mg3* (LC_{50} = 36.01 IJs/larva) at 48 h. In case of *S. litura*, H*gj* (LC_{50} = 23.49 IJs/larva) was yet again the most effective strain with least mortality at 36 h followed by H*ms1* (LC_{50} = 72.03 IJs/larva) and H*mg3* (LC_{50} = 98.50 IJs/larva). But, H*mg3* required only 10 IJs per larva by 48 h, followed by H*ms1* and H*gj* (LC_{50} = 12 and 14 IJs/larva respectively).

LT_{50} values of the strains *vs.* test insects were found to be rate dependent with IJ dose (Table 2a). LT_{50} values in *G. mellonella* ranged from 19.23 h to 45.89 h which was also the narrowest range among the test insects. LT_{50} of H*mg3* at 200 IJs was observed to be 19.23 h (least among all the doses as well as all the strains) and was significantly different from 10 and 20 IJs (38.87 h and 34.84 h respectively)(r = -0.876). While H*ms1* and H*gj* showed similar LT_{50} (20 h) at the dose of 200 IJs which was significantly different from 10 IJ dose (H*ms1* LT_{50} = 45.84 h and H*gj* LT_{50} = 45.89 h) towards *G. mellonella.*

In case of *H. armigera* as well, H*mg3* showed least LT_{50} (32.34 h) at the dose of 200 IJs followed by H*gj* and H*ms1* with 40.96 h and 44.08 h LT_{50}s respectively on the same dose (Table 2b). None of the strain was found to attain 50 % mortality at the 10 IJs concentration till 168 h against *H. armigera.* However LT_{50} @ 200 IJs for H*gj* was found to be significantly different from 50 IJ concentration, whereas the other two strains were found to be at par (Table 2b).

Lowest LT_{50} of 33.35 h was observed with strain H*gj* against *S. litura* followed by H*ms1* (LT_{50} = 40.02 h) and H*mg3* (LT_{50} = 56.24 h) at the 200 IJs, although found to be at par at these concentration (Table 2c). While 200 IJs in H*gj* was significantly different from 10, 20 and 50 IJs treatment with in the strain, it was found to be significantly different from 10, 20 50 and 100 IJs treatment among the rest two strains within the test insect.

In case of *G. mellonella,* for 10 fold decrease in the IJ concentration (10:100) 1.7-1.9 fold increase in LT_{50} was observed in among all the test strains. However in *H. armigera*, none of the strain able to attain 50% mortality @ 10 IJs till 7 days after treatment but in term of $LT_{50,}$ all the strains were found to be at par with the *G. mellonella* at the highest concentration tested (200 IJs) Strain H*gj* was most effective against *S. litura* in terms of infectivity with 3.4 fold decline with 10 times increase in IJ dose. While H*mg3* and H*ms1* strains showed only 1.5 and 1.7 fold decline respectively. But in term of $LT_{50,}$ all the strains were found to be significantly different with respect to *G. mellonella* at the highest concentration tested (200 IJs) In general for *Heterorhabditis* strains vs. the test insect analysis, LT_{50} was found to be negatively correlated with the rate of application.

