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IMPACT OF SOIL DWELLING ENTOMOPATHOGENIC NEMATODES, RECOVERED FROM THE SOIL OF UTTAR PRADESH (INDIA), ON COTTON BOLLWORM *Helicoverpa armigera* (Hübner) (LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT

High mobility, facultative diapause as pupae, rapid generation turnover, fecundity and ability to develop resistance against insecticides, make the management of *Helicoverpa armigera* very difficult. Entomopathogenic nematodes (EPNs) have wide host range and possess several positive attributes which make them one of the promising biocontrol agent. To establish their possible role in insect pest management, 12 EPN isolates, recovered from five districts of Uttar Pradesh (India), were used to test their virulence and biotic potential against the larvae of *H. armigera*. Based on morpho-molecular study they were identified as isolates of *Steinernema abbasi, S. siamkayai* and *Heterorhabditis indica.* All the tested isolates were virulent to kill *H. armigera* larvae within 24 to 60 h even at lowest dose (25 IJs larva^{-1}) and produced quantifiable infective juveniles. *H. indica* isolate CH_3 produced highest number of IJs (115.7±5 × 10^3 IJs larva^{-1}), followed by *S. abbasi* isolate CS_5 (100±8.5 × 10^3 IJs larva^{-1}); whereas lowest was recorded from *S. abbasi* isolate CS_3 (26.9±3×10^3 IJs larva^{-1}). Cent percent mortality with short LT_{50} values ranging from 17.02 to 44.77 h at all concentrations proved the pathogenicity of allied steinernematids and heterorhabditids against *H. armigera* under laboratory conditions.

Key words: Entomopathogenic nematodes, *Helicoverpa armigera Steinernema abbasi, S. siamkayai*, *Heterorhabditis indica*, virulence, progeny production

INTRODUCTION

Worldwide, complex of *Helicoverpa* species (Lepidoptera: Noctuidae) is listed as a devastating insect pest and caused a crop loss of 5-10% in temperate and 50-100% in tropical regions (Hussain and Ahmad, 2015). *Helicoverpa armigera* is a most serious polyphagous pest infesting over 181 species belonging to 45 plant families (Singh, 2005). Because of its high mobility, facultative diapause as pupae, rapid generation turnover, fecundity and ability to develop resistance against insecticides, its management is very difficult and life-history layouts make this insect one of the world's worst pest (Hussain and Ahmad, 2015). Serious yield losses have been reported in legumes due to *H. armigera* attack which range from 20 to 30% and sometime rise to 75% in chickpea alone with an estimate of US $ 927 million in chickpea and pigeon pea, and possibly $ 5 billion on different crops worldwide (Srivastava *et al.*, 2005; Wubneh, 2016).

Insecticides such as viruses (nuclear polyhedrosis virus), bacteria (*Bacillus thuringiensis*), fungi (*Trichoderma* spp.), protozoa (*Nosema locustae*) and entomopathogens nematodes may serve as an effective alternative to chemicals under IPM programme. The prospects of using microbial bio-insecticide entomopathogenic nematodes (EPNs) of families Steinernematidae and Heterorhabditidae against insect-pests in soil and hidden habitats against agricultural pest are better (Gaugler, 2002). Bedding *et al.* (1983) observed that the degree of infectivity of nematode species/ strains varied considerably in different hosts, and none of the nematodal species/strains infected all the insect species tested. This revealed the importance of testing a number of nematode species/ isolates against target insect under laboratory conditions prior to field evaluation. Our focus in this paper was on the manifestation of steinernematid and heterorhabditid nematodes in five districts of Western Uttar Pradesh, their virulence against larval stage of *H. armigera* and to assess their reproductive potential so that they may be utilized in *H. armigera* management commercially.

