Radio-sterilized *Spodoptera litura* (Fabr.) as a conducive host for *in vivo* safe transport of viable entomopathogenic nematodes, *Steinernema thermophilum* as potential parasitoids

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The potential of a radio-sterilized host, *Spodoptera litura* (Fabr.), an established noctuid pest, was ascertained for *in vivo* transport of the viable entomopathogenic nematodes (EPNs), *Steinernema thermophilum*. Radio-sterilization (70 Gy) of the host (pest) was done to avoid any pest population build-up from the host larvae that could inadvertently miss EPN infection. The infective juveniles (IJs) derived from a radio-sterilized host took 67.3 h to induce host mortality, 132 h for incubation, and showed 87.8% parasitization with 98.9 IJs harvesting per mg host body wt, indicating almost similar parasitizing behaviour of these IJs as control. The findings indicated the suitability of the radio-sterilized host, *S. litura*, for carrying the IJs (*in vivo*) in a safe mode, that could retain a substantial degree of infectivity to be utilized in the field for managing this serious noctuid pest using biocontrol measures.

Keywords: Biocontrol agents, entomopathogenic nematodes, host irradiation, pest management, *Spodoptera litura*, *Steinernema thermophilum*.

THE cotton leafworm *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is a dreadful cosmopolitan pest which infests over a hundred host plants, including soybean, tobacco, cotton, cabbage and chickpeas[1]. The outbursts of *S. litura* have become frequent lately in the Asian region due to insecticide resistance[2–4]. Amongst all the ecologically sound tactics applied to control of *Spodoptera* spp., biorational and biological control techniques are pre-eminent. One such potential biological control pest management approach is using entomopathogenic nematodes (EPNs)[5,6]. Recently, the application of EPNs belonging to families Steinernematidae and Heterorhabditidae has become prominent due to their safety to humans, immense reproductive potential and specificity[7–9]. EPNs mostly attack the pests present in the soil[10]. The infective juveniles (IJs) enter the insect body through natural openings and release their symbiotic bacteria into the insect hemocoel. The bacteria multiply inside the insect, release various toxins and cause the death of the insect within 48–72 h (ref. 11). The potential of EPNs as a biological control agent on noctuid lepidopteran insects has been studied broadly by applying *Steinernema* species against *Spodoptera* species[12–15], and *Helicoverpa zea*[16]. *Steinernema thermophilum* has been used for the control of many pests[17,18].

Nuclear technologies have been beneficial in advancing the biological and genetic traits of various insects, and radiation has been a powerful tool in managing insect pests[19]. Nuclear techniques have great relevance in the biological control of pests using different methods like reproductive sterilization of insects, secure transport of various parasitoids in the irradiated hosts and advancement in the suitability of insects for mass rearing[20]. Research has indicated that radio-genetic sterile insect technique can be integrated appropriately with different biological pest management approaches like disruption of pheromone[21], host-plant resistance[22], entomopathogens[23] and natural enemies[24].

Insects infected with EPNs could be released and pest suppression can be finally achieved by the progeny IJs which are released from the insect cadavers[25]. EPNs can survive in harsh and dry conditions for a long time inside the host cadaver. Better endurance of EPNs has been reported within the host cadaver in comparison to those present in the aqueous suspension[26,27]. It has been stated that EPNs can identify and rapidly kill their hosts in 48–72 h after entering them[28]. So, after a required duration of exposure to these nematodes, the host larvae can be transferred instantly to the field; however, this may not be fruitful if some of the host pests escape parasitization. This issue can be resolved by radio-sterilization of the host followed by exposure to IJs; then they can be transferred to the field before facing mortality. In this manner, the IJs emerging from the infected cadavers are released into the environment, and they seek new hosts in the field to multiply and maintain their population[25].

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The present study evaluated the parasitization performance of EPNs, *S. thermophilum* on radio-sterilized lepidopteran pest, *S. litura* (as host). It also ascertained the viability of *S. thermophilum* derived from the radio-sterilized host, *S. litura* in order to assess the potential of this sterilized lepidopteran pest as a feasible host to transport the viable parasitoid into the field in a safe mode.