Sl. No.	*Heterorhabditis* Strains used	LC_{50} IJs/larva	95 % Fiducial Limit		Slope ± SE	χ2	df	pc
			Lower	Upper				
G. mellonella at 36 hr								
1	HI(*Hmg3*)	13.83	2.71	25.4	1.60 ± 0.52	1.43	3	0.699
2	Nagpur(H*ms1*)	14.58	7.66	21.78	3.16 ± 1.02	2.63	3	0.452
3	Gujarat(H*gj*)	13.02	7.53	18.53	4.74 ± 1.79	0.07	3	0.995
G. mellonella at 48 hr								
1	HI(*Hmg3*)	7.59	0.17	14.68	1.91 ± 0.76	0.66	3	0.883
2	Nagpur (Hms1)	100% mortality was obtained at 48 hr in maximum concentrations						
3	Gujarat(Hgj)	100% mortality was obtained at 48 hr in maximum concentrations						
H. armigera at 36 hr								
1	HI(*Hmg3*)	189	72.1	36.6×10^6	0.94 ± 0.43	0.06	3	0.996
2	Nagpur (Hms1)	191.93	107.68	706.29	1.04 ± 0.25	3.29	3	0.349
3	Gujarat (Hgj)	115.24	72.71	261.18	2.21 ± 0.63	2.09	3	0.554
H. armigera at 48 hr								
1	HI(*Hmg3*)	36.01	20.5	52.57	3.24 ± 0.91	0.26	2	0.878
2	Nagpur (Hms1)	138.56	62.38	48.78×10^2	1.10 ± 0.44	0.14	3	0.743
3	Gujarat (Hgj)	55.52	31.22	96.13	1.90 ± 0.48	7.64	3	0.054
S. litura at 36 hr								
1	HI(*Hmg3*)	98.5	33.2	46.30×10^9	0.82 ± 0.40	0.02	3	0.999
2	Nagpur (Hms1)	72.03	42.76	142.42	1.86 ± 0.50	2.32	3	0.509
3	Gujarat (Hgj)	23.49	12.22	37.32	2.16 ± 0.56	0.82	3	0.845
S. litura at 48 hr								
1	HI(*Hmg3*)	10.91	0	28.98	0.92 ± 0.43	0.108	3	0.991
2	Nagpur (Hms1)	12.42	0.04	29.45	1.04 ± 0.44	3.31	3	0.346
3	Gujarat (Hgj)	14.66	3.8	25.83	1.73 ± 0.53	0.63	3	0.89
SE = standard error. χ2 = Pearson χ2 of the slope. df = degree of freedom for χ2 pc = critical probability of the slope.								

Table 1: Toxicity of three strains of *Heterorhabditis* sp. against last instar *Helicoverpa armigera, Spodoptera litura*, and *Galleria mellonella*.

Sl. No.	Heterorhabditis strain	No. of IJs	LT_{50} (hr)	Fiducial Limit		Slope ± S.E	χ2	DF	pc
				Lower	Upper				
1	HI(H*mg3*)	10	38.87	29.93	65.88	4.34 ± 1.56	0.55	2	0.76
		20	34.84	27.41	45.31	5.77 ± 1.87	0.17	2	0.919
		50	26.14	19.33	33.62	4.60 ± 1.23	1.2	2	0.549
		100	21.91	16.44	27.21	5.74 ± 1.44	1	2	0.607
		200	19.23	13.38	24.56	4.77 ± 1.26	0.94	2	0.625
r = -0.876									
2	Nagpur (H*ms1*)	10	45.84	36.27	60.29	4.78 ± 1.35	4.35	3	0.226
		20	30.8	23.38	37.73	5.32 ± 1.29	3.44	3	0.329
		50	27.79	24.83	30.54	15.62 ± 4.70	1.35	2	0.509
		100	27.07	23.14	30	13.15 ± 4.34	1.9	2	0.387
		200	20.03	15.17	25.16	6.60 ± 1.84	1.5	1	0.221
r = -0.790									
3	Gujarat (H*gj*)	10	45.89	35.78	63.47	4.19 ± 1.13	1.88	3	0.598
		20	30.8	23.38	37.73	5.32 ± 1.29	3.44	3	0.328
		50	24.49	18.98	32.19	6.08 ± 1.81	7.79	1	0.009
		100	23.29	17.91	30.15	6.11 ± 1.79	5.55	1	0.019
		200	20.03	15.17	25.16	6.59 ± 1.84	1.49	1	0.221
r = -0.736									
SE = standard error. χ2 = Pearson χ2 of the slope. df = degree of freedom for χ2. pc = critical probability of the slope.									

Table 2a: LT_{50} values calculated from dosage response assays conducted with three strains of *Heterorhabditis sp.* against last instar *Galleria mellonella.*

