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Biocontrol Potential of *Steinernema thermophilum* and Its Symbiont *Xenorhabdus indica* Against Lepidopteran Pests: Virulence to Egg and Larval Stages

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Abstract: Under laboratory conditions, the biocontrol potential of *Steinernema thermophilum* was tested against eggs and larval stages of two important lepidopteran insect pests, *Helicoverpa armigera* and *Spodoptera litura* (polyphagous pests), as well as *Galleria mellonella* (used as a model host). In terms of host susceptibility of lepidopteran larvae to *S. thermophilum*, based on the LC_{50} 36 hr after treatment, *G. mellonella* (LC_{50} = 16.28 IJ/larva) was found to be more susceptible than *S. litura* (LC_{50} = 85 IJ/larva), whereas neither host was found to be significantly different from *H. armigera* (LC_{50} = 54.68 IJ/larva). In addition to virulence to the larval stages, ovicidal activity up to 84% was observed at 200 IJ/50 and 100 eggs of *H. armigera* and *S. litura*, respectively. To our knowledge this is the first report of entomopathogenic nematode pathogenicity to lepidopteran eggs. Production of infective juvenile (IJ) nematodes/insect larva was also measured and found to be positively correlated with rate of IJ for *H. armigera* (r = 0.990), *S. litura* (r = 0.892), as well as *G. mellonella* (r = 0.834). Both Phase I and Phase II of symbiotic bacteria *Xenorhabdus indica* were tested separately against neonates of *H. armigera* and *S. litura* by feeding assays and found to be virulent to the target pests; phase variation did not affect the level of virulence. Thus *S. thermophilum* as well as the nematode's symbiotic bacteria applied separately have the potential to be developed as biocontrol agents for key lepidopteran pests.

Key words: entomopathogenic nematode, *Helicoverpa armigera*, ovicidal activity, Phase I and Phase II virulence, *Spodoptera litura*.

Safety and environmental issues surrounding the use of chemical insecticides has led to an emphasis on developing alternative control measures such as entomopathogens and their products. Entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae and Heterorhabditidae) occur naturally in soil environments and locate their host in response to chemical and physical cues (Shapiro-Ilan et al., 2012a). Species in these two families have been effectively used as biological insecticides in pest management programs (Grewal et al., 2005) as they are considered nontoxic to humans, relatively specific to their target pest(s) and can be applied with standard pesticide equipment (Shapiro-Ilan et al., 2006). Infective juveniles (IJ) nematodes search for and enter the host, then release symbiotic bacteria into the haemocoel; the bacteria is the primary agent responsible for killing the host (usually within 24 to 48 hr). *Xenorhabdus* spp. are entomopathogenic bacteria symbiotically associated with EPNs belonging to the family Steinernematidae. These bacteria are carried monoxenically within a bi-lobed vesicle in the anterior portion of the intestine of nonfeeding free-living infective stage nematodes (Bird and Akhurst, 1983; Martens et al., 2003). Once inside the host, the bacterial symbionts create a favorable environment for the nematode to propagate by suppressing the immune protein of the insect (Gotz et al., 1981) and providing nutrition for the development and reproduction of nematodes (Poinar, 1983).

EPNs can be used as inundative or inoculative biological control agents or the proteinaceous toxins produced by their symbionts can be transferred to or/and expressed in crop plants or other microorganisms (Shapiro-Ilan et al., 2012b). The IJ of EPNs are compatible with other biological and chemical pesticides, fertilizers, and soil amendments (Krishnayya and Grewal, 2002). The symbiotic association of bacteria with nematode makes it challenging for the insect to develop resistance.

The bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a global polyphagous pest imposing crop damage in India worth $1 billion (USD) annually and it attacks more than 200 crop species belonging to 45 families (Choudhury et al., 2010). In India, this insect occurs as a major pest in many economically important crops including cotton, pigeonpea, chickpea, tomato, okra, and blackgram. The tobacco armyworm *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is also an economically important polyphagous pest in India that causes considerable economic loss to many vegetable and field crops. This pest attacks more than 112 species of cultivated crops and causes severe losses of between 10% and 30% depending on the crop (Ferry et al., 2004; Baskar et al., 2011). The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), a major pest of bee honeycombs widely used as a model organism in laboratories studying EPNs being highly susceptible.