Materials and methods

Maintenance of insects

The culture of *S. litura* was maintained in the insectary under ambient conditions, viz. 27° ± 1°C temperature, 75 ± 5% relative humidity and 12 h light : 12 h dark period with lights on at 06:00 h and off at 18:00 h on a chickpea-based semi-synthetic diet[25]. The greater wax moth, *Galleria mellonela* (a factitious host of *S. thermophilum*) was reared in the laboratory on a semi-synthetic diet at 30 ± 2°C (ref. 29). The last instar larvae were used for parasitization to maintain the culture of *S. thermophilum*.

Maintenance of S. thermophilum EPNs

The culture of *S. thermophilum* was maintained on one of its factitious hosts *G. mellonela* and stored under optimal conditions, i.e. 25° ± 2°C temperature and 75 ± 5% relative humidity in Ringer's solution in a BOD incubator. Freshly harvested, 1–2-week-old EPNs were used for the experiments.

Irradiation of insects

The 0–1-day-old sixth instar larvae of *S. litura* were selected for all the experiments. The irradiation of larvae was performed at the Radiation Unit of the Institute of Nuclear Medicine and Allied Sciences (INMAS), Delhi, India, with cobalt-60 source at a dose rate 0.509–0.483 kGy/min. A dose of 70 Gy was used for the radio-sterilization of the last instar *S. litura* larvae[25].

Assessing the infective potential of S. thermophilum against radio-sterilized host, S. litura

Three experimental regimens were evaluated for the parasitization performance of *S. thermophilum*, viz. (i) N-IJs (normal infective juveniles) versus N-host, (ii) N-IJs versus Irr-host (radio-sterilized host at 70 Gy) and (iii) F1-IJs (IJs derived from Irr-host) versus N-host.

The 0–1-day-old sixth instar *S. litura* larvae (normal and irradiated with 70 Gy dose) were placed individually in glass petri dishes (50 × 15 mm) lined with a double layer of Whatman filter paper. The freshly harvested IJs (1–2-week-old) were used in all the experiments for inoculation. About 25–30 IJs per larva were inoculated and the petri dishes were sealed with parafilm to avoid any infection. The larvae were kept at 27° ± 2°C temperature and 75 ± 5% relative humidity. The host larvae were observed every 2–3 h to check the timings of morbidity and mortality. Morbidity was assessed as a primary behavioural response of the larvae caused due to release of bacterial toxins into the haemolymph of the host resulting in septicemia after the infection of EPNs. It included the slow response of the larvae to a probe (gently touching them with a pair of forceps), lethargic nature and lag in the resuming body posture when turned upside down. Larvae which died due to infection became slightly flaccid and dark brown in colour, which was not observed in natural mortality. After the death of the larvae, the cadavers were transferred into white traps[30] (prepared with petri dishes – 90 mm diameter). The IJs started releasing 5–6 days after the death of the host as the resources inside the host body started depleting. The time taken by the IJs to release from the host cadavers was recorded as incubation time. The IJs coming out of the cadavers were harvested daily, and the total harvesting profile of IJs, harvesting/mg body wt of host and harvesting period were recorded for each condition. The average reading of each cohort of ten host larvae constituted one replicate for morbidity, mortality, incubation time and harvesting parameters, and a cohort of 12–15 host larvae constituted one replicate for assessing per cent parasitization.

The parasitization performance of IJs derived from the radio-sterilized host was similarly assessed on the normal host (unirradiated host larvae). The experiment in each regimen was replicated five times.

Statistical analysis

Statistical analyses were performed using GraphPad Prism6 software (La Jolla, CA, USA). Student's *t*-test was used to compare the metamorphic potential and reproductive sterility in adult moths developed from normal and radio-sterilized hosts. One-way ANOVA followed by Tukey's multiple comparison test was used to analyse the bio-efficacy parameters of *S. litura* (as host) in different regimens.