Sl. No.	Heterorhabditis strain	No. of IJs	LT_{50}(hr)	Fiducial Limit		Slope ± S.E	χ2	DF	pc
				Lower	Upper				
1	Meghalaya HI(H*mg3*)	10	50% mortality was not observed till 168 hr						
		20	180.70*	Unable to calculate		0.76 ± 0.65	0.93	3	0.818
		50	140	34.81	74.13	2.98 ± 0.77	2	3	0.572
		100	70.69	26.72	53.07	3.87 ± 0.97	0.87	3	0.833
		200	32.34	22.37	42.01	4.77 ±1.37	1.48	2	0.477
r = -0.951									
2	Nagpur (H*ms1*)	10	50% mortality was not observed till 168 hr						
		20	129.56	81.92	409.59	1.93 ± 0.69	0.67	3	0.879
		50	117.6	72.57	360.41	1.83 ± 0.62	0.21	3	0.976
		100	84.49	39	214.98	1.55 ± 0.61	1.01	3	0.799
		200	44.08	20.76	87.09	2.93 ± 1.28	0.21	1	0.647
r = -0.982									
3	Gujarat (H*gj*)	10	50% mortality was not observed till 168 hr						
		20	180.70*	Unable to calculate		4.02 ± 2.15	0.52	2	0.773
		50	140	95.1	17.7	3.59 ± 1.80	0.09	2	0.956
		100	70.69	46.22	115.66	2.77 ± 0.88	0.24	2	0.889
		200	40.96	13.06	66.7	2.23 ± 0.68	0.04	2	0.98
r = -0.951									
* Values for 20 IJs in case of both Hmg3 and Hgj strains against *H. armigera* were obtained by extrapolation.									
SE = standard error. χ2 = Pearson χ2 of the slope. df = degree of freedom for χ2. pc = critical probability of the slope.									

Table 2b: LT_{50} values calculated from dosage response assays conducted with three strains of *Heterorhabditis sp.* against last instar *Helicoverpa armigera.*

Estimation of IJ yield

Perusal of (Table 3) H*ms1* (Nag) was the most effective strain for progeny production and yielded 160.38 × 10^3IJs /larva in *G. mellonella* and 190.39 × 10^3 /larva in *H. armigera* at the initial dose of 200 IJs/larva.

In *G. mellonella* lowest yield was 56.39 × 10^3 /larva, obtained in strain Hms1 at the dose of 10IJs/larva. IJ Yield in strain H*ms1* at 10 and 20 IJ/larva (56.39 × 10^3 and 64.33 × 10^3 respectively) and 50 and 100 IJ/larva (109.08 × 10^3 and 118.94 × 10^3 respectively) was found to be at par ($F_{4,14}$ = 246.85, p<0.0001). Second best IJ yield at the initial

Sl. No.	*Heterorhabditis* strain	No. of IJs	LT_{50}(hr)	Fiducial Limit		Slope ± S.E	χ2	DF	pc
				Lower	Upper				
1	H*mg3*	10	107.92	77.47	176.47	2.31 ± 0.68	2.32	5	0.803
		20	98.01	72.29	139.24	2.66 ± 0.71	1.91	5	0.861
		50	88.99	66.2	115.91	3.11 ± 0.78	2.65	5	0.754
		100	67.42	45.71	88.41	2.81 ± 0.67	1.46	5	0.918
		200	56.24	35.47	74.75	2.70 ± 0.65	1.04	5	0.959
r = -0.951									
2	H*ms1*	10	114.59	95.95	136.94	5.51 ± 1.38	7.12	5	0.212
		20	86.54	57.88	130.52	2.15 ± 0.62	4.85	5	0.434
		50	84.4	59.79	115.01	2.63 ± 0.68	3.25	5	0.662
		100	66.37	47.47	84.49	3.26 ± 0.72	0.32	5	0.997
		200	40.02	26.76	51.9	4.25 ± 1.16	1.46	2	0.482
r =-0.941									
3	H*gj*	10	143.63	112.93	253.65	3.34 ± 1.04	2.37	5	0.796
		20	91.86	67.64	123.37	2.89 ± 0.76	2.14	5	0.829
		50	62	44.04	77.74	3.64 ± 0.79	2.6	5	0.761
		100	42.24	23.63	57.38	2.83 ± 0.67	2.78	5	0.734
		200	33.35	24.29	42.45	6.27 ± 1.80	0.487	1	0.485
r = -0.800									
SE = standard error. χ^2 = Pearson χ^2 of the slope. df = degree of freedom for χ^2. p_c = critical probability of the slope.									