In developing biocontrol programs using EPNs, one mechanism to increase the chance of success is to screen novel nematode species or strains for potential efficacy against particular target pests (Shapiro-Ilan et al., 2012b). Nematodes that are native to the region

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associated with the target pests may be more suitable for biocontrol purposes because they will likely to be adapted to the climate and local environmental conditions and because regulatory issues are minimized when using endemic strains or species. *Steinernema thermophilum* is indigenous to India (Ganguly and Singh, 2000) and is thus adapted to local agro-climatic conditions, e.g., the nematode is capable of infecting insects at a wide range of temperatures (20°C to 35°C) including higher temperatures that are encountered in the region of the target pests (*H. armigera* and *S. litura*) (Ganguly and Singh, 2000). In this study, we investigated the biocontrol potential of *S. thermophilum*, against two major pests of India, i.e., *H. armigera* and *S. litura*, and one model insect *G. mellonella*. Specifically, virulence of the nematode and associated bacterial symbiont (tested separately) was determined in insect eggs and larval stages.

Materials and Methods

Test Insects: Larvae of *H. armigera* and *S. litura* were collected from the field and cultured on a chickpea-based semi-synthetic diet in the laboratory as described by Nagarkatti and Prakash (1974), and modified by Kalia et al. (2001) at 27 ± 2°C and 60 ± 5% relative humidity (RH). The adult moths were offered 10% honey solution fortified with multivitamins throughout their egg-laying period. In the present study, lab-reared eggs, neonates, and last instars were used.

G. mellonella was cultured in controlled laboratory conditions on semi-synthetic diet ingredients (wheat [*Triticum aestivum*] flour, 64 g; wheat bran, 64 g; maize [*Zea mays*] powder, 47.5 g; milk powder, 64 g; dried yeast powder, 47.5 g; wax powder, 12 g; honey, 75 ml; and glycerine, 75 ml) at 27 ± 2°C and 60 ± 5% RH. Adult moths were provided with 10% honey solution fortified with multivitamins in mating jars. In the mating jars, butter paper folded in a fan shape was provided for egg laying.

Nematodes: S. thermophilum Ganguly & Singh, 2000 was obtained from the Division of Nematology, IARI, New Delhi, and maintained at the Insect Physiology laboratory, Division of Entomology, IARI, New Delhi, in double-distilled water (ddw) at 10°C to 15°C. It was described based on morphological characters of its five life stages (Ganguly and Singh, 2000) as well as molecular parameters (Ganguly and Pandey, 2006). Nematodes were propagated by passage through last instar larvae *H. armigera* and *S. litura*. The IJ were harvested using White traps (White, 1927).

Bioassays with Steinernema thermophilum: Last instar *H. armigera*, *S. litura*, and *G. mellonella* were exposed individually in 12 well sterile plates (2.5-cm-diam. × 2-cm-depth, each well was padded with filter paper, Whatman No.1) to IJ suspended in 100 µl of sterile water applied at different rates i.e., 0, 5, 10, 20, 50, 100, and 200 IJ of *S. thermophilum*. Each test insect had 10 replicates per concentration and each treatment was repeated thrice, so 210 larvae per test insect were used. Incubation was performed at a constant temperature of 27 ± 2°C, and 60 ± 5% RH. Mortality was recorded at every 12 hr till 48 hr and afterward every 24 hr until pupation or 100% mortality was attained in most of the treatments, whatever was earlier. Mortality data at 36 hr as well as 48 hr was analyzed to calculate median lethal concentrations (LC_{50}). Median lethal time (LT_{50}) was calculated for the different concentrations.

Ovicidal activity assays: Freshly laid eggs from mating jars of *H. armigera* and *S. litura* were used to test for ovicidal activity in *S. thermophilum*. Fifty eggs of *H. armigera* and 100 eggs of *S. litura* per replicate were kept in each plastic container (5-cm-diam. × 2-cm depth), which had a filter paper lining. These eggs were overlaid with 100 µl of sterile water containing 0, 20, 50, 100, or 200 IJ. Each treatment was replicated thrice thus 750 to 1,500 eggs were used per test insect. The treated as well as control eggs were kept at a constant temperature of 27 ± 2°C and 60 ± 5% RH. Hatching was recorded every 24 hr until 96 hr. The number of unhatched and hatched eggs in each replicate was counted and LC_{50s} were calculated after 96 hr of treatment.

Table 1. Toxicity of *Steinernema thermophilum* against last instar *Helicoverpa armigera, Spodoptera litura*, and *Galleria mellonella*.