MATERIALS AND METHODS

Insect culture

The experiments were performed in the year 2015 and 2016. The larvae of *Galleria mellonella* were used for the isolation and maintenance of EPNs in laboratory as per David and Kurup (1988) with slightly modification that the amount of glycerol and honey in maize-based diet was reduced to 110 mL instead of 125 mL (just to reduce the moisture in diet). The paired adults were put in plastic jar covered with muslin cloth along with tissue paper and 10% honey solution fortified with multi-vitamin for feeding and better egg laying under laboratory conditions (27±1°C, 65±5% RH and 14 L:10D photoperiod). The 3rd and 4th stage larvae of same size were used in bioassays experiments. The eggs of *H. armigera* were procured from National Bureau of Agriculturally Important Insects (NBAII), Bangalore (National Accession No. NBAII-MP-NOC-01) and reared on chickpea-based diet (Nagarkatti and Prakash, 1974), modified by Kalia *et al*. (2001). The 4th stage larvae of *H. armigera* were used for further experimentations. The laboratory conditions were maintained as of *G. mellonella*.

Soil sampling and nematode culture

Random soil samples (250) were collected from various agricultural fields in different localities of Saharanpur, Muzaffarnagar, Meerut, Baghpat and Ghaziabad districts in Uttar Pradesh (India) for nematode isolation. Nematodes were isolated by soil baiting method (Bedding and Akhurst, 1975). Ten larvae of *G. mellonella* were placed in 250 mL polystyrene jars containing soil and kept in a BOD incubator at 27±1°C. Jars were observed daily for 7 days and dead larvae washed with double distilled water (DDW), disinfected with 0.1% sodium hypochlorite and transferred on modified white trap (Stock and Goodrich-Blair, 2012). Emerged 3rd stage juveniles (infective juveniles or IJs) were multiplied further to harvest new generations of nematodes on fully grown larvae of *G. mellonella* as per Stock and Goodrich-Blair (2012). Freshly emerged IJs were washed properly with DDW and disinfected with 0.1% sodium hypochlorite and stored in BOD at 15°C for further studies. However, freshly emerged (0- 6 days old) IJs were used in each experiment.

Larval mortality bioassays

Larval mortality bioassays were performed as previously described (Chaubey *et al.*, 2014; Istkhar *et al*., 2016). Four different concentrations of each isolate *viz*., 25, 50, 100 and 200 IJs were prepared in 400 µL DDW and released in wells of 6-well plates lined by double layer of Whatman Filter paper No. 1. Ten larvae of *G. mellonella* for each experiment along with control (only DDW) were placed in above prepared well plates. Observations were taken at every 12 h post-infection period

up to 72 h. Dead larvae of *G. mellonella* were placed on modified white trap (Stock and Goodrich-Blair, 2012) singly to observe the persistence of infection and emergence of IJs.

Lethal concentration and lethal time

To assess the concentrations needed for EPNs to kill 50 and 90% of *H. armigera* larvae, lethal concentrations (LC_{50} and LC_{90}) and lethal time (LT_{50} and LT_{90}) were calculated. The data obtained from larval mortality bioassays was utilized and presented in the form of IJs $larva^{-1}$ and hours for LC and LT values, respectively. For LC values, the mortality data was analyzed by retaining mortality data at 25, 50, 100 and 200 IJs $larva^{-1}$ doses through probit analysis inbuilt in SPSS (version 16.0). Values of LT were calculated at all the test concentrations individually as well as by combining the mortality data at all the concentrations applied through same software.

Progeny production

For the evaluation of IJs production for last instar larvae of same size and weight were exposed at a constant 100 IJs $larva^{-1}$ concentration prepared in DDW in 6-well plates along with control. For each isolate, 10 replicates were placed. Each cadaver was washed with DDW and disinfected with 0.1% sodium hypochlorite, placed on modified white trap and kept in BOD at 27 ±1°C. Emerged IJs were collected in tissue culture flask up to 15 days or till the emergence stopped and then number of IJs produced was calculated in counting dish in a Petri-dish. Data for progeny production was calculated by descriptive statistics and presented in number of IJs ± SE $larva^{-1}$.