Table 2c: LT_{50} values calculated from dosage response assays conducted with three strains of *Heterorhabditis sp*. against last instar *Spodoptera litura*.

dose of 200IJs/ larva was observed in strain H*gj* (139.01 × 10^3 /larva), which was significantly different from the yield among the other doses of this strain. It was also observed that production at 100IJs and 20IJs were significantly different from each other but both were equivalent to 50IJs. Least productivity for the strain H*gj* was observed at the dose of 10IJ (97.49 × 10^3 /larva), which was significantly different from rest of the doses ($F_{4,14}$ = 2087.1, p<0.0001). Lowest production at the dose of 200IJs/Larva (124.10 × 10^3 /larva) was observed in strain H*mg3*. Although a positive correlation (r =0.940) between the increase in concentration and the IJ production was observed, however there was a significant difference between 10, 20, 50 and 100 IJ/larva doses for this strain($F_{4,14}$ =370.46, p<0.0001).

In *H. armigera*, the IJs production was observed to be in the range of 40.65 x 10^3 /larva (10 IJ/larva dose) to 190.39 × 10^3 /larva (200 IJs per larva) with H*ms1* as the most efficient strain for IJ yield. In Hms1 all the concentrations were positive correlated (r = 0.952) to the IJ yield but were significantly different from each other ($F_{4,14}$ = 1125.81, p<0.0001). H*mg3* is the second best strain for production of IJs with 164.82 × 10^3 / larva at the concentration of 200IJs, followed by H*gj* with 160.63 x 10^3 / larva at the same dose. Strain H*mg3* yield at 10 and 50 IJs (40.65 x 10^3 / larva and 49.27 × 10^3 /larva respectively) is significantly different from each other but at par with 20 IJs (42.45 × 10^3 /larva), while production at 100IJ is different from rest of the concentrations ($F_{4,14}$ = 2559.84, p<0.0001). Although the relative yield at the concentrations of 10, 20 and 50 IJ for H*mg3* had been lowest as compared to the relative production from the prior mentioned concentrations of the other two strains. In contrast, the IJ reproduction levels in strain H*gj* had been observed to be moderately good at the concentrations of 100 (133.44 × 10^3 /larva) and 50IJ (103.69 × 10^3 /larva) which were significantly different from each other as well as from rest of the concentrations ($F_{4,14}$ =76.83 p<0.0001).

The ratio of yield per dose was calculated to evaluate production maxima from smallest dose of inoculum. With *G. mellonella* as the host for the *in vivo* IJ production the strain H*gj* was observed to be the best with the ratio of 6.75 × 10^3 yield/10IJs, followed by 6.05 × 10^3 yield/10IJs in strain H*mg3* and 5.64 × 10^3 yield/ 10IJ (significantly different from each other). While in case of *H. armigera*, H*ms1* was the best strain with 7.37 × 10^3 yield/ 10IJs which is found to be statistically at par with H*gj* having 6.92 × 10^3 yield/10IJs.

Based on the above results in terms of IJ production as well as yield/inoculation ratio H*ms1* is the most efficient strain in *H. armigera*. On contrary, in *G. mellonella* H*ms1* is best for production while H*gj* resulted in highest yield/inoculum ratio. Production of IJ was positively correlated with the IJ concentrations for all the three strains in both *H. armigera* and *G. mellonella*. Though, yield/ inoculum ratio was negatively correlated with the concentrations.

Isolation and bioefficacy of symbiotic *Photorhabdus luminescens* strains

Feeding assays of symbiotic bacteria *Photorhabdus luminescens* to *S. litura* neonates, revealed *P. luminescens* strain SG-*Ngp* as most effective by day 4 with 3.74 × 10^6 cells/ gm of diet (Table 4). While *P. luminescens* strain SG-*Mg3* was also effective with 8.49 × 10^7 cells/ gm of diet, *P. luminescens* strain SG-*gj* was observed to have 36.67% mortality even at the highest concentration tested (i.e. 2 × 10^8). A decrease in LC_{50} was observed at day 7 in all the strains, although SG-*Ngp* strain (LC_{50} = 4.06 × 10^5 cells/ gm) remained the most effective against *S. litura*. Strain SG-*Mg3* was moderately effective (LC_{50} = 2.96 × 10^6 cells/ gm) while SG-*gj* being least effective among the three (LC_{50} = 2.93 × 10^7 cells/ gm) against *S. litura*.