Sl. No.	Test insect	LC_{50} IJs/larva	95% Fiducial limit		Slope ± SE	χ^2	df	p_c
			Lower	Upper				
			At 36 hr					
1	*H. armigera*	54.68	27.82	128.36	1.44 ± 0.44	7.74	3	0.052
2	*S. litura*	85.08	58.66	129.54	3.36 ± 0.92	7.78	3	0.051
3	*G. mellonella*	16.28	1.54	33.44	1.23 ± 0.45	3.21	3	0.361
			At 48 hr					
1	*H. armigera*	17.89	8.13	28.51	2.20 ± 0.62	2.536	3	0.468
2	*S. litura*	21.91	5.25	42.11	1.36 ± 0.45	3.74	3	0.291
3	*G. mellonella*	100% mortality even at the lowest conc. of 10 IJs/larva						

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 2. LT_{50} values calculated from dosage response assays conducted with *Steinernema thermophilum* against last instar *Helicoverpa armigera*, *Spodoptera litura*, and *Galleria mellonella*.

Sl. No.	Insect	No. of IJs	LT_{50} (hr)	95% Fiducial limit		Slope ± SE	χ^2	df	p_c
				Lower	Upper				
1	*H. armigera*	10	65.20	45.50	110.66	2.10 ± 0.57	1.399	5	0.924
		20	39.87	29.05	52.30	3.02 ± 0.64	3.247	5	0.661
		50	34.97	25.46	45.85	3.32 ± 0.76	2.493	4	0.645
		100	31.92	25.32	39.12	6.76 ± 2.10	5.372	2	0.068
		200	18.54	13.18	24.06	5.32 ± 1.57	2.142	1	0.144
		r = -0.825							
2	*S. litura*	10	Maximum 40% mortality was obtained till 192 hr						
		20	99.64	81.46	120.28	4.07 ± 0.77	2.231	8	0.973
		50	52.03	35.91	70.24	2.26 ± 0.47	6.685	7	0.462
		100	50.32	40.88	62.94	5.38 ± 1.32	2.282	3	0.516
		200	41.40	30.88	55.94	5.91 ± 1.77	0.456	1	0.498
		r = -0.740							
3	*G. mellonella*	10	33.66	28.59	39.06	10.50 ± 2.99	1.897	2	0.387
		20	30.82	24.31	37.20	7.10 ± 2.17	3.633	2	0.163
		50	27.09	20.33	33.06	6.09 ± 1.75	3.826	2	0.147
		100	21.78	15.80	27.38	5.12 ± 1.31	1.178	2	0.554
		200	17.59	12.60	22.57	5.70 ± 1.66	1.168	2	0.434
		r = -0.957							

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.

Production of Steinernema thermophilum IJ: In the above experiment (Bioassays with *S. thermophilum*), ten nematode infected larvae were randomly selected and removed from plates, rinsed in ddw and transferred individually on to White traps. The IJ were harvested every 3 d up to a period of 30 d. Three replicates from pooled harvest/concentration were used to calculate the total number of IJ produced per ten larvae. The average yield/larva was then determined accordingly.

Isolation of symbiotic bacteria: Last instar larvae of *H. armigera* were exposed individually in sterile plates (5-cm-diam. × 2-cm-depth) lined with Whatman No.1 filter paper to 20 IJs of *S. thermophilum* suspended in 100 µl ddw. After 24 hr of exposure, fresh haemolymph was collected by making a lesion in a proleg, in precooled centrifuge tubes. Then 10 µl of this haemolymph was streaked on NBTA plates (Nutrient agar 7, Bromothymol blue 0.025; triphenyl-2,3,4-tetrazolium chloride 0.04 gl^{-1}). Plates were incubated at 28°C for 24 hr and primary form (Phase I) was differentiated from secondary form (Phase II) on the basis of standard characteristics (e.g., blue colony for Phase I and red colony for Phase II) (Akhurst, 1980). Phase I and II colonies were purified by subculturing 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 hr. 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 2×10^{10} cell ml^{-1} was prepared and appropriate serial dilutions were made as required by diluting with ddw for bioassays.

Bioassay with Phase I and Phase II of symbiotic bacteria Xenorhabdus indica: Both Phase I and II of symbiotic bacteria *X. indica* were tested separately against neonates of *H. armigera* and *S. litura* by feeding assays using diet incorporation methodin plastic

TABLE 3. Percentage mortality of last instar *Helicoverpa armigera*, *Spodoptera litura*, and *G. mellonella* exposed to different concentrations of the infective juveniles of the *Steinernema thermophilum*.