RESULTS AND DISCUSSION

In present study, no EPN was isolated directly from insect body but still the results showed beneficiary impact of soil dwelling nematodes on management of *H. armigera* by producing strong virulence against their larval stage. Only 21 soil samples yielded EPNs and their pathogenicity was confirmed by Koch's postulate using *Gallaria mellonella* larvae. Based on morpho-molecular analyses (unpublished data), these isolates were identified as *Steinernema abbasi S. siamkayai* and *Heterorhabditis indica*. Initially, these isolated were tested for their pathogenicity and reproductivity against *G. mellonella* in previous studies (Chaubey *et al*., 2014, Istkhar *et al*., 2016, Istkhar and Chaubey, 2016). Based on bioassays trials on *G. mellonella*, 12 highly pathogenic isolates were selected for pathogenicity and reproductivity trials against *H. armigera* under lab conditions. The entomopathogenic nematodes collected from different localities were as under:

Nematode species	Isolate[a]	Collection site	Field of soil collection[b]	Soil pH
Steinernema abbasi	CS_2	Meerut	Sugarcane	8.7
	CS_3	Saharanpur	Potato	7.6
	CS_4	Ghaziabad	Sugarcane	8.5
	CS_5	Baghpat	Open Field	7.8
	CS_{10}	Baghpat	Open Field	8.6
	CS_{15}	Muzaffarnagar	Barley	8.2
	CS_{21}	Saharanpur	Mango Garden	7.9
Steinernema siamkayai	CS_{16}	Muzaffarnagar	Wheat	7.9
	CS_{23}	Ghaziabad	Sugarcane	8.6
Heterorhabditis indica	CH_2	Muzaffarnagar	Wheat	7.8
	CH_3	Meerut	Wheat	8.5
	CH_6	Saharanpur	Mango	7.9

[a] Isolates names are laboratory tagging

[b] All nematode isolates were collected from soil and not directly from insect body.

Soil is a natural habitat of EPNs. Study showed that species of *Steinernema* were dominant (74.43%), followed by *Heterorhabditis* (28.57%) and was similar to finding of Pal *et al.* (2008). During soil sampling, all the nematodes were recovered from the soil having normal soil pH in the range of 7.6-8.7. The said pH has not been reported harmful to EPNs (Pal *et al.*, 2008). Most of the EPN isolates were recovered from cultivated habitats. Results suggest that there was higher prevalence of EPNs in cultivated habitats but presence of nematodes in non-cultivated habitat indicated that these areas should also be considered for EPN isolation.

Larval mortality bioassays showed that among the isolates of *S. abbasi* and *S. siamkayai* used, the isolate CS_2 killed all the larvae within 24 h of infection, followed by isolate CS_4, CS_5, CS_{10}, CS_{16} and CS_{21} in 36 h, CS_{15} in 48 h and CS_3 and CS_{23} in 60 h in all the respective concentrations. *H. indica* isolates CH_2 and CH_3 took 48 h followed by isolate CH_6 (60 h) (Fig. 1a-d). LC_{50} and LC_{90} values were calculated at three time intervals *viz.*, 24, 36 and 48 h post-treatment period where the lowest LC_{50} at 24 h was calculated in CS_{16} with 14 IJs larva^{-1} and least in CH_3 with 587 IJs larva^{-1}. *Steinernema* sp. isolate CS_2 killed all the larvae while 100% mortality was observed with isolate CS_{21} at this duration (Table 1).