Results

Radio-sterilization of host larvae of S. litura

The adults developed from irradiated sixth instar larvae (70 Gy) were significantly affected. The percentage of pupa formation had significantly decreased in the case of irradiated larvae compared to control larvae. In the control condition, the per cent pupa formation was found to be 93.7, while in the irradiated condition it was reduced to 40.6 (Table 1). The egg viability was nil when adults derived from irradiated larvae were crossed, in comparison to the

Table 1. Effect of gamma radiation on the metamorphosis and viability of the last instar larvae (L6) of *Spodoptera litura*

Host-stage irradiated	Dose given (Gy)	Pupa formation (%)	Malformed pupa[i] (%)	Adult emergence (%)	Malformed adult[ii] (%)	Egg viability (%)
L6	0	93.7 ± 2.5[a]	7.4 ± 0.87[a]	86.2 ± 4.1[a]	3.9 ± 0.53[a]	91.4 ± 3.1[a]
L6	70	40.6 ± 2.1[b]	34.9 ± 1.7[b]	06.1 ± 0.52[b]	3.2 ± 0.23[a]	0[b]

Means ± SE followed by the same alphabets within a column are not significantly different at $P < 0.05$ (Student's *t*-test); $n = 5$. Each replicate constitutes a cohort of 25 L6 with daily observations on growth and metamorphosis.

[i]Per cent malformed pupa was computed from the total pupae formation.

[ii]Per cent malformed adult was computed from the total adults enclosed. For egg viability, hatchability of the eggs laid due to self-cross of the adults derived from treated L6 was observed; the percentage data were transformed for biostatistics.

control, where egg viability was found to be 91.4%. It confirmed the complete sterilizing potential of 70 Gy gamma dose (Table 1).

Influence of host radiation on the infectivity potential of IJs

When *S. thermophilum* IJs were released on normal host larvae, the morbidity time was found to be 24 h and mortality time was recorded as 62 h. The time taken by the IJs to be released from the host cadavers (incubation time) was 136.8 h. Total harvesting of IJs per host was in the range 29,020–34,529. The harvesting could also be expressed as 121.9 IJs/mg host body wt and the harvesting period was recorded as 8–10 days (Table 2).

In response to the infection of *S. thermophilum* IJs in an irradiated host, the timings of morbidity and mortality had reduced by about 27% compared to control (normal IJs versus normal host). Due to radiation stress, the total harvesting of IJs/host was also affected and it decreased to 16,321–20,416 IJs; however, these IJs were in sufficient number to further parasitize other host larvae (potential pests) in the field, since these EPNs can multiply readily, possess the great reproductive capability and are extremely virulent. The harvesting period of these IJs was about 6–7 days (Table 2).

Bio-efficacy of S. thermophilum IJs derived from radio-sterilized host on normal host larvae of S. litura

IJs of *S. thermophilum* derived from the irradiated host showed almost similar timings for morbidity and mortality (Table 2), and exhibited no significant change in parasitization response compared to control (normal IJs vs normal host) (Figure 1), although the total IJs harvested from the infected host was about 20% less compared to the control (Table 2).

While comparing the performance of IJs in three regimens, viz. (i) N-IJs versus N-host, (ii) N-IJs versus Irr-host and (iii) F1-IJs (from Irr-host) versus N-host, the morbidity and mortality timings were significantly influenced in the second regimen ($F_{2,12} = 65.6$*, $P < 0.0001$ for morbidity timing; $F_{2,12} = 48.9$*, $P < 0.0001$ for mortality timing). Similarly, the incubation time of IJs decreased in the second regimen compared to the incubation period expressed by N-IJs and F1-IJs (derived from the irradiated host) on a normal host ($F_{2,12} = 31.07$*, $P < 0.0001$) (Table 2). Interestingly, no significant difference was found in the parasitization response of normal IJs of *S. thermophilum* among these three regimens ($F_{2,12} = 1.04$, $P = 0.372$), indicating that *S. thermophilum* was almost equally capable of infecting the normal and irradiated host larvae, and that host irradiation did not affect the parasitization potential of IJs of this EPN species (Figure 1).