Time (in hr)	No. of IJs/larva				
	10	20	50	100	200
	Percent mortality of *H. armigera*				
12	10	10	10	0	20
24	20	30	30	30	60
36	20	30	40	40	100
48	30	60	70	100	
72	60	70	80		
96	70	90	100		
120	70	100			
	Percent mortality of *S. litura*				
12	0	0	0	0	0
24	0	0	30	0	10
36	0	0	40	30	10
48	10	10	60	50	30
72	30	30	60	70	60
96	30	60	60	70	100
120	30	60	70	100	
144	40	80	80		
168	40	80	100		
192	40	80			
	Percent mortality of *G. mellonella*				
12	0	0	0	10	20
24	10	30	50	60	70
36	50	50	60	80	100
48	100	100	100	100	

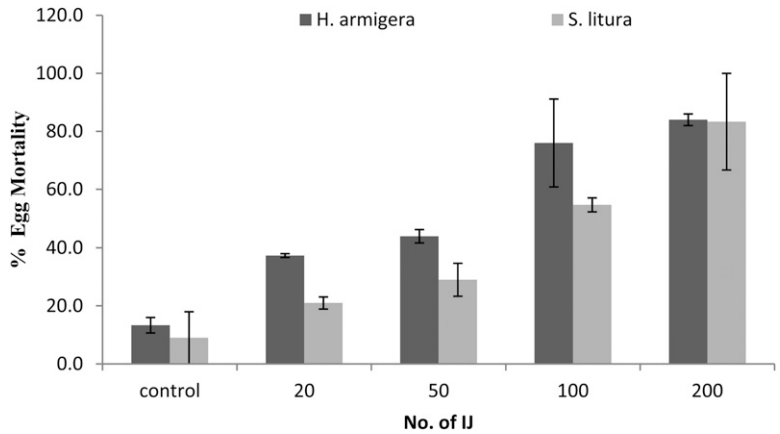

FIG. 1. Mean percentage mortality (± SE) of eggs of *Helicoverpa armigera* and *Spodoptera litura* by different infective juvenile (IJ) concentrations of *Steinernema thermophilum*.

containers (5-cm-diam. × 2-cm-depth) with 0.1 ml of solution incorporated at rates of 10, 1×10^2, 1×10^4, 1×10^6, or 1×10^8 bacterial cells/gm of diet. Each treatment was replicated thrice. In the control, the diet was incorporated with 0.1 ml of ddw alone. Ten neonates were released into each container, i.e., per replicate. A minimum of 180 neonates were used for each bioassay. Mortality was recorded every 24 hr till 96 hr, and LC_{50} was calculated after 96 hr of treatment.

Statistical analysis: The mortality data were analyzed using maximum likelihood program for probit analysis (Ross, 1977). The LC_{50s} in terms of IJs/larva or bacterial cells/gm diet or IJ/50 or 100 eggs, and LT_{50s} in terms of hr for different bioassays were considered significantly different if their 95% fiducial limits (FL) did not overlap. The percentage mortalities of test insects were taken up to 8 d at different intervals, i.e., at 12, 24, 36, 48, 72, 96, 120, 144, 168, and 192 hr. Data were corrected for control mortality (Abbott, 1925) and arc-sine transformed when required to meet assumptions of normality and homogeneity of variances. Treatment effects for IJ production and ovicidal data were determined by one way analysis of variance (ANOVA) (SAS 9.2, SAS Institute Inc., Cary, NC). Probability less than or equal to 5% (p value < 0.05) was accepted as statistically significant. Correlation between the parameters viz., LT_{50} vs. concentration, production vs. concentration and yield of IJ vs. inoculums was determined by regression analysis.

RESULTS

Bioassays with Steinernema thermophilum: In terms of host susceptibility of lepidopteran larvae to *S. thermophilum*, based on the LC_{50} 36 hr after treatment, *G. mellonella* (LC_{50} = 16.28 IJ/larva) was found to be more susceptible than *S. litura* (LC_{50} = 85 IJ/larva), whereas neither host was found to be significantly different from *H. armigera* (LC_{50} = 54.68 IJ/larva). Similarly at 48 hr, LC_{50} values vary from 17.89 IJ/larva (*H. armigera*) to 21.91 IJ/larva (*S. litura*) but not found to be significantly different (Table 1).