Table 1: LC_{50} and LC_{90} values (No. of infective juveniles larva^{-1}) of isolated *Steinernema* and *Heterorhabditis* spp. at different time intervals

	24 h					36 h					48 h				
Isolate	LC_{50}	LC_{90}	χ^2	df	Sig.	LC_{50}	LC_{90}	χ^2	df	Sig.	LC_{50}	LC_{90}	χ^2	Df	Sig.
CS_2	*a c*	*a c*	*a c*	*a c*	*a c*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*
CS_3	*b*	*b*	*b*	*b*	*b*	212	1765	0.0	2	1	16	227	0.0	2	1
CS_4	91	13520	0.12	2	0.94	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
CS_5	27	118	0.93	2	0.63	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
CS_{10}	22	47	0.10	2	0.95	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
CS_{15}	22	92	0.66	2	0.72	8	24	0.19	2	0.91	*c*	*c*	*c*	*c*	*c*
CS_{16}	14	78	0.48	2	0.79	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
CS_{21}	*a b*	*a b*	*a b*	*a b*	*a b*	*a c*	*a c*	*a c*	*a c*	*a c*	*a*	*a*	*a*	*a*	*a*
CS_{23}	159	843	1.96	2	0.38	33	82	0.77	2	0.68	22	39	0.40	2	0.82
CH_2	20	80	0.33	2	0.85	8	23	0.19	2	0.91	*c*	*c*	*c*	*c*	*c*
CH_3	587	93720	0.10	2	0.95	23	6544	0.09	2	0.96	*c*	*c*	*c*	*c*	*c*
CH_6	26	628	0.01	2	1.00	12	65	0.72	2	0.70	13	32	0.33	2	0.85

[a]No statistics were computed because the slope was zero.; df = degrees of freedom

[b]No mortality was initiated up to this time interval hence, statistics were not computed.

[c]100% Mortality achieved up to this time interval. hence, statistics were not computed.

Due to 100% mortality in *G. mellonella* larvae within 24 h by *S. abbasi* isolates CS_2 and CS_{21}, the LT values were not calculated. *S. siamkayai* isolate CS_{16} provided lowest LT_{50} value (14 IJs larva^{-1}) followed by *H. indica* isolate CH_2 (20 IJs larva^{-1}), *S. abbasi* isolate CS_{10} (22 IJs larva^{-1}), *S. abbasi* isolate CS_{15} (22 IJs larva^{-1}), *H. indica* isolate CH_6 (26 IJs larva^{-1}), *S. abbasi* isolate CS_5 (27 IJs larva^{-1}), *S. abbasi* isolate CS_4 (91 IJs larva^{-1}), *S. siamkayai* isolate CS_{23} (159 IJs larva^{-1}) and *H. indica* isolate CH_3 (587 IJs larva^{-1}) at 24 h (Table 2). In case of isolate *S. abbasi* isolate CS_3, no mortality was recorded at 24 h post-infection period, hence the LT values were unpredictable. The LT_{50} values ranged from 17.35 to 44.77 h in 25 IJs larva^{-1}, 17.35 to 42.43 h in 50 IJs larva^{-1}, 17.35 to 40.13 h in 100 IJs larva^{-1} and 17.35 to 36.76 h in 200 IJs larva while in combine LT_{50} the range was from 17.02 to 40.95 h in all the isolates tested. At all the applied doses, the LT_{50} was lowest with the isolate *S. abbasi* CS_2 (17. 35 h) and highest with *S. abbasi* isolate CS_3 (40.95 h) [Table 2].

The virulence of some EPN species has been documented against *H. armigera* and larvae of *H. armigera* were found susceptible to both the species of *Heterorhabditis* and *Steinernema* (Glazer and Navon 1989; Karunakar *et al.*, 1999; Navon *et al.* 2002; Hussain *et al.*, 2014). In response to different concentrations of infective juveniles *H. armigera* was found highly susceptible. In present study, all the isolates produced 100% mortality in 4th instar *H. armigera* larvae at all the respective