There was a significant effect on the total harvesting potential of IJs from the host cadavers in different experimental regimens ($F_{2,12} = 98.96$*, $P < 0.0001$). The harvesting of IJs was influenced in 70 Gy-irradiated hosts than in control (exhibited by N-IJs versus N-host), and F1-IJs (from Irr-host) versus N-host regimen, although the harvesting potential of dauers in the latter two regimens was quite comparable. Similarly, the harvesting period of IJs was markedly influenced in the experimental regimens compared to control (ANOVA, $F_{2,12} = 28.25$*, $P < 0.0001$).

Discussion and conclusion

In the present study, *S. thermophilum* was selected as a model EPN species because it had been isolated from India and was large in size compared to other EPN species. The pathogenicity of *S. thermophilum* has been tested previously against *Galleria mellonella* (Lepidoptera)[31], *Aplosonyx chalybaeus* (Coleoptera)[32], *Athalia lugens* (Hymenoptera)[17], *Helicoverpa armigera* and *S. litura* (Lepidoptera)[18]. Previous studies have shown that *S. litura* can be a potential host for different *Steinernema* species[13,33]. The infective potential of EPNs is influenced by external factors (temperature, moisture means of application, etc.[34]), internal (host-specific) factors and a few host-specific chemical cues which play a crucial role in finding an appropriate host for EPNs[35].

Stress-induced irradiation can be used for the radio-genetic control of the lepidopteran family pests[36,37]. Advancement has been made in this approach by integrating it with other ecologically safe pest management strategies, such as biological and parabiological control. The suppression of lepidopteran pests can be enhanced using host irradiation for

Table 2. Infective performance of entomopathogenic nematode *Steinernema thermophilum* on sixth instar larvae of *S. litura*

Regimen	Morbidity time (h)	Mortality time (h)	Incubation time (h)	Harvesting/mg body wt	Total harvesting	Harvesting period (days)
Normal infective juveniles (IJs) versus normal host	$24.53^{a} \pm 0.8$	$62.48^{a} \pm 2.41$	$136.8^{a} \pm 4.9$	$121.9^{a} \pm 6.8$	$31837^{a} \pm 121$	$9.2^{a} \pm 0.374$
Normal IJs versus irradiated (70 Gy) host	$17.88^{b} \pm 0.62$	$45.43^{b} \pm 2.1$	$109.6^{b} \pm 3.5$	$71.41^{c} \pm 4.1$	$18154^{c} \pm 820$	$6.6^{b} \pm 0.244$
F1-IJs (from irradiated host) versus normal host	$24.35^{a} \pm 0.82$	$67.36^{a} \pm 1.5$	$132.0^{a} \pm 4.6$	$98.97^{b} \pm 4.5$	$25486^{b} \pm 890$	$7.6^{b} \pm 0.244$
	$F = 65.62$*, $df = (2, 12)$, $P < 0.0001$	$F = 48.99$*, $df = (2, 12)$, $P < 0.0001$	$F = 31.07$*, $df = (2, 12)$, $P < 0.0001$	$F = 56.71$*, $df = (2, 12)$, $P < 0.0001$	$F = 98.96$*, $df = (2, 12)$, $P < 0.0001$	$F = 28.25$*, $df = (2, 12)$, $P < 0.0001$

The 0–1-day-old L6 of *S. litura* were exposed to 1–2-week-old IJs at a dose rate of 25–30 IJs/host for the bioassay. Means ± SE followed by same letter within a column are not significantly different at $P < 0.05$ (one-way ANOVA followed by Tukey's multiple comparison test); $n = 5$. Average reading of each cohort of ten host larvae constituted one replicate for morbidity, mortality, incubation time and harvesting parameters. *Significance at $P \leq 0.05$ level.