LT_{50} values for all the test insects were found to be rate dependent (Table 2). In *H. armigera* rates of 20, 50, and 100 IJ/larva were found to be at par with each other, whereas rate of 200IJ/larva was found to be significantly different from all other rates within this species. The lowest rate of 10 IJ/larva in *S. litura* was unable to attain 50% mortality, whereas 20IJ/larva was found to be significantly different from 50, 100, and 200 IJ/larva. In *G. mellonella* LT_{50} was found to be at par for all the treatment ranges from 10 IJ/larva to

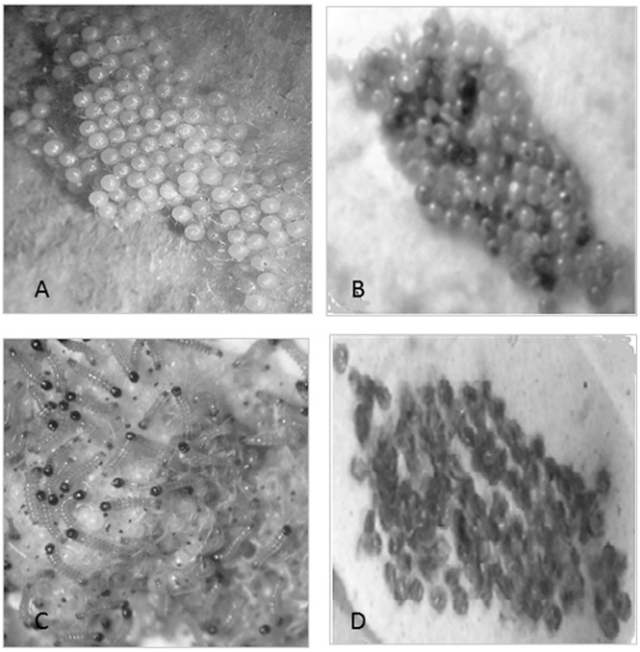

FIG. 2. Ovicidal activity of *Steinernema thermophilum* against eggs of *Spodoptera litura* A. Control after 24 h. B. Treatment after 24 h. C. Control after 96 h. D. Treatment after 96 h.

TABLE 4. Ovicidal activity of *Steinernema thermophilum* in *Helicoverpa armigera* and *Spodoptera litura*.

Sl. No.	Test insect	LC_{50} (No. of IJs/50 or 100 eggs, respectively)	95% Fiducial limit		Slope ± SE	χ^2	df	p_c
			Lower	Upper				
1	*H. armigera*	120	107	155	3.23 ± 0.54	12.16	2	0.002
2	*S. litura*	130	119	140	4.07 ± 0.40	6.21	2	0.045

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.

200 IJ/larva. When comparing virulence among the host insects, the LT_{50} of *G. mellonella* was found to be at par with *H. armigera* at the highest rate of 200IJ/larva and at the 100 IJ rate yet both insects differed significantly with *S. litura* at these rates (41.40 hr). At the lowest rate of 10 IJ/larva, the LT_{50} of *G. mellonella* (33.66 hr) was found to be significantly different from *H. armigera* (65.20 hr). At 20 IJs, there were no differences in LT_{50} among the hosts, whereas at the 50 IJ rate *G. mellonella* was more susceptible than *S. litura* but at par with *H. armigera*. *G. melonella* was found to be more susceptible than *H. armigera* at the 10 IJ rate. In all the test insects LT_{50} was found to be negatively correlated with the rate of application (Table 2).

Last instar of *H. armigera* and *G. mellonella* attained 100% mortality at 200 IJ/larva as early as 36 hr. However in *G. mellonella* 100% mortality was observed even at the lowest concentration of 10 IJ/larva within 48 hr (Table 3). On other hand in case of *S. litura*, even higher concentration viz., 200 IJ/larva and 100 IJ/larva had taken longer time, i.e., 96 hr and 120 hr, respectively, to kill 100% of the larvae. At the lowest concentration, 10 IJ/larva, only 70% and 40% mortality was attained even after 120 and 192 hr exposure in *H. armigera* and *S. litura*, respectively. Last instar *H. armigera* is nearly as susceptible as the model host *G. mellonella*.