It is noteworthy that the three strains showed lower LC_{50} against *S. litura* compared to *H. armigera* which concurrent with the results of IJ experiments.

Growth inhibition of *H. armigera* larvae after *P. luminescens* treatment

Perusal of (Table 5), a dose dependent inhibition in growth was observed in the 7th day larvae of *H. armigera*. The average weight of the larvae in control at day 7 was 41.7 mg. A decline of 20-31% was observed in the larval growth at the dose of 10^1 *P. luminescens* cells/

Sl. No.	Heterorhabditis strain	No. of IJS/larva	Avg yield/larva (x10^3) ± S.E.	Ratio of Yield (10^3)/ inoculated dose
G. mellonella				
1	H*mg3*	10	60.50^{e}± 1.05	6.05^{b}±0.10
		20	74.02^{d}± 0.77	3.70^{e}±0.04
		50	86.01^{c}± 1.12	1.72^{h}±0.02
		100	112.18^{b}± 2.14	1.12^{i}±0.02
		200	124.10^{a}± 1.41	0.62^{k}±0.01
			r = 0.940	r = -0.788
2	H*ms1*	10	56.39^{c}± 2.08	5.64^{c}±0.21
		20	64.33^{c}± 3.09	3.22^{f}±0.16
		50	109.08^{b}± 0.98	2.18^{g}±0.02
		100	118.94^{b}± 3.80	1.19^{j}±0.04
		200	160.38^{a}± 2.70	0.80^{jk}±0.01
			r = 0.952	r = -0.800
3	H*gj*	10	67.49^{d}± 0.71	6.75^{a}±0.07
		20	83.61^{c}± 1.89	4.18^{d}±0.09
		50	89.83^{bc}± 1.75	1.80^{h}±0.04
		100	95.24^{b}± 2.16	0.95^{ij}±0.02
		200	139.01^{a}± 1.88	0.70^{jk}±0.01
			r = 0.971	r = -0.774
H. armigera				
1	H*mg3*	10	40.65^{d}± 0.30	4.07^{b}±0.03
		20	42.45^{cd} ± 0.69	2.12^{c}±0.04
		50	49.27^{c}± 1.42	0.99^{e}±0.03
		100	86.54^{b}± 2.67	0.87^{e}±0.03
		200	164.82^{a}± 2.45	0.82^{e}±0.01
			r = 0.989	r = -0.673
2	H*ms1*	10	73.68^{e}± 0.32	7.37^{a}±0.03
		20	78.39^{d}± 3.19	3.92^{b}±0.16
		50	107.34^{c}± 2.59	2.15^{c}±0.05
		100	166.01^{b}± 1.52	1.66^{cd}±0.02
		200	190.39^{a}± 0.73	0.95^{e}±0.01
			r = 0.952	r = -0.757
3	H*gj*	10	69.22^{d}± 6.41	6.92^{a}± 0.64
		20	87.25^{d}± 1.42	4.36^{b}± 0.07
		50	103.69^{c}± 1.35	2.07^{c}± 0.03
		100	133.44^{b}± 1.52	1.33^{de}± 0.02
		200	160.63^{a}± 3.20	0.80^{e}± 0.02
			r = 0.968	r = -0.793

Table 3: Production of three strains of Heterorhabditis strains IJs in last instar *Helicoverpa armigera, Spodoptera litura*, and *Galleria mellonella*.

gm of diet. While average larval weight of ~23 mg was observed at the concentration of 10^2 cells/ gm of diet (43% growth reduction) for H*gj* and H*ms1*. H*gj* was able to reduce larval growth up to 13 mg (68%) while 7.4 mg (82.25%) was the average larval weight in H*ms1* treatment of 10^6 cells of the respective strains. Highest growth arrest of 98% was detected in H*ms1* ($F_{5,58}$ = 39.14, p<0.0001) at the dosage of 10^8 while at the same dosage 83% growth inhibition was exhibited byH*gj* ($F_{5,58}$ = 14.09, p<0.0001) treated larvae.