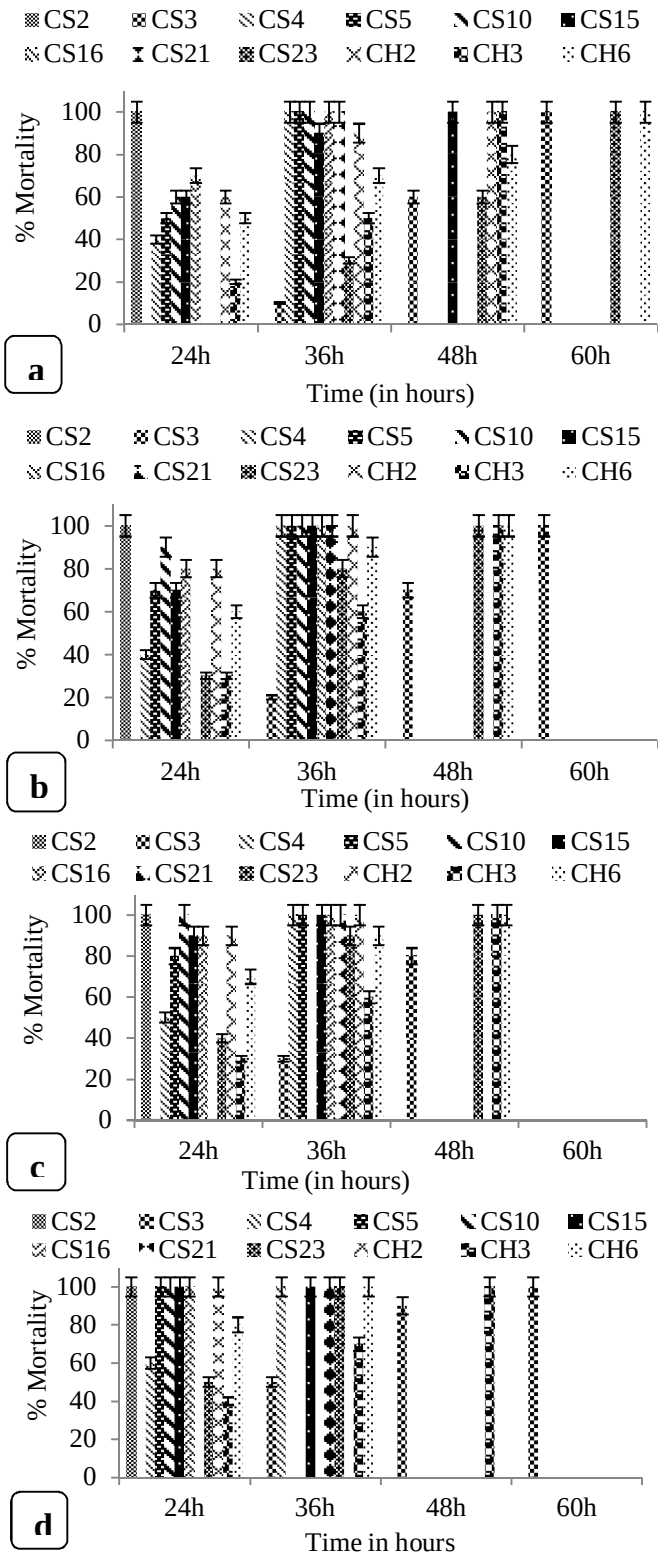

Fig. 1: Mortality in larvae of *Helicoverpa armigera* infected with various concentrations of IJs of nematodes at various post-infection periods a) 25 IJs larva^{-1}, b) 50 IJs larva^{-1}, c) 100 IJs larva^{-1}, and d) 200 IJs larva^{-1}