Ovicidal activity assays: The highest egg mortality (84%) was observed at 200 IJ/50 eggs of *H. armigera* and 200IJ/100 eggs of *S. litura*, respectively (Fig. 1). Low mortality was observed in the control, i.e., 9% and 14% was observed in *H. armigera* and *S. litura*, respectively (Fig. 2). There is a significant effect of IJ concentration on egg infection in both *H. armigera* ($F_{4,14} = 10.11$, $p < 0.0015$) and *S. litura* ($F_{4,14} = 10.57$, $p < 0.0013$) with a positive correlation of 0.97 and 0.91, respectively. Perusal of data in Table 4 shows that LC_{50} for *H. armigera* (120 IJ/150 eggs, $p_c = 0.002$) and *S. litura* (130 IJ/300 eggs, $p_c = 0.045$) is at par (Table 5). Specifically, LT_{50} was found to be negatively correlated with concentration of IJ/larva in *H. armigera* (r = -0.902) as well as *S. litura* (r = -0.890).

Production of IJ of Steinernema thermophilum: Following host mortality, the emerging IJ from host cadavers were collected and counted during a 30-d period. Data in Table 6 show a significant effect of concentration upon average yield of IJ/larva, although *H. armigera* average yield did not differ at 10, 20, and 50 IJ/larva. However, a significant difference between 100 IJ/ larva (25.8×10^3IJ/larva) and 200 IJ/larva (47.6×10^3 IJ/larva) was observed ($F_{4,14} = 161.39$, $p < 0.0001$). For S. *litura* ($F_{4,14} = 113.72$, $p < 0.0001$), IJ yield (39.2×10^3 IJ/larva) was found to be highest at 200 IJ/larva and found to be significantly different from other concentrations. Conversely yield at 10 and 20 IJ/larva (0.5×10^3 and 11.14×10^3, respectively) as well as 50 and 100 IJ/larva was found to be at par. In case of *G. mellonella,* yield was found to be at par at 50, 100, and 200 IJ/larva, significantly different at 10 and 20 IJ/larva ($F_{4,14} = 85.67$, $p < 0.0001$). Production of IJ was

TABLE 5. LT_{50} values calculated from dosage response assays conducted with *Steinernema thermophilum* against eggs of *Helicoverpa armigera* and *Spodoptera litura*.

Insect	No. of IJs	LT_{50} (hr)	95% Fiducial limit		Slope ± SE	χ^2	df	p_c
			Lower	Upper				
H. armigera	20	135.35	112.45	182.88	2.51 ± 0.347	0.801	2	0.669
	50	110.58	94.49	140.18	2.29 ± 0.303	0.211	2	0.899
	100	68.44	63.67	74.04	3.64 ± 0.306	13.28	2	0.001
	200	58.85	54.89	63.13	3.78 ± 0.300	7.410	2	0.024
r = -0.902								
S. litura	20	243.50	178.69	412.77	1.73 ± 0.240	5.191	2	0.074
	50	159.34	132.96	208.52	2.19 ± 0.240	3.107	2	0.211
	100	84.76	79.20	91.82	3.08 ± 0.225	9.422	2	0.008
	200	58.19	55.38	61.16	3.73 ± 0.207	9.878	2	0.007
r = -0.890								

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 6. Production of *Steinernema thermophilum* IJs in last instar *Helicoverpa armigera, Spodoptera litura*, and *Galleria mellonella*.

Sl. No.	No. of IJS/larva	Average yield/larva ($\times 10^3$) ± SE	Ratio of yield (10^3)/ inoculated dose
H. armigera			
1	10	3.70^{c} ± 0.24	0.37
2	20	9.29^{c} ± 1.10	0.47
3	50	9.46^{c} ± 0.65	0.19
4	100	25.82^{b} ± 2.87	0.26
5	200	47.61^{a} ± 0.22	0.24
S. litura			
1	10	0.5^{d} ± 0.20	0.05
2	20	11.14^{c} ± 1.08	0.55
3	50	27.04^{b} ± 0.54	0.54
4	100	31.25^{b} ± 1.78	0.31
5	200	39.21^{a} ± 0.54	0.19
G. mellonella			
1	10	21.11^{d} ± 0.91	2.11
2	20	24.41^{c} ± 0.83	1.22
3	50	31.88^{b} ± 0.27	0.63
4	100	34.23^{ab} ± 0.61	0.34
5	200	35.62^{b} ± 0.63	0.17

Different letters for mean percent mortality show significant differences after ANOVA and Tukey's Studentized Range (HSD) test ($P < 0.05$). SE = standard error.

positively correlated with the IJ concentrations for *H. armigera* (r = 0.990), *S. litura* (r = 0.871) as well as *G. mellonella* (r = 0.834). Though, yield/inoculum ratio was negatively correlated with the concentrations for *H. armigera* (r = -0.561), *S. litura* (r = -0.955) as well as *G. mellonella* (r = -0.795).