Discussion

The results obtained in the present study evidently showed that the virulence of the three strains of *Heterorhabditis* sp. tested to model insect *G. melonella vis a vis* polyphagous insects *H. armigera* and *S. litura* varied considerably, thus suggesting that each strain presents diverse virulence degrees in terms of LC_{50} as well as LT_{50}. This is substantially documented in literature [26-28]. However, the dosage of EPNs remain crucial, as a dosage that is too low results in low host mortality and a dosage that is too high may result in failed infections due to competition with secondary invaders [29]. Thus, LC_{50} values support in determining IJ dose for a particular insect host. Strains used in the present study have promising insecticidal action against *H. armigera* and *S. litura* at 48 h based on lower LC_{50} as well as LT_{50} values [30,31].

Similar to present results, [32] reported that the median lethal time was negatively correlated with increase in *H. indica* dose. They also reported the LT_{50} of the laboratory assay on *G. mellonella*, *H. armigera* and *S. litura* (36 h, 40 h and 48 h respectively), which deduces *S. litura* as the sturdiest of the three insects. In contrast, present study reported *G. mellonella* to be most susceptible for all the three strains, while strains H*ms1* and H*gj* exhibited lower LT_{50} in case of *S. litura* as compared to *H. armigera* which concurs LC_{50} and LT_{50} interpretations of [33,32]. Thus, H*ms1* is the most effective strain against *S. litura*, whether provided in IJ form or as symbiotic bacteria alone.

Besides, infectivity and mortality, mass production of IJs is also considered as an important criterion to assess EPN efficiency. Poor reproduction of EPNs may hamper their cost effectiveness in large-scale production systems [34]. In general nematode yield depends up on host size, nematode dosage and host density [35,26]. Several *Heterorhabditis* sp. had been reported to have *in vivo* production ranging from 8.0 × 10^4- 5.67 × 10^5 IJs per larva using *G. mellonella* as the host [36,35,28]. Our result validates these findings as the average yield of the three *Heterorhabditis* strains was found to be in this range.

Among the three strains, H*ms1* was most efficient IJ producer, using both *G. mellonella* and *H. armigera*. Although, when *H. armigera* was used as the host for this strain, the yield increased by 16%. It has been reported that, the quality as well as the composition of the lipids in the host insect play a major role in the production and yield of infective juveniles [37]. average IJs production using the same host in *Steinernema* sp. had been reported in 71 × 10^3 IJs per ml (*S. feltiae*) to 320 × 10^3 IJs per ml (*S. carpocapsae*) [38,39], which is lesser as compared to *Heterorhabditis*. The reproductive potential of *Heterorhabditis* strains was observed to be higher as compared to *S. thermophilum* at the same dosage range [22] Kalia et al., which concurs with the results of [40].

Photorhabdus species are known to be highly virulent towards a wide range of insect hosts. While there are several reports of both injectable as well oral activities of its purified toxin complex, however reports on oral toxicity of *P. luminescens* bacteria alone remain insufficient. As discussed by [22], using the symbiotic bacteria alone is a promising potential avenue for biological control, particularly because the bacteria are less expensive to produce than the nematode-bacteria complex. Oral toxicity on *P. luminescens* and *X. nematophila* against *Aedes aegypti* larvae was found to be 83% and 52 % respectively [41]. In addition, the EPN bacterial symbiont species culture suspensions have been used as immunosuppressant against *Aedes albopictus* and *Culex pipiens pallens, along with B. thuringiensis* [42]. *Xenorhabdus* sp. and *Photorhabdus temperate subsp. temperata* bacteria have been reported to cause high mortality of third-instar larvae of *Spodoptera exigua*, but not to the fifth-instar larvae when administrated orally [43]. The strains SG-NGP and SG-GJ, used in this study do exhibit growth dependent oral toxicity for the both *H. armigera* and *S. litura*, however SG-MG3 does not exhibit a dose dependent mortality for *H. armigera*.