concentrations. Even in case of *S. abbasi* isolate CS_2 the concentrations with 25, 50 100 and 200 IJs produced 100% mortality after 24 h post-treatment and in case of isolate CS_{21} in 36 h indicating that for pathogenicity lowest dose i.e. 25 IJs larva^{-1} is enough to kill the larval stage of *H. armigera*. Maximum time of 60 h was taken by *Steinernema* sp. isolate CS_3. *Heterorhabditis* sp. isolate CH_6 also indicated that all the doses applied were sufficiently virulent to kill all larvae within very short duration. High mortality of EPNs indicated low defense rate of *H. armigera* against the nematodes, therefore proved their applicability in insect's control. Comparatively, the isolates showed high mortality from *S. masoodi* (species *inquirenda* by Nguyen and Hunt, 2007) which produced 100% mortality in 4th instar larvae of *H. armigera* after 72 h post-infection of 100 IJs larva^{-1} dose (Hussain and Ahmad, 2015). Jothi and Mehta (2006) reported 83 to 100% mortality of *H. armigera* at 72 h at 40 IJs of *S. glaseri* larva^{-1} and 93% mortality at 72 h exposure by *S. abbasi* and *H. bacteriophora* in *H. armigera* larvae (Shoeb *et al*., 2006) was also less from the *S. abbasi*, *S. siamkayai* and *H. indica* isolates used in this study. A cent per cent mortality was observed in last instars of *H. armigera* when exposed to *S. feltiae* (Glazer and Navon, 1989) with 200 IJs larva^{-1} and 40 IJs of *S. glaseri* and *S. feltiae* (Karunakar *et al*., 1999) which was less from the isolates used in the study wherein only 25 IJs larva^{-1} was sufficient to produced 100% mortality within 24-60 h exposure. Prasad *et al.* (2012) in case of *H. indica* reported 73% mortality at doses ≥50 IJs larva^{-1} at 48 h with absolute mortality at dose ≥75 IJs larva^{-1} at 72 h with a LC_{50} value of 290 IJs larva^{-1} at 24 h (Prasad *et al*., 2012). High susceptibility of *H. armigera* larvae to *H. indica* was also reported by Banu *et al.* (2007) and Saravanapriya and Subramanian (2007) wherein they found only 4 and 9 IJs larva^{-1} sufficient to kill half population. *H. indica* isolates used in present study produced

Table 2: LT_{50} and LT_{90} values (in hours) of infective Juveniles of *Steinernema* and *Heterorhabditis* spp. at different concentrations

	Concentrations (No. of infective juveniles larva^{-1})																					
	Combine					25 IJs					50 IJs					100 IJs					200 IJs				
Isolate	LT_{50}	LT_{90}	$\chi 2$	df	Sig	LT_{50}	LT_{90}	$\chi 2$	df	Sig	LT_{50}	LT_{90}	$\chi 2$	df	Sig	LT_{50}	LT_{90}	$\chi 2$	df	Sig	LT_{50}	LT_{90}	$\chi 2$	df	Sig
CS_2	17.0	19.0	0	3	1	17.3	20.5	0.10	3	0.99	17.3	20.5	0.1	3	0.99	17.3	20.5	0.10	3	0.99	17.3	20.5	0.1	3	0.99
CS_3	40.9	51.9	1.84	3	0.61	44.8	54.9	0.58	3	0.90	42.4	53.0	0.50	3	0.92	40.1	50.6	0.30	3	0.96	36.8	46.5	0.34	3	0.95
CS_4	*A*	*a*	*a*	*a*	*a*	25.0	30.0	0.06	3	1	25.0	30.0	0.06	3	1	24.0	28.8	0.02	3	1	23.1	27.7	0.01	3	1
CS_5	*A*	*a*	*a*	*a*	*a*	24.0	28.8	0.02	3	1	22.1	26.8	0.01	3	1	20.9	25.6	0.01	3	1	17.3	20.5	0.1	3	0.99
CS_{10}	20.8	24.3	0	3	1.00	23.1	27.7	0.01	3	1	19.3	23.7	0.04	3	1	17.3	20.5	0.1	3	0.99	17.3	20.5	0.1	3	0.99
CS_{15}	20.5	27.5	2.59	3	0.46	23.2	33.5	0.52	3	0.91	22.1	26.8	0.01	3	0.91	19.3	23.7	0.1	3	1	17.4	20.5	0.1	3	0.99
CS_{16}	*A*	*a*	*a*	*a*	*a*	22.1	26.8	0.01	3	1	20.9	25.6	0.01	3	1	19.3	23.7	0.04	3	1	17.3	20.5	0.1	3	0.99
CS_{21}	*A*	*a*	*a*	*a*	*a*	21.6	26.6	0.00	3	1	21.6	26.6	0.0	3	1	21.6	26.6	0	3	1	21.6	26.6	0	3	1
CS_{23}	29.1	44.5	1.60	3	0.66	42.1	55.5	1.65	3	0.65	27.9	38.5	0.11	3	0.95	25.7	35.1	0.11	3	0.99	24.0	28.8	0.02	3	1
CH_2	20.2	27.1	3.55	3	0.31	23.2	33.5	0.52	3	0.91	20.9	25.6	0.01	3	1.0	19.3	23.7	0.04	3	1.0	17.3	20.5	0.1	3	0.99
CH_3	29.7	43.2	7.88	3	0.05	32.1	44.9	2.43	3	0.49	29.7	43.2	1.97	3	0.58	29.7	43.2	1.97	3	0.58	27.4	40.8	1.45	3	0.69
CH_6	23.1	36.5	5.27	3	0.15	27.6	49.1	2.28	3	0.52	23.2	33.5	0.52	3	0.91	22.1	32.3	1.12	3	0.77	20.9	25.6	0.01	3	1