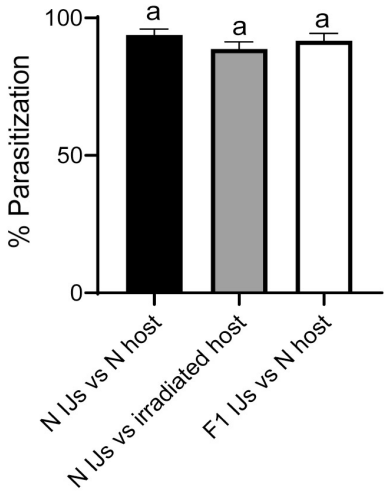

Figure 1. Parasitizing performance of entomopathogenic nematode (EPN) *Steinernema thermophilum* on sixth instar larvae of *Spodoptera litura* in different irradiated regimens. Average reading of each cohort of 12–15 host larvae constituted one replicate for per cent parasitization parameter.

augmenting the transport, quality and production of parasitoids, in conjunction with radiation-mediated sterile insect programmes, including the F1 sterility technique[38–41].

Thus, the feasibility of *in vivo* transport of EPN, *S. thermophilum* within host larvae of a serious pest, *S. litura* (against which this EPN species was found to act promisingly as a biocontrol agent) has been ascertained in the present study. *S. thermophilum* is best suited for parasitizing the hosts present in the soil with low mobility[42]. Generally, for *in vivo* transport of EPNs, inoculation of a host by IJs is done in large numbers and the infected host larvae are transported to the field immediately so that IJs can be released in the specific agro-field on time (in view of the limited 4–5 days of incubation) in order to encounter the pest (host) larvae in the field. It is considered that EPNs emerging from a host would be well adapted to act more effectively in terms of parasitization capability against the same host. For instance, *Steinernema glaseri* was assessed against *S. litura* in a similar mode[25]. Several studies have also suggested that the application of EPNs in infected cadavers was more efficient than the application of these IJs in aqueous suspensions[26,43–45].

To avoid any pest population build-up due to inadvertent missing of host inoculation by EPNs to the host (i.e. pest larvae), host irradiation was considered to exercise safe transport of IJs of *S. thermophilum* to the field.

The gamma dose of 70 Gy was reconfirmed for the radio-sterilization of *S. litura* host larvae (as reported by Seth and Barik[25]), and these irradiated host larvae were used for *S. thermophilum* infection and its proliferation potential. The relative bio-efficacy of *S. thermophilum* was evaluated against irradiated host *S. litura* in comparison with the control. The IJs (dauers) of *S. thermophilum* caused faster mortality in the irradiated host but showed similar parasitization capacity compared to the control, whereas harvesting was reduced. This indicated the substantial suitability of the radio-sterilized host compared to the control (unirradiated host). Further, the IJs which were harvested from the irradiated hosts were also evaluated against the normal host to predict their parasitization efficiency. The efficacy of IJs derived from the irradiated host (F1-IJs) on normal host was similar to the control (normal IJs versus the normal host), with a slight influence on the harvesting potential of EPNs.

In conclusion, the present study indicates that releasing *S. thermophilum* through radio-sterilized infected hosts might have immense potential for use as parasitoids to control the noctuid pest, *S. litura*. These findings support the use of irradiated host, *S. litura* larvae for the mass production of *S. thermophilum* and to facilitate their release into the field without any concern that the unintentionally or accidentally non-parasitized larvae would otherwise add

to the pest population. This study also reflects the reasonable parasitization and proliferation potential of *S. thermophilum* carried *in vivo* within the radio-sterilized host. It indicates that host irradiation does not influence the bio-efficacy of this EPN species which could be used in inundative mode or even in inoculative mode due to the substantial bio-efficacy and proliferation capacity of the IJs. Further studies on using the radio-genetic and biological control techniques in conjunction managing in *S. litura* are in progress.

Conflict of interest: The authors declare that there is no conflict of interest.

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