One of the important aspect of this study is the reduction in average larval weight gain in both *H. armigera* as well as *S. litura* upon administering the two *P. luminiscens* (i.e. SG-NGP andSG-

Test insect	Photorhabdus strain	LC_{50} (bacterial cells/gm of diet)	Fiducial limits (bacterial cells/ gm of diet) 95%	Slope ± SE	χ^2	Degree of freedom	p_c
After 4 day							
H. armigera	SG-MG3	Mortality was not dose dependent					
	SG-NGP	2.04×10^9	$2.56 \times 10^7 - 7.32 \times 10^{14}$	0.176 ± 0.049	1.341	3	0.719
	SG-GJ	6.85×10^9	$6.16 \times 10^7 - 1.03 \times 10^{17}$	0.179 ± 0.050	0.939	3	0.816
S. litura	SG-MG3	8.49×10^7	$2.8 \times 10^5 - 1.04 \times 10^{38}$	0.093 ± 0.043	0.514	3	0.916
	SG-NGP	3.74×10^6	$5.57 \times 10^4 - 7.56 \times 10^{12}$	0.118 ± 0.043	1.054	3	0.788
	SG-GJ	36.67% mortality at Highest conc. tested (1×10^8)					
After 7 day							
H. armigera	SG-MG3	Mortality was not dose dependent					
	SG-NGP	7.23×10^5	$1.26 \times 10^5 - 7.12 \times 10^8$	0.307 ± 0.050	1.398	3	0.706
	SG-GJ	8.56×10^7	$3.98 \times 10^6 - 5.86 \times 10^{10}$	0.210 ± 0.048	0.647	3	0.886
S. litura	SG-MG3	2.96×10^6	$20.9 \times 10^3 - 13.5 \times 10^{18}$	0.098 ± 0.042	1.744	3	0.627
	SG-NGP	4.06×10^5	$1.07 \times 10^4 - 5.50 \times 10^8$	0.135 ± 0.043	0.883	3	0.83
	SG-GJ	2.93×10^7	$1.69 \times 10^5 - 5.59 \times 10^{23}$	0.101 ± 0.043	1.365	3	0.714

SE = standard error. χ^2 = Pearson χ^2 of the slope. df = degree of freedom for χ^2. pc = critical probability of the slope.

Table 4: Efficacy of the three *Heterorhabditis* symbiotic bacteria *Photorhabdus strains* against neonates of *Helicoverpa armigera* and *Spodoptera litura* after 4 and 7 day of treatment.

	Average body weights (in mg)	% growth inhibition
Control	41.7 ± 72.4a	0
H*ms1*		
10^1	28.6 ± 3[a]	31.41
10^2	23.5 ± 2.1[b]	43.65
10^4	17.9 ± 3.2[c]	57.07
10^6	7.4 ± 1.7[d]	82.25
10^8	0.8 ± 0.3[d]	98.08
H*gj*		
10^1	33.1 ± 3.1[a]	20.6
10^2	23.6 ± 3.9[b]	43.4
10^4	20 ± 4.7[c]	52
10^6	13.3 ± 3.1[cd]	68.1
10^8	6.9 ± 2[d]	83.5
H*ms1* $F_{5,58}$ = 39.14, p<0.0001; H*gj* $F_{5,58}$ = 14.09, p<0.0001.		

Table 5: Growth Inhibition in *H. armigera.*

GJ) strains mixed at various concentrations in semi-synthetic diet. Several studies suggest restrained growth upon administration of EPN toxin in host [44]. Reported 10 strains of entomopathogenic bacteria exhibited over 75% antifeeding activity in 2nd instar larvae of diamondback moth, *Plutella xylostella* by using leaf-disc test**.** However, current study highlights the dose dependent effect of the SG-NGP and SG-G on both lepidopteran insects. This growth inhibition may have resulted from disruption of normal physiology of the insects due to various proteins as well as non-protein toxins for their potential insecticidal or growth inhibitory effects reported from EPN associated symbionts [45]. A 48kDa protein, Txp40, has been reported in 58 strains of *Photorhabditis* and *Xenorhabdus* sps., recombinant form of this protein caused cell growth inhibition *in vitro* cytotoxicity assay of *Aedes aegypti* cells [46]. Another study describes a 63kDa protein from *P. luminescens* having growth inhibition action towards *Manduca sexta* [47]. These studies suggest EPN associated symbionts to be effective feeding deterrents. Strain SG-MG3, however lacks growth inhibition effect in the two host insects. This study provides an important insight on the native EPN strains with possible insecticide potential and may be an addition to the prevalent pest management strategies. Further our studies suggest that not only EPN but also its associated symbiotic bacteria alone can be used for effective pest control.

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