[a]The data could not be computed due to linear dependence among covariate

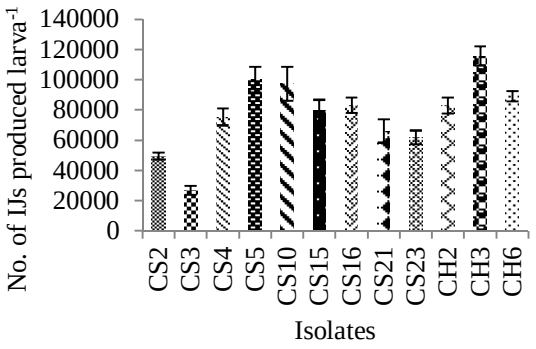

Fig. 2: Progeny production by nematodal isolates in host insect *Helicoverpa armigera* larvae

high pathogenicity with 100% mortality within 48-60 h in lowest dose. The LC_{50} values of other *H. indica* isolates were also effective.

The progeny production rate revealed that all the isolates were able to multiply in and kill the larvae; and were able to produce a good number of IJs (Fig. 2). The highest IJs produced larva^{-1} of *H. armigera* was noticed with *S. abbasi* isolate CS_5 ($100\pm8.5\times10^3$) and lowest with *S. abbasi* isolate CS_3 ($26.9\pm3\times10^3$). Other steinernematids also produced a good number of progeny. *H. indica* isolate CH_3 produced $115.7\pm6.3\times10^3$ IJs larva^{-1} which was highest among all the isolates tested.

The recycling potential of EPNs is an important factor for commercialization of these nematodes as biocontrol agent. Successful reproduction of EPNs within the body of *H. armigera* larvae in present study advocates that the nematodes have strong suppressive responses. However, the most pathogenic *S. abbasi* isolates CS_2 and CS_{21} showed lesser reproduction within the cadaver and the least pathogenic isolates produced better number of off-springs this may be the lesser time taken by the isolates to kill the host insect. Reports reveal that only a single penetrating IJ is enough to kill the host (Glazer & Lewis, 2000). For proper reproduction, the male and female ratio can also be responsible for low reproduction because of the inadequate number of IJs penetration (Lewis *et al*., 2006). High number of irregular relation within the virulence and progeny production in the host's body was estimated in similar studies (Shapiro-Ilan and Cottrell, 2005; Xu *et al.*, 2010).

Conclusion: EPNs have the potential to be deployed as a biopesticide with fast acting efficacious insecticidal properties against *H. armigera* apart from being safe to the environment. The test isolates, especially *S. abbasi* isolate CS_2, *S. siamkayai* isolate CS_{16} and *H. indica* isolate CH_2 were highly virulent to *H. armigera* and can be used and commercialized at a large scale because of good

reproductive potential. All other isolates also proved significantly effective with respect to their virulence properties against *H. armigera* under laboratory conditions. Future field evaluation studies will establish their possible role in formulating insect pest management strategies.

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