Bioassay with Phase I and Phase II of symbiotic bacteria Xenorhabdus indica: X. indica was pathogenic to *H. armigera* and *S. litura*. In comparing the susceptibility between hosts, *H. armigera* was found to be more susceptible to *X. indica* (both phases) than *S. litura* on the 4th and 7th day assessments (Table 7). The virulence of Phase I bacteria compared with Phase II bacteria did not differ in each of the host except in *S. litura*, for which 50% mortality could not be obtained by in the Phase II treatment by the 4th day even at the highest concentration. However, Phase II of symbiotic bacteria *X. indica* was found to be more virulent than Phase I against neonates of *H. armigera* ($F_{9,29} = 7.24$, $p < 0.0001$) and *vice versa* for *S. litura* ($F_{9,29} = 5.70$, $p < 0.0006$) (Fig. 3).

DISCUSSION

Virulence of *S. thermophillum* to two major polyphagous pests was determined and results indicate that susceptibility of *H. armigera* is similar to *G. mellonella*. In contrast *S. litura* was found to be less susceptible than *G. mellonella*. Numerous studies have made use of these assays to evaluate the efficacy of EPNs against various insect pests and also found a positive relationship between the dose of IJ and host mortality as found in the present study (Forschler and Nordin, 1988; Glazer and Navon, 1990; Sims et al., 1992; Peters and Ehlers, 1994; Bhatnagar et al., 2004; Phan et al., 2005; Ali et al., 2008; Adiroubane et al., 2010; Yadav and Lalramliana, 2012). *S. thermophilum* caused 100% mortality in *H. armigera* and *G. mellonella* within 36 hr through direct exposure of 200 IJ, which agrees with the studies of Ali et al., 2008. In contrast, in *S. litura* 100% mortality was observed later compared with the other two hosts tested. The reason for delayed mortality may be based on an inability of the IJs to penetrate the host or perhaps enzymes or toxins related to the symbiotic bacterium (*X. indica*) are less potent in *S. litura* relative to the other hosts. Thus, similar to numerous other studies, we observed that the efficacy of the EPNs against insect hosts varies with insect and nematode species (Shapiro-Ilan et al., 2002; Ansari et al., 2006).

Although it is not surprising that larvae were susceptible to *S. thermophilum*, a novel finding was that the EPN has the ovicidal activity as it was observed that IJ kills the eggs of *H. armigera* and *S. litura* by penetrating them. In *S. litura* eggs were laid in groups so single IJ was capable of penetrating and killing more eggs (2.31

TABLE 7. Efficacy of Phase I and Phase II of symbiotic bacteria *Xenorhabdus indica* against neonates of *Helicoverpa armigera* and *Spodoptera litura* after 4 and 7 d of treatment.

Test insect	Phase	LC_{50} (bacterial cells/gm of diet)	95% fiducial limits (lower - upper)	Slope ± SE[a]	χ^{2b}	df[c]	p_c[d]
			After 4 d				
H. armigera	Phase I	1.6×10^4	$0.65 \times 10^2 - 3.8 \times 10^6$	0.12 ± 0.04	0.313	3	0.958
	Phase II	2.0×10^2	$3.83\text{-}4.65 \times 10^3$	0.16 ± 0.04	0.648	3	0.885
S. litura	Phase I	9.9×10^{10}	$1.9 \times 10^8\text{-}1.9 \times 10^{26}$	0.12 ± 0.05	1.527	3	0.676
	Phase II	40% mortality at highest conc. tested ($1x10^8$)					
			After 7 d				
H. armigera	Phase I	5.3×10^3	$2.98\text{-}9.8 \times 10^5$	0.11 ± 0.04	0.605	3	0.895
	Phase II	2.0×10^2	$3.83\text{-}4.65 \times 10^3$	0.16 ± 0.04	0.648	3	0.885
S. litura	Phase I	1.7×10^8	$1.58 \times 10^6\text{-}1.15 \times 10^{16}$	0.13 ± 0.04	1.111	3	0.774
	Phase II	5.5×10^{10}	$7.2 \times 10^7\text{-}1.1 \times 10^{26}$	0.13 ± 0.05	1.110	3	0.775

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.

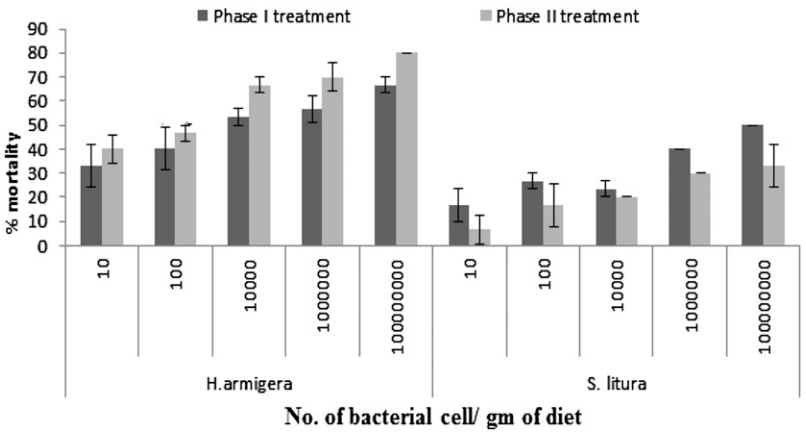

FIG. 3. Mean percentage mortality (± SE) of neonates of *Helicoverpa armigera* and *Spodoptera litura* by Phase I and Phase II of symbiotic bacteria *Xenorhabdus indica* on 7 d after treatment.

eggs) as compared with *H. armigera* (1.29 eggs), where eggs were laid sparsely. LT_{50} values for both the test insects were found to be rate dependent. When comparing the two hosts, LT_{50s} were similar at all rates except at the 100 IJ treatment in which *H. armigera* was virulent than *S. litura*. Other studies showed a lack of impact of EPN to insect eggs (De Doucet et al., 1998; Kim et al., 2004). However, Shahina et al. (2009) reported ovicidal activity of *Heterorhabditis bacteriophora* and *S. siamkayai* against *Rhynchophorus ferrugineus* (Coleoptera: Rhynchophoridae). Our finding of ovicidal activity by EPN intensifies their potential as biological control agents against lepidopteran pests.

In addition to a positive correlation between mortality and rate of the IJ applied, the present study also showed a positive correlation between the reproductive yield and the concentration of IJ used against *H. armigera*, *S. litura*, and *G. mellonella*. These results concur with Poinar and Thomas (1967), Reardon et al. (1986), and Abdel-Razek (2006). In contrast, Shapiro-Ilan et al. (2002) did not detect an impact of IJ inoculation rate on yield of progeny. Production of EPNs in the host can play a crucial role in their persistence in the environment because of recycling and thus affect the effectiveness of pest control applications (Harlan et al., 1971; Georgis and Hague, 1981). Furthermore, reproductive capacity is a critical component for optimum efficiency in commercial in vivo production ventures.

The basis of pathogenesis in EPNs is largely because of their symbiotic bacteria, which are carried in the alimentary tract of the IJ (Thomas and Poinar, 1979). According to Akhurst and Dunphy (1993) most species of *Xenorhabdus* are highly pathogenic when injected, e.g., with an LD_{50} of less than 20 cells. However, data on oral infection is lacking and hence this study was undertaken to determine toxicity of *X. indica* per *os*. We observed pathogenicity stemming from per os infection, but the level of virulence was affected by phase variation. Phase II bacteria of *X. indica* were found to be less effective than Phase I against neonates of *H. armigera,* which concurs with previous studies on *X. nematophila* (Volgyi et al., 1998), whereas an opposite trend in virulence was observed in our study for *S. litura*. Thus, efficacy of the two bacterial phases apparently varies among different insect hosts. Kumar et al. (2013) reported efficacy of a purified protease from *X. indica* in suppressing neonates of *H. armigera*. Similarly, Mahar et al. (2004) demonstrated the possibility of applying either bacterial suspensions containing cells of *X. nematophila* or cell-free solutions containing bacterial metabolites to control larvae of *P. xylostella*. This study extends previous finding by demonstrating that the EPN complex and symbiotic bacteria alone have the potential to be developed as biocontrol agents against key lepidopteran pests such as *H. armigera* and *S. litura*.

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